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Effects of *N*-ethylmaleimide on ouabain-insensitive cation fluxes in human red cell ghosts

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In red cells of several species, the sulfhydryl reagent *N*-ethylmaleimide activates a Cl^--dependent, ouabain-resistant K^+ transport pathway. Here we report our attempts to demonstrate ouabain-resistant Cl^--dependent K^+ fluxes stimulated by *N*-ethylmaleimide in resealed human red cell ghosts using Rb^+ as a K^+ analogue. In contrast to intact cells, the rate constants of the base level Rb^+ efflux in ghosts were similar in $NaNO_3$ and NaCl ($^{o}k_{Rb} = 0.535 \pm 0.079$ h^{-1} and 0.534 ± 0.085 h^{-1}, respectively), while 1 mM *N*-ethylmaleimide stimulated Rb^+ efflux strongly in $NaNO_3$ ($^{o}k_{Rb} = 14.26 \pm 1.32$ h^{-1}) and moderately in NaCl ($^{o}k_{Rb} = 2.73 \pm 0.54$ h^{-1}). This effect was dependent on the presence of internal ATP. Stimulation of Rb^+ efflux was observed in the presence of $\geqslant 0.2$ mM *N*-ethylmaleimide and increased at pH values approaching 8.0, consistent with titration of SH groups. *N*-Ethylmaleimide-stimulated Rb^+ efflux was approx. 50% inhibited by 100 μM quinine sulfate whereas 1 μM bumetanide had no effect. In NaCl the *N*-ethylmaleimide-stimulated efflux saturated with initial internal ghost Rb^+ concentration, but rates increased linearly in $NaNO_3$. Replacement of external Na^+ with glucamine or choline decreased the *N*-ethylmaleimide-stimulated Rb^+ efflux, suggesting a role for external Na^+. *N*-Ethylmaleimide-stimulated Rb^+ efflux was greater in buffers with lipophilic anions such as SCN^- or NO_3^- than in solutions with Cl^- or acetate. However, the cation selectivity of the pathway studied was low, as Li^+ efflux was also stimulated by *N*-ethylmaleimide. We conclude that the effect of *N*-ethylmaleimide on ouabain-resistant cation effluxes of human red cell ghosts is very different from the selective action of *N*-ethylmaleimide on Rb^+ influxes in intact red cells.

Introduction

The sulfhydryl reagent *N*-ethylmaleimide has been shown to stimulate ouabain-resistant Cl^--dependent K^+ transport in red cells from a variety of species [1–5]. In the human red cell, *N*-ethylmaleimide activated a latent $K^+ : Cl^-$ flux which was only partially inhibited by furosemide or bumetanide [4]; however, this activation was inhibited by depletion of intracellular ATP, suggesting involvement of cell metabolism [6,7]. Furthermore, *N*-ethylmaleimide at low concentrations abolished bumetanide-sensitive Na^+ flux with no effect on bumetanide-sensitive K^+ flux (both occurring through bumetanide-sensitive Na^+, K^+, $2Cl^-$ cotransport) [4]. In the present study, we have further investigated the mechanisms involved in *N*-ethylmaleimide stimulation of cation transport and its dependence on cellular metabolism, by examining the efflux of cations from resealed human red cell ghosts in the presence and absence

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

of *N*-ethylmaleimide, in solutions with Cl^- or Cl^- replacement by SCN^-, NO_3^-, or acetate. The data indicate that in ghosts *N*-ethylmaleimide dramatically activated cation effluxes in non-Cl^- media consistent with the hypothesis of the appearance of electrodiffusional leaks rather than activation of Cl^--dependent K^+ (or $Na^+ : K^+$) transport pathways. This work has been presented in preliminary form [8].

Materials and Methods

Materials. RbCl and $RbNO_3$ were obtained from Alfa Products (Danvers, MA). Bumetanide was kindly supplied by Hoffman-LaRoche (Nutley, NJ). All other chemicals were reagent grade products from either Sigma Chemical Company or Fisher Scientific.

Preparation of resealed ghosts. Human blood from healthy volunteers was collected by venipuncture into heparin. Cells were washed four times with 5 mM sodium phosphate buffer (pH 7.6), containing 145 mM $NaNO_3$, 0.1 mM EGTA, and 0.1 mM ouabain, and buffy coats were carefully removed. Although cells washed in buffer containing isotonic NaCl gave similar experimental results, cells were standardly washed in $NaNO_3$ solutions. Resealed ghosts were prepared by a modification of the procedures of Schwoch and Passow [9] and Funder and Wieth [10]. Red cells were osmotically lysed at 0°C in 30 volumes of 2.5 mM sodium phosphate buffer (pH 7.6), containing EGTA and ouabain as above. After 5 min at 0°C, $MgSO_4$ and ATP (sodium salt) were added to final concentrations of 4 mM and 2 mM, respectively, and isotonicity was restored by addition of the appropriate amount of 1 M RbCl, or in some cases 1 M LiCl. Ghosts were first kept at 0°C for 10 min with occasional mixing to allow ionic equilibration, and then transferred to a 37°C water bath for 60 min for resealing. Resealed ghosts were pelleted by centrifugation at $18\,000 \times g$ for 8–10 min and washed four times with the appropriate buffer for flux experiments. Such ghost preparations were found to contain approx. 95% resealed ghosts as defined by their ability to float when centrifuged onto a 43% sucrose cushion [11].

