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THE SUBCELLULAR DISTRIBUTION AND CHARACTERISATION OF ATPase ACTIVITY IN PIG PLATELETS

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SUMMARY

The distribution of ATPase activity has been studied in pig platelet subcellular fractions prepared by sucrose density gradient centrifugation. ATPase activities in the presence of Ca^{2+} and Mg^{2+} have been measured and the effect of monovalent cations, of ouabain, 2,4-dinitrophenol and Salyrgan investigated. ATPase activity was present in the soluble phase and in the membrane and granule fractions of the cell. The mean ratios for Ca^{2+}/Mg^{2+} activity being 2.6, 0.62 and 0.60 in these fractions. A large proportion of this divalent cation-activated ATPase can be accounted for by the contractile proteins of the platelet. The Mg^{2+}-ATPase of the granular fraction could be stimulated by 2,4-dinitrophenol. The effects of additions of Na^+ and K^+ and ouabain over a range of concentrations were most variable and the presence of a Mg^{2+}-activated $(Na^+ + K^+)$-ATPase in the cell could not be unequivocally demonstrated. High monovalent cation concentration (approx. 200 mM) showed a more consistent effect. Homogenate Mg^{2+}-ATPase was stimulated by high levels of Na^+ or K^+, Ca^{2+}-ATPase, however, was only slightly increased by 100 mM Na^+ and very markedly by 200 mM K^+. A similar effect with 200 mM K^+ was observed with all the subcellular fractions. The Ca^{2+}-ATPase of the membrane fraction could be increased for example, 2–3-fold by the addition of 200 mM K^+. Salyrgan was found to inhibit the ATPase activities of all fractions and the soluble phase activity was almost completely inhibited by this agent in the presence of either bivalent cation. The granule fraction was the least sensitive to Salyrgan. The findings well illustrate the difficulties in identifying other minor ATPase enzymes in the presence of a large contractile protein ATPase component of wider distribution in the cell.

INTRODUCTION

Using combined histochemical and electron micrographic procedures White and Krivit[1] demonstrated in the blood platelet an ATPase activity which appeared to be associated with the surface membrane, with mitochondria and possibly with certain other intracellular structures. An "ecto ATPase" has also been identified and partially characterised[2] on the outer surface of the intact platelet and its activity in the presence of sulphydryl inhibitors, ouabain and dinitrophenol has been studied[3].

A role for this "ecto enzyme" has been proposed in a number of platelet surface interactions, notably adhesiveness[4], aggregation[5,6,7] and in a variety of other activities of the cell such as pseudopodia formation, clot retraction and viscous metamorphosis, all of which involve changes in the characteristics of the surface membrane.

The earlier demonstration by Bettex-Galland and Lüscher[8–12] and Grette[13] of an actomyosin-like contractile protein complex (thrombosthenin) in platelet extracts, with a pronounced divalent cation-dependent ATPase activity, considered together with the more recent enzyme studies of a number of groups[14–17], has stimulated considerable speculation about the role of the ATP splitting enzymes in maintaining the structural integrity of the platelet membrane and in shape changes and other motile activities of the cell.

In an investigation of the ATPase activity of lysates and subcellular fractions prepared by density gradient centrifugation of platelet homogenates, French *et al.*[18] reported the presence of both Ca^{2+}- and Mg^{2+}-stimulated ATPase activity in granule-containing fractions, earlier shown to be rich in bound nucleotides and 5-hydroxytryptamine[19–21]. In addition to this granular location, French and his co-workers also reported considerable divalent cation-stimulated ATPase in other subcellular organelles and a significant soluble phase component too.

The "platelet release reaction", a phenomenon which has been extensively studied by Holmsen and his colleagues[22–24], also involves a divalent cation-dependent and highly selective extrusion of intracellular constituents through the platelet membrane. During this relase process, which can be triggered by cell contact with collagen or thrombin, intracellular stores of ATP are metabolically consumed and the suggestion has been made that the contractile proteins of the platelet may be involved in the movement of cell constituents towards the boundary membrane and their release into the extracellular environment. Divalent cation-activated ATPases have also been implicated in other cells which display a Ca^{2+}-dependent, stimulated secretion of intracellular granule constituents through their surface membrane. The leucocyte and the cells of the adrenal medulla are perhaps the best studied in this respect and recently contractile proteins with ATPase activity have been isolated from both these cell systems[25,26]. Recently too, micro-particles of platelet origin have been identified in plasma, and proteins extracted from these particles with high ionic strength salt solutions have an associated ATPase activity and physical properties similar to those of the platelet contractile protein complex "thrombosthenin"[27,28]. In this paper the ATPase activities of platelet subcellular fractions have been studied using a simple and highly reproducible density gradient procedure which has been earlier described[29]. With this procedure, separate membrane and granular fractions can be isolated free from the soluble phase of the cell and the subcellular distribution of the various ATPases present and their activity responses to certain activators and inhibitors has been investigated.

