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INTERACTION OF *N*-ETHYLMALEIMIDE AND Ca^{2+} WITH HUMAN ERYTHROCYTE MEMBRANE ATPase

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SUMMARY

Human erythrocyte membranes were treated with *N*-ethylmaleimide and then assayed for cation-sensitive components of ATPase activity. Inhibition of Na^+-activated ATPase and (Na^+–K^+)-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3), observed following *N*-ethylmaleimide treatment, was abolished when ethylene glycol bis-(β-aminoethyl)-*N*,*N*-tetraacetate (EGTA) was included in the assay system.

N-Ethylmaleimide pretreatment reduced Mg^{2+}-activated ATPase activity both in the absence and presence of EGTA. The Mg^{2+}-dependent components of [^{14}C]ADP-ATP exchange and membrane phosphorylation using [γ-^{32}P]ATP were reduced following *N*-ethylmaleimide pretreatment whereas the Na^+-stimulated components of these activities were unaffected.

At very low ATP concentration, chelation of endogenous Ca^{2+} increases the sensitivity of the ATPase system to stimulation by low concentrations of Na^+. In contrast a Ca^{2+}-requirement at a Na^+-dependent step of (Na^+ + K^+)-activated ATPase reaction sequence was observed at higher ATP concentration. These results suggest both inhibitory and activating effects of Ca^{2+} on alkali cation-sensitive ATPase.

INTRODUCTION

Human erythrocyte membrane ATPase activity has been shown to consist of at least two components[1,2]. One component requires Mg^{2+}, is not affected by cardiac glycosides and can be stimulated by low concentrations of Ca^{2+} (ref. 2). The other component is a Mg^{2+}-dependent (Na^+–K^+)-activated ATPase activity (ATP phosphohydrolase, EC 3.6.1.3) which is inhibited by cardiac glycosides and small amounts of Ca^{2+} (refs. 1–5).

Both components of ATPase activity are inhibited by sulfhydryl reagents such as *N*-ethylmaleimide[6–8]. With some membrane preparations, (Na^+–K^+)-ATPase is inhibited more than Mg^{2+}-ATPase activity[7–11]; in other preparations, such as ox brain[6], the reverse is true. During the course of experiments designed to test effects

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl)-*N*,*N*-tetraacetate; DTNB, dithionitrobenzoic acid; BAL, 2,3-dimercaptopropanol.

of *N*-ethylmaleimide and Ca^{2+} on cation-sensitive components of ATP hydrolysis, we observed that pretreatment of human erythrocyte membranes with *N*-ethylmaleimide inhibited (Na^+–K^+)-ATPase, only in the presence of small amounts of Ca^{2+}. In addition to this inhibition by Ca^{2+}, a Ca^{2+} requirement at some step(s) of the reaction sequence was also observed.

MATERIALS AND METHODS

Erythrocyte membranes were prepared from fresh, human erythrocytes and assays were carried out as described previously[12,13]. Unless indicated otherwise, 4 vol. membranes were incubated with 1 vol. of 5 mM *N*-ethylmaleimide in 0.01 M Tris–HCl buffer (pH 7.4–7.6) (*plus N*-ethylmaleimide) or 0.01 M Tris–HCl buffer (pH 7.4–7.6) (control) for 30 min at 37°. Following this preincubation, assays were started by adding an aliquot of the membrane suspension to the assay medium as described previously[12]. For assay periods longer than 2 min, mercaptoethanol was included in the medium, at a concentration 10 times the final *N*-ethylmaleimide concentration. Procedures for measuring ATP hydrolysis ($^{32}P_i$ released from [γ-^{32}P]-ATP), labeling with [γ-^{32}P]ATP and measuring [^{14}C]ADP–ATP exchange activity were determined as described[12,13]. All data presented are typical of three or more experiments.

The rate of ATP hydrolysis measured in the absence of added NaCl or KCl is referred to as Mg^{2+}-ATPase, the increment in rate effected by the addition of 50 mM NaCl, as Na^+-ATPase, and the increment in rate effected by the addition of 50 mM NaCl and 10 mM KCl, as (Na^+–K^+)-ATPase

RESULTS

Relationship between Ca^{2+} and N-ethylmaleimide inhibition

Erythrocyte membranes were preincubated with *N*-ethylmaleimide as indicated above and then the ATPase activity was assayed at two ATP concentrations to observe the following: (i) Maximal stimulation by Na^+ referred to as Na^+-ATPase using 2 μM ATP. At this low ATP concentration, K^+ addition does not further stimulate the activity observed with Mg^{2+} and Na^+ (ref. 13). (ii) Maximal stimulation with both Na^+ and K^+ present, referred to as (Na^+–K^+)-ATPase, using 40 μM ATP[13].

