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# ENZYMATIC REMOVAL OF ALKALINE PHOSPHATASE FROM RENAL BRUSH-BORDER MEMBRANES

## EFFECT ON PHOSPHATE TRANSPORT AND ON PHOSPHATE BINDING

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**Brush-border membrane vesicles prepared from rabbit kidney cortex were incubated at 37°C for 30 min with phosphatidylinositol-specific phospholipase C. This maneuver resulted in a release of approx. 85% of the brush-border membrane-linked enzyme alkaline phosphatase as determinated by its enzymatic activity. Transport of inorganic [$^{32}$P]phosphate (100 $\mu$M) by the PI-specific phospholipase C-treated brush-border membrane vesicles was measured at 20–22°C in the presence of an inwardly directed 100 mM $Na^+$ gradient. Neither initial uptake rates, as estimated from 10-s uptake values (103.5 ± 6.8%, $n = 7$ experiments), nor equilibrium uptake values, measured after 2 h (102 ± 3.4%) were different from controls (100%). Control and PI-specific phospholipase C-treated brush-border membrane vesicles were extracted with chloroform/methanol to obtain a proteolipid fraction which has been shown to bind $P_i$ with high affinity and specificity (Kessler, R.J., Vaughn, D.A. and Fanestil, D.D. (1982) J. Biol. Chem. 257, 14311–14317). Phosphate binding (at 10 $\mu$M $P_i$) by the extracted proteolipid was measured. No significant difference in binding was observed between the two types of preparations: 31.0 ± 9.37 in controls and 29.8 ± 8.3 nmol/mg protein in the proteolipid extracted from PI-specific phospholipase C-treated brush-border membrane vesicles. It appears therefore that alkaline phosphatase activity is essential neither for $P_i$ transport by brush-border membrane vesicles nor for $P_i$ binding by proteolipid extracted from brush-border membrane. These results dissociate alkaline phosphatase activity, but not brush-border membrane vesicle transport of phosphate, from phosphate binding by proteolipid.**

## Introduction

Alkaline phosphatase is a brush-border membrane-bound enzyme which has been proposed to be involved in the renal transport (reabsorption) of inorganic phosphate ($P_i$) [1]. In the past few years, however, considerable evidence has been presented against this hypothesis. Inhibition of alkaline phosphatase activity did not affect the phosphate transport by brush-border membrane vesicles prepared from kidney cortex [2–4], nor did it affect the phosphate reabsorption by isolated perfused proximal tubules [4]. A recent communication by Yusufi et al. [5] was even more convincing in this view. Enzymatic removal of alkaline phosphatase from brush-border membrane with phosphatidylinositol-specific phospholipase C also did not affect $P_i$ uptake into these brush-border membrane vesicles. These observa-

Abbreviations: Hepes, 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminoethane; Mes, 4-morpholineethanesulfonic acid; $P_i$, inorganic phosphate; PI, phosphatidylinositol.

tions strongly suggested that neither the alkaline phosphatase activity nor the alkaline phosphatase molecule is directly necessary for $P_i$ to be transported by the proximal tubular cells.

However, definition of the physiological role of alkaline phosphatase in the brush-border membrane still remains open. Storelli and Murer [3] proposed that alkaline phosphatase could act as a $P_i$-binding protein that facilitates $P_i$ transport. Evidence for such a binding has been presented by Beliveau and Brunette [6].

In our laboratory, Kessler et al. [7] extracted a proteolipid from rabbit kidney brush border membrane that binds $P_i$ with high affinity and specificity. The possibility exists, therefore, that our proteolipid could be, in fact, identical with the $P_i$-binding moiety of alkaline phosphatase. To investigate this possibility, we treated brush-border membrane vesicles with PI-specific phospholipase C to remove the alkaline phosphatase, then we extracted the brush-border membrane vesicles with chloroform/methanol and measured the $P_i$ binding by the extracted proteolipid. Our results indicate that neither the uptake of $P_i$ by treated brush-border membrane vesicles nor the binding of $P_i$ by the proteolipid extracted from these brush-border membrane vesicles were significantly different from control values.

