BBA 66106

ADENINE NUCLEOTIDE METABOLISM OF BLOOD PLATELETS

VII. ATPASES: SUBCELLULAR LOCALIZATION AND BEHAVIOUR DURING THE THROMBIN–PLATELET INTERACTION

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(Received December 10th, 1969)

SUMMARY

1. The subcellular localization of Ca^{2+}/Mg^{2+}-stimulated ATPase in human platelets was studied. The effect of thrombin on the enzyme was also investigated.

2. Characterization of the ATPase activity in platelet lysates revealed some similarities with actomyosin ATPase in its response to divalent cations.

3. Both Ca^{2+}- and Mg^{2+}-stimulated ATPases were found to be localized in a granular fraction containing the platelet-bound adenine nucleotides.

4. Thrombin did not cause the release of Ca^{2+}/Mg^{2+}-activated ATPase from washed human platelets.

5. A slight inhibition of Ca^{2+}-stimulated ATPase occurred 10 sec after exposure of washed human platelets to thrombin.

6. That Ca^{2+}/Mg^{2+}-stimulated ATPase occurs in granules containing adenine nucleotides seems established. However, although a slight drop in ATPase activity occurred after the addition of thrombin to washed platelets, no evidence of ATPase activation during the release phenomenon was obtained.

INTRODUCTION

Several substances located in the granules of human platelets are released by thrombin[1–5] and collagen[6,7], and the release of some of these constituents requires extracellular Ca^{2+} (refs. 1–5). During this process, intracellular metabolically active ATP is consumed[4,8]; and it has been suggested that thrombosthenin[9], the contractile platelet protein possessing Ca^{2+}/Mg^{2+}-stimulated ATPase activity[9] may be involved in this explosive platelet release reaction[1,10–12].

A possible presence of ATPase in the platelet granules and its activation during the release reaction might be pertinent to these findings. Recent evidence indicates that ATP and ATPase participate in the release of granule-located material from leucocytes[13], adrenal medulla[14] and posterior pituitary gland[15]. As thrombosthenin

with Mg^{2+}-stimulated ATPase activity has been extracted from platelet granules[16], the possibility exists that this ATPase also participates in the platelet release reaction.

The present paper reports the kinetics and subcellular localization of Ca^{2+}/Mg^{2+}-activated ATPase in human platelets as well as the behaviour of this enzymic activity during the interaction between washed platelets and thrombin.

MATERIALS

Chemicals

The following chemicals were obtained from Sigma Chemical Co. (St. Louis): ATP (disodium salt, crystalline), AMP (sodium salt, crystalline), IMP (sodium salt, crystalline), ouabain octahydrate 5-hydroxytryptamine (creatinine sulphate). ADP (sodium salt) was from the Nutritional Biochemical Corporation (Cleveland, Ohio). Cysteine · HCl was from British Drug Houses Ltd. (Poole, England). Bovine thrombin (EC 3.4.4.13) (Topostasine, "Roche") from Hoffman La Roche and Co. A.G. (Basle) was dissolved in Tris–NaCl buffer (pH 7.4) (50 units/ml), stored at −60°, and thawed immediately before use.

Radiochemicals

[$^{14}C_{10}$]ATP (ammonium salt) code CFB 91 from the Radiochemical Centre (Amersham) was stored at −60°, the specific activity used in the experiments being $1.2 \cdot 10^3$ disint./min per nmole. Carrier-free $^{32}P_i$ (Code FO) was supplied by the Institutt for Atomenergi Kjeller, Norway, and stored at 4°. [γ-^{32}P]ATP was prepared as the Tris salt (70 mM) by the method of GLYNN AND CHAPPELL[17] and stored at −60°. The specific activity of the preparations used in experiments varied between $1 \cdot 10^3$ and $7 \cdot 10^3$ disint./min per nmole ATP.

Solutions

Tris–NaCl buffer was 0.12 M NaCl in 0.03 M Tris–HCl (pH 7.4).

Washing Solution A consisted of 0.04 M NaH_2PO_4, 0.0047 M KH_2PO_4, 0.103 M NaCl, 0.005 M glucose and 0.005 M Na_2EDTA adjusted to pH 7.4 with NaOH (ref. 18).

