Effects of increased systolic Ca\textsuperscript{2+} and phospholamban phosphorylation during β-adrenergic stimulation on Ca\textsuperscript{2+} transient kinetics in cardiac myocytes

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Roof SR, Shannon TR, Janssen PM, Ziolo MT. Effects of increased systolic Ca\textsuperscript{2+} and phospholamban phosphorylation during β-adrenergic stimulation on Ca\textsuperscript{2+} transient kinetics in cardiac myocytes. Am J Physiol Heart Circ Physiol 301: H1570–H1578, 2011. First published July 15, 2011; doi:10.1152/ajpheart.00402.2011.—Previous studies demonstrated higher systolic intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) amplitudes result in faster [Ca\textsuperscript{2+}]\textsubscript{o} transient decline rates with ISO compared with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} or ISO but normalized ISO's time to peak with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o}. ISO increased Ser16 phosphorylation compared to Ca\textsuperscript{2+}, whereas Thr17 phosphorylation was similar. Ca\textsuperscript{2+} transient (CaT) (fluor 4) data were obtained from matched CaT amplitudes with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} and ISO. [Ca\textsuperscript{2+}]\textsubscript{i} decline was significantly faster with ISO compared with 3 mM [Ca\textsuperscript{2+}]\textsubscript{i}. Interestingly, the faster decline with ISO was only seen during the first 50% of the decline. CaT time to peak was significantly faster with ISO compared with 3 mM [Ca\textsuperscript{2+}]\textsubscript{i}. A Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CAMKII) inhibitor (KN-93) did not affect the CaT decline rates with 3 mM [Ca\textsuperscript{2+}]\textsubscript{i} or ISO but normalized ISO’s time to peak with 3 mM [Ca\textsuperscript{2+}]\textsubscript{i}. Thus, during β-AR stimulation, the major factor for the faster CaT decline is due to Ser16 phosphorylation, and faster time to peak is due to CAMKII activation.

phospholamban; phosphorylation; ryanodine receptor; sarcoplasmic reticulum calcium ion-adenosinetriphosphatase; calcium ion/calmodulin-dependent protein kinase

PRIMARY REGULATION OF INTRACELLULAR Ca\textsuperscript{2+} CONCENTRATION ([Ca\textsuperscript{2+}]\textsubscript{i}) UPTAKE IN THE SR

Ca\textsuperscript{2+} transient ([Ca\textsuperscript{2+}]\textsubscript{i}) uptake in the sarcoplasmic reticulum (SR) is performed by the SR Ca\textsuperscript{2+}-ATPase (SERCA) (2). SERCA’s function is modulated by the regulatory protein, phospholamban (PLB), which inhibits SERCA function (26). For example, transgenic mice with overexpression of PLB result in decreased SERCA activity and SR Ca\textsuperscript{2+} uptake, which results in a slower Ca\textsuperscript{2+} decline rate (19). The PLB-mediated inhibition of SERCA can be relieved by its dissociation. An increase in Ca\textsuperscript{2+} levels will cause this dissociation (20). Thus, increasing the peak amplitude of the [Ca\textsuperscript{2+}]\textsubscript{i} transient will increase SERCA activity, which results in faster [Ca\textsuperscript{2+}]\textsubscript{i} decline rates (3). PLB is also a major phosphoprotein in the myocyte. PLB can be phosphorylated on Ser\textsuperscript{16} through protein kinase A (PKA) or on Thr\textsuperscript{17} through Ca\textsuperscript{2+}/calmodulin-dependent protein kinase (CAMKII). Phosphorylation at either site also results in the dissociation of PLB from SERCA. In fact, phosphorylation at these sites with β-adrenergic (β-AR) stimulation in PLB overexpression mice normalizes the Ca\textsuperscript{2+} decline compared with the wild type (WT) (19). Furthermore, PLB knockout mice result in increased SERCA activity and SR Ca\textsuperscript{2+} uptake, which results in faster Ca\textsuperscript{2+} decline rates (25). Interestingly, there was no further acceleration of the Ca\textsuperscript{2+} decline due to β-AR stimulation in these myocytes (24). In addition, a previous study in mice that had mutated PLB, which could not be phosphorylated (both Ser\textsuperscript{16} and Thr\textsuperscript{17} mutated to alanine), had no lusitropic effect during β-AR stimulation (5). Thus, PLB is an important mediator of Ca\textsuperscript{2+} decline during β-AR stimulation. However, the faster [Ca\textsuperscript{2+}]\textsubscript{i} decline with β-AR stimulation may not only be due to PLB phosphorylation but the higher systolic Ca\textsuperscript{2+} levels as well. Unlike these mentioned studies, we matched Ca\textsuperscript{2+} transient peaks to best determine if the increase in systolic Ca\textsuperscript{2+} and/or PLB phosphorylation contributed to the lusitropic effect during β-AR stimulation.

Because lowering the [Ca\textsuperscript{2+}]\textsubscript{i} is the initiating event that permits relaxation, clearly defining the processes contributing to Ca\textsuperscript{2+} reserequestation in the SR is important. Relaxation of the cardiac muscle is governed by processes at the myofilament level (17, 18), and duration of activation is critically impacted by Ca\textsuperscript{2+} transient kinetics. Thus, governing of Ca\textsuperscript{2+} transient decline by the SR may have important clinical ramifications since relaxation is altered in many cardiomyopathies such as heart failure (31, 39).

