Biochimica et Biophysica Acta 904 (1987) 251–258
Elsevier

BBA 73746

Rat liver plasma membrane Ca^{2+}- or Mg^{2+}-activated ATPase. Evidence for proton movement in reconstituted vesicles

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(Received 26 March 1987)
(Revised manuscript received 6 August 1987)

Key words ATPase, Ca^{2+}-, ATPase, Mg^{2+}-, Proton movement, Fluorescence quenching, Proton pump

The Ca^{2+}- or Mg^{2+}-activated ATPase from rat liver plasma membrane was partly purified by treatments with sodium cholate and lysophosphatidylcholine, and by isopycnic centrifugation on sucrose gradients. The ATPase activity had high sensitivity to detergents, poor nucleotide specificity and broad tolerance for divalent cations. It was insensitive to mitochondrial ATPase inhibitors such as oligomycin and to transport ATPase inhibitors such as vanadate and ouabain. Using the cholate dialysis procedure, the partly purified enzyme was incorporated into asolectin vesicles. Upon addition of Mg^{2+}-ATP, fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) was observed. The quenching was abolished by a protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Asolectin vesicles or purified ATPase alone failed to promote quenching. These data suggest that the Ca^{2+}- or Mg^{2+}-activated ATPase from rat liver plasma membrane is able of H^+-translocation coupled to ATP hydrolysis.

Introduction

Emmelot and Bos [1] were the first to show the enrichment in various phosphohydrolase activities from purified rat liver plasma membrane preparations. Besides the $(Na^+ + K^+)$-ATPase, they described a Mg^{2+}-ATPase insensitive to ouabain, oligomycin, Ca^{2+} and -SH reagents. This ATPase exhibited an optimal activity at pH 8.5, required Ca^{2+} or Mg^{2+} and was active on GTP, UTP and CTP as well as on ATP. The function of this quantitatively important Ca^{2+}- or Mg^{2+}-activated ATPase activity (12–15 μmol ATP hydrolysed/min per g liver) remains unknown today.

A similar ATPase activity was identified in the plasma membranes of rat pancreatic [2] and mammary-gland cells [3], adipocytes [4], skeletal muscle cells [5], Ehrlich ascites carcinoma cells [6,7], human granulocytes [8] and platelets [9].

Recent studies have shown that next to the $(Na^+ + K^+)$-ATPase, rat liver plasma membranes contain an ATPase activity stimulated in the presence of Mg^{2+} by submicromolar concentrations of Ca^{2+} and by an endogenous activator distinct from calmodulin [10]. Lin and Fain [11] have purified this high-affinity Ca^{2+}-ATPase from rat liver which had been assumed by Chan and Junger [12] to be involved in Ca^{2+} transport. However, Lin has shown that this Ca^{2+}-ATPase differs from the liver plasma membrane Ca^{2+} pump [13,14]. Its function remains thus unknown today as well as

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine, FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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its relation with the Ca^{2+}- or Mg^{2+}-activated ATPase activity originally described.

On the other hand, H^+-ATPase activities have also been identified in plasma membranes of various cell types: yeast [15], plant cells [16,17], gastric mucosa [18], turtle bladder epithelial cells [19], the slime mould *Dictyostelium* [20], in calf brain coated vesicles [21], chromaffin granules [22], endosomes from cultured macrophages and babyhamster kidney fibroblasts [23] and in rat liver coated vesicles [24], multivesicular bodies [25], lysosomes [26–28], isolated Golgi [29] and even rough and smooth microsomes [30].

Since the plasma membrane belongs at least partly, to the vacuolar apparatus as well as the coated vesicles, the multivesicular bodies, the lysosomes and the Golgi apparatus, we decided to investigate the existence of a H^+ pump in rat liver plasma membranes and its relation with the Ca^{2+}- or Mg^{2+}-activated ATPase activity described by Emmelot and Bos [1].

