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Circ. Res. 2002;90;1181-1188; originally published online May 9, 2002;
DOI: 10.1161/01.RES.0000021115.24712.99

Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 75231
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Protein Kinase A Phosphorylates Titin’s Cardiac-Specific N2B Domain and Reduces Passive Tension in Rat Cardiac Myocytes

R. Yamasaki,* Y. Wu,* M. McNabb, M. Greaser, S. Labeit, H. Granzier

**Abstract**—β-Adrenergic stimulation of cardiac muscle activates protein kinase A (PKA), which is known to phosphorylate proteins on the thin and thick filaments of the sarcomere. Cardiac muscle sarcomeres contain a third filament system composed of titin, and here we demonstrate that titin is also phosphorylated by the β-adrenergic pathway. Titin phosphorylation was observed after β-receptor stimulation of intact cardiac myocytes and incubation of skinned cardiac myocytes with PKA. Mechanical experiments with isolated myocytes revealed that PKA significantly reduces passive tension. In vitro phosphorylation of recombinant titin fragments and immunoelectron microscopy suggest that PKA targets a subdomain of the elastic segment of titin, referred to as the N2B spring element. The N2B spring element is expressed only in cardiac titins, in which it plays an important role in determining the level of passive tension. Because titin-based passive tension is a determinant of diastolic function, these results suggest that titin phosphorylation may modulate cardiac function in vivo. (Circ Res. 2002;90:1181-1188.)

**Key Words:** connectin  ■  diastole  ■  myocyte mechanics  ■  β-adrenergic signaling  ■  resting tension

Stimulation of β-adrenergic receptors on cardiac myocytes initiates signaling pathways that enhance contractility and accelerate relaxation. These effects are mediated by cAMP-dependent protein kinase A (PKA), which phosphorylates a host of intracellular substrates, including accessory proteins on the thin and thick filaments. Phosphorylation of troponin I (TnI) on the thin filament reduces the myofibrillar Ca2⁺ sensitivity. On the thick filament, phosphorylation of myosin binding protein-C (MyBP-C) is thought to increase the flexibility of the myosin head by inhibiting a restraining interaction between MyBP-C and the S2 region of the myosin rod. The heart expresses specific isoforms of MyBP-C and TnI with sequence insertions containing PKA phosphorylation sites. The presence of these sites makes the cardiac isoforms of TnI and MyBP-C uniquely responsive to β-adrenergic stimulation relative to their skeletal muscle counterparts.

In addition to the thin and thick filaments, striated muscles also contain a third filament system composed of the giant protein titin. Individual titin molecules span the distance between the M line and the Z line to form an elastic connection between the A band and the ends of the sarcomere (see recent review and original citations). As the muscles are stretched from their slack length, the I-band–spanning segment of titin extends and generates an opposing force, referred to as passive tension. The titin-based passive tension is important in the heart, where it underlies the majority of the diastolic wall stress and may affect systolic function.

In the present work, we investigated whether the β-adrenergic pathway can also modulate the properties of titin. Our findings indicate that PKA phosphorylates titin and causes a significant decrease in passive tension in skinned rat cardiac myocytes. In vitro phosphorylation assays with recombinant titin fragments and immunoelectron microscopy (IEM) on ventricular muscle strips suggest that a large subdomain of the elastic region of titin, referred to as the N2B spring element, is the target site for PKA. The N2B spring element allows cardiac titins to function as adjustable springs by modulating the length of the elastic segment of titin in response to muscle stretch.

Because the N2B spring element is differentially spliced into only the cardiac titin isoforms, these findings demonstrate that like TnI and MyBP-C, titin contains a cardiac-specific domain that serves as a PKA substrate. We propose that when β-adrenergic stimulation enhances the heartbeat frequency and rate of contraction and relaxation, titin phosphorylation and the ensuing reduction in passive tension allow for more rapid and complete ventricular filling.
Materials and Methods