MATERIALS AND METHODS

Pig platelets were isolated from freshly drawn anticoagulated abattoir blood, homogenised and fractionated on sucrose density gradients exactly as previously described[29].

The isolated fractions from each platelet preparation were routinely monitored

for the marker enzymes previously used[29] and the same nomenclature employed for the major fractions and subfractions. Fraction A, the low density non-particulate zone was located at the top of the gradient above the membrane fraction and contained the major proportion of the soluble phase constituents of the cell. Fractions B and D are the membrane and granular zones, respectively, and these were subfractionated by high speed centrifugation (100000 × *g*, 60 min) to produce B_s and B_p and D_s and D_p, the soluble and particulate components of those two major zones. The intermediate zone, C and the highest density zone E, located below the granule layer, showed no particulate material sedimentable at 100000 × *g*, 60 min.

For measurements of ATPase activity the Tris ATP salt was used and the assay media were buffered to pH 7.4 by addition of 30 mM Tris–HCl. A concentration of 3 mM substrate was used in all determinations and the amounts of the various cations and inhibitors for the systems used in characterisation of the enzymes are listed in Table I. Incubations at 37 °C were carried out for periods of 15 or 20 min and the reactions terminated by the addition of ice-cold trichloroacetic acid (final concentration 7%). The liberated inorganic phosphate was determined by the Martin and Doty modification[30] of the procedure of Berenblüm and Chain[31]. Appropriate blanks and controls were processed with each assay. Activities have been expressed as μmoles P_i liberated per mg protein per h.

TABLE I

ASSAY MEDIA FOR ATPase ACTIVITIES

For details of the assay procedure and inorganic phosphate measurement see Materials and Methods section. In addition to the following cations and inhibitors, the assay media (final volume 2.0 ml) also contained 3 mM ATP and 30 mM Tris–HCl buffer (pH 7.4).

Assay system	*Bivalent cation*	*Other additions*
1	3 mM $CaCl_2$	None
2	3 mM $MgCl_2$	None
3	3 mM $CaCl_2$ and 3 mM $MgCl_2$	None
4	3 mM $MgCl_2$	100 mM NaCl, 30 mM KCl
5	3 mM $MgCl_2$	100 mM NaCl, 30 mM KCl, 0.25 mM ouabain
6	3 mM $MgCl_2$	0.25 mM ouabain
7	3 mM $MgCl_2$	0.25 mM dinitrophenol
8	3 mM $CaCl_2$	0.2 mM Salyrgan
9	3 mM $MgCl_2$	0.2 mM Salyrgan
10	3 mM $CaCl_2$	200 mM KCl
11	3 mM $MgCl_2$	200 mM KCl
12	3 mM $CaCl_2$	200 mM KCl, 0.2 mM Salyrgan

RESULTS

Distribution of ATPase in the subcellular fractions

ATPase activities of the homogenate, the major soluble fraction (A) and the membrane and granular fractions (B_p and D_p) were measured in a variety of incubation media. In addition to 30 mM Tris–HCl buffer (pH 7.4) and 3 mM Tris

TABLE II

EFFECT OF Ca^{2+} AND Mg^{2+} ON THE ATPase ACTIVITIES OF SUBCELLULAR FRACTIONS

All values are specific activities expressed as μmoles phosphate released per mg protein per h. Fractions were assayed using incubation media containing 30 mM Tris–HCl (pH 7.4), 3 mM ATP, and 3 mM $MgCl_2$ or 3 mM $CaCl_2$ or both cations each at 3 mM concentration (assay systems 1, 2 and 3, Table I). Dashes indicate no determination made. Mean ratios Ca^{2+}/Mg^{2+} activity expressed with 1 S.D., calculated from the figures in parentheses.

