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CYTOPLASMIC AND PLASMA MEMBRANE ADENOSINE TRIPHOSPHATASE OF POLYMORPHONUCLEAR NEUTROPHILS

COMPARISON OF THEIR ENZYMATIC PROPERTIES AND ATTEMPT FOR A DIRECT DETERMINATION OF MYOSIN ATPase ACTIVITY USING POLYMORPHONUCLEAR NEUTROPHIL EXTRACT

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Summary

Enzymatic properties of the ATPase of the plasma membrane and cytoplasmic myosin B from guinea-pig polymorphonuclear neutrophils were compared. In the plasma membrane, Mg^{2+}- and Ca^{2+}-activated ATPases showed the same dependence pattern on KCl concentration and pH, i.e., both ATPases increased with decreasing KCl concentration and with rising pH until pH 9.0. The maximum activation of Mg^{2+}-ATPase was observed at $1 \cdot 10^{-3}$ M Mg^{2+}. On the other hand, EDTA-activated ATPase activity was so low that no clear dependence curve was obtained. In myosin B, Mg^{2+}-ATPase activity was below one-tenth that of the plasma membrane ATPase with the maximum activation at $1 \cdot 10^{-2}$ M Mg^{2+} and pH 9.0. EDTA- and Ca^{2+}-activated ATPase exhibited almost the same activity and the same KCl-dependence curve, i.e., both ATPases increased with increasing KCl concentration. With regard to pH-dependence, Ca^{2+}-ATPase showed a U-shaped curve with the minimum at pH 7.0, whereas EDTA-activated ATPase indicated a bell-shaped curve with the maximum at pH 9.0.

Based on the findings that the EDTA-activated ATPase activity was hardly detected in the plasma membrane but high in myosin B, the distribution of ATPase activity on subcellular fractions was studied and the results obtained that the myosin-ATPase activity could be directly measured using the polymorphonuclear neutrophil extract if the EDTA-activated ATPase activity was used as an enzymatic marker for myosin.

Abbreviation: KIE, kallidinogenase-inactivator-einheiten, the same as kallikrein inactivator unit (KIU).

Introduction

Studies on the effect of a chemical modification on chemotaxis of polymorphonuclear neutrophils [1,2] have necessitated our examination of whether or not the chemotactic inhibition arose from a modification of myosin-ATPase, a contractile protein, in the cytoplasm. It has been already reported that the ATPase activity was localized on the plasma membrane and in the cytoplasm of polymorphonuclear neutrophils and both enzymes have been prepared independently from a different point of view, their properties being studied [3—11]. So we have no data concerning the comparison of enzymatic properties of both ATPases.

In this communication, therefore, our studies were initiated with the aim of obtaining complete and accurate information on enzymatic properties of the plasma membrane and cytoplasmic myosin ATPases, prepared simultaneously from the same source, and to see if myosin-ATPase is directly measured without purification, using homogenates or extracts of polymorphonuclear neutrophils, based on the information obtained.

Materials and Methods

Leukocytes. Polymorphonuclear neutrophils were obtained from glycogen-induced peritoneal exudates of guinea-pig as described previously [12].

