J. Membrane Biol. 35, 113–124 (1977)

ATPase and Phosphatase Activities from Human Red Cell Membranes: I. The Effects of N-Ethylmaleimide

D.E. Richards, A.F. Rega, and P.J. Garrahan

Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113–Buenos Aires, Argentina

Received 30 August 1976; revised 29 November 1976

Summary. (i) In human red cell membranes the sensitivity to N-ethylmaleimide of Ca^{2+}-dependent ATPase and phosphatase activities is at least ten times larger than the sensitivity to N-ethylmaleimide of $(Na^+ + K^+)$-ATPase and K^+-activated phosphatase activities. All activities are partially protected against N-ethylmaleimide by ATP but not by inorganic phosphate or by *p*-nitrophenylphosphate. (ii) Protection by ATP of $(Na^+ + K^+)$-ATPase is impeded by either Na^+ or K^+ whereas only K^+ impedes protection by ATP of K^+-activated phosphatase. On the other hand, Na^+ or K^+ slightly protects Ca^{2+}-dependent activities against N-ethylmaleimide, this effect being independent of ATP. (iii) The sensitivity to N-ethylmaleimide of Ca^{2+}-dependent ATPase and phosphatase activities is markedly enhanced by low concentrations of Ca^{2+}. This effect is half-maximal at less than 1 μM Ca^{2+} and does not require ATP, which suggests that sites with high affinity for Ca^{2+} exist in the Ca^{2+}-ATPase in the absence of ATP. (iv) Under all conditions tested the response to N-ethylmaleimide of the ATPase and phosphatase activites stimulated by K^+ or Na^+ in the presence of Ca^{2+} parallels that of the Ca^{2+}-dependent activities, suggesting that the Ca^{2+}-ATPase system possesses sites at which monovalent cations bind to increase its activity.

Red cell membranes of most mammalian species are able to drive not only active Na^+ and K^+ transport but also active Ca^{2+} transport (Schatzmann, 1975). Although it has been suggested that these two processes could be related (Schatzmann & Rossi, 1971; Rega, Richards & Garrahan, 1974), present experimental evidence favors the view that they are mediated by physically distinct systems. In fact, it is now known that the active center for ATP of the Ca^{2+} and the Na^{2+} pumps are carried by different membrane proteins, (Knauf, Proverbio & Hoffman, 1974) and that the antiserum that inhibits the Na^+ pump fails to inhibit the Ca^{2+}-ATPase (Glynn *et al.*, 1974).

Apart from ATPase and phosphatase activities which can be unequivocally related to one of the two active transport systems, red cell membranes also show ATPase and phosphatase activities which in the presence of Ca^{2+} are stimulated by either Na^+ or K^+ (Schatzmann & Rossi, 1971; Bond & Green, 1971; Garrahan, Pouchan & Rega, 1970; Rega, Richards & Garrahan, 1973). These activities are not observed in cell membranes possessing only Ca^{2+}-dependent or $(Na^+ + K^+)$-dependent ATPase activities (D.E. Richards, *unpublished*). It is not possible at this stage to establish whether the activities stimulated by monovalent cations in the presence of Ca^{2+} result from: (1) the stimulation of the Ca^{2+}-ATPase system, (ii) the modification by Ca^{2+} of the $(Na^+ + K^+)$-ATPase system or (iii) from the functioning of a third and yet unidentified enzymatic system.

Both $(Na^+ + K^+)$-ATPase (Skou & Hilberg, 1965; Skou, 1974) and Ca^{2+}-dependent ATPase (Bond, 1972) are inhibited by N-ethylmaleide and in both the effect of this reagent is modified by physiological ligands (Skou, 1974; Bond, 1972). A comparative study of the effects of N-ethylmaleimide on the different enzymic activities of human red cell membranes and of their modification by physiological ligands may provide useful information concerning the question posed above. The results of such study are presented in this paper.

Materials and Methods

Fragmented membranes from human red cells were obtained following the procedure already described (Garrahan, Pouchan & Rega, 1969).

