Smooth muscle expresses a cardiac/slow muscle isoform of the Ca^{2+} -transport ATPase in its endoplasmic reticulum

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Smooth muscle expresses in its endoplasmic reticulum an isoform of the Ca^{2+} -transport ATPase that is very similar to or identical with that of the cardiac-muscle/slow-twitch skeletal-muscle form. However, this enzyme differs from that found in fast-twitch skeletal muscle. This conclusion is based on two independent sets of observations, namely immunological observations and phosphorylation experiments. Immunoblot experiments show that two different antibody preparations against the Ca^{2+} -transport ATPase of cardiac-muscle sarcoplasmic reticulum also recognize the endoplasmic-reticulum/sarcoplasmic-reticulum enzyme of the smooth muscle and the slow-twitch skeletal muscle whereas they bind very weakly or not at all to the sarcoplasmic-reticulum Ca^{2+} -transport ATPase of the fast-twitch skeletal muscle. Conversely antibodies directed against the fast-twitch skeletal-muscle isoform of the sarcoplasmic-reticulum Ca^{2+} -transport ATPase do not bind to the cardiac-muscle, smooth-muscle or slow-twitch skeletal-muscle enzymes. The phosphorylated tryptic fragments A and A_1 of the sarcoplasmic-reticulum Ca^{2+} -transport ATPases have the same apparent M_r values in cardiac muscle, slow-twitch skeletal muscle and smooth muscle, whereas the corresponding fragments in fast-twitch skeletal muscle have lower apparent M_r values. This analytical procedure is a new and easy technique for discrimination between the isoforms of endoplasmic-reticulum/sarcoplasmic-reticulum Ca^{2+} -transport ATPases.

INTRODUCTION

It has by now become clear that smooth muscle contains two different Ca^{2+} -transport ATPases: a calmodulin-binding ATPase of M_r 130 000–140 000 in the plasma membrane (PM) [1] and an ATPase in the endoplasmic reticulum (ER) with an M_r of 100 000 [2–4].

Only the former enzyme has been successfully purified, by virtue of its binding to calmodulin, by using affinity chromatography [5]. Both polyclonal [6] and monoclonal [7] antibodies against this PM Ca^{2+} -transport ATPase were found to block the ATPase and the Ca^{2+} -transport activity competitively with ATP. Furthermore antibodies against the erythrocyte Ca^{2+} -transport ATPase were found to cross-react with the plasmalemmal Ca^{2+} -transport ATPase from smooth muscle [7].

The ER-type Ca^{2+} -transport ATPase from smooth muscle has as yet not been purified and it has therefore not been possible to raise antibodies against this protein. However, the existence of this ATPase has been indirectly demonstrated by means of its phosphoprotein intermediate [2–4]. This intermediate has a similar M_r to that of the Ca^{2+} -transport ATPase of skeletal-muscle sarcoplasmic reticulum (SR), and the steady-state concentration of its catalytic phosphoprotein intermediate is similarly decreased by La^{3+} . Moreover it presents tryptic fragments of comparable M_r values [2,3]. This enzyme differs in all these properties from the above-mentioned PM Ca^{2+} -transport ATPase. It was therefore unexpected that several groups [3,8] reported that antibodies raised

against the SR Ca^{2+} -transport ATPase of fast-twitch skeletal muscle did not bind to the Ca^{2+} -transport ATPase of smooth-muscle ER. This finding suggested that there exist important immunological differences between the Ca^{2+} -transport enzymes of the SR/ER of fast-twitch skeletal muscle and smooth muscle. However, there was recently a report that some monoclonal antibodies against the SR ATPase of fast-twitch skeletal muscle [9] or cardiac muscle [10] could also recognize epitopes on the smooth-muscle enzyme.

MATERIALS AND METHODS

Membranes

Membranes were prepared according to previously published methods from pig erythrocytes [11], skeletal muscle [12], cardiac muscle [12] and stomach (antral part) smooth muscle [13]. ER and PM were prepared from the post-mitochondrial supernatant of pig stomach smooth muscle in accordance with ref. [13]. In short, after the addition of digitonin to the post-mitochondrial supernatant in order to increase selectively the density of the PM fragments, ER and PM elements were separated by isopycnic equilibration on a sucrose density gradient containing 0.6 M-KCl.

