ORIGINAL

Immunohistochemical analyses of parathyroid hormone-dependent downregulation of renal type II Na-Pi co-transporters by cryobiopsy

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Abstract : . The "in vivo cryotechnique" (IVCT) is a new method of morphological analysis which has the advantage of freezing tissues in living animals without stopping their blood circulation. The purpose of this study was to investigate the effect of parathyroid hormone (PTH) on renal type II Na-Pi transporters (NaPi-IIa and NaPi-IIc) and "cryobiopsy" (CB) using special cryoforceps as a simple method of the IVCT. The kidney tissues were biopsied at various time points after PTH administration by CB using liquid nitrogen as the cryogen. By hematoxylin-eosin (HE) staining the kidney tissues, well-frozen areas without visible ice crystals were obtained in the tissue surface areas, and the brush border membrane (BBM) of proximal tubules was well preserved at a light microscopic level. Immunohistochemical evaluation showed that PTH downregulated NaPi-IIa and NaPi-IIc at the BBM, being controlled by a different mechanism. In this method, the PTH-induced internalization of NaPi-IIc from microvilli to subapical compartments was not observed in the tissue preparations. NaPi-IIc protein appears to be degraded in microvilli of the proximal tubular cells after the injection of PTH. We suggest that CB using liquid nitrogen is useful to investigate renal type II Na-Pi transporters at the light microscopic level. J. Med. Invest. 57 : 138-145, February, 2010

Keywords **:** *in vivo cryotechnique, cryobiopsy, kidney, type II Na-Pi transporter, PTH*

INTRODUCTION

Inorganic phosphate (Pi) is an essential nutrient in terms of both cellular metabolism and skeletal mineralization. The kidney is a major regulator of Pi homeostasis, and its Pi reabsorptive capacity increases or decreases to accommodate the Pi requirements. The solute carriers of two distinct renal Na-dependent Pi transporters (SLC34A1 [NaPi-IIa/NPT2a] and SLC34A3 [NaPi-IIc/NPT2c] are specifically expressed in the brush border membrane (BBM) of the renal proximal tubules (1-4). Important differences between them could influence their respective physiologic functions. For example,

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NaPi-IIa is electrogenic, coupling Pi transport with the transport of $3Na^+$ ions. In contrast, NaPi-IIc is electroneutral, only transporting 2 Na^+ for every Pi (5). NaPi-IIa plays a major role in renal Pi reabsorption, whereas NaPi-IIc is important for Pi reabsorption in weanling animals (1-4). In adult animals, however, NaPi-IIc mediates a very small percentage of Pi reabsorption (1, 5). Recently, several groups demonstrated that a lack of functional NaPi-IIc protein in patients with hereditary hypophosphatemic rickets with hypercalciuria (HHRH) results in severe renal Pi wasting, leading to hypophosphatemia, which indicates that the NaPi-IIc cotransporter plays an important role in renal Pi reabsorption and bone mineralization, and may be a key determinant of the plasma Pi concentration in humans (6, 7).

Other important differences between NaPi-IIa and NaPi-IIc could influence their respective physiologic regulation. Parathyroid hormone (PTH) is a major hormonal regulator of Pi reabsorption in the kidney, and the PTH-induced inhibition of Pi reabsorption is mediated by the endocytosis of NaPi-IIa from the BBM and subsequent lysosomal degradation (8-17). Upon the stimulation of PTH receptors, a decrease in apical NaPi-IIa occurs within minutes, without changes in the mRNA levels. In a previous study, cryosections of rat kidneys were immunohistochemically stained with a specific antibody directed against NaPi-IIa and NaPi-IIc transporters (1, 4). Immunohistochemical analysis demonstrated that NaPi-IIc is mainly localized in the subapical (or the base of the proximal tubule microvilli) regions after PTH injection (4). Several hours are required for a PTH-induced redistribution of NaPi-IIc. In addition, in rats with acute feeding of a high-Pi diet, NaPi-IIc is translocated from the entire brush border to the intracellular compartments (18). Immunoblotting and immunohistochemical analyses suggested that internalized NaPi-IIa is directed to the lysosomes for degradation, as described previously (1, 2, 18). In contrast, NaPi-IIc was internalized to the intracellular pool but not degraded in the lysosomes (18).

