Protein Kinase G Modulates Human Myocardial Passive Stiffness by Phosphorylation of the Titin Springs

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Abstract—The sarcomeric titin springs influence myocardial distensibility and passive stiffness. Titin isoform composition and protein kinase (PK)A-dependent titin phosphorylation are variables contributing to diastolic heart function. However, diastolic tone, relaxation speed, and left ventricular extensibility are also altered by PKG activation. We used back-phosphorylation assays to determine whether PKG can phosphorylate titin and affect titin-based stiffness in skinned myofibers and isolated myofibrils. PKG in the presence of 8-pCPT-cGMP (cGMP) phosphorylated the 2 main cardiac titin isoforms, N2BA and N2B, in human and canine left ventricles. In human myofibers/myofibrils dephosphorylated before mechanical analysis, passive stiffness dropped 10% to 20% on application of cGMP-PKG. Autoradiography and anti-phosphoserine blotting of recombinant human I-band titin domains established that PKG phosphorylates the N2-B and N2-A domains of titin. Using site-directed mutagenesis, serine residue S469 near the COOH terminus of the cardiac N2-B–unique sequence (N2-Bus) was identified as a PKG and PKA phosphorylation site. To address the mechanism of the PKG effect on titin stiffness, single-molecule atomic force microscopy force–extension experiments were performed on engineered N2-Bus–containing constructs. The presence of cGMP-PKG increased the bending rigidity of the N2-Bus to a degree that explained the overall PKG-mediated decrease in cardiomyofibrillar stiffness. Thus, the mechanically relevant site of PKG-induced titin phosphorylation is most likely in the N2-Bus; phosphorylation of other titin sites could affect protein–protein interactions. The results suggest that reducing titin stiffness by PKG-dependent phosphorylation of the N2-Bus can benefit diastolic function. Failing human hearts revealed a deficit for basal titin phosphorylation compared to donor hearts, which may contribute to diastolic dysfunction in heart failure. (Circ Res. 2009;104:87-94.)

Key Words: cGMP ■ nitric oxide ■ diastolic function ■ connectin ■ passive tension

Myocardial and chamber diastolic function are influenced by chamber geometry, hypertrophy, the extracellular matrix and the sarcomeric titin springs. Titins are giant proteins which exist in the heart in 2 main isoforms coexpressed in sarcomeres: a shorter, stiffer N2B-titin (3.0 MDa) and longer, more compliant N2BA isoforms (3.2 to 3.7 MDa). Differential expression of these isoforms is related to alternate gene splicing affecting the functionally elastic titin region, which is confined to the sarcomeric I-band.1 The springy titin segment comprises regions of serially linked immunoglobulin-like (Ig) domains separated by a cardiac-specific N2-B domain and a so-called PEVK segment (rich in proline, glutamate, valine, and lysine residues). The N2BA isoforms additionally have an N2-A domain and contain more Ig domains and PEVK-rich modules compared to the N2B isoform.

Differential expression of titin isoforms determines passive stiffness of the sarcomere.2,3 A low ratio of N2BA:N2B isoforms is found in sarcomeres with relatively high passive tension (PT), a high ratio in those with low PT. There is evidence for species-, age, transmural-, and chamber-specific differences in titin isoform expression.2,3 Patients with systolic heart failure (SHF) caused by coronary artery disease or dilated cardiomyopathy (DCM) express increased proportions of compliant N2BA isoforms in the left ventricles (LVs) and have lower-than-normal passive myofibrillar stiffness,4–6 possibly in an attempt to rescue diastolic function. In contrast, a recent study pooled endomyocardial biopsy specimens from patients with diastolic heart failure (DHF) and found reduced N2BA:N2B ratios as compared to SHF; whereas myocyte passive stiffness was high.7

Titin-based stiffness can also be modulated by posttranslational modification. Phosphorylation of a unique 572 amino acid sequence within the N2-B domain (N2-Bus) by cAMP-dependent protein kinase (PK)A lowers passive stiffness in rat, bovine, and human myocardium via yet unknown mech-
anisms.\textsuperscript{8–11} Interestingly, incubating skinned human cardiomyocytes with PKA produced a substantial reduction in passive stiffness in both SHF and DHF but more so in DHF,\textsuperscript{7} suggesting there may be a deficit for PKA-mediated titin phosphorylation which could explain part of the elevated passive stiffness of failing human hearts. The PKA effect on titin adds to the many other alterations in diastolic, as well as systolic function triggered by β-adrenergic stimulation.\textsuperscript{12,13}

The influence of cAMP signaling on cardiac function can in part be opposed by cGMP,\textsuperscript{14,15} which is generated by guanylyl cyclases in response to nitric oxide (NO) and natriuretic peptides. Cross-talk exists between cAMP and cGMP signaling with regard to the activity of the cyclic nucleotide-degrading enzymes, phosphodiesterases (PDEs), in that cGMP stimulated (PDE-2) or inhibited (PDE-3) PDEs hydrolyze both cGMP and cAMP.\textsuperscript{15,16} Importantly, inhibition of cGMP-hydrolyzing PDE-5 by sildenafil ameliorated cardiac hypertrophy, fibrosis and systolic dysfunction in a murine model of pressure overload.\textsuperscript{17} NO- or cGMP-diac hypertrophy, fibrosis and systolic dysfunction in a murine model of pressure overload.\textsuperscript{17} NO- or cGMP-enhancing therapies increased resting diastolic cell length in isolated myocytes and downward-shifted the diastolic pressure–volume relationship during filling of intact hearts, indicating decreased myocardial stiffness and enhanced left ventricular distensibility.\textsuperscript{18–21} Extensive in vitro and in vivo studies have demonstrated anti hypertrophic, antifibrotic, and proliferotic effects of natriuretic peptides.\textsuperscript{22–24} Thus, substantial evidence suggests that beneficial effects of cGMP can be augmented by exogenous natriuretic peptides or NO donors or inhibition of cGMP metabolism by a variety of PDEs. Although activating the cGMP effector PKG clearly affects mechanical properties known to be determined in part by the titin springs, such as diastolic tone (lowered by PKG), left ventricular extensibility (increased by PKG), and relaxation speed (accelerated by PKG), to date, data on a possible PKG-mediated influence on titin function have been lacking.