ATPase activity was assayed by measuring the amount of inorganic phosphate (Fiske & Subbarow, 1925) in the reaction mixture. The reaction was terminated by addition of 3 volumes of a freshly prepared solution of: sodium dodecylsufate 2% (w/v); SO_4H_2; 0.75 N; $(NH_4)_2MoO_4$, 0.04 N; 1-amino-2-naphthol-4-sulfonic acid, 0.008% (w/v); Na_2SO_3, 0.052% (w/v); and $NaHSO_3$, 0.052% (w/v). The absorbance at 690 nm was measured after 10 min. $(Na^+ + K^+)$-ATPase was assayed in a medium containing (mM): $MgCl_2$, 5; KCl, 20; NaCl, 80; ATP, 0.5; Tris-HCl (pH 7.8 at 37 °C), 50; and ethylene-glycol-1-bis(β-aminoethyl ether) N,N′-tetra-acetic acid (EGTA), 0.5. $(Na^+ + K^+)$-ATPase activity is the difference in the activities in the above mentioned medium and in a medium in which Na^+ and K^+ were replaced by an equivalent amount of Tris. K^+-activated phosphatase activity was estimated by the procedure already described using *p*-nitrophenylphosphate as substrate (Garrahan *et al.*, 1969).

Ca^{2+}-dependent ATPase activities were measured in a medium containing (mM): $MgCl_2$, 5; Tris-HCl, 140; EGTA, 0.5; $CaCl_2$, 0.7; ATP, 0.5; pH 7.8 at 37 °C. When present, 80mM KCl or NaCl replaced an equivalent amount of Tris-HCl. Ca^{2+}-dependent phosphatase activity was measured in the same medium except that 10 mM *p*-nitrophenyl-phosphate

was also present. Ca^{2+}-dependent activities are the difference between the activities measured in the above mentioned media and the activities measured in media in which $CaCl_2$ was omitted. All enzymatic assays were carried out at 37 °C for 30 min; the final volume of the reaction mixture was 0.3 ml. The quantity of fragmented ghosts in the reaction mixture was that which gave a hematocrit of 10%, calculated on the original volume of the cells (about 1 mg protein/ml).

Reaction with N-ethylmaleimide

Membrane were treated with N-ethylmaleimide by a modification of the procedure described by Skou (1974). Membranes (2.8 mg protein/ml) were incubated in media containing (mM): Tris-HCl, 150 (pH 7.8 at 37 °C); EGTA, 0.5; and variable amounts of N-ethylmaleimide. When present, KCl or NaCl replaced an equivalent amount of Tris-HCl. The reaction was initiated by the addition of a concentrated solution of N-ethylmaleimide and was carried out during 30 min at 37 °C. The reaction was stopped by the addition of enough β-mercaptoethanol to give a final concentration of 2% (v/v). After treatment with N-ethylmaleimide the suspension was spun down and the membranes were washed 3 times with about 50 volumes of 15 mM Tris-HCl (pH 7.4 at 0 °C). After the first wash the membranes were submitted to rapid freezing and thawing and after the last wash membranes were suspended in more of the wash solution ready for use. Control samples were treated in the same way except that N-ethylmaleimide was omitted from the incubation media.

Protein was determined by the method of Lowry *et al.* (1951). ATP, *p*-nitrophenylphosphate and N-ethylmaleimide were from Sigma Chemical Co. All other reagents were of A.R. degree. Solutions were prepared in doubly glass-distilled water.

Results

Fig. 1 shows the ATPase (1*a*) and phosphatase (1*b*) activities that remain after preincubation of red cell membranes during 30 min with increasing concentrations of N-ethylmaleimide. Ca^{2+}-dependent activities are expressed as percent of the activities measured in the presence of nonlimiting concentrations of Ca^{2+} plus K^+ in untreated membranes. Results make clear that: (i) Both $(Na^+ + K^+)$-ATPase activity and its related K^+-dependent phosphatase activity are reduced to one-half their control value by 2.5 mM N-ethylmaleimide; (ii) Ca^{2+}-dependent ATPase and phosphatase activities are also inhibited by N-ethylmaleimide. As judged by the concentration of N-ethylmaleimide needed to reduce to one-half their control values, the sensitivity of these activities to N-ethylmaleimide is at least 10 times larger than that of the activities related to the Na^+ pump; (iii) the effect of N-ethylmaleimide on the activities elicited by Ca^{2+} plus K^+ is almost the same as on the ATPase and phosphatase activities elicited by Ca^{2+} alone.

