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Variation of phospholamban in slow-twitch muscle sarcoplasmic reticulum between mammalian species and a link to the substrate specificity of endogenous Ca^{2+}-calmodulin-dependent protein kinase

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Abstract

Systematic immunological and biochemical studies indicate that the level of expression of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase regulatory protein phospholamban (PLB) in mammalian slow-twitch fibers varies from zero, in the rat, to significant levels in the rabbit, and even higher in humans. The lack of PLB expression in the rat, at the mRNA level, is shown to be exclusive to slow-twitch skeletal muscle, and not to be shared by the heart, thus suggesting a tissue-specific, in addition to a species-specific regulation of PLB. A comparison of sucrose density-purified SR of rat and rabbit slow-twitch muscle, with regard to protein compositional and phosphorylation properties, demonstrates that the biodiversity is two-fold, i.e. (a) in PLB membrane density; and (b) in the ability of membrane-bound Ca^{2+}-calmodulin (CaM)-dependent protein kinase II to phosphorylate both PLB and SERCA2a (slow-twitch isoform of Ca^{2+}-ATPase). The basal phosphorylation state of PLB at Thr-17 in isolated SR vesicles from rabbit slow-twitch muscle, colocalization of CaM K II with PLB and SERCA2a at the same membrane domain, and the divergent subcellular distribution of PKA, taken together, seem to argue for a differential heterogeneity in the regulation of Ca^{2+} transport between such muscle and heart muscle.

Keywords: Slow-twitch skeletal muscle; Sarcoplasmic reticulum; Phospholamban; Ca^{2+} pump; Ca^{2+}-calmodulin-dependent protein kinase II

1. Introduction

The calcium required for skeletal muscle contraction is derived from an intracellular store corresponding to the lumen of the sarcoplasmic reticulum (SR). In studies in the rat, resting contents of free Ca^{2+} within the SR [1] and the amplitude and time course of biphasic changes in myoplasmic free calcium ion concentration associated with the contraction-relaxation cycle, were shown to differ between fast-twitch and slow-twitch muscle fibers [2]. Ca^{2+} release and Ca^{2+} reuptake by the SR are tightly coupled processes, through changes in the Ca^{2+} concentration gradient across the SR membrane [3].

The different relaxation speeds of fast- and slow-twitch fibers have been mainly attributed to differences in SR membrane density, total number and basal functional state of Ca^{2+} pumps. Fast-twitch

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and slow-twitch skeletal muscle fibers express distinct SR Ca^{2+}-ATPase (SERCA) isoforms, which are the products of different genes [4]. Mammalian slow-twitch skeletal muscle and heart muscle express the same SERCA isoform (SERCA2a) [5]. Early studies in the cat [6] found that in vivo administration of epinephrine induced an acceleration of the rate of relaxation in both types of muscle. Regulation of SERCA2 activity by the state of phosphorylation of regulatory protein phospholamban (PLB) has been held until recently almost exclusively responsible for this effect. PLB is a membrane integral protein specific to cardiac and to slow-twitch SR and to ER of some smooth muscle tissues [7], that interacts with and reversibly inhibits SERCA2 in its native membrane environment. PLB is phosphorylated by cAMP-dependent protein kinase (PKA) and Ca^{2+}-CaM-dependent protein kinase (CaM K II), at Ser-16 and Thr-17 [8,9]. Phosphorylation of PLB disrupts the inhibitory interaction. Reversal of the inhibition was shown to be associated with an increase in affinity for Ca^{2+} of SERCA2a [10].

β-Adrenergic-induced cardiac relaxation, on account of recent work with perfused rat hearts, was explained with the sequential phosphorylation of PLB at Ser-16 and Thr-17, requiring the influx of Ca^{2+} through the L-type of Ca^{2+} channel [11]. Recent results with PLB-knocked out mice [12,13], and with mice over-expressing the PLB gene in skeletal muscle [14] seem to argue for a less important regulatory role of PLB in Ca^{2+} homeostasis of slow-twitch skeletal muscle fibers than of cardiac myocytes. PLB expression at the mRNA level [14] was found to be severalfold lower in mouse soleus, compared to that observed in the heart.

SR membrane-bound CaM K II, in addition to being able to phosphorylate PLB, was shown to phosphorylate the SERCA2a Ca^{2+}-ATPase isoform [15] at Ser-18 [16] in isolated SR vesicles from slow-twitch muscle and heart muscle of the rabbit. This substrate specificity is in agreement with evidence that the fast-twitch muscle SERCA1a lacks a phosphorylated serine or threonine residue at position 38. The main divergence between reports [17] lies in the functional consequences of CaM K II-mediated phosphorylation of SERCA2a. A recent study confirmed that phosphorylation of cardiac Ca^{2+}-ATPase, in the absence of PLB phosphorylation, resulted in a significant increase in the V_{max} of the SR Ca^{2+} transport system [18].

The present study addresses the problem of the biodiversity in PLB expression levels, according to the animal species and body size, as well as between slow-twitch skeletal and heart muscle of different mammalian species, despite extensive similarities in SR phenotype regarding the expression of SERCA2a. To this purpose, we used a wide variety of experimental approaches. Results reported here also deal with the problem of the functional interrelationship between the level of protein expression of PLB in mammalian slow-twitch muscle SR, and in the ability of membrane-bound CaM K II to phosphorylate both this protein substrate and SERCA2a within the same SR membrane domain.