Efflux measurements. At the start of efflux determinations ($t = 0$), ghosts were suspended at 1% v/v in the appropriate prewarmed (37°C) buffer containing 0.1 mM EGTA and 0.1 mM ouabain plus any other additions such as *N*-ethylmaleimide or the inhibitors bumetanide or quinine. At various times, 0.5 ml aliquots were removed and added to 0.5 ml buffer at 0°C. The sample was immediately centrifuged for 1 min in a Brinkmann Eppendorf centrifuge (approx. $20\,000 \times g$) and 0.9 ml of supernatant was removed without disturbing the ghost pellet. After addition of 1/100 volume of a concentrated hemolyzing solution containing 0.3 M CsCl, 0.8 M NH_4OH, and 0.2% acationic detergent (Acationox), cation concentration of the supernatant ($[Rb]_o^t$ or $[Li]_o^t$) was determined by flame emission or atomic obsorption spectrophotometry using a Perkin-Elmer model 5000 spectrophotometer. For spectrophotometric determination of the equilibrium cation concentration ($[Rb]_o^{t=\infty}$ or $[Li]_o^{t=\infty}$), 0.1 ml of the incubated 1% ghost suspension was diluted 100-fold with the above hemolyzing solution (diluted 1/100 before use). The rate constant for ouabain-resistant (OR) efflux (${}^{o}k_{Rb}^{OR}$ or ${}^{o}k_{Li}^{OR}$) was calculated as previously described [12] from the first order rate equation (shown below for Rb^+):

$$-\ln\left[1-\frac{[Rb]_o^t}{[Rb]_o^{t=\infty}}\right] = {}^{o}k_{Rb}^{OR}\cdot t$$

Ghost internal cation concentrations were calculated from supernatant cation concentrations assuming 96% fractional water content and 8% trapped extracellular volume, as determined previously for similar preparations [10,11] and assuming equal loading of every ghost. Initial internal ghost Rb^+ concentration calculated in this manner was usually approx. 100–120 mM. Ouabain-resistant flux in mmol/l ghost water per h (${}^{o}M_{Rb}^{OR}$) was calculated as ${}^{o}k_{Rb}^{OR}$ multiplied by initial internal ghost Rb^+ concentration ($[Rb]_i^{t=0}$). Possible loss of hemoglobin during the course of the experiments was monitored by absorbance of the supernatants at 412 nm.

Results

N-Ethylmaleimide stimulation of Rb efflux

Resealed ghosts loaded with RbCl were incubated at 37°C in isotonic $NaNO_3$ or NaCl buffer

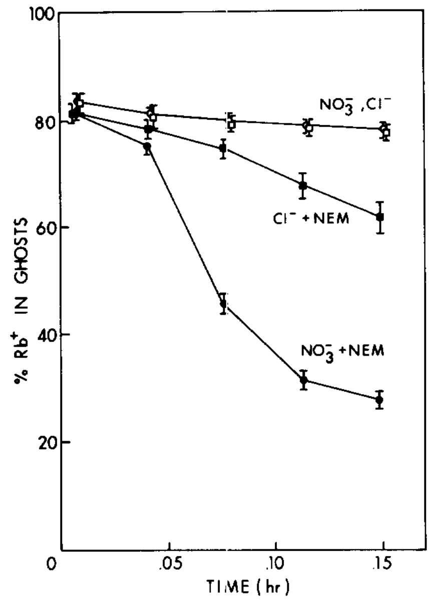

Fig. 1. Stimulation of Rb$^+$ efflux in resealed ghosts by *N*-ethylmaleimide (NEM). RbCl-loaded ghosts were incubated at 37°C in $NaNO_3$ (●, ○) or NaCl (■, □) with 1 mM *N*-ethylmaleimide (closed symbols) or without *N*-ethylmaleimide (open symbols). Percent Rb$^+$ in ghosts is calculated from appearance of Rb$^+$ in the medium as described in Materials and Methods. Points are means ±S.E., $n = 11$.

