Ectopic Expression of Phospholamban in Fast-Twitch Skeletal Muscle Alters Sarcoplasmic Reticulum Ca\(^{2+}\) Transport and Muscle Relaxation*

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There are three isoforms of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase; they are known as SERCA1, SERCA2, and SERCA3. Phospholamban is present in tissues that express the SERCA2 isoform and is an inhibitor of the affinity of SERCA2 for calcium. In vitro reconstitution and cell culture expression studies have shown that phospholamban can also regulate SERCA1, the fast-twitch skeletal muscle isoform. To determine whether regulation of SERCA1 by phospholamban can be of physiological relevance, we generated transgenic mice that ectopically express phospholamban in fast-twitch skeletal muscle, a tissue normally devoid of phospholamban. Ectopic expression of phospholamban was associated with a decrease in the affinity of SERCA1 for calcium. Assessment of isometric twitch contractions of intact fast-twitch skeletal muscles revealed depressed rates of relaxation in transgenic mice compared with wild-type cohorts. Furthermore, the prolongation of muscle relaxation appeared to correlate with the levels of phospholamban expressed in two transgenic mouse lines. These findings indicate that ectopic expression of phospholamban in fast-twitch skeletal muscle is associated with inhibition of SERCA1 activity and decreased relaxation rates of this muscle.

The sarcoplasmic reticulum (SR)\(^1\) is an internal membrane system that plays an important role in the initiation of muscle relaxation via the reduction of cytosolic calcium levels. The translocation of calcium from the cytosol into the SR is mediated by the SR Ca\(^{2+}\)-ATPase enzyme. There are currently three known isoforms of the SR Ca\(^{2+}\)-ATPase, which are the products of separate genes (1–5). The SERCA1 gene is expressed exclusively in adult fast-twitch skeletal muscle SR (1, 2, 5). The SERCA2 gene has two alternatively spliced isoforms, SERCA2a and SERCA2b. SERCA2a is expressed in cardiac, slow-twitch, and fetal fast-twitch skeletal muscles, while SERCA2b can be found in smooth muscle and nonmuscle tissues (4, 6, 7). The SERCA3 gene is expressed in a number of muscle and nonmuscle tissues and is considered the endoplasmic reticulum Ca\(^{2+}\)-ATPase isoform (3).

SERCA2a is the major isoform responsible for SR calcium transport in cardiac and slow-twitch skeletal muscles; it is regulated by phospholamban (8, 9). Dephosphorylated phospholamban is an inhibitor of the affinity of cardiac SR Ca\(^{2+}\)-ATPase for Ca\(^{2+}\), and phosphorylation relieves this inhibition (9). In vitro studies have demonstrated phosphorylation of phospholamban at distinct sites by cAMP-dependent, Ca\(^{2+}\)-calmodulin-dependent, and Ca\(^{2+}\)-phospholipid-dependent protein kinases (10). In vivo studies of isolated heart preparations have also shown that phospholamban can be phosphorylated during \(\beta\)-adrenergic stimulation and that this phosphorylation is associated with increases in the SR Ca\(^{2+}\)-ATPase activity and enhanced rates of cardiac relaxation (11–14). Furthermore, pretreatment of SR vesicles with an anti-phospholamban monoclonal antibody could relieve the inhibitory effects of phospholamban on the Ca\(^{2+}\) affinity of SERCA2 and enhance \(\mathrm{E}^\ast\mathrm{P}\) formation, while this antibody had no effect on SERCA1 activity (15).

SERCA1 mediates relaxation in fast-twitch skeletal muscle and although phospholamban is absent from this tissue, several in vitro studies suggest that SERCA1 can be regulated by phospholamban (8, 9, 16–18): (a) cross-linking of phospholamban to SERCA revealed a putative phospholamban-binding domain that is present in both SERCA1 and SERCA2 (8, 9, 16–18); (b) reconstitution of phospholamban with SERCA1 or SERCA2 in lipid bilayers was associated with similar inhibition of Ca\(^{2+}\) uptake rates using either isoform (9, 17); (c) co-expression studies of phospholamban with either SERCA1 or SERCA2 in COS-1 cells demonstrated that phospholamban could regulate either the SERCA1 or SERCA2 enzyme (8); and (d) stable expression of phospholamban in C2C12 cells, a fast-twitch skeletal muscle cell line that expresses SERCA1, resulted in an inhibition of SERCA1 activity in its native SR environment (16).

