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INHIBITION OF SODIUM-DEPENDENT TRANSPORT SYSTEMS IN RAT RENAL BRUSH-BORDER MEMBRANES WITH N,N'-DICYCLOHEXYLCARBODIIMIDE

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Treatment of rat renal brush-border membrane vesicles with N,N'-dicyclohexylcarbodiimide (DCCD) causes irreversible inhibition of the Na^+-coupled transport systems for D-glucose, L-phenylalanine, L-glutamate, and sulfate. The DCCD-reactive side groups of these transport systems differ in their sensitivity towards DCCD and protection by substrates. The D-glucose and L-glutamate transporters cannot be protected by their substrates. In contrast, Na^+ protects the transport systems for L-phenylalanine and sulfate from inactivation by DCCD. The data suggest covalent modification by DCCD of D-glucose and L-glutamate transporters apart from their substrate binding sites and of L-phenylalanine and sulfate transporters within their Na^+-binding regions.

N,N'-dicyclohexylcarbodiimide (DCCD) which reacts with mercapto-, tyrosyl-, or carboxyl groups in a hydrophobic environment is a well known inhibitor of proton translocating ATPases, Ca^{++}-ATPases and $(Na^+ + K^+)$ATPases (1,2). Carbodiimides were also found to irreversibly inhibit the Na^+/H^+ antiporter from rat and rabbit renal proximal tubules (3-8). Since the Na^+/H^+ antiporter could be protected by Na^+ from irreversible inactivation, DCCD must have modified the transport system at a side group closely related to cation exchange. In our previous studies (4) we noticed that DCCD not only reacted with the rat renal Na^+/H^+ exchanger but also inhibited irreversibly the Na^+-coupled transport systems for hexoses, neutral, acidic and basic amino acids, tricarboxylic acid cycle intermediates, and sulfate. A similar inhibition was reported for the Na^+-dependent phosphate transport system in rat renal membranes (7) and for the Na^+-coupled transport systems for hexoses and neutral amino acids in rabbit small intestinal membranes (9). These results could indicate that DCCD reacts with a Na^+-binding side group common to several Na^+-substrate cotransport systems. To further explore this possibility we performed substrate protection experiments on four representative cotransport systems present in rat renal brush-border membrane vesicles: the hexose carrier (test substrate: D-glucose), the transporters for neutral and acidic

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amino acids (test substrates: L-phenylalanine and L-glutamate, respectively) and the Na^+-sulfate cotransporter.

METHODS

Preparation of membrane vesicles: Brush-border membrane vesicles were prepared from the kidneys of male 180-250 g Wistar rats by a Mg^{++} precipitation technique (10). The vesicles were loaded with 150 mM KCl, 5 mM Hepes/Tris, pH 7.4, and stored at a protein concentration of 10 mg/ml in liquid nitrogen. Protein was determined according to Bradford (11) using bovine serum albumin as a standard.

Irreversible inhibition of the transport systems: Aliquots of frozen brush-border membrane vesicles (0.1 ml containing 1 mg protein) were quickly thawed in a 37°C water bath and diluted threefold with appropriate buffers. The composition of these buffers is indicated in the figure legends. All buffers had the same osmolarity and ionic strength. After an equilibration period of 30 min the membrane vesicles were incubated with 0.5 mM DCCD (final concentration) at room temperature for 15 min. The reaction was stopped by dilution of the suspension into 30 ml of 150 mM KCl, 5 mM Hepes/Tris, pH 7.0, and centrifugation at 20,000 rpm in a Sorvall SS 34 rotor for 20 min.

Transport studies: The pellets were taken up in 150 mM KCl, 5 mM Hepes/Tris, pH 7.0. Aliquots were diluted 10 fold for 15 s or 60 min into buffers containing 150 mM NaCl, 5 mM Hepes/Tris, pH 7.0, and 10 µM of the labeled substrates. Uptake of substrates was determined by the rapid filtration technique (12).

