Activation of $\beta_2$-Adrenergic Receptors Hastens Relaxation and Mediates Phosphorylation of Phospholamban, Troponin I, and C-Protein in Ventricular Myocardium From Patients With Terminal Heart Failure

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Background—Catecholamines hasten cardiac relaxation through $\beta$-adrenergic receptors, presumably by phosphorylation of several proteins, but it is unknown which receptor subtypes are involved in human ventricle. We assessed the role of $\beta_1$- and $\beta_2$-adrenergic receptors in phosphorylating proteins implicated in ventricular relaxation.

Methods and Results—Right ventricular trabeculae, obtained from freshly explanted hearts of patients with dilated cardiomyopathy ($n=5$) or ischemic cardiomyopathy ($n=5$), were paced at 60 bpm. After measurement of the contractile and relaxant effects of epinephrine (10 $\mu$mol/L) or zinterol (10 $\mu$mol/L), mediated through $\beta_1$-adrenergic receptors, and of norepinephrine (10 $\mu$mol/L), mediated through $\beta_2$-adrenergic receptors, tissues were freeze clamped. We assessed phosphorylation of phospholamban, troponin I, and C-protein, as well as specific phosphorylation of phospholamban at serine 16 and threonine 17. Data did not differ between the 2 disease groups and were therefore pooled. Epinephrine, zinterol, and norepinephrine increased contractile force to approximately the same extent, hastened the onset of relaxation by 15±3%, 5±2%, and 20±3%, respectively, and reduced the time to half-relaxation by 26±3%, 21±3%, and 37±3%. These effects of epinephrine, zinterol, and norepinephrine were associated with phosphorylation (pmol phosphate/mg protein) of phospholamban 14.0, 2.0, and 2.0; troponin I 40±7, 33±7, and 31±6; and C-protein 7.2±1.9, 9.3±1.4, and 7.5±2.0. Phosphorylation of phospholamban occurred at both Ser16 and Thr17 residues through both $\beta_1$- and $\beta_2$-adrenergic receptors.

Conclusions—Norepinephrine and epinephrine hasten human ventricular relaxation and promote phosphorylation of implicated proteins through both $\beta_1$- and $\beta_2$-adrenergic receptors, thereby potentially improving diastolic function. (Circulation. 1999;99:65-72.)

Key Words: heart failure $\beta$ receptors, adrenergic, beta 2 $\beta$ catecholamines $\beta$ phosphoproteins $\beta$ contractility, diastole
mediate positive lusitropic effects and whether implicated proteins are phosphorylated. This is not the case in a variety of animal models in which β₁ but not β₂-adrenergic receptors mediate hastening of ventricular relaxation. However, there is evidence in human atrium and human ventricular myocytes from normal and failing hearts that both β₁- and β₂-adrenergic receptors mediate not only inotropic but also lusitropic effects. We now confirm this in ventricular trabeculae from failing ischemic and cardiomyopathic hearts and show it to be associated with phosphorylation of phospholamban, troponin I, and C-protein for both β₁- and β₂-adrenergic receptors.

### Methods

#### Patients

Informed consent was obtained from all patients (Table 1). All patients had terminal heart failure and underwent cardiac transplant surgery. This study was approved by the ethics committees of the Papworth Hospital, Cambridgeshire (United Kingdom), and Alfred Hospital, Melbourne (Australia).

#### Isolated Right Ventricular Trabecula Carnae

Explanted hearts were obtained immediately (<1 minute) after removal from the patient. The endocardial layer of the right ventricular free wall was rapidly dissected in ice-cold, preoxygenated (95% O₂-5% CO₂) modified Krebs’ solution containing (mmol/L) Na¹ 125, K² 5, Ca⁴⁺ 2.25, Mg²⁺ 0.5, Cl⁻ 98.5, SO₄²⁻ 0.5, HCO₃⁻ 29, HPO₄⁻² 1, and EDTA 0.04 at the surgical theater. Trabeculae (width usually 1.3 mm) were dissected, set up at optimum length, and paced to contract isometrically at 60 bpm at 37°C in a bath containing the above solution supplemented with (mmol/L) Na¹ 15, fumarate 5, pyruvate 5, L-glutamate 5, and glucose 10 as described.

