Biochimica et Biophysica Acta, 394 (1975) 145–155

BBA 76966

EFFECT OF CARBONYLCYANIDE *m*-CHLOROPHENYLHYDRAZONE ON THE CALCIUM-STIMULATED ATPase ACTIVITY OF ERYTHROCYTE GHOSTS

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(Received October 29th, 1974)

SUMMARY

Incubation of erythrocyte ghosts with carbonylcyanide *m*-chlorophenylhydrazone (CCCP) plus Ca^{2+} resulted in inactivation of the Ca^{2+}-stimulated ATPase activity. Omission of Ca^{2+} or lowering of the temperature below 25 °C eliminated the inhibitory effect, as also did the presence of ATP during the incubation. On the other hand, the addition of β-mercaptoethanol did not influence the Ca^{2+}-dependent inhibition by CCCP. Compared with the level of CCCP which uncouples oxidative phosphorylation, a rather high level (0.5 mM) of CCCP was required to inhibit the ATPase activity in ghosts. However, once the inhibition had been accomplished, almost all of the CCCP could be removed from the ghost membrane by washing with a Ca^{2+}-containing solution, without affecting the inhibition of ATPase. If ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid was included in the washing medium, the inhibition of ATPase was nearly completely reversed by washing. The results indicate that only the Ca^{2+}-stimulated, Mg^{2+}-ATPase was inhibited by 0.5 mM CCCP, while the remaining components of the ATPase activity were slightly inhibited by higher levels of the uncoupler. Low levels of CCCP (0.1 mM) stimulated the Mg^{2+}-ATPase slightly. CCCP was much more specific for the Ca^{2+}-stimulated ATPases than *N*-(1-naphthyl)maleimide, an unusually effective sulfhydryl reagent, and the requirement of Ca^{2+} for inactivation was also quite specific.

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone (CAS Registry 555-60-2); EGTA, ethyleneglycol-bis(β-aminoethylether)-*N,N*-tetraacetic acid (CAS Registry 7365-44-8); TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid. In the interest of brevity, we will refer to the ATPase activity in the presence of various ions as $(Ca^{2+} + Mg^{2+})$-ATPase, etc. In each case this should be read as the ATPase activity in the presence of Ca^{2+} and Mg^{2+}, etc. This terminology does not attempt to deal with the question of the actual number of separate enzymes involved.

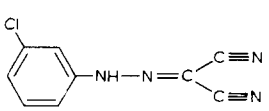
INTRODUCTION

ATPase (EC 3.6.1.3) activity in erythrocyte ghosts shows an absolute requirement for Mg^{2+}, with the basal activity in the presence of Mg^{2+} being stimulated by Ca^{2+} or by $Na^+ + K^+$. The $(Na^+ + K^+)$-stimulation of the ATPase is directly related to the active movement of K^+ and Na^+ across the plasma membrane [1–3]. In a similar fashion, the Ca^{2+}-stimulated component of the ATPase is directly related to the movement of Ca^{2+} out of the erythrocyte [4–6]. No role has definitely been assigned to the Mg^{2+}-ATPase, but it may be related to the energy-dependent endocytosis observed in erythrocyte ghosts [7, 8]. The $(Na^+ + K^+)$-stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase is not related to Na^+ and K^+ transport, since it occurs in the presence of ouabain and its role is unknown [9]. In the course of studies on the energy dependent endocytosis, the effects of an uncoupler of oxidative phosphorylation, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) [10] were investigated. This compound proved preferentially to inactivate the Ca^{2+}-transport ATPase, and only when the ghosts were preincubated with both CCCP and Ca^{2+}. The details of this phenomenon are described below.

METHODS

Erythrocyte ghosts were prepared from fresh porcine erythrocytes as previously described [8]. ATP hydrolysis was estimated on the basis of the amount of orthophosphate released as determined by the method of Martin and Doty [11]. Membrane protein was determined by the biuret method of Gornall et al. [12] with the modification that deoxycholate was present at 0.3 % in the final assay tube; bovine serum albumin was used as a standard.