We wanted to know whether cGMP-activated PKG can phosphorylate titin in mammalian, particularly human, heart and whether this posttranslational modification can improve diastolic function by reducing titin-based stiffness. Indeed, we detected phosphorylation sites for PKG in human cardiac titin and found a PKG-dependent decrease in myofibrillar stiffness. This mechanical effect is most likely mediated by phosphorylation of a serine residue in the cardiac-specific N2-Bus. Thus, cGMP-enhancing therapy could benefit diastolic function via increasing titin phosphorylation, thereby reducing titin stiffness.

Materials and Methods

Heart Tissue

Left ventricular tissue from human donor and failing DCM hearts was procured, deep-frozen in liquid nitrogen (LN\textsubscript{2}), and stored at −80°C (see the expanded Materials and Methods section in the online data supplement, available at http://cires.ahajournals.org). Left ventricular tissue of normal adult dogs was sampled as described.\textsuperscript{25} All procedures were conducted in full accordance with the institutional guidelines and were approved by the respective ethics committees.

Skinned Fiber Preparation

Left ventricular muscle strips were prepared from unfrozen human hearts and skinned in relaxing solution (for composition, see the expanded Materials and Methods section) supplemented with 1% Triton-X-100.\textsuperscript{4,26} The tissue was washed in relaxing solution and fiber bundles were dissected (diameter, 200 to 300 μm; length, 1.0 to 2.5 mm).

PT Recordings

Skinned human fibers were studied at 26°C using a muscle mechanics workstation (Scientific Instruments, Heidelberg, Germany).\textsuperscript{4,6,26} Force and sarcomere length (SL) were recorded on stepwise stretching the samples from slack SL (average, 1.9 μm) to a maximum SL of 2.6 to 2.7 μm. Another set of experiments was done after 30-minute incubation with 1.68×10\textsuperscript{-5} U/μL purified PKG and 300 μmol/L 8-pCPT-cGMP (cGMP) (Calbiochem). Alternatively, fibers were dephosphorylated by alkaline phosphatase (AP) (New England Biolabs) or protein phosphatase (PP)\textsubscript{2a} (Sigma-Aldrich, recombinant catalytic subunit, 0.3 U/μL) before incubation with PKG and cGMP in presence of phosphatase-inhibitor cocktail (Sigma-Aldrich).

Myofibril Force Measurements

Myofibrils isolated from left ventricular tissue of human donor heart as described\textsuperscript{6} were stretched-release in relaxing solution from slack SL by 50% at a frequency of 1 Hz for 10 seconds (room temperature). The force amplitudes in each cycle were measured and averaged to obtain passive stiffness. Recordings were taken before and after addition of cGMP-PKG.

Recombinant Titin Fragments

Human cardiac titin fragments were expressed in Escherichia coli and purified by the GST-Fusion System (Amersham Biosciences): I\textsubscript{2} to I\textsubscript{4}, N2-B (I\textsubscript{24/25–N2-Bus–I\textsubscript{26}), I\textsubscript{24/25}, N2-Bus, N terminus of N2-Bus (C411–572), middle part of N2-Bus (M190–410), C terminus of N2-Bus (C411–572), N-terminally truncated C terminus of N2-Bus (C420–572), C-terminally truncated C terminus of N2-Bus (C411–545), C terminus of N2-Bus with mutation at position 469 (CS469A), I26, N2-A, and PEVK (N2B-PEVK).

SDS-PAGE and 32P-Autoradiography

Phosphorylation by PKG and PKA was probed by autoradiography following 15% or 2% SDS-PAGE as reported.\textsuperscript{26} Recombinant titin fragments were analyzed after incubation with 1.68×10\textsuperscript{-5} U/μL PKG, cGMP, and [\textsuperscript{32}P]ATP (250 μCi/μM) for 20 minutes at 36°C. Some constructs were incubated with catalytic subunit of PKA (Biaffin, 1 U/μL) instead of cGMP-PKG. Skinned fibers were either directly phosphorylated or were first dephosphorylated by AP or PP\textsubscript{2a} (0.3 U/μL) before incubation with the respective kinase.

Dot Blot Analysis

Recombinant titin fragments were dephosphorylated by AP before incubation with cGMP and PKG, solubilized, and dotted onto a poly(vinylidene fluoride) membrane. The membrane was probed by monoclonal antibodies against phosphoserine residues (phosphoDetect anti-mouse mAb, clone 16B4, Calbiochem).

Atomic Force Microscopy Force Spectroscopy

Single-molecule force–extension traces were recorded (pulling rate, 500 nm sec\textsuperscript{-1}) at 22°C with an MFP-3D atomic force microscope (Atomic Force, Mannheim, Germany) using the engineered constructs I\textsubscript{25-I\textsubscript{26-N2-Bus-L26-I27} or N2-A (see the online data supplement), stretched under the same buffer conditions as in the fiber/myofibril measurements. Data were analyzed by Igor procedures (WaveMetrics, Portland, Ore) using the worm-like chain (WLC) model:\textsuperscript{27}

\[
F = \frac{k_N T p}{4} \left( \frac{1}{x - 2 + x^2} - \frac{1}{4 + x^2} \right)
\]

where \( F \) is entropic force; \( L_p \), persistence length; \( x \), end-to-end distance; \( L_c \), contour length; \( k_B \), Boltzmann constant; and \( T \), absolute temperature.
PKG Phosphorylates Cardiac Titin

Titin phosphorylation was probed in skinned cardiac fiber bundles of adult dog LV and human LV incubated with PKG and activator cGMP in the presence of radioactively labeled ATP. Back-phosphorylation was studied on fibers incubated with PKG only or with PKG following initial dephosphorylation by PP1, PP2a, or AP (all revealing similar results). The titin isoforms N2BA and N2B were both phosphorylated by PKG; some fibers showed very low intensity (example in Figure 1A, bottom right). Assuming the signal intensity following phosphatase pretreatment always represented the respective maximum phosphorylation, the relatively low back-phosphorylation signals obtained with PKG only in donor hearts suggest these hearts have rather high inherent (basal) levels of titin phosphorylation.

PKG Reduces PT in Skinned Human Cardiac Fibers

To test for possible functional consequences of PKG-mediated titin phosphorylation, we measured the passive force–SL relation of skinned fiber bundles from human donor LVs (n=4 nonfailing hearts, 3 fibers/tissue). Again, experiments were performed either on fibers incubated with PKG alone or on samples dephosphorylated before incubation with PKG (PP+PKG; Figure 1B). Data were normalized to those obtained for control fibers incubated without kinase (dashed lines), and mean values were fit by linear regression (Figure 1B). Incubation with PKG only did not cause a significant reduction in average PT, although a trend was apparent (Figure 1B, left). In phosphatase-pretreated fibers, PT decreased significantly by ~10% to 20% within the physiological SL-range (Figure 1B, right). The maximum average decrease was 13% at 2.2 μm SL.