2. Materials and methods

All chemicals were analytical grade. [^{32}P]ATP (3000–6000 Ci/mmol), was purchased from New England Nuclear (Du Pont De Nemours, Bad Homburg, Germany). The catalytic subunit of PKA (bovine heart, 1–2 picomolar units/μg) was obtained from Sigma. Molecular mass standards for SDS-PAGE were obtained from BDH Lab. Supplies (Poole, UK).

2.1. Biological sources and experiments with animals

New Zealand male adult rabbits, Wistar rats, Swiss mice and Hartley guinea pigs were the experimental animals used. Animals were killed by cervical dislocation. Human rectus abdominis was obtained during abdominal surgery. Unilateral denervation of rabbit soleus was obtained by transecting the common popliteal nerve and removing 0.5 cm to prevent reinnervation of the muscle. The muscle from the unoperated leg was used as control. Muscles from either side were removed from the killed animals after 2 weeks.

2.2. Preparation of skeletal muscle and cardiac SR

SR-enriched membrane preparations were isolated from homogenates of predominantly fast-twitch or slow-twitch (soleus and semitendinosus) hind leg

muscles of adult rabbits and fractionated by isopycnic sucrose density centrifugation using the method of Saito et al. [19], with slight modifications [20,21], to yield four distinct subfractions, labeled R1 to R4 from the top to the bottom of the gradient. An identical procedure was used for rabbit ventricular muscle and for rat soleus. Slow-twitch muscles were pooled from several animals, to yield about 5–10 g fresh tissue weight. Membrane fractions from the sucrose density gradient were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 μM PMSF, 1 μg/ml leupeptin, divided in aliquots and stored at −80°C until used. Protein concentration was determined by the Folin reaction [22], using BSA (Boehringer, Mannheim, Germany) as a standard.

2.3. Phosphorylation

2.3.1. Ca^{2+}-CaM-dependent phosphorylation

The standard assay medium (total volume 100 μl) for phosphorylation of SR membrane protein (100 μg) by endogenous Ca^{2+}-CaM K II contained 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 80 mM KCl, 1 mM EGTA, 1.1 mM $CaCl_2$ [21]. The free Ca^{2+} concentration of the assay medium, determined using the computer program of Fabiato [23], was 100 μM. Controls lacked 1 μM CaM. The reaction was started by adding 400 μM [γ-^{32}P]ATP (specific radioactivity 0.10 Ci/mmol). Incubation was at 0–4°C for 1–10 min [21,24].

2.3.2. Protein kinase A phosphorylation assay

The standard assay medium (total volume 100 μl) for phosphorylation of SR membrane protein (100 μg) by PKA contained 20 mM histidine, pH 7.0, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 20 mM NaF, 5 μM cAMP, and the catalytic subunit (2 μg) of PKA. Controls lacked cAMP, or both cAMP and PKA catalytic subunit. The reaction was started by adding 50 μM [γ-^{32}P]ATP. Incubation was at 30°C for 5 min.

For each kind of assay, reactions were quenched by adding 50 μl of Laemmli sample buffer to each sample. SDS-PAGE was carried out using the buffer system described by Laemmli [25]. 5–10% or 10–15% SDS gels were used, to verify the phosphorylation of SERCA2a and PLB. The gels were stained with Coomassie blue, dried and autoradiographed (16 h exposure), using Hyperfilm (Amersham), or by a Model GS-250 Molecular Imager (Bio-Rad Laboratories, Hercules, CA, USA), using a β-particle-sensitive screen (Imaging screen cassette BI), essentially as described previously [21].

2.4. Gel electrophoresis and Western blotting

SDS-PAGE was performed as reported in the legends to the figures. Protein loading of gels varied between 300 ng and 100 μg. After electrophoresis, proteins were transferred onto nitrocellulose. Incubation of blots with specific antibodies was performed as described by Damiani et al. [20]. Specific antibodies used were the following: (a) mouse monoclonal antibodies to (1) PLB (Affinity Bioreagents (ABR), Golden, CO, USA) (1/1000 dilution); (2) skeletal isoform of SR Ca^{2+} release channel/RyR1 (ABR) (1/1000 dilution); (3) skeletal isoform of triadin (ABR) (1/1000 dilution); (4) SERCA1 (ABR); (5) 53 kDa glycoprotein (ABR) (1/1000 dilution); (6) β isoform of CaM K II (Gibco BRL, Life Technologies, Paisley, UK) (1 μg/ml); (7) catalytic subunit of PKA (Transduction Laboratories, Lexington, KY, USA) (1/1000 dilution); (b) rabbit polyclonal antibodies to (1) SERCA2a (PhosphoProteinResearch (PPR), Bardsey, UK) (1/10 000 dilution); (2) phosphorylated PLB peptides (PThr^{17}-PLB and PSer^{16}-PLB) (PPR) (1/1000 dilution); (c) goat polyclonal antibody to δ and γ isoforms of CaM K II (Santa Cruz, CA, USA) (1 μg/ml). It was verified that, as indicated by the manufacturer, the anti-γ CaM K II antibody was cross-reactive with the β isoform. Antibody binding was detected by immunoenzymic staining [20]. Densitometric measurements of blotted proteins, after immunostaining, were carried out using a Bio-Rad Model GS-670 Imaging densitometer.

