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Effect of NaPi-mediated phosphate transport on intracellular pH

Received: 6 August 2000 / Received after revision and accepted: 18 October 2000 / Published online: 9 December 2000
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Abstract Extracellular pH has been shown previously to influence transport via type-II Na⁺/phosphate (NaPi) transporters by modifying the affinity of the carrier for Na⁺ and by altering the availability of divalent and monovalent phosphate. As the transport of monovalent phosphate would be expected to acidify, and that of divalent phosphate to alkalize the cell interior, the effect of phosphate transport on cytosolic pH was studied using ion selective microelectrodes in *Xenopus* oocytes expressing NaPi-3 or NaPi-5. At an alkaline extracellular pH (pH_e) of 8.0, addition of phosphate elicited a strong inward current, depolarization of the cell membrane and cytosolic alkalization. At pH_e 6.0 the phosphate-induced inward current and depolarization were reduced and the alkalization completely abolished. In conclusion, at alkaline pH_e phosphate transport is enhanced and the transport of divalent phosphate prevails. At pH_e 6.0, phosphate transport is attenuated and is accomplished by transport of both divalent and monovalent phosphate.

Keywords NaPi-3 · NaPi-5 · Phosphate · Proximal tubule · *Xenopus* oocytes

Introduction

Renal tubular phosphate transport is accomplished by phosphate uptake across the proximal tubular brush border and exit at the basolateral cell membrane [2, 13, 18]. Based on structural motifs, tissue distribution and func-

tional characteristics, three different types of Na⁺, phosphate transporters have been classified. The type-I transporter (NaPi-1) induces an anion channel [6], type IIa is the renal brush border Na⁺/phosphate cotransporter and type IIb is found apically in many tissues including the small intestine and the lung, but not the kidney [19]. Type-III transporters, originally identified as murine retroviral receptors [16] are expressed ubiquitously and related messenger ribonucleic acid (mRNA) has been identified in different renal structures [23]. Immunofluorescence studies show a basolateral location in the proximal tubule (D. Bacic, J. Biber, H. Murer, unpublished observation).

The type-II carrier in the rat renal brush border has been cloned and classified as NaPi-2 [17], that from humans as NaPi-3 [17], from opossum kidney cells as NaPi-4 [21], from flounder as NaPi-5 [11, 26], from the rabbit as NaPi-6 [24] and from mice as NaPi-7 [14]. All these carriers have similar functional properties. The transport of phosphate is coupled to Na⁺ that provides the electrochemical gradient for directed transport into the cell [2]. In the oocyte expressing system, a coupling ratio of 3:1 has been found [5, 6, 7,]. The type-II transporters in the renal brush border are rate limiting and thus determine renal phosphate reabsorption [13, 18].

The aim of numerous of studies has been the identification of the adequate phosphate moiety serving as substrate. Phosphate transport is enhanced in alkaline extracellular fluid, pointing to preferential transport of divalent phosphate [3, 5, 10, 20, 22]. On the other hand, pH also modifies the affinity of the carrier for Na⁺ [1, 4, 5, 11, 14]. Kinetic analysis and the comparison of the fluxes and currents has led to the conclusion that the carrier is able to transport both divalent and monovalent phosphate and that extracellular alkalization enhances the affinity for Na⁺ [4, 5, 11, 12, 14].

If the above model were correct, the contribution of divalent and monovalent phosphate to transport should vary as a function of extracellular pH (pH_e), which should be reflected by alterations of intracellular pH (pH_i). Given the apparent dissociation constant (pK_a) of

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Table 1 Effect of phosphate (1 mmol/l) on cell membrane potential (V_m) and intracellular pH (pH_i) in *Xenopus* oocytes expressing the Na-phosphate cotransporters NaPi-3 or NaPi-5 and exposed to an extracellular pH of 6.0 or 8.0

	V_m (mV)	ΔV_m (mV)		pH_i		ΔpH_i (pH/min)	
		pH 6	pH 8	pH 6	pH 8	pH 6	pH 8
NaPi3	-38.6±1.6	+3.8±0.7	+14.5±1.3	7.24±0.04	7.33±0.04	0.0025±0.0005	0.012±0.001
<i>n</i>	15	11	15	11	15	11	15
NaPi5	-37.2±1.7	+8.4±1.9	+37.6±5.4	7.40±0.04	7.42±0.04	0.0038±0.0008	0.028±0.005
<i>n</i>	10	7	10	7	10	7	10