Washing Solution B contained 0.15 M NaCl, 0.15 M Tris–HCl (pH 7.4) and 0.077 M EDTA (pH 6.8) (ref. 19) *plus* 0.3 M glucose in 45:4:1:20 proportions by volume.

Homogenization Solution was 0.44 M sucrose containing 0.01 M Tris–HCl and 0.001 M EDTA (pH 7.4).

Biological materials

Platelet-rich plasma was obtained from 500 ml of normal human blood collected either into the Fenwal Double Blood Pack Haemosystem containing 0.15 vol. of acid–citrate–dextrose solution followed by the addition of 0.05 vol. of 0.1 M Na_2EDTA (pH 7.4) (citrate–EDTA platelet-rich plasma), or into 0.075 vol. 0.077 M Na_2EDTA (pH 7.4) (EDTA platelet-rich plasma) followed by centrifugation at g_{max} 320 × g for 15 min at 15°.

Platelet lysates for ATPase characterization experiments were prepared from citrate–EDTA platelet-rich plasma. The platelets were harvested by centrifugation at $g_{max} = 2000 \times g$ for 30 min at 4°, and the platelets were washed twice with 40 ml

of Tris–NaCl buffer. The platelets were finally suspended in 0.01 M Tris–HCl (pH 7.4) and frozen–thawed twice to yield lysates containing 6 mg platelet protein per ml lysate. These were stored at −60° until required for assay.

Platelet suspensions for the subcellular fractionation were prepared from citrate–EDTA platelet-rich plasma, and washed with Washing Solution B as described previously[20]. The platelets were finally suspended in 1.5 ml of ice-cold Homogenization Solution.

Platelet suspensions for the release experiments were prepared from EDTA platelet-rich plasma and washed as described previously[20] except for the use of Washing Solution A (ref. 18). The platelets were finally suspended in various amounts of ice-cold Tris–NaCl buffer containing 0.003 M EDTA (pH 7.4) to give 3–4 mg of platelet protein per ml.

About 3 h elapsed between the collection of blood and the start of the subcellular fractionation or release experiments.

METHODS

Subcellular fractionation. The washed platelets suspended in Homogenization Solution were homogenized with a no-clearance Teflon pestle in a clear-walled glass tube and fractionated on three linear sucrose gradients as described elsewhere[21], except that the tube and pestle were washed with 1 ml of sucrose solution after homogenization, and the particles from the gradients were finally suspended in 0.7 ml of 0.05 M Tris–HCl (pH 7.4) with or without 0.003 M EDTA. The suspensions were stored at −60° until further analysis.

Release experiments. Basically, 2-ml aliquots of platelet suspensions were pre-incubated in plastic tubes at 37° for 5 min before various 0.4-ml additions. After incubation for various periods of time the tubes were immediately transferred to an ice-bath followed by centrifugation at 18 000 × *g* for 10 min at 4°. The supernatants (Extracellular Phase) were decanted, and the sediments resuspended in 2.5-ml of Tris–NaCl buffer containing 0.003 M EDTA (pH 7.4) (Intracellular Phase). All specimens were stored at −60° overnight prior to assay.

Experiments to ascertain the effect of thrombin on platelet Ca^{2+}-stimulated ATPase. Platelet suspensions of 1 ml containing 5.5 mM $CaCl_2$ were pre-incubated in plastic tubes for 5 min at 37° before the rapid addition with a syringe of 0.1 ml Tris–NaCl buffer or thrombin solution (50 units/ml). After various intervals (from 3 to 60 sec), the tube contents were rapidly transferred to glass tubes standing in acetone–dry-ice freezing mixtures where freezing was immediate. The mixtures were stored at −60° until assay.

Protein was determined by MILLER's[22] modification of the method of LOWRY *et al.*[23] with bovine serum albumin as standard. In the release experiments, the protein concentrations in the Extracellular Phases were corrected for added thrombin protein.

Serotonin was determined fluorometrically using a microadaptation of the method of CROSTI AND LUCCHIELLI[24]. To 0.15 ml of sample were added 0.15 ml of 0.62 M $ZnSO_4$ and 0.1 ml of 1 M NaOH. The precipitated protein was removed by centrifugation at 18 000 × *g*. To 0.2 ml of the supernatant was added 0.05 ml of 12 M HCl, and the fluorescence was then measured at emission 525 nm and excitation 295 nm in a Farrand spectrofluorometer specially adapted for square microcuvettes.

