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INHIBITION OF PHOSPHATE TRANSPORT IN HUMAN ERYTHROCYTES BY WATER-SOLUBLE CARBODIIMIDES

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Phosphate entry into human erythrocytes is irreversibly inhibited by treatment of the cells with the water-soluble carbodiimides 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMC) in the absence of added nucleophile. EDC is the more potent inhibitor (40% inhibition, 2 mM EDC, 5 min, 37°C, 50% hematocrit, pH 6.9), while more than 20 mM CMC is required to give the same inhibition under identical conditions. EDC inhibition is temperature-dependent, being complete in 5 min at 37°C, and sensitive to extracellular pH. At pH 6.9 only 50% of transport is rapidly inhibited by EDC, but at alkaline pH over 80% of transport is inhibited. Inhibition is not prevented by modification of membrane sulfhydryl groups but is decreased in the presence of 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), a reversible competitive inhibitor of anion transport. EDC treatment leads to crosslinking of erythrocyte membrane proteins, but differences between the time course of this action and inhibition of transport indicate that most transport inhibition is not due to crosslinking of membrane proteins.

Introduction

The Band 3 polypeptide, a major integral protein of the human erythrocyte membrane, mediates the rapid exchange of anions required for respiration [1,2]. Acid-base titrations of transport function have indicated that anion binding and translocation depend on the degree of protonation of two sets of exofacial groups with apparent pK values of about 12 [3] and 5 [4]. Exchange transport is tightly coupled and kinetic studies have suggested models in which positively charged sites within the molecule become alternately exposed to the intracellular and extracellular medium by movement of an 'anionic gate' driven by charge-repulsion effects of the transported anion [5]. Water-soluble carbodiimides react with carboxyl groups under mild conditions [6,7] and have been used both in the determination of essential residues in enzymes [8] and as 'zero length' protein crosslinking reagents [9].

In this study the effect of two water-soluble carbodiimides on phosphate entry into chloride-loaded erythrocytes is examined to provide a basis for the identification of carboxyl groups essential for the anion-transport function of Band 3. A preliminary report of partial inhibition of sulfate transport by EDC has been made [10].

Abbreviations: EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-sulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PCMBS, *p*-chloro-mercuribenzene sulfonate.

Materials and Methods

Materials

Carrier-free [^{32}P]phosphate was obtained from New England Nuclear. DNDS and CMC were purchased from Aldrich Chemical Co., Milwaukee, WI. 4,4'-Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and EDC were from Pierce Chemical Co., Rockford, IL. Outdated human red blood cells were kindly provided by the Canadian Red Cross. Other chemicals were of pure reagent grade from standard suppliers.

Methods

Preparation of erythrocytes and carbodiimide treatment. Red blood cells were washed four times with 10–15 vol. 0.9% w/v NaCl. 2-ml portions of packed cells were added to 2-ml volumes of 0.9% NaCl in 16 × 100 mm culture tubes and equilibrated to 37°C. 0.16 ml of 0.5 M phosphate buffer pH 7.5 was added and vortex-mixed. Carbodiimide freshly dissolved in phosphate buffer or saline was added and incubated for the required time. Carbodiimide concentrations were calculated for dilution into the total reaction volume assuming penetration into the cell interior.

After the appropriate time of incubation, 5 ml of ice-cold buffer A (28.5 mM sodium citrate, pH 7.4, 205.3 mM sucrose) were rapidly added and the tubes were centrifuged at 400 × *g* in a refrigerated centrifuge, at 4°C for 3 min. This washing procedure was repeated twice with 10-ml volumes of ice-cold buffer A.

In experiments where extracellular pH was varied small volumes of 0.9% w/v NaCl incubation medium were replaced by 5 M NaOH or 1 M HCl solution to achieve the required pH.

In view of a report that carbodiimide may react with phosphate [11], a control experiment was performed in which Mops replaced phosphate as buffer. No increase in transport inhibition at pH 6.9 by EDC was observed.

Cell lysis. Haemoglobin released on cell lysis was estimated by absorbance at 541 nm in 0.1% w/v sodium carbonate solution.