Myocyte Isolation

Left ventricular cardiac myocytes were enzymatically isolated from male Sprague-Dawley rats (as explained in an expanded Materials and Methods section, which can be found in the online data supplement available at http://www.circresaha.org/). To depress background PKA phosphorylation, rats were injected with (-)-propranolol (1 mg/200 g IP) 12 hours and 2 hours before euthanasia, and 2 μg/mL propranolol was included in all solutions through skinnning, as described previously.15,16 Furthermore, 1 μM L of the choliner-gic agonist acetylcholine16,17 was included in cell isolation solutions. Before skinning, cells were brought up in 1 mMol/L Ca2+ and incubated with either 0.1 μMol/L isoproterenol or 2 μMol/L propranolol for 6 minutes at 37°C. Myocytes were rapidly skinned and washed extensively to remove endogenous kinases. In earlier experiments, titin degradation occurred (an example is shown in Figure 1C), which was eliminated in subsequent experiments by suspending the cells in relaxing solution (RS) with a high concentration of EGTA before skinning (for ~60 seconds).

Myocyte Phosphorylation Studies

Suspensions of skinned myocytes were incubated for 40 minutes at room temperature in RS containing 1000 cpm/μmol [γ-32P]ATP either (1) alone, (2) with 1 U/μL of purified PKA (catalytic subunit from bovine heart, Sigma Chemical Co), or (3) with 1 U/μL PKA+15 μMol/L PKI (PKA-specific inhibitor TYANFISAGRT-GRNRML). The cells were solubilized and electrophoresed on 2% to 12% gradient gels, stained with Coomassie blue, destained, dried, and exposed for variable durations (~2 to 50 hours) to autoradiographic film with an intensifying screen at ~80°C. Coomassie blue-stained gels and their derived autoradiographs were scanned, and the integrated optical density (OD) of titin (T1), MyBP-C, and TnI bands was determined. For each sample, a range of loadings was electrophoresed on the same gel (for examples, see Figures 2A, 2B, and 2D), allowing us to determine integrated OD versus loading obtained from regression analysis. The slope ratio for samples treated with isoproterenol versus those treated with propranolol was then calculated (samples were run on the same gel). The ratio obtained from Coomassie blue-stained gels reflects differences in the relative phosphorylation levels of titin, MyBP-C, and Tn-I in the absence of PKA (control). These results are similar to those typically shown in studies of PKA-based myofibrillar protein phosphorylation. Titin phosphorylation is usually not assessed in these studies because its large size (M, ~3 MDa) prevents it from entering the pores of standard SDS gels.

On the 24-hour exposure (Figure 1B, right lane), with the use of low-percentage acrylamide gels and sensitive detection techniques, titin was shown to be phosphorylated by PKA in cardiac myocytes. In the absence of PKA (Figure 1B, left lane), no significant 32P incorporation was detected. In earlier experiments, titin degradation was present, an example of which is shown in Figure 1C. PKA treatment of cells with partially degraded titin revealed that the intact titin molecule (T1) was phosphorylated but that the T2 degradation product was not. Titin degradation was largely eliminated in subsequent experiments (see Materials and Methods).

In preliminary experiments, we also observed low phosphorylation levels of titin, MyBP-C, and Tn-I in the absence of exogenous PKA (results not shown). In these experiments, intact cells were suspended for ~1 hour in HEPES that was calcium free, suggesting that endogenous kinases have a higher activity or diffuse more slowly from such cells than

Statistical Analysis

Results are reported as mean ± SEM. Differences were assessed by using the Student t test, with P<0.05 indicating a significant difference.

Results

PKA Phosphorylates Titin in Skinned Myocytes

To mimic β-adrenergic stimulation, skinned rat left ventric- ular myocyte suspensions were incubated with the catalytic subunit of PKA, as described previously,17,19–21 in the presence of [γ-32P]ATP. Cells were then solubilized and electrophoresed, and phosphorylated proteins were detected via autoradiography. To reduce background PKA phosphorylation, hearts were perfused with propranolol and acetylcholine during cell isolation (see Materials and Methods). Figure 1A shows a Coomassie blue-stained gel along with autoradiographs from 2- and 24-hour exposures (Figure 1B). After a 2-hour exposure, phosphorylation of TnI and MyBP-C was clearly detected in the presence of PKA, with no detectable phosphorylation in the absence of PKA (control). These results are similar to those typically shown in studies of PKA-based myofibrillar protein phosphorylation. Titin phosphorylation is usually not assessed in these studies because its large size (M, ~3 MDa) prevents it from entering the pores of standard SDS gels.

