Substrate protection against inactivation of the mammalian polyamine-transport system by 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide

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Mammalian polyamine transporters have not thus far been biochemically characterized. Since essential carboxy groups in the polyamine carrier might participate in the transport process, the ability of two different carbodi-imides to affect [³H]spermidine uptake was assessed in Chinese hamster ovary cells. Both the hydrophobic 1,3-dicyclohexylcarbodi-imide (DCC) and the more polar 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) irreversibly inhibited spermidine transport with EC₅₀ values of 11 ± 4 and 96 ± 16 μM after 30 min at 22 °C respectively. Prior treatment with EDC in the absence of substrate decreased both the V_{max} and K_m for spermidine uptake in a time- and concentration-dependent manner. Spermidine-transport inactivation by EDC (1 mM) was temperature-dependent, with 60 and 90 % inhibition observed after 10 min at 22 and 37 °C respectively. Spermine (10 μM) almost fully protected against spermidine-transport inactivation by EDC at 22 °C, and decreased the rate of inactivation at 37 °C by about 80 %. Putrescine, spermidine and spermine were all effective in protecting against EDC-

mediated inactivation of [³H]spermidine and [³H]putrescine uptake at 22 °C with EC₅₀ values estimated at 10, 1 and less than 1 μM respectively. The nucleophile glycine ethyl ester (up to 50 mM) prevented the inhibition brought about by 1 mM EDC. Inhibition by 1 mM EDC was greater at pH 7.2 than at pH 5.8 (89 ± 3 compared with 44 ± 5 %), whereas the converse was true for 100 μM DCC (81 ± 3 compared with 92 ± 5 %). On the other hand, spermine did not protect against inactivation of spermidine uptake by DCC. Moreover, DCC, but not EDC, inhibited Na⁺-dependent amino acid uptake. The present data indicate that (i) EDC and DCC inhibit polyamine transport through distinct mechanisms, (ii) substrate binding occludes one or several carboxy groups lying in a polar environment of the carrier and (iii) these carboxyl residues might be activated by EDC to cross-link a neighbouring nucleophile side group, resulting in a conformation of the polyamine carrier which is inactive for transport.

INTRODUCTION

Specific transport systems responsible for the high-affinity uptake of putrescine and the natural polyamines are ubiquitously found in prokaryotes and eukaryotes [1,2]. Separate plasma-membrane carriers belonging to the ATP-binding cassette transporter family and with relative specificity for inward transport of spermidine [3] and putrescine [4] have been molecularly characterized in *Escherichia coli*. On the other hand, there is virtually no information on the structural features of eukaryotic polyamine transporters. In mammalian cells, polyamine-transport activity increases in response to various hormones [1,5,6] or after cell transformation [1,7], and is negatively regulated by intracellular polyamine levels [1,6,8]. The elevated polyamine uptake found in tumour cells is a major factor in the reduction of the efficacy *in vivo* of drugs targeted towards polyamine biosynthesis, such as the suicide inhibitor of ornithine decarboxylase, α-difluoromethylornithine [9,10].

Because of the polycationic characteristics of putrescine, spermidine and spermine, aspartate or glutamate residues might stabilize carrier–substrate interactions during the transport process. Carbodi-imides can be used as probes of the function of glutamate and aspartate residues since they readily react with protein carboxy groups [11–14]. Among other factors, the reactivity of carboxy groups toward various carbodi-imides is a function of their microenvironment. Thus 1,3-dicyclohexylcarbodi-imide (DCC), a hydrophobic carbodi-imide, has often been used to modify cation-transport ATPases [12,15–17] such as

bovine F₁F₀-ATPase, in which DCC reacts with a single acidic amino acid residue buried deep within the membrane [18]. On the other hand, more polar compounds such as 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) cannot modify carboxy groups present in hydrophobic domains, but can often react with residues more exposed to the solvent that are poorly reactive toward DCC [13,19].

In order to probe key biochemical features of the mammalian polyamine carrier, we have compared the effect of DCC and EDC on spermidine transport in Chinese hamster ovary (CHO) cells, as well as the ability of substrates of this carrier system to prevent inactivation by these agents in intact cell monolayers. We report that both EDC and DCC inhibit polyamine uptake in this system, although only modification by EDC can be antagonized by the simultaneous presence of polyamines at concentrations compatible with their affinity as substrates for transport. These data provide evidence that substrate binding to the polyamine carrier occludes solvent-exposed residues that are essential for the internalization function of the mammalian polyamine carrier.

