Regulation of Ca\(^{2+}\) handling by phosphorylation status in mouse fast- and slow-twitch skeletal muscle fibers

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Liu, Yeegei, Evangelia G. Kranias, and Martin F. Schneider. Regulation of Ca\(^{2+}\) handling by phosphorylation status in mouse fast- and slow-twitch skeletal muscle fibers. Am. J. Physiol. Cell Physiol. 42: C1915–C1924, 1997.—The effects of phosphorylation status on Ca\(^{2+}\) release and Ca\(^{2+}\) removal were studied in fast-twitch flexor digitorum brevis and slow-twitch soleus skeletal muscle fibers enzymatically isolated from wild-type and phospholamban knockout (PLBko) mice. In all fibers the adenosine 3',5'-cyclic monophosphate-dependent protein kinase (PKA) inhibitor H-89 decreased the peak amplitude of the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) transient for a single action potential, and the PKA activator dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP) reversed this effect, indicating modulation of Ca\(^{2+}\) release by phosphorylation status in all fibers. H-89 decreased the decay rate constant of the [Ca\(^{2+}\)] transient and DBcAMP reversed this effect only in phospholamban-expressing fibers (wild-type soleus), indicating modulation of Ca\(^{2+}\) removal only in the presence of phospholamban. A high basal level of PKA phosphorylation in soleus fibers maintained under our control conditions was indicated by the lack of effect of direct application of DBcAMP on Ca\(^{2+}\) release or Ca\(^{2+}\) removal in wild-type or PLBko soleus fibers and was confirmed by analysis of phospholamban from wild-type soleus fibers.

Phospholamban; sarcoplasmic reticulum; calcium release; excitation-contraction coupling

INTRACELLULAR CALCIUM RELEASE and removal play a central role in the activation and relaxation of skeletal muscle. The rapid release of Ca\(^{2+}\) from the sarcoplasmic reticulum in skeletal muscle is initiated by electrical depolarization of the transverse tubule membrane, which is sensed by charged intramembrane transverse tubule voltage sensors/dihydropyridine receptors (21). The resulting Ca\(^{2+}\) release occurs via Ca\(^{2+}\)-release channels in sarcoplasmic reticulum ryanodine receptors, which are located in the sarcoplasmic reticulum junctional face membrane (8). The ryanodine receptor has a large cytosolic domain that spans the gap between the sarcoplasmic reticulum and the densely opposed transverse tubule membrane, apparently contacting the transverse tubule cytosolic surface (3). Our knowledge about the regulation of sarcoplasmic reticulum Ca\(^{2+}\) release is mainly based on experiments with isolated sarcoplasmic reticulum vesicles and vesicles or release channels reconstituted into planar lipid bilayer membranes, which allow insight into the sarcoplasmic reticulum Ca\(^{2+}\) release on the single-channel level (12). Despite the clear demonstration of the effects of phosphorylation on the ryanodine receptor in vitro with the planar bilayer technique (12, 13), the functional impact of the phosphorylation reaction has not been clearly established in functioning skeletal muscle fibers.

Once Ca\(^{2+}\) has been released, it must be removed from the cytoplasm for relaxation to occur. Ca\(^{2+}\) uptake by the sarcoplasmic reticulum, which plays a major role in the control of muscle relaxation, is performed by sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (15, 20). There are three main isoforms of SERCAs. The SERCA1 gene is exclusively expressed in fast-twitch skeletal muscle, whereas the SERCA2 gene is expressed in cardiac muscle, slow-twitch skeletal muscle, and some other tissues (2). The activity of SERCAs in slow-twitch skeletal muscle and cardiac muscle is modulated by the regulatory, membrane-bound protein phospholamban. Phospholamban is a 25- to 27-kDa protein composed of five identical monomers present in slow-twitch fibers and cardiac myocytes but not in fast-twitch fibers (10). It can be phosphorylated by adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) and Ca\(^{2+}\)/calmodulin-dependent protein kinase at Ser-16 and Thr-17 (23). Phospholamban, in the dephosphorylated state, has been shown to be an inhibitor of the affinity of SERCA2 for Ca\(^{2+}\), and phosphorylation can relieve this inhibition (16, 17). In this way the sarcoplasmic reticulum Ca\(^{2+}\) pump can be dynamically regulated by phosphorylation-dephosphorylation of phospholamban.

A previous report used measurements of the rate of relaxation of whole soleus muscle from wild-type or phospholamban knockout mice to characterize the effects of phospholamban phosphorylation on the rate of Ca\(^{2+}\) uptake by the sarcoplasmic reticulum (27). In the present report, we use measurements of Ca\(^{2+}\) transients to study the effects of phosphorylation-dephosphorylation on both Ca\(^{2+}\) release and Ca\(^{2+}\) removal during excitation-contraction coupling in mouse single intact slow-twitch skeletal muscle fibers and compared them with the effects in fast-twitch skeletal muscle fibers, which lack phospholamban. We also used phospholamban knockout mice to further investigate the role of phospholamban in regulating SERCA function in functioning single slow-twitch skeletal muscle fibers.