ADP + ATP were determined after 50 μl of gradient fraction had been mixed with 50 μl EDTA–ethanol[25]. 25-μl portions of the resulting mixtures (Ethanol Extracts) were added to 3 ml "active" phosphoenolpyruvate–pyruvate kinase mixtures followed by boiling and determination of firefly luminescence[26].

Lactate dehydrogenase (EC 1.1.1.27), acid phosphatase (EC 3.1.3.2), β-glucuronidase (EC 3.2.1.31) and β-N-acetylglucosaminidase (EC 3.2.1.24) were assayed as described elsewhere[20].

ATPase (EC 3.6.1.3) was assayed by determination of $^{32}P_i$ formation from [γ-^{32}P]ATP in all experiments, except when [^{14}C]ATP breakdown products were determined.

The composition of the assay mixtures used for subcellular fractions and release experiments was based on information obtained from the kinetic experiments on platelet lysates (see RESULTS). 50 μl of sample were added to 50 μl of a medium containing: 0.8 mM [γ-^{32}P]ATP or [^{14}C]ATP, 60 mM Tris–HCl buffer (pH 7.5), 20 mM $MgCl_2$ or $CaCl_2$ and 1.5 mM ouabain. The mixtures were incubated at 37° for various time intervals and the reaction was stopped either by the addition of 0.5 ml of 6% trichloroacetic acid or by heating the mixtures in a boiling-water bath for 1.5 min. Precipitates were removed by centrifugation. Aliquots of the supernatants were either extracted for assay of $^{32}P_i$ (trichloroacetic acid mixtures) or subjected to electrophoresis to determine ATP metabolites (boiled mixtures).

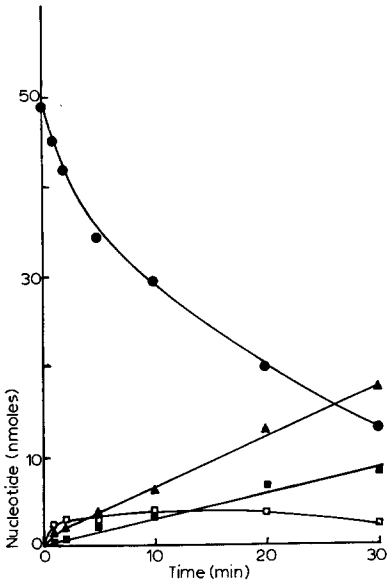
$^{32}P_i$ extraction procedure. This was done by a method based on the MARTIN AND DOTY[27] modification of the BERENBLUM AND CHAIN[28] method. To 0.3 ml of supernatant obtained from the trichloroacetic acid mixtures were added the following: 0.05 ml of 5 M H_2SO_4, 0.50 ml of an isobutanol–benzene mixture (1:1, by vol.) and 0.05 ml of 10% ammonium molybdate. The mixture was shaken for 15 sec, and the phases were separated by centrifugation. A 0.20-ml aliquot of the organic phase was applied to Whatman 3MM paper (2.5 cm × 2.5 cm), dried, and counted in scintillator solution as described below.

Electrophoresis. 20 μl of supernatant obtained from the boiled mixtures were applied as a band to a 2.5-cm wide strip of Whatman 3MM paper together with 90 nmoles each of non-radioactive ATP, ADP and IMP as markers (when [^{14}C]ATP was the substrate), or ATP, P_i and PP_i (when [γ-^{32}P]ATP was the substrate). The metabolites were separated at 60 V/cm for 60 min in 0.05 M sodium citrate buffer (pH 3.8) (ref. 29). The papers were dried at 100° and the bands observed under ultraviolet irradiation, P_i and PP_i after first spraying with ammonium molybdate reagent[30]. The [^{14}C]metabolite bands were cut out and counted in scintillation solution containing 2 g diphenyloxazole per l of toluene in a Beckman LS 200-B scintillation counter. The P_i and PP_i bands were dried at 50° before they were cut out and counted in a Beckman Lowbeta II counter.

