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Protein kinase A-dependent phosphorylation of ryanodine receptors increases Ca^{2+} leak in mouse heart ☆

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ABSTRACT

In heart failure, chronic catecholaminergic stimulation increases diastolic Ca^{2+} leak from ryanodine receptors (RyRs) of sarcoplasmic reticulum (SR), possibly due to the phosphorylation of RyRs through the activation of protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). In the present study, we attempted to identify which activated kinase is responsible for the enhanced Ca^{2+} leak caused by β-adrenergic stimulation. Trabeculae obtained from the hearts of adult male C57BL/6J mice were treated with isoproterenol and then permeabilized with saponin. To examine SR functions, Ca^{2+} in SR was released with caffeine and measured with fluo-3. The Ca^{2+} leak in isoproterenol-treated preparations was significantly increased when the PKA-dependent phosphorylation of RyR was increased without the involvement of CaMKII-dependent phosphorylation. Both the increase in Ca^{2+} leak and the phosphorylation of RyR were blocked by a PKA inhibitor. Our results show that β-adrenergic stimulation increases Ca^{2+} leak from SR through PKA-dependent phosphorylation of RyR.

Introduction

Sarcoplasmic reticulum (SR) plays an important role in excitation–contraction coupling as the main regulator of Ca^{2+} cycling in cardiac ventricular myocytes [1,2]. The functions of SR in Ca^{2+} handling are generally divided into two mechanisms: (1) Ca^{2+} uptake into SR by the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which is regulated by interaction with phospholamban (PLB); (2) Ca^{2+} release from SR through the Ca^{2+}-release channel called ryanodine receptor (RyR), which is activated by transsarcolemmal Ca^{2+} influx (Ca^{2+}-induced Ca^{2+} release mechanism) [1–3]. Luminal SR Ca^{2+} content is determined by the balance of the Ca^{2+} uptake and Ca^{2+} release functions of SR, and the SR Ca^{2+} content itself can also modulate these SR functions during systole and diastole [1–3].

We have reported that a small amount of Ca^{2+} leak from cardiac SR into the cytoplasm during the resting state, even under physiological conditions [4]. Recent reports have shown that the Ca^{2+} leak becomes more prominent in the failing heart, leading to contractile dysfunction via a reduction in SR Ca^{2+} content or arrhythmia [3,5]. The phosphorylation of RyR by protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) via the activation of β-adrenergic signaling has been proposed as a mechanism for the increase in Ca^{2+} leak under pathophysiological conditions, as has been shown in studies using the following preparations: (1) partially purified RyRs fused into bilayers [6,7], (2) isolated SR vesicles [8], and (3) isolated ventricular myocytes [9–11]. However, the functional significance of RyR phosphorylation by PKA or CaMKII under physiological conditions remains unclear.

We have previously reported a novel method for estimating SR functions (SR Ca^{2+} content, Ca^{2+} uptake, Ca^{2+} release and Ca^{2+} leak) in saponin-permeabilized multicellular preparations obtained from mammalian heart [4,12]. In the present study, we used this method to investigate (1) the increase in Ca^{2+} leak from SR due to β-adrenoceptor (β-AR) stimulation and (2) the involvement of kinases (PKA or CaMKII) in the increased Ca^{2+} leak.

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Materials and methods

All experiments were performed in accordance with the Guidelines on Animal Experimentation of The Jikei University School of Medicine, and the study protocol was approved by the Animal Care Committee of The Jikei University School of Medicine (approval Reference No.: H19-049). The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). An expanded Material and methods section is included in the online data supplement.

Preparations. Papillary muscles and trabeculae dissected from the left ventricles of adult male C57BL/6J mice (weight, 25–35 g) were prepared using a Langendorff apparatus as previously described [13] and used throughout all experiments.

Measurement of Ca^{2+} content in SR. To estimate SR functions, the papillary muscles or trabeculae of the left ventricle were first treated with Tyrode's solution with or without isoproterenol (1 μmol/L) for 30 min, and then the preparation was permeabilized with saponin (50 μg/ml) for 30 min in the relaxing solution.

