The endogenous cardiac sarcoplasmic reticulum Ca^{2+}/calmodulin-dependent kinase is activated in response to β-adrenergic stimulation and becomes Ca^{2+}-independent in intact beating hearts

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Abstract We investigated the effects of β-adrenergic stimulation on the activity of the endogenous cardiac sarcoplasmic reticulum Ca^{2+}/calmodulin-dependent protein kinase (SRCaM kinase) in Langendorff-perfused rat hearts. We found that isoproterenol induced generation of autonomous (Ca^{2+}-independent) SRCaM kinase activity to 28 ± 4.4% of the total activity. Moreover, dephosphorylation of the autonomous SRCaM kinase with protein phosphatase 2A resulted in an enzyme that was again dependent on Ca^{2+} and calmodulin for its activity. Activation of SRCaM kinase was coupled to phospholamban phosphorylation and activation of the cAMP-signaling system. Our results suggest that the cardiac SRCaM kinase is activated in response to β-adrenoceptor stimulation. This activation stimulates autophosphorylation at its regulatory domain and converts it to an active Ca^{2+}-independent species that may be the basis for potentiation of Ca^{2+} transients in the heart.

Key words: Calcium; Protein kinase; Catecholamine; Phospholamban; Sarcoplasmic reticulum; Rat heart

1. Introduction

β-Adrenergic stimulation of the heart is associated with increases in contractility and with augmentation of the rate of relaxation [1]. Phospholamban (PLB) is postulated to be the main protein involved in the transmission of cardiac β-adrenergic signaling, acting as an internal brake mechanism, allowing for rapid increases in cardiac contraction and relaxation [2]. PLB functions as a reversible inhibitor of the cardiac SR Ca^{2+} pump. Phosphorylation of PLB relieves the inhibition, resulting in an overall increase in the affinity of the Ca^{2+} pump for Ca^{2+}. During β-adrenergic stimulation, PLB is phosphorylated at Ser-16 and Thr-17 [3]. In vitro, PKA and the SR membrane-bound Ca^{2+}/CaM-dependent protein kinase (SRCaM kinase) are known to catalyze the phosphorylation of Ser-16 and Thr-17, respectively [3,4].

Although initially the cardiac SRCaM kinase was thought to be a dedicated kinase, i.e. PLB being the unique substrate, recent evidence suggests that it is CaM kinase isozyme, composed of δ-subunits [9]. CaM kinase is a ubiquitous multifunctional protein kinase controlled by a universal second messenger but relatively little is known about its specific cellular functions and activation in tissues other than brain [5]. This is true for cardiac muscle where there has been substantial speculation regarding roles for CaM kinase in controlling or modulating contractile function [6–8].

In addition to regulation of SR Ca^{2+} uptake (via PLB phosphorylation), the cardiac SRCaM kinase has been proposed to modulate calcium release from SR [10] and the L-type Ca^{2+} current [11]. Also, adenyl cyclase type III (present in heart) is inhibited by CaM kinase phosphorylation [12]. Experiments with cultured mouse myocytes, where incubation with KN-62 (a CaM kinase inhibitor) ceased the spontaneous beating which was recovered after washing with culture medium [8], support the view that SRCaM kinase plays a major role in excitation–contraction coupling.

A distinguishing feature of the cardiac SRCaM kinase is autophosphorylation which converts SRCaM kinase to an autonomous kinase [9]. It has been shown that Ca^{2+}/CaM-dependent autophosphorylation of a threonine residue within the regulatory domain (Thr-286/287 for the α/β-subunits of the neuronal CaM kinase, respectively) is responsible for the generation of autonomous activity [5] and that autophosphorylation precedes substrate phosphorylation and is required for full activation of the enzyme [13]. Studies in intact cells indicate that autophosphorylation accompanies physiological activation and autophosphorylation renders the kinase constitutively active [14,15]. Therefore, CaM kinase autophosphorylation and the generation of autonomous activity are used as a marker for CaM kinase activation in intact cells, in response to extracellular signals or during a cellular response [15–17].