RESULTS

Kinetic studies

ATP disappeared exponentially with time in platelet lysates (Fig. 1). The main products of this mixed enzyme system were AMP, ADP and IMP. Since no PP_i is formed and the adenylate kinase activity in such lysates is very high[25], the lysates most probably split ATP to ADP + P_i, and not to AMP + PP_i, as shown for plasma



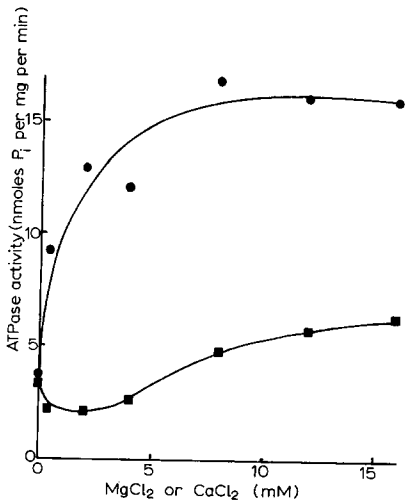


Fig. 1. Variations with the time of incubation of ATP (●), ADP (□), AMP (▲), and IMP (■) during the incubation of 0.4 mg platelet protein, 30 mM Tris–HCl (pH 7.5), 0.6 mM [^{14}C]ATP and 0.6 mM $MgCl_2$ at 37°. ATP breakdown products were assayed as described in METHODS.

Fig. 2. Effect of $MgCl_2$ (●) and $CaCl_2$ (■) on the ATPase activity of platelet lysate. Incubation mixtures containing 0.25 mg of platelet protein, 30 mM Tris–HCl (pH 7.5) and 0.4 mM [γ-^{32}P]ATP were incubated at 37°, for 3 min in the presence of $MgCl_2$, and 8 min in the presence of $CaCl_2$.

low-K_m ATPase[31]. The ATPase activity was linearly proportional to enzyme concentration.

The addition of Mg^{2+} to lysates gave a nearly 3-fold stimulation of ATPase activity compared with that observed with added Ca^{2+} (Fig. 2). Increasing concentrations of Mg^{2+} produced an increase in enzymic activity up to 6 mM Mg^{2+}, and above this concentration no change was observed, as found by CHAMBERS *et al.*[32]. In contrast, increasing concentrations of Ca^{2+} gave no significant changes below 5 mM. Above 5 mM, the ATPase activity increased with increasing Ca^{2+}, but did not level off below 15 mM.

The effects of Mg^{2+} and Ca^{2+} in combination were not additive (Table I), indicating that one enzyme and not two major ATPases were involved. With Ca^{2+} and Mg^{2+} in combination the activity was less than with either ion separately, suggesting antagonism between the ions. Addition of 8 mM EDTA almost completely inhibited the enzyme, probably by chelation of endogenous divalent cations. No effect of ouabain was observed (Table I), however it was introduced into the standard ATPase procedure.

Under the conditions used, the K_m was 0.2 mM (v_{max} = 25.6 nmoles P_i/min per mg protein) and 0.18 mM (v_{max} = 9.8 nmoles P_i/min per mg protein) for the Mg^{2+}- and Ca^{2+}-activated ATPase, respectively (Fig. 3). These values are of the same order as reported for microsomal and mitochondrial Mg^{2+}-stimulated ATPase[33–36] and rat liver mitochondrial Ca^{2+}-stimulated ATPase[37].

TABLE I

EFFECT OF IONS, EDTA AND OUABAIN ON PLATELET UNDIALYSED LYSATE ATPASE

Incubation mixtures, containing 0.25 mg undialysed lysate protein, 30 mM Tris–HCl (pH 7.5), 0.4 mM [γ-^{32}P]ATP and various additions, were incubated at 37° for 5 min.

Addition (mM)	*Activity*	
	nmoles P_i/min per mg protein	*%*
None	3.74	100
$MgCl_2$ (10)	14.60	390
$CaCl_2$ (10)	6.65	178
$MgCl_2$ + $CaCl_2$ (10)	4.01	107
EDTA (8)	0.47	13
$MgCl_2$ (10) + ouabain (0.75)	14.60	390

Subcellular localization of Ca^{2+}/Mg^{2+}-stimulated ATPase

Gradient centrifugation of platelet homogenates produced three distinguishable zones, the upper one particulate. The sucrose range and organelle content of nine fractions collected from the gradient are described in detail elsewhere[21]. Lactate dehydrogenase, acid phosphatase, β-glucuronidase and adenine nucleotides (ADP + ATP) were routinely used as markers for different fractions. Table II gives their percent distribution among the fractions.

