ELSEVIER

Biochimica et Biophysica Acta 1558 (2002) 187–197

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Molecular characteristics of small intestinal and renal brush border thiamin transporters in rats

Anna Verri [a], Umberto Laforenza [a,*], Giulia Gastaldi [a], Marisa Tosco [b], Gianguido Rindi [a]

[a] *Institute of Human Physiology, University of Pavia, Via Forlanini 6, 27100 Pavia, Italy*
[b] *Department of General Physiology and Biochemistry, University of Milan, Milan, Italy*

Received 6 June 2001; received in revised form 19 September 2001; accepted 21 September 2001

Abstract

The molecular characteristics of thiamin (T) transport were studied in the small intestinal and renal brush border membrane vesicles of rats, using [^{3}H]T at high specific activity. The effects of various chemical modifiers (amino acid blockers) on T uptake were examined and their specificity assessed. Treatment with the carboxylic specific blockers 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate, (1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride and *N*-ethyl-5-phenylisoaxolium-3′-sulfonate (Woodward's Reagent K) and with the sulfhydryl specific blocker *p*-chloromercuribenzene sulfonate inhibited T transport in both types of vesicles. Phenylglyoxal, but not ninhydrin, both reagents for arginine residues, and diethylpyrocarbonate, a reagent for histidine residues, specifically decreased T transport only in renal and small intestinal vesicles respectively. Similarly 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole reacted, but not *N*-acetylimidazole, both of which are reagents for tyrosine residues. However, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole inhibition was aspecific. Acetylsalicylic acid, a reagent for lysine and serine residues, decreased T transport, but the lysine effect was aspecific. Acetylsalicylic acid serine blockage also eliminated T/H$^+$ exchange in small intestinal vesicles. Taken together, these results suggest that for T transport carboxylic and sulfhydryl groups and serine residues are essential in both renal and small intestinal brush border membrane vesicles. In addition, arginine and histidine residues are also essential respectively for renal and small intestinal transporters. Serine was essential for the T/H$^+$ antiport mechanism. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Thiamin; Intestinal transport; Renal transport; Chemical modification; Brush border membrane vesicle; Carrier protein

1. Introduction

Thiamin (T) entry into rat enterocytes, as evaluated in brush border membrane vesicles (BBMVs), is a saturable, carrier mediated process at low (physiological) concentrations [1,2], which is Na$^+$ and K$^+$ independent and involves a T/H$^+$ antiport mechanism [3], inhibited by T analogs and derivatives.

T plasma level is, in part, regulated, like that of other organic cations, by the kidney. At high plasma levels organic cations are actively secreted [4], whereas at low plasma levels they can be actively reabsorbed [5,6]. T in rat plasma is not protein bound [7–9] and, under normal conditions, is reabsorbed, rather than secreted by the proximal tubule [10]. Recently a T/H$^+$ exchange mechanism has been identi-

* Corresponding author. Fax: +39-0382-507664.
E-mail address: lumberto@unipv.it (U. Laforenza).

0005-2736/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(01)00430-8

fied in rat renal BBMVs [11], with features resembling those of rat enterocytes.

The molecular characteristics of T microvillous intestinal and renal transporters have been little investigated. Komai and Shindo [12], using everted ring segments of the rat small intestine, put forward a preliminary hypothesis about the structure of the T transport system. On the basis of their results, both the amino group and the quaternary nitrogen atom of the vitamin have been recognized as being required for T transport, with 2′-methyl and 5-hydroxyethyl groups significantly influencing T binding to the carrier. Iwashima et al. [13] found a T binding protein located in the cytoplasmic membrane of yeast cells. Chemical modification of this protein suggests that an ionic interaction between a carboxyl residue and a quaternary nitrogen atom of the thiazole moiety of the T molecule could be involved in T binding to the protein. Recently this transporter has been cloned [14].

In the present study, we chemically modified some amino acid residues exposed to the right-side-out BBMVs of the rat small intestine and kidney, and examined the consequences on T transport. Preliminarily, the effect of various chemical modifiers on T uptake by intestinal and renal BBMVs was evaluated. The modifiers used were: CMC (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate), EDC (1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide hydrochloride) and WRK (or Woodward's Reagent K, *N*-ethyl-5-phenylisoaxolium-3′-sulfonate) for carboxylic groups; PCMBS (*p*-chloromercuribenzene sulfonate) and DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) for sulfhydryl groups; PGO (phenylglyoxal) and NIN (ninhydrin) for arginine residues; DEPC (diethyl pyrocarbonate) for histidine residues; NAI (*N*-acetylimidazole) and NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) for tyrosine residues; PLP (pyridoxal 5′-phosphate) and SAL (acetylsalicylic acid) for lysine and serine residues. Successively the specificity of action of each chemical modifier was also examined.

2. Materials and methods

2.1. Animals

Adult Wistar albino rats (250–300 g body weight) of either sex, reared on a complete standard diet containing 12 μg/g T [15], were used. Animals were killed by decapitation after 12 h fasting with water ad libitum.

2.2. Preparation of BBMVs

Intestinal BBMVs were prepared from the duodenal and jejunal mucosa of six adult rats using a Mg^{2+}/EGTA precipitation method as described by Rindi and Laforenza [16]. This method gives a lower purification, but it does not modify proton conductance [17]. All procedures were carried out at 0–4°C. The purity of the microvillous membrane was estimated by assessing the enrichment in alkaline phosphatase and K^+-phosphatase activities of BBMVs, determined according to Murer et al. [18], as compared to the initial mucosal homogenate. The enrichment of BBMVs was 15.1 ± 1.3 for alkaline phosphatase, and 1.6 ± 1.2 for K^+-phosphatase (mean ± S.E.M. of five different preparations).

Renal BBMVs were prepared from the kidney cortex of five or six rats using the Mg^{2+}/EGTA precipitation method of Biber et al. [19]. The enrichments were 15.5 ± 0.8 for alkaline phosphatase, and 1.6 ± 0.3 for K^+-phosphatase (mean ± S.E.M. of five different preparations).

Protein content was measured according to Lowry et al. [20], using bovine serum albumin as a standard.