Therefore, our purpose is to determine the major factor in the faster [Ca\textsuperscript{2+}]\textsubscript{i} decline during β-AR stimulation (i.e., the high systolic levels of [Ca\textsuperscript{2+}]\textsubscript{o}, or PLB phosphorylation). We hypothesize that the high systolic [Ca\textsuperscript{2+}]\textsubscript{o} levels seen during β-AR stimulation or high extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{o}) will be near the maximal velocity (V\textsubscript{max}) of the SERCA pump. Thus, matched systolic [Ca\textsuperscript{2+}]\textsubscript{o} levels with high [Ca\textsuperscript{2+}]\textsubscript{i} and β-AR stimulation will exhibit similar Ca\textsuperscript{2+} decline rates in the initial decline phase. In addition, PLB phosphorylation (i.e., β-AR stimulation) would then result in a faster Ca\textsuperscript{2+} transient decline during the final phase because of the leftward shift in the Michaelis constant at low [Ca\textsuperscript{2+}]\textsubscript{o}. To test this hypothesis, each myocyte will be superfused with high [Ca\textsuperscript{2+}]\textsubscript{o}, (3 mM) and with the β-AR agonist isoproterenol (ISO) to obtain data under these conditions with matched levels of Ca\textsuperscript{2+} transient amplitude.

β-AR stimulation also results in positive chronotropy. It is known that increasing frequency of stimulation also results in faster Ca\textsuperscript{2+} decline rates. However, this is independent of PLB phosphorylation and occurs via a separate mechanism (34). Hence the chronotropic effect (i.e., increasing pacing frequency) was not investigated. To our knowledge, no one has
measured [Ca\(^{2+}\)], decline rates comparing high [Ca\(^{2+}\)]o and ISO in the same myocyte using matched Ca\(^{2+}\) transient amplitudes. A previous study examined the effects of ISO and high [Ca\(^{2+}\)]o, in the same trabeculas (7). However, this study was specifically focused on these interventions on developed pressure. There was some examination of Ca\(^{2+}\) decline, in which they observed a faster Ca\(^{2+}\) decline with ISO vs. 20 mM [Ca\(^{2+}\)]o, but the amplitudes were not matched.

Our data suggest that, during β-AR stimulation, Ser\(^{16}\) phosphorylation is the major factor that drives the faster acceleration of the Ca\(^{2+}\) transient decline. Also, the faster Ca\(^{2+}\) transient time to peak is the result of CaMKII activation.

**MATERIALS AND METHODS**

**Cardiomyocyte Isolation**

Ventricular myocytes were isolated from mice (C57BL/6) (Jackson Laboratories, Bar Harbor, ME) as previously described (22). Briefly, the heart was cannulated and hung on a Langendorff apparatus. It was then perfused with Ca\(^{2+}\)-free Tyrode solution for 4 min. The solution was then switched to Tyrode solution containing Liberase Blendzyme II (0.077 mg/ml) (Roche Applied Science, Indianapolis, IN). After 3–5 min, the heart was taken down, the ventricles were minced, and myocytes were dissociated by trituration. Subsequently, the myocytes were filtered, centrifuged, and resuspended in Tyrode solution containing 200 μmol/l Ca\(^{2+}\). Myocytes were used within 6 h of isolation. All of the animal protocols and procedures were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

**Measurement of myocyte Ca\(^{2+}\) transients.** Ca\(^{2+}\) transient measurements were performed as previously described (22). Briefly, myocytes were loaded at room temperature with fluo-4 AM (10 μmol/l; Molecular Probes, Eugene, OR) for 30 min, and then another 30 min were allowed for intracellular deesterification. The solution for deesterification was Tyrode solution containing 200 μmol/l Ca\(^{2+}\). The instrumentation used for cell fluorescence measurements was a Cairn Research Limited (Faversham, UK) epifluorescence system. [Ca\(^{2+}\)]i was measured by fluo 4 epifluorescence with excitation at 480 nm and emission at 535 ± 25 nm. The illumination field was restricted to collect the emission of a single cell. Data were expressed as ΔF/Fo, where F is the fluorescence intensity, and Fo is the intensity at rest. Myocytes were stimulated at 1 Hz via platinum electrodes connected to a Grass Telefactor S48 stimulator (West Warwick, RI).

**Western blot analysis.** Homogenized myocytes were used to measure specific phosphorylation at Ser\(^{16}\) and Thr\(^{17}\) (Badrilla, Leeds, UK) with phosphospecific antibodies and normalized to calquestrin (ABR, Golden, CO) via Western blot analysis, as previously described (37).

**Ca\(^{2+}\) uptake rate.** SR Ca\(^{2+}\) uptake rate was determined as described previously (32) by converting the RT\(_{50}\) (time to 50% of the Ca\(^{2+}\) transient decline) into a tau. This value was then used to fit a declining single exponential curve, which was then converted to a total cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]) using well-characterized Ca\(^{2+}\)-buffering parameters. Next, we took the derivative over time (d[Ca\(^{2+}\)/dt). Finally, this derivative was fit into the Hill equation, and the uptake rate was calculated at 300 mM (roughly the RT\(_{50}\) [Ca\(^{2+}\)]; value).

**Solutions and drugs.** Normal Tyrode (NT) solution consisted of (in mmol/l): 140 NaCl, 4 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 glucose, and 5 HEPES, pH 7.4 adjusted with NaOH or HCl. ISO (1 μmol/l, a nonselective β-AR agonist) was prepared fresh each day. KN-93 (1 μmol/l, CAMKII inhibitor; EMD Chemicals, Gibbstown, NJ) was prepared each day from frozen aliquots. Zintelor (Zint, 100 μmol/l, β\(_2\)-AR agonist; Tocris Biosciences, Ellisville, MO) All chemicals were from Sigma (St. Louis, MO) except where indicated.

**Experimental protocol.** Our basic experimental protocol consisted of the myocyte first being perfused with control solution [NT with 1 mM Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]o)] until steady state was reached, and the myocyte was then superfused with the 3 mM [Ca\(^{2+}\)]o NT solution, which resulted in increased systolic Ca\(^{2+}\) levels. After steady state was reached, the solution was then switched back to control solution (1 mM [Ca\(^{2+}\)]o NT), resulting in the washout of 3 mM [Ca\(^{2+}\)]o, and the Ca\(^{2+}\) transient amplitude returning back to basal levels (~3 min). The myocyte was then perfused with 1 μM ISO (with 1 mM [Ca\(^{2+}\)]o), which increased myocyte systolic Ca\(^{2+}\) levels.