PKG-Mediated PT Reduction Occurs at the Level of the Sarcomere

Titin springiness dominates the PT development in mammalian heart in the SL range from 1.9 to ~2.2 μm, whereas collagen stiffness dominates at higher physiological SLs.²⁹ This fact could potentially explain our observation of a significant PKG-mediated PT reduction below 2.4 μm SL only (Figure 1B, right). To test whether the PT decrease is attributable to a direct PKG effect on titin-based stiffness, passive force in response to repetitive stretch-release cycles (bursts of ten 1-Hz oscillations between slack SL and 150% slack length) was studied in collagen-free myofibril preparations isolated from human donor LV (Figure 2). Passive stiffness on application of cGMP-activated PKG to myofibrils in relaxing buffer was compared to that before phosphorylation (Figure 2A). In myofibrils treated with cGMP-PKG passive stiffness remained unchanged, but in myofibrils dephosphorylated before mechanical analysis incubation with cGMP-PKG dropped stiffness significantly, on average by 12% (Figure 2B). This decrease is similar to that observed at shorter physiological SLs in skinned fibers (Figure 1B). Thus, PKG reduces passive stiffness via a direct effect on the sarcomeres, presumably on titin.

PKG Phosphorylates Several Sites Within the Spring Region of Titin

We hypothesized that PKG phosphorylates titin within its springy segment. To probe this hypothesis, we generated recombinant fragments of various domains along the human I-band-titin region (Figure 3A) and tested these constructs for cGMP-activated PKG-mediated phosphorylation. For comparison, constructs were also incubated with PKA (final concentration, 1 U/μL relaxing solution), which was previ-
analysis for PKG- or PKA-mediated phosphorylation (Figure 3B, bottom gels). Both kinases phosphorylated the N2-Bus, whereas PKG additionally phosphorylated I24/25. No signal was detected for I26. These results were supported by dot blots probing the PKG-phosphorylated recombinant fragments using monoclonal antibodies against phosphoserine residues (Figure 3C): cGMP-PKG again phosphorylated N2-B and I24/25, but not I26. Unlike in the autoradiography tests, Ig domains I2 to I4 showed no phosphorylation signal. Analysis of 3 subfragments of the N2-Bus (Nus, Mus, Cus) demonstrated the presence of PKG phosphorylation site(s) in the C-terminal part, but not in the other sections, of this unique sequence (Figure 3C).

Both PKG and PKA Phosphorylate Serine S469 of the Human N2-Bus

Comparing the amino acid sequence of the N2-Bus with online databases (NetphosK and Scansite Motif scanner), we found 5 potential consensus sequences for PKG and PKA phosphorylation, 1 each in the N-terminal (N1–189) and the middle portion (M190–410) and 3 in the C-terminal part (C411–572) (Figure 4A). However, using autoradiography, we readily confirmed the dot blot results (Figure 3C): PKG phosphorylated C411–572 (Cus) but not N1–189 (Nus) or M190–410 (Mus) (Figure 4B and 4C). We further generated 3 different mutants of the C411–572 construct, 1 with a truncation of the N-terminal potential phosphorylation site (C420–572), a second with a truncation of the C-terminal potential phosphorylation site (C411–545), and a third with the serine at position 469 of the N2-Bus exchanged by an alanine (C5469A) to inactivate this potential phosphorylation site. Of these 3 constructs, only the C5469A mutant was no longer phosphorylatable by PKG. These findings establish that PKG targets the N2-Bus of cardiac titin at position

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**Figure 2.** PKG effect on passive stiffness of isolated myofibrils from human donor heart. A, Top, Representative images of myofibril stretched repetitively from slack SL (1.85 μm) to ~150% slack (2.70 μm SL), while passive force was recorded. Scale bar, 5 μm. A, Bottom, Mechanics protocol (left) and change in passive stiffness relative to initial stiffness on application of cGMP-activated PKG (right). Symbols are means ± SEM for bursts of 10 oscillations per time point. ctrl indicates relative stiffness measured over time in absence of cGMP-PKG. B, Relative passive stiffness of myofibrils treated with PKG only or PKG after preincubation with phosphatase (PP pretreat. + PKG). Data are means ± SEM (n = 6 myofibrils; from 2 donor hearts). Control indicates average of “ctrl” measurements in (A). *P < 0.05 (Student’s t test).

**Figure 3.** Tests for PKG (and PKA)-mediated phosphorylation of recombinant constructs generated from the spring region of human titin. A, Domain architecture of human I-band titin (N2BA isoform from NH2 terminus to start of A-band). Dashed lines indicate splice pathways for cardiac N2B and skeletal muscle N2A isoforms. Horizontal bars indicate positions of constructs generated. B, SDS-PAGE and corresponding autoradiogram of the respective fragments after incubation with [γ-32P]ATP in absence (ctrl) or presence of PKA or cGMP-activated PKG. C, Dot blot analysis of recombinant fragments dephosphorylated before incubation with cGMP-PKG. Proteins were dotted onto a poly(vinylidene fluoride) membrane and probed by monoclonal antibodies against phosphoserine residues. Ctrl, positive control (Phosphodetection kit, Calbiochem).
S469. Notably, autoradiography of normal and mutation constructs showed that serine 469 is also phosphorylated by PKA (Figure 4B and 4C).

PKG-Mediated Phosphorylation Alters the Elasticity of the N2-Bus

The N2-Bus extends in the physiological SL range, in addition to Ig domain regions and the PEVK domain of titin.2 We wanted to know whether the elastic properties of the N2-Bus can be altered by PKG-mediated phosphorylation. Hence we generated a recombinant fragment in which the N2-Bus is bordered by the 2 naturally flanking Ig domains on either side, I24/25 and I26/27, respectively. The force–extension relationship of this N2-Bus construct was measured by single-molecule atomic force microscopy (AFM) (Figure 5A, top) and a possible mechanical effect owing to phosphorylation by cGMP-activated PKG was studied. The Ig domains unfold under forces of approximately 200 pN and provide characteristic sawtooth-like “unfolding” peaks (Figure 5A, bottom). At least 3 regularly spaced Ig-unfolding peaks must appear in the force trace to make sure the whole N2-Bus is stretched; recordings not fulfilling this requirement were not analyzed (see the expanded Materials and Methods section).