Transport measurements. 0.75 ml of packed erythrocytes (400 × *g*, 5 min, 4°C) in buffer A was added to 0.75 ml buffer A and equilibrated to 30°C in a shaking water bath. Transport was initiated by the addition of 50 μl 130 mM sodium phosphate pH 7.4 containing [^{32}P]phosphate with vigorous vortex-mixing. At suitable times 125-μl samples of the cell suspension were removed and immediately centrifuged for 15 s in a Fisher Microfuge model 235A. A 50 μl portion of the clear supernatant was taken for counting in a Beckman LS 7800 scintillation counter. Inhibition of transport was estimated from the change in half-time for the loss of radioactivity from the extracellular medium.

$$\text{Inhibition}\ (\%) = \left(1 - T_{1/2,\text{control}} / T_{1/2,\text{treated}}\right) \times 100$$

Preparation of red cell ghosts. Red cell ghosts were prepared by the method of Dodge et al. [12] using buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% v/v dimethylsulfoxide. Ghosts were washed at least five times to remove haemoglobin. This treatment gave white ghosts from untreated cells, but after long periods of incubation with EDC (60 min, 37°C, 10 mM EDC) ghosts were pink in colour, indicating that haemoglobin had crosslinked to the membrane. SDS-polyacrylamide gel electrophoresis was performed using the method of Laemmli [13]. Protein was determined according to Lowry et al. [14] except that all samples were assayed in the presence of 1% w/v SDS.

Results and Discussion

Carbodiimide inhibition of phosphate uptake

Treatment of erythrocytes with EDC gives inhibition of phosphate uptake which is not reversed by extensive washing of cells in buffer A (Fig. 1) with or without bovine serum albumin. Control experiments showed that transport inhibition is stable on incubation in buffer A for over 6 h at 37°C. Extensive washing of cells with 0.5% bovine serum albumin in 0.9% NaCl before washing with buffer A gave a slight reduction of inhibition, indicating that the standard washing procedure permits only a small amount of unreacted carbodiimide to remain associated with the cells. It is noteworthy that EDC inhibition occurs in the absence of added nucleophilic agents, and the addition of glycine ethyl ester (17.5 mM or 35 mM) to the reaction medium does not increase inhibition of transport.

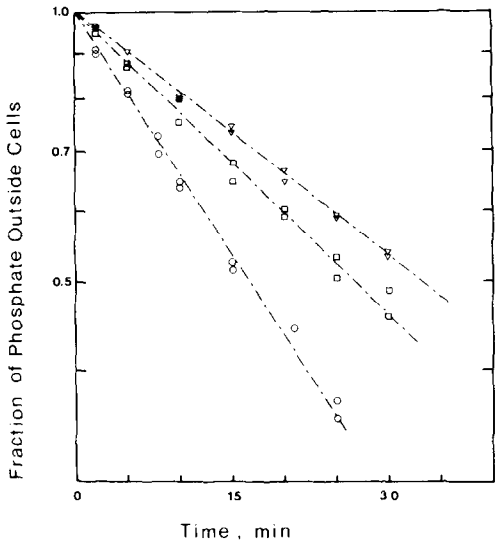

Fig. 1. Effect of pretreatment of erythrocytes with EDC for 5 min, 37°C, pH 6.9, as described in Materials and Methods. O- -O, No EDC treatment; □- -□, 2 mM EDC; △- -△, 10 mM EDC.