In Vitro Kinase Assay

Titin fragments (Figure 5) were incubated in a 20 μL total volume of kinase buffer (mmol/L: HEPES 25 [pH 7.4], KCl 100, MgCl2 10, ATP 2, and dithiothreitol 1) containing 555 cpm/μmol [γ-32P]ATP and 10 mU PKA. Reactions were incubated at 20°C to 22°C for 10 minutes and terminated with 90°C solubilization buffer. Samples were electrophoresed on 12% SDS gels, stained, destained, dried, and exposed, as described above.

Immunoelectron Microscopy

Skinned muscle strips were incubated with either 1 U/μL PKA or 1 U/μL PKA+15 μmol/L PKI for 40 minutes (RS in control experiments), washed four times with RS, and incubated for 100 minutes with RS containing 0.125 mg/mL FX-45, a recombinant fragment constituting the N-terminal half of human gelsolin. IEM was performed with the use of monoclonal antibodies to phosphoserine and phosphothreonine.

Western Blotting

Cells were solubilized, electrophoresed, and transferred to nitrocel-lulose membranes, as described earlier.18 Membranes were treated with anti-phosphoserine primary antibody and detected with horse-radish peroxidase and enhanced chemiluminescence.
It is well established that activation of the \(\beta\)-adrenergic pathway increases phosphorylation of TnI and MyBP-C.\(^{22,23}\) Whether phosphorylation of titin can also be enhanced by \(\beta\)-adrenergic agonists was investigated by using back-phosphorylation experiments. Cell suspensions were subjected to either the \(\beta\)-receptor antagonist propranolol or the \(\beta\)-receptor agonist isoproterenol, followed by rapid skinning and incubation with \(^{32}\text{P}\)ATP and PKA. Cell suspensions were then solubilized, electrophoresed, stained with Coomassie blue, and exposed to radiographic film. Individual gels were loaded with samples from both propranolol- and isoproterenol-treated cells, and a range of loadings was used to establish the relative protein content and \(^{32}\text{P}\) incorporation in each sample. With this method, protein phosphorylation in response to \(\beta\)-stimulation is detected by a decreased incorporation of \(^{32}\text{P}\) in isoproterenol-treated cells relative to propranolol-treated cells.

Figures 2A and 2B show \(^{32}\text{P}\) incorporation by MyBP-C and TnI after propranolol and isoproterenol treatment. Although a given loading of propranolol- or isoproterenol-treated samples contained similar quantities of MyBP-C and TnI (gel in Figures 2A and 2B), a smaller amount of \(^{32}\text{P}\) was incorporated into both proteins in isoproterenol-treated cells (radiograph in Figures 2A and 2B). To quantify differences in \(^{32}\text{P}\) incorporation, the integrated OD of radiographic bands was plotted as a function of loading (Figure 2C, left panel), and the slope of the linear range was calculated for isoproterenol- and propranolol-treated samples. The slopes were corrected for differences in protein content of the samples (differences were generally small), and the slope ratio of isoproterenol versus propranolol was then determined. Figure 2E shows that relative to propranolol, isoproterenol treatment decreased \(^{32}\text{P}\) incorporation by 47±9\% (\(n=5\)) and 42±9\% (\(n=5\)) for MyBP-C and TnI, respectively. These values are statistically significant (\(P<0.005\)) and similar to those reported by Strang et al.\(^{15}\)

\(^{32}\text{P}\) incorporation by titin is shown in Figure 2D, and the integrated OD versus loading relation is shown in Figure 2C, right panel. As observed for MyBP-C and Tn-I, isoproterenol treatment resulted in reduced \(^{32}\text{P}\) incorporation. The mean results of 6 experiments are shown in Figure 2E, right bar. Relative to propranolol, isoproterenol treatment decreased \(^{32}\text{P}\) incorporation by 52±12\% (\(P<0.01\)). These findings indicate that titin is phosphorylated by PKA in response to \(\beta\)-adrenergic stimulation in intact myocytes.