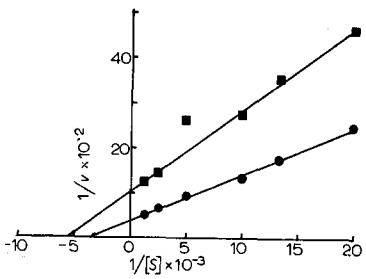
Mg^{2+}- and Ca^{2+}-stimulated ATPase activity showed similar distribution patterns (Fig. 4, Table II). Approx. 75% of the Mg^{2+}-stimulated ATPase activity and 60% of the Ca^{2+}-stimulated ATPase activity were bound to subcellular structures with the remainder in the soluble Fraction 1. The main parts of the particle-bound activity was present in Fractions 2–5, 8 and 9.

TABLE II

DISTRIBUTION OF PROTEIN, STANDARD MARKER ENZYMES, NUCLEOTIDES AND Mg^{2+}/Ca^{2+}-ACTIVATED ATPASE (PER CENT OF TOTAL ACTIVITY OR AMOUNTS) AMONG PLATELET SUBCELLULAR FRACTIONS

Values are means of four experiments.

Compound	*Fraction*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Protein	65.20	4.50	4.20	4.70	3.90	6.50	6.70	2.80	1.30
Lactate dehydrogenase	95.60	1.60	0.16	0.24	0.32	0.65	0.81	0.32	0.32
Acid phosphatase	38.24	11.67	10.96	10.25	8.48	9.55	8.28	1.47	1.09
β-Glucuronidase	35.30	9.40	14.70	10.60	6.40	10.60	9.80	2.20	0.90
Total nucleotides (ATP + ADP)	93.76	3.08	0.18	0.21	0.25	0.51	1.01	0.49	0.49
Mg^{2+}-activated ATPase	29.40	11.40	11.80	10.00	8.70	10.30	10.70	5.10	2.80
Ca^{2+}-activated ATPase	40.80	14.80	7.80	6.70	6.00	7.60	8.80	4.90	2.50



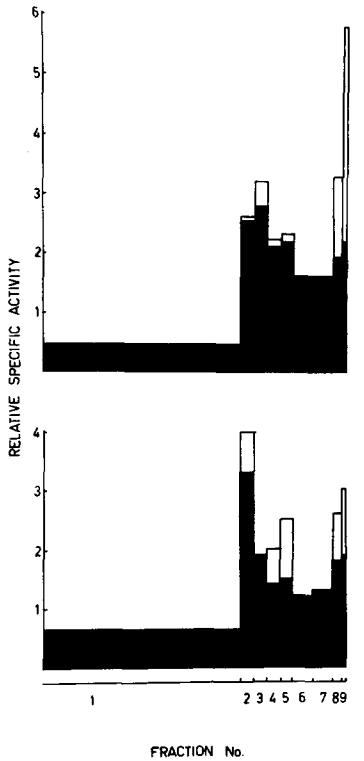


Fig. 3. Double reciprocal plot of ATPase in lysate *versus* ATP concentration. Incubation mixtures containing 0.25 mg platelet protein, 30 mM Tris–HCl (pH 7.5), and various concentrations of [γ^{32}P]ATP were incubated at 37° for 2 min in the presence of 20 mM $MgCl_2$ (●) and for 5 min in the presence of 20 mM $CaCl_2$ (■).

Fig. 4. Distribution patterns of Ca^{2+}-activated ATPase (lower) and Mg^{2+}-activated ATPase (top) (means of four experiments). Fractions are plotted in order of their collection, and their numbers are indicated on the abscissa. Each fraction is represented separately on the ordinate by its relative specific activity (% total activity/% protein). On the abscissa, each fraction is represented by its percentage of protein, the entire abscissa being 100%.

Whereas Ca^{2+} had stimulated the lysate ATPase to less than half the activity obtained in the presence of Mg^{2+} at pH 7.4 (Table I), the Ca^{2+}-stimulated ATPase activity was approximately equal to the Mg^{2+}-stimulated activity in the gradient fractions (Table III). This alteration in the response of the ATPase activity to these ions might be due to the effects of homogenization or other manipulations of the particles during the isolation procedure.