Each SR function (Ca^{2+} uptake, Ca^{2+} leak, and maximal Ca^{2+} content) was estimated with a Ca^{2+} indicator, fluo-3 (Dojindo Laboratories, Kumamoto, Japan), as described previously (Supplementary Figure I) [4]. The measured fluorescence change of fluo-3 was converted to Ca^{2+} concentration, and the SR Ca^{2+} content is expressed as micromoles per liter of cytoplasm [12]. Solutions used to estimate SR functions are listed in Table 1. All solutions contained 20 mmol/L NaN_3 to block the Ca^{2+} uptake of mitochondria and 20 mmol/L piperazine-*N*-*N*′-bis[2-ethanesulfonic acid] (PIPES) (ionic strength, 0.2 mol/L; temperature, 22 ℃; pH adjusted with KOH). In all protocols for estimating SR functions after β-AR stimulation, calyculin A (15 nmol/L; Biomol International, Plymouth Meeting, PA) was also added to all solutions to maintain the phosphorylation of proteins related to SR Ca^{2+} handling [14]. We confirmed that the phosphorylation levels of RyR were maintained throughout the period of our experimental protocol (approximately 2 h at room temperature) by adding calyculin A to the experimental solutions (data not shown). The Ca^{2+} leak was estimated by measuring Ca^{2+} in the SR after the preparation had been washed out in relaxing solution at various times (15–300 s) after Ca^{2+} loading (pCa 6.2 for 120 s) into SR. Because Ca^{2+} content in SR becomes saturated (designated "maximal Ca^{2+} content") after this loading protocol, the Ca^{2+} leak from SR can be measured with the same Ca^{2+} content. We also confirmed that the inhibition of RyR opening by ruthenium red significantly reduced Ca^{2+} leak from SR, which indicates Ca^{2+} leak through RyR (data not shown). To evaluate the maximal Ca^{2+} content of SR, curve-fitting of the Ca^{2+} leak data was performed and the value of Ca^{2+} content before washing was estimated as the maximal Ca^{2+} content [12].

Western immunoblotting. Western immunoblotting was performed as described previously [15,16]. The phosphorylation levels of RyR and PLB were normalized by means of the band intensities of the total amounts of the respective proteins. Antibodies against the PKA-dependent phosphorylation site (S2808) and the CaMKII-dependent phosphorylation site (S2814) of RyR were kindly donated by Dr. A.R. Marks (Columbia University College of Physicians and Surgeons, New York, NY), and other antibodies were purchased from commercial sources (see details in online supplement).

Statistical analysis. All data are presented as mean ± standard error of the mean (SEM). Statistical significance was estimated with nonparametric tests for two sets of data and with one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple comparisons, with the significance level set at $P < 0.05$.

Table 1
Composition of experimental solutions.

Period	Preload	Load	Wash	Preassay	Assay	Wash
A. Loading assay						
Time (s)	60	10–120	60	30	60	60
Ca^{2+} (pCa)	>8	8–5.6	>8	>8	>8	>8
KMs[a] (mmol/L)	128	126–128	154	162	103	154
Mg^{2+} (mmol/L)	1.5	1.5	1.5	0	0	1.5
EGTA[b] (mmol/L)	1	1	1	0	0	1
ATP[c] (mmol/L)	4	4	0	0	0	0
AMP[d] (mmol/L)	0	0	0	25	0	0
Caffeine (mmol/L)	0	0	0	0	50	0
Fluo-3 (μmol/L)	0	0	0	30	30	0
B. Leakage assay						
Time (s)	60	120	15–300	30	60	60
Ca^{2+} (pCa)	>8	6.2	>8	>8	>8	>8
KMs (mmol/L)	128	127	154	162	103	154
Mg^{2+} (mmol/L)	1.5	1.5	1.5	0	0	1.5
EGTA (mmol/L)	1	1	1	0	0	1
ATP (mmol/L)	4	4	0	0	0	0
AMP (mmol/L)	0	0	0	25	0	0
Caffeine (mmol/L)	0	0	0	0	50	0
Fluo-3 (μmol/L)	0	0	0	30	30	0

[a] MS, methanesulfonate.
[b] EGTA, ethylene glycol bis[β-aminoethylether]-*N*,*N*,*N*′,*N*′-tetraacetic acid.
[c] ATP, adenosine triphosphate.
[d] AMP, adenosine-5′-monophosphate.

