Reversal of the Ca^{2+} pump of blood platelets

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In this study, the endoplasmic Ca^{2+} transport ATPase of blood platelets was compared with the Ca^{2+} ATPase of sarcoplasmic reticulum skeletal muscle. Similar to the muscle enzyme, the Ca^{2+} ATPase from platelets was found to catalyse an $\text{ATP} \rightleftharpoons \text{P}_i$ exchange both in the presence and in the absence of a transmembrane Ca^{2+} gradient. When platelet vesicles are loaded with Ca^{2+} and diluted in medium containing ADP, P_i and EGTA, the ATPase catalyses Ca^{2+} efflux coupled to synthesis of

ATP. The stoichiometry between Ca^{2+} ion released and ATP synthesized by platelet Ca^{2+} ATPase is 1, while that of skeletal muscle is 2. Thapsigargin, a specific inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases, inhibited both the Ca^{2+} -dependent ATPase activity and the reversal of the platelet Ca^{2+} pump. The possibility is discussed that the differences observed between the two transport systems is related to the distinct amino acid sequences of the enzymes.

INTRODUCTION

In unstimulated platelets, the cytosolic Ca^{2+} concentration is limited to the nanomolar range by the transport of Ca^{2+} mediated by a Ca^{2+} ATPase located in the dense tubular system [1–3]. This is an active process that requires ATP as substrate and shares several similarities with the sarcoplasmic reticulum (SR) Ca^{2+} ATPase [4–9]. Platelet activation is promoted by an increase in cytosolic calcium concentration that is observed even in the absence of extracellular Ca^{2+} , suggesting that Ca^{2+} is released from internal calcium stores, most likely the dense tubular system. Ca^{2+} release within the cell is believed to be mediated by Ca^{2+} channels, such as an inositol 1,4,5-triphosphate-sensitive Ca^{2+} channel [10,11].

The Ca^{2+} ATPase from sarcoplasmic reticulum vesicles (SRV) catalyses both the hydrolysis and the synthesis of ATP. When the pump works in the forward direction, ATP is hydrolysed and Ca^{2+} is accumulated by the vesicles. In the reverse direction, ATP is synthesized and Ca^{2+} is released from the vesicles [12,13]. The stoichiometry between Ca^{2+} released and ATP synthesized by the Ca^{2+} ATPase from SR is well known to be 2 [14]. In this report, we show that the catalytic cycle of the human platelet Ca^{2+} ATPase can be reversed but exhibits a distinct stoichiometry between Ca^{2+} released/ATP synthesized when compared with the SR Ca^{2+} ATPase.

MATERIALS AND METHODS

Outdated platelets were obtained from a blood bank in concentrated form. Vesicles derived from the dense tubular system were prepared according to Le Peuch et al. [15]. SRV were prepared as described by Eletr and Inesi [16]. Red cell plasma membrane ATPase [17], ($\text{Na}^+ + \text{K}^+$) ATPase [18], plant H^+ ATPase [19] and soluble F_1 from bovine heart mitochondria [20] were prepared as previously described, and stored in liquid nitrogen.

Protein concentration was determined as described by Lowry et al. [21].

Calcium uptake was measured by filtration through Millipore

filters (0.45 μm) [22]. After filtration, the filters were washed four times with 5 ml volumes of 3 mM $\text{La}(\text{NO}_3)_3$ and the radioactivity remaining in the filters was counted on a scintillation counter.

ATP hydrolysis was determined by measuring the release of $[\gamma\text{-}^{32}\text{P}]\text{P}_i$ from $[\text{ATP-}^{32}\text{P}]$ [23]. Mg^{2+} -dependent ATPase activity was measured in the presence of 1 mM EGTA. Ca^{2+} -stimulated ATPase activity was determined by subtracting the Mg^{2+} -dependent ATPase activity from the ATPase activity measured in the presence of both Mg^{2+} and Ca^{2+} . The free- Ca^{2+} concentration was calculated from the apparent Ca-EGTA association constants provided by Schwartzbach et al. [24], using a computer program as described by Fabiato and Fabiato [25].

Synthesis of ATP from ADP and $^{32}\text{P}[\text{P}_i]$ was determined as previously described [23]. In some experiments, hexokinase (6 units/ml) and glucose (10 mM) were added to the assay media, so that all the ATP synthesized was converted into ^{32}P -labelled glucose 6-phosphate.