## Materials and Methods

*Brush-border membrane preparation*

Brush-border membrane vesicles were prepared from kidneys obtained from New Zealand white rabbits (3 kg) which had been killed by cervical dislocation after having been stunned with $CO_2$ gas. The cortices were dissected from the medullae and brush-border membranes were prepared by the $Mg^{2+}$ precipitation method according to Booth and Kenny [8]. Brush-border membrane vesicles were stored in liquid nitrogen in 300-$\mu$l aliquots until use, without significant loss of activity (see Results). The purity of the preparation was assessed by measuring the relative enrichment in alkaline phosphatase activity (routinely: 7–9-fold). Mitochondrial contamination was checked by measuring cytochrome *c* oxidase [15].

*Phosphate uptake measurement in brush-border membrane vesicles*

Uptake of $^{32}P$-labelled phosphate into brush-border membrane vesicles was measured according to the method of Kessler et al. [9], modified by Nord et al. [10]. The reaction was initiated by mixing 10 $\mu$l of brush-border membrane vesicles (40–60 $\mu$g protein in 300 mM mannitol/20 mM Hepes-Tris, pH 7.4) with 20 $\mu$l of incubation medium on a Thermolyne Maxi-Mix™ mixer. The final medium composition was 100 mM NaCl/100 mM mannitol/20 mM Hepes-Tris, pH 7.4/0.1 mM $KH_2PO_4$ and $^{32}P$ (approx. 25 $\mu$Ci/ml). After appropriate time intervals the reaction was terminated by adding 900 $\mu$l of ice-cold 'stop solution' (100 mM mannitol/150 mM NaCl/10 mM arsenate/10 mM Hepes-Tris, pH 7.0) and filtering the mixture over Millipore filters (type HAWP, 0.45 $\mu$m). The filters were washed twice with 3 ml of ice-cold stop solution.

*Enzymatic removal of alkaline phosphatase*

Alkaline phosphatase was released from brush-border membrane vesicles by treating (30 min at 37°C) the membrane vesicles with 2 $\mu$g/ml phosphatidylinositol-specific phospholipase C under gentle agitation. The vesicles were centrifuged for 1 h at $100\,000 \times g$ to separate the released alkaline phosphatase and phospholipase C from the brush-border membrane vesicles. The pellet was resuspended in 300 mM mannitol/20 mM Hepes, pH 7.4 and left on ice until uptake measurement or proteolipid extraction.

*Electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed by a modification of the method of Shapiro et al. [11]. Samples of PI-specific phospholipase C-treated and control brush-border membrane vesicles were made 1% in mercaptoethanol and 3% in SDS and placed in a boiling water bath for 5 min. After cooling on ice, the samples were made 10% in sucrose and applied on a 7.5% polyacrylamide (bisacrylamide/acrylamide 1 : 30) slab gel containing 0.1 M sodium phosphate, pH 6.8 and 0.1% SDS. The running buffer contained 0.1 M sodium phosphate and 0.1% SDS, pH 6.8. After electrophoresis at 5 V.cm, the gels were stained with Coomassie blue and the stain density

measured at 600 nm in a scanning densitometer RFT-II (Transidyne General Corp.).

*Proteolipid extraction*

Proteolipid was extracted into chloroform/methanol (1:2, v/v) at 50°C from both phospholipase C-treated and control brush-border membrane vesicles. The general extraction procedure described elsewhere was applied [7]. The dried extract was redissolved in a small amount of chloroform/methanol (2:1, v/v) and stored under $N_2$ at −20°C until used for the binding assay.

