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Inhibition of sodium-phosphate cotransport in renal brush-border membranes with the stilbenedisulfonate, H_2-DIDS

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Membrane proteins involved with sodium/phosphate cotransport across the renal brush border provide the sensitive control for phosphate homeostasis. The present study describes the inhibition of sodium/phosphate cotransport with the stilbenedisulfonate derivatives, DIDS and H_2-DIDS. Preincubation of the rat brush-border membrane vesicles with H_2-DIDS led to the inhibition of sodium-dependent phosphate uptake with a half maximal concentration, IC_{50}, of about 10 μM. The inhibition was irreversible supporting the notion that H_2-DIDS forms covalent bonds with the cotransporter. The cotransporter could be protected by excess sodium phosphate but not sodium chloride, sodium sulfate, sodium succinate, sodium bicarbonate, nor sodium phosphonoformate. These observations suggest that the stilbenedisulfonates may be useful in labeling the sodium/phosphate cotransporter within renal brush-border membranes.

Introduction

Phosphate is reabsorbed principally by the proximal tubule of the kidney [1,2]. The rate-limiting step in transepithelial transport is sodium/phosphate cotransport across the brush-border membrane of proximal tubular cells [3]. Sodium moves into the cell across the brush-border membrane down its electrochemical gradient coupled to phosphate which is concentrated in the cell [1,2]. This is also the step which is highly regulated by physiological controls [4]. Accordingly, a definition of the protein structure of this cotransporter is important in understanding function and control of phosphate transport.

A number of approaches have been used to label the sodium/phosphate cotransporter in brush-border membranes [5–9]. The purpose of labelling membranes in these studies was to facilitate the purification of the transporter in order to determine the biochemical structural and functional properties [10]. These approaches, to date, have not been totally successful.

Stilbenedisulfonate derivatives have proven to be useful agents in labelling anionic binding sites in various membranes [11–17]. Under appropriate conditions, DIDS is thought to bind to lysine residues in the transport protein and cause irreversible inhibition of anionic transport. Additionally, the reduced form of DIDS, H_2-DIDS, may be tritiated to facilitate membrane labeling. The disadvantage of the stilbenedisulfonates is that they form covalent bonds with many proteins of the membrane so that appropriate steps must be taken to specifically label only the sites of interest [12,16]. The present studies describe the specific inhibition of sodium/phosphate cotransport in renal brush-border membranes which may lead to a method of labelling the sodium/phosphate cotransporter with ^{3}H-DIDS.

Methods

Materials. H_2-DIDS, 4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonic acid was purchased from Molecular Probes Inc, Eugene, OR. DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid and other materials were purchased from Sigma or Fisher and were of highest quality available.

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Animals. Male Wistar rats (250–350 g) were anesthetized with pentobarbital sodium, and the kidneys were perfused with ice-cold saline and removed for preparation of brush-border membrane vesicles.

Vesicle preparation. Brush-border membrane vesicles were prepared from outer cortical tissue of the rat kidneys using the Mg^{2+} precipitation procedure as previously described [9]. The kidney slices were suspended in a homogenizing solution composed of 300 mM mannitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), and 18 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) (pH 7.4). The tissue was homogenized on ice at full speed with a glass-Teflon homogenizer. The homogenate was centrifuged at $3000 \times g$ for 10 min, the pellet was discarded, sufficient 1 M $MgCl_2$ was added to the supernatant to obtain a final concentration of 10 mM, and stirred for 20 min on ice, then centrifuged at $1900 \times g$ for 10 min. The resulting supernatant was centrifuged at $32500 \times g$ for 20 min. The pellet was resuspended in the homogenizing medium to half the original volume and treated with two additional $MgCl_2$ steps as follows. The suspension was treated with 10 mM $MgCl_2$ and after 20 min on ice was centrifuged at $4300 \times g$ for 12 min, and the resulting supernatant centrifuged at $32500 \times g$ for 20 min. The pellet was resuspended in homogenizing medium and treated with 10 mM $MgCl_2$ as before. After 20 min on ice the suspension was centrifuged at $6300 \times g$ for 12 min and the supernatant centrifuged at $32500 \times g$ for 20 min.

