J Muscle Res Cell Motil (2006) 27:435–444
DOI 10.1007/s10974-006-9090-5

ORIGINAL PAPER

Protein kinase-A phosphorylates titin in human heart muscle and reduces myofibrillar passive tension

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Received: 16 May 2006 / Accepted: 6 July 2006 / Published online: 4 August 2006

Abstract Protein kinase-A (PKA) is activated during β-adrenergic stimulation of the heart and is known to phosphorylate several sarcomeric proteins including the giant polypeptide titin. A PKA phosphorylation site on titin is located within the N2B-unique sequence, which is present in the elastic segment of the two major isoforms of cardiac titin, N2B and N2BA, but not in the skeletal-muscle isoforms of the N2A-type. In bovine and rat cardiomyocytes, PKA-mediated phosphorylation decreases passive tension (PT), an effect ascribed to titin phosphorylation. Whether titin is phosphorylated by PKA upon β-adrenergic stimulation in human heart has not been shown to date. Here we report that PKA induces phosphorylation of N2B and N2BA titin isoforms, as well as a characteristic proteolytic fragment of titin, T2, in human donor hearts. The PKA-induced phosphorylation signals were stronger when myofilaments were first de-phosphorylated by protein phosphatase-1, suggesting inherent phosphorylation of titin in human heart. Titin phosphorylation was associated with a reduction in PT of skinned human cardiac strips; the relative decrease was higher at shorter than at longer physiological sarcomere lengths. The PKA-dependent PT drop was substantially larger when fibers were pre-treated with protein phosphatase-1, indicating that inherent phosphorylation of titin is important for the basal myocardial PT level. Mechanical measurements on isolated myofibrils from rat heart confirmed the PKA effect on passive stiffness and also showed a more pronounced effect in the presence of reducing agent, DTT. In contrast, PKA did not alter the PT of single skinned rat diaphragm muscle fibers; however, the kinase was still able to phosphorylate the skeletal N2A-titin isoform, which lacks the N2B-unique sequence. Thus, an additional phosphorylation site in titin may exist outside the cardiac N2B-unique sequence. We conclude that PKA mediates phosphorylation of titin in normal human myocardium. Titin phosphorylation lowers titin-based passive stiffness in heart but not in skeletal muscle.

Keywords Connectin · Passive tension · Passive stiffness · Muscle mechanics · Phosphorylation

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Introduction

β-adrenergic stimulation is an important physiological mechanism to enhance cardiac performance during increased circulatory demands. Stimulation of the β-adrenergic receptors in cardiomyocytes activates the cAMP-dependent protein kinase-A (PKA). PKA mediates the phosphorylation of several muscle proteins involved either in intracellular Ca^{2+}-handling, e.g. ryanodine receptors, phospholamban, and the L-type Ca^{2+}-channel (Bers and Guo 2005; Bers 2006), or in regulation of actin–myosin interactions, e.g. cardiac troponin-I (cTnI) and myosin-binding protein C (MyBP-C) (Sumandea et al. 2004; Cazorla et al. 2006). Fast changes in intracellular Ca^{2+}-handling are thought to be largely responsible for the positive inotropic effect upon β-adrenergic stimulation. But posttranslational modifications of thin and thick filament components may play a role as well, as phosphorylation of

cTnI was shown to accelerate cross-bridge cycling (Hoh et al. 1988; Kentish et al. 2001) and reduce Ca^{2+}-sensitivity of force (Wattanapermpool et al. 1995; Fentzke et al. 1999), which likely contributes to the positive lusitropic and inotropic effects of β-adrenergic stimulation.

A recent study has identified the giant protein titin as a substrate of PKA (Yamasaki et al. 2002). Titin, often referred to as the third filament of the sarcomere, has a size of up to 3.7 MDa and spans a half-sarcomere from the Z-disc to the M-line. The protein has been recognized for being a major determinant of myocardial passive tension (PT) and elasticity (Opitz et al. 2003; Neagoe et al. 2003; Granzier and Labeit, 2004). The extensible segment of titin lies in the molecule's I-band portion, which encompasses a so-called PEVK-domain, a cardiac-specific N2B region, and two to three regions comprised of immunoglobulin-like (Ig) domains (Fig. 1A). Differential splicing in the mid-Ig region and the PEVK-domain gives rise to many titin isoform sizes (Freiburg et al. 2000). In comparison to the shortest cardiac isoform (termed N2B), the longer isoforms additionally contain a so-called N2A region and were thus named N2BA isoforms (Fig. 1A). When a sarcomere is stretched, the first elements that extend are the Ig-domain regions, whereas the PEVK-domain and a 572-residue unique sequence (N2B-Us) in the N2B region provide extensibility at longer physiological sarcomere lengths (SLs) (Linke et al. 1999). The N2B-Us was shown to target metabolic enzymes to the I-band via interaction with DRAL/FHL-2 (Lange et al. 2002) and also bind alpha-B-crystallin, which likely alters the mechanical properties of this element (Bullard et al. 2004).