2.5. Northern blot analysis

Northern blot analysis of total RNA isolated from the rat heart and soleus muscle using the ULTRASPECTM RNA isolation method (Biotecx, TX, USA) was performed as described by Zorzato et al. [26]. Denatured total RNA was electrophoresed on a 1% agarose gel, blotted onto Hybond$^+$ membranes (Amersham), and probed with a ^{32}P-labeled PLB

cDNA fragment (final concentration: 1×10^6 cpm/ml), derived from rat heart pRCPLB1 cDNA clone. Hybridization was performed overnight at 45°C. After hybridization, the blot was washed at high stringency.

2.6. Immunofluorescence microscopy

Rat heart and the soleus muscle of adult rabbits, rats, mice and guinea pigs were used. Preparations and immunofluorescence labeling of longitudinal cryosections of muscle fibers (8 μm in thickness) with monoclonal antibody to PLB were performed under the general conditions described by Sacchetto et al. [27] for immunolabeling of rabbit muscle fibers with other specific antibodies. Sections previously treated with antibody to PLB were incubated with the appropriate secondary antibodies conjugated with fluorescein, and examined in a Leitz DMR fluorescence microscope. Images were collected by a B/W chilled CCD camera (Hamamatsu, Japan), digitized (High Fish Beta, version 2.0) and analyzed using Image Processing software (Casti Imaging, version 3.4, Venice, Italy). The images were printed using a digital Mitsubishi (CP-D1E) color printer.

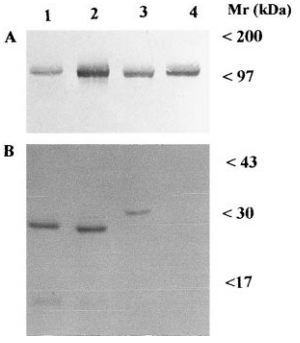

Fig. 1. Western blot analysis of whole muscle homogenates with antibodies to SERCA2a and PLB. Frozen tissue samples were thoroughly homogenized in 10 vols. of Laemmli sample buffer (total volume: 200 μl) containing 10 mM HEPES, pH 7.4, 20 mM KCl, 100 μM PMSF and 1 μg/ml of leupeptin, and were centrifuged for 30 min at $16000\times g$ using a Beckman Microfuge E. Supernatants were subjected to 10–15% polyacrylamide SDS-PAGE. Lanes: 1, human rectus abdominis; 2, rabbit soleus; 3, mouse soleus; 4, rat soleus. (A) Immunoblot (50 μg) with antibody to SERCA2a. (B) Immunoblot (200 μg) with antibody to PLB. Molecular mass standards are indicated.

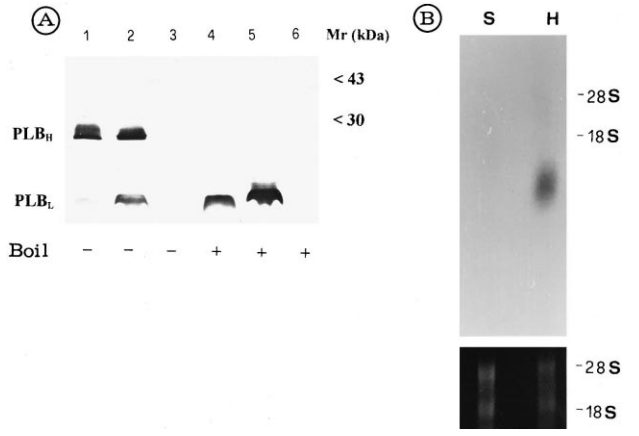

Fig. 2. Immunoblotting of muscle microsomes with antibody to PLB and Northern blot analysis of total RNA isolated from rat soleus and heart ventricular muscle. (A) Samples (100 μg) of microsomal fractions isolated by differential centrifugation [51] were analyzed by 10–15% SDS-PAGE, without boiling or after boiling for 5 min. Blots were immunostained with monoclonal antibody to PLB. Lanes: 1, 4, rabbit soleus; 2, 5, rat heart; 3, 6, rat soleus. Molecular mass standards are indicated. (B) Denatured total RNA (10 μg) isolated from rat heart (H) and soleus muscle (S), was blotted onto Hybond$^+$ membranes, and probed with a ^{32}P-labeled PLB cDNA fragment derived from rat heart pRCPLB1 cDNA clone. (Upper panel) Autoradiography after 6 days exposure at −80°C (Kodak X-OMAT film); (lower panel) ethidium bromide fluorescence of 28S and 18S ribosomal RNAs, indicating the amount of total RNA loaded onto the gel.

3. Results

3.1. Diversity in phospholamban expression levels shaping differences in slow-twitch muscle sarcoplasmic reticulum phenotype between different mammalian species