From the force–extension traces we could parameterize the mechanical properties of the N2-Bus (Figure 5A, bottom). Modeling the initial trace leading up to the first unfolding

Figure 4. Mapping the PKG and PKA phosphorylation site in the N2-Bus of titin. A, Amino acid sequence of the human N2-Bus. Shown are start positions (forward pointing arrows) and end positions (backward pointing arrows) of the N-terminal (N1–189), middle (M190–410), and C-terminal (C411–572) fragments generated. Black background, potential consensus sequences for PKG- or PKA-dependent phosphorylation (predicted by NetphosK and Scansite Motif scanner databases). B, Schematic representation of N2-Bus constructs generated, including 3 mutants of the C-terminal N2-Bus fragment (C420–572, C411–545, CS469A), each lacking 1 of the predicted potential phosphorylation sites. Results of autoradiography tests are listed on the right. C, SDS-PAGE and corresponding autoradiogram of the respective N2-Bus fragments after incubation with [γ-32P]ATP in absence (ctrl) or presence of cGMP-activated PKG or PKA.

Figure 5. Detecting mechanical changes in the N2-Bus of titin by single-molecule AFM force spectroscopy. A, Schematic of AFM setup and recombinant construct used (top) and representative force–extension trace for a single-molecule tether likely containing the whole N2-Bus and 3 Ig domains (bottom). Numbers 1, 2, and 3 refer to stretch stages 1, 2, and 3 in top schematic. Blue line shows fit according to WLC model (Equation) applied to data between the 2 pink circles representing extension of the N2-Bus. Here, the fit returned a persistence length (Lp) of 0.24 nm. B, Histogram of the distribution of persistence length values for the N2-Bus, in the absence (top) or presence (bottom) of cGMP-activated PKG. Lines are best Gaussian fits to data; numbers (mean±SD) indicate peak positions. C, Predicted change in force–SL curve of human cardiac titin, considering a persistence length increase for the N2-Bus from 0.35 nm (as for nonphosphorylated N2-Bus) to 0.67 nm (as for phosphorylated N2-Bus).
peak (corresponding to the stretching of the N2-Bus) using the WLC model (Equation) allowed extraction of the persistence length ($L_p$), a measure of the bending rigidity of the entropic spring. Histogram analyses showed that $L_p$ of N2-Bus was 0.39±0.26 nm (mean±SD) under control conditions (relaxing buffer) (Figure 5B, top). In the presence of cGMP-PKG, the majority of recordings revealed a significantly increased $L_p$ averaging 0.67±0.11 nm (Figure 5B, bottom). The contour length ($L_{cN2-Bus}$), another fitted parameter in the WLC model (Equation), was unchanged at ≈205 nm under all experimental conditions, and also the unfolding forces of the Ig domains remained unaltered (data not shown).

In another experimental series, we also stretched a recombinant N2-A construct by AFM. Although the N2-A domain is phosphorylatable by cGMP-PKG (Figure 3B), its mechanical properties were not altered by the kinase (Figure I in the online data supplement).

### Altered Bending Rigidity of the N2-Bus Explains the PKG-Induced Decrease in Titin-based Stiffness

Using the 2 average $L_p$ values measured for the N2-Bus (Figure 5B), we modeled the elastic force versus SL relationship (Figure 5C) as that of 3 independent WLCs corresponding to the titin Ig domain regions, the PEVK segment, and the N2-Bus. The prediction used the human titin sequence information and mechanical parameters of a cardiac-specific titin region, the N2-Bus. These PKG-dependent mechanical effects on titin could account for at least part of the observations that NO decreases myocardial stiffness, increases myocyte resting length, and downward shifts the diastolic filling curve in PV loops. Thus, PKG-mediated regulation of titin-based stiffness appears to be important in adjusting ventricular filling and regulating diastolic function in physiological and pathophysiologival settings.

Apart from NO, myocardial diastolic stiffness can also be modulated acutely by endothelin-1, β-adrenoceptor agonists that activate PKA, and angiotensin II, agents better known for their stimulating effects on cardiac contractility. These acute effects on diastolic function are distinct from the well-established long-term influences related to variables such as changes in chamber geometry, hypertrophy, extracellular matrix remodeling, and transitions in titin isoform composition. To date, mechanistic explanations for the acute effects on diastolic stiffness are sparse. Angiotensin II and endothelin-1 have been proposed to decrease myocardial passive stiffness through little understood mechanisms requiring the activation of G protein–coupled receptors and involving PKC and the Na+/H+ exchanger. In contrast, NO has been implicated in mediating these effects. In the PKA-induced PT reduction observed in rat, bovine, and human hearts, including cardiomyocytes from patients with SHF or DHF, has been explained by phosphorylation of the N2-Bus of titin, a suggestion supported by the present results (Figures 3 and 4). Although titin has long been known to contain phosphorylation sites and to be phosphorylat-

### Failing Human Hearts Have a Deficit in Basal Titin Phosphorylation

To address a possible pathophysiological significance of the PKG-mediated titin phosphorylation, we compared the titin back-phosphorylation signals on autoradiograms using human left ventricular tissue obtained from 3 nonfailing donors and 3 patients with end-stage DCM. Interestingly, PKG alone typically produced stronger back-phosphorylation signals in DCM than in donor hearts (Figure 6A; compare with Figure 1A). Measuring the difference in signal intensity between PKG-only and PP pretreated+PKG samples, the inherent (basal) phosphorylation levels were inferred (weighted for protein loading). There was a clear trend toward decreased basal titin phosphorylation in DCM compared to donor hearts (Figure 6B).

### Discussion

Cyclic GMP signaling, which is centrally involved in functional regulation of the cardiovascular system, can be promoted by natriuretic peptides (atrial natriuretic peptide and brain natriuretic peptide) activating the guanylyl cyclase A receptor and by stimulating NO synthase. The second messenger NO produces biphasic contractile effects in heart tissue, with augmentation at low levels and depression at high levels. NO participates not only in the control of contractility and heart rate but also limits cardiac remodeling after an infarction and contributes to the protective effect of ischemic pre- and postconditioning. Growing evidence suggests that activation of PKG (cGKI) via cGMP signaling can lower diastolic tone and increase LV extensibility.