Results

Effect of β-AR stimulation on Ca^{2+} leak from SR

We examined the effect of β-AR stimulation on Ca^{2+} leak from SR. First, we analyzed the Ca^{2+} content of SR in preparations that had or had not been treated with isoproterenol using a previously described protocol in which the SR is maximally loaded with Ca^{2+} (maximal Ca^{2+} content) [12]. Maximal Ca^{2+} contents of SR did not differ significantly between isoproterenol-treated preparations (362.3 ± 22.0 μmol/L cytosal, $n = 18$) and non-isoproterenol-treated preparations (380.8 ± 20.1 μmol/L cytosol, $n = 18$), indicating that β-AR stimulation had no effect on the maximal Ca^{2+} content of SR.

We investigated the effect of β-AR stimulation on Ca^{2+} leak from SR (Fig. 1). Because maximal Ca^{2+} content did not differ between preparations that had or had not been treated with isoproterenol, the time-dependent change in Ca^{2+} leak from SR can be estimated by measuring the Ca^{2+} content of the SR at different washing-out times (15–300 s) after Ca^{2+} loading. The Ca^{2+} leak in isoproterenol-treated preparations was significantly larger than that in non-isoproterenol-treated preparations (Fig. 1A) and a difference was clearly indicated by the time constant of Ca^{2+} leak (Fig. 1B).

The Ca^{2+} leak from SR is reportedly modulated by the phosphorylation of RyR by PKA or CaMKII, which are downstream kinases activated by β-AR stimulation [3], and these phosphorylation levels can be separately evaluated with antibodies against specific phosphorylation sites [7]. The PKA-dependent RyR phosphorylation at Ser2808 was significantly increased by treatment with isoproterenol (Fig. 1C). In contrast, the CaMKII-dependent RyR phosphorylation (Ser2814) was not significantly increased by treatment with isoproterenol (Fig. 1D). Thus, the increase in the RyR phosphorylation level at Ser2808 seems to be primarily responsible for the acceleration of Ca^{2+} leak from SR in isoproterenol-treated preparations.

To confirm the involvement of PKA activity in the changes in Ca^{2+} leak after β-AR stimulation, an inhibitor of PKA, H-89 (2 μmol/L), was added to the solution with isoproterenol before saponin permeabilization [17]. In the presence of the PKA inhibitor H-89, the maximal Ca^{2+} content of SR did not differ significantly

