Localization and identification of Ca^{2+} ATPases in highly purified human platelet plasma and intracellular membranes

Evidence that the monoclonal antibody PL/IM 430 recognizes the SERCA 3 Ca^{2+} ATPase in human platelets

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The Ca^{2+} ATPase activities of highly purified human platelet membranes prepared by high-voltage free-flow electrophoresis have been analysed by using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis, recognition by antibodies and phosphoenzyme-complex formation. The Ca^{2+} ATPase activity present in mixed membranes was found to be predominantly associated with intracellular membranes after subfractionation, with only a low level of activity associated with plasma membranes. The intracellular-membrane Ca^{2+} ATPase activity was inhibited totally with thapsigargin (Tg), whereas the plasma-membrane Ca^{2+} ATPase was not significantly affected, suggesting that the latter does not belong to the SERCA (sarco-endoplasmic-reticulum Ca^{2+} ATPase) class. A monoclonal antibody, 5F10, raised to the red-cell membrane Ca^{2+} ATPase [Cheng, Magocsi, Cooper, Penniston and Borke (1993) *Cell Physiol. Biochem.* 4, 31–43] recognized two bands at 135 and 150 kDa in mixed membranes and plasma membranes, and the corresponding bands in red-blood-cell membranes, confirming the Ca^{2+} ATPase to be of the PMCA (plasma-membrane Ca^{2+} ATPase) type. No recognition of any band was detected in intracellular membranes. Identification of the intracellular-mem-

brane Ca^{2+} ATPase activity was carried out with polyclonal antibodies with known specificity towards SERCA 2b (S.2b) and SERCA 3 (N89), and a monoclonal antibody, PL/IM 430, raised against platelet intracellular membranes. All of these antibodies recognized the 100 kDa Ca^{2+} ATPase in mixed membranes and intracellular membranes, with little or no recognition of the activity in the plasma membranes. In some membrane preparations the antibody PL/IM 430 and antiserum N89 recognized similar degradation products, of 74, 70 and 40 kDa, in the intracellular-membrane fraction. The Ca^{2+} ATPase recognized by PL/IM 430 was immunoprecipitated, and the immunoprecipitated protein was specifically recognized by the antiserum N89, but not by S.2b. Analysis of the phosphoenzyme-complex formation revealed potent phosphorylation of the 100 and 74 kDa peptides, both recognized by PL/IM 430 and N89. These studies report the presence of a PMCA in a purified plasma-membrane fraction from human platelets, and that the antibody PL/IM 430 recognizes the SERCA 3 Ca^{2+} ATPase in intracellular membranes.

INTRODUCTION

The maintenance of low cytosolic Ca^{2+} concentrations, necessary to keep platelets in a resting state and to re-establish a resting state after activation, is achieved by a combination of Ca^{2+} + Mg^{2+} ATPases (referred to as Ca^{2+} ATPases) present predominantly on the membranes containing intracellular stores and a $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger present on the plasma membranes (for a review see [1]). Recently there has been considerable interest in the identification and localization of Ca^{2+} ATPases present in human platelets. Most cells contain two types of Ca^{2+} ATPases: the plasma-membrane Ca^{2+} ATPases (PMCA types 1–4) [2,3] and those present on intracellular stores, known commonly as sarco-endoplasmic-reticulum Ca^{2+} ATPases (SERCA) [4,5]. The PMCA are known to be stimulated by calmodulin and have a larger molecular size (approx. 130–140 kDa on SDS/PAGE) than the SERCAs, which migrate as peptides of approx. 100–110 kDa. Two types of $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers have been identified, of which the platelet has been suggested to contain the retinal type [6].

The expression and identity of a functional PMCA in human platelets is still controversial, and only recently has the presence of low levels of immunologically identified PMCA been reported

using antibodies raised against the red-cell Ca^{2+} ATPase [7]. Its identity with respect to the subtype of PMCA (1–4) and its contribution to total Ca^{2+} efflux is still to be determined. In contrast with the PMCA, platelets possess high levels of intracellular-membrane Ca^{2+} ATPases that sequester Ca^{2+} into intracellular stores [8–10]. Recent studies have identified two forms belonging to the SERCA 2b and SERCA 3 subtypes [11–14]. The SERCA 2b shows characteristic tryptic digestion products of 55 and 35 kDa, and the formation of its phosphoenzyme complex is potentially inhibited by thapsigargin (Tg) [12]. The SERCA 3 Ca^{2+} ATPase is also characteristically cleaved by trypsin to yield a 80 kDa fragment [13], and formation of the phosphoenzyme complex is increased in the presence of La^{3+} [15]. The latter is inhibited relatively more potently by 2,5-di-(*t*-butyl)-1,4-benzohydroquinone [12]. Polyclonal antibodies directed towards specific regions of the two Ca^{2+} ATPases have also been described and have confirmed the identification [11,13].