*Phosphate binding assay*

The binding of inorganic phosphate ($P_i$) to the proteolipid was measured as described by Kessler et al. [7]. Appropriate amounts (3–5 μg protein/assay) of proteolipid were dissolved in 0.5 ml of 1-butanol/choloroform/methanol (150 : 50 : 25, v/v) in a microfuge tube. 10 μl of aqueous $P_i$ solution were added and the mixture shaken to achieve a single phase (10 μM $P_i$ in the single phase). After 10 min, 0.6 ml of 2 M ultrapure sucrose (Schwarz/Mann) were added, the mixture was shaken and centrifuged in a microfuge. 200 μl aliquots of the upper organic phase were sampled and $^{32}P$ was counted in a liquid scintillation photometer (Delta 300, Searle Analytic Inc). after mixing with 2 ml of scintillation fluid (Betaphase, Westchem Products, San Diego, CA). All binding experiments were run in duplicate along with a blank containing all the solvents but no proteolipid.

*Other methods*

Protein was measured by the method of Lowry et al. [12] in the presence of 1% SDS. In samples containing lipoprotein, the material was first solubilized in 5% SDS in a 60°C water bath until organic solvent had vaporized and the sample was clear. Alkaline phosphatase activity was measured by the method of Kelly and Hamilton [13] using 16 mM *p*-Nitrophenylphosphate as substrate [14].

*Materials*

Fresh kidneys were obtained from young adult New Zealand rabbits (2.5–3 kg), fed a standard diet. Carrier-free $^{32}P$ was obtained from ICN, Irvine, CA. Phosphatidylinositol-specific phospholipase C was generously provided by Dr. Martin Low, Oklahoma Medical Research Foundation, 825 N.E. 13th, Oklahoma City, OK, 73104. All other biochemicals (analytical reagent grade) were obtained from Sigma.

## Results

*Effect of storage of brush-border membrane vesicles in liquid $N_2$ on $P_i$ uptake*

The uptake of $P_i$ was measured in brush-border membrane vesicles which had been frozen in small aliquots (0.2–0.5 ml) in liquid nitrogen immediately after preparation. In Table I data of one representative experiment are shown where the samples were frozen for 1 and 20 days. Results were compared with unfrozen brush-border membrane vesicles in which the transport was mea-

TABLE I

EFFECT OF STORAGE OF BRUSH-BORDER MEMBRANE VESICLES IN LIQUID $N_2$ ON $P_i$ UPTAKE

Brush-border membrane vesicles were prepared as described in Methods. Uptake was measured at pH 7.4 at 20–22°C in presence of 200 μM $P_i$ and an inwardly directed 100 mM NaCl gradient. In controls, the transport was measured in freshly prepared brush-border membrane vesicles. 300 μl aliquots were frozen in liquid $N_2$. After 1 or 20 days, 1 h before the uptake experiment, the samples were thawed at 37°C and placed on ice until use. Data are from one representative experiment and are means ± S.E. of triplicate determinations. [a] pmol/mg protein; [b] % of control values. * indicates a significant difference from control ($p < 0.05$) (paired *t*-test).

| | Uptake | | | |
|---|---|---|---|---|
| | 10 s | 60 s | 120 s | 60 min |
| Control | 521 ± 12 [a] | 1555 ± 58 | 1852 ± 25 | 615 ± 32 |
| | 100 ± 2.3 [b] | 100 ± 3.7 | 100 ± 1.3 | 100 ± 5.2 |
| Day 1 | 107.5 ± 2.2 [b] | 83.4 ± 2.7 * | 95.5 ± 7.2 | 99.1 ± 3.1 |
| Day 20 | 106.0 ± 8.0 [b] | 96.7 ± 6.7 | 92.4 ± 6.4 | 110 ± 2.4 |

sured the day of preparation (Control). No significant differences were observed in the three experiments. In another membrane vesicle preparation, the $P_i$ uptake was still above 85% of control values after 2 months of storage in liquid $N_2$ (data not shown).