The resulting pellet containing the purified brush-border membrane vesicles was resuspended in the desired buffer at a final protein concentration of about 8 mg/ml. The vesicle suspension was passed twice through a 30-gauge needle and then stored in liquid nitrogen until uptake experiments were performed. The vesicles were suspended in buffer consisting of (in mM): 100, KCl; 20, mannitol; 21, TMA-OH; 42, Hepes-KOH (pH 8.2). All concentrations given here and elsewhere are final concentrations. No difference in uptake activity was observed between freshly made vesicles and vesicles stored up to 4 weeks in liquid nitrogen.

Enzyme assays. The purity of the final vesicle suspension was assessed by assaying the enzyme activity associated with the brush-border (δ-glutamyl transpeptidase) and basolateral membranes (ouabain-sensitive Na^+/K^+-ATPase). These enzyme activities were determined as previously reported [9]. Protein was determined by the method of Lowry after treatment of the membranes with 5% sodium dodecyl sulphate (w/v); dilutions of bovine serum albumin (BSA) were used as standards. The specific activity of δ-glutamyl transpeptidase was enriched 15-fold and the ouabain-sensitive Na^+/K^+-ATPase was enriched 1.2-fold in brush-border membrane fractions relative to the respective homogenates.

Uptake measurements. The final membrane pellet was resuspended in a buffer containing 100 mM KCl, 20 mM mannitol, 21 mM tetramethylammonium hydroxide (TMA-OH) and 42 mM Tris-Hepes (pH 8.2) (intravesicular buffer). The transport solution contained either 100 mM NaCl or 100 mM KCl and 20 mM mannitol, 21 mM TMA-OH, 42 mM Hepes-Tris (pH 8.2), 0.1 mM $NaH_2^{32}PO_4$ or $KH_2^{32}PO_4$ (10 μCi per ml).

Uptake measurements were done at 21°C in a 12×75 mm plastic test tube by adding 50 μl of incubation medium to 10 μl of membrane suspension. The reaction was started by mixing with a vortex and terminated by adding ice cold stop-solution containing (in mM): NaCl, 140; Na_2HAsO_4, 1.0; NaH_2PO_4, 0.5; Hepes, 2 (pH 7.5). The suspension was filtered on a Sartorius membrane filter (0.65 μM) and washed twice with 5 ml of cold stop-solution. The filters were dried and then dissolved in Filter Count (Packard Instruments), and counted in a liquid scintillation counter.

Irreversible binding of DIDS to sodium/phosphate cotransporter. In order for the stilbenedisulfonates to be useful in labeling the phosphate transporter, they must bind with high affinity, with specificity and irreversibly. The first studies determined the appropriate conditions whereby the stilbene disulfonates inhibit sodium-dependent phosphate transport. Preincubation of brush-border membranes with 0.1 mM H_2-DIDS for 25 min at 37°C was found to be optimal for inhibition. These membranes were washed three times with 100-fold (by volume) excess intravesicular buffer containing 0.1% bovine serum albumin (BSA) and 0.2 mM dithiothreitol (DTT) to remove the H_2-DIDS which was not irreversibly bound to the brush-border membrane. This was done by centrifugation at $48000 \times g$ followed by resuspension of the pellet.

Second, we determined that the presence of sodium and phosphate may protect the sodium/phosphate cotransporter from irreversible inhibition by H_2-DIDS. We used this approach to provide specificity of H_2-DIDS labelling. Brush-border membrane vesicles were incubated at 37°C for 25 min with 0.1 mM H_2-DIDS in the presence or absence of 50 mM NaH_2PO_4-Na_2HPO_4 (pH 8.2). The buffer solution consisted of (in mM): either 50, NaH_2PO_4-Na_2HPO_4 or 50, NaCl and 50, NaCl; 20, mannitol; 21, TMA-OH; 42, Hepes/KOH (pH 8.2). Following the incubation, the vesicles were washed three times with 100-fold excess vesicle buffer containing 0.1% BSA and 0.2 mM DTT to remove sodium, phosphate, and non-irreversibly bound H_2-DIDS. It was anticipated that this approach would result in anionic sites not involved with sodium/phosphate cotransport to be bound with cold H_2-DIDS; the transporter would be protected from H_2-DIDS

binding by the excess phosphate. Uptake assays were done at this point to confirm that these protection studies were effective.

