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Evidence that the platelet plasma membrane does not contain a $(Ca^{2+} + Mg^{2+})$-dependent ATPase

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The present study was designed to determine the subcellular distribution of the platelet $(Ca^{2+} + Mg^{2+})$-ATPase. Human platelets were surface labeled by the periodate-boro[^{3}H]hydride method. Plasma membrane vesicles were then isolated to a purity of approx. 90% by a procedure utilizing wheat germ agglutinin affinity chromatography. These membranes were found to be 2.6-fold enriched in surface glycoproteins compared to an unfractionated vesicle fraction and almost 7-fold enriched compared to intact platelets. In contrast, the isolated plasma membranes showed a decreased specific activity of the $(Ca^{2+} + Mg^{2+})$-ATPase compared to the unfractionated vesicle fraction. This decrease in specific activity was found to be similar to that of an endoplasmic reticulum marker, glucose-6-phosphatase, and to that of a platelet inner membrane marker, phospholipase A_2. We conclude, therefore, that the $(Ca^{2+} + Mg^{2+})$-ATPase is not located in the platelet plasma membrane but is restricted to membranes of intracellular origin.

Introduction

Blood platelets can be activated by a variety of stimuli such as ADP, thrombin, collagen and immune complexes. The responses of the platelet, however, are similar, resulting in platelet aggregation, release of material from storage organelles and manifestations of contractile activity. Although it has recently been shown that conditions exist under which platelets may be activated at very low Ca^{2+} concentrations [1,2], it remains undisputed that an elevated level of intracellular free Ca^{2+}, as produced by agonist-induced stimulation, will cause platelet activation and that platelets have the ability to regulate this concentration [3,4]. Accordingly, the resting state of the platelet is characterized by a low cytoplasmic free Ca^{2+} concentration and the reversal of activation therefore corresponds to the removal of this cation from the cytoplasm [5].

In an effort to identify this 'Ca^{2+} pump' activity within platelets, isolated membranes were tested for this activity. A membrane vesicle fraction was isolated from platelets which was devoid of mitochondria and secretory granules and accumulated calcium in the presence of ATP [6–10]. This membrane fraction consisted of vesicles derived from inner membranes as well as from the plasma membrane and showed $(Ca^{2+} + Mg^{2+})$-ATPase activity [8]. Ca^{2+} uptake by these vesicles was stimulated by cAMP and a protein kinase and it was suggested that this activity may be responsible for removal of Ca^{2+} from the platelet cytoplasm [8].

The question arises concerning the subcellular distribution of this 'Ca^{2+} pump' within the platelet. Previous studies suggested two possibilities. One is that the 'Ca^{2+} pump' has an intracellular location. Evidence has been provided showing that the dense tubular system in platelets, an internal

membrane structure comparable to the sarcoplasmic reticulum of muscle cells [11], contains a Ca^{2+}-transport system [8,12,13]. It may be, therefore, that this structure contains the (Ca^{2+} + Mg^{2+})-ATPase activity. The second possibility is that the 'Ca^{2+} pump' is of plasma membrane origin. Most animal cells have an outward-directed, ATP-dependent 'Ca^{2+} pump' in the plasma membrane that acts to maintain very low cytoplasmic Ca^{2+} concentrations [14–17].

The present study was designed to distinguish between these two possibilities. The Ca^{2+}-accumulating membrane vesicles from platelets were separated into fractions enriched either in plasma membrane or intracellular membranes in order to investigate whether the platelet plasma membrane also contains a (Ca^{2+} + Mg^{2+})-dependent ATPase involved in the transport of cytoplasmic Ca^{2+} to the outside of the cell.

Materials and Methods

Isolation and labeling of human platelets. Human blood platelets were isolated within 20 h after collection from citrated blood. The platelets were separated from plasma and washed once with 0.15 M NaCl, 2.7 mM EDTA (pH 6.8) by centrifugation at 2100 × *g* for 10 min at room temperature. The sediment was resuspended in the same solution and most of the remaining white and red blood cells were removed by centrifugation at 250 × *g* for 5 min. The supernatant was carefully decanted and recentrifuged at 2100 × *g* for 10 min to sediment the platelets.

Washed packed platelets (approx. $8 \cdot 10^{10}$/ml) were suspended in 0.13 M NaCl, 2.7 mM EDTA, 1 μM prostaglandin E_1 and 40 mM histidine (pH 6.5) to a concentration of $5 \cdot 10^9$/ml. Platelet surface glycoproteins were labeled with ^3H by a modification of the periodate-sodium boro[^3H]hydride ([^3H]$NaBH_4$) method as reported by Steiner et al. [18]. Briefly, the periodate oxidation was performed at pH 6.5, then usually $1.3 \cdot 10^{12}$ platelets were labeled with 5 mCi (185 MBq) [^3H]$NaBH_4$ at pH 8.0 in a total volume of 100 ml.