However, the analysis of NaPi-IIc was thought to have some technical limits on employing conventional methods using kidney tissues after tissue excision, because of the morphological damage to microvilli and other structures caused by ischemia and anoxia. Recently, Ohno et al. reported a new and advanced microscopic preparation technology, the "in vivo cryotechnique" (IVCT) (19-23). The IVCT has some advantages, through which we are able to directly observe the tissues or organs of living animals without stopping their blood circulation. Therefore, the tissues and organs can be observed in more natural states as compared with conventional chemical fixation or cryofixation after their excision. As some of the results of comparative studies between the IVCT and conventional methods, many morphological artifacts were shown to appear employing conventional methods using excised tissues. We considered the necessity of re-examine studies that employed conventional methods using the IVCT.

In this study, we investigated the effect of PTH injection on NaPi-IIa and NaPi-IIc, which was performed by CB using special hand-made cryoforceps as a simple IVCT method (24).

MATERIALS AND METHODS

Animals

Thyroparathyroidectomy (TPTX) was performed using male Wistar rats (6 weeks old), which were purchased from CLEA Japan Inc. (Tokyo, Japan). TPTX rats were maintained on standard chow for 1 week, and then intravenously administered bovine PTH (amino acid 1-34) at a dose of 7.5 μg/100 g body weight under anesthesia by a injection of pentobarbital sodium (100 mg/kg body wt) (4, 25). The protocol for this study was approved by the Animal Experimentation Committee of Kureha Special Laboratory.

Cryobiopsy

CB was performed using rats without PTH administration, and rats 15 min or 2, 4, 8, or 12 hr after PTH administration (n=4).

Special cryoforceps, which were previously reported by Fujii et al.(24), were used for CB (Fig. 1 a, b). The tips of the cryoforceps were round cups, which had sharpened edges. They had sponges just above the tips to absorb liquid nitrogen (-196°C), which flows down and directly cools the tips. The tips of the cryoforceps were pre-cooled in liquid nitrogen. The cryoforceps were used immediately for pinching a part of the kidney tissue in the rat anesthetized with pentobarbital sodium, which was quickly plunged into liquid nitrogen again (Fig. 1c, d).

The frozen kidney tissues were freeze-substituted

in acetone containing 2% paraformaldehyde at -80°C for 24 hrs. Then, the freeze-substitution solution containing the specimens was kept at -20°C for 2 hrs, at 4°C for 2 hrs, and finally at room temperature. They were washed in pure acetone, transferred into xylene, and routinely embedded in paraffin wax.

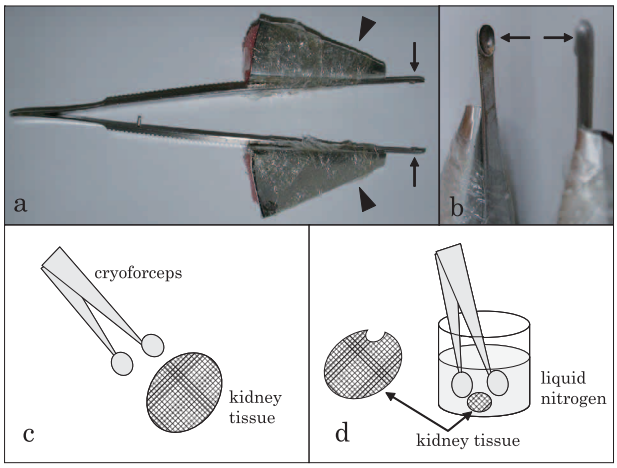

Fig. 1 (a, b) The hand-made cryoforceps. The tips of the cryoforceps are round cups (arrows), which have sharpened edges. They also have sponges just above the tips to absorb liquid nitrogen (arrowheads), which flows down and cools the tips.
(c, d) Schematic representation of cryobiopsy technique. The cryoforceps were used immediately for pinching a part of the kidney tissue, which was quickly plunged into liquid nitrogen again

Conventional chemical immersion-fixation

As the control of CB specimens, the conventional method was performed using the rats without PTH administration. The excised kidney tissues were fixed with 4% paraformaldehyde employing the immersion-fixation method overnight. After washing in PB, they were commonly dehydrated in a graded series of ethanol, immersed in xylene, and finally embedded in the paraffin wax.

Hematoxylin-eosin staining and immnohistochemistry

Three-micron-thick sections were cut, de-paraffinized, and routinely stained with hematoxylin-eosin (HE). For immunostaining, the paraffin sections were pretreated in citrate buffer (pH 6.0) with microwaves for 10 min. After the treatment, they were incubated with rabbit anti-NaPi-IIa or NaPi-IIc antibody (1 : 100) at room temperature for 1 hr. Thereafter, the sections were treated with EnVision Reagent (Dako Japan Inc.) at room temperature for 30 min. Normal rabbit serum was employed as an immunocontrol instead of each primary antibody.