PLB is a SR integral membrane protein, present as a pentamer of identical 52 amino acid subunits and an apparent molecular mass of 5–6 kDa on SDS-PAGE. Since PLB normally coexpresses and colocalizes with the Ca^{2+}-ATPase slow-twitch isoform (SERCA2a) [10] in the SR of skeletal muscle fibers of the corresponding type, we used the SERCA2a protein tissue level as the reference basis. Concentrations of slow-twitch Ca^{2+}-ATPase, like those in PLB, were evaluated by specific Western blotting after SDS-PAGE, using whole tissue homogenates. We chose the soleus as a representative slow-twitch muscle for making comparisons between the rabbit,

rat and mouse. The immunoblot data in Fig. 1 show that the level of expression of PLB in such muscle matches closely with that of SERCA2a protein in the case of the rabbit. On the same reference basis, PLB seems to be overexpressed in human rectus abdominis m. (Fig. 1), which is very interesting, given the mixed fiber composition of this and the large majority of skeletal muscles in the human species [28]. The depleted amount of PLB relative to SERCA2a in mouse soleus (Fig. 1) is consistent with earlier evidence of a markedly reduced level of PLB-specific mRNA in such muscle, compared to the heart [13]. The most interesting finding is that, on immunoblot evidence, rat soleus lacks PLB, and thus seems to be even more deviating from rabbit soleus, despite having been characterized as 92% composed of slow-twitch fibers on the basis of myosin isoform composition [29]. The immunoblot evidence presented in Fig. 2A, in which rat cardiac and soleus muscle microsomes are directly compared, shows that the PLB defect is truly specific to skeletal muscle fibers. More direct evidence in support of a selective block of PLB gene expression in rat slow-twitch muscle comes from comparative analysis of PLB mRNA between this type of muscle and heart muscle (Fig. 2B). Northern blot analysis of PLB mRNA demonstrates that the PLB cDNA probe hybridized with a single mRNA species in rat heart muscle, while the evi-

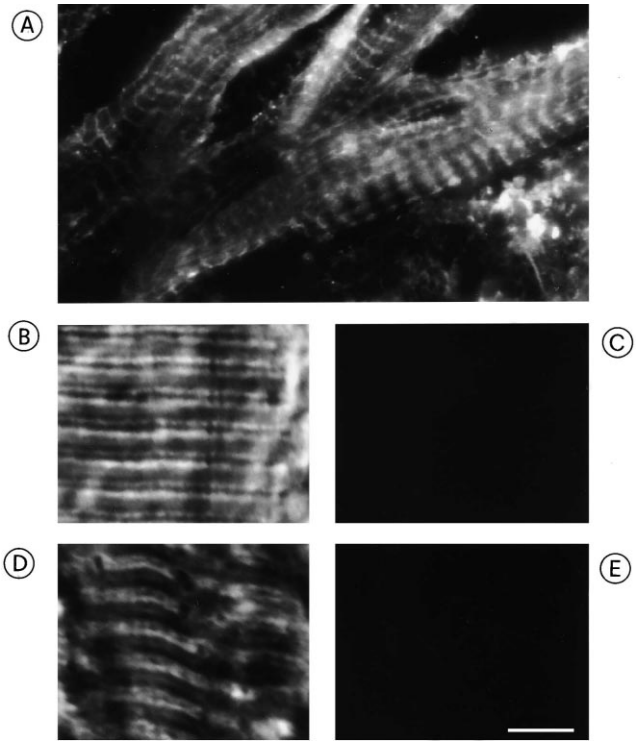

Fig. 3. Immunofluorescent localization of PLB in rat cardiac myocytes compared to rat soleus muscle fibers. Longitudinal cryosections of unfixed rat heart (A), unfixed (B,C), and paraformaldehyde-fixed (D,E) rat soleus fibers were incubated with monoclonal antibody to PLB. Antibody binding was visualized using anti-mouse IgG conjugated with fluorescein. (B,D) Phase contrast. (A,C,E) Fluorescent images. Bar, 10 μm.

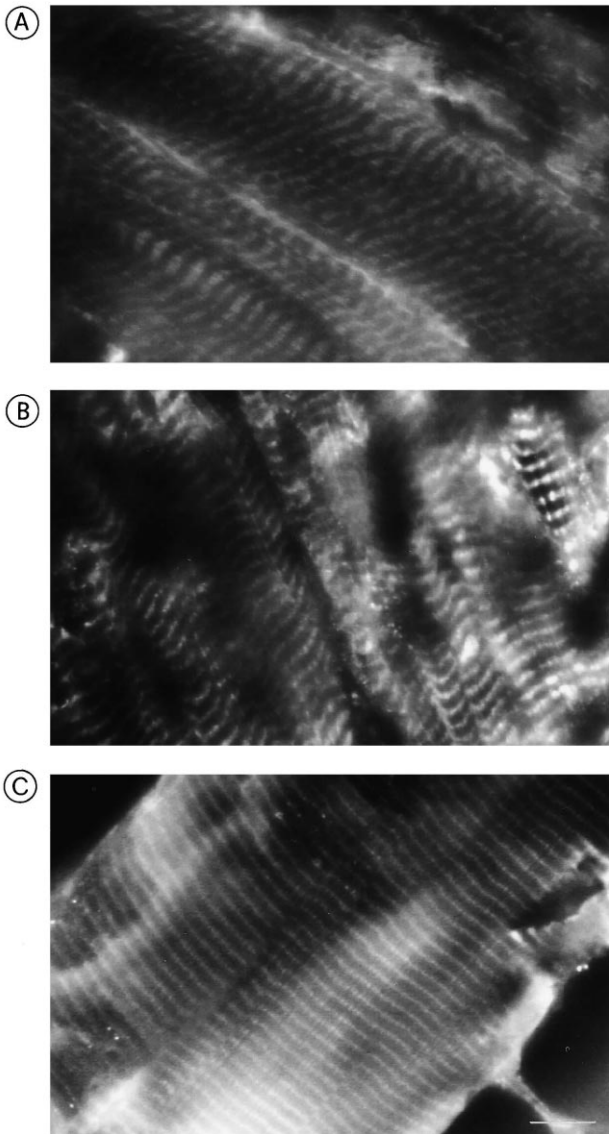

Fig. 4. Immunofluorescent localization of PLB in soleus fibers of the mouse, rabbit and guinea pig. Longitudinal cryosections of muscle fibers from of the soleus of the mouse (A), rabbit (B), and guinea pig (C) were stained with monoclonal antibody to PLB. Bar, 10 μm.

dence with rat soleus is found to be totally negative (Fig. 2B).