Preparation of antibodies against the Ca^{2+} -transport ATPase of cardiac-muscle SR

Antibodies against the Ca^{2+} -transport ATPase from the SR of pig cardiac muscle were obtained independently

Abbreviations used: PM, plasma membrane; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase, Ca^{2+} -stimulated Mg^{2+} -dependent ATPase.

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in the two laboratories (Leuven and Ulm), each following their own standard procedures and starting from locally obtained pig hearts.

In preparation L (Leuven) SR was prepared from pig ventricle muscle in accordance with ref. [12]. The ATPase was purified by preparative Laemmli-type SDS/polyacrylamide-gel electrophoresis on 3 mm-thick gels (7.5% acrylamide). The gels were superficially stained with Coomassie Brilliant Blue for 20 min and destained for 30 min. Bands corresponding to the 100000- M_r ATPase were excised and the protein was electroeluted overnight in the ISCO model 1750 electrophoretic sample concentrator at a setting of 1 W. The buffer in the sample cup was 4 mM-Tris/acetate buffer, pH 8.3, containing 1 mM-EDTA and 0.1% SDS. The inner and electrode compartments were filled with 40 mM-Tris/acetate buffer, pH 8.3, containing 1 mM-EDTA and 0.1% SDS. The immunization of a rabbit was done following a scheme as described in ref. [6] by three subcutaneous injections of 0.1 mg of protein in 0.2 ml of sample-cup buffer together with an equal volume of Freund's complete adjuvant.

In preparation U (Ulm) Ca^{2+} -transport ATPase was obtained from cardiac-muscle SR and prepared in accordance with ref. [14]. The ATPase was purified by preparative Laemmli-type SDS/polyacrylamide-gel electrophoresis on 3 mm-thick gels (10% acrylamide). Bands corresponding to the 100000- M_r ATPase were excised and homogenized in 10 mM-Mops/NaOH buffer, pH 7.5, containing 0.5 mM-sucrose. Then the gel particles were sedimented at 3000 g for 20 min and the supernatant was collected. This extraction procedure was repeated twice and the combined supernatants were concentrated by ultrafiltration on an Amicon XM50 membrane. In order to remove SDS, samples were subjected to a trichloroacetic acid/propan-2-ol precipitation [15] and resuspended in 0.9% NaCl to give a protein concentration of 0.3–0.5 mg/ml. The rabbit antiserum against the purified ATPase was obtained by subcutaneous injections repeated five times at weekly intervals and then eight times at 4-week intervals. Each injection consisted of 0.3–0.5 mg of protein in 1 ml of 0.9% NaCl mixed with 1 ml of Freund's complete adjuvant. Blood was drawn 2 weeks after the fifth booster injection, and this collection was repeated 2 weeks after each of the following injections. The immunoglobulin fractions were obtained from antiserum by repeated $(\text{NH}_4)_2\text{SO}_4$ precipitations [16] and redissolved in 5 mM-Mops/NaOH buffer, pH 7.4, containing 0.9% NaCl and 0.1% NaN_3 to give a protein concentration of 5 mg/ml.

Immunoblotting

Laemmli-type SDS/polyacrylamide-gel electrophoresis on 0.75 mm-thick slab gels containing 7.5% (w/w) acrylamide, electroblotting on to nitrocellulose and immunostaining with 4-chloro-1-naphthol were done as described previously [17]. In those experiments in which phosphorylation and immunostaining were combined, the blotting buffer contained 25 mM-sodium phosphate buffer, pH 6.5, but no methanol. Before electroblotting the gels were equilibrated in ice-cold blotting buffer for 30 min. Transfer was done in a Bio-Rad trans-blot cell for 16 h at 15 V (0.20 A) on to Immobilon membranes (Millipore IPVH 00010). After drying and autoradiography to demonstrate and quantify the phosphoprotein intermediates, the blots were immunostained with peroxidase-labelled secondary antibodies and 4-chloro-1-naph-

thol. Proteins on Immobilon membranes could after immunostaining still be stained with 0.1% Coomassie Brilliant Blue R250 in 50% (v/v) methanol/10% (v/v) acetic acid followed by a wash in the same solution without dye.