Following 30 min preincubation with *N*-ethylmaleimide (Fig. 1, clear bars), Mg^{2+}-ATPase was decreased more than either Na^+-ATPase (70 % compared to 51 %) or (Na^+-K^+)-ATPase (72 % compared to 44 %). Following *N*-ethylmaleimide pretreatment, but with 0.1 mM ethylene glycol bis-(β-aminoethyl)-*N*,*N*-tetraacetate (EGTA) included in the assay medium (Fig. 1, shaded bars), Mg^{2+}-ATPase was decreased but Na^+-ATPase or (Na^+-K^+)-ATPase activities were no longer inhibited.

Since *N*-ethylmaleimide pretreatment inhibited the alkali cation-sensitive ATPase activity measured in the absence but not presence of EGTA, the question arose whether *N*-ethylmaleimide sensitizes the system to inhibition by endogenous Ca^{2+} which is chelated by EGTA. Experiments were carried out at 2 μM ATP to ascertain whether (1) the EGTA effects were due to chelation of Ca^{2+} and (2) *N*-ethylmaleimide effects were due to reaction with membrane sulfhydryl groups. As shown in Fig. 2, with a constant concentration of 0.1 mM EGTA and an increasing amount

of added $CaCl_2$, Na^+-ATPase in the control samples was decreased 35 % only when an excess of 20 μM $CaCl_2$ was added. Following *N*-ethylmaleimide pretreatment 50 % inhibition was observed with equimolar EGTA and $CaCl_2$ and hence micromolar

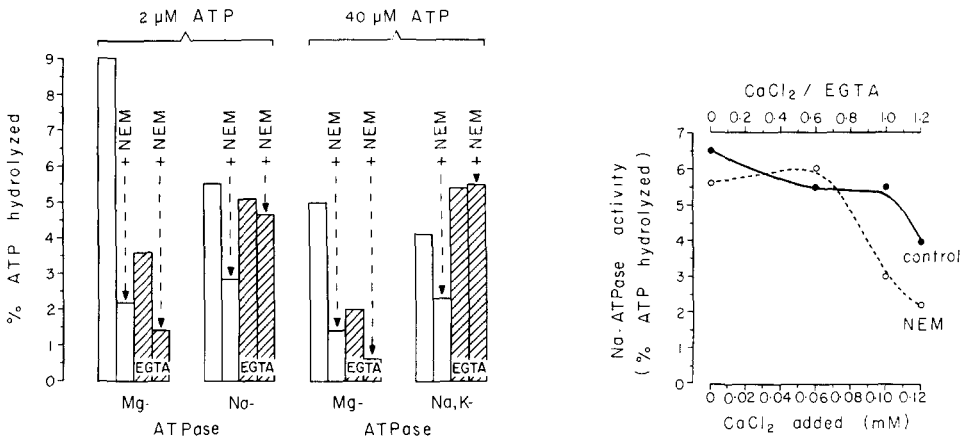

Fig. 1. Membranes were preincubated for 30 min at 37° without or with 1 mM *N*-ethylmaleimide (NEM) as indicated. At 2 μM ATP assays were carried out for 10 sec at 37° in a final volume of 0.5 ml containing 0.35 ml membranes (3.85 mg protein per ml) 0.002 mM [γ-^{32}P]ATP ($875 \cdot 10^3$ counts/min per 0.5 ml volume) 0.012 mM $MgCl_2$ and 40 mM Tris–HCl buffer (pH 7.4). At 40 μM ATP, assays were carried out for 5 min at 37° in a final volume of 0.25 ml containing 0.1 ml membranes (0.68 mg/ml) 0.04 mM [γ-^{32}P]ATP ($59 \cdot 10^3$ counts/min per 0.25 ml volume) 0.04 mM $MgCl_2$ and 8 mM mercaptoethanol. 0.1 mM Tris–EGTA buffer (pH 7.4), was absent (clear bars) or present (shaded bars) in the incubation medium. Na^+-ATPase and $(Na^+–K^+)$-ATPase activity refer to the increment in activity obtained in the presence of 50 mM NaCl or 50 mM NaCl *plus* 10 mM KCl, respectively, after subtraction of the activity observed without NaCl ("Mg^{2+}-ATPase").