The standard conditions for the ATPase assay were as follows. Ghosts were incubated at a concentration of 1 mg protein/ml in a solution which was 3 mM in $MgCl_2$ and 50 mM in *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer which had been adjusted to pH 7.4 at 37 °C with triethanolamine. All of the other reagents required for a given experiment, such as CCCP, $CaCl_2$, NaCl, KCl, etc., were also added and the ghosts preincubated for 5 min at 37 °C. Unless otherwise stated, $CaCl_2$ was present at 0.5 mM, NaCl at 100 mM and KCl at 20 mM. The ATPase assay was then initiated by the addition of 0.1 vol. of 30 mM ATP (Tris salt). After incubation (20 min where not otherwise indicated) the reaction was stopped by the addition of acidic silicotungstate and the determination of P_i was carried out. The CCCP utilized was obtained from Calbiochem, Lot 310090. The structural formula of CCCP is:



The $CaCl_2$ was obtained by titration of reagent grade $CaCO_3$ with HCl. When activities and concentrations are reported per mg, this is in terms of membrane protein, not total membrane weight.

Each type of experiment represented in the figures and tables was carried out

several times, and a typical representative experiment was chosen for publication. The aspects of the experiments discussed in this paper were consistently reliable but some other aspects of the ATPase activity were rather variable. The absolute level of the various ATPases varied from one preparation of ghosts to another, for example. The amount of the stimulation of the various ATPases at low CCCP concentrations varied from slight, as shown in Fig. 3 for the $(Na^+ + K^+ + Mg^{2+})$-ATPase, to substantial as shown in Fig. 1 for the same ATPase: the exact shape of the curves also differed somewhat, as appears in the small differences between the curves shown for the $(Na^+ + K^+ + Ca^{+2} + Mg^{2+})$-ATPase in Figs 1 and 2. No particular significance was assigned to these small deviations, which occurred between one batch of ghosts and another, but the study focussed on those aspects which were reproducible. Similarly, no significance is assigned to the apparent changes in the amount of the $(Na^+ + K^+)$-stimulation of the ATPase in the presence of CCCP shown in Fig. 4, since this represents a difference between two large numbers and was not seen in other experiments. The exact level of CCCP required for full inactivation of the Ca^{2+}-transport ATPase also varied somewhat between various ghost preparations, as may be seen from a comparison of Figs 1 and 2.

CCCP was dissolved in dimethylsulfoxide at concentrations of 100, 50 or 10 mM. The solution added was chosen so that the volume fraction of dimethylsulfoxide did not exceed 0.01. CCCP was quantitated by measuring the absorbance at 378 nm, using a molar extinction coefficient of $2.73 \cdot 10^4$ [10]. For this measurement, the ghost suspension was made 1 % (w/v) in sodium dodecylsulfate, which made the suspension sufficiently transparent for accurate absorbance measurements. The blank contained the same amount of ghosts and detergent, but no CCCP.

RESULTS

The effect of preincubation with CCCP on the ATPase activity of erythrocyte membranes is shown in Fig. 1. In the absence of Ca^{2+}, the effect of CCCP is not striking. Both the $(Na^+ + K^+ + Mg^{2+})$-ATPase and the Mg^{2+}-ATPase were moderately stimulated by low levels of CCCP. At higher levels of CCCP the stimulation was reversed, and in the case of the $(Na^+ + K^+ + Mg^{2+})$-ATPase a slight overall inhibition was observed. The effect of CCCP on the $(Ca^{2+} + Mg^{2+})$-ATPase was very marked; the Ca^{2+}-stimulated portion of the ATPase was nearly completely inhibited at 0.5 mM CCCP. The data of Fig. 1 suggested that the Ca^{2+}-stimulated ATPase was preferentially inactivated by the action of 0.5 mM CCCP, since the total ATPase activity in the presence of Ca^{2+} approached that obtained in the absence of Ca^{2+} at concentrations of CCCP above 0.5 mM.