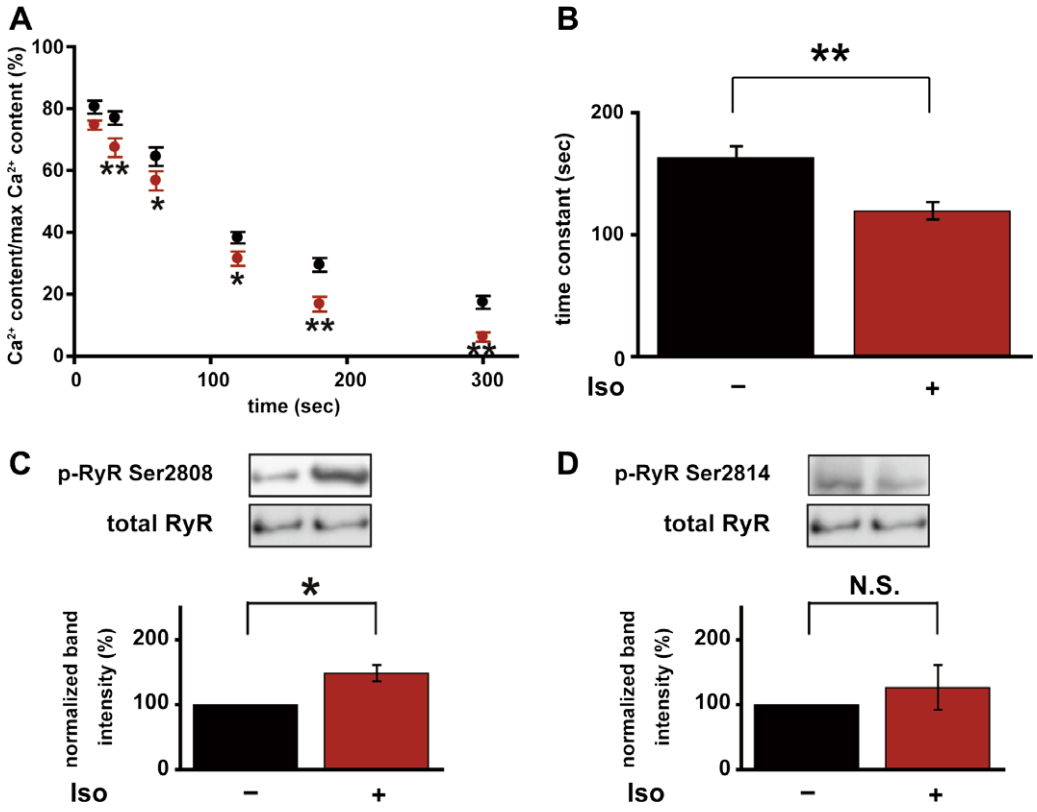

Fig. 1. Increase in Ca^{2+} leak from SR after β-AR stimulation. (A) Time-dependent changes in Ca^{2+} leak from SR before (black circles, $n = 18$) and after isoproterenol treatment (red circles, $n = 18$). Ordinate: Ca^{2+} content in SR was normalized by the maximal Ca^{2+} content in each preparation. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the value of Ca^{2+} content in SR before isoproterenol treatment at each time. (B) Time constant of Ca^{2+} leak from SR before (black bar, $n = 18$) and after isoproterenol treatment (red bar, $n = 18$). $^{**}P < 0.01$, compared with the time constant of Ca^{2+} leak from SR before isoproterenol treatment. (C,D) Western immunoblotting analysis of phosphorylation levels at RyR Ser2808 and at Ser2814 before (black bars) and after isoproterenol treatment (red bars, $n = 7$). Blots of total amounts of RyR protein are also shown ($n = 7$). $^{*}P < 0.05$, compared with phosphorylation levels at RyR Ser2808 before isoproterenol treatment.

between isoproterenol-treated preparations (374 ± 17.2 μmol/L cytosol, $n = 23$) and non-isoproterenol-treated preparations (347.8 ± 21.5 μmol/L cytosol, $n = 17$). Treatment with H-89 completely blocked the increase in Ca^{2+} leak observed after β-AR stimulation (Fig. 2A and B) and completely abolished the RyR phosphorylation at Ser2808 observed after β-AR stimulation (Fig. 2C). These results confirm that the increase in Ca^{2+} leak from SR after β-AR stimulation is mediated mainly through the PKA-dependent phosphorylation of RyR.

The acceleration of Ca^{2+} leak by β-AR stimulation was not blocked by CaMKII inhibition

To rule out the involvement of CaMKII-dependent RyR phosphorylation in the increased Ca^{2+} leak due to β-AR stimulation, the cell-permeable form of a specific CaMKII-inhibitory peptide, autocamtide 2-related inhibitory peptide (AIP) (100 μmol/L), was used [10], because the synthetic inhibitor of CaMKII (KN-93) is ineffective for multicellular preparations [18]. The preparations were pretreated with AIP for 30 min and then treated with isoproterenol in the continuous presence of AIP before saponin permeabilization. With this protocol, we confirmed that the CaMKII-dependent phosphorylation of PLB at Thr17 [19] and of RyR at Ser2814 were abolished by the CaMKII inhibitor AIP, even at their basal phosphorylation levels; however, the levels of PKA-dependent phosphorylation of PLB and RyR were not affected by AIP (data not shown). In the presence of AIP, the maximal Ca^{2+} content of SR did not differ significantly between isoproterenol-treated preparations (324.6 ± 29.4 μmol/L cytosol, $n = 12$) and non–isoproterenol-treated preparations (321.5 ± 17.9 μmol/L cytosol, $n = 10$). AIP did not block the isoproterenol-induced increase in Ca^{2+} leak (Fig. 3A and B) or the isoproterenol-induced RyR phosphorylation at Ser2808 (Fig. 3C).

Discussion

In the present study, we used saponin-permeabilized preparations of mouse left ventricle to examine SR functions modulated by β-AR stimulation. We found that the increase in Ca^{2+} leak from SR after β-AR stimulation is caused at least by the increase in PKA-dependent RyR phosphorylation at Ser2808 and not by CaMKII-dependent RyR phosphorylation under our experimental conditions.

The Ca^{2+} leak from cardiac SR occurs even under physiological conditions [4,12], and its importance under pathophysiological conditions, such as heart failure, has also recently been shown [5]. The increase in Ca^{2+} leak from RyR has been proposed as an important factor in the reduced Ca^{2+} content of SR in heart failure, which induces contractile dysfunctions [3]. Some groups have reported that the increased Ca^{2+} leak in the failing heart is caused by PKA-dependent hyperphosphorylation of RyR [6,8], but other groups have insisted that PKA-dependent phosphorylation does not increase Ca^{2+} leak from SR [11].

