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Characterization of membrane calcium pumps by simultaneous immunoblotting and ^{32}P radiography

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Calcium pumps of various plasma membrane, endoplasmic reticulum and sarcoplasmic reticulum preparations were visualized by simultaneous immunoblotting and autoradiography of the ^{32}P-labelled phosphoenzymes. The pump proteins and their fragments produced by a proteolytic pretreatment of the membranes were selectively phosphorylated by [γ-^{32}P]ATP, separated on an acidic SDS-polyacrylamide gel, blotted onto nitrocellulose and reacted with polyclonal antibodies raised against the purified human erythrocyte and rat skeletal muscle sarcoplasmic reticulum calcium pumps, respectively. The immuno-reaction was detected by peroxidase staining, while the phosphoproteins were shown by autoradiography of the same blot. An antibody against the erythrocyte calcium pump, reacting on the blot with the 140 kDa erythrocyte calcium pump and its 80 kDa proteolytic fragment, did not show a cross-reaction with the calcium pump of similar molecular mass in rat synaptosome membranes or with any of the endoplasmic- or sarcoplasmic-type calcium pumps. An anti-sarcoplasmic reticulum calcium pump antibody cross reacted with several sarcoplasmic and endoplasmic calcium pump proteins and their proteolytic fragments but with none of the plasma membrane pumps. This sensitive double-labelling method can be applied to study structural relationships and molecular alterations in various ion pump proteins.

Introduction

Selective phosphorylation of the in situ calcium pump proteins by [γ-^{32}P]ATP is a highly sensitive method of following the molecular characteristics of these transport systems, even in membranes where they appear only as minor components. As we recently demonstrated [1–4], by using appropriate phosphorylation conditions and a good resolution acidic SDS-polyacrylamide gel system, the calcium pumps and their proteolytic fragments in the erythrocyte or platelet membranes can be identified and various structure-function relationships can be elucidated. In the present communication, we extend the applicability of these methods by using an additional immunological tagging of the pump proteins and their fragments. We also demonstrate structural and immunological rela-

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ASRC antibody, polyclonal antibody raised against a purified rat skeletal muscle calcium pump protein.

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tionships between some plasma membrane calcium pumps and between various sarcoplasmic reticulum- and endoplasmic reticulum-type calcium pumps, respectively.

Experimental procedures

Membrane preparations. Membrane vesicles from human red cells, human peripheral blood lymphocytes and human platelets were prepared as described in Refs. 1, 5 and 2, respectively. Vesicles from synaptosome membranes were prepared as described in Ref. 6, while sarcoplasmic reticulum membranes were prepared according to Ref. 7.

Antibody preparations. Polyclonal antibody against the calmodulin-affinity purified human red cell calcium pump was prepared in rabbits, and this antibody has already been partially characterized [8]. For the preparation of anti sarcoplasmic reticulum-calcium pump antibody New Zealand rabbits were immunized with sarcoplasmic calcium ATPase prepared from the gastrocnemius muscle of rats by the method of MacLennan [9]. The purified enzyme was suspended in complete Freund adjuvant and injected four times subcutaneously at 3-week intervals. The reactivity of the sera was tested using an ELISA method. The antisera were used without further purification in the present study.

