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Modification of erythrocyte Na^+/Li^+ countertransport kinetics by two types of thiol group

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Abstract

Erythrocyte Na^+/Li^+ countertransport activity is decreased by reagents that react with thiol groups. An understanding of the role of these groups in control of Na^+/Li^+ countertransport may help to explain its association with disease states. The effect of thiol reactive agents on the kinetic parameters of Na^+/Li^+ countertransport has not previously been described. In choline medium, *N*-ethylmaleimide (NEM) and iodoacetamide (IAamide) cause a rapid decrease of about 40% in K_m for external sodium (K_m(So)) that is complete in 10 s with a much smaller change in V_{max} and an increase in the V_{max}/K_m ratio. In Na medium, NEM and IAamide both cause a rapid decrease in K_m(So) and V_{max}. With NEM the partial reduction in V_{max} is complete in 100 s although the NEM is sufficient to reduce V_{max} up to 15 min. With IAamide the decrease in V_{max} is initially slower but it continues apparently towards complete inhibition. These results indicate at least two types of thiol group controlling Na^+/Li^+ countertransport kinetics. The type 1 thiol reaction is Na independent and causes an increase in the apparent rate constant for Na association with the unloaded carrier so that V_{max}/K_m rises and K_m(So) decreases. The type 2 thiol reaction is facilitated by Na at the outside ion-binding site and causes a decrease in V_{max}, possibly by total blockage of carriers with IAamide but by a different mechanism with NEM such as reduced turnover rate.

Keywords: Erythrocyte membrane; *N*-Ethylmaleimide; Iodoacetamide; Sodium/lithium ion countertransport; Sulfydryl group; Thiol group; Transport kinetics

1. Introduction

Erythrocyte membranes show an obligatory, equimolar, exchange of intracellular lithium or sodium ions for extracellular lithium or sodium ions that is not inhibited by ouabain or furosemide and that does not require the presence of $HCO3^-$ ions; this activity has been called sodium-lithium countertransport. This transport mechanism obeys Michaelis-Menten kinetics and shows simple ping-pong kinetics [1]. It is not known what mediates this countertransport, but the fact that it is inhibitable by the alkylating agent *N*-ethylmaleimide (NEM) [2,3] suggests that it is a protein. As there is essentially no Li^+ in serum, Na^+/Li^+ countertransport must function in vivo as an equimolar Na^+/Na^+ exchange pathway which would appear to have no effect on cell sodium content and therefore no obvious physiological role [4].

Following some early studies on the nature of this transport process it became apparent that an increased activity is associated with some diseases such as essential hypertension and diabetic nephropathy [4], though there is still no proven pathophysiological link between the cause of these diseases and the altered Na^+/Li^+ countertransport activity. There have been in consequence many clinical studies of this countertransport activity using a single extracellular Na^+ concentration. Its role in relation to disease remains obscure and the suggestion that it is a mode of operation of the Na^+/H^+ exchanger now looks very unlikely [5–7]. We have recently shown that the difference in Na^+/Li^+ countertransport between normal and hypertensive subjects can be abolished by NEM treatment of the erythrocytes [8].

Na^+/Li^+ countertransport has been reported to be partially inhibited by NEM in a way that is dependent on the

Abbreviations: IAamide, iodoacetamide; NEM, *N*-ethylmaleimide; K_m(So) and V_{max}, Michaelis constant and maximum velocity with respect to external sodium assuming that the internal ion site is effectively saturated with lithium.

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presence of a transportable ion; either sodium or lithium [2]. A subsequent study confirmed much of these findings but disagreed on the apparent affinity for extracellular Na^+ to facilitate inhibition [3]. Neither of these studies investigated the kinetic mechanism by which the Na^+/Li^+ countertransport was reduced although it was suggested that the turnover rate of the carrier was affected [2]. Our initial study suggested that both K_m for external sodium (K_m(So)) and V_{max} were affected [9]. The target of NEM reaction is not known, neither as to it being a thiol group, nor as to it being the transmembrane carrier itself or an associated protein.

