Coordinate expression of Ca^{2+}-ATPase slow-twitch isoform and of β calmodulin-dependent protein kinase in phospholamban-deficient sarcoplasmic reticulum of rabbit masseter muscle

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Abstract Modulation of sarcoplasmic reticulum (SR) Ca^{2+} transport by endogenous calmodulin-dependent protein kinase II (CaM K II) involves covalent changes of regulatory protein phospholamban (PLB), as a common, but not the only mechanism, in limb slow-twitch muscles of certain mammalian species, such as the rabbit. Here, using immunofluorescent techniques in situ, and biochemical and immunological methods on the isolated SR, we have demonstrated that rabbit masseter, a muscle with a distinct embryological origin, lacks PLB. Accommodating embryological heterogeneity in the paradigm of neural-dependent expression of specific isogenes in skeletal muscle fibers, our results provide novel evidence for the differential expression in the SR of 72 kDa β components of CaM K II, together with the expression of a slow-twitch sarcoendoplasmic reticulum Ca^{2+}-ATPase isoform, both in limb muscle and in the masseter.

Key words: Phospholamban; Calmodulin protein kinase; Sarcoplasmic reticulum; Rabbit masseter; Skeletal muscle

1. Introduction

In mammalian skeletal muscles, sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) isogenes are differentially expressed in fast-twitch (SERCA1) and slow-twitch (SERCA2) fibers [1], depending on the rate and the pattern of firing of the innervating motoneurons [2,3]. The evidence for co-expression of SERCA2 and of phospholamban (PLB), acting as a Ca^{2+}-ATPase inhibitor in the dephosphorylated state in native cardiac and slow-twitch muscle sarcoplasmic reticulum (SR), first argued for a convergence in regulatory features of the SR Ca^{2+} transport system of these tissues [4,5]. The experimental evidence on which the convergence hypothesis rests [6] appears to be strengthened by observations in rabbit cardiac and slow-twitch muscle SR that SERCA2, in addition to PLB, is a specific substrate of endogenous calmodulin-dependent protein kinase II (CaM K II) [7–9]. This protein kinase is endowed with the significant property of retaining activity independently of Ca^{2+} and CaM, once autophosphorylated [10]. Correlative concepts, such as these, however, do not provide any clue to the suggested cause–effect relationship between specific motor innervation and the protein level of expression of PLB in skeletal muscle SR.

On account of studies of limb muscles of several mammalian species [11,12], it seems well established that the differential pattern of protein expression of SERCA isoforms, in relation to fiber types, matches closely the distribution between fibers of specific myosin heavy chain (MHC) isoforms. Whereas a high level of expression of PLB gene seems to be a constant feature of mammalian heart muscle [6,8,13], there is, on the other hand, emerging evidence of a wide range of differences in PLB expression in limb slow-twitch muscles of mammals, depending on the animal species [8]. That, in itself, seems to confute the validity of some of the assumptions that were made previously, regarding the neural dependency of PLB gene expression in skeletal muscle fibers.

In the present study, we purposely chose to investigate rabbit masseter, in comparison to limb muscles, because of its distinct embryological origin, and the well characterized fiber type composition of this muscle, in relation to MHC isoforms, including the peculiar expression of α-cardiac-like MHC (MHCI-α) [14,15], similarly to the homologous human muscle [16]. In an earlier study from our own laboratory using the isolated SR from rabbit masseter, the SR Ca^{2+}-ATPase protein had been characterized for catalytic as well as antigenic properties, reflecting its isoform composition [17].

In the present study, we report that the slow-twitch fiber population in rabbit masseter, here identified from the selective expression of SERCA2, and including also fibers expressing MHCI-α, lacks PLB. Expression of SERCA2, although not correlating with the expression of PLB in this particular muscle, significantly, appears to be associated with the differential expression of β-components of CaM protein kinase and of the slow-cardiac calsequestrin (CS) isoform in the isolated SR, similarly to limb slow-twitch muscle. These common properties seem to argue for the existence of specific molecular variants within the CaM K II β-γ-δ multigene family in relation to fiber type, rather than to the embryological origin and anatomical side of the muscle.

2. Materials and methods

All chemicals were analytical grade. [γ-^{32}P]ATP (3000–6000 Ci/mmol) was purchased from New England Nuclear (Du Pont De Nemours, Bad Homburg, Germany).