2.3. Transport efficiency of BBMVs

The transport efficiency of the small intestinal and renal vesicular preparations was evaluated by determining the time course profile of D-glucose uptake after incubation with 80 μM D-[U-^{14}C]glucose (specific activity, 0.31 GBq/mmol) as described by Casirola et al. [2]. The time course profile of D-glucose uptake in the presence of an initial 100 mM NaCl gradient (out > in) showed an overshoot at 6–45 s, which disappeared in the presence of a 100 mM KCl gradient. This time course was similar to that reported in the literature [21].

2.4. Thiamin uptake

BBMVs were incubated at 25°C in a solution containing (mM): 250 D-mannitol, 20 Tris–HEPES (pH

7.5) and 1 μM [^{3}H]T (specific activity, 27.75 GBq/mmol). 10 μl of vesicle suspension was rapidly mixed with 90 μl of transport medium, and the incubation was terminated by the addition of 3 ml of cold (0–4°C) stopping solution (150 mM NaCl, 1 mM Tris–HEPES, pH 7.5). The amount of T radioactivity taken up by the vesicles was radiometrically measured after their separation by a rapid filtration procedure using cellulose nitrate microfilters (Microfiltration System, Dublin, CA, USA; pore diameter, 0.65 μm) previously saturated with unlabelled T [2]. Blanks were prepared in each experiment to evaluate the radioactivity of [^{3}H]T non-specifically adsorbed in the filter. The values of blanks were subtracted from the total radioactivity retained in the filter. Radiometric measurements were made using a Packard Tri-Carb 2000 CA liquid scintillation counter (Packard Instrument, Downers Grove, IL, USA). Unless stated otherwise, all uptake values were means ± S.E.M. of at least triplicate determinations for each of the five different vesicle preparations.

2.5. Short-time incubations

For 15 s incubation time a STRUMA short-time incubation apparatus (Innovativ-Labor, Adliswill, Switzerland) was used.

2.6. Chemical modification of BBMVs

Control vesicles were always processed in the same conditions (time, pH, temperature and solutions) of BBMVs treated with specific blockers. Potential changes in osmolarity due to the addition of chemical modifiers, dithiothreitol (DTT), hydroxylamine and free amino acids were prevented by adding an isoosmotic amount of D-mannitol in control vesicle incubating solutions. In protection experiments, free amino acids were added in the incubation media in a molar ratio 40% higher than that of blockers.

Moreover, to distinguish between negative results and false negatives, when a chemical modifier did not affect T transport, higher concentrations of reagents were utilized.

2.6.1. Carboxylic groups

Carboxylic groups were blocked with 20 mM CMC or with 20 mM EDC following George and Borders [22], or even with 5 mM WRK following Dinur et al. [23].

2.6.2. Sulfhydryl groups

Sulfhydryl groups were blocked with 1.5 mM DTNB [24] or with 1 mM PCMBS [25,26]. In some experiments BBMVs pretreated with 1 mM PCMBS were further incubated at 25°C with 10 mM DTT, 20 mM HEPES–Tris (pH 7.0) and isoosmotic D-mannitol for 10 min [25].

2.6.3. Arginine residues

Arginine residues were blocked with PGO or NIN. BBMVs were incubated with 50 mM PGO at 25°C [27–29] or with 1 mM NIN at 37°C in the dark [30,31] (see legend to Fig. 3). In some experiments, BBMVs were incubated at 25°C with 50 mM PGO plus 70 mM L-histidine.

2.6.4. Histidine residues

Histidine residues were blocked with DEPC [32]. In some experiments, to test the specificity of the reaction, BBMVs pretreated with 5 mM DEPC were further incubated at 25°C with 200 mM hydroxylamine and 60 mM Tris–HEPES (pH 6.0) for 30 min, or with 10 mM DTT, 20 mM HEPES–Tris (pH 6.5) and isoosmotic D-mannitol for 10 min [26,33]. In addition, specificity was also evaluated by treating BBMVs with 5 mM DEPC, 7 mM L-arginine, 20 mM K_2HPO_4/KH_2PO_4 (pH 6.5) and isoosmotic D-mannitol at 25°C for 30 min.

2.6.5. Tyrosine residues

Tyrosine residues were blocked with 1 mM NBD-Cl following Lin et al. [34] or with 5 mM NAI following Hori et al. [26].

2.6.6. Lysine residues

Lysine residues were blocked with 2 mM PLP following Haghighi et al. [35].

2.6.7. Serine residues

The effect of the serine residue modifier SAL (10 mM) was tested following Han et al. [36]. For specificity, in some experiments BBMVs were treated at 25°C with 10 mM SAL, 14 mM L-serine, 70 mM Tris–HCl (pH 7.5) and isoosmotic D-mannitol for 20 min.

Finally, the effect of serine residue blockage on T/H^+ exchange was investigated using intestinal BBMVs treated with SAL and preequilibrated (see [3]) at pH 5 for 2 h at 4°C and 20 min at 25°C in solutions containing (mM): 280 D-mannitol, 2 $MgSO_4$, 20 MES–Tris, pH 5 (outwardly directed H^+ gradient) or 20 Tris–HEPES, pH 7.5 (H^+ equilibrium condition: controls). Both types of vesicles were then incubated as indicated above, with a solution containing 1 μM [^{3}H]T and (mM): 280 D-mannitol, 2 $MgSO_4$, 20 Tris–HEPES, pH 7.5.

2.7. Washing procedures

The reaction of BBMVs with the chemical modifiers was stopped by adding 10 volumes of an ice cold solution containing (mM): 250 D-mannitol, 20 Tris–HEPES, pH 7.5. The BBMVs were then centrifuged at 27 000×*g* for 20 min, repeating the step twice with 5 volumes of the same medium [26,28,37,38]. After washing, the vesicles were immediately utilized for uptake experiments.

2.8. Statistics

The significance of the differences in the means under different experimental conditions was evaluated using the following statistical methods: analysis of variance (ANOVA) followed by Newman–Keuls's *Q*-test; Student's *t*-test for paired data. All statistical methods were carried out using a computerized program [39].