In this report we show that treatment of intact erythrocytes with thiol reactive agents causes a marked decrease in both K_m(So) and V_{max} but, while the K_m(So) effect is very rapid and independent of extracellular Na^+, the V_{max} effect can be slower and is Na^+-dependent.

2. Materials and methods

2.1. Sodium-lithium countertransport assay

The method used was similar to that described by Canessa et al. [10]. Venous blood was collected from three normal subjects into tubes containing lithium heparin, centrifuged and the erythrocytes incubated in lithium-loading solution (140 mmol/l LiCl, 10 mmol/l Li_2CO_3, 10 mmol/l glucose, 10 mmol/l Tris-Mops, gassed with 95% O_2/5%CO_2 (pH 7.5), 290 ± 2 mosmol/kg) for 1.5 h. The

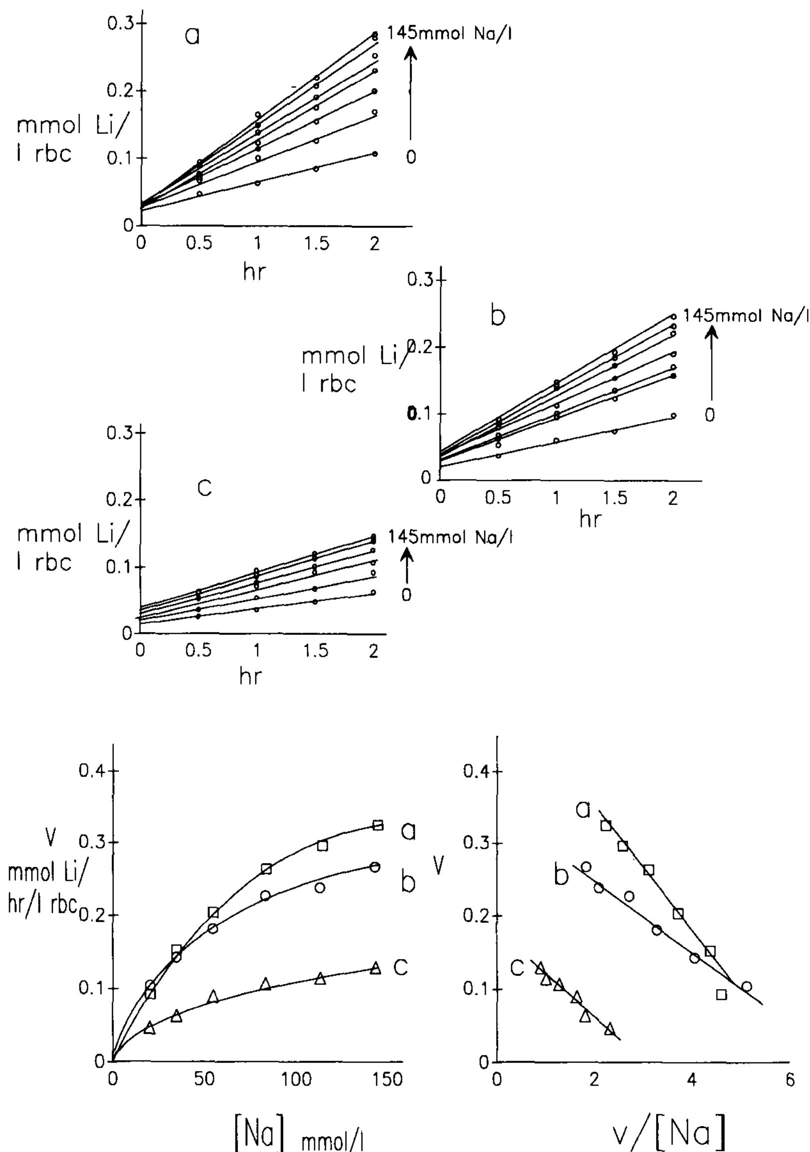

Fig. 1. Lithium efflux at varying external sodium concentrations from erythrocytes loaded with lithium after (a) no treatment, (b) NEM in choline medium, (c) NEM in sodium medium. The sodium concentrations used were 0, 20, 34, 58, 88, 115 and 145 mmol/l. The flux at 0 mmol/l sodium was subtracted from the other fluxes to obtain *v*. The Eadie-Hofstee plot for the results is also shown. Details are given in Materials and methods.

