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Expression of endoplasmic-reticulum Ca^{2+}-pump isoforms and of phospholamban in pig smooth-muscle tissues

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The expression of the gene 2 sarcoplasmic/endoplasmic-reticulum Ca^{2+}-pump isoforms (SERCA2a and SERCA2b) and of phospholamban was studied in pig smooth muscle of the stomach, longitudinal ileum, pulmonary artery and aorta. mRNA levels were determined using an RNAase protection assay. The SERCA2 isoforms and phospholamban were tested on Western blots with a panel of antibodies, some of which were isoform-specific. The pig smooth-muscle tissues all contained comparable SERCA2 mRNA levels, but these levels were 10–20-fold lower than SERCA2 mRNA levels in cardiac muscle. Of the SERCA2 mRNAs in smooth muscle, 72–81% encoded the non-muscle isoform (SERCA2b), and Western blot analysis with isoform-specific antibodies confirmed that the SERCA2b isoform is the predominant endoplasmic-reticulum Ca^{2+} pump in smooth muscle. In contrast with SERCA2 mRNA levels, phospholamban mRNA levels varied by 12-fold between the different pig smooth-muscle tissues, with low and very low levels in the pig pulmonary artery and the pig aorta respectively. The differential expression of phospholamban was also confirmed on Western blots. The finding that the phospholamban content varied between the different smooth-muscle tissues whereas the SERCA2 expression remained rather constant indicates that, in pig smooth muscle, the expression of phospholamban is not coupled with that of SERCA2.

INTRODUCTION

In the past few years, a series of sarcoplasmic/endoplasmic-reticulum (SR/ER) Ca^{2+}-pump isoforms has been described [1–12]. Three different genes (SERCA1, SERCA2 and SERCA3; the nomenclature for the SR/ER Ca^{2+} pumps is that of Burk *et al.* [12]) as well as alternative processing of the SERCA1 and SERCA2 transcripts result in the expression of at least five different SR/ER Ca^{2+}-pump isoforms. Molecular cloning of the smooth-muscle ER Ca^{2+} pump has demonstrated that smooth muscle expresses the same gene (SERCA2) as slow skeletal muscle, cardiac muscle and non-muscle tissues [7–9]. However, alternative processing of the SERCA2 primary gene transcript results in three different SERCA2-derived mRNAs in pig smooth muscle [13]. Class 1 mRNA encodes the SERCA2a Ca^{2+} pump isoform (M_r 110000; 997 aa) whereas class 2 and 3 mRNAs encode the SERCA2b Ca^{2+} pump isoform (M_r 115000; 1042 aa). The SERCA2a and SERCA2b isoforms differ only in their *C*-termini: the SERCA2a *C*-terminus (-NYLEP/*AILE*) is replaced by an extended tail of 49 amino acids in the SERCA2b protein (-NYLEP/*GKEC*..41*aa*..*MFWS*) [5–7]. The SERCA2a isoform corresponds to the Ca^{2+} pump in slow skeletal and cardiac SR. The SERCA2b isoform represents a Ca^{2+} pump which is also found in non-muscle tissues such as liver, kidney and brain [5–7, 9–13] and which corresponds to the Ca^{2+} pump of the intracellular Ca^{2+} store in smooth muscle and non-muscle tissues. Whether this Ca^{2+} store is part of the ER or whether it is located in a distinct intracellular organelle is, however, still a matter of uncertainty.

A second component of the Ca^{2+}-uptake system in slow skeletal and cardiac SR is phospholamban (PLB), a homopentameric phosphorylatable protein which functions as a regulatory protein of the SR Ca^{2+} pump. Dephosphorylated PLB inhibits the SR Ca^{2+} pump, but phosphorylation of PLB by cyclic AMP-, cyclic GMP- or Ca^{2+}/calmodulin-dependent protein kinases alleviates this inhibition [14,15]. A direct interaction between PLB and the SR CA^{2+} pump (SERCA2a and SERCA1 isoforms), which depends on the phosphorylation status of PLB, has recently been demonstrated [16]. PLB has also been demonstrated in some smooth-muscle tissues, and the molecular cloning of smooth-muscle PLB has revealed that it is identical to the cardiac protein [17–20].