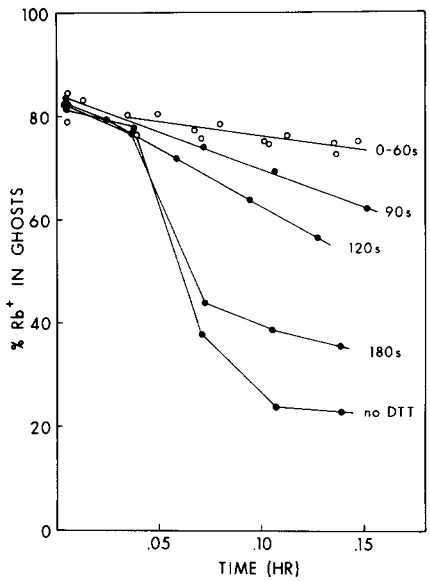

Fig. 2. Time-course of *N*-ethylmaleimide effects on efflux. Ghosts were incubated at 37°C in $NaNO_3$ solution containing 1 mM *N*-ethylmaleimide, and dithiothreitol (DTT) was added at 5 mM final concentration at the times indicated. Data are taken from one out of three similar experiments.

containing EGTA (to avoid Ca^{2+}-stimulated Rb$^+$ efflux via the Gardos channel [13]) and ouabain, with or without 1 mM *N*-ethylmaleimide (Fig. 1). There was a slow base level efflux which was similar in both $NaNO_3$ and NaCl. However, in the presence of *N*-ethylmaleimide, Rb$^+$ efflux was moderately stimulated in NaCl, and following a short lag phase, greatly stimulated in $NaNO_3$. This stimulation was not due to lysis of ghosts, as there was no loss of hemoglobin during the course of the experiments. Furthermore, the same results were obtained with resealed ghosts purified by centrifugation onto a 43% sucrose cushion [11]; therefore, *N*-ethylmaleimide-stimulated rapid loss of Rb$^+$ was not due to the presence of unsealed ghosts (type III, see Ref. 14).

In order to test whether the lag phase, most pronounced in $NaNO_3$, reflected the time necessary for *N*-ethylmaleimide to bind the appropriate site(s) or the time required for *N*-ethylmaleimide-binding to be translated into stimulation of efflux, ghosts were incubated in $NaNO_3$ as before. At various times, aliquots of ghosts were removed and added to a tube at 37°C containing 1 M dithiothreitol to give a final concentration of 5 mM dithiothreitol to quench unreacted *N*-ethylmaleimide, and efflux was continued (Fig. 2). The control, in which no dithiothreitol was added, was similar to efflux in $NaNO_3$ as in Fig. 1. Addition of dithiothreitol at times 0–60 s resulted in only base level efflux, and efflux rates increased as dithiothreitol was added at later times. Addition of dithiothreitol at 180 s had only a slight inhibitory effect, suggesting that *N*-ethylmaleimide had bound most of the relevant site(s) by this time. Thus, the lag appeared to be related to the time for *N*-ethylmaleimide to bind particular sites and effect stimulation of Rb$^+$ efflux in $NaNO_3$. Therefore, where possible, efflux rate constants were calculated from the relatively linear portion of the curves immediately following the lag. By these criteria, rate constants ($^{o}k^{OR}_{Rb}$) for *N*-ethylmaleimide-stimulated efflux were calculated to be 14.26 ± 1.32 h^{-1} in $NaNO_3$ and 2.73 ± 0.54 h^{-1} in NaCl. The rate constants for base level efflux were 0.535 ± 0.079 h^{-1} in $NaNO_3$ and 0.534 ± 0.085 h^{-1} in NaCl media.

N-Ethylmaleimide concentration dependence

RbCl-loaded ghosts were incubated at 37°C in $NaNO_3$ or NaCl in the presence of 0–2 mM *N*-ethylmaleimide, and rate constants for Rb^+ efflux were determined as above from the linear phase of efflux. Significant stimulation of ${}^{o}k_{Rb}^{OR}$ in $NaNO_3$ was seen at $\geqslant 0.2$ mM *N*-ethylmaleimide, approaching a plateau near 1 mM *N*-ethylmaleimide (Fig. 3). In contrast, the rate of Rb^+ efflux in NaCl showed a different dependence on *N*-ethylmaleimide concentration, with ${}^{o}k_{Rb}^{OR}$ gradually increasing only at *N*-ethylmaleimide > 0.5 mM. Moreover, the extent of *N*-ethylmaleimide stimulation of ${}^{o}k_{Rb}^{OR}$ in NaCl was much less than that in $NaNO_3$.

pH dependence of efflux

To examine the influence of pH on *N*-ethylmaleimide-induced Rb efflux, resealed ghosts were incubated with or without 1 mM *N*-ethylmaleimide in sodium phosphate buffers, at pH 6.0–7.8, containing EGTA, ouabain, and isotonic $NaNO_3$ or NaCl. *N*-Ethylmaleimide-stimulated efflux in $NaNO_3$ was markedly augmented at pH values approaching 8.0 (Fig. 4, upper panel), whereas rate constants of basal Rb^+ fluxes in either $NaNO_3$ or NaCl increased only slightly at higher pH (Fig. 4, lower panel). Efflux into NaCl, which was less than 1/10 of that measured in $NaNO_3$, exhibited a similar pH dependence (Fig. 4, lower panel). The pH dependence of Rb^+ efflux is consistent with increased *N*-ethylmaleimide titration of SH groups as they become deprotonated near their p*K*.