Recently the presence of another isoform of SERCA in human platelets has been suggested, on the basis of a distinct tryptic degradation pattern giving rise to peptides of molecular size 73, 68 and 40 kDa recognized by a monoclonal antibody to Ca^{2+} ATPase, PL/IM 430 [16]. This antibody was previously raised to highly purified platelet intracellular membranes pre-

Abbreviations used: SERCA, sarco-endoplasmic-reticulum Ca^{2+} ATPase; PMCA, plasma-membrane Ca^{2+} ATPase; Tg, thapsigargin; PMSF, phenylmethanesulphonyl fluoride.

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pared by high-voltage free-flow electrophoresis [17]. PL/IM 430 is a unique antibody that inhibits Ca^{2+} sequestration into both platelet membranes and permeabilized cells [17,18].

We now present studies on the localization and immunological identification of Ca^{2+} ATPases in purified platelet membranes prepared by free-flow electrophoresis and using antibodies specific for SERCA 2b and SERCA 3 and the antibody PL/IM 430. We report the near-exclusive localization of the SERCA pumps on intracellular membranes, and on the basis of immunoprecipitation experiments we suggest that PL/IM 430 recognizes the SERCA 3 isoform of the Ca^{2+} pump. In addition we report for the first time a Ca^{2+} ATPase activity in a purified plasma-membrane fraction that is insensitive to Tg, and identify this as belonging to the PMCA class, using an antibody, 5F10, raised to the red-cell Ca^{2+} ATPase.

METHODS

Materials

Soybean trypsin inhibitor, aprotinin, pepstatin, phenylmethane-sulphonyl fluoride (PMSF), dithiothreitol, ATP and neuraminidase (type X) were obtained from Sigma, Poole, Dorset, U.K. Protein A-Sepharose CL-4B was purchased from Pharmacia, Milton Keynes, U.K. Rabbit anti-mouse immunoglobulins and aminoethylcarbazole tablets were purchased from Sigma Immunochemicals, U.K. Affinity-isolated goat anti-mouse immunoglobulins, horseradish peroxidase conjugated to rabbit IgG and mouse IgG were from Dako (High Wycombe, Bucks., U.K.). [γ - ^{32}P]ATP (500 Ci/mmol) was from ICN Biochemicals, High Wycombe, Bucks., U.K. All the electrophoresis reagents were from Bio-Rad, Hemel Hempstead, Herts., U.K., and the nitrocellulose membrane (0.2 μm pore size) was from Schleicher and Schuell, Germany. All other reagents were purchased from Sigma.

Antibodies

The hybridoma cell line producing the PL/IM 430 antibody was grown in RPMI medium supplemented with 10% heat-inactivated fetal-calf serum, non-essential amino acids and glutamine at 37 °C. Antibody was purified from the cell supernatant by affinity chromatography on Protein A-Sepharose CL-4B according to the manufacturer's instructions (see [17]). Purified antibody was dialysed before use. The antibody 5F10 raised to red-cell PMCA was obtained from Affinity Bioreagents, Neshanic Station, NJ, U.S.A.

Polyclonal antibodies

N89 and S.2b have been shown to recognize specifically the SERCA 3 and SERCA 2b Ca^{2+} ATPases respectively. N89 was raised to a peptide sequence corresponding to residues 29–39 near the N-terminal region of the SERCA 3 pump [13], and S.2b was raised to a peptide corresponding to the specific portion of the C-terminal region of the SERCA 2b pump [19].