We have now analysed further the expression of the SERCA2 isoforms and of PLB in pig smooth muscle. mRNA levels were determined by means of an RNAase protection assay using a panel of anti-sense RNA probes. The expression of the SR/ER Ca^{2+}-pump isoforms was also investigated on Western blots by means of isoform-specific antibodies. This study shows that the SERCA2 gene is expressed similarly in different smooth-muscle tissues and that the SERCA2b isoform is the predominant ER Ca^{2+} pump in smooth muscle. In contrast, PLB levels differ markedly between smooth-muscle tissues. PLB expression is therefore not coupled to the expression of SERCA2 in pig smooth muscle.

MATERIALS AND METHODS

RNA isolation

Pig gastric smooth muscle (antral part) was prepared by selectively removing the gastric serosa and mucosa from the antrum. Pig ileal longitudinal smooth muscle was obtained by selectively stripping the outer longitudinal smooth-muscle layer from the rest of the ileum. Pig aorta and pulmonary artery were prepared by removing the adventitia. The tissues were frozen in liquid N_2 and stored at −80 °C until used. The total RNA of the pig ileal and gastric smooth-muscle layer and of pig aorta, pulmonary artery, liver and heart were prepared according to the Chirgwin procedure [21].

Abbreviations used: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} pump; PLB, phospholamban; aa, amino acid(s), nt, nucleotide(s).

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RNAase protection assay

mRNA levels were semi-quantitatively determined according to the method of Lee & Costlow [22]. Increasing amounts of tissue total RNA (0–50 μg) were mixed with carrier RNA (yeast total RNA pretreated with Proteinase K to remove RNAase activity) to a final amount of 50 μg of RNA. The vacuum-dried RNA pellet was resuspended in 20 μl of hybridization mixture consisting of formamide (80%), Pipes (25 mM), pH 6.8, NaCl (0.4 M), EDTA (5 mM) and 0.1–0.4 ng of a ^{32}P-labelled anti-sense RNA probe (specific radioactivity greater than 10^6 c.p.m./ng of RNA; see next section). After heating to 85 °C for 5 min, the samples were hybridized overnight at 50 °C. RNAase buffer (300 μl; NaCl, 375 mM, Tris/HCl, 75 mM, pH 8.0, and EDTA 5 mM) containing 100 μg of RNAase A and 300 units of RNAase T1 was then added, and the non-hybridized single-stranded RNA molecules were digested for 1 h at 37 °C. Finally 300 μg of yeast total RNA was added and the hybridized RNAase-resistant RNA was precipitated by adding 4.0 ml of ice-cold 10% trichloroacetic acid. The precipitation mixtures were kept on ice for 10 min and the precipitated RNA was collected on Whatman GF/C filters which were rinsed four times with 7.5 ml of ice-cold 10% trichloroacetic acid. The radioactivity retained on the dried filters was counted in a liquid scintillation counter. The protected c.p.m. were plotted as a function of input total RNA. The slope of this linear curve equals the protected c.p.m./μg of total RNA. This value was used to calculate the mol of transcripts/μg of total RNA, by taking into account the M_r of the probe and its specific activity.

Anti-sense RNA probes

Transcription vectors were constructed by subcloning the following restriction fragments in pGEM-7Z(f)+ (Promega, Madison, WI, U.S.A.): pSERCA2: *Cla*I–*Xho*I (nt 1519–2027) of pig SERCA2 class 1 cDNA [7]; pSERCA2b: *Xba*I–*Sca*I (nt 3250–3611) of pig SERCA2 class 2 cDNA [7]; pPLB: *Dra*I–*Eco*RI (nucleotides 551–737) of the pig PLB cDNA clone 8 [18]. Anti-sense RNA probes were synthesized by a transcription reaction *in vitro* by using a standard protocol [23], except that 240 μCi of [α-^{32}P]CTP (800 Ci/mmol; 40 mCi/ml) per transcription reaction was used and that the total concentration of CTP was adjusted to 20 μM with unlabelled CTP. SP6 RNA polymerase was used to synthesize pSERCA2b. T7 RNA polymerase was used for pSERCA2 and pPLB synthesis. After digestion with DNAase the probes were purified using a Sephadex G-50 spun column procedure [24]. RNA probes were labelled up to 10^6 c.p.m./ng of RNA. The integrity of the anti-sense RNA probes was checked on a 6% polyacrylamide/urea gel (more than 95% full-length RNA) and their specificity was evaluated by Northern blot analysis (see ref. [13])

Preparation of microsomes

Pig cardiac SR microsomes were prepared according to Jones & Cala [25]. Pig smooth-muscle ER microsomes of the stomach (antral part), pulmonary artery and aorta were prepared according to Raeymaekers *et al.* [26]. Pig liver microsomes were obtained as described by Gaetani *et al.* [27].

