Frequency-dependent acceleration of relaxation in mammalian heart: a property not relying on phospholamban and SERCA2a phosphorylation

Carlos A. Valverde, Cecilia Mundiña-Weilenmann, Matilde Said, Paola Ferrero, Leticia Vittone, Margarita Salas, Julieta Palomeque, Martín Vila Petroff and Alicia Mattiazzi

Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120, 1900 La Plata, Argentina

An increase in stimulation frequency causes an acceleration of myocardial relaxation (FDAR). Several mechanisms have been postulated to explain this effect, among which is the Ca^{2+}–calmodulin-dependent protein kinase (CaMKII)-dependent phosphorylation of the Thr^{17} site of phospholamban (PLN). To gain further insights into the mechanisms of FDAR, we studied the FDAR and the phosphorylation of PLN residues in perfused rat hearts, cat papillary muscles and isolated cat myocytes. This allowed us to sweep over a wide range of frequencies, in species with either positive or negative force–frequency relationships, as well as to explore the FDAR under isometric (or isovolumic) and isotonic conditions. Results were compared with those produced by isoprenaline, an intervention known to accelerate relaxation (IDAR) via PLN phosphorylation. While IDAR occurs tightly associated with a significant increase in the phosphorylation of Ser^{16} and Thr^{17} of PLN, FDAR occurs without significant changes in the phosphorylation of PLN residues in the intact heart and cat papillary muscles. Moreover, in intact hearts, FDAR was not associated with any significant change in the CaMKII-dependent phosphorylation of sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA2a), and was not affected by the presence of the CaMKII inhibitor, KN-93. In isolated myocytes, FDAR occurred associated with an increase in Thr^{17} phosphorylation. However, for a similar relaxant effect produced by isoprenaline, the phosphorylation of PLN (Ser^{16} and Thr^{17}) was significantly higher in the presence of the β-agonist. Moreover, the time course of Thr^{17} phosphorylation was significantly delayed with respect to the onset of FDAR. In contrast, the time course of Ser^{16} phosphorylation, the first residue that becomes phosphorylated with isoprenaline, was temporally associated with IDAR. Furthermore, KN-93 significantly decreased the phosphorylation of Thr^{17} that was evoked by increasing the stimulation frequency, but failed to affect FDAR. Taken together, the results provide direct evidence indicating that CaMKII phosphorylation pathways are not involved in FDAR and that FDAR and IDAR do not share a common underlying mechanism. More likely, a CaMKII-independent mechanism could be involved, whereby increasing stimulation frequency would disrupt the SERCA2a–PLN interaction, leading to an increase in SR Ca^{2+} uptake and myocardial relaxation.

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Corresponding author A. Mattiazzi: Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 y 120, 1900 La Plata, Argentina. Email: ramattia@atlas.med.unlp.edu.ar

The contraction frequency is a fundamental physiological modulator of myocardial function. When the rate of stimulation increases, there is normally an increase in contractility (positive force–frequency relationship). Although this is true for most mammalian species, rats and mice often show a flat or even negative force–frequency response. Moreover, lack of a positive force–frequency relationship is also found in the failing myocardium. Regardless of whether the force–frequency relationship is positive or negative, an increase in stimulation frequency is always associated with an acceleration of relaxation (frequency-dependent acceleration of relaxation, FDAR; Bers, 2000). This mechanism ensures the proper relaxation and diastolic filling of the ventricle at the higher rates. Schouten (1990) first hypothesized that the relaxant effect

C. A. Valverde and C. Mundiña-Weilenmann contributed equally to this work.

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of increasing stimulation frequency could be dependent on the activation of Ca^{2+}–calmodulin-dependent protein kinase (CaMKII) produced by an increase in Ca^{2+} cycling and phosphorylation of the sarcoplasmic reticulum (SR) protein, phospholamban (PLN). Two main findings converge to support this attractive hypothesis. First, the prerequisites for sustained CaMKII activation seem to be specifically met by the increase in stimulation frequency. In an already classical study, De Koninck & Schulman (1998) elegantly showed that CaMKII can decode the frequency of Ca^{2+} spikes into distinct amounts of kinase activity. Second, it has been shown that FDAR is dependent on the SR in different types of preparations and under different experimental conditions (Schouten, 1990; Bassani *et al.* 1995; Hussain *et al.* 1997). Since the main regulator of the SR Ca^{2+} uptake by sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA2a) is PLN, and the Thr^{17} site of PLN is specifically phosphorylated by CaMKII, PLN and the phosphorylation of its Thr^{17} site seems to be the natural candidate to explain FDAR, as suggested by Schouten (1990). However, the agreement regarding the first and second points mentioned above turned to controversy when either the dependence of CaMKII and/or the involvement of PLN were directly tested. While experiments from Bassani *et al.* (1995) and De Santiago *et al.* (2002) showed that FDAR is dependent on CaMKII activation and those from Hagemann *et al.* (2000) seemed to indicate that phosphorylation of the Thr^{17} residue of PLN is responsible for FDAR, other experiments failed to detect either an FDAR dependence on CaMKII activation (Hussain *et al.* 1997; Layland & Kentish, 1999; Kassiri *et al.* 2000) or a significant increase in PLN phosphorylation (Hussain *et al.* 1997). Experiments in hearts from transgenic animals with ablation of PLN did not help to clarify the controversy. Results from Li *et al.* (1998) and De Santiago *et al.* (2002) indicated that FDAR, although dependent on CaMKII activity, was not dependent on PLN, whereas results from Bluhm *et al.* (2000), and more recently from Champion *et al.* (2003) and Zhao *et al.* (2004), indicated that PLN did play a role in FDAR. Taken together, the possible participation of CaMKII-dependent Thr^{17} phosphorylation in the FDAR phenomenon is controversial and the underlying mechanisms of FDAR remain poorly understood.

The aim of the present study was to re-explore the mechanisms of FDAR using two different approaches. First, since the main mechanism of the isoprenaline-dependent acceleration of relaxation (IDAR) is the increase in PLN phosphorylation residues (Lindemann *et al.* 1983; Vittone *et al.* 1990; Mundiña-Weilenmann *et al.* 1996; Said *et al.* 2002), we aimed to compare the degree of PLN phosphorylation residues for a similar FDAR and IDAR. Second, we also aimed to compare the time course of the development of FDAR and IDAR with that of Ser^{16} and Thr^{17} phosphorylation of PLN. To these purposes three different preparations were used: isolated perfused rat hearts; cat papillary muscles; and cat myocytes. The different preparations allowed us to sweep over a wide range of frequencies, in species with either positive or negative force–frequency relationships, as well as to explore the FDAR under isometric (or isovolumic) and isotonic conditions.

Methods

Animal studies conformed to the guidelines for Animal Care of the Scientific Committee of the University of La Plata, School of Medicine and the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996). Cats (1–1.5 kg) and male Wistar rats (200–300 g) were anaesthetized by intraperitoneal injection of sodium pentobarbitone (3.5 and 14 mg (100 g body weight)$^{-1}$, respectively). Once anaesthetized, the chest was opened and the heart was removed for experimental use.

Mechanical studies

Rat heart perfusion. Isolated rat hearts were perfused according to the Langendorff technique at constant temperature (37°C) and flow (12–14 ml min^{-1}), as previously described (Mundiña-Weilenmann *et al.* 1996; Vittone *et al.* 1998). The composition of the physiological salt solution (PSS) was (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. To prevent interference from atrial beats on the electrical pacing, total heart blockade was performed by crushing the atrial septum. After this blockade, hearts were paced at different frequencies (Results). The mechanical activity of the heart was assessed by passing into the left ventricle a latex balloon connected to a pressure transducer (Perceptor DT, Namic, Glen Falls, NY, USA). The balloon was filled with aqueous solution to achieve a left ventricular end-diastolic pressure of approximately 10 mmHg.