Titin has long been known to contain phosphorylation sites and to be phosphorylatable (Somerville and Wang 1988; Takano-Ohmuro et al. 1992; Sebestyen et al. 1995; Labeit et al. 1997; Gautel et al. 2001). The phosphorylation of titin by PKA has been narrowed down to the N2B-Us (Yamasaki et al. 2002) (Fig. 1A) and evidence suggests that this modification decreases the PT in cardiomyocytes from both rat and cow heart (Yamasaki et al. 2002; Fukuda et al. 2005). Increased PKA activity was shown to lower passive stiffness also in engineered rat heart tissue (Zimmermann et al. 2002) and in failing human cardiac cells (Borbely et al. 2005; van Heerebeek et al. 2006). However, the molecular mechanisms underlying this effect remain unclear, because no changes in the mechanical properties of a recombinant human N2B-Us upon phosphorylation by PKA could be detected at the single-molecule level using atomic force spectroscopy (Leake et al. 2006). Possibly, the passive stiffness decrease upon titin phosphorylation in heart tissue is mediated not through a direct mechanical effect on the N2B-Us, but rather through changes in some binding properties of this titin segment. In any case, the PKA-mediated passive stiffness drop is interesting from a therapeutic point of view, because raising myocardial PKA activity by β-adrenoceptor stimulation could improve left ventricular diastolic function in patients with diastolic heart failure (van Heerebeek et al. 2006).

Although passive stiffness is lowered by PKA stimulation in human cardiomyocytes, information was lacking on whether titin is actually phosphorylated by PKA in human heart. Therefore, we performed titin phosphorylation tests on left ventricular tissue from normal human donor hearts and also used human cardiac tissue for testing the magnitude of the PKA-mediated passive stiffness decrease. Additionally, we wanted to know whether the PKA effect on passive stiffness is detectable only in cardiac tissue—as should be the case if this effect is mediated by phosphorylation of titin's cardiac-specific N2B region—i.e., whether titin and passive stiffness are indeed not modified by PKA in skeletal muscle. Results demonstrate a specific PKA effect on passive stiffness of human cardiac muscle, but also suggest that titin may contain additional PKA phosphorylation sites, also in the skeletal-muscle isoforms.

Materials and methods

Heart and skeletal muscle tissue

Left ventricular tissue from normal human donor hearts was a kind gift from Drs. Roger Hajjar, Federica del Monte and Judith Gwathmey (Massachusetts General Hospital, Boston, MA, USA) (for details, see Neagoe et al. 2002; Makarenko et al. 2004). Rat heart tissue was obtained from adult Sprague–Dawley rats from the department animal house at the University of Muenster. The animals were anesthetized with ether and killed by decapitation; the complete hearts were removed, immediately frozen in liquid nitrogen and stored at –80°C. The diaphragm was excised from adult rats and frozen at –80°C or sometimes used fresh to prepare skinned fibers (see below). All procedures were conducted in accordance with the guidelines of the local Animal Care and Use Committee.