Western blotting

The microsomal fractions of pig cardiac muscle, antrum, pulmonary artery, aorta and liver were separated on a Laemmli- [28] type polyacrylamide gel [7.5% (w/w) acrylamide for SERCA2 detection or 15.0% (w/w) acrylamide for PLB detection], blotted on to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA, U.S.A.) and immunostained as described previously [29]. The antisera, which were elicited against a peptide–BSA conjugate, were incubated for 1 h with 0.1% (w/v) BSA in 150 mM-NaCl/10 mM-Tris/HCl, pH 7.5, to neutralize the anti-BSA antibodies, and were subsequently added to the blot.

Antibodies

The following antibodies were used to detect the SERCA2 isoforms and PLB. The monoclonal antibody recognizing SERCA2a and SERCA2b isoforms and the monoclonal antibody against PLB were kindly provided by Dr. K. P. Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA, U.S.A.) and Dr. L. R. Jones (Krannert Institute of Cardiology, Indianapolis, IN, U.S.A.) respectively. The SERCA2b-specific polyclonal antiserum was elicited against the isoform-specific *C*-terminus of SERCA2b and has been described previously [30]. A SERCA2a-specific polyclonal antiserum was obtained by immunizing rabbits with a BSA-coupled peptide (PEP2a: NYLEPAILE) that corresponds to the nine *C*-terminal amino acids of the pig SERCA2a isoform (aa 989–997) [7]. Glutaraldehyde-mediated coupling of PEP2a to BSA and immunization of rabbits were performed using a previously described protocol [30].

SERCA2b *C*-tail fusion protein synthesis

A T7Gen10 fusion protein ending with the 54 *C*-terminal amino acids of the SERCA2b isoform (aa 989–1042: -NYLEP/GKEC..41aa..MFWS) was synthesized with the pGEMEX-1 expression system (Promega, Madison, WI, U.S.A.). The *Eco*RI–*Bam*HI fragment (nt 1–1121) of clone ER-15 [13], which encodes the 54 *C*-terminal amino acids of pig SERCA2b, was subcloned in the pGEMEX-1 vector. The open reading frame was restored by *Eco*RI digestion, Klenow fill-in and religation of the vector. An M_r-36000 fusion protein was induced in the JM109–DE3 strain and prepared according to the manufacturer's instructions. The fusion protein (approx. half of the total bacterial protein mass) was further purified on a preparative Laemmli- [28] type gel [12.0% (w/w) acrylamide] and electro-eluted with the ISCO Model 1750 sample concentrator (ISCO, Lincoln, NE, U.S.A.).

RESULTS

Characterization of the SERCA2a-specific antiserum

A major tool in the study of the expression of the SERCA2 Ca^{2+}-pump isoforms are isoform-specific antibodies. We have previously reported the synthesis of an antiserum specific for the SERCA2b isoform [30]. To obtain an antiserum specific for the SERCA2a isoform, we immunized rabbits with a peptide (PEP2a) corresponding to the nine *C*-terminal amino acids of the SERCA2a isoform (aa 989–997: -NYLEP/AILE) (see the Materials and methods section). The antiserum stained an M_r-100000 protein in cardiac SR which coincided perfectly with the protein detected with the non-discriminating anti-SERCA2 monoclonal antibody (Fig. 1*a*). However, as only the four ultimate amino acids (/AILE) of PEP2a are specific for the SERCA2a isoform, it could be that part of the antiserum is directed against the common NYLEP sequence. We therefore constructed a T7Gen10–SERCA2b fusion protein (see the Materials and methods section) in which the *C*-terminus consists of the 54 C-terminal amino acids of the SERCA2b isoform (aa 989–1042: NYLEP/GKEC..41aa..MFWS). This fusion protein contains the common NYLEP sequence, but it ends with the 49 C-terminal amino acids of the SERCA2b isoform. On Western blots, the anti-PEP2a antiserum did not react with the fusion protein, but the fusion protein was clearly detectable with the SERCA2b-specific antiserum (Fig. 1*b*). Therefore the anti-PEP2a