erythrocytes were then washed once with $MgCl_2$ (289 ± 1 mosmol/kg) and twice with choline medium (139 mmol/l choline chloride, 1 mmol/l $MgCl_2$, 10 mmol/l glucose, 10 mmol/l Tris-Mops (pH 7.4), 290 ± 2 mosmol/kg). After the final washing, the packed cell volume of the erythrocytes was measured using a micro-haematocrit and 0.2 or 0.25 ml of the packed cells was incubated in 4 ml of choline-ouabain medium (as above but containing 10^{-4} mol/l ouabain) or 4 ml of medium with a range of sodium concentrations (1 to 150 mmol/l) made by mixing the choline medium with sodium-ouabain medium (150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 10 mmol/l glucose, 10 mmol/l Tris-Mops, 10^{-4} mol/l ouabain (pH 7.4), 290 ± 2 mosmol/kg). Samples were taken after incubation for 30, 60, 90 and 120 min at 37° C. After centrifugation of incubation mixtures at 2000 × *g* for 3 min, 0.25–0.75 ml of supernatant was mixed with 0.75 ml of 3 mmol/l CsCl, made up to 1.5 ml with distilled water and the lithium content measured using an IL943 flame photometer using incubation media blanks. The sodium-lithium countertransport activity was determined by subtracting the rate of lithium efflux from erythrocytes in the choline medium from that in the sodium media. Erythrocytes after lithium loading and washing contained 8.5 ± 1.0 mmol Li/l rbc.

Osmolality was measured using a Camlab osmometer and adjusted as appropriate.

2.2. Kinetic parameters of sodium-lithium countertransport activity

To calculate the Michaelis-Menten constant for external sodium (K_m(So)) and maximum velocity (V_{max}) of sodium-lithium countertransport, the standard equation:

$$\text{flux rate} = V_{max} \times [Na^+]_e/[Na^+]_e + K_m(So)$$

relating these parameters to the flux rate and the sodium concentration of the medium ($[Na]_e$) was rearranged by the Eadie-Hofstee method to give;

$$\text{flux rate} = V_{max} - K_m(So) \times \text{flux rate}/[Na]_e$$

and the flux rate was plotted against the flux rate/$[Na]_e$. The K_m(So) was determined from the slope and the maximum velocity from the intercept on the *y*-axis.

Typical rate plots with the corresponding Eadie-Hofstee transformation for cells before and after NEM treatment are shown in Fig. 1. In a ping-pong mechanism the apparent K_m and V_{max} values for ions at the external site are affected by the degree of ion saturation of the internal