6.8 for $H_2PO_4^-/HPO_4^{2-}$, exclusive entry of divalent phosphate should lead to cytosolic alkalization and exclusive entry of monovalent phosphate to cytosolic acidification. At pH_i 6.8, pH should remain constant if monovalent and divalent phosphate enter at equal rates. The model would predict alkalization of pH_i following addition of phosphate in alkaline extracellular fluid as, under this condition, the carrier would transport almost exclusively divalent phosphate. The model predicts further that phosphate transport and the phosphate-induced current would be reduced at acidic pH_e and that, under such conditions, the cytosolic alkalization should be abolished or even reversed. The effect of phosphate transport through NaPi type-II transporters on pH_i , however, has never been studied systematically. The present study was performed to test the hypothesis that phosphate transport through NaPi-3 and NaPi-5 alters pH_i and, if so, whether such alterations are dependent on pH_e .

Materials and methods

Complementary RNA (cRNA) encoding the human phosphate transporter NaPi-3 or the flounder phosphate transporter NaPi-5 was synthesized in vitro as previously described [4, 11, 25]. Dissection of *Xenopus laevis* ovaries, collection and handling of the oocytes have been described in detail elsewhere [25]. Oocytes were injected with 10 ng cRNA in 50 nl water per oocyte; non-injected oocytes served as controls. All experiments were performed at room temperature 3–8 days after injection.

Two-electrode voltage-clamp recordings were performed at a holding potential of -50 mV. The data were filtered at 10 Hz and recorded with a MacLab analogue-to-digital converter and software for data acquisition and analysis (AD Instruments, Castle Hill, Australia). The external control solution (superfusate/ND96) contained (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 5 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES). The final solutions were titrated to the desired pH using HCl or KOH. The rate of superfusion was 20 ml/min and complete exchange of the bath solution took less than 10 s. The currents given are the maximal values measured during a 30-s substrate superfusion.

pH was measured using ion-selective microelectrodes prepared from pulled borosilicate electrodes, back-filled with 5% tributylchlorosilane in carbon tetrachloride (Fluka, Deisenhofen, Germany). To ensure a smooth coating and to evaporate the solvent the electrodes were baked at 400–450 °C for 5 min. A column of H^+ cocktail (proton ionophore II-cocktail A, Fluka) approximately 300 μm long was filled into the tip of the electrode and the electrode then back-filled with a solution of 100 mM Na citrate at pH 6.0. The electrodes were calibrated using solutions with pH 6.0, 7.0 and 8.0. Only electrodes with a linear slope of more than 50 mV/pH unit and stable calibration were used [25]. Signals were recorded with an electrometer (WPI model FD223, Sarasota, Fla., USA). On the basis of the calibration curve for the

Fig. 1 Response of membrane current (ΔI_p , dual-electrode voltage clamp, **A**) and potential (ΔV_m) and the electrochemical potential of an intracellular pH-sensitive electrode (V_{H^+}) (**B**) in *Xenopus* oocytes expressing the Na⁺/phosphate cotransporters NaPi-3 (left-hand traces) or NaPi-5 (right-hand traces) in response to addition of phosphate (1 mM) at extracellular pH (pH_e) of 6.0 or 8.0

pH-sensitive electrode, the chemical potential for H^+ (E_{H^+}) of oocytes was calculated as the difference between the membrane potential (V_m), measured simultaneously with a 3 M KCl micro-electrode, and the electrochemical potential of the pH-sensitive electrode (V_{H^+}).

Data are provided as means±SEM, *n* represents the number of oocytes investigated. The significance of differences between means was assessed using the paired Students *t*-test. *P*<0.05 was considered as significant.