Materials and Methods

Chemicals

ADP (disodium salt), ATP (disodium salt and magnesium salt), oligomycin, ouabain, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), *N*-ethylmaleimide, tributyltin, nigericin, L-α-phosphatidylcholine from soybean (asolectin) were obtained from Boehringer Mannheim, FCCP was from Janssen Pharmaceutica. ACMA was a generous gift from Dr. R. Kraayenhof (Amsterdam). All other reagents were purchased from Merck Darmstadt.

Isolation of rat liver plasma membranes

Liver plasma membranes were prepared from 250–300 g Wistar male rats according to Song et al. [31] as modified by Wibo et al. [32], except that 25 mM Tris-HCl (pH 7.4) was used instead of 1 mM $NaHCO_3$. The purified plasma membranes were suspended in 100 mM Tris-HCl buffer pH 7.4 (buffer A), frozen in liquid N_2 and stored at $-80\,^\circ C$ until used

Purification of Ca^{2+}- or Mg^{2+}-activated ATPase

The whole procedure was performed at 0–4 °C. Plasma membranes (1 mg/ml) were suspended in buffer A containing 1% sodium cholate, stirred for 10 min and centrifuged at 24000 rpm (80000 × *g*) in a SW-27 rotor for 30 min to remove the components solubilized by cholate. The pellet was suspended in buffer A at a concentration of 10 mg protein/ml. Lysophosphatidylcholine was added at the ratio of 0.15 mg/mg protein. The suspension was homogenized with 20 strokes of a Dounce homogenizer, then layered on a 1.3 ml discontinuous sucrose gradient made of 5, 15, 25% (w/w) solutions in buffer A, prepared in an eppendorf tube and centrifuged for 90 min at 24000 rpm in a SW-27 rotor; eppendorf tubes were floating in the 35 ml tubes filled with water.

The opalescent fractions located at the interface 5–15% sucrose were collected, diluted 5-fold with buffer A, layered over a continuous 5–25% sucrose gradient in a 15 ml tube and centrifuged at 24000 rpm for 18 h in a SW-27 rotor. Fractions of 0.5 ml were collected. Those enriched in Ca^{2+}- or Mg^{2+}-activated ATPase activities were frozen in liquid N_2 and stored at $-80\,^\circ C$ until used.

Incorporation of purified enzyme into phospholipid vesicles

Reconstituted asolectin vesicles were prepared according to the cholate dialysis method described by Kagawa and Racker [33] as modified by Villalobo et al. [34].

0 5 mg of ATPase was added to 1 ml homogeneous suspension of 20 mg asolectin in a 1% sodium cholate solution prepared in 3 mM Hepes, 1.5 mM Tris-HCl, 50 mM KCl, 50 mM NaCl and 10 mM $MgCl_2$ at pH 7.0. The suspension was sonicated three times for 10 s in a bath sonicator (Bransonic 150 W) at 4 °C. The resulting sonicated mixture was dialysed against 250 ml of the same buffer for 18 h at 4 °C with three buffer changes.

Measurement of ATP-dependent fluorescence quenching of ACMA in reconstituted vesicles

The procedure described by Dufour et al. [35] was used with some modifications. 100 μl of dialysed suspension were added to 1 3 ml of the above described dialysis buffer at 30 °C; 5 μl of ACMA (0,5 mM in ethanol) were added. The reaction was started by addition of 50 μl of 150

mM Na^+-ATP and 150 mM $MgSO_4$ previously adjusted to pH 7.0.

ACMA fluorescence was measured with a thermostated Beckman ACTA V spectrophotometer. Excitation wavelength was 400 nm and the emission light was screened by a PIL Lienenfilter (460 nm).

The fluorescence intensity was calibrated as 50% for full scale reading.

ATPase assay

ATPase activity was determined by incubating the preparations for 20 min at 37 °C in 200 µl of buffer A containing 5 mM $MgSO_4$ and 5 mM ATP (disodium salt) previously adjusted to pH 7.4. The reaction was started by the addition of ATP and stopped by addition of 600 µl of 1% (w/v) sodium dodecylsulfate as suggested by Dulley et al. [36]. Inorganic phosphate was determined as described by Fiske and SubbaRow [37].