Preparation of platelet membrane vesicles. Platelet membrane vesicles were prepared according to the method of Käser-Glanzmann et al. [8] with modifications as indicated below. Labeled packed platelets (approx. $8 \cdot 10^{10}$/ml) were suspended in three volumes of homogenizing medium [7,8] containing 30 mM KCl, 5 mM $MgCl_2$, 10 mM potassium oxalate, 2.7 mM Na_2ATP, 0.5 mM *N*-carbobenzoxyglutamyltyrosine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 40 mM histidine (pH 6.8). This suspension was sonicated in 20 ml aliquots on ice two times for 45 s at 30 s interval with a Branson Sonifier B-30 at position 5, 50% interval, pulsating. The disrupted platelets were centrifuged at 14000 × g_{av} for 20 min at 4°C. The supernatant was recentrifuged at 40000 × g_{av} for 60 min and this sediment was suspended in buffer A containing 50 mM KCl, 2 mM $MgCl_2$, 2 mM Na_2ATP, 2 mM EGTA and 40 mM histidine (pH 6.8) by gentle homogenization with a glass homogenizer. The resuspended 40000 × *g* sediment was then centrifuged at 4800 × g_{av} for 10 min to remove vesicle aggregates. The protein concentration of the supernatant, referred to as '40000 × *g* membranes' was adjusted to 10 mg/ml with buffer A.

Affinity chromatography on WGA-Sepharose 4B. Wheat germ agglutinin (WGA) was isolated from fresh wheat germs by the method of Bloch et al. [19] and coupled to Sepharose 4B (Pharmacia) according to March et al. [20]. This affinity gel contained 1 mg bound wheat germ agglutinin per ml gel.

In a typical experiment, 70 mg of the 40000 × *g* membranes were applied to 26 ml WGA-Sepharose 4B. The affinity chromatography was performed at 4°C, however, after the vesicle suspension had entered the gel, the flow was stopped and the column was incubated for 40 min at room temperature. Just before eluting the bound material with *N*-acetylglucosamine, the column was again incubated for 20 min at room temperature. For more details see legend to Fig. 1.

Enzyme assays. All membrane fractions were treated similarly to permit accurate comparison of enzyme activities. Fractions I and II as well as an aliquot of the 40000 × *g* membranes, diluted 1 : 2 with buffer A, were centrifuged at 150000 × g_{av} for 50 min at 4°C. The sediments were suspended in buffer B containing 80 mM NaCl, 15 mM KCl, 18 mM histidine and 18 mM imidazole (pH 7.1, 25°C) and homogenized with a glass homogenizer. The protein concentration of the membrane sus-

pensions was adjusted to 1 mg/ml. The suspensions were frozen at −70°C until analyzed. Triton X-100 was included in all assays to render the tested enzymes accessible for the respective substrates.

Mg^{2+}-ATPase, $(Na^+ + K^+ + Mg^{2+})$-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase. These three enzyme activities were determined in a set of six different assays according to Raess and Vincenzi [21]. The basic ATPase incubation medium contained in a final volume of 1 ml: 100 µl membrane vesicle suspension (0.1 mg protein), 0.018% (w/v) Triton X-100, 80 mM NaCl, 15 mM KCl, 0.1 mM EGTA, 18 mM histidine and 18 mM imidazole (pH 7.1, 25°C); in addition, according to the respective ATPase assay: 0.1 mM ouabain (Fluka), 0.15 mM $CaCl_2$, 3 mM MgATP or 3 mM Na_2ATP. The membranes were preincubated for 5 min in the above medium without ATP and the reaction was started by adding Na_2ATP or MgATP. After incubation for 30 or 60 min at 37°C under stirring the reaction was stopped by addition of 0.5 ml 10% (w/v) SDS solution.

The P_i content of each sample was determined by a modification of the method of Chen et al. [22]. Of the sample, 0.4 ml were added to 1 ml of a mixture containing 0.35% (w/v) ammonium heptamolybdic acid, 0.43 M sulfuric acid and 1.4% (w/v) ascorbic acid. After incubation for 20 min at 30°C the reaction was stopped by transferring the tubes into an ice bath. The absorption was measured at 825 nm within 2 h in a Cary 219-spectrophotometer. Potassium hydrogenphosphate (KH_2PO_4), in the presence of Triton X-100 and SDS, was used as a standard.

Glucose-6-phosphatase. This enzyme was assayed according to the method of Aronson et al. [23]. The enzyme activity was measured in the presence or absence of 0.009% (w/v) Triton X-100 at pH 6.1. The released P_i was determined as described for the ATPase assays.

Phospholipase A_2. The substrate used in this enzyme assay, L-α-1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine (New England Nuclear) with a specific activity of 54.5 mCi/mmol, was dissolved in toluene/ethanol (1 : 1) and stored at −20°C until used. The incubation medium contained in a final volume of 0.5 ml: 200 µg protein, 40 mM KCl, 0.072% (w/v) Triton X-100, 25 mM EDTA or 1 mM $CaCl_2$, 2.4 µM substrate and 60 mM Tris-HCl (pH 9.0). In some experiments calmodulin was added to the incubation medium. The mixture was incubated at 37°C for 1 h and the reaction was stopped by transferring the tubes to an ice bath. The released [1-^{14}C]arachidonic acid was then immediately analyzed by thin-layer chromatography according to the methods of Renkonen et al. [24] and Kramer et al. [25].