Demonstration of the phosphoprotein intermediates of the Ca^{2+} -transport ATPases

Phosphorylation was performed in a total volume of 200 μl at 0 °C for 10 s in a medium containing 100 mM-KCl, 30 mM-imidazole/HCl buffer, pH 6.8, and either 0.05 mM- CaCl_2 or 1 mM EGTA (potassium salt). The reaction was started by addition of 16.5 nM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (110 TBq/mmol) and stopped with stop medium: 10% (w/v) trichloroacetic acid in 50 mM-sodium phosphate containing 0.5 mM-ATP. The precipitated proteins were pelleted at 1000 g for 5 min, and the pellets were washed twice with ice-cold stop medium and once with ice-cold distilled water. The pellets were then dissolved in Laemmli sample buffer and shaken for 10 min at room temperature. A 40 μl portion of the sample was applied on 0.75 mm-thick slab gels (7.5% acrylamide) and electrophoresed at 10 °C for 1.5 h at 75 mA/two gels. After electroblotting of the gels as indicated above under 'Immunoblotting', autoradiography was performed at room temperature for 5 h on Kodak X-Omat S film. The autoradiograms were scanned with an LKB 2202 Ultra-scan laser densitometer.

Measurement of ATPase activity

$(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity was measured with an NADH-coupled enzyme assay at 37 °C in the presence of 10 μM - Ca^{2+} and 1 μg of ionophore A23187/ml as in ref. [5].

RESULTS

Binding of two different antibody preparations against the Ca^{2+} pump from pig cardiac SR

The antibodies against the Ca^{2+} -transport ATPase from the SR of pig cardiac muscle prepared in the Ulm laboratory were found to bind, on Western-type immunoblots, not only to their own antigen, but also to a protein band of similar M_r that was present in membranes from pig visceral (stomach antrum) smooth muscle. These antibodies reacted very weakly with the Ca^{2+} -transport ATPase of pig fast-twitch skeletal-muscle SR. However, the 130000- M_r Ca^{2+} -transport ATPase in pig erythrocytes or in pig smooth-muscle PM did not react with these antibodies.

Fig. 1 shows that the immunological resemblance between the Ca^{2+} -transport ATPases of the SR/ER from cardiac muscle and smooth muscle is more pronounced than that between the corresponding ATPases of cardiac-muscle and skeletal-muscle SR. Indeed, similar amounts of total activity of $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase that were electrophoresed and blotted presented at M_r 100000 almost the same intensity of immunostaining for cardiac SR and smooth-muscle ER, whereas the staining was much less for skeletal-muscle SR. The weak staining observed in the lower- M_r region varied in intensity from experiment to experiment and might be ascribed to some proteolytic degradation of the 100000- M_r Ca^{2+} -transport ATPase.

The above observations on the immunological relation-

Fig. 1. Binding of antibodies against the Ca^{2+} -transport ATPase from pig cardiac-muscle SR to equal activities of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of pig skeletal-muscle, cardiac-muscle and smooth-muscle SR/ER

Western-type immunoblots were made of Laemmli gels (7.5% acrylamide). The following fractions were applied: lane 1, SR of fast-twitch skeletal muscle (psoas) prepared in accordance with ref. [14]; lane 2, SR of psoas skeletal muscle prepared in accordance with ref. [12]; lanes 3 and 4, two different preparations of cardiac-muscle SR prepared in accordance with ref. [12]; lanes 5–7, three different preparations of ER from stomach smooth muscle. The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activities were determined at $10 \mu\text{M}$ - Ca^{2+} in the presence of $1 \mu\text{g}$ of Ca^{2+} -ionophore A23187/ml as described in ref. [5]. The amount of membranes applied to the gels corresponds to the same total $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity (17 nmol of P_i/min at 37°C). In this experiment the U antibody preparation was used.