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Abbreviations: CaM K II, calmodulin-dependent protein kinase II; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; MHC, myosin heavy chain; MHCI-α, α-cardiac-like myosin heavy chain; PLB, phospholamban; SR, sarcoplasmic reticulum

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2.1. Preparation of skeletal muscle SR

SR vesicles were isolated from homogenates of the masseter and of predominantly fast-twitch and slow-twitch (soleus and semitendinosus) hind-leg muscles, of New Zealand male adult rabbits, and were subjected to isopycnic sucrose-density centrifugation, using the method of Saito et al. [18], with slight modifications [19], to yield four distinct fractions (see figure legends). Membrane fractions from the sucrose-density gradient were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, divided into aliquots and stored at −80°C until used. Protein concentration was determined by the Folin reaction [20], using bovine serum albumin (Boehringer, Mannheim, Germany) as a standard.

2.2. Phosphorylation

The standard assay medium (total volume 100 μl) for phosphorylation of SR membrane proteins (1 mg/ml) by endogenous Ca^{2+}-CaM K II had a composition identical to that used in previous work [8]. The reaction was started by adding 400 μM [γ-^{32}P]ATP (specific radioactivity 0.10 Ci/mmol). After 5 min incubation at 0–4°C, the reaction was quenched by adding 50 μl of Laemmli sample buffer to each sample. 5–10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the buffer system described by Laemmli [21]. The gels were stained with Coomassie blue, dried and autoradiographed (16 h exposure), using Hyperfilm (Amersham), essentially as described previously [22].

2.3. Gel electrophoresis, immunoblotting and overlay with digoxigeninated-CaM

SDS–PAGE was carried out, according to Laemmli [21], as reported in the figure legends. Apparent M_rs were calculated from a graph of relative mobilities versus log M_r of standard proteins (BDH Lab. Supplies, Poole, UK; Electran Molecular weight marker, MW range 200–43 kDa, or 77–12 kDa range). After electrophoresis, proteins were transferred onto nitrocellulose.

Blots were incubated, as described by Damiani et al. [8] using:

1. Mouse monoclonal antibodies to: (1) skeletal isoform of SR Ca^{2+} release channel/RyR1 (Affinity Bioreagents (ABR), Golden, CO, USA) (1:1000); (2) skeletal isoform of triadin (ABR) (1:1000); (3) SERCA1 (ABR) (1:1000); (4) 53 kDa glycoprotein (ABR) (1:1000) (cross-reactive with sarcalumenin); (5) PLB (ABR) (1:1000); (6) β isoform of CaM K II (Gibco BRL, Life Technologies, Paisley, UK) (1 μg/ml).
2. Rabbit polyclonal antibodies to: (1) SERCA2a (PhosphoProtein-Research (PPR), Bardsey, UK) (1:10 000); (2) FKBP-12 (ABR) (1:2000).
3. Goat polyclonal antibody to δ and γ isoforms of CaM K II (Santa

