Calmodulin Kinase-Mediated Phosphorylation of Phospholamban in Skeletal Muscle Sarcoplasmic Reticulum. A Critical Reappraisal of the State of the Problem at the Light of New Findings with Human Normal and Diseased Muscle

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Abstract

The Ca^{2+}-transport system of the sarcoplasmic reticulum (SR) of mammalian hind-limb slow-twitch muscles has unique regulatory features, that have long been attributed to the special differentiating influence from the particular frequency and pattern of discharge of the innervating α motorneurons in the anterior horns of the spinal cord. This review summarizes the molecular mechanisms underlying such specific effects, focusing on phosphorylation of phospholamban (PLB) and of SR Ca^{2+}-ATPase by calmodulin kinase II (CaM K II). Regarding specifically the hypothesis according to which the neural control over the expression of fiber-type specific gene products in skeletal muscle SR encompasses the expression of PLB gene, there is divergent evidence, when the problem is examined in a wide range of mammalian species, from small rodent species to the human species. The discrepancy is underlined by observations, that the protein level of expression of PLB is virtually zero in rat slow-twitch muscle, and that it increases with animal body size; and with a consequent lack of correlation with the total amount of slow-twitch muscle Ca^{2+}-ATPase isoform found to be relatively immutable across different mammalian species. The experimental evidence appearing to be in conflict with the paradigma, is particularly striking in the case of human skeletal muscle having a mixed fiber composition. Most interestingly, transitions of Ca^{2+}-ATPase isoforms in human skeletal muscle fibers, under pathohological conditions leading to the appearance of intermediate fibers, were found not to be accompanied by down-regulation either of PLB or of CaM K II, suggesting that in such muscle expression of PLB and of the fast-twitch Ca^{2+}-ATPase isoform may be not mutually exclusive, or not in the absolute sense predicted by theory. The outstanding property that seems to link human skeletal and cardiac SR together, is the high PLB/Ca^{2+}-ATPase ratio. A common property with rabbit slow-twitch muscle SR appears to be the presence of a highly active, PLB-dedicated form of CaM K II. The postulated, complex interplay beween intracellular Ca^{2+} fluxes and the activity state of CaM K II, while adding interesting new facets to the regulatory features of SR Ca^{2+}-transport, inevitably invites to a number of questions, regarding the exact correlation between such mechanisms and the E-C characteristics and twitch properties of the muscle, depending also on the animal species.

Key words: Ca^{2+}-calmodulin dependent protein kinase II, skeletal muscle, sarcoplasmic reticulum, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), phospholamban.

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The perhaps most remarkable thing about the effects of motor innervation on skeletal muscle fibers, is how specific a transformation of the protein composition of myofibrils, it was found to be able to induce, depending on the frequency and the pattern of firing of α motorneurons. Similarly striking is the close matching of such changes with changes in protein compositional and biochemical-functional properties of sarcoplasmic reticulum (SR) membrane system. That is supported by: *a*) cross-reinnervation experiments with fast-twitch and slow-twitch hind-limb muscles of the cat and the rabbit [1, 5, 35], as well as of small rodent species, e.g. the rat

[22]; *b*) chronic electric stimulation of fast muscles at low frequency, i.e. at a frequency corresponding to the rate of discharge of slow motorneurons [26]; and, by *c*) comparative biochemical studies of pure fast-twitch and slow-twitch muscles at the stage of maturity, as well as in developing, and in old animals [27, 24].