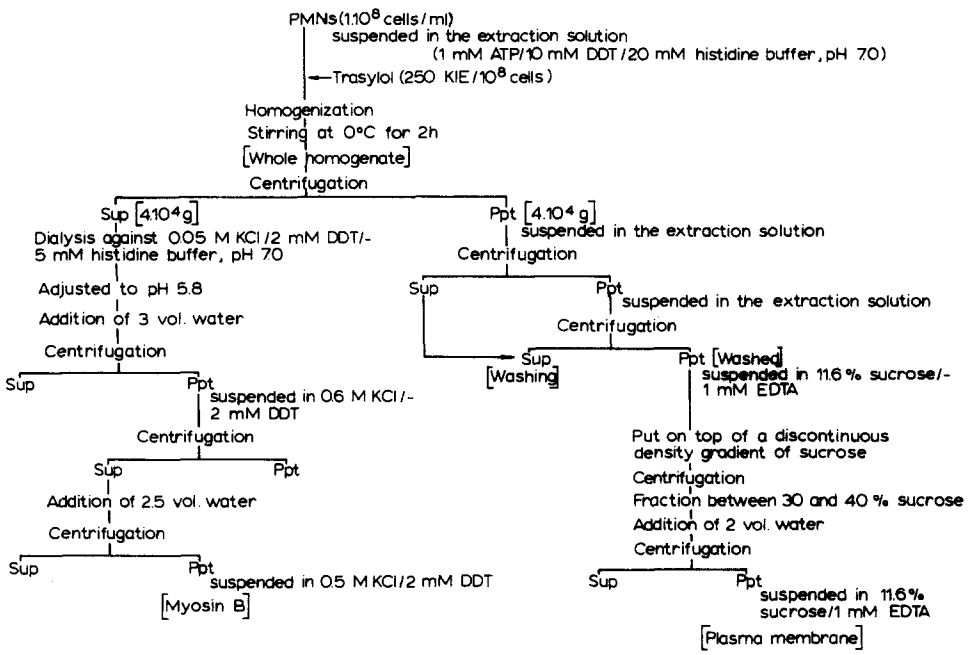
Preparation of myosin B and plasma membrane from polymorphonuclear neutrophils. Myosin B was extracted from polymorphonuclear neutrophils essentially according to the method of Shibata et al. [8] with modifications, and purified by our method used in the preparation of skeletal muscle myosin B [13]. Polymorphonuclear neutrophils were suspended in the extraction solution containing 1 mM ATP, 10 mM dithiothreitol and 20 mM histidine buffer (pH 7.0) to $1 \cdot 10^8$ cells per ml after washing once with the same solution. Trasylol (250 KIE/10^8 cells, 25 000 KIE/5-ml ampule) was added to the polymorphonuclear neutrophil suspension. Polymorphonuclear neutrophils were homogenized in a Polytron homogenizer at position 6 for 20 s where, at most, one-third of the cells were disrupted and further homogenized with 50 up-down strokes in a motor-driven Teflon-glass homogenizer to completely disrupt cells. The resulting homogenate was stirred for 2 h at 0° C and then centrifuged at 40 000 × *g* for 1 h. The supernatant was dialyzed overnight at 4° C against 0.05 M KCl/2 mM dithiothreitol/5 mM histidine buffer (pH 7.0), then adjusted to pH 5.8 with 0.1 N acetic acid and centrifuged at 10 000 × *g* for 15 min after the addition of 3 vols. of ice-cold, deionized distilled water. The precipitate was dissolved in 2 mM dithiothreitol/0.6 M KCl with gentle stirring and centrifuged at 1700 × *g* for 30 min. To the supernatant were added 2.5 vols. of ice-cold deionized distilled water and then spun down at 3000 × *g* for 30 min. The sediment, myosin B, was dissolved in a small volume of 2 mM dithiothreitol/0.5 M KCl (approx. 2 ml/10^9 cells). On the other hand, membrane fractions were obtained from pellets sedimented by centrifugation at 40 000 × *g* for 1 h essentially according to the method of Woodin and Wieneke [14]. Pellets were suspended in 2—3 ml of the extraction solution by homogenization with 25 up-down strokes in a Teflon-glass homogenizer and centrifuged at 40 000 × *g* for

1 h. After washing once more with the extraction solution, pellets were suspended in 5—6 ml of 11.6% sucrose/1 mM Na^+-EDTA solution (pH 7.2) and homogenized with 5 up-down strokes in a Teflon-glass homogenizer. The suspension was placed on top of a discontinuous density gradient, consisting of layers (8 ml each) of 30, 40 and 50% sucrose solution with 1 mM EDTA and centrifuged at 100 000 × *g* for 1 h. The fractions between 30 and 40% and between 40 and 50% sucrose were referred to as the plasma membrane fraction and cytoplasmic membrane fraction, respectively. The plasma membrane fraction was diluted with 2 vols. of ice-cold deionized distilled water and centrifuged at 200 000 × *g* for 30 min and resuspended in 11.6% sucrose/1 mM EDTA (approx. 1—2 ml). The procedure for preparation of myosin B and plasma membrane fractions is summarized in Scheme 1.