Preparation of human platelet membranes

Platelet plasma and intracellular membranes were prepared as described in detail in previous publications [9,10,17,20–22]. Briefly, platelets were separated from human blood, washed and treated with neuraminidase (0.05 unit/ml) for 20 min at 37 °C. Platelets were sonicated in 0.34 M sorbitol/10 mM Hepes, pH 7.4, with a cocktail of inhibitors, including aprotinin (0.3 unit/ml), pepstatin A (5 $\mu\text{g}/\text{ml}$), PMSF (0.2 mM), dithiothreitol (1 mM), soybean trypsin inhibitor (1 mg/ml) and E64 (1 μM).

Sonicated platelet preparations were centrifuged at 40000 g for 90 min on a linear (1–3.5 M) sorbitol density gradient to obtain a mixed membrane fraction (free of granular contamination). After concentration of the mixed membrane fraction by centrifugation (100000 g , 60 min), they were separated into plasma and intracellular membranes by application on to a Bender Hobein VAP 5 electrophoresis unit running at 750 V and 100 mA. Two discrete peaks of plasma and intracellular membranes were obtained, which have been well characterized with respect to marker enzymes, protein and lipid profiles, as well as Ca^{2+} -sequestration properties [22]. Peak fractions from the free-flow-electrophoresis profile were pooled, centrifuged (100000 g , 60 min) and resuspended in 0.34 M sorbitol/10 mM Hepes, pH 7.2, for subsequent studies.

Preparation of human red-blood-cell membranes

The red-blood-cell fraction obtained after separation of platelet-rich plasma from human blood was washed three times in a Hepes/saline buffer (130 mM KCl, 10 mM NaCl, 10 mM Hepes, pH 7.0) and then lysed in 1 litre of cold Tris/EGTA (10 mM Tris, 1 mM EGTA, pH 7.0, 4 °C). Lysis was allowed to take place for 20 min, after which the membranes were separated by centrifugation at 20000 g for 20 min at 4 °C. The membrane pellet was washed eight times or until the red colour was completely lost, and finally resuspended in Tris/EGTA and stored at –20 °C until use.

Electrophoresis and Western blotting

Membrane proteins (50 $\mu\text{g}/\text{well}$) were electrophoresed on an SDS/PAGE (5–15%) gel by the method of Laemmli [23] and transferred by semi-dry blotting to nitrocellulose membranes by using a current density of 0.8 mA/cm². After blocking the nitrocellulose membranes for 5 h with 5% dried milk in TBS/Tween buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween), they were probed with the corresponding primary antibody for 2 h (or overnight with 5F10), and then with either anti-rabbit or anti-mouse second antibody linked to horseradish peroxidase for 2 h. The blots were then developed with aminoethylcarbazole.

Immunoprecipitation

Immunoprecipitation was carried out on membranes lysed in buffer containing 1% Nonidet P40, 0.2% lithium dodecyl sulphate, 4 mM sodium deoxycholate, 10 mM NaF, 20 mM sodium pyrophosphate, 5 mM EDTA, 75 mM NaCl, 1 mM PMSF, 100 μM Na_2VO_4 , 0.2 mM leupeptin and 20 mM Tris, pH 7.4. Lysates were incubated with various amounts of PL/IM 430 linked to 100 μl of 10% Protein A-Sepharose via goat anti-mouse IgG. After incubation for 4 h at 4 °C, the immuno-adsorbant columns were extensively washed five times in 0.1% BSA/0.01% NaN_3 /0.5% Nonidet P40/0.1% SDS, once in buffer without BSA, and subsequently subjected to SDS/PAGE, transferred to nitrocellulose and subjected to Western blotting with appropriate antibodies (see the Results section).

Phosphoenzyme-intermediate formation

Intracellular-membrane Ca^{2+} ATPases were phosphorylated with [γ - ^{32}P]ATP (3 mCi/ μmol) with a final concentration of 3 μM ATP for 1 min at 4 °C. The reactions were stopped with the electrophoresis sample buffer and samples were subjected to SDS/PAGE, drying and autoradiography at –70 °C for 72 h.