We have exploited this approach to test whether β-adrenergic agonists, such as isoproterenol, which stimulate the cAMP-signaling system, are functionally coupled to activation of SRCaM kinase in intact heart. In addition, we wanted to determine whether the autoregulatory properties which have been shown to produce a Ca^{2+}-independent SRCaM kinase in vitro [9] are operational in a physiological system. We report for the first time activation of SRCaM kinase in the intact heart. Our results suggest that the cardiac SRCaM kinase autophosphorylates in a Ca^{2+}/CaM-dependent manner, becomes Ca^{2+}-independent and phosphorylates PLB at Thr-17 in response to β-adrenergic stimulation.

2. Materials and methods

2.1. Materials

Isoproterenol and secondary antibody (peroxidase-conjugated) mouse IgG were from Sigma. Secondary antibody (peroxidase-conju-

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Abbreviations: CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase II; SRCaM kinase, endogenous sarcoplasmic reticulum Ca^{2+}/calmodulin-dependent protein kinase; CaM, calmodulin; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; PLB, phospholamban; *Iso*-hearts, isoproterenol-stimulated hearts; *Iso*-SRCaM kinase, SRCaM kinase from isoproterenol- stimulated hearts; PVDF, polyvinylidene difluoride; PP, protein phosphatase

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gated) rabbit IgG was from Dianova. Biotinylated calmodulin was from Gibco-BRL, Life Technologies. Okadaic acid from Calbiochem. The Vectastain ABC kit was a product of Vector Labs (USA). Anti-phospholamban (mouse), IgG1, from Biomol. Phosphorylation site-specific antibodies PS-16 and PT-17 [18] were from Phosphoprotein research ELFORDLEA (UK). The phospholamban synthetic peptide PLB-24, calmodulin, rat forebrain CaM kinase and protein phosphatase 2A, from rabbit skeletal muscle, were as in [9]. Phosphorylase *b* and phosphorylase kinase as in [19]. All other materials as in [9].

2.2. Heart perfusions

Hearts from anesthetized (30 mg/kg sodium phenobarbital) and heparinized (500 U/kg) male Wistar rats (200–400 g body weight) were excised and cannulated for retrograde aortic perfusion with a modified Krebs-Henseleit solution containing (mM) NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, Na_2EDTA 0.05, KH_2PO_4 0.23 and glucose 11.1. The solution was saturated with 95% O_2/5% CO_2 pH 7.4. The perfusion apparatus and the corresponding software were from Hugho-Sachs Electronic (Germany). Systolic pressure was measured with a latex balloon, filled with ethanol/H_2O which was inserted into the left ventricle through the left atrium via a catheter to an Isotec transducer. The pressure in the balloon was set from 14–18 mm Hg. Signals were recorded on a Linearcorder mark 8WR3500. The recorded functional parameters were left ventricular pressure (LVP), +dp/dt, −dp/dt and coronary flow. The stimulation frequency was fixed at 340 beats/min. The perfusion was carried out at 37°C with a constant aortic pressure of 60 mm Hg. After a stabilizing period of 30 min (10 min without and 20 min with electrical stimulation), isoproterenol was added into the perfusion cannula by an infusion pump. The final concentration of the isoproterenol applied (1 μM) was calculated by the flow rate. In control hearts, isoproterenol was omitted. After 2 min perfusion, the hearts were freeze-clamped with pre-cooled (−196°C) Wollenberger clamps [20], pulverized with a pre-cooled mortar and pestle and stored under liquid nitrogen for further analysis.

2.3. Membrane vesicle preparation

Sarcoplasmic reticulum-enriched microsomal fractions were prepared according to previously published procedures [9,21] with minor modifications. Separate membrane vesicle preparations were made from each heart. All procedures were carried out at 4°C. Shortly, the powdered tissue from each heart was thawed and homogenized simultaneously in 6 vols. of sucrose 290 mM, NaN_3 3 mM, imidazole-HCl 10 mM pH 6.9, dithiothreitol 5 mM, NaF 10 mM, phenylmethylsulfonyl fluoride 0.2 mM, EDTA 2 mM, $Na_4P_2O_7$ 20 mM, leupeptin 2 mg/l, soyabean trypsin inhibitor 8 mg/l and okadaic acid 1 μM, 3× for 30 s with a Polytron (Brinkmann Instruments). The homogenate was centrifuged at 4000×*g* for 15 min. The supernatant was filtered 2× through glasswool and the filtrate was centrifuged at 14000×*g* for 15 min. The final supernatant was sedimented at 120000×*g* for 60 min. The soft pellet obtained was suspended in 650 mM KCl, 10 mM imidazole pH 6.8, 10 mM $Na_4P_2O_7$, okadaic acid 1 μM and incubated on ice for 60 min. The suspension was centrifuged first at 14000×*g* for 10 min and then at 120000×*g* for 60 min. The resulting pellet-containing sarcoplasmic reticulum vesicles was re-suspended in a small volume of 10 mM Hepes pH 7.2, 0.29 M sucrose, 0.5 mM EDTA, 0.1 M KCl, okadaic acid 1 μM and stored in liquid nitrogen.