MATERIALS AND METHODS

Cell culture and reagents

CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and routinely grown in α-minimal essential medium (Gibco/BRL Life Technologies, Burl-

Abbreviations used: DCC, 1,3-dicyclohexylcarbodi-imide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; CHO cells, Chinese hamster ovary cells; GEE, glycine ethyl ester; AIB, α-aminoisobutyrate.

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ington, Ont., Canada) supplemented with 10% (v/v) Cosmic® calf serum (Hyclone, Logan, UT, U.S.A.) in a 5% CO₂ atmosphere at 37 °C. [³H]Spermidine (1.53×10^4 Ci/mol), [³H]putrescine (3.0×10^4 Ci/mol) and α -amino[³H]isobutyric acid ([³H]AIB) (3.0×10^4 Ci/mol) were obtained from Dupont–New England Nuclear (Boston, MA, U.S.A.). Glycine ethyl ester (GEE) was obtained from Aldrich. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, *sym*-norspermidine, AIB, DCC and EDC were obtained from Sigma (St. Louis, MO, U.S.A.). Stock solutions of DCC and EDC were prepared freshly before use in DMSO and water respectively.

Carbodi-imide inactivation of polyamine uptake in intact cell monolayers

CHO-K1 cells grown in 24-well tissue culture plates to 80% confluence were washed twice with 1 ml of ice-cold PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 137 mM NaCl) before incubation with carbodi-imides. In experiments designed to assess protection by substrate from carbodi-imide-mediated inactivation, cell monolayers were preincubated for 10 min at 37 or 22 °C in Tris buffer (20 mM Tris/HCl, pH 7.4, 103 mM NaCl, 11 mM D-glucose, 5.4 mM KCl, 0.42 mM CaCl₂ and 0.41 mM MgCl₂) containing the indicated concentration of putrescine or polyamines before carbodi-imide addition. Carbodi-imides were then added to cell cultures in 400 μ l of Mes buffer (20 mM Mes/NaOH, pH 7.2, 103 mM NaCl, 11 mM D-glucose, 5.4 mM KCl, 0.42 mM CaCl₂ and 0.41 mM MgCl₂) [20] in the presence or absence of putrescine or polyamines, and cells were incubated at 22 °C or 37 °C for the indicated time. Wells were washed twice with 1 ml of cold PBS, and polyamine uptake was determined as described below.

When GEE was used as an exogenous nucleophile, cells were successively incubated with GEE for 10 min and then with 1 mM EDC for 30 min, or incubated for 30 min with both reagents in Mes buffer at 22 °C. Osmolality was kept constant by reducing the NaCl concentration in Mes and Tris buffers by an amount osmotically equivalent to the GEE concentration used.

Assay of [³H]spermidine, [³H]putrescine and [³H]AIB transport

After incubation with carbodi-imides, cell monolayers were washed twice with 1 ml of PBS; 400 μ l of Tris buffer containing 5 μ M [³H]spermidine or [³H]putrescine (each at 20 Ci/mol) was then added. Kinetic analysis of spermidine transport was similarly performed using 0.1 μ M [³H]spermidine plus increasing amounts of unlabelled spermidine. Standard incubation with radiolabelled substrate was carried out for 20 min at 37 °C, and then stopped by the addition of 1 ml of ice-cold PBS containing 5 mM *sym*-norspermidine (stop solution). The solution was aspirated, and cell monolayers were rinsed again twice with 1 ml of ice-cold stop solution. NaOH (1 M; 200 μ l) was then added and the cells were solubilized for 30 min at 60 °C. Radioactivity was determined from an aliquot of the homogenate after neutralization with 200 μ l of 1 M HCl. Protein content was determined [21] in cell homogenates obtained under identical conditions from parallel wells incubated in radiolabel free uptake medium, with BSA albumin (fraction V) as standard.