Cat papillary muscles. One or two papillary muscles were dissected from the right ventricle of a cat heart and mounted vertically in a chamber to contract isometrically. The muscles were stretched until they reached the length at which maximal developed tension (DT) occurred and then allowed to stabilize for 1 h at a constant frequency (30 pulses min^{-1}) and temperature (30°C) as previously described (Pedroni *et al.* 1990; Salas *et al.* 2001). Muscles were superfused with the same PSS as described above.

Isolation of cat myocytes. Myocytes were isolated according to the technique previously described

(Vila-Petroff *et al.* 2003). Briefly, excised cat hearts were mounted on a Langendorff perfusion apparatus. For enzymatic cell isolation, 118 units ml^{-1} collagenase type B (Worthington Biochemical Corp., Lakewood, NJ, USA) and 0.1 mg ml^{-1} pronase (Boerhinger Mannheim Corp., GmbH, Mannheim, Germany), were used. Perfusion solutions were similar to the PSS buffer described above, except that bicarbonate was replaced by 10 mM Hepes, pH adjusted to 7.4 (with NaOH) and the solution was continuously equilibrated with 100% O_2. The concentration of Ca^{2+} varied along the different steps in the isolation procedure. After enzymatic treatment, disaggregated myocytes were separated from the undigested tissue, rinsed several times with a Hepes solution containing 1% BSA and 500 μM $CaCl_2$ and kept in this solution at room temperature until use. Rod-shaped myocytes with clear and distinct striations and an obvious marked shortening and relaxation on stimulation were used. The experiments were performed at room temperature.

Indo-1 fluorescence and cell shortening measurements. Isolated myocytes were loaded with the cell-permeant acetomethyl ester form of indo-1 (17 μM for 9 min) according to the bulk method described by Spurgeon *et al.* (1990) and as we previously described (Vila-Petroff *et al.* 2003). Cells were placed on the stage of an inverted microscope (Nikon Diaphot 200) adapted for epifluorescence. Myocytes were continuously superfused at a constant flow (1 ml min^{-1}) and field stimulated via two platinum electrodes on either side of the bath (square waves, 2 ms duration and 20% above threshold). The excitation light was centred at 350 nm and the fluorescence emitted by the cell was recorded at 410 and 482 nm. Background fluorescence was subtracted from each signal before obtaining the 410:482 fluorescence ratio. Diastolic fluorescence ratio was measured as the mean value over a 100 ms period after the twitch was completed. Systolic fluorescence ratio was determined directly from the peak of the recorded ratio. Resting cell length and cell shortening were measured by a video-based motion detector (Crescent Electronics, Sandy, UT, USA) and stored by software for an off-line analysis. For phosphorylation studies an aliquot of the isolated myocyte suspension was placed on the stage of an optical microscope and field stimulated at room temperature. Laemmli sample buffer was added to terminate the experiment.

Biochemical assays

Preparation of rat heart SR membranes. Rat SR membrane vesicles were prepared as previously described (Mundiña-Weilenmann *et al.* 1996). Briefly, the pulverized ventricular tissue was homogenized in six volumes of homogenization buffer containing (mM): 5 Na_2EDTA, 25 NaF, 300 sucrose, 1 phenylmethanesulphonyl fluoride (PMSF), 1 benzamidine and 30 KH_2PO_4. The homogenate was centrifuged twice at 14 000 and 16 000*g* for 20 min. The resulting supernatant was centrifuged at 45 000*g* for 45 min. The pellet obtained was suspended in three volumes of buffer containing (mM): 10 Na_2EDTA, 25 NaF, 600 KCl, and 50 KH_2PO_4, and re-centrifuged as in the previous step. The resulting pellet was suspended in 10 mM Na_2EDTA, 10 mM NaF, 250 mM sucrose and 30 mM histidine.

Papillary muscles. Papillary muscles, pooled to get approximately 20 mg, were homogenized in 250 μl of a buffer containing (mM): 50 $Na_4P_2O_7$, 50 NaF, 50 NaCl, 5 Na_2EDTA, 5 EGTA, 0.1 Na_3VO_4, 10 Hepes, 1 PMSF, 1 benzamidine and 0.5 μg ml^{-1} *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), with a Teflon pestle and centrifuged at 11 500*g* for 20 min at 4°C. The supernatant was used for phosphorylation studies.

Solutions in the homogenization and membrane isolation procedure were pH 7.0 at 4°C. In all preparations, protein concentration was measured by the method of Bradford using bovine serum albumin as standard.

Electrophoresis and Western blot analysis. For immunological detection of PLN phosphorylation sites, 20–30 μg of SR membrane proteins, homogenized papillary muscles or myocyte suspension were electrophoresed per gel lane in a 10% SDS polyacrylamide gel, as previously described (Mundiña-Weilenmann *et al.* 1996). Proteins were then transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes and probed with the phosphorylation site-specific phosphorylated $PSer^{16}$ and $PThr^{17}$ PLN antibodies (1:5000; Badrilla, Leeds, UK). Immunoreactivity was visualized by peroxidase-conjugated antibodies using a peroxidase-based chemiluminescence detection kit (Amersham Bioscience). The signal intensity of the bands on the film was quantified using Scion Image software (based on NIH Image). Unless otherwise indicated, phosphorylation results are expressed as percentage of maximal Ser^{16} and Thr^{17} phosphorylation induced by isoprenaline, run in parallel. In additional experiments, 50 μg of SR membranes proteins were resolved in 8% acrylamide gels according to Laemmli. Proteins were transferred and probed with antiserum specific antibody for the Ca^{2+}-ATPase peptide phosphorylated on Ser^{38}, SERCA PS-38 (1:5000). As a positive standard, 0.1–2 pmol of a peptide containing the phosphorylated Ser^{38} site of SERCA2a, constructed as described by Rodriguez *et al.* (2004), was used. Both the antibody and the phosphopeptide were kindly provided by J. Colyer (University of Leeds, UK). Immunoreactivity was visualized as described above.

Back phosphorylation. Thirty micrograms of SR membranes, isolated as described above from hearts submitted to different frequencies, were phosphorylated in 50 μl of a reaction medium containing 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EGTA, 2 μM calmodulin and 0.1 mM $CaCl_2$. The reaction was initiated by the addition of 50 μM [γ-^{32}P]ATP, specific activity 1000–1600 counts min^{-1} pmol^{-1}, following preincubation of the rest of the assay components for 3 min at 30°C. The reaction was terminated after 5 min by the addition of SDS sample buffer, and the samples were subjected to SDS-PAGE in a 5–15% gradient gel. The gels were stained with Comassie Blue, dried and autoradiographed. ^{32}Phosphorus incorporation into the specific bands was quantified by analysing the signal intensity of the bands as described for the Western blot. Phosphorylation results were expressed as a percentage of the signal obtained at 120 beats min^{-1}.

Statistics

Data are expressed as means ± S.E.M. Statistical significance was determined by Student's *t* test for paired and unpaired observations, as appropriate. A *P* value < 0.05 was considered statistically significant.

