Further characterization of the plasma membrane- and intracellular membrane-associated platelet Ca^{2+} transport systems

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Biochemical characterization of the Ca^{2+} -ATPases isolated from human platelet intracellular and plasma membranes is reported. A comparative study of the previously partly described plasma membrane Ca^{2+} -ATPase [Enouf, Bredoux, Bourdeau & Levy-Toledano (1987) *J. Biol. Chem.* **261**, 9293–9297] and the intracellular membrane Ca^{2+} -ATPase obtained simultaneously shows differences in the following parameters: (1) different kinetics of the two enzymes; (2) similar apparent affinity towards Ca^{2+} (10^{-7} M), though the intracellular membrane enzyme was inhibited at Ca^{2+} concentrations above 10^{-6} M; (3) different pH dependence with an activity maximum at pH 7 for the intracellular membrane Ca^{2+} -ATPase and no detectable pH maximum for the plasma membrane Ca^{2+} -ATPase; (4) a 10-fold difference in the ATP requirement of the two Ca^{2+} -ATPases; (5) different patterns of inhibition by vanadate. Finally, the possible regulation of the Ca^{2+} -ATPases was examined by studying the effect of chlorpromazine on the two Ca^{2+} -ATPase activities, with only the plasma membrane enzyme being inhibited. It is concluded that the two platelet Ca^{2+} transport systems show biochemical differences in spite of the previously shown similarity in the molecular masses of their Ca^{2+} -ATPases, thus conferring a definite specificity to the platelet system.

INTRODUCTION

The regulation of the intracellular Ca^{2+} concentration in platelets has been thoroughly investigated during recent years and well-established data have been published. These include the increase in Ca^{2+} concentration associated with platelet activation as detected by fluorescent Ca^{2+} indicators [1]; the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release phenomenon shown by extensive studies on platelet membrane transduction [2–7]; and the existence of a Ca^{2+} pump (i.e. a Ca^{2+} -ATPase which mediates Ca^{2+} transport) located on intracellular membranes (dense tubular system) which has been demonstrated by the use of different membrane purification techniques [8–17].

In contrast, the presence of a Ca^{2+} pump on the plasma membrane of platelets, as in other cells, is still questionable. In fact, the first biochemical investigations on this subject concluded that such an enzyme does not exist [12–14]. However, cytochemical and immunological studies have yielded contradictory results [18,19]. Then, based on the differences in limited trypsin proteolysis of the phosphorylated intermediates of the Ca^{2+} pumps in intracellular membrane- or plasma membrane-enriched fractions, it was proposed that in platelets, a plasma membrane-associated Ca^{2+} pump might also be present [20]. Finally, we decided to investigate the possible presence of two Ca^{2+} pumps, associated with platelet plasma membranes and intracellular membranes, using a different approach. We first proceeded to the simultaneous isolation of both types of membranes and characterized their Ca^{2+} uptake [21], as well as the sensitivity of their Ca^{2+} transport to trypsin [22], which

allowed us to differentiate between the two Ca^{2+} transport systems.

However, these results also showed that the two Ca^{2+} -ATPases exhibited similarities in their molecular mass and in their recognition by an anti-(sarcoplasmic reticulum Ca^{2+} -ATPase) antibody. Thus it seemed important to further characterize the plasma membrane and intracellular membrane Ca^{2+} -ATPases. We have previously described a partial characterization of the Ca^{2+} -ATPase activity associated with the plasma membrane [21]. Because of some similarities between these characteristics and those of intracellular Ca^{2+} -ATPase [15–18], it seemed necessary to compare the plasma membrane and intracellular membrane Ca^{2+} -ATPases under our experimental conditions. The data thus obtained are completed by the study of the inhibition of the Ca^{2+} -ATPases by vanadate, and the comparison of their possible regulation by calmodulin.