Materials: All chemicals were of analytical grade. DCCD was obtained from Aldrich, Steinheim, FRG. D-[^{3}H]glucose, L-[^{3}H]glutamate, L-[^{3}H]phenylalanine, and [^{35}S]sulfate were from NEN/Du Pont, Dreieichenhain, FRG.

RESULTS AND DISCUSSION

D-Glucose transport. To determine the effect of DCCD on Na^+-dependent D-glucose uptake, rat renal brush-border membrane vesicles were preincubated with 0.5 mM DCCD for 30 min at room temperature. After removing unbound DCCD by washing the uptake of labeled D-glucose at 15 s and 60 min incubation time was determined in the presence of a Na^+ concentration difference ($[Na^+]_{out} > [Na^+]_{in}$). In accordance with earlier studies (13-15) this Na^+ concentration difference causes a transient accumulation of D-glucose in the vesicles as shown in Figure 1: uptake of D-[^{3}H]glucose into vesicles pretreated without DCCD (150mM KCl, -DCCD) at 15 s incubation time exceeds by a factor of 4 the uptake measured at 60 min, the presumed equilibrium. If vesicles were pretreated with DCCD, the 15 s uptake of D-glucose is reduced by 42%. D-glucose uptake at equilibrium is hardly changed ruling out the possibility that DCCD altered the intravesicular space and thereby non-specifically reduced 15 s D-glucose uptake. The data therefore indicate a covalent modification of the Na^+/D-glucose cotransporter by DCCD leading to an irreversible inhibition of substrate uptake. Comparable findings have been obtained previously with renal (4,7,16) and small intestinal brush-border membrane vesicles (9).

Covalent modification of the Na^+/hexose cotransporter by DCCD could occur directly at or apart from the binding sites for substrates (Na^+, sugar). If

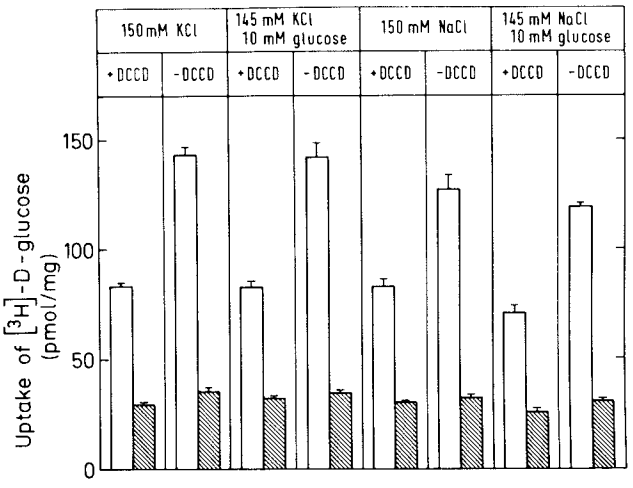

Figure 1. Irreversible inhibition of Na^+-dependent D-glucose transport by DCCD. Lacking effect of substrates. Eight aliquots of rat renal brush-border membrane vesicles containing 1 mg protein each were preincubated with 0.5 mM DCCD (+DCCD) or without DCCD (-DCCD) for 15 min in buffers containing 5 mM Hepes/Tris, pH 7.4, and salts and D-glucose as indicated above the columns. After washing and loading with 150 mM KCl, 5 mM Hepes/Tris, pH 7.4, the vesicles were incubated for 15 s (open columns) or 60 min (hatched columns) in buffers containing 150 mM NaCl, 5 mM Hepes/Tris, pH 7.4, and 10 µM D-[^{3}H]-glucose. The data represent means ± SD of four determinations.

the covalent modification occurs at the substrate binding sites, Na^+ and/or D-glucose should protect the transport system from inactivation. To test for possible protection, the vesicles were exposed to DCCD in the simultaneous presence of D-glucose, or of Na^+, or of both. Control vesicles were treated similarly, but in the absence of DCCD (-DCCD in Figure 1). It is obvious from an inspection of the data shown in Figure 1 that neither D-glucose, nor Na^+, nor both substrates together could restore 15 s D-glucose uptake in DCCD-treated vesicles to the levels reached in the respective controls. The different pretreatments had also no effect on the equilibrium uptake of D-glucose. Thus it is unlikely that non-specific changes in intravesicular space may have masked a possible protecting effect of Na^+ and D-glucose.