[³H]AIB-uptake activity was similarly determined in a 20 min assay in 400 μ l of Tris buffer containing 200 μ M [³H]AIB (30 Ci/mol). [³H]AIB-uptake was stopped by the addition of 1 ml of ice-cold PBS, and intracellular radioactivity was determined as outlined above after the monolayers had been rinsed twice with cold PBS. Na⁺-independent AIB uptake was measured in parallel by replacing NaCl with an equimolar concentration of choline chloride, and the Na⁺-dependent component of AIB

uptake was determined after subtracting Na⁺-independent from total [³H]AIB uptake.

Statistical analysis

Unless otherwise indicated, results are expressed as the mean $\pm$ S.D. for determinations from triplicate cell cultures. Statistical significance of differences between means was assessed by unpaired Student's *t* tests.

RESULTS

Effect of carbodi-imides on spermidine uptake in CHO cells

To evaluate the effect of carbodi-imides on spermidine transport in intact CHO cells, the relative abilities of DCC and the more polar EDC to inhibit subsequent spermidine uptake after a 30 min preincubation were first compared (Figure 1). DCC was clearly more potent than EDC in inactivating [³H]spermidine uptake, with EC₅₀ of 11.2 ± 4.1 and 96 ± 16 μ M respectively. Maximal inactivation for DCC was observed near its limit of solubility in water (100 μ M).

Inactivation of [³H]spermidine-uptake activity by EDC was reflected by an exponential decrease in the $V_{\max}$ with respect to time (Figure 2A) and carbodi-imide concentration (Figure 2B) (down to $88 \pm 3\%$ of the control value after 30 min of exposure to 1 mM EDC). Unexpectedly, however, the apparent K_m also decreased logarithmically to about 30% of the control value after a 30 min preincubation with 1 mM EDC.

Protection against carbodi-imide-mediated inactivation of spermidine transport by spermine

Useful information on the site(s) of action of protein-modifying reagents can be derived by protection experiments, in which the ability of substrates to prevent chemical modification of their cognate protein is assessed. We thus determined the effect of the simultaneous presence of spermine (10 μ M) during incubation with carbodi-imide on subsequent [³H]spermidine uptake activity

Figure 1 Concentration-dependence of spermidine-transport inactivation by carbodi-imides in intact CHO cell monolayers

Cells were treated with the indicated concentrations of EDC (○) or DCC (●) for 30 min at 22 °C before determination of the rate of [³H]spermidine uptake under standard conditions (see the Materials and methods section). Values represent the mean $\pm$ S.D. for triplicate determinations from three independent experiments.

Figure 2 Effect of EDC on the kinetic parameters of $[^3\text{H}]$ spermidine uptake

CHO cells were incubated under polyamine-free conditions (A) with 1 mM EDC for the time indicated at 22 °C or (B) with the indicated concentration of EDC for 30 min at 22 °C, before determination of the V_{max} (○) and K_m (●) for $[^3\text{H}]$ spermidine uptake in the absence of inhibitor by Lineweaver–Burk analysis. Values represent the mean $\pm$ S.D. for triplicate determinations.

Figure 3 Effect of spermine on the time course of EDC inactivation of spermidine uptake

CHO cells were treated with 1 mM EDC at 22 °C (A) or 37 °C (B) for the indicated period in the absence (○) or presence (●) of 10 μM spermine before measurement of spermidine uptake under standard conditions. Values represent the mean $\pm$ S.D. for triplicate determinations.

(Figure 3). Since carbodi-imide-coupled reactions are steeply temperature-dependent [12,20], incubations were performed at both 22 and 37 °C, and uptake was determined after various times of exposure to EDC. Indeed, maximal inactivation of spermidine transport by EDC (83 %) was observed after 20 min at 22 °C (Figure 3A), whereas an 88 % inhibition had already occurred after a 10 min incubation at 37 °C (Figure 3B). It is notable that spermine almost completely prevented the irreversible inhibition of $[^3\text{H}]$ spermidine uptake activity by EDC at 22 °C, and decreased the rate of carbodi-imide-mediated inactivation by 80 % or more at 37 °C.