Determination of ATPase activity. ATPase activity was determined as described previously [13] with a slight modification. Ca^{2+}- or EDTA-activated ATPase activity was determined by measuring the amount of P_i released in 1.5 h in 0.5 M KCl, pH 7.6 (20 mM histidine buffer), in the presence of 5 mM $CaCl_2$ or 1 mM EDTA at 37°C unless otherwise noted. Determination of Mg^{2+}-ATPase was carried out in 0 or 0.05 M KCl/25 mM Tris-maleate buffer (pH 7.0) in the presence of 1 mM $MgCl_2$ at 37°C for 1.5 h. ATP concentration was 2 mM. 0.1 ml of sample at a concentration of 1 mg/ml was used with myosin B and 0.2 mg/ml with plasma membrane.

Protein concentration. Protein was determined by the Biuret method [15] or by the method of Lowry et al. [16], using bovine serum albumin as the standard.

Scheme 1. Outline of preparation procedure for myosin B and plasma membrane from polymorphonuclear neutrophils (PMNs). DDT, dithiothreitol; Sup, supernatant; Ppt, precipitate.



Results

Myosin B ATPase

Fig. 1a shows the dependence of myosin-ATPase on KCl concentration. The EDTA-activated ATPase activity increased with increasing KCl concentration. Ca^{2+}-ATPase also increased with increasing KCl concentration and reached a maximum at approx. 0.4 M. On the other hand, Mg^{2+}-ATPase was hardly affected by KCl concentration from 0.8 to 0.1 M but activated a little at 0.05 M KCl. As can be seen in Fig. 1b, Ca^{2+}-ATPase gave a U-shaped curve with the minimum at pH 7.0, but the activity was markedly depressed at pH values higher than 9.0. The pH-dependence curve of EDTA-activated ATPase indicated that the activity increased rapidly with rising pH until pH 7.5 and then gradually to the maximum at pH 9.0, followed by the marked inhibition. Mg^{2+}-ATPase hardly changed until pH 7.5, but above this pH increased with rising pH with the maximum at pH 9.0. The dependence of myosin B-ATPase on Mg^{2+} concentration is shown in Fig. 1c. Addition of Mg^{2+} to the myosin B system brought about the depression of ATPase activity between $1 \cdot 10^{-5}$ and $1 \cdot 10^{-4}$ M and then activation with the maximum at $1 \cdot 10^{-2}$ M. These catalytic properties resembled those of smooth muscle myosin B [17].

Plasma membrane ATPase

The EDTA-activated ATPase activity of the plasma membrane was so low, contrary to myosin B, that no appreciable KCl-dependence curve was obtained as shown in Fig. 2a. Ca^{2+}- and Mg^{2+}-ATPases showed the same KCl-dependence curve, although Ca^{2+}-ATPase had somewhat lower activity than Mg^{2+}-ATPase, the activity of which was markedly higher compared to that of myosin B: both ATPases increased with decreasing KCl concentration. As seen in Fig. 2b, Ca^{2+}-ATPase increased with rising pH with the maximum at pH 9.0, followed by

