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Ca²⁺/calmodulin kinase II increases ryanodine binding and Ca²⁺-induced sarcoplasmic reticulum Ca²⁺ release kinetics during β -adrenergic stimulation

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Abstract

We aimed to define the relative contribution of both PKA and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) cascades to the phosphorylation of RyR2 and the activity of the channel during β -adrenergic receptor (β AR) stimulation. Rat hearts were perfused with increasing concentrations of the β -agonist isoproterenol in the absence and the presence of CaMKII inhibition. CaMKII was inhibited either by preventing the Ca²⁺ influx to the cell by low [Ca]_o plus nifedipine or by the specific inhibitor KN-93. We immunodetected RyR2 phosphorylated at Ser2809 (PKA and putative CaMKII site) and at Ser2815 (CaMKII site) and measured [³H]-ryanodine binding and fast Ca²⁺ release kinetics in sarcoplasmic reticulum (SR) vesicles. SR vesicles were isolated in conditions that preserved the phosphorylation levels achieved in the intact heart and were actively and equally loaded with Ca²⁺. Our results demonstrated that Ser2809 and Ser2815 of RyR2 were dose-dependently phosphorylated under β AR stimulation by PKA and CaMKII, respectively. The isoproterenol-induced increase in the phosphorylation of Ser2815 site was prevented by the PKA inhibitor H-89 and mimicked by forskolin. CaMKII-dependent phosphorylation of RyR2 (but not PKA-dependent phosphorylation) was responsible for the β -induced increase in the channel activity as indicated by the enhancement of the [³H]-ryanodine binding and the velocity of fast SR Ca²⁺ release. The present results show for the first time a dose-dependent increase in the phosphorylation of Ser2815 of RyR2 through the PKA-dependent activation of CaMKII and a predominant role of CaMKII-dependent phosphorylation of RyR2, over that of PKA-dependent phosphorylation, on SR-Ca²⁺ release during β AR stimulation.

Keywords

Ryanodine receptor; β -adrenergic stimulation; CaMKII-dependent phosphorylation; Sarcoplasmic reticulum Ca²⁺ release

1. Introduction

Activation of β -adrenergic receptors (β AR) plays a pivotal role in the modulation of cardiac function in response to stress or exercise. Stimulation of β AR by catecholamines results in the activation of cAMP-dependent protein kinase (PKA) cascade. However, the prevalent concept that cAMP/PKA pathway is solely responsible for β AR-mediated cardiac cellular responses

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has been challenged in the last few years. Evidence has grown supporting a critical role of Ca^{2+} /calmodulin-dependent protein kinase (CaMKII) in the effects induced by both acute and chronic β AR stimulation [1-6]. Similar to PKA, CaMKII phosphorylates the major components of the cardiac excitation-contraction coupling (ECC), including the L-type Ca^{2+} channel, the sarcoplasmic reticulum (SR) Ca^{2+} release channel or type 2 ryanodine receptor (RyR2) and the SR Ca^{2+} -ATPase regulator phospholamban (PLN).

In spite of the extensive studies on the effects of β AR stimulation on the individual components of the ECC, the physiological significance of the β -adrenergic regulation of RyR2 is not yet fully understood. Results from different laboratories aiming to answer this matter remain controversial [6-8]. One of the reasons for these disparate results probably arises from the difficulty inherent to measure the intrinsic function of the phosphorylated RyR2, since it is well established that Ca^{2+} release through the RyR2 in active cells is strongly dependent on the characteristics of the trigger Ca^{2+} current (I_{Ca}) and on SR Ca^{2+} load [8].

Three different phosphorylation sites at the RyR2 have been identified up to now in *in vitro* studies: Ser2809, Ser2815 (or Ser2808 and Ser2814, respectively, depending on the species) and Ser2030. Whereas Ser2815 has been shown to be the target site for CaMKII [9] and Ser2030 for PKA [10], results regarding the kinase involved in the phosphorylation of Ser2809 residue are controversial. Although originally described as a CaMKII site [11], Ser2809 was found to be exclusively phosphorylated by PKA [9,12], stoichiometrically phosphorylated by both PKA and CaMKII [13] or susceptible of being phosphorylated by cGMP-dependent protein kinase (PKG) and insensitive to either activation or inhibition of PKA [14].

During β AR stimulation, RyR2 was found to be phosphorylated at Ser2809 or at Ser2030 sites [9,12,14]. However, the possible CaMKII-dependent phosphorylation of Ser2809 (if any) and of Ser2815 sites of RyR2 and its physiological significance during β AR stimulation, is far from being clear. In an effort to further understand this issue, our research has focused on two aspects: first, to define the relative contribution of PKA and CaMKII to the phosphorylation of Ser2809 evoked by isoproterenol and to study the effects of β -agonists on the phosphorylation of Ser2815 site. Second, to determine the effect of CaMKII-dependent phosphorylation of RyR2 during β AR stimulation on the channel activity and on its capacity to release Ca^{2+} from the SR. To accomplish this purpose, we measured in SR vesicles, [^3H]-ryanodine binding and Ca^{2+} fluxes with a temporal resolution of ms (stopped flow technique), to better mimic the response of RyR2 channels to the sudden Ca^{2+} changes that occurred during L-type Ca^{2+} channel activation. The experiments were performed under careful control of SR Ca^{2+} load in the different situations explored. This strategy allowed us to dissect the functional role of the PKA and CaMKII pathways of RyR2 phosphorylation during β AR stimulation.

2. Materials and methods

2.1. Ex vivo experiments

2.1.1. Heart perfusions—Experiments were performed in isolated hearts from male Wistar rats (200–300 g body wt) perfused according to the Langendorff technique at constant temperature (37 °C), flow (12–14 ml/min) and heart rate (250 beats/min), as previously described [15]. The composition of the physiological salt solution was (in mM): 128.3 NaCl, 4.7 KCl, 1.35 CaCl_2 , 20.2 NaHCO_3 , 0.4 NaH_2PO_4 , 1.1 MgCl_2 , 11.1 glucose and 0.04 Na_2EDTA ; this solution was equilibrated with 95% O_2 –5% CO_2 to give a pH of 7.4. The mechanical activity of the heart was assessed by passing into the left ventricle a latex balloon connected to a pressure transducer (AD Instruments MLT9580, CO, USA). The balloon was filled with aqueous solution to achieve a left ventricular end-diastolic pressure of 7–13 mm Hg. The investigation conforms the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-234, revised 1996).

2.1.2. Experimental protocol—After stabilization, hearts were perfused for a 3 min period with either physiological salt solution with 1.35 mM $[Ca]_o$ or the same solution with 0.07 mM $[Ca]_o$ plus 0.4 μ M nifedipine (to inhibit the Ca^{2+} -influx to the cell), in the absence or the presence of different isoproterenol (Iso) concentrations between 0.3 and 300 nM. In additional experiments, hearts were perfused with: (a) 30–300 nM Iso in the absence and the presence of 5 μ M of the specific CaMKII-inhibitor, KN-93, and in the absence and the presence of 30 μ M of the PKA-inhibitor H-89 and (b) 1 μ M forskolin. At the end of the experiments, hearts were freeze-clamped and stored at $-80^\circ C$ until biochemical assays were performed.