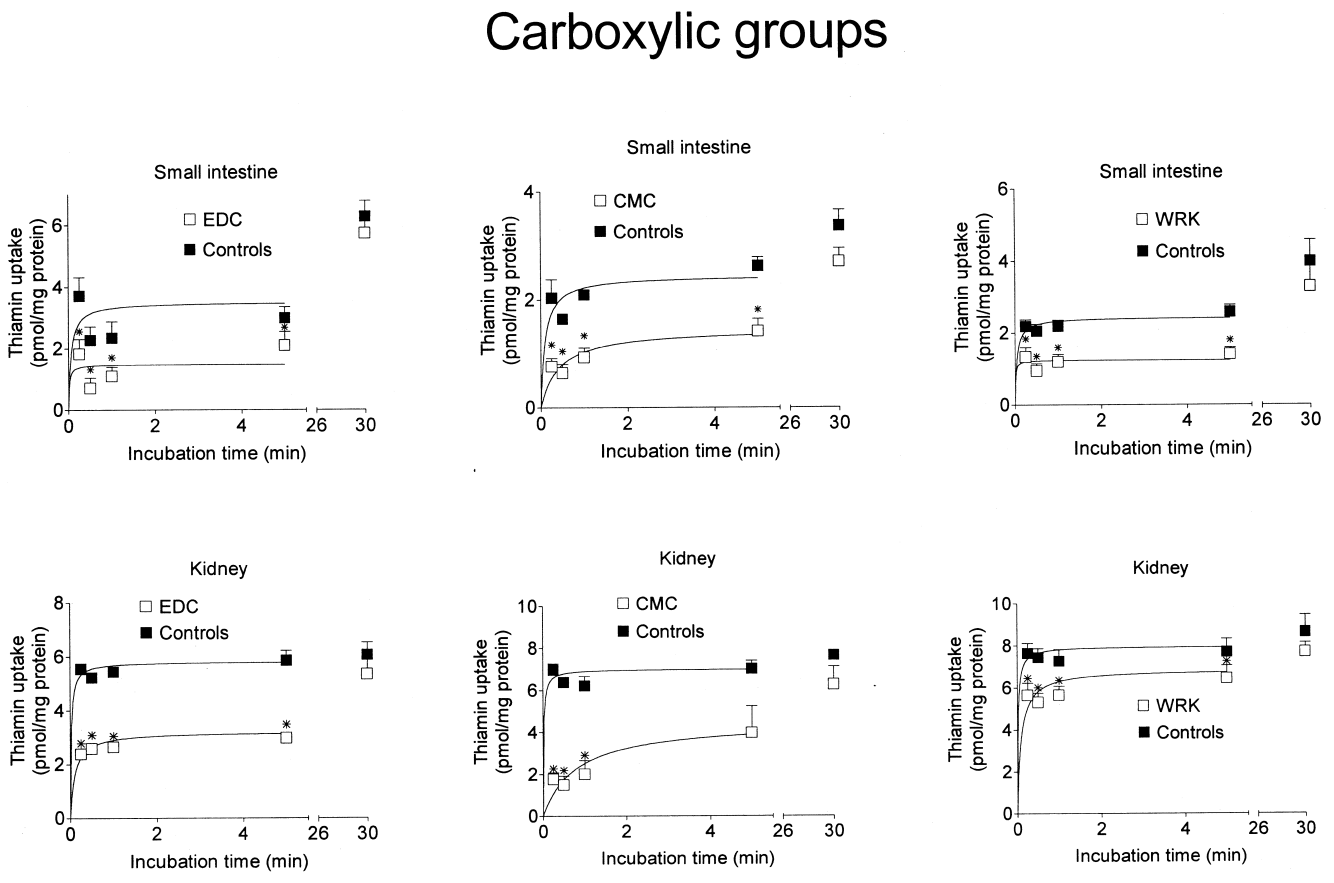

Fig. 1. Time course of T uptake by rat intestinal and renal BBMVs treated with carboxylic group blockers EDC, CMC and WRK. Vesicles treated with EDC, CMC and WRK (open squares) and control vesicles (filled squares) (see Section 2) were incubated at 25°C in a solution containing (mM): 250 D-mannitol, 20 Tris–HEPES, pH 7.5, and 1 μM [^{3}H]T (specific activity, 27.75 GBq/mmol). Symbols represent mean ± S.E.M. of triplicate determinations for each of six different vesicle preparations. $^*P < 0.05$ vs. controls (Student's *t*-test).

2.9. Reagents

Unlabelled T chloride hydrochloride was obtained from Prodotti Roche, Milan, Italy; EGTA and MES hydrate (β-morpholino-ethanesulfonic acid) from Aldrich Chimica, Milan, Italy. All other reagents were supplied by Sigma Chimica, Milan, Italy, and BDH, Poole, Dorset, UK.

2.10. Labelled compounds

[U-^{14}C]D-Glucose (specific activity, 10.8 GBq/mmol) and [^{3}H]T (specific activity, 429.2 GBq/mmol) were from Amersham Pharmacia Biotech UK, Amersham, UK.

3. Results

In preliminary experiments, the possible alteration of passive permeability due to vesicle treatment was tested by measuring T uptake at 0°C. Results showed that passive permeability of T was unaffected by all chemical modifiers utilized (A. Verri, unpublished data).

In treated and control vesicles from both kidney and small intestine T uptake reached equilibrium already after 15 s incubation. This can be, at least in part, ascribed to the presence of a binding component that, in the absence of a pH gradient, is about 50% of total T uptake [3,11].

3.1. Carboxylic groups

All the carboxylic group blockers significantly inhibited total T uptake in both small intestinal and renal BBMVs (Fig. 1). In renal BBMVs CMC and EDC showed inhibition potencies higher than WRK.