Sarcoplasmic Reticulum Membrane Specificity in Relation to Fiber Type

Differences between fast-twitch and slow-twitch SR phenotypes are quantitative, such as differences in the membrane density of Ca^{2+}-release channels (RyR1 receptors) and of Ca^{2+}-pumps, as well as qualitative. For instance, whereas a single isoform of RyR1, and of RyR1–associated proteins, FKBP-12 and triadin, are apparently expressed in fast-twitch and slow-twitch mammalian muscles [9, 28], each type of muscle expresses a distinct isoform of SR Ca^{2+}-ATPase protein [7], named SERCA1 for fast muscle and SERCA2 for slow muscle, respectively [3]. It is likewise known that the pattern of CS isoforms is not the same for fast-twitch and slow-twitch muscle, the slow-cardiac CS isoform being expressed exclusively in slow-twitch muscle, and that both in large and in small Mammals [9, 15]. Within this conceptual framework, this review, updated to present knowledge with new experimental data from our own laboratory, focuses on the regulatory features of SR Ca^{2+}-transport, regarded to be a nerve-activity dependent property of mammalian fast-twitch muscle, even though this assumption now seems not to be totally valid.

Molecular Components and Mechanisms in Regulation of Ca^{2+}-Transport in the Sarcoplasmic Reticulum of Mammalian Slow-Twitch Muscle

The SR Ca^{2+}-transport system of slow-twitch muscle has unique regulatory features that were originally attributed to the exclusive expression in this type of muscle of SR Ca^{2+}-ATPase regulatory protein phospholamban (PLB), acting as a substrate of endogenous protein kinases [34]. Mammalian fast-twitch muscles never express PLB, but can be induced to express PLB when chronically electrically stimulated at low frequency [20, 26], as shown in the rabbit and the dog. This is the main evidence on which the hypothesis of the neural-dependency of PLB expression lies. Nevertheless, there is emerging evidence of a previously unrecognized differential heterogeneity between different mammalian species, regarding the expression of PLB in slow-twitch muscle and which does not fit into the picture. We reported recently [12] that the level of expression of PLB in slow-twitch muscle varied from zero, in rat, and negligible levels in the mouse, to significant levels in the rabbit. The observation was also made that the PLB/SERCA2 ratio, was apparently the highest in human skeletal muscle, suggesting that the protein level of expression of PLB, in addition to being regulated by skeletal muscle-specific transcriptional regulatory elements of PLB gene [16], and to be stimulus-frequency dependent, might be related to the species mass size, and, then, inversely related to the speed of muscle contraction [6]; or, that it might be influenced by additional physiological variables linked to the animal species, such as differences in the type of posture and in the pattern of locomotion.

PLB was the earliest identified substrate of PKA in slow-twitch muscle SR [19, 23]. However, it should be pointed out that there is emerging evidence that endogenous PKA co-enriches with dihydropyridine receptor (DHPR) in isolated junctional TT [30]. Phosphorylation of DHPR α1 subunit by PKA was shown to modulate L-type Ca^{2+}-channel activity [33] and to affect the interaction of skeletal DHPR with RyR1 [21]. Indeed, there is a number of recent studies, foremost studies on the specific targeting of CaM K II to SR membranes [2], implying a more important role of CaM kinase II (CaM K II), rather than of PKA, regarding SR protein phosphorylation.

PKA-mediated and CaM K II-mediated phosphorylation of PLB, occurr at different sites, i.e. at Ser-16 or at Thr-17, respectively [34]. The evidence of phosphorylation of SERCA2 at Ser-38 by CaM K II, is more recent, and relies on experimental findings with rabbit cardiac and slow-twitch muscle SR [17, 36]. While phosphorylation of PLB disrupts its inhibitory interaction with SR Ca^{2+}-ATPase, and is manifested in an increase in Ca^{2+}-affinity of the Ca^{2+}-ATPase [34], the effect of phosphorylation of SERCA2 by CaM K II, apparently, is to increase the Vmax of Ca^{2+}-transport [17]. The existence of a dual mechanism by which SR membrane-bound CaM K II appears to be able to up-regulate SR Ca^{2+}-transport, is a further element arguing for a key control role of this protein kinase over transmembrane Ca^{2+}-fluxes in slow-twitch muscle fibers.