Our work now demonstrates that cGMP-activated PKG phosphorylates specific sites within the titin spring segment and decreases titin-based stiffness by altering the elastic properties of a cardiac-specific titin region, the N2-Bus. These PKG-dependent mechanical effects on titin could account for at least part of the observations that NO decreases myocardial stiffness, increases myocyte resting length, and downward shifts the diastolic filling curve in PV loops. Thus, PKG-mediated regulation of titin-based stiffness appears to be important in adjusting ventricular filling and regulating diastolic function in physiological and pathophysiologival settings.
able,\textsuperscript{2,3,35} the PKA-dependent phosphorylation of the N2-Bus was the only posttranslational modification of titin previously shown to alter titin stiffness.

The novel mechanism of diastolic stiffness regulation established here is mediated by phosphorylation of the human cardiac N2-Bus by PKG. The relative drop in passive stiffness induced by PKG was about half that seen elsewhere with PKA.\textsuperscript{7,9,11} We demonstrated a similar percentage of PKG-induced passive stiffness decrease (12\% to 17\%) in human cardiomyofibers (Figure 1B), single myofibrils (Figure 2), and single titin molecules (Figure 5), the latter being brought about solely by increasing the bending rigidity of the N2-Bus. Because the N2-Bus is part of the N2-B domain, which is not expressed in the skeletal muscle N2A titin isoforms, the mechanical effect should be cardiac-specific. The PKG effect was significant in phosphatase pretreated samples but almost disappeared when the dephosphorylation step was omitted, an observation that we attribute to the high basal levels of phosphorylation seen in the human donor hearts (Figure 6B).

However, the phosphorylation state of the human donor samples might have been altered by the pharmacological treatment of these hearts before transplantation surgery. In this context, inherent titin phosphorylation was found to be lower in healthy dog LV than in human donor LV. Follow-up studies on a larger cohort of human hearts and on animal models should help clarify this issue.

Using mutagenesis of recombinant constructs, we identified serine S469 of the human N2-Bus as the PKG phosphorylation site within that titin sequence. Our evidence suggests that it is most likely phosphorylation of S469 that alters N2-B-titin elasticity. We further demonstrated that S469 can also be phosphorylated by PKA. As the length and amino acid composition of the N2-Bus differ among mammalian species,\textsuperscript{36} it remains to be seen whether PKG (and PKA) phosphorylates the N2-Bus at the same and/or different sites in other species. By screening the N2-Bus sequence of rat cardiac titin,\textsuperscript{38} we found a sequence motif (QKTS) at position 556 to 559 of the N2-Bus that is very similar to the sequence motif (AKTS) at residues 466 to 469 of the human N2-Bus. Taken together with the observation that PKG phosphorylated both the N2BA and N2B isoforms in adult dog heart, we propose that PKG-mediated phosphorylation is a more general mechanism to regulate diastolic stiffness in mammalian heart.

We provide evidence that the PKG phosphorylation site relevant for the mechanical effect resides within the N2-Bus; however, PKG phosphorylated other domains in human titin as well, both in the N2-B (Ig domains I24/25) and N2-A segments (Figure 3). Because we found no influence of PKG on the mechanical properties of titin Ig domains in single-molecule AFM stretch experiments with engineered N2-B (Figure 5) or N2-A constructs (supplemental Figure I), we exclude that the PKG-mediated PT decrease in myofibrils results from an effect on these domains. The proximal Ig domains, I2 to I4, showed weak, if any, propensity to be phosphorylated by PKG, whereas no signal was detected for the Ig domain I26, which was suggested to be a substrate for PKG in rat uterus titin.\textsuperscript{37} The functional role(s) of the additional posttranslational modifications detected are currently unknown, but we speculate that phosphorylation at sites outside the N2-Bus could be important for regulating protein-protein interactions, because the titin springs interact with multiple structural and signaling molecules.\textsuperscript{2}

Because PKG phosphorylates various sites on human titin, a correlation between the titin phosphorylation level and the magnitude of PKG-induced mechanical effect was deemed not meaningful. This limitation notwithstanding, we observed a trend toward reduced basal levels of PKG-mediated titin phosphorylation in failing human DCM hearts compared to donor hearts (Figure 6). Thus, failing human hearts could have a titin phosphorylation deficit which includes the mechanically relevant phosphorylation site (although, as discussed above, the donor hearts could be hyperphosphorylated, which would make the observed low titin phosphorylation state in DCM less clinically relevant). One can then speculate that a titin phosphorylation deficit increases passive stiffness in failing human hearts. Indeed, skinned cardiomyocytes isolated from failing hearts of SHF or DHF patients showed elevated PT levels, which were substantially reduced by administration of PKA.\textsuperscript{7} The present findings suggest the intriguing possibility that a pathologically increased passive myocyte stiffness could also be normalized by PKG-mediated phosphorylation. Future work on this issue should consider the crosstalk between the PKA and PKG signaling systems in light of the fact that both kinases phosphorylate titin at site S469 of the N2-Bus. In any case, our results led us to suggest the following possible scenario: the cGMP-hydrolyzing PDE5 is present in the Z-disk and might regulate local pools of cGMP, which could then activate PKG.\textsuperscript{14} This kinase would reduce myofilament Ca\textsuperscript{2+} sensitivity and depress contraction but also lower titin-based stiffness. In the failing heart, this mechanism may be compromised and deficits in PKG-mediated titin phosphorylation could add to an increased passive stiffness. In turn, PKG-mediated regulation of titin stiffness could be a handle for pharmacological intervention, eg, via inhibition of PDE5\textsuperscript{14} by sildenafil.\textsuperscript{17} Finally, nitrates are already commonly used in the treatment of both SHF and DHF and their beneficial effects could well involve keeping titin stiffness low.

In summary, this study demonstrates that PKG reduces titin-based stiffness via phosphorylation of a serine residue (S469) within the N2-Bus of titin, thereby acting to improve diastolic function in human hearts.

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Disclosures

None.

References


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PROTEIN KINASE G MODULATES HUMAN MYOCARDIAL PASSIVE STIFFNESS BY PHOSPHORYLATION OF THE TITIN SPRINGS

by Krüger et al.