METHODS

Generation of phospholamban knockout mice. The phospholamban gene was targeted in embryonic stem cells, and...
phospholamban-deficient or knockout mice were generated as previously described (19).

Enzymatic dissociation and primary culture of flexor digitorum brevis or soleus muscle fibers. Single muscle fibers were dissociated from flexor digitorum brevis (FDB) muscles and sole muscles of 4- to 5-wk-old mice (CD-1, Charles River Laboratories) and soleus muscles of 7- to 8-wk-old phospholamban knockout mice and age-matched wild-type 129J mice essentially as described previously (1, 6), with slight modification. FDB or soleus muscles were dissected and pinned by the tendons to a Sylgard-lined dissecting chamber. The outer connective tissue was removed, and the muscles were immersed in 5 ml of minimum essential medium (MEM; Gibco BRL) with 10% fetal bovine serum (Biofluid) containing 0.2% collagenase (Sigma, type I). After incubation at 37°C in a water-saturated incubator with 5% CO2-95% air for ~3 h, the muscles were transferred with a wide-mouth Pasteur pipette into 5 ml of MEM, teased with forceps, and triturated to release single fibers.

Tissue culture plastic will not adequately transmit light in the near-ultraviolet (UV) wavelength range needed to excite fura 2. Therefore, fibers were cultured in 35-mm plastic petri dishes with a 10-mm-diameter hole through the center and a glass coverslip permanently glued to the bottom with silicone rubber sealant to form a well. All petri dishes were sterilized with UV light before use. Sterilized phosphate-buffered saline (PBS; 80 µl) was pipetted into the well, and 1 µl of laminin (Sigma, 1 µg/µl) was added to the PBS. The dishes were put in the culture hood at room temperature for at least 2 h. Extra solution was removed, and 2 ml MEM containing 10% fetal bovine serum and 50 µg/ml gentamicin sulfate were introduced into the dishes. About 10-20 dissociated single FDB fibers or 2-5 single soleus fibers were transferred directly to the bottom of the well. Fibers were cultured at 37°C in a water-saturated incubator in 5% CO2-95% air. Fiber cultures were used for Ca2+ recording within 24 h of plating.

Ca2+ recording. Intracellular Ca2+ concentration ([Ca2+]i) was measured with the fluorescent Ca2+-sensitive indicator fura 2 (Molecular Probes) as previously described (6, 18). Before loading with fura 2, the fibers in the culture dish were rinsed with Ringer solution, consisting of (in mM) 135 NaCl, 4 KCl, 1.6 MgCl2, 10 NaHCO3, 1.8 CaCl2, and 1 µM CaCl2 (pH 7.4) to wash out the culture medium. Fibers were then incubated with Ringer solution containing 1 µM acetoxyethyl ester of fura 2 (fura 2-AM) for 30 min at room temperature. After the fibers were loaded with fura 2, the solution was exchanged with Ringer solution to remove unincorporated fura 2-AM, and the fibers were allowed to deesterify for an additional 20 min. The low ester concentration and the low temperature during loading were chosen to minimize problems arising from compartmentalization of fura 2 within the fibers.

The experimental apparatus used for intracellular Ca2+ measurement was constructed around an inverted microscope using an Olympus ×40 fluor objective. The fiber culture chamber was placed on the microscope stage and studied at an ambient temperature of ~28°C in an enclosed space. Two platinum electrodes were connected to a computer-controlled stimulator placed into the fiber culture chamber to give field stimulation. UV light, emitted from a 75-W xenon arc lamp, was passed through an interference filter whereby the 380- or 358-nm excitation wavelength was selected. The diameter of the UV light-illuminated area could be altered by selecting different apertures so that only a short length of a single fiber was illuminated. An electronic shutter was used to restrict fluorescence excitation to the sampling interval, and the illuminating light was reduced by using a neutral density filter. We found no detectable change in the fluorescence due to photobleaching. Fluorescence emitted from a muscle fiber was collected by the objective lens, passed through a 40-nm band-pass filter centered at 510 nm, and transmitted to the photomultiplier tube. The signal from the photomultiplier tube was filtered at 430 Hz, digitized, and stored in a computer for off-line analysis. The signal was sampled repeatedly at 200-µs intervals, with each point in the stored records consisting of the average of five such successive determinations. Each stored record contained 450 points, corresponding to 450 ms of the sampled signal. The subtraction of background fluorescence was done by offsetting the photomultiplier tube outputs during the measurement of fluorescence by the fluorescence value measured from an area of coverslip without fibers at the beginning of each experiment.

Fibers were stimulated by a single pulse of 1-ms duration generated by a stimulator controlled by a computer. Each fiber was stimulated several times with the fiber illuminated alternatively at 380 nm (Ca2+-sensitive wavelength) or 358 nm (Ca2+-insensitive isosbestic wavelength) to give records of the fluorescence (F) for 380 or 358 nm excitation, F380 or F358, respectively. The ratio (R) F380/F358 was then calculated. F358 was used to correct possible movement artifacts (6, 18).