Skou (1974) has demonstrated that ATP protects the $(Na^+ + K^+)$-ATPase against inhibition by N-ethylmaleimide. Fig. 2 gives the result

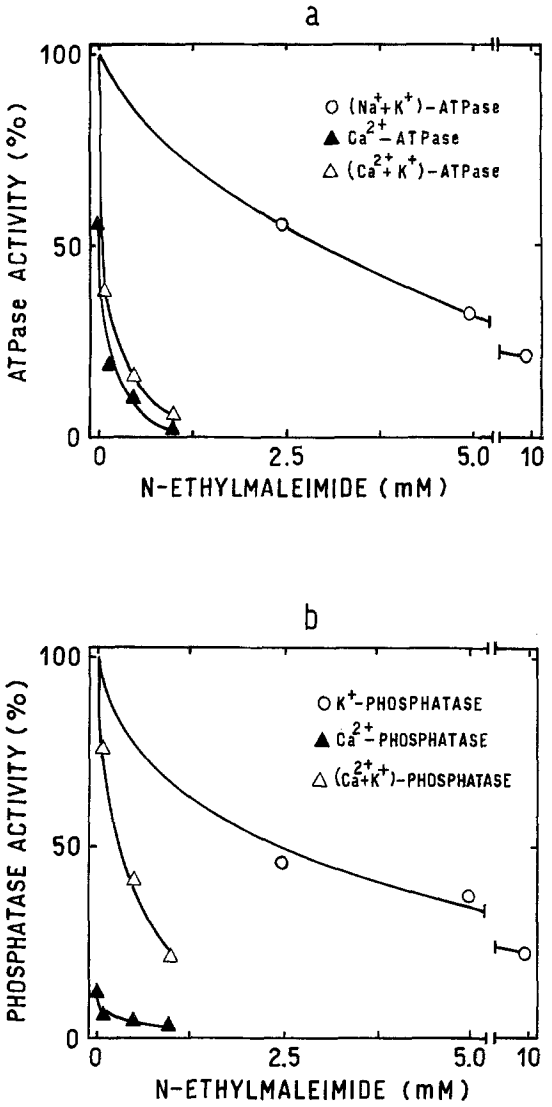
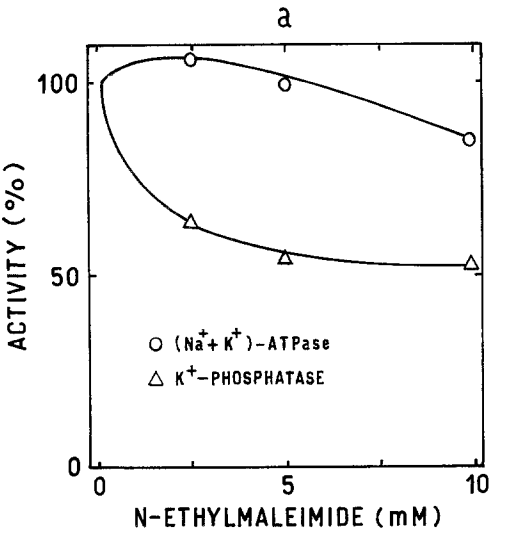
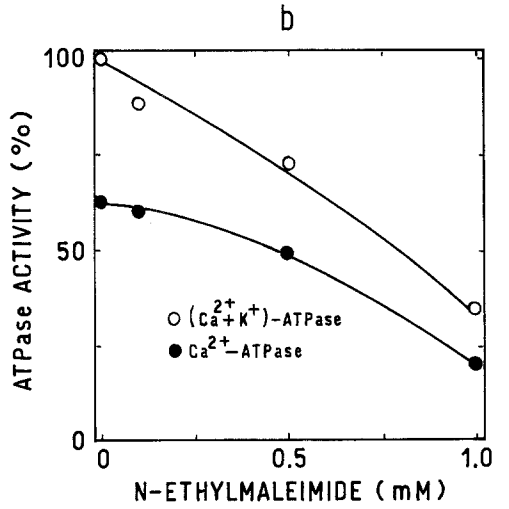