**Stoichiometry**

A stoichiometric estimate of phosphate incorporation by titin was obtained by comparing the level of titin phosphorylation in the presence of PKA with that of MyBP-C. We determined the slope of integrated OD versus loading for titin (T1) and MyBP-C bands on autoradiograms and calculated their ratio. This ratio was then corrected for the molar ratios of titin to MyBP-C, which are based on densitometry of protein gels and molecular weights of 2.97 MDa for titin (T1) and 140 kDa for MyBP-C. On a molar basis, titin incorporated 47±3\% (\(n=5\)) of the \(^{32}\text{P}\) incorporated by MyBP-C. Previous studies with cardiac muscle have estimated that PKA phosphorylates MyBP-C with a stoichiometry of \(\approx3\) mol phosphate per mole of substrate.\(^{5,23-25}\) A comparison of these results with the present findings suggests that \(\approx1.4\) mol phosphate is incorporated per mole of titin during PKA stimulation. The phosphate incorporation by titin was also determined with an alternative method. PKA-treated cell samples were electrophoresed, and the amount of protein in the T1 band was determined by using coelectrophoresed BSA protein standards (see online Materials and Methods section). In two separate experiments, liquid scintillation counting of the T1 bands revealed that 0.85 and 0.89 mol \(^{32}\text{P}\) were incorporated per mole of titin. Thus, the two different
methods suggest that PKA stimulation results in incorporation of \( \approx 1 \) mol phosphate per mole of titin.

**Effect of PKA on Passive Tension Generation**

To determine whether PKA phosphorylation affects the tension-generating properties of titin, passive tension was measured in skinned rat cardiac myocytes. Figure 3 shows representative force-SL traces before and after incubation with either PKA alone (Figure 3A) or PKA in the presence of PKi (Figure 3B). PKA decreased passive tension, whereas PKA in the presence of PKi had a minimal effect. Figure 4A shows mean passive tension values as a function of time (n=8 cells). Passive tension decreased during the first \( \approx 20 \) minutes of incubation, after which, passive tension attained a plateau. Figure 4B shows steady-state passive tension as a function of SL. Tension was reduced to \( \approx 40\% \) of the control value at 2.0 \( \mu m \) SL, and reductions of lesser magnitudes were observed at longer lengths. Passive tension was also decreased slightly in the presence of PKA-PKi (PKA inhibitor). Thus, we compared the data from PKA-treated cells with that from cells in the presence of PKA-PKi. Results in the presence of PKA were significantly less than those in the presence of PKA-PKi (Table). Because the extension of titin underlies almost all of the passive tension generated in skinned myocytes,\(^9\) we conclude that the observed decrease in passive tension is likely due to PKA-
based titin phosphorylation. For a discussion of alternatives, see below.

**N2B Spring Element Is Targeted by PKA In Vitro and In Situ**

The effect of PKA on passive tension suggests the presence of a phosphorylation site(s) within the extensible region of titin. This hypothesis was evaluated by expressing recombinant fragments representing the subdomains of cardiac N2B titin and testing them as PKA substrates in vitro. The fragments (Figure 5A) included the following: (1) the 572-residue unique sequence (the N2B spring element) from the cardiac-specific N2B splice element (uN2B), (2) an 8 immunoglobulin-like domain construct from the distal tandem immunoglobulin segment (I91-I98), and (3) the PEVK domain along with its flanking immunoglobulin-like domains (PEVK). In the absence of PKA, none of the fragments incorporated radioactive phosphate, whereas in the presence of PKA, only the N2B spring element was phosphorylated (Figure 5B). The N2B spring element plays an important role in passive tension generation in cardiac muscle, making it plausible that its phosphorylation by PKA could significantly affect passive tension, as shown in Figure 4.

To determine whether the N2B spring element is also targeted by PKA in the sarcomere, we used IEM. Skinned rat left ventricular muscle strips were incubated with the catalytic subunit of PKA, and phosphorylated residues were detected with anti-phosphoprotein antibodies. To eliminate labeling targets that might obscure I-band phosphorylation sites on titin (such as TnI), thin filaments were selectively extracted with a recombinant gelsolin fragment. Labeling was either absent or spotty in sarcomeres treated with PKA (Figure 6A, top), whereas well-defined epitopes were observed in the central I-band regions of PKA-treated strips labeled with anti-phosphothreonine and anti-phosphoserine antibodies (Figure 6A, middle and bottom). The position of the I-band epitopes varied with SL, indicating that the phosphorylated residues were located within the extensible segment of titin rather than the residual thin filament proteins or other sarcomeric components. Because the anti-phosphoprotein antibodies also react with MyBP-C (Figure 6B), A-band labeling was expected but not observed (Figure 6A). It appears that phosphorylated MyBP-C residues are accessible to antibodies when MyBP-C is blotted to nitrocellulose membrane but not when present in the A band.

