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Distribution of the Intracellular Ca^{2+}-ATPase Isoform 2b in Pig Brain Subcellular Fractions and Cross-Reaction with a Monoclonal Antibody Raised against the Enzyme Isoform 1[1]

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The presence and distribution of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) isoform 2b in microsomes and other subcellular fractions isolated from pig brain has been demonstrated by the combined use of a specific antibody raised against the SERCA2b isoform and ATP phosphorylation experiments. All subcellular fractions show an approximately 110 kDa phosphorylated protein, the band intensity being stronger in microsomes. Preliminary treatment of the samples with trypsin generates two phosphorylated fragments of about 57 and 33 kDa in the presence of Ca^{2+}. The observed fragments are typical trypsinized products of the SERCA2b isoform. The monoclonal antibody Y/1F4 raised against the sarcoplasmic reticulum Ca^{2+}-ATPase (isoform 1) binds to the 110 kDa band in membranes isolated from brain. The binding was stronger in microsomes than in other fractions. Furthermore, this antibody also recognizes a clear band at around 115 kDa. This band is always stronger in plasma membrane than in synaptosomes or microsomes and is unaffected by trypsin. Phosphorylation studies in the absence of Ca^{2+} suggest that the 115 kDa protein is not a Ca^{2+}-ATPase.

Key words: brain Ca^{2+} pumps, intracellular Ca^{2+}-ATPase, SERCA2b isoform.

The accumulation of Ca^{2+} into intracellular stores is mediated by Ca^{2+} transport ATPases of the sarco-endoplasmic reticulum Ca-ATPase (SERCA) family. Up to now, three different SERCA genes have been identified: SERCA1, SERCA2, and SERCA3 (*1–4*). The SERCA1 gene is expressed exclusively in fast-twitch skeletal muscle. Two different alternatively spliced messengers are transcribed from this gene. The SERCA2 gene is also alternatively spliced giving rise to the expression products SERCA2a, the muscle isoform present in cardiac/slow-twitch skeletal muscle, and SERCA2b, expressed in smooth muscle and non-muscle tissues. SERCA3 is present in different tissues, although its expression is not as ubiquitous as that of SERCA2b.

Although the presence of the SERCA2b isoform in brain was first proved by Gunteski-Humblin *et al.* (*4*) and Burk *et al.* (*5*) by identification of its mRNA, no study of its distribution after subcellular fractionation has yet been reported. We have previously shown the contribution of the intracellular (SERCA) and plasma membrane Ca^{2+}-ATPases to the enzymatic activity in brain fractions (*6*). Our aim in the present work has been to explore the distribution of SERCA isoforms in pig brain subcellular fractions, *i.e.*, microsomes, synaptosomes and synaptic plasma membrane (SPM). Moreover, the interaction of the SERCA2b isoform with an anti-SERCA1 monoclonal antibody provided additional information on a putative common consensus epitope present in both isoforms of the enzyme.

MATERIALS AND METHODS

Trypsin and soybean trypsin inhibitor were obtained from Boehringer Mannheim Biochemicals, Spain.The secondary antibodies conjugated with peroxidase were from DAKO A/S, Glostrup, Denmark, and BioRad, Spain. [γ-^{32}P]ATP was purchased from DuPont/New England Nuclear, Belgium. All other reagents were of the highest purity available.

Skeletal sarcoplasmic reticulum was prepared from rabbit as indicated in Champeil *et al.* (*7*). Microsomes isolated from COS-1 cells transfected with rabbit SERCA1 or pig stomach SERCA2a or SERCa2b cDNA, and with rat kidney SERCA3 cDNA were kindly provided by Prof. F. Wuytack (Laboratory of Physiology, University of Leuven, Belgium). The four primary antibodies, SERCA2a, SERCA2b, SERCA3-N89, and SERCA3-C90 polyclonal antibodies, were raised against specific peptides of these isoforms and also provided by Prof. F. Wuytack. Two monoclonal antibodies were also used as primary antibodies: the Y/1F4 antibody was raised against sarcoplasmic reticulum, which contains the SERCA1 isoform (Colyer *et al.*, 1989), and the non discriminating SERCA2 antibody IID8 was

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Abbreviations: EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis (β-aminoethylether)-*N,N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulfonic acid; PMSF, phenylmethanesulphonyl fluoride; PVDF, polyvinylidene difluoride; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SPM, synaptic plasma membrane; TBS, Tris-buffered saline.

purchased from Affinity Bioreagents, USA.