[Ca2+]i was calculated from fura 2 fluorescence considering the Ca2+ binding and unbinding kinetics in the Ca2+-fura 2 reaction using the equation (11, 18)

$$[Ca^{2+}] = K_d (R - R_{min} + k_{off} \cdot dR/dt)/(R_{max} - R)$$

where Rmin is the ratio under Ca2+-free conditions, Rmax is the ratio at saturating Ca2+ and Kd and koff are the dissociation constant and reverse rate constant, respectively, for the Ca2+-fura 2 reaction (3, 8, 19). The value of koff was set to 26 s−1, as determined in voltage-clamped cut rat extensor digitorum longus muscle fibers by fitting optical signals recorded simultaneously with antipyrylazo III and fura 2 in our laboratory (8). Kd is a scale factor in the above equation and will not alter the waveform of the Ca2+ transient. Here Kd was set to 70 nM. If the Kd for fura 2 in the fiber was actually higher than the 70 nM value assumed here, the [Ca2+]i transients calculated with a Kd of 70 nM and presented should be simply scaled up by the ratio of Kd to 70 nM. To determine the values of Rmax and Rmin, we performed in vivo calibrations. Rmax was determined by incubating fibers in Ringer solution and 10 µM ionomycin. Rmin was obtained by pressure injection of 0.5 M ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid into the fibers. Rmin was determined when the injection no longer significantly reduced the ratio of intracellular fluorescence. Intracellular resting [Ca2+]i was calculated from R in resting fibers with dR/dt set equal to zero.

For experiments concerning the effects of dibutylryl adenosine 3′,5′-cyclic monophosphate (DBcAMP), measurements were made on the same fiber before and after a 10-min exposure to DBcAMP, when a new steady-state condition was obtained. For experiments with N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinoliniumsulfonamide (H-89) and DBcAMP, a given individual fiber was studied under control conditions and after a 10-min exposure to H-89. The fiber was then rinsed with Ringer solution, DBcAMP was applied for 10 min, and a final set of measurements were made in the presence of DBcAMP.

Phospholamban immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Wegener and Jones (28) to detect the
relative amount of phospholamban phosphorylation by the resulting characteristic mobility shift. About 40 single dissociated soleus fibers from CD-1 mice, soleus fibers from wild-type 129 J mice, or FDB fibers from CD-1 mice were incubated in 0.5 ml of Ringer solution, with appropriate chemicals in the same concentrations as used for the Ca\(^{2+}\) measurement experiments. At the end of the incubation, 0.5 ml of 5% SDS (GIBCO BRL) containing 2 µM okadaic acid (LC Laboratories) was added to the fiber suspension to suppress phosphatase activity and possible consequent phospholamban dephosphorylation during sample preparation. The suspension was then quickly vortexed and frozen at −20°C. The fiber suspension was thawed and centrifuged at 14,000 g for 10 min at 4°C. The supernatant was assayed for protein concentration by the Bradford method, and 50 µg of protein were loaded per gel lane. Electrophoresis was conducted on 12% polyacrylamide gels (precast gels from NOVEX), and isolated protein was transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blotted with phospholamban monoclonal antibody (clone 2D12, from Affinity Bioreagent) at a 1:1,200 dilution followed by goat anti-mouse antibody conjugated with horseradish peroxidase (Amer sham). Chemiluminescence was detected with the enhanced chemiluminescence system (Amersham).

DBcAMP (Sigma) and H-89 (LC Laboratories) were dissolved in dimethyl sulfoxide (DMSO). Stock solutions were diluted in Ringer solution immediately before use. The concentration of DMSO in the Ringer solution did not exceed 0.1% (vol/vol).

All values are expressed as means ± SE for n observations. The paired Student's t-test was used to determine the significance of difference. A value of P < 0.05 was considered significant.

RESULTS

Fibers were stimulated by a single suprathreshold pulse of 1 ms duration (6). In response to the action potential resulting from field stimulation, [Ca\(^{2+}\)] increased rapidly to a peak and decayed first rapidly and then more slowly to the level before stimulation in FDB and soleus fibers from wild-type mice and in soleus fibers from phospholamban knockout mice (Fig. 1, full records).

The peak and final decline of the Ca\(^{2+}\) transient can be used, respectively, to assess the effects of phosphorylation by PKA on Ca\(^{2+}\) release and removal. The peak