Preparation of membrane fraction and immunoblotting

Methods for preparation of brush border membrane vesicles (BBMVs) with the use of calcium precipitation have been previously described (n=4-6) (4, 18). Protein samples were heated at 95°C for 5 min in sample buffer in the presence of 5% 2-mercaptoethanol and subjected to 8% SDS-PAGE. The separated proteins were transferred electrophoretically to polyvinylidene difluoride transfer membranes (hybond-P ; Amersham Pharmacia Biotech). The membranes were treated with diluted rabbit affinity-purified anti-NaPi-IIc (1 : 500) or anti-NaPi-IIa (1 : 4000) transporter antibodies (4), followed by treatment with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was utilized as the secondary antibody (Jackson Immuno Research Laboratories, Inc, West Grove, PA), and signals were detected using Immobilon Western (Millipore).

RESULTS

Effect of CB on kidney tissue

By HE staining of the kidney tissues prepared using CB, well-frozen areas without visible ice crystals were obtained in the tissue surface areas at a light microscopic level, which directly contacted the metal cups (Fig. 2a). However, some superficial compression artifacts caused by the mechanical forces were sometimes observed in the well-frozen

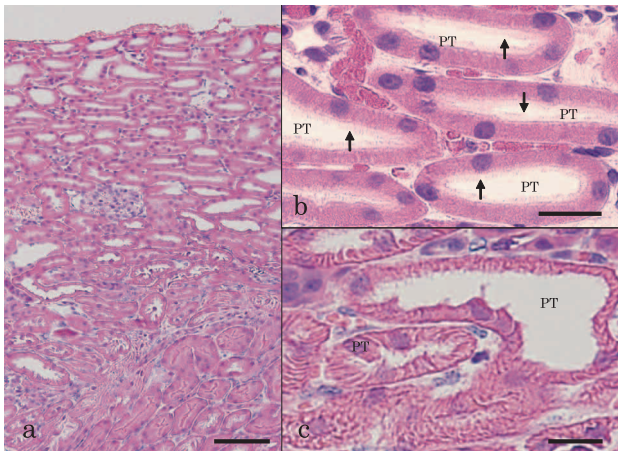

Fig. 2 HE staining of the TPTX-rat kidney tissues prepared using CB.
PT : proximal tubules.
(a) Lower magnification. Scale bar : 100 μm.
(b) Higher magnification of the surface areas. The arrows indicate the BBM of proximal tubules. Scale bar : 20 μm.
(c) Higher magnification of the deeper areas. Scale bar : 20 μm.

areas of the kidney tissue. In these well-frozen areas, most renal tubules were widely open and their outer surface was smooth. The BBM of proximal tubules was well-preserved (Fig. 2b). The tubular cells had uniform round nuclei, and their cytoplasm was evenly stained with eosin dye. The capillaries of peritubular spaces were widely open and congested with erythrocytes. In contrast, the deeper areas of frozen kidney tissue had many artifacts caused by damage due to inappropriate freezing. There were occasionally visible ice crystals, especially in the nuclei of the renal tubules. The renal tubular cells had shrank and showed distorted nuclei. Widened interdigitating spaces and disappearance of the BBM were noted in the proximal tubular cells due to ice-crystal damage (Fig. 2c).

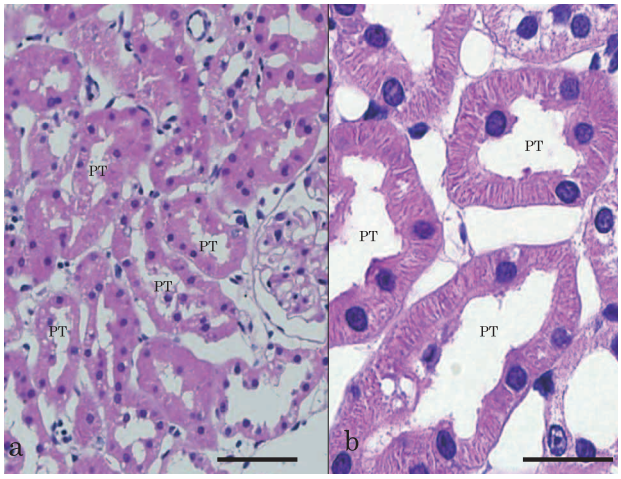

Fig. 3 HE staining of TPTX-rat kidney tissues prepared with the conventional chemical immersion-fixation.
PT : proximal tubules.
(a) Lower magnification. Scale bar : 50 μm.
(b) Higher magnification. Scale bar : 20 μm.