Preparation of Microsomes, Synaptosomes, and Synaptic Plasma Membranes from Pig Brain—Subcellular fractions were obtained from pig brain following the procedure described by Salvador and Mata (*8*). Briefly, a fresh brain (approx. 80 g) was homogenized in 10 volumes of 10 mM Hepes/KOH, pH 7.4, 0.32 M sucrose, 0.5 mM $MgSO_4$, 0.1 mM PMSF, and 2 mM β-mercaptoethanol (Buffer I). After two centrifugation steps at low and high speed (see 6 for details) we obtained a supernatant and a pellet. Microsomes were isolated from the supernatant by the procedure of Black *et al.* (*9*), slightly modified to obtain a better yield. Synaptosomes were obtained from the pellet using a 20–40% (w/v) sucrose gradient and collected at the interface, followed by washing in 10 mM Hepes/KOH, pH 7.4. Synaptic plasma membranes were prepared from synaptosomes by osmotic lysis in 10 mM Hepes/KOH, pH 7.4, 1 mM EDTA, and 2 mM β-mercaptoethanol, with subsequent centrifugation at 20,000 ×*g* for 30 min. The synaptic plasma membrane fraction, obtained in the pellet, was resuspended in 10 mM Hepes/KOH, pH 7.4, and 0.32 M sucrose and stored at −70°C.

Protein Determination—The protein content was evaluated by the method of Bradford (*10*) using bovine serum albumin as a standard.

Electrophoresis and Immunoblotting—Electrophoresis was performed by the method of Laemmli (*11*) in 7.5% polyacrylamide gels. Protein transfer to a PVDF membrane was carried out in a semi-dry system from Bio-Rad. The PVDF membrane was quenched for 30 min in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 containing 2% non-fat dry milk (TBS-milk). In one set of experiments, immunological detection was done by first incubating the PVDF membrane for 1 h at room temperature with the primary antiserum in TBS containing 0.05% Tween 20, and afterward, for 1 h with peroxidase-conjugated secondary immunoglobulins. Staining was carried out in medium containing 100 mM Mops, 60 mM Tris, pH 7.2, 1.4 mM 3,3′-diaminobenzidine, 6 mM $NiCl_2$, 0.0003% H_2O_2. In another set of experiments, blotting was done by successive incubation of the PVDF membrane with the primary antibody in TBS-milk for 2 h at room temperature or overnight on ice, with the secondary antibody (peroxidase conjugated to goat anti-mouse or anti-rabbit) for 90 min, and then with substrate 4-chloro-1-methoxi-1-naphthol plus 0.03% H_2O_2 and 10% methanol until color development was achieved. The PVDF membrane was washed extensively between steps with TBS-milk.

Enzyme Phosphorylation by ATP—Microsomes, synaptosomes, or SPM from pig brain (100 µg of protein), or microsomal membranes from COS-1 cells transiently transfected with SERCA2b or SERCA3 cDNA (15 µg) were incubated on ice in a total volume of 0.1 ml in the presence of 20 mM Mops/KOH, pH 6.8, 100 mM KCl, and 100 µM $CaCl_2$. The phosphorylation reaction was started by adding [γ-^{32}P]ATP (33 nM, 111,000 GBq/mmol) and stopped 15 s later by the addition of 1 ml ice-cold 7% (w/v) trichloroacetic acid. After acid quenching, the samples were washed three times with stopping solution and then resuspended in electrophoresis sample buffer. When indicated, COS-1 cells microsomes were pretreated in phosphorylation buffer (10 min on ice) with 5 µg of trypsin (0.05 mg/ml). Alternatively, microsomes, synaptosomes, or SPM from pig brain were preincubated in the presence of 10 µg trypsin (0.1 mg/ml). Trypsinization was stopped by adding 20 µg of trypsin inhibitor (from turkey egg) (0.2 mg/ml), and phosphorylation was started immediately as described above by the addition of radioactive ATP. ^{32}P-phosphorylated ATPases were separated according to the method of Laemmli (*11*) in a 7.5% polyacrylamide gel. Radioactive phosphoproteins were transferred to a PVDF membrane and detected by autoradiography on Hyperfilm RPN-6 (Amersham Ibérica, Spain). Films were exposed for 24 h at −70°C in the presence of a Lightning Plus intensifying screen. In other set of experiments, microsomes from pig brain (50 µg of membrane protein plus 50 µg of bovine serum albumin as a carrier) were phosphorylated in assay medium containing 30 mM Mops/KOH, pH 7.0, 75 mM KCl, 100 µM $CaCl_2$, and 50.9 µM EGTA (50 µM free Ca^{2+}). The phosphorylation reaction was started by adding 50 nM [γ-^{32}P]ATP (~11,100 MBq/mmol) and stopped 15 s later by the addition of 1 ml of ice-cold 12% (w/v) trichloroacetic acid, 20 mM phosphoric acid (*12, 13*). The subsequent electrophoresis was performed in 6.5% polyacrylamide gels by the method of Laemmli (*11*).

