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Tyrosine residues are essential for the activity of the human placental taurine transporter

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Treatment of human placental brush-border membrane vesicles with four tyrosine group-specific reagents, *N*-acetylimidazole, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), tetranitromethane and *p*-nitrobenzenesulfonyl fluoride, inhibited NaCl gradient-driven taurine uptake in these vesicles without affecting the vesicle integrity. The relative potency of these reagents to inhibit the transporter was in the following order: tetranitromethane > NBD-Cl > *p*-nitrobenzenesulfonyl fluoride > *N*-acetylimidazole. The inhibition by *N*-acetylimidazole was reversible with hydroxylamine and the inhibition by NBD-Cl was reversible with 2-mercaptoethanol. Kinetic analysis of taurine uptake in control and in *N*-acetylimidazole-treated membrane vesicles revealed that the inhibition was primarily due to a reduction in the maximal velocity. There was no change in the affinity of the transporter for taurine in control and treated vesicles. The transporter could be protected from the *N*-acetylimidazole-induced inhibition by Na^+. The dependence of taurine uptake rate on extravesicular Na^+ concentration was sigmoidal and analysis of the data revealed that two Na^+ ions were involved per transport of one taurine molecule. It is concluded that tyrosine residues are essential for optimal transport function of the human placental taurine transporter and that these critical tyrosine residues are located at or near the Na^+-binding site of the transporter.

Introduction

Transport of taurine across the plasma membrane of a variety of cells occurs via the β-amino acid carrier which accepts, in addition to taurine, other β-amino acids such as β-alanine and hypotaurine as preferred substrates (for reviews, see Refs. 1–3). The carrier is coupled to both Na^+ and Cl^- [4–9]. This dependence on Cl^- renders the β-amino acid carrier unique among the well-characterized Na^+-coupled amino acid carriers. We have recently identified the presence of the β-amino acid transport system in brush-border membranes isolated from normal term human placentas [8]. This transporter exhibits characteristics which are very similar to those described for the system in other tissues.

Even though the β-amino acid transporter has been characterized in detail in various tissues with respect to ion-dependence, substrate specificity, stoichiometry and kinetics, no attempt has been made in any of these tissues to gain insight into the transport system at the protein level. In this investigation, we have attempted to elucidate the chemical nature of the human placental taurine transporter. The results presented here show that tyrosine residues play an important role in the catalytic activity of the transporter and that these critical tyrosine residues are located at or near the Na^+-binding site of the transporter.

Methods and Materials

Isolation of human placental brush-border membrane vesicles

Brush-border membrane vesicles from normal term human placentas were prepared by a Mg^{2+}-aggregation method described previously [10,11]. These membranes were highly enriched (20–25-fold) in the brush-border marker enzymes, alkaline phosphatase and 5′-nucleotidase in comparison with the homogenate of the washed

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PNBSF, *p*-nitrobenzenesulfonyl fluoride.

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placental tissue [11,12]. The membrane vesicles were preloaded with a suitable buffer and the protein concentration of the membrane suspension was adjusted to 5 mg/ml before storing the membranes in liquid N_2. The composition of the preloading buffer varied depending upon the individual experiment.

Treatment with reagents

N-Acetylimidazole. Membrane vesicles were treated with *N*-acetylimidazole at room temperature (21-22°C) for 1 h in 10 mM Hepes-Tris buffer (pH 7.4) containing 100 mM K_2SO_4. Protein concentration during treatment was 1 mg/ml. Stock solutions of *N*-acetylimidazole were prepared in the treatment buffer. After treatment, the excess reagent was removed by dilution and centrifugation as follows. The membranes were diluted 5-fold with the preloading buffer (10 mM Hepes-Tris (pH 7.4) containing 300 mM mannitol) and centrifuged at $60000 \times g$ for 30 min. The supernatants were discarded and the pellets suspended in the same buffer. In experiments where the reversal by hydroxylamine of the *N*-acetylimidazole-induced inhibition was investigated, after removal of the excess *N*-acetylimidazole, control and treated membranes were again incubated with or without 150 mM hydroxylamine for 1 h at 37°C. Hydroxylamine solution was prepared by dissolving hydroxylamine hydrochloride in 10 mM Hepes and adjusting the pH to 7.4 with Tris. After incubation, the reagent was removed by dilution and centrifugation.