Other procedures. SDS-polyacrylamide gel electrophoresis: The proteins of the platelet membrane fractions were prepared for electrophoresis as described by Clemetson et al. [26]. Proteins were electrophoresed through 1.5 mm thick 5 to 20% polyacrylamide exponential gels according to O'Farrell [27] with 40 mA constant current for 4 h. The gels were then stained for 2 h with 0.03% Coomassie blue in the presence of 25% isopropanol and 10% acetic acid. Some gels were also prepared for fluorography as described previously by Steiner et al. [18].

The free Ca^{2+} concentration of different assay mixtures was calculated with an iterative computer program using pH, total concentrations of Ca^{2+}, Mg^{2+}, EGTA, and Na_2ATP and the appropriate association constants [28] for the interactions of EGTA and ATP with H^+, Ca^{2+} and Mg^{2+} ions.

The protein content was determined by a modification of the method of Bradford [29]. Membrane containing fractions were first solubilized with 1.06% (w/v) Triton X-100, then diluted to 0.106% Triton X-100. Of this solution, 0.1 ml were added to 1 ml 1 : 5 diluted dye reagent concentrate (Bio-Rad). The absorbance was read at 595 nm against a blank containing the same final Triton X-100 concentration as the samples to correct for the background color caused by the detergent itself. For the quantitation of protein a standard curve was prepared according to Gogstad et al. [30] using a dilution series of human gamma globulin in the presence of the same amount of Triton X-100. The obtained curve was linear up to a protein concentration of 0.12 mg/ml.

The ^{3}H-radioactivity was measured as described previously by Steiner et al. [18].

Other materials. Calmodulin, isolated from bovine brain, was a gift from Drs. J. and S. Stieger-Luterbacher, University of Berne, Switzerland.

Results

Fractionation of the 40 000 × g membranes on WGA-Sepharose 4B

Theoretically, four types of vesicles could be expected in the calcium-accumulating membrane fraction referred to as '40 000 × g membranes'; rightside-out and inside-out vesicles derived from the plasma membrane and rightside-out and inside-out vesicles originating from inner membranes. In order to separate the vesicles with sugar residues on their outside from all other vesicles, the 40 000 × g membranes were further fractionated by WGA-affinity chromatography. As shown in Fig. 1, most of the applied protein (approx. 80%) did not bind to the affinity gel and was eluted in fractions 9 to 23. Fractions 9 to 13 (Fraction I) were turbid and contained therefore mainly membrane bound proteins, wheras fractions 14 to 23 were clear and contained soluble proteins. A subsequent wash of the column with 0.6 M KCl to remove nonspecifically bound material usually eluted less than 1% of the initially

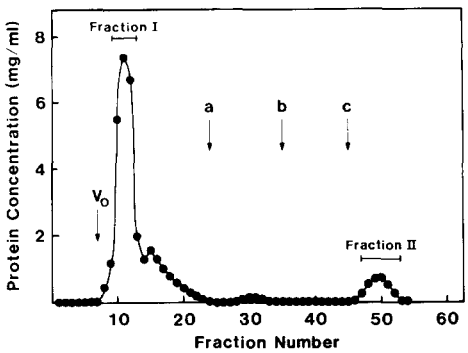

Fig. 1. WGA-Sepharose 4B affinity chromatography of 40 000 × g membranes. 40 000 × g membranes were applied to a column (5.3 cm^2 × 5 cm) containing WGA-Sepharose 4B previously equilibrated with buffer A at 4°C. The effluent was monitored by absorbance measurements at 280 nm and collected in 2 ml fractions. The protein content of each fraction was determined. The turbid, membrane-containing fractions 9–13 were pooled (→ Fraction I) as well as the bound material eluted in fractions 47–53 (→ Fraction II). After elution of the nonbinding material, the flow rate was increased from 3.0 $ml \cdot h^{-1} \cdot cm^{-2}$ to 8.4 $ml \cdot h^{-1} \cdot cm^{-2}$. The elution buffer was changed from buffer A to (a) buffer A containing 0.6 M KCl; (b) back to buffer A and (c) to buffer A containing 30 mg/ml *N*-acetylglucosamine.

TABLE I

PROTEIN AND RADIOACTIVITY CONTENT OF FRACTIONS OBTAINED DURING PURIFICATION OF MEMBRANE VESICLES

Sialic acid containing glycoproteins of the platelet surface were labeled by a modification of the periodate-boro[^{3}H]hydride method. Fractions I and II were isolated by affinity chromatography on WGA-Sepharose 4B as described in Material and Methods.

Fraction	Protein		Radioactivity		Spec. activity	Factor of PM-enrichment [a]
	mg	(%)	cpm (×10^{-6})	(%)	cpm/mg	
Fractionation of platelets:						
Platelet lysate	1900	(100)	257	(100)	135000	1.0
14000 × g sediment	900	(47)	122	(47)	135000	
14000 × g supernatant	1000		137		137000	1.0
40000 × g sediment	161	(8)	56	(22)	350000	2.6
40000 × g supernatant	830	(44)	82	(32)	99000	
Affinity chromatography:						
40000 × g membranes [b]	73	(100)	26	(100)	359000	2.7
Fraction I	48	(66)	18	(69)	376000	2.8
Pool of fractions 14–35	10	(14)	1	(3)	80000	
Fraction II	5	(7)	4	(17)	859000	6.4
After centrifugation (for enzyme assays):						
40000 × g membranes					574000	4.3
Fraction I					529000	3.9
Fraction II					927000	6.9

[a] Enrichment of plasma membrane (PM)-protein compared to whole platelets.