The inconsistent results seem to be due to limitations of methods used in previous studies. The Ca^{2+} leak from SR induced by PKA-dependent phosphorylation has most often been observed with single-channel recording in lipid bilayers [5,6], $^{45}Ca^{2+}$ flux measurement in SR vesicles [8], and Ca^{2+} spark measurement

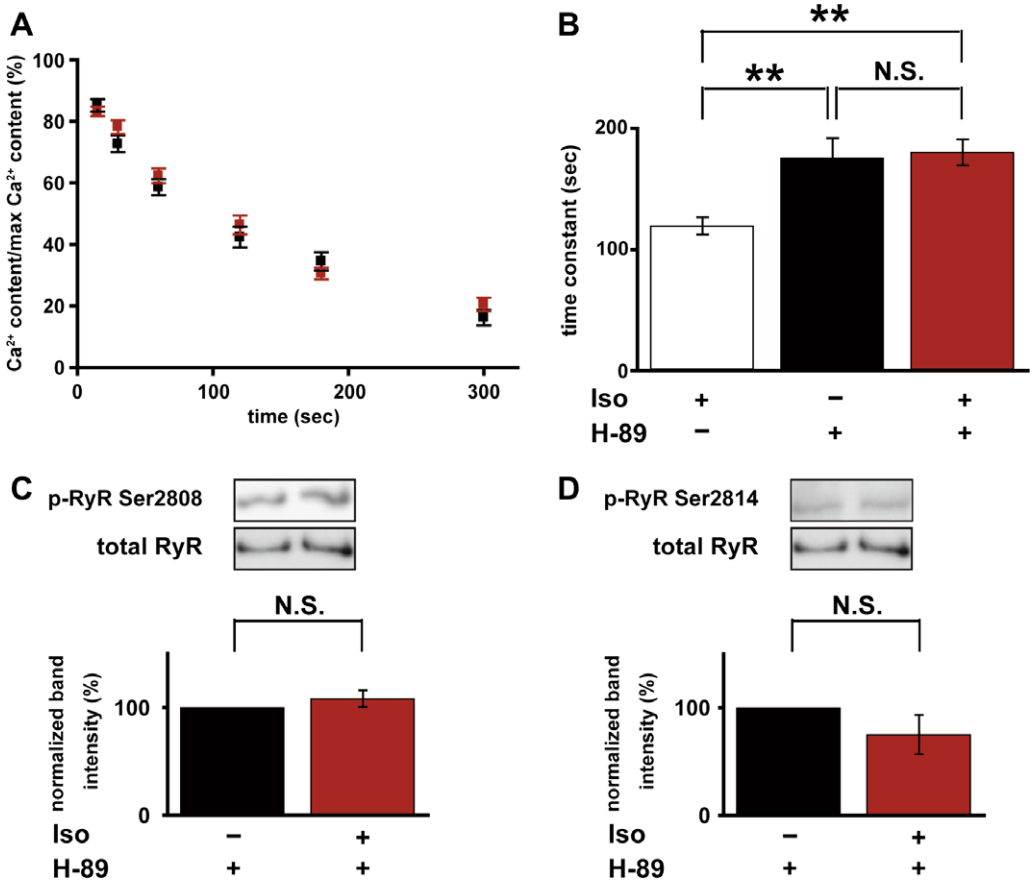

Fig. 2. Ca^{2+} leak from SR after β-AR stimulation in the presence of H-89. (A) Time-dependent changes in Ca^{2+} leak from SR before (black squares, $n = 17$) and after isoproterenol treatment (red squares, $n = 23$) in the presence of H-89. (B) Time constant of Ca^{2+} leak from SR before (black bar, $n = 17$) and after (red bar, $n = 23$) isoproterenol treatment in the presence of H-89. Time constant of Ca^{2+} leak from SR after isoproterenol treatment in the absence of H-89 is also shown (white bar, $n = 7$). $^{**}P < 0.01$, compared with the time constant of Ca^{2+} leak from SR after isoproterenol treatment in the absence of H-89 (one-way ANOVA). (C,D) Western immunoblotting analysis of phosphorylation levels at RyR Ser2808 and at Ser2814 before (black bars) and after (red bars, $n = 7$) isoproterenol treatment in the presence of H-89. Blots of total amounts of RyR protein of are also shown ($n = 7$).