Fig. 1. The effect of preincubation with increasing concentrations of N-ethylmaleimide on ATPase (*a*) and phosphatase (*b*) activities of red cell membranes. $(Na^+ + K^+)$-ATPase and K^+-phosphatase activities are expressed as percent of the activities of membranes pretreated in media without N-ethylmaleimide. Ca^{2+}-dependent and $(Ca^{2+} + K^+)$-dependent activities are expressed as percent of the $(Ca^{2+} + K^+)$-dependent activities of membranes pretreated in the absence of N-ethylmaleimide

Fig. 2. (*a*), (*b*) and (*c*): The effect of preincubation with increasing concentrations of N-ethylmaleimide in the presence of 3 mM ATP on ATPase and phosphatase activities of red cell membranes. Results are expressed as in Fig. 1


a
100
50
0
ACTIVITY (%)
○ (Na+ + K+)-ATPase
△ K+-PHOSPHATASE
0
5
10
N-ETHYLMALEIMIDE (mM)


b
100
50
0
ATPase ACTIVITY (%)
○ (Ca2+ + K+)-ATPase
● Ca2+-ATPase
0
0.5
1.0
N-ETHYLMALEIMIDE (mM)

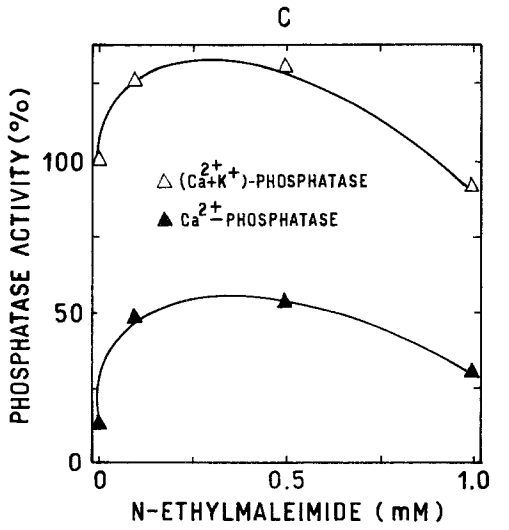

c
100
50
0
PHOSPHATASE ACTIVITY (%)
△ (Ca2+ + K+)-PHOSPHATASE
▲ Ca2+-PHOSPHATASE
0
0.5
1.0
N-ETHYLMALEIMIDE (mM)

Fig. 2a–c

of an experiment similar to that shown in Fig. 1, except that 3 mM ATP was present during preincubation with N-ethylmaleimide. Comparison of these results with those in Fig. 1 indicates that for the activities related to the Na^+ pump (Fig. 2*a*) protection by ATP is much more manifest in the ATPase than in the phosphatase activity. Ca^{2+}-dependent activities are also protected against N-ethylmaleimide by ATP (Fig. 2*b* and *c*), but in this case while ATP partially protects Ca^{2+}-dependent ATPase, it reverses the inhibition of Ca^{2+}-dependent phosphatase activities by N-ethylmaleimide. Fig. 2*b* and *c* also show that the parallelism between the effects of N-ethylmaleimide on Ca^{2+}-dependent and $(Ca^{2+}+K^+)$-dependent activities is preserved when ATP is present during treatment with N-ethylmaleimide. Control experiments (not shown) demonstrated that neither *p*-nitrophenyl-phosphate, a substrate of the phosphatase, nor orthophosphate can substitute ATP in its protective effect.