Prepn No.	*Homogenate*			*Fraction A*			*Fraction B_p*			*Fraction D_p*		
	Ca^{2+}	Mg^{2+}	$Ca^{2+}+Mg^{2+}$	Ca^{2+}	Mg^{2+}	$Ca^{2+}+Mg^{2+}$	Ca^{2+}	Mg^{2+}	$Ca^{2+}+Mg^{2+}$	Ca^{2+}	Mg^{2+}	$Ca^{2+}+Mg^{2+}$
1	0.41	0.50 (0.82)	0.11	0.93	0.21 (4.4)	—	0.61	1.66 (0.37)	0.56	1.28	2.08 (0.62)	1.62
2	0.45	0.75 (0.60)	0.38	0.92	0.16 (5.8)	—	0.91	1.73 (0.53)	1.02	1.15	1.33 (0.86)	0.75
3	0.51	0.54 (0.94)	0.51				0.62	0.96 (0.65)	0.50	1.13	3.13 (0.36)	1.93
4	0.36	0.59 (0.61)	0.34	0.16	0.08 (2.0)	0.0	1.26	1.86 (0.68)	1.08	2.51	5.89 (0.43)	3.02
5	0.56	0.57 (0.98)	0.15	0.23	0.14 (1.6)	0.07	0.34	0.78 (0.44)	0.72	1.18	2.58 (0.46)	1.38
6	0.48	0.52 (0.92)	0.36	0.12	0.08 (1.5)	0.02	0.70	0.99 (0.71)	0.35	1.78	1.62 (1.10)	0.94
7	0.37	0.73 (0.51)	0.52				2.66	3.01 (0.88)	2.30	4.33	5.14 (0.84)	4.37
12	0.48	0.60 (0.80)	0.57	1.54	0.97 (1.6)	—	0.86	1.37 (0.63)	—	0.48	1.03 (0.47)	—
13	0.33	0.22 (1.50)	0.30	0.55	0.42 (1.3)	—	0.29	0.42 (0.69)	—	0.45	1.07 (0.42)	—
Mean Ca^{2+}/Mg^{2+} activity ratio	0.85 ± 0.29			2.6 ± 1.8			0.62 ± 0.14			0.60 ± 0.25		

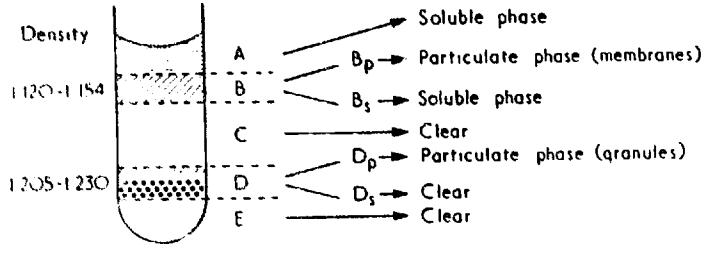

TABLE III

EFFECT OF SODIUM, POTASSIUM AND OUABAIN ON Mg^{2+}-ATPase ACTIVITIES OF SUBCELLULAR FRACTIONS

Assay media, in addition to 30 mM Tris–HCl (pH 7.4), 3 mM ATP, 3 mM $MgCl_2$: medium 2, no other additions; medium 4, 100 mM NaCl, 30 mM KCl; medium 5, 100 mM NaCl, 30 mM KCl, 0.25 mM ouabain; medium 6, 0.25 mM ouabain. Values in parentheses between the results for assay systems 4 and 5 represent percentage reduction by ouabain of the Mg^{2+}-activated ($Na^+ + K^+$)-ATPase activity of each sample. Specific activities are expressed as μmoles phosphate released per h per mg protein. Dashes in columns indicate that no measurements were made.

Prepn No.	*Homogenate Assay medium*				*Fraction A Assay medium*				*Fraction B_p Assay medium*				*Fraction D_p Assay medium*			
	2	*4*	*5*	*6*	*2*	*4*	*5*	*6*	*2*	*4*	*5*	*6*	*2*	*4*	*5*	*6*
2	0.50	0.67 (66)	0.23	0.58	0.21	—	—	—	1.66	1.12 (29)	0.79	1.01	2.08	3.08 (17)	2.51	3.01
3	0.75	0.67 (28)	0.48	0.62	0.16	0.25 (100)	0.00	—	1.73	1.55 (14)	1.33	1.43	1.33	1.21 (24)	0.92	1.29
4	0.54	0.57 (—)	0.63	0.71					0.96	1.08 (18)	0.88	1.01	3.12	2.78 (1)	2.76	3.92
5	0.59	0.73 (12)	0.64	0.73	0.08	0.13 (54)	0.06	0.12	1.86	1.57 (8)	1.44	1.40	5.89	4.14 (—)	4.27	5.67
6	0.57	0.20 (—)	0.30	0.28	0.14	0.16 (31)	0.11	0.15	0.78	0.82 (22)	0.64	0.34	2.58	2.87 (2)	2.82	2.60
7	0.52	0.60 (3)	0.58	0.55	0.08	0.04 (—)	0.05	0.09	0.99	1.21 (29)	0.86	1.01	1.62	1.55 (—)	1.59	1.58
8	0.73	0.74 (—)	0.76	0.83	—	—	—	—	3.01	2.50 (8)	2.29	2.82	5.14	5.47 (5)	5.17	5.20
Mean	0.60	0.60	0.52	0.60	0.13	0.14	0.06	0.12	1.57	1.40	1.17	1.29	3.11	3.01	2.86	3.32

ATP, the activators and inhibitors listed in Table I were included in the routine spectrum of assay systems at the final concentrations indicated. Most determinations were applied to at least seven and in some cases nine different preparations of homogenates and subfractions from different platelet pools. All these density gradient preparations produced the five major zones shown in the diagram accompanying Table II with clearly delineated membrane and granule bands. The positions of these zones within the gradient tubes were extremely reproducible.