RESULTS

In order to identify SERCA isoforms in subcellular fractions isolated from pig brain, we started by studying its binding to SERCA-specific polyclonal antibodies. Figure 1 shows the immunoreaction with different antigens of SERCA2a (Fig. 1A), SERCA2b (Fig.1B), and N-SERCA3 (Fig. 1C) antibodies. The antigens used were: microsomes (lane 1), synaptosomes (lane 2), SPM (lane 3), control microsomes from COS-1 cells (lane 4), or COS-1 microsomes overexpressing SERCA2a (lane 5), SERCA2b (lane 6), SERCA3 (lane 7), or SERCA1 (lane 8) isoforms. The SERCA2a antibody only recognized this isoform in COS-1

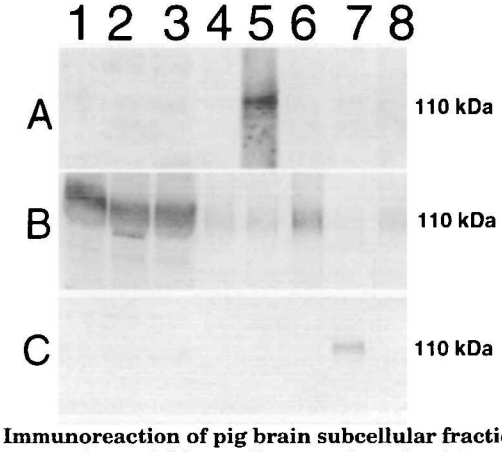

Fig. 1. **Immunoreaction of pig brain subcellular fractions and microsomes from COS-1 cells transfected with cDNA of SERCA isoforms with specific antibodies raised against SERCA2a, SERCA2b, and SERCA3 isoforms.** Lanes 1, 2, and 3: 80 µg of microsomes, synaptosomes and SPM from pig brain, respectively. Lane 4: microsomes from non-transfected COS-1 cells. Lanes 5, 6, 7, and 8: 10 µg of microsomal membranes from COS-1 cells overexpressing the following cDNAs: pig SERCA2a (lane 5), pig SERCA2b (lane 6), rat SERCA3 (lane 7), rabbit SERCA1 (lane 8). The PVDF membrane was incubated with the specific polyclonal antibodies: (A) anti-SERCA2a (1/200), (B) anti-SERCA2b (1/100), or (C) anti-SERCA3-N89 (1/1,000). Immunodetection was done with diaminobenzidine as described in "MATERIALS AND METHODS."

cells microsomes (Fig. 1A, lane 5), while the specific SERCA2b antibody bound strongly to a band protein around 110 kDa in all fractions isolated from pig brain (Fig. 1B, lanes 1, 2, and 3) in addition to the isoform expressed in COS-1 cells microsomes (lane 6). Finally, the anti-SERCA3 antibody bound only to this protein in microsomes from COS-1 cells transfected with the SERCA3 isoform (Fig. 1C, lane 7).