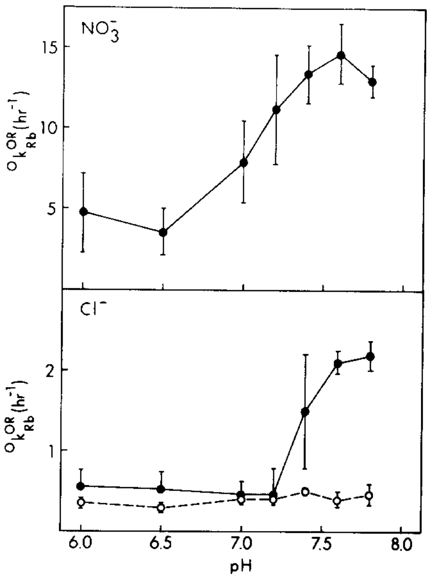

Fig. 4. pH dependence of *N*-ethylmaleimide-stimulated Rb^+ efflux. Ouabain-resistent Rb^+ efflux rate constants (${}^{o}k_{Rb}^{OR}$) were measured in solutions of $NaNO_3$ (upper panel) or NaCl (lower panel) at pH 6.0–7.8, either with 1 mM *N*-ethylmaleimide (●) or without *N*-ethylmaleimide (○). Efflux rates in the absence of *N*-ethylmaleimide were very similar in NO_3^- and Cl^-, as shown in the lower panel. Points represent means ± S.E., $n = 3$.

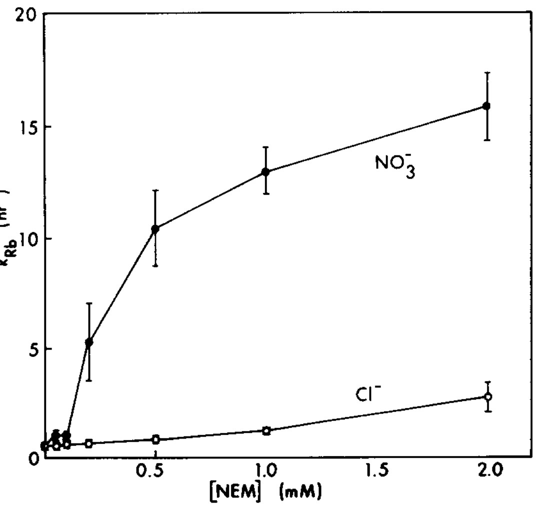

Fig. 3. *N*-Ethylmaleimide (NEM) concentration dependence of Rb^+-efflux stimulation. Ouabain-resistant Rb^+ efflux was measured at 37°C in the presence of 0–2 mM *N*-ethylmaleimide in either $NaNO_3$ (●) or NaCl (○). Efflux rate constants (${}^{o}k_{Rb}^{OR}$) were calculated as in Materials and Methods. Points represent means ± S.E., $n = 4$.

Cation selectivity of N-ethylmaleimide-stimulated efflux

In order to determine whether cation efflux induced by *N*-ethylmaleimide was specific for Rb^+, ghosts were loaded with LiCl instead of RbCl. Resealed ghosts were then incubated at 37°C as before, in sodium phosphate buffer (pH 7.6), containing EGTA, ouabain and either isotonic $NaNO_3$ or NaCl plus or minus 1 mM *N*-ethylmaleimide. Results are summarized in Table I. Base level efflux of Li^+ was similar to that of Rb^+ in both $NaNO_3$ and NaCl, and efflux of Li^+ was stimulated by *N*-ethylmaleimide in a similar fashion to

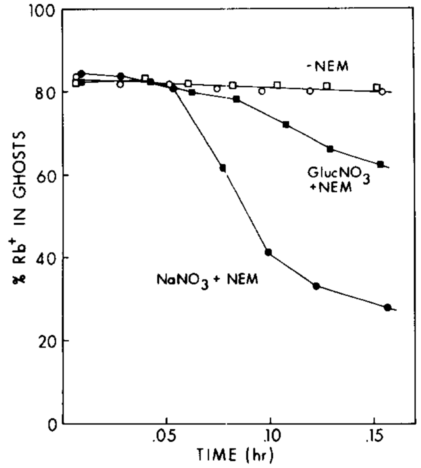

Fig. 5. N-Ethylmaleimide-stimulated Rb^+ efflux in Na^+-free media. Ghosts were washed and incubated in either $NaNO_3$ (●, ○) or glucamine nitrate ($GlucNO_3$) (■, □) with 1 mM N-ethylmaleimide (NEM) (closed cymbols) or without N-ethylmaleimide (open symbols). Data represent one of three similar experiments.