The failure of Na^+ and D-glucose to protect from irreversible inactivation indicates that DCCD binds to the transporter protein apart from the substrate binding sites. Thereby, DCCD binding must lead to a conformational change, e. g. by intra- or intermolecular crosslinking which hinders either the binding or the translocation of the substrates. Working with small intestinal brush-border membranes Weber et al. (9) also found no protection by D-glucose and Na^+ from inactivation of the hexose carrier by DCCD. Turner (16), on the other hand, found a protection of the renal D-glucose transport system by all sugars that are accepted by the system, and by phlorizin. In this study, however, a different carbodiimide, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), was used which may react with side groups not attacked by

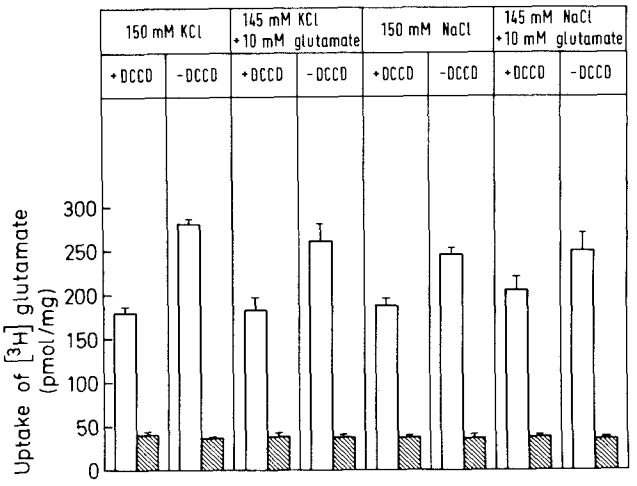

Figure 2. Effects of DCCD and substrates on Na^+-dependent L-glutamate transport in rat renal brush-border membrane vesicles. The experimental conditions were similar to those detailed in the legend of Figure 1 except that D-glucose was replaced by L-glutamate.

DCCD. Na^+ did not afford protection in Turner's experiments (16) similar to our investigations with DCCD. Thus it seems unlikely that DCCD-reactive side groups are involved in binding and transport of Na^+ and hexoses. Available evidence suggests that sodium ions are bound to a tyrosyl residue within the renal and small intestinal Na^+/D-glucose cotransporter (17,18). For the small intestinal Na^+/D-glucose amino groups have been shown to be located at the hexose binding site (19).

L-Glutamate transport. In the next experiment we tested for the effect of DCCD on Na^+-dependent L-glutamate uptake into rat renal brush-border membrane vesicles. Again in accordance with previous results (20) a Na^+ concentration difference ($[Na^+]_{out} > [Na^+]_{in}$) causes concentrative L-glutamate uptake into control vesicles (Figure 2, 150 mM KCl, -DCCD, compare open and hatched columns). Pretreatment with 0.5 mM DCCD for 15 min causes an inhibition by 36% of the 15 s L-glutamate uptake indicating a covalent modification of the transport system for acidic amino acids. Inclusion of L-glutamate during the pretreatment with DCCD does not restore L-glutamate uptake showing that this amino acid cannot protect the transport system from irreversible inactivation. When vesicles were pretreated with DCCD in the presence of Na^+, subsequently determined Na^+-driven, absolute L-glutamate uptake is hardly changed. The relative inhibition by DCCD, however, is attenuated to 18-23% of the respective controls, because L-glutamate uptake is decreased in vesicles pretreated with Na^+ (-DCCD). Based on relative inhibitions Na^+ affords some protection of the transport system against inactivation by DCCD. The effects, however are small which would indicate that, similar to the Na^+/D-glucose cotransporter, DCCD-sensitive side groups may not be involved in the binding