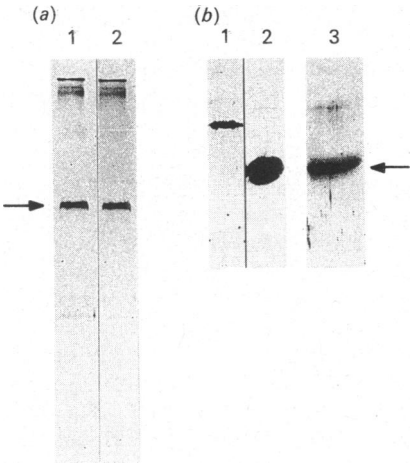

Fig. 1. The anti-PEP2a antiserum recognizes the SERCA2a isoform but not the SERCA2b isoform

(*a*) Western blots containing membrane fractions of pig cardiac SR were stained with the anti-PEP2a antiserum (lane 1) and subsequently overstained with a non-discriminating anti-SERCA2 monoclonal antibody (lane 2). Note that the M_r-100000 band stained with the anti-PEP2a antiserum (indicated by the arrow) coincides perfectly with the anti-SERCA2-stained band. (*b*) A Western blot containing the T7Gen10–SERCA2b fusion protein was incubated with either the anti-PEP2a antiserum (lane 1) or the anti-SERCA2b antiserum (lane 2). The former antiserum does not react with the SERCA2b *C*-tail fusion protein. Lane 3 shows the purified T7Gen10–SERCA2b fusion protein (indicated by the arrow) after Laemmli- [28] type gel electrophoresis and Coomassie Brilliant Blue staining. The band in lane 1 corresponds to an aspecific reaction with a bacterial contaminant.

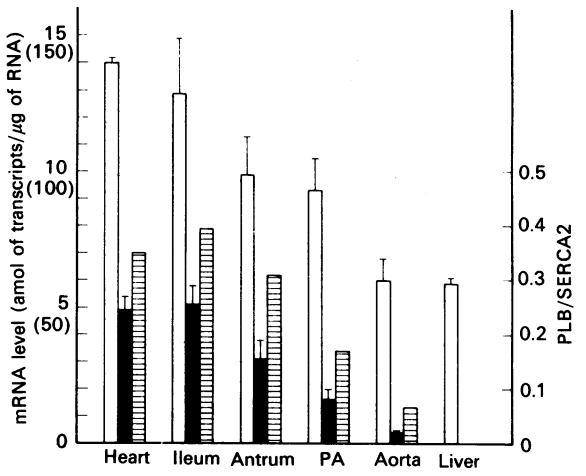

Fig. 2. mRNA levels of SERCA2 and PLB and the ratio of PLB to SERCA2

mRNA levels in pig cardiac muscle, in pig smooth muscle of longitudinal ileum, antrum, pulmonary artery (PA), aorta and in pig liver were determined with an RNAase protection assay. □, SERCA2 mRNA levels determined with a non-discriminating anti-sense RNA probe (pSERCA2); ■, PLB mRNA levels; ▤, ratio of PLB mRNA to SERCA2 mRNA. The SERCA2 and PLB bars correspond to mean mRNA levels ($n = 5$; S.E.M. indicated by the vertical line) which are expressed in 10^{-18} mol (amol) of transcripts/μg of total RNA, except for the cardiac mRNA levels, for which the scale has been compressed 10-fold (values in parentheses apply to the cardiac mRNA levels). The PLB/SERCA2 ratio is expressed on a relative scale.

antiserum is specific for the SERCA2a isoform of the SR/ER Ca^{2+} pump.