The SERCA isoform content and distribution in all pig fractions was determined by studying the SERCA phosphorylation pattern provided by different membrane sources, and assayed under different experimental conditions (Fig. 2). For this, as a control, we used the phosphorylation bands given by microsomal membranes from COS-1 cells transiently transfected with SERCA2b cDNA in the intact (lane 1) and digested (lane 2) form, or with SERCA3 cDNA in the intact (lane 3) and digested (lane 4) state. Our data indicate the absence of SERCA phosphoproteins from pig brain microsomes when the phosphorylation reaction was carried out in the absence of Ca^{2+} (lane 5). However, a strong band corresponding to the 110 kDa SERCA phosphoprotein was evident when Ca^{2+} was present in the reaction medium (lane 6). On the other hand, when brain microsomes were pretreated with trypsin before phosphorylation in the presence of Ca^{2+} (lane 7), two different radioactive fragments (around 57 and 33 kDa) were observed. It should be noted that this digestion pattern corresponds to that of SERCA2b (lane 2). The synaptosomal membrane fraction (lanes 8–10) and the synaptic plasma membrane (lanes 11–13) were also phosphorylated under similar assay conditions, *i.e.*, in Ca^{2+}-free medium (lanes 8 and 11), in the presence of Ca^{2+} (lanes 9 and 12), or after preliminary trypsin digestion (lanes 10 and 13). As can be seen, the phosphorylation patterns are similar to those described for brain microsomes, although the band intensities are lower in the synaptosomal samples and even weaker in the synaptic plasma membrane fraction.

Subsequent immunological detection was done by immunoblotting with the aid of the SERCA2-specific antibody, IID8. Figure 3 shows that the monoclonal antibody selectively binds to the 110 kDa Ca^{2+}-ATPase protein in microsomes (lane 1), synaptosomes (lane 2), and also in the synaptic plasma membrane (lane 3), giving a similar distribution to that described above. Interestingly, the antibody also binds to a protein band located at about 200 kDa (asterisk) that likely corresponds to the typical SERCA2b

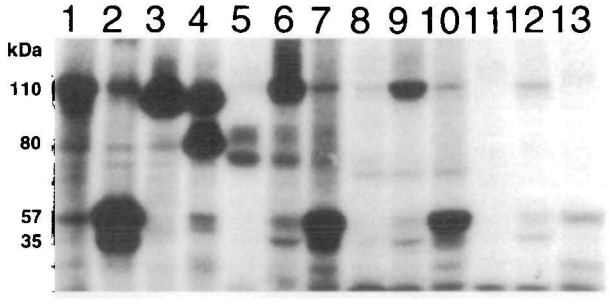

Fig. 2. **Autoradiogram of SERCA phosphoproteins from different sources obtained under different assay conditions.** Trypsin digestion and phosphorylation were performed as indicated in "MATERIALS AND METHODS." Lanes 1 and 2: 15 μg of microsomal membranes from COS-1 cells transiently transfected with SERCA2b cDNA. Lanes 3 and 4: 15 μg of microsomal membranes from COS-1 cells transiently transfected with SERCA3 cDNA. Lanes 5, 6, and 7: 80 μg of pig brain microsomes. Lanes 8, 9, and 10: 80 μg of synaptosomes. Lanes 11, 12, and 13: 80 μg of synaptic plasma membrane. Samples phosphorylated in the absence of Ca^{2+} (lanes 5, 8, and 11), in the presence of 100 μM Ca^{2+} (lanes 6, 9, and 12). Samples pretreated with trypsin before phosphorylation in the presence of 100 μM Ca^{2+} (lanes 2, 4, 7, 10, and 13).

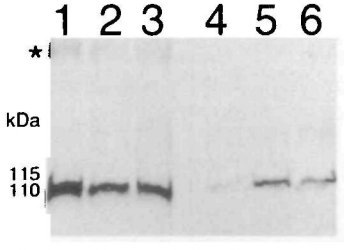

Fig. 3. **Western blot analysis of fractionated pig brain membranes in the presence of the SERCA2-specific monoclonal antibody IID8 and the SERCA1 monoclonal antibody Y/1F4.** Lanes 1 and 4: 80 μg of microsomes. Lanes 2 and 5: 80 μg of synaptosomes. Lanes 3 and 6: 80 μg of synaptic plasma membrane from pig brain. Blots from 7.5% Laemmli gels were incubated with the SERCA2 antibody IID8 (1/5,000, lanes 1, 2, and 3) and the SERCA1 antibody Y/1F4 (1/100, lanes 4, 5, and 6), and immunostained with diaminobenzidine as described in "MATERIALS AND METHODS." Asterisk indicates the SERCA2b dimer.