It has been shown in brain microsomes that protection by ATP against inhibition by N-ethylmaleimide of the $(Na^+ + K^+)$-ATPase system is partially prevented when either Na^+ or K^+ occupy their sites in the $(Na^+ + K^+)$-ATPase system (Skou, 1974). Results in Fig. 3*a* show that a similar effect can be observed in the $(Na^+ + K^+)$-ATPase from red cells. If the ATPase and phosphatase activities stimulated by monovalent cations in the presence of Ca^{2+} were catalyzed by the $(Na^+ + K^+)$-ATPase it is predictable that the protective effect of ATP on these activities would also be reversed by Na^+ or K^+. Fig. 3*a* shows in sharp contrast with this prediction, monovalent cations in the preincubation media lead to a slight decrease in the inhibitory effect of N-ethylmaleimide on $(Ca^{2+}+K^+)$-dependent ATPase activity. Furthermore, and in contrast with the $(Na^+ + K^+)$-ATPase, the effect is exerted with equal effectiveness by Na^+ or K^+ and it is independent on the presence of ATP during preincubation with N-ethylmaleimide. The effects of Na^+ or K^+ during preincubation with N-ethylmaleimide were also tested on Ca^{2+}-dependent ATPase with results almost identical to those shown in Fig. 3*a* for $(Ca^{2+}+K^+)$-dependent ATPase.

The studies on the effects of monovalent cations during preincubation with N-ethylmaleimide were extended to the phosphatase activities of red cell membranes. Results in Fig. 3*b* show that K^+ but not Na^+, increases the inhibitory effect of N-ethylmaleimide on K^+-dependent phosphatase activity. The effect of K^+ is dependent on the presence of ATP during preincubation with N-ethylmaleimide, suggesting that K^+ acts on the phosphatase through a mechanism similar to that proposed for the $(Na^+ + K^+)$-ATPase by Skou (1974). Fig. 3*b* also shows

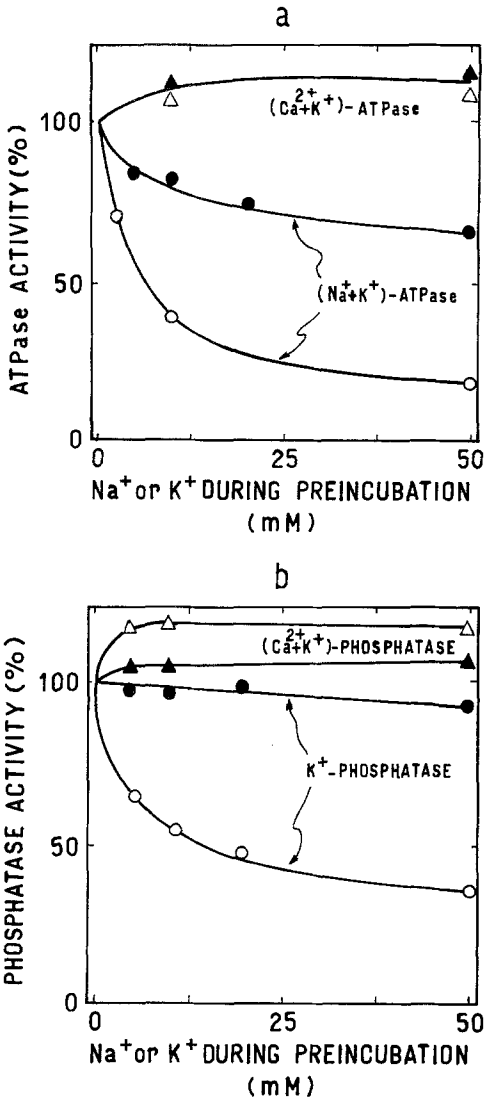


Fig. 3. The effect of increasing concentrations of either Na^+ (●, ▲) or K^+ (○, △) during preincubation with N-ethylmaleimide in media containing 3 mM ATP on ATPase (*a*) or phosphatase (*b*) activities of red cell membranes. The concentration of N-ethylmaleimide was 5.0 mM for $(Na^+ + K^+)$-ATPase and K^+-phosphatase, and 0.5 mM for Ca^{2+}-dependent activities. All results are expressed as percent of the activity remaining after treatment with N-ethylmaleimide in media without Na^+ or K^+

that K^+ during preincubation slightly decreases the inhibitory effect of N-ethylmaleimide on $(Ca^{2+} + K^+)$-dependent phosphatase activity, Na^+ being much less effective in this respect. As in the case of the