Results

H_2DIDS inhibition of sodium-phosphate cotransport

To determine if the stilbenedisulfonates inhibit sodium/phosphate cotransport in brush-border membrane vesicles prepared from rat kidneys, membranes were pretreated with 100 μM H_2-DIDS by incubation at 37°C for 30 min. The results are shown in Fig. 1. The presence of H_2-DIDS inhibited sodium phosphate uptake into brush-border membrane vesicles with no effect on phosphate uptake in the absence of an external sodium gradient. The addition of H_2-DIDS to the transport solution inhibited phosphate transport by about 25%. However, if the vesicles were preincubated with H_2-DIDS for 25 min at 37°C prior to uptake determinations, phosphate uptake was nearly abolished.

We next investigated the dose-response relationship of irreversible H_2-DIDS inhibition of sodium/phosphate cotransport. As shown in Fig. 2, brush-border membranes were preincubated with various H_2-DIDS concentrations followed by washing the vesicles to remove unbound H_2-DIDS; and uptakes were performed with a sodium-gradient in the presence of 100 μM phosphate. The IC_{50} for H_2-DIDS inhibition

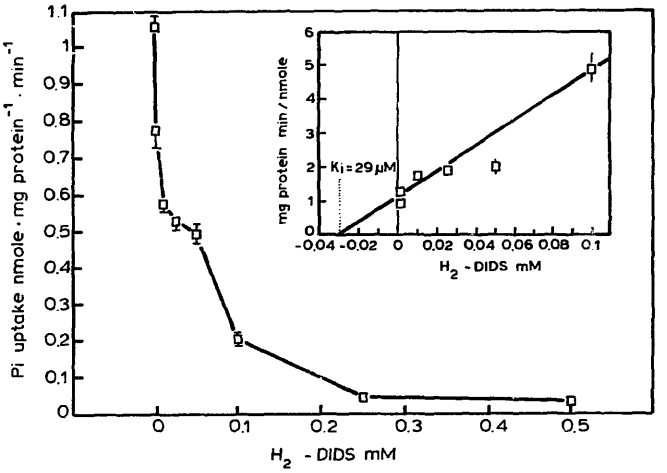

Fig. 2. Concentration-dependent H_2-DIDS inhibition of sodium/phosphate cotransport. Brush-border membrane vesicles were pretreated with various concentrations of H_2-DIDS at 37 °C for 25 min as indicated. Unbound H_2-DIDS was removed by washing three times and transport was performed as given in legend to Fig. 1. Values are means ± S.E. of four observations.