Fig. 2 shows that at pH 6.9 EDC is a more potent inhibitor of transport that CMC. 20 mM CMC causes significant cell lysis during incubation at 37°C. The inhibition of anion transport by

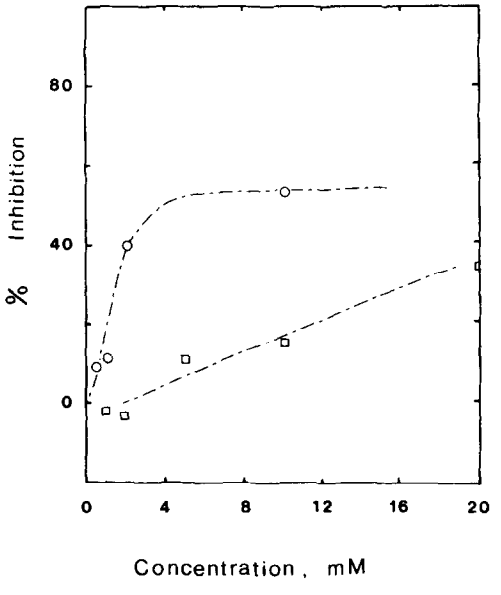

Fig. 2. Concentration-dependence of the inhibition of phosphate transport by carbodiimides. Incubations for 5 min, 37°C, pH 6.9 in phosphate-buffered saline. O- -O, EDC; □- -□, CMC.

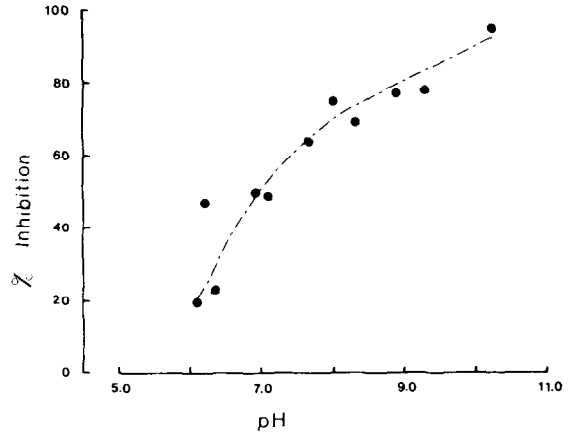

Fig. 3. Effect of extracellular pH on transport inhibition by 10 mM EDC, 4 min, 37°C in phosphate-buffered saline solution. Transport was assayed at 30°C in buffer A as described in Materials and Methods.

EDC was further characterized and it is apparent that an inhibition of approx. 50% of transport is readily achieved. Prolonged incubation and subsequent treatments with fresh carbodiimide did not greatly increase the inhibition of transport. Wieth et al. [15] noted a similar inhibition of chloride fluxes in erythrocyte ghost preparations after treatment with a non-penetrating carbodiimide.

After treatment with EDC intact erythrocytes still exhibit concentrative uptake of phosphate driven by high intracellular chloride concentrations, indicating that remaining transport activity is still coupled and that inhibition is not due to nonspecific changes in membrane permeability resulting from carbodiimide treatment of cells.

Fig. 3 shows that EDC inhibition of transport is strongly influenced by the pH of the extracellular

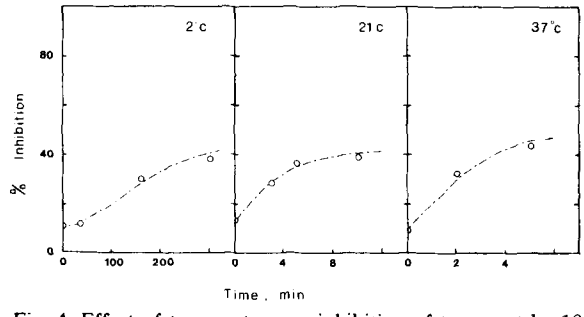

Fig. 4. Effect of temperature on inhibition of transport by 10 mM EDC at pH 6.9 in phosphate-buffered saline solution.

medium. Higher pH gave more complete inhibition, suggesting that attack by the carbodiimide on carboxyl residues which is optimal at lower pH values is not rate-limiting for the irreversible inhibition of transport. The irreversible inhibition may be due to subsequent nucleophile attack or intramolecular rearrangement, both of which are enhanced at alkaline pH [7,21].

Transport inhibition shows a strong temperature-dependence (Fig. 4). The small inhibition at zero time at all temperatures probably reflects EDC which remains associated with the cells throughout the washing procedure and then reacts at the higher temperature used for the transport assay.