^{45}Ca was purchased from Du Pont-New England Nuclear (Wilmington, DE, U.S.A.). Other reagents were of analytical grade.

RESULTS

Ca^{2+} uptake and ATP hydrolysis

Platelet vesicles (PV) accumulate Ca^{2+} in the presence of ATP (Figure 1a and [1–3]), generating a Ca^{2+} gradient across the vesicle membrane. Addition of the Ca^{2+} ionophore A23187 causes the release of the accumulated Ca^{2+} .

The sesquiterpene thapsigargin has been shown to specifically inhibit the Ca^{2+} ATPases that are located in internal membranes [sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCA)], such as those found in SRV and in the platelet's dense tubules [26–28], while having practically no effect on the plasma membrane Ca^{2+} ATPase activity. The Ca^{2+} -dependent ATPase activity of the PV was practically abolished by 1 μM thapsigargin, indicating that all of the ATPase activity of the vesicles we used was derived from dense tubules, with practically no plasma

Abbreviations used: SR, sarcoplasmic reticulum; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SRV, sarcoplasmic reticulum vesicles; PV, platelet vesicles.

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Figure 1 Ca^{2+} uptake and $\text{ATP} \rightleftharpoons \text{P}_i$ exchange catalysed by platelet Ca^{2+} ATPase

(a) The Ca^{2+} uptake was measured at 35 °C in a medium containing 50 mM MOPS/Tris (pH 7.0), 0.4 mM ATP, 10 mM P_i , 5 mM MgCl_2 , 50 μM $^{45}\text{CaCl}_2$, 80 mM KCl and 0.1 mg/ml of PV. At the time indicated in the Figure (arrow), A23187 to a final concentration of 5 μM was added. (b) The reaction was as in (a) except that the medium contained non-radioactive CaCl_2 and 10 mM $^{32}\text{P}\text{P}_i$. The values are the average of three different experiments performed with three different vesicle preparations.

Figure 2 Effect of thapsigargin on the activity of different ATPases

The medium composition was 50 mM MOPS/Tris (pH 7.0), 3 mM MgCl_2 , 1 mM ATP and (□) 0.1 mM CaCl_2 , 0.1 $\mu\text{g/ml}$ calmodulin and 0.2 mg/ml red cell plasma membrane; (○) 0.1 mM CaCl_2 , 0.1 mg/ml SRV and 0.1 mg/ml Triton X-100; (●) 0.1 mM CaCl_2 , 0.1 mg/ml PV and 0.1 mg/ml Triton X-100; (■) 2 mM KCl, 10 mM NaCl and 0.04 mg/ml ($\text{Na}^+ + \text{K}^+$) ATPase; (△) 0.02 mg/ml soluble F_1 ATPase; and (▲) 0.1 mg/ml plant H^+ ATPase. The values are the average of three different experiments performed with three different vesicle preparations.

membrane Ca^{2+} -dependent ATPase contaminant (Figure 2). The concentration dependence observed for thapsigargin inhibition of the platelet Ca^{2+} ATPase was equal to that observed for the SR Ca^{2+} ATPase. The ($\text{Na}^+ + \text{K}^+$) ATPase, F_1 ATPase, plasma membrane Ca^{2+} ATPase and the plant H^+ ATPase were not significantly affected by thapsigargin concentrations of up to 200 μM (Figure 2). The Ca^{2+} -stimulated ATPase activity observed in Figure 2 was unaffected by the mitochondrial inhibitors sodium azide and oligomycin and was totally inhibited by 100 μM vanadate (results not shown).

Reversal of the Ca^{2+} pump

Earlier reports have shown that the Ca^{2+} ATPase from SR catalyses both the hydrolysis and the synthesis of ATP (' $\text{ATP} \rightleftharpoons \text{P}_i$ exchange') when a Ca^{2+} gradient is formed across the vesicles membrane [29,30]. During this exchange, some enzyme units use the energy derived from the hydrolysis of ATP to pump Ca^{2+} to

Figure 3 $\text{ATP} \rightleftharpoons \text{P}_i$ exchange in the absence of Ca^{2+} gradient catalysed by platelet Ca^{2+} ATPase

The reaction medium contained 50 mM Mops/Tris (pH 7.0), 5 mM MgCl_2 , 0.4 mM ATP, 6 mM P_i , 0.2 mM ADP, 5 μM A23187, 0.1 mg PV/ml and either $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (○) or $^{32}\text{P}\text{P}_i$ (●). The reaction was stopped after 1 h at 35 °C by addition of trichloroacetic acid. The values are the average of three different experiments performed with three different vesicle preparations.