3.2. Sulfhydryl groups

PCMBS produced on T transport an average inhibition of 50% and 44% in renal and intestinal BBMVs respectively, while DTNB was ineffective (Fig. 2). Moreover, at 15 s incubation PCMBS inhibition (65%±4 and 44%±14 for renal and small intestinal vesicles respectively) of T uptake was reversed by further treatment of both types of vesicles

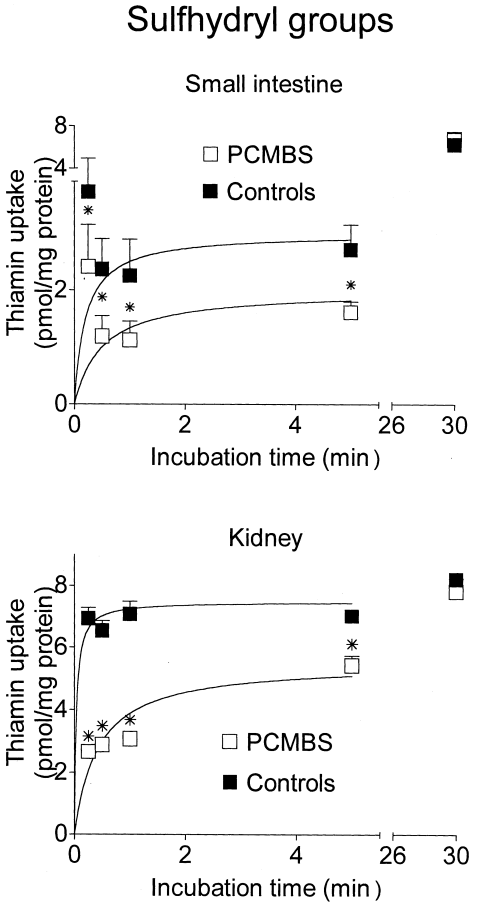

Fig. 2. Time course of T uptake by rat intestinal and renal BBMVs treated with the sulfhydryl group blocker PCMBS. Vesicles treated with PCMBS (open squares) and control vesicles (filled squares) (see Section 2) were incubated as described in Fig. 1. Numbers of experiments for each symbol as in Fig. 1. *$P < 0.05$ vs. controls (Student's t-test).

with 10 mM DTT, which can reverse the inhibition of sulfhydryl groups. Percent inhibition of T uptake, 2.5±3 and 0.6±7 in renal and small intestinal vesicles respectively, became negligible.

3.3. Arginine residues

In renal and small intestinal BBMVs T uptake was inhibited by PGO to about 55% and 30% respectively (Fig. 3); in contrast, NIN failed to inhibit. Since NIN was ineffective and PGO could also interact with histidine residues [40], in some experiments, both renal and small intestinal vesicles were treated with PGO in the presence or absence of 70 mM of free L-histidine. The excess of L-histidine protected

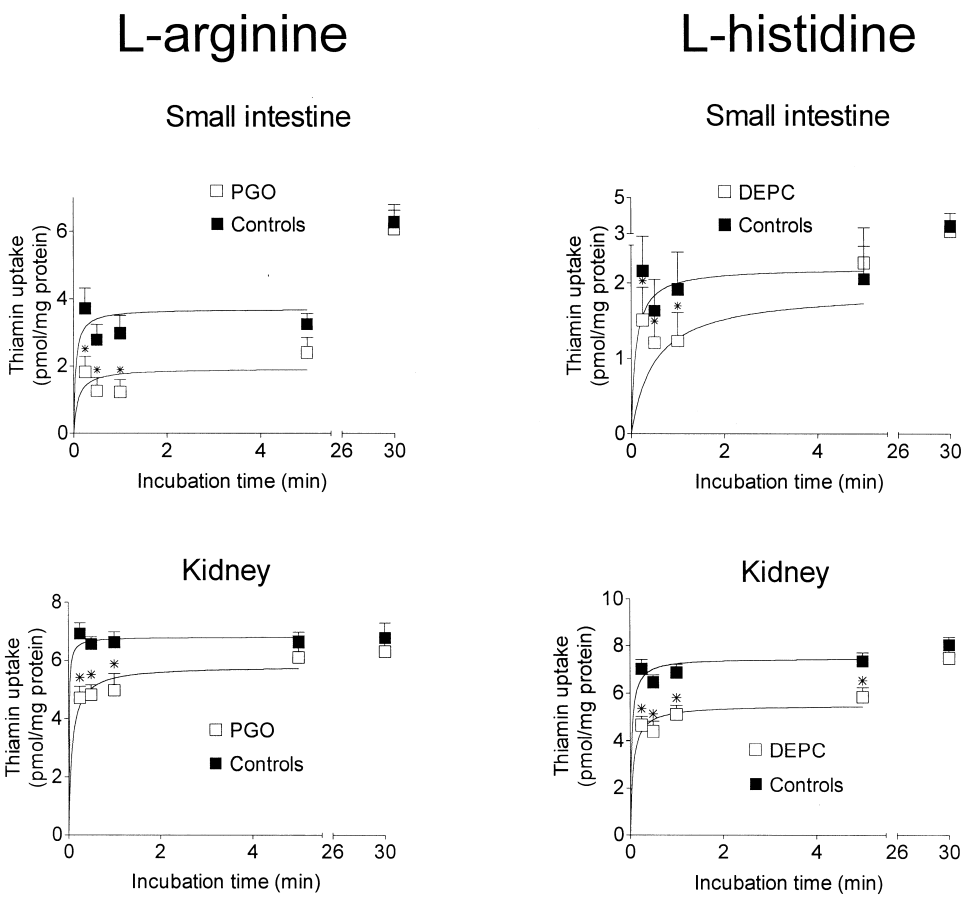

Fig. 3. Time course of T uptake by rat intestinal and renal BBMVs treated with the L-arginine and L-histidine blockers PGO and DEPC respectively. Vesicles treated with PGO or DEPC (open squares) and control vesicles (filled squares) (see Section 2) were incubated as described in Fig. 1. Numbers of experiments for each symbol as in Fig. 1. $*P < 0.05$ vs. controls (Student's t-test).

intestinal, but not renal vesicles (Fig. 4). Therefore, the small intestinal BBMV inhibition was probably caused by PGO reacting with histidine rather than with arginine residues.

3.4. Histidine residues

Treatment of both renal and intestinal BBMVs with DEPC significantly lowered T uptake by about 30% (Fig. 3). The inhibitory effect of DEPC at 15 s incubation (amounting to 53% ± 5 and 38% ± 6 for renal and small intestinal vesicles respectively) was not reversed by treatment with either 10 mM DTT (i) or 200 mM hydroxylamine (ii), which are capable of reversing the inhibition of sulfhydryl groups and tyrosine residues respectively [26,33]. Using these reagents, percent inhibition was (i) 67% ± 5 and 31% ± 9, and (ii) 43% ± 4 and 53% ± 10 vs. respective controls (vesicles incubated with DTT or hydroxylamine without DEPC) for renal and small intestinal vesicles respectively. Thus, under our experimental conditions, DEPC did not react with sulfhydryl groups and tyrosine residues, but only with histidine residues.