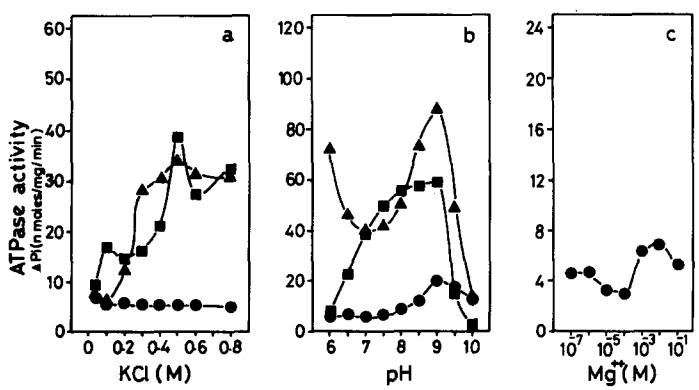


Fig. 1. Catalytic parameters of the ATPase activity of myosin B. (a) Dependence of ATPase activity on KCl concentration. The ATPase activity was measured at various KCl concentrations at pH 7.6 in the presence of 1 mM EDTA (■) or 5 mM $CaCl_2$ (▲) and at pH 7.0 in the presence of 1 mM $MgCl_2$ (●). (b) pH-dependence. The ATPase activity was measured in 20 mM Tris-maleate buffer at pH values 6.0—10.0 containing 0.5 M KCl in the presence of 1 mM EDTA (■) or 5 mM $CaCl_2$ (▲) or 0.05 M KCl in the presence of 1 mM $MgCl_2$ (●). (c) Effect of Mg^{2+} concentration. The ATPase activity was measured in 0.05 M KCl (pH 7.0) in the presence of $MgCl_2$ at the indicated concentration. Values are the average of three to six experiments.

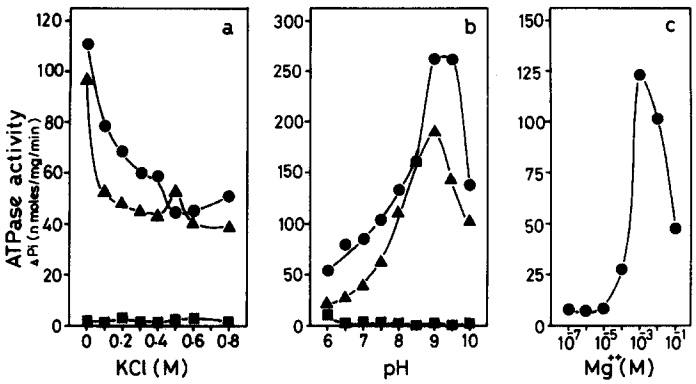


Fig. 2. Catalytic parameters of ATPase activity of the plasma membrane. (a) KCl-dependence. The ATPase activity was measured at various KCl concentrations at pH 7.6 in the presence of EDTA (■) or $CaCl_2$ (▲) and at pH 7.0 in the presence of $MgCl_2$ (●) at 37°C for 3 h. (b) pH-dependence. The ATPase activity was measured at various pH values (20 mM Tris-maleate buffer) in 0.5 M KCl in the presence of EDTA (■) or $CaCl_2$ (▲) and 0 M KCl in the presence of $MgCl_2$ (●) at 37°C for 3 h. (c) Effect of Mg^{2+} concentration. The ATPase activity was measured in 0 M KCl (pH 7.0) in the presence of $MgCl_2$ at various concentration at 37°C for 3 h. Values are the average of three to four experiments.

inhibition. Mg^{2+}-ATPase exhibited the pH-dependence curve similar to that of Ca^{2+}-ATPase with the maximum at pH 9.0—9.5. No clear dependence curve was obtained with EDTA-activated ATPase due to the very low activity. Plasma membrane ATPase was scarcely affected by the addition of Mg^{2+} to $1 \cdot 10^{-5}$ M, as indicated in Fig. 2c, and then increased markedly with increasing Mg^{2+} concentration with maximum activation at $1 \cdot 10^{-3}$ M, followed by a decrease in the activity.

