Long-term Beta Adrenoceptor-Mediated Alteration in Contractility and Expression of Phospholamban and Sarcoplasmic Reticulum Ca$$^{++}$$-ATPase in Mammalian Ventricle

ABSTRACT

We studied the influence of prolonged administration of the beta adrenoceptor agonist isoproterenol on contractile parameters and expression of sarcoplasmic reticulum (SR) Ca$$^{++}$$-ATPase and phosphorylamban, genes important for Ca$$^{++}$$ uptake into the SR. Iso isoproterenol (Iso), 0.9% NaCl (Ctrl), propranolol (Prop) or Iso plus Prop were administered to rats by subcutaneous infusion with osmotic minipumps for 1, 2, 3, 4, 8, 13 and 26 days, respectively. The positive inotropic effect of Iso was impaired in rats pretreated with Iso in vivo. Iso pretreatment shortened time to peak tension (TPT) by 28%, time of relaxation (RT) by 27% and total contraction time (TCT) by 27% compared to the appropriate controls (day 2). The shortening of time-dependent contractile indices started after 1 day of Iso pre-treatment, reached a maximum after 2 days and remained reduced for 4 days. Longer treatment by Iso failed to affect time parameters, whereas the positive inotropic effect of Iso added to the isolated muscles persisted. The shorted contractile time parameters were accompanied by diminished mRNA and protein expression of phosphorylamban (PLB) and SR-Ca$$^{++}$$-ATPase (SERCA). The mRNA levels for PLB and SERCA were maximally reduced by 31 ± 1.3% and 41 ± 1.4% in the Iso-pretreated group (2 days) respectively. The reduced mRNA levels were accompanied by reduced levels of the corresponding proteins. It is concluded that altered levels of PLB and SERCA probably account for the noted changes in contractile time parameters in the mammalian heart.

Cardiac hypertrophy is an important step leading to heart failure in patients. Heart failure is accompanied by prolonged relaxation, which is deemed detrimental to cardiac performance. Relaxation is mediated by the removal of Ca$$^{++}$$ from the sarcoplasm through the combined action of Na$$^{+}$$/Ca$$^{++}$$ exchanger in the sarcolemma and SERCA 2a. The physiological inhibitor of SERCA is PLB, an intrinsic protein of the SR. PLB lowers the affinity of SERCA for Ca$$^{++}$$ at low Ca$$^{++}$$ concentrations (Odermatt et al., 1996). Indeed, overexpression of PLB prolongs the duration of contraction in the heart of transgenic mice (Kadambi et al., 1996) and ablation of the PLB gene shortens contraction duration (Luo et al., 1994). To elucidate a possible causal relationship between hypertrophy and altered relaxation several animal models have been used. For instance, pressure overload or thyroid hormone induced hypertrophy, changed relaxation and altered expression of SR proteins (Lompre et al., 1989; Nagai et al., 1989; De la Bastie et al., 1990; Kiss et al., 1994; Matsui et al., 1995).

We focused on changes in cardiac function after stimulation of beta adrenoceptors in an in vivo model (Mende et al., 1992). Chronic beta adrenoceptor stimulation leads to cardiac hypertrophy in this model. This is accompanied by biochemical and mechanical alterations. The density of beta-1 and beta-2 adrenoceptors was reduced, the expression of the alpha subunit of inhibitory G-proteins was increased and the positive inotropic effect of beta adrenoceptor stimulation was attenuated (Chang et al., 1982; Eschenhagen et al., 1992).

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ABBREVIATIONS: Iso, (-)-isoproterenol; TCT, total contraction time; TPT, time to peak tension; RT, time of relaxation; PLB, phosphorylamban, Prop, propranolol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis, SERCA, sarcoplasmic reticulum Ca$$^{++}$$-ATPase.
These alterations were in part reversible by beta adrenoceptor blockade (Eschenhagen et al., 1992). One could hypothesize that this model might mimic important alterations that occur in progressive human heart failure, which is characterized by hypertrophy and subsequent decline in cardiac function leading ultimately to terminal heart failure accompanied by increasing adrenergic stimulation. There is strong negative correlation between catecholamine levels and life expectancy.