ship between the different SR/ER Ca^{2+} -transport ATPases were confirmed by using the L antibody against the pig cardiac SR Ca^{2+} -transport ATPase prepared in the Leuven laboratory. We furthermore in the latter experiments quantified the total amount of SR/ER Ca^{2+} -transport ATPase present on the blot in a different way, namely by determining the amount of their phosphoprotein intermediates rather than by determining their total activity. The relationship between the intensity of immunostaining of the SR/ER ATPases with L antibodies and the amount of phosphoprotein intermediate is depicted in Fig. 2. Pig cardiac-muscle SR and antrum ER ATPases presented almost the same reaction. However, the enzyme of the SR from pig psoas, a skeletal muscle with mainly fast-twitch fibres, did not react. The low immunostaining of the ER Ca^{2+} -transport ATPase of the bovine pulmonary artery might be ascribed to a species difference. The antibodies that were raised against pig antigens showed a weaker cross-reaction with the corresponding enzymes of bovine origin.

The L antibodies against the cardiac SR Ca^{2+} -transport ATPase used for the experiments shown in Fig. 2 did not bind to ATPase from fast-twitch skeletal muscle and hence appear to be more selective for the Ca^{2+} -transport enzymes of cardiac-muscle SR, slow-twitch skeletal-muscle SR and smooth-muscle ER than the preparation U used in Fig. 1.

Fig. 2. Relationship between the amount of phosphoprotein intermediate and immunostaining with antibodies against the Ca^{2+} -transport ATPase from cardiac-muscle SR

SR/ER membranes from pig cardiac muscle ($\bullet$), pig antrum smooth muscle (Δ), pig psoas muscle ($\circ$) and bovine pulmonary artery ($\times$) were phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the proteins were separated on Laemmli SDS/polyacrylamide-gel electrophoresis. After Western blotting the blots were first autoradiographed and then immunostained. The autoradiograms and immunoblots were scanned with a laser densitometer and the values of immunostaining were plotted as a function of the amounts of phosphoprotein intermediate (both relative units). The L antibody preparation was used in these experiments.

Binding of antibodies against the Ca^{2+} -transport ATPase of pig fast-twitch skeletal-muscle SR

Antibodies raised against the SR Ca^{2+} -transport ATPase from pig fast-twitch skeletal muscle did not bind to the Ca^{2+} -transport ATPase of antrum smooth muscle or of cardiac muscle (Fig. 3b; see also ref. [8]).

Isoform typing by means of tryptic phosphoprotein fragments

The conclusion, deduced from the studies with antibodies, that the Ca^{2+} -pump isoform expressed in the SR of fast-twitch skeletal-muscle fibres appears to be different from that expressed in cardiac-muscle, smooth-muscle and slow-twitch skeletal-muscle cells could be further substantiated by the analysis of their phosphoprotein intermediates. For this approach we used the well-known property of the Ca^{2+} -transport ATPase of skeletal-muscle SR to persist as a catalytically active enzyme after partial tryptic digestion [18,19]. Trypsin first splits the enzyme into two fragments of similar M_r , which are designated as A and B fragments. The A fragment is subsequently split into A_1 and A_2 fragments. The aspartic acid residue that is the acceptor for P_i in the catalytic cycle is localized in the A_1 fragment. The $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase activity is preserved and the enzyme can still be phosphorylated after splitting the Ca^{2+} pump by trypsin into the A_1 , A_2 and B fragments. Fig. 3 shows autoradiograms displaying the native phosphoprotein intermediates and the phosphorylated tryptic fragments A and A_1 of the SR Ca^{2+} -transport ATPases from psoas, soleus, cardiac muscle and antrum muscle. Two A fragments and two A_1 fragments of different apparent M_r values can be recognized. Both the A fragment (M_r 55000) and the A_1 fragment (M_r 33000)

Fig. 3. Tryptic phosphoprotein fragments of different phosphorylated Ca^{2+} -transport ATPases from the SR/ER and their reaction with antibodies