Figure 4 ATP synthesis and Ca^{2+} efflux in PV

(a) Ca^{2+} efflux. The vesicles were first loaded using a medium composition identical with that in Figure 1 and after 40 min the vesicles were pelleted by centrifugation and the supernatant was discarded. The Ca^{2+} -loaded vesicles were suspended in cold water and immediately diluted in medium containing 50 mM Mops/Tris (pH 7.0), 10 mM glucose, 20 $\mu\text{g/ml}$ hexokinase, 2 mM EGTA, 5 mM MgCl_2 , 10 mM P_i , 0.05 mg/ml PV, either in the absence (○) or in the presence (●) of 0.2 mM ADP. (b) ATP synthesis was measured in the same efflux medium as in (a) but using 10 mM $^{32}\text{P}\text{P}_i$ and vesicles loaded with non-radioactive Ca^{2+} . ATP synthesis was measured either in the absence (○) or in the presence (●) of 0.2 mM ADP. Active Ca^{2+} efflux (△), calculated by subtracting the effluxes measured in (a) with and without ADP. The values are the average of three different experiments performed with three different vesicle preparations.

the lumen of the vesicles, while other units use the energy derived from the Ca^{2+} gradient to synthesize ATP from ADP and P_i . The present study shows that the platelet ATPase can also catalyse an $\text{ATP} \rightleftharpoons \text{P}_i$ exchange (Figure 1b). Under the conditions used in Figure 1(b), addition of the ionophore A23187 collapses the Ca^{2+} gradient and abolishes the $\text{ATP} \rightleftharpoons \text{P}_i$ exchange reaction.

The SRV ATPase encompasses two distinct classes of Ca^{2+} -binding sites: high-affinity Ca^{2+} sites that face the external surface of the vesicles and bind Ca^{2+} in the micromolar range, and low-affinity Ca^{2+} -binding sites which are saturated by Ca^{2+} in the millimolar range and face the vesicles' lumen [30]. It has been shown [14,30,31] that the ATPase from SRV can catalyse an ' $\text{ATP} \rightleftharpoons \text{P}_i$ exchange' even in the absence of a Ca^{2+} gradient. This occurs when Ca^{2+} -permeable vesicles ('leaky vesicles') are incubated in a medium containing sufficient Ca^{2+} to saturate both the high- and low-affinity Ca^{2+} sites of the enzyme.

Figure 5 Inhibition by thapsigargin on the reversal of PV Ca²⁺ ATPase

The conditions were identical with those used in Figure 4 with either none (●) or 1 μM (△) thapsigargin for (a) active Ca²⁺ efflux and (b) ATP synthesis. (c) Concentration dependence for inhibition of ATP synthesis by thapsigargin. The reaction time was 10 min. The values are the average of three different experiments performed with three different vesicle preparations.

Saturation of the low-affinity Ca²⁺-binding sites simultaneously inhibits the hydrolysis of ATP and activates the synthesis of ATP. As observed with SRV, the Ca²⁺ ATPase activity of PV, permeabilized by the Ca²⁺ ionophore, exhibits a biphasic pattern: activation of ATP hydrolysis is observed when Ca²⁺ is varied in the low micromolar range (1–10 μM), whereas inhibition of ATP hydrolysis and activation of ATP synthesis is observed when the free-Ca²⁺ concentration is increased to values higher than 200 μM (Figure 3).

The catalytic cycle of SRV Ca²⁺ ATPase can be reversed and the enzyme can catalyse net synthesis of ATP from ADP and P_i [12]. This is also observed in PV (Figure 4). When PV are loaded with Ca²⁺ and diluted in medium containing EGTA, an efflux of Ca²⁺ is observed (Figure 4a). ADP, P_i and Mg²⁺ promote an increase in the Ca²⁺ efflux (Figure 4a). This increment is referred to as the active Ca²⁺ efflux [14] and is coupled to synthesis of ATP. The active Ca²⁺ efflux coupled to the synthesis of ATP is not observed in the absence of added ADP (Figure 4b). The stoichiometry found between Ca²⁺ released and ATP synthesized by platelet Ca²⁺ ATPase was 0.98 ± 0.17 ($n = 5$), whereas the Ca²⁺/ATP stoichiometry of SRV is 2 [14,30].