Moreover, we have shown that after chronic beta adrenoceptor stimulation, the relaxation of papillary muscles was enhanced and the expression of PLB and SERCA on protein levels was reduced (Stein et al., 1996). This was in part compensated by reduced phosphorylation of PLB (Stein et al., 1996). Moreover, the expression of mRNA for SERCA was accompanied by changes in Ca transient or persistent? Is it preceded by alterations in SERCA and/or PLB expression?

Thus, we addressed the following questions. Is the enhanced relaxation in hypertrophy due to chronic beta adrenoceptor stimulation transient or persistent? Is it preceded or followed by alterations in SERCA and/or PLB expression? Are alterations in SERCA and PLB expression accompanied by changes in Ca+ uptake? What is the temporal relationship between mRNA and protein levels of PLB and SERCA?

Materials and Methods

Animals. Male Wistar rats (270–300 g) were treated with subcutaneous infusions by osmotic minipumps (Alzet osmotic pump type ML2; Alza, Palo Alto, CA) as described before (Eschenhagen et al., 1992; Mende et al., 1992). Minipumps were implanted subcutaneously in the neck of the animals under short-term ether anesthesia. Mean rate of infusion was 5 μl/hr. Rats were treated 1, 2, 3, 4, 8, 13 or 26 days with either 0.9% NaCl as control, (±)-Iso HCl alone (2.4 mg/kg/day dissolved in 0.002 M HCl; Boehringer-Ingelheim, Ingelheim, Germany), (±)-Prop HCl alone (29.7 mg/kg/day; Sigma, St. Louis, MO) or a combination (Iso and Prop). The animals had free access to food and tap water. For the 26 days' treatment, minipumps were removed after 13 days under short-term ether anesthesia, and a new freshly filled pump was implanted. Because drug delivery was stable for 14 days, pumps were reimplanted into another rat when the total time of drug delivery was <13 days. The rats were killed by a blow to the neck and bleeding from the carotid arteries. Hearts were rapidly removed and exsanguinated in ice-cold 0.9% NaCl. Papillary muscles were used for contraction experiments, and ventricles were frozen immediately in liquid nitrogen for further analysis.

Contraction experiments. The experiments were performed on electrically driven (frequency, 1 Hz; duration, 5 msec; intensity, 20% greater than threshold) papillary muscles from the left ventricles of the pretreated rats (Eschenhagen et al., 1992; Mende et al., 1992). The preparations were isolated, mounted and suspended individually in glass tissue chambers for recording isometric contractions.

The bathing solution (10 ml) was a modified Tyrode's solution containing (mM) NaCl 119.8, KCl 5.4, CaCl2 1.8, MgCl2 1.05, Na2HPO4 0.42, NaHCO3 22.6, Na2EDTA 0.05, ascorbic acid 0.28 and glucose 5.0. It was continuously gassed with 95% O2 and 5% CO2 and maintained at 35°C. Force of contraction was measured with an inductive force transducer. Concentration-response curves were obtained cumulatively and expressed as increases in force of contraction (mN).

Immunohistochemical determinations. Frozen cross sections (5 μm) of each heart were mounted on Silan-coated glass slides and fixed in 4°C cold acetone for 90 sec. For histological examination, the slides were stained with hematoxylin and eosin; for immunohistochemical examination, the slides were incubated with the monoclonal antibody A1 against PLB (Drago and Colyer, 1994) in a dilution of 1:200 in 0.6% bovine serum albumin, followed by a rabbit anti-mouse bridging antibody (1:30 in phosphate-buffered solution; 30 min at room temperature; Dako Diagnostika, Hamburg, Germany) and a polyclonal mouse APAAP complex (1:100 in RPMI; 60 min at room temperature; Dianova, Hamburg, Germany). The enzyme reaction was developed for 25 min at room temperature in a freshly prepared new fuchsin solution containing naphthol-bisphosphate and levamisole. Finally, the sections were counterstained with hematoxylin and mounted with Kayser's glycerin gelatin. All slides were stained in one preparation. Omission of the primary antibody served as negative control.

