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Chemical modification of the Na^+/H^+ exchanger of thymic lymphocytes. Inhibition by *N*-ethylmaleimide

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A Na^+/H^+ exchanger is involved in the regulation of cytoplasmic pH and cellular volume in a variety of cells. Little is known about the molecular nature of this exchanger. The purpose of this study was to survey a variety of group-specific covalent reagents as potential inhibitors of the exchanger. Na^+/H^+ countertransport activity was assayed as the amiloride-sensitive rate of Na^+-induced alkalinization in acid-loaded lymphocytes, or as the rate of swelling in cells suspended in sodium propionate medium. Activity was not affected by proteinases or by carboxyl-group and amino-group specific reagents. A significant inhibition was produced by diethylpyrocarbonate, a histidine-specific reagent and by *N*-ethylmaleimide, a sulfhydryl group reagent. A similarly reactive but nonpermeating sulfhydryl agent, glutathione-maleimide, failed to inhibit Na^+-H^+ exchange. Moreover, the reaction with *N*-ethylmaleimide was sensitive to changes in the cytoplasmic pH. The data suggest that the chemically reactive groups of the Na^+/H^+ exchanger of lymphocytes have limited exposure to the extracellular medium but that an internally located sulfhydryl group is critical for the cation-exchange activity.

Introduction

A transport system that exchanges Na^+ for H^+ has been described in the plasma membrane of a variety of mammalian cells [1–8]. The exchange is electrically silent [1,2,6] and is inhibited by the pyrazine derivative amiloride, a potassium-sparing diuretic [9]. Na^+/H^+ countertransport is involved in transepithelial ion transport [1], in the regulation of cellular volume [2,3], in cytoplasmic pH (pH_i) homeostasis [5,6], and is activated by serum and growth factors [10,11], prompting suggestions of a causal role in mitogenesis. In spite of the clear physiological relevance of the Na^+/H^+ exchanger, its molecular identity has not yet been elucidated, nor is much known about its disposition in the bilayer. By analogy with other ion-transporting system [12,13], it is likely to be an intrinsic transmembrane protein or glycoprotein.

The modification of protein side-chains by means of group-specific reagents has been successfully used in characterizing the functionally relevant groups of several membrane proteins [14]. Moreover, covalent reagents have been helpful in the study of the transmembrane symmetry [15] and the molecular identification of transport proteins [12]. Another approach involves the use of proteolytic enzymes applied to membranes to cleave exposed protein domains [12,14]. The pur-

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; DMSO, dimethylsulfoxide.

pose of the present study was to survey a series of group-specific covalent reagents and proteolytic enzymes as potential inhibitors of the lymphocyte Na^+/H^+ exchanger, in order to gain understanding of its functional groups and their locations. Because the functional assays used in this study were performed in intact cells, the survey was limited to agents that are reactive under physiological conditions, and that do not compromise the integrity of the cells. The results indicate that a cytoplasmically oriented sulfhydryl group is essential for the operation of the exchanger. Moreover, countertransport is also inhibited by reaction with a histidine side-chain. The external aspect of the transporter appears to be resistant to the agents tested.

Materials and methods

Amiloride was the kind gift of Merck, Sharp and Dohme, Canada; 5,6-dicarboxyfluorescein acetoxymethyl ester was the generous gift of the Dr. T.J. Rink, Cambridge University, U.K.; glutathione/maleimide and 2′-7′-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester were synthesized by Dr. M. Ramjeesingh of the Hospital for Sick Children, Toronto by the methods of Abbott and Schachter [16] and Rink et al. [17], respectively. Nigericin was purchased from Calbiochem; propionic acid was from BDH; *N*-ethylmaleimide, tri-*n*-butyltin chloride, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1,3-dicyclohexylcarbodiimide and phenylglyoxal monohydrate were from Aldrich; diazinedicarboxylic acid bis[*N*,*N*-dimethylamide] (diamide), bovine serum albumin, trypsin, chymotrypsin, *p*-chloromercuribenzoic acid, *p*-chloromercuribenzenesulfonic acid, 2,4,6-trinitrobenzenesulfonic acid, monensin, diethylpyrocarbonate, sodium propionate and pyridoxal 5-phosphate were from Sigma.

Solutions. Phosphate-buffered saline contained (in mM): 137 NaCl, 2.7 KCl, 1.2 KH_2PO_4, 8.1 Na_2HPO_4, 10 glucose, 0.68 $CaCl_2$ and 0.49 $MgCl_2$. Choline$^+$-solution contained (in mM): 140 choline chloride (recrystallized from methanol), 10 glucose, 1 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 20 Tris-Mes (pH 7.2). Na^+-solution and K^+-solution were prepared by isoosmotic replacement of choline$^+$ by Na^+ or K^+, respectively, but were otherwise identical. Sodium propionate medium contained (in mM): 140 sodium propionate, 1 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes (pH 6.7).

Stock solutions of nigericin and monensin were made in ethanol and kept at −20°C. The pH-sensitive carboxyfluorescein derivatives were stored frozen in DMSO. A 10 mM aqueous stock of amiloride was kept at 4°C. Solutions of all other reagents were freshly made on the day of the experiment.