*Phospholipase C treatment of brush-border membrane vesicles*

Brush-border membrane vesicles were incubated for 30 min at 37°C in the absence (control) or the presence of 2 μg/ml of PI-specific phospholipase C to release alkaline phosphatase. Enzyme activity was measured in the pelleted brush-border membrane vesicles and supernatants after 1 h centrifugation at 100000 × *g* and results are presented in Table II. Pellets of PI-specific phospholipase C-treated membranes lost 83.7% of activity compared to control-treated brush-border membrane vesicles. At the same time, while unsignificant amounts of alkaline phosphatase activity were found in the control supernatants (< 1%), the PI-specific phospholipase C-treated supernatants contained 84.2% of the total activity. There was no significant loss of total alkaline phosphatase activity in the PI-specific phospholipase C-treated preparation. These results indicate that the enzymatic activity was effectively released from the brush-border membrane vesicles. Moreover, PI-specific phospholipase C appeared to exert a rather specific effect on alkaline phosphatase without altering substantially the protein composition of the brush-border membrane vesicles (Fig. 1).

TABLE II

ENZYMATIC RELEASE OF ALKALINE PHOSPHATASE ACTIVITY BY TREATMENT OF BRUSH-BORDER MEMBRANE VESICLES WITH PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C (PL-C)

Brush-border membrane vesicles were incubated for 30 min at 37°C without (controls) or with PI-specific phospholipase C (2 μg/ml). The suspension was centrifuged 1 h at 100000 × *g* and alkaline phosphatase activity measured in the pellets and in the supernatants. Data are means ± S.E. of six experiments. Total activity = activity in pellet + activity in supernatant.

| | Alkaline phosphatase activity | | |
|---|---|---|---|
| | Pellet | | Supernatant |
| | μmol/min per mg protein | % of control | % of total activity |
| Controls | 1.154 ± 0.149 | 100 ± 12.9 | 0.55 ± 0.15 |
| PLC-treated | 0.193 ± 0.019 | 16.7 ± 1.61 | 84.2 ± 1.6 |

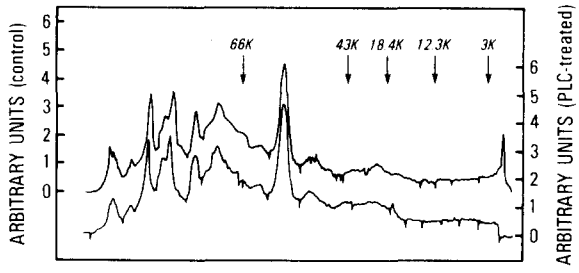


Fig. 1. Densitometry tracings of SDS-polyacrylamide gel electrophoresis of PI-specific phospholipase C-treated and control brush-border membrane vesicles. The protein composition of PI-specific phospholipase C-treated (lower tracing) and control brush-border membrane vesicles (upper tracing) was analyzed by SDS-polyacrylamide gel electrophoresis. The Coomassie blue stained slab gels were scanned at 600 nm, and the recorded tracings are shown. The ordinates of the two tracings are offset to allow easy comparison. Arrows indicate the migration region of molecular weight markers. 66 K, albumin; 43 K, ovalbumin; 18.4 K, β-lactoglobulin; 12.3 K, cytochrome *c*; 3 K, insulin.

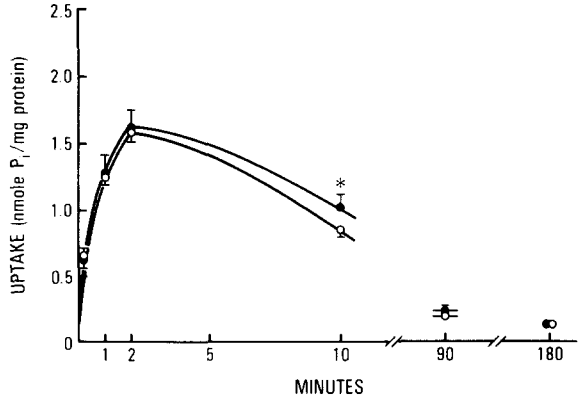


Fig. 2. Uptake of $P_i$ into control (●) and PI-specific phospholipase C-treated (○) brush-border membrane vesicles from rabbit kidney cortex. Brush-border membrane vesicles were incubated for 30 min at 37°C without (control) or with 2 μg/ml PI-specific phospholipase C, and centrifuged 1 h at 100000 × *g*. Pellets were resuspended in 300 mM mannitol/20 mM Hepes-Tris, pH 7.4 and uptake was started by mixing 10 μl of brush-border membrane vesicles with 20 μl of uptake buffer. Final incubation medium contained 100 μM $P_i$/100 mM mannitol/20 mM Hepes-Tris, pH 7.4 and 100 mM NaCl gradient (out > in). After appropriate time intervals, the reaction was terminated (see Methods). Data are means ± S.E. (vertical bars) of six experiments run in triplicate.