Results

FDAR is dependent on SR

Several previous studies indicate that the SR is involved in the relaxant effect of increasing contraction frequency (FDAR; Schouten, 1990; Bassani *et al.* 1995; Hussain *et al.* 1997). Experiments were performed to confirm this issue in our experimental conditions. Figure 1 shows typical records of the left ventricular developed pressure (LVDP) of a perfused rat heart. The increase in contraction frequency from 60 to 120 beats min^{-1} decreased LVDP (negative staircase, Fig. 1*A*). This negative inotropic effect was associated with a FDAR, which is better appreciated in the normalized records of Fig. 1*B*. Perfusion with ryanodine and thapsigargin, to functionally block the SR, abolished the FDAR as shown in the original and normalized records of Fig. 1*C* and *D*, respectively. The low range of the force–frequency relationship explored in these experiments was due to the fact that higher, more physiological frequencies for the rat, cause contracture of the hearts in the absence of a functional SR. As an additional index of FDAR, we used the ratio between the half-relaxation time ($t_{1/2}$) at the high and the low frequency studied ($t_{1/2}$ at 120 beats min^{-1}/$t_{1/2}$ at 60 beats min^{-1}; Fig. 1*E*; De Santiago *et al.* 2002). A significant decrease of

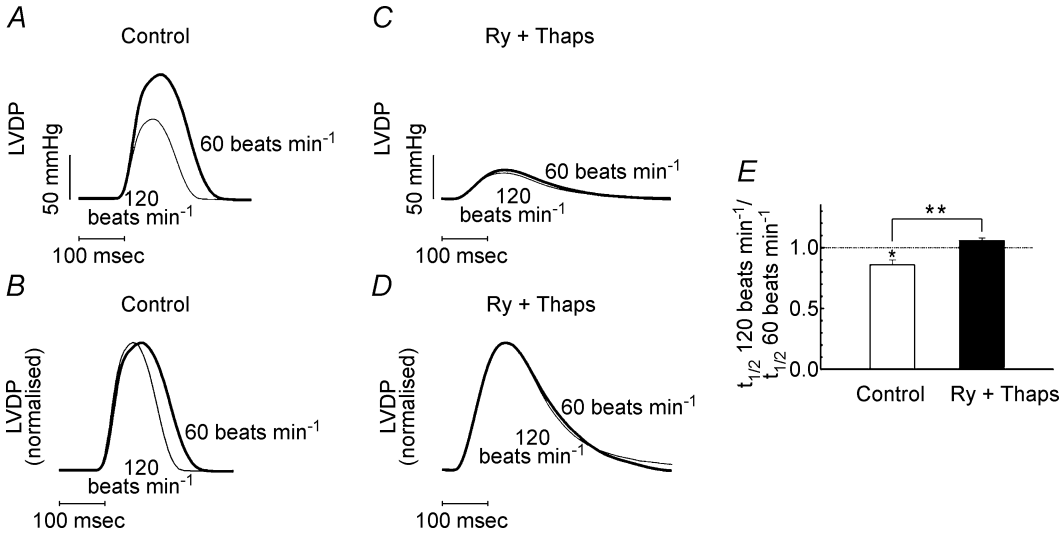

Figure 1. Frequency-dependent acceleration of relaxation (FDAR) depends on a functional sarcoplasmic reticulum (SR)

A, records of left ventricular developed pressure (LVDP) obtained from a perfused rat heart. The increase of the contraction frequency produced a decrease in LVDP (negative staircase). *B*, normalized records of LVDP showing that this force–frequency relationship is associated with an accelerated relaxation. *C*, inhibition of SR function with the treatment of 0.5 μM ryanodine (Ry) plus 2 μM thapsigargin (Thaps) produced a negative inotropic effect at both stimulation frequencies and suppressed both the negative staircase observed in *A* and FDAR. *D*, normalized records of LVDP to illustrate better the suppression of FDAR in the presence of Ry and Thaps. *E*, bars show the ratio between the time to half-relaxation ($t_{1/2}$) at the high and low frequencies (FDAR index) obtained in the absence (Control) or the presence of an inhibited SR (Ry + Thaps). Inhibition of SR function abolished the decrease of the ratio below 1 that was produced by FDAR. Results are expressed as means ± S.E.M. of $n = 4$–6 experiments. $^*P < 0.05$ with respect a ratio of 1; $^{**}P < 0.05$ with respect to control.

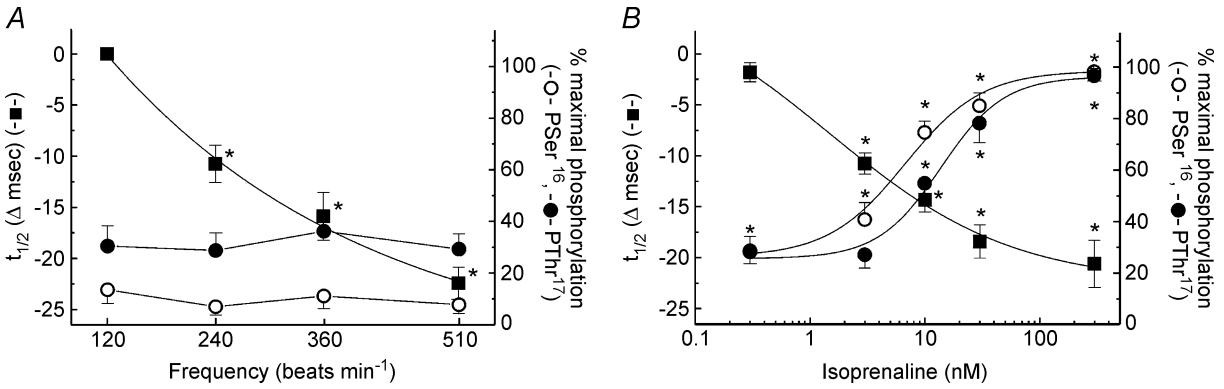

Figure 2. Effects of increasing contraction frequency (*A*) and isoprenaline (*B*) on relaxation and phosphorylation of Ser^{16} and Thr^{17} residues of PLN in the perfused rat heart
The increase in the contraction frequency from 120 to 510 beats min^{-1} produced a decrease in $t_{1/2}$ (■ in *A*) similar to that induced by increasing isoprenaline from 0.3 to 300 nM (■ in *B*). While the relaxant effect of isoprenaline was associated with the enhancement of the phosphorylation of both PLN residues, the increase in the contraction frequency did not modify PLN phosphorylation either at Ser^{16} (○) or Thr^{17} (●) residues. Results are expressed as means ± S.E.M. of $n = 4$–12 experiments; $t_{1/2}$ is expressed as change (Δms) with respect to 120 beats min^{-1} (*A*) or the absence of drug (*B*). $^*P < 0.05$ with respect to 120 beats min^{-1} (*A*) or the absence of drug (*B*).

this index below a ratio of 1 is indicative of FDAR. It can be seen that in the absence of ryanodine and thapsigargin, the increase in the stimulation frequency reduced the FDAR index to 0.86 ± 0.04, $n = 4$ ($P < 0.05$), whereas in the presence of these drugs, FDAR was abolished. Thus, it seems undoubted that the underlying mechanism of FDAR either resides at the SR level or at least needs a functionally intact SR.

Does Thr^{17} of PLN participate in the FDAR?

Figure 2 illustrates the relaxant effect and the average PLN phosphorylation at Ser^{16} and Thr^{17} sites, observed with incremental heart rates (Fig. 2*A*) and isoprenaline concentrations (Fig. 2*B*) in the perfused rat heart. Both the FDAR and the isoprenaline-dependent acceleration of relaxation (IDAR) were similar, as indicated by the reduction of $t_{1/2}$. However, while increasing stimulation frequency from 120 to 510 beats min^{-1} failed to increase the phosphorylation of Ser^{16} and Thr^{17} of PLN, addition of isoprenaline from 0.3 to 300 nM produced a significant increase in the phosphorylation of both PLN residues. Similar results were obtained in another multicellular preparation of a different species, the cat papillary muscle. Again, while the relaxant effect of 1 μM isoprenaline was associated with a significant increase in the phosphorylation of Ser^{16} and Thr^{17} residues of PLN, no significant changes in the phosphorylation of both residues were observed, for a similar relaxant effect produced by raising stimulation frequency from 10 to 60 beats min^{-1} (Fig. 3).