The specificity of the effect of CCCP is further shown by the data of Fig. 2. The presence of ouabain in this experiment inactivated the $(Na^+ + K^+)$-transport ATPase. This allowed the observation of the effect of $Na^+ + K^+$ on the Ca^{2+}-transport ATPase [9]. The data clearly show that this $(Na^+ + K^+)$-stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase, like the $(Na^+ + K^+ + Mg^{2+})$-ATPase and Mg^{2+}-ATPase, was slightly stimulated by low levels of CCCP. At higher CCCP concentrations, the $(Na^+ + K^+)$ stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase was somewhat more sensitive to the effect of CCCP than were the $(Na^+ + K^+ + Mg^{2+})$-ATPase and the Mg^{2+}-ATPase. However, it was clearly much less sensitive to CCCP than was the Ca^{2+}-

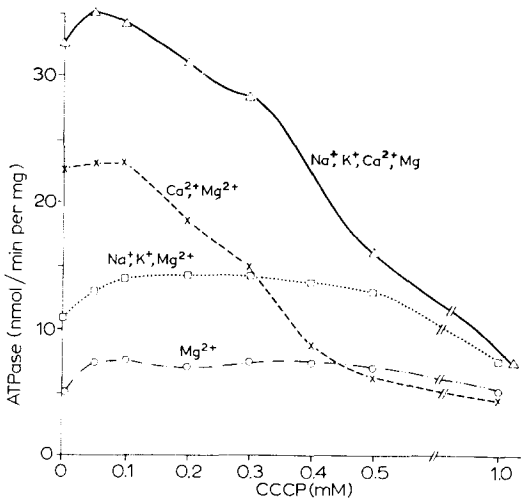


Fig. 1. Inhibition of Ca^{2+}-stimulated ATPase activity by CCCP. Ghosts were preincubated for 5 min at 37 °C with the entire medium except ATP, and the reaction was then started by addition of ATP. Ca^{2+} was present at 0.5 mM.

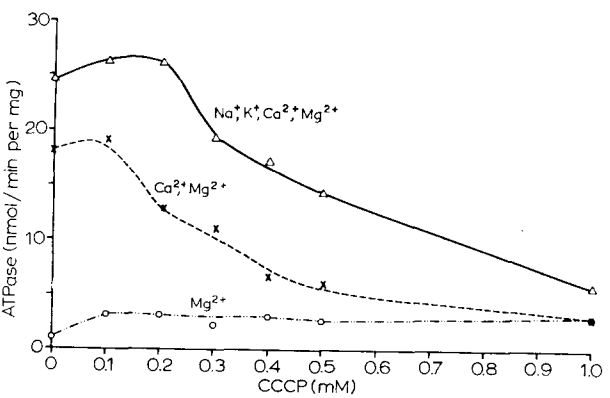


Fig. 2. Effect of CCCP on ATPase activities in the presence of ouabain. Experiments were done in the same manner as those of Fig. 1, except that ouabain was present at a concentration of 0.5 mM.

stimulated component of the $(Ca^{2+}+Mg^{2+})$-ATPase. As the figure shows, this Ca^{2+}-transport ATPase was reduced to 20 % of its original value at 0.4 mM CCCP, while the $(Na^+ + K^+)$-stimulated component of this ATPase remained essentially uninhibited. Thus, of the four observable ATPase activities of erythrocyte membranes, the Ca^{2+}-transport ATPase was uniquely sensitive to inhibition by CCCP.

The inhibition of the Ca^{2+}-stimulated component of the $(Ca^{2+}+Mg^{2+})$-ATPases was dependent upon the presence of Ca^{2+} in the preincubation medium. Table I shows that the presence of CCCP without Ca^{2+} in the preincubation medium led to no inhibition of the $(Ca^{2+}+Mg^{2+})$-ATPase. Stating this requirement in another way, it was necessary to add Ca^{2+} before ATP in order for inhibition of the

TABLE I

INHIBITION OF $(Ca^{2+}+Mg^{2+})$-ATPase REQUIRED BOTH Ca^{2+} AND CCCP; WASHING IN PRESENCE OF Ca^{2+} ONLY DID NOT REMOVE THE INHIBITION

Ghosts, at 10 mg membrane protein per 10 ml, were preincubated for 10 min at 37 °C in 50 mM TES · triethanolamine (pH 7.4), 3 mM $MgCl_2$ and CCCP as indicated. After the reaction, the suspension was cooled and the ghosts pelleted by centrifuging at 17 000 rev./min in a Sorvall SS-34 rotor. The pellet was resuspended in 10 ml of the same medium as was used for the preincubation, except that CCCP was omitted, and washed the number of times indicated. $(Ca^{2+}+Mg^{2+})$-ATPase was assayed as described in Methods; Ca^{2+} was present at a concentration of 0.5 mM, but no more CCCP was added.