2.1.3. Preparation of SR membrane vesicles for Western blot, [3H]-ryanodine binding and Ca^{2+} release kinetic assays—SR membrane vesicles for Western blot were prepared following the protocol previously described [1]. Briefly, frozen hearts were homogenized in 6 volumes of ice-cold solution which contained: 5 mM Na_2EDTA , 25 mM NaF, 300 mM sucrose, 30 mM KH_2PO_4 (pH 7.0 at $4^\circ C$) and 1 mM PMSF, 1 mM benzamidine, 0.5 μ g/ml leupeptin and 1 μ g/ml pepstatin as protease inhibitors. The homogenate was sedimented twice at $14,000\times g$ and $16,000\times g$ for 20 min. The resulting supernatant was centrifuged at $45,000\times g$ for 45 min. The pellet obtained was suspended in 3 volumes of the above buffer containing 600 mM KCl and centrifuged as in the previous step. The final pellet was resuspended in small volumes of 10 mM Na_2EDTA , 10 mM NaF, 250 mM sucrose and 30 mM histidine (pH 7.0 at $4^\circ C$). SR vesicles for [3H]-ryanodine binding assay were isolated as above except that Na_2EDTA was omitted and NaF was replaced by 1 μ M okadaic acid as phosphatase inhibitor, in all the buffers. SR preparations were enriched approximately threefold with respect to homogenates as estimated by Western blot analysis of RyR2 and [3H]-ryanodine binding assay. SR vesicles for Ca^{2+} release kinetic assays were prepared following the protocol previously described [16] except that the homogenization buffer contained 20 mM MOPS/Tris (pH 6.8), 300 mM sucrose, 1 mM okadaic acid and protease inhibitors as above. Protein was measured by the method of Bradford, using bovine serum albumin as standard.

2.1.4. Electrophoresis and Western Blot analysis—For immunological detection of RyR2 and phosphorylated RyR2, SDS-PAGE was performed according to the method of Laemmli in 6% acrylamide gels [17]. Twelve and 50 μ g of SR membrane protein were electrophoresed per gel lane for the *in vitro* assay and for the assessment of phosphorylation level in the perfused heart, respectively. Separated proteins were transferred to PVDF membranes (Immobilon-P, Millipore) and probed with antibodies RyR2-PS2809 (1:5000; Badrilla, Leeds, UK), RyR2-PS2809 (1:1000) and RyR2-PS2815 (1:1000), both kindly provided by A. Marks and X. Wehrens (Columbia University, USA), and RyR2 antibody (1:2500; Affinity Bioreagents Inc.). Immunoreactivity was visualized by peroxidase-conjugated antibodies using a peroxidase-based chemiluminescence detection kit (ECL, Amersham). The signal intensity of the bands was quantified using ImageJ (NIH). Results of phosphorylation of RyR2 normalized by the total RyR2 content were expressed as percentage of the maximal phosphorylation induced by the β agonist, isoproterenol, which is achieved at 30–300 nM. Phosphorylation of Thr17 of PLN was detected as previously described [1].

2.1.5. In vitro phosphorylation/dephosphorylation assays—Phosphorylation reactions of SR vesicles (0.6 mg/ml) were conducted for 10 min at room temperature in a buffer containing 50 mM histidine (pH 7.0), 5 mM $MgSO_4$, 5 mM NaF and 2.5 μ M okadaic acid. For PKA phosphorylation, 1.5 U of the catalytic subunit per μ g of SR protein, 1 mM EGTA and 1 μ M of the CaMKII inhibitor, KN-93, were added to the assay medium. For phosphorylation by the endogenous CaMKII, 0.5 mM $CaCl_2$, 50 μ g/ml calmodulin (CaM) and 1 μ M of the PKA inhibitor PKI were added. For the control samples, Ca^{2+} (1 mM EGTA present), CaM and PKA were omitted from the medium. Phosphorylation reactions were initiated by the addition

of 0.2 mM ATP. For dephosphorylation reactions, cardiac SR vesicles (1 mg/ml) were incubated for 30 min at 37 °C in a solution containing (in mM): 50 histidine (pH 7.0), 5 MgSO₄ and 1 EGTA with 2–4 U of alkaline phosphatase per µg of SR protein. After incubations, all reactions were stopped by the addition to the medium of SDS sample buffer.

2.1.6. [³H]-ryanodine binding—[³H]-ryanodine binding was performed in SR cardiac membranes (60 µg) assayed in 0.25 ml of incubation medium according to Lokutta et al. [18]. The medium contained 1 mM benzamidine, 1 mM PMSF, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin A as protease inhibitors and 1 µM okadaic acid as phosphatase inhibitor, without addition of Mg²⁺ or ATP. The necessary CaCl₂ to set free [Ca²⁺] in the range of 0.1 to 100 µM was calculated using the stability constants of Fabiato [19]. [³H]-ryanodine was diluted directly into the incubation medium to achieve a final concentration of 10 nM. Incubation lasted 90 min at 36 °C. Samples were run in duplicate, filtered onto glass fiber filters (Munktell MGB) and washed four times with 5 ml cold water. The filters were placed in vials containing scintillation liquid cocktail and retained radioactivity was measured in a β-counter. The specific binding was defined as the difference between the binding in the absence (total binding) and in the presence (non-specific binding) of 10 µM unlabeled ryanodine.

2.1.7. Active Ca²⁺ loading of cardiac SR vesicles—SR vesicles were actively loaded with Ca²⁺ in the presence of an ATP-regenerating system as previously described [16]. Briefly, SR vesicles (1 mg/ml) were added to a solution containing: 0.05 mM CaCl₂, 100 mM KCl, 2 mM ATP, 3 mM MgCl₂, 10 mM phosphocreatine, 15 U/ml creatine kinase and 20 mM imidazole MOPS (pH 7.2 at 4 °C). To avoid changes in the redox and phosphorylation status of SR vesicles during Ca²⁺ uptake, we included a redox buffer (3 mM of reduced glutathione/ glutathione oxidized (GSSG/GSH) in a ratio 1/100), 1 µM okadaic acid and 0.5 µM staurosporine as phosphatase and kinase inhibitors, respectively. The active Ca²⁺ loading of these vesicles was determined by measuring the decay in extravesicular free [Ca²⁺] [16]. Active Ca²⁺ uptake was completed in 25 min at 30 °C. At this time all SR vesicles were equally loaded with Ca²⁺ (extravesicular [Ca²⁺] stabilized at 0.59±0.11 µM Ca²⁺, n=6).

2.1.8. Ca²⁺ release kinetics—Ca²⁺ release kinetics were determined in a SX.18MV fluorescence stopped-flow spectrometer from Applied Photo-physics Ltd. (Leatherhead, U.K.). The increase in extravesicular [Ca²⁺] was measured by the increase in fluorescence of the Ca²⁺ indicator Calcium Green-5N (Molecular Probes, Eugene, OR). The fluorescent emission of the dye was measured through a 515 nm cut-off long-pass filter, using an excitation wavelength of 488 nm. Ca²⁺ release was initiated by rapidly mixing (less than 2 ms) 1 volume of the solution containing Ca²⁺ loaded SR vesicles with 10 volumes of releasing solution containing (in mM): 100 KCl, 1.6 ATP, 20 imidazole-MOPS, pH 7.2 and 1 µM Calcium Green-5N. After mixing, this solution produced free concentrations of 1.2 mM ATP, 17 µM Mg²⁺ and pCa 6 [16].