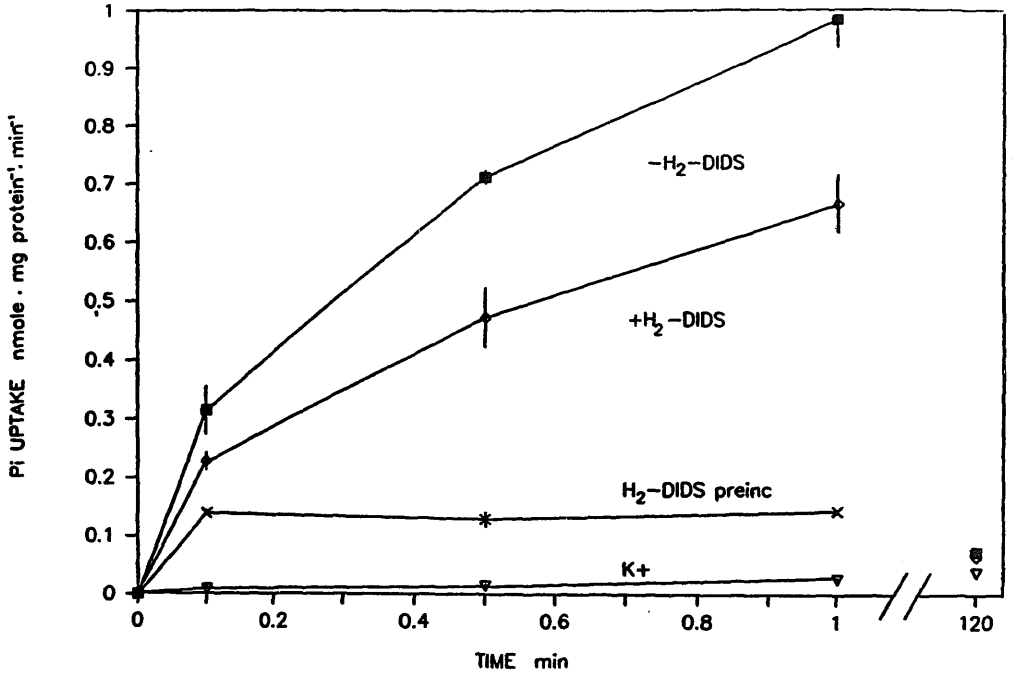

Fig. 1. H_2-DIDS inhibition of sodium-phosphate uptake into renal brush-border membrane vesicles. Brush-border membrane vesicles were prepared and transport measured as described under Methods. Vesicles were suspended in buffer consisting of (in mM): 100, KCl; 20, mannitol; 21, TMA-OH; 42, Hepes-KOH (pH 8.2). [^{32}P]Phosphate uptake was performed in the presence of sodium or potassium and with and without 100 μM H_2-DIDS in the transport solution. The transport solution consisted of (in mM): 100 NaCl or 100 KCl; 20, mannitol; 21, TMA-OH; 42, Hepes-KOH (pH 8.2). In some studies, where indicated, membranes were preincubated with 100 μM H_2-DIDS at 37°C for 25 min prior to performing uptake determinations. The preincubation solution consisted of (in mM): 50 NaH_2PO_4 or 50, NaCl; 50 NaCl; 20, mannitol; 21, TMA-OH; 42, Hepes-KCl (pH 8.2). In these latter studies the unbound H_2-DIDS was removed by washing three times with vesicle suspension solution before transport measurements. Values are means ± S.E. of a minimum of four observations and the figure is representative of at least three independent experiments.

of sodium-dependent phosphate uptake was 10 μM for three separate preparations, showing that H_2-DIDS inhibition occurred at low concentrations. These data also suggested that H_2-DIDS, a water soluble molecule which labels plasma membranes [18,19] may be competing with external phosphate for binding to a site (or sites) on the renal brush-border membrane. These dose-response inhibition curves are similar to those obtained for inhibition of the erythrocyte anion transporter, Band 3, in which the stilbenedisulfonates do interact with putative anion binding or recognition sites [19], and thus support the idea of a specific interaction of H_2-DIDS with a structural site on the surface of the brush-border membrane.

H_2-DIDS differs from the parent DIDS molecule by having a reduced double bond connecting the two benzene rings. H_2-DIDS was as effective as DIDS, IC_{50} 25 μM, in blocking sodium-dependent phosphate uptake into renal brush-border membrane vesicles (data not shown).