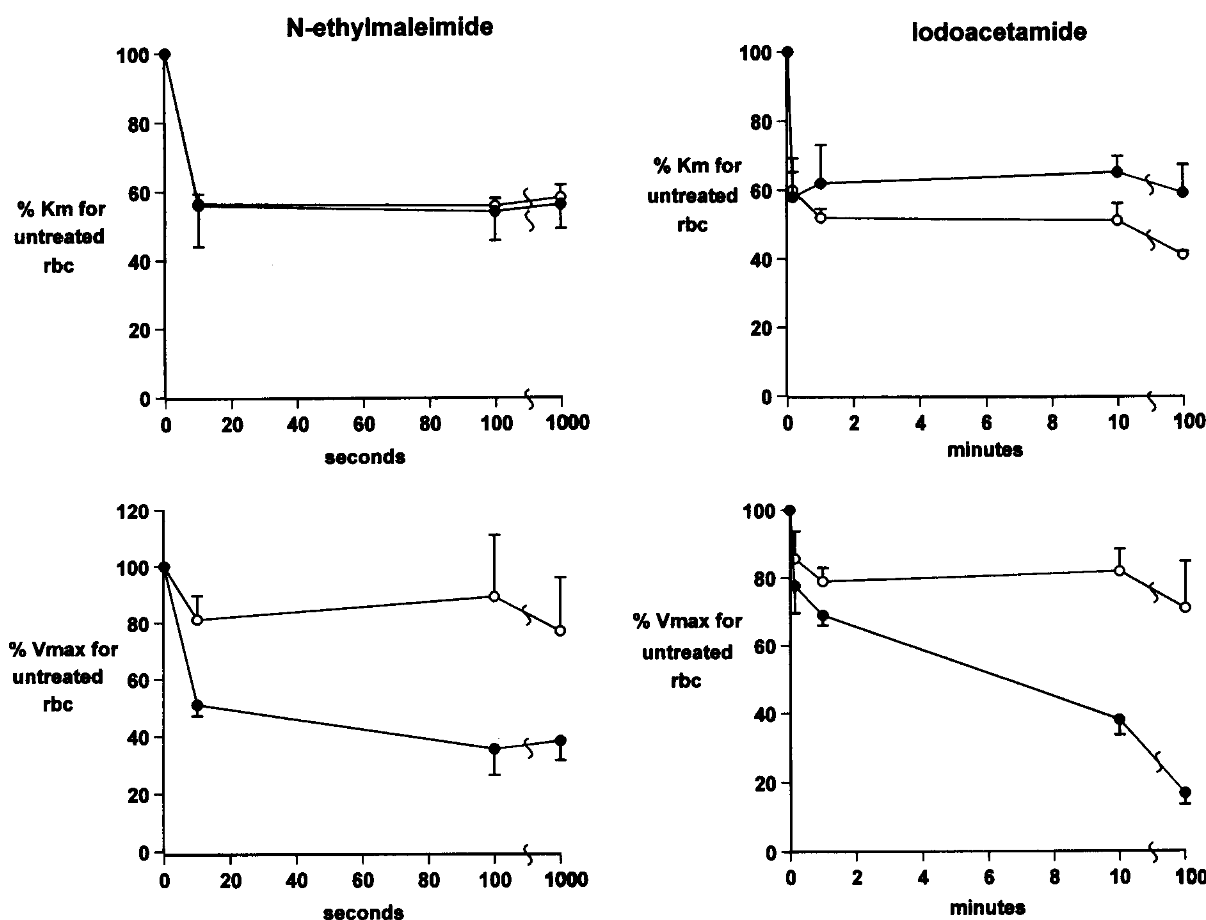

Fig. 2. The effect of *N*-ethylmaleimide and iodoacetamide in choline medium (○) or sodium medium (●) on the K_m for external sodium and Vmax of Na^+/Li^+ countertransport.

site and this was shown for Na^+/Li^+ countertransport by Hannaert and Garay [1]. In the present experiments the internal lithium concentrations were intended to effectively saturate the internal ion site.

On the Eadie-Hofstee plots for individual subjects the standard errors of slopes (for the calculation of K_m) were 5% in untreated cells and 14% after NEM treatment. The corresponding values for the intercept (for the calculation of V_{max}) were 3% in untreated cells and 8% after NEM treatment.

2.3. N-Ethylmaleimide (NEM) and iodoacetamide (IAamide) treatment of erythrocytes

Erythrocytes (0.5 ml) were suspended in 3 ml (for NEM) or 5 ml (for IAamide) of choline or sodium medium (as sodium-ouabain above but without ouabain). NEM (3 μmol in 100 μl of choline medium) or IAamide (150 μmol in 1 ml of choline or sodium medium) was added to the prewarmed suspension and incubated at 37° C for between 10 s and 100 min. The reaction was stopped by the addition of a 5-fold (NEM) or 2-fold (IAamide) excess of mercaptoethanol in choline medium. Erythrocytes were then washed with choline medium and used to determine K_m(So) and V_{max}.

