Demonstration of Two Forms of Calcium Pumps by Thapsigargin Inhibition and Radioimmunoblotting in Platelet Membrane Vesicles*

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Calcium transporting ATPases (Ca²⁺ pumps) translocate calcium ions through cellular membranes at the expense of ATP hydrolysis to the extracellular space or into intracellular calcium storage sites. Plasma membrane-type Ca²⁺ pumps are approximately 140-kDa calmodulin-binding proteins encoded by several genes which, by alternative splicing, give rise to several structurally related but distinct proteins (1–3).

For the approximately 100-kDa sarco/endoplasmic reticulum-type calcium pumps (SERCA),³ three genes have already been identified. The SERCA 1 gene codes for the skeletal muscle sarcoplasmic reticulum calcium-pump; the SERCA 1a and 1b are alternatively spliced forms corresponding to the adult and neonatal pump isoforms, respectively. The SERCA 2 gene also produces alternative spliced species, SERCA 2a and 2b. These are identical, except for a tetrapeptide C-terminal part, present in SERCA 2a, but replaced by a 49-amino acid tail in the SERCA 2b isoform. The first one is expressed in heart and slow skeletal sarcoplasmic reticulum, the second is predominantly expressed in smooth muscle and several nonmuscular tissues. A third gene product, SERCA 3, has also been detected by Northern blotting in heart, skeletal muscle, and in a variety of nonmuscular tissues (4–10).

The maintenance of low cytosolic calcium concentrations by calcium pumps is essential for the control of platelet activation. Although Ca²⁺ homeostasis plays an important role in platelet functions, the exact nature of platelet calcium pumps and their regulation is not known. Our previous work, based on the distinct autophosphorylation and transport kinetic characteristics of tryptic fragments, as well as subcellular membrane fractionation and immunoprecipitation experiments indicated the presence of two Ca²⁺ pump entities in human platelets (11, 13, 25, 29–30). However, direct evidence for the presence of two distinct calcium pumps in intact platelet membranes was lacking.

In this work calcium pumps were specifically labeled in intact or trypsinized platelet vesicles by [³²P]ATP. Upon formation of the aspartylphosphate intermediate of the ATPase, electrophoresis in an acidic polyacrylamide electrophoresis system, previously developed to preserve the phosphoenzyme intermediates (11, 12), was carried out. The proteins were blotted onto nitrocellulose, autoradiographed, and immunostained. Since our recent cloning work indicated² that platelets contain the SERCA 2b isoform, the blots were treated with an antiserum raised against a synthetic peptide, corresponding to the unique C-terminal sequence of this isoform (14). We also examined the immunoreaction with an anti rat-sarcoplasmic reticulum calcium pump antiserum of broad specificity toward calcium pumps of sarco- or endoplasmic reticulum origin (12). In order to differentiate the calcium pump isoforms, the effect of thapsigargin, a recently identified inhibitor of endoplasmic reticulum calcium uptake and tumor promoter (18–21, 23), was studied on the platelet Ca²⁺ pumps. Inhibition of active calcium uptake into platelet vesicles was measured and compared with the differential inhibitory effect of thapsigargin on the phosphoenzyme formation of the two platelet calcium pumps. In addition, in order to assign the tryptic fragments of the platelet calcium pumps to the respective intact ones, the effect of thapsigargin on the Ca²⁺ pump fragments in trypsinized platelet membrane
vesicles was examined. Moreover, the effect of Mg²⁺ and La³⁺ on the inhibition by thapsigargin was characterized.

The results obtained indicate the presence of two endoplasmic-reticulum-type Ca²⁺ pumps in platelets of 100 and 97 kDa apparent molecular mass, respectively, having different autophosphorylation characteristics, thapsigargin sensitivity, and tryptic proteolytic fragmentation pattern.