NBD-Cl. Membrane vesicles were treated with NBD-Cl at room temperature for 1 h in 10 mM Hepes-Tris buffer (pH 7.4) containing 100 mM K_2SO_4. Stock solutions of NBD-Cl were prepared in ethanol. Control membrane vesicles were treated similarly, but in the presence of an equal concentration of ethanol alone (final concentration, 1%, v/v). After treatment, unreacted NBD-Cl was removed by dilution and centrifugation. In experiments where the reversal of inhibition by 2-mercaptoethanol was studied, control and treated membranes were again incubated with or without 10 mM 2-mercaptoethanol at room temperature for 30 min. The solution of 2-mercaptoethanol was prepared in 10 mM Hepes-Tris buffer (pH 7.4) containing 100 mM K_2SO_4. After incubation, the reagent was removed by dilution and centrifugation.

Tetranitromethane and PNBSF. Membrane vesicles were treated with tetranitromethane or PNBSF at room temperature for 30 min in 20 mM K_2HPO_4-KH_2PO_4 buffer (pH 8.8) containing 100 mM K_2SO_4. Stock solutions of the reagents were prepared in ethanol. Control membrane vesicles were treated similarly but in the presence of an equal concentration of ethanol alone (final concentration, 1% (v/v)). After treatment, unreacted reagents were removed by dilution and centrifugation.

In all cases, the membrane vesicles were finally preloaded with 10 mM Hepes-Tris (pH 7.5) containing 300 mM mannitol. Protein concentration of the membrane suspension was adjusted to 5 mg/ml before use in uptake experiments.

Protection with Na^+. Membrane vesicles were preincubated with 200 mM K_2SO_4 or 200 mM Na_2SO_4 for 1 h and then treated with *N*-acetylimidazole. Control membrane vesicles were treated similarly, but in the absence of *N*-acetylimidazole. After treatment, salts and unreacted reagent were removed by dilution and centrifugation.

Uptake measurements

Uptake measurements were made at room temperature using a rapid filtration technique [13]. Millipore filters (DAWP type; pore size 0.65 μm) were used in these assays. Uptake was initiated by rapidly mixing 40 μl of membrane suspension (0.2 mg protein) with 160 μl of uptake buffer containing radiolabeled taurine. The uptake buffer was 10 mM Hepes-Tris (pH 7.5) containing 150 mM NaCl. Under these conditions, there was an inwardly directed NaCl gradient across the brush-border membrane ($[NaCl]_o = 120$ mM; $[NaCl]_i = 0$). Uptake was terminated by adding 3 ml of ice-cold stop buffer (5 mM Hepes-Tris, 155 mM KCl (pH 7.5)) and the mixture was filtered. After washing with the stop buffer (3×5 ml), the radioactivity associated with the filter was counted by liquid scintillation spectrometry. In those experiments where protection by Na^+ was investigated, the membrane vesicles were suspended in 10 mM Hepes-Tris buffer (pH 7.0) containing 600 mM mannitol. The uptake buffer was 10 mM Hepes-Tris (pH 7.0) containing 300 mM NaCl. The composition of the stop buffer was 5 mM Hepes-Tris, 310 mM KCl (pH 7.0).

Statistics

Uptake measurements were routinely done in duplicate or triplicate and the variation among the replicate values was always less than ±10% of the mean value. Each experiment was repeated with two or three different membrane preparations. Results are given as the mean ± S.E. Statistical differences were determined by Student's *t*-test and a *P* value less than 0.05 was considered significant.

Materials

[2(n)-^{3}H]Taurine (specific radioactivity, 20.9 Ci/mmol) and L-[1-^{14}C]glucose (specific radioactivity, 54.8 mCi/mmol) were purchased from Du Pont-New England Nuclear, Boston, MA, U.S.A. Taurine, NBD-Cl, *N*-acetylimidazole, PNBSF, hydroxylamine and 2-mercaptoethanol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Tetranitromethane was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. All other chemicals were of analytical grade.