2.4. Sodium ion loading of erythrocytes

For experiments to determine the effect of intracellular sodium on NEM inhibition of Na^+/Li^+ countertransport, erythrocytes were loaded with sodium using nystatin. Erythrocytes (1 ml) were suspended in 5 ml of sodium medium containing 0.25 mg of nystatin for 20 min at 4° C. Erythrocytes were then washed with sodium medium containing 1 mg/ml albumin and four times with choline medium. The total sodium efflux rate constant (3–5 mmol/l $[Na]_i$) into sodium-free medium was then 0.63 S.D. 0.06 h^{-1} compared with 0.54 S.D. 0.08 h^{-1} in the original cells. Erythrocytes were then treated with NEM in choline or sodium medium as described above for 15 min, loaded with lithium and Na^+/Li^+ countertransport activity at 150 mmol/l Na^+ determined.

3. Results

When intact, washed, red cells from normal, healthy, individuals were briefly exposed to 1 mM N-ethylmaleimide (NEM) at pH 7.5 prior to washing and Li^+-loading, the kinetic parameters of the subsequently measured Na^+/Li^+ countertransport were modified. After 10 s, and possibly much more rapidly, the K_m(So) parameter had decreased to approximately 60% of the initial value (Fig. 2). Prolonged exposure to NEM caused no further change in K_m(So). The time-course of this effect of NEM on K_m(So) was the same in the presence or in the absence of Na^+ during the treatment (Fig. 2).

Table 1
Dependence of Na^+/Li^+ countertransport inhibition by NEM on intra- and extracellular sodium

Intracellular Na (mmol/kg rbc)	Extracellular Na (mmol/l)	% inhibition
2.9±0.9	136.7±3.2	59.5±5.7
90.5±1.2	3.0±0.5	3.2±1.6

Means ± S.E., n = 5.

Treatment with NEM in the presence of Na^+ also reduced V_{max}. The decrease in V_{max} to about 50% occurred in 10 s followed by a slower decline to 35% up to 100 s but no further effect up to 1000 s (Fig. 2). Treatment in the absence of Na^+ (choline medium) characteristically caused little or no decline in V_{max} (Fig. 2). The dependence of the effect on sodium was entirely at the outside ion-binding site, since, if erythrocytes were loaded to 90.5 mmol/l intracellular sodium but with very low extracellular sodium, NEM did not cause inhibition (Table 1).

The NEM concentration used in the present experiments (6 μmol/ml rbc) was relatively low compared with the total concentration of reactive erythrocyte thiol groups and would be expected to decline during the incubation. However after 15 min incubation in choline medium with NEM at 6 μmol/ml rbc, the addition of an equal volume of sodium medium showed that NEM remained at a high enough concentration to give the maximum Na-facilitated decrease in Na^+/Li^+ countertransport activity (Fig. 3). Therefore, it seems reasonable to assume that the events up to 1000 s were not limited by the NEM concentration.

The effect of another thiol-reactive agent; iodoacetamide (IAamide) on Na^+/Li^+ countertransport kinetics was also investigated. This reagent did not cause widespread membrane damage over the time of the incuba-

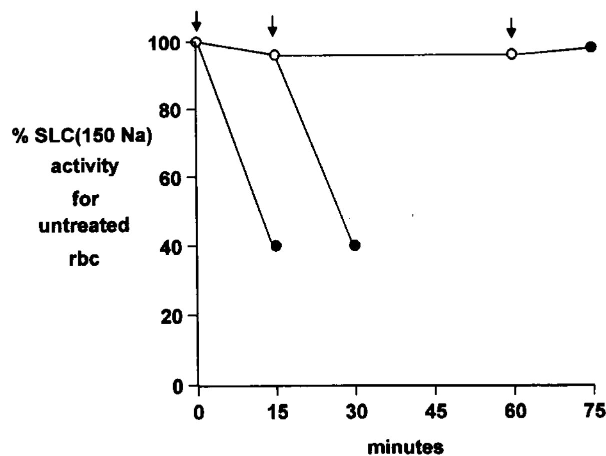

Fig. 3. The effect of addition of sodium at various times after N-ethylmaleimide on the ability to decrease Na^+-Li^+ countertransport activity. Erythrocytes were initially in choline medium (○) with 6 μmol N-ethylmaleimide/ml rbc and sodium was added at ↓ (●).