Concentrations of spermine required to prevent inhibition of spermidine transport by EDC were consistent with its interaction

Figure 4 Concentration-dependence for protection of spermidine transport by spermine against carbodi-imide-mediated inactivation

CHO cells were incubated for 30 min with 1 mM EDC at 37 °C (○), 1 mM EDC at 22 °C (●) or 100 μM DCC at 37 °C (□) in the presence of the indicated concentrations of spermine before determination of spermidine uptake under standard conditions. Values represent the mean $\pm$ S.D. for triplicate determinations from three independent experiments.

as a substrate of this transport system in CHO cells ($K_m \approx 1.8 \mu\text{M}$ [22] (Figure 4). Thus a half-maximal protection against a 30 min inactivation reaction with EDC at both 22 and 37 °C was obtained at about 0.5 and 0.9 μM spermine respectively. In contrast with inhibition by EDC, no significant ($P > 0.25$) protection against inactivation by 100 μM DCC was afforded by spermine (up to 100 μM ; Figure 4). The lack of protection by spermine against transport inhibition by DCC was not due to a difference in the rate of inactivation between EDC and DCC, since the kinetics of inhibition of spermidine-uptake activity by the two carbodi-imides at 22 °C and 37 °C were very similar (results not shown).

Relative potency of substrates of the polyamine-transport system for protection against EDC-mediated inactivation

If spermine protects the polyamine-transport system against inhibition by EDC by occluding the site(s) of modification on the carrier, one would predict that other substrates of the transport system would prevent inhibition with a relative potency paralleling their respective affinity for uptake. Indeed, Figure 5(A) shows that, in addition to spermine, putrescine and spermidine were also effective in protecting against EDC-mediated inactivation of $[^3\text{H}]$ spermidine transport, with EC_{50} values estimated at 9.9 ± 2.0 and $0.90 \pm 0.22 \mu\text{M}$ respectively. However, spermidine maximally protected only 56 % of spermidine transport activity, as compared with the preservation of 84 % of uptake activity by spermine. Interestingly, the relative abilities of putrescine, spermidine and spermine to protect against EDC-mediated inactivation of spermidine transport were consistent with their relative affinities for the polyamine-transport system in CHO cells (7.0, 3.7 and 1.8 μM respectively) [22]. Furthermore $[^3\text{H}]$ putrescine transport was also strongly inactivated by EDC, and co-incubation with putrescine, spermidine and spermine protected diamine-uptake activity against this inhibition at approximate EC_{50} values of 6 ± 2 , 0.8 ± 0.3 and $0.6 \pm 0.2 \mu\text{M}$ respectively (Figure 5B). In contrast with their relative effects on $[^3\text{H}]$ spermidine-uptake activity, maximal protection of putrescine transport by spermine (62.7 ± 4.8 % of control levels) against EDC-mediated inactivation was significantly ($P < 0.05$) lower than that observed with putrescine or spermidine (78.4 ± 4.3 and 78.0 ± 5.2 % respectively).

Figure 5 Protection of [^3H]spermidine- and [^3H]putrescine-uptake activities by various substrates against EDC-mediated inactivation

CHO cells were treated for 30 min with 1 mM EDC at 22 °C in the presence of the indicated concentrations of putrescine (○), spermidine (●) or spermine (□) before determination of the rate of uptake of (A) [^3H]spermidine and (B) [^3H]putrescine under standard conditions. Values represent the mean $\pm$ S.D. from triplicate determinations.

Table 1 Effect of pH on carbodi-imide inhibition of [^3H]spermidine uptake

Before the uptake assay, cells were treated with the indicated carbodi-imide at pH 5.8 or pH 7.2 in Mes buffer for 30 min. Cell monolayers were washed twice with PBS, and [^3H]spermidine uptake activity was then determined. * $P < 0.01$, ** $P < 0.05$ compared with value at pH 5.8.