While evidence obtained in these in vitro studies suggests that phospholamban can regulate the SERCA1 enzyme, it is not presently clear whether this regulation would lead to alterations of skeletal muscle kinetics. The advent of transgenesis allows for tissue-specific expression of a protein of interest and assessment of its physiological consequences in that particular tissue. Thus, transgenic mice provide ideal systems for testing phospholamban-mediated regulation of SERCA1 activity in vivo and its effects on fast-twitch skeletal muscle function. In this study, we generated a transgenic mouse model, which ectopically expresses phospholamban exclusively in fast-twitch skeletal muscle under the control of the rat myosin light chain 1f (MLC1f) promoter and enhancer elements. Introduc-
tion of phospholamban into fast-twitch skeletal muscle resulted in inhibition of SERCA1 activity in its native lipid environment and in alterations in muscle relaxation kinetics.

**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice by Pronuclear Injection**—The MLC1f/PLB (Fig. 1A) construct was generated by combining the 5′-flanking promoter (1.5 kilobases (kb)) and the 920-base pair (bp) enhancer region of the rat myosin light chain 1/3 locus, isolated from NRCAT900 (1B), with an 853-bp cassette that included the mouse PLB cDNA (603-bp fragment as well as a 250-bp fragment coding for the SV40 polyadenylation signal). The 603-bp phospholamban cDNA fragment, isolated from the α-MHC/PLB transgene construct (20), encompassed 13 bp of the phospholamban 5′-untranslated region, the entire phospholamban-coding region, as well as 434 bp of the 3′-untranslated region including the first polyadenylation signal. The resulting 3.2-kb phospholamban-coding region, as well as 434 bp of the 3′ untranslated region of the phospholamban 5′ untranslated region, was addressed by pronuclear microinjection of fertilized mouse eggs to generate transgenic mice.

Transgenic mice carrying the MLC1f-driven phospholamban transgene were identified using polymerase chain reaction and Southern analysis fragment as well as a 250-bp fragment coding for the SV40 polyadenylation signal. The resulting 3.2-kb phospholamban-coding region, as well as 434 bp of the 3′ untranslated region of the phospholamban 5′ untranslated region, was addressed by pronuclear microinjection of fertilized mouse eggs to generate transgenic mice.

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**Northern blot analysis** (21). Mice positive for the transgene were bred, and expression of the transgene was determined using Northern blot analysis of total RNA from gastrocnemius, tibialis anterior, and EDL muscles. After establishing transgene copy number was determined relative to the phospholamban gene (copy number = 2), using the PhosphorImager and ImageQuant analysis system (Molecular Dynamics, Sunnyvale, CA). Mice positive for the transgene were bred, and expression of the transgene was determined using Northern blot analysis (21).

**Quantitative Immunoblot Analysis**—Quantitative immunoblotting of transgenic fast-twitch skeletal homogenates was carried out as described previously (22). The transgenic phospholamban was detected using anti-phospholamban polyclonal anti sera (1:25 dilution) isolated from rabbits that had been immunized with a peptide corresponding to amino acids 1–25 of phospholamban (22). The SR Ca2+/ATPase levels were determined relative to the phospholamban gene (copy number = 2), using the PhosphorImager and ImageQuant analysis system (Molecular Dynamics, Sunnyvale, CA). Mice positive for the transgene were bred, and expression of the transgene was determined using Northern blot analysis (21).

