Inhibitory Effect of Zinc on PEPT1-Mediated Transport of Glycylsarcosine and β-Lactam Antibiotics in Human Intestinal Cell Line Caco-2

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Purpose. The aim of this study was to examine the effects of zinc on the intestinal peptide transporters (PEPT1 and basolateral peptide transporter) and to elucidate the mechanism of the interactions.

Methods. Caco-2 cells were pretreated with zinc, and the uptake studies were carried out.

Results. Zinc treatment resulted in the inhibition of [^{14}C]glycylsarcosine (Gly-Sar) uptake via PEPT1 in a concentration-dependent manner, whereas it showed moderate inhibitory effect on the basolateral peptide transporter. Zinc also inhibited the uptake of oral β-lactam antibiotics such as ceftibuten and cephradine by PEPT1. Kinetic analysis showed that zinc treatment increased K_m values without affecting V_{max} values of the [^{14}C]Gly-Sar uptake. The inhibition of [^{14}C]Gly-Sar uptake induced by zinc was observed in the presence of an H^+ gradient but not in the absence of an H^+ gradient.

Conclusions. These results indicate that zinc is a competitive inhibitor of PEPT1. Zinc inhibited the PEPT1 function, possibly by interacting with histidine residues of PEPT1 that are part of an H^+-binding site. These findings would provide important information for clinical, physiologic, and biochemical aspects of peptide transporters.

KEY WORDS: zinc modulation; peptide transporter; Caco-2 cells; small intestine; drug interaction.

INTRODUCTION

Zinc is an essential metal ion necessary for growth, metabolism, and maintenance of cell function. Zinc deficiency in humans results in growth retardation, male hypogonadism, skin changes, poor appetite, mental lethargy, and lack of taste and smell (1). Because these symptoms are reversed when zinc is administered to the body, zinc has been clinically used for its deficiency. Previously, Bettger and O'Dell (2) proposed that pharmacologic doses of zinc may exert some of their actions directly on cellular membranes, either by altering permeability or by modulating the activity of membrane-bound enzymes. Thereafter, numerous studies reported that zinc showed an inhibitory effect on the intestinal membrane proteins such as glucose transporter (3), threonine transporter (4), and Na^+,K^+-ATPase (5). In addition, zinc has been shown to modulate the activity of a number of membrane proteins expressed in the brain, such as Ca^{2+} channels (6), N-methyl-D-aspartate (NMDA) subtypes of glutamate receptors (7), and glutamate transporters (8).

The H^+-coupled peptide transporters (PEPT1 and PEPT2) expressed in the brush border membranes of the intestinal and renal epithelial cells mediate the absorption of di- and tripeptides and play important nutritional roles for protein homeostasis (9). Furthermore, because both peptide transporters have a broad substrate specificity, peptide-like drugs structurally related to small peptides are also transported by these transporters (10). For example, β-lactam antibiotics (11), the anticancer agent bestatin (12), the antivirus agent valacyclovir (13), and the photodynamic therapy agent δ-aminolevulinic acid (14) are included as substrates of PEPT1 and PEPT2. We also found that another peptide transporter was expressed in the basolateral membrane of intestinal epithelial cells and that both PEPT1 and the basolateral peptide transporter cooperate in the efficient transepithelial transport of small peptides and various drugs (15,16). Thus, the intestinal peptide transporters play important nutritional and pharmacokinetic roles.

There are increasing numbers of people who are taking zinc as a drug or a supplement for zinc deficiency, and its clinical effects have been confirmed (17,18). Because zinc is often administered orally and has shown inhibitory effects on various membrane proteins, we hypothesized that the functions of the PEPT1 and basolateral peptide transporter might be affected by orally taken zinc. It has been considered that the intestinal peptide transporters greatly contribute to protein homeostasis and the therapeutic efficacy of peptide-like drugs (9,10). Therefore, it would be important to predict and avoid the food or drug interaction for patients treated with zinc. Furthermore, there have been numerous studies on the interaction of organic compounds with peptide transporters to clarify the substrate recognition mechanisms of peptide transporters (19,20). However, there is little information concerning the interaction of inorganic compounds with peptide transporters. Based on the above background, in the present study, we examined the effects of zinc on peptide transporters, mainly focusing on PEPT1, using the human intestinal cell line Caco-2.