MATERIALS AND METHODS

Preparation of platelet plasma membrane and intracellular membrane fractions

Human platelets were freshly isolated and washed in modified Tyrode's buffer. The cells were then disrupted by controlled ultrasonication in a lysis buffer containing 0.8 mg of benzamidine/ml, 0.1 unit of aprotinin/ml, 4.8 μg of leupeptin/ml, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-dithiothreitol and 1 mM-EGTA. Lysed platelets were centrifuged at 19000 *g* to eliminate unlysed platelets, mitochondria and α -granules. The supernatant

Abbreviation used: Ca^{2+} -ATPase, Ca^{2+} -activated, Mg^{2+} -dependent adenosine triphosphatase.

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was centrifuged at 100000 *g* and the pellet obtained, consisting of mixed membrane vesicles [11,23], was then layered over a 40 % (w/v) sucrose solution and centrifuged again at 100000 *g* for 120 min. The two membrane subfractions (interface and pellet fractions) obtained were characterized by immunological and biochemical parameters [24]. Briefly, weak [³H]concanavalin A labeling, a low phosphodiesterase activity and a lower ratio of cholesterol/phospholipid were observed in the pellet membrane fraction. Electrophoresis of the two membrane subfractions resulted in different protein profiles with different proportions of high and low molecular mass constituents. A significant difference between the membrane subfractions was the enrichment of the pellet membrane fractions in protein(s) of about 55 kDa. Such an observation is also made in the protein profile of intracellular membranes isolated by free-flow electrophoresis using Coomassie Blue [25] or silver staining [26]. Finally, by using a quantitative immunofluorescence technique [24], the IgG L antibody which specifically recognizes the platelet surface oriented glycoprotein GP IIb-IIIa was shown not to react with the pellet membrane fraction. Similar results were obtained with the glycoprotein-IIb-recognizing anti-LEK^a antibody. Finally, recent investigations gave evidence for the presence on the interface membrane fraction of GTP-binding proteins which mediate phospholipase C activity (J. Enouf, E. Corvazier & S. Levy-Toledano, unpublished work). Thus, based on these different characteristics, the two interface and pellet membrane subfractions appeared to consist of plasma membranes and intracellular membranes respectively.

Measurement of Ca²⁺-ATPase activity

The enzymic activity was tested using the Malachite Green reagent [27] as previously described. A previous characterization of this Ca²⁺-ATPase activity, performed on the mixed 100000 *g* membrane fraction, showed that the enzyme was Mg²⁺-dependent, Ca²⁺-activated and regulated by membrane phospholipids [23].

RESULTS

In order to obtain easy and complete comparison of the enzymes, we completed the previously published characterization of the plasma membrane Ca²⁺-ATPase [21] by the study of new parameters, and compared these data with those on the intracellular membrane Ca²⁺-ATPase obtained by our simultaneous isolation procedure of plasma membranes and intracellular membranes.

The Ca²⁺-activated ATPase activities of the two membrane fractions were first determined using conditions previously described for measuring the Ca²⁺-ATPase activity of the 100000 *g* membrane fraction [21], i.e. at pH 8 with 0.5 mM-ATP. The Ca²⁺-ATPase that we measured was identified as a Mg²⁺-dependent Ca²⁺-activated ATPase activity, i.e. no Ca²⁺-ATPase was detected in the absence of Mg²⁺. Thus in each experiment we calculated the Ca²⁺ activation of the Mg²⁺-ATPase activity. Plasma membranes exhibited a mean specific activity (ten different consecutive preparations) of 41 ± 12 (mean $\pm$ S.D.) nmol of ATP hydrolysed $\cdot$ min⁻¹ $\cdot$ mg⁻¹, and intracellular membranes exhibited a mean specific activity (six different consecutive preparations) of 15 ± 5 nmol of ATP hydrolysed $\cdot$ min⁻¹ $\cdot$ mg⁻¹.