Determination of Ca^{2+} ATPase activities

Ca^{2+} ATPase activities were measured by using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ exactly as described previously [24]. Final incubations contained 120 mM KCl, 5 mM MgSO_4 , 20 mM Hepes, pH 7.2, 1 mM MgATP containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 50 μM CaCl_2 and membranes (50–100 μg of protein). Reactions were incubated for 15 min at 37 °C and stopped by addition of 2.5 ml of Norit A (activated charcoal, 25 mg/ml in 0.1 M H_3PO_4). After centrifugation (1500 g for 20 min), 0.5 ml of the supernatant was taken, and the radioactivity representing released $[\text{P}^{32}]\text{P}_i$ was measured by liquid-scintillation spectrometry. The basal Mg^{2+} ATPase activity was estimated in the presence of 5 mM EGTA. For the inhibition of Ca^{2+} ATPase activity, 3 μM Tg was used in the assay.

All results shown are means $\pm$ S.D., or, for Western blots, are typical of at least three separate determinations.

RESULTS

Table 1(a) shows the basal bivalent-cation (both Mg^{2+} and Ca^{2+})-activated ATPase activities of all three membrane fractions. Mixed membranes were taken directly from sorbitol density gradients; intracellular and plasma membranes were obtained by subjecting the mixed membranes to free-flow electrophoresis. Basal Mg^{2+} ATPase activities, measured in the presence of 5 mM EGTA, of all the membranes did not show any significant difference from one another. However, Ca^{2+} ATPase activities showed a predominant presence in both mixed and intracellular membranes, with a lower activity in plasma membranes. Intracellular-membrane Ca^{2+} ATPase was only slightly increased compared with the activity in mixed membranes. This is probably due to unavoidable partial degradation during free-flow electrophoresis, which is carried out at 7 °C (see below). However, this activity was 2–3-fold higher as compared with plasma membranes. Previous studies indicated that plasma membranes obtained by free-flow electrophoresis had little or no ability to sequester Ca^{2+} , nor did they show an increase in Ca^{2+} ATPase activity above the Mg^{2+} -stimulated component [9,10]. This may have been due to insufficient protection with proteolytic

Table 1 Ca^{2+} - and Mg^{2+} -ATPase activities of purified platelet membranes, and the effects of Tg

(a) Ca^{2+} - and Mg^{2+} -ATPase activities (nmol of P_i /min per mg of protein) were measured as described in the Methods section. Activities shown are means $\pm$ S.D. of three separate preparations. Ca^{2+} Mg^{2+} ATPase activities were estimated in the presence of 50 μM Ca^{2+} ; Mg^{2+} ATPase activities were estimated in the presence of 5 mM EGTA. Abbreviations: MM, mixed membranes; PM, plasma membranes; IM, intracellular membranes. (b) Effect of thapsigargin (3 μM) on PM and IM ATPase activities. All other conditions were as for (a).

(a) Distribution of activities		
	Ca^{2+} ATPase	Mg^{2+} ATPase
MM	80 $\pm$ 9.3	16.5 $\pm$ 9.6
PM	44.8 $\pm$ 13.2	22.8 $\pm$ 8.3
IM	85.5 $\pm$ 19.0	20.6 $\pm$ 6.8
(b) Effects of Tg on PM and IM Ca^{2+} ATPase activities		
	Ca^{2+} ATPase	Mg^{2+} ATPase
PM	37.2 $\pm$ 4.9	24.7 $\pm$ 8.3
PM + Tg	34.1 $\pm$ 4.3	22.6 $\pm$ 2.2
IM	87.4 $\pm$ 2.4	24.1 $\pm$ 3.3
IM + Tg	21.3 $\pm$ 4.3	22.9 $\pm$ 2.6

Figure 1 Immunological identification of a PMCA-type Ca^{2+} ATPase in purified platelet membranes, using the antibody 5F10

Platelet membranes (80 μg , lanes 2, 3, 4) and red-blood-cell membranes (60 μg , lane 1) were subjected to SDS/PAGE, followed by transfer to nitrocellulose and Western blotting using the antibody 5F10. The blot shown is typical of three. Key: rbc, human red-blood-cell membranes; m, platelet mixed membranes; p, platelet plasma membranes; i, platelet intracellular membranes; st, molecular-size standards with values (kDa) as indicated on the right. Arrows indicate the positions of 150, 135 and 75 kDa bands.

inhibitors. In the present studies we have therefore additionally used dithiothreitol, trypsin inhibitors and the calpain inhibitor E64 during the sonication of platelets.