**Quantitative Immunoblot Analysis of Transgene Expression**—The MLC1f/PLB transgenic construct (Fig. 1A) was generated to direct ectopic expression of the mouse phospholamban cDNA in fast-twitch skeletal muscle. Sixteen transgenic lines were identified by polymerase chain reaction and Southern blot analyses of genomic DNA from tail biopsies. Transgene expression was assessed by Northern blot analysis of total RNA from gastrocnemius, tibialis anterior (TA) and EDL muscles. Abundant expression of the 1-kb phospholamban transcript was detected in all three muscles and in two separate lines, 75 and 76 (Fig. 1B), consistent with previous observations (30). Southern blot analysis of genomic DNA indicated that the transgene copy number was 12 and 7 in lines 75 and 76, respectively. Both of these lines were propagated for further characterization. The tibialis anterior and EDL muscles, which are composed of 98% fast-twitch fibers (31), were used for the biochemical and physiological studies, respectively. However, due to the limited size of these muscles, the gastrocnemius, which is 80% fast-twitch (31), was used for immunofluorescence studies to examine transgene insertion into the SR membranes. Western blot analysis of these three muscles using monoclonal antibodies specific for either SERCA1 or SERCA2 indicated that the TA and EDL muscles express only SERCA1, while the gastrocnemius expresses SERCA1 and nominal (~15%) levels of SERCA2 (data not shown).

**Statistical Analysis**—Data are presented as mean ± S.E. The number (n) of mice used is indicated. Statistical differences between the means were analyzed using analysis of variance and the Student Newman-Keuls test for multiple comparisons. Values with p < 0.05 were considered statistically significant.

**RESULTS**

**Generation of Transgenic Mice**—The MLC1f/PLB transgenic construct (Fig. 1A) was generated to direct ectopic expression of the mouse phospholamban cDNA in fast-twitch skeletal muscle. Sixteen transgenic lines were identified by polymerase chain reaction and Southern blot analyses of genomic DNA from tail biopsies. Transgene expression was assessed by Northern blot analysis of total RNA from gastrocnemius, tibialis anterior (TA) and EDL muscles. Abundant expression of the 1-kb phospholamban transcript was detected in all three muscles and in two separate lines, 75 and 76 (Fig. 1B), consistent with previous observations (30). Southern blot analysis of genomic DNA indicated that the transgene copy number was 12 and 7 in lines 75 and 76, respectively. Both of these lines were propagated for further characterization. The tibialis anterior and EDL muscles, which are composed of 98% fast-twitch fibers (31), were used for the biochemical and physiological studies, respectively. However, due to the limited size of these muscles, the gastrocnemius, which is 80% fast-twitch (31), was used for immunofluorescence studies to examine transgene insertion into the SR membranes. Western blot analysis of these three muscles using monoclonal antibodies specific for either SERCA1 or SERCA2 indicated that the TA and EDL muscles express only SERCA1, while the gastrocnemius expresses SERCA1 and nominal (~15%) levels of SERCA2 (data not shown).

**Quantitative Immunoblot Analysis of Transgene Expression**—To determine the levels of phospholamban expression relative to the levels of the SR Ca2+/ATPase, we performed quantitative immunoblotting using homogenates of tibialis anterior and EDL muscles isolated from line 75-derived mice. Cardiac homogenates were also processed in parallel to compare
the relative levels of phospholamban expression in the transgenic skeletal muscles. We observed phospholamban expression levels that were ~70% of those present in the heart (Fig. 2A), while the SR Ca\(^{2+}\)-ATPase levels were ~2-fold higher in the skeletal homogenates than those in the heart (Fig. 2B). Thus, the relative ratio of phospholamban to SR Ca\(^{2+}\)-ATPase was ~1:3 in the transgenic fast-twitch skeletal muscles, when compared with a relative value of 1:1 in cardiac muscle (Fig. 2C). The actual ratio of phospholamban to SR Ca\(^{2+}\)-ATPase in cardiac muscle is currently unclear, but in this study it was set as 1:1. Examination of the phospholamban expression levels in animals from line 76 indicated that they were 40% of those present in the heart (data not shown). Furthermore, there was no phospholamban detected in the gastrocnemius, tibialis anterior or EDL muscles from the wild-type, nontransgenic cohorts (data not shown).