Rb^+ efflux, with approx. 50-fold increase in ${}^{o}k_{Li}^{OR}$ in $NaNO_3$ and approx. 7-fold increase in ${}^{o}k_{Li}^{OR}$ in NaCl. Therefore, N-ethylmaleimide-stimulated efflux was not specific for Rb^+.

In order to determine whether external Na^+ was required for the observed N-ethylmaleimide-stimulated efflux, Rb^+-loaded ghosts were also incubated plus or minus N-ethylmaleimide in Na^+-free media consisting of 10 mM Tris-Mops buffer (pH 7.4), EGTA, ouabain, and isotonic glucamine chloride or glucamine nitrate. In the experiment shown in Fig. 5, Rb^+ efflux was greatly decreased in glucamine media, suggesting a role for extracellular Na^+ in the observed N-ethylmaleimide-stimulated Rb^+ efflux. Decreased efflux was also seen in two other identical experiments, although the extent of inhibition was somewhat variable. Lack of N-ethylmaleimide effects were not due to inactivation of N-ethylmaleimide in glucamine as monitored by absorbance at 300 nm to detect unreacted N-ethylmaleimide. Preliminary experiments with choline chloride suggested that N-ethylmaleimide-dependent Rb^+ efflux could be observed in Na^+-free media at a higher pH ($>$ 7.6).

TABLE I

CATION SELECTIVITY OF N-ETHYLMALEIMIDE-STIMULATED EFFLUX

Ghosts were loaded with either RbCl or LiCl and cation efflux was measured at 37°C in either $NaNO_3$ or NaCl. Figures are expressed as means ± S.E., $n = 3$, NEM, N-ethylmaleimide.

Efflux medium	NEM	${}^{o}k_{Rb}^{OR}$ (h^{-1})	${}^{o}k_{Li}^{OR}$ (h^{-1})
$NaNO_3$	−	0.31 ± 0.08	0.35 ± 0.21
$NaNO_3$	+	13.08 ± 2.12	17.62 ± 3.25
NaCl	−	0.30 ± 0.02	0.50 ± 0.15
NaCl	+	3.95 ± 0.15	3.57 ± 0.54

Dependence of efflux on internal Rb^+ concentration

We examined the kinetic properties of N-ethylmaleimide-stimulated Rb^+ efflux by measuring the rate of efflux as a function of the Rb^+ concentration within the ghosts. Ghosts were prepared containing increasing concentrations of RbCl, plus the appropriate amount of glucamineCl to maintain internal isotonicity, and then incubated in either $NaNO_3$ or NaCl with or without N-ethylmaleimide for Rb^+ efflux measurement. For the graphic representation of Fig. 6, ouabain-resistant efflux was calculated from the internal Rb^+ concentration multiplied by the efflux rate constant. N-Ethylmaleimide-stimulated ouabain-resistant efflux appeared to be a linear function of initial internal Rb^+ concentration, with a correlation coefficient of 0.973. Base fluxes also fit a linear relationship with initial internal Rb^+ concentration, with a correlation coefficient of 0.812. However, N-ethylmaleimide-stimulated Rb^+ efflux in NaCl buffer did not exhibit a linear relationship, but appeared to plateau. (Attempts to fit these points to a line gave a correlation coefficient of 0.557, and produced a line which clearly did not represent the data.) This suggested that N-ethylmaleimide-stimulated efflux might occur via different mechanisms in NaCl and $NaNO_3$.

Effects of anion substitution

Since it was clear that the rate of N-ethylmaleimide-stimulated efflux of cations from resealed ghosts was dependent on the extracellular anion composition (i.e., NO_3^- vs. Cl^-), we examined Rb^+ efflux in solutions with two other