As an additional experimental approach in our survey, we used immunofluorescent techniques. Immunofluorescent labeling with monoclonal antibody to PLB of longitudinal cryostat sections of rat cardiac myocytes (Fig. 3A) gave a characteristic pattern consisting of transverse striations. Using this technique, the total absence of PLB from rat soleus fibers appears to be confirmed (Fig. 3B–E). Vice versa, PLB could be easily immunodetected not only in slow-twitch fibers from rabbit soleus, but also in the corresponding type of fibers of the guinea pig, as well as, significantly, of the mouse (Fig. 4). As previously described for slow-twitch fibers of several skeletal muscles of the dog [30], the immunofluorescent cross-striations seen in longitudinal sections of such fibers appear to consist of short rods extending over the length of the I-band along the major axis of the fibers (Fig. 4).

3.2. *Differential PLB expression, and matched differences in the pattern of protein phosphorylation by CaM K II and PKA between slow-twitch muscle SR of the rat and the rabbit*

In further experiments, comparative analysis of slow-twitch muscle SR between the rat and the rabbit was refined, using purified SR fractions following isopycnic sucrose density centrifugation. All membrane fractions were characterized for density of diagnostic markers of junctional (RyR1, triadin) and of free (SERCA2a, GP53) SR membrane domains, using Western blot techniques and specific antibodies. A pairwise comparison between immunoblot data for slow-twitch muscle of the rat and rabbit indicated a close correspondence in membrane protein composition between fractions having the same buoyant density, and the absence of PLB from rat SR as the only difference. Fraction R2, enriched in Ca^{2+} pump membrane, was tested for endogenous CaM K II phosphorylating activity, by incubating in the presence of [γ-^{32}P]ATP and of Ca^{2+}-CaM. Identification of PLB relied on its phosphorylation by this protein kinase, and when using a Ca^{2+}-free assay medium, on phosphorylation by added PKA catalytic subunit, in addition to being assessed by dissociation of PLB pentamers into monomers after boiling (Fig. 5A). Phosphorylation of rabbit SR PLB, when catalyzed by membrane-bound CaM K II, is shown to be characteristically coupled to incorporation of radioactivity into a band of about 60 kDa (Fig. 5B), corresponding to the autophosphorylated protein kinase, on account of studies

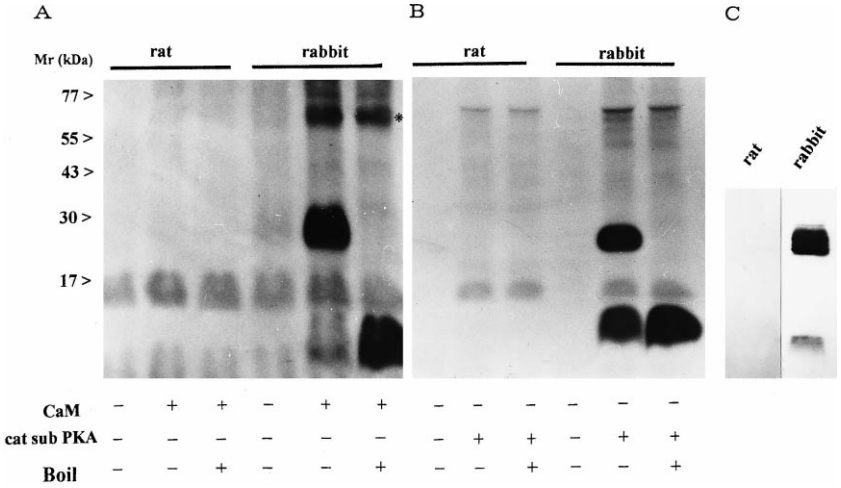

Fig. 5. Comparison between purified SR from slow-twitch muscle of the rabbit and the rat with regard to the pattern of protein phosphorylation and specific phosphorylation of PLB by CaM K II and PKA. (A,B) Phosphorylation of a sucrose density-purified SR R2 fraction was performed as described for CaM K II (A) and PKA (B) assays in Section 2. Incubation times were 3 min (A) and 5 min (B), respectively. SR protein was resolved by 10–15% SDS-PAGE, without and with previous boiling. The protein loading was 100 μg. Gels were dried and ^{32}P-labeled proteins were detected by autoradiography. Asterisk at about 60 kDa indicates autophosphorylated CaM K II. Pentameric PLB is shown to undergo dissociation into monomers after boiling. (C) The blot transfer (100 μg of protein for rat, 20 μg of protein for rabbit), without previous boiling, was incubated with monoclonal antibody to PLB.

with the isolated SR from rabbit fast-twitch muscle [31].

In contrast, phosphorylation of SR vesicles from rat slow-twitch muscle, regardless of experimental assay conditions, detected only a single radioactive band of approx. 15 kDa after SDS-PAGE (Fig. 5), perhaps corresponding to early described phosphoprotein phospholemman, on account of electrophoretic mobility [32], and indistinguishable from a rabbit SR minor component. Phosphorylation of such component, interestingly, seems not to be enhanced by Ca^{2+}-CaM or by exogenous PKA in the absence of Ca^{2+}.