**EXPERIMENTAL PROCEDURES**

**Materials**—-[γ-[³²P]ATP (110 TBq/mmol, 370 MBq/mg), ^{45}Ca (45 GBq/mmol, 72 MBq/ml), and radioactive protein molecular weight standard mixture was from Amersham International, United Kingdom. Trypsin, trypsin inhibitor, and aprotinin were purchased from Sigma. Thapsigargin was isolated as previously described (26) and dissolved in dimethyl sulfoxide before application. In control experiments dimethyl sulfoxide (0.5-5%) was used alone and did not interfere with the assays. Nitrocellulose membrane (0.45-µm pore size) was from Schleicher & Schuell. The preparation of anti-SERCA 2b antipeptide antiserum was described in Ref. 14.

Platelet membrane vesicles were isolated from fresh platelet-rich plasma, based on the modified version (11) of the method of Käser-Glanzmann (24). Platelets were washed three times with Tyrode buffer. The washed platelets were resuspended (10⁹ cell/ml) in a solution containing 100 mM KCl, 15 mM NaCl, 2 mM MgCl₂, 12 mM sodium citrate, 10 mM glucose, 25 mM HEPES-K, pH 7.4, 0.1 mM dithiothreitol, and 0.4 mg/ml aprotinin, sonicated three times with an MSE sonicator (maximum amplitude), and centrifuged for 20 min at 35,000 × g at 4 °C. The collected supernatant was sedimented at 16,000 × g. The pellet was resuspended in 30 mM KCl, 20 mM Tris-HCl, pH = 7.4, and 0.1 mM dithiothreitol, homogenized with a Teflon-glass homogenizer, and diluted to a concentration of about 10–15 mg of membrane protein/ml.

**Calcium Transport Measurements**—Ca²⁺ influx into platelet membrane vesicles was measured for 3 min at 37 °C by the rapid filtration technique described in Ref. 27. The transport medium contained 130 mM KCl, 34 mM HEPES-K, pH = 7.2, 2 mM MgCl₂, 5 mM K-oxalate, 100 µM CaCl₂ (labeled with ^{45}Ca²⁺), 110 µM EGTA (free Ca²⁺ concentration being 1.6 µM) and 140 or 500 µg of membrane protein/ml, as indicated. Platelet membrane vesicles were preincubated with the required amount of thapsigargin for 3 min at 37 °C before initiating calcium uptake by the addition of 0.5 mM ATP.

Limited proteolysis of platelet membranes was carried out on ice in the presence of 50 µg/ml trypsin (11). The proteolysis medium contained 130 mM KCl, 34 mM HEPES-K, pH = 7.0, 0.5 mM dithiothreitol, 50 µM CaCl₂, and 0.5–2 mg of membrane protein/ml. After 10 min of digestion, the reaction was stopped with 5-fold excess of soybean trypsin inhibitor. Membrane phosphorylation experiments were performed immediately after proteolysis.

**Phosphorylation of the Calcium Pump(s) in Platelet Membrane Vesicles**—Phosphorylation was carried out on ice in the medium used for proteolysis, containing 50 µM CaCl₂, 100 µM LaCl₃, 1 mM MgCl₂, and thapsigargin as indicated. The reaction was started by the addition of 0.05 µM ATP (including [γ³²P]ATP). After 1 min of incubation, the reaction was stopped by the addition of ice-cold trichloroacetic acid (5% final concentration) containing 1 mM ATP and 10 mM phosphoric acid, and the samples were washed three times with the same solution. The precipitates were dissolved in the electrophoresis sample buffer and either counted for radioactivity or analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Ref. 28.

**Electrophoresis and Immunoblotting**—Acidic polyacrylamide gel electrophoresis and Western blotting was performed (12, 28) with the following modifications: electrophoresis was done in a Bio-Rad Protein 16CM electrophoresis unit at 4 °C at 120 V constant voltage. Sample buffer contained 150 mM Tris, 8 mM EDTA-Na₂, 3% (w/v) sodium dodecyl sulfate, 20% (w/v) Saccharose, 0.14 mg/ml bromphenol blue, 1% dithiothreitol, and was adjusted to pH = 7.4 with HCl. After dissolution of the trichloroacetic acid-precipitated protein pellet of sample buffer, samples were realkalinized with 1.7 M Tris, and 20-µl samples, corresponding to 100–300 µg of membrane protein, were deposited per well. After electrophoresis and immunoblotting, membranes were autoradiographed using KODAK X-OMAT AR films.

