Age-Related Alterations in the Phosphorylation of Sarcoplasmic Reticulum and Myofibrillar Proteins and Diminished Contractile Response to Isoproterenol in Intact Rat Ventricle

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Previous studies have shown that the inotropic response of the heart to β-adrenergic stimulation declines with aging. This alteration has been attributed partly to an age-related impairment in the activation of the β-adrenocceptor–G protein–adenylate cyclase complex. To further understand the mechanisms underlying the age-related deficit, the present study compared β-adrenergic-mediated contractile response, cAMP accumulation, and phosphorylation of sarcoplasmic reticulum and myofibrillar proteins in isolated perfused hearts from adult (6–8 months) and aged (28–30 months) Fischer 344 rats. In isometrically contracting, electrically paced (240 beats per minute) hearts perfused at constant flow rate (9 ml/min per gram ventricle), the baseline contractile performance differed significantly between adult and aged hearts. Thus, contraction duration was prolonged (15%, p<0.001) in the aged relative to the adult heart, and this was due to increases in time to peak tension and relaxation time. Further, developed peak tension, normalized per gram ventricular wet weight, was significantly lower (20%, p<0.05) in the aged compared with the adult heart. In these isolated perfused heart preparations, β-adrenergic stimulation with isoproterenol (ISO, 0.001–1 μM) evoked concentration-dependent positive inotropic and lusitropic responses, both of which were significantly lower (15–20%, p<0.05–0.001) in the aged compared with the adult heart. These age-related differences were manifested as relatively smaller ISO-induced increases in 1) developed peak tension, 2) maximum rate of tension development (+dT/dt), and 3) maximum rate of relaxation (−dT/dt) in the aged compared with the adult heart. The ISO-induced abbreviation of time to half relaxation was also less marked in the aged heart. Under similar experimental conditions, ISO (0.1 μM)–induced increase in tissue cAMP content was also lower (18%, p<0.05) in the aged heart. ISO (0.1 μM)–induced phosphorylation of the sarcoplasmic reticulum protein phospholamban and myofibrillar protein troponin I was significantly diminished (38% and 25% decline, respectively, for phospholamban and troponin I; p<0.05–0.001) in the aged compared with the adult heart. No significant age-related difference was, however, evident in ISO-induced phosphorylation of C protein of myofibrils. These data suggest that age-related decrements in β-adrenergic–mediated cAMP accumulation and phosphorylation of phospholamban and troponin I contribute to the diminished contractile responses of the aged heart to β-adrenergic stimulation. (Circulation Research 1993;72:102–111)

KEY WORDS • heart • protein phosphorylation • β-adrenoceptors • aging • cAMP

It is well recognized that, in the mammalian heart, β-adrenergic stimulation results in enhanced contractile force as well as in increases in the rates of force development and relaxation. Most effects of β-adrenergic stimulation are likely mediated by cAMP-dependent mechanisms. Increased cytosolic cAMP, as a result of the activation of the β-receptor–G protein–adenylate cyclase complex by adrenergic stimulation, activates cAMP-dependent protein kinases, which induce phosphorylation of membrane and contractile proteins. Phosphorylation of an integral sarcoplasmic reticulum (SR) protein phospholamban (PL) results in increased Ca++ uptake by SR Ca++-ATPase in vitro and enhanced myocardial relaxation in the intact heart. Sarcolemmal proteins that are phosphorylated include calcium channels and a 15-kd protein. Phosphorylation of calcium channels results in an increased opening of voltage-dependent Ca++ channels and augments Ca++ inflow during the cardiac action potential. Troponin I (TnI) and C protein in myofilaments are phosphorylated by cAMP-dependent mechanisms both in isolated myofibrils and in intact heart. The contribution of TnI phosphorylation to cardiac contractility is not entirely clear; a functional role based on the alteration of myofilament sensitivity to Ca++, resulting in an enhanced rate of...
relaxation, has been proposed.12,13 The function of C protein remains unknown.