Cell isolation and characterization. Thymic lymphocytes were isolated from male Wistar rats as previously described [6] and were maintained for up to 8 h in Hepes-buffered solution RPMI 1640 (HCO_3^--free). Cell viability, determined by trypan blue exclusion, was greater than 95% throughout this period. Cell sizing and counting were performed with a Coulter Counter (Model ZM) and Channelyzer combination, using the shape factor determined by Segel et al. [18] for human lymphocytes.

Intracellular pH (pH_i) measurement and manipulation. Measurement of pH_i was performed fluorimetrically using dicarboxyfluorescein or bis(carboxyethyl)carboxyfluorescein-loaded cells essentially as described [6,19]. Acid-loading of the cells was accomplished by addition of the K^+-H^+ exchange ionophore, nigericin, to cells suspended in choline$^+$-solution (see Ref. 6 and Results for details). Acid-loading could be terminated at the desired pH_i by addition of serum albumin (5 mg/ml final) to scavenge the ionophore [6]. The buffering power of the cells was determined by the NH_4^+-pulse technique [20, as desribed earlier [6].

Other methods. Na^+ and K^+ contents were estimated by flame photometry using Li^+ as an internal standard, as described [19]. Unless otherwise indicated, the experiments were performed at 20–22°C. The results are presented in the tables either as the mean ± S.E. of the number of determinations indicated in parenthesis, or in the figures as representative traces of at least three similar experiments.

Results

Na^+-H^+ exchange assay: a survey of potential inhibitors

The Na^+/H^+ exchanger of thymic lymphocytes

is virtually quiescent at physiological pH_i (7.0–7.1), but is greatly stimulated upon cytoplasmic acidification [6]. In acid-loaded cells, its operation can be conveniently detected as a Na^+-induced alkalinization of the cytoplasm. In order to assess the effects of a number of reagents on Na^+-H^+ exchange activity, lymphocytes were acid-loaded and their pH_i monitored fluorimetrically, using carboxyfluorescein derivatives. Acid-loading was accomplished by resuspending cells in choline$^+$-solution and addition of nigericin, which catalyzes the exchange of intracellular K^+ for external protons. Once the desired level of pH_i was reached, the ionophore was scavenged by addition of albumin and Na^+-H^+ exchange activity was tested by addition of Na^+ to the medium. A typical experiment is illustrated in Fig. 1 in which the acid-loading and ionophore-scavenging sections are omitted for simplicity, (see Refs. 6 and 19 for illustrations of the full procedure). As reported earlier [6], a rapid alkalinization is recorded upon addition of extracellular Na (Na_o^+), which can be completely abolished by amiloride (Fig. 1a and b). At pH_i 6.3–6.4, and using 70 mM Na_o^+, the maximal of rate of alkalinization approaches 0.3 pH units/min. Considering the cytoplasmic buffering power (25 mmol/l per pH unit), this is equivalent to a H^+ extrusion rate of 7.5 $mmol \cdot l^{-1} \cdot min^{-1}$.

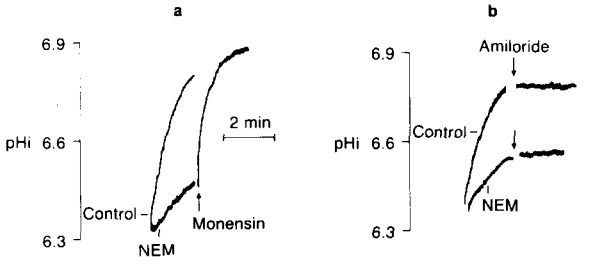

Fig. 1. Effects of *N*-ethylmaleimide (NEM) and amiloride on Na^+-induced alkalinization. Rat thymocytes were loaded with dicarboxyfluorescein, resuspended in phosphate-buffered saline and incubated for 15 min in the presence or absence of 1 mM *N*-ethylmaleimide. The cells were then sedimented and resuspended in choline$^+$-solution for acid-loading, which was accomplished by addition of nigericin as described in Materials and Methods (not illustrated). After termination of acid-loading by scavenging the ionophore with albumin, the activity of the Na^+/H^+ exchanger was tested by suspending the cells in medium containing 70 mM Na^+ (1 vol. Na^+-medium and 1 vol. choline$^+$-medium). The fluorescence traces shown commence upon resuspension of the cells in the Na^+-containing medium. (a) Comparison of the response of control and maleimide-treated cells. At the time indicated, 1 μM monensin was added to the *N*-ethylmaleimide-treated sample. (b) Effect of amiloride on the total and maleimide-resistant Na^+-H^+ exchange activity. Where indicated by the arrows, 50 μM amiloride was added to both samples. The traces are representative of at least five similar experiments. See Materials and Methods for details of pH_i measurement and calibration.