Immunostaining was done by an avidin-biotin-peroxidase staining system as in Ref. 22. For staining with the SERCA 2b antiserum, membranes were quenched in 6 mM Tris, 0.5 mM NaCl, 5% (w/v) bovine serum albumin, pH = 7.4, supplemented with 20% heat-inactivated human serum, added to increase specificity of immunostaining. The anti-SERCA 2b rabbit antisera was diluted 120-fold in the same solution.

**RESULTS**

**Electrophoretic Resolution of Two Ca²⁺ Pumps in Platelet Membrane Vesicles**—Ca²⁺ pumps in human platelet membrane vesicles were labeled by [γ-[³²P]ATP in the presence of Ca²⁺. After electrophoresis of the membrane proteins and blotting onto nitrocellulose, two closely migrating but distinct phosphorylated bands of molecular mass of about 100 and 97 kDa, respectively, were obtained upon autoradiography (Fig. 1, panel A, lane 1). In the presence of 50 µM Ca²⁺, the phosphorylation of the upper band (100 kDa, upper arrow-
TGbM) pump-specific autophosphorylation reaction on platelet membranes. Calcium phosphorylation was performed at 1 pM thapsigargin in the presence of 50 pM concentrations of 250 pmol/mg membrane protein. When Ca²⁺ pump autophosphorylation was performed at 1 mM thapsigargin, the presence of 50 µM Ca²⁺ alone (△) inhibition was less pronounced and addition of 100 µM La³⁺ (○) abolished the inhibition in the presence, as well as in the absence of Mg²⁺.

**Inhibition by Thapsigargin of Active Ca²⁺ Uptake into Platelet Membrane Vesicles**—As shown on Fig. 2, thapsigargin inhibited active Ca²⁺ uptake into platelet membrane vesicles. Inhibition by thapsigargin appeared to be dependent on the inhibitor to membrane protein ratio, half-maximal inhibition obtained at 120–140 nM at 0.5 mg/ml, and at 35–40 nM thapsigargin at 0.14 mg/ml membrane protein concentration, respectively. IC₅₀ was about 250 pmol of thapsigargin/mg of membrane protein.

**Inhibition by Thapsigargin of Phosphoenzyme Intermediate Formation of Ca²⁺ Pumps in Platelet Membrane Vesicles**—As shown on Fig. 3, thapsigargin also caused a dose-dependent inhibition of total phosphoenzyme formation, with a half-maximal concentration of about 100 nM at 0.4 mg/ml membrane protein concentration, corresponding again to an IC₅₀ of about 250 pmol of thapsigargin/mg of membrane protein. This inhibition was more pronounced in the presence of Mg²⁺ plus Ca²⁺ than with Ca²⁺ alone and abolished by La³⁺ in the presence, as well as in the absence of Mg²⁺. Since Mg²⁺ decreases steady state phosphoenzyme level by facilitating the hydrolysis of the aspartylphosphate group, it can be concluded that thapsigargin interferes with the phosphorylation step of the transport cycle, rather than inhibiting the dephosphorylation step, as in the case of lanthanum, which blocks the enzyme in the phosphorylated state.

**Differential Inhibition of Phosphoenzyme Intermediate Formation of the Two Platelet Ca²⁺ Pumps by Thapsigargin**—Fig. 4 shows the effect of thapsigargin on the appearance of the phosphorylated bands after the membrane proteins were resolved by acidic polyacrylamide gel electrophoresis. Thapsigargin inhibited the phosphoenzyme formation predominantly in the case of the higher molecular mass (100 kDa) enzyme (SERCA 2b). While in the absence of thapsigargin under the reaction conditions used (i.e. in the presence of Ca²⁺ and Mg²⁺, without La³⁺, at 0.4 mg of membrane protein/ml) the upper band appears to be more phosphorylated (Fig. 4, lane 1), the addition of thapsigargin results in an almost complete disappearance of this band in the submicromolar thapsigargin concentration range (Fig. 4, lanes 2–4); at 2 µM thapsigargin concentration practically only the lower 97-kDa pump species remained phosphorylated, although to a lesser extent, than in the absence of the inhibitor. At this thapsigargin concentration, the Ca²⁺-uptake is almost completely inhibited. This slight discrepancy between transport and phosphorylation experiments is probably due to the different assay conditions used in the two types of experiments (see "Experimental Procedures").

