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CaMKII inhibition targeted to the sarcoplasmic reticulum inhibits frequency dependent acceleration of relaxation and Ca^{2+} current facilitation

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Abstract

Cardiac Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) in heart has been implicated in Ca^{2+} current (I_{Ca}) facilitation, enhanced sarcoplasmic reticulum (SR) Ca^{2+} release and frequency dependent acceleration of relaxation (FDAR) via enhanced SR Ca^{2+} uptake. However, questions remain about how CaMKII may work in these three processes. Here we tested the role of CaM-KII in these processes using transgenic mice (SR-AIP) that express four concatenated repeats of the CaMKII inhibitory peptide AIP selectively in the SR membrane. Wild type mice (WT) and mice expressing AIP exclusively in the nucleus (NLS-AIP) served as controls. Increasing stimulation frequency produced typical FDAR in WT and NLS-AIP, but FDAR was markedly inhibited in SR-AIP. Quantitative analysis of cytosolic Ca^{2+} removal during $[\text{Ca}^{2+}]_i$ decline revealed that FDAR is due to an increased apparent V_{max} of SERCA. CaMKII dependent RyR phosphorylation at Ser2815 and SR Ca^{2+} leak were both decreased in SR-AIP vs. WT. This decrease in SR Ca^{2+} leak may partly balance the reduced SERCA activity leading to relatively unaltered SR- Ca^{2+} load in SR-AIP vs. WT myocytes. Surprisingly, CaMKII regulation of the L-type Ca^{2+} channel (I_{Ca} facilitation and recovery from inactivation) was abolished by the SR-targeted CaM-KII inhibition in SR-AIP mice. Inhibition of CaMKII effects on I_{Ca} and RyR function by the SR-localized AIP places physical constraints on the localization of these proteins at the junctional microdomain. Thus SR-targeted CaMKII inhibition can directly inhibit the activation of SR Ca^{2+} uptake, SR Ca^{2+} release and I_{Ca} by CaMKII, effects which have all been implicated in triggered arrhythmias.

Keywords

Calcium; CaMKII; L-type Ca^{2+} channel; sarcoplasmic reticulum; relaxation

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Introduction

Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) has many cellular targets including those involved in cardiac excitation-contraction coupling (ECC), ion channel function, pump activity and gene expression [1,2]. CaMKII is known to associate with ryanodine receptors (RyRs) [3,4], L-type Ca^{2+} channels [5-7] and possibly phospholamban (PLB) [8]. This basal localization near targets may be important for efficient and specific regulation of these CaMKII effector proteins.

In particular, CaMKII has been functionally implicated in the mediation of 1) frequency-dependent acceleration of relaxation (FDAR), 2) RyR-mediated SR Ca^{2+} leak and 3) Ca^{2+} current (I_{Ca}) facilitation. These are all important processes in myocyte Ca^{2+} handling, but there are open questions about the role of CaMKII each. Here we focus on testing whether a peptide CaMKII inhibitor (AIP) specifically targeted to the SR membrane (in transgenic mice) can inhibit 1) FDAR, 2) RyR-mediated SR Ca^{2+} leak and 3) I_{Ca} facilitation.

FDAR contributes to optimal diastolic ventricular filling at increased heart rates. Both relaxation and $[\text{Ca}^{2+}]_i$ decline rates are accelerated at higher frequency in all species studied and enhanced rate of SR Ca^{2+} uptake appears to drive both effects [9–12]. Some reports have shown that FDAR can be strongly suppressed by CaMKII inhibitors (KN-93, KN-62, AIP) [9,12–14], while other reports could not detect effects on FDAR of organic inhibitors KN-93 or KN-62 [11,15–17]. CaMKII-dependent PLB phosphorylation seemed a plausible FDAR mediator and some data seemed to support that [14,18]. However, FDAR is robustly present in PLB knockout mice, where it remains sensitive to CaMKII inhibitors [12,13]. Thus, CaMKII involvement in FDAR is incompletely resolved. Peptide CaMKII inhibitors like AIP are likely to be much more selective than KN-93 and KN-62, and having AIP genetically targeted to the SR avoids having to use peptide variants made membrane permeant (which adds technical limitations as well). The present studies should help to clarify this controversial area.

CaMKII associates with the cardiac RyR [4,19], and the effects of RyR phosphorylation on diastolic SR Ca^{2+} leak is another controversial area [20]. The question is important because such a leak can reduce SR Ca^{2+} content available for release and can also be arrhythmogenic. Again, an endogenous SR-targeted selective CaMKII inhibitor allows a unique opportunity to test for this effect in intact ventricular myocytes.