Heart muscle tissue. Left ventricular (LV) tissue from human donor and failing hearts was procured and deep-frozen in liquid nitrogen in the operating theater within minutes of the loss of coronary circulation. Tissue was stored at -80°C. Non-failing human heart samples were obtained from brain-dead human donors with normal LV function. These heart donors suffered head trauma (2×), sub-arachnoid hemorrhage, or cerebrovascular accident (2×). Tissue samples from failing hearts were obtained from patients with idiopathic dilated cardiomyopathy (IDCM) who underwent heart transplantation due to severe systolic dysfunction (NYHA class III or IV) with severely impaired LV ejection fractions (Online Table I). These DCM patients showed no sign of ischemic heart disease by coronary angiography. Characteristics of the human hearts used and the individual titin isoform composition by gel electrophoresis (SDS-PAGE; see below) are shown in Online Table I. All human tissue collection was in accordance with Australian National Health Medical Research guidelines and approved by the Human Research Ethics Committee of the University of Sydney (reference 7326). Tissue from healthy adult dog (8-12 years) left ventricles was sampled at the Mayo Clinic (Rochester, Minnesota) as described1 and was also stored at -80°C. All animal procedures were conducted in full accordance with the institutional guidelines and were approved by the Mayo Clinic ethics committee.

Online Table I. Human heart samples.

<table>
<thead>
<tr>
<th>No. / Type</th>
<th>Gender</th>
<th>Age</th>
<th>LVEF %</th>
<th>Titin N2BA %</th>
<th>Titin N2B %</th>
<th>SEM %</th>
<th>N (Titin gels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Donor</td>
<td>female</td>
<td>48</td>
<td>46</td>
<td>37</td>
<td>63</td>
<td>3.5</td>
<td>4</td>
</tr>
<tr>
<td>2 Donor</td>
<td>female</td>
<td>53</td>
<td>30</td>
<td>70</td>
<td>3.0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>3 Donor</td>
<td>male</td>
<td>27</td>
<td>35</td>
<td>65</td>
<td>2.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>4 Donor</td>
<td>male</td>
<td>13</td>
<td>35</td>
<td>65</td>
<td>4.0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>5 Donor</td>
<td>male</td>
<td>26</td>
<td>28</td>
<td>72</td>
<td>5.0</td>
<td>4</td>
<td>4</td>
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<tr>
<td>1 IDCM</td>
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<td>63</td>
<td>15-20</td>
<td>45</td>
<td>55</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
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<td>35</td>
<td>49</td>
<td>51</td>
<td>0.7</td>
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<tr>
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<td>male</td>
<td>58</td>
<td>&lt;40</td>
<td>38</td>
<td>62</td>
<td>4.0</td>
<td>4</td>
</tr>
</tbody>
</table>
Preparation of skinned cardiac fiber bundles. Small muscle strips were prepared from the left ventricles of de-frozen human hearts and skinned for 3 hrs on ice in relaxing solution (7.8 mM ATP, 20 mM creatine phosphate, 20 mM imidazole, 4 mM EGTA, 12 mM Mg-propionate, 97.6 mM K-propionate, pH 7.0, 40 µg/ml leupeptin, 30 mM 2,3-butanedione monoxime (BDM), 1 mM DTT) supplemented with 1% w/v Triton-X-100. The skinned tissue was extensively washed in relaxing buffer without Triton-X-100 and small fiber bundles with a diameter of 200–300 µm and a length of 1.0–2.5 mm were dissected.

Passive tension measurements on fibers. Force measurements on skinned human fiber bundles were performed with a muscle mechanics workstation (Scientific Instruments, Heidelberg, Germany) as described. Samples were bathed in relaxing solution at 26°C and mounted between stainless steel clips attached to a motor arm and a force transducer. Sarcomere length (SL) was measured by laser diffraction using a 670-nm He-Ne-Laser. Only fiber bundles showing well discernible first-order diffraction bands were used for mechanical measurements. Fibers were stretched from slack SL (average, 1.9 µm) in six steps of 0.1–0.2 µm/sarcomere (each step completed in 1 s) to a maximum SL of 2.6-2.7 µm. In between the stretches a 1-min-hold period was observed to wait for stress relaxation. Following the last stretch-hold, fibers were released back to slack SL to test for possible shifts of baseline force. From the recordings we analyzed the force at the end of each hold period (near steady-state force). A second set of stretch experiments was done in relaxing solution containing purified protein kinase-G (PKG; purified from bovine lung as described) at a final concentration of 1.68 × 10^{-5} U/µl. These experiments were performed in the presence of 300 µM selective PKG-activator, 8-(4-chlorophenylthio)guanosine-3', 5'-cyclic monophosphate (8-pCPT-cGMP (cGMP), Calbiochem). Cyclic GMP and PKG were incubated for 30 min at 26°C before the stretch series commenced. In a third set of experiments, we first added alkaline phosphatase (AP; New England Biolabs) or protein phosphatase-2a (PP2a, Sigma-Aldrich, recombinant catalytic subunit, α-isofom from rabbit; final concentration 0.3 U/µl) for 20 min to the relaxing solution at 30°C. In a few measurements we instead used protein phosphatase-1 (PP1, Sigma-Aldrich, recombinant catalytic subunit, α-isofom from rabbit, final concentration 0.3 U/µl) supplemented with 1 mM MnCl₂, with essentially the same results. The PP2a/AP/PP1 was then removed in several washing steps with relaxing solution, before fibers were bathed in relaxing buffer supplemented with protein phosphatase inhibitor cocktail (Sigma-Aldrich) and PKG in presence of cGMP for 30 min at 26°C. Then the stretch protocol was performed again. Force data were expressed relative to the values measured after
de-phosphorylation of the fibers by phosphatase but before phosphorylation by PKG. In control experiments we confirmed full reproducibility of the force levels at all SLs in successive stretch-hold series conducted in relaxing buffer only, for a given fiber bundle.

Isolation of myofibrils. Left ventricular tissue from human donor heart was skinned as described above. The skinned muscle strips were extensively washed and homogenized using an Ultra-Turrax (IKA). The myofibrillar suspension was kept on ice and was used for a maximum of 10 h.

Passive stiffness measurements on isolated myofibrils. Passive stiffness measurements were performed using an optic fiber-based force transducer (homebuilt) with nanoNewton resolution (~5 nN).\textsuperscript{7} Custom-written LabView software was used for motor control, data acquisition and analysis.\textsuperscript{8} Single myofibrils or small myofibrillar bundles were mounted under a Zeiss Axiovert-135 inverted microscope between the force transducer and the tip of a glass microneedle connected to a piezoelectric actuator (Physik Instrumente). A typical experimental protocol consisted in repeatedly stretching and releasing the myofibril from slack SL (average, 1.85 µm) to 150% slack length at a frequency of 1 Hz for 10 s, at room temperature. SL was detected by video microscopy. The peak-to-valley force amplitude in each cycle was measured and the amplitudes of the 10 cycles per burst were averaged using an automated LabView algorithm. Measurements were performed in relaxing solution 5 and 0 min before and 5, 10, and 15 min after addition of $1.68 \times 10^{-5}$ U/µl PKG in presence of cGMP. The mean force amplitude at each time point was then related to the mean amplitude before addition of kinase. In control experiments no kinase was added to the myofibrils but the force amplitudes were measured every 5 min for up to 30 min to confirm there is no significant mechanical fatigue.