Fig. 1. Time course of Ca²⁺-ATPase activities associated with plasma membranes and intracellular membranes

The membrane vesicles (10 μ g of protein) were incubated at 37 °C in 0.2 ml of reaction mixture containing 20 mM-Hepes, pH 8/100 mM-KCl/2 mM-MgCl₂/0.001 % Triton X-100/0.5 mM-EGTA in the presence or absence of 0.5 mM-CaCl₂. Both incubations were initiated by the addition of 0.5 mM-ATP (final concentration) and stopped after different times with 2 ml of Malachite Green reagent. Control experiments in the absence of Ca²⁺ were conducted without ATP addition. The Ca²⁺-activated ATPase activity was calculated by subtracting the values obtained with MgCl₂ and without adding CaCl₂ from those obtained with MgCl₂ plus added CaCl₂. The determination of P_i was obtained by a standard P_i solution. ●, Plasma membranes; ○, intracellular membranes. Results are the means $\pm$ S.E.M. for five different experiments.

Time courses of the Ca²⁺-ATPase activities

Fig. 1 shows the time courses of the plasma membrane- and intracellular membrane-associated Ca²⁺-ATPase activities. A plateau value was reached within 15 min for the intracellular Ca²⁺-ATPase, whereas 30 min was needed for the plasma membrane enzyme.

Ca²⁺ activation of the Ca²⁺-ATPase activities

Fig. 2 shows the Ca²⁺ dependence of the Ca²⁺-ATPase activities of the two different membrane fractions. It can be seen that the Ca²⁺ activation of the two Mg²⁺-ATPase activities occurs at about the same Ca²⁺ concentration (10⁻⁷ M). At higher Ca²⁺ concentrations, a negligible inhibition of the enzyme appeared with the plasma membranes when compared with the mixed membranes [21] but the inhibition was greater in the case of the intracellular membranes, leading to the enzyme inactivation at 10⁻⁴ M.

These data support the proposed localization of the two enzymes, because in intact cells, intracellular membranes are limited in their calcium content, whereas extrusion of Ca²⁺ through the plasma membrane results in its dilution in extracellular medium.

pH-dependence of the Ca²⁺-ATPase activities

Fig. 3 shows the pH sensitivity of the two enzyme activities. The plasma membrane Ca²⁺-ATPase did not appear highly sensitive to the variations in pH in the range tested, but the maximum enzyme activity seemed to be detected at pH 8 [21], whereas an optimum at

Fig. 2. Effect of Ca^{2+} on the Ca^{2+} -ATPase activities

The membrane vesicles were incubated for 10 min at 37°C under the experimental conditions described in the legend to Fig. 1 with the exception that CaCl_2 was added at different concentrations. The Ca^{2+} concentrations were calculated as reported in [23] using apparent association constants of 6.02×10^8 , 9.7×10^2 , 9.55×10^3 and $4.57 \times 10^4 \text{ M}^{-1}$ for CaEGTA , MgEGTA , CaATP and MgATP respectively. Calculations were made as described in Fig. 1 legend and results are expressed as Ca^{2+} -ATPase activity versus pCa. Δ , Plasma membranes; $\bullet$, intracellular membranes. Results are the means $\pm$ S.E.M. for three different experiments.

Fig. 3. Effect of pH on the Ca^{2+} -ATPase activities

The membrane vesicles were incubated for 10 min at 37°C in 20 mM-Hepes buffer (at different pH values)/100 mM-KCl/0.5 mM-EGTA in the presence or absence of 0.5 mM- CaCl_2 , 2 mM- MgCl_2 and 0.001 % Triton X-100. The reactions were conducted as described in the legend to Fig. 1. $\bullet$, Plasma membranes; $\circ$, intracellular membranes. Results are the means $\pm$ S.E.M. for five different experiments.

pH 7 could be observed for the intracellular membrane enzyme. In addition, the pH dependence of the intracellular Ca^{2+} pump appears as a bell shaped curve but that of the plasma membrane pump did not. This results in different ratios between the enzymic activities of the supernatant and pellet membrane fractions at different pH values. When the enzyme activities were tested at pH 8 and pH 7 for plasma and intracellular membranes respectively, the mean ratio was 1.5 (four experiments). It increased to 2 when both measurements were made at a pH above 7.5.