**Statistics.** Data were presented as means ± SE. Differences between groups were evaluated for statistical significance (P < 0.05) by ANOVA for multiple groups or paired Student’s t-tests for two groups.

**RESULTS**

To compare [Ca\(^{2+}\)], decline rates of comparable [Ca\(^{2+}\)], with low and high PLB phosphorylation, a NT solution made with 3 mM [Ca\(^{2+}\)]o, was used to achieve similar [Ca\(^{2+}\)], peaks as during β-AR stimulation with ISO. Variability of Ca\(^{2+}\)+ handling between cells being exposed to 3 mM [Ca\(^{2+}\)]o and ISO was minimized by superfusing each myocyte with both solutions.

**Ca\(^{2+}\) transient kinetics experimental protocol.** Shown in Fig. 1A is a representative experiment showing Ca\(^{2+}\) transients over time with the various solutions as explained in MATERIALS AND METHODS. Shown in Fig. 1B, perfusing the myocytes with 3 mM [Ca\(^{2+}\)]o and ISO resulted in a significantly increased maximal peak systolic [Ca\(^{2+}\)]i (1 mM [Ca\(^{2+}\)]o; 1.6 ± 0.1; 3 mM [Ca\(^{2+}\)]o; 3.6 ± 0.2; ISO: 3.9 ± 0.2 ΔF/Fo; n = 21 cells/10 hearts). The majority of myocytes reached a higher maximal systolic [Ca\(^{2+}\)]o with ISO compared with 3 mM [Ca\(^{2+}\)]o, although not significant. Shown in Fig. 1C, perfusion with 3 mM [Ca\(^{2+}\)]o and ISO also resulted in a significantly faster Ca\(^{2+}\) transient rate measured as RT\(_{50}\) (1 mM [Ca\(^{2+}\)]o; 225 ± 7; 3 mM [Ca\(^{2+}\)]o; 154 ± 5; ISO: 127 ± 3 ms).

![Fig. 1. Experimental protocol. A: representative time plot of the experimental protocol. B: pooled data (means ± SE) of maximum Ca\(^{2+}\) transient amplitude with 1 mM extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]o, open bar), 3 mM [Ca\(^{2+}\)], (black bar), or isoproterenol (ISO, gray bar). C: pooled data (means ± SE) of Ca\(^{2+}\) transient decline measured as the time to 50% relaxation (RT\(_{50}\). P < 0.05 vs. 1 mM [Ca\(^{2+}\)]o (*) and vs. 1 and 3 mM [Ca\(^{2+}\)]o (**) (n = 21 cells/10 hearts).](http://ajpheart.physiology.org/10.22033.1)}
Interestingly, the ISO Ca\(^{2+}\) transient decay rate was significantly faster than the 3 mM [Ca\(^{2+}\)]\(_o\).

**Ca\(^{2+}\) transient kinetics with matched amplitudes.** Although the maximum systolic Ca\(^{2+}\) levels were not statistically different between 3 mM [Ca\(^{2+}\)]\(_o\) and ISO, the slightly higher peak with ISO may have resulted in the faster Ca\(^{2+}\) transient decline. Thus, for better comparisons of Ca\(^{2+}\) transient decline rates, we used matched Ca\(^{2+}\) transient amplitude levels. Representative matched individual [Ca\(^{2+}\)] transient traces are shown in Fig. 2A. Shown in Fig. 2B are the matched peak values of 3 mM [Ca\(^{2+}\)]\(_o\), (3.5 ± 0.2 ΔF/F\(_o\)) and ISO (3.5 ± 0.2 ΔF/F\(_o\)).

Similar results were observed when we switched the order of solutions. That is, after initial equilibration at the basal condition of 1 mM [Ca\(^{2+}\)]\(_o\), the myocytes were first superfused with ISO, washed out (~15 min), and then perfused with 3 mM [Ca\(^{2+}\)]\(_o\) (data not shown). Thus, the effects of these interventions on Ca\(^{2+}\) transient kinetics are independent of the order of the solutions.

We also measured the Ser\(^{16}\) and Thr\(^{17}\) phosphorylation under similar experimental conditions (i.e., myocyte perfusion with 3 mM [Ca\(^{2+}\)]\(_o\), or ISO for 3 min, which is a similar time point in which we matched the Ca\(^{2+}\) transient amplitudes). Shown in Fig. 2C, myocytes perfused with ISO had significantly increased Ser\(^{16}\) phosphorylation compared with myocytes perfused with 3 mM [Ca\(^{2+}\)]\(_o\) [4.4 ± 0.5 vs. 0.9 ± 0.3 arbitrary units (AU) (n = 4)]. However, there was no difference in Thr\(^{17}\) phosphorylation between ISO and 3 mM [Ca\(^{2+}\)]\(_o\) [1.5 ± 0.6 vs. 1.7 ± 0.6 AU (Fig. 2D)]. Thus, with our experimental protocol and matching Ca\(^{2+}\) transient amplitudes, the only difference between ISO and 3 mM [Ca\(^{2+}\)]\(_o\) is the Ser\(^{16}\) phosphorylation levels.

Using matched-amplitudes Ca\(^{2+}\) transient data, we explored the effects of 3 mM [Ca\(^{2+}\)]\(_o\) and ISO on Ca\(^{2+}\) decline by analyzing the time it takes the Ca\(^{2+}\) transient to decline by 25% (RT25), 50% (RT50), 75% (RT75), and 90% (RT90) from its peak amplitude. As shown in Fig. 3A, at each time point, the Ca\(^{2+}\) decline with ISO was significantly faster compared with 3 mM [Ca\(^{2+}\)]\(_o\). We further analyzed the effects of ISO and 3 mM [Ca\(^{2+}\)]\(_o\) on the Ca\(^{2+}\) transient decline by examining various time intervals. By breaking the decline into intervals, we can better determine at which point in the [Ca\(^{2+}\)] transient PLB phosphorylation will result in different decline rates compared with matched systolic [Ca\(^{2+}\)] levels with 3 mM [Ca\(^{2+}\)]\(_o\). Thus, we divided the declining Ca\(^{2+}\) transient into intervals: RT25–25, RT75–50, and RT90–75. For example, we subtracted the RT25 from the RT50 to get the RT50–25 interval. This is the time it took for Ca\(^{2+}\) to decline 25% from the peak amplitude to the 50% point. Data are shown in Fig. 3B. The RT50–25 interval was the only interval significantly different between 3 mM [Ca\(^{2+}\)]\(_o\) and ISO. Thus, the faster Ca\(^{2+}\) transient decline with ISO compared with 3 mM [Ca\(^{2+}\)]\(_o\) occurs only in the first 50%.

