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Diethylpyrocarbonate inhibition of sodium-glucose cotransport in kidney brush-border membrane vesicles

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Exposure of kidney brush-border membrane vesicles to the acylating reagent diethylpyrocarbonate resulted in inactivation of the glucose transporter, as demonstrated by inhibition of sodium-coupled D-glucose transport and phlorizin binding. The transport site(s) was protected against inactivation by the simultaneous presence of sodium ions and D-glucose, and were partially protected by phlorizin. Transport activity was not restored by hydroxylamine; this rules out the possibility of diethylpyrocarbonate interaction with histidine, serine or tyrosine transporter residues. Dithiothreitol, a thiol protector, slightly prevented diethylpyrocarbonate inactivation. It is therefore suggested that (an) amino group(s) in the translocation complex is involved, at the level of the sugar transport site and the preferential protection of D-glucose against diethylpyrocarbonate inactivation related to a conformation change caused by the simultaneous binding of sodium and D-glucose to the cotransporter.

Protein modification using selective or semi-selective reagents has provided information on the structure-function relationships of the Na^+-glucose cotransporter from kidney brush-border membranes. Use of such techniques has demonstrated that thiol, disulfide and carboxyl groups [1–4] closely related to the sugar binding site and tyrosine residues [5] related to the sodium site are essential to Na^+-glucose cotransporter function. The present study documents inactivation of coupled sodium and glucose translocation by diethylpyrocarbonate. The results highly suggest either involvement of (an) amino group(s) related to the glucose transport site(s) or a change in the conformation of the cotransporter.

Brush-border membrane vesicles from pig kidney outer cortex (3–4 mm slices) were prepared as previously described [6] and suspended in 150 mM NaCl, 1 mM Tris-Hepes (pH 7.4) at a final concentration of 10 mg/ml membrane protein. 1 ml aliquots were frozen and stored in liquid nitrogen. Membrane vesicles thawed at 37°C were dialyzed during 12 h against 100 mM KCl or 100 mM NaCl buffered by 20 mM Tris-HCl (pH 6) and adjusted to a final concentration of 5 mg of protein per ml by adding appropriate volumes of KCl or NaCl buffer (pH 6). Diethylpyrocarbonate (freshly prepared in absolute ethanol) was added to the membrane suspension such that the final concentration of ethanol was never more than 0.5% (no effect on transport experiments). Incubation was carried out at 25°C for 15 min, after which membrane vesicles were suspended in 30 vols. of 100 mM mannitol, 10 mM Tris-Hepes (pH 7.4) buffer and immediately centrifuged at 50000 $\times g$ for 30 min at 4°C. Pellets were resuspended

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in the same buffer and centrifuged again in the same conditions; this step was repeated twice and final pellets were homogenized in the same buffer at the final concentration of 2.5 mg/ml.

In experiments concerning reactivation of diethylpyrocarbonate-treated vesicles by hydroxylamine, both control and treated membranes were washed twice in 100 mM mannitol, 10 mM Tris-Hepes (pH 7.4) buffer, as described above, and resuspended in the same buffer at a final concentration of 3 mg protein/ml. Hydroxylamine (titrated to pH 7 with KOH) was added to a final concentration of 0.5 M and membrane vesicles were incubated at 25 °C for various times, washed twice in 100 mM mannitol, 10 mM Tris-Hepes (pH 7.4) and resuspended in the same medium at 2.5 mg/ml membrane protein.

D-Glucose uptake by membrane vesicles was measured in the presence of an inwardly directed 100 mM K^+ or Na^+ gradient. Uptake was initiated by adding membrane vesicles (40 μl) to 1.5 mM radioactive D-glucose in 100 mM mannitol, 150 mM KCl or NaCl, 1 mM Tris-Hepes (pH 7.4) (80 μl). After incubation at 25 °C, transport was stopped with 2 ml of cold stopping solution (100 mM KCl or NaCl, 1 mM Tris-Hepes (pH 7.4) containing unlabelled D-glucose), and membrane vesicles were retained on a presoaked 0.65 μm Sartorius filter; the filter was rinsed and the radioactivity retained was measured after addition of Instagel scintillation fluid (Packard).

Phlorizin binding was carried out by adding 30 μM radioactive phlorizin (10 μl) to membrane vesicles (30 μl) preincubated for 60 min at 25 °C in 150 mM NaCl, 1 mM Tris-Hepes (pH 7.4) (60 μl) containing either 30 mM mannitol or 30 mM D-glucose. After incubation for 1 min at 25 °C, membrane vesicles were retained on a Sartorius filter and the radioactivity was measured as described previously. Specific D-glucose-inhibitable phlorizin binding was calculated by subtracting binding in the presence of D-glucose from binding in the presence of mannitol.