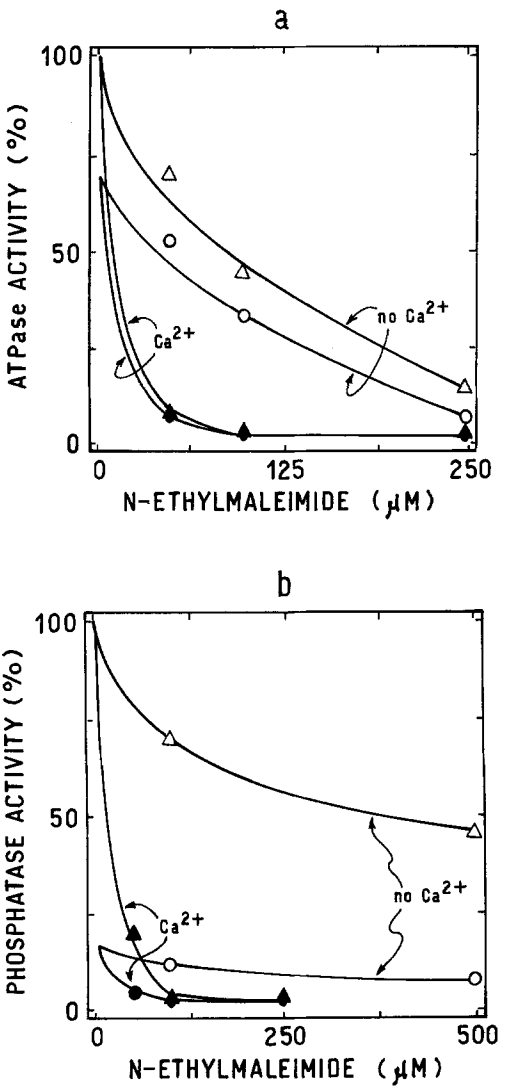


Fig. 4. The effect of increasing concentrations of N-ethylmaleimide on Ca^{2+}- (○, ●) and $Ca^{2+}+K^+$)-dependent (△, ▲) ATPase (*a*) and Ca^{2+}- (○, ●) and ($Ca^{2+}+K^+$)-dependent (△, ▲) phosphatase (*b*) activities of red cell membranes treated in media containing either 0.5 mM EGTA (no Ca^{2+}) or 0.5 mM EGTA plus 0.6 mM $CaCl_2$ (Ca^{2+}). Activities are expressed as in Fig. 1

($Ca^{2+}+K^+$)-dependent ATPase, the effects of monovalent cations on the ($Ca^{2+}+K^+$)-dependent phosphatase do not require ATP during preincubation with N-ethylmaleimide and are also apparent on the (Ca^{2+})-dependent phosphatase.

Table 1. The effect of Ca^{2+} on the sensitivity to N-ethylmaleimide of ATPase and phosphatase activities of red cell membranes

	Concentration of Ca^{2+} for half-maximal effect (μM)
Ca^{2+}-dependent ATPase	0.75
$(Ca^{2+}+K^+)$-dependent ATPase	0.75
Ca^{2+}-dependent phosphatase	0.33
$(Ca^{2+}+K^+)$-dependent phosphatase	0.60
$(Na^+ + K^+)$-activated ATPase	10^3
K^+-activated phosphatase	10^3

For Ca^{2+}-dependent activities pretreatment was carried in media containing 0.05 mM N-ethylmaleimide. For $(Na^+ + K^+)$-dependent activities the concentration of N-ethylmaleimide was 0.6 mM. The preincubation media contained different concentrations of Ca^{2+} which for values below 10 μM were adjusted with Ca-EGTA buffers (Wolf, 1973). The concentrations of Ca^{2+} for half-maximal effect were calculated by graphical interpolation as those reducing to one-half the activities remaining after treatment with N-ethylmaleimide in media containing 0 μM Ca^{2+}.