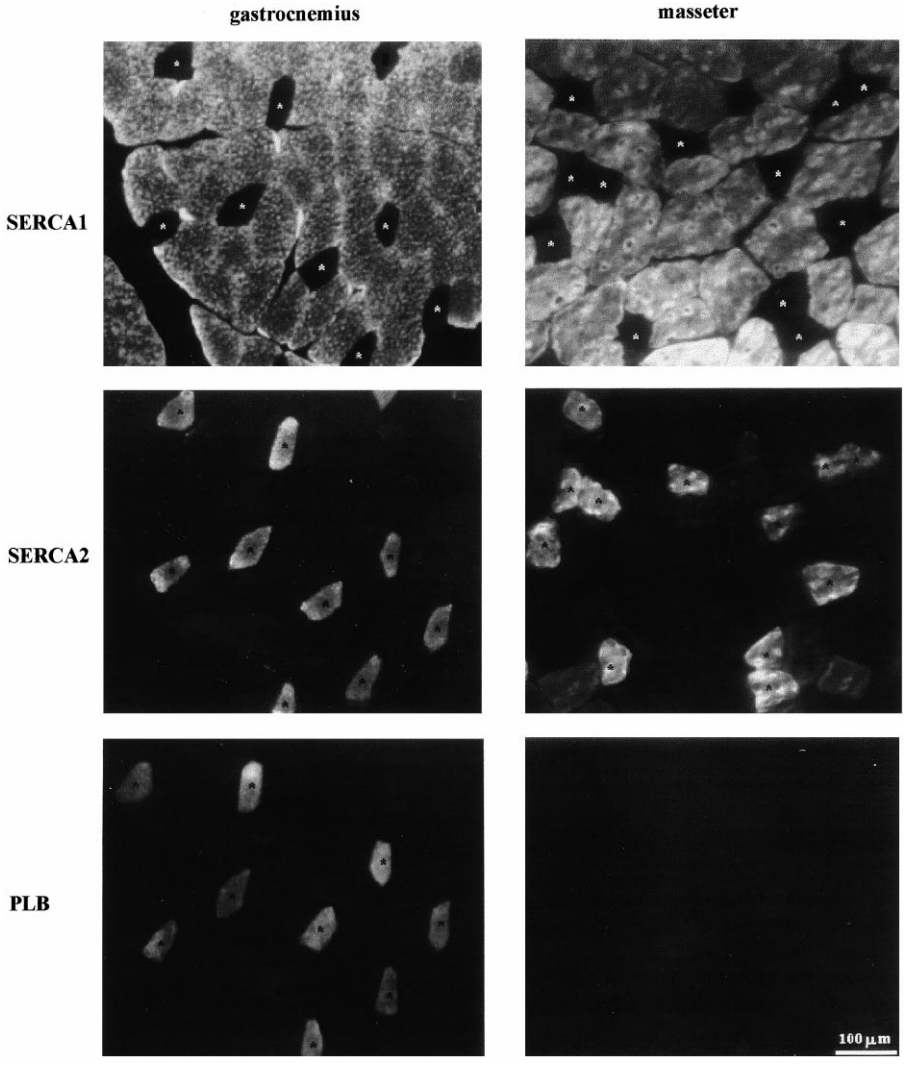

Fig. 1. Comparison between immunostaining of rabbit gastrocnemius and of masseter muscle fibers with antibody to SERCA1, SERCA2 and PLB. Serial transverse cryosections of rabbit gastrocnemius and of masseter muscle fibers were incubated with mouse monoclonal antibody to SERCA1, SERCA2 and PLB. Antibody binding was visualized using anti-mouse IgG conjugated with fluorescein. Asterisks mark slow-twitch fibers expressing SERCA2, which are immunostained also with the antibody to PLB in the case of gastrocnemius, but not of the masseter muscle.

Cruz, CA, USA) (1 μg/ml) [8,23]. Antibody binding was detected by immunoenzymatic staining [19].

Overlay with digoxigeninated CaM was carried out under experimental conditions identical to those described previously by Damiani et al. [22].

2.4. Immunofluorescence microscopy

The general conditions for immunofluorescence labeling of transverse cryosections of muscle fibers (8 μm in thickness) from rabbit masseter and the gastrocnemius muscle were identical to those described previously [8,24]. Sections, after being incubated with specific antibodies including a monoclonal antibody to MHCI-α (antibody F88112F8, see [16], kind gift of Professor S. Schiaffino, Department of Experimental Biomedical Sciences, University of Padua, Padua, Italy), were incubated with the appropriate secondary antibodies conjugated with fluorescein, and then 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.

3. Results and discussion

Immunofluorescent staining of serial cross-sections of rabbit gastrocnemius (superficial layer) with specific antibodies to SERCA1, SERCA2 and to PLB gave a characteristic picture by showing that PLB was localized to only slow-twitch fibers expressing SERCA2, as expected (Fig. 1). Application of these antibodies to serial sections of rabbit masseter, as shown in Fig. 1, similarly resulted in the identification of only two types of fibers expressing either SERCA1 or SERCA2, but not both, and which, like in gastrocnemius muscle, were found to differ in relative diameter (Fig. 1). Fiber typing for SR Ca^{2+}-ATPase isoforms of rabbit masseter appears to be consistent with immunologic and biochemical characterization of the Ca^{2+}-ATPase in the isolated SR from rabbit masseter [17], as about 60% was composed of the fast-twitch protein isoform. By comparison, the percentage content of fast MHC in rabbit masseter was estimated to be approximately 50% of the total MHC content, on the basis of SDS–PAGE analysis [25]. The relatively greater development of the SR in fast-twitch fibers may account for the difference.