2.4. Renaturation of SR proteins and in situ autophosphorylation

Fractionated SR membrane proteins transferred from SDS-PAGE to Immobilon membranes were denatured in situ with guanidine-HCl (7 M), renatured overnight (20 h at 4°C) in a buffer containing NP-40 (0.1% v/v) and autophosphorylated with [γ-^{32}P]ATP, 10 μCi/ml, in the presence or absence of Ca^{2+}/CaM, as described in [22]. Throughout the procedure, care should be taken that the membrane does not become dry and it is always kept in solution. Autophosphorylation was detected by autoradiography using an intensifying screen at −70°C for ≈22 h. Autoradiographs were scanned using a PDI (USA) densitometer with The Discovery Series software.

2.5. Phosphorylation of PLB-peptide following in situ renaturation

Blotted SR membrane proteins were denatured and renatured (as above) and the membrane piece corresponding to 54–52 kDa (≈0.6 cm^2) was excised and blocked for 1 h with 5% BSA in 30 mM Tris-HCl pH 7.5. Next, it was placed into a microcentrifuge tube with 40 mM Hepes buffer pH 7.3. The buffer was removed and the SRCaM kinase activity was assayed using a synthetic phospholamban-like peptide, PLB-24, as substrate (50 μM), with 70 μl of assay mixture as described in [9]. The reaction temperature was 30°C and after 15 min 60 μl of the reaction mixture were spotted onto phosphocellulose P-81 paper and the sample was processed for scintillation counting. 100% activity is defined as that determined in presence of Ca^{2+}/CaM (total activity) for SRCaM kinase from control hearts, and corresponds to 45 nmol Pi/min/mg of blotted SRCaM kinase (after in situ denaturation and renaturation). To determine the amount of SRCaM kinase present in the 54–52 kDa blot, CaM-binding assay was performed and the peak area of the signal was quantified by densitometry (O.D. relative units). The corresponding amount of protein was determined from a standard curve (μg of purified rat forebrain CaM kinase vs. O.D. relative units from CaM-binding assays).

2.6. Dephosphorylation of blotted SRCaM kinase from Iso-hearts

Blotted SR membrane proteins from isoproterenol-stimulated hearts were in situ denatured and renatured and the piece of the membrane corresponding to 54–52 kDa was excised from the lane, blocked with BSA and placed into a microcentrifuge tube with 40 mM Tris-HCl pH 8.0, 5% ethylene glycol (v/v), 1 mg/ml BSA (buffer A) and PP2A (10 U/ml). Control incubations received phosphatase dilution buffer (50 mM Tris-HCl pH 7.0, 30 mM dithiothreitol, 20 mM caffeine, 1 mg/ml BSA) instead of PP2A. Reaction time was 2 min at 30°C, the reactions were terminated with 50-fold dilution into ice-cold buffer A and the blot was washed 2× with excess of 40 mM Hepes buffer pH 7.3 and used for peptide PLB-24 phosphorylation (as above).

2.7. Immunoblotting of phospholamban

SR membrane proteins were solubilized in sample buffer [23] and boiled at 95°C for 5 min (to completely dissociate PLB into its monomers). 20 μg of SR proteins were fractionated on a urea/SDS gel system [23] electrotransferred onto PVDF membranes and probed with the general anti-PLB monoclonal antibody (IgG1) [24]. For site-specific discrimination of PLB phosphorylation, the phosphorylation site-specific antibodies PS-16 and PT-17, raised against PLB-phosphorylated peptides [18], were used (at 1 : 10000 dilution) according to manufacturer's recommendations.