Labelling and identifying the calcium pumps. Selective phosphorylation of the calcium pump proteins was carried out as described in Ref. 1, in brief: the membranes were phosphorylated at 4°C by [γ-^{32}P]ATP (0.2 μM final concentration) in a medium containing 75 mM KCl, 30 mM Hepes-potassium, 20 μM $CaCl_2$ and 50 μM $LaCl_3$. The reaction was stopped by the addition of ATP- and P_i-containing trichloroacetic acid, the precipitate was washed with the same solution and then dissolved in the electrophoresis buffer. The acidic SDS-polyacrylamide gel system for resolving the phosphorylated pump proteins and conserving the phosphoenzyme labelling is described in Ref. 1. After electrophoresis, in the present experiments, the wet slab gels (8 × 15 cm and 1.5 mm thick) were placed on nitrocellulose filter sheets and the proteins were electroblotted (35 V, 150–200 mA) overnight at 8°C in a buffer containing 30 mM Tris-glycine buffer (pH 8.3) and 20% methanol. The blots were saturated with bovine serum albumin in a NaCl (300 mM) and Tris (30 mM, pH 7.0) buffer containing 5% bovine serum albumin, for 1 h at 22°C. The first antibody, dissolved in the same buffer, was allowed to react for 1 h, then the blot was washed with the above NaCl-Tris buffer containing 0.01% Tween-20 (two washes of 10 min each). The second antibody was a horseradish-peroxidase conjugated anti-rabbit IgG (Nordic), incubated for 1 h in the same NaCl-Tris-bovine serum albumin buffer with the blot. The second antibody was washed out with the NaCl-Tris-Tween buffer and the peroxidase staining was developed in the NaCl-Tris buffer containing 1 mg/ml diaminobenzidine and 0.001% H_2O_2. The dried, stained blot was exposed to an X-ray film (MEDIFORT) for 24–72 h at −30°C to obtain the autoradiogram of the labelled proteins. The molecular weights of the labelled proteins were estimated by running phosphorylated red cell membrane proteins as markers on the same blots.

Limited proteolysis of the membranes was carried out at the trypsin concentrations indicated in the figure legends at 4°C in the buffer used for membrane phosphorylation. Proteolysis was terminated by the addition of excess trypsin inhibitor to the media.

Results

Fig. 1 shows the autoradiogram (A) and the horseradish peroxidase (HRP)-stained immunoblot (B) of human red cell, rat synaptosome, rat skeletal muscle sarcoplasmic reticulum, and human platelet membrane preparations. The membranes were phosphorylated by [^{32}P]ATP and tagged by a polyclonal anti-erythrocyte calcium pump antibody. Every second lane shows similar experiments with the same membranes pretreated with trypsin.

Membrane phosphorylation (panel A) was carried out by [γ-^{32}P]ATP at 4°C in the presence of calcium plus lanthanum (but no magnesium), which resulted in a selective labelling of both plasma membrane- and endoplasmic reticulum- or sarcoplasmic reticulum-type calcium pumps. In the case of plasma membrane calcium pumps these

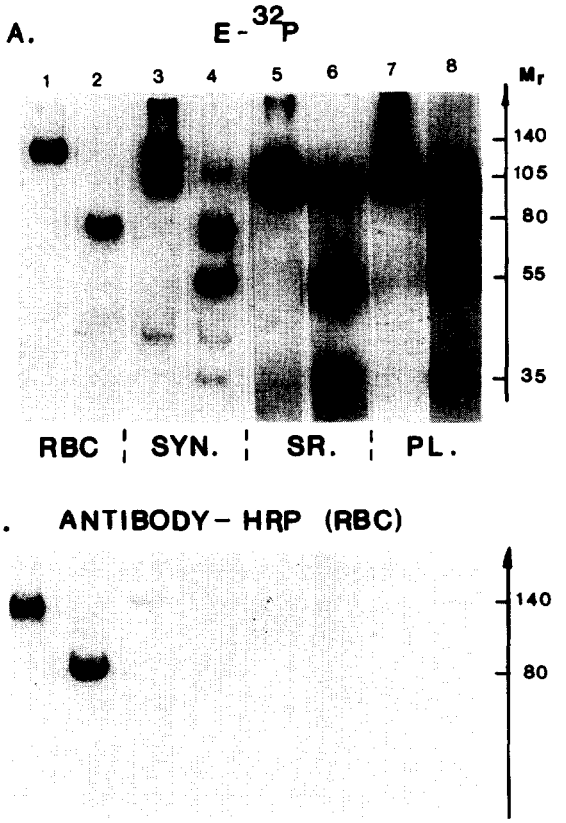

Fig. 1. Autoradiographed (A) and peroxidase-stained (B) immunoblot of ^{32}P-labelled calcium pump proteins examined with an anti-erythrocyte calcium pump antibody. The membranes from human erythrocytes (lanes 1, 2), rat synaptosomes (3, 4), rat skeletal muscle sarcoplasmic reticulum (5, 6) and human platelets (7, 8) were phosphorylated in the presence of calcium and lanthanum, as described in Experimental procedures. The samples run in lanes 2, 4, 6 and 8 were treated with 30 μg/ml trypsin for 10 min at 4° C before phosphorylation.