Results

Effect of N-acetylimidazole treatment on taurine uptake

The time courses of NaCl gradient-dependent taurine uptake in control and *N*-acetylimidazole (7.5 mM)-treated membrane vesicles are given in Fig. 1. In both cases, uptake of 0.2 μM taurine was measured in the presence of an inwardly directed NaCl gradient ($[NaCl]_o = 120$ mM; $[NaCl]_i = 0$). Taurine uptake was markedly reduced in treated membrane vesicles compared to control vesicles. Initial uptake rates measured with a 30 s incubation were reduced by 70% following treatment with *N*-acetylimidazole (2.56 ± 0.19 and 0.74 ± 0.04 pmol/mg of protein in control and treated vesicles, respectively). The uptake values measured at 3 h incubation were not the same in control and treated membrane vesicles and thus did not represent equilibrium values. It has been shown earlier in this laboratory that equilibrium values for taurine uptake in the presence of a NaCl gradient were not obtained in intestinal [9] or placental [8] brush-border membrane vesicles unless the vesicles were incubated for periods longer than 3 h. Since we could not use the taurine uptake values at 3 h incubation as a measure of intravesicular volume and thus of vesicle integrity, we measured the uptake of L-glucose in these membrane vesicles to calculate the intravesicular volume. The intravesicular volume measured by this method was similar in control and *N*-acetylimidazole-treated membrane vesicles (1.77 ± 0.01 versus 1.73 ± 0.03 μl/mg of protein; $P > 0.05$). These results demonstrate that the integrity of the membrane vesicles was not affected by the treatment.

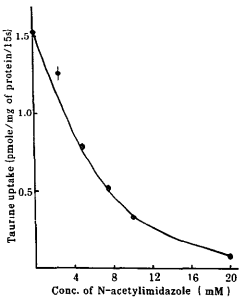

Fig. 2. Dose-dependence of inhibition of taurine uptake by *N*-acetylimidazole. Membrane vesicles were treated with varying concentrations of *N*-acetylimidazole (0–20 mM) for 1 h at room temperature at pH 7.4 in 10 mM Hepes-Tris buffer containing 100 mM K_2SO_4. After treatment, the vesicles were washed free of the reagent and preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol. Uptake of radiolabeled taurine (final concentration, 0.3 μM) was measured with a 15 s incubation in the presence of an inwardly directed NaCl gradient. The results are given as the mean ± S.E. ($n = 6$, two membrane preparations).

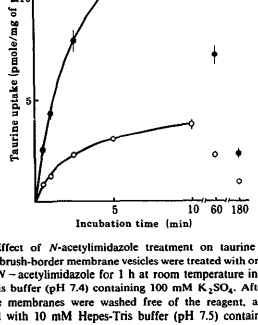

Fig. 1. Effect of *N*-acetylimidazole treatment on taurine uptake. Placental brush-border membrane vesicles were treated with or without 7.5 mM *N*-acetylimidazole for 1 h at room temperature in 10 mM Hepes-Tris buffer (pH 7.4) containing 100 mM K_2SO_4. After treatment, the membranes were washed free of the reagent, and then preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol. Uptake of radiolabeled taurine (final concentration, 0.2 μM) was measured in the presence of an inwardly directed NaCl gradient ($[NaCl]_o = 120$ mM; $[NaCl]_i = 0$). The results are given as the mean ± S.E. ($n = 4$, two membrane preparations). ●——●, Control vesicles; ○——○, *N*-acetylimidazole-treated vesicles.

Fig. 2 describes the effect of increasing concentrations of *N*-acetylimidazole during treatment on the initial rates (15 s incubation) of 0.3 μM taurine uptake. The inhibition of taurine uptake progressively increased with increasing concentrations of the reagent. A 50% inhibition was observed when the concentration of *N*-acetylimidazole was 5 mM and more than 90% inhibition was obtained with 20 mM reagent.