[b] Supernatant after the 40000 × g sediment was resuspended and centrifuged at 4800 × g_{av} for 10 min.

applied protein. After the column was reequilibrated in buffer A, the specifically bound material, representing about 7% of the total protein, was eluted in fractions 47 to 53 (Fraction II) by including *N*-acetylglucosamine in buffer A. The results of a typical fractionation experiment are shown in Table I. The specific activity of the surface label was 6.4-fold higher in Fraction II as compared to the platelet lysate. In five independent experiments the enrichment of plasma membrane protein in Fraction II was 6.3 ± 0.5. Since the carbohydrate of plasma membranes is exclusively confined to the outer membrane surface [31], the plasma membrane vesicles in Fraction II were judged to have a rightside-out orientation.

About 70% of the ^{3}H-labeled plasma membrane vesicles of the $40\,000 \times g$ membranes were unable to bind to the lectin, perhaps because of their inside-out orientation or because of other factors which exclude the retention in the column. Almost 90% of the protein and the radioactivity applied to the WGA-Sepharose 4B column were usually recovered. Without EGTA in buffer A and without purification of the $40\,000 \times g$ sediment the percentage of protein recovered in the flow through decreased to about 55% instead of 80%, probably due to vesicle aggregates, whereas the percentage of protein recovered in Fraction II was approximately the same.

In a control experiment, the $40\,000 \times g$ membranes were applied to a Sepharose 4B column; no binding was detected but the recovery of protein in this particular experiment was 100%. In another set of control experiments, different amounts of the $40\,000 \times g$ membranes (20–70 mg) were applied to the WGA-affinity column. The protein content of the bound material (Fraction II) was always between 5 and 8% of the applied protein (data not shown). Therefore, the separation of $40\,000 \times g$ membranes in two plasma membrane containing fractions (Fraction I and II) was due to specific binding to the immobilized wheat germ agglutinin. A similar percentage of bound material was obtained using Con A-Sepharose 4B as affinity gel. Since concanavalin A requires the presence of Ca^{2+} for binding activity, it could not be used together with EGTA-containing buffers.

For further characterization an aliquot of the $40\,000 \times g$ membranes as well as Fraction I and Fraction II were centrifuged and the resulting sediments were resuspended. As shown in Table I all three analyzed fractions revealed an increase of the specific activity of the surface label. The plasma membrane proteins in Fraction II were almost 7-fold enriched compared to intact platelets.

Fig. 2 shows the SDS-polyacrylamide gel separations of these three fractions. The glycoproteins (GP) IIb and IIIa were most enriched in Fraction II, whereas this fraction contained less actin (M_r

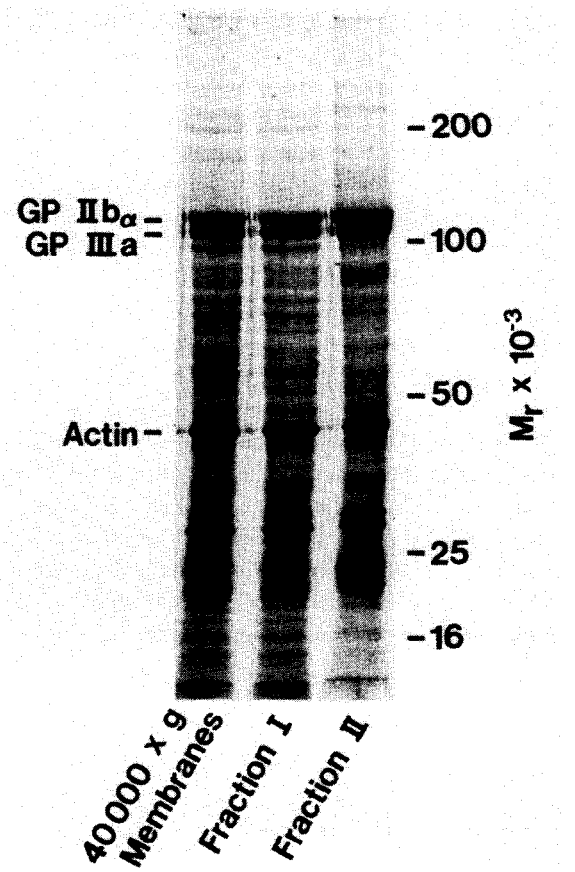

Fig. 2. SDS-polyacrylamide gel electrophoretic separations of the proteins of the $40\,000 \times g$ membranes, of Fraction I and Fraction II. After fractionation of the $40\,000 \times g$ membranes, Fraction I and Fraction II as well as an aliquot of the $40\,000 \times g$ membranes were centrifuged at $150\,000 \times g_{av}$ for 50 min at 4°C and resuspended in buffer B. 60 μg of protein of each fraction were treated with SDS, reduced with dithiothreitol, electrophoresed through 5 to 20% polyacrylamide exponential gels and stained with Coomassie brilliant blue. The positions of the glycoproteins (GP) IIb and IIIa, of actin and of some molecular weight standards are indicated.