ATP-dependence of the Ca^{2+} -ATPase activities

Fig. 4(a) shows the effect of different concentrations of MgATP {calculated according to the method of Fabiato & Fabiato [28] using a computer program kindly provided by Dr. L. Brass (Hematology-Oncology Section, Hospital of the University of Pennsylvania, PA, U.S.A.)} on the Ca^{2+} -activated Mg^{2+} -ATPase of the

Fig. 4. Effect of ATP on the basal Mg^{2+} -ATPase and on the Ca^{2+} -ATPase activities

The membrane fractions were incubated for 10 min at 37°C under the experimental conditions described in the legend to Fig. 1 with two modifications: (1) the incubations were conducted in the presence or absence of 0.001 % Triton X-100 and (2) the incubations were conducted at pH 7 for intracellular membranes (a) and at pH 8 for plasma membranes (b) according to the results of the experiment shown in Fig. 3. The reactions were initiated by adding different ATP concentrations and conducted as described in Fig. 1. Basal Mg^{2+} -ATPase was calculated by subtracting the values obtained in the presence of MgCl_2 and ATP from those obtained in the presence of MgCl_2 without ATP. $\circ$, Ca^{2+} -ATPase activity in the presence of Triton; Δ , basal Mg^{2+} -ATPase activity in the presence of Triton. Results are the means $\pm$ S.E.M. for four different experiments.

intracellular membrane in the presence of detergent, and also on the membrane-associated basal Mg^{2+} -ATPase activity. A maximum Ca^{2+} -ATPase activity occurred at about $5 \mu\text{M}$ -MgATP ($20 \mu\text{M}$ -ATP) in the absence of Triton X-100 (results not shown), corresponding to an apparent K_m of $1.25 \mu\text{M}$ -MgATP. The response differed when enzyme activity was measured in the presence of 0.001 % Triton X-100; although the same curves were obtained up to $5 \mu\text{M}$ -MgATP, the Ca^{2+} -ATPase activity increased further at higher MgATP concentrations. This may reflect a second, low-affinity, site for ATP on the Ca^{2+} -ATPase which became apparent under these conditions. The Mg^{2+} -ATPase activity also depended on the ATP concentration and no saturation was reached, in contrast with the Ca^{2+} -activated Mg^{2+} -ATPase, even at the highest ATP concentrations used (0.1–0.2 mM).

The most striking difference for the plasma membrane Ca^{2+} -ATPase (Fig. 4b) was the shift of the response of the enzyme activity as a function of ATP concentration: the maximum response was reached in the range of 12–30 μM -MgATP, i.e. 0.2–0.5 mM-ATP, a concentration similar to that required for the membrane-associated basal Mg^{2+} -ATPase activity [21]. This corresponded to an apparent K_m of about $10 \mu\text{M}$ -MgATP. Furthermore, the addition of Triton X-100 did not modify the enzyme activity of this membrane fraction (results not shown).

The difference in reactivity of the Ca^{2+} -ATPases towards ATP can also be deduced from the following observations. Resuspension of membranes in a buffer with or without ATP gave different estimations of the Mg^{2+} -ATPase activity, calculated by subtracting the values obtained in the presence of both Mg^{2+} and ATP from those obtained without ATP. As these latter values were increased in the presence of ATP, the Mg^{2+} -ATPase activity was underestimated. Consequently, the ratio between the specific activities of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase and the Mg^{2+} -ATPase (measured with $12 \mu\text{M}$ -MgATP) reached 2.88 for plasma membranes resuspended in buffer with ATP (results not shown), and 0.5 (see Fig. 4b) when resuspended in the absence of ATP. This is explained by the fact that the specific activities of the Ca^{2+} -activated Mg^{2+} -dependent ATPases are unchanged (calculated by subtracting the values obtained without adding CaCl_2 from those obtained with added CaCl_2). The same ratio calculated for intracellular membranes gave similar values (about 0.7) in the presence or absence of ATP in membrane resuspension buffer (see Fig. 4a). Therefore we could formulate the following explanation: based on the dependence on ATP, we suggest that the two Mg^{2+} -ATPase activities are similar in the two membrane fractions. This conclusion was also proposed by Hack *et al.* [29]. The difference would come from the dependence of the Ca^{2+} -activated ATPase on ATP.