Ca^{2+} and Mg^{2+}-dependent activities, and activities in the presence of both cations (assay systems 1, 2 and 3, respectively, Table I) were measured for the homogenates, the membrane and granule fractions (B_p and D_p) and fraction A, representing the soluble phase, in nine different experiments. The specific activity results are shown in Table II. The Ca^{2+}/Mg^{2+} activity ratios for the various fractions are presented in Table III.

The homogenates from all preparations gave essentially similar activities in the assays performed in the presence of either Ca^{2+} or Mg^{2+} (Table II), the mean Ca^{2+}/Mg^{2+} activity ratio for the homogenate (Table III) being 0.85 ± 0.29. The range of activities for these ATPases of homogenates form nine different preparations of platelets was remarkably narrow. Specific activity values for the Ca^{2+}-enzymes varied only between 0.33 and 0.56 μmole of P_i per mg protein per h and for the Mg^{2+}-enzyme between 0.22 and 0.75 μmole/mg protein per h. With both cations present the range of activity lay between 0.11 and 0.57 μmole/mg protein per h. Both the membrane and granule fractions B_p and D_p consistently displayed significantly higher activities with Mg^{2+} than Ca^{2+}. The mean Ca^{2+}/Mg^{2+} activity ratios for these two particulate fractions were 0.62 ± 0.14 and 0.60 ± 0.25, respectively. The soluble phase fractions (fraction A) however, were quite different and showed ATPase activities of up to six times higher in the presence of Ca^{2+} than with Mg^{2+}. The mean activity ratio for this fraction Ca^{2+}/Mg^{2+} was 2.6 ± 1.8.

Ca^{2+} and Mg^{2+} added together in the assay media, gave with all the fractions and homogenates much lower activities than was recorded in the presence of Mg^{2+} alone. Frequently these were lower than the activities measured in the presence of only Ca^{2+}.

In the presence of Mg^{2+}, Na^+ and K^+ added (assay medium 4) at concentrations usually considered optimal for the Mg^{2+}-activated $(Na^+ + K^+)$-ATPase of other tissues (100 mM NaCl and 30 mM KCl), gave very variable results (Table III). In some preparations of a particular fraction, the presence of the two cations very slightly activated the ATPase, above values measured in their absence (assay medium 2), whilst in other preparations of the same fraction from a different platelet pool their presence was inhibitory. Measurements of the Mg^{2+}-ATPase and the Mg^{2+}-activated $(Na^+ + K^+)$-ATPase with and without the addition of ouabain (0.25 mM) were compared on homogenate and particulate and soluble fractions from seven different platelet preparations. These results are also shown in Table III. The effect of ouabain was generally to slightly inhibit the particulate fraction ATPase. However, an activation effect was occasionally observed with both the Mg^{2+}-ATPase and the Mg^{2+}-activated $(Na^+ + K^+)$-ATPase in a number of the homogenate preparations. The membrane (B_p) fraction enzymes were the most consistent in their response to ouabain and the Mg^{2+}-activated $(Na^+ + K^+)$-ATPase activity of this fraction was inhibited significantly in all preparations. Ouabain reduced these activities by between

8 and 30% (mean 18.6%) for the seven membrane fractions. Dinitrophenol (assay system 7, Table IV) had a highly variable effect upon the Mg^{2+}-ATPase activities of the membrane fractions. Two fractions were inhibited by 0.25 mM 2,4-dinitrophenol, four fractions were activated and the remaining three fractions were not

TABLE IV

EFFECT OF 2,4-DINITROPHENOL ON Mg^{2+}-ATPase ACTIVITIES OF HOMOGENATES AND GRANULAR FRACTIONS D_p

Results are expressed as specific activity (μmoles P_i per mg protein per h) and percentage increase in activity with 0.25 mM dinitrophenol additions to the assays. The figures in parentheses above each column refer to the assay media used (see code Table I).