D-[U-^{14}C]Glucose (230 mCi/mmol) was obtained from Amersham International (U.K.) and [^{3}H]phlorizin (35–55 Ci/mmol) from New England Nuclear. Diethylpyrocarbonate and hydroxylamine were from Sigma. Other chemicals were of the highest available grade.

Brush-border membrane vesicles treated for 15 min with increasing concentrations of diethylpyrocarbonate (1 to 10 mM) were assayed for D-glucose transport activity after 90 s of incubation in the presence of a sodium gradient. 50% of maximum D-glucose inactivation was observed with a diethylpyrocarbonate concentration of 1 mM; maximal inhibition was reached at a concentration of 5 mM (Fig. 1A). No modification in the D-glucose equilibrium value (after 60 min of incubation) was noted with any of the inhibitor concentrations tested. A diethylpyrocarbonate concentration of 2 mM was used in all subsequent experiments.

Measurement of D-glucose uptake as a function of time in diethylpyrocarbonate-treated membrane vesicles in the presence of a sodium gradient resulted in abolition of the 'overshoot' phenomenon (Fig. 1B). A D-glucose substrate with a final con-

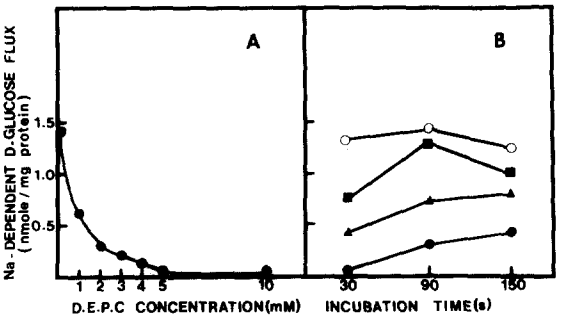

Fig. 1. (A) Inhibition of sodium-dependent D-glucose uptake by diethylpyrocarbonate. Membrane vesicles were incubated with diethylpyrocarbonate concentrations ranging from 1 to 10 mM for 15 min at 25 °C (pH 6), then washed with diethylpyrocarbonate-free buffer (pH 7.4). D-Glucose uptake was measured after 90 s of incubation. Sodium-dependent D-glucose uptake was calculated by subtracting KCl values from NaCl values. The results represent mean values of three experiments. (B) Protection of D-glucose transport against diethylpyrocarbonate inactivation by D-glucose and phlorizin. Membrane vesicles preincubated in 100 mM KCl or NaCl buffer (pH 6) without or with D-glucose (100 mM) or phlorizin (5 μM) for 15 min at 25°c were incubated for 15 min at 25 °C with 2 mM diethylpyrocarbonate. The vesicles were washed free of reagent salts and sugars, and sodium-dependent D-glucose was calculated at various times during incubation by subtracting D-glucose uptake in the presence of KCl gradient from the value in the presence of NaCl gradient. Control vesicles (○), diethylpyrocarbonate-treated vesicles without protection (●) or protected by 100 mM D-glucose (■) or 5 μM phlorizin (▲). Points represent mean values of three experiments.

TABLE I

PROTECTION OF D-GLUCOSE TRANSPORT AGAINST DIETHYLPYROCARBONATE INACTIVATION BY VARIOUS SUBSTRATES

Membrane vesicles preincubated for 15 min at 25 °C in 100 mM KCl or NaCl buffer (pH 6) without or with indicated compounds were then incubated with 2 mM diethylpyrocarbonate for 15 min at 25 °C. D-Glucose uptake and phlorizin binding were measured. Each value represents the mean of three separate experiments. The errors indicated are standard deviations.