Studies were then carried out to determine the Tg-sensitivity of the Ca^{2+} ATPase of the two membrane fractions. Tg has been shown to inhibit platelet Ca^{2+} ATPase activity [24] and all SERCA-type Ca^{2+} pumps, but not the PMCA [25]. Table 1(b) shows that Tg (3 μM) totally inhibited the Ca^{2+} ATPase activity of the intracellular-membrane fraction to basal levels as measured with EGTA, but that the Ca^{2+} ATPase activity of the plasma membrane was not significantly affected. The Mg^{2+} ATPase activity of both membrane fractions was not affected. These results confirm that the low level of Ca^{2+} ATPase activity detected in the plasma-membrane fractions was not a contaminant of the SERCA intracellular Ca^{2+} ATPase, and might be PMCA-type. Low levels of a PMCA in human platelets have been reported recently by Cheng et al. [7], using a range of antibodies to the red-cell PMCA. We have used one of these antibodies, 5F10, and have observed recognition of two bands with migration at 135 and 150 kDa in platelet mixed-membrane and plasma-membrane fractions (Figure 1). The antibody also recognized the corresponding bands in red-blood-cell membranes. No detection of any band was seen in platelet intracellular membranes, confirming the exclusive plasma-membrane location of the Ca^{2+} ATPase. In mixed membranes and plasma membranes another faint band at 75 kDa was also seen in some preparations, which may reflect an antibody-recognized degradation product. In the red-cell membrane preparation additional degradation products at 91, 59, 48 and 38 kDa were also observed that were not detected with platelet membranes. At present the isoform specificity of 5F10 has not been described, and hence the identity of these fragments requires further elucidation.

The identities of the intracellular-membrane Ca^{2+} ATPase activities were also analysed by using antibodies. Previously the Ca^{2+} ATPase recognized by PL/IM 430 was localized on intracellular membranes prepared by free-flow electrophoresis [17];

Figure 2 Immunological identification of Ca^{2+} -ATPases in platelet membranes, using antibodies PL/IM 430, N89 and S.2b

Platelet membranes (50 μg) were subjected to SDS/PAGE, followed by transfer to nitrocellulose and Western blotting (for details, see the Methods section). (a), (d), immunodetection with monoclonal antibody PL/IM 430; (b), immunodetection with antiserum N89; (c), antiserum S.2b. Results shown are typical Western blots, repeated on at least three other membrane preparations. Key: m, mixed membranes (lanes 1, 4, 7, 10), p, plasma membranes (lanes 2, 5, 8, 11); i, intracellular membranes (lanes 3, 6, 9, 12). Arrows denote 100 kDa Ca^{2+} -ATPase.

Figure 3 Immunoprecipitation of Ca^{2+} -ATPase by PL/IM 430

(a) Intracellular membranes were solubilized and immunoprecipitated with various amounts of PL/IM 430, then subjected to SDS/PAGE, transferred to nitrocellulose and probed with PL/IM 430; see the Methods section for full details. Arrows indicate migration position of parent 100 kDa Ca^{2+} -ATPase. Prominent degradation products of 74 and 70 kDa are also observed. Western blots are representative of at least three other observations. (b) Recognition of PL/IM 430 immunoprecipitates with antibodies PL/IM 430, N89 and S.2b. Parallel immunoprecipitates from solubilized mixed (m) and intracellular (i) membranes were carried out with 50 μl of PL/IM 430 antibody as described in (a) and, after SDS/PAGE and blotting, were probed with PL/IM 430 (lanes 1, 2), N89 (lanes 3, 4) and S.2b (lanes 5, 6). Arrows represent the position of 100 kDa Ca^{2+} -ATPase, with degradation products also observed at 74, 70 and 40 kDa (lanes 1–4). Western blots shown are typical of at least three separate determinations on different membrane preparations.

Figure 4 Phosphoenzyme-complex formation of Ca^{2+} -ATPase in intracellular membranes

Intracellular membranes were incubated with [γ - ^{32}P]ATP, followed by SDS/PAGE, Western blotting and autoradiography of the blots. Lanes 1 and 3 are autoradiographs of Western blots probed with PL/IM 430 (lane 2) and N89 (lane 4). Arrows represent positions of peptides of 100 (parent Ca^{2+} -ATPase), 74, 55 and 40 kDa. Results shown are typical of six separate determinations.