Protein determination

Protein was determined as described by Lowry et al. [38] and modified by Schacterle et al. [39], with bovine serum albumin as reference.

Results

Purification of rat liver plasma membrane Ca^{2+}- or Mg^{2+}-activated ATPase

The purification of membrane-bound enzymes requires the use of an appropriate detergent in the right conditions In the case of rat liver plasma membrane ATPase, currently used detergents, such as Emulgen 913, Triton X-100 or Zwittergent 3-08 had very little influence on the activity but failed to solubilize the enzyme even at high detergent-to-protein ratio. Other ones, like Brij 35, Zwittergent 3-12, Zwittergent 3-16, sodium cholate, polidocanol and lysophosphatidylcholine had good solubilization properties but were inhibitory at high concentrations. We observed, however, with lysophosphatidylcholine that decreasing the detergent-to-protein ratio reduced concommitantly the inhibition of the activity (Fig. 1) and that it was possible to obtain conditions where satisfactory solubilization was achieved without excessive inhibition of activity.

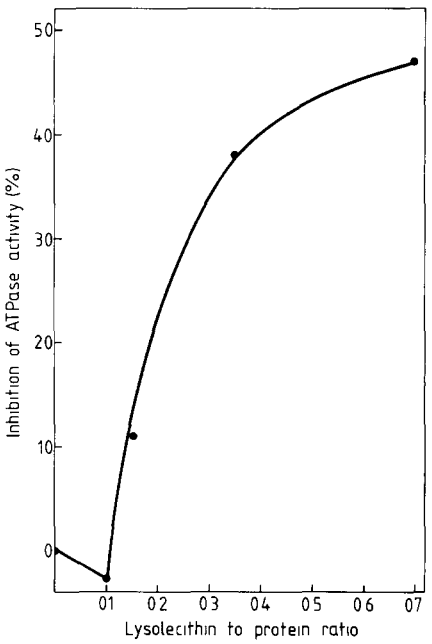

Fig 1 Influence of lysophosphatidylcholine to protein ratio on Ca^{2+}- or Mg^{2+}-activated ATPase activity Cholate washed membranes were suspended in buffer A at a concentration of 10 mg protein per ml. Lysolecithin dissolved in water was added at indicated ratios After incubation for 15 min at 0 °C, ATPase activity was assayed as described in Materials and Methods

These observations led us to develop a procedure for the obtention of an ATPase-enriched membranous fraction by two successive detergent treatments, which each stripped the membrane from non-ATPase proteins (Table I). Cholate washed membranes were treated by a relatively low concentration of lysophosphatidylcholine at a high concentration of protein. The stripped membranes were separated by centrifugation through a discontinuous sucrose gradient giving a 3.8-fold purification of the enzyme activity with 29.3% yield.

A subsequent isopycnic centrifugation of this fraction on linear sucrose gradient (5–25%) allowed to eliminate minor contaminants and the bulk of another plasma membrane marker, alkaline phosphatase. The final membranous preparation was enriched 12.5-times in ATPase activity (yield 16%).

Effect of bivalent cations on ATPase activity

As shown in Table II, ATPase from the enriched

TABLE I

PURIFICATION OF Ca^{2+}- or Mg^{2+}-ACTIVATED ATPase FROM RAT LIVER PLASMA MEMBRANES

The procedure was described in Materials and Methods

Purification step	Total protein (mg)	Total activity (μmol P_i/min)	Specific activity (μmol P_i/min per mg protein)	Purification factor	Yield (%)
1 Plasma membranes	35 3	30 0	0 85 [a]	1	100
2 Cholate washing	19 7	21 8	1 11	1 3	72 7
3 Lysolecithin stripping and discontinuous gradient	2 7	8 8	3 26	3 8	29 3
4 Isopycnic centrifugation	0 45	4 8	10 67	12 5	16 0

[a] ATPase activity was enriched 11 7 times in plasma membrane preparations when compared to total liver homogenates

fractions had broad divalent cations tolerance in the following order of efficiency: Ca > Mg > Zn > Fe > Mn > Cu > Cd > Co > Ni. As observed on the plasma membrane preparations, ATPase activity was higher in the presence of Ca^{2+}-ATP than with Mg^{2+}-ATP. It was not stimulated by Ca^{2+} when assayed with Mg^{2+}-ATP.