2.1.9. Statistics—For comparison among different groups, ANOVA was used followed by Tukey test. A *P* value <0.05 was considered statistically significant.

3. Results

3.1. Phosphorylation studies

3.1.1. In vitro phosphorylation/dephosphorylation of Ser2809 and Ser2815 sites of RyR2—The goals of the *in vitro* phosphorylation/dephosphorylation assays were twofold: (1) to verify the specificity and the ability of the antibodies used to detect the phosphorylation level of RyR2; (2) to elucidate the controversial issue regarding the phosphorylation of Ser2809 site: two independent groups using phospho-specific-antibodies reported that this site was

either exclusively phosphorylated by PKA [9,12] or phosphorylated by both PKA and CaMKII [13]. We therefore performed experiments using the antibodies from both laboratories under the same experimental conditions to determine whether discrepancies in the results could be ascribed to the different methodology and source of the antibodies used. Representative immunoblots and overall results are shown in Fig. 1. Treatment of the SR membrane preparation with alkaline phosphatase reduced the immunorecognition by the phospho-antibodies with respect to a non-treated sample. Densitometric analysis with the two phospho-Ser2809 antibodies provided equivalent results: the signal increased when SR membrane vesicles were exposed to the catalytic subunit of PKA and after the addition of Ca^{2+} and CaM to activate the endogenous CaMKII. The signal with the phospho-Ser2815 antibody only increased in the Ca^{2+} /CaM-treated samples and did not show any significant increase in the PKA-treated samples. In all cases, immunodetection with an antibody against RyR2 showed no difference in the content of RyR2 among the different treatments. Taken together the results indicated that the antibodies were faithfully reporting on the phosphorylation level of RyR2 and confirmed that Ser2809 can be phosphorylated by either PKA or CaMKII and that Ser2815 site can be exclusively phosphorylated by CaMKII.

3.1.2. β -adrenergic-dependent phosphorylation of Ser2809 and Ser2815 sites of RyR2 in the intact heart—The concentration-dependence of the phosphorylation of Ser2809 and Ser2815 in response to isoproterenol stimulation was studied in the absence and presence of low extracellular Ca^{2+} concentration ($[\text{Ca}]_o$) and nifedipine to avoid Ca^{2+} -influx to the cell and the consequent activation of CaMKII. Under this latter condition, basal contractile activity as well as the response to isoproterenol were completely suppressed indicating that the maneuver is an effective experimental tool to decrease intracellular Ca^{2+} (Fig. 2A).

Fig. 2B shows that basal phosphorylation levels of Ser2809 were high, in agreement with previous findings [10,13,14,20,21]. The figure also shows that 0.3 nM, the lowest isoproterenol concentration studied, produced a significant increase in the phosphorylation of Ser2809 site. Higher isoproterenol concentrations produced a slight increase in the phosphorylation of this residue, which did not attain, however, significant levels when compared with 0.3 nM isoproterenol. Thus, in agreement with previous results [14,22], Ser2809 was virtually maximally phosphorylated at the lowest isoproterenol concentration used. Low $[\text{Ca}]_o$ plus nifedipine produced a modest albeit significant decrease in the basal phosphorylation of Ser2809 and also in the phosphorylation produced by the lowest isoproterenol concentrations (0.3 and 3 nM). These results would indicate that under basal conditions and at the lower isoproterenol concentrations, PKA and CaMKII pathways both contribute to the phosphorylation of Ser2809 site. In contrast, low $[\text{Ca}]_o$ plus nifedipine did not affect the phosphorylation evoked by the highest concentrations of the β -agonist, which would indicate that at the higher isoproterenol concentrations, Ser2809 site is phosphorylated only by PKA. Thus, the procedure of avoiding Ca^{2+} entry to the cell allows us to dissect the relative participation of PKA and CaMKII in the phosphorylation of Ser2809 site under β AR stimulation. The maneuver further reveals that the PKA phosphorylation of Ser2809 increased in a concentration-dependent manner with the concentration of the β -agonist.

Fig. 2C shows that basal phosphorylation values of Ser2815 were high as in the case of Ser2809. However, and in contrast to what was observed with Ser2809 site, basal Ser2815 phosphorylation was not affected by low $[\text{Ca}]_o$ plus nifedipine. Moreover, phosphorylation of Ser2815 increased with the increase in isoproterenol concentration, attaining significant levels at 30 and 300 nM. At all isoproterenol concentrations the presence of low $[\text{Ca}]_o$ plus nifedipine decreased the phosphorylation of Ser2815 residue to basal levels. Of interest, the dose-response curve to PKA-dependent phosphorylation of RyR2 (Fig. 2B) was shifted to lower isoproterenol concentrations with respect to the dose-response curve to CaMKII-dependent

phosphorylation (Fig. 2C). A similar shift has been previously described for the PKA and CaMKII-dependent PLN phosphorylation [2,23]. Since CaMKII-dependent RyR2 phosphorylation at Ser2815 site was shown to be enhanced in isolated rabbit hearts submitted to increased heart rates [9], and the maneuver of reducing Ca^{2+} plus nifedipine renders a non-beating heart (see Fig. 2A), we repeated the experiments with 300 nM isoproterenol in the absence and the presence of the CaMKII inhibitor KN-93. In these two conditions contraction frequency was the same, *thus ruling out* any possible frequency-dependent CaMKII activity change. As shown in Fig. 2C, 5 μM KN-93 was able to mimic the effects of low $[\text{Ca}]_o$ plus nifedipine on the isoproterenol-induced phosphorylation of Ser2815. The results suggest that Ser2815 site of RyR2 is concentration-dependently phosphorylated by the activation of the CaMKII-dependent pathway of the β -adrenergic stimulation. KN-93 also mimicked the effect of low $[\text{Ca}]_o$ plus nifedipine on Ser2809 site, failing to reduce the phosphorylation of Ser2809 produced by 300 nM isoproterenol (Fig. 2B).

Recent experiments by Curran et al. [6] indicated that activation of CaMKII by a PKA-independent pathway was responsible for the enhancement of isoproterenol-induced SR Ca^{2+} leak. We therefore explored whether the increase in the phosphorylation of Ser2815 site of RyR2 observed during β AR stimulation here described was also due to a PKA-independent activation of CaMKII. For this purpose, we inhibited PKA with H-89 during β AR stimulation and we directly activated the adenylyl cyclase and therefore PKA, bypassing the β AR, with forskolin. Thirty micromolar H-89 was required to achieve the complete inhibition of the positive inotropic effect of isoproterenol (results not shown). Forskolin was used at 1 μM , a concentration that produced an increase in contractility and relaxation not significantly different from that evoked by isoproterenol (maximal rate of rise of pressure: 174.5 ± 9.3 vs. $181.3 \pm 7.1\%$ of control and half relaxation time -10.4 ± 2.8 vs. -12.1 ± 2.3 Δ ms from control, for forskolin and isoproterenol, respectively). Fig. 3 shows typical immunoblots (upper panels) and average data (lower panels) of the phosphorylation results obtained in this experimental series. H-89 blunted the isoproterenol-induced increase in the phosphorylation of Ser2815 of RyR2. Moreover, forskolin evoked a phosphorylation of Ser2815 of RyR2 similar to that produced by isoproterenol (Fig. 3A). Thus, in our conditions, the CaMKII-dependent phosphorylation of Ser2815 of RyR2 is dependent on the increase in PKA activity evoked by β AR stimulation. β AR stimulation also phosphorylated Thr17 residue of PLN, a well known intracellular marker of increased CaMKII activity, through a PKA-dependent pathway (Fig. 3B).