In a further attempt to search for the underlying mechanisms of FDAR, we compared the effect of elevating contraction frequency in the absence and the presence of the β-agonist in the perfused rat heart (Fig. 4*A*). At each stimulation frequency, the presence of 30 nM isoprenaline produced a further decrease in $t_{1/2}$. However, both curves were parallel and the FDAR index did not reveal any differences between the values measured in the absence and the presence of the β-agonist ($t_{1/2}$ at 510 beats min^{-1}/$t_{1/2}$ at 120 beats min^{-1} $= 0.59 \pm 0.02$ and 0.54 ± 0.06, respectively, Fig. 4*B*). We next tested the variation of heart rate at a maximal isoprenaline stimulation (300 nM, Fig. 4*C*). At this maximal concentration, stimulation frequency was increased from 120 to 590 beats min^{-1}. At a fixed stimulation

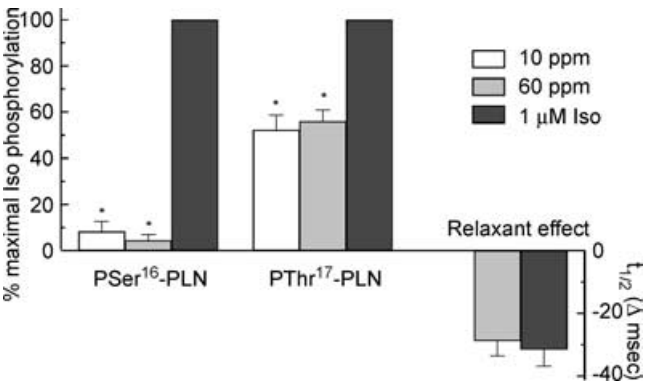

Figure 3. Phosphorylation of Ser^{16} and Thr^{17} residues of PLN at a similar relaxant effect produced by either increasing contraction frequency or addition of appropriate isoprenaline concentration in cat papillary muscles
A similar decrease of $t_{1/2}$ was obtained when cat papillary muscles stimulated at 10 beats min^{-1} were then stimulated at 60 beats min^{-1} or perfused with 1 μM isoprenaline. Only the relaxant effect produced by the β-agonist was associated with enhancement of PLN phosphorylation at the Ser^{16} and Thr^{17} residues. Phosphorylation of Ser^{16} and Thr^{17} residues was maximal at this concentration of isoprenaline. Results are expressed as means ± S.E.M. of $n = 6$–13 experiments. $^*P < 0.05$ with respect to 1 μM isoprenaline.

frequency of 120 beats min^{-1}, isoprenaline produced a concentration-dependent increase in the relaxant effect. At a maximal isoprenaline concentration (300 nM), an abrupt change to 510 beats min^{-1} in the continuous presence of the β-agonist produced a further decrease in $t_{1/2}$ that was not associated with any additional increase in the phosphorylation of PLN sites (Fig. 4*D*).

Taken together, the results in multicellular preparations argue in favour of the hypothesis that IDAR and FDAR are supported by different mechanisms: while IDAR occurs tightly associated with the phosphorylation of PLN residues, FDAR occurs in the absence of any significant increase in the phosphorylation of PLN sites.

Is SERCA2a the target of the CaMKII activation induced by increasing stimulation frequency responsible of FDAR?

The results presented so far are compelling evidence indicating the lack of any important physiological role of PLN in the FDAR phenomenon. To test the possible participation of other CaMKII substrates in the FDAR, the status of phosphorylation of SERCA2a, the major alternative candidate, was explored. Figure 5*A* shows a typical autoradiogram and overall results of the phosphorylation of SERCA2a, studied by the back phosphorylation technique in perfused rat hearts,

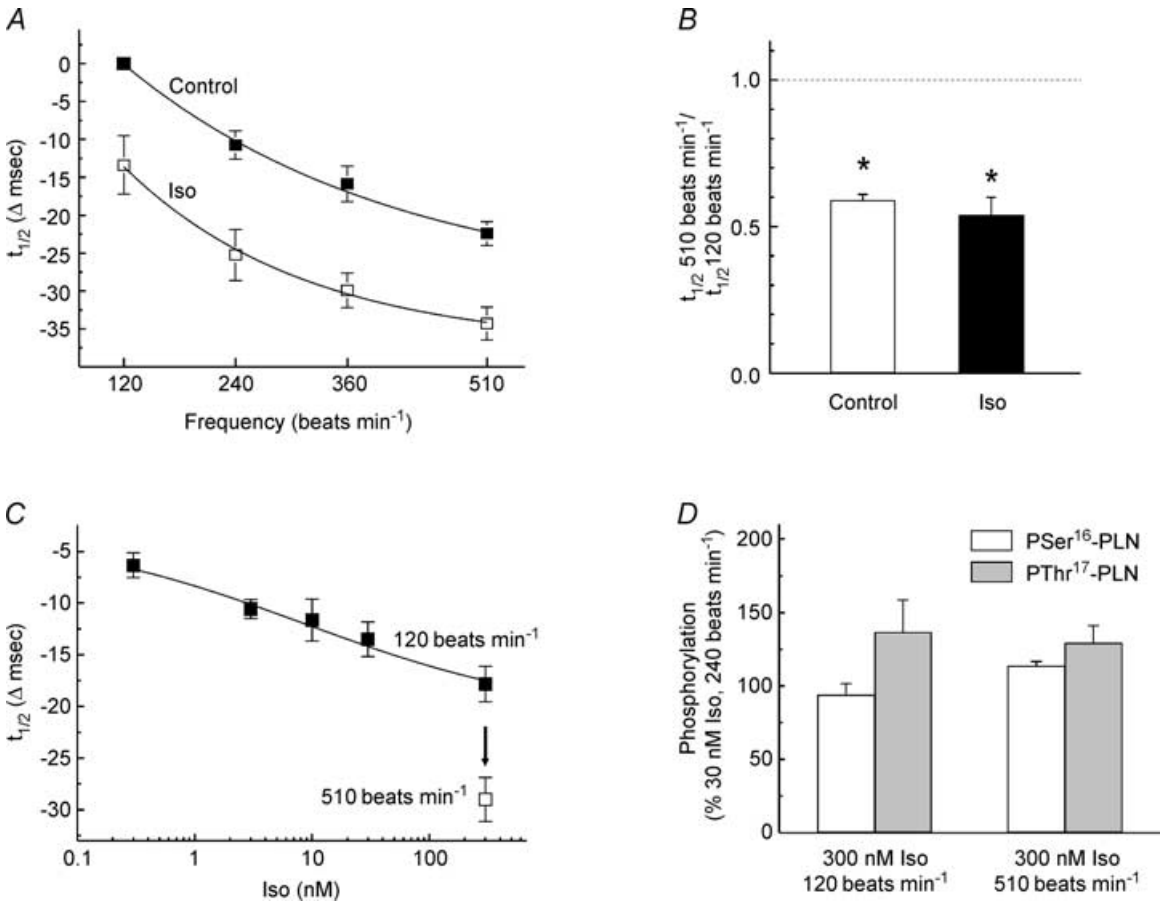

Figure 4. Do FDAR and IDAR share a common mechanism?
A and *B* illustrate the effects of β-adrenergic stimulation on FDAR. *A*, rat hearts were stimulated at increasing frequencies in the absence (Control) and in the presence of 30 nM isoprenaline (Iso). A similar decrease of $t_{1/2}$ to that induced by increasing contraction frequency from 120 to 510 beats min^{-1} was attained in the absence and in the presence of the β-agonist. *B*, the lack of action of isoprenaline upon FDAR was also detected in the ratio between $t_{1/2}$ at 510 and 120 beats min^{-1}. The significant decrease of this ratio below 1 was not different between control and isoprenaline-stimulated hearts. Results are expressed as means ± S.E.M. of $n = 5$–12 experiments. $^*P < 0.05$ with respect to a ratio of 1. *C* and *D* illustrate the effects of the enhancement of contraction frequency on IDAR. *C*, hearts, stimulated at 120 beats min^{-1}, were perfused with increasing isoprenaline concentrations. At the maximal concentration-dependent relaxant effect of isoprenaline (300 nM), increasing the contraction frequency to 510 beats min^{-1} produced an additional decrease in $t_{1/2}$. *D*, phosphorylation levels of the Ser^{16} and Thr^{17} residues of PLN induced by 300 nM isoprenaline were not modified by a change in stimulation frequency from 120 to 510 beats min^{-1}. Results are expressed as means ± S.E.M. of $n = 4$ experiments.