CaMKII also mediates I_{Ca} facilitation, where repeated depolarizations cause a Ca^{2+} - and CaMKII-dependent increase in I_{Ca} amplitude and slowing of inactivation [21–24]. As the L-type Ca^{2+} channel is in the sarcolemma, one might expect that SR localized AIP would not interfere with CaMKII mediated I_{Ca} regulation. However, it is known that most of the L-type Ca^{2+} channels in ventricular myocytes are localized at the junctions where the sarcolemma and SR are in very close apposition (~12 nm apart) [25]. Thus, if the SR-targeted CaMKII inhibitor peptide could influence I_{Ca} regulation, it might provide novel spatial constraints about the localization of the Ca^{2+} channels and kinases that regulate them.

To investigate these three topics, we used transgenic mice with SR-targeted CaMKII inhibition (SR-AIP). The transgene consisted of four copies of the CaMKII inhibitor peptide, AIP, connected to a PLB trans-SR membrane domain, which served as the SR localization signal [26,27]. Prior work has indicated that this peptide is exclusively localized to the SR and no other cellular compartment in adult ventricular myocytes [26,27]. Two loss-of-function mutations in the PLB sequence prevent direct SERCA inhibition by the fusion protein. Whole heart function and diastolic relaxation is slightly impaired however, these mice do not show overt cardiac failure [26,27]. Effectiveness and specificity of CaMKII inhibition is evident by virtual abolition of CaMKII dependent PLB phosphorylation at Thr17, while PKA dependent phosphorylation at Ser16 was normal. RyR phosphorylation at Ser2809 is slightly reduced, but

Ser2815 may be the main site of CaMKII-dependent phosphorylation [19] regulating diastolic SR Ca^{2+} leak [1,2].

We tested the hypotheses that SR-restricted CaMKII inhibition in SR-AIP mice inhibits FDAR, reduces diastolic SR Ca^{2+} leak (and RyR phosphorylation at Ser2815) and leaves I_{Ca} facilitation unaltered. We used isolated cardiac myocytes from SR-AIP vs. Wild type (WT) mice and as an additional control group, transgenic mice in which a fusion protein of four AIP repeats is specifically expressed in the nucleus using a nuclear localization signal (NLS-AIP).

We found that FDAR, RyR phosphorylation at Ser2815 and SR Ca^{2+} leak were inhibited in SR-AIP mice, and surprisingly that I_{Ca} facilitation was inhibited as well. Thus, the CaMKII inhibitory peptide anchored in the SR membrane can inhibit SERCA stimulation during FDAR, and CaMKII effects on the RyR and L-type Ca^{2+} channel.

Materials and Methods

Transgenic mice

Design and generation of SR-AIP mice was previously described [26]. Briefly, four copies of the DNA sequence encoding the 13 amino acid highly specific and potent CaMKII inhibitor AIP (autocamtide-2-related inhibitory peptide) was placed 5' to a sequence encoding an 8 amino acid long FLAG epitope. For SR targeting, a synthetic gene encoding a truncated PLB transmembrane domain (amino acids 23–52) was inserted with loss of function mutations L31A and N34A, which prevents SERCA inhibition. Nuclear localization in the NLS-AIP mice was achieved via the SV40 large T-antigen nuclear location signal [28]. 3–4 months old mice of both genders were used. None of the female mice had given birth previous to the experiments and no signs of heart failure were present. Previous studies have confirmed that expression of the CaMKII inhibiting peptide is limited to the SR [26] and to the nucleus [29] in SR-AIP and NLS-AIP, respectively.

Cardiac myocyte isolation, shortening and Ca^{2+} transients

Single ventricular myocytes were isolated using standard enzymatic technique as previously described [12]. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. Field stimulated myocyte shortening and $[\text{Ca}^{2+}]_i$ were measured as previously described (at 23°C), using fluo-4 [13]. Fractional shortening was measured as difference between diastolic and systolic cell length divided by the diastolic cell length at the frequency measured. SR- Ca^{2+} load was measured as the $\Delta[\text{Ca}^{2+}]_i$ amplitude upon rapid exposure to 10 mM caffeine. At higher frequencies where twitch relaxation and Ca^{2+} transient decline was incomplete, the time constant was calculated from the last transient after cessation of electrical stimulation, therefore allowing complete relaxation and Ca^{2+} transient decline and accurate calculation of τ .

In a subset of cells, the maximal attainable cytosolic fluo-4 fluorescence was measured by rupturing the cell membrane after the experiments with a sharp glass electrode. By doing so, Ca^{2+} from the bath solution (1 mM) enters the cytosol and saturates the indicator. In all cells in which this procedure was performed, the maximal attainable fluorescence was substantially higher than the fluorescence of the caffeine-evoked Ca^{2+} transient that was previously measured in the same cell. This shows that the fluo dye is not saturated during the measurement of SR Ca^{2+} load and gives sufficient sensitivity to detect differences in SR Ca^{2+} load. Data were analyzed offline with custom-made software.