Fig. 2. Dependence of Na^+-ATPase on Ca^{2+} concentration. Incubation was carried out as described in Fig. 1 (2 μM [γ-^{32}P]ATP), except that assays were for 2 min in a final volume of 0.25 ml containing 0.1 ml membranes (0.72 mg/ml), 8 mM mercaptoethanol, 0.1 mM Tris–EGTA buffer and $CaCl_2$ as indicated. NEM stands for *N*-ethylmaleimide.

amounts of free Ca^{2+} (ref. 14); 70 % inhibition was observed with 20 μM excess $CaCl_2$ (0.12 mM $CaCl_2$ added). With a $CaCl_2$/EGTA ratio of 0.6, and hence a free Ca^{2+} concentration approx. 0.1 μM at pH 7.4 (ref. 14), no inhibition was observed following *N*-ethylmaleimide treatment.

The data in Fig. 3 support the conclusion that the effects of *N*-ethylmaleimide are due to reaction with sulfhydryl groups. When the membranes were treated with the sulfhydryl reagent dithionitrobenzoic acid (DTNB), the pattern of inhibition was similar to that observed with *N*-ethylmaleimide *i.e.* Na^+-ATPase activity was completely inhibited in the control (EGTA omitted); with 0.1 mM EGTA in the assay, inhibition was reduced to 48 %.

^{32}P *incorporation and* [^{14}C]*ADP–ATP exchange*

The effects of *N*-ethylmaleimide on the transfer of ^{32}P from [γ-^{32}P]ATP to membrane components at 37° and on the [^{14}C]ADP–ATP exchange activity at 0° were tested. The Na^+-stimulated components of these activities have been shown to participate in Na^+-ATPase activity of erythrocyte membranes[12,13]. The results in

Table I indicate that *N*-ethylmaleimide treatment resulted in a marked decrease in both the level of ^{32}P bound and the exchange activity observed without added Na^+. In contrast, Na^+-stimulated phosphorylation and exchange were not significantly affected. Thus *N*-ethylmaleimide pretreatment resulted in an increased ratio of Na^+-dependent/Mg^{2+}-dependent labeling and exchange. Na^+-stimulated phosphorylation was decreased in the presence of K^+, both in the controls and following *N*-ethylmaleimide pretreatment.

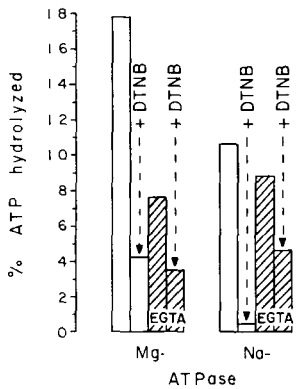

Fig. 3. Membranes were preincubated for 30 min at 37° without or with 1 mM DTNB as indicated. Assays were carried out for 2 min at 37° in a final volume of 0.25 ml containing 0.1 ml membranes (1.41 mg/ml), 0.002 mM [γ-^{32}P]ATP, 0.012 mM $MgCl_2$ and 50 mM NaCl as required, as described in Fig. 1.

TABLE I

EFFECTS OF *N*-ETHYLMALEIMIDE TREATMENT ON MEMBRANE PHOSPHORYLATION AND [^{14}C]ADP–ATP EXCHANGE

For phosphorylation determinations, membranes 0.35 ml (3.6 mg membrane protein per ml), were initially incubated for 30 min at 37° without or with 1 mM *N*-ethylmaleimide (NEM) as described in MATERIALS AND METHODS and were then incubated for 10 sec at 37° in a final volume of 0.5 ml containing 0.002 mM [γ-^{32}P]ATP ($1700 \cdot 10^3$ counts/min per 0.5 ml volume), 0.012 mM $MgCl_2$ and 30 mM Tris–HCl buffer (pH 7.4). NaCl (50 mM), KCl (10 mM) and Tris–EGTA buffer (pH 7.4) (0.1 mM) were included as indicated. For [^{14}C]ADP–ATP exchange, membranes, 0.1 ml (2.0 mg protein per ml), were incubated for 1 h at 0° in a final volume of 0.25 ml containing 1 μM [^{14}C]ADP (0.025 μC per 0.25 ml volume) and 2 μM ATP, under the conditions described previously[13].