![Fig. 1. Ca\(^{2+}\) transients were recorded from a flexor digitorum brevis (FDB) fiber from CD-1 mouse (1st row), a soleus fiber from CD-1 mouse (2nd row), a soleus fiber from wild-type 129 J mouse (3rd row), and a soleus fiber from phospholamban knockout mouse (4th row), before, during exposure to 1 µM H-89, and during exposure to 10 µM dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP; left to right), respectively. Record segments between the vertical marks below each full record were vertically expanded and are shown (insets) as dots with single exponential fits superimposed as solid lines. First point of each expanded record is 10th ms after the stimulation.](http://ajpcell.physiology.org/issue)
amplitude of the Ca\textsuperscript{2+} transient was used as an index of the function of all steps in the Ca\textsuperscript{2+}-release process during excitation-contraction coupling. The plasma membrane and the transverse tubule ion channels underlying the action potential, the transverse tubule voltage sensors controlling the sarcoplasmic reticulum Ca\textsuperscript{2+}-release channels, and the sarcoplasmic reticulum Ca\textsuperscript{2+}-release channels, as well as the sarcoplasmic reticulum Ca\textsuperscript{2+} content, all contribute to the amount of Ca\textsuperscript{2+} released and the amplitude of the resulting Ca\textsuperscript{2+} transient. The slow phase of decline of the change in [Ca\textsuperscript{2+}] after an action potential (Fig. 1, vertically expanded partial records) occurs after Ca\textsuperscript{2+} release is completed and is determined by the parallel action of myoplasmic binding sites and membrane transport systems. Considering the limited role of Ca\textsuperscript{2+} flux into or out of a fiber during and after activation of skeletal muscle, the major contributions to Ca\textsuperscript{2+} binding and transport would be given by troponin C, the sarcoplasmic reticulum Ca\textsuperscript{2+}-pump, and, in a fast-twitch fiber, parvalbumin. The final decay of the Ca\textsuperscript{2+} transients after stimulation can be well fitted by a single exponential plus a constant (6, 11), as shown by the fits superimposed on the Ca\textsuperscript{2+} transients in Fig. 1. The interval for the exponential fit began 10 ms after stimulation and lasted 380 ms. The decay rate constant from the fit provides an empirical characterization of the decay of Δ[Ca\textsuperscript{2+}] after stimulation, giving an estimate of the rate of uptake of Ca\textsuperscript{2+} from the myoplasm by the sarcoplasmic reticulum Ca\textsuperscript{2+}-pump in slow-twitch fibers, which lack parvalbumin (7), and of the combined effects of Ca\textsuperscript{2+} uptake by the sarcoplasmic reticulum Ca\textsuperscript{2+}-pump and Ca\textsuperscript{2+} binding to parvalbumin in fast-twitch fibers. Ca\textsuperscript{2+} binding to troponin C is relatively rapid, so that troponin C is likely to be in equilibrium with the myoplasmic [Ca\textsuperscript{2+}] during the slower phase of decay of [Ca\textsuperscript{2+}] (11, 20).

Effects of phosphorylation state on Ca\textsuperscript{2+} handling in FDB fibers. We first studied the effects of 10 µM DBcAMP on the peak amplitude and the decay rate constant of Ca\textsuperscript{2+} transients in FDB fibers from wild-type CD-1 mice (Fig. 2, first and second bars of each panel). The peak amplitude of the Ca\textsuperscript{2+} transient (Fig. 2, A and C) was increased from 2.19 ± 0.46 µM in control to 2.66 ± 0.37 µM in 10 µM DBcAMP (n = 7, P < 0.05; Fig. 2A). To remove the effects of fiber-to-fiber variability, each value of peak Δ[Ca\textsuperscript{2+}] after drug treatment in a particular fiber was normalized to the value of peak Δ[Ca\textsuperscript{2+}] in the same fiber under control conditions before any drug application. A similar normalization procedure was applied to the decay rate constant of the slow decay of [Ca\textsuperscript{2+}] after the pulse in each fiber. With the use of such normalized values, the mean percentage increase in the peak of the Ca\textsuperscript{2+} transient in the individual fibers was 30 ± 11% (Fig. 2C). The rate constants for decay of Δ[Ca\textsuperscript{2+}] (Fig. 2, B and D) were 95 ± 6 and 92 ± 9 s\textsuperscript{-1} (n = 7, P > 0.05; Fig. 2D) before and after the application of 10 µM DBcAMP, respectively, and the mean of the percentage change in the normalized value was 4 ± 7% (Fig. 2D). Thus, application of DBcAMP to FDB fibers caused a modest...
increase in Ca\(^{2+}\) release, as indicated by the significant increase in the peak of the \([\text{Ca}^{2+}]\) transient, but caused no change in the rate of Ca\(^{2+}\) binding and transport during the slow decay of \([\text{Ca}^{2+}]\) after Ca\(^{2+}\) release.

In another group of FDB fibers, the effects of the specific PKA inhibitor H-89 were examined (Fig. 2, third and fourth bars of each panel). As shown in Fig. 2, A and C, 1 µM H-89 decreased the amplitude of Ca\(^{2+}\) transients from 2.60 ± 0.43 µM in control fibers (third bar) to 1.38 ± 0.32 µM (fourth bar; n = 7, P < 0.05). After dishes were rinsed with Ringer solution, the fibers were exposed to 10 µM DBcAMP (fifth bar). DBcAMP partially reversed the inhibitory effects of H-89 on the amplitude of the Ca\(^{2+}\) transients, increasing the peak of the Ca\(^{2+}\) transients to 2.17 ± 0.25 µM (P < 0.05, compared with in the presence of 1 µM H-89).