Comparison of morphology between CB and conventional chemical immersion-fixation

By employing the conventional method, the shrinkage of renal tissues was usually observed, which was indicated by the irregular outer surface of renal tubules, and their condensed smaller nuclei (Fig. 3a). As compared with the cytoplasm of renal tubules by CB, the cytoplasm of renal tubules prepared using the conventional method was more weakly stained with eosin, and occasionally contained some vacuoles (Fig. 3b). The BBM of proximal tubules was extensively destroyed.

Effect of PTH on the localization of renal NaPi-IIa and NaPi-IIc

In TPTX rat kidneys without PTH administration, immunoreactive signals of NaPi-IIa and NaPi-IIc transporters were localized at the BBM of proximal convoluted tubules (Fig. 4a, 5a). In TPTX rat kidneys at 15 min after PTH injection, immunoreactive signals of NaPi-IIa protein markedly decreased at the BBM of proximal convoluted tubules, which changed to weakly detectable in the subapical cytoplasm beneath the BBM (Fig. 4b). Similar findings were observed after 2 and 4 hr (Fig. 4c, d). The intensity of immunoreactive signals of NaPi-IIa protein at 8 hr after PTH injection became weaker than that in the untreated TPTX rats, and recovered to the baseline level after 12 hr (Fig. 4e, f).

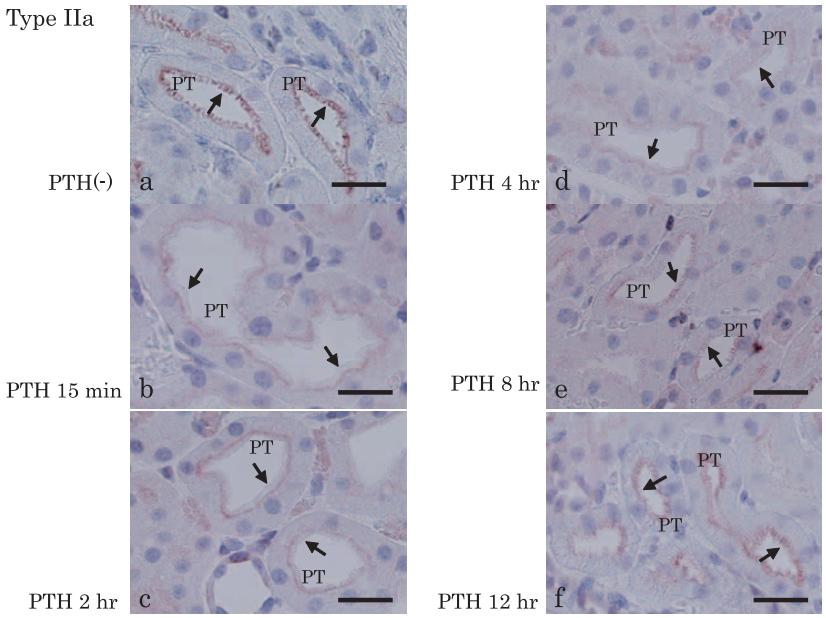

Fig. 4 Immunochemical analyses of the type IIa Na-Pi cotransporter of TPTX-rat kidney tissues treated with PTH. The arrows indicate the BBM of proximal tubules. PT : proximal tubules. Scale bar : 20 μm.
(a) Untreated TPTX-rat kidney.
(b-f) TPTX-rat kidney tissues 15 min or 2, 4, 8, or 12 hr after PTH administration.

In contrast, the immunoreactive signals of NaPi-IIc remained at the BBM of proximal convoluted tubules at 15 min and 2 and 4 hr after PTH administration (Fig. 5b-d). The levels of type IIc at the BBM were slightly decreased after 8 hr, and returned to the baseline level at 12 hr after PTH treatment (Fig. 5e, f).

Effect of PTH on NaPi-IIa and NaPi-IIc protein levels in the TPTX rat

The effect of the injection of PTH on the expression of NaPi-IIa and NaPi-IIc was examined using Western blot analysis in TPTX rats. Fifteen minutes after the injection of PTH, NaPi-IIa protein levels were significantly decreased in the apical membrane. NaPi-IIa protein levels remained low until 8 hr after the injection of PTH, and gradually increased from 12 hr after injection. NaPi-IIc protein levels remained unchanged at 2 hr after injection. Eight hours after PTH injection, NaPi-IIc protein levels decreased to 85% of the initial values in the BBM fraction. Thereafter, the amounts of NaPi-IIc protein gradually increased in the BBM fractions (Fig. 6).