MATERIALS AND METHODS

Materials

[^{14}C]Glycylsarcosine (Gly-Sar) (1.78 GBq/mmole) was obtained from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). Ceftibuten (Shionogi, Osaka, Japan), cephradine (Sankyo, Tokyo, Japan), and cefotiam (Takeda, Osaka, Japan) were gifts from the respective suppliers. $ZnSO_4$ and diethylpyrocarbonate (DEPC) were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals used were of the highest purity available.

Cell Culture

Caco-2 cells are cultured as described previously (15). For uptake studies from the apical side, Caco-2 cells were seeded on 12-well cluster plates or 60-mm plastic dishes. For uptake studies from the basolateral side, the cells were seeded

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ABBREVIATIONS: DEPC, diethylpyrocarbonate; Gly-Sar, glycylsarcosine; NMDA, N-methyl-D-aspartate; PEPT, peptide transporter.

on microporous membrane filters inside Transwell cell culture chambers (Costar, Cambridge, MA). The cell monolayers were given fresh medium every 2 to 4 days and were used on the 14th or 15th day for uptake experiments.

Uptake Studies by Cell Monolayers

The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 2-(N-morpholino)ethanesulfonic acid (MES; adjusted for pH 5.5 and 6.0) or N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; adjusted for pH 6.5, 7.0, and 7.4). For uptake studies, Caco-2 cells were washed with incubation medium (pH 7.4) and pretreated with $ZnSO_4$ (pH 6.0 or 6.5) for 30 min. Preliminary experiments showed that the inhibitory effect of zinc was maximal after 30 min of treatment. Thereafter, the cells were washed with incubation medium (pH 7.4), and the uptake studies were carried out. The uptakes of radiolabeled substrates (15) and β-lactam antibiotics (21) were determined as described previously.

Functional Expression of Rat PEPT1 and PEPT2 in *Xenopus* Oocytes

The capped cRNA of rat PEPT1 or PEPT2 was synthesized *in vitro* and injected into *Xenopus* oocytes as described previously (12). Three days after the injection of water or cRNAs, the uptake experiments were initiated in a 24-well plate. The composition of the uptake buffer was as follows (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 MES (pH 6.0) or HEPES (pH 6.5 and 7.4). The oocytes were pretreated with $ZnSO_4$ (pH 6.5) for 30 min and then incubated with [^{14}C]Gly-Sar for 30 min. The uptake was terminated by adding 2 ml of ice-cold uptake buffer to each well, and the oocytes were washed five times with 2 ml of the buffer. After washing, each oocyte was transferred to a scintillation counting vial and solubilized in 500 μl of sodium lauryl sulfate. Five milliliters of ACSII (Amersham Biosciences AB, Uppsala, Sweden) was added to each solubilized oocyte, and the radioactivity was determined in a liquid scintillation counter.

Data Analysis

Each experimental point or column shown in the figures represents the mean ± SE of 4 to 11 measurements from one to three separate experiments. When the error bars are not shown, they are smaller than the symbols. Data were analyzed statistically by the nonpaired *t* test. The half-maximal inhibition (IC_{50}) values were determined by nonlinear regression analysis.

RESULTS

To prevent the direct interaction of zinc and Gly-Sar, we treated cells with zinc before [^{14}C]Gly-Sar uptake. At first, we examined the effect of the zinc concentration on [^{14}C]Gly-Sar uptake by PEPT1 and the basolateral peptide transporter in Caco-2 cells. As shown in Fig. 1A, [^{14}C]Gly-Sar uptake via PEPT1 was decreased by zinc in a concentration-dependent manner, and the IC_{50} value was observed at 3.4 ± 0.3 mM. In contrast, zinc treatment did not show much effect on [^{14}C]Gly-Sar uptake by the basolateral peptide transporter but had a moderate inhibitory effect at higher concentrations (Fig. 1B).