ATP-dependent ACMA fluorescence quenching in reconstituted vesicles of purified Ca^{2+}- or Mg^{2+}-activated ATPase

The membrane fraction enriched in Ca^{2+}- or Mg^{2+}-activated ATPase activity was vesiculated in the presence of asolectin using the cholate-dialysis method. Proton movements across the vesiculated membranes were measured using the ΔpH probe ACMA [40]. As shown in Fig. 2, addition of 5 mM Mg^{2+}-ATP to the reconstituted ATPase asolectin vesicles induced ACMA fluorescence quenching (trace A) Similar quenching could not be promoted neither with pure asolectin vesicles nor with

TABLE II

EFFECT OF DIVALENT CATIONS ON ATPase ACTIVITY

ATPase activity was determined on purified fractions as described in Materials and Methods in the presence of ATP alone or added with EDTA or Mg^{2+} or another divalent cation EDTA or the cations were 5 mM The salts were provided in the form of Cl^- The activity in the presence of Mg^{2+} is taken as 100%, it was 11 3 μmol P_i/min per mg protein

Cations	Relative ATPase activity
-	22
EDTA	2
Mg^{2+}	100
Ca^{2+}	125
Zn^{2+}	71
Fe^{2+}	65
Mn^{2+}	60
Cu^{2+}	52
Cd^{2+}	43
Co^{2+}	34
Ni^{2+}	28

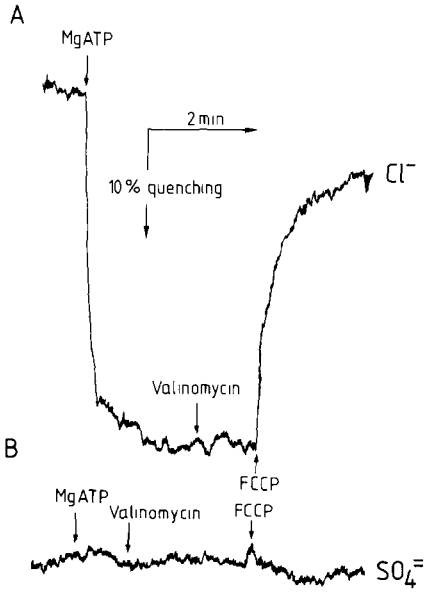

Fig 2 ATP-dependent fluorescence quenching of ACMA in reconstituted vesicles Fluorescence quenching measurements were carried out as described in Materials and Methods In A the salts were in form of chloride, in B in form of sulfate In A and B, 0 5 μg of valinomycin and 4 4 μM FCCP were successively added to the energized reconstituted vesicles

TABLE III

EFFECT OF DIFFERENT CHEMICALS ON THE ATPase ACTIVITY AND ACMA-FLUORESCENCE QUENCHING

ATPase activity and ACMA fluorescence quenching were determined as described in Materials and Methods in the presence of $MgCl_2$ NEM, *N*-ethylmaleimide.

Chemicals [a]	% of control	
	ATPase [b] activity	ACMA fluorescence quenching
Control	100	100
Oligomycin (5 μg/ml)	93	93
NEM 500 μM	54	81
DCCD 500 μM	34	7
Tributyltin 100 μM	34	21
NaN_3 10 mM	81	85
Ouabain 1 mM	103	97
Vanadate 50 μM	85	–
NBD-Cl 100 μM	90	103
FCCP [c]	121	<10

[a] When chemicals were dissolved in ethanol, concentration in the test did not exceed 1%

[b] Activity of control was 11.2 μmol P_i/min per mg protein.