To exclude any cross reaction of DEPC with L-arginine, intestinal and renal BBMV were treated with DEPC in the presence or absence of 7 mM of free L-arginine: L-arginine protected only renal vesicles (Fig. 4).

3.5. Tyrosine residues

T uptake by both renal and intestinal BBMVs was significantly inhibited by NBD-Cl to about 60% up

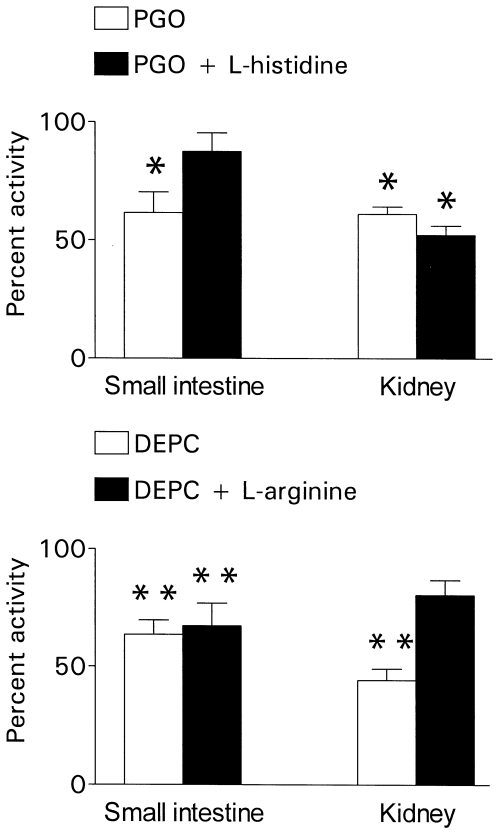

Fig. 4. Effect of L-histidine and L-arginine on T uptake by rat intestinal and renal BBMVs treated with the L-arginine and L-histidine blockers PGO and DEPC respectively. Vesicles, treated with the blockers in the presence or absence of a large amount of the free amino acid target, were incubated as described in Fig. 1 for 15 s. Each bar represents mean ± S.E.M. of at least triplicate determinations for each of four different preparations. $*P < 0.05$ vs. intestinal vesicles treated with PGO+L-histidine; $**P < 0.05$ vs. renal vesicles treated with DEPC+L-arginine (ANOVA followed by Newman–Keuls's Q-test before transformation of the data as percent activity).

to 1 min incubation; the intestinal uptake was also reduced (by 25% decrease) at 5 min incubation (Fig. 5). In contrast, the preincubation of the vesicles with the more specific tyrosine blocker NAI did not inhibit T uptake [41,42].

3.6. Lysine and serine residues

SAL is a reagent for either lysine [43] or serine residues [44]. SAL treatment significantly reduced renal and intestinal vesicular T uptake by about 30–40% (Fig. 5). In order to distinguish between L-lysine and L-serine involvement, BBMVs were treated with PLP, a lysine specific blocker, in separate experiments [35]. T vesicular transport was not affected by this treatment, showing that SAL specifically interacted with serine residues.

In other experiments, renal and intestinal BBMVs were treated with SAL in the presence or in the absence of 14 mM free L-serine. L-Serine protected the vesicles from SAL action and T uptake did not differ significantly from that of vesicles treated with L-serine in the absence of SAL (controls). Percent inhibition accounted to only 2.8 ± 1 and 13 ± 4 in renal and small intestinal vesicles respectively.

3.7. Serine residues and T/H^+ exchange

The time course of T uptake by intestinal BBMVs in the presence of an outwardly directed H^+ gradient (pH_{in} 5:pH_{out} 7.5) is shown in Fig. 6. As can be seen, the overshoot uptake of T was virtually eliminated by vesicle pretreatment with 10 mM SAL, indicating that serine plays an essential role in the T/H^+ exchange mechanism.

4. Discussion

Several reports indicate that a carrier-mediated T transport system is present in the apical membrane of the small intestine and kidney of mammals [1–3,11,12,45–48], but little is known about the molecular structure of the T transporter, and what is known is confined to the small intestine [12]. The present study is the first approach towards identifying the amino acids involved in the intestinal and renal transmembrane T transporter by inducing modifications in the proteins of renal and small intestinal rat BBMVs. The functional efficiency of our BBMV preparations, evaluated as the time course of D-glucose uptake (A. Verri, unpublished data), and their purity, evaluated as the enrichment of alkaline phosphatase and K^+-activated phosphatase activities, were similar to those reported in the literature and are considered sound for transport studies.

Chemically, T is an organic cation which exists as a monovalent cation at physiological pHs (range, 5–7.4) due to the presence of a quaternary nitrogen at the 3 position of the thiazole moiety. At pHs high-