= 43 000) than the 40 000 × *g* membranes or Fraction I. There are some other major differences between these fractions, e.g. three proteins, with apparent molecular weights of about 200 000, 100 000 and 55 000 are strongly reduced in Fraction II compared to the two other fractions. In the molecular weight range of about 30 000 an intense double band can be seen in Fraction II, whereas in the two other fractions the upper of these two bands is only faint.

In a control experiment the same amount of radioactivity from each of the three fractions was loaded on 7.5% polyacrylamide gels. Fluorograms of these gels showed that the ^{3}H-labeled glycoproteins were present in the same total and relative quantities, even though the samples contained different amounts of total protein (data not shown). This indicates that the membrane fraction isolated by affinity chromatography (Fraction II) had a similar glycoprotein-composition as the 40 000 × *g* membranes and Fraction I.

Enzyme activities

In order to open sealed vesicles, Triton X-100 was included in all assay mixtures. For each enzyme assay the optimal Triton X-100 to protein ratio (w/w) was determined. As an example, the specific activity of the Mg^{2+}-ATPase is shown in dependence of this ratio (Fig. 3). At a ratio of 1.8, the Mg^{2+}-ATPase activity reached a maximum, about 1.5-fold higher than in the absence of detergent. At higher ratios the enzyme activity was inhibited by Triton X-100. Therefore all ATPase activities were measured in the presence of 0.018% (w/v) Triton X-100 (→ ratio: 1.8). This concentration is slightly lower than the cmc (critical micellar concentration) of Triton X-100 [32]. From these data, however, it is not possible to conclude, whether the increase of the Mg^{2+}-ATPase activity is only achieved through opening of sealed vesicles or also through stimulation of the enzyme activity by the detergent itself.

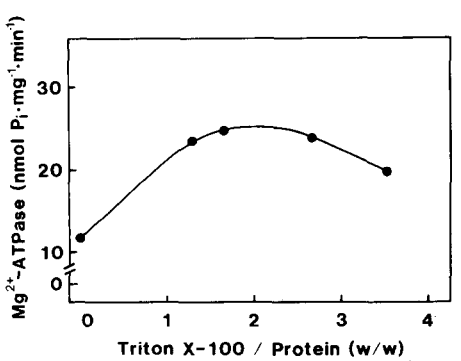

Fig. 3. Effect of Triton X-100 on Mg^{2+}-ATPase activity. The Mg^{2+}-ATPase activity of the 40 000 × *g* membranes was measured in the presence of different Triton X-100 concentrations. The enzyme activity is shown in dependence on the detergent to protein ratio.

The three ATPase activities of platelet membranes were differentiated according to their requirement for mono- and/or divalent cations. The ouabain-sensitive ($Na^+ + K^+ + Mg^{2+}$)-dependent ATPase as well as the surface label were used as markers for the plasma membrane. Glucose-6-phosphatase, an enzyme of the endoplasmic reticulum [33], and phospholipase A_2, an enzyme located exclusively in platelet inner membranes [34], were used as inner membrane markers. Glucose-6-phosphatase activity was inhibited by all Triton X-100 concentrations tested. With 0.009% (w/v) Triton X-100 in the incubation medium the inhibition of the specific activity was about 50%. However, higher Triton X-100 concentrations did not further inhibit this enzyme activity and the percentage of inhibition at a given Triton X-100 concentration was independent of the enzyme concentration used.

Table II shows the distribution of these markers in subcellular platelet fractions. The ($Na^+ + K^+ + Mg^{2+}$)-ATPase is in fact a useful marker for the platelet plasma membrane; its distribution is very similar to that of the surface label. It appears that the percentage of plasma membrane protein to total protein is almost twice as high in Fraction II compared to that in Fraction I. According to the results obtained for the glucose-6-phosphatase and the phospholipase A_2 activities, the percentage of membrane proteins derived from inner membranes is about 3-times lower in Fraction II compared to that in Fraction I. When glucose-6-phosphatase activity was measured in the absence of inhibiting Triton X-100, its specific activity in Fraction II was 25%, instead of 38%, of that measured in Fraction I. In some experiments the sealed vesicles were opened by the 'freeze-thaw' method described by Kaulen and Gross [35], whereupon the activity rose to about 31%, indicating, that after

TABLE II

DISTRIBUTION OF ENZYME ACTIVITIES AND OF SPECIFIC ACTIVITY OF THE SURFACE LABEL IN SUBCELLULAR FRACTIONS OF PLATELETS

The highest specific activity of one particular 'marker' found in the three analyzed fractions was referred to 100%. The specific activities of the same 'marker' measured in the two other fractions are expressed as a percentage to this highest specific activity. Details of each enzyme assay are given in Materials and Methods. Each value is the mean ± SD of 3–5 independent experiments.