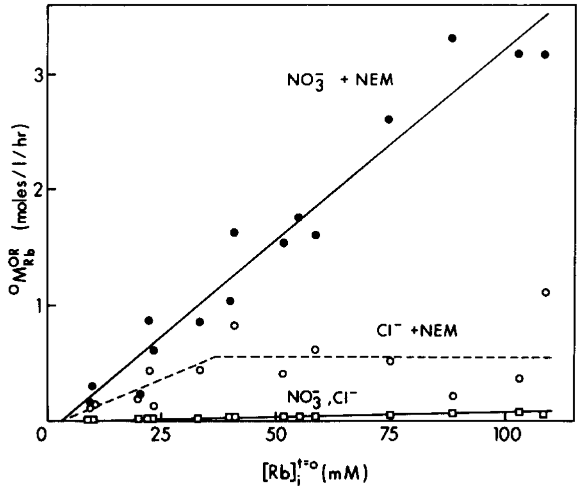

Fig. 6. Dependence of ghost Rb^+ efflux on initial internal Rb^+ concentration. Ghosts were prepared containing increasing concentrations of RbCl plus glucamine chloride sufficient to maintain constant internal osmolality. Ouabain-resistant Rb^+ efflux was measured in $NaNO_3$ or NaCl with or without 1 mM *N*-ethylmaleimide (NEM). Data is a composite of three similar experiments and is expressed as moles Rb^+ efflux/liter ghost water per h ($^oM^{OR}_{Rb}$) calculated as in Materials and Methods. The solid lines were generated by linear regression analysis of data. The open circles did not fit a straight line, therefore the dotted line is a visual approximation. Symbols: □, $NaNO_3$ or NaCl without *N*-ethylmaleimide; ●, $NaNO_3$ + *N*-ethylmaleimide; ○, NaCl + *N*-ethylmaleimide.

TABLE II

EFFECTS OF ANION SUBSTITUTION

Efflux of Rb^+ was measured at 37°C in various anionic media. Data are presented as means ± S.E., $n = 3$. Large standard errors are due to individual donor variability, NEM, *N*-ethylmaleimide.

Efflux medium	$^ok^{OR}_{Rb}$ (h^{-1})	
	−NEM	+NEM
NaSCN	0.53 ± 0.10	38.49 ± 14.99
$NaNO_3$	0.34 ± 0.08	16.83 ± 2.81
NaCl	0.28 ± 0.09	3.73 ± 0.52
NaAc	0.25 ± 0.04	2.93 ± 0.48

anions, acetate (Ac^-) and thiocyanate (SCN^-). Ouabain-resistant Rb^+ efflux in the presence and absence of 1 mM *N*-ethylmaleimide was measured as before in either isotonic $NaNO_3$, NaCl, NaAc, or NaSCN (Table II). *N*-Ethylmaleimide-stimulated efflux was much faster in $NaNO_3$ or NaSCN than in NaCl or NaAc buffers, suggesting a faster cation movement across the membrane in the presence of more lipophilic anions.

Effects of inhibitors

We examined *N*-ethylmaleimide-activated Rb^+efflux in the presence of inhibitors of ouabain-resistant cation pathways present in intact red cells, such as the Ca^{2+}-activated K^+ (Gardos) channel or $Na^+ : K^+ : 2Cl^-$ cotransport. Rb^+-loaded ghosts were incubated with *N*-ethylmaleimide in $NaNO_3$ or NaCl in the presence of either 100 µM quinine sulfate (an inhibitor of the Gardos channel, Ref. 15) or 1 or 100 µM bumetanide (a loop diuretic which inhibits $Na^+ : K^+ : 2Cl^-$ cotransport, even at the lower concentration, Refs. 4, 16 and 17), and efflux rate constants are shown in Table III. Quinine inhibited *N*-ethylmaleimide-activated Rb^+ efflux by 20–70% in $NaNO_3$ or NaCl in several experiments (mean ± S.E. = 50.0 ± 5.5% inhibition) with no effect on basal flux rates. Confirming other reports [15], we found the same concentration of quinine to completely inhibit Ca^{2+}-activated K^+ efflux in human red cell ghosts (at approx. 1 µM free Ca^{2+}). Bumetanide at 1 µM had no significant effect on *N*-ethylmaleimide-stimulated Rb^+, but 100 µM bumetanide caused a variable inhibition of efflux. This higher concentration of bumetanide

TABLE III

INHIBITOR EFFECTS ON *N*-ETHYLMALEIMIDE-STIMULATION OF EFFLUX

Rb^+ efflux rates in ghosts were determined in the presence of compounds known to inhibit cation pathways in the intact cell. Figures are means ± S.E., $n = 3$. NEM, *N*-ethylmaleimide.

Efflux conditions	$^ok^{OR}_{Rb}$ (h^{-1}), efflux medium	
	$NaNO_3$	NaCl
−NEM	0.23 ± 0.06	0.29 ± 0.06
+NEM	20.39 ± 3.29	1.96 ± 0.78
+NEM + quinine (100 µM)	9.91 ± 2.83	1.01 ± 0.61
−NEM	0.30 ± 0.06	0.27 ± 0.03
+NEM	14.12 ± 1.57	1.95 ± 0.88
+NEM + bumetanide (1 µM)	17.49 ± 1.88	1.91 ± 0.88
+NEM + bumetanide (100 µM)	12.94 ± 1.90	0.69 ± 0.24

is sufficient to inhibit *N*-ethylmaleimide-activated Cl^--dependent $K^+ + Cl^-$ transport as well as $Na^+ : K^+ : 2Cl^-$ cotransport in the human red cell [4].