Ca+ uptake measurement. Frozen hearts were homogenized in 250 mM sucrose, 10 μM cantharidin and 30 mM histidine (pH 7.0). Cantharidin was added to inhibit protein phosphatases. Cantharidin (10 μM) inhibits completely type 1 and type 2A phosphatases (Neumann et al., 1995). Ca+ uptake in homogenates was measured by the microfiltration technique (Martonosi and Feretos, 1964). The reaction buffer contained 50 mM MOPS (N-morpholino)propanesulfonic acid; pH 7.0), 3 mM MgCl2, 100 mM KCl, 5 mM NaF, 10 mM potassium oxalate, 0.5 mM EGTA, 10 μM cantharidin and different CaCl2 concentrations to give pCa values of 7.49. Free calcium concentrations were calculated by the method of Bers (1994). Ca+ uptake was measured by preincubation of homogenates with antibody for 20 min on ice. Ca+ uptake could be stimulated by preincubation of homogenates with an anti-PLB antibody (2D12) for 20 min on ice. Ca+ uptake was initiated by the addition of 3 mM ATP and then performed at 37°C. Aliquots of 100 μl were filtered at various time points on 0.22-μm filters (GS type, Millipore) and washed twice with 5 ml of 150 mM NaCl. Quantification of 45Ca+ was measured by scintillation counting.

Total RNA preparation. Total RNA was isolated from ventricular tissue (frozen immediately after death) according to the protocol of Chomczynski and Sacchi (1987). RNA concentration was determined by absorbance at 260 nm in triplicate. RNA was denatured with 42% formamide and 5.8% formaldehyde at 95°C for 2 min and size-fractionated by electrophoresis in 1% agarose gels containing 0.5 μg/ml ethidium bromide (Fluka Chemie, Buchs, Switzerland). RNA was transferred to Hybond N nylon membranes overnight (Amer sham, Braunschweig, Germany) by Northern blot capillary transfer using 20× SSC (3 M NaCl and 0.3 M sodium citrate, pH 7.0) as the transfer medium. Transfer was controlled on an ultraviolet transilluminator.

cDNA probes. The plasmids (pBS) with cDNA inserts for rat SERCA 2a and PLB were kindly provided from Dr. K. R. Boheler (Lompre et al., 1989). Plasmid (pGEM-2) with cDNA insert for rat Gαs was a kind gift from Dr. R. R. Reed. The plasmids were transformed into Escherichia coli, and positive clones were picked and grown in rich medium. Plasmids were obtained by large-scale preparation. Inserts were isolated by digestion with EcoRI for SERCA 2a, PLB and Gαs. The cDNA inserts were purified from 1% agarose gels. Sizes were ~2000 bp for SERCA 2a, ~1100 bp for PLB and ~1100 bp for Gαs. The cDNA probes were labeled (Meg-Prime kit; Amersham Buchler, Braunschweig, Germany) with [32P]dCTP (3.000 Ci/mmol, New England Nuclear-Dupont, Bad Homburg, Germany) to a specific activity of 3.5 to 8.0×106 dpm/μg. Unbound
radioactivity was separated by gel filtration with Sephadex G-50 DNA grade (Pharmacia Fine Chemicals, Uppsala, Sweden).

**Hybridization procedure.** Hybond N nylon membranes were prehybridized for 2 to 6 hr at 42°C in a prehybridization solution containing 50% formamide, 5× Denhardt’s solution (1 mg/ml Ficoll, polyvinylpyrrolidone and bovine serum albumin), 0.9 M NaCl, 0.06 M NaH₂PO₄, 0.006 M EDTA, 0.1% SDS and 400 μg/ml tRNA from yeast. The 3²P-labeled probes were added to the prehybridization solution in a concentration of 0.5 to 1× ¹⁰⁶ dpm/ml. Hybridization of the membrane was performed at 42°C for 16 to 20 hr. The membranes were washed twice in 2× SSC, 0.1% SDS at room temperature followed by 15 min washing in 2× SSC, 0.1% SDS at 65°C. The blots were washed three times to a final stringency in 0.2× SSC, 0.1% SDS at 65°C. Wet blots were sealed in plastic wrap and exposed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for 24 hr, followed by a subsequent exposure to X-ray films (X-OMAT AR; Kodak) for 2 to 5 days at −80°C by using intensifier screens. For further hybridizations with other radiolabeled probes, the blot membrane was washed with boiling in 0.1% SDS. After a control exposure in the PhosphorImager to assess loss of label, membranes were then used for subsequent hybridizations.