Inclusion of Triton X-100 greatly increased the Ca^{2+}-stimulated ATPase activity in Fractions 8 and 9. The detergent did not affect either Mg^{2+}-stimulated or Ca^{2+}-stimulated ATPase in Fractions 6 and 7, but stimulated both activities in the remaining fractions (Fig. 4).

ATPase activity was also present in the fractions in the absence of added ion, and was probably due to the presence of endogenous divalent cations in the particles (Table III). This activity occurred mainly in fractions principally containing plasma and endoplasmic membranes and mitochondria.

ABLE III

FECT OF DIVALENT CATIONS ON THE SPECIFIC ACTIVITY OF ATPASE AMONG SUBCELLULAR FRACTIONS

ecific activity is expressed in nmoles P_i/min per mg protein. Values are means of four experiments. The values parentheses indicate the range.

ldition	*Fraction*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
one	0.32 (0.2–0.8)	1.60 (0.8–2.4)	3.80 (0.5–4.2)	0.99 (0.2–1.8)	2.40 (0.6–2.5)	1.23 (0.6–2.0)	2.20 (1.2–2.5)	0.90 (0.4–1.3)	0.66 (0.3–0.8)
gCl_2	1.25 (0.8–1.6)	7.00 (5.1–9.4)	7.70 (5.2–10.9)	5.90 (4.6–7.3)	6.20 (4.6–7.6)	4.40 (4.1–5.3)	4.40 (3.9–4.7)	5.00 (4.5–5.9)	6.00 (5.4–7.2)
aCl_2	1.50 (1.2–2.1)	13.50 (11.1–16.4)	7.60 (6.4–9.2)	5.90 (4.4–6.5)	6.40 (4.2–6.8)	4.80 (3.9–5.7)	5.40 (3.2–6.5)	7.10 (5.3–8.1)	7.70 (6.3–8.5)
$gCl_2 + CaCl_2$*	0.85	6.40	4.50	3.60	3.40	2.60	2.60	2.90	2.16

* From two fractionation experiments.

The effect of thrombin on the release of Ca^{2+}/Mg^{2+}-stimulated ATPase

The effect of thrombin in the presence and absence of extracellular Ca^{2+} on the distribution in cells and the environment of Ca^{2+}/Mg^{2+}-stimulated ATPases was compared with that of lactic dehydrogenase, serotonin and *N*-acetylglucosaminidase. Ca^{2+}/Mg^{2+}-stimulated ATPases were not released from the platelets to the Extracellular Phase on exposure to thrombin (Table IV). In no instance did the specific

ABLE IV

FECT OF THROMBIN ON THE INTRACELLULAR AND EXTRACELLULAR DISTRIBUTIONS IN PLATELET SUSPENSIONS · Ca^{2+}/Mg^{2+}-ACTIVATED ATPASE AND OTHER PLATELET CONSTITUENTS

atelet suspensions were incubated with Tris–NaCl buffer or 5 NIH units thrombin/ml for 5 min at 37° in the sence or presence of $CaCl_2$ (2.5 mM Ca^{2+} above the concentration of EDTA in the medium). For experimental tails see METHODS. Amounts of enzymes and substances are expressed as per cent distribution between Intracellular and Extracellular Phases. Specific activity is expressed in nmoles/min per mg. Values are means of ree experiments.

bstance or zyme	*Amount*	*Intracellular Phase*				*Extracellular Phase*			
		$-Ca^{2+}$		$+Ca^{2+}$		$-Ca^{2+}$		$+Ca^{2+}$	
		Buffer	*Thrombin*	*Buffer*	*Thrombin*	*Buffer*	*Thrombin*	*Buffer*	*Thrombin*
$^{2+}$-activated ATPase	% Total	99	98	97	97	1	2	3	3
	Specific activity	8.9	8.0	8.5	9.3	1.7	0.7	3.2	2.3
g^{2+}-activated ATPase	% Total	98	98	97	98	2	2	3	2
	Specific activity	11.2	11.8	10.4	11.2	4.3	1.8	3.5	1.1
ctate dehydrogenase	% Total	97	96	96	94	3	4	4	6
	Specific activity ($\times 10^2$)	59.5	61.5	49.6	43.0	39.0	18.2	29.0	25.0
rotonin	% Total	90	36	91	30	10	64	9	70
	Specific activity	2.2	0.9	2.6	0.8	4.4	12.7	3.6	12.7
-Acetyl-glucosaminidase	% Total	93	74	93	48	7	26	7	52
	Specific activity	21.0	17.5	20.2	11.8	13.0	41.0	17.3	96.0

activity of the ATPase in the Extracellular Phase reach the level of the Intracellular Phase. That the "release reaction"[12] had occurred in these reactions was shown by the release to the Extracellular Phase of serotonin and *N*-acetylglucosaminidase while lactate dehydrogenase was retained (Table IV).