We also investigated the effects of 3 mM [Ca\(^{2+}\)]\(_o\) and ISO on diastolic Ca\(^{2+}\) levels. We observed no difference in diastolic Ca\(^{2+}\) values between 1 mM [Ca\(^{2+}\)]\(_o\) and 3 mM [Ca\(^{2+}\)]\(_o\) (103% of 1 mM [Ca\(^{2+}\)]\(_o\)). There was a slight but significant decrease in diastolic Ca\(^{2+}\) values with ISO compared with 1 mM [Ca\(^{2+}\)]\(_o\) (91% of 1 mM [Ca\(^{2+}\)]\(_o\)). We believe these results further strengthen our argument that Ser\(^{16}\) phosphorylation results in a greater Ca\(^{2+}\) decline, which results in a decreased diastolic [Ca\(^{2+}\)]\(_i\). We further analyzed the relationship between the systolic Ca\(^{2+}\) levels and RT50. Shown in Fig. 4 are the maximal steady-state Ca\(^{2+}\) transient amplitudes plotted against their respective RT50. Myocytes with low PLB phosphorylation are shown in black (NT with 1 and 3 mM [Ca\(^{2+}\)]\(_o\)). Consistent with previous studies (3), the higher the peak [Ca\(^{2+}\)]\(_i\), the faster the rate of decline. Myocytes with ISO (i.e., high Ser\(^{16}\) phosphorylation) are shown in gray. In addition to the 1 μM ISO (1 mM [Ca\(^{2+}\)]\(_o\)) group, we were also able to obtain smaller peak Ca\(^{2+}\) transient amplitudes by perfusing myocytes with 1 μM ISO and 0.25 mM [Ca\(^{2+}\)]\(_o\). We then analyzed the slope of these lines. Our data show that the phosphorylation of Ser\(^{16}\) resulted in a weaker correlation (slope of −14.3 ± 3.4) compared with myocytes with low phosphorylated PLB (slope of −38.3 ± 2.8). Thus, our data suggest that the PLB phosphorylation is more effective at
Increasing the rate of \([Ca^{2+}]_i\) decline is much less dependent on peak \([Ca^{2+}]_i\) transient amplitudes. Thus, with \(\beta\)-AR stimulation (and PLB phosphorylation), the cardiomyocyte is less dependent on the peak \([Ca^{2+}]_i\) levels for the rate of \([Ca^{2+}]_i\) decline.

![Graph of Ca\(^{2+}\) transient decline](image1)

**Fig. 3.** Effects of 3 mM \([Ca^{2+}]_o\) and ISO on \([Ca^{2+}]_i\) transient decline with matched peaks. A: pooled data (means ± SE) of \([Ca^{2+}]_i\) decline with 3 mM \([Ca^{2+}]_o\) (black) or ISO (gray), RT\(_{25}, RT\(_{75}, RT\(_{90}\), the time it takes the \([Ca^{2+}]_i\) transient to decline by 25, 75, and 90%, respectively, from its peak amplitude. B: pooled data (means ± SE) of \([Ca^{2+}]_i\) transient decline time intervals with 3 mM \([Ca^{2+}]_o\), or ISO, RT\(_{25}, RT\(_{75}, RT\(_{90}, the time for \([Ca^{2+}]_i\) to decline 25% from the peak amplitude to the 50% point; RT\(_{75}, RT\(_{90}, the time for \([Ca^{2+}]_i\) to decline 50% from the peak amplitude to the 75% point; RT\(_{90}, the time for \([Ca^{2+}]_i\) to decline 75% from the peak amplitude to the 90% point. *P < 0.05 vs. corresponding 3 mM \([Ca^{2+}]_o\). (n = 21 cells/10 hearts).

We further investigated this point by grouping the myocytes that had a higher maximum response to ISO together (n = 14) and the myocytes that had a higher response to 3 mM \([Ca^{2+}]_o\) together (n = 7). Shown in Fig. 5A, when grouping the data, the myocytes that had a higher maximum peak systolic \([Ca^{2+}]_o\) with ISO (4.0 ± 0.2 vs. 3.5 ± 0.2 ΔF/F\(_o\), P < 0.05) also had a faster \([Ca^{2+}]_i\) transient decline rate (126 ± 3 vs. 161 ± 7 ms, P < 0.05). It is unknown if the major factor in the faster \([Ca^{2+}]_i\) decline is the higher systolic \([Ca^{2+}]_o\) levels or PLB Ser\(^{16}\) phosphorylation. However, the myocytes that a higher maximum peak systolic \([Ca^{2+}]_i\) with 3 mM \([Ca^{2+}]_o\) (4.1 ± 0.1 vs. 3.7 ± 0.1 ΔF/F\(_o\), P < 0.05) had a slower \([Ca^{2+}]_i\) transient decline rate (141 ± 3 vs. 130 ± 5 ms, P < 0.05) (Fig. 5B). We also calculated \([Ca^{2+}]_i\) uptake rates with 3 mM \([Ca^{2+}]_o\) and ISO. Shown in Fig. 5C, ISO had a greater \([Ca^{2+}]_i\) uptake rate at 300 nm \([Ca^{2+}]_o\) (4.1 ± 0.1 vs. 468 ± 28 μM/s, P < 0.05). Thus these data suggest that PLB Ser\(^{16}\) phosphorylation is the major factor responsible for the faster \([Ca^{2+}]_i\) transient decline rate during \(\beta\)-AR stimulation.