Ca²⁺ fluxes during relaxation and SR Ca²⁺ leak

Relative contributions of NCX and SERCA to [Ca²⁺]_i decline were calculated as previously described [12,30,31]. Briefly, mean values for Ca²⁺ transient amplitudes and time constants of Ca²⁺ transient decline during twitches and caffeine exposure were used, creating noise-free curves matching mean experimental data. These values were converted to intracellular free [Ca²⁺] using $[Ca^{2+}]_i = (K_d * F/F_0)/(K_d / [Ca^{2+}]_{rest} + 1 - F/F_0)$, with $K_d = 1.144 \mu M$ and $[Ca^{2+}]_{rest} = 150 nM$. Free [Ca²⁺]_i was further converted to total cytosolic Ca²⁺ ([Ca²⁺]_{tot}) using cellular passive cytosolic Ca²⁺-buffering measurements [32]. The rate of change in total cytosolic Ca²⁺ ($d[Ca^{2+}]_{tot}/dt$) is plotted as a function of [Ca²⁺]_i. Then [Ca²⁺]_{tot} removal during a twitch is attributed to the sum of fluxes by individual Ca²⁺ removal systems ($J_{Twitch} = J_{SERCA} + J_{NCX} + J_{Slow}$). The flux via the slow removal pathways (J_{Slow}) is known to be very small and was therefore lumped with J_{NCX} . Ca²⁺ decline during sustained exposure to Caffeine is exclusively due to J_{NCX} . The NCX curve (Figure 4A) is derived from Ca²⁺ decline during caffeine exposure; the SERCA curve (Figure 4B) is the twitch curve minus the caffeine curve ($J_{SERCA} = J_{Twitch} - J_{NCX}$).

SR Ca²⁺ leak was measured as tetracaine-sensitive shift in [Ca²⁺]_i [33]. Briefly, SR Ca²⁺ leak is abruptly blocked by application of 1 mM tetracaine in Na⁺-free, Ca²⁺-free solution to block changes in cellular Ca²⁺. The abrupt block of SR Ca²⁺ leak causes SR Ca²⁺ content to rise and cytosolic Ca²⁺ to decline, such that the [Ca²⁺]_i decline is directly indicative of SR Ca²⁺ leak. Since SR Ca²⁺ leak depends strongly on SR Ca²⁺ content [33], the latter was also measured.

Measurement of I_{Ca}

I_{Ca} was measured under voltage clamp in ruptured patch configuration similar as described previously [34]. I_{Ca} facilitation was measured by applying a 50 ms prepulse to -50 mV and a 200 ms test pulse to 0 mV at 0.5 Hz for 20 times. Recovery from inactivation was measured using a modification of a two-pulse protocol described previously [35] and shown in Figure 7C (inset). I_{Ca} was normalized to the peak current measured at the first pulse.

Western blot analysis

Quantitative immunoblot analysis was carried out following standard protocols. Protein (quantified via a modified Lowry assay; Pierce, Rockford, IL) was separated on 6% (RyR) or 10% (Calsequestrin (CSQ); GAPDH) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Primary antibodies used were anti-RyR 5029 (RyR), anti-phospho-RyR Ser2815 (PS2815) and anti-phospho-RyR Ser2809 (PS2809) (generously provided by Dr. A. Marks, Columbia University, NY). Anti-Calsequestrin antibody was from Affinity BioReagents, Golden, CO and anti-GAPDH antibody was from Abcam Inc, Cambridge, MA.

Reagents and solutions

Unless otherwise stated, reagents used were of analytical grade (Sigma; St. Louis, MO). Normal Tyrode's solution (NT) contained (in mmol/L) NaCl 140, KCl 4, MgCl₂ 1, CaCl₂ 1, glucose 10, and HEPES 5 (pH adjusted to 7.4 with NaOH). For I_{Ca} NT was used with KCl replaced with 4 CsCl. The pipette solution contained (in mmol/L) CsCl 125, TEA-Cl 20, EGTA 10, HEPES 10, Phosphocreatine 5, ATP 5, and GTP 0.3 (pH adjusted to 7.2 with CsOH).

Statistical analysis

Data are presented as means ± S.E. Student's unpaired *t*-test (with Bonferroni correction for multiple comparison where applicable) was used to determine significance (at $P < 0.05$).