Crosslinking of erythrocyte membrane proteins by EDC

SDS-polyacrylamide gel electrophoresis shows that 10 mM EDC at 37°C, pH 6.9, gives crosslinking of most membrane proteins which increases with incubation time (Fig. 5). After 5 min the amount of Band 3 seen in the separating gel is decreased by about 10% while phosphate uptake is reduced by 40% (Fig. 3). Extensive crosslinking of spectrin and the pink colour of ghost membranes indicate that EDC is able to penetrate the erythrocyte membranes and react at the cytoplasmic surface. Treatment of intact erythrocytes for 2 min with 10 mM EDC, pH 6.9, 37°C in phosphate-buffered saline gave over 30% inhibition of transport (Fig. 4) without significant loss of Band 3 from the separating gel in SDS-polyacrylamide gel electrophoresis. Chymotrypsin treatment (0.5 mg/ml, 37°C, 60 min) of these cells quantitatively generates a 60 kDa polypeptide from Band 3, indicating that transport inhibition is not a result of intramolecular crosslinking between this portion of Band 3 and the rest of the molecule. On prolonged incubation with EDC (Fig. 5) both Band 3 and the 60 kDa fragment generated by chymotrypsin treatment were much reduced in 7.5% acrylamide SDS-electrophoresis separating gels. 340 μM DNDS does not significantly reduce crosslinking of membrane protein (10 mM EDC, 15 min, 37°C, pH 6.9).

Effect of sulfhydryl modification on EDC inhibition

The Band 3 molecule contains six sulfhydryl groups, five of which may be modified by incubation of intact erythrocytes with 10 mM *N*-ethylmaleimide for 1 h at 37°C [16]. A sixth sulfhydryl located in the membrane-spanning portion of the molecule [17] is unreactive to *N*-ethylmaleimide but can be modified by *p*-chloromercuribenzene sulfonate (PCMBS) [18].

Treatment of erythrocytes with *N*-ethylmaleimide or PCMBS does not significantly affect anion transport [16,19]. Cells treated for 60 min, 36°C, pH 6.9 in phosphate-buffered saline medium before EDC treatment (10 mM, 10 min, 37°C) showed 5–9% more transport inhibition than cells not exposed to sulfhydryl-modifying agents. If EDC inhibition were generated by reaction of a

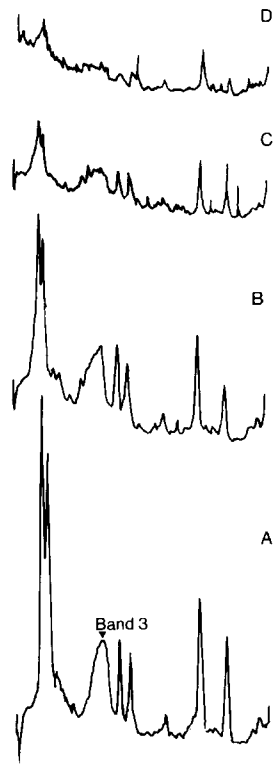

Fig. 5. Effect of EDC treatment on erythrocyte membrane proteins. Cells were incubated at 37°C with 10 mM EDC, pH 6.9, in phosphate-buffered saline for various times. (A) Control (EDC absent); (B) 5 min; (C) 15 min; (D) 60 min. Haemolysis was minimal (B, 0.75%; D, 1.25%) determined from haemoglobin in the incubation medium. Erythrocyte ghosts were prepared from treated cells as described in Materials and Methods. Samples of ghost membranes (25 μg protein) were run on an SDS-7.5% acrylamide gel. The gel was scanned at 595 nm after Coomassie blue staining.

sulfhydryl group substantial protection from inhibition would have been observed. Therefore modification of sulfhydryl groups by EDC does not play a role in the inhibition of phosphate transport.

Effect of occupation of the stilbene binding site on EDC inhibition

Anion transport in human erythrocytes is readily inhibited by a variety of stilbene derivatives capable of binding with high affinity to a site on Band 3 accessible from the extracellular medium [20]. Fluorescence energy transfer measurements have indicated that stilbene binding sites on the subunits of Band 3 dimers are close together [5] and that the observed negative cooperativity of stilbene binding might be a result of the mutual proximity of these binding sites.