Several studies have shown that the responsiveness of the heart to β-adrenergic stimulation declines with aging in humans and animals.14,15 Evidence of an age-related deficit intrinsic to the myocardium has been provided by observations showing diminished contractile response to β-adrenergic agonists in isolated cardiac muscles16,17 and perfused hearts18 from aged compared with adult rats. The mechanisms underlying the impaired β-adrenergic response of the aging heart have not as yet been clearly understood. Previous studies from this and other laboratories have documented age-related alterations in the functional integrity of cardiac β-receptor–G protein–adenylate cyclase system; thus, although the number of β-receptors remains unaltered in the aged heart,17,19,20 activation of adenylate cyclase by β-receptor agonists is significantly diminished, apparently because of impairments in agonist–receptor interaction, G protein function, and receptor–effector (adenylate cyclase) coupling.19–21 These findings suggest that cAMP-dependent control of cardiac function is likely compromised in the aging heart. However, the effects of aging on cAMP-dependent cellular processes (i.e., events distal to the β-receptor–G protein–adenylate cyclase system) remain less well defined. Studies using rat cardiac tissue have reported lack of age-related alteration in protein kinase activity17 and reduced translocation of membrane-bound protein kinase21 after β-adrenergic stimulation. An age-related decrease in the phosphorylation of contractile proteins TnI and C protein was shown in isolated rat ventricular myocytes stimulated by norepinephrine or isoproterenol (ISO).22 Using isolated rat cardiac SR membrane, we have demonstrated that the ability of PL to undergo cAMP-dependent phosphorylation and the relative responsiveness of the Ca2+ pump to PL phosphorylation, as manifested by relative increase in Ca2+ uptake in vitro, are not altered in aging.23 However, the effect of aging on β-adrenergic–mediated phosphorylation of PL and myofibrils in the intact functioning myocardium has not yet been established. Therefore, in this study, we have evaluated the effect of aging on protein phosphorylation and contractile responses to the β-receptor agonist ISO in intact rat myocardium. The results demonstrate selective age-related changes in the phosphorylation of SR and myofibrils as well as reduced contractile function of aged myocardium in response to ISO stimulation.

Materials and Methods

Chemicals

L-ISO, histone (type VI-S, from calf thymus), and cAMP-dependent protein kinase (catalytic subunit) were purchased from Sigma Chemical Co., St. Louis, Mo. [3H]cAMP, [γ-32P]ATP, and 32P in Na2HPO4 were obtained from New England Nuclear, Montreal, Canada. Electrophoresis reagents were from Bio-Rad Laboratories, Mississauga, Ontario. All other chemicals were of the highest purity available from Sigma or BDH Chemicals, Toronto.

Animals

Male virgin Fischer 344 rats aged 6–8 months (adult rats, 310–380 g body weight) and 28–30 months (aged rats, 290–420 g body weight) were obtained from the National Institute on Aging rat colony maintained by Harlan Industries, Indianapolis, Ind. On arrival, the rats were housed individually in the Health Sciences Animal Care Facility at the University of Western Ontario with free access to food (Purina chow containing 20% protein) and water and used for experiments within 2 weeks.

Heart Perfusion

Heart perfusion was performed using the Langendorff technique. Briefly, the animals were decapitated, hearts were quickly excised, and retrograde aortic perfusion was started with modified Krebs-Henseleit buffer (K-H buffer). The K-H buffer contained (mM) NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, CaCl2 1.5, and glucose 11. The buffer was equilibrated with 95% O2-5% CO2, which maintained a pH of 7.4. The temperature was kept at 37±0.2°C through a water-jacketed system, and perfusion flow rate was set constant at 9 ml/min per gram ventricular mass. Because the wet ventricular mass is approximately 22% higher in aged heart compared with adult heart (0.75±0.05 g for adult and 0.92±0.06 g for aged heart, n=12–15, authors’ unpublished observation), the flow rate was adjusted accordingly. The hearts were allowed to stabilize for 20–30 minutes and used in the following protocols.

Protocol A. In this protocol, the effect of aging on contractile function was evaluated. Both atria were removed, and the ventricular myocardium was electrically paced at 240 beats per minute with an SD-9 stimulator (Grass Instrument Co., Quincy, Mass.) via platinum-wire electrodes inserted into the epicardium at two times threshold voltage with a pulse duration of 5 msec. The pacing rate was slightly higher than the intrinsic heart rate in vitro for both adult and aged hearts. A hook was placed in the apex, and isometric contraction was measured as the apical basal displacement with a Grass FT.03C force displacement transducer. The first derivatives for the maximum rate of tension development (+dT/dt) and the maximum rate of relaxation (−dT/dt) were obtained electronically by means of a Grass 7P20C differentiator. Resting tension was set at 2 g. All parameters were recorded on a Grass 7D polygraph system. Parameters of myocardial contractile function were recorded before and during continuous perfusion of ISO (0.001–1 μM) dissolved in K-H buffer containing 0.2 mM dithiothreitol as an antioxidant. The hearts were challenged with varying concentrations of ISO for 1 minute (0.001–0.1 μM ISO) or 30 seconds (0.5–1 μM ISO) in ascending order, with a washing out period of 5–10 minutes between each concentration. The following parameters were measured from recordings obtained at a paper speed of 100 mm/sec: 1) contraction duration, which includes time to peak tension and time to half relaxation, 2) peak developed tension, 3) +dT/dt, and 4) −dT/dt. ISO-induced peak responses were expressed as percent changes.