**Immunofluorescence Co-Localization of Phospholamban and the SR Ca\(^{2+}\)-ATPase**—To determine whether the transgenic phospholamban protein was localized in the SR membranes, we utilized indirect immunofluorescence labeling of both phospholamban and the SR Ca\(^{2+}\)-ATPase in the same cryosections of transgenic muscles. Gastrocnemius muscles were excised from wild-type and transgenic animals, sectioned using a cryotome, and mounted on glass slides. The cryosections were then reacted with antisera to either phospholamban or the SR Ca\(^{2+}\)-ATPase. The binding of antisera to phospholamban was detected using a rhodamine-conjugated secondary antibody, while binding to the SR Ca\(^{2+}\)-ATPase was detected with a fluorescein-conjugated secondary antibody. The use of differentially labeled secondary antibodies allows for the detection of both proteins in the same cryosection. Similar staining patterns for the SR Ca\(^{2+}\)-ATPase were observed in wild-type and transgenic muscles. However, we could detect staining for phospholamban expression only in transgenic muscles (Figs. 3 and 4). The lack of phospholamban detection in the wild-type muscles, which are composed of 80% slow-twitch fibers (31), is likely due to the low levels of phospholamban expression in slow-twitch fibers (27). To determine the extent of overlap present in the staining patterns for the two different antibodies, the images obtained in the transgenic muscles for both phospholamban and the SR Ca\(^{2+}\)-ATPase were then digitized and electronically superimposed. In regions of the superimposed images where the staining patterns were not overlapping, the color was either red or green, representing rhodamine or fluorescein staining, respectively (Fig. 4A). The regions that appeared yellow represent areas in which the staining for both antigens were co-localized. As shown in Fig. 4B, merging of images for phospholamban and the SR Ca\(^{2+}\)-ATPase resulted in a pattern that was predominantly yellow, suggesting that the transgenic phospholamban was co-distributed with the Ca\(^{2+}\)-ATPase in the SR membranes.

**Effects of Ectopic Phospholamban Expression on SR Ca\(^{2+}\)-Uptake**—The effects of ectopic phospholamban expression on the apparent initial rates of ATP-dependent, oxalate-facilitated SR Ca\(^{2+}\)-uptake were assessed in whole muscle homogenates using tibialis anterior muscles from wild-type and transgenic animals (line 75). The advantages of using homogenates rather than SR vesicles and the validity of this approach have been previously defined (32, 33). SR Ca\(^{2+}\)-uptake was determined over a wide range of calcium concentrations in each preparation. Measurements of Ca\(^{2+}\)-uptake at each calcium concentration were taken at time intervals of 0.5, 1.0, and 1.5 min to
determine initial rates. We observed very high rates of Ca\(^{2+}\)-uptake at the 0.5-min interval but these rates decreased at subsequent time points (data not shown). This phenomenon has been previously observed in studies of fast-twitch skeletal SR membranes by Feher et al. (34), who suggested that the nonlinearity observed beyond 0.7 min was due to rupture of the SR vesicles. On the basis of these findings, we chose to determine the apparent initial rates of SR Ca\(^{2+}\)-uptake at the 0.5-min interval. Ectopic expression of PLB was associated with a significant decrease in SR Ca\(^{2+}\) uptake rates at low Ca\(^{2+}\), with no apparent change in the maximal velocity (V\(_{\text{max}}\)) of Ca\(^{2+}\) uptake (Fig. 5). Analysis of these data indicated that the EC\(_{50}\) values of the SR Ca\(^{2+}\) uptake for Ca\(^{2+}\) were significantly (p < 0.05) higher in transgenic muscles (0.284 \(\mu\)M ± 0.01; n = 5) than in wild-type muscles (0.189 \(\mu\)M ± 0.01; n = 5). Thus, expression of phospholamban in fast-twitch skeletal muscle resulted in a decrease in the affinity of the SR Ca\(^{2+}\)-ATPase for calcium, consistent with its inhibitory effects previously reported in cardiac muscle (32, 33).