3.2. Functional studies

Functional studies were performed in SR membrane vesicles isolated in conditions which preserved the phosphorylation levels achieved under the different experimental situations in the perfused rat heart. We studied the β -adrenergic effect at 300 nM isoproterenol, a concentration at which Ser2809 was exclusively phosphorylated by PKA and Ser2815 by CaMKII (see Figs. 2B and C). Thus at this concentration, the inhibition of CaMKII activity either by preventing the Ca^{2+} influx to the cell by low $[\text{Ca}]_o$ plus nifedipine or by the specific inhibitor KN-93, allowed to dissect the relative role of both kinases, PKA and CaMKII, during β -adrenergic stimulation.

3.2.1. [^3H]-ryanodine binding—Fig. 4 shows the $[\text{Ca}^{2+}]$ dependence of [^3H]-ryanodine binding to SR vesicles isolated from the differently treated hearts. Specific binding under control conditions showed a graded dependence of $[\text{Ca}^{2+}]$, which was maximal at 10 μM $[\text{Ca}^{2+}]$. Membrane vesicles isolated from isoproterenol perfused hearts, showed an increased [^3H]-ryanodine binding, reaching statistical significance at 1, 10 and 100 μM $[\text{Ca}^{2+}]$. This upward shift in the [^3H]-ryanodine binding produced by isoproterenol, indicated an increased Ca^{2+} -induced activation of RyR2 compared with control. The isoproterenol-induced

stimulation of the binding was abolished when either KN-93 or low $[Ca]_o$ plus nifedipine was perfused simultaneously with isoproterenol. Neither KN-93 nor low $[Ca]_o$ plus nifedipine perfused in the absence of the β -agonist, modified $[^3H]$ -ryanodine binding. At 10 μM $[Ca^{2+}]_o$, the ryanodine binding was 0.21 ± 0.02 (control), 0.22 ± 0.02 (KN) and 0.20 ± 0.03 (low Ca) pmol/mg protein in agreement with previous findings [18].

3.2.2. Ca^{2+} release kinetics—Fig. 5 shows the time course of Ca^{2+} release from SR vesicles isolated from hearts perfused under the different experimental conditions. In every case the increase in fluorescence followed a double exponential function: $A_1 [1 - \exp(-k_1 t)] + A_2 [1 - \exp(-k_2 t)]$, characterized by a fast rate constant k_1 and a slower rate constant k_2 , which differed in magnitude by approximately 20-fold under control conditions. Previous studies in isolated SR vesicles have demonstrated that the faster rate constant of Ca^{2+} release, k_1 , correlates with the average open probability of a population of channels in response to a sudden $[Ca^{2+}]_o$ change, while k_2 is most likely due to a time-dependent decrease in channel activity following rapid activation by Ca^{2+} [16,24,25]. Overall results in Fig. 6A show that k_1 increased 5.8-fold in isoproterenol-treated hearts and that this increase was prevented in hearts simultaneously perfused with either low $[Ca]_o$ plus nifedipine or the CaMKII inhibitor KN-93. This behavior was similar to the one observed in the phosphorylation of Ser2815 site as shown for comparison in the same figure. The fractional Ca^{2+} release that occurs with the faster rate constant ($A_1 / (A_1 + A_2)$) did not significantly change among the different experimental conditions (control: 0.28 ± 0.09 ; 300 nM isoproterenol: 0.23 ± 0.02 ; 300 nM isoproterenol in low $[Ca]_o$ plus nifedipine: 0.21 ± 0.07 and 300 nM isoproterenol in the presence of KN-93, 0.22 ± 0.07). In addition, SR vesicles from isoproterenol-treated hearts showed a significant increase in k_2 with respect to control values. Of interest this increase in k_2 was not significantly affected by low $[Ca]_o$ plus nifedipine or KN-93 and it would therefore represent a PKA-dependent effect of βAR stimulation. These results paralleled the results obtained with the PKA-dependent phosphorylation of Ser2809 site of RyR2 (Fig. 6B).

4. Discussion

The functional role of RyR2 phosphorylation has been a controversial issue over the years. Numerous and contradictory results have been reported regarding the physiological and pathophysiological relevance not only of PKA-dependent phosphorylation but also of CaMKII-dependent phosphorylation of RyR2 [6-14,18,26-35]. In spite of this profusion of work regarding RyR2 phosphorylation and function, the PKA and CaMKII-dependent phosphorylations of RyR2 under βAR stimulation and their functional consequences are not well defined. The main goal of the present experiments was to investigate this issue.

Our results demonstrated that: (1) Ser2809 and Ser2815 of RyR2 are dose-dependently phosphorylated under βAR stimulation by PKA and CaMKII respectively; (2) CaMKII-dependent phosphorylation of Ser2815 during βAR stimulation is secondary to PKA activation; (3) CaMKII contributes to the phosphorylation of Ser2809 of RyR2 under basal conditions and at the lowest isoproterenol concentrations and (4) CaMKII-dependent phosphorylation of RyR2 (but not PKA-dependent phosphorylation), is responsible for the β -induced increase in the channel activity as indicated by $[^3H]$ -ryanodine binding and fast SR Ca^{2+} release kinetic studies. To the best of our knowledge, the present results reveal for the first time the predominant role of CaMKII-dependent phosphorylation of RyR2 on the Ca^{2+} release response of the RyR2 to a sudden Ca^{2+} change over that of PKA-dependent phosphorylation during βAR stimulation. Our approach is unique in the sense that we used for our functional assays SR membrane vesicles that preserved the phosphorylation level achieved under the different experimental conditions in the perfused intact heart, but that were equally loaded with Ca^{2+} immediately before the functional assay.

4.1. The CaMKII pathway of β AR stimulation on RyR2 phosphorylation and activity

The functional effects of β AR stimulation have been naturally linked to the cAMP-PKA intracellular cascade and the associated PKA-dependent phosphorylations. However, β AR stimulation also triggers the activity of Ca^{2+} -dependent kinases and phosphatases, among which is CaMKII. The effects of these secondary cascades, which may modify the classic cAMP-PKA pathway during β AR stimulation, are frequently dismissed. Our results showed that CaMKII phosphorylates RyR2 at Ser2815 site during β AR stimulation in a dose-dependent manner. These results differed from those of Wehrens et al. [9], who failed to detect the isoproterenol-induced increase in Ser2815 phosphorylation. The reason for this discrepancy is difficult to explain. Since their experiments also failed to show a significant increase in the phosphorylation of Thr17 of PLN which is known to increase under β AR stimulation [1,2, 23], one might speculate that either CaMKII was not sufficiently activated by isoproterenol in their experimental conditions or that the activity of phosphatases relative to that of CaMKII in their preparations is too high, avoiding the detection of the phosphorylation of Thr17 of PLN and Ser2815 of RyR2.