Preincubation with CCCP (mM)	Ca^{2+} (mM)	No. of washes	CCCP in washed ghosts, (nmol/mg)	$(Ca^{2+}+Mg^{2+})$-ATPase (nmol/mg per min)
0	0	0	—	20.5
0.5	0	0	—	21.0
0.5	0.5	0	—	7.0
0	0	2	0	19.3
0.5	0	2	23	18.2
0.5	0.5	2	26	7.1
0	0	5	0	19.0
0.5	0	5	6.8	17.9
0.5	0.5	5	8.0	7.3
0.5	0	5*	5.9	18.0
0.5	0.5	5*	8.1	14.6

* In addition to the other components, the medium was made 1 mM in EGTA.

Ca^{2+}-stimulated ATPase to occur. Table I also shows that when both Ca^{2+} and CCCP were present during the preincubation, the activity was lowered to the level expected for the Mg^{2+}-ATPase alone, in agreement with Fig. 1.

Since the presence of ATP would lower the level of free Ca^{2+} by chelating a part of the Ca^{2+}, the level of free Ca^{2+} was calculated under the conditions of preincubation. In this calculation, the presence of the ghosts was ignored and it was assumed that ATP^{3-} made no contribution. Even though these simplifying assumptions were made, a cubic equation resulted; this equation was solved algebraically [13]. The free Ca^{2+} concentration was calculated to be 0.21 mM when the total concentrations were: ATP, 3.0 mM; Mg^{2+}, 3.0 mM; Ca^{2+}, 0.5 mM (the conditions of our normal experiments). The same type of calculation showed that a total Ca^{2+} concentration of 1.04 mM was required to yield a free Ca^{2+} concentration of 0.50 mM in the presence of the above concentrations of ATP and Mg^{2+}. An experiment was then carried out, under the conditions of Table I, to test the effect of this level of Ca^{2+}. Preincubation with 0.5 mM Ca^{2+} alone resulted in a $(Ca^{2+}+Mg^{2+})$-ATPase of 15.5 nmol/mg per min, while preincubation with 0.5 mM Ca^{2+} and 0.5 mM CCCP resulted in inhibition of this ATPase to 6.2 nmol/mg per min. When the solution was made 1.04 mM in Ca^{2+} simultaneously with the addition of ATP, the ATPase was not significantly changed from that observed at 0.5 mM Ca^{2+}, being 15.0 nmol/mg per min, but combination of preincubation with 0.5 mM CCCP with addition of this level of Ca^{2+} with the ATP resulted in a partial inhibition, to 10.7 nmol/mg per

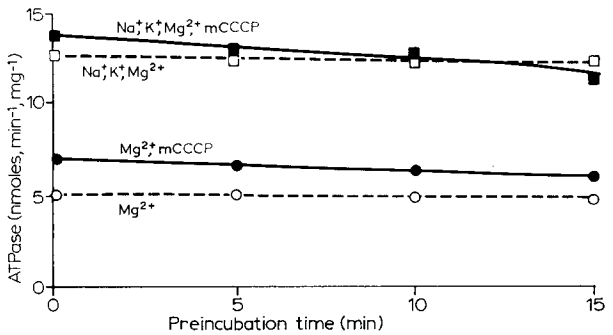


Fig. 3. Effect of preincubation time on the non-Ca^{2+}-ATPases. Preincubations were carried out as in Fig. 1 except that the level of CCCP was 0.5 mM in the experiments shown by the filled symbols, and the time of preincubation was varied. Open symbols represent experiments from which CCCP was omitted; circles, Mg^{2+}-ATPase; squares, $(Na^+ + K^+ + Mg^{2+})$-ATPase.

min. Thus a higher total Ca^{2+}, added with ATP, resulted in some CCCP inhibition of the Ca^{2+}-transport ATPase, not seen when 0.5 mM Ca^{2+} was added with ATP.