Protocol B. In this protocol, the effect of aging on protein phosphorylation in SR and myofibrils in response to ISO challenge was evaluated. Based on the results of the studies in protocol A, an ISO concentration of 0.1 μM was used for these experiments. This
concentration of ISO produced significant contractile responses in adult and aged hearts, revealed age-related differences in the inotropic response, and did not elicit severe arrhythmias or ventricular fibrillation. After stabilization for 20 minutes, the isolated perfused spontaneously beating hearts (200–220 beats per minute) from adult and aged rats were switched to a recirculating perfusion system containing 1 mCi 32P in 100 ml K-H buffer (with 0.26 mM KH2PO4) for 40 minutes. The hearts were then perfused with nonradioactive K-H buffer for 2 minutes and were either challenged with 0.1 μM ISO or vehicle buffer (control hearts) for 1 minute. At the end of the challenge, the atri were removed, and the hearts were freeze-clamped with Wollenberger clamps precooled in liquid nitrogen. The hearts were then pulverized in liquid nitrogen and stored at −70°C for future assay.

**Membrane and Myofibrillar Preparations**

Membrane vesicles enriched in SR were prepared according to the procedure of Harigaya and Schwartz,24 with some modifications.25–27 Briefly, the frozen powdered tissue was thawed and homogenized in 20 vol medium I three times for 15 seconds each at 30-second intervals with a Polytron PT-10 homogenizer (setting 6, Brinkman Instruments, Rexpale, Ontario). Medium I consisted of 30 mM KH2PO4 (pH 7.0), 10 mM NaF, 5 mM EDTA, 0.3 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. The homogenate was centrifuged at 4,300g for 10 minutes. The supernatant was then centrifuged at 15,000g for 20 minutes. The supernatant obtained was filtered through four layers of cheesecloth, and solid KCl was then added to the supernatant to bring the final concentration to 0.6 M. After incubation for ice for 25 minutes, the supernatant was centrifuged at 39,000g for 60 minutes, and the final pellet was suspended in medium I. The yield was approximately 2 mg microsomal protein per gram wet tissue. No age-related difference was seen in the SR protein yield. Enzyme markers were assayed as described previously.25 Mitochondrial contamination was approximately 15%, as assessed from azide-sensitive Ca2+-Mg2+-ATPase activity measured in the presence of 3.9 μM free Ca2+, 5 mM MgCl2, 2.5 mM ATP, and 5 mM NaN3. Sarcolemmal contamination was approximately 10%, as assessed from ouabain-sensitive Na+,K+-ATPase activity measured in the presence of 100 mM NaCl, 5 mM MgCl2, 5 mM ATP, 1 mM ouabain, and 125 μM sodium dodecyl sulfate (SDS) per milligram SR protein. The Ca2+-stimulated ATPase activity of SR membranes (measured in the presence of 3.9 μM free Ca2+, 5 mM MgCl2, and 2.5 mM ATP) amounted to 220±18 nmol inorganic phosphate (P1) per milligram protein per minute. No age-related difference was observed in these enzyme markers. Further, as documented extensively in our previous studies,25,26 the polypeptide composition and relative purity of SR membranes isolated from adult and aged rat hearts are essentially similar (also see “Results”). Myofibrils were prepared from the pellet obtained after the first centrifugation of heart homogenate (see above) according to the procedure of Rapundalo et al.27 Briefly, the pellet was resuspended in medium II (mM: KH2PO4 100, NaF 50, and EDTA 5, pH 7.0), homogenized, and centrifuged at 3,500g for 10 minutes. This procedure was repeated once. The resulting pellet was resuspended in medium II supplemented with 1% Triton X-100 and sedimented at 3,500g for 10 minutes. The pellet was subsequently washed four times by resuspension in medium III (mM: imidazole 30, KCl 60, MgCl2 2, and NaF 10, pH 7.7) followed by centrifugation as above. The washed pellet was digested with 2% SDS in 50 mM Tris-HCl (pH 7.5) at room temperature overnight before protein fractionation by electrophoresis. No age-related difference was evident in the relative purity of the myofibril preparations, as judged from the polypeptide composition and density of individual peptide bands after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (see “Results”). Protein concentration was determined according to the method of Lowry et al.28 using defatted bovine serum albumin as standard.

**Gel Electrophoresis and Western Immunoblotting**

SDS-PAGE was performed, as detailed elsewhere,25 with two identical 8–18% gradient acrylamide slab gels. Both SR and myofibrils were solubilized with an equal volume of sample buffer containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue. An equal amount of protein was applied to each lane (40–70 μg for gel 1, 100–180 μg for gel 2), and electrophoresis was conducted at 16 mA per gel through the stacking gel and 25 mA per gel through the separating gel. Both gels were fixed overnight in 25% isopropanol alcohol/10% acetic acid. Gel 1 was stained with Coomassie blue, destained, and dried and was used for molecular mass estimation. The dried gel 1 and gel 2, wrapped in plastic film, were used for autoradiography by placing in contact with Kodak X-Omat AR film in a Kodak Lanex regular cassette (with intensifying screen) at −80°C for 48–72 hours. Radioactive bands corresponding to PL, TnI, and C protein were identified according to their molecular mass range and specific 32P incorporation in response to ISO. These bands were cut from gel 2 and counted in Beckman aqueous liquid scintillation cocktail. The amount of phosphate incorporation was quantified by dividing 32P incorporation by the specific radioactivity of [γ-32P]ATP determined for each heart (see below) and expressed as picomoles 32P incorporated per milligram protein.