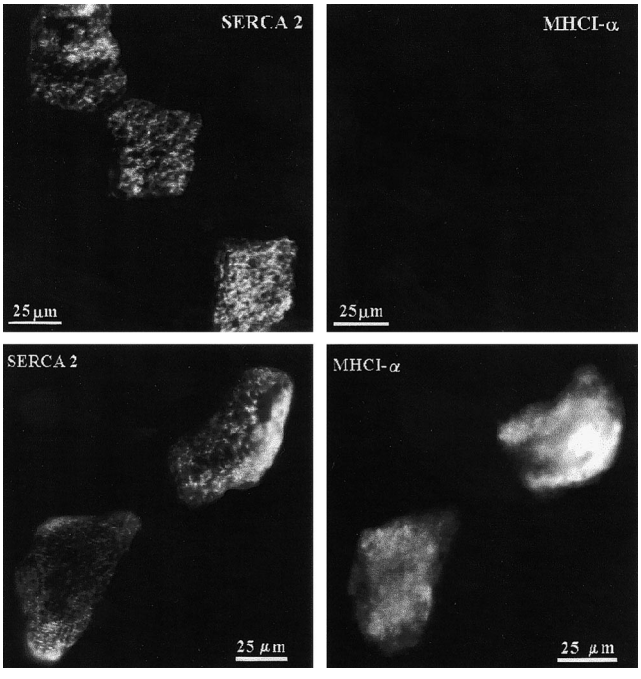

Fig. 2. Immunostaining of rabbit masseter muscle fibers with antibody to MHCI-α and to SERCA2. Serial transverse cryostat sections of rabbit masseter muscle fibers were labeled with specific monoclonal antibodies to SERCA2 and to MHCI-α [16]. Immunostaining with the anti-MHCI-α antibody, appears to be confined to SERCA2-positive fibers.

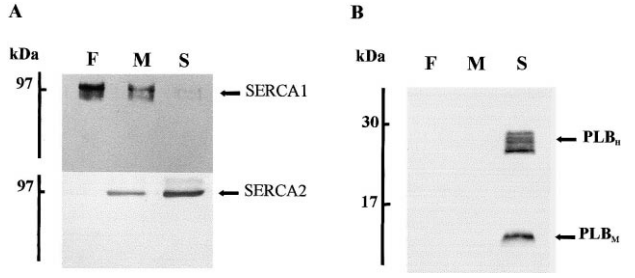

Fig. 3. Comparison between sucrose-density purified SR isolated from fast-twitch, slow-twitch and masseter muscle, with regard to immunoreactivity with antibodies to SERCA1, SERCA2a and PLB. Samples (20 μg) of sucrose-density purified R2 membrane fractions isolated from fast-twitch (F), slow-twitch (S) and from the masseter muscle (M), were analyzed by 10–15% SDS–PAGE. Blots were immunostained with monoclonal antibody to SERCA1, SERCA2 (A) or to PLB (B). Molecular mass standards are indicated on the left.

When sections of rabbit masseter serial to those used for the immunolocalization of SERCA1 and SERCA2 were tested with antibody to PLB, the puzzling observation was made that none of the fibers was stained by this antibody (Fig. 1). The abnormality, of course, lies in the lack of expression of PLB in fibers expressing SERCA2, including also a fiber subtype in which MHCI-α appears to be co-expressed (Fig. 2), which is in agreement with previous immunocytochemical studies on rabbit masseter [14,15].