Effects of treatment with NBD-Cl, tetranitromethane and PNBSF on taurine uptake

N-Acetylimidazole is a reagent which is fairly specific for modification of tyrosine residues [14] and the inhibition of taurine uptake in human placental brush-border membrane vesicles by treatment with the reagent indicates that the taurine transporter in these membranes may possess tyrosine residues which are critical for the transport activity of the carrier protein. To provide further supporting evidence for the participation of tyrosine residues in the transport function, we investigated the effects of three other reagents which are

TABLE I

Comparative potency of tyrosine group-specific reagents to inhibit taurine uptake in human placental brush-border membrane vesicles

Reagent	IC_{50} (μM)
Tetranitromethane	10
NBD-Cl	16
PNBSF	175
N-Acetylimidazole	4800

commonly employed for modification of tyrosine residues. These reagents are NBD-Cl, tetranitromethane and PNBSF. Treatment of the membrane vesicles with all three reagents effectively inhibited taurine uptake in a dose-dependent manner (data not shown). The approximate values for the concentration of these reagents to produce 50% inhibition of taurine uptake are given in Table I and the inhibitory potency appears to be in the following order: tetranitromethane > NBD-Cl > PNBSF > *N*-acetylimidazole.

Reversibility of inhibition

Incubation of *N*-acetylimidazole-treated membranes with hydroxylamine would deacetylate the modified tyrosine residues and regenerate the phenolic hydroxyl groups [15,16]. Therefore, we tested the ability of hydroxylamine to reverse the *N*-acetylimidazole-induced inhibition of taurine uptake. In these experiments, it was found that incubation of the membrane vesicles with 150 mM hydroxylamine alone caused a considerable inhibition (41 ± 7%) of taurine uptake. Therefore, we measured taurine uptake in membrane vesicles which were treated in four different ways to provide proper controls for the assessment of reversal of the inhibition (Fig. 3): control vesicles (A), vesicles treated with *N*-acetylimidazole (B), vesicles treated with hydroxylamine (C) and vesicles treated with *N*-acetylimidazole followed by treatment with hydroxylamine (D). Treatment of membrane vesicles with 6 mM *N*-acetylimidazole caused 80% inhibition of taurine uptake (B versus A). But, this inhibition was reduced to 30% after incubation with hydroxylamine (D versus C). It is clear from these results that hydroxylamine reverses the *N*-acetylimidazole-induced inhibition of taurine uptake.

Similarly, NBD-Cl-induced inhibition of taurine uptake should also be reversible with thiols such as 2-mercaptoethanol because the phenolic hydroxyl group of tyrosine residues which has been modified by NBD-Cl can be regenerated by thiols [17–19]. We therefore assessed the ability of 2-mercaptoethanol to reverse the NBD-Cl-induced inhibition of taurine uptake (Table II). NBD-Cl at 25 μM caused 70% inhibition of taurine uptake, but this inhibition was reduced to 43% after treatment with 10 mM 2-mercaptoethanol. Treatment of the membrane vesicles with 10 mM 2-mercaptoethanol

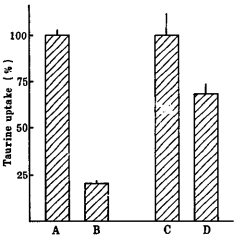

Fig. 3. Reversibility of *N*-acetylimidazole inhibition by hydroxylamine. Membrane vesicles were treated with 6 mM *N*-acetylimidazole for 1 h at room temperature at pH 7.4. After treatment, unreacted *N*-acetylimidazole was removed by dilution and centrifugation. These vesicles as well as the vesicles which were treated similarly but in the absence of *N*-acetylimidazole were then incubated for 1 h at 37°C with or without 150 mM hydroxylamine-HCl in 10 mM Hepes-Tris buffer, (pH 7.4). In each case, the vesicles were washed free of the reagent and preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol before used in uptake experiments. Uptake of taurine (0.3 μM) was measured with a 30 s incubation in the presence of an inwardly directed NaCl gradient. Uptake in vesicles which were treated with *N*-acetylimidazole alone (B) is expressed as percent of uptake in vesicles which were treated in the absence of *N*-acetylimidazole and hydroxylamine (A) (1.07 ± 0.03 pmol/mg protein per 30 s = 100 percent). Uptake in vesicles which were treated with *N*-acetylimidazole followed by hydroxylamine (D) is expressed as percent of uptake in vesicles which were treated with hydroxylamine alone (C) (0.63 ± 0.07 pmol/mg protein per 30 s = 100 percent). The results are given as the mean ± S.E. (n = 9, three membrane preparations).