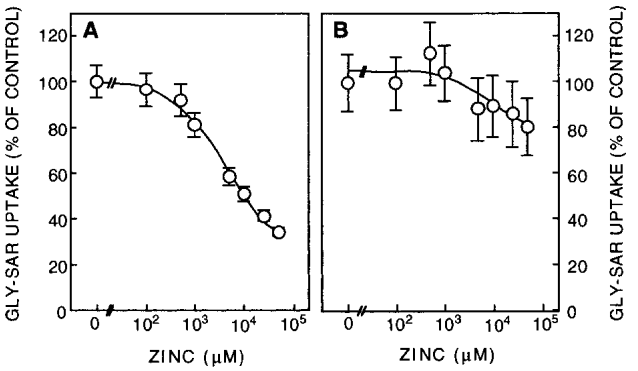

Fig. 1. Effect of zinc concentration on [^{14}C]Gly-Sar uptake by PEPT1 (A) and the basolateral peptide transporter (B) in Caco-2 cells. Caco-2 monolayers were preincubated at 37°C for 30 min with various concentrations of $ZnSO_4$ (pH 6.0). After preincubation, the cells were rinsed once with the incubation medium (pH 7.4) and then incubated at 37°C for 15 min with [^{14}C]Gly-Sar (20 μM) added to either the apical (pH 6.0) or the basolateral side (pH 7.4). Thereafter, the radioactivity of the solubilized cells was determined. Each point represents the mean of nine independent monolayers from three separate experiments.

We also examined the effect of zinc on the uptake of various β-lactam antibiotics by PEPT1 (Fig. 2). Similarly, the uptake of the oral β-lactam antibiotics (ceftibuten and cephradine) via PEPT1 was inhibited by zinc [ceftibuten, 4.53 ± 0.28 (control) vs. 3.25 ± 0.13 (5 mM zinc treatment); cephradine, 3.43 ± 0.12 (control) vs. 2.87 ± 0.09 (5 mM zinc treatment) nmole/mg protein/15 min]. On the other hand, the uptake of cefotiam, which is a parenteral β-lactam and not a substrate for PEPT1, was not inhibited by zinc [1.96 ± 0.14 (control) vs. 2.31 ± 0.16 (5 mM zinc treatment) nmole/mg protein/15 min].

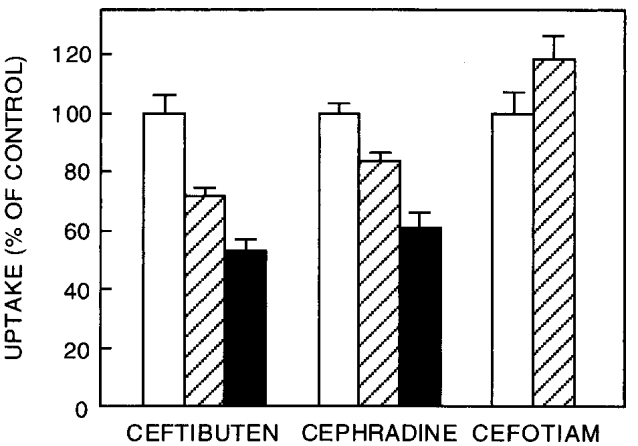

Fig. 2. Effect of zinc on the uptake of various β-lactam antibiotics by PEPT1 in Caco-2 cells. Caco-2 monolayers were preincubated at 37°C for 30 min with $ZnSO_4$ (pH 6.5) at the concentration of 0 (open column), 5 (hatched column), or 20 mM (filled column). The cells were incubated at 37°C for 15 min with each drug (1 mM, pH 6.0). Thereafter, each β-lactam antibiotic extracted from the cell monolayers was measured by high-performance liquid chromatography. Each column represents the mean of eight to nine independent monolayers from three separate experiments.