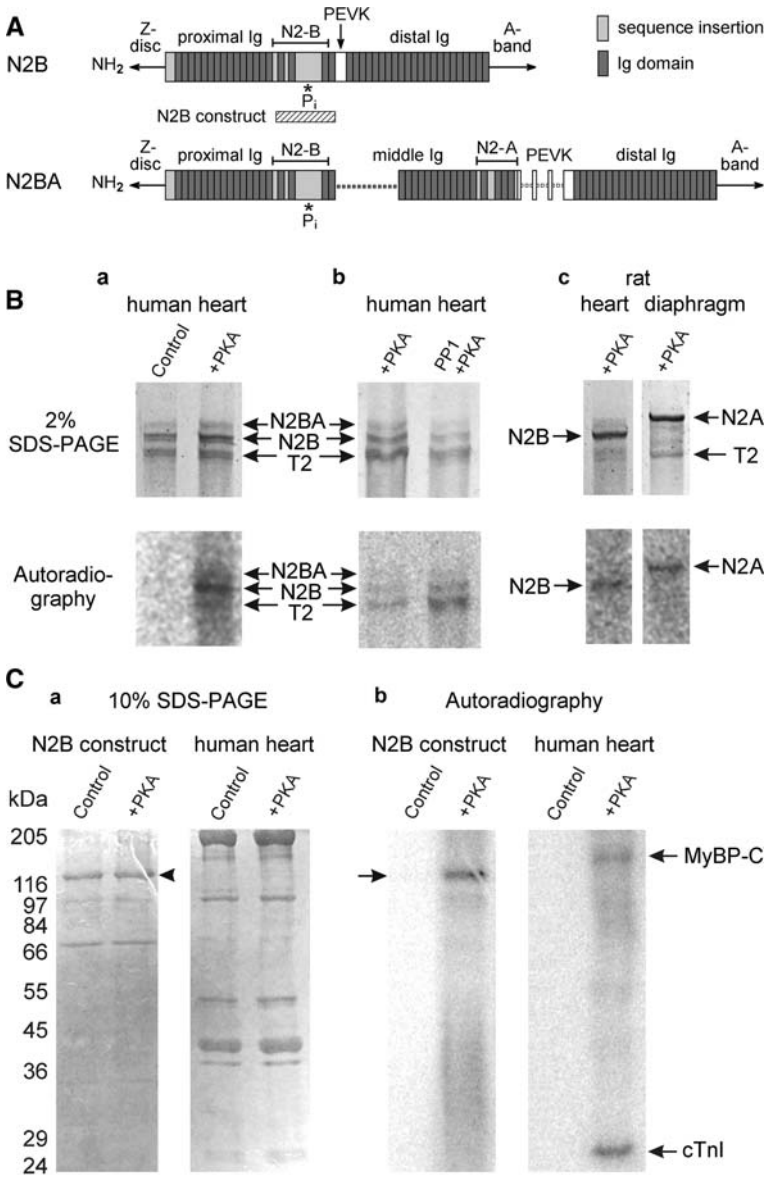

Fig. 1 PKA-mediated titin phosphorylation in human and rat hearts and rat diaphragm muscle. (**A**) Domain architecture of the elastic I-band segments in the titin isoforms, N2B and N2BA. Asterisks indicate site of phosphorylation by PKA within the N2B-unique sequence insertion. Horizontal bar (striped pattern) denotes a recombinant construct containing the entire human N2B-region (N2B-construct). (**B**) Coomassie-stained 2% SDS-PAGE and autoradiography of human heart (**a**, **b**), rat left ventricular (**c**), and rat diaphragm (**c**) skinned fibers incubated at 30°C with [γ-^{32}P]ATP in the absence (control) or presence of PKA (+PKA). Human heart was also exposed to PKA following treatment with protein phosphatase-1 (PP1 + PKA). In (**b**) titin was purposely degraded by long-term incubation with PKA and clearly shows phosphorylation of the T2 band (titin proteolytic fragment). (**C**) 10% SDS-PAGE (**a**) and autoradiogram (**b**) to test for PKA-mediated phosphorylation of the recombinant N2B-construct (arrowhead) and of lower molecular-mass proteins in skinned human heart fibers. Arrows indicate strong phosphorylation signals

Preparation of skinned cardiac fiber bundles and single skeletal muscle fibers

For tension measurements, small muscle strips were prepared from the left ventricles of frozen human donor hearts and skinned overnight in relaxing solution (7.8 mM ATP, 20 mM creatine phosphate, 20 mM imidazole, 4 mM EGTA, 12 mM Mg-proprionate, 97.6 mM K-proprionate, pH 7.0, 40 μg/ml leupeptin, 30 mM BDM, pCa 8) supplemented with 0.5% w/v Triton-X-100 on ice (Neagoe et al. 2002; Makarenko et al. 2004). The skinned tissue was extensively washed in the same buffer without Triton-X-100 and small fiber bundles with diameters of 200–300 μm and a

length of 1.0–2.5 mm were dissected. The same skinning protocol was applied to rat diaphragm muscle, from which single skeletal fibers were dissected (Prado et al. 2005).

Fiber mechanical measurements

Passive force measurements. Force measurements were performed with a muscle mechanics workstation (Scientific Instruments, Heidelberg, Germany) (Neagoe et al. 2002). Skinned human cardiac fiber bundles or single rat diaphragm muscle fibers were bathed in relaxing solution at room temperature and mounted between stainless steel clips attached to the motor arm and the force transducer. SL was measured by laser diffraction (Makarenko et al. 2004) using a 670-nm He–Ne-Laser. Only fibers/fiber bundles showing well detectable first-order diffraction bands were used for mechanical measurements. Fibers were stretched from slack SL (average, 1.85 μm for human cardiac fiber bundles; ~2.5 μm for rat diaphragm fibers) usually in six steps of 0.1–0.3 μm/sarcomere (completed in 1 s) to a maximum desired SL. 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. As a control, we repeated the stretch protocol on the same preparation after a 20-min rest period to test whether the force values were reproducible. Reproducibility within the experimental error (±5% at any stretch state) could indeed be confirmed. From the recordings we analyzed the force at the end of each step-hold period (= near steady-state force). These values were used to calculate force per cross-sectional area, the latter of which was estimated from the diameter of the preparations (at slack SL) assuming a circular shape.

A second set of stretch experiments was done in relaxing solution containing PKA (recombinant catalytic subunit, BIAFFIN) at a final concentration of 1 U/μl. PKA was incubated for at least 20 min at room temperature before the stretch series commenced. In a third set of experiments, we first added protein phosphatase-1 (PP1, SIGMA-ALDRICH, recombinant catalytic subunit, α-isoform from rabbit, final concentration 0.3 U/μl) for 20 min to the relaxing solution supplemented with 1 mM $MnCl_2$ at room temperature. Then, the PP1 was removed in several washing steps with relaxing solution, before the skinned fibers were bathed in relaxing buffer containing PKA (final concentration, 1 U/μl) for 20 min at room temperature. Then the stretch protocol was performed again. Force data were expressed relative to the values measured before phosphorylation by PKA.