**Quantification of specific mRNA.** Hybridization intensity of autoradiographic signals on Northern blots were measured quantitatively by two-dimensional densitometry (ImageQuant; Molecular Dynamics). Then, 20 μg total RNA was used for quantitative analysis. Northern blots were hybridized subsequently against SERCA 2α, PLB and Gₛα₁,₆ as described above. Gₛα₁,₆ was used to correct the amount of total RNA bound to the membrane (Eschenhagen et al., 1992). Furthermore, an external standard (microscales; Amer sham) was used to normalize methodical variation. Total RNA of several rat hearts was isolated, and 20 μg was used for each Northern blot as an internal standard. The final result was obtained by normalizing to Gₛα₁,₆, external standard and internal standard.

**Preparation of homogenate.** Next, 50 mg of powdered heart tissue were homogenized at 4°C three times for 30 sec each with a Polytron PT-10 (Kinematica, Luzern, Switzerland) in 300 μl of 10 mM NaHCO₃. Then, 600 μl of 20% SDS was added (Movsesian et al., 1994; Linck et al., 1996). Mixtures were incubated at 25°C for 20 min before centrifugation. Supernatants were collected and assayed for protein, according to the method of Lowry (1951) after trichloroacetic acid precipitation.

**SDS-PAGE and autoradiography.** SDS extracts made as described above were thawed, and additional SDS buffer made according to Laemmli (1970) was added. Samples were heat-treated for 10 min at 95°C to convert the high-molecular-weight form of PLB into the low-molecular-weight form. Next, 50 μg of homogenate sample protein were loaded per lane. These amounts were in the linear range for PLB. A similar linearity was obtained for SERCA in homogenates (data not shown) as has been published before (Linck et al., 1996).

Gels were run according to Lindemann and Watanabe (1985) using 10% acrylamide separating gels. Gel after electrophoresis, separated proteins were transferred to nitrocellulose membranes as described (Neumann et al., 1991). Nitrocellulose sheets were incubated with monoclonal antibody A1 raised against PLB (Drago and Colyer, 1994) and monoclonal antibody AB 465 to SERCA (MacDougall et al., 1991). Protein binding antibodies were visualized using [¹²⁵I]protein A. Radioactive bands identified by autoradiography were excised from the nitrocellulose sheet, and bound radioactivity was quantified by γ counting. Background counts, which were <15% of total counts for each band, were subtracted from all measurements.

Plasma catecholamine and Iso levels were determined using high-performance liquid chromatography with electrochemical detection. Blood was collected from the carotid arteries and centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was stored at −80°C until use. Then, 200 μl of serum and 100 μl of 10 μg/ml dihydroxybenzylamine as internal standard were transferred into sample car-
(table 1). The first new finding of the present work is the transient alteration in time parameters. TCT (fig. 2A), TPT (fig. 2B) and RT (fig. 2C) were shortened after Iso pretreatment for 1, 2, 3 and 4 days in comparison with NaCl-pretreated animals. Time parameters were maximally reduced after 2 days of Iso pretreatment with a reduction of TCT from 136 ± 1.7 to 99 ± 3.4 msec (27%), of TPT from 57 ± 1.2 to 41 ± 1.1 msec (28%) and of RT from 79 ± 1.5 to 58 ± 2.8 msec (27%). In contrast to the persistently bluntened inotropy, the effect on time parameter was thus transient. The Iso-induced reduction of contraction time parameters was blocked in the presence of Prop, demonstrating beta adrenergic-induced effects (day 2, table 1).

Next, we studied whether Ca++ uptake was altered. The Ca++ uptake was time and Ca++ dependent (data not shown). At linear conditions (pCa 7.5), the maximum rate of uptake amounted to 0.44 ± 0.10 and 0.98 ± 0.14 (nmol/mg of protein/5 min) in NaCl- and Iso-treated (2 days) ventricles, respectively (fig. 3). This corresponds to an increase by 123% in the Iso-pretreated rats vs. control (P < 0.05, n = 6).