Portions (50 μg) of SR/ER membranes from pig psoas, soleus, cardiac muscle and antral smooth muscle at 1 mg of protein/ml were digested with 5 μg of trypsin for 10 min on ice in a solution of 30 mM-imidazole/HCl buffer, pH 6.8, containing 100 mM-KCl. The digestion was stopped with 10 μg of trypsin inhibitor. CaCl_2 (50 μM) was added and phosphorylation was performed as indicated in the Materials and methods section. After Laemmli SDS/polyacrylamide-gel electrophoresis and Western electroblotting the blots were autoradiographed and then stained with antibodies. The + and - signs above the lanes indicate the presence and the absence respectively of trypsin treatment before phosphorylation A_s , A_{1s} , A_f and A_{1f} indicate the positions of the A and A_1 tryptic fragments of the respectively slow-twitch and fast-twitch isoforms. L antibodies against cardiac-muscle SR Ca^{2+} -transport ATPase (a) and antibodies against the SR Ca^{2+} -transport ATPase of fast-twitch skeletal muscle (b) were used in this experiment.

obtained from the fast-twitch skeletal muscle (psoas) have slightly lower apparent M_r values than those obtained from cardiac muscle and smooth muscle (M_r 58000 and 35000 respectively). Both types of A and A_1 fragments were observed in the soleus muscle, which is known to contain both slow-twitch and fast-twitch fibres. Each of the lanes in Figs. 3(a) and 3(b) received the same amount (50 μg) of membrane protein. Therefore the intensities of the spots on the autoradiogram are proportional to the relative amounts of ATPase or ATPase

fragments present in the different membrane preparations.

Binding of antibodies to the tryptic fragments

In agreement with the above-described selective recognition of the different native Ca^{2+} -pump isoforms by our antibodies against the fast-twitch skeletal-muscle and cardiac-muscle Ca^{2+} -transport ATPases, we also observed that the antibodies against the fast-twitch skeletal-muscle Ca^{2+} -transport ATPase only recognize the tryptic

fragment of the fast-twitch skeletal-muscle enzyme whereas the antibodies against the cardiac-muscle Ca^{2+} -transport ATPase recognized the tryptic fragments of the cardiac-muscle, smooth-muscle and slow-twitch soleus-muscle Ca^{2+} pumps.

A study of the binding of antibodies to these tryptic fragments of the ATPase revealed that the L antibodies against the cardiac-muscle SR Ca^{2+} -transport ATPase are mainly directed against epitopes present on the A and A_1 fragments. In contrast, our antibodies against the fast-twitch skeletal-muscle Ca^{2+} -transport ATPase reacted mainly with the B tryptic fragment of the fast-twitch skeletal-muscle Ca^{2+} -transport ATPase. It should be mentioned that, because the tryptic fragments A and B of the skeletal-muscle SR ATPase migrate on the gels so closely together, it requires a careful analysis before these conclusions can be drawn unambiguously. After restaining the immunostained blot with Coomassie Brilliant Blue to show up all tryptic fragments and then overlaying it with the autoradiogram, we could unambiguously identify the A and B fragments. It then became clear that the antibodies against the enzyme from skeletal muscle bind to the B fragment of the fast-twitch-skeletal-muscle enzyme, which migrates slightly faster than the phosphorylated A fragment (fragment B_r has M_r 54000, fragment A_r has M_r 55000).

Binding of antibodies against cardiac-muscle SR Ca^{2+} -transport ATPase to proteins in sucrose-density-gradient fractions of smooth muscle

We tested the reaction of the U antibodies against the cardiac-muscle SR Ca^{2+} -transport ATPase with the different subcellular fractions from pig antrum. These antibodies bind to peptides of M_r 100000 in the membrane fractions enriched in ER fragments (i.e. at the low-density end of the gradient) (Fig. 4). The very limited binding of the antibodies occurring in the high-density fraction, which contains largely PM, may be explained by its contamination with ER membranes [13]. A similar pattern of antibody binding is observed in subcellular fractions obtained from pig aorta, pig coronary artery or bovine pulmonary artery separated by the same density-gradient procedure (results not shown here, but see ref. [20]). In the last-mentioned case, however, the antibodies, which were raised against pig antigens, showed a much weaker cross-reaction with the corresponding enzymes of bovine origin. The stained polypeptide bands of M_r below 100000 might represent proteolytic fragments of the Ca^{2+} -transport ATPase. We had previously shown that antibodies against the 130000- M_r PM Ca^{2+} -transport ATPase mainly bind to a 130000- M_r band at the opposite end of the gradient, i.e. at the high-density fractions where the plasma membranes equilibrate [6].