*Uptake of $P_i$ by brush-border membrane vesicles treated with PI-specific phospholipase C*

Uptake of $P_i$ (100 $\mu$M) by brush-border membrane vesicles treated with or without PI-specific phospholipase C was measured at pH 7.4 and at 20–22°C in the presence of a 100 mM NaCl gradient (out > in). Data from 6 experiments are summarized in Fig. 2. Neither initial rates of uptake measured after 10 s, nor equilibrium (180 min) values were significantly affected by the removal of alkaline phosphatase from the brush-border membrane vesicles. The uptake after 10 min was, however, significantly lower in PI-specific phospholipase C-treated membranes than in controls.

*Binding of $P_i$ to proteolipid extracted from PI-specific phospholipase C-treated brush-border membrane vesicles*

Binding of 10 $\mu$M $P_i$ to proteolipids extracted from brush-border membrane vesicles treated without (controls) or with 2 $\mu$g/ml PI-specific phospholipase C was measured. In controls, $P_i$ binding was $31.0 \pm 9.4$ nmol/mg protein (mean $\pm$ S.E. of eight separate binding experiments run in duplicate on three different proteolipid preparations: $n = 8$ (3)). This value was not significantly different from that obtained on proteolipids extracted from brush-border membrane vesicles treated with PI-specific phospholipase C, in which case the $P_i$ binding was $29.8 \pm 8.3$ nmol $P_i$/mg protein ($n = 8$ (3)). Furthermore, addition to the assay system of 20 $\mu$M $Mn^{2+}$ 10 min prior to the phosphate stimulated the $P_i$ binding to the same extent in both types of preparations: in controls, $P_i$ binding was $89.2 \pm 14.2$ vs. $108.1 \pm 21.1$ nmol $P_i$/mg protein ($n = 8$ (3)) in proteolipids extracted from PI-specific phospholipase C-treated brush-border membrane vesicles.

## Discussion

Phosphate is reabsorbed from the renal proximal tubular fluid via a sodium-dependent, carrier-mediated transport mechanism. In contrast to the mitochondrial or bacterial phosphate-transport systems, where several investigators have already proposed possible molecular candidates for the $P_i$ transporter [16–19], only little is known about the renal phosphate carrier. Kessler et al. [7] presented data about a proteolipid extracted from renal brush-border membrane vesicles which binds phosphate with high affinity and specificity. Arsenate inhibited the $P_i$ binding; it also inhibits $P_i$ uptake by brush-border membrane vesicles [20]. Kessler et al. [7] proposed this proteolipid as a possible candidate for a component of the renal $P_i$ transporter. On the other hand, Peticlerc and Plante [1] proposed that brush-border membrane-bound alkaline phosphatase is involved in $P_i$ transport, but this hypothesis has lost considerable credit during the last few years (see Introduction). Recently, however, Béliveau and Brunette [6] presented evidence that alkaline phosphatase could be a phosphate-binding protein. Since alkaline phosphatase appears capable of binding $P_i$ and since Kessler et al. [7], proposed a $P_i$-binding proteolipid as a possible candidate for the $P_i$ carrier, it was of particular interest to elucidate how removal of alkaline phosphatase from the brush-border membrane vesicles prior to extraction of the proteolipid might affect the $P_i$-binding to the extract. In fact, the rather ungentle extraction procedure proposed by Kessler et al. [7] and used also in the present experiments, might have disrupted alkaline phosphatase into smaller peptides, so that the $P_i$-binding moiety of the alkaline phosphatase and proteolipid could have been the same initial molecule.