at two different stimulation frequencies, 120 and 510 beats min^{-1}. The back phosphorylation method shows the mirror phosphorylation image of the actual *in vivo* phosphorylation. Phosphorylation of SERCA2a was the same at the two frequencies explored. Moreover, and in agreement with the experiments performed with site-specific phosphorylation antibodies (Fig. 2*A*), no significant changes in the phosphorylation of PLN were observed. Similar results were obtained in the presence and absence of added exogenous CaMKII, which would indicate that possible variations in the CaMKII activity in the different experimental situations are not responsible for the results obtained. In an additional group of experiments, we used a recently described polyclonal antibody specific for the phosphorylated Ser^{38} peptide of SERCA2a (Rodriguez *et al.* 2004) to evaluate the phosphorylation of SERCA2a in SR membrane vesicles isolated from hearts contracting at 120 and 510 beats min^{-1}. The immunoblot of Fig. 5*B* shows that no significant phosphorylation of SERCA2a could be detected in these membrane preparations, in spite of the high immunodetection sensitivity of the assay (0.5 pmol of the phosphopeptide used for calibration, see Methods). Assuming that SERCA2a is 10% of total SR protein (Hawkins *et al.* 1994), 50 pmol SERCA2a should be expected in 50 μg of SR protein loaded per gel lane. The lack of detection of SERCA2a phosphorylation would indicate that, if phosphorylation occurred, it would result in the generation of less than 1% of Ser^{38} phosphoprotein.

Effects of CaMKII inhibition on FDAR

In a further attempt to study the possible involvement of the CaMKII-dependent phosphorylations in the FDAR, we performed experiments with the specific CaMKII inhibitor, KN-93. Since KN-93 is unable to inhibit the autophosphorylated form of CaMKII (Tokumitsu *et al.* 1990), hearts were perfused with 5 μM of the inhibitor in the presence of low extracellular Ca^{2+} (0.25 mM) for 10 min, to diminish Ca^{2+} entry to the cell and allow for dephosphorylation of the enzyme (Bassani *et al.* 1995). Increasing the stimulation frequency from 120 to 510 beats min^{-1} decreased $t_{1/2}$ by 20.10 ± 2.83 ms and the time constant of the Ca^{2+} transient decay (τ) by 7.40 ± 2.52 ms ($n = 5$) in the absence of KN-93. In the presence of the inhibitor, $t_{1/2}$ and τ decreased by 17.60 ± 3.31 and 5.10 ± 4.11 ms, respectively ($n = 5$). Of note, despite the lack of effect of KN-93 on relaxation, the CaMKII inhibitor produced a significant enhancement of the negative staircase observed in the perfused rat heart. Raising the stimulation frequency from 120 to 510 beats min^{-1} diminished LVDP by 38.83 ± 2.39 and 76.28 ± 2.93% in the absence and presence, respectively, of KN-93. This negative inotropic effect may be the consequence of KN-93 inhibition of L-type Ca^{2+} current (especially at higher frequencies), or ryanodyne receptor gating (Li *et al.* 1997). These results indicate that the failure of KN-93 to significantly impair FDAR is not due to either an inadequate concentration of KN-93 or to the protocol of administration of the inhibitor.

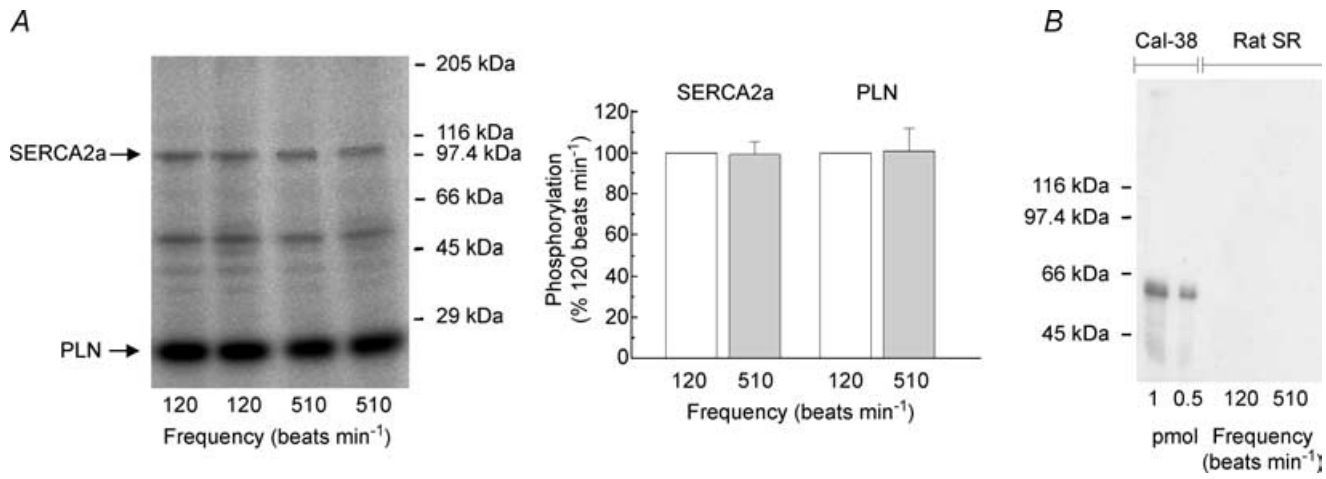

Figure 5. Effect of stimulation frequency on CaMKII phosphorylation of SR membranes
A, SR membranes isolated from perfused rat hearts stimulated at 120 and 510 beats min^{-1} were back phosphorylated with the endogenous CaMKII and [γ-^{32}P]-ATP. Left panel, autoradiogram; right panel, overall results, expressed as means ± S.E.M. of $n = 9$ experiments. Note that the *in situ* treatment (frequency change) did not alter the ability of SERCA2a and PLN to serve as substrate for the *in vitro* phosphorylation. *B*, immunoblot of SR membranes probed with SERCA PS-38 antibody. The antibody did not detect any significant phosphorylation of SERCA2a in SR membranes, although it was able to recognize the phosphoepitope peptide conjugated to a scaffolding protein (calibration standard, Cal-38) run in parallel.

Phosphorylation of Thr^{17} of PLN in myocytes

The lack of detection of any significant increase in PLN phosphorylation after increasing the stimulation frequency in multicellular preparations is hard to reconcile with the findings of Hagemann *et al.* (2000), who reported a close association between FDAR and the phosphorylation of Thr^{17} of PLN in isolated rat myocytes. To examine whether the cause of the discrepant results resides in the different preparations used, we performed experiments in isolated cat myocytes. Stimulation frequencies ranging from 10 to 60 beats min^{-1} produced an FDAR, indexed by the decrease in the time from the peak to half of the Ca^{2+} transient decline ($t_{1/2}$ transient), and a significant increase in the phosphorylation of Thr^{17} at faster stimulation rates, independently of that of Ser^{16} (Fig. 6*A*). Figure 6*B* shows that the frequency-dependent abbreviation of the $t_{1/2}$ transient tightly correlates with the frequency-dependent increase in Thr^{17} phosphorylation. However, when the degree of PLN phosphorylation obtained at the maximal FDAR was compared with the isoprenaline-induced PLN phosphorylation at a similar relaxant effect, the latter intervention (3 nM isoprenaline) significantly enhanced the phosphorylation of both PLN residues (49.1 ± 7.8 and 56.6 ± 16.6%, $n = 7$, above basal values for Ser^{16} and Thr^{17}, respectively), whereas the increase in stimulation frequency only enhanced the phosphorylation of Thr^{17} site by 29.6 ± 7.2%, $n = 9$, without modifications in the phosphorylation of Ser^{16} (Fig. 7).