conditions produce a maximum labelling, while the sarcoplasmic reticulum or endoplasmic reticulum calcium pumps are at near maximum phosphorylation levels (see Ref. 2). In the erythrocyte membrane (lanes 1 and 2) the only proteins phosphorylated are the 140 kDa calcium pump and its 80 kDa tryptic limit polypeptide. In rat brain synaptosome membrane vesicles (lanes 3 and 4), which show a calmodulin-stimulated calcium uptake (data not shown), the major phosphorylated bands are 140 kDa and 105 kDa proteins, while trypsin treatment produces 80, 76, 55 and 35 kDa phosphoproteins. In the rat sarcoplasmic reticulum membrane the phosphorylated protein is the 105 kDa calcium pump (some dimers and tetramers of this protein also appear) and its labelled proteolytic fragments have molecular masses of about 55 and 35 kDa. In a human platelet membrane preparation, containing both plasma membrane and endoplasmic reticulum, but no mitochondrial or granule membranes (see Ref. 2), the 100–105 kDa calcium pump phosphoprotein(s) yield(s) tryptic fragments of 80, 55 and 35 kDa. Thus, the erythrocyte membrane contains only a plasma membrane-type calcium pump, the sarcoplasmic reticulum membrane contains a different kind of calcium pump, and the synaptosome and platelet membrane preparations appear to contain a mixture of these proteins. Since in the platelet membranes no 140 kDa pump protein is seen, the 80 kDa fragment may originate from a partially proteolyzed plasma membrane-type pump or from a third kind of calcium pump protein (see Ref. 2).

Panel B in Fig. 1 shows that the only proteins reacting with the anti-erythrocyte calcium pump antibody on this blot are the 140 kDa erythrocyte membrane calcium pump and its tryptic fragment of about 80 kDa. No immuno-reaction is seen with the calcium pumps in synaptosomes, sarcoplasmic reticulum, or platelet membranes (Fig. 1B), while the anti-erythrocyte calcium pump antibody cross-reacts with the 140 kDa calcium pump seen in human lymphocyte membrane preparations (data not shown). In parallel experiments we have found that there was no difference in the immuno-reactivity of the 140 kDa erythrocyte calcium pump on the blot if (1) no ^{32}P-phosphoenzyme-forming phosphorylation was applied, (2) phosphorylation was carried out at 37° C for 5 min in the presence of 5 mM magnesium (producing protein-kinase type phosphorylation of various membrane proteins) or (3) when the trichloroacetic acid precipitation step was omitted.

Fig. 2 shows the autoradiogram (A) and the horseradish peroxidase-stained immunoblot (B) of rat sarcoplasmic reticulum, platelet and synaptosome membranes reacting with a polyclonal antibody raised against a purified rat skeletal muscle calcium pump protein (ASRC antibody). Similar to the experiments shown in Fig. 1, phosphorylation was carried out in the presence of calcium