**Isometric Twitch Contractions in Isolated Skeletal Muscles**—To determine whether the decreased affinity of the SR Ca\(^{2+}\)-uptake for Ca\(^{2+}\) was associated with alterations of muscle function, isometric twitch contractions were assessed in EDL muscles from wild-type and transgenic animals (line 75). The EDL muscle was utilized for these studies since its small size allows for complete oxygenation of the muscle, which is critical for maintenance of muscle function. Although the transgenic mice appeared phenotypically indistinguishable from their wild-type cohorts at the gross level, the transgenic EDL muscles exhibited a significantly altered isometric twitch contraction profile, which was associated with a prolonged relaxation phase (Fig. 6A). Quantitative assessment of isometric twitch contractions of EDL muscles at L\(_{\text{max}}\) revealed that ectopic phospholamban expression in the EDL muscle resulted in a significant increase in the half-relaxation times (12.5 ± 0.6 ms, n = 7 in transgenics versus 7.3 ± 0.9 ms, n = 4 in wild types, p < 0.05) without a change in the contraction times (19.8 ± 0.3 ms, n = 7 in transgenics versus 18.9 ± 0.4 ms, n = 4 in wild types) of transgenic EDL muscles compared with wild-type EDL muscles. We also characterized the contractile parameters of EDL muscles from another transgenic line (line 76), which expressed lower levels (40% versus heart; Fig. 1B) of phospholamban than those present in line 75 (70% versus heart). Consistent with the results obtained in line 75, we observed a prolonged half-relaxation time (9.7 ± 0.97 ms; n = 3) but no effect on contraction time (18.8 ± 0.2 ms; n = 3) in EDL muscles from line 76. When the relative levels of phospholamban in the EDL muscles from wild-type (0%), line 76 (40%), and line 75 (70%) were plotted against the half-relaxation times in these muscles, a close linear correlation was observed (Fig. 6B), indicating that phospholamban is a major regulator of relaxation in these muscles.

To determine whether the prolonged relaxation observed in the transgenic EDL muscles could be reversed by \(\beta\)-adrenergic

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**Fig. 3. Double immunofluorescence labeling of the SR Ca\(^{2+}\)-ATPase but not phospholamban in the same wild-type gastrocnemius cryosection.** The SR Ca\(^{2+}\)-ATPase was detected using an anti-SR Ca\(^{2+}\)-ATPase polyclonal antibody in conjunction with a fluorescein-conjugated anti-rabbit secondary antibody, while phospholamban was detected using an anti-phospholamban monoclonal antibody in conjunction with a rhodamine-conjugated anti-mouse secondary antibody. Images were produced using laser confocal scanning microscopy. Bar, 10 \(\mu\)m.

**Fig. 4. Double immunofluorescence labeling of phospholamban and SR Ca\(^{2+}\)-ATPase in the same transgenic gastrocnemius cryosection.** In A, phospholamban was detected using an anti-phospholamban monoclonal antibody in conjunction with a rhodamine-conjugated anti-mouse secondary antibody and appears red, while the SR Ca\(^{2+}\)-ATPase was detected using an anti-SR Ca\(^{2+}\)-ATPase polyclonal antibody in conjunction with a fluorescein-conjugated anti-rabbit secondary antibody and appears green. B, digital overlay of staining patterns for both proteins. Regions, where staining for both phospholamban and the SR Ca\(^{2+}\)-ATPase are superimposed, appear yellow and indicate that the two antigens are co-distributed, at the limits of resolution of light microscopy. Regions in which they are not superimposed appear either red or green. Control experiments were performed in which the primary antibody was omitted and no fluorescence was detected. Images were produced using laser confocal scanning microscopy. Bar, 10 \(\mu\)m.
stimulation, we exposed both wild-type and transgenic muscles to cumulative doses of isoproterenol. Isoproterenol stimulated force production by 30% in both wild-type and transgenic muscles (data not shown) but had no effect on either the contraction or the half-relaxation times of wild-type and transgenic EDL muscles (Fig. 7). Thus, isoproterenol could not reverse the depressed rates of relaxation observed in the transgenic EDL muscles on phosphorylamban expression. To determine whether this lack of isoproterenol stimulation was due to defects in the transgenic protein, which prevented its phosphorylation by cAMP-dependent protein kinase, transgenic homogenates were phosphorylated in vitro with the catalytic subunit of protein kinase A. Phosphorylation of transgenic phosphorylamban was associated with alterations in its electrophoretic mobility in SDS-polyacrylamide gels (data not shown), consistent with previous reports (35), indicating that there were no inherent defects that precluded phosphorylation of phosphorylamban in vivo.