The lack of detection of PLB in cryostat sections of rabbit masseter, using immunofluorescent techniques, agrees with the negative immunoblot evidence for the presence of PLB in the isolated SR, in experiments in which sucrose-density purified SR fractions from this muscle, and from representative slow-twitch and fast-twitch muscles of the hind-limbs, were characterized for the relative content in PLB, SERCA1, and SERCA2 using specific Western blotting (Fig. 3). These results (Fig. 3) highlight the deficiency in PLB of SR membranes from rabbit masseter, despite the presence of a significant proportion of SERCA2, as expected. Basic differences in protein composition between the free (Ca^{2+} pump) and junctional membrane domain of the SR appear to be preserved in rabbit masseter. That is supported by Western blot analysis of sucrose-density purified SR membrane fractions, using antibodies to specific markers of either membrane domain. These data clearly show (Fig. 4) that membrane fractions enriched in longitudinal SR derivatives are richer in the Ca^{2+} pump-associated glycoproteins sarcalumenin and GP53, and that, conversely, fractions enriched in junctional TC display the highest membrane density of RyR1 and RyR1-associated proteins, FKBP-12 and triadin. The specificity of the PLB defect in masseter SR is further underlined by the observation that the complement in CS isoforms, as identified by Stains All blue staining after SDS–PAGE, is reminiscent of that observed with typical slow-twitch muscles [19,26], in that the slow-cardiac isoform of CS, in addition to the main skeletal

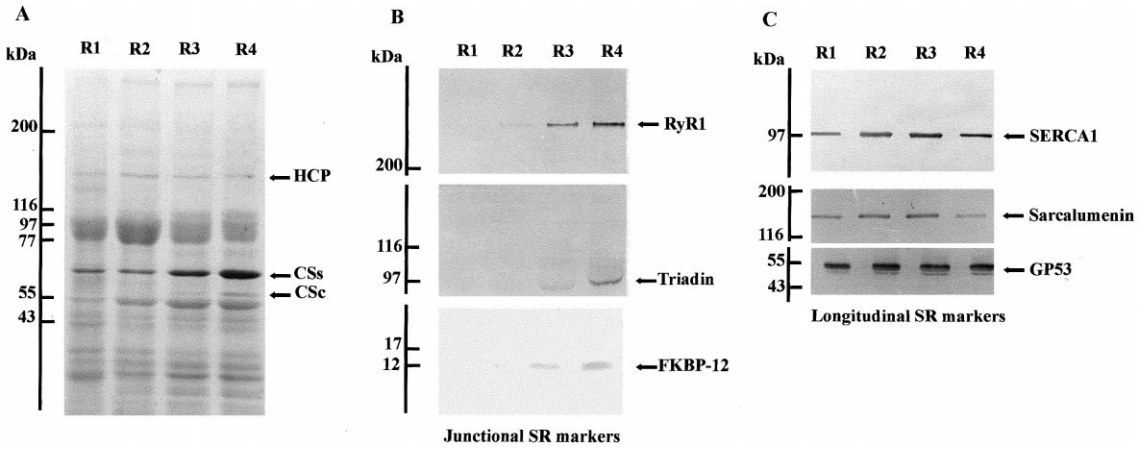

Fig. 4. Distribution of longitudinal and junctional SR protein markers between membrane subfractions isolated from rabbit masseter muscle, following SDS–PAGE and immunoblot. SR membrane fractions were isolated from rabbit masseter muscle by isopycnic sucrose-density centrifugation, as described in Section 2, and are labeled R1 to R4 from top to bottom of the gradient. Samples (30 μg) were analyzed by 5–10% SDS–PAGE, followed by either staining with Stains All (A), or immunoblotting with the indicated antibody (B). Position of individual proteins is marked by arrows. The size of molecular mass standards is indicated on the left. HCP: histidine-rich, Ca^{2+} binding protein; CSs: skeletal isoform of CS; CSc: slow/cardiac isoform of CS.

CS isoform, appears to be expressed and to be enriched in junctional TC (Fig. 4A).

Looking for the possible expression of the cardiac isoform of RyR (RyR2) in rabbit masseter, given the expression of MHCI-α in a subpopulation of slow-twitch fibers, we used Western blot techniques and specific antibody to this protein isoform. However, the evidence with junctional TC was totally negative. Although we cannot exclude the possibility that RyR3 might be expressed at a very low level in masseter SR, as reported for rabbit diaphragm SR [27], the presence of a significant amount of this specific isoform seems to have to be excluded, since the pattern of the Ca^{2+} activation/Ca^{2+} inactivation profile of [^{3}H]ryanodine binding to isolated TC from rabbit masseter was found to be closely similar to that of fast-twitch muscle TC, using identical experimental assay conditions (results not shown). These findings also confirm that SR assemblies of proteins are basically the same in the masseter and in limb muscles.