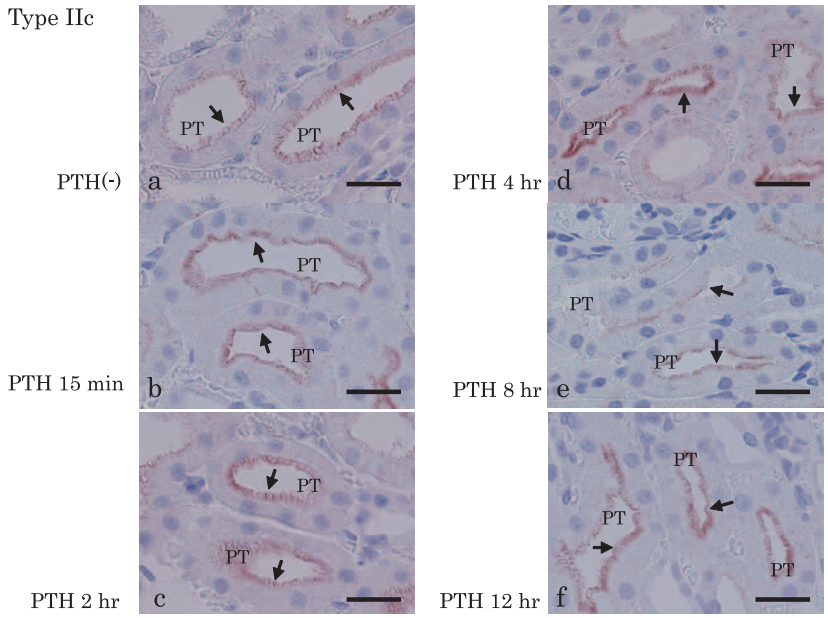

Fig. 5 Immunochemical analyses of the type IIc Na-Pi cotransporter of TPTX-rat kidney tissues treated with PTH. The arrows indicate the BBM of proximal tubules. PT : proximal tubules. Scale bar : 20 μm.
(a) Untreated TPTX-rat kidney.
(b-f) TPTX-rat kidney tissues 15 min or 2, 4, 8, or 12 hr after PTH administration.

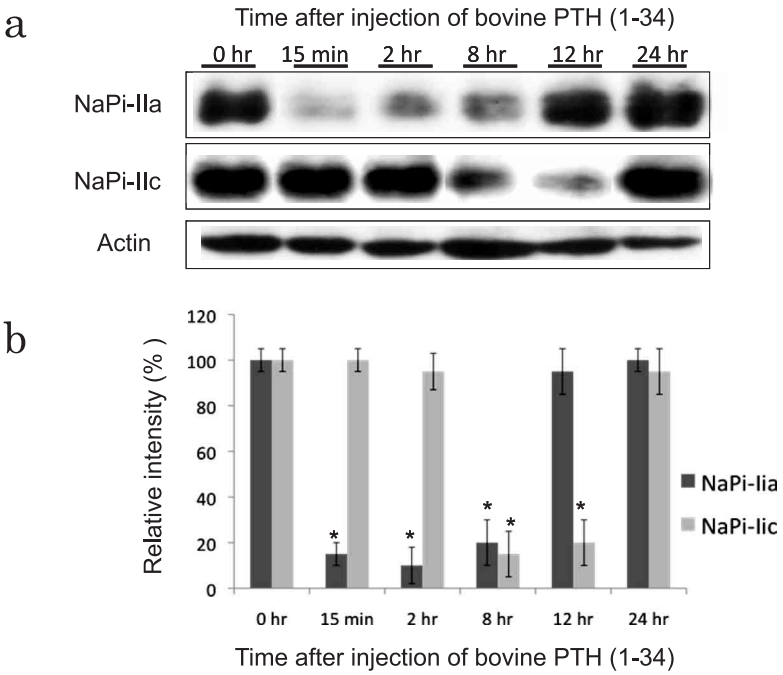

Fig. 6 Effect of PTH on NaPi-IIa and NaPi-IIc protein level in TPTX-rats.
(a) Immunoblots were performed with the use of renal BBMVs from TPTX rats treated with PTH. Actin was used as internal control. (b) Visualized band intensity for the TPTX rats was designated as 100%. Values are means± S.D. *$p < 0.05$ compared with TPTX-rat (0 hr) : n=4-6 rats