In all experiments, contractile force and its first derivative were recorded simultaneously. Cross-sectional area of trabeculae that were not snap-frozen was determined from the length and weight of the muscle at the end of the experiment, assuming a density of 1.063. Rapid freezing of trabeculae prevented measurements of length and weight under conditions of contraction.

#### Specific Activation of β₁- and β₂-Adrenergic Receptors

To irreversibly block tissue uptake of catecholamines and α-adrenergic receptors, trabeculae were incubated for 90 minutes with phenoxybenzamine followed by washout.

To establish conditions for selective activation of β₁- and β₂-adrenergic receptors, experiments were carried out as described previously on ventricular preparations from hearts without advanced failure and ventricular myocytes from a donor heart and hearts in terminal failure. To determine β₁-selective activation, inotropic concentration-effect curves to (−)-isoproterenol were determined in the absence and presence of the β₁-blocker CGP20712A. For β₂-selective activation, inotropic concentration-effect curves to (−)-epinephrine in the presence of CGP20712A (300 mmol/L) were determined in the absence and presence of ICI118551 (50 mmol/L). In these studies, only a single concentration-effect curve was determined on each trabeculum. After equilibrium effects were reached with the highest catecholamine concentration, (−)-isoproterenol was added at a concentration (200 μmol/L) that surmounts the blockade caused by CGP20712A and ICI118551. The experiments were terminated by increasing the CaCl₂ concentration to 9.25 mmol/L in the presence of catecholamines. In 4 additional patients, the effects of 9.25 mmol/L CaCl₂ were also investigated in the absence of catecholamines but in the presence of CGP20712A.

To determine relaxation and protein phosphorylation mediated by β₁-adrenergic receptors, trabeculae were exposed for 2 hours to ICI118551 (50 mmol/L) followed by incubation for 5 minutes with...
10 μmol/L (−)-norepinephrine. To assess β2-adrenergic receptor-mediated effects for tissues from the same patient, trabeculae exposed for 2 hours to CGP20712A (300 nmol/L) were incubated for 5 minutes with 10 μmol/L (−)-epinephrine or 10 μmol/L zintrol. To assess maximal effects mediated through both β1- and β2-adrenergic receptors, CGP20712A-treated trabeculae were exposed for 5 minutes to 200 μmol/L (−)-isoproterenol. Basal protein phosphorylation was determined in trabeculae from the same patient incubated with either CGP20712A or ICI118551 but not agonist.

**Processing of Trabeculae for Protein Phosphorylation and Immunodetection**

Freeze-clamped tissue derived from contracting trabeculae was homogenized in a histidine buffer containing NaF 25 mmol/L and phenylmethylsulfonyl fluoride 100 μmol/L as described. The homogenates were centrifuged at 100 000g; the pellet contained phosphorylamban, and the supernatant contained troponin I and C-protein.

**Protein Phosphorylation**

The method used, back-phosphorylation, has previously been described and adapted to human cardiac tissue. The phosphorylation reaction was started by the addition of [γ-32P]ATP and the catalytic subunit of cAMP-dependent protein kinase. We used 30 μg of protein per assay. After 5 minutes of incubation, the reaction was terminated with trichloroacetic acid, the resulting pellets were solubilized and boiled, and proteins were separated by PAGE. Radioactive bands corresponding to phosphorylamban, troponin I, and C-protein were identified according to molecular mass and by immunodetection. The calculated difference in 32P incorporation reflected agonist-induced endogenous phosphate incorporation into the intact trabeculae and is expressed as picomoles of phosphate per 1 mg protein. Protein was determined with BSA as the standard.