Expression of the SERCA2 isoforms in smooth muscle

We have analysed the expression of SERCA2 in four different pig smooth-muscle tissues (longitudinal ileum, antrum, pulmonary artery and aorta) as well as in pig cardiac muscle and pig liver. The mRNA levels were first semi-quantitatively determined by means of an RNAase protection assay (see the Materials and methods section). To compare the SERCA2 mRNA levels we used an anti-sense RNA probe (pSERCA2) which corresponded to the common part of the SERCA2 transcripts (nt 1519–2027 [7]) and which therefore did not discriminate between the different SERCA2-derived mRNAs. SERCA2 mRNAs were expressed at comparable levels in the four smooth-muscle tissues and in liver (Fig. 2) and there was only a 2-fold difference between longitudinal ileum and aorta [$(12.9 \pm 2.0) \times 10^{-18}$ versus $(6.0 \pm 0.8 \times 10^{-18}$ mol of transcripts/μg of RNA (mean ± S.E.M.; $n = 5$)]. However, the SERCA2 mRNA level was 10–20-fold higher in cardiac muscle than in the smooth-muscle tissues or in the liver. As pig smooth muscle contains three different SERCA2 mRNAs, one of which (class 1) encodes the SERCA2a isoform, whereas the other two (class 2 and 3) encode the SERCA2b isoform (see the Introduction section), we subsequently determined the relative proportions of SERCA2b mRNA with pSERCA2b. This anti-sense RNA probe corresponds to nucleotides 3250–3611 of the class 2 cDNA [7] and recognizes only the class 2 and 3 mRNAs. In longitudinal ileum, antrum and aorta, a similar fraction of the SERCA2 mRNA pool was of the SERCA2b type, i.e. class 2 and 3 mRNAs: 81% in the longitudinal ileum and 72% in the aorta (Fig. 3). Consequently, only 19–28% of the

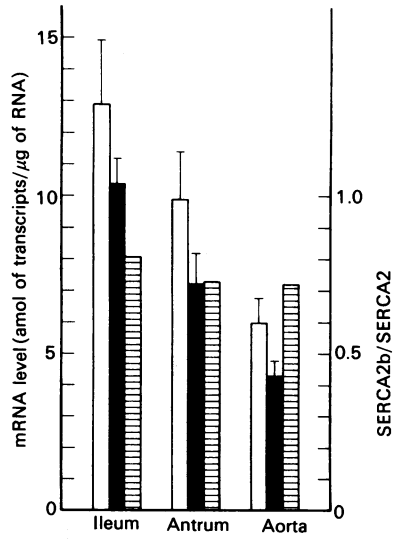

Fig. 3. Relative proportions of the SERCA2b-encoding mRNAs in pig smooth muscle

SERCA2- and SERCA2b-encoding mRNA levels in pig smooth muscle of longitudinal ileum, antrum and aorta were determined with a RNAase protection assay. □, SERCA2 mRNA levels determined with a non-discriminating anti-sense RNA probe (pSERCA2); ■, SERCA2b-encoding mRNA levels determined with an anti-sense RNA probe which recognizes only the SERCA2b-encoding mRNAs, i.e. class 2 and 3 mRNA in smooth muscle; ▤, ratio of SERCA2b mRNA to SERCA2 mRNA. SERCA2 and SERCA2b bars corespond to mean mRNA levels ($n = 5$; S.E.M. indicated by the vertical line) which are expressed in amol of transcripts/μg of total RNA. The SERCA2b/SERCA2 ratio is expressed on a relative scale.

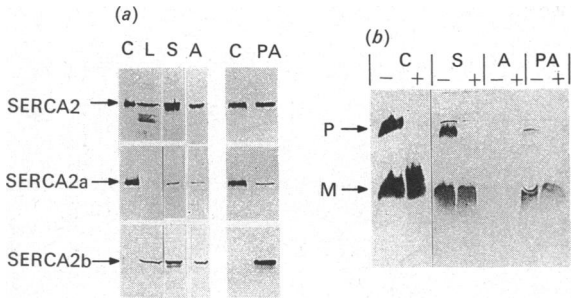

Fig. 4. Western blots showing the relative expression of the SERCA2 isoforms and of PLB in pig smooth muscle