Bond (1972) has shown that the rate of onset of inhibition of $(Ca^{2+}+Na^+)$-dependent ATPase by N-ethylmaleimide is significantly increased by Ca^{2+}, the effect of Ca^{2+} being half-maximal at 5 μM. Fig. 4*a* shows that if Ca^{2+} is present during preincubation there is a marked increase in the sensitivity of both Ca^{2+}-dependent and $(Ca^{2+}+K^+)$-dependent ATPase activities towards N-ethylmaleimide, the concentration of the inhibitor for half-maximal effect dropping from 100 μM when Ca^{2+} is absent during preincubation to about 14 μM when 100 μM free-Ca^{2+} is present. The effect of Ca^{2+} is even larger when tested on the Ca^{2+}-dependent and $(Ca^{2+}+K^+)$-dependent phosphatase activities. When Ca^{2+} is added to the preincubation medium the concentration of N-ethylmaleimide for half-inhibition decreases from 400 to about 20 μM (Fig. 4*b*). The sensitizing effect of Ca^{2+} is also apparent in the presence of ATP and cannot be mimicked by Mg^{2+} at concentrations up to 30 mM.

Although Ca^{2+} also sensitizes the activities related to the Na^+ pump, in this case 100 μM Ca^{2+} only decreases from 2.5 to 1.7 mM the concentration of N-ethylmaleimide for half-maximal inhibition (experiment not shown).

Results in Table 1 allow a comparison of effectiveness of Ca^{2+} in increasing the sensitivity towards N-ethylmaleimide of all the activities tested. Results show that for Ca^{2+}-dependent activities half-maximal

increase in sensitivity to N-ethylmaleimide is reached at less than 1 μM Ca^{2+}, while 1 mM Ca^{2+} is needed for the same effect on the activities related to the Na^+-pump. This result suggests that the increase in the sensitivity of the $(Na^+ + K^+)$-ATPase to N-ethylmaleimide results from the combination of Ca^{2+} at sites that are different from those at which Ca^{2+} combines to exert this effect on Ca^{2+}-dependent activities.

Discussion

Results presented in this paper show that in human red cell membranes under all conditions tested the response to N-ethylmaleimide of the enzymatic activities related to the Na^+ pump differs from that of the Ca^{2+}-dependent enzymatic activities. More specifically: (i) The concentration of N-ethylmaleimide required to reduce to one-half Ca^{2+}-dependent activities after a 30-min preincubation with the agent is at least 10 times less than that required to obtain the same effect on the activities related to the Na^+ pump; (ii) Ca^{2+} increases the sensitivity towards N-ethylmaleimide of all activities but the apparent affinity of Ca^{2+} for this effect is about 1000 times higher for the Ca^{2+}-dependent activities than for the activities related to the Na^+ pump; (iii) in contrast with the increase in the sensitivity to N-ethylmaleimide that Na^+ or K^+, in the presence of ATP, elicit on the activities related to the Na^+ pump, these cations slightly protect Ca^{2+}-dependent activities against N-ethylmaleimide, this effect being independent of ATP.

These results made it possible to use the inhibition by N-ethylmaleimide as a tool to obtain information about the relation of the Ca^{2+}-dependent and the $(Na^+ + K^+)$-dependent enzymic systems to the activities that Na^+ or K^+ alone elicit in the presence of Ca^{2+}. Results are clear in showing that under the experimental conditions tested the response of these activities to N-ethylmaleimide differs significantly from that of the activities related to the Na^+ pump and approaches closely the response to N-ethylmaleimide of the enzymatic activities catalyzed by the Ca^{2+}-ATPase system.

The results therefore are in favor of the view that stimulation by Na^+ or K^+ of the hydrolysis of ATP or the hydrolysis of *p*-nitrophenylphosphate that red cell membranes catalize in the presence of Ca^{2+} is caused by the activation of the Ca^{2+}-dependent ATPase system. This view implies that the Ca^{2+}-ATPase system possesses sites to which monovalent cations are able to bind and whose physiological meaning is obscure

since no effect of monovalent cations has yet been demonstrated on the rate of active Ca^{2+} extrusion from red cells (Schatzmann, 1975).