**Effects of \(\beta_2\)-AR stimulation on \([Ca^{2+}]_i\) transient decline rates with 3 mM \([Ca^{2+}]_o\) and Zint.** The functional response to \(\beta\)-AR stimulation is primarily regulated through \(\beta_1\)-AR and \(\beta_2\)-AR receptors. \(\beta_1\)-AR stimulation leads to high PLB Ser\(^{16}\) phosphorylation by PKA. Interestingly, \(\beta_2\)-AR stimulation does not increase Ser\(^{16}\) PLB phosphorylation (23). To further test the contribution of Ser\(^{16}\) phosphorylation on \([Ca^{2+}]_i\) decline, we performed our experimental protocol but substituted Zint, a

![Graph of Ca\(^{2+}\) transient amplitudes and declines](image2)

**Fig. 4.** Effects of PLB Ser\(^{16}\) phosphorylation on the relationship between maximal steady-state \([Ca^{2+}]_i\) transient amplitude and decline. Individual values of peak \([Ca^{2+}]_i\) vs. RT\(_{90}\) with low (black, \(r^2 = 0.77\)) and high (gray, \(r^2 = 0.37\)) Ser\(^{16}\) phosphorylation.

![Graph of Ca\(^{2+}\) transient response to ISO](image3)

**Fig. 5.** Grouped \([Ca^{2+}]_i\) transient amplitudes and declines. A: pooled data (means ± SE) of \([Ca^{2+}]_i\) transient amplitude (left) and decline (right) in myocytes that had a higher response to ISO (n = 14). B: pooled data (means ± SE) of \([Ca^{2+}]_i\) transient amplitude (left) and decline (right) in myocytes that had a higher response to 3 mM \([Ca^{2+}]_o\) (n = 7). C: pooled data (means ± SE) of calculated \([Ca^{2+}]_i\) uptake rate at 300 nM \([Ca^{2+}]_o\). (*P < 0.05 vs. corresponding 3 mM \([Ca^{2+}]_o\). (n = 21 cells/10 hearts).
β₂-AR, agonist, for ISO. Matched Ca²⁺ transient amplitudes for 3 mM [Ca²⁺]₀ (2.2 ± 0.2 ΔF/F₀) and Zint (2.2 ± 0.2 ΔF/F₀) (n = 5 cells/3 hearts) were analyzed. Figure 6A displays representative matched Ca²⁺ transient traces of 3 mM [Ca²⁺]₀ and Zint. Shown in Fig. 6B are the Ca²⁺ transient decline rates (RT₂₅, RT₅₀, RT₇₅, and RT₉₀). We also analyzed the time intervals between 25–50%, 50–75%, and 75–90% (Fig. 6C), as described above. The data in Fig. 6, B and C, show that the Ca²⁺ transient decline is not significantly different at any time points or between intervals. Thus, with matched [Ca²⁺]₀, β₂-AR stimulation does not result in faster Ca²⁺ decline.

Effects of CAMKII inhibition on Ca²⁺ transient decline rates with 3 mM [Ca²⁺]₀, and ISO. β-AR stimulation and high [Ca²⁺]₀ can lead to CAMKII activation and PLB Thr¹⁷ phosphorylation. Although we observed no difference in the Thr¹⁷ phosphorylation status with 3 mM [Ca²⁺]₀ and ISO, we wanted to determine if Thr¹⁷ phosphorylation played a role in the Ca²⁺ transient decline. Hence, we repeated our experiments in the presence of the CAMKII inhibitor KN-93 (1 μm/l).

KN-93 resulted in no significant changes in maximum steady-state Ca²⁺ transient amplitudes (Fig. 7A) or RT₅₀ (Fig. 7C) during superfusion with 3 mM [Ca²⁺]₀. We also investigated the effect of ISO with KN-93 on maximum steady-state amplitudes (Fig. 7B) and RT₅₀ (Fig. 7D). KN-93 did not have a significant effect on the response to ISO.

Figure 8A displays representative matched Ca²⁺ transient traces during superfusion with 3 mM [Ca²⁺]₀ and ISO in the presence of KN-93. Shown in Fig. 8A are the Ca²⁺ transient decline rates (RT₂₅, RT₅₀, RT₇₅, and RT₉₀) in the presence of KN-93. Results with and without KN-93 were similar, that is, the Ca²⁺ transient decline at each time point was faster with ISO superfusion compared with 3 mM [Ca²⁺]₀.

We also analyzed the time intervals with KN-93 between 25–50%, 50–75%, and 75–90%, as described above. The data in Fig. 8C show that only the Ca²⁺ transient decline in the first 50% is significantly different. Thus, CAMKII inhibition (and PLB Thr¹⁷ phosphorylation) had no effect on the ISO- or 3 mM [Ca²⁺]₀-induced Ca²⁺ decline. Thus, these data suggest that Ser¹⁶ is the major phosphorylation site responsible for the faster Ca²⁺ transient decline during β-AR stimulation.

Effects of 3 mM [Ca²⁺]₀, and ISO on Ca²⁺ transient time to peak. Ca²⁺ transient time to peak, the time it takes for the myocyte to reach its maximum systolic level, was compared with 3 mM [Ca²⁺]₀ and ISO. Shown in Fig. 9A, ISO reached its peak significantly faster than 3 mM [Ca²⁺]₀ (71 ± 2 vs. 79 ± 2 ms, P < 0.05). However, in the presence of KN-93, the time to peak with 3 mM [Ca²⁺]₀ and ISO is similar (78 ± 3 vs. 76 ± 3 ms) (Fig. 9B). Therefore, the faster time to peak during β-AR stimulation with ISO is likely because of the activation of CAMKII.