(*a*) SR- or ER-enriched membrane fractions of cardiac muscle (lanes C), liver (lane L), stomach smooth muscle (lane S), aorta (lane A) and pulmonary artery (lane PA) were analysed on Western blots using a non-discriminating anti-SERCA2 monoclonal antibody or a polyclonal antiserum specific for the SERCA2a or the SERCA2b isoform, as indicated. Approximately equal amounts of SERCA2 protein (as judged by the reaction with the anti-SERCA2 antibody) were applied per lane. Note that the SERCA2a band in smooth muscle is much weaker than that in cardiac muscle, whereas the SERCA2b signal in smooth muscle is comparable with that in liver. Lanes 1–4 are a composite of the same blot, whereas lanes 5 and 6 were taken from a separate experiment. (*b*) SR- or ER-enriched membrane fractions of pig cardiac muscle (lane C), stomach smooth muscle (lane S), aorta (lane A) and pulmonary artery (lane PA) were analysed on Western blots using a monoclonal antibody against PLB. As in (*a*), approximately equal amounts of SERCA2-immunoreactive protein were applied. Samples were (+) or were not (−) boiled in sample buffer before application. P denotes the PLB pentamer, which decomposes into the monomer (M) upon boiling. Note that the PLB signal varied between the different smooth-muscle tissues, in spite of the fact that an approximately equal amount of SERCA2 protein was applied.

smooth-muscle SERCA2 mRNAs should code for the SERCA2a isoform. A direct assessment of the class 1 mRNA level was not possible, however, as any probe for the SERCA2a-specific part also reacts with the class 3 mRNAs. Similar conclusions based on S1 nuclease mapping experiments were drawn for rabbit and rat smooth muscle, although in some rat smooth-muscle tissues the SERCA2a mRNA accounted for up to 70% of the total SERCA2 mRNA [8,10].

These data were complemented at the protein level. SERCA2 Ca^{2+} pumps were revealed on Western blots using the non-discriminating anti-SERCA2 monoclonal antibody or polyclonal antisera specific for either the SERCA2a isoform or the SERCA2b isoform. When a Western blot containing SR- or ER-enriched microsomes of cardiac muscle, antrum, pulmonary artery, aorta or liver was probed with the non-discriminating monoclonal antibody, an immunoreactive band of M_r 100000 was detected in all tissues (Fig. 4*a*). However, a different picture was obtained when the isoform-specific antisera were used. The SERCA2a isoform was clearly present in cardiac muscle and to a lesser extent in the smooth-muscle tissues, but it could not be detected in liver. The SERCA2b isoform was present in liver and smooth muscle, but it could not be detected in cardiac muscle (Fig. 4*a*). The relative proportions of the SERCA2a and SERCA2b isoforms in smooth muscle were estimated by comparing the intensities of the SERCA2a and SERCA2b signals in smooth muscle with those in cardiac muscle and liver. When approximately equal amounts of the SERCA2 proteins were applied (as judged by the immunoreaction with the non-discriminating antibody), the SERCA2b signal in smooth muscle was comparable with that in liver, whereas the SERCA2a signal in smooth muscle was much less intense than that in cardiac muscle (Fig. 4*a*). Therefore the SERCA2b isoform constitutes the majority of the ER Ca^{2+} pumps in smooth muscle.

Expression of PLB in smooth muscle

We next examined the expression of PLB in smooth muscle. PLB mRNA levels were determined by the RNAase protection assay. In contrast with the SERCA2 levels, PLB mRNA levels clearly differed between the smooth-muscle tissues, with a 12-fold difference between longitudinal ileum and aorta [$(5.1 \pm 0.7) \times 10^{-18}$ versus $(0.4 \pm 0.07) \times 10^{-18}$ mol of transcripts/μg of RNA (means ± S.E.M.; $n = 5$)]. When compared with cardiac muscle, PLB mRNAs were 10 (longitudinal ileum) to 122 times (aorta) less abundant. PLB mRNAs were not detected in liver. The expression of PLB relative to that of the SR/ER Ca^{2+} pump was estimated by calculating the ratio of PLB mRNA to SERCA2 mRNA. This ratio was almost identical in cardiac muscle, longitudinal ileum and antrum (PLB/SERCA2 = 0.35, 0.39 and 0.31 respectively), but it was appreciably lower in the pulmonary artery (PLB/SERCA2 = 0.17) and lower still in the aorta (PLB/SERCA2 = 0.07), reflecting the lower level of PLB expression in proportion to SERCA2 in the latter tissues. It should be noted that the values obtained for the longitudinal ileum cannot necessarily be extrapolated to the circular smooth-muscle layer of ileum.