[c] Activity of control and treated samples was measured on asolectin reconstituted vesicles

non-vesiculated membranes. Addition of the protonophore FCCP to the reconstituted vesicles reversed the fluorescence quenching induced by Mg^{2+}-ATP (A). These results suggested that the ATPase activity was involved in a H^+ translocation coupled to ATP hydrolysis.

In the presence of chloride ions, the rate of ACMA quenching was not increased by the addition of valinomycin and potassium which collapse the membrane potential. On the other hand when asolectin vesicles were reconstituted in buffer containing only impermeant anions such as sulfate (trace B), fluorescence quenching did not occur.

Effect of inhibitors on ATPase activity and fluorescence quenching

As shown in Table III, the plasma membrane Ca^{2+}- or Mg^{2+}-activated ATPase activity was insensitive to oligomycin, a mitochondrial ATPase inhibitor up to a concentration of 5 μg per ml. Neither ouabain (1 mM) nor vanadate (50 μM), both being inhibitors of the $(Na^+ + K^+)$-ATPase nor 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (100 μM), an inhibitor of the clathrin-coated vesicles, [42] had any significant effect.

Inhibitors of poor specificity such as the sulfhydryl reagent, *N*-ethylmaleimide, and *N,N'*-dicyclohexylcarbodiimide (DCCD) had little effect at 100 μM, but at 500 μM they inhibited 46% and 66%, respectively. Tributyltin inhibited 66% at 100 μM. Similarly to the ATPase activity in non-reconstituted purified plasma membrane fractions, the ACMA fluorescence quenching of recon-

TABLE IV

SUBSTRATE SPECIFICITY OF THE ATPase ACTIVITY AND ACMA FLUORESCENCE QUENCHING

Substrate concentration was 5 mM; all nucleotide triphosphates were in form of Na salts. Mg^{2+}-ATPase activities and ACMA fluorescence quenching were determined as described in Materials and Methods Where indicated, ATP was replaced by corresponding nucleotide triphosphate

Substrates	% of control			
	ATPase activity of the purified enzyme		Reconstituted asolectin vesicles	
	Mg^{2+}-stimulated	Ca^{2+}-stimulated	Mg^{2+}-ATPase activity	ACMA fluorescence quenching
ATP	100	100	100 [a]	100
ITP	92	104	119	4
CTP	122	110	116	14
GTP	81	81	108	68
UTP	99	112	105	33
ADP	51	51	n t [b]	n t [b]

[a] Activity of control was 1 6 μmol P_i/min per ml

[b] n.t., not tested

stituted vesicles was not sensitive to oligomycin, ouabain, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, *N*-ethylmaleimide, NaN_3. The sensitivity to high concentrations of *N,N'*-dicyclohexylcarbodiimide (93% inhibition at 500 μM), and to tributyltin (79% inhibition at 100 μM) is probably of little mechanistic significance due to the low specificity of these reagents at high concentrations.

Substrate specificity of Ca^{2+}- or Mg^{2+}-activated ATPase activity and fluorescence quenching

Ca^{2+}- or Mg^{2+}-activated ATPase activity and ACMA fluorescence quenching were tested with different nucleotides at a 5 mM concentration As shown in Table IV, both Ca^{2+}- and Mg^{2+}-dependent ATPase activities had poor substrate specificity. Indeed the enzyme showed no or little preference for ATP over ITP, CTP, GTP or UTP. The enzyme preparation also catalysed the hydrolysis of ADP (51%). These results are similar to those obtained by Lin et al. [14].

In contrast to the hydrolytic activities, ACMA fluorescence quenching showed some specificity for ATP Indeed GTP was able to support ACMA fluorescence quenching for 68% only and ITP could not

Conclusion

It is quite likely that the ATPase activity that we have greatly enriched from rat liver originates from the plasma membrane and not from contaminating components. Indeed, it cosedimented during isopycnic centrifugation with 5'-nucleotidase the usual plasma membrane enzyme marker [1] and it was resistant to specific inhibitors such as oligomycin, ouabain, vanadate, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole acting on mitochondrial-, $(Na^+ + K^+)$-, and clathrin-coated vesicles ATPases, respectively.