**Assignment of Tryptic Fragments to the Respective Nonproteolysed Ca²⁺ Pumps by Differential La³⁺ and Thapsigargin Sensitivity**—Upon mild trypsin proteolysis (Fig. 4, panel B), platelet membrane vesicles give rise to 80-, 55-, and 35-kDa autoprophosphorylatable Ca²⁺ pump fragments (11). Out of these fragments, the autoprophosphorylation of the 80-kDa fragment

**Different Reactivity of the Two Ca²⁺ Pumps with Anti-Ca²⁺ Pump Antibodies**—Both bands were recognized upon immunostaining (Fig. 1, panel B) by a polyclonal antiserum (lane 3), raised against rat sarcoplasmic reticulum Ca²⁺ pump, whereas only the upper (100 kDa) band was stained by an antiserum, specific for the SERCA 2b isoform (lane 4). Thus, two distinct Ca²⁺ pump proteins can be discerned in platelet membrane vesicles, having different molecular masses, lanthanum sensitivities of phosphoenzyme formation, and immunoreactivities toward anti-Ca²⁺ pump antibodies. To further characterize these two calcium transport systems, the effect of thapsigargin on the phosphoenzyme formation of the Ca²⁺ pumps and on the ATP-dependent Ca²⁺ uptake into platelet membrane vesicles was studied.

**FIG. 4. Differential inhibition of phosphoenzyme formation of the two platelet Ca²⁺ pumps by thapsigargin.** Platelet membrane vesicle proteins, after autophosphorylation by [γ³²P]ATP of the Ca²⁺ pumps, were resolved by electrophoresis, blotted onto nitrocellulose, and autoradiographed. Panel A, calcium pumps were autophosphorylated by [γ³²P]ATP in platelet membrane vesicles, in the presence of various thapsigargin concentrations. Thapsigargin specifically inhibited the autophosphorylation of the upper (100 kDa) Ca²⁺ pump species in the submicromolar concentration range (lanes 1–4). Addition of lanthanum abolished the inhibition caused by thapsigargin (lane 5). Panel B, platelet membrane vesicles were digested with trypsin as under "Experimental Procedures," and phosphoenzyme formation was induced in the absence (lane 6) or presence of various thapsigargin concentrations. 200 nM thapsigargin (lane 7) preferentially inhibited the 55-kDa Ca²⁺ pump fragment (lower arrowhead), without affecting the phosphoenzyme formation of the 80-kDa fragment (upper arrowhead). At 2 µM thapsigargin, both fragments were inhibited (lane 8), and addition of La³⁺ abolished the inhibition (lane 9). head) was more pronounced (Fig. 1, panel A, lane 1). Addition of 100 µM La³⁺ during phosphorylation resulted in an increase of radiolabelling of the lower band (97 kDa, lane 2, open arrowhead), while it did not affect the labeling of the 100-kDa protein.

**FIG. 3. Inhibition by thapsigargin of the total Ca²⁺ pump phosphoenzyme formation in platelet membranes.** Calcium pump-specific autophosphorylation reaction on platelet membranes (400 µg/ml) was performed as under “Experimental Procedures,” in the presence of 1 mM Mg²⁺, 50 µM Ca²⁺, and increasing thapsigargin concentrations (■). Thapsigargin inhibited phosphoenzyme formation half-maximally at about 100 nM, which corresponds to an IC₅₀ of 250 pmol/mg membrane protein. When Ca²⁺ pump autophosphorylation was performed at 1 µM thapsigargin, the presence of 50 µM Ca²⁺ alone (△) inhibition was less pronounced and addition of 100 µM La³⁺ (○) abolished the inhibition in the presence, as well as in the absence of Mg²⁺.