3.3. *Endogenous CaM K II-mediated versus PKA-mediated phosphorylation of PLB in rabbit slow-twitch muscle, in comparison to the heart*

The evidence showing that rabbit slow-twitch muscle PLB is phosphorylated in vitro, to a similar extent, by membrane-bound CaM K II and by a large excess of added PKA catalytic subunit may provide misleading information, regarding the effectiveness of PLB phosphorylation by PKA endogenously present in the isolated SR. In agreement with earlier findings [33], omitting the addition of exogenous PKA resulted in a relatively low level of ^{32}P incorporation into PLB, in the presence of activatory concentrations of cAMP (not shown). Site-specific phosphorylation of PLB at Ser-16 under these conditions is contrasted in Fig. 6A with PLB phosphorylation at Thr-17, under the action of endogenous CaM K II. The two protein kinases, when analyzed in the several membrane fractions from the sucrose density gradient, displayed a distinct distribution. Endogenous PKA, when identified by specific Western blotting with antibody to human PKA catalytic subunit, is found enriched in fraction R1 of very low buoyant density (Fig. 6B), and having a depleted amount of SR-specific markers (not shown). In contrast, immunocharacterization of CaM K II for β, γ and δ subunits, using antibodies against the γ class (cross-reactive with β) and δ class of CaM K II subunits, is indicating a diffuse distribution in membrane fractions of truly SR origin (Fig. 6B), in agreement with recent evidence [34]. The correspond-

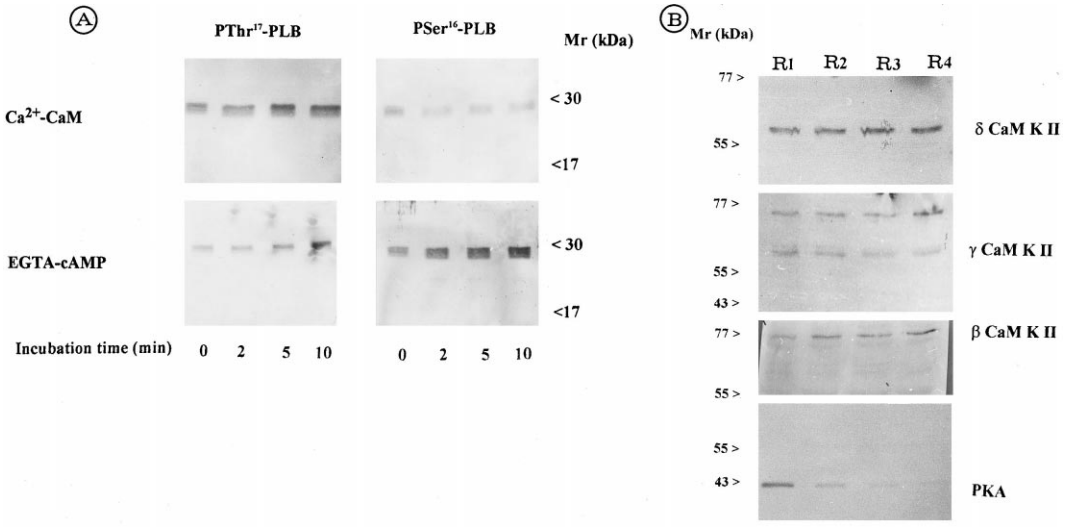

Fig. 6. (A) Western blot demonstration of PLB phosphorylation state and discrimination between Ser-16- and Thr-17-phosphorylated PLB, during incubation of SR vesicles. Slow-twitch muscle SR was phosphorylated for 2–10 min at 0–4°C with non-radioactive ATP, in the presence of either Ca^{2+}-CaM and under assay conditions otherwise identical to those described for CaM K II assay (upper panels), or EGTA and cAMP (lower panels), as described in Section 2. Samples (30 μg) were analyzed by 10–15% SDS-PAGE. Blots were incubated with antibody raised against PLB peptides phosphorylated at Ser-16 ($PSer^{16}$-PLB) or at Thr-17 ($PThr^{17}$-PLB). (B) Comparison between endogenous PKA and CaM K II for distribution within the SR. Samples (30 μg) were analyzed by 5–10% SDS-PAGE. Molecular mass standards are indicated. The anti-CaM K II γ isoform polyclonal antibody partially cross-reacts with the β isoform recognized by specific anti-CaM K II β isoform monoclonal antibody.