In order to be useful, the covalent binding of H_2-DIDS to the membranes must be specific for the sodium/phosphate transporter. To test this, we preincubated brush-border membrane vesicles with excess sodium phosphate, 50 mM, in an attempt to protect the phosphate transporter from H_2-DIDS interaction but not other anionic sites of the membrane. Subsequently, sodium and phosphate and unbound H_2-DIDS were removed by rigorous washing and transport function was determined. Fig. 3 illustrates that the presence of excess phosphate completely protects transport function as reflected by sodium-dependent [^{32}P]phosphate uptake. Protection was 25% more effective in the presence of sodium phosphate than with potassium phosphate (data not shown). In seven separate membrane preparations, sodium-dependant phosphate uptake was 989 ± 218 pmol mg^{-1} min^{-1}. Preincubation with 100 μM H_2-DIDS for 25 min, 37°C, and subsequent removal with extensive washing inhibited uptake by 80%, uptake was 160 ± 40 pmol mg^{-1} min^{-1}, $n = 7$. The presence of 50 mM sodium phosphate fully protected the transporter; uptake was 932 ± 253 pmol mg^{-1} min^{-1}, $n = 7$. It is not likely that phosphate simply precipitates with H_2-DIDS in the protection studies, because H_2-DIDS inhibits sodium-sulfate uptake in the presence of 50 mM KH_2PO_4. (data not shown).

In order to support these findings, we next performed similar protection studies with other anions that may protect the sodium/phosphate cotransporter from H_2-DIDS labelling. Preincubation with 50 mM sodium chloride, sodium sulfate, sodium succinate, sodium bicarbonate, or sodium phosphonoformate with H_2-DIDS for 25 min was without protective effect (Table I). However, if the inhibition studies were performed for 5 min, sodium arsenate, sodium bicarbonate, sodium succinate and sodium phosphonoformate were weakly protective of H_2-DIDS inhibition; 26%, 22%, 11%, and 13%, respectively (Table I). Sodium

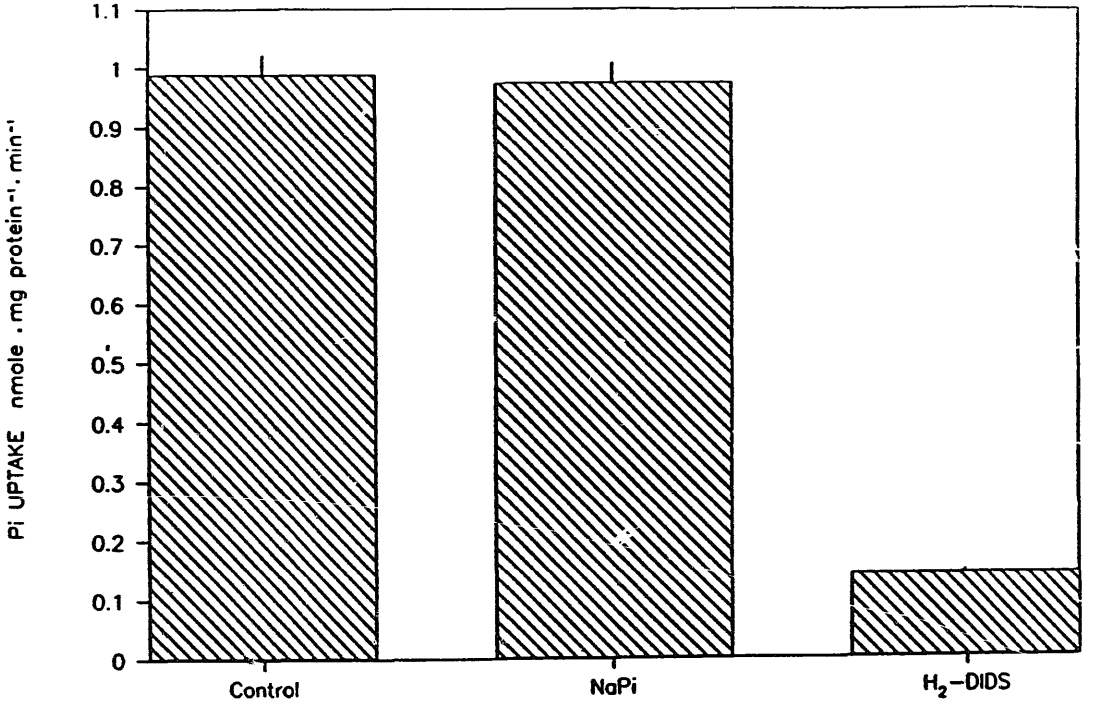

Fig. 3. Protection of H_2-DIDS inhibition of sodium/phosphate cotransport with sodium phosphate. Brush-border membrane vesicles were pretreated with 40 μM H_2-DIDS, 37°C for 25 min, in the presence and absence of 50 mM sodium phosphate. The membranes were subsequently washed with vesicle buffer to remove sodium phosphate and unbound H_2-DIDS and transport was performed as described under Methods.