**Site-Specific Western Blot Analysis of Phosphorylamban**

Specific antibodies against phosphorylamban phosphoserine 16 and phosphorylamban phosphothreonine 17 were used as reported. Crude membrane fractions were solubilized at room temperature in a lysis buffer containing SDS. Blots were incubated with antibodies raised against a synthetic oligopeptide sequence of phosphorylamban with either a phosphorylated serine 16 or threonine 17 residue and visualized with an enhanced chemiluminescence-based detection system. To demonstrate the specificity of the immunological reaction, the procedures were also performed in the presence of 0.1 μmol/L of the corresponding 11–amino-acid-residue oligopeptides of phosphorylamban, phosphorylated at either Ser16 or Thr17.

**Statistical Analysis**

Data are expressed as sample mean ± SE. Student’s paired t test or 1-way ANOVA followed by the Bonferroni method was used for multiple comparisons by use of InStat (GraphPad software version 2.0). We used P<0.05 as the limit for statistical significance.

**Results**

**Positive Inotropic and Lusitropic Effects Mediated Through β1- and β2-Adrenergic Receptors**

(−)-Norepinephrine caused positive inotropic effects in the presence of the β2-selective blocker ICI118551 that were antagonized with high potency by the β2-selective blocker CGP20712A. The threshold concentrations of (−)-norepinephrine to increase contractile force were 300 nmol/L and 60 μmol/L in the absence and presence of CGP20712A (300 nmol/L), respectively, and were therefore mostly mediated through β2-adrenergic receptors. (−)-Epinephrine caused positive inotropic effects in the presence of the β1-selective blocker CGP20712A that were antagonized by the β2-selective ICI118551. The threshold concentrations of (−)-epinephrine were 60 nmol/L and 20 μmol/L in the absence and presence of ICI118551 (50 nmol/L), respectively, consistent with mediation through β2-adrenergic receptors. These results from hearts in terminal failure (data from 22 trabeculae of patients 1 through 3 of Table 1; not shown) agree with previous data from ventricular tissue from hearts in mild failure27 and ventricular myocytes.

To obtain robust phosphorylation signals, we selected a relatively high catecholamine concentration, 10 μmol/L, which, however, under the conditions of the present and previous studies, is receptor subtype–specific and produces nearly maximal effects through β1- or β2-adrenergic receptors. Marked positive inotropic and lusitropic effects of (−)-norepinephrine and (−)-epinephrine, mediated through β2- and β2-adrenergic receptors, respectively, were similar to those of (−)-isoproterenol (Figures 1 and 2). Zintrol, a β2-selective partial agonist effective in human ventricle32 and atrium,23 also caused positive inotropic and lusitropic effects, but the lusitropic effects tended to be smaller than those of the catecholamines (Figures 1 and 2) and the onset of response tended to be slower than with catecholamines (Figure 1).

**Comparison of Inotropic and Lusitropic Potencies of (−)-Norepinephrine and (−)-Epinephrine Through β1- and β2-Adrenergic Receptors**

To compare the inotropic and lusitropic potency of (−)-norepinephrine through β2-adrenergic receptors with the corresponding potencies of (−)-epinephrine through β2-adrenergic receptors, we determined concentration-effect curves for the 2 catecholamines under receptor-selective conditions. The lusitropic potencies of both (−)-norepinephrine and (−)-epinephrine were significantly greater than the corresponding inotropic potencies (Figure 3 and Table 2).

**High Ca2+ Does Not Hasten Relaxation**

High extracellular calcium concentration has been shown to abbreviate the duration of Ca2+ transients in canine cardiomyocytes23 and to hasten relaxation in guinea pig cardiomyocytes. To examine the effects of a high Ca2+ concentration, we compared the effects of 9.25 mmol/L CaCl2 with those of basal conditions (2.25 mmol/L) in trabeculae from patients 8 and 15 through 17 in Table 1. Increasing extracellular Ca2+ to 9.25 mmol/L failed to hasten relaxation, whereas (−)-norepinephrine, administered as a positive control, hastened relaxation (Figure 4). In 11 trabeculae from 4 patients, Ca2+ 9.25 mmol/L increased contractile force from 1.2±0.4 (SE from 4 patients) to 6.6±2.0 mN/mm2 but did not change relaxation parameters. The t60 and time to peak values were 127±7 and 126±5 ms, and 179±15 and 186±7 ms at 2.25 and 9.25 mmol/L CaCl2, respectively.