Active force measurements. Skinned human cardiac fiber bundles or single fibers from rat diaphragm were first mounted in relaxing solution (pCa 8.0) without BDM; cardiac muscle strips were pre-stretched by 10% of their slack length. Then pCa was decreased stepwise (see Krüger et al. 2006) until a maximum force was reached (usually at pCa 4.5) before fibers were returned to pCa 8.0. On the same specimens we also tested the effect of PKA (1 U/μl) on force generation. First, recombinant catalytic subunit of PP1 (final concentration, 0.3 U/μl) was added to the relaxing buffer supplemented with 1 mM $MnCl_2$ for 20 min at room temperature to de-phosphorylate the myofilaments. After washout of PP1, fibers were incubated with recombinant catalytic subunit of PKA (in pCa 8.0 buffer) for 30 min at room temperature before a next activation series at different pCa values commenced. Measured forces were related to cross-sectional area (see above) and the mean tensions (passive and active) were calculated at pCa 8.0 and pCa 4.5. In control measurements we confirmed that two successive activation series in the absence of PKA revealed reproducible tension data.

Isolation of myofibrillar bundles

Rat left ventricular muscle tissue was dissected and 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 myofibrillar bundles

Passive stiffness measurements on isolated myofibrillar bundles were performed using an optic fiber-based force transducer (homebuilt (Fearn et al. 1993)) with nanonewton resolution (~5 nN). The operating principle is based on the deflection of an optical fiber and the detection of the emitted light by two optical receive fibers, each of which transmits light to a phototransistor. Custom-written LabView software is used for motor control, data acquisition and analysis (Linke and Fernandez 2002). Small myofibrillar bundles were mounted between the free tip of an optical fiber and the tip of a glass micropipette connected to a piezoelectric actuator (Physik Instrumente). Experiments were performed under a Zeiss Axiovert-135 inverted microscope.

Force measurements were carried out at room temperature in relaxing buffer. A typical experimental protocol consisted of stretching and releasing a myofibrillar bundle by 30% from its slack length with a frequency of 5 Hz, for a period of 5 s. The maximum force amplitude in each cycle was detected and the amplitudes of the 25 cycles per burst were averaged using an automated LabView algorithm. Measurements were performed in relaxing solution before and immediately after addition of 1 U/µl PKA, in the presence or absence of 1 mM DTT. Five to ten 5 s-bursts were applied under each experimental condition and the mean force amplitude and standard deviation was calculated. The mean force amplitude was then related to the mean amplitude before addition of PKA.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and ^{32}P autoradiography

Protein phosphorylation by PKA was probed by standard autoradiography (Witt et al. 2001) following SDS-PAGE with concentrations of either 10% (Leake et al. 2006) or 2% (Makarenko et al. 2004) polyacrylamide. A recombinant human N2B-construct (Fig. 1A), the expression of which was reported previously (Leake et al. 2006), was analyzed together with human heart tissue after incubation with the catalytic subunit of PKA (BIAFFIN, final concentration 1 U/µl in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM ATP, 0.06% NaF) in the presence of [γ-^{32}P]ATP (specific activity, 250 μCi/µM) for 60 min at 30°C. The proteins were denatured, dissolved, electrophoresed on 10% SDS-polyacrylamide gels, and identified by coomassie blue staining. The gel was dried and exposed to autoradiographic film (Fujifilm BAS-1800 II) for 8 h at room temperature.

A 2% SDS-PAGE was performed to test for titin phosphorylation. Skinned fibers were either directly phosphorylated by adding PKA or first de-phosphorylated by PP1 before incubation with PKA. Incubation with PKA (BIAFFIN, recombinant catalytic subunit) at a final concentration of 1 U/µl in relaxing buffer containing 0.5 mM ATP, 20 mM creatine phosphate, 20 mM imidazole, 4 mM EGTA, 12 mM Mg-proprionate, 97.6 mM K-proprionate, pH 7.0, 40 µg/ml leupeptin, pCa 8) was done in the presence of [γ-^{32}P]ATP (specific activity, 250 μCi/µM) for 30 min at 30°C. Fibers in relaxing solution to which [γ-^{32}P]ATP but no PKA was added, served as controls. To de-phosphorylate the myofilaments, untreated skinned fibers were incubated with the catalytic subunit of PP1 (SIGMA-ALDRICH) at a final concentration of 0.3 U/µl in relaxing buffer supplemented with 1 mM $MnCl_2$ for 20 min at room temperature. After a washing step with relaxing solution to remove the PP1, fibers were then exposed to 1 U/µl PKA in relaxing buffer in the presence of [γ-^{32}P]ATP under the same conditions as described above. Protein bands were separated on 2% SDS-polyacrylamide gels, stained with coomassie blue (Opitz and Linke 2005), and gels were dried before exposure to autoradiographic film usually for 24 h at –80°C. ^{32}P-incorporation was visualized by phosphoimaging.