In previous work with rat cardiac muscle, the extensible behaviors of the subdomains constituting the elastic region of N2B titin were characterized, including the N2B spring element. Sequence-specific antibodies were used to label the boundaries of each subsegment, and the positions of the

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<tr>
<td>Passive tension: after PKA treatment (mN/mm²; n=8)</td>
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<td>PKA-induced tension reduction (% of control)</td>
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<td>PKA+PKI-induced tension reduction (% of control; n=8)</td>
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*Passive tension before treatment.
†Comparison of PKA and PKA+PKI results.

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**Figure 5.** In vitro PKA phosphorylation survey of extensible segment of titin. A, Schematic diagram showing domain organization of elastic region of titin (N2B isoform) and location of recombinant fragments representing the 572-residue unique sequence within the N2B element (uN2B), the PEVK domain (PEVK), and the tandem immunoglobulin segments (I91-I98). B, Coomassie blue-stained gel of recombinant fragments incubated with γ-32P]ATP in the absence (−) and presence (+) of PKA (left) and autoradiogram showing phosphorylation of uN2B by PKA (right).
epitopes relative to the Z line were measured as a function of SL by using IEM. A comparison of these data with the data produced from a similar analysis of the phosphothreonine and phosphoserine epitopes allowed us to estimate their positions within the titin filament. Figure 6C shows the mobility of these epitopes in an overlay with those of the Un and Uc antibodies, which label the N- and C-terminal ends, respectively, of the N2B spring element. The phosphoepitopes occupied positions between Un and Uc as sarcomeres were stretched, indicating that they are located within the N2B spring element. The presence of phosphorylated residues in the N2B spring element in PKA-treated samples is consistent with our in vitro findings (Figure 5) and further implicates this region of titin as a target for PKA.

Discussion

Numerous studies have shown that in addition to its role in passive tension generation, titin also participates in cell-signaling pathways. Titin contains a serine/threonine kinase domain located at the periphery of the M line in the sarcomere. The titin kinase is thought to phosphorylate the Z-line–associated protein telethonin during myofibrillogenesis and may have additional roles in adult tissues. Titin is also targeted by kinases in muscle cells. Skeletal muscle titins have been identified as phosphoproteins in situ, and titin phosphorylation by cdc2 kinase and extracellular signal–regulated kinase 1 has been demonstrated in vitro. In the present study, we report titin phosphorylation on β-receptor stimulation of intact cardiac myocytes as well as incubation of skinned cardiac myocytes with PKA. Mechanical experiments with isolated myocytes have revealed that PKA significantly reduces passive tension. We have also demonstrated that (as with the well-characterized myofibrillar PKA substrates MyBP-C and TnI) titin contains a PKA-responsive domain expressed only in cardiac muscle. Because the activation of PKA via β-adrenergic stimulation constitutes a major regulatory pathway in the heart, our findings may have implications for the modulation of diastolic function in vivo.

Titin Phosphorylation

We found that only T1, not T2, is phosphorylated by PKA (Figure 1C). T2 is the product of proteolytic cleavage within the PEVK domain and constitutes the C-terminal portion of the molecule. These findings suggest that the PKA-based titin phosphorylation observed in Figures 1 and 2 is targeted to site(s) N-terminal of the PEVK domain. This notion is supported by our in vitro experiments with recombinant I-band titin fragments (Figure 5), which detected PKA phosphorylation only N-terminally to the PEVK domain, in the N2B spring element. Moreover, the detection of phosphorylated residues within the N2B spring element in situ by IEM (Figure 6) provides further evidence that this region of titin serves as a PKA substrate. A recent sequence comparison has revealed multiple short insertions/deletions in the N2B element of different species (M. Greaser, unpublished data, 2002). The human N2B spring element contains a threonine PKA consensus phosphorylation site (QRVT), as defined by Kennelly and Krebs, whereas a different consensus PKA phosphorylation site is found in rat (M. Greaser, unpublished data, 2002). Considering that PKA may also phosphorylate nonconsensus sites, future experimental work is required to precisely establish which residues of the N2B sequence are phosphorylated by PKA.