Inhibition by vanadate

Fig. 5 shows that both Ca^{2+} -ATPase activities were inhibited by vanadate. However the curves appeared different in two respects: first, the Ca^{2+} -ATPase inhibition observed in intracellular membranes reached 100 %, but it did not exceed 60 % in the case of the plasma membrane-associated enzyme. Secondly, vanadate inhibited the enzyme from plasma membranes in a biphasic way: half-maximal inhibition occurred with about $0.5 \mu\text{M}$ -vanadate for the plasma membrane Ca^{2+} -ATPase and with $5 \mu\text{M}$ -vanadate for the intracellular Ca^{2+} -ATP-

Fig. 5. Effect of vanadate on Ca^{2+} -ATPase activities

The membrane vesicles were incubated for 10 min at 37°C under the experimental conditions described in the legend to Fig. 1. The experiments were conducted at pH 7 for intracellular membranes and at pH 8 for plasma membranes. Different concentrations of inhibitor were added to the incubation mixture. The reactions were initiated by the addition of 0.2 mM-ATP for intracellular membranes and 0.5 mM-ATP for plasma membranes according to the results shown in Fig. 4. The reactions were stopped and the Ca^{2+} -ATPase activities determined as described in the legend to Fig. 1. $\circ$, Intracellular membranes; $\bullet$, plasma membranes. Results are the means $\pm$ S.E.M. for four different experiments.

ase. Thus in these terms the plasma membrane Ca^{2+} -ATPase exhibited a greater sensitivity to vanadate.

Regulation of Ca^{2+} -ATPase activities: effect of chlorpromazine

Despite its non-selective effect, the phenothiazine analogue chlorpromazine has been used by different authors to test a possible regulatory role for calmodulin in platelet Ca^{2+} transport. Thus we compared the effect of chlorpromazine on both plasma membrane and intracellular membrane Ca^{2+} -ATPase activities. Fig. 6 shows that the intracellular membrane Ca^{2+} -ATPase was not sensitive to the action of between 0 and $25 \mu\text{M}$ -chlorpromazine. In contrast, when using the same chlorpromazine concentrations, a small stimulation of the plasma membrane Ca^{2+} -ATPase occurred between 1 and $5 \mu\text{M}$, which was followed by a significant inhibition of the enzyme activity which reached 30–35 % on increasing the chlorpromazine concentrations to 25 – $50 \mu\text{M}$. Thus, the slight regulatory effect of chlorpromazine occurred with the plasma membrane enzyme.

DISCUSSION

This report further characterizes the platelet plasma membrane- and intracellular membrane-associated Ca^{2+} transport systems. The comparative biochemical characterization of the Ca^{2+} -ATPases emphasizes their differences, especially the specific inhibition by high Ca^{2+} concentrations of the intracellular membrane Ca^{2+} -ATPase, the different requirements for the phosphate donor ATP, the different sensitivities towards vanadate and the specific effect of chlorpromazine on the plasma membrane Ca^{2+} -ATPase.

Based essentially on the time course of Ca^{2+} -ATPase activities and their Ca^{2+} concentration dependence, we

Fig 6. Effect of chlorpromazine on Ca^{2+} -ATPase activities

The membrane vesicles were incubated under the experimental conditions described in the legend to Fig. 5. Reactions were conducted in the absence or presence of different chlorpromazine concentrations diluted in water immediately before use. The experiments were conducted as previously described in Fig. 5 legend. ○, Intracellular membranes; ●, plasma membranes. Results are the means $\pm$ S.E.M. for five different experiments

suggest that both enzymes can contribute to the Ca^{2+} homeostasis of the 'resting' platelets, but the plasma membrane enzyme would continue to function after the intracellular enzyme had stopped.