	40000 × *g* membranes (%)	Fraction I (%)	Fraction II (%)
Mg^{2+}-ATPase	86 ± 15	100 ± 2	77 ± 14
$(Na^+ + K^+ + Mg^{2+})$-ATPase	66 ± 6	56 ± 6	100 ± 0
$(Ca^{2+} + Mg^{2+})$-ATPase	90 ± 7	100 ± 4	29 ± 5
Glucose-6-phosphatase	91 ± 5	100 ± 0	38 ± 3
Phospholipase A_2	86 ± 2	100 ± 0	27 ± 3
Surface label	62 ± 4	57 ± 3	100 ± 0

this treatment more of the enzyme molecules in Fraction II were accessible for the substrate. The Mg^{2+}-ATPase activity seems to be present in both the plasma membrane and the inner membranes.

Among the three analyzed fractions, the specific activity of the $(Ca^{2+} + Mg^{2+})$-ATPase showed a distribution quite similar to the two inner membrane markers, glucose-6-phosphatase and phospholipase A_2. It appears therefore, that the platelet $(Ca^{2+} + Mg^{2+})$-dependent ATPase is located exclusively in membranes of intracellular origin.

In some experiments Ca^{2+} uptake by Fraction I and II, rather than $(Ca^{2+} + Mg^{2+})$-ATPase activity, was measured immediately after the fractionation of the 40000 × *g* membranes by WGA-affinity chromatography. Fraction I revealed normal Ca^{2+}-uptake activity, i.e. 100–150 nmol $Ca^{2+} \cdot mg^{-1} \cdot h^{-1}$ as reported previously [8], whereas Fraction II showed no Ca^{2+}-accumulating activity.

Kinetic properties of the $(Ca^{2+} + Mg^{2+})$-ATPase in the presence of Triton X-100

In order to investigate whether the kinetic properties of the $(Ca^{2+} + Mg^{2+})$-ATPase are affected by the isolation and assay conditions, the dependence of this enzyme activity on the free Ca^{2+} concentration as well as the effect of the calcium-modulator calmodulin were studied. In the presence of Triton X-100 maximal activation of $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured at a free Ca^{2+} concentration of 15 μM (Fig. 4). Kinetic analysis revealed a K_m for free Ca^{2+} of 5.6 μM and a V_{max} of 15 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$. It has been reported that the apparent K_m for Ca^{2+}-activation of the ATPase is in the range of 0.1–1 μM and that the specific activity in platelet membranes is between 20 and 50 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$ [8,36]. The similarity of these values suggests that the enzyme was only slightly affected by the prolonged EGTA-treatment of the membranes during

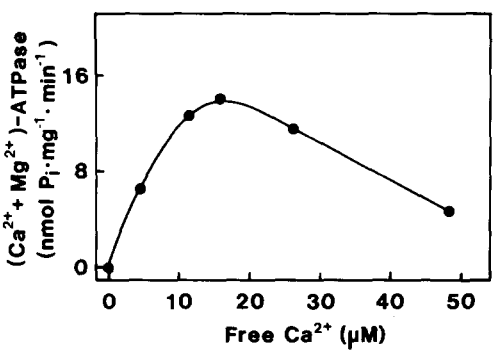

Fig. 4. $(Ca^{2+} + Mg^{2+})$-ATPase activity of platelet inner membranes: dependence on free calcium concentration in the presence of Triton X-100. This ATPase activity of Fraction I was assayed in a medium containing 80 mM NaCl, 15 mM KCl, 0.1 mM ouabain, 0.018% (w/v) Triton X-100, 0.1 mM EGTA, 3 mM Mg-ATP, 18 mM histidine, 18 mM imidazole, (pH 7.1), varying free Ca^{2+} concentrations from 0 to 50 μM (calculated as described in Materials and Methods) and 100 μg of membrane protein in a volume of 1 ml at 37°C. The membranes were preincubated for 5 min in the above medium without ATP and the reaction was started by addition of 3 mM MgATP. After 60 min, the reaction was stopped by addition of 0.5 ml 10% (w/v) SDS and the P_i released was determined as described in the text. The ordinate represents net $(Ca^{2+} + Mg^{2+})$-dependent ATPase, i.e. the difference between ATPase activity in the presence and absence of Ca^{2+}.

isolation and by the inclusion of Triton X-100 in the enzyme assays. Bovine brain calmodulin in the range of 2–10 μg/ml was unable to stimulate the $(Ca^{2+} + Mg^{2+})$-ATPase over a range of 10^{-4} M to 10^{-8} M Ca^{2+}. In some experiments Triton X-100 was omitted, because it is known that detergents can alter the calmodulin sensitivity of enzymes [37]. In none of these assays a stimulation by calmodulin was detectable. This supports the observation of Dean et al. [36] who found no significant stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase by calmodulin after washing platelet membranes with 2 mM EGTA for 20 min.

The phospholipase A_2 activity in our membrane fractions could be stimulated by the addition of calmodulin. At a Ca^{2+} concentration of 0.2 mM, 1.2 μM calmodulin from bovine brain stimulated the enzyme activity more than 3-fold in the presence of Triton X-100, without reaching the activity measured at 1 mM Ca^{2+} in the absence of calmodulin. The observed calmodulin-dependence of the phospholipase A_2 is in agreement with the findings reported by Wong et al. [38].