Carbodi-imide	Spermidine uptake (% control)	
	pH 5.8	pH 7.2
EDC (1 mM)	56.4 $\pm$ 5.3	10.7 $\pm$ 3.0*
DCC (100 μM)	7.8 $\pm$ 5.3	19.0 $\pm$ 3.0**

Differential pH-dependence of inactivation of spermidine transport by EDC and DCC

Carbodi-imide-coupled reactions involving carboxylic acids in solution are favoured at pH values close to the pK_a of the reactive groups involved, since the protonated carboxy group is believed to be the reactive species [12]. Thus, if EDC-mediated inactivation of the polyamine carrier only involved the irreversible modification of carboxy groups through the formation of *N*-acylureas [12,13], one would expect an increased inhibition of [^3H]spermidine uptake if the modification reaction was carried out at a lower pH. Preliminary experiments indicated that subsequent polyamine uptake remained unaffected by the exposure of CHO-K1 cells to Mes buffer down to pH 5.8 for 30 min at 22 °C (results not shown). We thus compared the extent of inactivation of spermidine transport after a 30 min incubation with maximally

Figure 6 Prevention of EDC-mediated inhibition of [^3H]spermidine transport by GEE

CHO cells were incubated for 30 min with 1 mM EDC and the indicated concentrations of GEE at 22 °C with (●) or without (○) a 10 min preincubation period at 22 °C with GEE, before determination of spermidine-uptake activity under standard conditions. Values represent the mean $\pm$ S.D. from triplicate determinations.

effective concentrations of carbodi-imides (1 mM EDC or 100 μM DCC) at pH 5.8 and 7.2 in order to assess this hypothesis.

Interestingly, the irreversible inactivation of spermidine-transport activity by EDC was much greater when the modification reaction was carried out at pH 7.2 than at 5.8 (44 ± 5 compared with 89 ± 3 % inhibition respectively; $P < 0.01$) (Table 1). On the other hand, lowering the pH had the expected effect on the DCC-mediated inactivation (81.0 ± 3.0 and 92.2 ± 5.3 % inhibition at pH 7.2 and 5.8 respectively; $P < 0.05$). In time-course experiments not shown here, the half-life of spermidine uptake activity in the presence of 100 μM DCC was determined to be 6.7 min at pH 5.8, as compared with 10.9 min at pH 7.2. Thus pH reproducibly influenced the rate of inactivation of the spermidine carrier activity by DCC in this system, albeit to a lower extent and in a manner diametrically opposed to the corresponding effect on the EDC-mediated reaction. The striking qualitative differences between the two carbodi-imides in their pH-dependence for the inhibition of polyamine transport reinforce the notion that EDC and DCC inhibit carrier activity through distinct mechanisms.

Effect of GEE on EDC inhibition of [^3H]spermidine uptake

Nucleophiles such as GEE have been used to probe the nature of the functional groups involved in modifications catalysed by carbodi-imides [16,23]. A lysine residue close to the EDC modification site on the polyamine carrier could behave as an endogenous nucleophile and participate in a zero-length cross-linking reaction to an acidic residue [13,23]. Addition of an exogenous nucleophile should provide a competing substrate for that potential modification and prevent inactivation of the carrier by EDC if irreversible inhibition is due to zero-length cross-linking. Indeed, GEE relieved up to 30–35 % of spermidine transport inhibition exerted by 1 mM EDC at 22 °C whether the nucleophile was added before or simultaneously with EDC (Figure 6).

Effect of DCC and EDC on [^3H]AIB uptake

Since several other plasma-membrane transport systems are known to be modified by carbodi-imides [24–26], we next

Table 2 Effect of pretreatment with EDC and DCC on Na⁺-independent and -dependent [³H]AIB uptake in CHO cells

CHO cells were preincubated with the indicated concentration of carbodi-imide for 30 min at 22 °C before the determination of Na⁺-independent and Na⁺-dependent [³H]AIB uptake as described in the Materials and methods section. Values represent the mean $\pm$ S.D. for triplicate determinations. * $P < 0.05$, ** $P < 0.01$ compared with untreated cells.

Pretreatment	[³ H]AIB uptake (nmol/h per mg of protein)	
	Na ⁺ -independent	Na ⁺ -dependent
None	13.5 $\pm$ 1.1	83.1 $\pm$ 14.7
100 μ M EDC	14.9 $\pm$ 1.2	64.2 $\pm$ 2.7
300 μ M EDC	15.3 $\pm$ 1.0	61.6 $\pm$ 5.2
1000 μ M EDC	16.0 $\pm$ 1.0	68.3 $\pm$ 2.9
10 μ M DCC	12.9 $\pm$ 1.1	56.7 $\pm$ 5.2*
30 μ M DCC	11.2 $\pm$ 1.1	44.6 $\pm$ 4.5**
100 μ M DCC	4.9 $\pm$ 1.2**	22.6 $\pm$ 2.9**