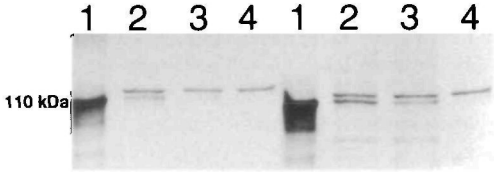

Fig. 4. **Immunological detection of SERCA2b in pig brain microsomes using the SERCA1-monoclonal antibody Y/1F4 (panel A) and the SERCA2-specific monoclonal antibody IID8 (panel B).** Two similar semi-dry Western blots from 6.5% Laemmli gels are shown. Lane 1: 0.5 μg of sarcoplasmic reticulum from rabbit skeletal muscle. Lanes 2, 3, and 4: 30 μg of pig brain microsomes. Microsomes in lanes 3 and 4 were pretreated with trypsin for 1 or 10 min, respectively (1/10; trypsin/protein). The blot was first reacted with the SERCA1-monoclonal antibody Y/1F4 (1/25). The same blot was subsequently incubated with the SERCA2-specific monoclonal antibody IID8 (1/2,000). The immunostaining was performed with 4-methoxy-1-naphthol as described in "MATERIALS AND METHODS."

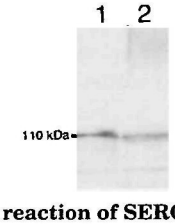

Fig. 5. **Immunological reaction of SERCA2b in COS-1 cells using the monoclonal antibody Y/1F4.** A semi-dry Western blot from a 7.5% Laemmli gel is shown. Lanes 1 and 2: 10 and 20 μg of microsomes from COS-1 cells overexpressing SERCA 2b, respectively. The PVDF membrane was incubated overnight with Y/1F4 (1/25) and subsequently with the secondary antibody (1/3,000) and 4-methoxy-1-naphthol as described in "MATERIALS AND METHODS."

dimer previously reported by Verboomen *et al.* (*3*). We also tested the immunoreaction of a monoclonal antibody (Y/1F4) raised against sarcoplasmic reticulum from rabbit skeletal muscle (SERCA1 isoform) (*14*). The Y/1F4 antibody at the dilution used (1/100) binds to a protein located at around 115 kDa. The binding was weaker in microsomes (lane 4) than in the other fractions (lanes 5 and 6). Nevertheless, the antibody at lower dilution (1/25) also binds to SERCA2b (Fig. 4A). This panel shows the strong binding of the antibody to SERCA 1, used as a control (lane 1), and its cross-reaction between this isoform, the 110 kDa SERCA2b isoform, and the 115 kDa protein mentioned above (lane 2). When brain microsomes were digested with trypsin (lane 3), the 110 kDa protein was proteolyzed and the antibody did not recognize the fragments generated. However, the 115 kDa protein appears to be resistant to proteolysis, even after a 10 min digestion period (lane 4). Further incubation of the PVDF membrane with the IID8 antibody (Fig. 4B) corroborated the binding of IID8 to the 110 kDa protein (as shown in Fig. 3).

The binding of Y/1F4 to the SERCA2b isoform was also shown in COS-1 cells microsomes overexpressing SERCA-2b (Fig. 5). A well defined band around 110 kDa appeared with 10 μg (lane 1) or 20 μg (lane 2) of protein. At higher amounts of microsomal protein, it was also possible to see a reaction with a wide band located at around 200 kDa, corresponding to the SERCA2b dimer previously reported by Verboomen *et al.* (*3*).