Table 2
Effect of β-maleimidopropionic acid (MPA) compared with NEM on the kinetics of Na+/Li+ countertransport in 2 subjects after 10 min incubation at 6 μmol/ml rbc in Na medium

Subject		Untreated	NEM	MPA
A	K_m	72	45	67
	V_{max}	0.37	0.12	0.38
B	K_m	96	47	96
	V_{max}	0.50	0.16	0.46

Units: K_m mmol Na+/l; V_{max} mmol Li/h per l rbc.

tions and it could therefore be used at a higher concentration (50 mM in the following experiments) than NEM. Therefore, the concentration of IAamide would remain more nearly constant throughout the treatment period.

The experiments with iodoacetamide showed the same features as those with *N*-ethylmaleimide. The K_m(So) decreased within 10 s independently of Na^+ (Fig. 2) and the decrease in V_{max} was Na^+-dependent (Fig. 2). However, with IAamide V_{max} initially decreased more slowly than with NEM but the inhibition appeared to continue towards completion (Fig. 2).

β-Maleimidopropionic acid appeared to have little effect on either K_m(So) or V_{max} (Table 2).

4. Discussion

The results reported here suggest that there are at least two classes of NEM reactive groups, presumably protein thiols, that govern the kinetics of Na^+/Li^+ countertransport. These thiol groups will be referred to provisionally as type 1 and type 2 thiols, respectively. The main evidence for the existence of the two types of thiol group comes from the different characteristics of the changes in K_m(So) and V_{max} with NEM and IAamide treatment. In chemical modification studies, if two process occur simultaneously it is possible to conclude that they are linked or dependent on a single reaction. However, if they can be shown to occur separately as in the present case, it must be concluded that two different reactions are involved. There is no obvious way in which the reaction of a single class of thiol group could lead to both the abrupt, Na^+-independent, change in K_m(So) and the more prolonged Na^+-dependent change of V_{max}.

Previous workers have investigated inhibition of sodium-lithium countertransport by NEM and IAamide and reported (a) its partial nature and (b) its Na^+-dependence [2,3]. However, these earlier investigations did not examine the kinetic parameters after partial inhibition and thus were not able to detect separate affects on K_m(So) and V_{max}. These previous studies would only have been able to detect the effects on V_{max} of the type 2 thiol. The type 1 thiol effect on V_{max}/K_m would have been effectively silent when ion flux was measured at about 140 mmol/l Na^+. Consequently this is the first indication of the involvement of two classes of thiol group.

Levy and Livne [3], from an examination of the dependence of inhibition rate on NEM concentration, did conclude that each molecule of porter reacted with 1.5 molecules NEM at NEM concentrations above 0.4 mM, but their analysis seems too simplistic in view of the complexity of the system with many competing reactions that might involve NEM.

1 ml of packed red blood cells contains 2 to 3 μmol of reduced glutathione and a total of 45 μmol of thiol groups reactive to mercury [11] of which about 12.5 μmol would be reactive to NEM under the conditions used here [12]. In our experiments we were adding 6 μmol NEM and 300 μmol IAamide (ml packed cell)$^{-1}$. It is clear that in the NEM experiments the NEM concentration would not remain constant; indeed we know it falls to essentially zero in 60 min (Fig. 3). However, there was enough NEM remaining after 15 min to give the maximum decrease in V_{max}. Therefore, in the time-course experiment the failure of NEM to cause a further decrease in V_{max} after 100 s, despite a much more rapid initial reaction than IAamide, cannot be explained on an inadequate supply of NEM.

If pH and reactant concentrations can be held constant, a study of the kinetics of alkylation can be used as a sensitive probe of conformation (or hydration) in the immediate environment of the reactive thiol. In this respect IAamide is preferable since its concentration can be assumed to be more or less constant in the time range of these experiments. However it remains possible that NEM and IAamide are reacting differently or even with different thiol groups in the presence of sodium. There is precedent for this in for example the reactivity of thiol groups of the anion exchanger [13] and glucose transporter [14].