The effects of preincubation in the presence of various ions are also shown in Figs 3 and 4. Fig. 3 shows that the level of the ATPase measured in the absence of Ca^{2+} is independent of the time of preincubation up to 15 min, whether CCCP is present during the preincubation or not. On the other hand, Fig. 4 shows that, in

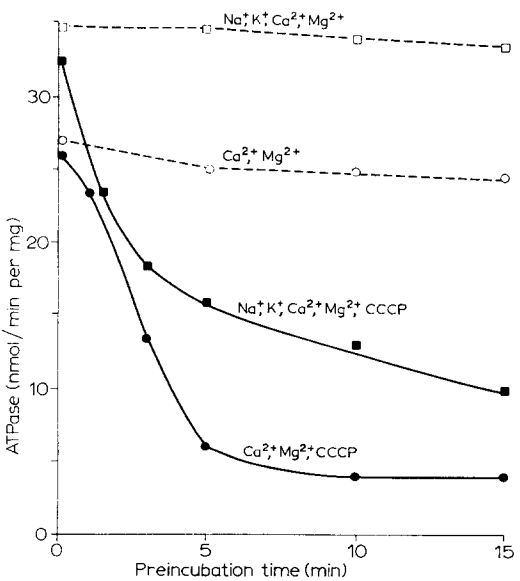


Fig. 4. Effect of preincubation time on the Ca^{2+}-ATPases. The same sample of ghosts and the same conditions were used as in Fig. 3. Comparison with Fig. 3 will show that the residual activity after preincubation corresponded to the activity of the non-Ca^{2+}-ATPase obtained under the same conditions. Symbols have the same meaning as in Fig. 3, except that 0.5 mM Ca^{2+} was present throughout each experiment.

the presence of Ca^{2+}, the inhibition of the Ca^{2+}-stimulated component of the ATPase developed during the first 5 min of preincubation at 37 °C. The Ca^{2+}-stimulated component of the $(Na^+ + K^+ + Mg^{2+})$-ATPase was inhibited more effectively when a longer preincubation was used (Fig. 4). Comparison of Figs 3 and 4 shows that this stimulation was completely inhibited after a 15 min preincubation. This was consistent with the lesser sensitivity of this activity already discussed with regard to Fig. 2. In the absence of CCCP the activity of the ATPase in the presence of Ca^{2+} was not affected significantly by the length of time of preincubation. The inhibition effect of CCCP was very dependent upon the temperature of preincubation. At temperatures of 0–25 °C no inhibitory effect of CCCP was observed after a 5-min preincubation. The inhibitory effect of CCCP was not reversed by higher concentrations of Ca^{2+} in the assay medium, showing that the effect of CCCP was not due to chelation of Ca^{2+}.

Although both CCCP and Ca^{2+} were required for the inhibition of the Ca^{2+}-stimulated component of the ATPase, it was not possible to detect any specific binding of CCCP in the presence of Ca^{2+}. Table II shows a comparison of the binding of CCCP to porcine red cell ghosts in the presence and absence of Ca^{2+}. A complete balance sheet is presented and it is clear that no detectable differences occurred, whether Ca^{2+} was present or not during the preliminary incubation. After several washes, only a very small amount of CCCP remained bound to the ghosts, but Table I shows that even this small amount of remaining CCCP was sufficient completely to inactivate the Ca^{2+}-stimulated component of the ATPase. Even five washes led to no reactivation of the Ca^{2+}-stimulated ATPase. However, if EGTA was included in the wash medium, the ATPase was substantially reactivated by washing.

TABLE II

LACK OF EFFECT OF Ca^{2+} ON DETECTABLE CCCP BINDING

Samples were preincubated and washed as described in Table I. Consecutive washes were designated I, II, etc. and the amount of CCCP determined in each and also in the ghosts remaining. Since the ghost pellet occupied only 10 % of the total volume of each wash, the possible trapping of CCCP would be insignificant compared with the observed amount bound. At an initial CCCP concentration of 1000 nmol/ml, 0.1 nmol/mg would be trapped after the fourth wash. The total recovery represents the sum of that bound and that washed out: the deviations from 100 % recovery are regarded as within the limits of error of the method. The Ca^{2+} concentration was 0.5 mM.