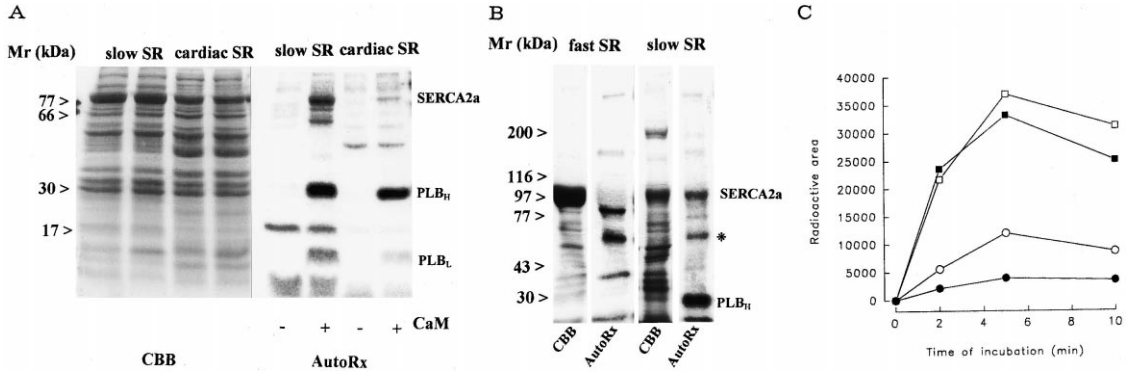

Fig. 7. Comparison between rabbit slow-twitch, fast-twitch and cardiac muscles for SR membrane protein phosphorylation by endogenous Ca^{2+}-CaM dependent phosphorylating system. Phosphorylation reactions were performed under standard assay conditions for CaM K II, in the absence and in the presence of 1 μM CaM, for 3 min at 0–4°C, and using a sucrose density-purified SR fraction (R2) for all muscles. Samples (50 μg) were analyzed using either a 10–15% (A), or a 5–10% SDS-PAGE (B). ^{32}P-Labeled proteins were detected by exposing Coomassie blue-stained (CBB) dried gels onto β-particle-sensitive screens for 120 min. Screens were scanned using a Bio-Rad GS-250 Molecular Imager, and images were collected and analyzed using Phosphor Analyst/PC Image analysis software (Bio-Rad). Original autoradiograms (AutoRx) were printed using a digital Mitsubishi (CP-D1E) color printer. Asterisk indicates autophosphorylated 60 kDa CaM K II. (C) Time course of ^{32}P incorporation into SERCA2a (circles) and PLB (squares). SR vesicles from rabbit heart (filled symbols) and slow-twitch muscle (open symbols) were phosphorylated by endogenous CaM K II in the presence of 1 μM CaM for the indicated time. After SDS-PAGE, ^{32}P-labeled proteins were analyzed and quantified using β-particle-sensitive screens and appropriate software. Densitometric data are expressed as radioactive area (counts × mm).

ence in apparent molecular mass between the γ and δ subunit and the ^{32}P-labeled protein (60 kDa) appears to be the closest, thus allowing for a direct comparison with phosphorylation data (Fig. 5). Together, these findings suggest that phosphorylation of PLB by endogenous PKA, unlike that mediated by CaM K II, might be due to the exposure of PLB phosphorylation sites in SR vesicles to the action of TT-bound, rather than SR-bound PKA.

Ten discrete phosphorylation-induced shifts in electrophoretic mobility in SDS-PAGE of PLB pentamer [10] have been reported after graded phosphorylation by PKA (at Ser-16) and CaM K II (at Thr-17), corresponding to the existence of two phosphorylation sites per monomer [9]. Western blot analysis of PLB phosphorylation state, on the basis of these criteria, together with the immunoblot evidence using PLB phosphorylation site-specific antibodies, was in support of the presence of PLB pentamer in unphosphorylated as well as in various phosphorylation states, at mainly Thr-17, in the case of stored-frozen SR vesicles from rabbit slow-twitch muscle. That was in contrast with the negative evidence for identically stored, cardiac SR vesicles (not shown).

3.4. *Phosphorylation of SERCA2a, coupled to phosphorylation of PLB in rabbit slow-twitch muscle SR*

It is still controversial [10] whether native SERCA2a (i.e. in isolated longitudinal SR from mammalian cardiac and slow-twitch muscle) may be phosphorylated by membrane-bound CaM K II, although this seems to be strongly supported by recent evidence using rabbit cardiac SR [18].

We have reinvestigated this problem in experiments where the dual ability of endogenous CaM K II to phosphorylate PLB and SERCA2a was tested in isolated SR vesicles from rabbit slow-twitch muscle, by direct comparison with cardiac SR vesicles (Fig. 7A), and using SR vesicles from rabbit fast-twitch muscle as a negative control (Fig. 7B). Normalized values for the incorporation of ^{32}P into pentameric PLB protein band indicated non-significant differences between slow-skeletal and cardiac SR (Fig. 7A,C).

There was straightforward evidence for the concomitant incorporation of radioactivity into a protein band having mobility corresponding to that of SR Ca^{2+}-ATPase protein, in the case of slow-twitch

muscle SR (Fig. 7A,B), which is consistent with the SERCA2a isoform specificity of CaM K II [15]. In this connection, it should be noted that when SR vesicles, either from slow-twitch or fast-twitch muscle, were incubated in the presence of Ca^{2+}-CaM, incorporation of radioactivity into the 60 kDa autophosphorylated CaM K II protein kinase band was found to be similarly high (Fig. 7B). In native fast-twitch muscle SR, CaM K II is shown to phosphorylate a protein electrophoretic component, moving ahead of the Ca^{2+} pump protein, and which may probably correspond to tightly membrane-bound phosphofructokinase [35,24]. As a point of interest, in the experiment illustrated in Fig. 7C, endogenous CaM K II associated with cardiac SR appears to be comparatively less able to phosphorylate SERCA2a than its slow-twitch muscle counterpart. This difference, if confirmed, will have to be explained.