Results

Ca²⁺-frequency and Shortening-frequency relationships

Cytosolic Ca²⁺ transients and contractility were measured in field-stimulated myocytes paced stepwise from 0.2 to 4 Hz. Figure 1A shows an original dataset of Ca²⁺ transients (top panel) and cell shortening (bottom panel) obtained in a myocyte isolated from SR-AIP. Average data for Ca²⁺ transient amplitudes are shown in Figure 1B. Amplitudes were similar among groups at 0.2 Hz and decreased in all three groups with increasing stimulation frequency to a comparable extent. From 0.2 to 4 Hz transient amplitude in WT decreased from 2.9 ± 0.2 to $1.3 \pm 0.1 \Delta F/F_0$ ($P < 0.05$). Similarly, NLS-AIP decreased from 2.8 ± 0.2 at 0.2 Hz to $1.7 \pm 0.1 \Delta F/F_0$ at 4 Hz ($P < 0.05$) and SR-AIP decreased from 2.7 ± 0.3 to $1.1 \pm 0.2 \Delta F/F_0$ ($P < 0.05$). Quantitatively similar results were obtained for fractional shortening however, SR-AIP showed a significantly more negative shortening-frequency relation (Figure 1C). After an initial decline in fractional shortening from 0.2 to 0.5 Hz, WT and NLS-AIP exhibited a fairly flat shortening-frequency relation upon further increase of stimulation frequency whereas, SR-AIP myocytes showed a negative shortening-frequency relation over the whole range of stimulation frequencies. It is possible that the more pronounced negative shortening-frequency relation in SR-AIP myocytes relates to reduced FDAR and limited ability to stimulate SR Ca²⁺ uptake in SR-AIP (as discussed below). However, this effect was less apparent in the Ca²⁺ transients, and the complex kinetic factors which contribute to the amplitude of contraction and Ca²⁺ transients makes such inferences more complicated. To more directly evaluate the effects of SR-AIP specifically on SR Ca²⁺-ATPase function we examined FDAR in more detail.

Frequency-dependent acceleration of relaxation (FDAR)

Normally SERCA mediated [Ca²⁺]_i uptake increases with increased stimulation frequency, and accordingly Ca²⁺ transient decline and twitch relaxation were faster in all groups at higher frequencies (FDAR; Figure 2). However, FDAR was significantly inhibited in SR-AIP vs. WT or NLS-AIP. Normalized Ca²⁺ transients and cell shortening from WT and SR-AIP at 0.2 and 4 Hz are shown in Figure 2A and C, respectively, showing faster Ca²⁺ transient decline and twitch relaxation at 4 Hz vs. 0.2 Hz and the blunted response in SR-AIP. Note that the last twitch of a train at a given frequency was used to avoid complications due to elevated diastolic [Ca²⁺]_i at higher frequencies.

Mean data for the exponential time constant τ of Ca²⁺ transient decline are shown in Figure 2B. WT and NLS-AIP showed typical FDAR: upon increasing stimulation frequency from 0.2 Hz to 4 Hz τ decreased by 59 % in WT (from 247 ± 16 to 101 ± 5 ms; $P < 0.05$) and by 46 % in NLS-AIP (from 245 ± 20 to 131 ± 8 ms; $P < 0.05$). In contrast, in SR-AIP FDAR was strongly suppressed and τ fell only by 24 %, from 251 ± 10 only to 192 ± 4 ms; $P < 0.05$ vs. 0.5 Hz and $P < 0.05$ vs. NLS-AIP and WT. Global CaMKII inhibition by 10 min preincubation with 1 μ M of the CaMKII inhibitor KN-93 reduced FDAR in NLS-AIP myocytes to a similar extent as seen in SR-AIP (Figure 2B inset showing the FDAR index as the ratio of $\tau_{4 \text{ Hz}} / \tau_{0.2 \text{ Hz}}$).

Quantitatively similar data were obtained from cell shortening measurements. Mean data for τ of twitch relaxation are shown in Figure 2D. At 0.2 Hz τ of twitch relaxation was comparable in WT, NLS-AIP and SR-AIP. In WT increasing stimulation frequency from 0.2 to 4 Hz decreased τ by 75% (from 158 ± 20 to 39 ± 11 ms; $P < 0.05$), and comparably in NLS-AIP (from 157 ± 24 to 41 ± 7 ms; $P < 0.05$). In contrast, in SR-AIP τ only fell by only 35% (from 168 ± 17 to 108 ± 7 ms; $P < 0.05$ vs. 0.2 Hz and $P < 0.05$ vs. NLS-AIP and WT). The inset in Figure 2B shows the FDAR index for twitch relaxation. These results are consistent with previous data in WT mice [13] and suggest that FDAR is in large part due to CaMKII activity.