To test the hypothesis that alterations in SERCA and PLB, proteins important for Ca++ uptake into the SR, are responsible for the altered contraction time parameters, we determined the expression on both mRNA and protein levels. A typical Northern blot is seen in figure 4. Iso pretreatment induced a reduction in SERCA mRNA level (figs. 4 and 5A) at days 1 and 2 of Iso pretreatment by 35% and 41% in comparison to NaCl pretreatment, respectively. The maximal decrease occurred after 2 days of Iso pretreatment and the effect vanished after 3 days of treatment and longer. Iso pretreatment for 2 days decreased SERCA mRNA level from 1.1 ± 0.06 (NaCl) density units to 0.64 ± 0.05 (Iso) density units (by 41%). When Iso and Prop were simultaneously applied, SERCA mRNA level amounted to 1.0 ± 0.04 (n = 5). Thus, the reduction of SERCA mRNA levels were antagonized by simultaneously administered Prop. Prop alone had no effect on SERCA mRNA levels (table 1). Iso pretreatment induced a reduction in SERCA protein level from day 1 to day 4 compared with NaCl pretreatment (figs. 4 and 5B). The protein decreased by 19% after 1 day, by 21% after 2 days, by 19% after 3 days and by 24% after 4 days of Iso pretreatment. The Iso-induced effect vanished after treatment of ≥8 days. The effect was blocked by simultaneously administered Prop, whereas Prop alone did not affect protein expression (table 1).

SERCA-mediated Ca++ uptake into the SR is regulated by PLB. Therefore, we determined also the PLB mRNA and protein expression. The hybridization with the PLB probe revealed two mRNAs for PLB at 3.3 and 1.3 kb, although both mRNAs encode for the same protein (fig. 4). The different mRNAs for the same protein could be caused by distinct polyadenylation sites (Toyofuku and Zak, 1991). These polyadenylation sites might be important as a protection against endonucleases. Thus, it was conceivable that the PLB mRNA expression were differently regulated between both mRNAs and therefore resulted in differential alterations. Hence, we quantified both PLB mRNAs either together or each band separately. However, no differences were apparent (data not shown) and the sum of both transcripts is plotted in figure 6A. The PLB mRNA levels were reduced in 1- and 2-day Iso-pretreated rats. The mRNA levels decreased from 1.88 ± 0.10 to 1.33 ± 0.15 (29%) density units after 1 day and from 1.99 ± 0.19 to 1.38 ± 0.06 (31%) density units after 2 days, respectively. A longer Iso pretreatment of 3 days and more was not accompanied by altered PLB mRNA expression. Moreover, a 2-day pretreatment with Iso plus Prop abolished the Iso-induced effect (table 1). Furthermore, the corresponding PLB protein levels were decreased after 1 to 4 days of Iso pretreatment in comparison with NaCl pretreatment (fig. 6B). The decrease started after 1 day reached a maximum after 2 days and remained reduced for 4 days. PLB protein level was maximally reduced from 8839 ± 312 to 5182 ± 367 cpm (41%) after 2 days of Iso pretreatment in comparison to NaCl-pretreated rats. The Iso effect on PLB protein levels

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<th>Parameter</th>
<th>NaCl</th>
<th>Iso</th>
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<td>TPT (msec)</td>
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Differences between treatment groups were tested by analysis of variance followed by Bonferroni's t test. * P < .05 vs. NaCl.
was antagonized by simultaneously administered Prop (table 1).

Furthermore, the tissue distribution of PLB was investigated immunohistochemically (fig. 7). The control hearts (A) showed a strong specific cytoplasmic staining for PLB in cardiomyocytes as well as in smooth muscle cells of the media of coronary arteries, whereas the interstitial cells were negative. This is consistent with the muscle-specific expression of PLB and supports the specificity of the antibody used in the present study. Moreover, the Iso-pretreated rat hearts (B) exhibited a weaker cytoplasmic staining for PLB in cardiomyocytes compared with control (A), whereas the smooth muscle cells of the arteries revealed the same staining intensity regardless of treatment. This suggests that the regulation of PLB expression occurs mainly in cardiomyocytes. Sections from Iso-pretreated hearts displayed interstitial edema.