Moreover, this work also explains why the specific activity of the intracellular membrane Ca^{2+} -ATPase is partially inhibited in our membrane preparation. Indeed, the first studies on the two enzymic activities used the experimental procedure described for the determination of the enzyme activity present in a mixed membrane fraction [23]. The additional studies on the pH sensitivity, Ca^{2+} dependence and ATP requirement show that these were not the optimum conditions for determination of the intracellular membrane enzyme activity. Indeed, the pH used was 8, whereas this study shows that the appropriate condition for studying the enzyme present in intracellular membranes is pH 7. Also, we used $\approx 10^{-6}$ M- Ca^{2+} , as determined by the previous studies, and there is a possibility that some Ca^{2+} uptake has occurred into the membrane vesicles during the centrifugation process, thus leading to partial inhibition of the enzyme [21]. The ATP requirement studies of the enzymes show that they differ by a factor of 10, in contrast with the associated Mg^{2+} -ATPase activities, causing an increased difficulty in detection of the intracellular Ca^{2+} -ATPase. These observations, together with the possible inhibition of the enzyme after an additional centrifugation step, can explain the inhibition of the intracellular membrane Ca^{2+} -ATPase [29].

The properties of the plasma membrane and intracellular membrane platelet Ca^{2+} -ATPases agree with those generally described for plasma and intracellular Ca^{2+} -ATPases from other cell types. Thus, the difference in pH sensitivity appears similar to that described for cardiac sarcolemmal and sarcoplasmic reticulum Ca^{2+} -ATPases [30]. The response to high Ca^{2+} concentrations agrees with that of rabbit sarcoplasmic reticulum [31]

which can be explained by the binding of Ca^{2+} to low-affinity sites located on the internal face of the membrane vesicles [32]. The ATP requirement of the two Ca^{2+} -ATPase activities also shows similarities with the erythrocyte Ca^{2+} -ATPase [33] and the muscle intracellular Ca^{2+} -ATPase [32]. The vanadate-induced inhibition of the two Ca^{2+} -ATPases also gives dose-response curves comparable with those obtained with synaptosome and synaptic membrane Ca^{2+} -ATPases [34]. Moreover, we have observed an inhibition of only the plasma membrane Ca^{2+} -ATPase by chlorpromazine. This may be due to interference with phospholipid bilayer structure or inhibition of the endogenous calmodulin kinase present in that membrane preparation. Another alternative is a direct inhibition of the enzyme activity due to calmodulin inactivation, as for erythrocyte Ca^{2+} -ATPase, which was recently shown to possess a calmodulin-binding site located on the C-terminal chain [35].

In addition to these general characteristics of the plasma membrane and intracellular membrane Ca^{2+} -ATPases [32,36], our previous work showed their similarities in molecular mass as well as their different sensitivities to trypsin. Consequently, our results enhanced the specificity of the platelet Ca^{2+} transport systems, for which we formulate the hypothesis that the two enzymes are distinct but similar, their differentiations resulting from their respective membrane environments. This hypothesis is now supported by a recent paper showing immunolocalization of platelet Ca^{2+} -ATPases using anti-(intracellular platelet Ca^{2+} -ATPase) antibody [37]. This work reveals the presence of such an enzyme type, both in the open canalicular system (i.e. particular plasma membranes) and in the dense tubular system (i.e. intracellular membranes). Such a localization, together with the similarities in the two types of Ca^{2+} -ATPases, can offer some other possible explanation of the different results in the literature. Platelets can undergo some cell activation leading to fusion of open canalicular system membranes with those of internal structures. As a consequence, the two types of membranes might be obtained together depending on the isolation method used, thus explaining the interpretation of a single Ca^{2+} -ATPase associated with intracellular membranes. By using fresh platelets without any activation process during the membrane isolation procedure, it is possible to show the two transport systems associated with intracellular and plasma membranes, the latter possibly being a mixture of open canalicular system and plasma membranes.

Finally, although these hypotheses appear possible, we also cannot exclude the presence of different isoforms of the platelet Ca^{2+} -ATPases associated with the different membrane fractions, as more and more frequently reported in the literature [38–42]. Further work is needed to distinguish between these different possibilities.

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