To evaluate the inhibitory potency of a series of reagents, cells ($(20–25) \cdot 10^6$/ml) were suspended in phosphate-buffered saline and incubated for 15 min at room temperature with the concentrations of the reagents indicated in Table I. The cells were then sedimented and resuspended in choline$^+$-solution for acid-loading and testing of amiloride-sensitive Na^+-induced alkalinization. An aliquot of the treated cells was used for viability determinations. Only those samples with acceptable viability (over 80%) were assayed for Na^+/H^+ activity.

A typical experiment in which Na^+-induced alkalinization was determined following incubation with one of the potential covalent inhibitors is illustrated in Fig. 1. In this case, cells were pretreated with 1 mM *N*-ethylmaleimide prior to the pH_i determination. Under the conditions used, this concentration of *N*-ethylmaleimide reduced the rate of alkalinization by over 50%. Also shown in Fig. 1 is the effect of monensin, an exogenous Na^+-H^+ exchange ionophore. Addition of the ionophore to *N*-ethylmaleimide-treated cells produced a rapid alkalinization, indicating that their reduced response is due to inhibition of the intrinsic Na^+-H^+ exchange mechanism, rather than to indirect effects associated with alterations of the ionic gradients (see below).

The effect on Na^+-H^+ exchange of a number of inhibitors could not be assessed by means of the fluorescence assay, since they interfered with the probe or affected pH_i significantly. In these instances, an alternative assay, illustrated in Fig. 2, was employed. This method detects the operation of the transporter as changes in cellular volume, following resuspension of the lymphocytes in sodium propionate solutions [21]. Penetration of the protonated lipid-soluble acid leads to cytoplasmic acidification and activation of Na^+-H^+ exchange. The continued operation of the antiport upon more prolonged exposure to sodium propionate results in a considerable increase in Na^+

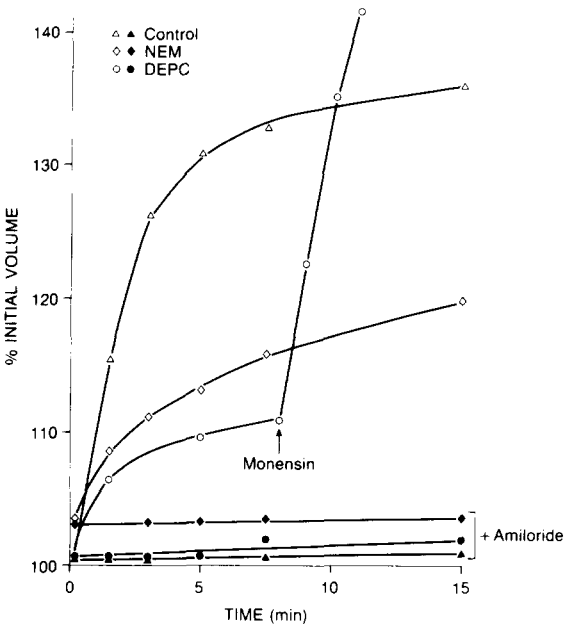

Fig. 2. Effects of *N*-ethylmaleimide (NEM), diethylpyrocarbonate (DEPC) and amiloride on Na^+-H^+ exchange detected as cell swelling. Cells were preincubated in phosphate-buffered saline for 15 min in the presence or absence of either 0.5 mM *N*-ethylmaleimide (◇, ◆) or 1 mM diethylpyrocarbonate (○, ●) at room temperature (20–22°C). The cells were then sedimented and resuspended in sodium propionate solution with (solide symbols) or without (empty symbols) 100 μM amiloride. Cellular volume was measured at the indicated times after resuspension using the Coulter Counter-Channelyzer combination. The median volume of control thymocytes ranged between 110 and 114 μm^3. Where indicated, 5 μM monensin was added to the diethylpyrocarbonate-treated sample. The results are representative of three or more similar experiments.

(and propionate$^-$) content. This is accompanied by an osmotic water shift and cell swelling, measurable by electronic cell sizing using the Coulter-Channelyzer system (Fig. 2). As shown in Fig. 2, and in agreement with earlier results [21], this swelling is virtually abolished by amiloride, an inhibitor of the antiport. The usefulness of this assay to quantitate inhibition of Na^+-H^+ exchange was established by using *N*-ethylmaleimide, which was previously shown to inhibit the exchanger by the more direct pH_i assay (Fig. 1). As shown in Fig. 2, pretreatment with this sulfhydryl reagent also inhibits swelling in sodium propionate medium to about the same degree (approx. 60%).

A summary of the results obtained with a variety of reagents is presented in Tables I and II. Briefly, the amiloride-sensitive Na^+-induced alkalinization (Table I) was unaffected by amino-, carboxyl- or arginine-specific reagents, or by the proteolytic enzymes trypsin and chymotrypsin. In contrast, significant inhibition was detected in the case of *N*-ethylmaleimide, *p*-chloromercuribenzoic acid and *p*-chloromercuribenzenesulfonic acid, whereas the sulfhydryl-oxidizing agent, diamide [22], had little effect.