Although we did not observe labeling at sites other than the N2B spring element, the work of Somerville and Wang suggests that there may be additional PKA target sites in skeletal muscle titin. The authors observed an increase of phosphate incorporation into titin on β-adrenergic stimulation of intact skeletal muscle. Because skeletal muscle titin does not contain the N2B element, a possible site for phosphorylation is the N2A element, which is expressed in the central I-band region in skeletal titins but is absent in the myocardium of rodents, which express predominantly the N2B titin...
isoform. To determine whether the N2A element could provide an additional PKA target, we tested it as a PKA substrate in vitro. No phosphorylation was observed under conditions in which the N2B spring element was phosphorylated (data not shown), suggesting that the phosphorylation observed by Somerville and Wang occurred at a site(s) other than the N2A or N2B element. Possibilities are the additional immunoglobulin-like domains and longer PEVK domain present in skeletal titins.

**Effect on Passive Tension**

Incubation of skinned myocytes with the catalytic subunit of PKA significantly reduced passive tension in an SL-dependent manner, with an \( \approx 60\% \) decrease observed at an SL of 2.0 \( \mu \)m and lesser reductions at SLs of >2.0 \( \mu \)m (Figure 4B). The \( \approx 20\% \) reduction that we observed at a SL of \( \approx 2.3 \mu \)m is consistent with the results of Strang et al., who measured an \( \approx 25\% \) to 45\% reduction in the resting tension of skinned rat myocytes at an SL of 2.3 \( \mu \)m on incubation with the catalytic subunit of PKA. Because passive tension of myocytes is derived mainly from titin, the effect of PKA on passive tension is probably titin-based. Although intermediate filaments also contribute to the passive tension along the physiological SL range in the heart, this contribution is minor (approximately a few percent of that of titin). The reduction in passive tension that we observed may result from interactions between titin and TnI or MyBP-C that are modulated by phosphorylation of TnI or MyBP-C. However, this possibility is not likely because extracting TnI via removal of the thin filament does not significantly affect passive tension at SLs \( \leq 2.2 \mu \)m, where we found a large reduction in passive tension after PKA treatment. A role for MyBP-C is also improbable because it is found in the A-band region of the sarcomere, where titin is inextensible. Therefore, we conclude that the reduction of passive tension after PKA treatment likely results from an intrinsic change in the elasticity of titin.

**Mechanism**

The SL dependence of the passive tension decrease observed in our experiments may be a function of the nonlinear extension-SL relation of the N2B spring element. At SLs \( \leq 2.2 \mu \)m, titin extends predominantly within the tandem immunoglobulin and PEVK spring elements, and the N2B spring element is relatively inextensible. At longer SLs, at which the extensibility of the tandem immunoglobulin and PEVK springs are nearly exhausted, the N2B spring is extended and provides the majority of the extensibility of titin and determines the level of passive tension. The passive tension decrease measured in the presence of PKA was largest at short SLs and decreased at longer SLs (Figure 4). This suggests that phosphorylation may affect N2B extension at low degrees of sarcomere stretch, at which it likely assumes a compact folded conformation. Phosphorylation could, for example, destabilize native structures within the N2B element, causing it to extend. This would give the tandem immunoglobulin segments and PEVK domain lower fractional extensions at a given SL, leading to a decrease in passive tension. At longer SLs, these structures will be denatured because of stretch; therefore, phosphorylation will have less impact on the extension of the N2B element and, consequently, on passive tension.

**Conclusions**

Results indicate that the N2B spring element adjusts the force of titin at short to intermediate SLs in response to \( \beta \)-adrenergic stimulation. We speculate that when \( \beta \)-adrenergic stimulation enhances the heartbeat frequency and rate of contraction and relaxation, the reduction in the force of titin resulting from N2B phosphorylation allows for more rapid and complete ventricular filling. Changes in ventricular filling due to titin phosphorylation and possible modifications of this mechanism in heart diseases with altered \( \beta \)-adrenergic signaling are areas for further investigation.

**Acknowledgments**

This study was supported by the American Heart Association, Northwest Affiliate (predoctoral fellowship to R. Yamasaki), by Deutsche Forschungsgemeinschaft (La La668/5-2 to Dr Labeit), and by the National Institutes of Health (HL-61497 and HL-62881 to Dr Granzier and HL-62466 to Dr Greaser).

**References**