To investigate whether Thr^{17} phosphorylation of PLN can account at least in part for the rate-dependent abbreviation of the $t_{1/2}$ transient in myocytes, we studied the time course of the appearance of the FDAR and Thr^{17} phosphorylation (Fig. 8*A*). As early as in the second beat (2.5 s) after the frequency step (from 10 to 50 beats min^{-1}), the $t_{1/2}$ transient was significantly decreased, reaching its lowest value. However, phosphorylation of PLN at the Thr^{17} residue significantly increased 15 s after the frequency change. Thus, phosphorylation of Thr^{17} occurred at a slower rate than the FDAR, arguing against the participation of PLN phosphorylation in the determination of this effect. In contrast, the onset of IDAR was temporally associated with the increase in the phosphorylation of Ser^{16} (Fig. 8*B*). Phosphorylation of Thr^{17} became significant only after 2 min of isoprenaline administration (66.0 ± 15.3%). This increase was associated with a further enhancement of IDAR,

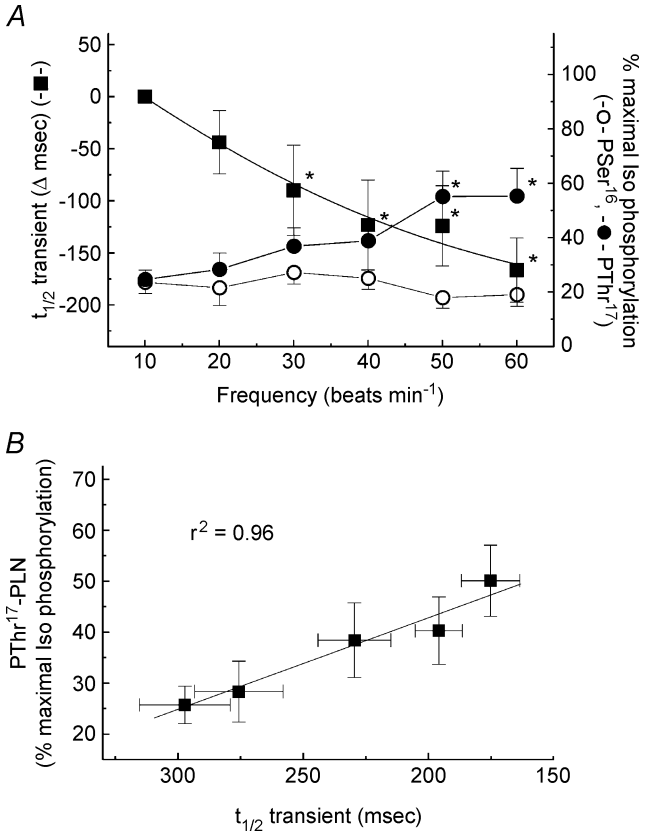

Figure 6. Effects of increasing contraction frequency on relaxation and phosphorylation of Ser^{16} and Thr^{17} residues of PLN in cat myocytes

A, average data of isolated cat myocytes stimulated for 3 min at increasing frequencies. The decrease in the half-relaxation time of the Ca^{2+} transient ($t_{1/2}$ transient, ■) was associated with an increase in the phosphorylation of Thr^{17} (●) independently of that of Ser^{16} (○), detected as in Fig. 2. *B*, correlation between Thr^{17} phosphorylation and $t_{1/2}$. $t_{1/2}$ is expressed as change (Δms) with respect to 10 beats min^{-1}. Results are expressed as means ± S.E.M. of $n = 4$–9 experiments.

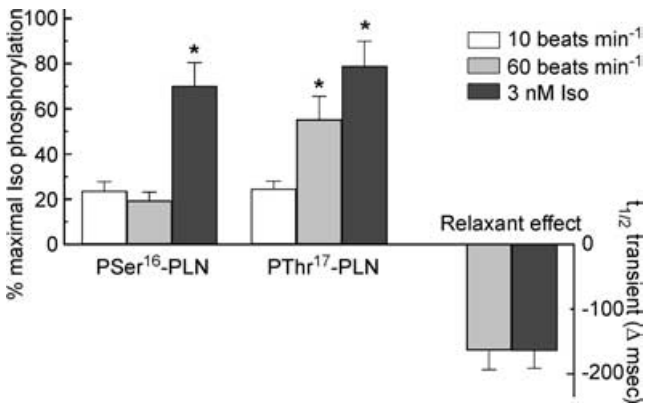

Figure 7. Phosphorylation of Ser^{16} and Thr^{17} residues of PLN at a similar relaxant effect produced by either increasing contraction frequency or increasing isoprenaline concentration in cat myocytes

A similar decrease in $t_{1/2}$ transient was obtained when isolated cat myocytes were either submitted to a frequency change from 10 to 60 beats min^{-1} or superfused with 3 nM isoprenaline. While IDAR and FDAR were associated with a significant increase in the phosphorylation of Thr^{17}, IDAR was also associated with a significant increase in the phosphorylation of Ser^{16}. Results are expressed as means ± S.E.M. of $n = 8$–10 experiments. $^*P < 0.05$ with respect to 10 beats min^{-1}.

which occurred in the absence of any significant increase in the phosphorylation of Ser[16]. The time-delayed Thr[17] phosphorylation is consistent with former results in rat and guinea-pig hearts (Wegener *et al.* 1989; Kuschel *et al.* 1999) and might indicate that the PKA-dependent increase in intracellular Ca^{2+} is not large enough at the very beginning of isoprenaline administration to activate CaMKII (Said *et al.* 2002).

Figure 9 shows the results of a group of experiments in which we studied the effects of inhibiting Thr[17] phosphorylation with the specific inhibitor of CaMKII, KN-93, on the FDAR. Although 1 μM KN-93 inhibited the frequency-dependent increase in the phosphorylation of Thr[17], the decrease in half-relaxation time ($t_{1/2}$) and the time constant of the Ca^{2+} transient decay (τ) associated with a frequency step from 10 to 50 beats min^{-1}, was not different in the presence and in the absence of the CaMKII inhibitor. Another CaMKII inhibitor (autocamtide-2 related inhibitory peptide, AIP, 10 μM) also failed to diminish the relaxant effect of the increase in contraction frequency. Thus, even in a preparation in which the increase in stimulation frequency increases the phosphorylation of the Thr[17] site of PLN, this phosphorylation cannot explain the FDAR.

Discussion

Although the FDAR phenomenon has been explored in several laboratories, the conflicting results obtained in the different studies conspire against a complete understanding of the underlying mechanisms involved. The aim of the present study was to gain further insight into the comprehension of these mechanisms. The use of different species and preparations allowed us to explore a wide range of stimulation frequencies under various experimental conditions, i.e. isometric or isotonic contractions, different temperatures, and even different inotropic response to the increase in stimulation frequency.