Swelling in sodium propionate (Table II) was not affected by pretreating the cells with the amino-group reagent trinitrobenzene sulfonate or with dicyclohexylcarbodiimide, a lipid-soluble carboxyl-group reagent. In contrast, a marked inhibition was found using diethylpyrocarbonate (Fig. 2 and Table II). Cells partially inhibited with this agent could swell rapidly upon addition of monensin, indicating that the cytoplasmic acidification and the inward Na^+ gradient persisted. Importantly, complete inhibition was obtained when amiloride was added to diethylpyrocarbonate-treated cells, suggesting that the residual swelling is also mediated by the diuretic-sensitive system.

Studies of the mechanism of inhibition of Na^+-H^+ exchange

Diethylpyrocarbonate spontaneously hydrolyses in aqueous solutions to yield ethanol and carbon dioxide. This results in a marked cytoplasmic acidification and complicates the study of the concentration dependence of the inhibitory effect. For these reasons, the mechanism of diethylpyrocarbonate inhibition was not investigated further. The sulfhydryl reagents *p*-chloromercuribenzoate and *p*-chloromercuribenzenesulfonate produced substantial spontaneous increases in cellular volume with associated gain of cellular Na^+ and loss of K^+ (data not shown), which resemble the effects of organomercurials in red cells [23]. Because both the ionic gradients and cellular volume have critical effects on the activity of the Na^+/H^+ exchanger [6,19], the inhibitory mechanism of the organomercurials was not further explored. Instead, a more detailed investigation of the mode of action of *N*-ethylmaleimide was undertaken.

TABLE I

EFFECTS OF COVALENT REAGENTS ON Na^+-H^+ EXCHANGE ACTIVITY DETECTED AS Na^+-INDUCED ALKALINIZATION

The Na^+-H^+ exchange activity was assessed as a Na^+-induced alkalinization of acid-loaded cells (see Materials and Methods). Viability was determined by Trypan blue exclusion. Treatment with the reagents was for 15 min at 20–22°C, except in the case of the proteinases in which case the temperature was 37°C. The data are the means ± S.E. of the number of experiments in parentheses. n.d., not determined.

Reagent	Type [a]	Concentration (mM)	Effect on Na^+-H^+ exchange (% inhibition)	Viability (%)
Pyridoxal phosphate	amino group	0.5	n.d.	≥ 90
		1.5	5.0; 8.7 [b]	≥ 90
Dimethylaminopropylethyl-carbodiimide	carboxyl group	0.5	n.d.	≥ 90
		2.0	1.7; 0.5 [b]	≥ 90
Phenylglyoxal	arginine	1	8.3 ± 8 (3)	≥ 90
		3	n.d.	85
N-Ethylmaleimide	sulfhydryl group	1.0	64.7 ± 5.3 (5)	≥ 90
p-Chloromercuribenzoic acid	sulfhydryl group	0.1	28.5 ± 5.9 (4)	75–90
		0.5	n.d.	< 20
p-Chloromercuribenzene-sulfonic acid	sulfhydryl group	0.25	31.2 ± 7.4 (4)	≥ 90
Diamide	sulfhydryl group	2	n.d.	≥ 90
		6	3.7 ± 2.1 (4)	≥ 90
Trypsin	proteinase	$6.2 \cdot 10^{-3}$	4.3 ± 4 (3)	≥ 80
Chymotrypsin	proteinase	$5.9 \cdot 10^{-3}$	6.0 ± 3 (3)	≥ 90

[a] Refers to the primary, though not necessarily the only, target group for the reagent.
[b] Range of two experiments.

The time-course of inhibition of Na^+-H^+ exchange (measured as Na^+-induced alkalinization) by *N*-ethylmaleimide is illustrated in Fig. 3a. In these experiments, the cells were preincubated with 0.5 mM *N*-ethylmaleimide for the indicated periods in phosphate-buffered saline, followed by washing and fluorimetric assay of Na^+-H^+ exchange activity. During the assay, pH_i was 6.25–6.35, pH_o was 7.2 and Na_o^+ was 70 mM. The inhibition had a relatively rapid onset (it was detectable by 5 min), but was incomplete even after 1 h. Fig. 3B shows the concentration depen-

TABLE II

EFFECTS OF COVALENT REAGENTS ON Na^+-H^+ EXCHANGE ACTIVITY MEASURED AS SWELLING IN SODIUM-PROPIONATE

Na^+-H^+ exchange activity was assessed as cellular swelling of cells suspended in sodium propionate solution. Viability was defined as the percent cells excluding Trypan blue. Treatment with the reagents was for 15 min at 20–22°C. Where the inhibition was sufficiently large for quantitation, the data are given as means ± S.E. of the number of experiments in parentheses. n.d., not determined.