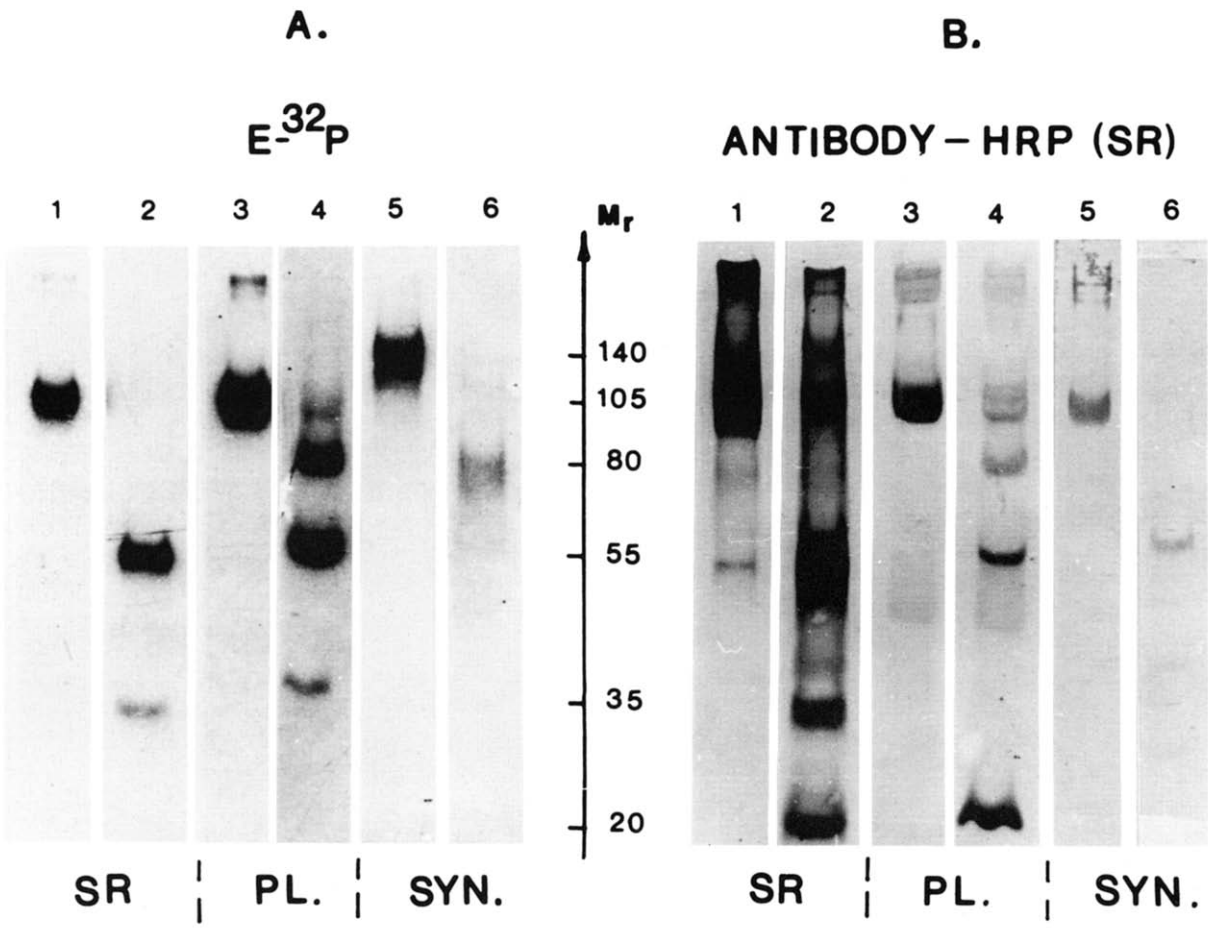

Fig. 2. Autoradiographed (A) and peroxidase-stained (B) immunoblot of ^{32}P-labelled calcium pump proteins examined with ASRC antibody. The membranes form rat skeletal muscle (fast) sarcoplasmic reticulum (lanes 1, 2), human platelets (3, 4) and rat synaptosomes (5, 6) were phosphorylated in the presence of calcium and lanthanum as described in Experimental procedures. The samples run in lanes 2, 4 and 6 were treated with 30 μg/ml trypsin for 10 min at 4° C before phosphorylation.

plus lanthanum and every second lane represents trypsin-pretreated membranes.

The phosphorylation pattern is similar to that seen in Fig. 1, except that in this synaptosome membrane preparation the 105 kDa protein and its proteolytic fragments are less abundant. In panel B, the immunoblot shows a strong reaction of the ASRC antibody with the 105 kDa rat sarcoplasmic reticulum calcium pump protein and with its proteolytic fragments of 55, 50, 35 and 20 kDa. In the proteolysed sarcoplasmic reticulum a weak band at about 150 kDa appears, probably produced from the dimeric sarcoplasmic reticulum calcium pump proteins seen in the undigested samples. The ASRC antibody also reacts with the 100–105 kDa pump proteins in the platelet membrane (actually showing a double band in this region), and in the trypsin-pretreated platelet membrane additional reactions with 80, 55, 35 and 20 kDa fragments become clearly visible. In the synaptosome membrane the ASRC antibody does not react with the 140 kDa calcium pump but depicts the much less abundant (see panel A) 105 kDa calcium pump and its 55 kDa proteolytic fragment. There is absolutely no cross-reaction of the ASRC antibody with the 140 kDa calcium pump in the erythrocyte membrane or with the proteolytic fragments of this protein (data not shown).