With the use of normalized values for each fiber, it was shown that H-89 decreased the Ca\(^{2+}\) transient amplitude to 53 ± 7% of the control value (P < 0.05) and that subsequent application of DBcAMP returned it to 93 ± 10% of control. The observation that the Ca\(^{2+}\) transients in DBcAMP after exposure to and washout of H-89 (third and fifth bars in Fig. 2, A and C) were not increased to a level similar to that obtained after direct application of DBcAMP without prior H-89 (first and second bars) indicates that the inhibitory effects of H-89 were probably not completely reversed after washout. In these experiments extensive washing of fibers was not practical because of possible detachment of muscle fibers from the laminin-coated coverslip of the experimental chamber.

There were no significant changes in decay rate constant of the Ca\(^{2+}\) transient in FDB fibers either after application of H-89 or after application of DBcAMP (Fig. 2, B and D), even though the changes in Ca\(^{2+}\) release in the same fiber indicated that PKA activity had been modified in these fibers (Fig. 2, A and C). This is consistent with a lack of direct modulation of the SERCA by phosphorylation in the FDB fibers, which lack the sarcoplasmic reticulum modulatory protein phospholamban.

Effects of phosphorylation state on Ca\(^{2+}\) handling in soleus fibers. Unlike the situation in fast-twitch skeletal muscle fibers, the SERCA in slow-twitch fibers may be regulated by phosphorylation of phospholamban, which is present in slow-twitch fibers. The effects of activating PKA on Ca\(^{2+}\) release and Ca\(^{2+}\) uptake were therefore examined in soleus fibers (Fig. 3). Surprisingly, direct application of 10 µM DBcAMP had little or no effect on the amplitude or decay rate constant of Ca\(^{2+}\) transients in soleus fibers (Fig. 3, first and second bars of each panel). Although the Ca\(^{2+}\) transient amplitudes were slightly increased, from 1.29 ± 0.08 to 1.38 ± 0.10 µM (n = 10, P < 0.05; Fig. 3A), after application of 10 µM DBcAMP, the average increase was only 7% (Fig. 3C). The decay rate constants were 39 ± 5 and 40 ± 5 s\(^{-1}\) (n = 10, P > 0.05; Fig. 3B) before and after DBcAMP, respectively. A possible explanation for the lack of effects of direct application of DBcAMP on Ca\(^{2+}\) release or transport in soleus fibers could be that the relevant proteins were already maximally phosphorylated in soleus fibers before the application
of DBcAMP to the isolated soleus fibers under our experimental conditions. As in FDB fibers, H-89 was used to examine this possibility. As depicted in Fig. 3, H-89 caused a 27 ± 7% decrease in the amplitude of the Ca\(^{2+}\) transient (Fig. 3C) and a 26 ± 7% decrease in the decay rate constant (Fig. 3D) of the Ca\(^{2+}\) transient in soleus fibers. After H-89 was washed out and DBcAMP was added, recovery in the peak amplitude and in the decay rate constant of the Ca\(^{2+}\) transients was consistently observed (Fig. 3, third through fifth bars of each panel).

To verify our interpretation of the high basal phosphorylation under control conditions in soleus fibers, we directly examined the phosphorylation state of phospholamban in parallel biochemical studies on soleus fibers under similar experimental conditions. Phosphorylation of phospholamban induces a conformational change in the protein, resulting in a mobility shift of phospholamban on SDS-PAGE, which was used as an indication of phospholamban phosphorylation (28, 29). Figure 4 illustrates phospholamban mobility in protein samples from soleus fibers under control conditions (lane 1), in the presence of 1 µM H-89 (lane 2), and in the presence of 10 µM DBcAMP after washout of H-89 (lane 3). In lane 2 the apparent molecular mass of phospholamban was ~25 kDa, indicating predominantly unphosphorylated phospholamban. In contrast, in lanes 1 and 3, the apparent molecular mass of phospholamban was ~27 kDa, indicating phosphorylated phospholamban. These results indicate that both in the control condition and in the presence of 10 µM DBcAMP after H-89, phospholamban was in the phosphorylated state. Phospholamban remained in the unphosphorylated state only in the presence of H-89. Similar changes in phospholamban phosphorylation status were observed in soleus fibers from 129 J wild-type mice (lanes 4–6) under the same experimental conditions. These observations are consistent with the physiological results concerning the decay of [Ca\(^{2+}\)] in soleus fibers illustrated in Fig. 3.

As expected, phospholamban was not detected in FDB fibers under control or experimental conditions (Fig. 4, lanes 7–9) or in soleus fibers from phospholamban knockout mice (not shown).

Ca\(^{2+}\) handling in phospholamban knockout mice. In soleus muscle of phospholamban knockout mice, all fibers must lack phospholamban. In such fibers any observed response of the rate of sarcoplasmic reticulum Ca\(^{2+}\) uptake to protein kinase activators or inhibitors could have nothing to do with phospholamban. Figure 5 shows the effects of applying H-89 and then DBcAMP on the Ca\(^{2+}\) transient amplitude and decay rate constants of soleus fibers from phospholamban knockout mice. As in the case of soleus fibers from wild-type mice, 1 µM H-89 inhibited the Ca\(^{2+}\) transient amplitude to 76 ± 9% of control. After washing out H-89, DBcAMP (10 µM) reversed the inhibitory effects of H-89 and increased the Ca\(^{2+}\) transient amplitude to 98 ± 8% of the control value (Fig. 5, A and C). Based on the observed effects of these agents, PKA was apparently activated to a similar extent in knockout mice as in wild-type mice. However, unlike the situation in soleus fibers of wild-type mice, neither 1 µM H-89 nor 10 µM DBcAMP had any effects on the rate of decay of Ca\(^{2+}\) transients in soleus fibers from phospholamban knockout mice (Fig. 5, B and D). Note that the three bars in Figs. 5 and 6 represent the same pharmacological conditions as represented by the third through fifth bars, respectively, in Figs. 2 and 3.