Prepn No.	*Mg^{2+}-ATPase (2)*	*% increase with dinitrophenol (2 and 7)*	*Mg^{2+}-ATPase (2)*	*% increase with dinitrophenol (2 and 7)*
1	2.08	44.7	0.50	46.0
2	1.33	Inhibition	0.75	Inhibition
3	3.13	37.7	0.54	20.4
4	5.89	9.5	0.59	25.4
5	2.58	2.3	0.57	9.6
6	1.62	9.3	0.52	29.0
7	5.14	15.2	0.73	1.4
12	1.03	8.7	0.60	5.0
13	1.07	10.3	0.22	27.3
Mean % increase	—	16.8	—	20.5

significantly altered. Overall there was no statistically significant effect. In the homogenates and granule fractions however, the Mg^{2+}-ATPase was activated by the addition of dinitrophenol in eight out of the nine platelet preparations. This activation was significant ($P<0.05$). The highest activations recorded were 45% and 46% for the granules and for the homogenate, respectively. When increasing amounts of Na^+ were added alone to the assay system for the measurement of Mg^{2+}-ATPase in platelet homogenates, these were shown to reduce the Mg^{2+} activity by 50–60% (Fig. 1). This reduction occurred sharply between 100 and 200 mM Na^+ and was maintained at higher Na^+ levels. Ca^{2+}-ATPase activity, however, (also Fig. 1) showed a slight increase in activity, reaching a maximum around 100 mM Na^+ and this level was maintained at high Na^+ concentrations.

The Mg^{2+}-ATPase activity responded in the same way to K^+ as to Na^+ (Fig. 2) and reduction occurred at approximately the same level of monovalent cation concentration. In contrast, however, ATPase activities of homogenates were markedly increased in the presence of K^+, showing peak activation around 200 mM K^+ and a sharp fall at higher K^+ levels (also Fig. 2). A similar activation was observed with K^+ towards the Ca^{2+}-ATPase of the membrane fractions (Fig. 3) rising to a maximum at 200–250 mM K^+, but the decrease at higher K^+ levels was less pronounced with these fractions. As a result of these findings the effect of the addition

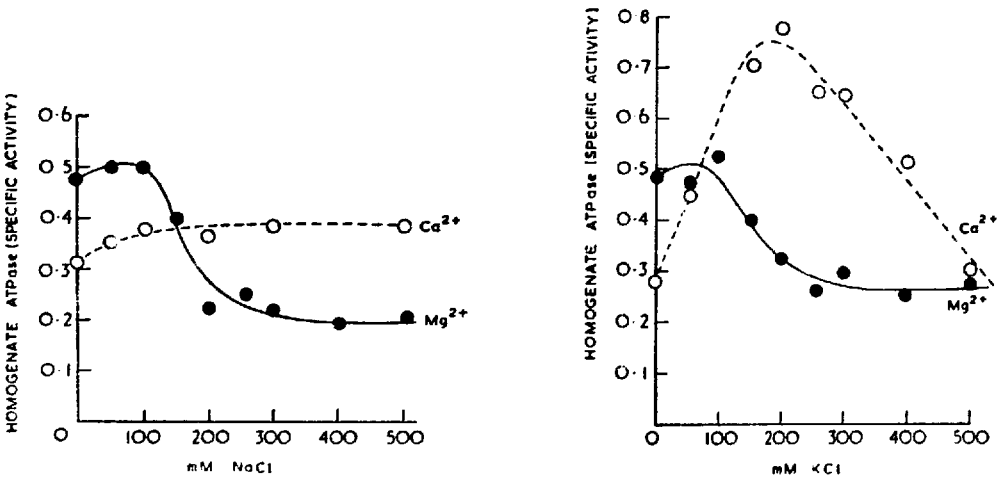

Fig. 1. Effect of increasing amounts of Na^+ on the Mg^{2+}-ATPase activity (●—●) and Ca^{2+}-ATPase activity (○--- ○) of platelet homogenate.

Fig. 2. Effect of increasing amounts of K^+ on the Mg^{2+}-ATPase activity (●—●) and Ca^{2+}-ATPase activity (○--- ○) of platelet homogenate.

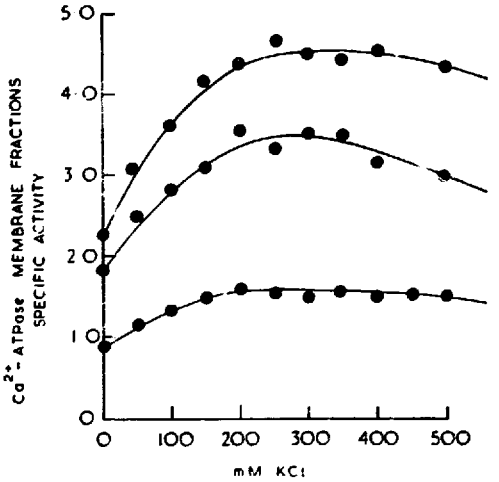

Fig. 3. Effect of increasing amounts of K^+ on the Ca^{2+}-ATPase activity of three membrane fraction preparations.