Since isoproterenol is an activator of both β1- and β2-adrenergic receptors (36), we investigated whether the isoproterenol-elicited increases in developed force in the EDL muscle were due to β1- or β2-adrenergic receptor activation. Isolated, intact EDL muscles were preincubated with either CGP-210712A or ICI-118551, which are specific β1- and β2-adrenergic receptor antagonists, respectively (35). Pretreatment with CGP-210712A had no effect, while ICI-118551 completely abolished the isoproterenol-induced increases in developed force (data not shown). These findings suggest that the effects of isoproterenol in the mouse fast-twitch skeletal muscle are mediated through the β2-adrenergic receptor pathway and that its inability to reverse the depressed rates of relaxation in the transgenic muscles may be due to the lack of phosphorylamban phosphorylation.

To determine whether phosphorylamban is phosphorylated during isoproterenol stimulation, we exposed isolated transgenic EDL muscles to 6 μM isoproterenol and then subjected the muscle homogenates to Western blot analysis. Two site-specific polyclonal antibodies, which recognize phosphorylated serine-16 or threonine-17 residues in phosphorylamban (24, 25), were used to determine the phosphorylation status of phosphorylamban. We could not detect phosphorylation of either serine or threonine residues in phosphorylamban after isoproterenol stimulation in the transgenic skeletal muscles (Fig. 8). However, in parallel studies both phosphorylated residues of endogenous phosphorylamban could be identified after isoproterenol stimulation of mouse hearts (Fig. 8). These findings suggest that activation of β2-adrenergic signaling pathways in mouse EDL muscles does not promote phosphorylamban phosphorylation, in agreement with previous observations in rat cardiomyocytes (35).

**DISCUSSION**

This study presents the first evidence that SERCA1 activity can be regulated by phosphorylamban in vivo, resulting in depressed rates of fast-twitch skeletal muscle relaxation. A transgenic mouse model was generated, which ectopically expressed phosphorylamban exclusively in fast-twitch skeletal muscle under the control of the rat myosin light chain 1f promoter and enhancer elements. Indirect immunofluorescence labeling of phosphorylamban and SR Ca2+-ATPase in transgenic gastrocnemius tissues indicated that the two proteins were co-distributed, suggesting that the transgenic phosphorylamban was incorporated into the SR membranes. This ectopic phosphorylamban expression was associated with decreases in the affinity of the SR Ca2+-pump for Ca2+ in agreement with previous studies involving phospholipid bilayer reconstitution (9, 18) or cell culture co-expression studies (8, 16) of phosphorylamban and SERCA1. Although we cannot presently exclude the possibility that modulation of SR Ca2+ uptake activity occurs through the
formation of Ca\(^{2+}\)-selective phospholamban channels (37), our findings indicate that the affinity of the SR transport system for calcium was altered without any effects on its maximal velocity.

The generation of this ectopic expression model allowed us to extend our previous studies on phospholamban-mediated regulation of SERCA1 activity in vitro (9, 16, 18) and to examine the functional implications of this modulation in the intact muscle. The inhibitory effects of phospholamban expression on SR Ca\(^{2+}\) uptake reflected alterations in the contractile properties of isolated fast-twitch skeletal muscles. Examination of isometric twitch dynamics of transgenic EDL muscles revealed a prolongation in the half-relaxation time, without alterations in the contraction time, as compared with the wild-type EDL muscles. The observed changes in relaxation rates were not due to a reduction in SR Ca\(^{2+}\)-ATPase expression levels, since the changes in V\(_{\text{max}}\) of the Ca\(^{2+}\)-ATPase activity was similar in transgenic and nontransgenic muscles. Furthermore, the decreases in the rates of relaxation appeared to correlate with the levels of phospholamban expressed in the transgenic fast-twitch skeletal muscles. However, it is not currently known whether the observed physiological alterations were only due to phospholamban expression or whether an unknown compensatory response also occurred in the transgenic mice, which led to an increase in passive muscle stiffness.