DISCUSSION

In the present study, we examined the two following points. The first involved our modified CB method using liquid nitrogen alone as the cryogen. The second involved the localization of NaPi-IIa and NaPi-IIc protein using TPTX rats. Regarding the first point : for observation of the kidney tissues, we performed CB using special cryoforceps as a simple IVCT method, which was previously reported by Fujii et al. (24). For the quick-freezing method, selection of the cryogen is important, and they used an isopentane-propane mixture in their original report. We could easily prepare the isopentane-propane mixture without the need for special apparatus, and the running cost was relatively low (26). In the present study, we used liquid nitrogen alone as the cryogen, because we are not permitted to use propane gas in our experimental institution. The cooling ability of liquid nitrogen was much lower than the isopentane-propane cryogen, therefore, the tissue damage caused by ice crystals was carefully examined using HE-stained kidney tissues of CB specimens. CB specimens were also compared with paraformaldehyde-fixed specimens employing the conventional method using tissue excision. In our results, satisfactory areas suitable for critical observation were obtained in some superficial areas of the frozen tissues by this CB method, and the microvilli of proximal tubules were well maintained. In these areas, there was no shrinkage of the renal tubules due to chemical fixation and dehydration. We observed no vacuolar changes and also lightly eosin-stained cytoplasm of the renal tubular epithelium, which was probably caysed by anoxic or ischemic changes due to the excision of kidney tissues. Compared with isopentane-propane, acceptable tissue areas without visible ice crystals were thought to be decreased using liquid nitrogen, but morphological images in some superficial areas of frozen kidney tissues were maintained very well even though liquid nitrogen was used as the cryogen in the present study. The results indicated that specimens prepared by CB using liquid nitrogen alone were of sufficient quality to observe the living rat kidney tissue at the light microscopic level.

Concerning the second point : we focused on NaPi-IIa and NaPi-IIc transporters, which mediate Pi reabsorption in the renal proximal tubules. NaPi-IIa is a major one, responsible for about 70% of the Pi reabsorption in adult rodent kidneys (1, 2, 27), whereas the remaining 30% is handled by NaPi-IIc (1, 4, 5). The PTH-induced inhibition of Pi reabsorption is known to be mediated by the endocytosis of NaPi-IIa (8-17). The light and electron microscopic examinations already demonstrated that NaPi-IIa moved from the BBM to apical cytoplasm, and, subsequently, was degraded by lysosomal enzymes. In the present study, immunohistochemical evaluation at various time points following PTH administration after TPTX showed that PTH downregulated NaPi-IIa at the BBM. This finding also supported the previous reports.

Recently, it has been reported that genetic mutations in NaPi-IIc (NPT2c) cause human hereditary hypophosphatemic rickets with hypercalciuria (6, 7). and NaPi-IIc may be critical for determining the serum Pi levels and urinary Pi excretion in humans. The present study indicated the downregulation of NaPi-IIc at the BBM on PTH treatment. However, the immunolocalization of NaPi-IIc was not observed in the subapical cytoplasm beneath the BBM, as observed for NaPi-IIa. Although the regulation mechanism of NaPi-IIc is still unclear, these findings suggest the possibility that the downregulation of NaPi-IIc is not lysosomal degradation, but involves another mechanism at the BBM (4). Segawa et al. reported a PTH-induced downregulation of NaPi-IIc in an immunofluorescence study employing the conventional method, and concluded that NaPi-IIc was localized in a subapical compartment of the microvilli at 12 hr after PTH treatment (4). In the present study with the IVCT, the accumulation of NaPi-IIc in the subapical region was not observed. Regarding this, the conventional method may damage microvilli and subapical compartments in the proximal tubules. Further study is needed to analyze the immunolocalization of NaPi-IIc using electron microscopy.

The IVCT is thought to be important as a new method to capture the dynamic morphology of living animal cells and tissues, and is actually useful for morphological analyses including the immunohistochemical changes of renal transporters. The present study indicates that CB using liquid nitrogen alone, which is a simple IVCT method, is also useful to investigate NaPi-IIa and NaPi-IIc at the light microscopic level. CB performance is easier and can be applied for the repeated sampling of tissues or organs in living animals. We now consider that the CB method will be useful to investigate other renal transporters.

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