Table I shows that DNDS, which binds reversibly to the stilbene site, can reduce EDC inhibition of phosphate transport. Residual transport activity after EDC treatment of intact cells is readily inhibited by 50 μM DIDS, showing that the carbodiimide treatment has not blocked access to stilbene binding sites of uninhibited Band 3 molecules. These results are consistent with the reaction of the EDC at or near the transport site, access to which may be hindered if the stilbene binding site is occupied. The lack of complete protection by DNDS may indicate that inhibition of transport can also result from reaction of the carbodiimide at other sites in the Band 3 molecule.

TABLE I

EFFECT OF DNDS ON EDC INHIBITION OF PHOSPHATE TRANSPORT

EDC 10 mM, 10 min, 37°C, pH 6.9. Washing, 1×5 ml and 2×10 ml 0.5% w/v bovine serum albumin in 0.9% NaCl and 1×10 ml 0.9% NaCl before standard wash with buffer A and transport assay.

[DNDS] (μ)	EDC inhibition (%)		Protection(%)
	−DNDS	+DNDS	
85	27.9	28.2	nil
170	39.7	32.8	6.9
	43.9	37.7	6.2
340	36.0	20.4	15.6
		26.1	9.9
425	39.7	23.4	16.3

Conclusions

Carbodiimides can activate carboxylic acids towards nucleophilic attack by forming *o*-acylureas. In the absence of a suitable nucleophile water may hydrolyze the isourea to free urea and regenerate the free carboxylic acid. The isourea may undergo rearrangement to a stable *n*-acylurea [6]. Carbodiimides are also reported to form stable adducts with the sulfhydryl of cysteine or the hydroxyl of tyrosine [7].

This study shows that the stable inhibition of phosphate uptake by EDC in the absence of exogenous nucleophile is not due to modification of membrane sulfhydryl groups. The increased inhibition at alkaline pH values indicates that the limiting step for inhibition is not attack by the carbodiimide on carboxylic acid groups but the rearrangement of the resulting isourea to *n*-acylurea, a process which is base-catalyzed [21], or to intramolecular or intermolecular attack by amino groups leading to protein crosslinking. Inhibition appears to be due to reaction at a specific site or sites on the Band 3 molecule rather than nonspecific crosslinking of membrane proteins in view of the rapidity of inhibition and partial protection by DNDS.

Band 3 exists in the membrane as dimers or associations of dimers [5,21], so the maximal 50% inhibition of transport observed after reaction of EDC at pH 6.9 may imply that modification of one subunit hinders carbodiimide reaction with the other subunit by steric or allosteric interactions, yet leaves transport unimpaired and inhibitable by DIDS. A similar effect has been reported by Wieth et al. [15] following treatment of erythrocyte ghosts at pH 6.0 with a non-penetrating carbodiimide. An alternative explanation might be that an allosteric effect of carbodiimide reaction renders further carbodiimide modification labile by increasing accessibility of the latter site to water molecules. A third possibility is that EDC modification reduces the turnover of each monomer by 50%. This appears less likely since Wieth and Bjerrum [3] have shown that a 50% decrease in chloride transport in resealed ghosts after treatment with a non-penetrating carbodiimide is a result of modification of only 50% of the transport sites.

It is not clear whether the greater inhibition at alkaline pH is due to a change in protein conformation exposing different inhibitable sites on the Band 3 molecule or to a change in the mechanism of carbodiimide inhibition. Protein crosslinking and generation of stable *n*-acylurea derivatives of carboxylic acid would be promoted at higher pH values and it is also possible that a more rapid penetration of EDC through the erythrocyte membrane could promote inhibition by reaction at an internal site or sites.

The results in this study indicate that EDC irreversibly inhibits anion transport and this reagent may allow identification of a specific site or sites in the Band 3 molecule essential for the anion-transport function.

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