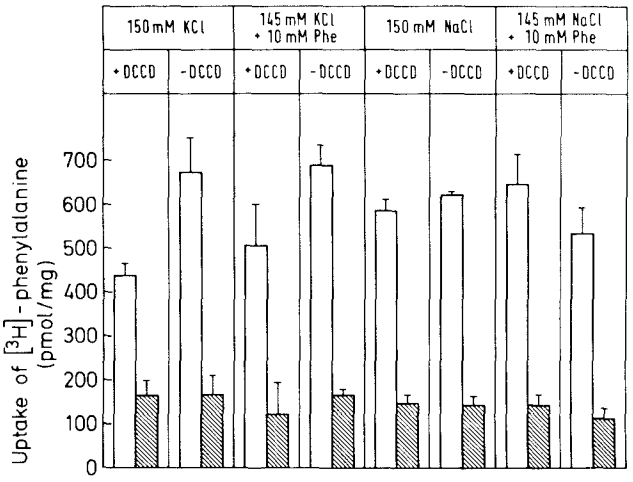

Figure 3. Irreversible inactivation of the Na^+-coupled transport system for L-phenylalanine and effects of substrates on the inhibition. For experimental details see legend to Figure 1. D-glucose has been replaced by L-phenylalanine.

of Na^+ and L-glutamate. Again, the irreversible inhibition may reflect covalent modification of the transporter protein outside of the substrate binding sites and subsequent steric hindrance of substrate binding and translocation.

L-Phenylalanine uptake. As a third system we tested the transporter for neutral amino acids using L-phenylalanine as substrate. The results are shown in Figure 3. As with D-glucose and L-glutamate we find an overshooting uptake of L-phenylalanine in the presence of an inward Na^+ gradient (21). The 15 s uptake is decreased by 35% when the vesicles were pretreated with DCCD. L-phenylalanine present during the preincubation together with DCCD does not significantly restore uptake. However, when Na^+ was present during the preincuabtion together with DCCD, a complete restoration of L-phenylalanine transport is seen. These results suggest that DCCD binds to the transporter for neutral amino acids at or close to the Na^+ binding site. Thereby, the transport system for neutral amino acids differs from those for hexoses and acidic amino acids which showed no or only borderline protection by Na^+.

Sulfate transport. Finally, we investigated the effect of DCCD and substrates on the $(Na^+)_2$/sulfate cotransporter (22). Figure 4 shows that sulfate at 15 s incubation time is highly enriched in control vesicles over the equilibrium value (150 mM KCl, -DCCD, compare open and hatched columns). DCCD causes a drastic decrease in 15 s sulfate uptake without decreasing equilibrium uptake. Thereby, the sulfate transport system is the most DCCD-sensitive transport system as noted earlier in our studies (4). Whereas the inclusion of sulfate into the preincubation buffer does not restore transport activity we find a significant decrease in inhibition by DCCD when the preincubation

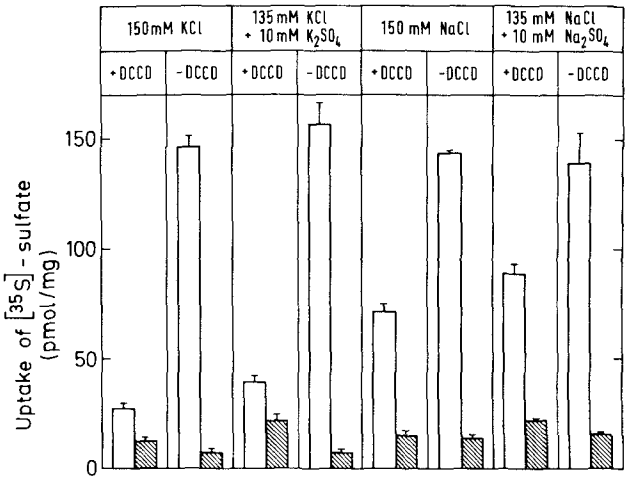

Figure 4. Irreversible inhibition of the $(Na^+)_2$-sulfate cotransport system in rat renal brush-border membrane vesicles by DCCD and protection by Na^+. The experiments were performed as described in the legend to Figure 1 except that D-glucose was replaced by sulfate.