Fig. 3 shows the phosphorylation pattern (A)

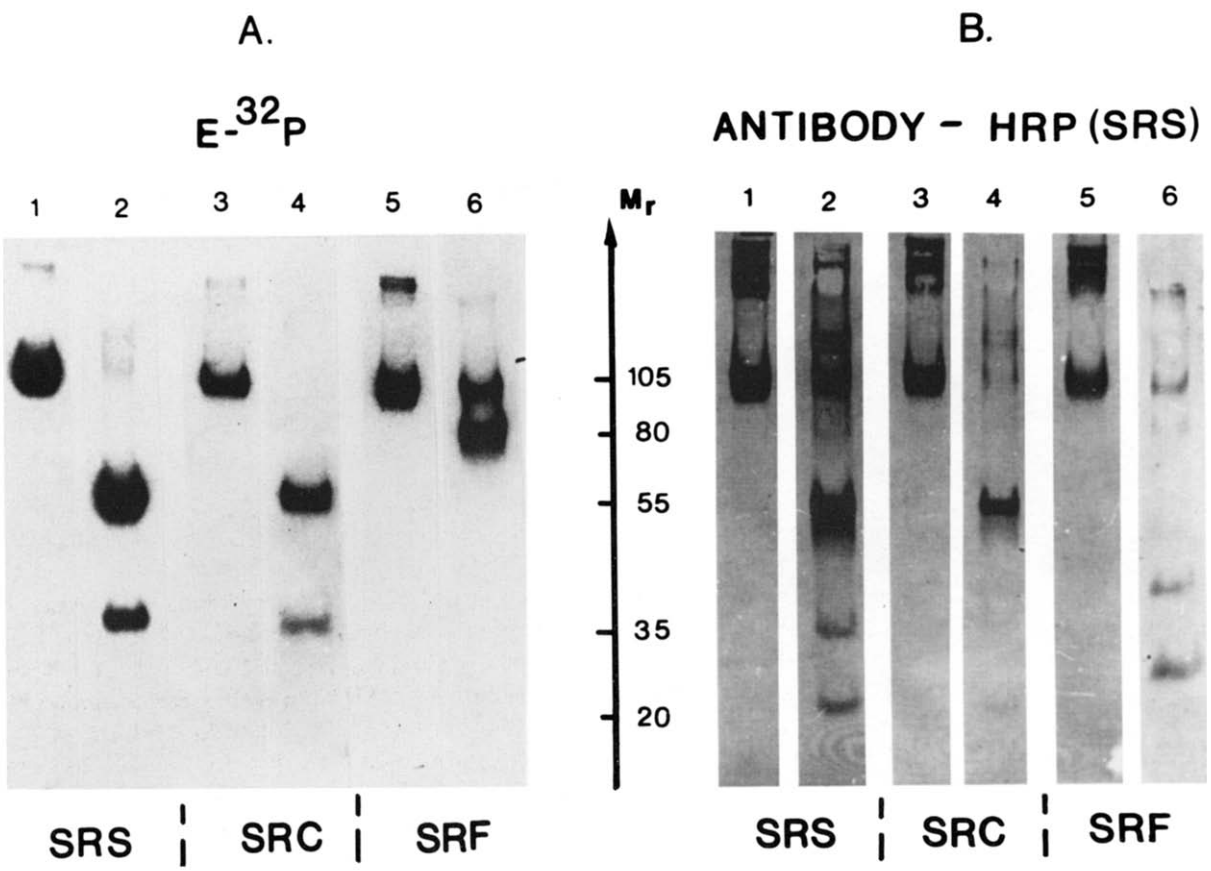

Fig. 3. Autoradiographed (A) and peroxidase-stained (B) imunoblot of ^{32}P-labelled calcium pump proteins examined with ASRC antibody. The membranes from rabbit skeletal muscle (lanes 1, 2), rabbit cardiac muscle (3, 4) and fish (carp – *Cyprianus carpio*) skeletal muscle (5, 6) were phosphorylated in the presence of calcium and lanthanum as described in Experimental procedures. The samples run in lanes 2, 4 and 6 were treated with 30 μg/ml trypsin for 10 min at 4° C before phosphorylation.