Our major finding is that the phosphorylation of PLN residues does not participate in FDAR and that FDAR and IDAR should be explained by different mechanisms. While IDAR occurs closely associated with a significant increase in PLN phosphorylation, no significant increases in the phosphorylation of Thr[17] residue of PLN, the site that would be expected to be phosphorylated by increasing stimulation frequency, could be detected associated with FDAR in the perfused rat heart and in cat papillary muscles (Figs 2 and 3). Furthermore, the

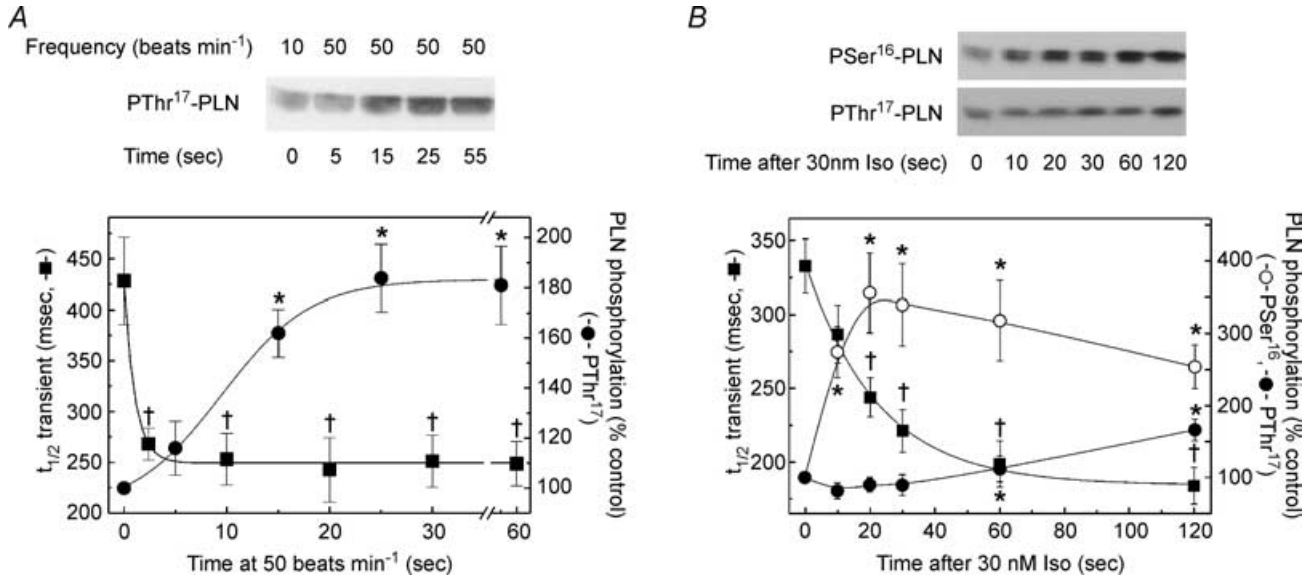

Figure 8. Time course of relaxation and phosphorylation of PLN residues during an increase in stimulation frequency and administration of isoprenaline in cat myocytes

A, upper panel shows a representative immunoblot of Thr[17] PLN phosphorylation during an increase in stimulation frequency from 10 to 50 beasts min^{-1}. Lower panel shows the average data of Thr[17] PLN phosphorylation ($n = 5$ for each data point, ●) and of the decrease in $t_{1/2}$ transient ($n = 3$) for the same frequency step. The decrease in $t_{1/2}$ transient (■) became significant 2.5 s after the change of frequency, while phosphorylation of Thr[17] occurred more slowly. *B*, upper panel shows a representative immunoblot of Ser[16] and Thr[17] phosphorylation during administration of 30 nM isoprenaline. Lower panel shows the average data of Ser[16] (○) and Thr[17] (●) PLN phosphorylation ($n = 5$ for each data point) and of the decrease in $t_{1/2}$ transient ($n = 6$, ■) for the same isoprenaline concentration. The phosphorylation of Ser[16] of PLN occurred temporally associated with the onset of IDAR. Phosphorylation of Thr[17] attained significant levels after 2 min of isoprenaline administration and was associated with a further enhancement of IDAR. $*P < 0.05$ with respect to the phosphorylation of Ser[16] or Thr[17] at 10 beats min^{-1} (*A*) and before isoprenaline administration (*B*). †$P < 0.05$ with respect to the $t_{1/2}$ value at 10 beats min^{-1} (*A*) and before isoprenaline administration (*B*).

increase in stimulation frequency produced a similar and parallel relaxant effect when hearts were perfused in the presence and the absence of a high isoprenaline concentration, without any significant increase in the level of PLN phosphorylation above the value observed at the lowest frequency (Fig. 4). Although FDAR was associated with an increase in Thr^{17} phosphorylation in isolated myocytes, this increase occurred after the maximal FDAR was reached (Fig. 8). In contrast, the increase in the phosphorylation of the Ser^{16} residue of PLN, the first site that becomes phosphorylated upon isoprenaline administration, was temporally associated with a relaxant effect. This relaxant effect was further enhanced when phosphorylation of Thr^{17} site attained significant levels. These results clearly indicate that FDAR is not dependent on the phosphorylation of Thr^{17}, and that IDAR and FDAR do not share any common underlying mechanism. In addition, FDAR occurs in the absence of any detectable increase in SERCA2a phosphorylation (Fig. 5).

In the intact ventricle, activation of CaMKII and inhibition of PP1, the phosphatase that dephosphorylates PLN, have been shown to be necessary conditions for detection of the phosphorylation of Thr^{17} of PLN (Mundiña-Weilenmann *et al.* 1996; Vittone *et al.* 1998; Said *et al.* 2002). These prerequisites are achieved after β-adrenoceptor stimulation, in which the activation of PKA increases intracellular calcium (by phosphorylation of the Ser^{16} residue of PLN and L-type Ca^{2+} channels; Tsien *et al.* 1986; Mundiña-Weilenmann *et al.* 1996) and inhibits PP1 (Cohen & Cohen, 1989; Gupta *et al.* 2002), or by mimicking these conditions by increasing extracellular calcium in the presence of either acidic pH or okadaic acid, to inhibit PP1 (Mundiña-Weilenmann *et al.* 1996; Vittone *et al.* 1998; Said *et al.* 2002). Indeed, we have previously shown that increasing extracellular calcium, which by itself failed to increase the phosphorylation of Thr^{17}, significantly increased the phosphorylation of this residue in the presence of either high