alone did not have any deleterious effect on taurine uptake (1.58 ± 0.02 versus 1.56 ± 0.03 pmol/mg protein per 30 s).

TABLE II

Reversal of NBD-Cl-induced inhibition of taurine uptake by 2-mercaptoethanol

Membrane vesicles were treated with 25 μM NBD-Cl for 1 h at room temperature at pH 7.4. After treatment, the unreacted NBD-Cl was removed by dilution and centrifugation. These vesicles as well as the vesicles which were treated similarly but in the absence of NBD-Cl were then incubated at pH 7.4 for 30 min at room temperature with or without 10 mM 2-mercaptoethanol. In each case, the vesicles were then washed free of the reagent and preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol before used in uptake experiments. Uptake of 0.3 μM taurine was measured with a 30 s incubation in the presence of an inwardly directed NaCl gradient. The results are presented as the mean ± S.E. (n = 6).

Experimental condition	Taurine uptake	
	pmol/mg per 30 s	%
Control	1.58 ± 0.02	100
NBD-Cl	0.48 ± 0.01	30
2-Mercaptoethanol	1.56 ± 0.03	100
NBD-Cl + 2-mercaptoethanol	0.89 ± 0.01	57

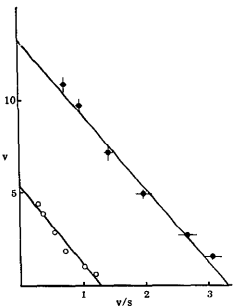

Fig. 4. Effect of *N*-acetylimidazole treatment on the influence of taurine concentration on the uptake. Membrane vesicles were treated with or without 6 mM *N*-acetylimidazole for 1 h at room temperature in 10 mM Hepes-Tris buffer (pH 7.4) containing 100 mM K_2SO_4. After treatment, the vesicles were washed free of the reagent and then preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 300 mM mannitol. Uptake of taurine was measured with a 15 s incubation in the presence of an inwardly directed NaCl gradient ($[NaCl]_o = 120$ mM; $[NaCl]_i = 0$). Concentration of taurine was varied between 0.5 and 15 μM. The results are given as Eadie-Hofstee plots (uptake rate/substrate concentration versus uptake rate). Each datum represents the mean ± S.E. ($n = 4$, two membrane preparations). Where not shown, the standard error is within the data point. *v*, uptake rate in pmol/mg protein per 15 s; *s*, taurine concentration in μM. ●——●, Control vesicles; ○——○, *N*-acetylimidazole-treated vesicles.

Effect of N-acetylimidazole treatment on the kinetics of taurine uptake

The kinetics of taurine uptake in control and *N*-acetylimidazole-treated membrane vesicles were studied to determine whether the *N*-acetylimidazole-induced inhibition was due to a reduction in the affinity of the transporter for taurine or to a decrease in the maximal velocity of the transporter. In these experiments, membrane vesicles were treated in the presence or absence of 6 mM *N*-acetylimidazole. After removal of the unreacted reagent, initial rates (15 s incubation) of taurine uptake were measured in control and treated membrane vesicles in the presence of an inwardly directed NaCl gradient ($[NaCl]_o = 120$ mM; $[NaCl]_i = 0$). The concentration of taurine was varied between 0.5 and 15 μM. The results in Fig. 4, given as Eadie-Hofstee plots (initial uptake rate/taurine concentration versus initial uptake rate), demonstrate that the uptake system obeyed Michaelis-Menten kinetics describing a single transport system in control as well as treated membrane vesicles (i.e., the plots were linear in both cases; $r^2 > 0.97$). The Michaelis-Menten constant (K_t) for the taurine uptake system was 4.0 ± 0.1 μM. But, the value for the maximal velocity (V_{max}) was markedly reduced in treated vesicles compared to control vesicles (5.4 ± 0.1 versus 13.3 ± 0.6 pmol/mg protein per 15 s).