**Phosphorylation of Phosphorylamban, Troponin I, and C-Protein**

Both β1- and β2-adrenergic agonists reduced the capacity of tissues to incorporate 32P-phosphate into phosphorylamban, troponin I, and C-protein in vitro, as assessed after back-phosphorylation and shown representatively in Figure 5. This
Figure 1. Positive inotropic and lusitropic effects of β-adrenergic receptor agonists on 4 right ventricular trabeculae from patient 7 in Table 1. Tracings show recordings of contractile force and, immediately underneath, first derivative. Right-side diagrams are superimposed fast-speed recordings before and 5 minutes after agonist administration. Trabeclae were incubated for 5 minutes with 300 nmol/L CGP20712A and 10 μmol/L (-)-epinephrine or 10 μmol/L zinterol to selectively stimulate β2-adrenergic receptors, 300 nmol/L CGP20712A and 200 μmol/L (-)-isoproterenol to stimulate β1- and β2-adrenergic receptors, or 50 nmol/L ICI118551 and 10 μmol/L (-)-norepinephrine to selectively stimulate β1-adrenergic receptors. Agonist administration is indicated by arrow. Each agonist caused a positive inotropic response associated with reduction in time to reach peak force and hastened relaxation. Shown are fast-speed recordings of individual contractions (refer to bar calibrated at 100 ms) and slow-speed recordings (refer to bar calibrated at 1 minute).

Figure 2. Effects of β-adrenergic agonists on contractile force, time to peak force, and time to reach 50% relaxation (t50). Trabeculae were incubated with 10 μmol/L (-)-epinephrine (EPI) or 10 μmol/L zinterol (ZINT) in presence of 300 nmol/L CGP20712A to selectively stimulate β2-adrenergic receptors, 200 μmol/L (-)-isoproterenol (ISO) in presence of 300 nmol/L CGP20712A to stimulate β1- and β2-adrenergic receptors, or 10 μmol/L (-)-norepinephrine (NE) in presence of 50 nmol/L ICI118551 to stimulate β1-adrenergic receptors. Agonist-evoked changes are depicted by black. There were no differences in basal values for force, time to peak force, or t50 between groups (P>0.1). Numbers of patients are given in parentheses under the number of trabeculae. Values given are sample mean±SE. *P<0.05 for effects of β-adrenergic agonists.

Both (−)-norepinephrine and (−)-epinephrine induced site-specific phosphorylation of phospholamban through β1- (2 patients) and β2-adrenergic receptors (5 patients), respectively (Figure 7), as demonstrated in competition assays with synthetic phospho-oligopeptides (Figure 7). The (−)-epinephrine-evoked phosphorylation of phospholamban at both Ser16 and Thr17 was prevented by ICI118551 (50 nmol/L) (Western blots from 1 patient; not shown).

A trabeculum of 1 patient (patient 8 in Table 1) was exposed to high Ca²⁺ (9.25 mmol/L), which increased contractile force (basal, Ca²⁺ 2.25 mmol/L) from 1.4 to 10.5 mN but did not shorten the t50 of relaxation, shorten the time to the onset of relaxation, or produce phosphorylation at Ser16 or Thr17 of phospholamban (not shown).

Discussion

Both β1- and β2-Adrenergic Receptors Hasten Relaxation Through cAMP Pathways

Our results show that both the β1- and β2-adrenergic receptors mediate hastening of relaxation of human ventricular myocardium, consistent with an involvement of cAMP-dependent pathways for both receptors. (−)-Norepinephrine and (−)-epinephrine had similar lusitropic potencies through β1- and β2-adrenergic receptors, respectively, and the lusitropic intrinsic activity of (−)-epinephrine was nearly that of (−)-nor-
epinephrine. As expected from cAMP pathways, the PKA substrates implicated in relaxation, 34 suggesting similar coupling of these 2 receptors, but reluctant to couple to the cAMP pathway that hastens relaxation, and stress the importance of direct work on human myocardium.