Expression of recombinant titin fragments. Human cardiac titin fragments (NCBI accession NM_003319.3) were expressed in Escherichia coli XL1-Blue and purified by the GST-Fusion System (Amersham Biosciences). The following constructs were generated: I2-4, Ig-domains ubiquitously expressed in all skeletal and cardiac titin isoforms; N2-B, cardiac-specific domain encoded by titin-exon 49 (I24/25—N2-Bus—I26); Ig-domains I24/25 and I26, which are part of the N2-B domain; N2-Bus, the 572-residue unique sequence of the N2-B domain, as well as three subfragments of it (Nus, Mus, Cus): N-terminus (N1-189), middle portion (M190-410), and C-terminus (C411-572); N2-A, N2-A domain (encoded by titin exons 102-109);
PEVK, constitutively expressed PEVK region present in all muscle titins and encoded by titin exons 219-225. Of the N2-Bus we also generated the following mutants: C420-572, N-terminally truncated C-terminus; C411-545, C-terminally truncated C-terminus; and CS469A, C-terminus of N2-Bus with amino acid exchange, serine→alanine, at position 469.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and ^32P autoradiography.** Protein phosphorylation by PKG (and PKA) was probed by standard autoradiography following SDS-PAGE with concentrations of 15% or 2% polyacrylamide, as previously reported. Recombinant fragments of human I-band titin were analyzed after incubation with $1.68 \times 10^{-5}$ U/µl PKG and cGMP in the presence of $[^{\gamma}32P]$ATP (specific activity, 250 µCi/µM) for 20 min at 36°C. Some constructs were incubated with the catalytic subunit of PKA (Biaffin, final concentration 1 U/µl in relaxing solution) instead of cGMP-PKG. The peptides were denatured, dissolved, electrophoresed on 15% SDS-polyacrylamide gels, and identified by coomassie blue staining. The gel was dried and exposed to autoradiographic film (Fujifilm BAS-1800 II) for up to 24 hours at room temperature.

2% SDS-PAGE was performed to test for titin phosphorylation. Skinned fibers were either directly phosphorylated by adding PKG ($1.68 \times 10^{-5}$ U/µl) cGMP and $[^{\gamma}32P]$ATP or were first de-phosphorylated by AP or PP2a (0.3 U/µl) for 30 min at 30°C before incubation with the kinase. After a washing step with relaxing solution to remove the phosphatase the fibers were incubated with cGMP-PKG for 30 min at 30°C in relaxing solution supplemented with phosphatase inhibitor cocktail (Sigma-Aldrich) and $[^{\gamma}32P]$ATP (specific activity, 250 µCi/lM). Fibers in relaxing solution to which $[^{\gamma}32P]$ATP but no kinase was added, served as controls. Protein bands were stained with coomassie blue and gels were dried before exposure to autoradiographic film usually for 24 h at room temperature. $^{32}$P-incorporation was again visualized by phosphoimaging (Fujifilm BAS-1800 II).

**Dot blot analysis.** Recombinant titin fragments were first de-phosphorylated by phosphatase (alkaline phosphatase, 0.001 U/µl) for 1 h at 37°C. Phosphatase activity was then blocked by addition of excess phosphatase inhibitor cocktail (Sigma-Aldrich). De-phosphorylated fragments were incubated with PKG ($4 \times 10^{-5}$ U/µl) and cGMP (300 µM) for 15 min at 37°C before they were solubilized in sample buffer and dotted onto a PVDF membrane. The membrane was then probed by monoclonal antibodies against phosphoserine residues (phosphoDetect™ anti-mouse mAB, clone 16B4, Calbiochem). Goat anti-mouse IgG conjugated with horseradish peroxidase served as secondary antibody.
**AFM force measurements and analysis.** Single-molecule AFM force spectroscopy was done using an MFP-3D atomic force microscope (Atomic Force F&E GmbH, Mannheim, Germany). Shaped silicon nitride cantilevers (MSCT-AUHW, Veeco Metrology Group, Santa Barbara, CA) were used, whose individual spring constants (~40 pN nm\(^{-1}\)) were calibrated before each experiment using the equipartition theorem. Force-extension experiments were performed on a recombinant construct containing the N2-Bus flanked on either side by two Ig domains (I24—I25—N2-Bus—I26—I27)\(^9\) or on a construct comprising the human titin N2-A domain (Ig-domains I80-I83). The same buffer conditions were used as in fiber and myofibril stretch measurements (relaxing solution alone or relaxing solution containing cGMP-PKG). In additional control experiments, bovine serum albumin was added to the buffer at a concentration equaling that of the PKG, but had no effect on titin mechanics.

The likelihood that a single protein, especially the full-length protein, attaches to the cantilever tip is very low. Thus, a critical step in the analysis is to find a way of detecting those events when truly a single peptide is stretched. This is achieved by analyzing the characteristic sawtooth-like unfolding peaks of the flanking Ig-domains. Each peak in the force-extension curve indicates unfolding of one Ig domain. Regular peak spacing suggests a single protein is pulled. For the (I24—I25—N2-Bus—I26—I27) construct we analyzed only those force-extension curves showing at least three regularly spaced Ig unfolding peaks, because only then could we be sure that the full N2-Bus was stretched (“fingerprinting”).