Cations added	*Membrane phosphorylation (pmoles ^{32}P bound per mg)*	
	Control	*NEM-treated*
(a) Mg^{2+}	0.72	0.23
(b) Mg^{2+} + Na^+	1.49	0.93
(c) Mg^{2+}, Na^+, K^+	0.67	0.30
Na^+-dependent phosphorylation (b—a)	0.77	0.70
K^+-dependent dephosphorylation (c—b)	0.82	0.63
	[^{14}C]ADP–ATP exchange (pmoles [^{14}C]ATP formed per mg per h)	
(d) Mg^{2+}	82	44
(e) Mg^{2+} + Na^+	184	157
Na^+-dependent exchange (e—d)	102	113

TABLE II

EFFECTS OF EGTA

For measurements of ATP hydrolysis at 40 μM ATP, 0.1 ml membranes (0.67 mg protein per ml) were incubated for 5 min in a final volume of 0.25 ml containing 40 μM [γ-^{32}P]ATP ($79 \cdot 10^3$ counts/min per 0.25 ml volume) 40 μM $MgCl_2$, 40 mM Tris–HCl buffer (pH 7.4), and NaCl and KCl as indicated. For measurements of ATP hydrolysis at 2 μM ATP, 0.1 ml membranes (0.96 mg/ml) were incubated for 2 min in a final volume of 0.25 ml containing 2 μM [γ-^{32}P]ATP ($140 \cdot 10^3$ counts/min per 0.25 ml volume) 25 μM $MgCl_2$, 40 mM Tris–HCl buffer (pH 7.4), and NaCl as indicated. Measurements of phosphorylation were carried out as described in Table I.

ATP concn. (μM)	*Quantity measured*	*Additions*	*ATP hydrolyzed (%)*	
			−EGTA	*+EGTA*
40	Mg^{2+}-ATPase	None	8.1	4.4
	Na^+-ATPase	50 mM NaCl	3.7	0.2
	$(Na^+–K^+)$-ATPase	50 mM NaCl + 10 mM KCl	7.7	6.8
2	Mg^{2+}-ATPase	None	19.6	7.1
	Na^+-ATPase	0.5 mM NaCl	0.4	6.7
		5.0 mM NaCl	5.1	8.5
		50 mM NaCl	12.8	11.9
			^{32}P bound (pmoles/mg)	
			−EGTA	*+EGTA*
2	Mg^{2+}-dependent phosphorylation	None	0.67	0.76
	$(Mg^{2+} + Na^+)$-dependent phosphorylation	50 mM NaCl	1.46	1.34

Effects of EGTA

During the course of these studies, we observed that EGTA addition did not change Na^+-ATPase activity measured at 2 μM ATP and 50 mM NaCl. However, EGTA had marked effects on (1) the Na^+ concentration required to stimulate ATP hydrolysis measured at very low concentrations of ATP, *e.g.* 2 μM, and on (2) the relative sensitivity of ATPase to activation by Na^+ alone *versus* Na^+ *plus* K^+ measured at higher ATP concentrations, *e.g.* 40 μM. Typical results are shown in Table II; results were similar whether 0.1 mM EGTA was omitted or 0.1 mM EGTA *plus* 0.1 mM $CaCl_2$ were added. As indicated, at 2 μM ATP the NaCl concentration required to induce approximately half-maximal Na^+-ATPase activity was reduced approx. 10-fold with 0.1 mM EGTA present. At higher ATP concentrations (40 μM), the Na^+-ATPase, but not $(Na^+–K^+)$-ATPase was decreased markedly (95 %) by EGTA. This suggests that chelation of endogenous Ca^{2+} by EGTA probably alters the relative sensitivity of the ATPase to activation by Na^+ alone *versus* Na^+ *plus* K^+.

As shown in Table II, EGTA did not affect either Mg^{2+}- or Mg^{2+}- *plus* Na^+-dependent phosphorylation of membranes using 2 μM [γ-^{32}P]ATP.

DISCUSSION

Mg^{2+}-dependent $(Na^+–K^+)$-ATPase appears to be a multistage reaction sequence consisting of (1) Na^+-stimulated phosphorylation of a membrane component E_1 (refs.