Ginsburg and Bers [8] studied the effects of the β -agonist isoproterenol under conditions of controlled I_{Ca} trigger and SR Ca^{2+} load. They found that β AR stimulation did cause a significant increase in the maximal rate of SR Ca^{2+} release. Moreover, Lindegger and Niggli elegantly demonstrated that β AR stimulation may directly increase the SR Ca^{2+} release [33]. Both studies, however, failed to discriminate whether the stimulatory effect of isoproterenol was due to PKA and/or CaMKII-dependent phosphorylation of the RyR2. The present findings are in line with these previous observations and further show that CaMKII-dependent phosphorylation of RyR2 is responsible for the increased activation of SR Ca^{2+} channels in this situation. Of note, the functional consequences of β -adrenergic-induced CaMKII-dependent phosphorylation may not be exclusively attributed to the phosphorylation of Ser2815 site, since *in vitro* results revealed other putative CaMKII-dependent sites as yet not identified [13].

The faster SR Ca^{2+} release kinetics produced by the CaMKII-dependent phosphorylation of RyR2 cannot be attributed to the β -adrenergic-induced increase in SR Ca^{2+} load associated with the phosphorylation of PLN [34,35], since SR vesicles isolated from hearts submitted to the different experimental conditions were equally loaded with Ca^{2+} before the functional assay. Obviously under *in vivo* conditions, the increased I_{Ca} trigger and SR Ca^{2+} load should contribute to the overall action of β AR stimulation on SR Ca^{2+} release.

An additional finding of our experiments is that in spite of the increase in the faster rate constant (k_1) associated with the CaMKII-dependent phosphorylation of RyR2 during β -adrenergic stimulation, the fractional Ca^{2+} release that occurs with this constant ($A_1 / [A_1 + A_2]$) did not significantly change among the different experimental conditions. A possible explanation for this finding might be that the decrease in intravesicular Ca^{2+} in the isoproterenol-treated vesicles leads to an anticipated end of the fast Ca^{2+} release, resulting in an unvarying fraction of Ca^{2+} released. Alternatively, CaMKII-dependent phosphorylation of the channel could enhance not only the transition to a high opening probability mode but also diminish the time at which the channel remains in this state. In line with these findings, experiments of Ginsburg and Bers [8] demonstrated in isolated myocytes that isoproterenol did systematically increase the maximal rate of Ca^{2+} release for a given I_{Ca} and SR Ca^{2+} load without increasing the release amplitude, suggesting that isoproterenol must also hasten the turn-off of Ca^{2+} release.

Recent experiments indicated that β AR stimulation enhances diastolic Ca^{2+} leak by an increase in the activity of CaMKII that was independent of PKA activation [6]. The present findings complement those previous experiments indicating that CaMKII also regulates Ca^{2+} -induced systolic Ca^{2+} release during β AR stimulation. However, our experiments failed to support the

conclusion that the increase in CaMKII activity was independent of PKA activation. We showed that CaMKII-dependent phosphorylation of Ser2815 site of RyR2 as well as that of Thr17 site of PLN, was blunted by a concentration of the PKA-inhibitor H-89, at which the mechanical effects of the β -agonist were also blocked. We are aware of the fact that the concentration of H-89 required for the suppression of the isoproterenol-induced increase in contractility in the intact heart was rather high (30 μ M) which would limit the value of this drug as a useful tool in dissecting signaling pathways (See also Bracken et al. [36]). Indeed it has been shown that 30 μ M H-89 is the IC50 for CaMKII in *in vitro* conditions. However, this concentration failed to affect CaMKII activity in more intact preparations [37]. Moreover, our experiments also showed that activation of PKA by forskolin was able to mimic the mechanical and CaMKII-dependent phosphorylation effects of isoproterenol, strongly suggesting that the CaMKII-dependent phosphorylation of Ser2815 site of RyR2 and Thr17 residue of PLN is secondary to PKA activation. Further studies are warranted to solve the discrepancy regarding the pathways of activation of CaMKII during β AR stimulation.

4.2. The PKA pathway of β AR stimulation on RyR2 phosphorylation

The phosphorylation of Ser2809 site of RyR2 has been controversial over the years [9,11-13]. *In vitro* experiments by Witcher et al. first described Ser2809 residue of RyR2 as a CaMKII-dependent site [11]. This notion was challenged by a large piece of evidence provided by Marks' group, both in *in vitro* and *ex vivo* experiments, which indicates that Ser2809 site is solely phosphorylated by PKA [9,12]. Subsequent studies from Colyer's group [13] indicated that both kinases equally contribute to the phosphorylation of this site. Our experiments showed that Ser2809 could be phosphorylated by both PKA and CaMKII when interrogated by the specific antibodies used by both groups. Furthermore, although CaMKII seems to contribute to the basal phosphorylation of Ser2809, PKA is the main kinase that phosphorylates this site under β AR stimulation. In fact, at maximal isoproterenol concentrations, the strong PKA activation can make up for any loss in CaMKII activation when CaMKII is blocked. Our final major finding is that PKA-dependent phosphorylation of RyR2 during β AR stimulation does not seem to directly contribute to the fast Ca^{2+} release during the stimulation with the β -agonist. These results are in line with the findings from Li et al. [35] and Guo et al. [30] that suggest that PKA effects on SR Ca^{2+} release are secondary to the increased in SR Ca^{2+} load produced by the kinase. The present findings suggest, however, that PKA-dependent phosphorylation of the channel would increase the slow rate constant of Ca^{2+} release, k_2 . Studies on Ca^{2+} release kinetics in canine cardiac SR vesicles using the same protocol of the present experiments, suggested that k_2 may most probably represent a time-dependent decrease in RyR2 channel open probability following Ca^{2+} activation, compatible with an increase in the inactivation/adaptation processes [16]. If these results can be extrapolated to the rat heart, our findings would indicate that the increase in k_2 produced by isoproterenol represents a decrease in time-dependent channel inactivation/adaptation. This conclusion differs from that reported by Valdivia et al. [38] who found that PKA accelerates the kinetics of adaptation of RyR2 incorporated in planar lipid bilayers. Alternatively the increase in k_2 might represent a PKA-dependent subconductance opening of the channel as described by Wehrens et al. [9]. Unfortunately our results did not allow to discriminate between these two attractive possibilities.