Protection of the taurine transporter from N-acetylimidazole-induced inhibition by Na^+

Tyrosine residues have been shown to be located at or near the Na^+-binding site of several Na^+-dependent transport systems in small intestine, kidney [20–25] and placenta (unpublished data). In all cases, the transport activity is inhibited by treatment of purified brush-border membrane vesicles with tyrosine group-specific reagents, but the transporters can be protected from the inhibition by Na^+. Therefore, to determine whether the tyrosine residues which are critical for the transport function of the human placental taurine transporter are located at or near the Na^+-binding site of the transporter, we compared the *N*-acetylimidazole-induced inhibition of taurine uptake in membrane vesicles which were treated with the reagent in the presence of K^+ with the inhibition in vesicles treated with the reagent in the presence of Na^+. The rationale for this experiment was that if the critical tyrosine residues are located at or near the Na^+-binding site, Na^+ would mask these residues and prevent the chemical modification of these residues by *N*-acetylimdiazole. In order to ensure proper controls, two sets of experiments were performed. In one set, membrane vesicles were treated with or without 5 mM *N*-acetylimidazole in a medium containing 200 mM K_2SO_4. In another set, membrane vesicles were treated with or without 5 mM *N*-acetylimidazole in a medium containing 200 mM Na_2SO_4. In all cases, after incubation the reagent and salts were removed by dilution and centrifugation and the membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 600 mM mannitol. Uptake rates of taurine (0.3 μM) were measured with a 30 s incubation in these vesicles in the presence of an inwardly directed NaCl gradient ($[NaCl]_o = 240$ mM; $[NaCl]_i = 0$). Taurine uptake in membrane vesicles which were treated in the presence of Na_2SO_4 but in the absence of *N*-acetylimidazole were lower than the uptake in membrane vesicles which were treated in the presence of K_2SO_4 but in the absence of *N*-acetylimidazole (5.17 ± 0.28 versus 3.49 ± 0.12 pmol/mg protein per 30 s). This difference is most likely due to the presence of unwashed residual Na^+ within the vesicles treated with Na_2SO_4. This would result in a decrease in the magnitude of the Na^+ gradient across the membrane during uptake measurement and hence taurine uptake would be reduced. However, as seen in Table III, when the uptake rates of taurine in *N*-acetylimidazole-treated vesicles were compared with the uptake rates in respective controls, it was found that *N*-acetylimidazole treatment caused 44% inhibition in K_2SO_4 medium, but the inhibition was reduced to 33% in Na_2SO_4 medium. The

protection offered by Na^+ was small (25%), but statistically significant ($P < 0.001$) and highly reproducible ($n = 9$; three different membrane preparations).

Dependence of taurine uptake on Na^+ concentration

Placental taurine transporter exhibits an absolute requirement for Na^+ [8]. We therefore studied the dependence of the initial rates of taurine uptake on the concentration of external Na^+ (trans-zero-conditions) while keeping the external Cl^- concentration constant. This experiment allowed us to calculate the number of Na^+ ions involved per transport of one taurine molecule by a procedure described by Turner [26]. Since the experiment involves activation of the transporter by external Na^+, the procedure has been termed as 'activation' method [26]. The initial rates of taurine uptake increased with increasing concentrations of Na^+ (Fig. 5). The sigmoidal shape of the curve indicates that the taurine transporter interacts with more than one Na^+ ion. To determine the number of Na^+ ions interacting with the transporter, the data were fit to a Hill-type equation

$$v = \frac{V_{max}[Na^+]^n}{K'^n_{t(Na^+)} + [Na^+]^n}$$

and plots of v versus $v/[Na^+]^n$ were constructed for a Na^+ concentration range of 10–200 mM, assigning n a value of 1, 2 or 3. When $n = 1$ or 3, the plots were curvilinear (results not shown), but when $n = 2$, the plot was very close to a straight line ($r = -0.94$) (Fig. 5, inset). The exact value of n for the best fit to a straight line was 1.75 ($r = -0.99$). Thus, two Na^+ ions appear to be involved per transport of one taurine molecule in these vesicles.