The effects of stimulating or inhibiting PKA on Ca\(^{2+}\) handling were also examined in soleus fibers from age-matched 129 J wild-type mice (Fig. 6) and were compared with results from the phospholamban knockout mouse (Fig. 5). Consistent with a high basal phosphorylation, neither the Ca\(^{2+}\) transient amplitudes (1.63 ± 0.25 and 1.78 ± 0.30 µM; n = 7, P > 0.05) nor the decay rate constants (50 ± 8 and 49 ± 8 s\(^{-1}\); n = 7, P > 0.05) were significantly changed after the application of 10 µM DBcAMP. As shown in Fig. 6, H-89 decreased the amplitude of the Ca\(^{2+}\) transient by 24 ± 5% (Fig. 6, A and C), a similar percentage and amount as in soleus fibers from phospholamban knockout mice (Fig. 5). In contrast, H-89 decreased the decay rate constant of the Ca\(^{2+}\) transient by 31 ± 8% (Fig. 6, B and D) in soleus fibers from 129 J wild-type mice (Fig. 6), whereas H-89 had no effects on the Ca\(^{2+}\) decay rate constant in soleus fibers from phospholamban knockout mice (Fig. 5). Subsequent application of 10 µM DBcAMP antagonized both effects of H-89 on soleus fibers from 129 J wild-type mice (Fig. 6). Indeed, as can be seen from comparison of Figs. 5 and 6 under control conditions, the basal values of the amplitude and decay rate constants of Ca\(^{2+}\) transients in soleus fibers of phospholamban knockout mice were similar to the

![Fig. 4. Western blot demonstration of phosphorylation state of phospholamban from dissociated soleus and FDB fibers. Fibers were dissociated from soleus muscles of CD-1 mouse and 129 J wild-type mouse and from FDB muscle of CD-1 mouse by same method as in Ca\(^{2+}\) measurements. Lanes 1, 2, and 3: soleus fibers from CD-1 mouse; lanes 4, 5, and 6: soleus fibers from 129 J wild-type mouse; lanes 7, 8, and 9: FDB fibers from CD-1 mouse. Lanes 1, 4, and 7 show proteins from fibers under control conditions. Lanes 2, 5, and 8 are from fibers treated with 1 µM H-89 for 10 min. Lanes 3, 6, and 9 are from fibers treated with 10 µM DBcAMP for 10 min after washout of H-89. In lanes 1, 3, 4, and 6 the mobility of phospholamban was shifted from 25 to 27 kDa.](http://ajpcell.physiology.org/)

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Fig. 5. Effect of H-89 and DBcAMP after H-89 on Ca\(^{2+}\) transient amplitude (A and C) and decay rate constant (B and D) in phospholamban knockout mouse soleus fibers (n = 9). A and C give mean values and C and D present mean value of each parameter relative to control conditions in same fiber. The three bars in this figure represent the same conditions as in the 3rd through 5th bars, respectively, in Figs. 2 and 3.

Fig. 6. Effect of H-89 and DBcAMP on Ca\(^{2+}\) transient amplitude (A and C) and decay rate constant (B and D) in wild-type 129 J mouse soleus fibers (n = 7); same format as in Fig. 5.
values from wild-type mice, despite the respective absence or presence of phospholamban. This suggests that there was a high basal level of phospholamban phosphorylation in cultured, enzymatically isolated wild-type soleus fibers under these experimental conditions. As a result of this kind of basal phosphorylation, DBcAMP cannot increase the decay rate constant in control wild-type soleus muscle, since there is no inhibition from phospholamban on sarcoplasmic reticulum ATPases in the basal state in our experiments on enzymatically dissociated soleus fibers maintained in culture for ~20 h before the experiments. In contrast, the basal state of phosphorylation of phospholamban does not appear to be complete in whole soleus muscles isolated from wild-type mice and studied under other experimental conditions after ~1 h of equilibration in Krebs-Henseleit solution (26).

For FDB fibers from phospholamban knockout mice, the peak of the Ca\textsuperscript{2+} transient was 2.63 ± 0.34, 1.35 ± 0.28, and 2.35 ± 0.44 µM (n = 10) in control, H-89, and DBcAMP after H-89, respectively. The mean relative values in the presence of H-89 and DBcAMP after H-89 compared with control conditions were 55 ± 10% and 91 ± 13%, similar to the values from the FDB fibers of wild-type CD-1 mice. The values for the decay rate constant of the Ca\textsuperscript{2+} transient were 96 ± 6, 97 ± 7, and 98 ± 7 s\textsuperscript{-1} in control, H-89, and DBcAMP after H-89, respectively.