ATP-dependence of N-ethylmaleimide-activated Rb$^+$ efflux

N-Ethylmaleimide-stimulated Cl^--dependent K^+ fluxes in human red cells require an intact cellular metabolism, as shown by loss of *N*-ethylmaleimide stimulation of efflux in ATP-depleted cells [6,7,18]. Therefore, we investigated the relationship between ATP and *N*-ethylmaleimide-stimulated Rb^+ efflux in ghosts which were loaded with or without 2 mM NaATP during the resealing process. (All ghost preparations used in previously described experiments contained ATP.) Rb^+ efflux was measured in the presence and absence of 1 mM *N*-ethylmaleimide, in $NaNO_3$ or NaCl as before, and the results are shown in Table IV. First, base fluxes in $NaNO_3$ and NaCl were generally lower in ghosts resealed with ATP than in ghosts without ATP, consistent with greater leakiness of ghosts resealed in the absence of ATP. Furthermore, ghosts without ATP lost approximately half the loaded Rb^+ during the washing procedure prior to the efflux experiment (not shown). Ghosts prepared without ATP, however, were sealed as defined by their ability to float when sedimented onto a 43% sucrose cushion [11]; resealed ghosts purified by this method gave similar results compared to unpurified resealed ghosts in identical efflux experiments. Second, there was no significant stimulation of Rb^+ efflux by *N*-ethylmaleimide in ghosts resealed in the absence of ATP in either $NaNO_3$ or NaCl solutions. Therefore the ability of *N*-ethylmaleimide to activate ouabain-resistant Rb^+ efflux in ghosts was apparently dependent on cytoplasmic ATP. In preliminary experiments, we found that addition of NaATP (as usual) before resealing of ghosts prepared from ATP-depleted cells at least partially restored *N*-ethylmaleimide stimulation of Rb^+ efflux in ghosts in both $NaNO_3$ and NaCl media (not shown). These findings may suggest a direct role for ATP in *N*-ethylmaleimide-stimulated cation permeability in resealed ghosts.

TABLE IV

N-ETHYLMALEIMIDE-STIMULATED EFFLUX IN GHOSTS RESALED ±ATP

Ghosts were resealed in the presence or absence of 2 mM NaATP, and Rb^+-efflux rates were determined as before. Data are expressed as means ± S.E., $n = 3$. NEM, *N*-ethylmaleimide.

Efflux medium	NEM	${}^{o}k^{OR}_{Rb}$ (h^{-1}), ghosts resealed	
		+ATP	−ATP
$NaNO_3$	−	0.39 ± 0.20	0.68 ± 0.14
$NaNO_3$	+	14.04 ± 2.14	0.89 ± 0.06
NaCl	−	0.41 ± 0.15	0.59 ± 0.22
NaCl	+	2.43 ± 1.17	0.77 ± 0.10

Discussion

Incubation of RbCl-loaded resealed ghosts with the sulfhydryl reagent *N*-ethylmaleimide resulted in a marked stimulation of ouabain-resistant Rb^+ efflux; many properties of this efflux, however, were very different from those of *N*-ethylmaleimide-activated Cl^--dependent K^+ efflux in intact human red cells or low-K^+ red cells of sheep [4,6,7,12,18]. First, the anion dependence of *N*-ethylmaleimide-stimulated efflux in ghosts was opposite to that in intact cells, with ${}^{o}k^{OR}_{Rb}$ in ghosts greatest in the presence of more lipophilic anions ($SCN^- > NO_3^- \gg Cl^- \geqslant$ acetate), more consistent with a conductive pathway [19–21]. In addition, rates of *N*-ethylmaleimide-stimulated efflux in ghosts were higher in buffers at pH values approaching 8.0, consistent with increased *N*-ethylmaleimide titration of SH groups near their p*K*, whereas in intact ovine erythrocytes, *N*-ethylmaleimide-activated K^+ transport was maximal at pH ≈ 6.0 [22]. This may reflect titration of different SH groups and/or activation of different efflux pathways. (The pH dependence of *N*-ethylmaleimide-stimulated Cl^--dependent K^+ permeability in the intact human red cell has not yet been studied; therefore, species differences may exist in properties of these SH sites.) Furthermore, *N*-ethylmaleimide-stimulated Rb^+ efflux in resealed ghosts was not sensitive to the loop diuretic bumetanide at concentrations which have been shown to significantly inhibit $Na^+ : K^+ : 2Cl^-$ cotransport in the red cell [4,16,17].