In addition to its high resistance to ATPase inhibitors, the purified ATPase activity exhibits the other properties of the Ca^{2+}- or Mg^{2+}-activated ATPase· poor substrate specificity and broad tolerance for divalent cations.

We report here that when incorporated into asolectin vesicles, the purified Ca^{2+}- or Mg^{2+}-activated ATPase is able to promote ATP-dependent ACMA fluorescence quenching which is reversed by addition of a protonophore, FCCP. Moreover FCCP increased by 20% the ATPase activity of reconstituted vesicles. These results indicate that the Ca^{2+}- or Mg^{2+}-activated ATPase might be involved in a H^+ translocation coupled to ATP hydrolysis. Indeed ACMA, a fluorescent weak base, has been used to assess proton transport in phospholipid vesicles. It has been demonstrated that the fluorescence of low concentrations of ACMA is quenched when a pH gradient acid-inside developes across vesicle membranes [35,40]. As expected, the ACMA quenching promoted by the reconstituted Ca^{2+}- or Mg^{2+}-activated ATPase is abolished by a detergent which destroys the osmotic barrier. The fact that valinomycin in the presence of K^+ does not stimulate the ACMA fluorescence quenching and that protonophores rapidly reverse the fluorescence changes induced by ATP in the absence of valinomycin indicates that another ion movement accompanies the H^+ influx observed in the ATPase reconstituted vesicles. Since quenching is not obtained when SO_4^{2-} replaces Cl^- even in the presence of valinomycin it can be concluded that either the sulfate ion inhibits directly ACMA quenching, or that a chloride influx might be coupled to H^+ influx in the reconstituted vesicles. This result might also reflect the presence of an anion-sensitive regulation site on the ATPase molecule as suggested by Van Dyke [42] for rat liver multivesicular bodies ATPase.

Although ACMA fluorescence quenching of reconstituted vesicles shows, like the non-vesiculated Ca^{2+}- or Mg^{2+}-activated ATPase activity, high resistance to ATPase inhibitors, it exhibited more specificity for ATP and GTP than the nucleotide hydrolytic activity and increased sensitivity to *N,N'*-dicyclohexylcarbodiimide and tributyltin. Similar observations have been obtained by Van Dyke et al. on rat liver coated vesicles [24] and multivesicular bodies [25]. In both cases, H^+-pumping was specific for ATP whereas hydrolytic activity was not The H^+-pumping was also slightly differently affected by *N,N'*-dicyclohexylcarbodiimide and *N*-ethylmaleimide than the enzyme activity.

Our results also resemble those obtained by Schneider [27] and by Moryama et al. [28] with rat liver lysosomes. This is not surprising in view of

the fact that their granules were prepared according to Trouet et al. [43] after injection of Triton WR-1339. Thinès-Sempoux [44] reported indeed that the membranes of these phagolysosomes possess some chemical and enzymatic similarities with the pericellular membrane.

Oertle et al. [45] characterized an oubain-insensitive ATPase activity of rat liver plasma membranes. Its sensitivity to *N,N'*-dicyclohexylcarbodiimide and its resistance to oligomycin led the authors to the conclusion of the possible existence in rat liver plasma membrane of an H^+-ATPase similar to that described in other plasma membranes. We confirmed their observations and supported their conclusion by showing the ACMA-fluorescence quenching linked to ATP hydrolysis and the stimulation of the ATPase activity by the protonophore FCCP in reconstituted vesicles.

Our observations introduce a new view to the study of the functions of the Ca^{2+}- or Mg^{2+}-activated ATPase activity from rat liver plasma membrane by showing its capability of H^+-pumping in reconstituted vesicles and its similarity to that found in organelles involved in receptor-mediated endocytosis and in secretory processes.

Acknowledgements

The work reported here was supported by grants from the Belgian Fonds National de la Recherche Scientifique (FNRS) and Ministère de la Politique et Programmation Scientifiques.

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