Western immunoblotting was used to localize and quantify Ca2+-ATPase of cardiac SR. The SR membranes (30 μg protein) were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes according to the method of Towbin et al.29 The membranes were blocked with 3% gelatin in Tris-buffered saline, incubated with rabbit polyclonal antisera against cardiac SR Ca2+-ATPase (kindly provided by Dr. A.K. Grover, McMaster University, Hamilton, Canada), and then incubated further with alkaline phosphatase–conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:2,000 dilution). Color development was performed using a Bio-Rad assay kit. The immunoreactive protein band representing Ca2+-ATPase was quantified by laser scanning densitometry using an LKB Ultrascan XL laser densitometer.
Determination of Specific Radioactivity of \( [\gamma-^3\text{P}]\text{ATP} \)

The specific radioactivity of \( [\gamma-^3\text{P}]\text{ATP} \) in each heart was determined using the procedure of Hawkins et al\(^{10} \) with minor modifications. ATP was extracted from \( ^3\text{P} \)-labeled cardiac tissue as follows: Generally, 200 \( \mu \text{g} \) frozen tissue was thawed and homogenized in 0.8 ml of 0.5 M HClO\(_4\) using a Polytron PT-10 homogenizer and setting 8, with four 10-second bursts at 30-second intervals. The homogenate was centrifuged at 39,000g for 20 minutes, and the supernatant was neutralized with saturated K\(_2\text{CO}_3\) in the presence of BDH universal indicator (20 \( \mu \text{L} \)/ml). The mixture was kept ice cold for 30 minutes, and precipitated KClO\(_4\) was removed by centrifugation at 39,000g for 20 minutes. To determine the specific radioactivity of the \( \gamma \)-phosphate of \( [\gamma-^3\text{P}]\text{ATP} \), aliquots of the supernatant were used in a histone phosphorylation assay as follows: A 40-\( \mu \text{L} \) portion of the supernatant was added to a reaction mixture (total volume, 100 \( \mu \text{L} \)) containing 50 mM Tris-HCl (pH 6.8), 0.4 mg histone H2A/ml, 10 mM magnesium acetate, 30 mM dithiothreitol, 30 mM NaF, and 5 \( \mu \text{g} \) cAMP-dependent protein kinase (catalytic subunit). The reaction mixture was incubated at 30\(^\circ\)C for 140 minutes. Under these conditions, histone phosphorylation reached a plateau within 90 minutes and remained stable at least until 140 minutes. The reaction was terminated by transferring 45 \( \mu \text{L} \) reaction mixture to a 4-cm\(^2\) piece of filter paper (No. 1, Whatman International Ltd., Maidstone, England). The filter squares were washed four times for 15 minutes each in ice-cold 10% trichloroacetic acid, rinsed once in ethanol, and dried, and the radioactivity was determined by liquid scintillation spectrometry. A sample of pure \( [\gamma-^3\text{P}]\text{ATP} \) with known specific radioactivity was included in the assay as a standard. The specific radioactivity of each heart was expressed as the ratio between the unknown and the standard. The specific radioactivity of \( [\gamma-^3\text{P}]\text{ATP} \) was usually lower (10–15%) for aged heart compared with adult heart perfused on a particular day with the same stock solution of \( ^3\text{P} \).

cAMP Assay

Tissue cAMP content was determined by radioimmunoassay using a \(^{125}\text{I}\)cAMP radioimmunoassay kit (RIANEN, Dupont, Mass.). Cardiac samples from freeze-clamped control and ISO (0.1 \( \mu \text{M} \), 1 minute)–stimulated hearts were used. Some tissues from \( ^3\text{P} \)-perfused hearts that had decayed longer than 6 months were also included in the assay. Samples (100 mg wet weight) of frozen powdered tissue were homogenized in 6% (wt/vol) trichloroacetic acid, extracted with ether, acetylated, and assayed in triplicate. The extent of myocardial swelling during perfusion was similar (=15% in 1 hour) in adult and aged hearts; hence, the cAMP content was expressed in picomoles per milligram wet weight. Results were corrected for recoveries, which were monitored with \(^{3}\text{H}\)cAMP and routinely determined to be 80–90%.