Statistical analysis

Statistical significance was evaluated by Student's *t*-test. Differences were taken as significant when $P < 0.05$.

Results and discussion

PKA phosphorylates titin in human cardiac muscle

Previous studies suggested that PKA phosphorylates titin from rat and bovine heart muscle within the cardiac-specific N2B-Us (Fig. 1A) (Yamasaki et al. 2002; Fukuda et al. 2005). Therefore we asked (i) whether titin can be phosphorylated by PKA also in human heart muscle and (ii) whether skeletal muscle titin really is not phosphorylatable by PKA.

Autoradiography on electrophoresed tissue obtained from human donor hearts confirmed that PKA preferentially phosphorylates the two sarcomeric proteins, cTnI and myosin binding protein-C (MyBP-C) (Fig. 1C), as reported numerous times (for reviews, see e.g., Kögler and Rüegg 1997; Murphy, 2006). Typical results of the titin phosphorylation tests are shown in Fig. 1B. Using skinned human cardiac fibers (panels a, b), [γ-^{32}P] was readily incorporated in the presence (+PKA), but not in the absence (control), of PKA into the two cardiac titin isoforms, N2BA (>3.2 MDa) and N2B (3.0 MDa). To study whether titin in human donor hearts may be inherently phosphorylated, some samples were first de-phosphorylated using PP1 and then back-phosphorylated with PKA. The titin phosphorylation signals were substantially higher in PP1 + PKA-treated myocardium than in samples exposed to PKA only (Fig. 1B, panel b). These analyses were repeated at least three times with tissue from four different human hearts and revealed reproducible results. A PKA-dependent phosphorylation site must indeed be present in the cardiac-specific

N2B region, as the kinase phosphorylated a recombinant human N2B-construct containing the N2B-Us (Fig. 1C), confirming earlier work by Yamasaki et al. (2002) and ourselves (Leake et al. 2006). In conclusion, titin in human myocardium shows some basal level of phosphorylation but is readily phosphorylatable by PKA. These findings hint at the possibility that phosphorylation of human cardiac titin is increased in vivo upon β-adrenergic stimulation.

A faint phosphorylation signal was also obtained from the T2-titin band (Fig. 1B, panel a) representing a proteolytic fragment of intact titin usually considered to encompass the molecule's A-band region plus the distal portion of the I-band segment (Opitz et al. 2004). The T2 titin was more clearly seen to be phosphorylatable (Fig. 1B, panel b) when titin degradation was purposely aggravated (at a temperature of 30°C, at which the phosphorylation assays were usually performed, titin degraded more quickly than at room temperature). This result differs from that reported by Yamasaki et al. (2002), who found no phosphorylation of the T2-titin band in rat heart. In our hands, PKA phosphorylated intact rat cardiac titin (3.0 MDa, N2B isoform) but perhaps less expectedly, also a skeletal titin isoform (N2A-type, ~3.6 MDa) in rat diaphragm (Fig. 1B, panel c). Our findings are consistent with earlier work by Somerville and Wang (1988) showing that titin phosphorylation occurs in mouse diaphragm muscle and can be increased by β-adrenergic agonists. The fact that both skeletal titin and the T2 degradation product of human heart can be phosphorylated, suggests the presence of an additional PKA-dependent phosphorylation site outside the cardiac N2B-unique sequence, possibly within titin's A-band region.

Phosphorylation of titin reduces active and PT in human heart but not in skeletal muscle

Skinned fibers from human donor hearts and rat diaphragm were mechanically studied for PT at diastolic [Ca^{2+}] (pCa 8) and total (passive plus maximum isometric) tension during Ca^{2+}-activation at pCa 4.5 (Fig. 2). Tension development was measured before and after a 30-min long incubation with PKA (after PP1 pre-treatment) in relaxing buffer. In fiber bundles from human donor hearts (Fig. 2A) PT was significantly decreased from 2.50 ± 0.62 mN/mm^2 before PKA to 0.90 ± 0.25 mN/mm^2 after PKA (mean ± SEM, $n = 3$–7), a reduction by 64%. Phosphorylation had less relative effect on total (passive + active) tension at pCa 4.5 (the decrease was 10 ± 2%), but in absolute terms the reduction was somewhat greater than under relaxing conditions (Fig. 2A). Because the PKA effect on PT was shown to persist even after extraction of muscle thin filaments (Fukuda et al. 2005), the force reduction at pCa 8.0 must be unrelated to actin–myosin interactions or actin–titin interactions (Kulke et al. 2001).

For comparison we performed similar experiments using skinned single skeletal fibers from rat diaphragm (Fig. 2B). Here, tension levels remained unchanged upon PKA-dependent phosphorylation, at both pCa 8.0 (PT) and pCa 4.5 (total tension (passive + developed)). These findings suggest that the reduction of passive and active isometric tension through PKA-mediated phosphorylation results from a β-adrenergic signaling cascade specific to cardiac muscle.