DISCUSSION

Smooth muscle expresses in its ER an isoform of the Ca^{2+} -transport ATPase that is closely related to or identical with the one in cardiac muscle or slow-twitch skeletal-muscle SR. However, this enzyme differs from that in fast-twitch skeletal-muscle SR, as can be deduced from a comparison of the immunoblotting intensity relative to the amount of antigen present on Western-type blots. The immunostaining intensity was found to be linearly proportional to the amount of antigen over the range used in those experiments. Because the relative

Fig. 4. Binding of antibodies against the Ca^{2+} -transport ATPase from pig cardiac-muscle SR to polypeptides in subcellular fractions from pig antrum smooth muscle

Western-type immunoblots were made of Laemmli gels (7.5% acrylamide). The fractions (40 μg /lane) applied on the gel were obtained as indicated under 'Membranes' in the Materials and methods section. The sucrose concentrations (% w/w) at which the different fractions equilibrated are indicated above the lanes. The density-gradient separations were obtained as described in ref. [13] and we refer to this for details concerning marker enzymes. In this type of gradient the ER fragments equilibrate at the lower densities (i.e. in lanes most to the left). The U antibody preparation was used in this experiment.

amounts of Ca^{2+} -transport ATPase differ for the different SR/ER preparations, we could not rely on the concentration of membrane protein to quantify the antigen concentration. We have instead used two different procedures to assess the amount of the different Ca^{2+} -transport ATPases on the blots. In the experiment depicted in Fig. 1 we have adapted the protein concentrations in such a way that each lane received the same total amount of Ca^{2+} -transport ATPase assuming that the kinetic parameters of the enzymes are similar [21,22]. In the second procedure we have quantified the amount of Ca^{2+} -pump protein by measuring the phosphoprotein intermediate (Fig. 2). Both methods lead to the same conclusion that the Ca^{2+} -transport ATPases of the cardiac-muscle and smooth-muscle SR/ER are more closely related to each other than to the enzyme of the fast-twitch skeletal-muscle SR. It should also be stressed that our observations were made with different antibody preparations obtained in the two laboratories following different strategies. Both antibody preparations gave similar results, although the antibody prepared in Ulm appears to bind weakly to the fast-twitch skeletal-muscle Ca^{2+} -transport ATPase whereas the Leuven antibody is more selective and does not bind to the enzyme of the fast-twitch skeletal-muscle Ca^{2+} -pump.

Our conclusion that the Ca^{2+} -transport ATPases of the smooth-muscle, cardiac-muscle and slow-twitch skeletal-muscle SR are related and differ from that of the fast-twitch skeletal-muscle isoform is confirmed by comparing

their respective A and A₁ tryptic phosphoprotein fragments.

The difference in electrophoretic mobility of the respective A and A₁ tryptic fragments of the SR Ca²⁺-pump isoforms in fast-twitch skeletal and slow-twitch skeletal (or cardiac) muscle has not been reported up until now and yet it presents an easy way to discriminate both isoforms. The reason why this difference in electrophoretic mobility of the tryptic phosphoprotein fragments of the different SR/ER Ca²⁺-transport ATPase isoforms escaped attention in previous investigations must probably be sought in the use of acidic gels. In order to increase the stability of the phosphoprotein intermediates acid gels are generally used. We have used in our procedure the Laemmli SDS/polyacrylamide-gel system with its high resolving power rather than the acidic gels. Although Laemmli electrophoresis leads to a much more pronounced hydrolysis of the acyl phosphate bond in the intermediate compared with the acid gels, enough of the phosphoprotein survives the procedure to allow its detection. Moreover the blotting technique has allowed us the use of immunostaining.