Discussion

The major aim of this study was to determine whether the platelet plasma membrane contains an energy-dependent 'calcium pump'. Such activity would be capable of actively expelling Ca^{2+} ions to the outside of platelets, as has been demonstrated in many other cells. Previously, it has been shown that membrane vesicles from platelets have Ca^{2+}-accumulating activity [6–10]. Since this membrane preparation contains inner membranes as well as considerable amounts of plasma membrane [9], it was not known whether this Ca^{2+}-uptake activity was associated with inner membranes and/or the plasma membrane. Vesicles derived from the plasma membrane could only contribute to Ca^{2+}-transport if their configuration is inside-out. However, inside-out plasma membrane vesicles could easily result during homogenization by resealing of fragments of the open canalicular system. In order to distinguish between these possibilities, we designed a procedure to isolate pure platelet plasma membrane and tested this preparation for $(Ca^{2+} + Mg^{2+})$-dependent ATPase activity, which is a prerequisite of an energy-dependent Ca^{2+}-transport.

Isolation of platelet plasma membrane

Platelet plasma membrane has been isolated by many different techniques (for review, see Ref. 33; also Refs. 39–42). Kambayashi et al. [42] used WGA-agarose affinity chromatography of lysed platelets and found this an easy way to obtain a sample that was enriched in plasma membrane. This sample, however, showed a higher specific activity of glucose-6-phosphatase than the lysed platelets used as the starting material, indicating that it was contaminated with endoplasmic reticulum. In contrast, we observed that this enzyme was primarily associated with Fraction I, i.e. the inner membranes (cf. Table II). A similar discrepancy was found with respect to the plasma membrane marker, $(Na^+ + K^+ + Mg^{2+})$-ATPase. Kambayashi et al. [42] found the highest specific activity in lysed platelets. In the present study, this enzyme clearly showed an accumulation in the plasma membrane fraction. Such discrepancies could arise either from differences in methods employed or because enzyme activities were either stimulated or inhibited by the procedures used in membrane isolation.

The development of mild procedures to label the surface of cells, such as the periodate-boro[^{3}H]hydride method [43] or the lactoperoxidase-iodination technique [44], has provided a useful tool to evaluate the plasma membrane enrichment in isolated fractions. This evaluation method appears to circumvent the mentioned stimulation-inhibition problem with enzyme activity measurements. In our study the platelet surface was labeled by the periodate-boro[^{3}H]hydride method. This labeling procedure has advantages compared to others: (1) the radioactivity is covalently incorporated into glycoproteins and can therefore not easily be exchanged during fractionation; and (2) the labeling method does not change the surface charge of the cell. Fraction II showed an almost 7-fold higher specific activity of the radioactive label than whole platelets. It appears unlikely that this enrichment in surface label is caused by elution of unlabelled membrane proteins by repeated washing because the specific activity of whole labeled platelets, i.e. after the platelets have been washed eight times, was used as reference value. In fact, the plasma membrane enrichment in Fraction II may even be slightly higher, since during the

first steps of fractionation no attempt was made to completely block the Ca^{2+}-activated proteinase [45], which is known to cleave soluble glycocalicin from GP Ib [46]. Although some of the GP Ib may have been lost during lysis by ultrasonication, even though the EDTA concentration was 0.7 mM, it was still the most intensely labeled glycoprotein in all three analyzed fractions.

Surface labeling can be used to determine the plasma membrane enrichment in a particular fraction compared to whole platelets, however, this evaluation method by itself does not allow the assessment of the absolute purity of an isolated membrane fraction. We therefore developed a mathematical approach to analyze the obtained distribution of several membrane marker activities in the three analyzed fractions. These three fractions were treated similarly in order to permit accurate comparison of enzyme activities. From the distribution of the two plasma membrane markers, $(Na^+ + K^+ + Mg^{2+})$-ATPase and surface label, and the three markers for inner membranes, glucose-6-phosphatase, phospholipase A_2 and $(Ca^{2+} + Mg^{2+})$-ATPase, shown in Table II, two independent functions of the form $y = f(x)$ can be generated:

For the plasma membrane markers: * $y_1 = 0.64 \cdot x_1$

For the inner membrane markers: ** $y_2 = 2.94 \cdot x_2 - 204$

* On average the specific activity of a plasma membrane marker found in the $40000 \times g$ membranes is 64% compared to that in Fraction II. The function for the plasma membrane markers can therefore be expressed as

$y_1 = 0.64 \cdot x_1$.

** On average the specific activity of an inner membrane marker found in Fraction II is 34% compared to that in the $40000 \times g$ membranes. By referring 'a' to the percentage of inner membrane protein in Fraction II and 'b' to the percentage of inner membrane protein in the $40000 \times g$ membranes, the following function can be generated

$a = 0.34 \cdot b$

x can be substituted for a by using the relation $x = 100\% - a$, assuming that Fraction II only contains proteins derived from the plasma membrane and the inner membrane. y can be substituted for b by using the relation $y = 90\% - b$, assuming that 10% of the total protein in the $40000 \times g$ membranes (as prepared for the enzyme assays) originate from other sources than the plasma membrane and inner membranes. The above function can now be expressed as

$y_2 = 2.94 \cdot x_2 - 204$

x: percentage of plasma membrane protein to total protein in Fraction II;
y: percentage of plasma membrane protein to total protein in the $40000 \times g$ membranes.