The effect of thrombin on the "Intracellular" Ca^{2+} ATPase

It having been established that Ca^{2+}-activated ATPase is not released during the "release reaction"[12], the effect of thrombin and Ca^{2+} on the intracellular Ca^{2+}-activated ATPase during the initial stages of the release was investigated by cooling and freezing of the platelet–thrombin mixtures without prior separation of the cells and medium. In the four experiments performed, the only highly significant difference from the buffer control value was an 8% drop in activity 10 sec after the addition of thrombin, *i.e.* from 5.06 nmoles P_i/min per mg protein (control) to 4.65 nmoles P_i/min per mg protein (thrombin), the significance of the difference being $0.001 < P < 0.01$.

DISCUSSION

By electronmicroscopic histochemistry, platelet ATPase has been demonstrated in plasma and endoplasmic membranes, mitochondria and possibly some granules[39], and the extraction of thrombosthenin with Mg^{2+}-stimulated ATPase activity from membranes (plasma and endoplasmic reticulum) and granule fractions has been described previously[16]. These granule fractions were heterogeneous, however, consisting of mitochondria and several types of α-granules. The fractionation method used in the present work permits sub-division of the "granule" fraction (lower particulate fraction) into a mixture of well-preserved mitochondria and α-granules (Fractions 4–6) and α-granules with "bull's eye" (Fractions 7–9)[20]. Clearly, both Mg^{2+}- and Ca^{2+}-activated ATPase were present in Fractions 7–9 which contained particles with serotonin and metabolically inactive nucleotides[8]. Owing to their function, these subcellular particles might be termed "secrosomes"[39] and the platelet secrosomes thus resemble those of other secretory cells[12–15] in their ATPase content.

The function of platelet secrosome ATPase during the release reaction is not apparent from the present work. It is not released by thrombin under conditions giving pronounced release of serotonin and *N*-acetylglucosaminidase but is retained similarly to lactate dehydrogenase and other enzymes located in cytosol, plasma-endoplasmic reticulum membranes and mitochondria[40]. The failure to find any release of ATPase to the Extracellular Phase might be evidence for the retention of platelet secrosomes during the release reaction. Since only 16% of platelet ATPase was present in Fractions 7–9, possible variations in this ATPase during the initial phase (0–3 sec) of the release reaction[40] could hardly be demonstrated when measuring "bulk" ATPase. Nevertheless, the small drop in ATPase activity noted 10 sec after the addition of thrombin might be related to the secrosomal ATPase only, and further evidence for such a relation is greatly required.

Apart from its location in secrosomes, platelet ATPase showed a similar distribution pattern to that of hexokinase[21] *i.e.* in fractions containing membrane fragments (Fraction 2) mainly derived from plasma membranes, endoplasmic reticulum, organelles ruptured during homogenization and in fractions containing only mitochondria and α-granules (Fractions 4–9). Although the association of ATPase with the

mitochondrial and/or granular structures seems substantiated in the present work, the definite presence of ATPase in the plasma membrane remains uncertain.

The extent to which thrombosthenin contributes to the ATPase activity in the different fractions is also uncertain. We have previously[20] isolated a fibrous material from the soluble fraction which could as well be superprecipitated thrombosthenin, and such material was found in all subcellular fractions. Any progress in the understanding of the possible participation of ATPase in the release reaction thus requires a further subdivision and characterization of the ATPase of the different fractions, especially the exact quantitation of the amount of thrombosthenin in each fraction. As an indirect approach, the amount of "typical mitochondrial" ATPase, as evaluated with drug action, is under study in this laboratory.

ACKNOWLEDGEMENTS

We wish to thank Mrs Anne-Carine Østvold for skilful technical assistance. Financial support was provided by Anders Jahres Fond til Vitenskapens Fremme, De Norske Livsforsikringsselskapers Forening and Nasjonalforeningen Det Norske Råd For Hjerte- og Karsykdommer.

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