Figure 3 shows the effect of zinc concentration on [^{14}C]Gly-Sar uptake by rat PEPT1- and rat PEPT2-expressing oocytes. The [^{14}C]Gly-Sar uptake via both transporters was also inhibited by zinc treatment in a concentration-dependent manner. The IC_{50} values of zinc for PEPT1 and PEPT2 were 1.7 and 0.56 mM, respectively.

Next, to clarify whether the inhibitory effect of zinc is specific to PEPTs, the effect of zinc on [^{3}H]alanine uptake in Caco-2 cells was examined. Alanine uptake by Caco-2 cells was reported to be Na^+-dependent and carrier-mediated (22). As shown in Fig. 4, [^{3}H]alanine uptake from the apical or the basolateral side was not inhibited by zinc in the range of concentrations 1–10 mM.

We examined whether other divalent cations showed a similar effect on peptide transport via PEPT1 in Caco-2 cells. Cells were preincubated with $ZnSO_4$, $MnSO_4$, $FeSO_4$, or $CuSO_4$ (1–5 mM), and then [^{14}C]Gly-Sar uptake was measured (Fig. 5). The data showed that Mn^{2+} had little effect on [^{14}C]Gly-Sar uptake, whereas Fe^{2+} and Cu^{2+} inhibited [^{14}C]Gly-Sar uptake in a concentration-dependent manner.

Figure 6 shows the concentration dependence of [^{14}C]Gly-Sar uptake with or without treatment with zinc. The specific uptake was calculated by subtracting the nonspecific uptake, which was estimated in the presence of excess unlabeled dipeptide, from the total uptake, and kinetic parameters were calculated according to the Michaelis-Menten equation. The treatment of zinc significantly decreased the affinity of Gly-Sar to PEPT1 [K_m values 1.00 ± 0.03 (control) vs. 1.83 ± 0.11 (zinc treatment) mM, $p < 0.005$], without affecting V_{max} values [9.93 ± 0.97 (control) vs. 8.27 ± 0.52 (zinc treatment) nmole/mg protein/15 min, $p = 0.204$]. These results suggest that zinc competitively inhibited [^{14}C]Gly-Sar uptake by PEPT1.

We then examined the pH dependence of [^{14}C]Gly-Sar uptake by PEPT1 with or without zinc treatment (Fig. 7A). The uptake of [^{14}C]Gly-Sar at pH 6.0 was greatly inhibited by the zinc pretreatment, compared with the uptake of [^{14}C]Gly-Sar at other pHs. Interestingly, [^{14}C]Gly-Sar uptake at pH 7.4 was not significantly inhibited by zinc.

Zinc was reported to interact with histidine residues of various membrane proteins (7,8,23). It would be interesting to compare the inhibitory effect of zinc with that of diethylpyrocarbonate (DEPC), which is a histidine residue modifier, on [^{14}C]Gly-Sar uptake by PEPT1. Figure 7B shows the pH dependence of [^{14}C]Gly-Sar uptake by PEPT1 in Caco-2 cells treated with or without DEPC. In contrast to zinc treatment, [^{14}C]Gly-Sar uptake was inhibited by the treatment of DEPC at all pH values examined including pH 7.4.

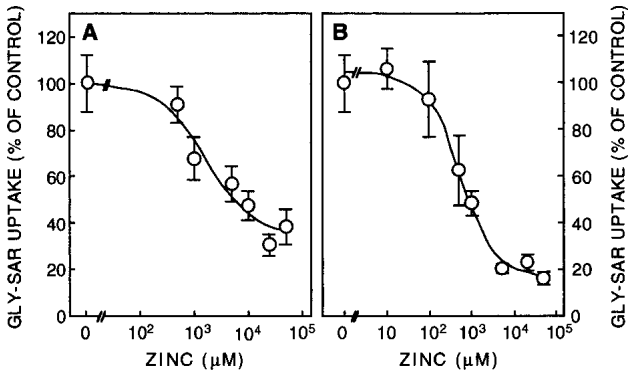

Fig. 3. Effect of zinc concentration on [^{14}C]Gly-Sar uptake by oocytes expressing rat PEPT1 (A) and rat PEPT2 (B). The oocytes were preincubated at 25°C for 30 min with various concentrations of $ZnSO_4$ (pH 6.5). After preincubation, the oocytes were rinsed three times with the uptake buffer (pH 7.4) and then incubated at 25°C for 30 min with [^{14}C]Gly-Sar (100 μM, pH 6.0). The radioactivity of the solubilized oocytes was determined. Each point represents the mean of 8 to 11 independent oocytes.