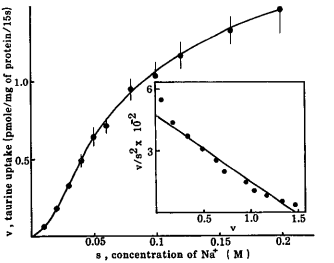

Fig. 5. Dependence of taurine uptake on Na^+ concentration. Membrane vesicles were preloaded with 10 mM Hepes-Tris buffer (pH 7.5) containing 600 mM mannitol. Uptake of 0.3 μM taurine was measured in these vesicles with a 15 s incubation in the presence of increasing concentrations of Na^+ (0–200 mM). Concentration of Cl^- was kept constant at 200 mM by the addition of KCl. Osmolality was maintained by varying the concentration of mannitol. The results are given as the mean ± S.E. ($n = 4$, two membrane preparations). Inset: Hill-type plot (v versus $v/[Na^+]^n$) with the value of $n = 2$.

TABLE III

Protection of the taurine transporter from N-acetylimidazole-induced inhibition by Na^+

Membrane vesicles were treated with or without 5 mM *N*-acetylimidazole in the presence of either 200 mM K_2SO_4 or 200 mM Na_2SO_4. After treatment, the vesicles were washed free of the reagent and salts by dilution and centrifugation. The washing procedure was repeated thrice, and the buffer used during washing was 10 mM Hepes-Tris (pH 7.0) containing 600 mM mannitol. The membranes were finally suspended in the same buffer. Uptake of 0.3 μM taurine was measured with a 30 s incubation in these vesicles in the presence of an inwardly directed NaCl gradient ($[NaCl]_o = 240$ mM; $[NaCl]_i = 0$). The data represent the mean ± S.E. ($n = 9$; three different membrane preparations).

Treatment condition	Taurine uptake (pmol/mg protein per 30 s)	Inhibition (%)
K^+	5.17 ± 0.28	
K^+ and *N*-acetylimidazole	2.91 ± 0.17	44
Na^+	3.49 ± 0.12	
Na^+ and *N*-acetylimidazole	2.33 ± 0.09	33 ($P < 0.001$)

Discussion

Tyrosine residues have been shown to be critical for the activity of many Na^+-dependent transport systems [20–25]. The taurine transporter of the human placental brush-border membrane also exhibits an absolute dependence on Na^+ and hence it was of interest to investigate the role of tyrosine residues in the catalytic activity of this transporter. We have employed in this study four reagents which are specific for tyrosine residues and they are *N*-acetylimidazole, NBD-Cl, PNBSF and tetranitromethane. Treatment of placental membrane vesicles with all four reagents resulted in inhibition of NaCl gradient-dependent taurine uptake in these vesicles. The integrity of the vesicles was not affected by the treatment as assessed by the measurement of the intravesicular volume. There is no evidence that these reagents increase ion permeabilities of the membrane vesicles because *N*-acetylimidazole did not alter the Na^+ uptake driven by a H^+ gradient via the Na^+–H^+ exchanger in human placental brush-border membrane vesicles [27] nor did NBD-Cl alter the Na^+ permeability in renal brush-border membrane vesicles [20]. The inhibition observed in the present investigation was re-