The intersection of these two linear functions has the following coordinates: 89% for 'x' and 57% for 'y'. That means, 89% of the total protein in Fraction II originated from the plasma membrane and accordingly 11% from the inner membranes. The contamination by inner membranes is probably due to multilamellar vesicles where a inner membrane vesicle is enclosed within a plasma membrane vesicle. Such multiple vesicles would bind to wheat germ agglutinin but could not take up calcium. For the calculations shown above we assumed that 10% of the total protein in the $40000 \times g$ membranes, as prepared for the enzyme assays, originated from other sources than the plasma membrane or the inner membranes (cf. footnote **). Under the assumption that the $40000 \times g$ membranes contain no other such proteins, the purity of plasma membrane proteins in Fraction II (x) would be 84%; with a contamination of 20% of other proteins, x would be 93%. This indicates, that approx. 90% of the proteins in Fraction II originated from the plasma membrane and 10% from inner membranes.

Since the surface label in Fraction II was found to be 7-fold enriched and, according to our mathematical evaluation method, about 90% of the total protein in Fraction II are plasma membrane proteins, a plasma membrane marker can be enriched about 8-fold and the percentage of plasma membrane protein to total platelet protein appears to be approx. 12%. In a different approach, Sixma et al. [33] have estimated that 20 to 25% of the total platelet protein are present in the plasma membrane.

Localization of the platelet $(Ca^{2+} + Mg^{2+})$-ATPase

Analysis of the obtained distribution of marker enzyme activities in subcellular fractions indicate that the plasma membrane of human platelets, unlike that of most other cells, contains no $(Ca^{2+}$

+ Mg^{2+})-dependent ATPase. It appears likely, that the platelet 'Ca^{2+} pump' is located in the dense tubular system. This pump activity is believed to actively remove Ca^{2+} from the cytoplasm and to sequester it within this internal membrane structure [47]. This conclusion is based on the following observations: (1) among the three analyzed fractions the (Ca^{2+} + Mg^{2+})-ATPase showed a similar distribution pattern as the two inner membrane markers, phospholipase A_2 and glucose-6-phosphatase; (2) glucose-6-phosphatase is a specific marker for endoplasmic reticulum or dense tubular system; (3) the $40000 \times g$ membranes are devoid of mitochondria and secretory granules [8] and do therefore mainly contain plasma membrane vesicles and vesicles derived from the dense tubular system.

Histochemical studies of platelets demonstrated, that the (Ca^{2+} + Mg^{2+})-ATPase is located in the dense tubular system as well as in the invaginations of the platelet plasma membrane, the open canalicular system [13]. Since this membrane system probably formed inside-out plasma membrane vesicles after sonication of whole platelets, these vesicles could not bind to the lectin and were therefore eluted in Fraction I together with the dense tubular system membrane vesicles. However, if these inside-out plasma membrane vesicles would contribute to the total (Ca^{2+} + Mg^{2+})-ATPase activity measured in Fraction I, the difference between the specific activities of Fraction I and Fraction II should be greater for this enzyme than that for the two inner membrane markers, glucose-6-phosphatase and phospholipase A_2. Since these differences are similar for all three enzymes, as shown in Table II, it appears rather unlikely that the open canalicular system also contains (Ca^{2+} + Mg^{2+})-ATPase activity. This conclusion is based on the assumption that the ATPase of the open canalicular system shows the same properties than the one which is located in the dense tubular system. However, if one assumed, that only certain domains of the plasma membrane would contain a (Ca^{2+} + Mg^{2+})-ATPase and that these domains, unlike the open canalicular system, would constitute only a very low percentage of the total surface area of platelets, the distribution of this enzyme (Table II), would probably not be altered significantly. Therefore, this possibility cannot be excluded, although no evidence for an uneven distribution of plasma membrane proteins has as yet been provided.

Menashi et al. [12] presented evidence that the ATP-dependent Ca^{2+}-accumulating activity is almost exclusively associated with an intracellular membrane fraction. The low percentage of Ca^{2+}-transport associated with their plasma membrane fraction is probably due to a slight contamination by inner membranes [41]. However, the absence of Ca^{2+}-uptake activity in this particular fraction does not yet prove that the plasma membrane has no 'Ca^{2+} pump', because a membrane vesicle has to be sealed as well as in the right configuration to be able to take up calcium.

The data of this study suggest that the platelet (Ca^{2+} + Mg^{2+})-dependent ATPase mediates Ca^{2+}-removal from the cytoplasm by transporting Ca^{2+} into the dense tubular system, but not by expelling Ca^{2+} to the outside of the cell. Ca^{2+} efflux across the plasma membrane, as recently demonstrated by Brass [47], must therefore be mediated by another mechanism. Na^+-Ca^{2+} exchange, a common transport mechanism in other tissues, appears to be a minor contributor to the Ca^{2+} efflux, at least in resting platelets [48]. The glycoprotein IIb-IIIa complex may be involved in Ca^{2+} transport, because platelets from patients with Glanzmann's thrombasthenia showed a markedly decreased Ca^{2+} exchange [49]. These platelets have no or just a few GP IIb-GP IIIa complexes in the plasma membrane. The mechanism of this interesting Ca^{2+} transport, however, is not yet understood.

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