Thapsigargin inhibits the active Ca²⁺ efflux and ATP synthesis in PV (Figure 5). The $K_{0.5}$ found for inhibition of ATP synthesis

Figure 6 Effect of thapsigargin on Ca²⁺ efflux in the absence of ADP

Vesicles were loaded with Ca²⁺ as in Figure 4 and diluted in media containing 50 mM Mops/Tris (pH 7.0) and 2 mM EGTA, either in the absence (●) or presence (△) of 2 μM thapsigargin. (a) PV; (b) SRV. The Ca²⁺ load was 80 nmol Ca²⁺/mg protein in (a) and 2 μmol Ca²⁺/mg protein in (b). The values are the average of three different experiments performed with three different vesicle preparations.

was 10 nM (Figure 5c). The efflux of Ca²⁺ from PV, measured in the absence of substrates required for ATP synthesis, was unaffected by thapsigargin at concentrations up to 1 μM (Figure 6a). This is different from that observed in SRV [32] in which thapsigargin blocks Ca²⁺ efflux both in the absence and in the presence of substrates of ATP synthesis (compare Figures 6a and 6b). Note that the rate of Ca²⁺ efflux from PV is 50 times slower than that observed in SRV.

DISCUSSION

The vesicles derived from human platelets used in the present study contain, in addition to dense tubules, plasma membrane contaminants [33,34]. Fauvel et al. [33] showed that plasma membrane contaminants accumulate negligible amounts of Ca²⁺ [33]. This is in agreement with our present findings that Ca²⁺ ATPase activity and the synthesis of ATP are totally suppressed by the specific inhibitor thapsigargin (Figures 2 and 5). Thapsigargin is a highly specific inhibitor of SERCA and has no effect on plasma membrane Ca²⁺ ATPase [26–28].

The amino acid sequence of the PV and SRV Ca²⁺ ATPases are different [35–37] and the two enzymes exhibit different tryptic digestion patterns [38]. Recently, it was reported that human platelets express both the SERCA_{2-b} isoform (smooth-muscle/non-muscle type endoplasmic reticulum Ca²⁺ ATPase) and SERCA₃ isoform, whereas the SERCA₁ gene encodes the fast skeletal-muscle SR ATPase [35–37]. Human platelet Ca²⁺ ATPase activity is inhibited by thapsigargin to a similar degree to that observed with SERCA₁ (Figure 2). This indicates that thapsigargin inhibition of ATPase activity does not distinguish between the platelet and SERCA₁ isoforms.

There is no consensus in the literature regarding the stoichiometry between the degree of Ca²⁺ ion transport and ATP hydrolysis by platelet Ca²⁺ ATPase. Some authors have found values close to 1 [15] while others have reported a value of 2 [6]. This stoichiometry should be the same regardless of whether the pump operates forwards (ATP hydrolysis) or backwards (ATP synthesis). The ATP ⇌ P_i exchange catalysed by PV was found to be similar to that observed in SRV (Figures 1 and 3). The mechanism by which the binding of Ca²⁺ triggers the synthesis of ATP has been examined in detail in previous studies [14,30]. We found a value of 1 for the Ca/ATP coupling ratio measured during the reversal of platelet Ca²⁺ ATPase (Figure 4). The value is half of that previously described for SR Ca²⁺ ATPase [14]. This

indicates that the synthesis of ATP is mediated by the binding of one Ca^{2+} ion to an internal Ca^{2+} -binding site of platelet Ca^{2+} ATPase and not two Ca^{2+} ions as described for SRV [14]. It might be possible that the different Ca^{2+} /ATP ratios are related to the different amino acid sequences of the two ATPases. It is however noteworthy that in SRV, a different Ca^{2+} /ATP ratio has been measured using non-physiological substrates, such as one Ca^{2+} transported per molecule of substrate hydrolysed [39].

It has been shown that the SR Ca^{2+} ATPase mediates Ca^{2+} efflux even in the absence of substrates required for ATP synthesis [40]. This efflux of Ca^{2+} is enhanced by several drugs that interact with the ATPase [41,42] and is decreased by thapsigargin ([32] and Figure 6b). Although thapsigargin inhibits the reversal of the Ca^{2+} pump (Figure 5), it does not modify the rate of Ca^{2+} efflux from PV in the absence of substrates required for ATP synthesis (Figure 6), which is different from observations with SRV. It is possible that the differences between the two Ca^{2+} transport systems are also related to the different amino acid sequences of the enzymes.

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