SR Ca²⁺ load

To test whether decreased SERCA activity in SR-AIP myocytes limits SR Ca²⁺ load (especially at higher frequency where FDAR is apparent) SR Ca²⁺ content was measured after steady-state stimulation at 0.2, 1 and 4 Hz in WT and SR-AIP (Figure 3A). Caffeine remained present until [Ca²⁺]_i declined to diastolic levels. Averaged data (Figure 3B) show that SR Ca²⁺ load tended to decrease in both groups at higher frequency (and more so in SR-AIP), but not significantly. Similarly, fractional SR Ca²⁺ release tended to be slightly lower at higher frequency and in SR-AIP (not shown).

Quantification of cytosolic Ca²⁺ removal

The [Ca²⁺]_i-dependence of Ca²⁺ removal via SERCA and NCX was quantified in WT and SR-AIP cells at 0.2 and 4 Hz using the amplitude and τ values for twitch and caffeine-induced Ca²⁺ transients from Figures 1–3 (see Methods). Figure 4A and 4B shows NCX and SERCA mediated removal of total cytosolic Ca²⁺ as a function of free [Ca²⁺]_i, respectively. At both stimulation frequencies Ca²⁺ removal rates via NCX did not differ between WT and SR-AIP (Figure 4A). However, NCX mediated Ca²⁺ removal was slower at 4 Hz than at 0.2 Hz, which is readily explained by the higher [Na⁺]_i at higher frequency. In WT, Ca²⁺ removal rate via SERCA (Figure 4B) was greatly elevated at 4 Hz, entirely due to increased $V_{\max}$ without change in K_m ($V_{\max}$ = 697 and 1486 μ M/s at 0.2 and 4 Hz, respectively; K_m = 412 μ M at both 0.2 and 4 Hz). In SR-AIP, in contrast, the frequency dependent enhancement of SERCA Ca²⁺ activity was markedly attenuated. $V_{\max}$ increased only slightly from 730 μ M/s at 0.2 Hz to 836 μ M/s at 4 Hz without change in K_m (K_m = 416 μ M and 415 μ M for 0.2 and 4 Hz, respectively). Thus according to our analysis, the enhancement of SR Ca²⁺ uptake at higher frequencies is mediated by enhancement of the apparent SERCA $V_{\max}$ and this effect is largely sensitive to CaMKII inhibition.

CaMKII dependent RyR phosphorylation

CaMKII dependent RyR phosphorylation was quantified in four pairs of WT and SR-AIP hearts using immunoblots with phospho-specific antibodies (Figure 5). RyR expression was reduced by ~25 % in SR-AIP vs. WT ($P < 0.05$) when normalized to GAPDH but unchanged when normalized to CSQ. After normalization to RyR expression level, CaMKII dependent phosphorylation at Ser2815 was decreased by 62 % in SR-AIP vs. WT ($P < 0.05$). As previously described (using a different RyR-phospho2809 antibody) [26], the mean phosphorylation level at Ser2809 was lower in SR-AIP vs. WT, although not significantly. This data, particularly on Ser2815 phosphorylation, demonstrates that the SR-localized CaMKII inhibition includes junctional proteins and is not restricted to the longitudinal SR.

Reduced SR Ca²⁺ leak in SR-AIP mice

Since CaMKII dependent RyR phosphorylation has been associated with increased diastolic SR Ca²⁺ leak, we measured leak as tetracaine-induced shifts in cytosolic Ca²⁺ in intact WT and SR-AIP myocytes (Figure 6) [33]. Diastolic SR Ca²⁺ leak was significantly lower in SR-AIP vs. WT mice at both 0.2 Hz (tetracaine-sensitive Δ [Ca²⁺]_i = 15 ± 2 nM in WT vs. 9 ± 2 nM in SR-AIP, $P < 0.05$) and 4 Hz (13 ± 1 nM in WT and 8 ± 2 nM in SR-AIP, $P < 0.05$) (Figure 6B). These results suggest that SR-AIP mice have a reduced SR Ca²⁺ leak that may contribute to limiting the decrease in SR Ca²⁺ load (which was not significantly decreased; Figure 3B) in SR-AIP mice. Possibly the combination of reduced SR Ca²⁺ leak, a tendency for reduced fractional release and reduced SERCA function in SR-AIP result in a relatively unaltered SR Ca²⁺ content.

I_{Ca} facilitation and recovery from inactivation

We next tested whether *I_{Ca}* facilitation and recovery from inactivation are altered by SR-AIP. Both processes depend on CaMKII [21,22,24,36,37] but if the AIP moieties in the fusion protein anchored in the SR membrane cannot reach the sarcolemma these processes should be unaltered in SR-AIP mice. To rule out secondary effects on *I_{Ca}* facilitation due to changes in intracellular Ca²⁺ handling, we performed these experiments in the presence of 10 mM EGTA, therefore abolishing intracellular Ca²⁺ transients.