2.8. Other methods

Tissue levels of cAMP were determined as in [23]. The phosphorylase *b* to *a* transformation (% of total activity) was estimated as in [25]. Activities of PKA were analyzed by a modified method of Murray et al. [26]. CaM-binding assays were performed as in [27], using 0.5 μg/ml biotinylated CaM in the presence of 1 mM $CaCl_2$. Bound CaM was visualized using Vectastain ABC reagents and the ECL detection kit. Protein concentration was determined by the Biorad protein-binding assay using γ-globulin as standard.

3. Results and discussion

The present study was undertaken as a first approach toward assessing the activation of SRCaM kinase in intact beating hearts following isoproterenol stimulation (1 μM for 2 min). In order to accurately measure the autophosphorylation state of SRCaM kinase in vivo, control and *Iso*-hearts were freeze-clamped with Wollenberger clamps and cardiac SR membrane proteins were prepared in a homogenization medium that preserves the endogenous protein-phosphorylation state [14]. Next, the technique of in situ denaturation and renaturation of blotted SR membrane proteins was employed to assess SRCaM kinase substrate phosphorylation and autophosphorylation activities. Using this technique, we circumvent potential pitfalls associated with measurement of CaM kinase activities in crude lysates: (i) contributions from overlapping (in substrate specificity) Ca^{2+}/CaM-dependent protein kinases; and (ii) artifactual activities that reflect alterations which occurred during the assaying procedure, i.e. activity modulation by cellular components (kept apart in vivo) car-

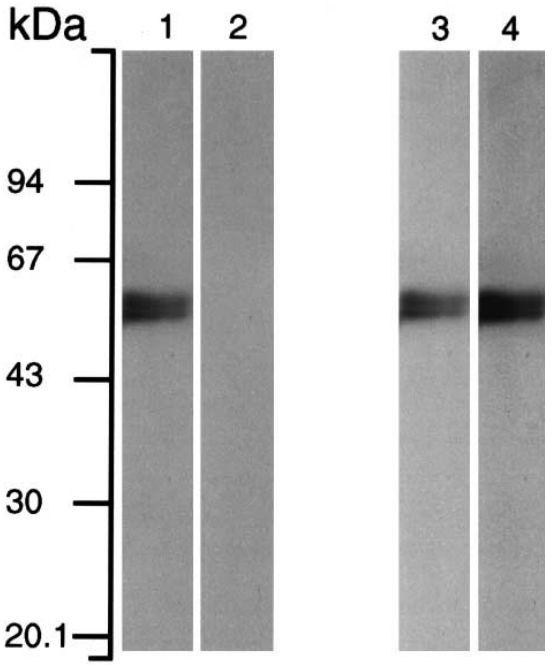

Fig. 1. In situ denaturation, renaturation and autophosphorylation of blotted cardiac SR proteins. SR membrane proteins from isoproterenol stimulated (1 μM, for 2 min) rat hearts (lanes 3 and 4) and control hearts (lanes 1 and 2) were prepared as described in Section 2. Next, they were fractionated on 10% SDS-PAGE (50 μg/lane), electrotransferred to Immobilon-P membranes and were in situ denaturated and renaturated. The blots were incubated with kinase reaction buffer with [γ-^{32}P]ATP in presence (lanes 1 and 3) or absence (lanes 2 and 4) of Ca^{2+}/calmodulin. Autophosphorylation was detected by autoradiography and peak areas were quantified (O.D.) by densitometry.

ried through the assaying procedure. The latter complication is of particular concern in cardiomyocyte lysates where intracellular compartments/structures maintain compartmentation of second messengers and responses to them [28].