The 115 kDa protein reacting with Y/IF4 can tentatively be considered to be a member of the ATPase family. We tried to determine whether or not this protein is a Ca^{2+}-ATPase by performing phosphorylation experiments. We used intact or trypsin-digested microsomes from pig brain with [γ-^{32}P]ATP as the phosphorylating substrate. The results obtained are presented in Fig. 6. Lane 1 and lane 5 show the 110 kDa phosphoprotein band corresponding to intact microsomes, whereas lane 2, lane 3, and lane 4 display the phosphorylated fragments appearing after 0.5, 1, or 5 min of trypsin digestion, respectively. Moreover, when the phosphorylation experiments were carried out in the presence of Mg^{2+} and absence of Ca^{2+} (lane 6), the strong band at 110 kDa disappeared, whereas the upper protein (approx. 115 kDa) was still phosphorylated. When Mg^{2+} was absent in the reaction medium the protein was not longer phosphorylated (lane 7).

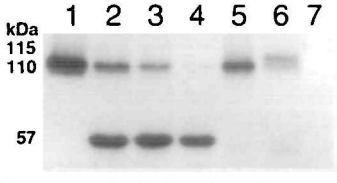

Fig. 6. **Autoradiogram of pig brain microsomes phosphorylated in the presence or absence of Ca^{2+} and Mg^{2+}.** Pig brain microsomes (50 μg of protein) were phosphorylated for 15 s as described in "MATERIALS AND METHODS" in the presence of 50 μM Ca^{2+} without pretreatment with trypsin (lane 1) or after incubation with trypsin for 30 s (lane 2), 1 min (lane 3), or 5 min (lane 4). The phosphorylation reaction was also carried out in the presence of 50 μM Ca^{2+} and 2 mM Mg^{2+} (lane 5), in the presence of 2 mM Mg^{2+} without Ca^{2+} (lane 6), or in the absence of both Ca^{2+} and Mg^{2+} (lane 7). Each lane was loaded with 30 μg of sample protein.

Table I summarizes the cross-reaction of various antibodies with different brain subcellular fractions and microsomal membranes from COS-1 cells transfected with SERCA isoforms. As can be seen, the monoclonal Y/1F4 could also bind to the SERCA isoform overexpressed in COS-1 cells.

DISCUSSION

We show in the present report that the SERCA2b isoform has a different distribution in pig brain subcellular fractions. It is also evident that the monoclonal antibody Y/1F4 raised against SERCA1 is able to react with the 110 kDa SERCA2b protein.

The immunoreaction of brain fractions with SERCA isoform-specific antibodies (Fig. 1) shows that the 110 kDa protein is only recognized by the SERCA2b-specific antibody. This indicates the presence of the SERCA2b isoform in microsomes, synaptosomes and SPM from pig brain, and the absence of any other SERCA isoforms in these fractions.

The autoradiogram of subcellular fractions from pig brain obtained under SERCA phosphorylation conditions shows the presence of a phosphoprotein at approximately 110 kDa belonging to the SERCA type of Ca^{2+}-pumps. The identification and distribution of the SERCA isoform was based on the analysis of phosphoprotein fragments (Fig. 2). The tryptic digestion of SERCA2b is characterized by the rapid generation of two phosphoprotein fragments at 57 and 33 kDa (lane 2), whereas the tryptic pattern of SERCA3 produces a single 80 kDa phosphoprotein band (lane 4). Therefore, the detected proteolytic pattern of SERCA present in brain subcellular membranes (lanes 7, 10, and 13) corresponds to the SERCA2b isoform. Furthermore, a perusal of band intensities corresponding to the phosphorylated bands confirmed that the relative abundance of the detected SERCA2b protein decreases from microsomes to synaptosomes and SPM. These results are in good agreement with the SERCA distribution previously outlined in our laboratory based on kinetic studies and phosphorylation experiments in the presence of SERCA inhibitors (*6*).