Species-dependent differences between the functions of β1- and β2-adrenergic receptors may possibly be due to differences in coupling to the Gs-protein/cAMP pathway and differential coupling to Gs and Gt guanine nucleotide-sensitive transducer proteins that cause activation and inhibition of the cAMP pathway, respectively. In cat heart, stimulation of the Gs/cAMP pathway through β1- and β2-adrenergic receptors is proportional to the corresponding receptor densities, 34 suggesting similar coupling of these 2 receptors, but only β1-adrenergic receptors hasten relaxation. 18 In contrast, human cardiac β2-adrenergic receptors are more tightly coupled to the Gs/cAMP pathway 27,28,35,36 than are β1-adrenergic receptors, and this was later confirmed with human recombinant receptors. 37,38 The selective coupling of human β2-adrenergic receptors probably contributes to the marked lusitropic effects of (−)-epinephrine. 36 In ventricular myocytes from nonfailing rat hearts, the β2-adrenergic–Gs/cAMP pathway leading to relaxation can be demonstrated only in the presence of pertussis toxin, 25 presumably after inactivation of functional Gi. This suggests that coupling of the rat cardio-

### TABLE 2. Inotropic and Lusitropic Potencies and Intrinsic Activities of (−)-Norepinephrine and (−)-Epinephrine Through β1- and β2-Adrenergic Receptors

<table>
<thead>
<tr>
<th></th>
<th>(−)-Norepinephrine (β1)</th>
<th>(−)-Epinephrine Through β1- and β2-Adrenergic Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Log EC50</td>
<td>IA</td>
</tr>
<tr>
<td>Force</td>
<td>5.93±0.13</td>
<td>1.03±0.17</td>
</tr>
<tr>
<td>Time to peak force</td>
<td>6.18±0.19</td>
<td>0.96±0.03</td>
</tr>
<tr>
<td>t50</td>
<td>6.43±0.17*</td>
<td>0.99±0.01</td>
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</tbody>
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Data obtained from 18 and 19 individual trabeculae for (−)-norepinephrine and (−)-epinephrine, respectively, from 11 patients (patients 4 through 14; Table 1). Intrinsic activity (IA) was calculated as the fraction of the maximum effect of (−)-norepinephrine and (−)-epinephrine vs response of (−)-isoproterenol (200 μmol/L).

*P<0.05 vs force; †P<0.05 vs (−)-norepinephrine.
myocyte $\beta_2$-adrenergic receptor is tighter to $G_i$ than to $G_s$. The tighter coupling of human $\beta_2$-adrenergic receptors to the $G_i/cAMP$ pathway compared with $\beta_1$-adrenergic receptors may explain why, even in failing human ventricle (this work) in which $G_i$ function and mRNA levels ($G_{ic}$) are increased, $^{39-41}$ $\beta_2$-adrenergic receptor–mediated relaxation can still occur through the $G_i/cAMP$ pathway. It is thus possible that in nonfailing hearts with unaltered $G_i$ function, $\beta_2$-adrenergic receptors may mediate even more marked relaxant effects than in failing hearts.

One limitation of this study is that the magnitude of lusitropic effects did not correlate (not shown) with the degree of protein phosphorylation in the same ventricular preparation. Protein phosphorylation peaks may have preceded the observed lusitropic response, an issue that can be clarified only with future kinetic experiments. Our findings of significant PKA-dependent phosphorylation of phospholamban, troponin I, and C-protein through both $\beta_1$- and $\beta_2$-adrenergic receptors agree, however, with an earlier report of PKA stimulation $^{42}$ suggesting a causal relationship. Unlike the situation in many other species, $^{18-23}$ human ventricular (this work) and atrial $^{25}$ $\beta_2$-adrenergic receptors appear to function mainly through a PKA-dependent pathway. Support for this concept has also recently been provided for the $\beta_2$-adrenergic receptor–mediated increases in L-type $\mathrm{Ca}^{2+}$ current with an obligatory involvement of PKA in human atrial myocytes. $^{43}$ Zinterol increased the L-type $\mathrm{Ca}^{2+}$ current and appeared to dissociate slowly from the $\beta_2$-adrenergic receptors. $^{43}$ Correspondingly, we attribute the relatively slow inotropic and lusitropic onset observed in human ventricular trabeculae (Figure 1) to slow equilibration of zinterol with $\beta_2$-adrenergic receptors.