	Balance sheet of CCCP removal by washing						
	nmol/ml CCCP originally added	nmol/ml CCCP in supernatant				nmol/mg CCCP bound to ghosts	Total nmol/ml CCCP recovered
		I	II	III	IV		
No Ca^{2+}	100	70	22	10	4	6.8	116
	250	168	46	18	8	10.6	249
	500	392	70	24	10	12.4	508
	1000	814	96	36	14	18.6	979
Plus Ca^{2+}	100	64	22	10	4	7.8	108
	250	178	46	16	8	10.6	259
	500	384	74	24	10	14.0	506
	1000	804	96	40	14	18.8	973

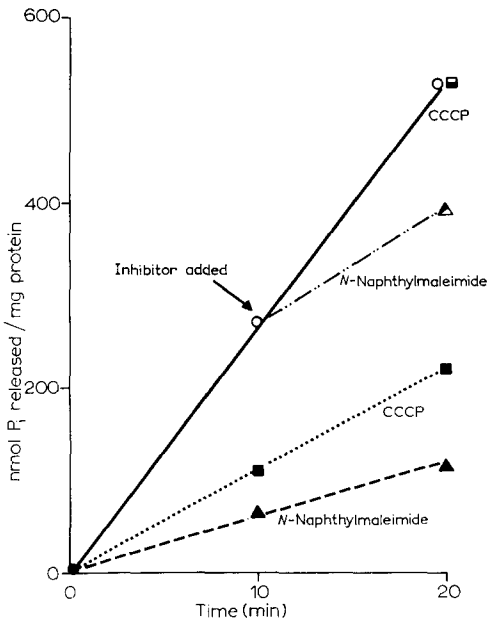


Fig. 5. When added after ATP, CCCP inhibits the $(Mg^{2+} + Ca^{2+})$-ATPase less than *N*-(1-naphthyl)-maleimide does. The preincubation was carried out for 5 min at 37 °C. The closed symbols indicate experiments in which inhibitor was present during the incubation, the half-closed symbols indicate experiments in which inhibitor was added 10 min after ATP, and the open circles indicate an experiment in which no inhibitor was present. Triangles indicate *N*-(1-naphthyl)-maleimide (0.02 mM) and squares indicate CCCP (0.5 mM). Ca^{2+} was present at 0.5 mM.

It is interesting to compare the effects of CCCP with those of another effective inhibitor of red cell membrane ATPase, *N*-(1-naphthyl)maleimide. *N*-(1-Naphthyl)-maleimide has been shown to be about 100 times more effective than *N*-ethylmaleimide as an inhibitor of red cell membrane ATPase [8]. Fig. 5 shows the effect of adding these two inhibitors before and after ATP was added. In the case of the *N*-(1-naphthyl) maleimide, the prior presence of ATP did not prevent a partial inhibition of the enzyme.

TABLE III

COMPARISON OF CCCP AND *N*-NAPHTHYLMALEIMIDE

Preincubation was for 20 min at 37 °C and pH 7.4, in the appropriate ATPase assay medium.

Additive	ATPase activity (nmol/mg per min)			
	Mg^{2+}	$(Na^+ + K^+ + Mg^{2+})$	$(Ca^{2+} + Mg^{2+})$	$(Na^+ + K^+ + Ca^{2+} + Mg^{2+})$
None	5.6	16.3	21.4	37.6
CCCP, 0.5 mM	7.5	15.3	5.1	12.2
N-(1-Naphthyl)-maleimide, 0.05 mM	3.8	6.0	3.8	3.8

TABLE IV

SPECIFICITY OF Ca^{2+} AS AN INACTIVATION COFACTOR

In these experiments, ghosts, at a protein concentration of 4 mg/ml, were preincubated in 0.5 mM CCCP, 50 mM TES · triethanolamine (pH 7.4), for 5 min at 37 °C, with additives also present at the following concentrations: $MgCl_2$, 3 mM; ATP, 3 mM; Ca^{2+}, 0.5 mM; NaCl, 100 mM; EDTA, 1 mM; KCl, 20 mM. When ATP was present during the preincubation, the inorganic phosphate present after the preincubation was measured and subtracted from the final phosphate value.