compared the relative ability of EDC and DCC pretreatment to modify the subsequent uptake of AIB, which is chiefly a substrate of the Na⁺-dependent amino acid-transport system found in CHO cells [27]. DCC inactivated Na⁺-dependent [³H]AIB uptake in a concentration-dependent manner, whereas the Na⁺-independent component was affected only at the maximal concentration used (100 μ M) (Table 2). On the other hand, EDC pretreatment had virtually no effect on either Na⁺-dependent or -independent [³H]AIB-uptake activity even at a maximal concentration of 1 mM.

DISCUSSION

The present data provide strong evidence that amino acid residues critically important for the internalization of both putrescine and spermidine are located in a solvent-accessible exposed domain of the polyamine transporter that becomes occluded on substrate binding. To our knowledge, this is the first identification of a substrate-protected chemically modifiable domain of a eukaryotic polyamine carrier. Previous reports have pointed to the presence of thiol groups functionally important for polyamine transport, as determined with thiol-reactive agents such as *N*-ethylmaleimide [1,28]. However, we have found no protection by substrates against inhibition of polyamine-uptake activity by such agents (K. Torossian, M. Audette and R. Poulin, unpublished work), and whether the inactivation observed directly involves the polyamine carrier remains uncertain.

Natural substrates of the polyamine-transport system (putrescine, spermidine and spermine) afforded protection of both putrescine- and spermidine-uptake processes against EDC-mediated inhibition with absolute as well as relative potencies consistent with their known affinity for the polyamine-transport system in CHO cells [22]. These observations thus strongly favour the view that inactivation of polyamine transport by EDC primarily involves chemical modification of residues present on the carrier itself. Interestingly, spermine afforded better protection of spermidine transport against EDC-mediated inactivation than putrescine. It is as yet unclear whether this reflects the presence of distinct carriers for putrescine and spermidine uptake. On the basis of kinetic differences, some groups have proposed the existence of at least two different types of mammalian transporters with different specificity for putrescine and polyamine uptake [1,22,29], but support for a single class of

diamine/polyamine transporter has also been presented [5,6,30]. Notwithstanding these uncertainties, the structure of the substrate interacting with the carrier(s) involved in putrescine and spermidine transport clearly influences the reactivity toward EDC of critical side groups that are differentially required for the specific uptake of these two substrates.

Carbodi-imides can react not only with carboxylic acids, but with other side groups such as thiols and phenols. However, only in the case of carboxy groups can nucleophilic reagents modify the rate of chemical inactivation by carbodi-imides [11,12]. Thus the observation that high concentrations of GEE could partly block inactivation of the spermidine transporter by EDC indicates that aspartate and/or glutamate residues are indeed the reactive species involved. On the other hand, the fact that inactivation of spermidine uptake by EDC was strongly enhanced by increasing pH from 5.8 to 7.2 cannot be explained by the classical low pH-dependence of carbodi-imide reactions [12]. This suggests that carbodi-imide attack on carboxylic residues is not the rate-limiting step for polyamine-transport inhibition by EDC. Carboxyl modification by carbodi-imides proceeds through the formation of an unstable *O*-acylurea adduct of the protonated carboxy group and subsequent rearrangement leading to (i) regeneration of the original carboxy group through hydrolysis, (ii) formation of a stable *N*-acylurea or (iii) cross-linking with an added or neighbouring nucleophile such as a lysyl ϵ -amino group [11–13,16,31,32]. Reversal of EDC-mediated inactivation of spermidine uptake by GEE might be best explained by the third mechanism, whereby GEE would compete for carbodi-imide-activated cross-linking with a nucleophilic group present on the polyamine carrier itself (Figure 7). Thus, the internal cross-linking between a carboxyl residue and an adjacent nucleophilic side group catalysed by EDC might lead to an inactive conformation of the polyamine transporter (Figure 7C), whereby amide formation between the same carboxy group and an added free nucleophile such as GEE would preserve a substantial fraction of transport activity (Figure 7D). Although the strong pH-dependence between 5.8 and 7.2 would appear to rule out a lysine side chain as the nucleophilic group involved in a 'zero cross-link' reaction, abnormally low p*K*_a values for lysine ϵ -amino groups can result from interaction with neighbouring carboxyl side chains [33]. It is also possible that the anomalous pH-dependence of EDC inactivation reflects a protonation-dependent change in conformation of the polyamine carrier resulting in a differential accessibility of the reactive carboxy groups.