The catecholamine ($\sim$)-isoproterenol can induce phosphorylation of phospholamban at both Ser16 (through PKA) and Thr17 (through CaMKII) in rodent ventricle. $^{7,44}$ The CaMKII–catalyzed phosphorylation of phospholamban can contribute to increased contractility and hastened relaxation, provided dephosphorylation is negligible. $^{44}$ Type 1 phosphatase catalyzes this dephosphorylation, and the activity of type 1 phosphatase can, in turn, be inhibited by isoproterenol through PKA–catalyzed phosphorylation of protein phosphatase inhibitor-1 in guinea pig ventricular myocytes. $^{45}$ Our results show for the first time that Thr17 phosphorylation of phospholamban occurs in failing human
ventricle through both β1- and β2-adrenergic receptors. We suggest that the CaM kinase–catalyzed phosphorylation of Thr17 of phospholamban in human ventricle can be demonstrated because its dephosphorylation is retarded by simultaneous inhibition of type 1 phosphatase by the β1- or β2-adrenergic receptor–mediated phosphorylation of protein phosphatase inhibitor-1. High extracellular Ca2+ concentration does not appear to result in activation of sarcoplasmic reticulum CaM kinase because, in contrast to the catecholamines, it does not hasten relaxation in trabeculae from nonfailing or failing hearts (Figure 4) and can actually prolong contractions and Ca2+ transients of trabeculae from failing human ventricle.12 In agreement with this, we have seen in the trabeculae of patient 8 in Table 1 that high Ca2+ concentration did not hasten relaxation and did not induce phosphorylation at Thr17 or Ser16 of phospholamban, whereas (−)-epinephrine caused these effects through β2-adrenergic receptors, in line with an indirect role of PKA but not necessarily of high Ca2+ concentration per se. In contrast, concentrations of (−)-norepinephrine (through β1-adrenergic receptors) and (−)-epinephrine (through β2-adrenergic receptors) and Ca2+, which cause matching increases in contractile force, are associated with marked hastening of relaxation with the catecholamines only (even in the presence of high Ca2+ concentration; Figure 3) but not with Ca2+ alone.

**Possible Clinical Relevance**

We have conclusively shown that (−)-epinephrine, acting through β2-adrenergic receptors, and (−)-norepinephrine, acting through β1-adrenergic receptors, hasten relaxation with similar potency and efficacy and cause phosphorylation of proteins implicated in the relaxation process. These results require verification in myocardium from normal hearts of donors not treated with drugs, such as ACE inhibitors. It seems reasonable, however, to suggest that diastolic function of failing heart may be improved by the action of endogenous catecholamines, mediated through both β1- and β2-adrenergic receptors. For example, it is conceivable that during stress endogenous plasma epinephrine surges elicit not only tachycardia but also beneficial hastening of ventricular relaxation, mediated at least partly through β2-adrenergic receptors, thus producing a relative lengthening of diastole.

Because β2-adrenergic receptors hasten relaxation in failing human ventricular myocardium, it could be clinically desirable to selectively improve diastolic function under conditions in which β2-adrenergic receptors are blocked. This may happen in patients with chronic heart failure undergoing treatment with β1-selective blockers.46 It is plausible that in these patients endogenous epinephrine may actually contribute to an improvement in diastolic function via β2-adrenergic receptors. The likelihood of this occurring is enhanced by the observation that at least in human atrium β2-adrenergic receptor function is increased by long-term β1-adrenergic receptor blockade.24

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**References**


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