Reagent	Type	Concentration (mM)	Effect on Na^+-H^+ exchange (% inhibition)	Viability (%)
Trinitrobenzene sulfonate	amino group	0.1	< 10	≥ 90
		0.5	< 10	≥ 90
Dicyclohexyl-carbodiimide	carboxyl group	0.05	< 10	≥ 90
		0.3	n.d.	20
Diethylpyrocarbonate	histidine	1.0	64.2 ± 8.2 (5)	≥ 90
N-Ethylmaleimide	sulfhydryl group	1.0	58.3 ± 11 (3)	≥ 90

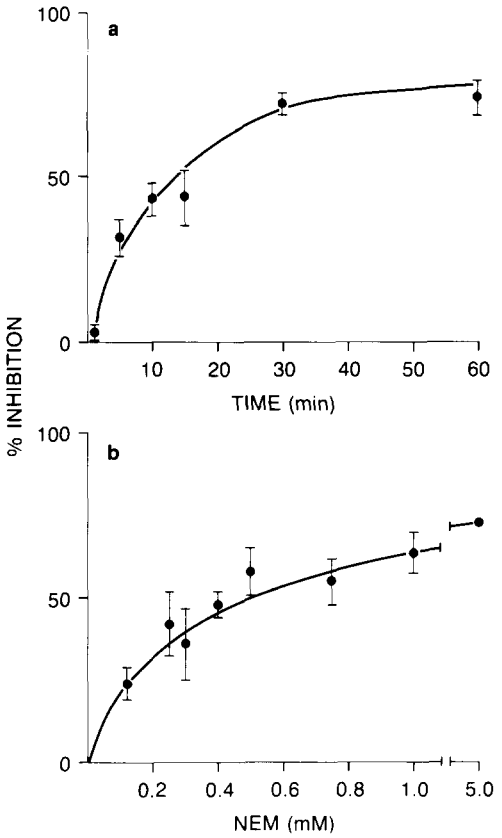

Fig. 3. Time and concentration dependence of the inhibition of Na^+-H^+ exchange by *N*-ethylmaleimide (NEM). Na^+-H^+ exchange was measured as the amiloride-sensitive Na^+-induced alkalinization of acid-loaded cells. The conditions were pH_i = 6.3–6.4, pH_o = 7.2, $[Na^+]_o$ = 70 mM. (a) Time dependence. The cells (approx. $2.5 \cdot 10^6$/ml) were incubated with 0.5 mM *N*-ethylmaleimide in phosphate-buffered saline for the periods of time indicated in the abscissa. This was followed by sedimentation, acid-loading and assay of Na^+-induced alkalinization as described for Fig. 1. Ordinate: percent inhibition of Na^+-H^+ exchange activity. The data are means ± S.E. of three experiments. (b) Concentration dependence. The cells were incubated for 15 min in the presence of the concentrations of *N*-ethylmaleimide indicated in the abscissa, followed by sedimentation and assay of Na^+-induced alkalinization as above. The data are means ± S.E. of three experiments, except for the 5 mM point, which is the mean of only two determinations.

dence of the inhibitory response, measured after a 15 min incubation in phosphate-buffered saline. Importantly, inhibition was incomplete even at comparatively high concentrations of the reagent, with little change between 1 and 5 mM. In all instances, viability, defined as the ability to exclude Trypan blue, was not less than 80% and the residual Na^+-H^+ exchange activity could be completely blocked by 50 μM amiloride.

As mentioned above and discussed in detail elsewhere [6], the activity of the antiport is critically dependent on the cytoplasmic Na^+ concentration and on cellular volume. It was therefore essential to establish if *N*-ethylmaleimide was affecting these parameters or whether inhibition resulted from direct reaction with the exchanger. In four experiments, the median cellular volume was not affected significantly by concentrations of *N*-ethylmaleimide ranging from 5 μM to 5 mM, over a period of at least 15 min. Results for 0.5 mM are summarized in Table III.

In ten control experiments, the intracellular concentrations of Na^+ and K^+ were found to be 29.8 and 138.1 mM, respectively (Table III). After a 30 min incubation in the presence of 0.5 mM

TABLE III

EFFECTS OF *N*-ETHYLMALEIMIDE ON CELLULAR VOLUME AND CONTENTS

Cell volume was measured electronically using the Coulter-Channelyzer combination. Buffering power was measured fluorimetrically in the pH_i 6.4–7.1 range by the NH_4^+-pulse technique. Na^+ and K^+ contents were determined by flame photometry. Where indicated, cells were treated with 0.5 mM *N*-ethylmaleimide for 15 min. Data are means ± S.E. of (*n*) experiments. ns., not significantly different ($P > 0.1$). *P* was calculated using Student's *t*-test for nonpaired data.