we have now used the polyclonal antibodies specific to SERCA 2b (S.2b) and SERCA 3 (N89). Figure 2 shows a typical Western-blot analysis of the purified membranes. All three antibodies recognize the corresponding 100 kDa peptides in mixed and intracellular membranes, with little or no recognition of the corresponding protein in plasma membrane. With a number of membrane preparations (Figures 2a and 2b, but not Figure 2d), PL/IM 430, and to a lesser extent N89, recognized a 40 kDa peptide with mixed membranes along with 74 and 70 kDa peptides in intracellular membranes. These are very likely to be partial degradation products, as their levels increased as the membrane purification procedure continued. The S.2b antibody showed prominent recognition of the 100 kDa peptide, and in one membrane preparation (from five analysed with the antibody) a faint recognition of a 30 kDa fragment was observed, implying that this ATPase may be more resistant to endogenous protease action during the preparation. All of these antibodies recognized only the 100 kDa peptide with platelet lysates (results not shown).

Although the identities of the Ca^{2+} pumps recognized by the S.2b and the N89 antibodies have been established [13,19], the Ca^{2+} -ATPase isoform recognized by PL/IM 430 is still to be definitively assigned. To study this further, we have carried out immunoprecipitation analysis using PL/IM 430. Figure 3(a) shows a dose-related immunoprecipitation of lysed intracellular membranes using PL/IM 430, followed by SDS/PAGE, transfer to nitrocellulose and re-probing with PL/IM 430. The antibody immunoprecipitated the 100 kDa peptide along with the lower-molecular-mass degradation products. When immunoprecipitated proteins from both mixed- and intracellular-membrane lysates were re-probed with N89 or S.2b antibodies, the N89 antibody recognized products identical with those recognized by PL/IM 430, whereas S.2b showed no recognition of any product (Figure 3b). Both S.2b and N89 are rabbit polyclonal antibodies; this clearly rules out any possibility of non-specific staining. This strongly suggests that PL/IM 430 recognizes the SERCA 3 Ca^{2+} -ATPase.

We have also examined the phosphoenzyme-complex formation of the Ca^{2+} -ATPase in intracellular membranes incubated with [γ - ^{32}P]ATP. Figure 4 shows a typical result obtained

(repeated six times) showing an autoradiograph of Western blots probed with PL/IM 430 and N89. Phosphoenzyme complex formation was observed with the 100 kDa and 74 kDa peptides, both of which were recognized along with the 70 and 40 kDa degradation products by PL/IM 430 and N89 antibodies. Low levels of phosphorylation were also detected with 55 and 40 kDa peptides (Figure 4). It is likely that the 55 kDa fragment is a degradative product of the SERCA 2b Ca^{2+} ATPase (see [11]). The S.2b antibody, which is raised to a C-terminal peptide of the Ca^{2+} pump, only recognized the 100 kDa protein on Western blots (results not shown). It would not bind to an N-terminal 55 kDa phosphoprotein fragment.