Results

As shown in Fig. 1, the application of 1 mM phosphate caused an inwards current, depolarization and cytosolic alkalization in *Xenopus* oocytes expressing either NaPi-3 or NaPi-5. The mean phosphate-induced current in NaPi-3-expressing oocytes was 10.6±1.6 nA (*n*=5) at

Fig. 2 Correlation between phosphate (1 mM)-induced ΔV_m and alteration of cytosolic pH (ΔpH_i) at pH_e 8.0 (squares) or 6.0 (circles). Upper and lower panels show data from NaPi-3- and NaPi-5-expressing oocytes, respectively. The lines are the respective least-squares fits of the data

pH_e 8.0 and 8.5 ± 1.4 nA ($n=5$) at pH_e 6.0. In NaPi-5-expressing oocytes the inwards currents were significantly larger: 121 ± 10 nA ($n=10$) at pH_e 8.0 and 108 ± 8 nA ($n=10$) at pH_e 6.0. In the current-clamp mode, V_m was -38.6 ± 1.6 mV ($n=15$) in NaPi-3-expressing oocytes and -37.2 ± 1.7 mV ($n=10$) in NaPi-5-expressing oocytes in the absence of extracellular phosphate. The addition of 1 mmol/l phosphate in NaPi-3-expressing oocytes led to depolarization of V_m by 14.5 ± 1.3 mV ($n=15$) at pH_e 8.0 and by 3.8 ± 0.7 mV ($n=11$) at pH_e 6.0 and in NaPi-5-expressing oocytes by 37.6 ± 5.4 mV ($n=10$) at pH_e 8.0 and 8.4 ± 1.9 mV ($n=7$) at pH_e 6.0.

Prior to the extracellular addition of phosphate pH_i was not significantly different between NaPi-3- and NaPi-5-expressing oocytes. Mean pH_i in NaPi-3-expressing oocytes was 7.33 ± 0.04 ($n=15$) at pH_e 8.0 and 7.24 ± 0.04 ($n=11$) at pH_e 6.0 and in NaPi-5-expressing oocytes 7.42 ± 0.04 ($n=10$) at pH_e 8.0 and 7.40 ± 0.04 ($n=7$) at pH_e 6.0. Addition of phosphate led to cytosolic alkalinization at pH_e 8.0 that was almost abolished at pH_e 6.0 (Fig. 1, Table 1). As shown in Fig. 2, the phos-

phate-induced alteration of pH_i (ΔpH_i , pH units per minute) was a linear function of the depolarization (ΔV , in volts) at pH_e 8.0 but not at pH_e 6.0. Linear regression analysis yielded the following least-squares fits at pH_e 6.0:

$$\Delta pH_i = 0.0013 \pm 0.0008 + 0.1 \pm 0.3 \cdot \Delta V \quad (R=0.2)$$

for NaPi-3-expressing oocytes and

$$\Delta pH_i = 0.0034 \pm 0.0018 + 0.1 \pm 0.2 \cdot \Delta V \quad (R=0.1)$$

for NaPi-5-expressing oocytes. Neither the intercepts nor the slopes were significantly different from zero.

At pH_e 8.0 the corresponding equations were:

$$\Delta pH_i = 0.0001 \pm 0.0068 + 0.8 \pm 0.2 \cdot \Delta V \quad (R=0.8)$$

and

$$\Delta pH_i = 0.0001 \pm 0.0026 + 0.8 \pm 0.2 \cdot \Delta V \quad (R=0.8)$$

for NaPi-3- and NaPi-5-expressing oocytes respectively. The slopes, but not the intercepts, were significantly different from zero and from the respective values at pH_e 6.0.

Discussion

As reported earlier [5], phosphate induces a larger current when added to alkaline extracellular fluid than at acidic pH_e. The differences in current are reflected by similar differences of phosphate-induced depolarization of the cell membrane and reflect differences in phosphate transport. Three mechanisms contribute to pH dependence of phosphate transport. First, protons bind to the same transmembrane domain as Na⁺, possibly altering the conformation of the carrier [15] and hence the affinity of the carrier for Na⁺ [4, 5, 11, 14]. Accordingly, at acidic pH Na⁺ binding is impaired and the maximal transport rate for phosphate is decreased. Second, the carrier accepts divalent phosphate, the form prevailing in alkaline extracellular fluid, more readily [3, 10, 20, 22]. Third, protons alter the transition of the empty carrier from the intracellular to the extracellular side of the membrane [13]. A comparison of flux studies with currents revealed, somewhat surprisingly, that the flux declined more markedly than did the current, i.e. that the induced current per transported phosphate was greater at acidic pH than at alkaline pH [5]. This apparent discrepancy can be explained by a shift of transported phosphate from the divalent to the monovalent form [5]. The transport of divalent phosphate with three Na⁺ is equivalent to the transport of one unit charge, the transport of three Na⁺ with one monovalent phosphate is equivalent to the transport of two unit charges.