was performed in the presence of Na^+ with or without sulfate. The relative inhibitions decrease from 82% (KCl) to 50% (NaCl) and 36% (NaCl + sulfate). That Na^+ does not completely protect from inactivation is probably due to the high affinity of the transport system for DCCD. From these results we conclude that the $(Na^+)_2$/sulfate cotransporter can be modified with DCCD. This reagent probably binds directly at or closely to the Na^+ binding site. Binding to the sulfate binding site is not likely because i) sulfate does not protect and ii) anions bind generally to positively charged side groups which are not DCCD-reactive.

Conclusions. The inhibition of several Na^+-dependent transport systems by a single side group reagent, DCCD, could indicate that all these transport systems are equipped with identical binding sites for sodium ions. However, different sensitivities of the transport systems to DCCD and different protections by Na^+ rather favour the view that sodium-coupled transport systems in rat renal brush-border membrane vesicles do not contain Na^+ binding sites of similar molecular architecture. DCCD-reactive side groups (carboxyl-, tyrosyl-, or mercapto groups in a hydrophobic environment) seem to be related to Na^+ binding in the transport systems for neutral amino acids and for sulfate whereas the Na^+ binding sites in the D-glucose and the L-glutamate carriers show no DCCD-sensitivity.

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REFERENCES

1. Azzi, A., Casey, R.P., and Nalecz, M.J. (1984) Biochim. Biophys. Acta 769, 209-226
2. Schoner, W., and Schmidt, H. (1969) FEBS-Lett. 5, 285-287
3. Burnham, C., Munzesheimer, C., Rabon, E., and Sachs, G. (1982) Biochim. Biophys. Acta 685, 260-272
4. Friedrich, T., Sablotni, J., and Burckhardt, G. (1986) J. Membrane Biol. 94, 253-266
5. Friedrich, T., Sablotni, J., and Burckhardt, G. (1987) Biochem. Biophys. Res. Commun. 144, 869-875
6. Igarashi, P., and Aronson, P.S. (1987) J. Biol. Chem. 262, 860-868
7. Kinsella, J.L., Wehrle, J., Wilkins, N., and Sacktor, B. (1987) J. Biol. Chem. 262, 7092-7097
8. Rocco, V.K., Cragoe, E.J.jr., and Warnock, D. G.(1987) Am. J. Physiol. 252, F517-F524
9. Weber, J., Siewinski, M., and Semenza G. (1987) Biochim. Biophys. Acta 900, 249-257
10. Biber, J., Stieger, B., Haase, W., and Murer H. (1981) Biochim. Biophys. Acta 647, 169-176
11. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254
12. Hopfer, U., Nelson, K. Perotto, J., and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25-32
13. Aronson, P.S., and Sacktor, B. (1975) J. Biol. Chem. 250, 6032-6039
14. Beck, J.C., and Sacktor, B. (1975) J. Biol. Chem. 250, 8674-8680
15. Kinne, R., Murer, H., Kinne-Saffran, E., Thees, M., and Sachs, G. (1975) J. Membrane Biol. 21, 375-395
16. Turner, R.J. (1986) J. Biol. Chem. 261, 1041-1047
17. Lin, J.T., Stroh, A., and Kinne, R. (1982) Biochim. Biophys. Acta 692, 210-217
18. Peerce, B.E., and Wright, E.M. (1985) J. Biol. Chem. 260, 6026-6031
19. Peerce, B.E., and Wright, E.M. (1984) Proc. Natl. Acad. Sci. USA 81, 2223-2226
20. Burckhardt, G., Kinne, R., Stange, G., and Murer, H. (1980) Biochim. Biophys. Acta 599, 191-201
21. Evers, J., Murer, H., and Kinne, R. (1976) Biochim. Biophys. Acta 426, 598-615
22. Lücke, H., Stange, G., and Murer, H. (1979) Biochem. J. 182, 223-229