TABLE I

Protection of sodium / phosphate cotransport with external anions

Brush-border membrane vesicles were preincubated with the various sodium salts of the anions (50 mM) prior to treatment with 100 μM H_2-DIDS. Vesicles were suspended in buffer solution consisting of (in mM): 100, KCl; 20, mannitol, 21, TMA-OH; 42, Hepes-KOH (pH 8.2). The incubation solution used in the protection studies consisted (in mM): 50, Na anion, 50 NaCl, 20 mannitol, 20, TMA-OH; 42 Hepes-KOH (pH 8.2). The vesicles were incubated for 5 min or 25 min at 37°C and the vesicles were subsequently washed three times with vesicle buffer to remove sodium, anions, and unbound H_2-DIDS. Sodium-phosphate uptake was performed by techniques given in text. The transport solution consisted of (in mM): 100, NaCl or 100 KCl; 20, mannitol, 21, TMA-OH; 42, Hepes-KOH (pH 8.2). The ability of the anions to protect against 100 μM H_2-DIDS is given as a percentage of the inhibitable phosphate uptake; i.e. difference between control uptake in the absence of H_2-DIDS and in the presence of 100 μM H_2-DIDS, both performed with NaCl buffer. Values are means ± S.E. for at least four observations performed on one membrane preparation prepared from ten different rats. * indicates significance, $P < 0.001$, from control transport rates.

Anions	5 min preincubation		25 min preincubation	
	sodium-phosphate uptake (pmol min^{-1} (mg protein)$^{-1}$)	protection (%)	sodium-phosphate uptake (pmol min^{-1} (mg protein)$^{-1}$)	protection (%)
Control ($-H_2$-DIDS)	1423±50	100	899±49	100
Na_2HPO_4	1330±42	91±4	937±65	100±7
NaH_2AsO_4	643± 8 *	26±2 *	212± 5 *	3±1 *
Na_2SO_4	417±50 *	4±4 *	211±19 *	3±2 *
$NaHCO_3$	504±35 *	22±3 *	210± 6 *	3±1 *
Sodium succinate	490±30 *	11±3 *	209± 6	3±1 *
Sodium phosphonoformate	516±32 *	13±3 *	210± 6 *	3±1 *
NaCl	372±12 *	0	195± 9 *	0

sulfate was without effect. H_2-DIDS, 100 μM, did not inhibit sodium-glucose uptake suggesting that it binds predominently to anion transporters [11].

Finally, we determined the effect of prolonged treatment of H_2-DIDS with and without protection with sodium phosphate. Brush-border membrane vesi-