Additive	ATPase (nmol/mg per min)			
	Mg^{2+}	$(Na^+ + K^+ + Mg^{2+})$	$(Ca^{2+} + Mg^{2+})$	$(Na^+ + K^+ + Ca^{2+} + Mg^{2+})$
Less CCCP	5.1	15.1	20.6	38.4
None	7.7	18.1	22.4	35.1
EDTA	7.3	18.5	22.1	37.2
Mg^{2+}	7.1	18.3	21.5	36.0
Mg^{2+} and ATP	5.1	15.4	30.6	40.0
Mg^{2+}, Na^+ and K^+	7.3	16.3	24.5	33.9
Mg^{2+} and Ca^{2+}	7.9	20.0	10.6	25.0
Ca^{2+}	7.0	16.9	14.3	24.7

N-(1-naphthyl)maleimide is apparently less specific as an inhibitor of ATPase: this is evident from Table III, which shows that *N*-(1-naphthyl)maleimide inhibited not only the Ca^{2+}-stimulated ATPases, but also the $(Na^+ + K^+ + Mg^{2+})$-ATPase and Mg^{2+}-ATPase. The inhibitory effect of *N*-(1-naphthyl)maleimide was prevented by prior addition of β-mercaptoethanol. On the other hand, the effect of CCCP was not altered by the addition of sulfhydryl protecting reagents.

The uniqueness of Ca^{2+} in potentiating the inhibition of ATPase by CCCP is shown in Table IV. The presence of Mg^{2+}, Na^+ and K^+, or ATP did not lead to any inhibition of the ATPase in the presence of CCCP. Incubation of the ghosts with Mg^{2+} · ATP plus CCCP seemed to enhance the $(Ma^{2+} + Ca^{2+})$-ATPase activity, for reasons that are not clear.

DISCUSSION

In view of the relative unspecificity of most inhibitors of erythrocyte membrane ATPases, the specific effect of CCCP is particularly interesting. Previous studies [14, 15] have focused primarily on the effects of *N*-ethylmaleimide and proteolytic enzymes. It is particularly instructive to compare the results reported here with those reported by Bond [15], who reported that incubation at 47 °C, exposure to trypsin and reaction with *N*-ethylmaleimide inhibited the Mg^{2+}-ATPase, the $(Mg^{2+} + Na^+ + K^+)$-ATPase, and the $(Mg^{2+} + Ca^{2+})$-ATPase. He further observed that the presence of Ca^{2+} during any of these three inactivating treatments greatly increased the inactivation of the $(Mg^{2+} + Ca^{2+})$-ATPase by any of these treatments. In his study, the effect of Ca^{2+} was strong but not unique, since Mg^{2+} or Mg^{2+} · ATP also potentiated the inactivation of the $(Mg^{2+} + Ca^{2+})$-ATPase by trypsin or by *N*-ethylmaleimide. He interpreted these results as evidence for a conformational change in the $(Mg^{2+} + Ca^{2+})$-ATPase brought about by the presence of Ca^{2+}. The effect of CCCP on the

erythrocyte membrane ATPases is much more specific than the effects reported by Bond. CCCP inactivates completely the Ca^{2+}-stimulated portion of ATPase while slightly stimulating the Mg^{2+}-ATPase. The stimulation of the $(Na^+ + K^+ + Mg^{2+})$-ATPase was probably due to stimulation of its Mg^{2+}-component. The requirement for Ca^{2+} as a cofactor in the inactivation of the enzyme by CCCP is also more specific. As shown in Table IV, Mg^{2+}, $Mg^{2+} \cdot$ ATP or EDTA in combination with CCCP did not lead to inactivation of any of the ATPases.