As shown in Fig. 1, SRCaM kinase from stimulated hearts (lanes 3 and 4) exhibits Ca^{2+}/CaM-dependent and Ca^{2+}/CaM-independent autophosphorylation while, under similar conditions, SRCaM kinase from non-stimulated hearts (lanes 1 and 2) autophosphorylates only in a Ca^{2+}/CaM-dependent manner. Quantitation by densitometry of the level of in situ autophosphorylation on the blot indicates that isoproterenol stimulation induces: (i) decrease of the Ca^{2+}/CaM-dependent autophosphorylation activity (lane 3, 65% of lane 1, $P<0.05$, $n=3$ separate SR preparations); and (ii) generation of significant Ca^{2+}/CaM-independent autophosphorylation activity (lane 4, 210% of lane 1, $P<0.05$, $n=3$, as above). Immunoreaction of lanes 1 and 3 from Fig. 1 (control and *Iso*-treated SR membrane proteins) with the CK2-DELTA antibody, specific for δ-isoforms of CaM kinase [9,15], indicates similar SRCaM kinase protein levels for control and *Iso*-SRCaM kinase (not shown).

Next, SRCaM kinase activity was measured as incorporation of phosphate into exogenous PLB-24 peptide, a good substrate for the cardiac SRCaM kinase [4,9]. Control and isoproterenol-treated SR proteins were in situ denatured, renatured and the piece of the membrane corresponding to SRCaM kinase (54–52 kDa) was used to phosphorylate the PLB-24 peptide. In Fig. 2, *Iso*, it is shown that isoprotenol-treated SRCaM kinase has become Ca^{2+}/CaM-independent to 28 ± 4.4% of the total activity. Using syntide-2 as substrate, similar increase in autonomous activity was obtained. The slight decrease in total activity, 81.2 ± 6.2% for *Iso*-SRCaM kinase (Fig. 2), is due to thermal instability of the autophosphorylated SRCaM kinase (Dr. H. Schulman, personal communication and [9]). To provide further evidence that the Ca^{2+}-independent form of SRCaM kinase was generated by autophosphorylation rather than due to other post-translational modifications, the effect of PP2A on this enzymatic activity was examined using SRCaM kinase from stimulated hearts. We found that PP2A treatment reduced the Ca^{2+}-independent activity to 8 ± 1.8% of the total activity while the Ca^{2+}-independent activity of SRCaM kinase from control hearts was 4.5 ± 0.8%. (Fig. 2, *PP2A* and *Control*). It might be argued that incomplete dephosphorylation can be due to steric hindrance, resulting from immobilization of SRCaM kinase on the membrane. Thus, we propose that autophosphorylation is responsible for the Ca^{2+}-independent activity,

Table 1
Consequences of β-adrenergic stimulation in Langendorff-perfused rat myocardium
A

Experimental groups	cAMP (pmol/mg of protein)	Phosphorylase *a*	PKA activity (−cAMP/+cAMP)	
			Cytosolic	Particulate
Control	4.49 ± 0.49 (5)	8.03 ± 1.18 (4)	0.08 ± 0.01 (5)	0.24 ± 0.05 (5)
Isoproterenol	14.40 ± 2.71 (3)	56.80 ± 2.20 (3)	0.54 ± 0.04 (4)	0.37 ± 0.03 (4)

B

t (min)	+dp/d*t* (mm Hg/s)	−dp/d*t* (mm Hg/s)	LVP (mm Hg)	Coronary flow (ml/min)
0	1245 ± 79	943 ± 47	53.3 ± 3.3	7.6 ± 1.4
2	4615 ± 217	4119 ± 166	186.7 ± 7.6	12.4 ± 1.3

Table A: Effects of isoproterenol stimulation on the cAMP-signaling system. Rat hearts were stimulated with isoproterenol (1 μM, for 2 min) as described in Section 2. Control hearts were perfused under similar conditions in absence of isoproterenol. Hearts were freeze-clamped and the biochemical parameters were determined in control and isoproterenol-stimulated hearts. The PKA activity is expressed as the ratio of malantide phosphorylation in the presence and absence of 2.8 μM cAMP. Values represent means ± S.E.M. Numbers in parentheses indicate independent experiments (tissue sample for one experiment was from 1 heart).
Table B: Effects of isoproterenol stimulation on hemodynamics. Rat hearts were stimulated with 1 μM isoproterenol as described in Section 2. Stimulation frequency was fixed at 340 beats/min. Isoproterenol infusion (2 min) was initiated after a stabilizing period of 30 min ($t=0$ min). Inotropy and rate of relaxation were measured from the first derivatives of LVP (+dp/d*t* and −dp/d*t*, respectively). Values represent means ± S.E.M. of at least 4 hearts.