and the ASRC antibody immunoblot (B) of sarcoplasmic reticulum membranes isolated from rabbit skeletal muscle [1,2], rabbit heart muscle [3,4] and fish (carp) skeletal muscle [5,6]. In all these membranes, the calcium pumps have molecular masses of about 105 kDa, but the phosphorylated proteolytic fragments seen in panel A are different: while both the rabbit skeletal and heart muscle sarcoplasmic reticulum pumps have fragments similar to that of the rat sarcoplasmic reticulum, in fish sarcoplasmic reticulum the only phosphorylated tryptic fragment is a peptide of about 80 kDa. Immuno-reaction of the rabbit skeletal or heart muscle sarcoplasmic reticulum is also very similar to that seen in the rat sarcoplasmic reticulum, and the same is true for the slow-type rat muscle examined (data not shown). However, in the fish sarcoplasmic reticulum preparation the immuno-reactive fragments have relative molecular masses of about 80, 40 and 25 (the immuno-reaction in the 80 kDa fragment is much smaller than expected by its phosphorylation).

Discussion

During the past few years, immunological techniques for identifying membrane transport proteins have become available and give new perspectives for studying structure-function relationships in these systems. Polyclonal and monoclonal antibodies have been prepared against purified plasma membrane [8,10–14] and endoplasmic or sarcoplasmic reticulum [15–19] calcium pumps and

various precipitation, enzyme-assay and Western blot techniques have been applied to follow their reactions [8–19]. In this communication, we present results obtained with a double-labelling technique: selective phosphorylation of the in situ calcium pumps and an immunoblot of the radioactively labelled samples. Using this method, we have demonstrated differences and similarities in the molecular structure of these pumps and shown that a proteolytic pretreatment of the membranes further increases the sensitivity of this detection. For the immunoblots here we used polyclonal antibodies to obtain a relatively wide range of recognition.

The anti-erythrocyte calcium pump antibody used in this study reacts at the cytoplasmic membrane surface [8,14]. Here we show that this antibody reacts on the blot with the 140 kDa erythrocyte calcium pump protein independent of its state of phosphorylation, and a variable reaction is seen with the phosphorylated limit polypeptide (see Refs. 1, 3, 4 and 20) of 76–80 kDa. This antibody cross-reacted with the calcium pump of the lymphocyte plasma membrane but did not recognize either the 140 kDa calmodulin-sensitive calcium pump in synaptosomes, or the calcium pumps in platelet and sarcoplasmic reticulum membranes.

The polyclonal anti-sarcoplasmic reticulum calcium pump (ASRC) antibody did not react with any of the 140 kDa plasma membrane calcium pumps examined but recognized the sarcoplasmic- and endoplasmic-type pumps and most of their proteolytic fragments. In this case, again, antibody recognition was independent of the phosphorylation of the proteins or their fragments. The platelet membrane, which seems to contain a mixture of calcium pumps has several ASRC-reactive polypeptide fragments (see also Ref. 19), and this finding still leaves the origin of these pumps uncertain. Although the coding genes for slow and cardiac muscle sarcoplasmic reticulum calcium pumps are different from the gene of the fast-type sarcoplasmic reticulum calcium pump [21], the tryptic fragments are similar and are all recognized by the ASRC antibody. Even the fish sarcoplasmic reticulum calcium pump was found to react with this antibody, although it shows an unexpected pattern of tryptic fragmentation, essentially similar to that reported for the lobster sarcoplasmic reticulum [22].

In membrane preparations in which the calcium pump is a minor fraction of the proteins, or when the biological sample is available in limited amounts only, this double-labelling technique allows a reassuring identification of the polypeptides belonging to these pump systems. Further investigation of the structural relationships and molecular alterations in the calcium pumps using both polyclonal and monoclonal antibodies is currently under way in our laboratories.

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