The possibility of characterizing easily a Ca²⁺-transport ATPase of the SR as a slow-twitch or fast-twitch type by the study of its A and A₁ tryptic phosphoprotein intermediates also provides a procedure for monitoring the expression and the changes in expression of the isoforms in different skeletal muscles or during myogenesis. On the basis of our analysis we conclude that the soleus muscle preparation of the pig contains similar amounts of the slow-twitch and fast-twitch isoforms of the Ca²⁺-transport ATPase. This finding might indicate that this muscle contains slow-twitch fibres and fast-twitch fibres, as has been found in the soleus muscle of the rat by the immunohistochemical staining of slow-twitch and fast-twitch types of myosin [23]. It is likely that each fibre type expresses its own Ca²⁺-transport ATPase isoform. However, during the development *in ovo* of chicken thigh muscles co-expression of both the fast-twitch and slow-twitch Ca²⁺-transport ATPase isoforms in the same fibre has also been demonstrated [24] but their expression was not correlated with that of the myosin heavy-chain isoforms.

Our observations confirm and extend these of several other groups indicating that there exists a pronounced immunological similarity between the Ca²⁺-transport ATPases of cardiac muscle and slow-twitch skeletal muscle whereas the enzyme of the fast-twitch skeletal muscle differs in this respect [21,25,26]. More specifically Jorgensen & Campbell [27] reported that a monoclonal antibody against dog cardiac-muscle SR Ca²⁺-transport ATPase cross-reacted with the Ca²⁺-transport ATPase of the slow-twitch skeletal-muscle fibres and not with that of the fast-twitch skeletal-muscle fibres. Kaprielian & Fambrough [24] obtained a monoclonal antibody against chicken cardiac-muscle SR ATPase that also recognized specifically the slow-twitch ATPase but that did not bind to the fast-twitch skeletal-muscle isoform. We can now extend these observations and conclude that also smooth muscle expresses a Ca²⁺-transport ATPase that is similar to that of slow-twitch skeletal-muscle fibres or cardiac muscle.

Two closely cross-hybridizing genes for Ca²⁺-transport ATPases have been demonstrated in the rabbit genomic DNA [28]. This finding was considered to indicate that distinct forms of the Ca²⁺-transport ATPase exist for

fast-twitch skeletal and for slow-twitch skeletal muscle or cardiac muscle. From the work in the same laboratory it became also clear that possible alternative splicing of the 3' exons in neonatal and mature transcripts of the Ca²⁺-transport ATPase of the fast-twitch skeletal muscle might lead to an additional diversity of the post-translational isoforms [29]. We propose that such minor modifications might be responsible for differences between the isotypes expressed in the different muscles that have not been detected as yet.

It was recently shown that the mRNA for the SR Ca²⁺-transport ATPase expressed in fast-twitch skeletal muscle differs from that expressed in cardiac muscle and smooth muscle by its size. S₁-nuclease mapping has moreover shown that the cardiac-muscle and smooth-muscle mRNAs possess different 3'-end sequences [30]. It is however, not yet clear whether those differences are only confined to the 3'-end non-coding region or whether they extend into the translated region.

Because it has not been possible up until now to raise specific antibodies against the 100000-M_r ER Ca²⁺-transport ATPase from smooth muscle, antibodies against the cardiac-muscle SR ATPase are of considerable value for the study of the distribution of the ER Ca²⁺-transport ATPase in smooth muscle.

The close resemblance between the Ca²⁺-transport ATPases in cardiac-muscle SR, slow-twitch skeletal-muscle SR and smooth-muscle ER, and their difference from the fast-twitch skeletal-muscle form, may be another indication that smooth muscle and cardiac muscle deal differently with Ca²⁺ as compared with fast-twitch skeletal muscle. It has been previously shown that smooth muscle and cardiac muscle, unlike fast-twitch skeletal muscle, contain phospholamban, a protein interacting with the ATPase and regulating its activity [31]. Also, the calsequestrins, which are involved in the sequestration of Ca²⁺ in the SR/ER, are very similar or identical in cardiac muscle and smooth muscle but different from that in fast-twitch skeletal-muscle [32].