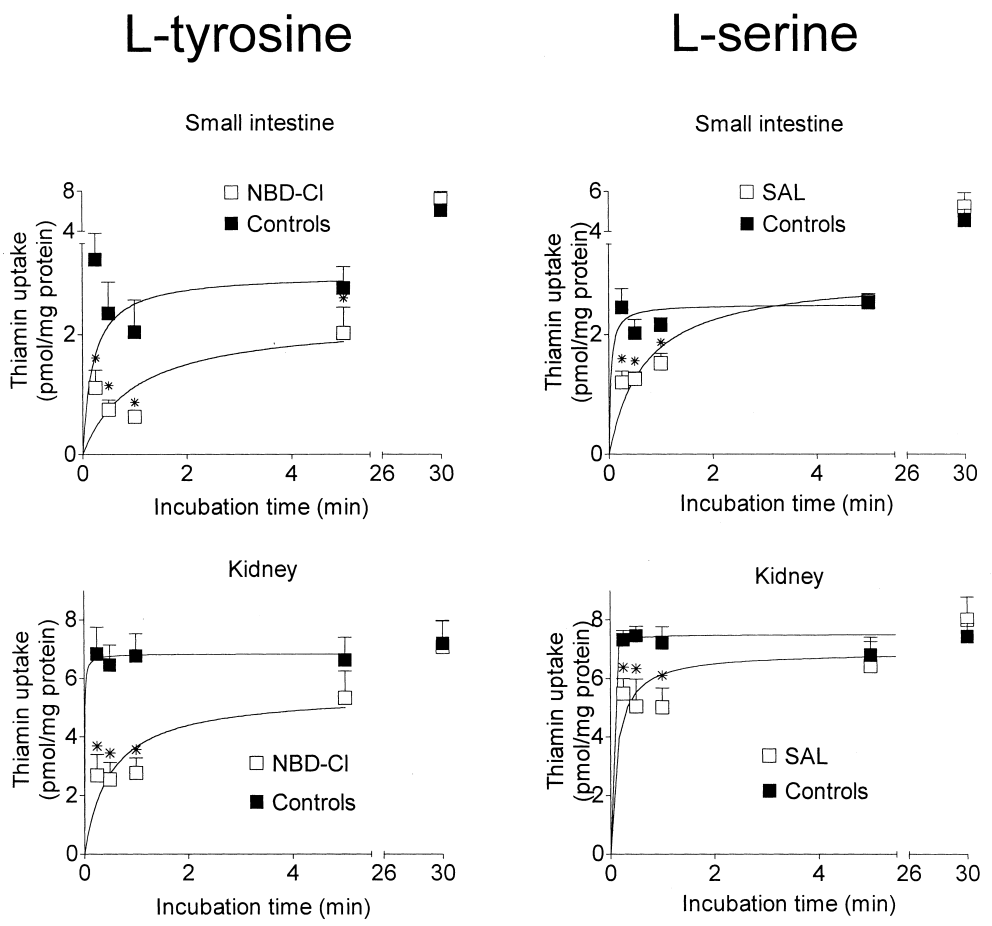

Fig. 5. Time course of T uptake by rat intestinal and renal BBMVs treated with the L-tyrosine and L-serine blockers NBD-Cl and SAL respectively. Vesicles treated with NBD-Cl and SAL (open squares) and control vesicles (filled squares) (see Section 2) were incubated as described in Fig. 1. Numbers of experiments for each symbol as in Fig. 1. $*P < 0.05$ vs. controls (Student's t-test).

er than 9.0, T is undissociated with the thiazole ring open, whereas, at pHs lower than 4.0, T exists as a divalent cation with nitrogen at the 1′ position of the pyrimidine moiety protonated [12]. These data suggest the involvement of the carboxylic groups of T transporters in T binding [12,13].

Our results showed that carboxylic blockers inhibited either small intestinal or renal T uptake by about 50% (Fig. 1). Since the carbodiimides CMC and EDC as well as the WRK are hydrophilic compounds [31], which cannot cross biological membranes, it can be assumed that T can bind its transporter at the external cellular side. Moreover, as previously suggested [12,13], the quaternary nitrogen of the T thiazole moiety is probably responsible for the binding of T to the carboxylic groups of T transporters, it being the only quaternary nitrogen present at physiological pH.

By using DTNB and PCMBS, the presence of sulfhydryl groups in the molecular structure of the T transporter could be assessed. Only PCMBS inhibited the time course of T uptake in both types of vesicles (Fig. 2). The inhibition was specific, since DTT was able to remove the linkage with sulfhydryl groups, suggesting that the sulfhydryl blocker PCMBS probably inhibited the functional sulfhydryl groups of the T transporter.

DTNB, another sulfhydryl groups blocker, was unable to inhibit T uptake since it is an aromatic disulfide with a greater steric impediment, preferring to react with denatured proteins [49], which is not our case. An important role of sulfhydryl groups was observed for organic cation transporters [25,50] and for H^+/ATPase [51]. Interestingly, our results would be in keeping with the hypothesis that T could cross the biological membranes as a thiazolium ring

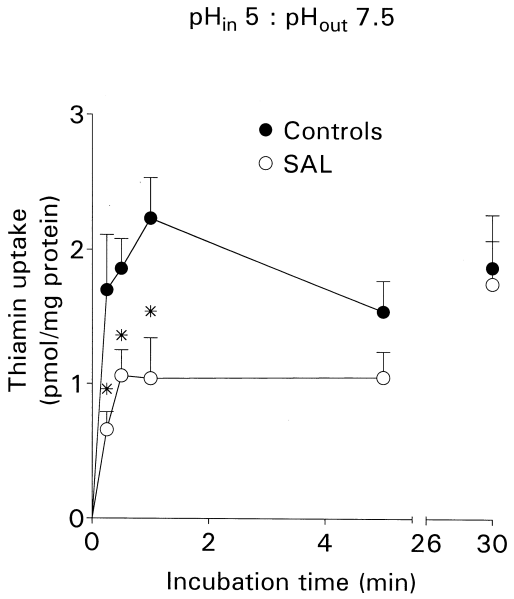

Fig. 6. Time course of T/H$^+$ exchange (under pH gradient, pH$_{in}$ 5:pH$_{out}$ 7.5) in rat intestinal BBMVs. Vesicles treated with SAL and controls (see Section 2) were preincubated (2 h at 4°C and 20 min at 25°C) in a medium containing (mM): 280 D-mannitol, 2 $MgSO_4$, 20 MES–Tris, pH 5. Vesicles were then incubated at 25°C with a solution containing (mM): 280 D-mannitol, 2 $MgSO_4$, 20 Tris–HEPES, pH 7.5 and 1 μM [^{3}H]T (specific activity, 27.75 GBq/mmol). Symbols represent mean ± S.E.M. of at least triplicate determinations for each of five different preparations. *$P < 0.05$ vs. control (Student's *t*-test).

opened thiol species, interacting with membrane sulfhydryl groups during translocation [52–55].

The role played by arginine and histidine residues in the structure of T transporters was also investigated by using either PGO or DEPC. Both blockers inhibited renal and intestinal T uptake (Fig. 3). Specificity experiments, while excluding any cross reaction by DEPC with sulfhydryl groups and tyrosine residues, suggested that PGO in the small intestinal BBMVs blocked histidine rather than arginine residues. In fact, vesicles preincubated with PGO in the presence of an L-histidine excess normally took up T (Fig. 4). Vice versa, the presence of an L-arginine excess during DEPC treatment could protect renal but not small intestinal BBMVs (Fig. 4). Taken together, these results suggest that the T transporter has essential arginine or histidine residues in the kidney and in the small intestine BBMVs respectively.