As expected for the transport of divalent phosphate, addition of phosphate to alkaline extracellular fluid led to cytosolic alkalinization. At pH_e 8.0, approximately 94% of phosphate is in the divalent form, which, at this pH, is the prevailing form transported. At a pH_i of 7.0, only some 61% of phosphate is in the divalent form.

Divalent phosphate entering the cytosol fluid at pH_i 7.0 will thus bind H^+ and lead to cytosolic alkalinization. In theory, the same result would be obtained if Na^+ , H_2PO_4^- entry were paralleled by H^+ exit. The present experiments cannot distinguish between translocation of free and phosphate-bound H^+ .

With an acidic extracellular fluid (pH_e 6.0), approximately 86% of phosphate is in the monovalent form, whilst at pH_i 7.0 only 39% of phosphate is monovalent. Monovalent phosphate entering the cytosolic fluid would thus be expected to release H^+ . Instead, a slight, but significant, alkalinization of pH_i is still observed (Table 1), indicating that phosphate is transported at a ratio slightly, but significantly, greater than that prevailing in the cytosol, i.e. greater than $1.6\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$. In other words, more phosphate entered the cells in the divalent form despite the marked prevalence of monovalent phosphate at acidic extracellular pH. This finding suggests that the transporter binds or translocates divalent phosphate preferentially. On the other hand, if only divalent phosphate were transported, the slope of $\Delta\text{pH}_i/\Delta V_m$ would have remained constant, irrespective of pH_e . This is clearly not the case. It is thus safe to conclude that both moieties are transported.

As recognized earlier, phosphate transport, and thus phosphate-induced currents, are larger in NaPi-5- [11] than in NaPi-3-expressing *Xenopus* oocytes [18]. Accordingly, the induced pH changes are also larger in NaPi-5- than in NaPi-3-expressing oocytes. *Xenopus* oocytes may express the more closely related flounder carrier more readily than the mammalian transporter. Similar observations have been made for the $\text{Na}^+/\text{cotransporter}$ NBC [9]. In addition, the present experiments were performed at room temperature, which is adequate for experiments in *Xenopus* oocytes with the flounder transporter but possibly not for the mammalian carrier. Despite the different rates of transport rates achieved with NaPi3 and NaPi5, the correlation between induced alkalinization and current is virtually identical, pointing to functional similarity of the two carriers.

In conclusion, pH_i measurements confirmed that NaPi-3 and NaPi-5 are capable of transporting both divalent and monovalent phosphate. Even at acidic pH_e more divalent than monovalent phosphate is transported or, alternatively H^+ is extruded in parallel to entry of monovalent phosphate. Despite the fact that the transport of divalent phosphate would be expected to be accompanied by transport of less charge per phosphate, the current produced is larger at alkaline pH_e due to stimulation of transport. Thus, the results of the present study are consistent with and support the current model of phosphate transport via NaPi-3 and NaPi-5.

Acknowledgements This study was supported by the Deutsche Forschungsgemeinschaft Bu 704/7-1, by the EC, contract number ERBCHRX-CT94-0595 and by a grant from the Federal Ministry of Education, Science, Research and Technology to F. Lang (Fö 01KS9602, Interdisciplinary Centre for Clinical Research).