Preincubation medium containing	Incubation medium containing	Na^+-dependent glucose uptake (pmol/mg per 30 s)	Remaining transport (%)	Phlorizin binding (pmol/mg per min)	Remaining binding (%)
KCl or NaCl	No addition	1325 ± 50	100	30.8 ± 4	100
KCl or NaCl	Diethylpyrocarbonate	93 ± 35	7	6.8 ± 2	22
KCl + D-glucose 100 mM	Diethylpyrocarbonate	412 ± 40	31	–	–
NaCl + D-glucose 100 mM	Diethylpyrocarbonate	745 ± 29	56	18.8 ± 3	61
KCl + phlorizin 5 μM	Diethylpyrocarbonate	395 ± 80	31	–	–
NaCl + phlorizin 5 μM	Diethylpyrocarbonate	412 ± 60	31	4.3 ± 2.5	14
NaCl + L-glucose 100 mM	Diethylpyrocarbonate	150 ± 15	11	–	–
NaCl 400 mM	Diethylpyrocarbonate	44 ± 10	4	–	–
NaCl + dithiothreitol 20 mM	Diethylpyrocarbonate	471 ± 45	35	–	–
KCl or NaCl	Diethylpyrocarbonate, then reversion by hydroxylamine	301 ± 40	23	–	–

centration of 100 mM protected the glucose transporter from inactivation by diethylpyrocarbonate (Fig. 1B). Protection was reduced when potassium ions were used instead of sodium ions; however, protection was not due to the sodium ions themselves because they failed to prevent diethylpyrocarbonate inactivation of the glucose transporter even at a concentration of 400 mM (Table I). Phlorizin (5 μM), a non-transported competitive inhibitor of D-glucose, provided poor protection of the D-glucose transporter against diethylpyrocarbonate inactivation (Fig. 1B); the protection afforded by phlorizin was identical in the presence of Na^+ and K^+ ions, and was similar to that observed when D-glucose and K^+ ions were added simultaneously (Table I). Nevertheless, proof of diethylpyrocarbonate interaction with a glucose transport site of the cotransporter was provided by the absence of protection with stereoisomer L-glucose (Table I). Similar results have been reported in small intestinal brush-border membranes using amino group reagents [7]. Phlorizin binding was investigated in brush-border membranes treated with 2 mM diethylpyrocarbonate in the absence or presence of D-glucose or phlorizin and compared to a control without diethylpyrocarbonate. The phlorizin binding inhibition noted was partly reversed by D-glucose, but not by phlorizin (Table I).

It has been demonstrated that diethylpyrocarbonate reacts principally with the amino and imidazole groups of proteins [8]. Diethylpyrocarbonate inactivation of an enzyme may be correlated with modification of a histidine residue if hydroxylamine reactivates the enzyme. When kidney brush-border membranes were treated with 2 mM diethylpyrocarbonate and subsequently subjected to 0.5 M hydroxylamine at +4 °C or +20 °C, D-glucose transport inhibition was slightly reversed, even after 20 h incubation (Table I). Failure of hydroxylamine to restore D-glucose transport activity can be ascribed to the modification of other protein residues. Occasionally diethylpyrocarbonate has been shown to react with a variety other nucleophilic residues which occur in proteins, including tyrosine, serine, sulfhydryl residues [9]. Ethoxyformylation of tyrosine and serine residues is reversed by hydroxylamine. However, glucose transport inhibition by diethylpyrocarbonate in kidney brush-border membranes does not seem to be caused by ethoxyformylation of thiol residues because the presence of 20 mM dithiothreitol poorly protected the transporter from inactivation by diethylpyrocarbonate (Table

I). The failure of hydroxylamine to restore transport activity and the low protection afforded by dithiothreitol suggest that at least one amino group has been modified by ethoxyformylation. It can be assumed, that diethylpyrocarbonate blocks (an) amino group residue(s) essential for the translocation of D-glucose substrate, possibly a lysine residue known to have secondary effects on enzyme activity or conformation [9] and previously identified in the binding site of small intestinal brush-border membranes [10].

Finally, the mechanism of Na^+-glucose cotransporter inactivation by diethylpyrocarbonate reagent might affect the sugar transport site directly, after sodium activation of the transporter, or might affect another transporter site leading to inactivation of sugar transport. As previously suggested [4,11] the transporter with phlorizin bound is presumably looked in a single conformation with the substrate binding site outwardly exposed, whereas the transporter with D-glucose bound can be exposed alternately outwardly and inwardly; this could explain the differential protection afforded by D-glucose and phlorizin in the presence of sodium ions. Thus D-glucose, but not phlorizin, might induce a conformational change in the D-glucose transporter, resulting in a transporter configuration providing better protection against diethylpyrocarbonate inactivation. This hypothesis is supported by diethylpyrocarbonate inhibition of phlorizin binding, prevented by D-glucose, but not phlorizin protection. In addition, the protection observed with D-glucose or phlorizin in the presence of potassium ions already noted in small intestinal brush-border membranes with amino group reagents [7] could affect other nucleophilic transporter residue(s); this would explain the low degree of reversion induced by hydroxylamine as well as the protection provided by dithiothreitol.

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