DISCUSSION

The present study illustrates the use of highly purified platelet membrane fractions and isoform-specific antibodies to Ca^{2+} ATPases in order to identify and localize the different Ca^{2+} pumps. Previous studies using these membranes demonstrated that the platelet Ca^{2+} ATPase activities were predominantly on intracellular membranes [8,9]. Subsequently a number of studies indicated that two distinct types of Ca^{2+} ATPases were present in platelets. These conclusions were based on isoform-specific tryptic fragmentation patterns, on the ability of La^{3+} to enhance the phosphoenzyme complex formation of one of the isoforms, and a differential inhibition of the phosphoenzyme-complex formation by Tg and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone [12,15,26]. These two isoforms have recently been identified to be the SERCA 2b and SERCA 3 Ca^{2+} ATPases, which can be resolved in acidic gels as 100 and 97 kDa peptides respectively [11,13]. Trypsin digestion of the SERCA 2b isoform results in the formation of a 55 kDa and a 35 kDa fragment, whereas the SERCA 3 isoform is proteolysed with trypsin to give a 80 kDa fragment. For SERCA 3 the formation of the phosphoenzyme complex of both the parent 97 kDa peptide and the 80 kDa fragment is enhanced in the presence of La^{3+} . In this study we have shown that antibodies specifically directed against the SERCA 2b and SERCA 3 Ca^{2+} ATPases detect the proteins in intracellular membranes, with little or no binding in plasma membranes. In addition, we have used the monoclonal antibody PL/IM 430, which was raised to intracellular membranes and which has been shown to inhibit Ca^{2+} sequestration by more than 50% into these stores [17,18]. This unique antibody recognizes an as yet unknown epitope, leading to inhibition of Ca^{2+} sequestration without any apparent effect on hydrolysis of ATP [17]. In addition to that of human platelets, it recognizes the Ca^{2+} ATPase pump in a number of human megakaryoblastoid and lymphoid cell lines [12]. PL/IM 430 does not bind to rat SERCA 3 expressed in COS cells, nor does it recognise a band of 97 kDa in rat platelets [13]. Therefore it is important to determine the identity of the SERCA pump recognized by PL/IM 430, as it represents a major Ca^{2+} ATPase isoform in human platelets. Papp et al. [12] reported that PL/IM 430 recognized the native 97 kDa peptide which gave rise to the 80 kDa proteolytic fragment whose phosphorylation was inhibited by 2,5-di-(*t*-butyl)-1,4-benzohydroquinone. The 80 kDa fragment is not recognized by PL/IM 430, but Wuytack et al. [13] identified this as a SERCA 3 product in COS cells over-expressing this Ca^{2+} pump. However, in contrast with these findings, Kovacs et al. [16] recently suggested that PL/IM 430 recognizes a Ca^{2+} ATPase distinct from either SERCA 2b or SERCA 3. This suggestion was based on the formation of PL/IM 430-recognizable 73, 68 and 40 kDa peptides by mild trypsin treatment of platelet membranes. It is of considerable interest that in a number of membrane preparations we detect the appearance of similar

peptides of 74, 70 and 40 kDa during our relatively long membrane-purification procedure (Figure 2), even though soybean trypsin inhibitors and aprotinin are present throughout. The possibility that trypsin-like proteases are contaminating our preparations cannot be discounted. In phosphorylation experiments both the parent 100 kDa protein and the 74 kDa fragment can be phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Figure 4), which is in agreement with the studies of Kovacs et al. [16]. As regards the identity of the Ca^{2+} ATPase recognized by PL/IM 430, our Western-blotting experiments with this antibody show a striking similarity to that observed with the SERCA 3-specific N89 antibody. This was strongly supported by the observation that PL/IM 430-immunoprecipitated Ca^{2+} ATPase showed near-identical recognition of both the parent (100 kDa) and degradation products (74, 70 and 40 kDa) with N89, and, as expected, no cross-reactivity with the S.2b antibody. This suggests that PL/IM 430 recognizes the SERCA 3 Ca^{2+} ATPase, or at least (but unlikely) that it may co-immunoprecipitate a SERCA 3 Ca^{2+} ATPase. From the present evidence we favour the former possibility, and suggest that platelet intracellular Ca^{2+} stores contain two distinct SERCA Ca^{2+} ATPases, not three. Further studies are underway for epitope-mapping of the site recognized by PL/IM 430.

Our studies also demonstrate for the first time a Ca^{2+} ATPase present on a purified plasma membrane that is insensitive to Tg. The insensitivity to Tg confirms that this ATPase is not a contaminant from the intracellular membranes. Previous attempts to observe a Ca^{2+} ATPase activity in purified plasma membranes proved unsuccessful, and may indicate the highly labile nature of this activity [10,26–28]. Only recently Cheng et al. [7] reported immunological identification of low levels of a 135 and 120 kDa protein using antibodies raised to a red-cell Ca^{2+} ATPase. The isoform specificities of the antibodies used were not described, and thus the isoform(s) expressed in platelets still needs to be determined. We have used one of these antibodies, 5F10, and have observed it to show staining of peptides at 135 and 150 kDa migration in both mixed membranes and plasma membranes. Whether this reflects two isoforms of the PMCA present in platelets, or whether the smaller-molecular-size species is a product of the larger, is not known, but both peptides were also detected in red-cell membranes. From a number of determinations, the immune reaction of 5F10 towards platelet membranes was less than that observed with red-cell membranes, highlighting the need to develop new antibodies that would exhibit higher affinities towards the PMCA in human platelets. Significantly, the demonstration of a Ca^{2+} ATPase activity in platelet plasma membranes will now allow its characterization, and also determination of its relative contribution to the removal of Ca^{2+} from the platelet cytosol.