Statistical Analysis of Data

Results are presented as mean±SEM. Comparisons of myocardial contractile parameters between the two age groups were performed using nonpaired Student’s \( t \)

### Table 1. Comparison of Baseline Contractile Function Parameters in Adult and Aged Rat Hearts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Adult hearts</th>
<th>Aged hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T ) (g/g)</td>
<td>7.1±0.6</td>
<td>5.7±0.3*</td>
</tr>
<tr>
<td>TPT (msec)</td>
<td>94±2</td>
<td>104±2†</td>
</tr>
<tr>
<td>( \text{RT}_{1/2} ) (msec)</td>
<td>37±3</td>
<td>46±2†</td>
</tr>
<tr>
<td>CD (msec)</td>
<td>131±2</td>
<td>150±3†</td>
</tr>
<tr>
<td>+dT/dt (g/sec)</td>
<td>137±8</td>
<td>100±8*</td>
</tr>
<tr>
<td>−dT/dt (g/sec)</td>
<td>154±8</td>
<td>107±10†</td>
</tr>
</tbody>
</table>

\( T \), peak developed tension; TPT, time to peak tension; \( \text{RT}_{1/2} \), time to half relaxation; CD, contraction duration (TPT+\( \text{RT}_{1/2} \)); +dT/dt, maximum rate of tension development; −dT/dt, maximum rate of relaxation. Values are mean±SEM (\( n=15 \)).

Isometric cardiac contractions were recorded from isolated perfused and electrically paced (4 Hz) hearts from adult and aged rats. \( *p<0.05 \) and \( †p<0.001 \) vs. adult hearts.

### Results

**Baseline Contractile Function Parameters in Adult and Aged Hearts**

The results presented in Table 1 compare the baseline (control) contractile function parameters in isolated, constant flow–perfused, isometrically contracting adult and aged rat hearts paced at 4 Hz. Peak tension, \(+dT/dt\), and \(−dT/dt\) were significantly lower in the aged compared with the adult heart. The contraction duration was significantly prolonged in the aged heart as a result of age-related increases in the time to peak tension and relaxation time. This age-associated prolongation of contraction duration in the intact heart conforms to previous observations using isolated, isometrically contracting muscle preparations such as trabeculae carneae, interventricular septum, and papillary muscle.\(^{16,17,32}\) However, in contrast to the present finding with intact heart, previous studies using isolated cardiac muscle preparations did not reveal age-related differences in peak tension.\(^{16,17,32}\) In other studies, we have noted that the age-related decrease in peak tension is clearly manifested when intact hearts are stimulated at frequencies greater than 120 per minute (B. Bell, M.T. Jiang, and N. Narayanan, unpublished results). Therefore, the low stimulation frequencies (<100 per minute) used in previous studies\(^{16,17,32}\) may have masked the age-related difference in peak tension.

**Effect of Aging on Contractile Responses of the Heart to ISO**

Typical recordings of isometric contractions of adult and aged hearts before (control) and after stimulation with a selected concentration of ISO (0.1 \( \mu \text{M} \)) are presented in Figure 1. Generally, the maximum contractile response occurred within 20–30 seconds after ISO stimulation in adult and aged hearts; in both age groups, the maximum contractile response was maintained for approximately 15–20 seconds thereafter. The results in Figure 1 depict the maximum contractile response to 0.1 \( \mu \text{M} \) ISO; the magnitude of the positive inotropic response was clearly lower in the aged compared with the
adult heart. Disproportionately larger increases in $-\Delta t/\Delta t$ over $+\Delta t/\Delta t$, as seen in Figure 1, were observed in the adult heart after stimulation with varying concentrations (0.1–1 μM) of ISO, and this is in accordance with the previously reported effects of β-adrenergic stimulation in mammalian myocardium.33,34 Such a trend, however, was less prominent in the aged heart. The age-related decrease in the positive inotropic response to ISO was observed at varying concentrations of ISO (Figure 2a). Although the ISO-induced increase in $+\Delta t/\Delta t$ tended to be lower in the aged compared with the adult heart at varying concentrations of ISO (Figure 2b), this age-related difference was not statistically significant. The relative increases in $-\Delta t/\Delta t$ at 0.1–1 μM ISO were significantly lower in the aged compared with the adult heart (Figure 3a). Similarly, the relative abbreviation of the time to half relaxation at 0.1–0.5 μM ISO was significantly less marked in the aged heart (Figure 3b). Thus, the positive lusitropic response to ISO is compromised in the aged heart.

**Effect of Aging on ISO-Induced cAMP Accumulation and Protein Phosphorylation in the Myocardium**

As shown in Figure 4, the tissue cAMP levels measured under control (basal) conditions did not differ significantly between adult and aged hearts. Stimulation with 0.1 μM ISO resulted in approximately a 2.8-fold increase in cAMP in the adult heart as compared with a 2.3-fold increase in the aged heart. Consequently, ISO-induced cAMP accumulation was significantly lower (≈18% less, p<0.05) in the aged heart.