Another pertinent feature of the effect of CCCP is the low amount of bound CCCP which was necessary to cause inactivation of the Ca^{2+}-stimulated ATPase. Table I shows that less than 5 nmol of CCCP per mg of membrane protein was required to inactivate the Ca^{2+}-stimulated ATPase. Although this is a very low level of CCCP, the CCCP is still in considerable excess compared with the probable number of molecules of $(Ca^{2+} + Mg^{2+})$-ATPase present in the erythrocyte membrane. A recent study [16] has directly demonstrated the existence of a protein subunit which is phosphorylated only in the presence of Ca^{2+}. The maximum amount of phosphate incorporated is about 1.6 pmol of phosphate per mg of erythrocyte membrane protein, and this is a reasonable estimate for the amount of $(Ca^{2+} + Mg^{2+})$-ATPase present in the erythrocyte membrane. Thus, even 5 nmol of CCCP bound to the erythrocyte membrane is a substantial molar excess over the approx. 2 pmol of $(Ca^{2+} + Mg^{2+})$-ATPase responsible for the observed activity. It was not possible to detect any difference in the amount of CCCP bound upon addition of Ca^{2+} in the preincubation mixture, but this is not surprising in the light of the above calculations. The number of sites on the $(Ca^{2+} + Mg^{2+})$-ATPase (assuming one CCCP bound per $(Ca^{2+} + Mg^{2+})$-ATPase molecule) would correspond to only about 0.2 % change in the amount of CCCP bound after 5 washings. Such a change would be undetectable with the experimental accuracy available.

The combination of uncoupler and Ca^{2+} appeared to be quite firmly bound to the active site of the enzyme. However, when Ca^{2+} was removed from the washing buffer and was chelated by addition of EGTA, then the CCCP which specifically inhibited the Ca^{2+}-stimulated ATPase was easily removed and full activity was restored, suggesting that both Ca^{2+} and CCCP were necessary for the type of binding observed.

The lack of inhibition when Ca^{2+} was added after or with ATP was somewhat ambiguous, since the membrane was exposed to a lower free Ca^{2+} concentration when ATP was present. The effort to further test this, by raising the Ca^{2+} concentration added with ATP, showed that a partial inhibition occurred when the free Ca^{2+} concentration was 0.5 mM in the presence of ATP. Thus, the protective effect of ATP was partially due to chelation of Ca^{2+}.

It is interesting to note that, of the various forms of the enzyme studied, only one is maximally susceptible to CCCP inhibition. Of the four forms it is necessary to consider in analysis of the enzyme's function (free enzyme, enzyme $\cdot$ Ca^{2+} complex, enzyme $\cdot$ ATP complex, and enzyme $\cdot$ Ca^{2+} $\cdot$ ATP complex), only the enzyme $\cdot$ Ca^{2+} complex, and to a lesser extent, the enzyme $\cdot$ Ca^{2+} $\cdot$ ATP complex, appear to be affected by the presence of CCCP. This suggests that a unique combination of factors is necessary for the inhibition of the enzyme by this uncoupler. Since Ca^{2+} and ATP specifically affect the inactivation of the enzyme by CCCP, the uncoupler is probably acting near the active site of the enzyme. In order to explain the observed results it is necessary to postulate the following.

(1) That the binding of Ca^{2+} to the enzyme creates the conditions which make it possible for CCCP to bind at a site which inactivates the Ca^{2+}-stimulated ATPase.

(2) That the binding of ATP (or $Mg^{2+} \cdot$ ATP) further changes the conditions so that CCCP is less effective in inactivating the enzyme.

If we accept the contention of Wolf [17] that $Mg^{2+} \cdot$ ATP is the substrate for the enzyme and that Ca^{2+} is an autosteric effector, then the following explanation seems likely. The binding of Ca^{2+} alters the active site of the enzyme, making it capable of binding CCCP or splitting $Mg^{2+} \cdot$ ATP. The binding of CCCP could prevent the binding of $Mg^{2+} \cdot$ ATP to the catalytic site in the correct way and thus prevent its hydrolysis. If the active site were saturated with $Mg^{2+} \cdot$ ATP, the binding of CCCP would be more difficult and inhibition less complete.

The electron-rich nitrogens of the cyano groups in the CCCP might be expected to have some tendency to chelate Ca^{2+}. It might be suggested that the effect of CCCP on the Ca^{2+}-ATPase was due to the chelation of the Ca^{2+} by CCCP. However, this is clearly not the case, since in the washed samples of ghosts Ca^{2+} is greatly in excess both of the amount needed to stimulate the Ca^{2+}-ATPase and of the amount of CCCP which remains bound to the ghosts. Thus, if any chelation occurs it must occur only at the site of the action of CCCP.

ACKNOWLEDGEMENTS

We wish to acknowledge the excellent technical assistance of Mr Michael McKinney. This work was supported by grants from the National Science Foundation (GB-33807) and the National Institutes of Health (DE-02668, RR-05333 and AM-17588).

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