Some H$^+$ driven transport systems (symports and antiports), similar to the renal and small intestinal T/H$^+$ antiport, possess essential histidine or arginine residues for their functioning [32,56]. Probably, arginine and/or histidine are located at the H$^+$ binding sites rather than at the organic and inorganic compound binding sites [26,56–59]. As previously suggested [12,13], the nitrogen at the 1′ position of the pyrimidine moiety of T can react with a positive charge present in the amino acid sequence of the transporter. At physiological pH, both histidine and arginine residues meet this requirement [60].

Blockers NBD-Cl and NAI allowed us to investigate the role of tyrosine residues in the T transport system. T uptake was inhibited by NBD-Cl, but not by NAI (Fig. 5), an apparent discrepancy that could be explained by considering the specificity and the sensitivity of the two tyrosine blockers. NBD-Cl potentially can react with tyrosine, lysine and cysteine residues [42]. In our experiments, NBD-Cl interaction with L-lysine residues could be excluded, since at pH 7.4, the reaction of NBD-Cl with the amino group is almost negligible, taking place at pHs higher than 8 [61]. In addition, BBMV treatment with PLP, a specific lysine blocker, did not modify T transport. Thus, the sites chemically modified by NBD-Cl could only be tyrosine and cysteine residues. Since NAI did not inhibit T uptake, the conclusion can be reached that NBD-Cl reacted with cysteine residues. This hypothesis was confirmed following the use of the sulfhydryl specific reagent PCMBS, which significantly inhibited T uptake in both renal and small intestinal vesicles (Fig. 2). However, it has previously been shown that NAI is the most selective tyrosine reagent, even though its inhibitory potency appears to be lower than that of NBD-Cl [41,42].

SAL, a blocker of serine residues, reduced T uptake in both renal and small intestinal BBMVs (Fig. 5). The presence of a large amount of free L-serine during SAL treatment protected the vesicles, indicating strong competition for SAL between exogenous free L-serine and the transporter sites modified by SAL. However, SAL inhibition could not be ascribed to the blockage of L-lysine residues, the other amino acid target for SAL [36,43,44,62], since PLP, a specific lysine blocker, was ineffective. It should be emphasized that the blockage of serine residues also impeded T/H$^+$ exchange in intestinal (and in renal, A. Verri, unpublished data) BBMVs as was shown by the disappearance of the typical overshoot [3],

observed in untreated vesicles in the presence of an outwardly directed pH gradient (Fig. 6).

In conclusion, carboxyl groups, sulfhydryl groups and serine residues should be essential for T transport in both renal and small intestinal BBMVs. In addition, the renal transporter has essential arginine and the small intestinal histidine residues. The differences in the sensitivity toward the chemical modifiers could also indicate that the renal transporter of T is different from the transporter present in small intestine. Thus, the existence of different transporters could account for the differences in the transport capacity and specificity previously observed in kidney and small intestine [3,11].

It is interesting to compare our results on rats with those by Katsura et al. [50], who applied our experimental approach to the same animals but with tetraethylammonium (TEA) instead of T as an organic cation. Here, the renal BBMV transporter of TEA was found to possess the essential carboxyl and sulfhydryl groups and histidine and arginine residues. Thus the renal T transporter would appear to be different from the renal organic cation transporter. However, no definitive conclusions can be drawn, since our study is much more extensive, including also serine, tyrosine and lysine residues, which were not investigated by Katsura et al. [50].

It must be emphasized that, in general, the results of experiments with chemical blockers have often been found to have a larger influence on activity than with mutagenesis, probably because of the steric effects caused by the relatively bulky labeling reagents [63,64]. Thus the results reported here will be fully appreciated only after the amino acid sequences of the T intestinal and renal transporters have been found and mutagenesis experiments performed. For example, the involvement of essential arginine residues in the Na^+ dependent renal transport of glucose was previously suggested by Strevey et al. [65] by using PGO treated BBMVs. Cloned sequences for Na^+ dependent glucose transporters SGLT1 and SGLT2 confirmed important arginine groups in at least two different domains [66].

Acknowledgements

We wish to express our gratitude to Prodotti Roche, Milan, Italy, for their generous gift of unlabeled thiamin. Part of the present results were presented by A.V. in partial fulfillment of the Italian Dottorato di Ricerca (PhD) in Physiology (academic year 1996–1997).

References

[1] G. Rindi, G. Gastaldi, D. Casirola, G. Ferrari, IRCS Med. Sci. 13 (1985) 234–235.
[2] D. Casirola, G. Ferrari, G. Gastaldi, C. Patrini, G. Rindi, J. Physiol. 398 (1988) 329–339.
[3] U. Laforenza, M.N. Orsenigo, G. Rindi, J. Membr. Biol. 161 (1998) 151–162.
[4] B.R. Rennick, J. Pharmacol. Exp. Ther. 122 (1958) 449–465.
[5] M. Acara, B.R. Rennick, Am. J. Physiol. 225 (1973) 1123–1128.
[6] M. Acara, B.R. Rennick, J. Pharmacol. Exp. Ther. 199 (1976) 32–40.
[7] G. Malnic, A. Carvalho Da Silva, R.C. De Angelis, Z.J. Gomes, Am. J. Physiol. 198 (1960) 1274–1278.
[8] G. Rindi, L. De Giuseppe, G. Sciorelli, J. Nutr. 94 (1968) 447–454.
[9] J.Y. Thom, R.E. Davis, G.C. Icke, Int. J. Vitam. Nutr. Res. 55 (1985) 269–273.
[10] W. Weber, M. Nitz, M. Looby, J. Pharmacokinet. Biopharm. 18 (1990) 501–523.
[11] G. Gastaldi, E. Cova, A. Verri, U. Laforenza, A. Faelli, G. Rindi, Kidney Int. 57 (2000) 2043–2054.
[12] T. Komai, H. Shindo, J. Nutr. Sci. Vitaminol. 20 (1974) 179–187.
[13] A. Iwashima, H. Nishimura, H. Nishino, Vitamins (Japan) 58 (1984) 577–587.
[14] F. Enjo, K. Nosaka, M. Ogata, A. Iwashima, H. Nishimura, J. Biol. Chem. 272 (1997) 19165–19170.
[15] L. Randoin, J. Causeret, Bull. Soc. Hyg. Aliment. 35 (1947) 14–18.
[16] G. Rindi, U. Laforenza, Methods Enzymol. 279 (1997) 118–131.
[17] I. Sabolic, G. Burckhardt, Biochim. Biophys. Acta 772 (1984) 140–148.
[18] H. Murer, E. Ammann, J. Biber, U. Hopfer, Biochim. Biophys. Acta 433 (1976) 509–519.
[19] J. Biber, B. Stieger, W. Haase, H. Murer, Biochim. Biophys. Acta 647 (1981) 169–176.
[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[21] P.S. Aronson, B. Sacktor, J. Biol. Chem. 250 (1975) 6032–6039.
[22] A.L. George, C.L. Borders Jr., Biochem. Biophys. Res. Commun. 87 (1979) 59–65.
[23] D. Dinur, E.R. Kantrowits, J. Hajdu, Biochem. Biophys. Res. Commun. 100 (1981) 785–792.
[24] T. Nakayama, H. Tanabe, Y. Deyashiki, M. Shinoda, A.