Figure 5 shows typical protein profiles of SR (lanes 1 and 2) and myofibrils (lanes 3 and 4) isolated from adult and aged hearts after $^{32}$P perfusion without ISO stimulation (i.e., control hearts). No age-related difference was evident in the polypeptide composition of SR or myofibrils. Also, Western immunoblot analysis of four separate SR preparations from adult and aged hearts showed no statistically significant age-related difference in the density of immunoreactive Ca$^{2+}$-ATPase band in these membrane preparations (Figure 6). The protein profiles of SR and myofibrils isolated from ISO-stimulated hearts did not differ from those of control hearts. Figure 7 compares ISO-induced protein phosphorylation in SR of adult and aged hearts. No appreciable protein phosphorylation was evident in SR from adult and aged hearts not subjected to ISO stimulation. Stimulation with 0.1 μM ISO for 1 minute resulted in phosphorylation of three major peptide bands with apparent molecular masses of 24, 15, and 12 kd. The 24- and 12-kd proteins represent the high and low molecular mass forms of PL as determined by their specific response to β-adrenergic stimulation, by the molecular mass range, and by conversion of the high molecular mass form into low molecular mass form after boiling the samples in SDS before electrophoresis (compare left and right panels in Figure 7; also, see Reference 23). The magnitude of ISO-induced phosphorylation of PL (low and high molecular mass forms) was strikingly lower in the aged compared with the adult heart. Cumulative data on $^{32}$P incorporation into PL in control and ISO-stimulated hearts are presented in Figure 8. The control (basal) level of $^{32}$P incorporation into PL did not differ in adult and aged hearts. On stimulation with 0.1 μM ISO for 1 minute, $^{32}$P incorporation into PL increased ninefold in the adult and sixfold in the aged heart, reflecting a significant age-related decline (≈38%, p<0.001) in PL phosphorylation in response to ISO. It appears that age-related decline also occurs in the phosphorylation of the 15-kd protein and a 42-kd protein (Figure 7); the latter likely represents membrane-associated protein kinase capable of undergoing autophosphorylation.

Figure 9 compares ISO-induced protein phosphorylation in myofibrils of adult and aged hearts. Stimulation with 0.1 μM ISO caused a marked increase in the phosphorylation of two peptide bands with apparent molecular masses of 130–140 kd, designated C protein, and 29 kd, designated TnI. The magnitude of ISO-induced phosphorylation of TnI, but not C protein, appeared to be lower in the aged compared with the.
adult heart. Cumulative data on 32P incorporation revealed a significant age-related decrease (≈25%, p<0.05) in the ISO-induced phosphorylation of TnI (Figure 10a), whereas no significant age-related difference was evident in ISO-induced phosphorylation of C protein (Figure 10b).

**Discussion**

The results presented here demonstrate that 1) in isolated perfused isometrically contracting rat hearts, the positive inotropic and lusitropic responses to β-adrenergic stimulation are significantly lower in the aged compared with the adult heart and 2) the diminished contractile responses in the aged heart are accompanied by attenuation of the β-adrenergic-mediated increase in tissue cAMP levels as well as phosphorylation of PL in SR and TnI in myofibrils. To our knowledge, this is the first report documenting age-associated alterations in β-adrenergic modulation of protein phosphorylation and contractile function in the intact beating heart.

The reduced positive inotropic response of the intact aged heart to β-adrenergic stimulation confirms previous findings by others using isometric cardiac muscle preparations, such as left ventricular trabeculae carnea.16 and interventricular septum.17 Interestingly, however, the age-associated decline in the relaxant effect (lusitropic response) of β-adrenergic stimulation (manifested as diminished relative increase in −dT/dt and shortening of the time to half relaxation in the aged compared with the adult heart) documented in the present study using intact heart was not observed in earlier studies using cardiac muscle preparations.16,17 The reason for this disparity is not clear, but it could be due to various factors such as age-related regional differences.

**Figure 3.** Graphs comparing the relaxation-promoting effect of isoproterenol in adult and aged rat hearts. Experimental details were as described in the legend to Figure 2. Panel a: Maximum rate of relaxation (−dT/dt). Panel b: Time to half relaxation (RT ½). *p<0.05 compared with adult (n=8–10 except at 1 μM isoproterenol, where n=4).

**Figure 4.** Bar graph comparing isoproterenol (ISO)-induced cAMP accumulation in adult and aged rat hearts. Isolated perfused rat hearts were stimulated with 0.1 μM ISO or vehicle buffer (control [CON]) for 1 minute and then freeze-clamped with Wollenberger clamps precooled in liquid nitrogen. cAMP content in tissue extracts was determined by radioimmunoassay. The increase in tissue cAMP level in response to ISO stimulation was significantly lower (18%, *p<0.05) in aged compared with adult hearts (n=9 per group).