In contrast, the anion dependence of *N*-ethylmaleimide-stimulated Rb^+ efflux in ghosts was parallel to that reported for Ca^{2+}-stimulated K^+ efflux (the Gardos channel) in red blood cells [23]; moreover, a similar dependence of Ca^{2+} activated Rb^+ efflux on anion composition of the medium was found in these same preparations of resealed ghosts (not shown). This may suggest that *N*-ethylmaleimide-stimulation of Rb^+ efflux involves the opening of a conductive pathway for Rb^+, similar to the Gardos channel. Consistent with this, *N*-ethylmaleimide-stimulated Rb^+-efflux rates in $NaNO_3$ or NaCl were similar to K^+-efflux rates described in valinomycin- or gramicidin-treated cells [20,21]. In such ionophore-treated cells, the K^+ permeability was shown to be limited by the anion conductance, the rate of K^+ efflux in $NaNO_3$ is approx. 6–10-times that in NaCl [21]. In our studies, Rb^+ was approx. 6-times more rapid in $NaNO_3$ than in NaCl. Furthermore, *N*-ethylmaleimide-stimulated Rb^+ efflux was inhibited by quinine at concentrations previously shown to inhibit Ca^{2+}-activated K^+ efflux [15] and confirmed in these same ghost preparations. However, the *N*-ethylmaleimide-induced pathway did not exhibit the strict cation specificity of the Gardos channel, as *N*-ethylmaleimide stimulated the efflux of Li^+ as well as Rb^+.

A surprising finding (and another distinction from the Gardos channel) was the ATP-dependence of *N*-ethylmaleimide-stimulated cation efflux in ghosts, which was reminiscent of the dependence on cellular metabolism found for *N*-ethylmaleimide-activation of K^+ fluxes in human and low-K^+ sheep erythrocytes [6,7,18]. Although the mechanism of potentiation of the *N*-ethylmaleimide effect by cellular metabolism is unclear, a direct involvement of cytoplasmic ATP was suggested by our preliminary experiments showing that ghosts prepared from ATP depleted cells, when resealed in the presence of NaATP, exhibited *N*-ethylmaleimide-stimulation of cation efflux similar to ghosts derived from fresh cells. Whether there is a similar involvement of ATP in *N*-ethylmaleimide activation of cation flux in ghosts and intact cells is also uncertain.

The rate of *N*-ethylmaleimide-stimulated Rb^+ efflux in $NaNO_3$ was found to be a linear function of initial internal ghost Rb^+ concentration, whereas the rate of efflux in NaCl appeared to plateau. This may be evidence for different mechanisms of *N*-ethylmaleimide-stimulated flux in the presence of NO_3^- versus Cl^-, with efflux in $NaNO_3$ more resembling free diffusion through a large 'pore' or leak, and efflux in NaCl resembling movement via a saturable pathway. The observed properties of *N*-ethylmaleimide-stimulated cation efflux may therefore be related to the simultaneous functioning of multiple mechanisms, which may vary in proportion in media of different anionic composition.

Deuticke and co-workers [24–26] have described the formation of leaks with the properties of aqueous pores in the membranes of red cells treated with the bivalent oxidant diamide. However, *N*-ethylmaleimide was reported to suppress membrane protein oxidation resulting in pore formation [24–26], suggesting that our results reflect different phenomena.

The lack of *N*-ethylmaleimide-stimulation in Na^+-free media suggested a requirement for external Na^+ for either the action of *N*-ethylmaleimide or the movement of Rb^+ across the membrane. This apparent involvement of Na^+ is of particular interest in light of the finding that *N*-ethylmaleimide inhibits Na^+-Li^+ exchange in the intact cell [27,28]. The time necessary for *N*-ethylmaleimide to inhibit the Na^+-Li^+ (Na^+-Na^+) exchanger is approx. 3 min [28], which is the same time as that needed for *N*-ethylmaleimide stimulation of Rb^+ efflux. Also, inhibition of the exchanger by *N*-ethylmaleimide, like stimulation of ghost Rb^+ efflux, is not seen in choline media without external Na^+ [28].

It is evident that reconstitution of *N*-ethylmaleimide-stimulated Cl^--dependent K^+ transport in resealed ghosts is not straightforward. Difficulties may include behavior of the ghost as a swollen red cell with maximally activated fluxes (due to maximal swelling and lysis used to prepare ghosts), loss of a soluble component of the system, alteration of *N*-ethylmaleimide sites of action, disruption of the system, or activation of other pathways during ghost preparation (see for example, Refs. 29 and 30).

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