of 200 mM KCl to the assay systems for Ca^{2+}- and Mg^{2+}-ATPase determination (assay media 10 and 11) was routinely investigated with homogenates and subcellular fractions. The results for nine different platelet preparations are presented in Table V. With Mg^{2+} as the activating cation, the effect of the addition of 200 mM KCl was generally to depress the activity in all fractions examined. The only exceptions to this finding occurred with the granular fractions D_p. In three preparations, KCl had an activating effect, the specific activities being increased by 1.9, 3.5 and 52.9%. The most consistent response to KCl occurred in the membrane fraction (B_p), with reduction in Mg^{2+}-ATPase activity of between 11 and 52% (mean 31%) in the presence of 200 mM KCl. In contrast KCl additions to the Ca^{2+}-ATPase were generally stimulatory, the membrane fraction in particular showing increase in specific activity of between 40 and 390% (mean 177%).

TABLE V

EFFECT OF 200 mM KCl ON Ca^{2+}-ATPase AND Mg^{2+}-ATPase ACTIVITIES OF THE SUBCELLULAR FRACTIONS

Results expressed as specific activity (μmoles P_i per mg protein per h) and percentage change with 200 mM KCl in the assay media. For ATPase determinations see Material and Methods section.

Prepn No.	*Fraction A*				*Fraction B_p*			
	Mg^{2+} spec. act.	*$Mg^{2+}+K^+$ %change*	*Ca^{2+} spec. act.*	*$Ca^{2+}+K^+$ %change*	*Mg^{2+} spec. act.*	*$Mg^{2+}+K^+$ %change*	*Ca^{2+} spec. act.*	*$Ca^{2+}+K^+$ %change*
1	0.21		0.93	+61.7	1.66	−51.8	0.61	+170.1
2	0.16		0.92	+23.6	1.73	−46.2	0.91	+169.5
3					0.96	−11.5	0.62	+ 95.2
4	0.08	−12.5	0.16	+58.2	1.86	−32.3	1.26	+208.2
5	0.14	− 7.1	0.23	+69.7	0.78	−21.8	0.34	+390.4
6	0.08	−25.0	0.12	+56.4	0.99	−18.2	0.70	+ 47.8
7					3.01	−35.5	2.66	+ 95.5
12	0.97		1.54	+50.0	1.37		0.86	+311.7
13	0.42		0.55	+77.5	0.42		0.29	+104.2
Mean % change		−12.7%		+56.7%		−30.9%		+177.0%

TABLE VI

EFFECT OF SALYRGAN ON Ca^{2+}-ATPase AND Mg^{2+}-ATPase ACTIVITIES OF SUBCELLULAR FRACTIONS

Results expressed as specific activities (μmoles P_i per mg protein per h) for each assay either Ca^{2+} or Mg^{2+} added to the media and as percentage change in activity with the further addition of Salyrgan to the assays. (The negative sign represents decreases in activity and the positive sign an increase.) Dashes in fraction A columns represent no analysis.

Prepn No.	*Fraction A*				*Fraction B_p*			
	Mg^{2+} spec. act.	*Mg^{2+} + Sal. %change*	*Ca^{2+} spec. act.*	*Ca^{2+} + Sal. %change*	*Mg^{2+} spec. act.*	*Mg^{2+} + Sal. %change*	*Ca^{2+} spec. act.*	*Ca^{2+} + Sal. %change*
1	0.21	−100	0.93	−100	1.66	−78.9	0.61	−74.7
2	0.16	−100	0.92	−100	1.73	−83.2	0.91	−80.9
3	—	—	—	—	0.96	−70.2	0.62	−85.0
4	0.08	−100	0.16	−100	1.86	−34.7	1.26	−67.8
5	0.14	− 57.1	0.23	−100	0.78	−63.5	0.34	−71.5
6	0.08	− 87.5	0.12	−100	0.99	−61.9	0.70	−86.3
7	—	—	—	—	3.01	−41.8	2.66	−68.5
12	0.97	− 93.8	1.54	− 91.6	1.37	−90.9	0.86	−94.6
13	0.42	− 85.7	0.55	− 90.9	0.42	−96.9	0.29	−86.1
Mean % change		− 89.8%		− 97.4%		−69.2%		−79.5%