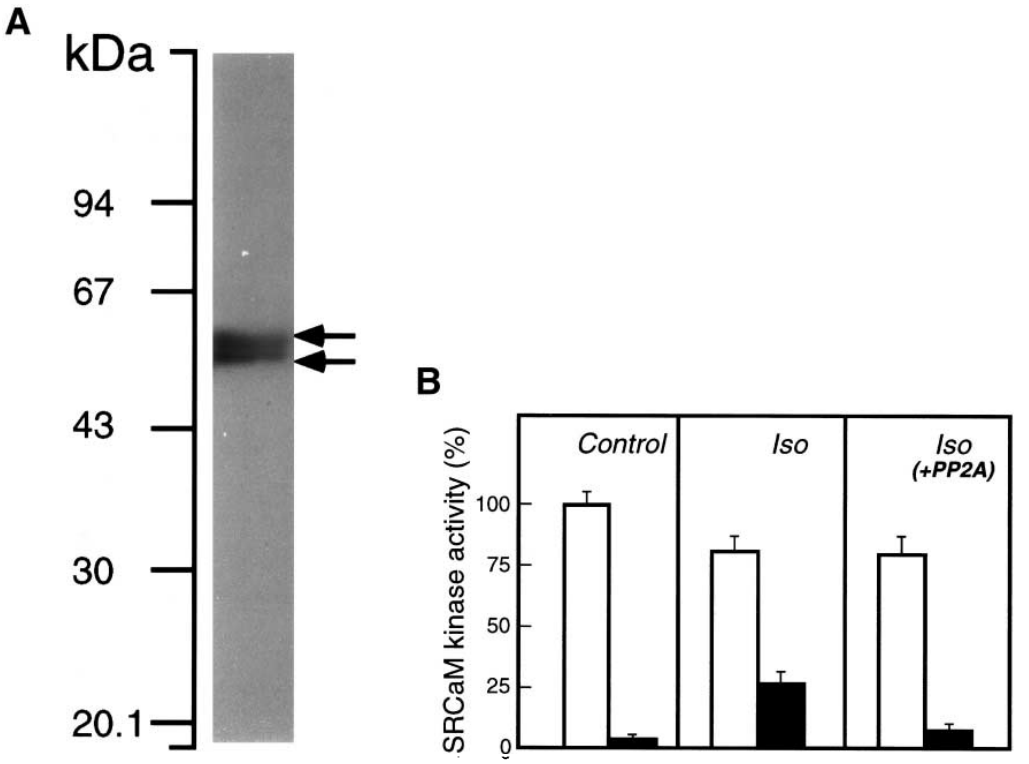

Fig. 2. Phosphorylation of the peptide PLB-24 by renaturable SRCaM kinase from control hearts, *Iso*-hearts, and by renaturable *Iso*-SRCaM kinase dephosphorylated in vitro. Following in situ denaturation and renaturation of blotted SR proteins (70 μg/lane) from control and *Iso*-hearts, the piece of the blot corresponding to the SRCaM kinase (54–52 kDa) was excised from the lane, blocked with BSA, and (1) incubated in an assay mixture with peptide PLB-24 in presence (open bars) or absence (solid bars) of Ca^{2+}/CaM or (2) the *Iso*-SRCaM kinase was treated with PP2A (10 U/ml) and sequentially assayed for peptide PLB-24 phosphorylation (*PP2A*). 100% activity is the activity in presence of Ca^{2+}/calmodulin of renaturable SRCaM kinase from control hearts as described in Section 2. Values are means ± S.D. of three determinations (representing three different hearts). Duplicate aliquots (70 μg) from control and *Iso*-hearts were analyzed by in situ renaturation and phosphorylation as in Fig. 1 to confirm the presence of SRCaM kinase in the 54–52 kDa area (left side of Fig. 2).

in particular within the regulatory domain, i.e. Thr-287, since the other putative sites for autophosphorylation (Thr-306 and Ser-315) are protected in vivo by the bound calmodulin.