Distribution of ATPase activity on subcellular fractions

A comparison of enzymatic properties of myosin B and plasma membrane ATPases suggested the possibility that myosin-ATPase could be directly determined using homogenate without purification of myosin B, if EDTA-activated ATPase were measured, especially at high KCl concentrations, as an enzymatic marker for myosin. Then we examined the distribution of ATPase activities on subcellular fractions fractionated according to the procedure as indicated in Scheme 1. As seen in Table I, the majority of Mg^{2+}-ATPase activity of whole homogenate was found in the $4 \cdot 10^4$ g precipitate fraction. The Ca^{2+}-ATPase activity also was mostly recovered in the $4 \cdot 10^4$ g precipitate fraction when measured at 0 M KCl. If the ATPase measurement was carried out at 0.5 M KCl, however, the Ca^{2+}-ATPase activity appeared not only in the $4 \cdot 10^4$ g precipitate fraction but also to a fair extent in the $4 \cdot 10^4$ g supernatant fraction, as deduced from KCl-dependence curves in Figs. 1a and 2a. As for EDTA-activated ATPase, a large percentage of the whole homogenate activity was observed in the $4 \cdot 10^4$ g supernatant fraction and approx. 60% of homogenate ATPase activity was found in the myosin B fraction with the high specific activity, suggesting the possibility that EDTA-activated ATPase in the whole homogenate or the $4 \cdot 10^4$ g supernatant represents the myosin-ATPase in polymorphonuclear neutrophils. To ascertain the viability of this idea, KCl- and pH-dependence patterns of EDTA- and Ca^{2+}-activated ATPases were studied with

TABLE I

DISTRIBUTION OF ATPase ACTIVITY IN FRACTIONS

ATPase activity was measured at the indicated KCl concentrations at 37°C under standard conditions. Values represent the mean ± S.D. of four to five separate experiments. Details of the procedure are found in the text and Scheme 1.

Fraction	KCl (M)	Total ATPase activity (nmol P_i/min per 10^9 cells)			Specific ATPase activity (nmol P_i/min per mg protein)		
		Mg^{2+}	Ca^{2+}	EDTA	Mg^{2+}	Ca^{2+}	EDTA
Whole homogenate	0	611 ± 181	499 ± 162	68 ± 21	9.8 ± 2.0	8.0 ± 1.8	1.0 ± 0.2
	0.5	427 ± 151	488 ± 142	280 ± 77	6.6 ± 1.5	7.8 ± 1.6	4.4 ± 0.7
$4 \cdot 10^4$ g supernatant	0	51 ± 17	41 ± 13	49 ± 13	2.0 ± 0.5	1.6 ± 0.4	1.4 ± 0.5
	0.5	59 ± 25	211 ± 73	243 ± 84	2.4 ± 0.4	7.8 ± 1.3	8.8 ± 3.4
$4 \cdot 10^4$ g precipitate	0	561 ± 154	461 ± 134	—	15.4 ± 3.3	12.4 ± 1.6	—
	0.5	383 ± 119	358 ± 102	83 ± 17	10.4 ± 1.9	9.8 ± 1.9	3.0 ± 0.8
Washed precipitate	0	383 ± 175	333 ± 172	—	14.2 ± 3.6	11.0 ± 2.5	—
	0.5	262 ± 134	254 ± 113	31 ± 19	9.0 ± 3.2	8.8 ± 2.3	1.4 ± 1.2
Washing	0	16 ± 6	16 ± 5	—	7.2 ± 1.0	7.6 ± 1.2	—
	0.5	13 ± 4	30 ± 12	34 ± 11	5.8 ± 0.8	13.6 ± 2.7	15.0 ± 2.1
Myosin B fraction	0.05	17 ± 2	19 ± 11	28 ± 3	6.0 ± 3.5	6.9 ± 6.3	16.8 ± 6.1
	0.5	14 ± 4	115 ± 30	171 ± 6	5.5 ± 3.8	51.3 ± 18.0	59.5 ± 11.5

whole homogenate, $4 \cdot 10^4$ g supernatant and $4 \cdot 10^4$ g precipitate fractions (Fig. 3). The whole homogenate fraction showed KCl- and pH-dependence curves similar to those of myosin B with EDTA-activated ATPase but seemed to indicate the combined pattern of myosin B and plasma membrane especially in the KCl-dependence curve with Ca^{2+}-ATPase (Fig. 3a). Ca^{2+}-ATPase of the $4 \cdot 10^4$ g precipitate fraction (Fig. 3c) was essentially similar to that of the