**Figure 5.** Protein profiles of sarcoplasmic reticulum and myofibrils isolated from adult and aged rat hearts. The proteins (70 μg each of sarcoplasmic reticulum and myofibrils) were fractionated by sodium dodecyl sulfate-polyacrylamide (8–18% gradient) gel electrophoresis. For details see “Materials and Methods.” Lanes 1 and 2 refer to sarcoplasmic reticulum isolated from adult and aged hearts, respectively. Lanes 3 and 4 refer to myofibrils isolated from adult and aged hearts, respectively.
differences in the sensitivity of the heart to β-adrenergic agonists, the age (22–25 versus 28–30-month-old aged rats) and strain (Wistar versus Fischer 344) of rats used, and the contraction frequencies (24–80 versus 240 beats per minute) selected. The critical importance of the latter factor in unmasking age-related differences in contractile performance is underscored by our observation that a significant decrease in developed tension (under baseline conditions) in the aged compared with the adult heart is seen only at contraction frequencies >120 beats per minute (see “Results”). This is not surprising, because restitution of cardiac excitation–contraction coupling may be slower in the aged compared with the adult heart,14,15 and this may be partially due to an age-related decline in the Ca2+-sequestering activity of SR.25,26 Thus, the negative force–frequency relation seen in the rat heart becomes apparently more pronounced with aging, and such an age-related difference becomes clearly manifested at heart rates closer to the physiological range.

In analyzing the mechanisms responsible for the diminished contractile responses of the aged heart to β-adrenergic stimulation, the present study focused on β-adrenergic–mediated changes in tissue cAMP level and protein phosphorylation in SR and myofibrils. The finding that β-adrenergic stimulation elicits lesser net cAMP accumulation in the aged compared with the adult heart is consistent with previous observations showing diminished β-adrenergic activation of adenylate cyclase in cardiac membranes from aged compared with adult rats19–21 and apparently lower net cAMP production in isolated cardiomyocytes from senescent compared with adult rats stimulated with norepinephrine.22 Thus, decline in β-adrenergic activation of adenylyl cyclase (and consequent reduction in cAMP production) is one of the likely factors underlying the diminished contractile responses of the aged heart to β-adrenergic stimulation. The precise nature of the age-associated deficit in the β-receptor–G protein–adenylyl cyclase system is not yet understood, but evidence suggesting impairments of agonist–receptor interaction,19–21 signal transduction by G protein,19 and alteration in the activity of adenylate cyclase catalytic unit has been obtained in previous studies. It must be noted, however, that age-related changes in more than one locus may contribute to the diminished net cAMP accumulation in response to β-adrenergic stimulation in the aged heart. For example, in the aged heart, cAMP phosphodiesterase activity may be enhanced,22 and the antiadrenergic action of adenosine may be exaggerated because of the age-associated increase in cardiac tissue levels of adenosine. The potential contribution of these factors in attenuation of the net cAMP levels cannot be ignored. Also, there exists considerable evidence suggesting structural and functional compartmentalization of cAMP and cAMP-dependent protein kinase in cardiomyocytes and selective or preferential modulation of cAMP level and protein kinase activity in these compartments by different hormonal stimuli.35–38 Hence, the age-related difference in the total-tissue cAMP pool observed after β-adrenergic activation does not necessarily imply that changes of a similar magnitude occur in the cAMP content of different subcellular/functional compartments. Further study of the subcellular distribution of cAMP after β-adrenergic stimulation is needed to clarify whether the age-related difference is selectively more pronounced in specific compartments.

Our results demonstrate that the age-related diminution in β-adrenergic-mediated cAMP accumulation was accompanied by selective and nonuniform changes in protein phosphorylation in SR and myofibrils. Thus, in the aged heart, approximately an 18% decrease in cAMP accumulation resulted in a nearly 25% decrease in the phosphorylation of myofilamental protein TnI, whereas phosphorylation of C protein of myofilaments was unaltered. On the other hand, in SR, phosphorylation of PL was reduced to a much greater extent (≈38%) than TnI. As discussed below, the interplay of several factors may underlie such selective and disproportionate age-related differences in β-adrenergic–mediated protein phosphorylation in the intact heart.

Factor 1: Differential sensitivity due to compartmentalization of cAMP signal. Differential sensitivity of TnI and PL phosphorylation toward the β-adrenergic agonist ISO in isolated perfused rat hearts has been demonstrated in a recent study. Thus, it was shown that half-maximal phosphorylation of TnI occurs with a 20-fold lower concentration of ISO or 10-fold increase in tissue cAMP content compared with that required for half-maximal phosphorylation of PL.28 These findings imply the participation of distinct cAMP signal cascades in the phosphorylation of TnI and PL and/or differential accessibility of these proteins to activated cAMP-dependent protein kinase in the intact heart. Therefore,
it is possible that differential age-related alterations in distinct cAMP signal cascades may result in disparate levels of phosphorylation in TnI and PL. Alternatively, because TnI can undergo maximal phosphorylation with a much smaller increase in cAMP level than that required by PL, it is possible that a moderate decrease in total cAMP level may have minimal effect on the phosphorylation of TnI and more pronounced effect on the phosphorylation of PL. This may explain, at least in part, the difference in the degree of age-related reduction in phosphorylation in PL and TnI and perhaps the lack of age-related change in C protein phosphorylation as well.