WT and NLS-AIP showed typical *I_{Ca}* facilitation (Figure 7A). Peak *I_{Ca}* amplitude increased over the first 4–5 depolarizations (Figure 7B) reaching $106 \pm 1.8\%$ in WT ($n = 15$) and $107 \pm 2.3\%$ in NLS-AIP ($n = 10$). In contrast *I_{Ca}* facilitation was blocked in SR-AIP and peak amplitude decreased to $92 \pm 2.4\%$ ($n = 12$) where it stabilized.

A two-pulse protocol (Figure 7C, inset) was used to measure *I_{Ca}* recovery from inactivation. In WT, the recovery of *I_{Ca}* availability was well described by a single exponential function with $\tau = 230$ ms (Figure 7C). In SR-AIP, the fit for recovery required a second, much slower time constant of 1276 ms in addition to a fast time constant of 193 ms. The slow phase of recovery contributed 33% to the overall recovery in SR-AIP myocytes.

Discussion

In cardiomyocytes, CaMKII regulates critical components involved in ECC and CICR, including Ca²⁺ influx, release and reuptake [1,2]. The activity of CaMKII is regulated in response to frequency and amplitude of Ca²⁺ transients [38,39]. At low stimulation frequencies CaMKII mediated phosphorylation is small but increases substantially at high frequencies [14,40,41]. Given the large number of effector proteins, specific targeting of CaMKII activity is critical to coordinate the dynamic functional requirements of the cardiomyocyte. We found that a specific CaM-KII inhibitory peptide exclusively expressed in the SR membrane in transgenic mice (SR-AIP [26,27]) resulted in inhibition of FDAR, RyR phosphorylation, SR Ca²⁺ leak and *I_{Ca}* facilitation.

FDAR and cytosolic Ca²⁺ removal

FDAR is present in all ventricular muscle and ensures appropriate diastolic filling at higher heart rates. It results from accelerated SR Ca²⁺ uptake and does not depend on Ca²⁺ removal out of the cell [9,11,17]. The contribution of PLB and CaMKII to FDAR remains controversial. CaMKII inhibition has been reported to suppress FDAR in several studies [9,12,13], but other studies did not detect FDAR inhibition with KN-93 or KN-62 [11,15–17]. In isolated myocytes CaMKII dependent phosphorylation of PLB at Thr17 occurs in a frequency dependent manner [14,17]. However, FDAR develops substantially faster than does PLB Thr17 phosphorylation [17]. Furthermore, FDAR is present in mice lacking the PLB CaMKII phosphorylation site [42] and in PLB knockout mice (where FDAR was still inhibited by CaMKII inhibitors) [12, 13,18]. These studies suggest a PLB independent, but at least partially CaMKII dependent mechanism for FDAR. In the present study, both control groups showed robust FDAR whereas in SR-AIP it was strongly, but incompletely inhibited (Figure 2). This result indicates that CaMKII contributes to FDAR. Our quantitative analysis of [Ca²⁺]_i decline indicated that FDAR in WT and NLS-AIP was due to increased SERCA *V_{max}* without altered *K_m* (Figure 4). The frequency- and CaMKII-dependent enhancement of SERCA *V_{max}* reported here in isolated cells is consistent with *in vitro* Ca²⁺ uptake measurements in ventricular homogenates from SR-AIP mice [26], and also with Ca²⁺ uptake into permeabilized rat myocytes where CaMKII inhibitors reduced *V_{max}* [9]. Some reports attributed enhanced SERCA *V_{max}* to CaMKII dependent SERCA phosphorylation [43,44]. However, such a direct CaMKII-dependent SERCA phosphorylation has been strongly challenged by others [45–47].

Regardless of whether SERCA is the CaMKII target our findings suggest that a CaMKII dependent enhancement of SERCA $V_{\max}$ at least partly mediates FDAR.

NCX mediated Ca^{2+} extrusion counteracts faster SR Ca^{2+} uptake

Faster SR Ca^{2+} uptake due to FDAR should, in principle, increase SR Ca^{2+} content and hence steady-state Ca^{2+} transient amplitude. However, what we found was, if anything, a frequency-dependent decrease in SR Ca^{2+} content in SR-AIP and WT. This is presumably due to Ca^{2+} extrusion via NCX during the Ca^{2+} transient and facilitated by the short action potential in mouse [10], which promotes Ca^{2+} efflux. Although NCX mediated Ca^{2+} removal is slightly slower at higher frequencies in both groups (Figure 4A, due to increased $[\text{Na}^+]_i$ at higher frequencies) the more frequent NCX mediated Ca^{2+} extrusions at higher frequencies may compensate or even overcome the effect of faster SR Ca^{2+} uptake (resulting in unaltered or even reduced SR- Ca^{2+} load). Our results therefore emphasize the importance of functional CaMKII modulation of the SR Ca^{2+} uptake in the very basic cellular response to increased frequency.