	Control	*N*-Ethylmaleimide-treated	*P*
Cell volume (μm^3)	115.2 ± 1.1 (8)	115.8 ± 2.3 (4)	n.s.
Buffering power (mmol/l per pH unit)	23.2 ± 2.3 (21)	22.3 ± 2.6 (18)	n.s.
Na^+ content ($mmol \cdot l^{-1}$)	29.8 ± 5.3 (10)	62.2 ± 2.6 (4)	< 0.005
K^+ content ($mmol \cdot l^{-1}$)	138.1 ± 6.7 (10)	110.2 ± 8.1 (4)	≤ 0.02

N-ethylmaleimide, Na^+ content increased to 62 mM, while K^+ content dropped to 110.2 mM (Table III). Because Na^+ an conceivably with H^+ for the cytoplasmic site on the exchanger, it was important to determine whether the changes in ionic content underlie the inhibition. For this purpose, similar changes in cellular Na^+ were induced by preincubation of cells with ouabain (2.5 mM), an inhibitor of the Na^+/K^+ pump. These cells were then used for determinations of Na^+-H^+ exchange activity by either the Na^+-induced alkalinization or propionate-mediated swelling assays. After a 90 min incubation with the glycoside, the Na^+ concentration increased to 52 mM (average of two determinations), compared to 22 mM in the paired controls. In four determinations, the Na^+-induced H^+ extrusion rates in controls averaged 11.2 ± 0.4 mmol $\cdot l^{-1} \cdot min^{-1}$. A 15-min treatment of these cells with 0.5 mM *N*-ethylmaleimide reduced this rate by 49% (average of two determinations), consistent with the results of Fig. 3. The H^+ extrusion rate in ouabain-treated cells was 10.6 ± 0.2 mmol $\cdot$ (l cells)$^{-1} \cdot$ min^{-1} ($n = 5$), which is not significantly different from the control rate *. Treatment of these Na^+-loaded cells with *N*-ethylmaleimide resulted in the expected inhibition (48%, average of two experiments). Using the swelling assay in cells loaded with Na^+ to comparable or even higher (up to 89 mM) levels, the results were similar. The *N*-ethylmaleimide inhibition was as high in Na^+-loaded as in control cells.

That the change in ion content does not mediate the inhibitory effect of *N*-ethylmaleimide was also shown by treatment with inhibitor in Na^+-free K^+-solution. Under these conditions, the intracellular Na^+ concentration does not increase during the incubations (21 mM after 15 min, average of two determinations), but the inhibition persists: 75% inhibition when using 0.5 mM maleimide. The Na^+-H^+ exchange in untreated controls was slightly stimulated by preincubation in K^+-solution, in agreement with earlier results [6].

The determination of the rate of H^+ extrusion, as well as the rate of swelling in propionate$^-$ media depend critically on the buffering power of the cells. It is conceivable that *N*-ethylmaleimide could alter this parameter, resulting in an apparent inhibition of Na^+-H^+ exchange. To rule out this possibility, buffering-power determinations were carried out in control and *N*-ethylmaleimide-treated cells by the NH_4^+-pulse technique [20] as described elsewhere [6]. The results are summarized in Table III. In the pH_i range of 6.4–7.1, the buffering capacity of control cells was 23.2 ± 2.3 mmol/l per pH unit. After treatment with *N*-ethylmaleimide (0.5 mM for 15 min) the buffering power was found to be 22.3 ± 2.6 mmol/l per pH unit, which is not significantly different from the control value. Therefore, a change in buffering power cannot explain the inhibitory effects of the maleimide.

Sidedness of the N-ethylmaleimide inhibition

N-Ethylmaleimide readily permeates biological membranes and could therefore be acting at either the internal or external surface of the plasma membrane, or within its hydrophobic core. To gain knowledge of its site of action, the inhibitory potency of *N*-ethylmaleimide was compared with that of glutathione-maleimide, a nonpermeating analog with comparable sulfhydryl reactivity [16].

Paired samples were treated with both maleimides and their Na^+/H^+ countertransport activity was compared to that of controls using the fluorescence assay. In three experiments, a 15-min incubation with 1 mM *N*-ethylmaleimide produced 63.3 ± 8.9% inhibition of Na^+-induced H^+ extrusion. In the same batches of cells, freshly dissolved glutathione-maleimide at concentrations of 0.5, 2.5 and 5 mM inhibited exchange by 0%, 13.3 ± 3.6% and 15.3 ± 7.7%, respectively. The small and variable inhibition observed at high concentrations may be due to contamination of the glutathione-maleimide with a permeable maleimide derivative. The failure of the impermeant maleimide to block Na^+-H^+ exchange was confirmed using the propionate-swelling assay (data not shown). These data suggest that the reactive sulfhydryl groups are exposed only at the cytoplasmic surface.

Protection experiments

A series of experiments were performed to de-

* Increasing intracellular Na^+ failed to competitively inhibit H^+ efflux under these conditions. This may be explained by the low pH_i used for these determinations (approx 6.3). Given the comparatively low affinity of the transport site for Na^+ (apparent $K_m = 51$ mM), the internal face of the transporter might have been saturated with H^+ at pH_i 6.3.

termine whether the extent or rate of inhibition could be affected by the functional state of the exchanger. Cells were exposed to the inhibitor under conditions where the exchanger is largely quiescent ($pH_i \geq 7.0$) or activated ($pH_i = 6.3$). This was followed by a comparison of the residual Na^+-H^+ exchange activity under identical conditions: $pH_i = 6.3–6.4$, $pH_o = 7.2$ and $Na_o = 70$ mM. In three experiments, the maleimide inhibited exchange by $67.4 \pm 6.7\%$ (mean $\pm$ S.E.) when incubated at pH_i 7.0, but only by $46.2 \pm 7.8\%$ (mean $\pm$ S.E.) at pH_i 6.3–6.4. Thus, activation of countertransport by acid-loading did not increase the exposure of maleimide-sensitive sites. The reduced inhibition may be due to the decreased reactivity of sulfhydryl groups at reduced pH_i (see Discussion).