Fig. 2. Effect of Iso pretreatment (●) in comparison of NaCl pretreatment (○) on contraction time parameter in isolated electrically driven papillary muscles. A, Total contraction time. B, Time to peak tension. C, Time of relaxation. Abscissae, duration of treatment (0–26 days). Ordinates, time parameter in msec. *p < 0.05 vs. control (0). Crosses depict significant differences vs. NaCl pretreatment.

Fig. 3. Effect of Iso pretreatment (2 days; filled bars) in comparison with NaCl pretreatment (2 days; open bars) on Ca\(^{++}\) uptake by SR vesicles isolated from the pretreated hearts. Ca\(^{++}\) uptake was determined in the absence and presence of the anti-PLB antibody (2D12). Numbers are indicated in brackets. *p < 0.05 vs. NaCl pretreatment.

Fig. 4. Detection of PLB and SERCA mRNA (Northern blot, left) in cardiac extracts from NaCl pretreated (2 days; lane 1) or Iso pretreated (2 days, lane 2) rats. Membranes were hybridized with \(^{32}\)P-labeled specific rat probes for PLB or SERCA. Radioactivity bound to membranes was identified after exposure to PhosphorImager screens. The autoradiograms are depicted. Both 18S and 28S ribosomal RNA are labeled. Detection of PLB and SERCA protein levels (Western blot, right) in cardiac homogenates from NaCl pretreated (2 days; lane 1) or Iso pretreated (2 days; lane 2) rats. Homogenates were subjected to electrophoresis and electrophoresis transferred to nitrocellulose sheets. Nitrocellulose sheets were cut into two strips to separate high- and low-molecular-weight proteins. The strips were incubated with antibodies against PLB or SERCA. Protein bound to strips was identified by incubation with a second radioactive antibody and exposure to PhosphorImager screens. The autoradiograms are depicted. Molecular weights are indicated.

Discussion

In the present study, we demonstrate that chronic beta adrenoceptor stimulation in vivo leads to persistent hypertrophy but to transiently enhanced relaxation in the mammalian heart. We studied the altered relaxation in ventricular preparations (fig. 2) but similarly shortened relaxation was also noted in electrically driven left atria (2 days, data not shown). It was important to show that hastened relaxation was accompanied by enhanced Ca\(^{++}\) uptake in this
model. This supports the hypothesis that alterations in SERCA and/or PLB may underlie the altered relaxation observed.

**Alterations in SERCA and PLB levels in various model systems.** Alterations of SERCA and PLB expression have been noted in postnatal development, with drug treatment and mechanical intervention. The expression of only one protein or both was altered in previous publications. In postnatal development, only SERCA was increased but PLB remained unchanged (for review see Lompre et al., 1994). A discoordinate regulation has been shown for hormonal stimulation. For instance, thyroid hormone treatment led to a decrease of PLB (mRNA and protein) and an increase in SERCA (mRNA and protein) expression. Concomitantly, the Ca$^{2+}$ uptake was increased and time of relaxation was shortened (Rohrer and Dillmann, 1988; Nagai et al., 1989; Arai et al., 1991; Kiss et al., 1994; for extensive review, Lompre et al., 1994). Drug-induced hypothyroidism increased the ratio of PLB to SERCA (mRNA and protein level), decreased Ca$^{2+}$ uptake and prolonged relaxation (Nagai et al., 1989; Kiss et al., 1994). These studies tend to indicate that the ratio of PLB to SERCA is inversely related to Ca$^{2+}$ uptake by the SR, regardless which intervention and species are studied. Moreover, we have presented evidence that PLB is dephosphorylated after chronic beta adrenergic stimulation (Stein et al., 1996). This would at least in part compensate for the reduced level of PLB because only unphosphorylated PLB inhibits SERCA activity. Finally, there are several examples for coordinate reduction of PLB and SERCA levels. Cardiac hypertrophy due to pressure overload (pulmonary artery banding) was accompanied by reduced levels of PLB (mRNA) and SERCA (mRNA and protein) and reduced Ca$^{2+}$ uptake (Matsui et al., 1995). PLB was not measured on protein level, and therefore the ratio of PLB to SERCA was not given. In another model of pressure hypertrophy (aortic banding leading to pulmonary congestion), both PLB and SERCA were reduced at the protein level. The relaxation was impaired, Ca$^{2+}$ uptake was reduced, and the ratio of PLB to SERCA was diminished (Kiss et al., 1995). Of note, in a subgroup of animals with cardiac hypertrophy without heart failure, no changes in PLB or SERCA were noted. Thus, the findings by Kiss et al. (1995) obtained in a completely different model are in agreement with the present finding.