In vitro, the active, autonomous SRCaM kinase (or neuronal CaM kinase) can autophosphorylate in absence of Ca^{2+}/CaM at sites that become accessible following dissociation of CaM from the holoenzyme, i.e. Ser-314/315, Thr-305/306 for α/β-subunits [9,27]. In agreement, our data suggest that the observed large Ca^{2+}/CaM-independent autophosphorylation activity (Fig. 1, lane 4) is at sites masked by CaM in intact heart and the limited Ca^{2+}/CaM-dependent autophosphorylation activity (Fig. 1, lane 3) mostly reflects the pool of SRCaM kinase molecules which were not autophosphorylated in vivo at the autonomous site.

Immunoblots of SR membrane proteins from control and stimulated hearts with the antibodies PS-16 or PT-17 (discriminating between PLB phosphorylated at Ser-16 or Thr-17, respectively) showed that PLB from *Iso*-hearts was phosphorylated at Thr-17 and Ser-16 (Fig. 3, lanes 1 and 2, respectively). Under similar exposure times, PLB from control hearts did not react with the antibodies PS-16 and PT-17 (not shown). Phosphorylation of PLB at Ser-16 has been postulated to be mediated by PKA, reflecting activation of the cAMP-signaling system in response to β-agonist stimulation [3,23]. In agreement, we found that in *Iso*-hearts: cAMP levels and PKA activities (cytosolic and particulate) were elevated (Table 1A). Also, the phosphorylase *b* to *a* conversion was increased (Table 1A), reflecting acceleration of glycogenolysis in response to higher energy demands due to enhanced contractility (Table 1B).

The protein kinase, which is known to catalyze in vitro phosphorylation of the Thr-17 site of PLB, is CaM kinase [4,9]. The cytosolic cardiac CaM kinase could not be the physiologically relevant kinase for the Thr-17 site of PLB since (i) it has K_m for PLB in the micromolar range [29], (ii) kinase and substrate exist in the heart in submicromolar amounts, implying that V_{max} conditions would not exist in the intact cell, unless we assume localization of these two proteins in the same subcellular compartment, e.g. sarcoplasmic reticulum. Therefore, we propose that phosphorylation of PLB at Thr-17 is mediated mostly by SRCaM kinase. Activation of SRCaM kinase reflects increase in intracellular Ca^{2+} levels. The nature of calcium that activates CaM kinase in intact cells can be either from influx through the L-type Ca^{2+} channel [16,30] or from intracellular Ca^{2+} pools [15,31]. Stimulation of β-adrenoceptors leads to an enhanced calcium influx which is the result of PKA-mediated phosphorylation of the L-type Ca^{2+} channel [32]. Experiments with verapamil, a Ca^{2+} channel blocker, showed that verapamil treatment of isoproterenol-stimulated cardiac myocytes resulted in large reduction of PLB phosphorylation levels [33]. This indicates that Ca^{2+} influx through the L-type Ca^{2+} channel is required for activation of the cardiac SRCaM kinase. Interestingly, Thr-17 PLB phosphorylation in the intact heart requires elevated tissue cAMP levels; conditions that increase Ca^{2+} but do not augment cAMP fail to produce PLB phos-