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REFERENCES

- Authi, K. S. (1994) *Adv. Exp. Biol. Med.* **344**, 83–104
- Carafoli, E. (1992) *J. Biol. Chem.* **267**, 2115–2118
- Wuytack, F. and Raeymaekers, L. (1992) *J. Bioenerg. Biomembr.* **24**, 285–300
- Burk, S. E., Lytton, J., MacLennan, D. H. and Shull, G. E. (1989) *J. Biol. Chem.* **264**, 18561–18568
- MacLennan, D. H., Toyofuku, T. and Lytton, J. (1992) *Ann. N.Y. Acad. Sci.* **671**, 1–10
- Kimura, M., Aviv, A. and Reeves, J. P. (1993) *J. Biol. Chem.* **268**, 6874–6877
- Cheng, H. Y., Magocsi, M., Cooper, R. S., Penniston, J. T. and Borke, J. L. (1993) *Cell Physiol. Biochem.* **4**, 31–43

- 8 Dean, W. L. (1984) *J. Biol. Chem.* **259**, 7343–7348
- 9 Menashi, S., Authi, K. S., Carey, F. and Crawford, N. (1984) *Biochem. J.* **222**, 413–417
- 10 Hack, N., Croset, M. and Crawford, N. (1986) *Biochem. J.* **233**, 661–668
- 11 Enouf, J., Bredoux, R., Papp, B., Djaffar, I., Lompre, A. M., Kieffer, N., Gayet, O., Clemetson, K., Wuytack, F. and Rosa, J. P. (1992) *Biochem. J.* **286**, 135–140
- 12 Papp, B., Enyedi, A., Paszty, K., Kovacs, T., Sarkadi, B., Gardos, G., Magnier, C., Wuytack, F. and Enouf, J. (1992) *Biochem. J.* **288**, 297–302
- 13 Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K. S. and Casteels, R. (1994) *J. Biol. Chem.* **269**, 1410–1416
- 14 Bobe, R., Bredoux, R., Wuytack, F., Quarck, R., Kovacs, T., Papp, B., Corvazier, E., Magnier, C. and Enouf, J. (1994) *J. Biol. Chem.* **269**, 1417–1424
- 15 Papp, B., Enyedi, A., Kovacs, T., Sarkadi, B., Wuytack, F., Thastrup, O., Gardos, G., Bredoux, R., Levy-Toledano, S. and Enouf, J. (1991) *J. Biol. Chem.* **266**, 14593–14596
- 16 Kovacs, T., Corvazier, E., Papp, B., Magnier, C., Bredoux, R., Enyedi, A., Sarkadi, B. and Enouf, J. (1994) *J. Biol. Chem.* **269**, 6177–6184
- 17 Hack, N., Wilkinson, J. M. and Crawford, N. (1988) *Biochem. J.* **250**, 355–361
- 18 Hack, N., Authi, K. S. and Crawford, N. (1988) *Biosci. Rep.* **8**, 379–388
- 19 Wuytack, F., Eggermont, J. A., Raeymaekers, L., Plessers, L. and Casteels, R. (1989) *Biochem. J.* **264**, 765–769
- 20 Authi, K. S. and Crawford, N. (1985) *Biochem. J.* **230**, 247–253
- 21 Authi, K. S. (1992) *FEBS Lett.* **298**, 173–176
- 22 Crawford, N., Hack, N. and Authi, K. S. (1992) *Methods Enzymol.* **245**, 5–20
- 23 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 24 Authi, K. S., Bokkala, S., Patel, Y., Kakkar, V. V. and Munkonge, F. (1993) *Biochem. J.* **294**, 119–126
- 25 Lytton, J., Westlin, M. and Hanley, M. R. (1991) *J. Biol. Chem.* **266**, 17067–17071
- 26 Enyedi, A., Sarkadi, B., Foldes-Papp, Z., Monostory, S. and Gardos, G. (1986) *J. Biol. Chem.* **261**, 9558–9564
- 27 Steiner, B. and Lusher, L. F. (1985) *Biochim. Biophys. Acta* **818**, 299–309
- 28 Fauvel, J., Chap, H., Rogues, V., Levy-Toledano, S. and Douste-Blazy, L. (1986) *Biochim. Biophys. Acta* **856**, 155–164