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REFERENCES

1. Wuytack, F., De Schutter, G. & Casteels, R. (1981) FEBS Lett. **129**, 297–300
2. Wuytack, F., Raeymaekers, L., Verbist, J., De Smedt, H. & Casteels, R. (1984) Biochem. J. **224**, 445–451
3. Chiesi, M., Gasser, J. & Carafoli, E. (1984) Biochem. Biophys. Res. Commun. **124**, 797–806
4. Carsten, M. E. & Miller, J. D. (1984) Arch. Biochem. Biophys. **232**, 616–623
5. De Schutter, G., Wuytack, F., Verbist, J. & Casteels, R. (1984) Biochim. Biophys. Acta **773**, 1–10
6. Verbist, J., Wuytack, F., Raeymaekers, L. & Casteels, R. (1985) Biochem. J. **231**, 737–742
7. Verbist, J., Wuytack, F., Raeymaekers, L., Van Leuven, F., Cassiman, J. J. & Casteels, R. (1986) Biochem. J. **240**, 633–640
8. Wuytack, F., Raeymaekers, L. & Casteels, R. (1985) Experientia **41**, 900–905
9. Zubrzycka-Gaarn, E., MacDonald, G., Phillips, L., Jorgensen, A. O. & MacLennan, D. H. (1984) J. Bioenerg. Biomembr. **16**, 441–462

10. Levitsky, D. O., Syrbu, S. I. Cherepakhin, V. V. & Rokhlin, O. V. (1987) *Eur. J. Biochem.* **164**, 477–484
11. Steck, T. L. & Kant, J. A. (1974) *Methods Enzymol.* **31**, 172–180
12. Jones, L. R. & Cala, S. E. (1981) *J. Biol. Chem.* **256**, 11809–11818
13. Raeymaekers, L., Wuytack, F. & Casteels, R. (1985) *Biochim. Biophys. Acta* **815**, 441–458.
14. Suko, J. & Hasselbach, W. (1976) *Eur. J. Biochem.* **74**, 123–130
15. Gietzen, K. & Kolandt, J. (1982) *Biochem. J.* **207**, 155–159
16. Gietzen, K. & Kolandt, J. (1985) *Biochem. J.* **228**, 479–485
17. Wuytack, F., De Schutter, G., Verbist, J. & Casteels, R. (1983) *FEBS Lett.* **154**, 191–195
18. Stewart, P. S., MacLennan, D. M. & Shamoo, A. E. (1976) *J. Biol. Chem.* **251**, 712–719
19. Andersen, J. P., Vilsen, B., Collins, J. H. & Jørgensen, P. L. (1986) *J. Membr. Biol.* **93**, 85–92
20. Eggermont, J. A., Vrolix, M., Raeymaekers, L., Wuytack, F. & Casteels, R. (1988) *Circ. Res.* **62**, 266–278
21. DeFoor, P. M., Levitsky, D., Biryukova, T. & Fleischer, S. (1980) *Arch. Biochem. Biophys.* **200**, 196–205
22. Dulhunty, A. F., Banyard, M. R. C. & Medveczky, C. J. (1987) *J. Membr. Biol.* **99**, 79–92
23. Müntener, M., Van Hardeveld, C., Everts, M. E. & Heizmann, C. W. (1987) *Exp. Neurol.* **98**, 529–541
24. Kaprielian, Z. & Fambrough, D. M. (1987) *Dev. Biol.* **124**, 490–503
25. Damiani, E., Betto, R., Salvator, S., Volpe, P., Salvati, G. & Margreth, A. (1981) *Biochem. J.* **197**, 245–248
26. Leberer, E. & Pette, D. (1986) *Eur. J. Biochem.* **156**, 489–496
27. Jorgensen, A. O. & Campbell, K. P. (1986) *Biophys. J.* **49**, 589a
28. Brandl, C. J., Green, N. M., Korczak, B. & MacLennan, D. H. (1986) *Cell* **44**, 597–607
29. Brandl, C. J., DeLeon, S., Martin, D. R. & MacLennan, D. H. (1987) *J. Biol. Chem.* **262**, 3768–3774
30. de la Bastie, D., Wisnewsky, C., Schwartz, K. & Lompre, A. M. (1988) *FEBS Lett.* **229**, 45–48
31. Raeymaekers, L. & Jones, L. R. (1986) *Biochim. Biophys. Acta* **882**, 258–265
32. Wuytack, F., Raeymaekers, L., Verbist, J., Jones, L. R. & Casteels, R. (1987) *Biochim. Biophys. Acta* **899**, 151–158

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