Fraction D_p				*Homogenate*			
Mg^{2+} spec. act.	*$Mg^{2+}+K^+$ %change*	*Ca^{2+} spec. act.*	*$Ca^{2+}+K^+$ %change*	*Mg^{2+} spec. act.*	*$Mg^{2+}+K^+$ %change*	*Ca^{2+} spec. act.*	*$Ca^{2+}+K^+$ %change*
2.08	+52.9	1.28	−24.7	0.50	−23.1	0.41	+ 88.6
1.33	−46.6	1.15	+36.5	0.75	−39.5	0.45	+183.2
3.13	−16.6	1.13	−12.0	0.54	− 3.0	0.51	+ 79.3
5.89	−30.2	2.51	+22.2	0.59	0	0.36	+ 41.7
2.58	+ 1.9	1.18	+26.1	0.57	−23.0	0.56	+ 16.3
1.62	−13.6	1.78	+62.5	0.52	− 2.5	0.48	− 11.4
5.14	+ 3.5	4.33	−29.3	0.73	− 9.9	0.37	+ 22.7
1.03		0.48	+ 7.9	0.60		0.48	− 81.7
1.07		0.45	+14.3	0.22		0.33	+ 92.0
	− 9.9%		+12.6%		−14.3%		+ 66.0%

Fraction D_p				*Homogenate*			
Mg^{2+} spec. act.	*$Mg^{2+}+Sal.$ %change*	*Ca^{2+} spec. act.*	*$Ca^{2+}+Sal.$ %change*	*Mg^{2+} spec. act.*	*$Mg^{2+}+Sal.$ %change*	*Ca^{2+} spec. act.*	*$Ca^{2+}+Sal.$ %change*
2.08	− 1.9	1.28	−58.0	0.50	−100	0.41	−100
1.33	−50.5	1.15	−74.3	0.75	− 78.8	0.45	− 90.7
3.13	− 5.6	1.13	−34.9	0.54	− 67.8	0.51	− 90.9
−5.89	−18.7	−2.51	−42.5	0.59	− 37.0	0.36	− 70.6
2.58	−26.5	1.18	−27.6	0.57	− 57.8	0.56	− 77.3
1.62	−38.1	1.78	−72.8	0.52	− 43.3	0.48	− 76.4
5.14	−21.5	4.33	−23.5	0.73	− 40.4	0.37	− 38.1
1.03	−25.4	0.48	−53.2	0.60	− 72.1	0.48	− 72.0
1.07	−15.2	0.45	+ 1.8	0.22	− 70.1	0.33	− 77.9
	−22.2%		−47.7%		− 63.0%		− 77.1%

With one single exception (platelet preparation 13, fraction D_p), the Mg^{2+}- and the Ca^{2+}-ATPase activities of the homogenates and the fractions from the nine gradient preparations showed inhibition by the sulphydryl agent Salyrgan (Table VI). The greatest sensitivity to this agent was displayed by the soluble phase fractions in all preparations. In the presence of either of the bivalent cations, Salyrgan almost completely inhibited the soluble phase ATPase activity (mean inhibition $94.4 \pm 4.2\%$ for Ca^{2+} activity, and $89.8 \pm 14.7\%$ for the Mg^{2+} activity). Slightly less inhibition by Salyrgan was recorded for the bivalent cation ATPases present in the membrane fractions, being $79.5 \pm 9.3\%$ inhibited with Ca^{2+} present and $69.2 \pm 21.1\%$ with Mg^{2+}. The ATPases of the granule fraction were the least sensitive to Salyrgan with a mean percentage inhibition of only $22.2 \pm 14.4\%$ for the Mg^{2+}-ATPase and $47.7 \pm 23.9\%$ for the Ca^{2+}-dependent enzyme. The residual insensitive activity of the granule fraction was up to four times higher with Mg^{2+} present than with Ca^{2+}.

DISCUSSION

Much divalent cation-activated ATPase activity was present in all fractions studied but the unequivocal demonstration of a Mg^{2+}-dependent enzyme, activated synergistically by Na^+ and K^+, has not been made. In most preparations of homogenate and subcellular fractions the changes in activity with the added monovalent cations were small and their significance difficult to assess. Moreover, in some fractions a pronounced inhibition of activity was observed on the addition of Na^+ and K^+ at 100 and 30 mM concentration, respectively. Additions of ouabain to the Mg^{2+} $(Na^+ + K^+)$-ATPase assays also gave variable effects and only the Mg^{2+} $(Na^+ + K^+)$-ATPase of the membrane fraction showed a consistent response to this agent with between 8 and 30% inhibition recorded for seven platelet preparations. This finding does, however, compare fairly well with the figures reported by Saba and co-workers[3,14] for their light microsomal fraction prepared from human platelets. They recorded 14% inhibition with 0.1 mM ouabain and 23% inhibition with 0.001 mM concentration with this fraction but ouabain was always stimulatory towards the enzyme of the heavy microsomal fraction in their studies. The identity of this enzyme and its relation to the ecto-ATPase described by Chambers *et al.*[2] and Mason and Saba[3] must await improved techniques for the isolation of platelet plasma membrane components. Although it is noted that Chambers *et al.*[2] were unable to demonstrate ouabain inhibition with their ecto-ATPase, changes in the degree and direction of ouabain sensitivity of $(Na^+ + K^+)$-ATPase is a well documented phenomenon with red cell membranes and certain microsomal preparations, and is believed to be related to the preparative procedures. Shrier *et al.*[32] commented upon significant variations in the effectiveness of ouabain towards red cell membrane $(Na^+ + K^+)$-ATPase with inhibitory effects being lower the more vigorous the preparative procedures. Brown[33,34] also found similar ouabain sensitivity changes with rabbit heart preparations and a plant ATPase and also suggested that ouabain affects enzyme activity by alteration of the conformation of the protein in a way largely determined by the pre-reaction conformation, either increasing or decreasing the accessibility of the binding sites for K^+. These and many other observations, which include the effects of ultrasound, detergents, pH changes and the presence of divalent cations during preparation[35–37] underline the complexity of the Mg^{2+}-