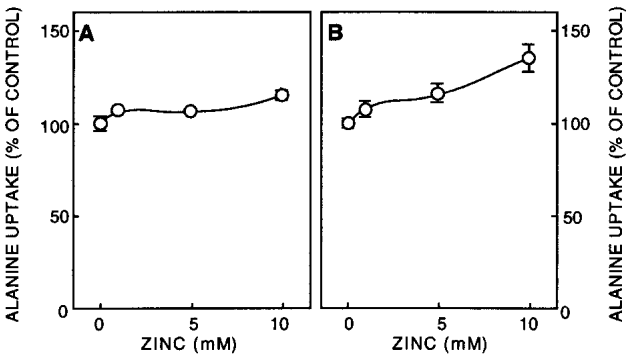

Fig. 4. Effect of zinc on [^{3}H]alanine uptake from the apical (A) and the basolateral (B) side in Caco-2 cells. Caco-2 monolayers were preincubated at 37°C for 30 min with various concentrations of $ZnSO_4$ (pH 6.5). The cells were incubated at 37°C for 15 min with [^{3}H]alanine (100 μM) added to either the apical (pH 6.0) or the basolateral side (pH 7.4). The radioactivity of solubilized cells was determined. Each point represents the mean of nine independent monolayers from three separate experiments.

DISCUSSION

In the present study, we have clearly demonstrated that zinc is a competitive inhibitor of the intestinal peptide transporter PEPT1 and shows moderate inhibitory effect on the

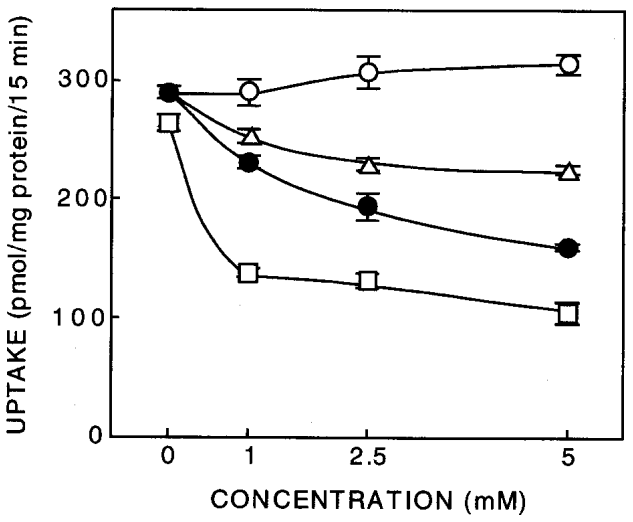

Fig. 5. Effect of various divalent cations on [^{14}C]Gly-Sar uptake by PEPT1 in Caco-2 cells. Caco-2 monolayers were preincubated at 37°C for 30 min with $ZnSO_4$ (●), $MnSO_4$ (○), $FeSO_4$ (Δ), and $CuSO_4$ (□) (pH 6.5 except for pH 5.5 for $CuSO_4$). The cells were incubated at 37°C for 15 min with [^{14}C]Gly-Sar (20 μM, pH 6.0). The radioactivity of the solubilized cells was determined. Each point represents the mean of four to seven independent monolayers from three separate experiments.