Phosphorylation of PLB by CaM K II in the Sarcoplasmic Reticulum of Human Skeletal Muscle

The most precise knowledge of the fiber type composition of human skeletal muscle was obtained from characterization studies of fast and slow isomyosins and of troponin and tropomyosin isoforms in single, chemically skinned fibers [31]. That has helped enormously to interpret biochemical data on isolated SR membranes, in terms of the relative contribuiton of fast and slow fibers to the average protein composition, and which was further refined using specific markers of the free and the junctional membrane domain of the SR [8, 11]. All these data, taken together with other kinds of information, including studies in the electron microscope [18], are stressing the mixed fiber composition of human skeletal muscles at the several

anatomical sites, as well as less marked differences in morphological ultrastructure between fast and slow fibres in comparison to muscles of experimental animals.

Using immunofluorescent techniques and a deltoid muscle sample from a normal individual, we verified (Fig. 1) that SERCA1 and SERCA2 were expressed in distinct types of fibers and that intermediate fibers were not present. On the same evidence, one can see extensive similarities between human skeletal muscle and a representative fiber bundle from rabbit gastrocnemius, i.e. composed of fast and of slow fibers. As revealed by immunostaining of serial sections with antibody to PLB, the evidence appears to be also that in rabbit gastrocnemius, PLB is co-expressed with SERCA2, only. In contrast, human deltoid muscle, in addition to expressing PLB in the corresponding type of fibers, is found to express PLB in a discrete subpopulation of fast-twitch fibers (Fig. 1, marked with F), although at a much lower level. Such property, which in itself, is in conflict with the dogma that the expression of SERCA1 gene and of PLB gene are mutually exclusive, argues that the less restricted human fast fibers, might be more versatile in their ability to adjust SR Ca^{2+}-transport, depending on the potential interaction of SERCA1 with PLB in the native membrane enviroment.

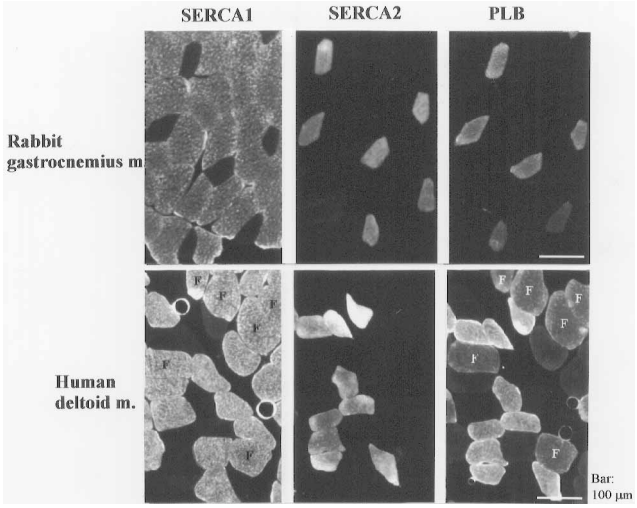

Figure 1: Immunofluorescent localization of phospholamban, SERCA1 and SERCA2 in fast-twitch and slow-twitch fibers of rabbit and human skeletal muscle. Serial transverse cryosections of unfixed rabbit gastrocnemius and of normal human deltoid were incubated with mouse monoclonal antibody to SERCA1, SERCA2 and PLB. Antibody binding was visualized using anti-mouse IgG conjugated with fluorescein. Muscle fibers immunostained with antibodies both to SERCA1 and PLB are marked with F.

In biochemical studies of the isolated SR from human skeletal muscle, in which the vastus lateralis was the particular muscle used and was compared with rabbit soleus SR, we have used Western blot techniques and specific antibodies to SERCA1, SERCA2 and PLB, after SDS-PAGE of sucrose-density purified SR. SERCA1 was not detectably present in rabbit soleus SR, whereas both SERCA1 and SERCA2 could be detected in the isolated SR from human skeletal, vastus lateralis muscle. Comparing the PLB/SERCA2 ratio between human vastus lateralis and rabbit soleus (Fig. 2), is very striking, in that it shows a threefold difference between the two types of muscle.