**Time course studies.** To address the question of whether chronic beta adrenergic stimulation leads to transient or persistent alterations in relaxation and PLB and/or SERCA gene expression, a complete time course of beta adrenergic stimulation has been studied in the present work. Gene ex-
pression in eukaryotes is usually primarily regulated at the transcriptional level. However, there are a number of exceptions (Kozak, 1991), even for PLB and SERCA. A physiological intervention (electrically stimulation of fast twitch skeletal muscle) led to enhanced PLB and SERCA gene expression (Hu et al., 1995). However, transcription rates were not directly measured by Hu et al. (1995). Moreover, SERCA mRNA alterations are not always faithfully followed by SERCA protein levels. After unloading of rat soleus muscle SERCA mRNA increased, whereas SERCA protein was unchanged indicating that SERCA can be under translational control (Schulte et al., 1993). The parallel decrease in mRNA and protein levels for SERCA and PLB (day 1 and 2) in the present study might suggest transcriptional control. The persistent decline in protein levels for SERCA and PLB at times (3 and 4 days) where mRNA for SERCA has reached control values can have various reasons. Based on work in skeletal muscle, one might speculate on a nontranscriptional control. The quotient of PLB to SERCA mRNA stayed constant throughout the time investigated, indicating a parallel regulation of these levels. The easiest explanation would be a parallel change in the transcriptional activity of these genes. Promoter analysis will be required to more rigorously address these questions.

Moreover, it can be asked whether isoprenaline is acting directly on the heart or by means of mediators. Preliminary data indicate a reduction of mRNA levels for PLB and SERCA in forskolin-stimulated rat neonatal cardiomyocytes (J. Neumann, I. Gombosova, B. Linck, unpublished observations), indicating that the effects might be brought about by direct stimulation of cAMP in cardiomyocytes.

SERCA and PLB protein levels showed a concordant decrease of both. However, the PLB/SERCA ratio was reduced after 2 days of beta adrenergic stimulation. Thus, the reduction in PLB protein level was more pronounced than the reduction in SERCA protein expression.

A causal relationship between protein levels of PLB and SERCA and time parameters is supported by the parallel normalization of relaxation and levels of PLB and SERCA. It remains the subject of future work why PLB reductions are transient, whereas alterations in other genes are permanent. However, these observations are indications of an interesting dissociation of inotropy and lusitropy in this model. The transient effect on lusitropy could be related to SR protein expression, whereas the persistent diminished inotropy by Iso might result from the well-described persistent change in sarcolemmal proteins (e.g., elevated G_i proteins; Eschenhagen et al., 1992).

Finally, it can be asked whether the changes in PLB are generalized or occur only in ventricles. Actually, at 2 days of beta adrenergic stimulation PLB protein level was reduced in left and right atria (data not shown). This indicates a general underlying mechanism in atria and ventricles.

In summary, we report here a transient concordant reduction in PLB and SERCA expression at the protein level. Their altered ratio is suggested to underlie at least in part the transient shortened time of relaxation in chronic beta adrenergic stimulation. Thus, chronic beta adrenoceptor stimulation in vivo is accompanied by complex transient and persistent alterations in cardiac function and gene expression.
References


Send reprint requests to: Dr. Bettina Linck, Institut für Pharmakologie und Toxikologie der Westfälischen Wilhelms-Universität, Domagkstraße 12, D-48149 Münster, Germany.