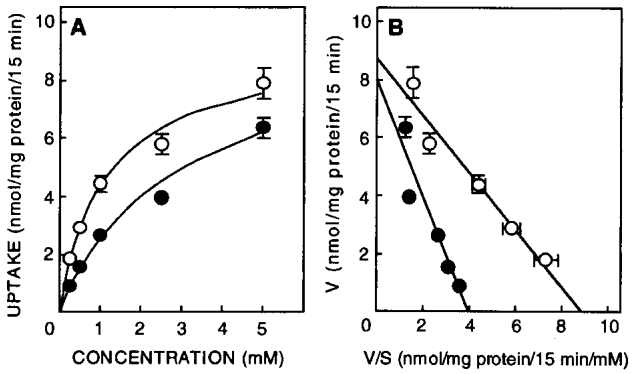

Fig. 6. Concentration dependence of [^{14}C]Gly-Sar uptake by PEPT1 in Caco-2 cells with or without the pretreatment of zinc. Caco-2 monolayers were preincubated at 37°C for 30 min in the absence (○) or the presence (●) of 5 mM $ZnSO_4$ (pH 6.5). A, The cells were incubated at 37°C for 15 min with various concentrations of [^{14}C]Gly-Sar (pH 6.0). Nonspecific uptake was evaluated by measuring [^{14}C]Gly-Sar uptake in the presence of 50 mM glycylleucine, and the results are shown after correction for the nonsaturable component. The radioactivity of the solubilized cells was determined. Each point represents the mean of nine independent monolayers from three separate experiments. B, Eadie-Hofstee plots of [^{14}C]Gly-Sar uptake after correction for the nonsaturable component.

intestinal basolateral peptide transporter. [^{14}C]Gly-Sar uptake by PEPT2 was also inhibited by zinc in a concentration-dependent manner. The present observations may show negative effects on the nutritional and clinical aspects of the small intestinal functions. For example, if zinc is coadministered with peptide-like drugs such as oral β-lactam antibiotics, there is a possibility that the oral bioavailabilities of the peptide-like drugs may be reduced. When zinc is taken as a supplement or medication, a dose of 10 to 30 mg of zinc is usually administered orally. Therefore, it is likely that intestinal zinc concentration is increased to the millimolar range, which is shown to inhibit the PEPT1 activity in the present study. It is well known that divalent and trivalent pharmaceutic cations (e.g., Zn^{2+}, Mg^{2+}, Fe^{2+}, and Al^{3+}) interact with antibacterials such as new quinolones and tetracycline antibiotics. They form chelates with antibacterials and reduce the intestinal absorption of these drugs. Thus, it is likely that zinc can affect drug absorption not only by chelating with drugs but also by modulating the function of PEPT1. Therefore, it would be important to take this information into consideration when zinc is administered as a drug or a supplement for its deficiency.

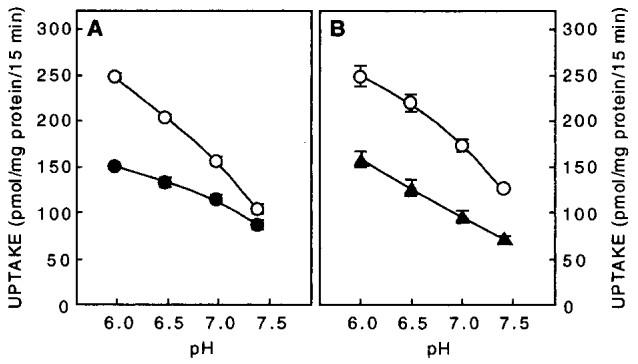

Fig. 7. pH dependence of [^{14}C]Gly-Sar uptake by PEPT1 in Caco-2 cells with or without the pretreatment with zinc (A) or DEPC (B). A, Caco-2 monolayers were preincubated at 37°C for 30 min in the absence (○) or the presence (●) of 5 mM $ZnSO_4$ (pH 6.5). B, Caco-2 monolayers were preincubated at 25°C for 10 min in the absence (○) or the presence (▲) of 0.5 mM DEPC (pH 6.5). The cells were incubated at 37°C for 15 min with [^{14}C]Gly-Sar (20 μM) at various pH values. The radioactivity of the solubilized cells was determined. Each point represents the mean of five to eight independent monolayers from two to three separate experiments.