PLB expression can be induced in hind leg fast-twitch muscles of the dog [36] and the rabbit [37] by chronic low-frequency stimulation. In contrast, denervation of the soleus of 8-day-old rabbits was found to cause only a 50% decrease in SERCA2 mRNA 27 days postoperatively and no further changes thereafter [38], suggesting that continuous innervation was not required for muscle postnatal differentiation. We found the protein expression level both of SERCA2a and of PLB to be unchanged in the isolated SR from 2 week denervated soleus of adult animals. We also failed to observe any denervation-induced abnormality in CaM K II-mediated phosphorylation of either protein substrate (not shown).

4. Discussion

The PLB gene is highly conserved in higher vertebrates and codes for a single 52 amino acid protein product in all species investigated and in the several tissues. The coexpression of PLB with the SERCA2 gene in striated cardiac and slow-twitch skeletal muscles and smooth muscle of mammals is considered to be a dogma [7,8,10]. Nevertheless, there is increased awareness now, on account of comparative evidence from different mammalian species, regarding vascular smooth muscle in particular that the PLB expression levels do not match closely with those of SERCA2a and, actually, can vary considerably from species to species [39].

The present study focuses on the heterogeneous expression levels of PLB in hind limb slow-twitch muscles of small rodent species, compared to the rabbit, and highlights the observation that PLB mRNA is not expressed in rat soleus, which is the normal condition found in fast-twitch muscles of this and other mammalian species, including the rabbit [10]. The significance of this finding is strengthened by the evidence, both at PLB protein and specific mRNA level, that, in the rat, PLB is not at all expressed in slow-twitch muscle, while being expressed in the heart, thus extending earlier partial evidence [40] (see Fig. 2). Our present evidence strongly argues for the existence of tissue-specific transcriptional regulatory elements of PLB gene [41]. The lack of PLB in rat soleus muscle seems to link this species to the mouse, even though the lack of PLB in mouse homologous muscle is partial rather being complete. A differential level of expression of PLB in mouse slow-twitch and heart muscle had been reported [13]. It seems well established from studies in the rabbit and dog [39] that the PLB/SERCA2a ratio is lower for slow-twitch than for heart muscle, which, therefore, seems to be a general rule. The rat and mouse results, taken together with these previous results and our present findings with rabbit and human skeletal muscle, imply that the level of expression of PLB in slow-twitch skeletal muscle fibers varies according to the species body size.

It should be pointed out that the switching on of PLB gene induced by chronic low-frequency stimulation of fast-twitch muscle, i.e with an impulse pattern similar to that normally delivered to a slow-twitch muscle by its own motor nerve, is an effect so far described only for the rabbit [37] and the dog [36]. In these animal species, the induced transcription of PLB gene was shown to lag behind the rise in the rate of transcription of SERCA2 gene [42]. Chronic low-frequency stimulation of rat fast-twitch muscle induces much smaller changes in muscle twitch properties [43] and in Ca^{2+} uptake by the isolated SR [44] than those observed in the rabbit (see for a review [45]). Such differential heterogeneity seems to be consistent with the observed species-specific differences in basal PLB levels in slow-twitch muscle.

Some of our experimental results touch on the problem of whether cAMP-activated phosphorylation of PLB may be the only mechanism for acceleration of the rate of muscle relaxation, following β-adrenergic stimulation in vivo [6], or, rather, diverse mechanisms might be involved in skeletal compared to heart muscle, as well as depending on the animal species. Study of the effects of PKA inhibitors and activators on the intracellular Ca^{2+} transient in isolated soleus fibers of normal and PLB-deficient mice [12] was not particularly revealing of a critical modulatory role of PLB on SR Ca^{2+} transport. Two key proteins in the E-C coupling process, that were shown to be phosphorylated by PKA in isolated skeletal muscle membranes, are the DHPR in junctional TT [46,47] and RyR1 in SR TC [48]. The present results with rabbit slow-twitch muscle, taken together with our own previous findings [46], are in support of the colocalization of PKA and the DHP receptor in junctional transverse tubules, as a common property for fast and slow muscle in this species. Since PLB seems to keep memory of the action of CaM K II during the SR isolation procedure, as shown here, that also argues against PKA having a prominent role in rabbit slow-twitch muscle. A recent study found that CaM K II was selectively targeted to rat skeletal muscle SR through binding to anchoring protein αKAP [34].

The phosphorylation of SERCA2a, in addition to PLB, by endogenous CaM K II, as shown here, is in excellent agreement with the reports by Hawkins et al. [15,49], and together with this previous evidence argues that CaM K II can modulate Ca^{2+} transport in slow-twitch muscle SR by a dual mechanism. The SERCA2a isoform substrate specificity of SR-bound CaM K II is an important element on which the hypothesis of the regulatory role of SERCA2a phosphorylation state on the speed of cardiac muscle relaxation is based [15,18]. The validity of this hypothesis was questioned since purified, reconstituted cardiac SR was found not to be phosphorylated by rat brain CaM K II [17]. However, it should be recognized that in mammals, a number of alternative spliced isoforms of CaM K II are generated from the closely related α, β, γ and δ genes, and that members of the α and β classes are expressed only in neuronal tissues [34]. In rabbit slow-twitch muscle SR, our immunoblot data revealed the presence of β, γ and δ subunits. Differentiation-dependent expression of the δ class of skeletal muscle CaM K II [50] and a novel β variant of the protein kinase [37] have been described recently. One therefore wonders whether functionally specialized, although not necessarily identical, PLB- and SERCA2a-dedicated protein isoforms of CaM K II may be selectively expressed in the SR of slow-twitch and heart muscle of mammals of larger body size.

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