Factor 2: Age-related alteration in calmodulin-dependent phosphorylation of PL. Although phosphorylation of TnI after β-adrenergic stimulation may be exclusively through a cAMP-dependent mechanism, a Ca²⁺-calmodulin-dependent mechanism is likely involved in the phosphorylation of PL in the intact heart secondary to an increase in cytosolic Ca²⁺. Although the ability of PL to undergo cAMP-dependent phosphorylation in vitro is not altered in aging, as shown by our previous study, the possibility of an age-related decline in the phosphorylation of PL by a Ca²⁺-calmodulin-dependent mechanism cannot be excluded.

Factor 3: Age-related alteration in protein dephosphorylation. Age-related differences in the rates of protein dephosphorylation due to altered protein phosphatase activity can give rise to apparent differences in the extent of phosphorylation of different protein substrates. The present study did not examine the effect of aging on protein phosphatase activity in heart muscle.

However, a recent study reported identical values for time to half dephosphorylation of TnI in isolated cardiomyocytes from adult and aged rats subjected to β-adrenergic stimulation, suggesting lack of age-related changes in protein phosphatase activity. Thus, it seems unlikely that the age-related alteration in protein phosphatase activity contributes to the selective age-associated changes in protein phosphorylation in SR and myofibrils.
When the physiological effects thought to underlie PL and TnI phosphorylation are considered, the age-related decline in the phosphorylation of these proteins can be mechanistically linked to the diminished contractile responses of the aged heart to β-adrenergic stimulation. Phosphorylation of PL is recognized to play a key role in acceleration of myocardial relaxation upon β-adrenergic stimulation by increasing the velocity of Ca\(^{2+}\) sequestration from the myoplasm by the SR Ca\(^{2+}\) pump.\(^{2-6}\) This effect of PL phosphorylation on the SR Ca\(^{2+}\) pump also leads to augmentation of contractile Ca\(^{2+}\) reserve within the SR lumen, which, in turn, would enable greater Ca\(^{2+}\) release from SR during subsequent excitations, thus promoting the positive inotropic effect of β-adrenergic stimulation as well.\(^{7}\) Therefore, the age-related decline in PL phosphorylation can account, at least in part, for the diminished lusitropic and inotropic responses of the aged heart to β-adrenergic stimulation. The physiological effects of TnI phosphorylation are less clearly understood. Phosphorylation of TnI has been found to cause decreased affinity of troponin C for Ca\(^{2+}\), suggesting that TnI phosphorylation upon β-adrenergic stimulation may also help to promote myocardial relaxation by favoring dissociation of Ca\(^{2+}\) from the myofilaments.\(^{12,13}\) However, the time course of TnI dephosphorylation upon β-receptor blockade has been found to be slower than the reversal of the relaxant effect.\(^{42,43}\) As noted earlier, a recent study using isolated perfused rat hearts has documented a markedly higher sensitivity of TnI phosphorylation (compared with PL phosphorylation) toward β-adrenergic agonist ISO; further, this study also showed a strong correlation between TnI phosphorylation and the lusitropic (−dF/dt) and inotropic (+dF/dt) changes in response to ISO stimulation.\(^{44}\) Thus, it is possible that the age-related decline in TnI phosphorylation may also be an underlying factor responsible for the diminished inotropic and lusitropic responses of the aged heart to β-adrenergic stimulation. Other target proteins of cAMP-dependent protein kinase known to influence cytosolic Ca\(^{2+}\) transients, and therefore cardiac contractile responses to β-adrenergic stimulation, include the sarcolemmal Ca\(^{2+}\) channel\(^{7,9}\) and a 15-kd sarcolemmal protein.\(^9\) Phosphorylation of these sarcolemmal substrates is thought to enhance transsarcolemmal Ca\(^{2+}\) influx, thus promoting the positive inotropic response to β-adrenergic stimulation.\(^7,9\) The possibility of age-related alterations in β-adrenergic-mediated protein phosphorylation in cardiac sarcolemma and their potential relevance to the diminished contractile responses of the aging heart to β-adrenergic stimulation remain to be investigated.

Finally, it should be noted that the age-related changes in ISO-induced contractile responses and biochemical events observed in intact ventricles in our study do not necessarily reflect uniform alterations in both right and left ventricles since 1) functional, mechanical, and structural differences exist between the right and left side of the heart\(^{44}\) and 2) the impact of aging on the two ventricles may be dissimilar.\(^{44,45}\) Furthermore, recent studies have shown significant struc-
tural and morphological changes with advanced aging in rat myocardium\(^{15,46}\); therefore, the potential contribution of these factors to the age-related alterations in protein phosphorylation and cardiac contractile function seen in the current study cannot be overlooked.

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