4.3. Implications

The increase in SR Ca^{2+} release and intracellular Ca^{2+} transient produced by isoproterenol depends primarily on the increased I_{Ca} trigger and SR Ca^{2+} load. These two effects are regulated by CaMKII-activity [28] and cannot be avoided in the intact heart. It is therefore difficult to dissect whether the increase in the velocity of the fast SR Ca^{2+} release described here has any significant contribution to the overall increase in intracellular Ca^{2+} transient and contractility evoked by β AR stimulation. Experiments from Eisner's group [39-41] indicated

that in conditions where the SR Ca^{2+} content is not controlled, factors that alter the sensitivity of RyR2 to trigger Ca^{2+} , as is the case of CaMKII-dependent phosphorylation, will have only transitory effects on the amplitude of Ca^{2+} transients. This is because the increase in Ca^{2+} transient amplitude evoked by the increase in Ca^{2+} release due to RyR2 sensitization, decreases Ca^{2+} influx via the L-type Ca^{2+} channel and increases Ca^{2+} efflux via NCX. Ultimately this would decrease SR Ca^{2+} content, counteracting the initial effect of RyR2 sensitization. It is noteworthy, however, that under these conditions, the maintenance of Ca^{2+} transient amplitude occurs in spite of a lower SR Ca^{2+} content and I_{Ca} , which would actually indicate an enhanced ECC gain. Thus, even though the enhancement of I_{Ca} and SR Ca^{2+} uptake through the phosphorylation of PLN appear as the primary mechanisms of the positive inotropic effect of β -agonists [8,34,35], the CaMKII-dependent enhancement of Ca^{2+} -induced SR fast Ca^{2+} release may also play a significant role in this positive inotropic action. Recent experiments by Curran et al. [6] indicated that CaMKII activation during β AR stimulation enhanced SR Ca^{2+} leak, which would tend to decrease SR Ca^{2+} load and reduce the positive inotropic effect of isoproterenol. The CaMKII-dependent stimulation of systolic SR Ca^{2+} release during β AR stimulation here described may constitute a mechanism able to counteract, at the same RyR2 level, the negative action of SR Ca^{2+} leak on the positive inotropy of β AR stimulation. In this scenario, it is interesting to mention that a CaMKII-dependent increase in diastolic Ca^{2+} leak was described in a rabbit heart failure model [31]. In this model, CaMKII blockade restored SR Ca^{2+} content but has only modest effects on intracellular Ca^{2+} transient amplitude. Our experiments support the view that a CaMKII-dependent increase in systolic SR Ca^{2+} release may effectively counteract the deleterious effects of SR Ca^{2+} leak in this heart failure model.

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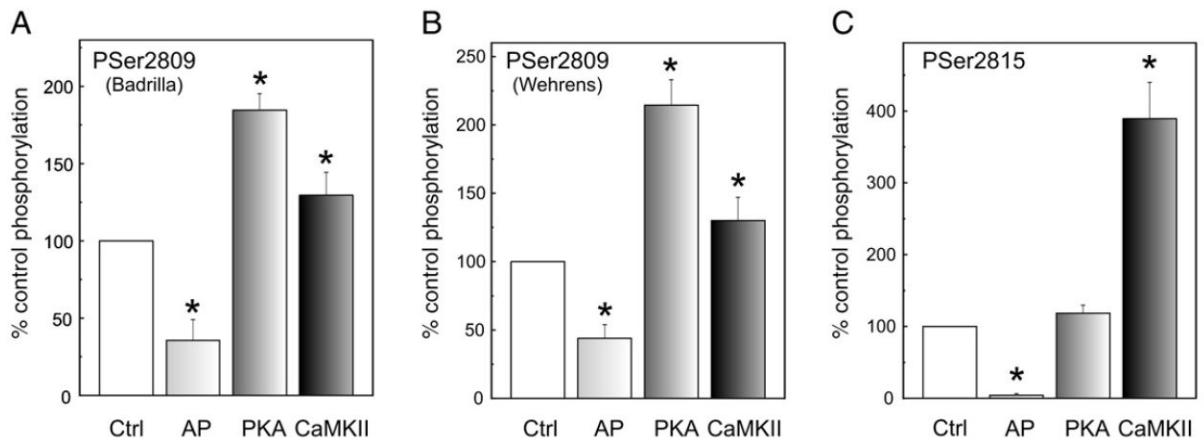
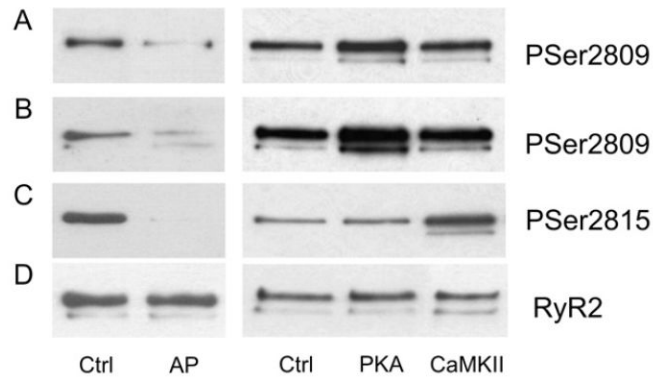


Fig. 1. Site-specific phosphorylation of RyR2. Representative immunoblots and overall results of SR membrane vesicles dephosphorylated by treatment with alkaline phosphatase (AP) and phosphorylated *in vitro* by the catalytic subunit of PKA in the presence of EGTA (PKA) or by the endogenous SR-associated CaMKII in the presence of the PKA inhibitor PKI (CaMKII) as described under Materials and methods. For the control samples, Ca²⁺ (1 mM EGTA present), calmodulin and the catalytic subunit of PKA were omitted (Ctrl). SR proteins were separated on SDS-PAGE and transferred to PVDF membranes. Blots were probed with anti-PS2809-RyR2 from Badrilla (A), anti-PS2809-RyR2 (B) and anti-PS2815-RyR2 (C) from X. Wehrens and A. Marks laboratories and anti-RyR2 from Affinity Bioreagents (D). Results are expressed as percentage of the phosphorylation obtained in Ctrl. **p*<0.05 with respect to Ctrl.

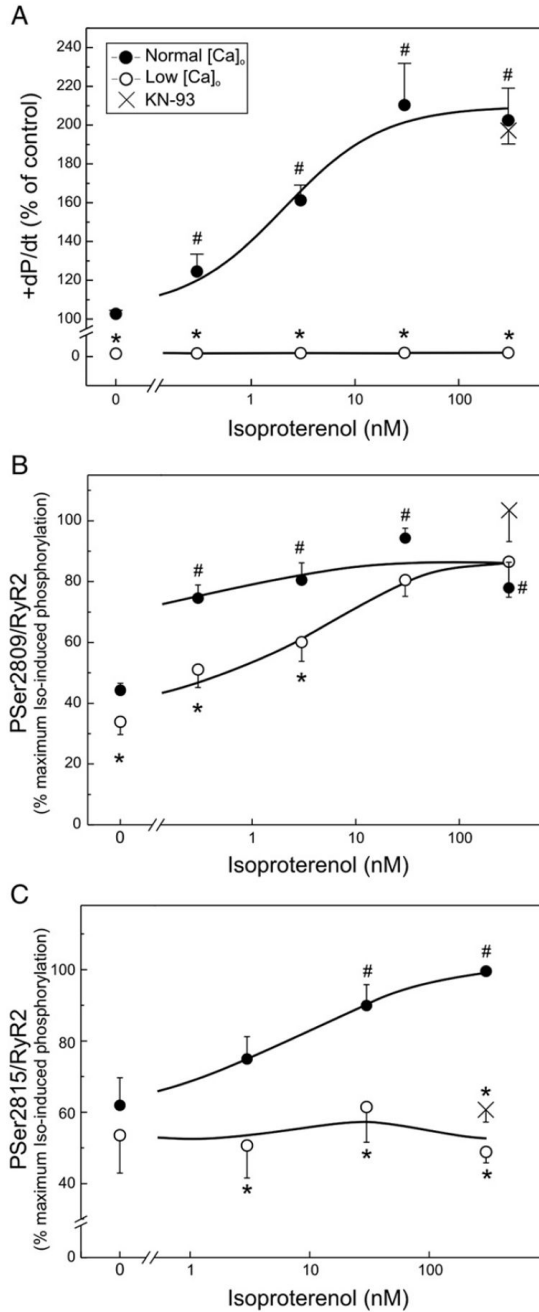


Fig. 2.