**DISCUSSION**

Our data show that, when we compared matched systolic [Ca²⁺]₀ levels with ISO and 3 mM [Ca²⁺]₀, PLB Ser¹⁶ phosphorylation is the major factor for the faster Ca²⁺ decline. Interestingly, this faster decline was only observed during the first 50% of the decline. β-AR stimulation also resulted in a faster Ca²⁺ transient time to peak, which was prevented by CAMKII inhibition.

**β-AR stimulation.** Stimulation of the β-AR pathway results in the classical positive inotropic and lusitropic effect (4), that is, in terms of Ca²⁺ handling, an increase in systolic [Ca²⁺], levels with a faster time to peak and decline. In mouse myocytes, the majority (>93%) of the Ca²⁺ decline is the result of SR uptake via SERCA (2). SERCA is regulated by PLB. Ca²⁺ binding to SERCA results in the dissociation of PLB from SERCA, which will result in enhanced activation. The higher [Ca²⁺] levels during β-AR stimulation should result in further dissociation of PLB from SERCA and faster Ca²⁺ decline. It has been observed that, with higher systolic Ca²⁺ levels, there is a faster Ca²⁺ transient decline (3). PLB is also a phosphoprotein and a key end target of the β-AR pathway. Thus, PKA phosphorylates PLB on Ser¹⁶ (21), and CAMKII phosphorylates PLB on Thr¹⁷. Phosphorylation of PLB also results in dissociation of PLB from SERCA. Hence, it is unknown which is the major factor accounting for the faster Ca²⁺ transient decline during β-AR stimulation: PLB phosphorylation or the greater systolic Ca²⁺ levels.

**High extracellular Ca²⁺ vs. ISO.** We investigated Ca²⁺ transient kinetics with ISO and 3 mM [Ca²⁺]₀, ISO and 3 mM [Ca²⁺]₀, increased peak systolic Ca²⁺ levels and increased the
Ca\(^{2+}\) decline rate (Fig. 1). Interestingly, the decline rate with ISO was faster compared with 3 mM [Ca\(^{2+}\)]. To be assured that the faster decline observed with ISO was the result of PLB phosphorylation and not the small difference in systolic Ca\(^{2+}\) levels, we examined matched Ca\(^{2+}\) transient amplitudes (Fig. 2).

Using the matched amplitudes, we investigated the time it took the Ca\(^{2+}\) transient decline to reach 25, 50, 75, and 90% decline from its peak (Fig. 3A). Our data show that, at each time point, the Ca\(^{2+}\) decline rate was faster with ISO. We further analyzed these effects by examining various time intervals of the Ca\(^{2+}\) transient decline. We measured the interval from 25–50%, 50–75%, and 75–90% decline from its peak (Fig. 3B). Interestingly, our data show that ISO resulted in a faster decline rate during the initial 50% of the decline. These results do not match our hypothesis that PLB phosphorylation would result in a faster decline during the final phase of the decline.

Therefore, we now propose that systolic Ca\(^{2+}\) levels in mice myocytes, even during β-AR stimulation, do not reach the V\(_{\text{max}}\) of the pump, but, during the first 50% of the decline, the dissociation constant between 3 mM [Ca\(^{2+}\)], and ISO is at its greatest difference at this point. Furthermore, we were able to show that, at 300 nm [Ca\(^{2+}\)], ISO has a greater SR Ca\(^{2+}\) uptake rate compared with 3 mM [Ca\(^{2+}\)]. (Fig. 5C). Thus, ISO and, more specifically, Ser\(^{16}\) phosphorylation, accelerates the SR Ca\(^{2+}\) uptake at [Ca\(^{2+}\)] levels at our RT\(_{50}\).

**PLB phosphorylation with high extracellular Ca\(^{2+}\) and ISO.**

As mentioned, β-AR stimulation leads to the phosphorylation of Ser\(^{16}\) by PKA. It has also been shown that β-AR stimulation and increased [Ca\(^{2+}\)] can activate CAMKII and PLB Thr\(^{17}\) phosphorylation (28, 35). Thus, it has been suggested that Thr\(^{17}\) phosphorylation is also involved in the faster Ca\(^{2+}\) decline rates. Our data show that there was a large increase in Ser\(^{16}\) phosphorylation with ISO compared with 3 mM [Ca\(^{2+}\)]; however, Thr\(^{17}\) phosphorylation levels were similar (Fig. 2). Because our Ca\(^{2+}\) transient amplitudes were matched, the only difference between our two datasets is the level of Ser\(^{16}\) phosphorylation. Thus, we believe our faster Ca\(^{2+}\) decline with ISO compared with 3 mM [Ca\(^{2+}\)] is the result of Ser\(^{16}\) phosphorylation.

**Effect of PLB Ser\(^{16}\) phosphorylation on the relationship between systolic Ca\(^{2+}\) levels and [Ca\(^{2+}\)], decline rates.** We also examined the relationship between maximum systolic Ca\(^{2+}\) levels and the Ca\(^{2+}\) transient decline rate measured as RT\(_{50}\) (Fig. 4). Our data show that, with low Ser\(^{16}\) phosphorylation, as systolic Ca\(^{2+}\) levels increased there was a direct relationship for a faster Ca\(^{2+}\) transient decline rate. This is consistent with a previous study (3). There was a similar tendency when PLB Ser\(^{16}\) was phosphorylated. However, when we examined the slopes of each line, the ISO value was considerably less than the 3 mM [Ca\(^{2+}\)] value. Thus, the ISO group is less dependent upon Ca\(^{2+}\) transient peak. In addition, the r\(^{2}\) value for the ISO data indicated that we did not have a good fit. Thus, there is less of a correlation between Ca\(^{2+}\) transient amplitude and RT\(_{50}\) in the ISO group. Our data suggests that the Ca\(^{2+}\) transient decline rate with PLB Ser\(^{16}\) phosphorylation is much less dependent on systolic Ca\(^{2+}\) levels. We further analyzed this by grouping together the myocytes that had a higher response to 3 mM [Ca\(^{2+}\)] and an increased [Ca\(^{2+}\)], to examine the slopes of each line. The ISO group had a significantly higher Ca\(^{2+}\) transient amplitude, this group had a significantly slower Ca\(^{2+}\) decline rate. Taken together, our data suggest that Ser\(^{16}\) phosphorylation is the major factor responsible for the faster Ca\(^{2+}\) transient decline rate with β-AR stimulation.