The distribution of SERCA2b in subcellular fractions was

TABLE I. **Binding of a-SERCA antibodies to calcium pumps from different tissues.**

	Mic[a]	Syn[a]	SPM[a]	SERCA2a[b]	SERCA2b[b]	SERCA3[b]	SERCA1[b]	SR
a-SERCA2a	–	–	–	+++	–	–	–	–
a-SERCA2b	+++	++	+	–	+++	–	–	–
a-SERCA3 N89	–	–	–	–	–	+++	–	–
a-SERCA3 C90	–	–	–	–	–	+++	–	–
Y/1F4	+++	++	+	–	+++	+	+++	+++
IID8	+++	++	+	+++	+++	–	–	–

[a]Microsomes, synaptosomes, and synaptic plasma membrane from pig brain. [b]Microsomes from COS-1 cells transfected with cDNA from pig stomach SERCA2a or SERCA2b, rat kidney SERCA3, or rabbit SERCA1. SR, rabbit skeletal muscle sarcoplasmic reticulum.

also shown using an anti-SERCA2 monoclonal antibody, IID8. This antibody reacts with all of the isolated pig brain subcellular fractions, although the band intensity is stronger in microsomes than in synaptosomes or SPM (Fig. 3, lanes 1, 2, and 3). The monoclonal antibody Y/1F4 raised against the sarcoplasmic reticulum SERCA1 (*14*) did not bind to this protein at the concentration used, but it showed clear binding to another protein around 115 kDa. The band intensity decreased in the microsomal fraction (Fig. 3, lane 4).

Immunoreactivity of the monoclonal antibody Y/1F4 with the 110 kDa SERCA2b present in pig brain was observed when the antibody was used at higher concentration (Fig. 4A). The band intensity disappeared when this protein was trypsinized. This binding suggests the presence of common amino acids in both isoforms (SERCA1 and SERCA2b) that are involved in antibody binding. The SERCA1 epitope responsible for detection with Y/1F4 has been attributed to amino acid residues N^{510}KMFVK^{515} (*15*), although not all amino acid residues in a given epitope are equally involved in antibody binding (*16*, *17*). According to the primary structures of different SERCA isoforms given by Gunteski-Hamblin *et al.* (*4*) and Burk *et al.* (*5*), all of them have the sequence K^{511}MFVK^{515} in common. Therefore, the antibody could cross-react with these isoforms (see Table I). Nevertheless, the phosphorylation experiments showed that only SERCA2b is present in pig brain fractions (Fig. 1). Furthermore, Y/1F4 also binds to microsomes from COS-1 cells transfected with SERCA2b (Fig. 5). From these studies we can conclude that the monoclonal antibody Y/1F4 can be used to specifically recognize the ubiquitous SERCA2b isoform in non-muscle tissues. Similar results were obtained with another monoclonal antibody raised against the skeletal sarcoplasmic reticulum (*18*), although the lack of an epitope sequence for this antibody does not provide further information about a possible common epitope in SERCA1 and SERCA2b isoforms.

We have also observed that Y/1F4 binds to a 115 kDa protein that seems to be resistant to trypsin proteolysis (Figs. 3 and 4). This protein is phosphorylated in the absence of Ca^{2+}, but not in the absence of Mg^{2+} (Fig. 6) . The presence of this protein has been also observed in adrenal medulla microsomes (*13*). It was concluded that this protein cannot correspond to the phosphorylated form of SERCA, although this issue was not investigated further.

Our results shown in Fig. 6 also confirm that the 115 kDa phosphoprotein is not a Ca^{2+}-ATPase. When the phosphorylation experiments were performed in the absence of Ca^{2+} and presence of Mg^{2+}, the strong band at 110 kDa disappeared, but the upper protein was still present. However, the phosphoprotein was not formed in the absence of Mg^{2+}. These results suggest that the 115 kDa band may correspond to a Mg^{2+}-ATPase protein. Further experiments seem to support this idea (*19*, *20*). Currently, experiments are underway to identify the physiological role of this protein.

Table I summarizes the immunoreaction of all antibodies used in this study with fractions from brain and other sources. Specific antibodies show the presence of SERCA2b as a unique SERCA isoform. It is important to pinpoint the binding of Y/1F4 to SERCA2b in this isoform expressed in COS-1 cells, which makes this monoclonal antibody effective for the identification of this isoform in non-muscle tissues.

We thank Prof. Frank Wuytack and Dr. Hilde Verboomen (Laboratory of Physiology, University of Leuven, Belgium) for the gift of SERCA isoforms specific antibodies, and microsomes from COS-1 cells expressing the SERCA isoforms, and for their collaboration in some experiments shown in this work.

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