For these experiments we needed large amounts of brush-border membrane vesicles. Since daily preparation of vesicles is time-consuming, we checked the stability of brush-border membrane vesicles by preserving small aliquots in liquid nitrogen. We found that phosphate transport is not significantly altered by storage at very low temperatures (Table I). This is similar to the results with several other transport systems present in the kidney brush-border membrane vesicles as shown by Stevens et al. [21].

Alkaline phosphatase appears to be attached to the brush-border membrane vesicles by strong interactions with phosphatidylinositol [22]. Disruption of these interactions can be successfully achieved by treatment of brush-border membrane vesicles with phosphatidylinositol-specific phospholipase C [5,22,23]. Effective release of the enzyme from the brush-border membrane vesicles is evident from the data presented in Table II. Ap-

proximately the same amount of enzymatic activity which disappeared from the brush-border membrane vesicles (85%) appeared in the supernatant of the PI-specific phospholipase C-treated membranes. Yusufi et al. [5] presented electrophoretic evidence of the alkaline phosphatase release from the brush-border membrane vesicles. Furthermore, they showed that the activity of other typical brush-border membrane enzyme was unaltered after PI-specific phospholipase C treatment. In the present study, SDS-polyacrylamide gel electrophoresis (Fig. 1) of control and PI-specific phospholipase C-treated brush-border membrane vesicles did not show any major difference in their protein composition, thus confirming the rather specific effect of PI-specific phospholipase C on alkaline phosphatase, without disturbing significantly the other membrane proteins. Thus, PI-specific phospholipase C is a powerful tool for enzymatically removing alkaline phosphatase from the native membrane.

Uptake of $P_i$ into PI-specific phospholipase C-treated brush-border membrane vesicles was similar to that observed in control membranes (Fig. 2). The 10-min uptake value, which was 16.5% lower in PI-specific phospholipase C treated membranes ($p < 0.05$), could result from a slightly altered nonspecific permeability of the treated brush-border membrane vesicles, which allows equilibrium to be achieved more quickly after the initial transient overshoot. Our results confirm the data presented by Yusufi et al. [5] or those obtained by inhibiting the alkaline phosphatase activity with non-specific inhibitors, namely L-Bromotetramisole [4], levamisole [1,2] or by EDTA [3]. One could argue that the 15% of alkaline phosphatase activity still present in the PI-specific phospholipase C-treated membranes might be sufficient to transport $P_i$ with 100% efficiency. However, Yusufi et al. [5] showed an over 90% removal of alkaline phosphatase activity by PI-specific phospholipase C without affecting the $P_i$ uptake. Thus less than 10% of total alkaline phosphatase activity would have to be responsible for the entire $P_i$ transport. This appears unlikely, but theoretically possible.

PI-specific phospholipase C-treated and control brush-border membrane vesicles were extracted with chloroform/methanol, and the resulting proteolipids were studied for their $P_i$-binding activity. Our data indicate that the specific binding of $P_i$ was similar in both preparations. Kessler and Vaughn (unpublished observations) showed evidence for a requirement of divalent metals for both $Na^+$-gradient driven phosphate transport in brush-border membrane vesicles and phosphate binding to the proteolipid. Manganese was the most effective metal ion. Therefore, we added $Mn^{2+}$ to our binding assay to determine whether its presence also stimulated the $P_i$ binding to the proteolipid extracted from PI-specific phospholipase C-treated brush-border membrane vesicles. The stimulation we observed was similar in both proteolipids and binding represented about 300% of the values obtained in the absence of $Mn^{2+}$. These data indicate that the presence of alkaline phosphatase activity in brush-border membrane vesicles is not necessary for the binding of $P_i$ by the extracted proteolipid. Therefore, identity of the proteolipid with the $P_i$-binding moiety of alkaline phosphatase appears to be unlikely.

In summary, PI-specific phospholipase C released approx. 85% of alkaline phosphatase activity from the brush-border membrane vesicles. This treatment did not affect either the transport of phosphate by brush-border membrane vesicles or the binding of phosphate by the proteolipid extracted from the treated brush-border membrane vesicles.

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