References

1. Amstutz M, Mohrmann M, Gmaj P, Murer H (1985) Effect of pH on phosphate transport in rat renal brush border membrane vesicles. *Am J Physiol* 248:F705–F710
2. Biber J, Murer H (1994) Renal epithelial apical Na/Pi cotransporter. *Cell Physiol Biochem* 4:185–197
3. Bindels RJ, Van den Broek LA, Van Os CH (1987) Effect of pH on the kinetics of Na^+ -dependent phosphate transport in rat renal brush-border membranes. *Biochim Biophys Acta* 897: 83–92
4. Busch A, Waldegger S, Herzer T, Biber J, Markovich D, Hayes G, Murer H, Lang F (1994) Electrophysiological analysis of Na^+/P_i cotransport mediated by a transporter cloned from rat kidney and expressed in *Xenopus* oocytes. *Proc Natl Acad Sci USA* 91:8205–8208
5. Busch AE, Wagner CA, Schuster A, Waldegger S, Biber J, Murer H, Lang F (1995) Properties of electrogenic Pi transport by a human renal brush border Na^+/P_i transporter. *J Am Soc Nephrol* 6:1547–1551
6. Busch AE, Waldegger S, Murer H, Lang F (1996) The molecules of proximal tubular transport – insights from electrophysiology. *Nephron* 72:1–8
7. Busch AE, Biber J, Murer H, Lang F (1996) Electrophysiological insights of type I and II Na^+/P_i transporters. *Kidney Int* 49: 986–987
8. Busch AE, Schuster A, Waldegger S, Wagner CA, Zempel G, Broer S, Biber J, Murer H, Lang F (1996) Expression of a renal type I sodium/phosphate-transporter (NaPi-1) induces a conductance in *Xenopus* oocytes permeable for organic and inorganic anions. *Proc Natl Acad Sci USA* 93:5347–5351
9. Boron WF, Hediger MA, Boulpaep EL, Romero MF (1997) The renal electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter. *J Exp Biol* 200:263–268
10. Cheng L, Sacktor B (1981) Sodium gradient-dependent phosphate transport in renal brush border membrane vesicles. *J Biol Chem* 256:1556–1564
11. Forster IC, Wagner CA, Busch AE, Lang F, Biber J, Hernando N, Murer H, Werner A (1997) Electrophysiological characterization of the flounder type II Na^+/P_i cotransporter (NaPi-5) expressed in *Xenopus laevis* oocytes. *J Membr Biol* 160:9–25
12. Forster IC, Biber J, Murer H (2000) Proton-sensitive transitions of renal type II Na^+ -coupled phosphate cotransporter kinetics. *Biophys J* 79:215–230
13. Gmaj P, Murer H (1986) Cellular mechanisms of inorganic phosphate transport in kidney. *Physiol Rev* 66:36–70
14. Hartmann CM, Wagner CA, Busch AE, Markovich D, Biber J, Lang F, Murer H (1995) Transport characteristics of a murine renal Na/Pi-cotransporter. *Pflügers Arch* 430:830–836
15. Horra C de la, Hernando N, Lambert G, Forster I, Biber J, Murer H (2000) Molecular determinants of pH sensitivity of the type IIa Na/P(i) cotransporter. *J Biol Chem* 275: 6284–6287
16. Kavanaugh MP, Kabat D (1996) Identification and characterization of a widely expressed phosphate transporter/retrovirus receptor family. *Kidney Int* 49:959–963
17. Magagnin S, Werner A, Markovich D, Sorribas V, Stange G, Biber J, Murer H (1993) Expression cloning of human and rat renal cortex Na/Pi cotransport. *Proc Natl Acad Sci USA* 90: 5979–83
18. Murer H (1992) Homer Smith Award. Cellular mechanisms in proximal tubular Pi reabsorption: some answers and more questions. *J Am Soc Nephrol* 2:1649–65
19. Murer H, Hernandez N, Forster I, Biber J (2000) Proximal tubular phosphate reabsorption: molecular mechanisms. *Physiol Rev* (in press)
20. Sacktor B, Cheng L (1981) Sodium gradient-dependent phosphate transport in renal brush border membrane vesicles. Effect of an intravesicular greater than extravesicular proton gradient. *J Biol Chem* 256:8080–8084

21. Sorribas V, Markovich D, Hayes G, Stange G, Forgo J, Biber J, Murer H (1994) Cloning of a Na/Pi cotransporter from opossum kidney cells. *J Biol Chem* 269:6615–6621
22. Strevè J, Giroux S, Beliveau R (1990) pH gradient as an additional driving force in the renal reabsorption of phosphate. *Biochem J* 271:687–692
23. Tenenhouse HS, Roy S, Martel J, Gauthier C (1998) Differential expression, abundance and regulation of Na-phosphate cotransporter genes in murine kidney. *Am J Physiol* F527–F534
24. Verri T, Markovich D, Perego C, Norbis F, Stange G, Sorribas V, Biber J, Murer H (1995) Cloning of a rabbit renal Na-Pi cotransporter, which is regulated by dietary phosphate. *Am J Physiol* 268:F626–F633
25. Wagner CA, Friedrich B, Setiawan I, Lang F, Bröer S (2000) The use of *Xenopus laevis* oocytes for the functional characterization of heterologously expressed membrane proteins. *Cell Physiol Biochem* 10:1–12
26. Werner A, Murer H, Kinne RK (1994) Cloning and expression of a renal Na-Pi cotransport system from flounder. *Am J Physiol* 267:F311–F317