[11] or cytosolic Ca^{2+} shift in cardiomyocytes [9,10]. When Ca^{2+} leak from SR is measured with lipid bilayers or SR vesicles, the structures around SR are not preserved. The T-tubule, one such structure around SR, plays a crucial role in cardiac excitation–contraction coupling [1,20], and the spatial dispersion of T tubules in cardiomyocytes from the failing heart has recently been reported [21]. When Ca^{2+} leak from SR is measured in cardiomyocytes, separately measuring each SR function (Ca^{2+} uptake, Ca^{2+} release, and Ca^{2+} leak) is difficult. In addition, Ca^{2+} content in SR, which is an important regulator of RyR activity (Ca^{2+} leak and Ca^{2+} release) [22,23], is not controlled in most studies. The advantages of using saponin-treated preparations to investigate SR functions are that: (1) each SR function can be measured separately; (2) Ca^{2+} leak can be estimated with the same SR Ca^{2+} content; and (3) the intracellular environment, including the ion concentration, can be manipulated easily while integrated structures around SR are preserved. Thus, our preparation is suitable for evaluating SR functions under conditions more closely resembling the physiological state.

Our present results in normal mice heart are consistent with those of previous studies showing that PKA-dependent phosphorylation of RyR is responsible for the increase in Ca^{2+} leak from SR [5]. On the other hand, another β-AR-signaling component, CaMKII activity, has also been reported as a possible mechanism of the increase in Ca^{2+} leak from SR [9,10]. In addition, a genetically modified mouse model of the PKA phosphorylation site of RyR has been established; however, the results of studies with this model have been controversial [6,24]. Because the CaMKII-dependent phosphorylation level at Ser2814 was not affected by β-AR stimulation in our preparations (Fig. 3), whether phosphorylation at Ser2814 itself increases Ca^{2+} leak from SR remains unclear. However, our present results strongly suggest that PKA-dependent phosphorylation of RyR increases Ca^{2+} leak from SR without CaMKII-dependent phosphorylation.

A previous study has found that electrical stimulation is required for the CaMKII-dependent phosphorylation of RyR [7]. Because we did not stimulate the preparation while it was being treated with isoproterenol, we might have underestimated CaMKII-dependent phosphorylation under our experimental conditions. Chelu et al. have recently shown, in a genetically modified mouse model lacking the CaMKII-dependent phosphorylation site of RyR, that CaMKII-dependent phosphorylation of RyR promotes atrial fibrillation [25]. Their result shows that CaMKII-dependent phosphorylation is primarily important for inducing fibrillation in atrial muscles. Our results do not rule out a possible role for CaMKII-dependent phosphorylation of RyR in Ca^{2+} leak in ventricular muscles. However, the increase in Ca^{2+} leak by PKA-dependent phosphorylation of RyR might play an important role in concert with CaMKII-dependent phosphorylation under pathophysiological conditions accompanying an increase in the catecholamine concentration.