Our kinetic data do not warrant sophisticated analysis, but some interpretation is possible. From the equation:

$$v = V_{max} \times [Na^+]_e / [Na^+]_e + K_m(So)$$

as $[Na^+]_e$ approaches zero then $v = (V_{max}/K_m) \times [Na^+]_e$. Thus, V_{max}/K_m may be interpreted as the pseudo-first-order rate constant for reaction between extracellular Na^+ and unloaded carrier. If V_{max}/K_m is interpreted in this way then it appears that the rapid type 1 reaction modifies the carriers into a faster loading form (Fig. 4). However, V_{max}/K_m is to some degree dependent on rate constants that affect the delivery of unloaded carrier to the sodium loading side of the membrane [15]. In this case it would be possible to suggest that alkylation of type 1 thiols could reduce the delivery of transporter to the external face of the membrane. In the case of the glucose transporter, an NEM induced decrease in K_d measured by competitive binding was interpreted as a decrease in turnover of the transporter rather than a change in glucose binding [14]. However, in that study measurements of transport rate were not made to test this assumption further.

The type 1 thiol must be highly accessible to both NEM

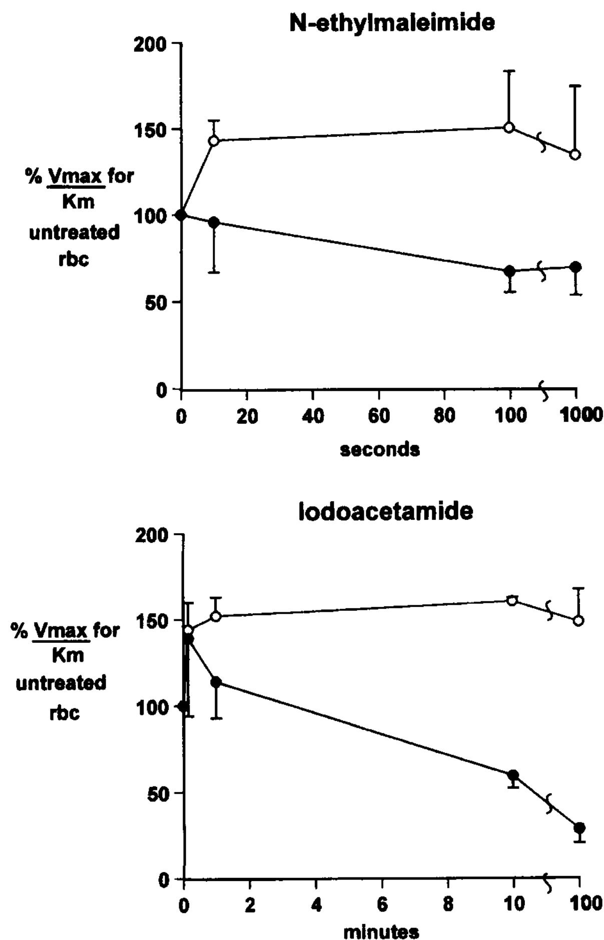

Fig. 4. The effect of *N*-ethylmaleimide and iodoacetamide in choline medium (○) or sodium medium (●) on the V_{max}/K_m ratio of Na^+/Li^+ countertransport.

and IAamide. However, preliminary experiments suggest that this thiol does not react with β-maleimidopropionic acid. The type 2 thiol was shown to be inaccessible extracellularly by the earlier studies [2] and this thiol is also unaffected by β-maleimidopropionic acid. Therefore, either the thiols have a specific reactivity to NEM or β-maleimidopropionic acid cannot penetrate to their site. Evidence from depletion of erythrocyte reduced glutathione by these agents suggests that the latter reason is more probable.

The Na^+-dependent type 2 effect on V_{max} is most easily interpreted as a progressive complete inhibition of transport occurring by alkylation of a thiol that is somewhat protected in Na^+-free medium. Presumably extracellular Na^+ favours a carrier conformation that allows much more rapid alkylation of the thiol group(s). However, there are differences in the way that NEM and IAamide affect V_{max} and it cannot at present be excluded that these two agents are affecting V_{max} by different mechanisms and that only IAamide is able to cause total inhibition.

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