In order to assess the relative membrane density of CaM K II in human muscle SR, we used Western blot techniques and polyclonal antibodies to the kinase δ subunit, and which identified a peptide having a slightly faster electrophoretic mobility. Autoradiographic evidence (Fig. 3), indicates that human 58 kDa and rabbit 60 kDa peptide both were intensely phosphorylated after incubating SR vesicles with [γ-^{32}P]-ATP, in the presence of Ca^{2+}-CaM, and that the self-phosphorylation of CaM K II was associated with a

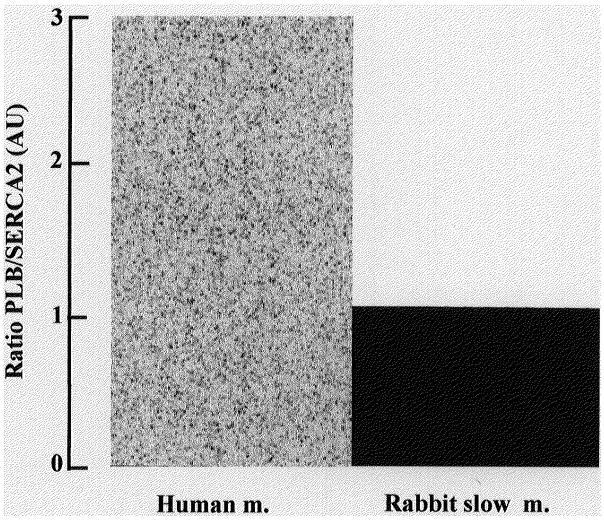

Figure 2. Phospholamban and SERCA2 protein levels in rabbit and human isolated SR. SR membranes were isolated from rabbit slow-twitch muscles and from human vastus lateralis by isopycnic sucrose-density centrifugation, following the method of Saito et al. [29], with the modifications reported by Damiani and Margreth [9]. PLB and SERCA2 levels were quantified immunochemically, by incubating blots of electrophoretically-resolved SR proteins with monoclonal antibodies to PLB and SERCA2, respectively. The protein loading was identical for rabbit and human SR membranes. Densitometric measurements of immunostained proteins were carried out using a Bio-Rad Model GS-670 Imaging densitometer.

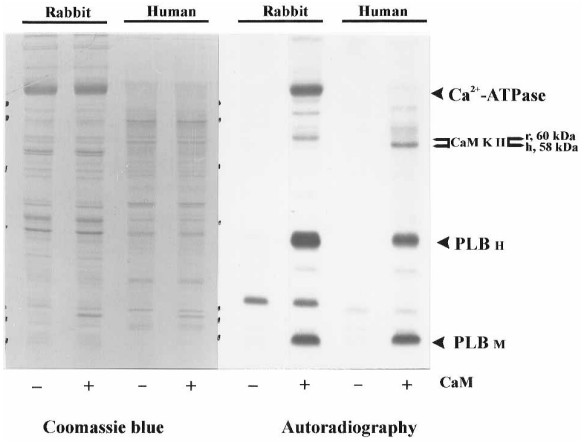

Figure 3. Phosphorylation of phospholamban by endogenous CaM K II. Sucrose-density purified free SR membranes from rabbit slow-twitch muscles and from human vastus lateralis were incubated for 5 min at 0° C with 400 μM [γ^{32}P]-ATP, in the presence of 100 μM free Ca^{2+} and in the absence or in the presence of 1 μM CaM, as indicated. SR protein (100 μg) was resolved by 10-15% SDS-PAGE. Gels were dried and ^{32}P-labelled proteins were detected by autoradiography.

comparable extent of phosporylation of PLB (Fig. 3). Characterization of the phosphorylation status of PLB in store-frozen SR vesicles before incubation, was done using phosphorylation-site specific antibodies, and revealed little or no phosphorylation of PLB at Ser-16 and the presence of phosphorylated Thr-17 in PLB monomer and, in a varying number, in PLB pentamer.