12, 13, 15–18), and Na^+-stimulated [^{14}C]ADP–ATP exchange[19,20], (2) Mg^{2+}-activated transformation of phosphorylated intermediate ($E_1P \rightleftharpoons E_2P$)[9,21], (3) K^+-stimulated dephosphorylation ($E_2P \rightarrow E_2$)[22–26] and, (presumably) (4) reversion to the original form ($E_2 \rightleftharpoons E_1$)[21]. Inhibition of Na^+-stimulated ATPase activity, associated with increased Na^+-sensitive [^{14}C]ADP–ATP exchange activity and loss of sensitivity to K^+-stimulated hydrolysis of phosphorylated intermediate has been effected by treating membrane preparations with *N*-ethylmaleimide[7–9] or oligomycin[8,27,28]. Similar results were obtained with human red cell membranes incubated with oligomycin[13]. These results suggested that the site of inhibition was at a step following initial phosphorylation of intermediate, *e.g.* $E_1P \rightleftharpoons E_2P$. In the present study *N*-ethylmaleimide did not increase exchange activity or block K^+-stimulated decrease in bound ^{32}P. It is possible, therefore, that with the present conditions of low ATP and Mg^{2+} concentration used, the partial inhibition by *N*-ethylmaleimide (50–70 %) was mainly at another site, *e.g.* transformation of unphosphorylated form of intermediate, *i.e.* $E_2 \rightleftharpoons E_1$, as discussed by SIEGEL AND ALBERS[21] for 2,3-dimercaptopropanol (BAL)–arsenite effects. Our data suggest that the inhibition may not be due directly to *N*-ethylmaleimide but to Ca^{2+}, *i.e.* the *N*-ethylmaleimide-treated system may be more sensitive to Ca^{2+} inhibition than the untreated system.

In our experiments, blockage (alkylation) of sulfhydryl groups would have occurred during *N*-ethylmaleimide pretreatment and should have been negligible in the subsequent assay. Either the assay period was short (< 2 min) compared to the pretreatment period (30 min) or excess mercaptoethanol was present during the assay. Thus the Ca^{2+} effects do not reflect modification of the reaction between sulfhydryl reagent and membrane protein, although such effects have been described[29]. Under the conditions of our experiments blockage of sulfhydryl groups appeared to increase the sensitivity of the enzyme system to inhibition by Ca^{2+}. In experiments with ox brain preparations, SKOU AND HILBERG[30] described ATP protection against inhibition by sulfhydryl reagents; it is possible that their observations are related also to Ca^{2+} binding by ATP.

Competition between Ca^{2+} and Na^+ has been described extensively in studies with excitable tissues. Although EPSTEIN AND WHITTAM[31] provided kinetic evidence indicating that Ca^{2+} inhibition of (Na^+–K^+)-ATPase is due to CaATP acting as a competitive inhibitor of MgATP and probably not due to Ca^{2+}–Na^+ competition, other studies have favoured the latter possibility[32–34]. More recently[35], Na^+ stimulation of Ca^{2+} efflux from subcellular vesicles has been shown to be blocked by low concentrations of certain sulfhydryl reagents, including *N*-ethylmaleimide. The present observations may be explained similarly. Thus, alkylation of certain sulfhydryl groups may decrease the ability of Na^+ to displace Ca^{2+} from 'Na^+ sites' resulting in decreased Na^+-ATPase activity. Moreover, chelation of Ca^{2+} by EGTA markedly increases the sensitivity of ATPase to stimulation by low concentrations of Na^+, as shown in Table II.

The present study provides evidence for both inhibitory and activating effects of Ca^{2+} on the transport ATPase system. With changing (increasing) ATP concentration, the ATPase system appears to change from being Na^+-dependent to Na^+- *plus* K^+-dependent[13]. This change probably reflects the activity of different reaction steps which are evident when they become rate-limiting as the ATP concentration is increased. At 40 μM ATP, both Na^+ and Na^+ *plus* K^+ activation are observed. At this

concentration and in contrast to results at 2 μM ATP, removal of endogenous Ca^{2+} reduced markedly the Na^+-sensitive component without affecting the Na^+- *plus* K^+-stimulated component. This observation suggests also that the Na^+-stimulated component observed at 40 μM ATP reflects the activity of a step distinct from, *e.g.* subsequent to, the Na^+-stimulated component observed at 2 μM ATP.

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