dependent ATPase activities in cell membranes and lend support to the view of Skou[38] that the $(Na^+ + K^+)$-ATPase and the Mg^{2+}-ATPase which generally accompanies it in membrane systems, may well be the same enzyme functioning in different ways due to conformational changes produced in the isolation procedure. The platelet would certainly be expected to exhibit active monovalent cation transport since the intracellular concentration of K^+ (86.4 mequiv/l cell water) is high[39]. This value is close to the levels reported for the erythrocyte, and intact platelets have been shown to take up K^+ from the medium by a process which is inhibited by anaerobic conditions and iodoacetate[40]. However, the maintenance of such an ionic gradient and a monovalent cation activated ATPase have not yet been definitively linked as a functional entity in the platelet.

A comparison of the Mg^{2+}-ATPase activity of the homogenates and fractions with and without the addition of 0.25 mM dinitrophenol revealed that in addition to the homogenate the only fractions which showed significant change with the agent were the granule containing fractions D_p. Increase in activity of between 2 and 45% were recorded with mean increase of 17% for the granule fraction and 20% for the homogenates (eight preparations).

Lavis and Letchworth[41] reported a red cell membrane ATPase, distinct from the $(Na^+ + K^+)$-ATPase which could be stimulated by dinitrophenol and a similar enzyme believed to be associated with the mitochondria has been demonstrated in the blood platelet[42].

In the present investigation a considerable divalent cation-activated ATPase without the characteristics of the Na^+, K^+, translocatory enzyme or a mitochondrial ATPase has been demonstrated in all the subcellular fractions. The ecto-ATPase reported in human red cells[43,44] and the ecto-enzymes of platelets investigated by Robinson *et al.*[5] and Chambers *et al.*[2] were all shown to be unaffected by ouabain or Na^+ and K^+. The latter authors suggested the possibility that their ATPase, with its variable dependence upon Ca^{2+} or Mg^{2+}, may be the contractile protein complex (thrombosthenin) of the platelet. The properties of this enzyme in the present study strongly indicate the presence of a contractile protein ATPase complex with a predominant myosin-like component in the soluble phase, whose response to divalent cations and Salyrgan is conditioned by ionic strength changes, presence of other cations, and possibly too, the degree of interaction with an actin-like moiety also present in the cells. The soluble fraction ATPase is certainly myosin-like in its properties and high Ca^{2+}-/Mg^{2+}-ATPase activity ratios were recorded even under low ionic strength conditions of measurement. The Ca^{2+}-ATPase of this fraction was also significantly inhibited by Mg^{2+}. The location and extent of association of the two major protein components of the contractile complex in other cell fractions was more difficult to assess and the diffuse distribution of these proteins with high divalent cation-dependent activity greatly affected the interpretation of the results for other ATP hydrolysing enzymes present in these fractions.

During the course of these investigations it was noticed that a white fluffy precipitate often appeared in the soluble phase after storage for a few days at 4 °C. Although gradient centrifugation and subfractionation was always performed on the same day as homogenisation and enzymatic and analytical studies made as soon as possible thereafter, the formation of this precipitate, which was very rich in Ca^{2+}-ATPase activity, was an uncontrollable factor in these studies. It is possible that this

material was beginning to aggregate unseen during gradient centrifugation, and one must consider that the trapping out of this protein ATPase during membrane vesiculation may well account for some of the difficulties in the present study of defining the intracellular localisation of the various ATPases present. This soluble phase, spontaneously precipitating, protein has formed the basis of a further study concerned with the subcellular localisation of the myosin-like protein of the platelet contractile system.

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