Experiments were also performed to determine whether the occupancy of the outside transport site by substrates or inhibitors would affect the inhibition by *N*-ethylmaleimide. Cells were incubated with the inhibitor in the presence or absence of Na^+ (K^+ substitution) or in the presence of amiloride. This was followed by washing, acid-loading and measurement of Na^+-induced alkalinization as described above. Preincubation in K^+-solution with or without amiloride in the absence of *N*-ethylmaleimide did not significantly affect transport, and the maleimide inhibited exchange to comparable levels when incubated in Na^+-solution, K^+-solution or K^+-solution with 200 μM amiloride ($52.2 \pm 1.7\%$, $50.5 \pm 3.7\%$ and $46.7 \pm 6.4\%$, respectively, $n = 4$).

Nature of the N-ethylmaleimide-resistant Na^+-H^+ exchange

A fraction of the Na^+-H^+ exchange activity was resistant to *N*-ethylmaleimide (Fig. 3). This flux could reflect a separate population of exchangers which, although insensitive to the maleimide, are inhibited by amiloride. Alternatively, it could represent a more slowly transporting mode of a homogeneous population. In the former case, the resistant population might be expected to differ from the rest of the exchangers not only in its sensitivity towards the maleimide, but perhaps in other parameters as well. To distinguish between the above possibilities, we compared the external pH dependence and amiloride sensitivity of the total and *N*-ethylmaleimide-resistant fluxes. Fig. 4 illustrates a comparison of the external pH sensitivity of untreated and maleimide-treated cells in the 6.2–7.8 range. As reported earlier [6], Na^+-induced alkalinization in intact cells is minimal at pH 6.0–6.2 and increases linearly between 6.2 and 7.8. When the residual flux (37% of the control) is normalized, an identical dependence on external pH_o was found (open symbols, Fig. 4).

The amiloride sensitivity of the total and residual fluxes was also virtually identical. Addition of 7.5 μM amiloride inhibited the control by 50.4% and the residual flux by 48.4% (averages of two experiments). In these experiments, the residual flux was 33% of the initial flux.

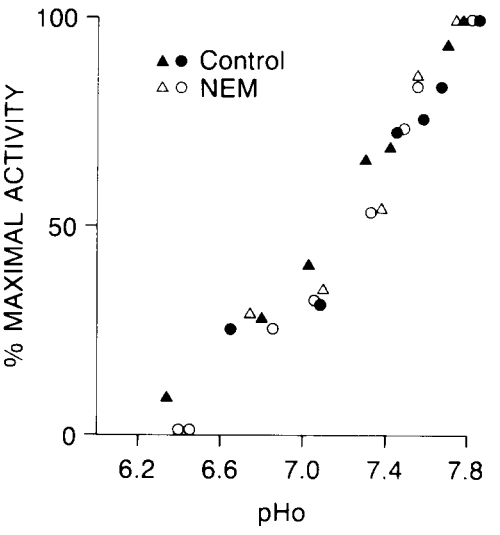

Fig. 4. External pH (pH_o) dependence of Na^+-H^+ exchange in untreated (solid symbols) and *N*-ethylmaleimide-treated (NEM, open symbols) lymphocytes. The cells were preincubated in the presence or absence of 0.5 mM *N*-ethylmaleimide for 15 min at room temperature, followed by sedimentation and acid-loading in choline$^+$-solution. Na^+/H^+ countertransport was assayed as the Na^+-induced alkalinization, detected fluorimetrically in dicarboxyfluorescein-loaded cells. The assay medium was buffered with Tris-Mes and the pH was varied in the 6.2–7.8 range by changing their ratio, while keeping their total concentration at 20 mM. To allow comparison, the data are normalized as percentage of maximal activity, which was observed at pH 7.7–7.8. The points are individual measurements from two experiments, represented by the two symbols. In these experiments, *N*-ethylmaleimide inhibited Na^+-H^+ exchange by 77% (average of two determinations at pH_o 7.3).

Discussion

Relatively little is known about the molecular properties of the Na^+/H^+ exchanger. From functional studies, it appears that transport sites that can bind Na^+ and H^+ are exposed at both the inside and outside faces of the membrane [1,6–8]. An additional regulator site that also binds H^+ and perhaps Na^+ is exposed only at the cytoplasmic face [6,24]. The inhibitor amiloride appears to bind to the outward-facing transport site, in competition with Na^+ [1,6,7]. Thus, the transporter is functionally asymmetric and is exposed at both surfaces.