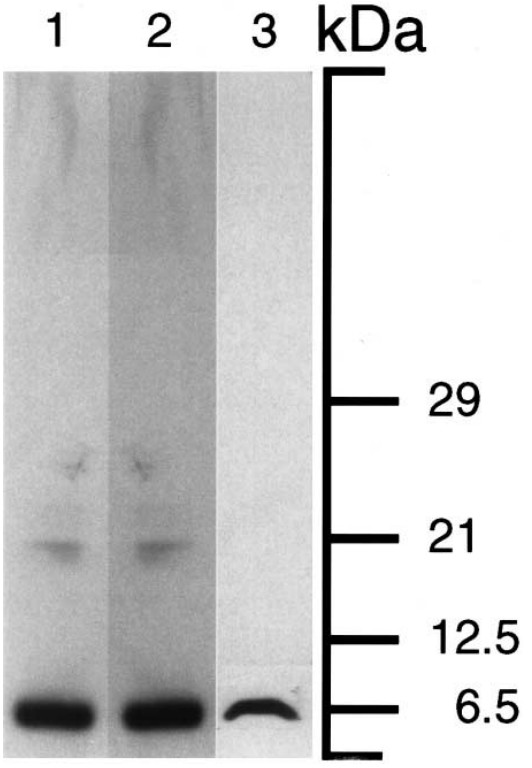

Fig. 3. Immunoblotting of cardiac SR proteins from *Iso*-stimulated hearts with phosphorylation site-specific antibodies against PLB phosphorylated at Ser-16 or Thr-17. SR membrane proteins from *Iso*-hearts (20 μg/lane) were separated on an urea/SDS gel system, electrotransferred to membranes, and probed for immunoreaction with the antibodies PT-17 (lane 1) or PS-16 (lane 2), discriminating between PLB phosphorylated at Ser-16 or Thr-17, respectively. Lane 3 was probed with the general anti-PLB monoclonal antibody (not discriminating between phospho and dephospho-PLB) to indicate the position of the PLB monomer.

phorylation at Thr-17 [3,7]. Several studies support the view that in intact heart the PPs are active. Incubation of ventricular cardiomyocytes with okadaic acid (a PP1 and PP2A inhibitor) increased the phosphorylation of PLB and troponin I [33,34] while termination of isoproterenol infusion of intact hearts results in dephosphorylation of PLB and troponin I [35]. Omitting PP inhibitors from the media during cardiac SR vesicle preparation greatly reduced the levels of autonomous SRCaM kinase activity and phosphate incorporation into Thr-17 of PLB (personal observation). It has been shown that the major cardiac SR-PP (70%) is PP1 type [36]. Stimulation of intact hearts with isoproterenol increased inhibitor-1 activity (inhibitor of PP1) and reduced drastically SR PP1 activity [37]. It is known that PP1 dephosphorylates and deactivates the autonomous CaM kinase [38]. Thus, increase in Ca^{2+} alone would not result in activation of SRCaM kinase and PLB phosphorylation since the SR-PP1 is active in the intact heart and dephosphorylates the kinase. Therefore, the β-adrenoceptor-induced increase in cAMP, which probably leads to PKA-mediated inhibition of the SR-PP1, is required for the activation of SRCaM kinase to become manifest as PLB phosphorylation at Thr-17. Talosi et al. found that, after termination of *Iso* infusion of intact hearts, PLB was dephosphorylated but the rate of Ser-16 dephosphorylation (which correlated with decreases in cAMP levels) was faster than the Thr-17 dephosphorylation [35] although the PLB-PP dephosphorylates with the same efficiency phospho-Ser or phospho-Thr in PLB. These results comply with our data: the finding that Thr-17 remains phosphorylated for a longer period reflects SRCaM kinase phosphorylation in the autonomous mode and supports a role for SRCaM kinase autophosphorylation in short-term potentiation of the Ca^{2+} signal in the heart.

PP2A and PP2C (a Mg^{2+}-dependent PP) are the other two SR-PPs (30%) [36]. Both PP2A and PP2C in vitro dephosphorylate and deactivate the autophosphorylated SRCaM kinase/CaM kinase [9,39]. Since okadaic acid alone does not completely inhibit the dephosphorylation of SRCaM kinase (personal observation) and additional PP inhibitors are required, we propose that all three PPs are involved in the intact heart. Further studies are required to examine the particular contributions of the SR PP2A and PP2C (in addition to PP1) in the reversal of the cellular functions induced by the autophosphorylation of SRCaM kinase as well as how the synergistic actions of the two second messengers (cAMP and Ca^{2+}) result in inhibition of SR-PPs during β-adrenergic signaling.

In conclusion, our study identifies the cardiac SRCaM kinase as one of the targets of β-adrenergic stimulation. We propose that the mechanism of activation of the cardiac SRCaM kinase in response to β-adrenergic stimulation involves Ca^{2+} influx through the L-type Ca^{2+} channel and concomitant inhibition of the SR-PP1 activity (by cAMP-dependent mechanisms) that results in SRCaM kinase activation to become evident. The activated SRCaM kinase autophosphorylates within the regulatory domain and becomes Ca^{2+}-independent. One manifestation of this activation is phosphorylation of PLB at Thr-17 which contributes to further increase in myocardial relaxation.

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