In addition to zinc, we examined the effect of other divalent cations on peptide transport via PEPT1 (Fig. 5). Zn^{2+}, Fe^{2+}, and Cu^{2+} inhibited [^{14}C]Gly-Sar uptake in a concentration-dependent manner, though the inhibitory potency is different among these divalent cations. Moreover, Mn^{2+} had no inhibitory effect on [^{14}C]Gly-Sar uptake. These results suggest that inhibitory effect of divalent cations on Gly-Sar uptake varies with the divalent cation used.

The present study also showed that zinc competitively inhibited the [^{14}C]Gly-Sar uptake by PEPT1. Because zinc is not structurally similar to Gly-Sar, it appears to be difficult to expect zinc to interact with the substrate binding site of PEPT1. Thus, we speculated that zinc competitively inhibited the H^+ binding to PEPT1 based on the following reasons. First, it was demonstrated that histidine residues were identified as zinc-binding sites of various transporters and receptors, such as NMDA receptors (7), glutamate transporters (8), or dopamine transporters (23). X-ray crystallography of zinc-binding proteins also revealed that the imidazole group of histidine can interact directly with zinc (24). Second, the histidine residue located in transmembrane domain 2 or 4 of PEPT1 was suggested to be the H^+ binding site (21,25,26). These histidine residues are also conserved in PEPT2 and considered to play a pivotal role in transport activity (25,26). Indeed, [^{14}C]Gly-Sar uptake via PEPT2 was also inhibited by zinc treatment (Fig. 3). Third, zinc did not inhibit the [^{14}C]Gly-Sar uptake in the absence of an H^+ gradient, although the histidine residue modifier DEPC exhibited the inhibitory effect in that condition (Fig. 7). Because DEPC may interfere with the H^+–histidine interaction and Gly-Sar–histidine interaction (21,25,26), [^{14}C]Gly-Sar uptake in the absence of an H^+ gradient was inhibited by DEPC. Fourth, [^{14}C]Gly-Sar uptake via the basolateral peptide transporter was not markedly inhibited by zinc (Fig. 1). The intestinal basolateral peptide transporter was not driven by the H^+ gradient (15,27), suggesting that the histidine residue for H^+-binding does not exist in the basolateral peptide transporter. This may cause moderate inhibition by zinc for the basolateral peptide transporter. From all these findings, it seems likely that interaction of zinc with PEPT1 occurred in the histidine residue of PEPT1 proteins working as an H^+-binding site.

It was demonstrated that zinc exhibited the inhibitory effect on the glucose transporter (3), threonine transporter (4), and Na^+,K^+-ATPase (5). These reports showed that zinc competitively inhibited their activities and suggested that zinc may interact with the transporter by binding to thiol groups. On the other hand, the present study demonstrated that the inhibitory effect of zinc on H^+/peptide cotransporters seems to result from the interaction with histidine residues. It would be interesting to examine the effect of zinc on other H^+-coupled transport systems to clarify the mechanism of interactions between Zn^{2+} and H^+ via histidine residues.

Daniel and Adibi (28) reported that zinc stimulated the uphill transport of dipeptides into renal brush-border membrane vesicles. On the other hand, we failed to show the stimulatory effect of zinc on [^{14}C]Gly-Sar uptake via PEPT1 or PEPT2. The reason for this discrepancy between the findings of Daniel and Adibi and those of the present study is unknown, but it may be related to the experimental systems (membrane vesicles and cultured cell lines) or to the differences in the zinc concentrations used in the experiments. Daniel and Adibi used lower concentrations of zinc, although we applied clinically used concentrations of zinc to this study.

In conclusion, the present findings suggest that zinc inhibits [^{14}C]Gly-Sar uptake via PEPT1 in a competitive fashion. Zinc is suggested to interact with a histidine residue of PEPT1 working as an H^+ binding site. This inorganic ion should be a useful compound to examine the transport mechanism of peptide transporters. In addition, investigation of zinc with peptide transporters *in vivo* may provide appropriate information for clinical usage of zinc for its deficiency.

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