**FIG. 2. Differential inhibition of phosphoenzyme formation of the Ca²⁺ pump proteins by thapsigargin.** A, platelet membrane vesicle proteins (400 µg/ml) were autophosphorylated by [γ³²P]ATP in the presence of various thapsigargin concentrations, resolved by acidic polyacrylamide gel electrophoresis, blotted onto nitrocellulose and autoradiographed. Lane 1, buffer control; lane 2, 0.14 mg/ml membrane protein; lane 3, 0.5 mg/ml membrane protein; lane 4, 2 mg/ml membrane protein. In (A), thapsigargin inhibited phosphorylation of both the upper (100 kDa) and lower (97 kDa) phosphoenzyme species, the latter being more pronounced in the presence of thapsigargin. In (B), thapsigargin was added at 0.4 µM/mg of membrane protein, in the presence of various thapsigargin concentrations. Lane 1, buffer control; lane 2, 0.14 mg/ml membrane protein; lane 3, 0.5 mg/ml membrane protein; lane 4, 2 mg/ml membrane protein. In (B), thapsigargin inhibited phosphorylation of both the upper (100 kDa) and lower (97 kDa) phosphoenzyme species, the latter being more pronounced in the presence of thapsigargin.
is enhanced by lanthanum (11), similarly to that observed in the case of the 97-kDa non-proteolyzed pump, in this work (Fig. 1, lane 2). This suggests that the 80-kDa fragment may be the cleavage product of the 97-kDa Ca²⁺ pump species, the 55- and 35-kDa fragments coming from the 100 kDa form.

In order to test this hypothesis, platelet membrane vesicles were subjected to limited trypsin proteolysis, and thapsigargin inhibition of the phosphoenzyme formation of the Ca²⁺ pump fragments was studied. As seen on Fig. 4, lane 7, at 200 nM thapsigargin concentration the phosphoenzyme level of the 55-kDa Ca²⁺ pump fragment was decreased, whereas that of the 80-kDa fragment remained unchanged, as compared to the control (lane 6). The autophosphorylation of the 35-kDa fragment was also inhibited by 200 nM thapsigargin (not shown). The same pattern of inhibition of phosphoenzyme formation was obtained for the non-proteolyzed Ca²⁺ pumps, the 100 kDa form being inhibited, and the 97 kDa form being relatively insensitive to this thapsigargin concentration (Fig. 4, lane 4). At 2 µM thapsigargin both fragments (80 and 55 kDa) appeared to be inhibited (Fig. 4, lane 8), although to a lesser extent than the respective nonproteolyzed ones, probably due to differences in the affinity of thapsigargin to the more or less truncated pump fragments. Addition of lanthanum abolished thapsigargin inhibition of calcium pump autophosphorylation in the case of proteolysed, as well as nonproteolysed platelet membranes (Fig 4, lanes 5 and 9).

**DISCUSSION**

In the present work direct evidence is presented for the existence of two separate calcium transporting ATPases in human platelets. Two closely migrating endoplasmic reticulum-type pump species were resolved on polyacrylamide gels and identified by their capacity of phosphoenzyme intermediate formation and reactivity with anti-Ca²⁺ pump antibodies. This approach has already been widely used in the study of calcium pumps in different cell types (11, 12, 22), including platelets, with success for the resolution of calcium pump fragments after trypsinolysis (11, 12).

The higher molecular weight band is identified as the SERCA 2b isoform, recently cloned from platelet libraries in our laboratory. It has been shown that this isoform produces (11). The 80-kDa tryptic fragment was also inhibited by 200 nM thapsigargin (not shown). The same pattern of inhibition of phosphoenzyme formation was obtained for the non-proteolyzed Ca²⁺ pumps, the 100 kDa form being inhibited, and the 97 kDa form being relatively insensitive to this thapsigargin concentration (Fig. 4, lane 7), although at a lesser extent than the respective nonproteolyzed ones, probably due to differences in the affinity of thapsigargin to the more or less truncated pump fragments. Addition of lanthanum abolished thapsigargin inhibition of calcium pump autophosphorylation in the case of proteolysed, as well as nonproteolysed platelet membranes (Fig 4, lanes 5 and 9).

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