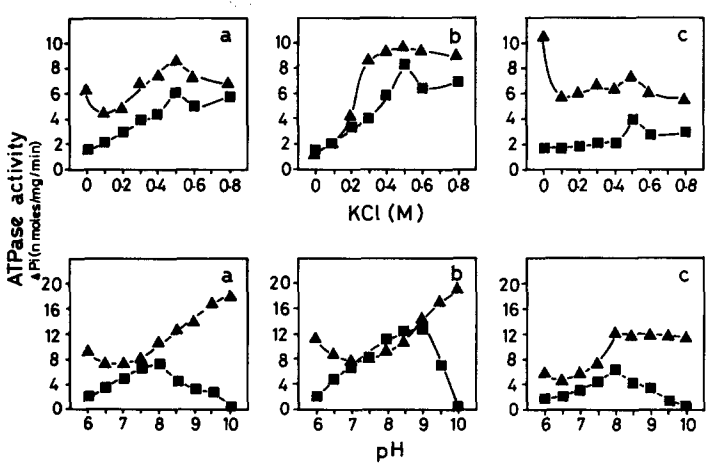


Fig. 3. KCl- and pH-dependence of the ATPase of whole homogenate, $4 \cdot 10^4$ g supernatant and $4 \cdot 10^4$ g precipitate fractions. The ATPase activity of each fraction was measured at various KCl concentrations at pH 7.6 and at various pH values in 0.5 M KCl in the presence of EDTA (■) or $CaCl_2$ (▲), using 0.1 ml of sample at concentrations of 3—4 mg protein/ml. (a) Whole homogenate, (b) $4 \cdot 10^4$ g supernatant, (c) $4 \cdot 10^4$ g precipitate.

plasma membrane in KCl- and pH-dependence, but the dependence of the EDTA-activated ATPase activity on pH and KCl concentration was observed to some extent unlike the plasma membrane, indicating that this fraction was contaminated somewhat with contractile proteins, which would be deduced from the ATPase activity on the washing fraction in Table I. The $4 \cdot 10^4$ g supernatant fraction showed almost the same KCl- and pH-dependence curves as those of myosin B with EDTA- and Ca^{2+}-activated ATPases (Fig. 3b), suggesting that this fraction was representative of myosin-ATPase and that the measurement of EDTA-activated ATPase of the $4 \cdot 10^4$ g supernatant fraction would be superior to that of the whole homogenate fraction for the direct determination of myosin-ATPase in polymorphonuclear neutrophils.

Discussion

The most distinct difference in enzyme activity between myosin B and plasma membrane was observed with EDTA- and Mg^{2+}-activated ATPases. The EDTA-activated ATPase activity was hardly observed in the plasma membrane but was considerably higher in myosin B especially at a high KCl concentration. Therefore, it seemed possible that myosin B-ATPase could be monitored directly in the whole homogenate fraction if the ATPase activity were measured at 0.5 M KCl in the presence of EDTA. In fact, the whole homogenate fraction already showed essentially the same KCl- and pH-dependence in the presence of EDTA as that of myosin B-ATPase, although the KCl- and pH-dependence patterns of the $4 \cdot 10^4$ g supernatant fraction were much more similar to those of myosin B. These results support the possibility that EDTA-activated ATPase activity of the whole homogenate fraction is representative of myosin-ATPase in polymorphonuclear neutrophils, so that we could see simply by checking the change in EDTA-activated ATPase of the whole homogenate fraction without further purification if the chemotactic inhibition of polymorphonuclear neutrophils by a chemical modification is based on the modification of myosin, which is in progress. On the other hand, Mg^{2+}-ATPase activity of the plasma membrane was very high especially at low KCl concentrations compared with that of myosin B as deduced from the KCl-dependence curves (Figs. 1a and 2a), so that it could be envisaged that Mg^{2+}-ATPase activity at low KCl concentrations of the $4 \cdot 10^4$ g precipitate fraction would be derived from the plasma membrane. However, the $4 \cdot 10^4$ g precipitate fraction contained not only the plasma membrane but also the cytoplasmic membrane, and information on the ratio of plasma to cytoplasmic membrane is not available. So, it is impossible at the present stage to determine directly the plasma membrane ATPase activity using the $4 \cdot 10^4$ g precipitate.

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