EDC-mediated inactivation mostly affected the $V_{\max}$ of spermidine transport, but also paradoxically reduced its K_m by almost 4-fold. This pattern of inhibition suggests that the carboxy groups attacked by EDC are not critically involved in substrate recognition. This situation would arise if EDC specifically modifies carboxyl side chains residing in a polar environment but outside the polyamine-binding domain. In that case, substrate binding might induce a conformational change leading to the occlusion of the carboxy groups targeted by EDC and thus decrease the rate of carbodi-imide modification. The steady decrease in the apparent K_m for spermidine uptake induced by EDC might be interpreted as reflecting either (i) the preferential inactivation of a major subpopulation of polyamine carrier(s) with a slightly lower affinity for spermidine or (ii) a change in conformation of the polyamine carrier into a form that is inactive for the internalization process, but which binds substrate with an increased affinity. The current data do not allow discrimination between these two possibilities.

In marked contrast with EDC, DCC potently inactivated spermidine transport without protection by the substrate, while

Figure 7 Hypothetical scheme for the inactivation of the polyamine transporter by EDC

(A) The carbodi-imide EDC reacts with the carboxy side group of at least one aspartate or glutamate residue in a solvent-accessible domain of the transporter outside the substrate-binding domain, forming an *O*-acylurea adduct in (B). This unstable intermediate promotes (C) the formation of a 'zero cross-link' with a nucleophilic residue, such as an internal amide with the ϵ -amino group of a neighbouring lysine residue, leading to an inactive conformation of the carrier or (D) reaction to an amide with an added free nucleophile such as GEE, which preserves a substantial fraction of the transport activity.

exhibiting enhanced reactivity with a decrease in pH characteristic for carboxy group modification by carbodi-imides [11,12]. It is thus clear that these reagents inactivate polyamine transport through widely different mechanisms. Lack of cross-reactivity between DCC and hydrophilic carbodi-imides with given peptide residues is not uncommon [13,25] and essentially reflects the basic differences in the hydrophobicity of these reagents. Such divergent reactivities is also shown here by the lack of sensitivity of Na^+ -dependent AIB uptake toward EDC, but its potent inactivation by DCC. It cannot be ascertained from the present data whether DCC inactivates spermidine transport via a direct modification of the transporter. In this context, DCC is a well-known inactivator of cellular cation ATPases [14], including the Na^+/K^+ -ATPase [16,17]. Inactivation of the latter by DCC might indeed be directly responsible for the inhibition of Na^+ -dependent AIB transport [34]. However, the profound and rapid suppression of spermidine transport by DCC cannot be solely attributed to inactivation of Na^+/K^+ -ATPase, since ouabain has a much more limited effect on spermidine uptake [1,29,35].

In conclusion, we have identified at least one unique carboxyl side chain on the mammalian polyamine transporter that can specifically react with a water-soluble carbodi-imide, subsequently resulting in its apparent cross-linking with a neighbouring nucleophile and inactivation of carrier function. This site resides in a polar environment distinct from the polyamine-binding

domain of the transporter, and its accessibility is masked by a conformational change brought about by substrate binding to the polyamine carrier. A very similar mechanism of modification by EDC has been postulated for the dog kidney Na^+/K^+ -ATPase [16]. Another essential but more hydrophobic site is modified by DCC but its direct association with the polyamine transporter is as yet uncertain. Identification of the residues modified by carbodi-imides on the polyamine carrier will await the purification and reconstitution of this membrane transporter. Nevertheless, the present data suggest a novel approach for specifically labelling the mammalian polyamine transporter by chemical modification. Thus, by using an appropriate radiolabelled nucleophile such as [^{14}C]GEE, it should be possible to detect specific labelling of the transporter by comparing the pattern of membrane proteins labelled by the nucleophile via EDC activation in the presence or absence of substrate protection.

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