We next investigated the subunit composition pattern of CaM K II tightly associated to the SR. Immunoblotting with antibodies to the δ and γ subunits [8,23], having a similar molecular size (about 60 kDa) [23], revealed distinct CaM K II phenotypes for the isolated SR from fast-twitch muscle, on the one hand, and slow-twitch muscle and the masseter, on the other hand. Immunoblot evidence using antibodies to the γ subunit is particularly revealing of differences according to the type of muscle (Fig. 5A). Following the distribution of β CaM K II between SR membrane fractions from limb fast-twitch and slow-twitch muscle (Fig. 5B), using a monoclonal antibody to the 72 kDa β subunit [23], we have found previously unreported striking differences. The evidence suggested that the β subunit identified by antibody was specific to slow-twitch muscle SR. This interpretation agrees with the lower, but detectable level of 72 kDa immunostained protein in muscle SR (Fig. 5C).

In complementary experiments, we used overlay techniques and digoxigeninated CaM as the ligand, as an additional criterion for identifying CaM K II subunits [22,28], and, moreover, we assessed the ability of the protein kinase to undergo self-phosphorylation, by incubating SR vesicles with [γ-^{32}P]-ATP, at optimizing concentrations of Ca^{2+} and of CaM (Fig. 6). The results in Fig. 6 demonstrate a binding interaction of Ca^{2+}-CaM with a 60 kDa protein band, which had been already tentatively identified as the main isoform of CaM K II

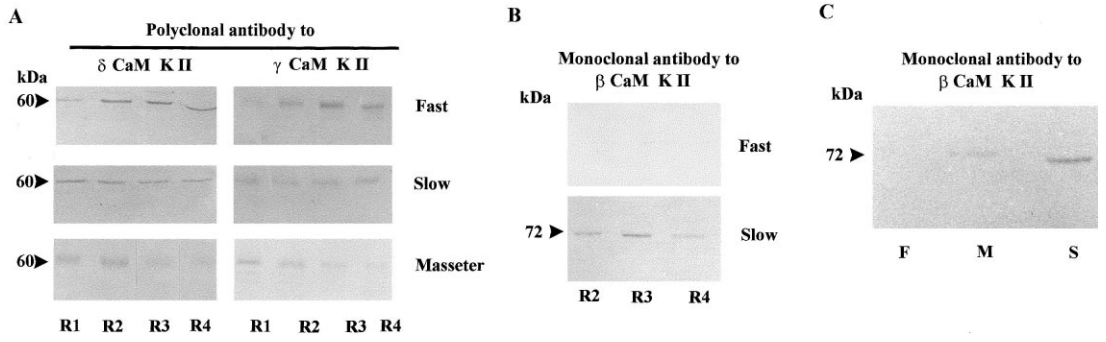

Fig. 5. Comparison between SR membrane fractions isolated from hind-leg fast-twitch and slow-twitch muscles and from the masseter, with regard to the subunit composition of CaM K II. Samples (40 μg) of SR membranes isolated from fast-twitch, slow-twitch and from the masseter muscle were analyzed by 5–10% SDS–PAGE. Immunoblots were labeled with polyclonal antibodies to the δ isoform or to the γ isoform of CaM K II (A), or with a monoclonal antibody to the β isoform of CaM K II (B, C). In panel C, R2 membranes isolated from fast-twitch (F), slow-twitch (S) and masseter muscle (M) are compared. The apparent molecular masses of the immunostained proteins, as calculated from molecular mass standards (see Section 2), are indicated on the left.