The observed changes in tension after PKA-mediated protein phosphorylation may be due to multiple effects at the myofilament level, as PKA phosphorylates not only titin but also cTnI and cMyBP-C (Fig. 1C; Patel et al. 2001; Murphy, 2006). Phosphorylation of cTnI by PKA is well known to reduce myofilament Ca^{2+}-sensitivity, promote cross-bridge cycling, increase cTnI binding to the thin filament, and enhance Ca^{2+} dissociation from TnC (Kranias and Solaro 1982; Zhang et al. 1995; Kentish et al. 2001; Gomes et al. 2005; van der Velden et al. 2006). These cTnI-mediated effects contribute to accelerated relaxation and β-agonist-induced lusitropy, and are considered downstream mechanisms of β-adrenergic stimulation during increased circulatory demands. Further, phosphorylation of cMyBP-C may contribute to the regulation of myocardial contraction, as PKA-triggered myofilament desensitization requires the presence of cMyBP-C (Cazorla et al. 2006). Whether the PKA-induced drop in total tension of human fibers at pCa 4.5 (Fig. 2A; also cf. Borbely et al. 2005; van Heerebeek et al. 2006) is related to a decreased Ca^{2+}-sensitivity via the phosphorylation effects on cTnI and cMyBP-C, cannot be answered straightforwardly, since changes in Ca^{2+} sensitivity are not always associated with altered maximum active-tension development (Wolff et al. 1996; Witt et al. 2001). However, we propose that at least part of the PKA-induced reduction in total tension at high calcium, which was reported in other species as well (Bartel et al. 1994; Gomes et al. 2005), is caused by phosphorylation of titin. Whereas we and others (Yamasaki et al. 2002; van Heerebeek et al. 2006) have shown a phosphorylation-induced decrease in titin-based PT, there is no evidence at this time that titin also affects developed tension in a PKA-dependent manner. We conclude that most of the observed drop in total tension at pCa 4.5 following phosphorylation (Fig. 2A) may be caused by reduced basal diastolic force levels.

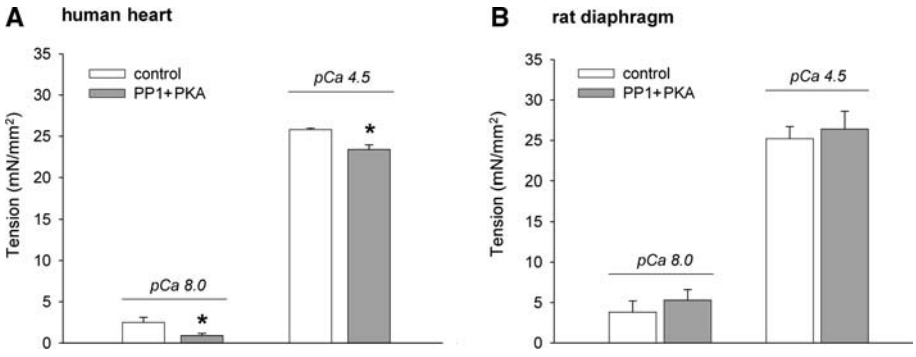

Fig. 2 PKA-effect on passive and developed tension in muscle fibers. Tension at pCa 8.0 (relaxing buffer) and pCa 4.5 (developed + PT) in (**A**) skinned fiber bundles from human donor heart and (**B**) skinned single muscle fibers from rat diaphragm. Human-heart preparations were pre-stretched by 10% from their slack length before activation. Bars represent the tension before (control, white bars) and after (gray bars) 30-min long treatment with PKA following pre-incubation in PP1-containing relaxing buffer. Data are means ± SEM; $n = 3$–7 experiments. $^*P < 0.05$ in Student's t-test

Titin phosphorylation in human heart lowers PT in a SL-dependent manner

The PKA effect on PT of rat heart fibers was reported to be larger at short SLs than at long SLs (Strang et al. 1994; Yamasaki et al. 2002). We find that this phenomenon is also detectable in skinned fiber bundles from human donor hearts (Fig. 3). Figure 3A shows representative passive SL-tension relationships before and after titin phosphorylation (induced by incubating the fibers in PKA-containing relaxing buffer following pre-treatment with PP1). In this example, the relative decrease in PT was 52% at SL 2.05 μm, much greater than the 21% at SL 2.45 μm. A summary graph (Fig. 3B) highlights the average relative drop in PT as a function of SL (mean of 6 fibers per data point). The full range of the phosphorylation-mediated PT decrease is evident from a set of experiments in which the fibers were de-phosphorylated with PP1 before addition of PKA (Fig. 3B, triangles and dashed regression line). Under these conditions, PKA decreased PT on average by ~40% at SL = 2.0–2.15 μm and ~20% above 2.4 μm SL. When the step of pre-treatment with PP1 was omitted in another set of experiments, a significantly smaller average drop in PT was apparent, although the SL-dependency was preserved (Fig. 3B, squares and dashed-dotted regression line): the mean reduction now was 25% at 2.05 μm SL and 17% or less above 2.3 μm SL. As a control, human cardiac fibers were incubated with [γ ^{32}P]ATP for 30 min in the absence of PKA, but