Results presented in this paper confirm the observation by Bond (1972) that Ca^{2+} increases the reactivity to N-ethylmaleimide of Ca^{2+}-dependent ATPase activity and show that this effect is also apparent in all other Ca^{2+}-dependent enzymatic activities of red cell membranes. Since the increase in the reactivity to N-ethylmaleimide is exerted at a range of Ca^{2+} concentrations similar to that required for activation of Ca^{2+}-dependent enzymatic activities and in both phenomena Mg^{2+} cannot replace Ca^{2+}, it seems economical to assume, in agreement with Bond's view (1972), that binding of Ca^{2+} to the same site results in both sensitization to N-ethylmaleimide and stimulation of Ca^{2+}-dependent enzymatic activities. If this were true, the fact that sensitization to N-ethylmaleimide by Ca^{2+} does not require ATP implies that the existence of sites with high affinity for Ca^{2+} in the Ca^{2+}-dependent ATPase is independent of ATP. This is of importance in interpreting the mechanism of the absolute dependence on ATP of Ca^{2+}-dependent phosphatase activity (Garrahan *et al.*, 1970), since it rules out the possibility that such dependence is caused by a requirement of ATP for the binding of Ca^{2+} to the system.

PJG and AFR are established investigators from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. This work was supported by grants from the CONICET. The authors are grateful to the Instituto de Transfusiones Luis Agote, Universidad de Buenos Aires, for providing the blood used.

References

Bond, G.H. 1972. Ligand-induced conformational changes in the (Mg^{2+} + Ca^{2+})-dependent ATPase of red cell membranes. *Biochim. Biophys. Acta* **288**:423

Bond, G.H., Green, J.W. 1971. Effects of monovalent cations on the (Mg^{2+} + Ca^{2+})-dependent ATPase of the red cell membrane. *Biochim. Biophys. Acta* **241**:393

Fiske, C.H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375

Garrahan, P.J., Pouchan, M.I., Rega, A.F. 1969. Potassium activated phosphatase from human red cells. The mechanism of potassium activation. *J. Physiol. (London)* **202**:777

Garrahan, P.J., Pouchan, M.I., Rega, A.F. 1970. Potassium activated phosphatase from human red blood cells. The effects of adenosine triphosphate. *J. Membrane Biol.* **3**:14

Glynn, I.M., Karlish, S.J.D., Cavieres, J.D., Ellory, J.C., Lew, V.L., Joergensen, P.L. 1974. The effects of an antiserum to Na^+, K^+-ATPase on the ion transporting and hydrolytic activities of the enzyme. *Ann. N.Y. Acad. Sci.* **242**:357

Knauf, P.A., Proverbio, F., Hoffman, J.F. 1974. Electrophoretic separation of different phosphoproteins associated with Ca-ATPase and Na, K-ATPase in human red cell ghosts. *J. Gen. Physiol.* **63**:324

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265

Rega, A.F., Richards, D.E., Garrahan, P.J. 1973. Calcium ion-dependent *p*-nitrophenyl phosphate phosphatase activity and calcium ion-dependent adenosine triphosphatase activity from human erythrocyte membranes. *Biochem. J.* **136:**185

Rega, A.F., Richards, D.E., Garrahan, P.J. 1974. The effects of Ca^{2+} on ATPase and phosphatase activities of erythrocyte membranes. *Ann. N.Y. Acad. Sci.* **242:**317

Schatzmann, H.J. 1975. Active calcium transport and Ca^{2+}-activated ATPase in human red cells. *Curr. Top. Memb. Trans.* **6:**125

Schatzmann, H.J., Rossi, G.L. 1971. $(Ca^{2+}+Mg^{2+})$-activated membrane ATPase in human red cells and their possible relation to cation transport. *Biochim. Biophys. Acta* **241:**379

Skou, J.C. 1974. Effect of ATP on the intermediate steps of the reaction of the $(Na^{+}+K^{+})$-activated enzyme system. I. Studied by the use of N-ethylmaleimide inhibition as a tool. *Biochim. Biophys. Acta* **339:**246

Skou, J.C., Hilberg, C. 1965. The effect of sulfhydril-blocking reagents and of urea on the $(Na^{+}+K^{+})$-activated enzyme system. *Biochim. Biophys. Acta* **110:**359

Wolf, H.U. 1973. Divalent metal ion buffers with low pH-sensitivity. *Experientia* **29:**241