In a typical experiment 2 µl of a 2 nM protein solution were pipetted onto a clean glass surface and protein fragments were allowed to adsorb to the surface, which was then rinsed with relaxing buffer after 5 min. Following another 5 min for equilibration, proteins were picked up randomly by pressing the cantilever tip onto the sample for 1 s at high force (typically 1.8 nN) and letting the protein adsorb to the tip. The usual pulling rate for all force-extension traces was 500 nm s\(^{-1}\). Surface protein density was optimized to ensure a low probability of attachments to the cantilever tip (~1 in 500 attempts) thereby minimizing the possibility of catching two or more molecules in the same pulling event. Only experiments showing a regular sawtooth pattern characteristic for a single protein were used for further analyses.\(^{10}\) Experiments were performed at 22°C.
Recorded force-extension traces were analyzed by fitting individual unfolding events by an Igor procedure (Wave Metrics, Portland, OR) using the worm-like chain (WLC) model. This model describes the pure-entropic elasticity of a polymer \(^{11}\) by

\[ F = \left( \frac{k_B T}{L_p} \right) \left[ \frac{1}{4(1 - x/L_c)^2} - \frac{1}{4} \frac{x}{L_c} \right], \tag{1} \]

where \( F \) is the entropic force, \( L_p \) the persistence length, \( x \) the end-to-end extension, \( L_c \) the contour length, \( k_B \) the Boltzmann constant and \( T \) the absolute temperature. The fitting parameters are \( L_p \) and \( L_c \). The contour length difference, \( \Delta L_c \), between two Ig-domain unfolding peaks was 24-34 nm.\(^{10}\)

**Modeling titin-based force.** The titin force versus SL relationship was modeled using a force-extension curve generated from the weighted sum of three WLC force-extension relations corresponding to the different extensible regions in cardiac titin \(^{3,11}\): segments of tandem-Ig, the PEVK-region and the N2-Bus. These segments are characterized by different contour and persistence lengths, namely the total titin extension is given by:

\[ X = \sum_{i=1}^{3} x_i \tag{2} \]

where the extension \( x_i \) at a force \( F \) of the \( i \)-th spring satisfies Eq. 1. Solutions for the equations were found using a standard numerical interpolation technique.

Using the same model as in Ref. 3, unfolding of Ig-domains was modeled as a 2-state process (folded and unfolded) for proximal and mid Ig’s, with a probability of \( P_{\text{prox/mid}} = A/(A+B) \), where \( A \) and \( B \) are force-dependent unfolding and folding rate constants. Unfolding of distal Ig-domains was modeled as a 3-state process (folded, intermediate and unfolded), with a probability of \( P_{\text{dist}} = A_1A_2/(A_1A_2+B_1B_2+A_1B_2) \), with \( A_1 \) and \( B_1 \) being force-dependent unfolding and folding rate constants between the fully-folded state and an intermediate state; \( A_2 \) and \( B_2 \) are the equivalent rate constants between the intermediate state and the fully-unfolded state. Upon unfolding of an Ig-domain the contour length of the WLC representing the Ig-region was assumed to decrease by 4.4 nm, the length of a single folded Ig-domain,\(^{12}\) with a simultaneous increase in the contour length of the WLC by 32.5 nm. The result is three different functional force-extension relations, \( F_i(X) \).
All kinetic parameters, including values for A, B, A₁, A₂, B₁ and B₂, were taken from Ref. 3 according to data published. Values for the number of Ig’s and PEVK residues as well as those for L_p and L_c for each WLC are shown in Online Table II, including the different L_p values for the N2-Bus measured by AFM force spectroscopy with and without PKG-dependent phosphorylation.

To predict the force-extension curves of titin using the two different measured L_p values for the N2-Bus, we used a mean expression ratio for N2BA:N2B isoforms in human donor heart of 35:65. The integral under each curve was calculated and the difference between the integrals reported as the stiffness change caused by altered elastic properties of the N2-Bus.

**Online Table II. Parameters used in the prediction of the force-extension relation of titin. For kinetic parameters used for Ig-domain unfolding/refolding, see Ref. 3.**

<table>
<thead>
<tr>
<th>N2B titin isoform</th>
<th>n</th>
<th>L_p/ nm</th>
<th>L_c/ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>proximal Ig’s</td>
<td>15</td>
<td>10 **</td>
<td>66</td>
</tr>
<tr>
<td>distal Ig’s</td>
<td>22</td>
<td>10 **</td>
<td>97</td>
</tr>
<tr>
<td>mid Ig’s (N2-B)</td>
<td>4</td>
<td>10 **</td>
<td>18</td>
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<tr>
<td>PEVK</td>
<td>186</td>
<td>0.91</td>
<td>67</td>
</tr>
<tr>
<td>N2-Bus (control)</td>
<td>572</td>
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<td>206</td>
</tr>
<tr>
<td>N2-Bus (PKG)</td>
<td>572</td>
<td>0.67</td>
<td>206</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>N2BA titin isoform *</th>
<th>n</th>
<th>L_p/ nm</th>
<th>L_c/ nm</th>
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</thead>
<tbody>
<tr>
<td>proximal Ig’s</td>
<td>15</td>
<td>10 **</td>
<td>66</td>
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<tr>
<td>distal Ig’s</td>
<td>22</td>
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<tr>
<td>mid Ig’s (including N2-B)</td>
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<td>10 **</td>
<td>106</td>
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<td>PEVK</td>
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<td>283</td>
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<tr>
<td>N2-Bus (control)</td>
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<td>206</td>
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<tr>
<td>N2-Bus (PKG)</td>
<td>572</td>
<td>0.67</td>
<td>206</td>
</tr>
</tbody>
</table>

* The increase in molecular weight from the N2B (3000 kDa) to the N2BA isoforms (3300 kDa) was assumed to be due to extra insertions of both PEVK residues and Ig-domains. The additional quantity of PEVK in the N2BA isoforms was assumed to be a constant 600 residues. The additional quantity of Ig’s in the N2BA isoforms was assumed to be an average of 20 domains. Since the persistence lengths for distal and proximal Ig-regions are equal they can be represented as a single WLC whose contour length is the sum of those for the two separate regions. For the model, mid-Ig’s were assumed to have properties equivalent to distal Ig’s.

** For region with folded Ig-domains.
References:


Online Figure I. Results of single-molecule AFM force-spectroscopy measurements on a recombinant fragment comprising titin’s N2-A region (human titin exons 102-109), stretched in
the absence (left panels) or presence (right panels) of PKG (1.68 × 10⁻⁵ U/µl) and activator cGMP (300 µM). The engineered construct contained four Ig-domains (I80-I83 of human I-band titin), some of which are connected by short linker sequences. Force-extension traces were analyzed using the wormlike-chain (WLC) model of entropic titin elasticity (Eq. 1 in the main manuscript) and WLC fits were done for every Ig-unfolding peak. Shown are histograms for the distribution of Ig-domain unfolding forces (A), the contour-length increase upon unfolding of an Ig-domain (B), and the persistence-length values of the polypeptide chain after unfolding of at least one Ig-domain (C). Lines are best Gaussian fits to data; numbers (mean±SD) indicate peak positions. No significant changes in the mechanical parameters of the N2-A domain induced by cGMP-PKG were apparent.