These several findings, together, thus seem to provide a mechanistic link betwen CaM K II and PLB in human skeletal muscle, in a way similar to that reported for rabbit and dog slow-twitch skeletal muscle, while there seems to be a wide divergence between human vastus lateralis and rabbit soleus, concerning the ability of endogenous CaM K II to phosphorylate SR Ca^{2+}-ATPase (Fig. 3). The perhaps simplest explanation for the observed difference may be that, in the isolated SR from human skeletal muscle, the Ca^{2+}-ATPase protein is comprised of SERCA1 (which is not phosphorylated by CaM K II, [17]), and SERCA2, and, in addition to that, the total membrane density of SR Ca^{2+}-ATPase is very low, in comparison to rabbit soleus muscle SR.

CaM K II-Dependent Phosphorylation of PLB, in Human Diseased Muscle, Under Conditions Associated with a Slow-to-Fast-Transformation of SR Ca^{2+}-ATPase

Trying to clarify the question of whether expression of PLB in human skeletal muscle may be not restricted to slow-twitch fibers, we also examined some selected cases of human myopathies. In a previous work [11], in which muscle biopsy specimens from patients with Myotonic dystrophy (DM) were compared with adult control muscle, we had observed an increase in Ca^{2+}-ATPase activity and reciprocal changes in the membrane density of the SERCA2 isoform of Ca^{2+}-ATPase in the isolated SR and without any accompanying marked change of PLB. These results have been confirmed and extended.

On reinvestigating this problem, using immunofluorescent techniques, in recent collaborative work (S. Servidei and P. Tonali, University of Rome), we have found that in some, although not all DM cases, the SERCA2-SERCA1 transition at the isolated SR level, reflected the occurrence of intermediate muscle fibers, i.e. fibers containing an admixture of SERCA1 and SERCA2. This is a new finding, although not totally surprising given the evidence of fibres containing an admixture of fast and slow isomyosins in the same disease [32]. Intermediate muscle fibers were likewise identified in deltoid muscle biopsy material from one patient with Thomsen's disease (Fig. 4, marked with asterisk). Regardless of the exact genesis of the transforming event, appearing not to be specific to the type of disease and rather to be apparently connected with the onset of the myotonic reaction, as such, it is interesting to note that slow-twitch fibers "en route" to the other type (Fig. 4), seem to maintain the capability of synthesising PLB. Immunostaining with specific antibody to PLB is also revealing a depleted amount of PLB in a proportion of fast-twitch fibers (Fig. 4, marked with F), like in normal human muscle. The slow-to-fast change of SR Ca^{2+}-ATPase, as investigated in several cases of DM, was found not to impair the phosphorylation of PLB, and rather to lead to an

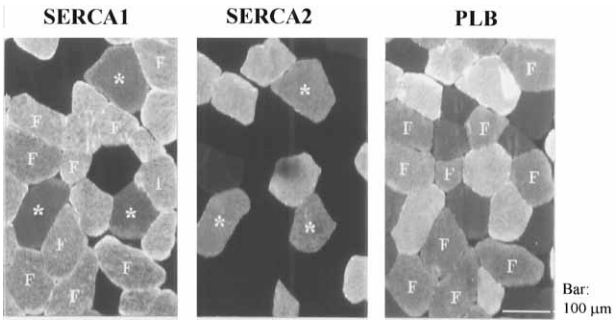

Figure 4. Immunofluorescent localization of SERCA1, SERCA2 and phospholamban in muscle fibers from human pathological muscle. Serial transverse cryosections of unfixed human deltoid muscle fibers from a biopsy from a patient with Thomsen's disease, were stained with monoclonal antibody to SERCA1, SERCA2 and PLB, as described in the legend to Fig. 1. SERCA1-positive muscle fibers immunostained also with antibody to PLB are marked with F. Intermediate fibers coexpressing SERCA1 and SERCA2 are marked with an asterisk.