β -adrenergic-dependent effects on left ventricular function and phosphorylation of RyR2 in the perfused rat heart in the absence and the presence of low [Ca]_o and KN-93. Maximal rate of rise of pressure (+dP/dt, A), phosphorylation of Ser2809 (B) and of Ser2815 (C) of RyR2 in response to isoproterenol in the absence (closed circles) and presence of low [Ca]_o and nifedipine (open circles), to avoid Ca²⁺ influx to the cells. Isoproterenol increased contractility and the phosphorylation of both Ser2809 and Ser2815 sites of RyR2. Low [Ca]_o plus nifedipine (Low [Ca]_o) abolished contractility in the absence and presence of isoproterenol (+dP/dt, A) and significantly diminished basal Ser2809 phosphorylation and that produced by the lowest isoproterenol concentrations (0.3 and 3 nM) without significantly affecting the phosphorylation

evoked by the highest concentrations of the β -agonist (B). Low $[Ca]_o$ plus nifedipine did not decrease basal phosphorylation of Ser2815 but significantly diminished to basal values the phosphorylation of Ser2815 at all isoproterenol concentrations tested (C). 5 μ M of the CaMKII inhibitor KN-93 (crosses) did not affect the increase in contractility and phosphorylation of Ser2809 induced by 300 nM isoproterenol, but mimicked the effects of low $[Ca]_o$ plus nifedipine in the phosphorylation of Ser2815 induced by 300 nM isoproterenol. Mean \pm SEM, $n=3-10$ experiments. [#] $p<0.05$ with respect to hearts perfused with normal $[Ca]_o$ in the absence of drugs. * $p<0.05$ with respect to the corresponding situation in the presence of normal $[Ca]_o$.

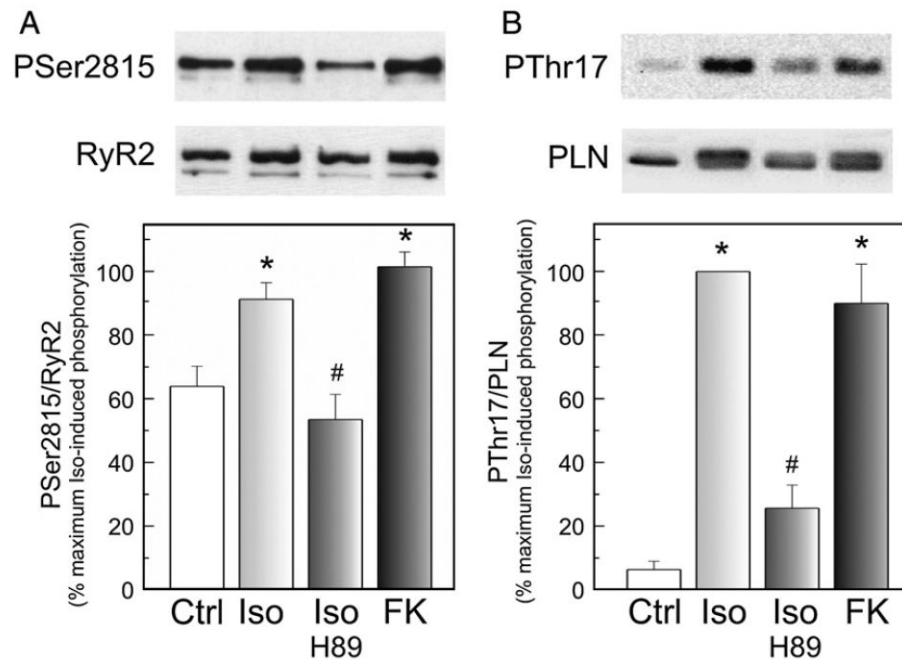


Fig. 3.

Activation of PKA is required for the CaMKII-dependent phosphorylation of Ser2815 of RyR2 and Thr17 of PLN. Representative immunoblots and overall results of SR vesicles isolated from hearts perfused without (Ctrl) and with 30 nM isoproterenol in the absence (Iso) and the presence of 30 μ M of the PKA inhibitor H-89 (Iso-H-89) and with 1 μ M of forskolin (FK). Phosphorylation of Ser2815 of RyR2 and Thr17 of PLN is shown as ratio between the signals of the site-specific phospho-antibody and the antibody used to recognize the non-phosphorylated form. * p <0.05 with respect to Ctrl. # p <0.05 with respect to Iso-treated hearts.

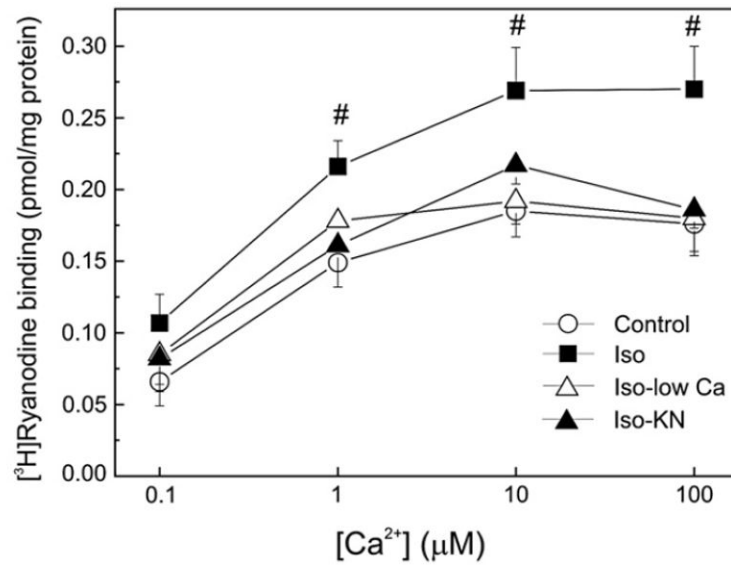


Fig. 4. [³H]-ryanodine binding in SR vesicles isolated from treated hearts. Ca²⁺ dependence of [³H]-ryanodine binding in cardiac SR vesicles isolated from hearts perfused in the absence (Control) and the presence of 300 nM isoproterenol (Iso) and in the presence of Iso, either with low [Ca]_o plus nifedipine (Iso-low Ca) or with 5 μM KN-93 (Iso-KN). Isoproterenol increased [³H]-ryanodine binding. This increase did not occur in the presence of KN-93 or low [Ca]_o plus nifedipine. Of note, at 300 nM isoproterenol, the decrease in Ca²⁺ influx to the cell or the inhibition of CaMKII did not affect the phosphorylation of Ser2809 site but significantly decreased the phosphorylation of Ser2815 site (see Fig. 2). Neither KN-93 nor low [Ca]_o plus nifedipine modified [³H]-ryanodine binding in the absence of isoproterenol. #*p*<0.05 with respect to control hearts.