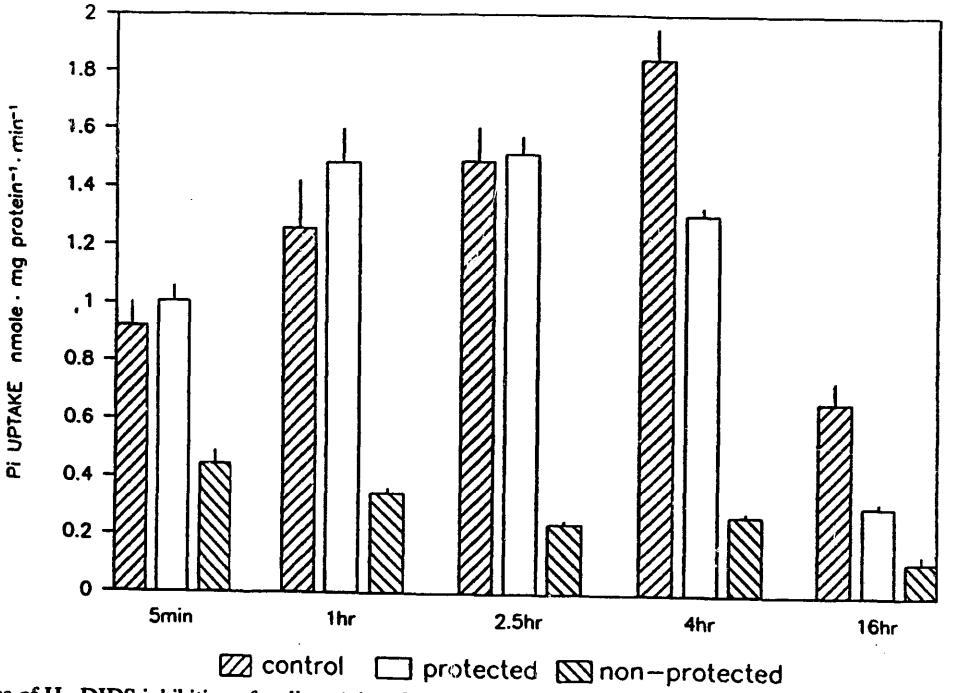

Fig. 4. Time-dependence of H_2-DIDS inhibition of sodium/phosphate cotransport and protection with external sodium phosphate. Brush-border membrane vesicles were treated with 100 μM H_2-DIDS, at 37°C for the time indicated, in the presence and absence of 50 mM sodium phosphate. H_2-DIDS and sodium phosphate was removed by extensive washing and sodium-dependent [^{32}P]phosphate uptake studies performed with 0.1 mM Na_2HPO_4 at 30 s. Values at each time period are mean values of two independent membrane preparations each with four observations. The S.E. indicated is the average of S.E. for the two membrane preparations.

cles were treated with 100 μM H_2-DIDS in the presence and absence of 50 mM sodium phosphate for 5 min to 16 h. Fig. 4 summarizes these results. The presence of sodium-phosphate completely protected the transporter up to about 3 h. After this time period, sodium-phosphate was not entirely protective which may be a reflection of continued covalent binding of H_2-DIDS with the phosphate transporter. Sodium-dependent phosphate uptake increased with time of incubation in the control vesicles to 4 h; we have no explanation for this observation.

Discussion

In this report we have shown that the stilbene disulfonate, H_2-DIDS, a well-characterized inhibitor of membrane anion transport, blocks sodium-dependent phosphate transport in brush-border membranes of the kidney cortex. Inhibition of sodium-phosphate cotransport is irreversible as extensive washings with buffer solutions containing BSA were not effective in enhancing phosphate uptake (Fig. 1). As the IC_{50} is of the order 10 μM in the presence of 0.1 mM phosphate, H_2-DIDS is useful as a label for the cotransporter. Moreover, it is possible to protect the sodium/phosphate cotransporter from H_2-DIDS binding by preincubating the membranes in high concentrations of sodium phosphate. This may allow one to design a method of specifically labeling sodium phosphate protectable sites of brush-border membranes and in so doing it may be a tool to use in protein isolation.

The stilbene derivatives, H_2-DIDS and DIDS have been used extensively to evaluate the role of membrane proteins In the major anion transporter of the red blood cell [11,13,14,19–21]. These reagents have also been shown to inhibit anionic transport in epithelial cells. DIDS has been reported to inhibit Na- and H-sulfate coupled transporter in basolateral membranes of the kidney and intestine [22–25] and ileal brush-border membranes [22,26,27]. Stilbene-sensitive anion transport systems, most likely Cl^-/OH^- exchanger, have been observed in rat ileal and renal brush-border membranes [16,27–29] and in Ehrlich cells [12,15,30]. H_2-DIDS has been used to identify Na^+/HCO_3^- cotransport in pancreatic acinar cells [31]. H_2-DIDS also inhibits bile acid transport across plasma membranes of hepatic cells [32] and has been used to label and reconstitute the Cl^- channel of thick ascending limb cells [33]. The present studies indicate that H_2-DIDS also inhibits the sodium/phosphate cotransporter in renal brush-border membranes. It likely inhibits sodium/phosphate cotransport in other epithelial cells as well, such as the intestine.