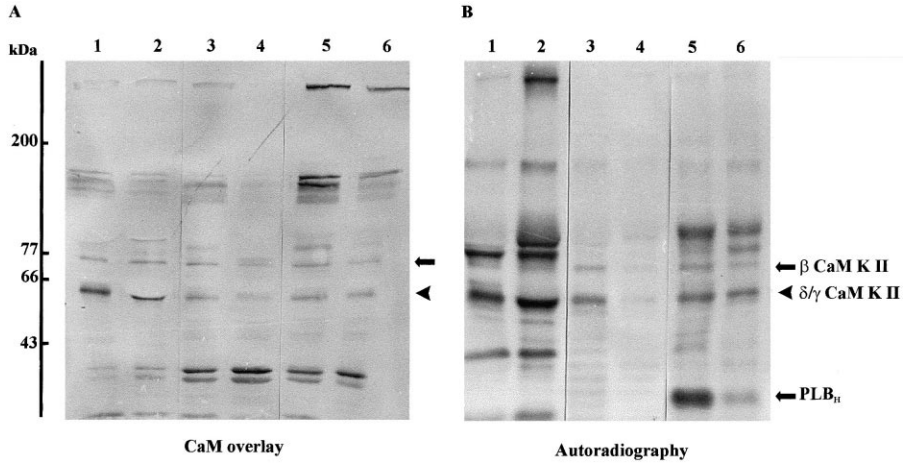

Fig. 6. Comparison between SR fractions from hind-leg fast-twitch and slow-twitch muscles and from the masseter muscle, with regard to the pattern of protein phosphorylation by endogenous CaM K II vis-à-vis ligand overlay with digoxigeninated CaM. Samples (100 μg) of sucrose-density-purified R2 (lanes 1, 3, 5) and R4 (lanes 2, 4, 6) fractions were analyzed by 5–10% SDS–PAGE. Lanes 1 and 2: fast-twitch; lanes 3 and 4: slow-twitch; lanes 5 and 6: masseter. A: Blots were incubated with digoxigeninated-CaM, in the presence of 1 mM $CaCl_2$. B: Phosphorylation was carried out, as described in Section 2, in the presence of 1 μM CaM. Gels were dried and ^{32}P-labelled proteins were detected by autoradiography. Arrow and arrowhead indicate the 72 kDa β subunit and the 60 kDa protein band, comprised of the δ and γ of CaM K II subunits, respectively.

associated to the SR of rabbit fast-twitch muscle [22,29], and that, accordingly, is found to be most abundant in longitudinal SR and the junctional TC fraction from such muscle. Also in correlation with immunoblot data for CaM K II γ subunit, these results indicate that the same subunit is present in decreased amounts in masseter muscle SR, as well as in the isolated SR from slow-twitch muscle. Self-phosphorylation of 60 kDa protein CaM K II in the presence of Ca^{2+}-CaM is supported by autoradiograms in Fig. 6B, and in the same order of potency, as for the other data, concerning longitudinal SR. In fast-twitch muscle junctional TC, self-phosphorylation of CaM K II appears to be associated with intense phosphorylation of junctional SR-specific proteins, among which triadin and histidine-rich Ca^{2+} binding protein, as reported previously [22,28]. Conversely, incorporation of radioactivity from [γ-^{32}P]ATP into protein at 72 kDa, likely corresponding to the CaM K II β subunit, is found to be the weakest in fast-twitch muscle SR, compared to the SR from slow-twitch muscle and from masseter muscle.

These results, taken together with previous results on CaM K II in rabbit fast-twitch muscle SR [22,28,29], and the recent finding that a β_M CaM K II skeletal muscle-specific isoform associates to rat hind-limb skeletal muscle SR, via αKAP anchor protein [23], are relevant to the still open question of whether specific molecular variants of the CaM K II multigene (γ, δ, β) family might be associated with the SR, in relation to fiber types. This possibility is strongly supported by the evidence reported here. In particular, the observed similarities in the ratio between CaM K II γ and β subunits, between the masseter and slow-twitch muscle of the rabbit, seem to argue that such a pattern of subunits might be dictated by the same neural influences affecting the expression of the SERCA2 gene, as well as the cardiac CS gene.

As implicated by the regulatory characteristics of CaM K II [10], and also supported by recent experimental findings [30], the mode of action of this protein kinase, would allow for a prolongation of the effect of brief Ca^{2+} transients. Given the SERCA2 specificity of SR-bound CaM K II [7–9], that is particularly interesting. The missing evidence in the present study is the demonstration of the ability of endogenous CaM K II to phosphorylate SERCA2 in masseter SR, as in the SR of slow-twitch muscle. However, that might be dependent on the experimental method, also because of the promiscuous presence of SERCA2 and SERCA1 in masseter SR, reducing the level of detection of ^{32}P-phosphoprotein. Future studies, using different experimental approaches, might be able to clarify this important issue.

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