Reduced SR Ca^{2+} leak explains unaltered SR Ca^{2+} load despite decreased SERCA activity

CaMKII associates with and directly phosphorylates the cardiac RyR [19,34,48,49]. This leads to increased RyR channel opening in bilayers and to an increased Ca^{2+} spark frequency in myocytes at a given SR Ca^{2+} content [3,19,34,48–50]. Controversy remains about which RyR amino acid is the critical CaMKII target (Ser2809 vs. Ser2815) [19,48,49]. Our results show a major reduction in Ser2815 phosphorylation in SR-AIP vs. WT (with only a marginal effect at Ser2809), and are thus consistent with Ser2815 being a significant CaMKII phosphorylation target.

SR Ca^{2+} load is a balance of Ca^{2+} uptake and SR Ca^{2+} leak. We find reduced SR Ca^{2+} leak in SR-AIP, which is in line with reduced CaMKII dependent RyR phosphorylation. The fact that SR Ca^{2+} leak is slightly smaller at 4 Hz vs. 0.2 Hz in both groups may be secondary to the slight reduction in SR Ca^{2+} load (Figure 3B) and the very steep relationship between leak and load, especially at high SR Ca^{2+} load [33]. However, the increased frequency is expected to enhance CaMKII-dependent RyR leak. Because our results show that SR Ca^{2+} leak is reduced at 4 Hz, the effect of decreased SR Ca^{2+} load likely dominates the effect of increased RyR phosphorylation. The reduced leak in SR-AIP (Figure 6) and lower fractional release seen with CaMKII inhibition at given I_{Ca} and SR Ca^{2+} load [35] may explain why SR Ca^{2+} content is not significantly altered vs. WT at 4 Hz, despite the lower SERCA function (Figure 4B). In WT, on the other hand, high SR Ca^{2+} leak and fractional release may counteract the enhanced SR Ca^{2+} uptake via SERCA.

I_{Ca} facilitation is inhibited by SR-targeted CaMKII inhibition

Ca^{2+} -dependent I_{Ca} facilitation is characterized by a stepwise increase of I_{Ca} amplitude and a slowing of inactivation. It requires CaMKII-dependent phosphorylation, presumably of the α_{1c} -subunit or an associated protein [21,23,24]. Consequently, global CaMKII inhibition abolishes facilitation [22,24,34,51]. Since immunofluorescent and biochemical results showed that the CaMKII inhibitory peptide construct is exclusively located in the SR [26], we expected to find unaltered I_{Ca} facilitation in SR-AIP. In WT and NLS-AIP I_{Ca} facilitation was consistently present. Surprisingly, I_{Ca} facilitation was completely abolished in SR-AIP. In addition, I_{Ca} recovery from inactivation, a process slowed by AIP [36,37] and by the synthetic CaMKII inhibitor KN-93 [35], was also markedly slowed in SR-AIP. Therefore our results suggest that the CaMKII inhibiting peptide located in the SR membrane inhibits CaMKII-dependent phosphorylation of the L-type Ca^{2+} channel. Different mechanisms could be responsible. The distance between the SR and the plasma membrane in the dyadic cleft is ~15 nm [52]. The cytosolic part of the L-type Ca^{2+} channel protrudes approximately 3–5 nm into

the cell [53,54], which leads to an effective distance between the SR membrane and a putative CaMKII interaction site on the channel of 10–12 nm. The cytosolic part of the CaMKII inhibitory peptide construct (4 copies of AIP and FLAG protein) consists of 60 amino acids. Assuming either an elongated structure (e.g. β -strand) or a shorter α -helix, this would result in a length of 9–21 nm. Thus, SR-AIP at the junctional SR could reach to inhibit a prepositioned CaMKII near the Ca^{2+} channel [5]. Additionally, the AIP fusion protein may restrict access of CaMKII to the junction and therefore limit CaMKII dependent regulation of the L-type Ca^{2+} channel. The complete lack of I_{Ca} facilitation in SR-AIP also suggests that most of the L-type Ca^{2+} channels are indeed close enough to the junctional SR to be subject to AIP effects. This is consistent with ultrastructural data showing that L-type Ca^{2+} channels are highly localized at these SR junctions [25].

In summary, SR-localized AIP inhibits CaMKII dependent regulation of SR Ca^{2+} uptake via SERCA, SR Ca^{2+} release via RyR and even Ca^{2+} entry via I_{Ca} . This emphasizes an important multifaceted regulation of cardiac myocytes Ca^{2+} transport by CaMKII associated with or extremely close to the SR.