Grillo and Aronson [25] suggested that an external histidine residue is involved in the transport mechanism in renal brush-border vesicles. This conclusion was based on the inhibition resulting from Rose-bengal-catalyzed photo-oxidation and from treatment with diethylpyrocarbonate. Our results confirm the inhibitory properties of diethylpyrocarbonate (Table I and Fig.2). Unfortunately, the chemical instability of this reagent prevented further characterization of its reaction.

Parker [26] reported recently that glutaraldehyde can stabilize or 'lock in' the volume-activated exchanger of dog red cells. In this case, the regulatory function of the molecule has been impaired, but the transporting function remains unaffected, in spite of the avid reaction of the aldehyde with amino groups. The present results are consistent with this view, since a number of amino-specific reagents failed to alter the rate of Na^+/H^+ coutertransport (Table I). In addition, the present data suggest that the exchanger has a very limited exposure to the external milieu. A number of nonpenetrating reagents with varied chemical specificities failed to affect the countertransport rate. These include amino-group reagents (such as trinitrobenzene-sulfonate and diisothiocyanostilbene disulfonate), carboxyl-group reagents (such as dimethylaminopropylethylcarbodiimide), sulfhydryl reagents (like glutathione maleimide and dithiobisnitrobenzoic acid) and proteinases (trypsin and chymotrypsin).

The activity was markedly inhibited by *N*-ethylmaleimide. Although potentially reactive with amino and imidazole groups, maleimides preferentially react with sulfhydryl side-chains under physiological conditions [27]. The reactive sulfhydryl groups involved in Na^+-H^+ exchange appear to be located on the cytoplasmic side of the membrane, based on two lines of evidence. First, the lipid-soluble *N*-ethylmaleimide was much more inhibitory than the nonpermeant glutathione-maleimide. Second, the inhibition was more pronounced when the incubation with the maleimide was at $pH_i \simeq 7.0$ than at pH_i 6.3–6.4, at constant external pH. This is likely due to the increased availability at higher pH_i of the mercaptide ion, which is the chemical species reactive with the alkylating agent [27]. The internal location of the critical sulfhydryl group(s) would also account for the failure of extracellular Na^+ and amiloride to protect the transporter from inhibition by the maleimide.

The results also provide some information concerning the mechanism of inhibition. The maleimide did not change the apparent affinity of the exchanger for extracellular H^+ or amiloride, indicating the intactness of the externally facing transport site. Moreover, transport was inhibited throughout the pH_i 6.3–6.9 range, and the fractional inhibition was approximately constant throughout this range (data not shown), suggesting that the affinity of the internally facing site was not altered. Finally, the inhibition was never complete, regardless of the time or concentration of inhibitor used. The simplest hypotheses compatible with these observations are that the alkylating agent acts on all of the exchangers producing a partial inhibition, or that there are two species of exchangers, both being amiloride-sensitive but only one being susceptible to N – ethylmaleimide inhibition. Given that no independent evidence of two types of exchanger was found, the first explanation appears to be preferable. Since the residual transport activity involves no change in apparent affinities for H_o^+ or amiloride, it is unlikely that the maleimide reacted with the transport site. The same conclusion arises from the observation that the reaction is not modulated by the presence of Na^+ or amiloride. Therefore, the observed partial inhibition may result from the modification of a sulfhydryl group that reduces the translocation rate of the transporter.

In view of the complexity inherent to whole cell systems, indirect mechanisms of inhibition cannot

be entirely excluded. Changes in buffering power, cell volume or ion content were ruled out as possible causes of the inhibition. On the other hand, a reduction in the number of transport sites could result from impaired insertion of sites into the membrane. Inhibition of protein synthesis by the maleimide is unlikely to underlie the inhibition since: (a) the onset of the blocking effect is detectable by 5 min and (b) preincubation of cells with concentrations of puromycin, cycloheximide or actinomycin D which abolish protein synthesis had no inhibitory effect on Na^+-H^+ exchange (Goetz, J.D. and Grinstein, S., unpublished data).

Inhibition of membrane recycling could also reduce the number of exchangers in the plasma membrane. Although this possibility cannot be presently ruled out, we have evidence that at least part of the effect must occur on exchangers already inserted in the plasmalemma. This conclusion is based on experiments where amiloride-sensitive $^{22}Na^+$ uptake was measured in isolated membrane vesicles (prepared as in Ref. 28) while imposing a pH gradient (pH 8 outside, pH 6 inside). In five experiments, pretreatment of the vesicles with 2–10 μmol *N*-ethylmaleimide per mg membrane protein (comparable to the reagent-to-protein ratios used for intact cells) inhibited Na^+ uptake by $65.2 \pm 12.5\%$. This indicates that the alkylating agent is capable of direct partial inhibition of the exchanger in a system devoid of membrane recycling.

In summary, the data indicate that in thymocytes, the integrity of histidine and sulfhydryl side-chains is essential for the optimal activity of the Na^+-H^+ exchange system. Moreover, the relevant sulfhydryl group(s) are internally located and their reactivity is unaffected by changes in the functional state of the protein. As in other transport systems [14,16], the maleimides may be useful agents of identifying the transport protein and for determining its arrangement in the bilayer.

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