No significant changes in resting [Ca\textsuperscript{2+}] were observed after application of 10 µM DBcAMP, 1 µM H-89, or 10 µM DBcAMP after washout of H-89 in FDB or soleus fibers from wild-type or phospholamban knockout mice.

**DISCUSSION**

Protein phosphorylation and dephosphorylation are important mechanisms underlying regulation of excitation-contraction coupling in skeletal and cardiac muscle. Kinases in skeletal muscle include PKA and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase. Proteins of both the sarcolemma and sarcoplasmic reticulum may be regulated by phosphorylation (12, 13, 25). Using intact single fast-twitch and slow-twitch skeletal muscle fibers, we analyzed the effects of inhibition or activation of PKA on Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} removal under conditions in which cellular integrity is maintained close to in vivo conditions.

Specific protein kinase inhibitors can be useful tools in determining the physiological significance of particular protein kinase systems. H-89 is a relatively specific inhibitor of PKA (14). The K_i value for PKA is 0.048 µM. The K_i value of H-89 for guanosine 3',5'-cyclic monophosphate-dependent protein kinase is 10 times higher, showing that the inhibitor is relatively selective for PKA. H-89 was shown to have a much less potent effect on Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II, with K_i of 70 µM. Because the concentration of H-89 used in our experiment was ~20 times higher than its K_i on PKA, we expected near-maximum inhibition of PKA.

Dissection of the effects of phosphorylation status on the sarcoplasmic reticulum Ca\textsuperscript{2+}-release system from those that may be due to the sarcoplasmic reticulum Ca\textsuperscript{2+} pump presents a major challenge. Any change in activity of protein kinases that affects the phosphorylation state of proteins controlling Ca\textsuperscript{2+} release may also result in changes in the phosphorylation state of phospholamban. Thus changes in SERCA activity, which could possibly cause changes in sarcoplasmic reticulum Ca\textsuperscript{2+} content and, in turn, alter the amount of Ca\textsuperscript{2+} released, could occur concomitantly with changes due to the direct effects of phosphorylation of components of the Ca\textsuperscript{2+}-release system. Fast-twitch muscle fibers, as well as slow-twitch fibers from phospholamban knockout mice, are ideal models to use for investigation of direct effects of phosphorylation on Ca\textsuperscript{2+} release, since, in contrast to slow-twitch muscle from wild-type mice, they lack phospholamban. In such fibers SERCA activity is unaffected by PKA (Figs. 2 and 5) because of the absence of phospholamban (Fig. 4). Thus changes in Ca\textsuperscript{2+} transients in fast-twitch muscle fibers or in slow-twitch fibers from phospholamban knockout mice cannot be attributed to changes in sarcoplasmic reticulum Ca\textsuperscript{2+} content resulting from modulation of sarcoplasmic reticulum pump activity.

Voltage-dependent Na\textsuperscript{+} channels are modulated by PKA (9). To ensure that the effects of H-89 and DBcAMP on Ca\textsuperscript{2+} transients observed in our study had no relation with Na\textsuperscript{+} channels, we observed the possible effects of H-89 or DBcAMP on the voltage threshold of stimulation. The Ca\textsuperscript{2+} transient amplitude in the presence or absence of H-89 or DBcAMP was monitored. After 10 min of application of H-89 or DBcAMP, increasing stimulation voltage did not result in any change of Ca\textsuperscript{2+} transient amplitude (data not shown). These findings suggest that the inhibitory effects of H-89 or stimulatory effects of DBcAMP on Ca\textsuperscript{2+} transients are not due to their possible action on Na\textsuperscript{+} channels, at least for the concentrations we used.

It has been shown that the sarcolemmal dihydropyridine receptor/Ca\textsuperscript{2+} channel in cardiac muscle is a substrate of PKA and that phosphorylation enhances Ca\textsuperscript{2+} influx. In skeletal muscle the dihydropyridine receptor acts as a voltage sensor instead of a Ca\textsuperscript{2+} channel (23). The transient increase of Ca\textsuperscript{2+} in cytoplasm in response to depolarization of skeletal muscle fibers is contributed almost exclusively by influx from the sarcoplasmic reticulum, without significant Ca\textsuperscript{2+} influx. Although there are reports that the dihydropyridine receptor in skeletal muscle may be phosphorylated by PKA, its functional consequence are unknown (22).