The relative expression of PLB compared with that of the SR/ER Ca^{2+} pump was investigated further on Western blots. SR- or ER-enriched membrane fractions of cardiac muscle and smooth muscle containing approximately equal amounts of immunoreactive SERCA2 protein were stained with a monoclonal antibody against PLB (Fig. 4*b*). PLB was clearly present in the stomach smooth muscle, was barely detectable in the pulmonary artery, and could not be detected at all in the aorta. This confirmed that the PLB content was not proportional to the SERCA2 content in the different smooth-muscle tissues. The fact that PLB could not be detected in the pig aorta correlates with previous observations that PLB could not be demonstrated in pig aortic ER by cyclic AMP-dependent phosphorylation [17]. Presumably PLB expression in pig aorta is too low to be detected at the protein level via either immunostaining or phosphorylation.

DISCUSSION

Pig smooth muscle expresses the SERCA2 gene, and alternative processing yields three mRNA types encoding either the SERCA2a (class 1) or the SERCA2b (class 2 and 3) isoform [7, 13]. Semi-quantitative analysis of the SERCA2 mRNA levels by means of an RNAase protection assay shows that the SERCA2 gene is expressed to a similar extent in four different smooth-muscle tissues and in liver. However, when compared with cardiac muscle, the SERCA2 mRNA levels in smooth muscle are 10–20-fold lower. In pig smooth muscle, 72% (aorta) to 81% (longitudinal ileum) of the SERCA2 mRNAs encode the SERCA2b isoform. Analysis of smooth-muscle membrane fractions with isoform-specific polyclonal antisera confirms that the SERCA2b isoform is the predominant Ca^{2+} pump in smooth muscle, whereas it cannot be detected in cardiac muscle. Therefore SERCA2 expression in different smooth-muscle tissues is fairly similar, but it differs both quantitatively (a 10–20-fold difference in mRNA level) and qualitatively (alternative processing of the SERCA2 transcripts leading to either the SERCA2a or the SERCA2b isoform) from that in cardiac muscle.

In contrast with SERCA2 expression, the expression of PLB varies markedly between different pig smooth-muscle tissues (longitudinal ileum > antrum > pulmonary artery > aorta). In

the pig aorta, the mRNA level for PLB is very low, and PLB cannot be detected by immunostaining. However, in canine and bovine aorta and in bovine pulmonary artery, PLB can be readily detected either via immunostaining or via cyclic AMP-dependent phosphorylation [17,18] Therefore PLB expression in the aorta and pulmonary artery seems to vary in a species-dependent way. Whether the low PLB/SERCA2 ratio in the pig arterial smooth muscle tissues has any functional implication still remains to be shown. As the cyclic AMP- or cyclic GMP-dependent regulation of the SR/ER Ca^{2+} uptake can be at least partially mediated via the phosphorylation of PLB [14,15], one might expect that the ER Ca^{2+} uptake in smooth-muscle tissues with a low PLB level is relatively insensitive to modulation by catecholamines, endothelium-derived relaxation factor and organic nitro-compounds. One should, however, bear in mind that the predominant Ca^{2+} pump in pig smooth muscle is the SERCA2b isoform, and that up until now no experimental data on SERCA2b regulation by PLB have been published. Therefore the impact of the various PLB levels on ER Ca^{2+} uptake in pig smooth muscle cannot be adequately assessed as long as the sensitivity of the SERCA2b protein for phospholamban has not been established.

In view of the co-expression of SERCA2a and PLB in slow skeletal and cardiac muscle [3,31] and of the direct interaction between SERCA2a and PLB [16], one might expect a coupling between the expression of PLB and that of SERCA2a. Indeed, parallel changes in SERCA2a and PLB mRNA levels have been observed in cardiac muscle during arterial pressure overload [32] and in skeletal muscle during chronic low-frequency stimulation [33]. However, experimentally induced hyperthyroidism increases the SERCA2a mRNA levels in cardiac muscle but down-regulates PLB mRNA [32]. Likewise, the PLB content of the four smooth-muscle tissues differs markedly, in spite of the similar SERCA2a levels. Therefore, although PLB and SERCA2a are co-ordinately expressed in some circumstances, their expression is not strictly coupled.

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