**Effects of β\(_{2}\)-AR stimulation on Ca\(^{2+}\) transient decline.** To further investigate the role of Ser\(^{16}\) phosphorylation on accelerating Ca\(^{2+}\) decline, we tested if β\(_{2}\)-AR stimulation would change Ca\(^{2+}\) transient decline rates. β\(_{2}\)-AR stimulation is known to increase Ca\(^{2+}\) transients via local regulation of Ca\(^{2+}\) entry, but, because of its coupling with Gi, its compartmentalization, β\(_{2}\)-AR stimulation does not phosphorylate Ser\(^{16}\)
Ca\textsuperscript{2+} transient kinetics during \beta-AR stimulation

PLB to the extent of \beta\textsubscript{1}-AR (23). Thus, if Ser\textsuperscript{16} phosphorylation is the driving force behind the accelerated Ca\textsuperscript{2+} decline, \beta\textsubscript{2}-AR stimulation should not result in faster Ca\textsuperscript{2+} decline with matching Ca\textsuperscript{2+} transient peaks. Our data demonstrate that this was indeed the case. That is, matched Ca\textsuperscript{2+} transient peaks from 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} and Zint had similar Ca\textsuperscript{2+} decline rates. Thus, this provided additional evidence that Ser\textsuperscript{16} phosphorylation is the driving force behind the accelerated Ca\textsuperscript{2+} decline.

Effects of CAMKII inhibition on Ca\textsuperscript{2+} transient decline. Because we did observe Thr\textsuperscript{17} phosphorylation with ISO and 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 7), although not significant, CAMKII inhibition blunted the Ca\textsuperscript{2+} transient amplitude with \beta-AR stimulation, consistent with previous data (6). We also examined the effects of Ca\textsuperscript{2+} transient decline with matched Ca\textsuperscript{2+} transient amplitudes during ISO and 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} perfusion with KN-93. We observed a similar effect, that is, even with CAMKII inhibition, ISO resulted in a faster Ca\textsuperscript{2+} decline during the first 50% (Fig. 8).

Under our experimental conditions, Thr\textsuperscript{17} phosphorylation does not play a role in enhancing the Ca\textsuperscript{2+} transient decline rate. Previous studies have found, for CAMKII-mediated PLB Thr\textsuperscript{17} phosphorylation, there must be concomitant phosphatase inhibition or acidosis (28, 35). Because we did not inhibit phosphatases with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o}, we would get minimal phosphorylation. During \beta-AR stimulation, there is an inhibition of the phosphatases (29). However, this phosphorylation step occurred much slower (~5 min) than Ser\textsuperscript{16} phosphorylation. Given that we were using Ca\textsuperscript{2+} transients that had matched amplitudes, we picked ISO-stimulated Ca\textsuperscript{2+} transients before the peak was reached (~2–3 min). With the short perfusion time and the lack of phosphatase inhibition or acidosis, CAMKII did not phosphorylate PLB and hence had no effect on ISO-stimulated Ca\textsuperscript{2+} transient decline. Furthermore, similar to our conclusions, these authors showed that the lusitropic effect of \beta-AR stimulation correlated with PLB Ser\textsuperscript{16} phosphorylation.

Other factors that may contribute to Ca\textsuperscript{2+} decline could be the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) or troponin (Tn) I phosphorylation. NCX contributes to the Ca\textsuperscript{2+} transient decline (2). Studies have found that \beta-AR stimulation is able to enhance NCX current (30). However, this is controversial, since others did not observe this effect (10). Under our experimental conditions, we believe that NCX does not play a role in the effects of \beta-AR stimulation on Ca\textsuperscript{2+} transient decay. NCX plays a minor role in the Ca\textsuperscript{2+} decline rates in mouse myocytes (<5%); thus, this effect, if any, would be minor at best.

![Graph A](http://ajpheart.physiology.org/)  
![Graph B](http://ajpheart.physiology.org/)  
![Graph C](http://ajpheart.physiology.org/)

Fig. 8. Effects of 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} and ISO on Ca\textsuperscript{2+} transient decline in the presence of KN-93. A: representative traces of matched [Ca\textsuperscript{2+}]\textsubscript{o} amplitudes with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} + KN-93 (black) and ISO + KN-93 (gray). B: pooled data (means ± SE) of Ca\textsuperscript{2+} decline with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} + KN-93 (black) or ISO + KN-93 (gray). C: pooled data (means ± SE) of Ca\textsuperscript{2+} transient decline time intervals with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} + KN-93 or ISO + KN-93. *P < 0.05 vs. corresponding 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} (n = 15 cells/4 hearts).

![Graph A](http://ajpheart.physiology.org/)  
![Graph B](http://ajpheart.physiology.org/)

Fig. 9. Effects of 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} and ISO on Ca\textsuperscript{2+} transient time to peak. A: pooled data (means ± SE) of Ca\textsuperscript{2+} transient time to peak with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} (black) or ISO (gray). B: pooled data (means ± SE) of Ca\textsuperscript{2+} transient time to peak with 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} + KN-93 or ISO + KN-93. *P < 0.05 vs. 3 mM [Ca\textsuperscript{2+}]\textsubscript{o} (−KN-93: n = 21 cells/10 hearts, +KN-93: n = 15 cells/4 hearts).
Furthermore, with the use of the NCX knockout mouse, there was no difference in Ca\(^{2+}\) transient decline rates with \(\beta\)-AR stimulation in WT compared with knockout myocytes (14). In larger species (e.g., rabbit, human, etc.) NCX plays a greater role in the Ca\(^{2+}\) transient decline rate (~30%) (2). Consequently, there may be a species difference on the role of NCX and regulation of Ca\(^{2+}\) transient decay during \(\beta\)-AR stimulation. However, in rabbit myocytes during \(\beta\)-AR stimulation, the SR accounts for ~90% of the Ca\(^{2+}\) removal (36). Thus, the effect of NCX would still be minor. Therefore, during \(\beta\)-AR stimulation, we believe the major stimulus for the increased acceleration of the Ca\(^{2+}\) transient decline is PLB Ser\(^{16}\) phosphorylation.