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Figure 1.

Frequency dependence of Ca^{2+} transients and fractional shortening. A, Original trace of simultaneously measured Ca^{2+} transients (top) and fractional shortening (bottom) in a cell isolated from SR-AIP. B, Ca^{2+} transient amplitude in WT (●, n = 7), NLS-AIP (▼, n = 10) and SR-AIP (○, n = 6) (**P* < 0.05 vs. 0.2 Hz). C, Fractional shortening in WT (●, n = 5), NLS-AIP (▼, n = 12) and SR-AIP (○, n = 9) (**P* < 0.05 vs. 0.2 Hz).

Figure 2.

Frequency dependent acceleration of relaxation is blunted in SR-AIP. A, Normalized Ca^{2+} transients at 0.2 and 4 Hz in WT and SR-AIP. B, Time constant (τ) of Ca^{2+} transient decline in WT ($\bullet$, $n = 7$), NLS-AIP ($\blacktriangledown$, $n = 10$) and SR-AIP ($\circ$, $n = 6$) ($P < 0.05$ vs. WT and NLS-AIP at the same frequency, $*P < 0.05$ vs. 0.5 Hz in the same group). Inset shows FDAR index ($\tau_{4Hz}/\tau_{0.2Hz}$) for WT, NLS-AIP, SR-AIP and NLS-AIP + KN-93 ($*P < 0.05$ vs. SR-AIP and NLS-AIP + KN-93). C, Normalized cell shortening at 0.2 and 4 Hz in WT and SR-AIP. D, τ of twitch relaxation in WT ($\bullet$, $n = 6$), NLS-AIP ($\blacktriangledown$, $n = 10$) and SR-AIP ($\circ$, $n = 6$) ($P < 0.05$ vs. WT and NLS-AIP at the same frequency, $*P < 0.05$ vs. 0.5 Hz in the same group).

Figure 3. SR- Ca^{2+} load in WT and SR-AIP at 0.2, 1 and 4 Hz stimulation frequency. A, Typical experiment obtained in WT. After steady-state stimulation 10 mM caffeine was rapidly applied and remained present until $[\text{Ca}^{2+}]_i$ reached diastolic levels. B, Average data of SR Ca^{2+} load in WT (n = 7) and SR-AIP (n = 5) at 0.2, 1 and 4 Hz stimulation frequency.

Figure 4.

Frequency dependent increase in SERCA activity is markedly inhibited in SR-AIP. A, Ca^{2+} removal via NCX is bigger at 0.2 than at 4 Hz but is not different between WT and SR-AIP. B, SERCA V_{max} is substantially increased at 4 Hz in WT but shows only a small increase in SR-AIP. K_m is not frequency sensitive.

Figure 5.

Quantitative immunoblot analysis of RyR expression and phosphorylation. A, Original Western Blots showing total RyR expression and phosphorylation at site Ser2809 and Ser2815. Calsequestrin (CSQ) expression was measured in the same samples as loading control. B, Average data for four pairs of mice. SR-AIP mice have significantly decreased phosphorylation at site Ser2815 (* $P < 0.05$ vs. WT).

Figure 6.

SR Ca^{2+} leak is reduced in SR-AIP vs. WT. A, Protocol used to measure SR Ca^{2+} leak. Shift in diastolic $[Ca^{2+}]_i$ upon removal of tetracaine was used to quantify SR Ca^{2+} leak (NT: normal Tyrode's solution; 0Na 0Ca 1Tetra: Sodium-free, calcium-free Tyrode's solution with 1 mM Tetracaine; 0Na 0Ca: Sodium-free, calcium-free Tyrode's solution; Cf: Tyrode's solution with 10 mM Caffeine). B, Average data for tetracaine sensitive shift in diastolic $[Ca^{2+}]_i$ in WT ($n = 16$) and SR-AIP ($n = 10$) at 0.2 and 4 Hz (* $P < 0.05$ vs. WT at the same stimulation frequency).

Figure 7.

SR targeted CaMKII inhibition abolishes I_{Ca} facilitation and slows recovery from inactivation. A, Original I_{Ca} recordings obtained in WT and SR-AIP. I_{Ca} in response to the first (I_1) and to the fifth testpulse (I_5) is shown. B, Average data for I_{Ca} peak current normalized to the first testpulse for WT (●, $n = 15$), NLS-AIP (▼, $n = 10$) and SR-AIP (○, $n = 12$). C, Recovery from inactivation in WT (●, $n = 5$) could be well fit to a monoexponential function, whereas SR-AIP (○, $n = 5$) required a fit with an additional slow component. The inset shows the two-pulse voltage protocol that was applied to test availability. Time gaps between the pulses were 100–3000 ms.