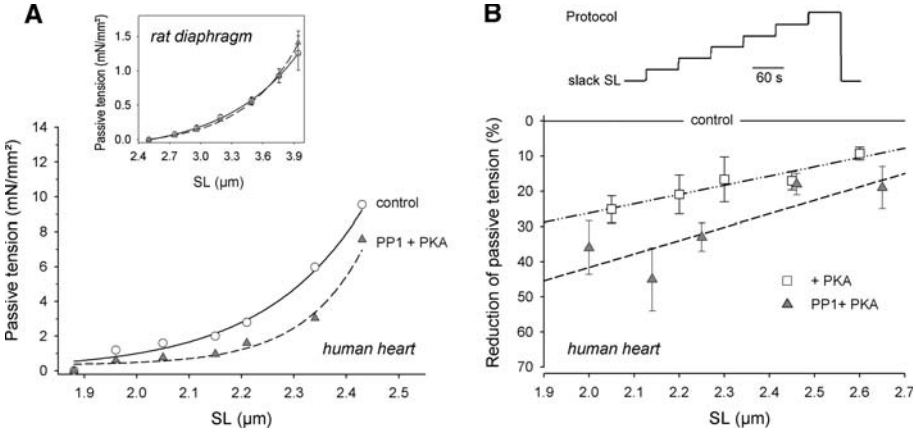

Fig. 3 Impact of PKA treatment on SL dependent PT of skinned fibers. (**A**) Near steady-state PT levels of human cardiac muscle strips before (control, open circles) and after exposure to PP1 and PKA (30 min) (PP1 + PKA, gray filled triangles). Data were fitted with two-order exponential functions. Inset: PT in single fibers from rat diaphragm before (open circles) and after treatment with PP1 + PKA (gray filled triangles). (**B**) PKA-mediated reduction in PT as a function of SL in human cardiac strips. Data were obtained after incubation of samples in PKA only (open squares, dashed-dotted line) or in PP1 followed by PKA (gray filled triangles, dashed line). Data points (mean ± SEM; $n = 6$ fibers from at least two different hearts) for each set of experiments were fitted by linear regression. Inset: Protocol for the experiments in (**A**) and (**B**)

no changes in the passive length-tension relationships were found (data not shown). In summary, titin phosphorylation by PKA reduces PT in human cardiac muscle. This reduction is larger when fibers are pretreated with protein phosphatase-1, suggesting that inherent phosphorylation of titin is involved in determining the basal PT level in human heart tissue.

When another experimental series of this kind was performed on single skinned muscle fibers from rat diaphragm, we detected no PKA-induced alteration in PT, at all SLs (Fig. 3A, inset). Rat diaphragm expresses a rather long N2A-titin isoform (3.6–3.7 MDa (Fig. 1B), which likely contains a large number of differentially spliced Ig domains and PEVK exons (Freiburg et al. 2000; Greaser, 2001; Prado et al. 2005). Clearly, these differentially spliced segments, even if they contained a still-unknown phosphorylation site, cannot be mediators of a PKA-effect on PT. The absence of any effect of titin phosphorylation on PT in skeletal muscle again suggests that the phosphorylation site on titin that is mechanically relevant is in a cardiac-specific segment, the N2B-region.

The N2B-Us within the cardiac N2B-region is an extensible element that elongates predominantly at long physiological SLs (>2.3 μm) when the extension of titin's other stretchable elements, the Ig-domain regions and the PEVK-domain, is largely exhausted (Linke et al. 1999). A possible explanation for the PT decrease upon PKA treatment is altered extensibility of the N2B-Us. It was speculated (Yamasaki et al. 2002) that phosphorylation could alter the contour length and/or persistence length of the N2B-Us, thereby reducing PT at a given SL. However, we recently showed in a single-molecule mechanics approach that PKA-dependent phosphorylation of recombinant human N2B-segment does not modify the force-extension behavior of the N2B-Us (Leake et al. 2006). We concluded that PKA phosphorylation does not directly affect the mechanical characteristics of the N2B spring element. Instead, phosphorylation of the N2B-Us could alter as yet unknown protein–protein interactions (e.g., homophilic binding between N2B-regions from adjacent titin filaments), which may then affect PT. However, the molecular basis for the PKA effect on titin-based PT, as well as the SL-dependency of this effect, as yet remains unresolved.

Reducing conditions enhance the effect of titin phosphorylation on PT

Cardiac titin's N2B-Us has the potential for disulphide bridge formation, at least under oxidative stress conditions (Leake et al. 2006). Whether or not the PKA effect on PT requires reducing conditions—which are the conditions present in the natural environment of muscle cells (Rutten et al. 2005)—was tested here. For these experiments we used isolated myofibrils from rat cardiac muscle (Linke et al. 1996) and measured changes in passive stiffness on PKA treatment in both non-reducing and reducing environments (Fig. 4).