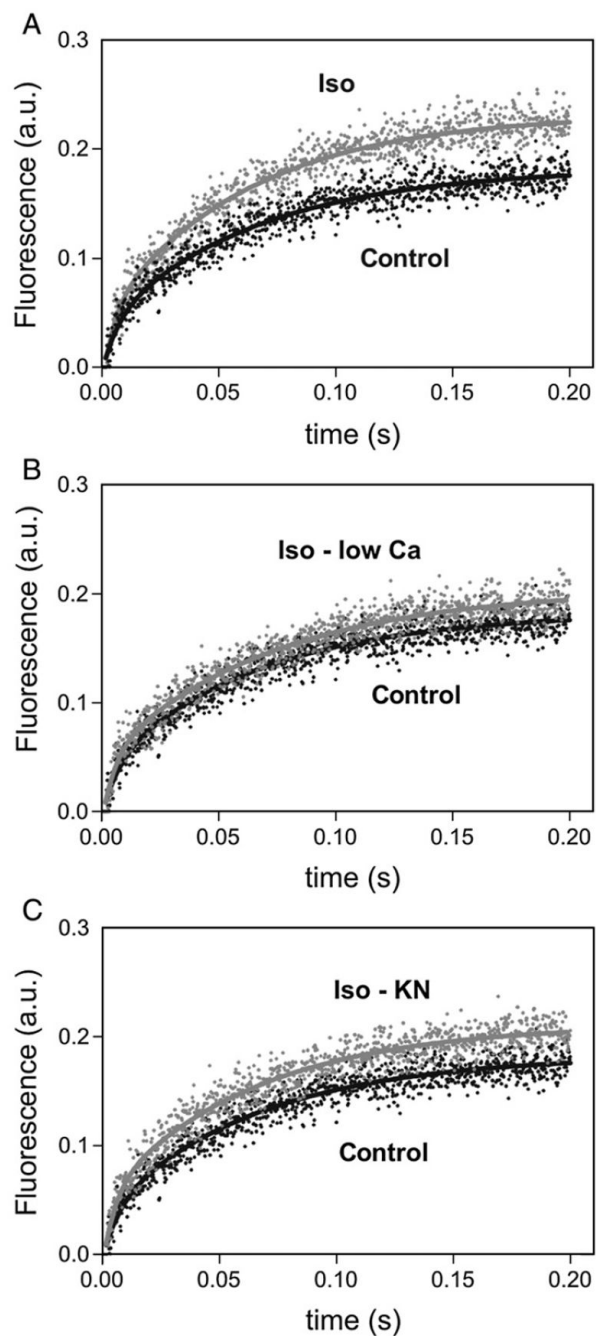


Fig. 5. Effect of isoproterenol on Ca^{2+} release kinetics in isolated hearts. Release kinetic was measured following the change in fluorescence of Calcium Green 5 and was fitted to the double exponential function: $A_1 [1 - \exp(-k_1 t)] + A_2 [1 - \exp(-k_2 t)]$. Representative Ca^{2+} release records in SR vesicles isolated from hearts submitted to the different experimental protocols. The increase in Ca^{2+} release kinetics produced by isoproterenol (Iso, A) was significantly decreased by low $[\text{Ca}]_o$ plus nifedipine (Iso-low Ca, B) or by CaMKII-inhibition (Iso-KN, C). Records shown are the average of 5 to 7 determinations.

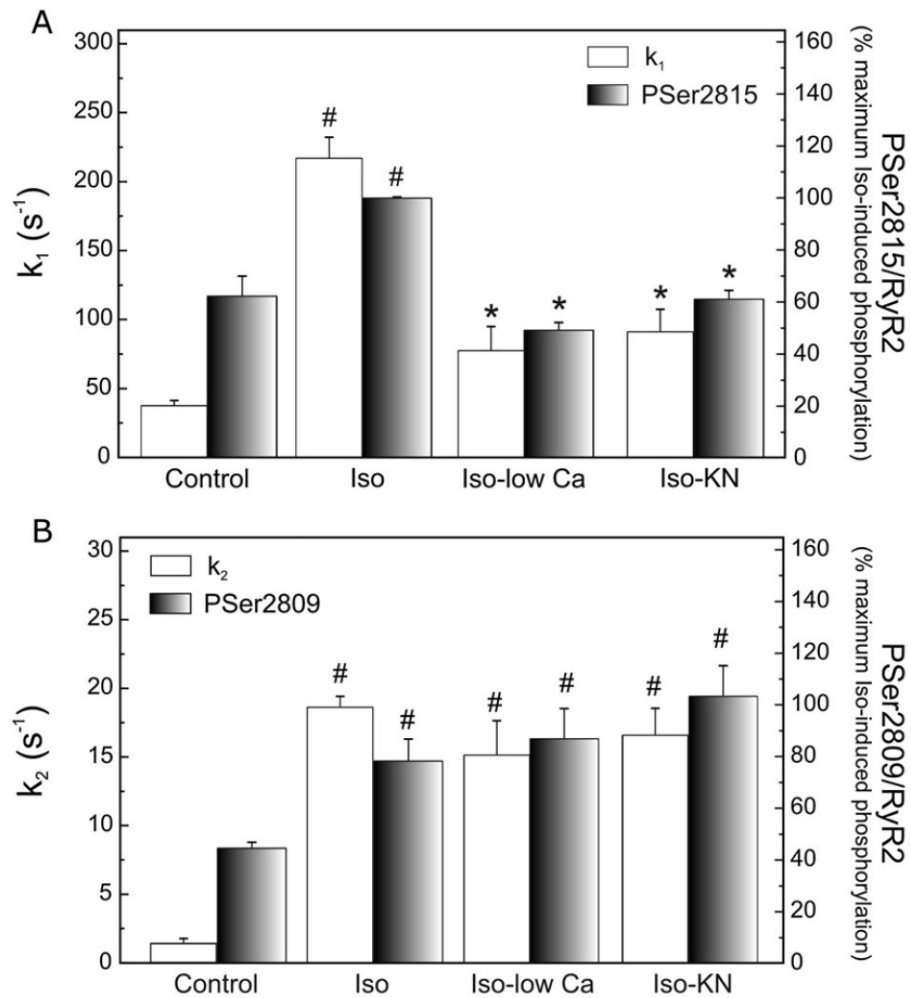


Fig. 6. Ca^{2+} release rate constants (k_1 and k_2) and phosphorylation of RyR2 sites. The rate constant of the fast-exponential release (k_1 , A) increased 5.8-fold in isoproterenol-treated hearts (Iso) and this increase was prevented in hearts perfused with low $[\text{Ca}]_o$ plus nifedipine (Iso-low Ca) or the CaMKII inhibitor KN-93 (Iso-KN). k_2 increased with Iso and it did no decrease by inhibition of CaMKII (B). Changes in the phosphorylation of P-Ser2815 (A) and P-Ser2809 sites (B) in the same experimental conditions are shown for comparison. # $p < 0.05$ with respect to control hearts; * $p < 0.05$ with respect to Iso.