Since the inhibitory effects of phospholamban in cardiac and slow-twitch skeletal muscles can be relieved during \(\beta\)-adrenergic stimulation (11, 20, 27, 28, 32), we exposed the wild-type and transgenic EDL muscles to cumulative doses of isoproterenol, a mixed \(\beta\)-adrenergic agonist. Isoproterenol stimulated force production by 30% but had no effect on either the contraction or half-relaxation times in wild-type EDL muscles, in agreement with previous findings (38). The observed stimulation of force production by isoproterenol in this study may be due to cAMP-dependent and/or Ca\(^{2+}\) -calmodulin-dependent phosphorylation of key calcium-handling proteins such as the sarcoplasmic reticulum calcium channel (39), the ryanodine receptor (40, 41), and the SR Ca\(^{2+}\)-ATPase (42). While the functional implications of these phosphorylations is currently unclear, it is possible that isoproterenol stimulation may alter the activities of these proteins, thereby increasing the cytosolic calcium available for contraction and leading to enhanced force production. In the transgenic EDL muscles, isoproterenol stimulation was also associated with a 30% increase in developed force and had no effect on the contraction or half-relaxation times, consistent with its effects in the wild-type EDL muscles. Thus, isoproterenol could not reverse the prolonged half-relaxation time associated with phospholamban expression in this muscle. These findings in fast-twitch skeletal muscle appear to be in contrast with our recent observations in the murine soleus, which suggested that phospholamban was a key modulator of the effects of isoproterenol on the half-relaxation time of mouse slow-twitch skeletal muscle (27). Therefore, we postulated that the failure of isoproterenol to reverse the inhibitory effects of phospholamban might be due to the lack of phospholamban phosphorylation in the transgenic muscles. Actually, previous studies in rat cardiomyocytes suggested that phosphorylation of phospholamban may occur during \(\beta_1\)- but not \(\beta_2\)-adrenergic receptor activation (36). To determine which receptor subtype mediates the effects of isoproterenol in the EDL muscle, we used specific \(\beta_1\)- and \(\beta_2\)-adrenergic receptor antagonists. Our results indicate that the effects of isoproterenol in mouse fast-twitch skeletal muscle are mediated via the \(\beta_2\)-adrenergic receptor cascade. In addition, Western blot analysis of transgenic muscles, using antibodies that specifically recognize phosphoserine-16 or phosphothreonine-17 in phospholamban (24, 25), revealed that isoproterenol stimulation was not associated with any detectable phosphorylation of phospholamban. Together, these findings suggest that the effects of isoproterenol in mouse fast-twitch skeletal muscle occur through activation of \(\beta_2\)-adrenergic receptors, which are not coupled to phospholamban phosphorylation. These results in fast-twitch skeletal muscle are consistent with those of Xiao et al. (35), who reported that specific activation of \(\beta_2\)-adrenergic receptors in rat cardiac myocytes was not associated with changes in Ca\(^{2+}\) dynamics, contractility, and phospholamban phosphorylation.

In summary, our findings indicate that the fast-twitch skel-
etal muscle isoform of the SR Ca\(^{2+}\)-ATPase may be regulated by phospholamban in vivo and is associated with significant decreases in the rate of relaxation of transgenic muscles. Thus, induction of phospholamban expression in fast-twitch skeletal muscle would result in some functional alterations resembling its slow-twitch counterpart and therefore compromise the physiological responsiveness of fast-twitch fibers especially in “fight or flight” situations. Future studies designed to elucidate the mechanisms involved in dictating tissue-specific expression of phospholamban may identify the factors/elements missing in fast-twitch skeletal muscle, which prevent expression of this important regulatory protein in vivo.

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