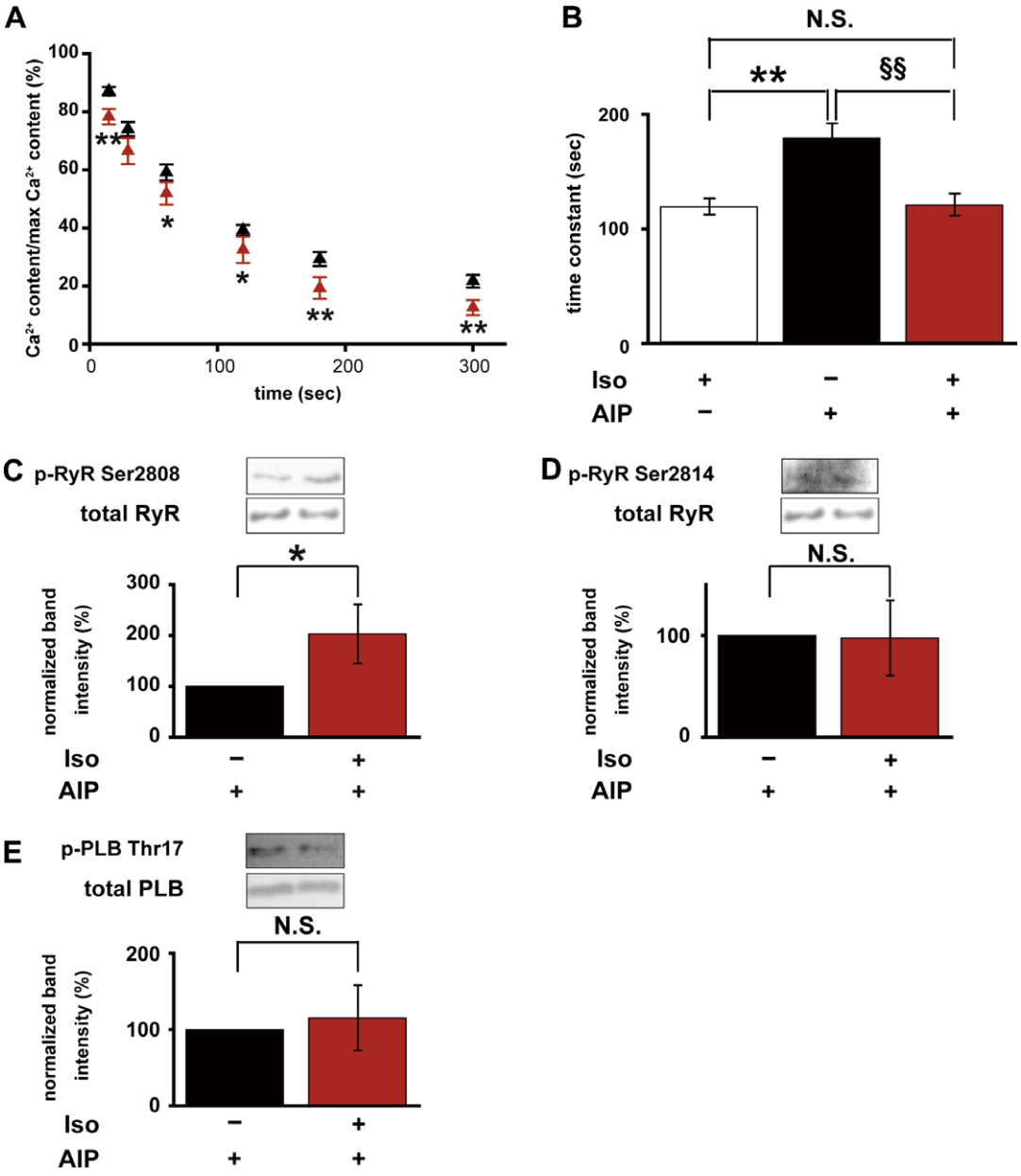

Fig. 3. Effects of β-AR stimulation on Ca^{2+} leak from SR in the presence of AIP. (A) Time-dependent changes in Ca^{2+} leak from SR before (black triangles, $n = 10$) and after isoproterenol treatment (red triangles, $n = 12$) in the presence of AIP. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with Ca^{2+} leak from SR before isoproterenol treatment in the presence of AIP at each time. (B) Time constant of Ca^{2+} leak from SR before (black bar, $n = 10$) and after (red bar, $n = 12$) isoproterenol treatment in the presence of AIP. Time constant of Ca^{2+} leak from SR after isoproterenol treatment in the absence of AIP is also shown (white bar, $n = 7$). $^{**}P < 0.01$, compared with the time constant of Ca^{2+} leak from SR after isoproterenol treatment in the absence of AIP. $^{\$\$}P < 0.01$, compared with the time constant of Ca^{2+} leak from SR in the presence of AIP (one-way ANOVA). (C) Western immunoblotting analysis of phosphorylation levels at RyR S2808 before (black bar, $n = 4$) and after (red bar, $n = 4$) isoproterenol treatment in the presence of AIP. The blots of total amounts of RyR protein are also shown. $^{*}P < 0.05$, compared with phosphorylation levels at RyR Ser2808 before isoproterenol treatment. D, Western immunoblotting analysis of phosphorylation levels at RyR S2814 before (black bar, $n = 4$) and after isoproterenol treatment (red bar, $n = 4$) in the presence of AIP. The blots of the total amounts of RyR protein are also shown. E, Western immunoblotting analysis of phosphorylation levels at PLB Thr17 before (black bar, $n = 4$) and after (red bar, $n = 4$) isoproterenol treatment in the presence of AIP. The blots of the total amounts of PLB protein are also shown.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.09.071.

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