versible and occurred as a consequence of reagent-induced chemical modification of essential amino acid residues in the taurine transport protein. One of the major difficulties in interpreting the data from experiments involving group-specific reagents is the lack of absolute specificity of the reagents employed. NBD-Cl has been employed to modify tyrosine residues in enzymes and transport proteins [17,18,20,23,28,29] but this reagent can also interact with thiol groups [30,31]. Even though there is evidence that amino groups can also react with NBD-Cl [32,33], this reaction is seen only at alkaline pH and is almost negligible at neutral or near neutral pH values [33]. Since the placental brush-border membrane vesicles were treated with NBD-Cl at pH 7.4 in the present study, it is unlikely that amino groups were modified under these conditions. But, under similar experimental conditions, reaction with tyrosine [33] and sulfhydryl residues [30] is extensive. In both cases, the reaction can be reversed and the phenolic hydroxyl and sulfhydryl groups regenerated by subsequent incubation with free thiols [17–19,28]. It was found in the present investigation that the incubation of NBD-Cl-treated membranes with 2-mercaptoethanol caused significant reversal of the NBD-Cl-induced inhibition of the taurine transporter. Therefore, it is concluded from these results that the inhibition of taurine uptake seen in human placental membrane vesicles upon treatment with NBD-Cl is due to chemical modification of tyrosine and/or sulfhydryl groups of the trasnport protein.

Tetranitromethane is one of the most widely used reagents to modify tyrosine residues in proteins [34,35]. However, this reagent has also been shown to react with thiol groups [36,37]. Treatment of placental brush-border membrane vesicles with tetranitromethane resulted in effective inhibition of the taurine transporter, suggesting the involvement of tyrosine and/or sulfhydryl groups in the catalytic function of the transporter. PMSF, another tyrosine group-specific reagent [38], also caused drastic inhibition of taurine uptake in these membrane vesicles.

The results with the above-mentioned reagents thus suggest, but do not necessarily prove, that tyrosine residues are essential for the function of the placental taurine transporter. It would be most useful to have a reagent which is highly specific for tyrosine residues. Although such a reagent is not available yet, *N*-acetylimidazole, of all reagents, appears to be best suited for this purpose [14]. This reagent, to some extent, can also modify sulfhydryl and amino groups. However, there are ways to differentiate between the reactions of sulfhydryl, amino and phenolic hydroxyl groups with this reagent. *N*-Acetylimidazole acetylates sulfhydryl groups in proteins, but the products immediately hydrolyze in aqueous solutions [16,39]. Amino groups and phenolic hydroxyl groups are also acetylated by the reagent. But, hydroxylamine at neutral pH or mild alkaline conditions deacetylates phenolic hydroxyl groups [15,16] whereas deacetylation of amino groups by hydroxylamine requires hot acidic or alkaline conditions [39].

It was found in the present investigation that *N*-Acetylimidazole inhibited taurine uptake in placental brush-border membrane vesicles. The inhibition could be demonstrated even after washing the membranes two or three times indicating that the inhibition was not due to acetylation of sulfhydryl groups. Since the *N*-acetylimidazole-induced inhibition could be reversed with hydroxylamine under mild alkaline conditions, it is the acetylation of tyrosine residues, and not the acetylation of amino groups, which is responsible for the inhibition.

Table I shows that tetranitromethane and NBD-Cl were more potent than PMSF and *N*-acetylimidazole in inhibiting the taurine transporter. Whether the greater inhibitory potency of these two reagents is due to their capability to react with tyrosine as well as thiol groups is yet to be determined because there is no available data at present on the essentiality of thiol groups for the catalytic activity of the placental taurine transporter.

We also investigated the influence of the chemical modification of the critical tyrosine residues on the kinetic parameters of the uptake system. Modification of tyrosine residues with *N*-acetylimidazole resulted in a decrease in V_{max}, with no effect on K_t. These results are consistent with a reversible noncompetitive inhibition or an irreversible inactivation. Since the reagent is known to acetylate the phenolic hydroxyl group of tyrosine residues, it appears that such a modification irreversibly inactivates the transporter. To determine whether the tyrosine residues which are critical for the activity of the placental taurine transporter are located at or near the Na^+-binding site of the transporter, we compared the efficacies of *N*-acetylimidazole to inhibit the transporter activity when the treatment with the reagent was performed in the presence of K^+ or Na^+. The transporter was significantly protected from the inhibition by Na^+. These data suggest, but do not prove, that the essential tyrosyl residues are present at the Na^+-binding site. It is possible that these reactive residues are located at a site which is spatially distinct from the Na^+-binding site but whose conformation is influenced by the binding of Na^+ to the transporter.

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