A number of reagents have been used to label the sodium/phosphate cotransporter. The most widely used have been the phosphonocarboxylic acids [34–36]. Dousa and colleagues have reported that the phosphonocarboxylic acids, phosphonoformic and phosphonoacetic acid, inhibit sodium-dependent phosphate uptake in renal cortical brush-border membrane vesicles [5,37–39]. These competitive inhibitors are fully reversible, reasonably selective, and have an apparent inhibition constant (K_i) of 1–2 mM [5,10]. More recently, Szczepanska-Konkel et al. have used a brominated derivative of phosphonoacetic acid to improve the affinity, $K_i = 0.33$ mM, and to provide irreversible inhibition of the sodium/phosphate transporter [8]. This analogue apparently forms covalent bonds at alkaline pH with the sodium/phosphate cotransporter. Preliminary results suggest that when δ-bromophosphonoacetic acid is incubated at alkaline pH a covalent bond is formed between the δ-carbon and a nucleophilic group, such as NH_2 or SH, located in the vicinity of the sodium/phosphate cotransporter which irreversibly inhibits transport. Interestingly, as the authors point out, the reaction does not proceed to completion, as only about 35% of the cotransport is irreversibly inhibited whereas 70% is competitive and reversible. Nevertheless, this approach could serve as a useful method of labelling the transporter in brush-border membranes.

Tenenhouse and Lee have reported that sulfate inhibits [^{14}C]phosphonoformate binding, as does phosphate [40]. Accordingly, the sulfate anion is interacting with some binding sites on the membrane similar to phosphonoformate binding sites. In the present studies, both these anions, sulfate and phosphonoformate, were without a protective effect of H_2-DIDS binding to the transporter. This supports the notion that H_2-DIDS is forming covalent bonds with the sodium/phosphate cotransporter which sulfate and phosphonoformate are not able to effectively protect.

Other studies, have recently implicated tyrosine and lysine residues in the sodium/phosphate cotransporter. Wuarin et al. used the group-specific reagent, *N*-acetylimidazole, to inhibit phosphate uptake in an established renal epithelial OK cell line [41]. The preincubation of 10 mM *N*-acetylimidazole irreversibly inhibited sodium-dependent phosphate uptake by 70%. The presence of 5 mM phosphate but not 5 mM sulfate protected the transporter. They tritiated *N*-acetylimidazole and demonstrated that this agent labels four proteins of 31, 53, 104 and 176 kDa. As parathyroid hormone diminished the incorporation of *N*-[^{3}H]-acetylimidazole into the brush-border membranes, they concluded that these proteins were involved in cotransporter function and that tyrosine and/or lysine plays a critical role in this function.

Phenylglyoxal, an organic reactive reagent, has been used by Béliveau and Strevey to inhibit sodium/phosphate cotransport in renal brush-border membranes [42]. Phenylglyoxal, 50 mM, inhibited sodium-

dependent phosphate influx by 60%. Pre-equilibration with sodium or phosphate protected the transporter by about 55% whereas the pre-equilibration with both sodium and phosphate, 150 mM, were fully protective. These studies supported the contention of Béliveau and Strevey that the sodium electrochemical gradient induces conformational changes in the phosphate transporter that may lead to changes in the accessibility of a reactive arginine in the sodium/phosphate cotransporter. The use of amino acid reactive agents may be useful in labelling the proteins involved in sodium/phosphate cotransport of renal brush-border membranes.

In summary, the stilbene analogue H_2-DIDS, or its oxidized form, DIDS, may be useful in labelling proteins. However, as H_2-DIDS also covalently binds to many membrane proteins appropriate protection studies must be included in the protocol to ensure selectivity for the phosphate transporter.

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