Hara, H. Sawada, Biochim. Biophys. Acta 1120 (1992) 144–150.
[25] R. Hori, H. Maegawa, T. Okano, M. Takano, K. Inui, J. Pharmacol. Exp. Ther. 241 (1987) 1010–1016.
[26] R. Hori, H. Maegawa, M. Kato, T. Katsura, K. Inui, J. Biol. Chem. 264 (1989) 12232–12237.
[27] J. Strevey, M.G. Brunette, R. Béliveau, Biochem. J. 223 (1984) 793–802.
[28] J. Strevey, V. Vachon, B. Beaumier, S. Giroux, R. Bèliveau, Biochim. Biophys. Acta 1106 (1992) 110–116.
[29] R. Béliveau, J. Strevey, J. Biol. Chem. 262 (1987) 16885–16891.
[30] K. Takahashi, J. Biochem. 80 (1976) 1173–1176.
[31] R.L. Lundblad, C.M. Noyes, in: Chemical Reagents for Protein Modification, CRC Press, Boca Raton, FL, 1984, Vol. 2.
[32] H.M. Said, R. Mohammadkhani, Biochem. J. 290 (1993) 237–240.
[33] N. Bindlev, E.M. Wright, J. Membr. Biol. 81 (1984) 159–170.
[34] J.T. Lin, A. Stroh, R. Kinne, Biochim. Biophys. Acta 692 (1982) 210–217.
[35] B. Haghighi, G. Flynn, H.R. Levy, Biochemistry 21 (1982) 6415–6420.
[36] P.F. Han, G.Y. Han, H.C. McBay, J. Johnson Jr., Experientia 36 (1980) 1149–1150.
[37] B. Beaumier, R. Béliveau, Biochim. Biophys. Acta 1068 (1991) 142–148.
[38] J. Bertran, A. Roca, E. Pola, X. Testar, A. Zorzano, M. Palacin, J. Biol. Chem. 266 (1991) 798–802.
[39] S.A. Glantz, Statistica per Discipline Bio-mediche: Programma Applicativo, 2nd edn., McGraw-Hill Libri Italia, Milan, 1987.
[40] M. Gonzáles-Sepúlveda, M.T. Núñez, J. Membr. Biol. 141 (1994) 225–230.
[41] L.A. Cohen, Annu. Rev. Biochem. 37 (1968) 695–726.
[42] P. Kulanthaivel, J.B. Simon, G. Burckhardt, V.B. Mahesh, F.H. Leibach, V. Ganapathy, Biochemistry 29 (1990) 10807–10813.
[43] J.E. Walker, FEBS Lett. 66 (1976) 173–175.
[44] F.J. Van der Ouderaa, M. Buytenhek, D.H. Nugteren, D.A. Van Dorp, Eur. J. Biochem. 109 (1980) 1–8.
[45] T. Komai, K. Kawai, H. Shindo, J. Nutr. Sci. Vitaminol. 20 (1974) 163–177.
[46] M.A. Mahajan, M.J. Acara, Pharmacol. Exp. Ther. 268 (1994) 1311–1315.
[47] G. Gastaldi, E. Cova, A. Verri, Pflügers Arch. 434 (1997) R33.
[48] G. Rindi, U. Laforenza, Proc. Soc. Exp. Biol. Med. 224 (2000) 246–255.
[49] A.F.S.A. Habeeb, Methods Enzymol. 25 (1972) 457–464.
[50] T. Katsura, M. Takano, Y. Tomita, M. Yasuhara, K. Inui, R. Hori, Biochim. Biophys. Acta 1146 (1993) 197–202.
[51] B.J. Simon, G. Burckhardt, J. Membr. Biol. 117 (1990) 141–151.
[52] P. Haake, L.P. Bausher, J.P. McNeal, J. Am. Chem. Soc. 93 (1971) 7045–7049.
[53] J.M. Duclos, P. Haake, Biochemistry 13 (1974) 5358–5362.
[54] R.D. Brown, Ann. NY Acad. Sci. 378 (1982) 442–448.
[55] R.D. Brown, J. Theor. Biol. 143 (1990) 565–573.
[56] R. Suchi, Y. Stern-Bach, S. Schuldiner, Biochemistry 31 (1992) 12500–12503.
[57] E. Padan, L. Patel, H.R. Kaback, Proc. Natl. Acad. Sci. USA 76 (1979) 6221–6225.
[58] F.G. Grillo, P.S. Aronson, J. Gen. Physiol. 82 (1983) 27a.
[59] D. Meredith, C.A.R. Boyd, Am. J. Physiol. 269 (1995) L137–L143.
[60] L. Patthy, J. Thesz, Eur. J. Biochem. 105 (1980) 387–393.
[61] A.A. Aboderin, E. Boedefeld, P.L. Luisi, Biochim. Biophys. Acta 328 (1973) 20–30.
[62] J. Jeffery, L. Hobbs, H. Jörnvall, Biochemistry 24 (1985) 666–671.
[63] R.J. Webb, Y.M. Khan, J.M. East, A.G. Lee, J. Biol. Chem. 275 (2000) 977–982.
[64] H.R. Kaback, J. Wu, Q. Rev. Biophys. 30 (1997) 333–364.
[65] J. Strevey, M.G. Brunette, R. Béliveau, Biochem. J. 223 (1984) 793–802.
[66] M.A. Hediger, D.B. Rhoads, Physiol. Rev. 74 (1994) 993–1026.