increased recovery of phosphorylated PLB at Thr-17. Immunoblot evidence indicated an unchanged membrane density of CaM K II in the same SR membrane preparations. Together, these findings seem to argue that, the ongoing slow-to-fast transformation of the muscle does not basically alter the effectiveness of CaM K II-dependent phosphorylation of PLB, suggesting that this regulatory mechanism might be able to act synergistically with the SERCA2-SERCA1 transition, to the effect of augmenting the maximal rate of muscle relaxation.

Concluding Remarks and Prospects

In summary, it is the hypothesis of a differential regulatory complexity of SERCA1 versus SERCA2, based on work with pure slow-twitch and fast-twitch muscles of experimental animals [18, 36], that cannot be completely reconciled with our present data with human muscle SR, rather being the biochemical properties of the regulatory system of SR Ca^{2+}-transport to divide. Nevertheless, our present study identifies PLB as the only specific substrate of endogenous CaM K II in the isolated SR from human skeletal muscle. Morevoer, human skeletal muscle SR, by virtue of its high membrane density of PLB, and the large excess of PLB molecules over the number of Ca^{2+}-pumps, appears to have properties somewhat intermediate between those of typical slow-twitch muscle SR and of cardiac muscle SR [25].

Mechanisms involved in targeting of CaM K II to SR membrane, have been shown to involve anchor protein αKAP [2], in the case of rat skeletal muscle. However, from knowledge that SR-bound CaM K II is able to phosphorylate triadin in human skeletal muscle (A. Pallanca), similarly to TC from rabbit fast-twitch muscle [10], an heterogeneity in CaM K II isoforms and in specific targeting of CaM K II to free and to junctional SR cannot be entirely excluded, at the present time. CaM K II, in addition to having being implicated in regulation of SR Ca^{2+}-transport in mammalian cardiac muscle and slow-twitch muscle, seems to have other important physiological effects. A recent study with cardiac myocites found that a Ca^{2+}-CaM K II-mediated phosphorylation of unknown membrane proteins is the trigger for Ca^{2+}-dependent facilitation of L-type Ca^{2+}-channels in cardiac myocites [14].

The perhaps most significant aspect of CaM K II action, is that the enzyme, once activated, undergoes autophosphorylation at Thr-286 and retains activity independently of Ca^{2+} and CaM (CaM K II autonomous form) [4]. Such regulatory features prolong the effects of brief Ca^{2+} transients. In our experiments with isolated TC from rabbit fast-twitch muscle, self-phosphorylation of endogenous CaM K II was found to be optimal at pCa 4-5 [10]. As predicted by computer simulations, in experiments of rapid superfusion of immobilized Ca^{2+} and CaM K II [13], it was shown that enzyme can decode the frequency of Ca^{2+} spikes, and that the frequency response is modulated by the amplitude and duration of individual spikes. On account of these properties, it would be tempting to speculate that CaM K II may play an important role in a number of physiological processes, from the E-C coupling process to the functional coupling between SR Ca^{2+}-release and Ca^{2+}-transport in the course of the Ca^{2+}-transient upon muscle depolarization. Further progress in knowledge of CaM K II signalling pathway should be useful in a broad range of studies on skeletal muscle, including also study of the molecular mechanisms involved in translation and processing of the information arising from the specific pattern of discharge of motorneurons. The analysis of the activity status of CaM K II in the SR membrane system, depending on the concentration of Ca^{2+}, and on the balance between self-phosphorylation and dephosphorylation reactions, seems to be particularly timely, as it is the problem of the differential heterogeneity in Ca^{2+}-CaM modulated reactions in the SR membrane system, according to the type of muscle and to the animal species.

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