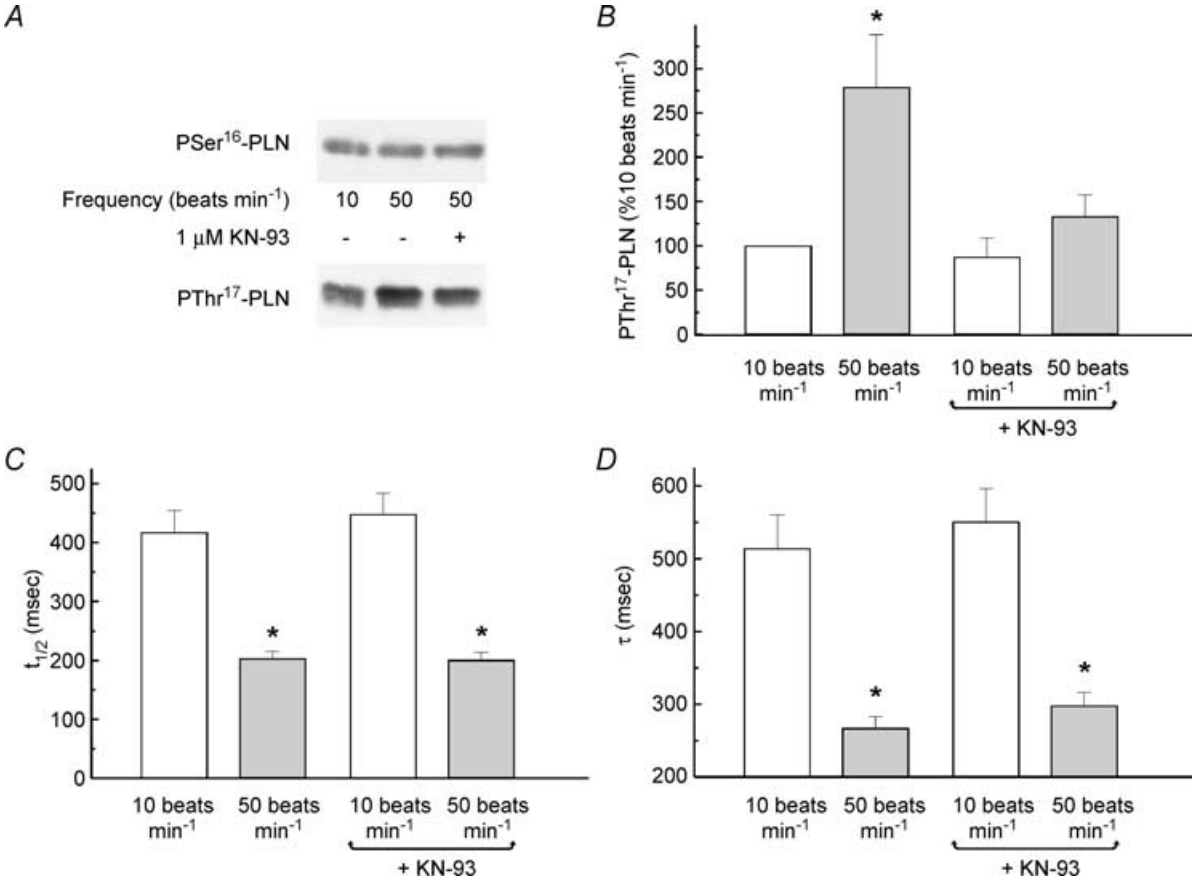

Figure 9. Effects of CaMKII inhibition on the relaxation and Thr^{17} phosphorylation induced by a frequency step from 10 to 50 beats min^{-1}

A, representative immunoblot of Ser^{16} PLN phosphorylation ($PSer^{16}$-PLN) and Thr^{17} PLN phosphorylation ($PThr^{17}$-PLN) at 10 and 50 beats min^{-1} in the absence and the presence of 1 μM KN-93 to inhibit CaMKII. Inhibition of CaMKII reduced the phosphorylation of Thr^{17} attained at 50 beats min^{-1}. *B*, bars show the overall results ($n = 6$–9 experiments). *C* and *D*, the diminished Thr^{17} phosphorylation did not occur associated with either a reduction in $t_{1/2}$ or in the time constant of the Ca^{2+} transient decline (τ). Results are expressed as means ± S.E.M. of $n = 6$–10 experiments. $^*P < 0.05$ with respect to 10 beats min^{-1}.

isoprenaline concentrations or phosphatase inhibitors (Mundiña-Weilenmann *et al.* 1996; Vittone *et al.* 1998; Said *et al.* 2002). In the present experiments, it was shown that the increase in stimulation frequency did not further increase the phosphorylation of Thr^{17}, even in the presence of a high isoprenaline concentration. These results would indicate that the increase in contraction frequency either failed to enhance CaMKII activation above that evoked by isoprenaline or produced an increase in phosphatase activity that would counteract the β-agonist-induced phosphatase inhibition. The latter possibility seems unlikely, however, since Ser^{16} phosphorylation was not decreased by the increase in stimulation frequency. In any case, our results demonstrated that the increase in frequency produced a pronounced and significant relaxant effect, under conditions in which the hearts already showed an accelerated relaxation owing to the presence of isoprenaline and in the absence of any significant change in Thr^{17} phosphorylation.

The present experiments also showed, however, that in isolated myocytes, FDAR was associated with an increase in Thr^{17} phosphorylation. This finding is in agreement with the experiments of Hagemann *et al.* (2000), which showed a good correlation between the phosphorylation of Thr^{17} and FDAR. However, a good correlation coefficient does not necessarily imply a causal relationship. We showed that the increase in PLN phosphorylation observed with FDAR, i.e. Ser^{16} and Thr^{17} sites, was significantly lower than that associated with IDAR, for the same relaxant effect. More importantly, the CaMKII-induced phosphorylation of the Thr^{17} site of PLN observed in the intact myocytes was temporally dissociated from the relaxant effect of increasing stimulation frequency. As further support for the lack of participation of Thr^{17} phosphorylation on FDAR, specific inhibition of CaMKII by KN-93 failed to affect FDAR. Thus, our results do not support the conclusion of Hagemann *et al.* (2000), in the sense that the relaxant effect of increasing stimulation frequency was determined by the degree of Thr^{17} phosphorylation.

The cause of the contradictory findings that Thr^{17} was phosphorylated by increasing stimulation frequency in isolated myocytes but not in the perfused rat heart or the papillary muscle is not apparent to us. The degree of phosphorylation of any protein depends on the balance between the activities of kinases and phosphatases involved in this particular phosphorylation. The increase in the phosphorylation of Thr^{17} of PLN in the isolated myocytes associated with FDAR, as opposed to the lack of phosphorylation in the intact heart and the papillary muscles, may indicate either a higher CaMKII activity or a lower phosphatase activity in the isolated myocytes compared with the multicellular preparations. Although we did not explore these possibilities, it is tempting to speculate that the lower temperature used in the experiments with isolated myocytes may produce a relatively higher inhibition of phosphatases than of CaMKII, leading to the necessary imbalance responsible for the increase in phosphorylation of Thr^{17} observed in isolated myocytes. Evidence suggesting a higher inactivation of phosphatases relative to kinases has been found in both intact and permeabilized rat myocytes (Mattiazzi *et al.* 1994; Bassani *et al.* 1995), working also at low temperature.

Experiments from Narayanan's group (Xu *et al.* 1993; Xu & Narayanan, 1999; 2000) were the first to describe a direct CaMKII-dependent phosphorylation of SERCA2a. This phosphorylation, occurring at Ser^{38}, resulted in activation of the V_{max} of Ca^{2+} transport (Xu *et al.* 1993; Toyofuku *et al.* 1994; Xu & Narayanan, 1999). Other results, however, argue against this finding. While Reddy *et al.* (1996) failed to demonstrate a CaMKII-dependent phosphorylation of SERCA2a, Odermatt *et al.* (1996) did show a significant increase in Ser^{38} phosphorylation that was, however, not associated with any significant change in the activity of SERCA2a. The present experiments failed to demonstrate any significant increase in SERCA2a phosphorylation from increasing stimulation frequency. A possible source of error to be considered is that the lack of SERCA2a phosphorylation with increasing stimulation frequency might arise from the fact that the phosphorylation of a single residue in a huge protein, although present, cannot be detected with the method used. However, in agreement with previous findings (Rodriguez *et al.* 2004), phosphorylation of the Ser^{38} residue of SERCA2a was also undetected when using a sensitive specific antibody raised against this site. In addition, the fact that KN-93 failed to affect FDAR both in intact myocytes and perfused hearts would further support the contention that the CaMKII-dependent phosphorylation of SERCA2a is not involved in the FDAR.

The lack of dependence of FDAR on CaMKII shown in the present results is in agreement with previous findings (Hussain *et al.* 1997; Layland & Kentish, 1999; Kassiri *et al.* 2000). In contrast, other experiments were able to demonstrate a CaMKII dependence of FDAR (Bassani *et al.* 1995; Li *et al.* 1997, 1998; De Santiago *et al.* 2002). The cause of these opposite results is not readily apparent. The different experimental protocols (steady state *versus* post-rest contractions) used in some of these previous studies (Bassani *et al.* 1995; Li *et al.* 1997, 1998) could be partly responsible for the discrepant results. This issue, however, requires further investigation.

Kimura *et al.* (1997) first proposed that elevated Ca^{2+}, by itself, enhances the PLN–SERCA2a heterodimer dissociation, thereby increasing SERCA2a activity. More recent experiments from the same group further showed that high Ca^{2+} disrupts the physical interaction between PLN and SERCA2a in HEK cells, overexpressing SERCA2a

and PLN (Asahi *et al.* 2000). Although it is difficult to explore this model *in vivo*, it is tempting to speculate that FDAR can be explained by a direct effect of high intracellular Ca^{2+} on the interaction between PLN and SERCA2a, a possibility that does not require CaMKII activation.

In any case, our results provided direct evidence indicating that the FDAR phenomenon is not dependent on the phosphorylation of any of the two main proteins involved in the SR calcium uptake. The results show in addition that IDAR and FDAR do not share a common underlying mechanism.

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