Ser-2843 in the skeletal muscle sarcoplasmic reticulum Ca\textsuperscript{2+}-release channel/ryanodine receptor is a major target for PKA (13, 27). Using the planar lipid bilayer technique, Hain et al. (12) found that channel recovery from Mg\textsuperscript{2+} block was obtained by exogenous PKA or by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II and that channel block by millimolar Mg\textsuperscript{2+}, which is believed to be physiological concentration, was restored by dephosphorylation using protein phosphatase. Their results suggest that only the phosphorylated Ca\textsuperscript{2+} channel is active under physiological conditions. Our findings concerning Ca\textsuperscript{2+} release from intact fibers are consis-
tent with these results from single-channel recordings. In our system the response of FDB fibers to 10 µM DBcAMP was only a 23% increase of the peak of the Ca\textsuperscript{2+} transient. Considering the relatively high concentration of DBcAMP that we used, the negligible or relatively moderate increase could mean that, in the steady state under our experimental conditions, all or most of the Ca\textsuperscript{2+}-release channels were already in the phosphorylated state in soleus or FDB fibers, respectively. In a few of the FDB fibers we found that, after application of 1 µM H-89, there was no Ca\textsuperscript{2+} transient on stimulation, even if much higher voltage was used. But after the fibers were thoroughly rinsed and 10 µM DBcAMP was added, the Ca\textsuperscript{2+} transients were restored. Fibers exhibiting this behavior were not included in our results because we cannot obtain Ca\textsuperscript{2+} transient decay rate constants from them. However, these observations support the idea that the phosphorylated state may be essential for the Ca\textsuperscript{2+}-release channel to open in response to membrane depolarization during excitation-contraction coupling.

In intact cardiac myocytes, loading the cells with specific antibody to phospholamban greatly enhanced Ca\textsuperscript{2+} uptake by sarcoplasmic reticulum. B-Receptor agonists could not increase the rate of relaxation in such antibody-loaded cells (24). Compared with the role played in cardiac cells, phospholamban may be much less important in slow-twitch skeletal muscle. Briggs et al. (4) noted that antibody to phospholamban produced a much weaker stimulation of Ca\textsuperscript{2+} uptake in sarcoplasmic reticulum vesicles from slow-twitch muscle than in cardiac sarcoplasmic reticulum vesicles. Although the antibody increased the rate of Ca\textsuperscript{2+} uptake by cardiac sarcoplasmic reticulum vesicles 15-fold, Ca\textsuperscript{2+} uptake by vastus intermedius (slow-twitch muscle) sarcoplasmic reticulum vesicles was only increased 1.5-fold. In our experiments we found that inhibition of PKA causes a 26% suppression of the rate of Ca\textsuperscript{2+} uptake in soleus fibers from wild-type CD-1 mice and a 31% suppression in soleus fibers from wild-type 129J mice. Both effects were largely reversed by subsequent treatment with DBcAMP. Assuming that, in soleus fibers, which lack the Ca\textsuperscript{2+} binding protein parvalbumin, the rate of decay of the Ca\textsuperscript{2+} transient directly reflects sarcoplasmic reticulum pump activity, these results indicate that there appears to be ~30% modulation of sarcoplasmic reticulum pump activity by phosphorylation in wild-type mouse soleus fibers. Soleus fibers from phospholamban knockout mice fail to show these effects of phosphorylation on Ca\textsuperscript{2+} transport activity.

Although we used a relatively high concentration (10 µM) of DBcAMP to activate protein kinase in isolated muscle fibers, this concentration was without effect on the Ca\textsuperscript{2+} decay rate constant in soleus fibers isolated from wild-type mice maintained and studied under our experimental conditions. Considering that 10 µM DBcAMP should be high enough to achieve near-maximal activation of PKA and that 10 µM DBcAMP did in fact increase the amplitude of Ca\textsuperscript{2+} transients in isolated FDB fibers under the same experimental conditions, our observation of lack of stimulation of Ca\textsuperscript{2+} uptake by DBcAMP in soleus fibers from wild-type mice suggests that most phospholamban is phosphorylated in the resting state in our wild-type soleus fibers. Consistent with this interpretation, application of the PKA inhibitor H-89, which should favor dephosphorylation of phospholamban, led to partial suppression of the sarcoplasmic reticulum Ca\textsuperscript{2+}-uptake rate in wild-type mouse slow-twitch fibers but not in soleus fibers from phospholamban knockout mice. Biochemical assays of phospholamban phosphorylation in parallel studies of soleus fibers confirmed the near-maximal phosphorylation of phospholamban under control conditions. The maximal basal state phosphorylation of phospholamban found here in wild-type soleus fibers under our experimental conditions is in contrast to the apparent incomplete phosphorylation of phospholamban in whole soleus muscles isolated from wild-type mice (26). In such muscles the rate of force relaxation has been found to increase after application of isoproterenol, indicating an acceleration of Ca\textsuperscript{2+} uptake that was presumably due to phospholamban phosphorylation (26). This difference in basal state phosphorylation of phospholamban in isolated single slow-twitch fibers and isolated whole muscles raises interesting questions for possible future studies of the control of phosphorylation levels in muscles and muscle fibers.

The present results clearly establish that modulation of the level of phosphorylation of phospholamban can alter the rate of decay of Ca\textsuperscript{2+} after Ca\textsuperscript{2+} release in wild-type soleus fibers. In contrast, modulation of phosphorylation status has no effect on the rate of decay of Ca\textsuperscript{2+} in phospholamban knockout mice or in wild-type FDB fibers, both of which lack phospholamban. Variation in the phosphorylation status of other proteins underlies the observed changes in peak amplitude of the Ca\textsuperscript{2+} transient with changes in kinase or phosphatase activity.

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