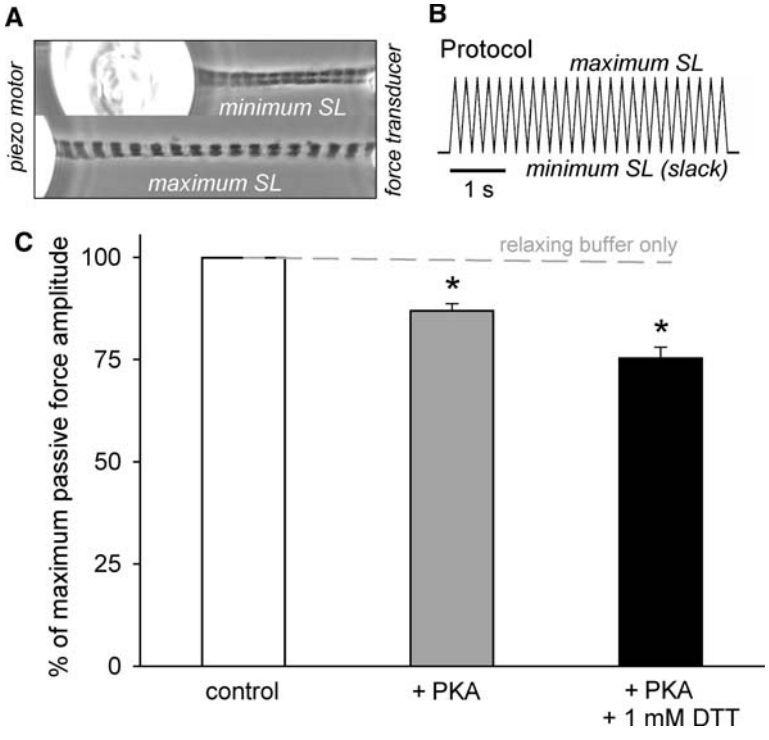

Fig. 4 PKA effect on passive stiffness of isolated rat cardiac myofibrils. (**A**) Images of a myofibrillar preparation at slack SL (minimum SL) and at maximally stretched SL. (**B**) Mechanics protocol, repeated 5–10 times per experimental condition. (**C**) Effect of PKA treatment on the passive-force amplitude of mechanically perturbed myofibrils in the absence (gray bar) and presence of 1 mM DTT (black bar). Data are means ± SEM of $n = 3$ experiments per group and are expressed relative to the maximum force amplitude before addition of PKA (white bar). The dashed gray line indicates control measurement if no PKA/DTT was included in the relaxing buffer. *Significantly different at $P < 0.05$ in Student's *t*-test.

The myofibrils in relaxing buffer (Fig. 4A) were subjected to bursts of 5 Hz stretch-release cycles beginning from slack SL (Fig. 4B) and force responses were recorded before and immediately after PKA treatment. As shown in Fig. 4C, phosphorylation in a non-reducing environment lowered the peak-to-peak passive-force amplitude on average by 13 ± 2% (mean ± SEM, $n = 3$). When reducing agent DTT (1 mM) was present during preparation of the myofibrils and throughout the phosphorylation protocol, the decrease in passive-force amplitude after addition of PKA almost doubled to 24 ± 3%. Thus, the effects of DTT and PKA on passive stiffness are additive. Possibly then, reducing conditions may promote the extensibility of the N2B-Us upon phosphorylation, perhaps by affecting some binding properties of this titin region, but the molecular mechanisms of this putative effect remain to be elucidated.

In summary, we demonstrated that PKA mediates phosphorylation of cardiac-titin isoforms, most probably within the N2B-region, in human donor hearts. PKA reduced the PT in skinned fiber bundles from human left ventricles and in isolated myofibrils from rat heart, an effect that was enhanced under reducing conditions. PKA-mediated titin phosphorylation was stronger and caused a larger drop in PT when fibers were first de-phosphorylated by protein phosphatase-1, suggesting that titin is inherently phosphorylated to some degree in human heart. Skeletal-muscle titin isoform could still be phosphorylated by PKA, but skinned single fibers from rat diaphragm did not respond to PKA with changes in PT. These results let us conclude that β-adrenergic stimulation induces a signaling cascade that, in addition to many other downstream actions, can lower diastolic stiffness in normal human heart via titin phosphorylation.

Acknowledgements We thank Patrick Lang, Dennis Bein and Pascal Michelis for performing mechanical measurements. We are grateful to Drs. Roger Hajjar, Federica del Monte and Judith Gwathmey (Massachusetts General Hospital, Boston) for the kind gift of human heart tissue. This work was supported by grants from the German Research Foundation (Li 690/7-1, SFB 629 to W.A. Linke).

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