Phosphorylation of TnI during \(\beta\)-AR stimulation desensitizes the myofilaments to Ca\(^{2+}\) and results in a greater dissociation of Ca\(^{2+}\) from TnC. This may contribute to the faster Ca\(^{2+}\) decline observed with ISO. However, a previous study has shown that TnI phosphorylation does not play a role in Ca\(^{2+}\) decline in an unloaded preparation (i.e., isolated myocytes) (24). Thus, we do not believe TnI phosphorylation plays a role in the faster Ca\(^{2+}\) decline with ISO under our experimental conditions.

Ca\(^{2+}\) transient time to peak with high extracellular Ca\(^{2+}\) and ISO. We also measured the time it took the Ca\(^{2+}\) transient to reach its peak point (i.e., time from stimulation to peak). We compared the effects of ISO and 3 mM [Ca\(^{2+}\)]\(_{o}\) on Ca\(^{2+}\) transient time to peak. ISO perfusion resulted in a significantly faster Ca\(^{2+}\) transient time to peak, and this effect was prevented by CAMKII inhibition (Fig. 9). A previous study also observed that, with matched SR Ca\(^{2+}\) loads and L-type Ca\(^{2+}\) current, ISO also resulted in a faster rate of [Ca\(^{2+}\)]\(_{o}\) rise and implicated the ryanoxydine receptor (RyR) (11). Studies have shown that CAMKII can phosphorylate the RyR (12, 15, 27). Additionally, studies have shown that, during \(\beta\)-AR stimulation, CAMKII phosphorylates the RyR to increase its activity (6, 8). Our results with KN-93 may seem surprising in that CAMKII can affect Ca\(^{2+}\) transient upstroke but not decline. However, a previous study found that there was a time course disparity in Thr\(^{17}\) phosphorylation and RyR phosphorylation by CAMKII (16). That is, with increasing frequency of stimulation, PLB Thr\(^{17}\) phosphorylation did not change in the first 40 s and then linearly increased for 5 min. RyR Ser\(^{2814}\), the CaMKII site (although controversial), reached maximum phosphorylation after 90 s of increasing the frequency of stimulation. Although these measurements were performed using a different protocol than ours (force frequency vs. \(\beta\)-AR stimulation), there does appear to be a different time dependence to PLB and RyR CaMKII phosphorylation. Our data using \(\beta\)-AR stimulation are consistent with these results in that we observed an apparent effect of CaMKII on RyR function but not PLB function after 3 min. Thus, \(\beta\)-AR stimulation will activate CAMKII to likely increase RyR activity, which results in a faster Ca\(^{2+}\) transient time to peak. The faster time to peak may also be because of faster SR Ca\(^{2+}\) uptake. Nevertheless, a previous study found that transgenic mice overexpressing SERCA2a did not result in a faster Ca\(^{2+}\) transient time to peak (13). Our data are consistent with these observations, since our faster time to peak with ISO was prevented by CaMKII inhibition with KN-93. Yet, KN-93 had no effect on Ca\(^{2+}\) transient decline rates. Thus, if increased SERCA activity was responsible for the faster time to peak, this effect would not have been prevented by KN-93.

Our study also has implications for other mediators of PLB phosphorylation in addition to the \(\beta\)-AR/PKA pathway [i.e., phosphatases, SERCA activators via glutathionylation (1), nitroxyll-induced nitrosylation of PLB (9), reactive nitrogen species, etc.]. For example, nitric oxide (NO) is an important regulator of cardiac contraction (38). We have previously shown that neuronal NO synthase (NOS1) signaling targets PLB to modulate myocyte contraction (37). We have shown that NOS1 knockout myocytes had decreased PLB Ser\(^{16}\) phosphorylation, depressed Ca\(^{2+}\) transient amplitude, and a slower Ca\(^{2+}\) transient decline. It was unknown if the slower [Ca\(^{2+}\)], decline in these myocytes was because of the reduced PLB Ser\(^{16}\) phosphorylation and/or blunted Ca\(^{2+}\) transient amplitude. Our current results would suggest that the NOS1-mediated PLB Ser\(^{16}\) phosphorylation is the major factor on Ca\(^{2+}\) transient decline rather than the amplitude of the Ca\(^{2+}\) transient.

Some limitations of our study include only using one mouse strain. However, our previous work has shown no difference in force decline during \(\beta\)-AR stimulation in three different mouse strains (33). Thus, we believe that our observations are valid for most (if not all) mouse strains. \(\beta\)-AR stimulation also increases heart rate. Increasing pacing frequency by itself also accelerates the Ca\(^{2+}\) decline. However, this is independent of PLB phosphorylation and a separate mechanism (34). Thus, we did not investigate the effects of pacing. In addition, we only used one ISO concentration. We believe using lower ISO concentrations will produce the same qualitative results, but not as great quantitatively. Furthermore, we believe that performing these experiments will not change our conclusion that PLB Ser\(^{16}\) is the major factor for the faster Ca\(^{2+}\) transient decline during \(\beta\)-AR stimulation.

In conclusion, we have shown that the major factor for the faster Ca\(^{2+}\) decline during \(\beta\)-AR stimulation is PLB Ser\(^{16}\) phosphorylation, which only occurs during the initial 50% \(\beta\)-AR stimulation also results in a faster Ca\(^{2+}\) transient time to peak that was dependent on CAMKII.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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