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Changes in Isoform Composition, Structure, and Functional Properties of Titin from Mongolian Gerbil (*Meriones unguiculatus*) Cardiac Muscle after Space Flight

I. M. Vikhlyantsev[1]*, A. D. Okuneva[1,2], M. D. Shpagina[1], Yu. V. Shumilina[1], N. V. Molochkov[1], N. N. Salmov[1], and Z. A. Podlubnaya[1,2]

[1]*Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia; fax: (4967) 33-05-53; E-mail: vikhlyantsev@iteb.ru*
[2]*Pushchino State University, 142290 Pushchino, Moscow Region, Russia*

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Abstract—Changes in isoform composition, secondary structure, and titin phosphorylation in Mongolian gerbil (*Meriones unguiculatus*) cardiac muscle were studied after 12-day-long space flight onboard the Russian spacecraft Foton-M3. The effect of titin on the actin-activated myosin ATPase activity at pCa 7.5 and 4.6 was also studied. Almost twofold increase in titin long N2BA isoform content relative to that of short N2B isoform was found on electrophoregrams of cardiac muscle left ventricle of the flight group gerbils. Differences in secondary structure of titin isolated from cardiac muscle of control and flight groups of gerbils were found. An increase in phosphorylation (1.30-1.35-fold) of titin of cardiac muscle of the flight group gerbils was found. A decrease in activating effect of titin of cardiac muscle of the flight group gerbils on actomyosin ATPase activity *in vitro* was also found. The observed changes are discussed in the context of *M. unguiculatus* cardiac muscle adaptation to conditions of weightlessness.

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Key words: Mongolian gerbils *Meriones unguiculatus*, cardiac muscle, weightlessness, titin isoforms, actin-activated ATPase activity of myosin

It is known that the human or animal exposure to authentic or modeled microgravitation (anti-orthostatic bed hypokinesia, dry immersion, suspension model) results in development in skeletal muscle of "hypogravitational muscle syndrome" [1] accompanied by significant morphofunctional changes: muscle fiber atrophy of both slow and fast type [2, 3], destructive changes in thin fibers [3], increased degradation of myosin heavy chains [4], lowered content of sarcomere cytoskeleton proteins, in particular, of titin N2A isoform [5-10], distortion of sarcolemmal membrane dystrophin layer integrity [11], disorganization of desmin filaments [12], increased volume of extracellular matrix [13], and myosin phenotype transformation towards increased fraction of fast isoforms of heavy myosin chains [3, 14]. We also found decreased content or complete destruction of a new higher molecular mass titin isoform, first discovered by us [15], in human and rat *m. soleus* [16, 17] under conditions of modeled microgravitation.

There are few data on morphological alterations in human and animal myocardium and on structure–functional changes in cardiac muscle proteins under conditions of microgravitation. There are data indicative of both human and animal cardiac muscle atrophy under conditions of authentic or modeled microgravitation [18-21] and concerning its absence [22, 23]. It was shown that in rat myocardium after 14-day-long modeling gravitation unloading (suspension model) the decrease in connexin 43 (protein of intercellular gap junctions) content which, according to authors' opinion, contributes to development of cardiac arrythmia [24]. In another work, an increased content of troponin I proteolytic fragment was found in cardiac muscle of suspended rats, which was accompanied by lowered force and rate of cardiac muscle

Abbreviations: AM, actomyosin; ATPase, adenosine triphosphatase; MHC, myosin heavy chains; LC, myosin light chains; LV, left ventricle of the heart; P_i, inorganic phosphate; S1, myosin subfragment 1.

* To whom correspondence should be addressed.

contractions [25]. In this case no changes were revealed in expression of heavy chains of myosin, tropomyosin, and troponin T in cardiac muscle of suspended rats. Earlier, we did not find changes in contents of MyBP-C, light (LC1 and LC2), and heavy (α and β isoforms) myosin chains in myocardium of *Meriones unguiculatus* after their 12-day-long stay conditions of authentic microgravitation [26]. The aim of this work was to study changes in isoform composition and structure–functional properties of titin of cardiac muscle of *M. unguiculatus* after 12-day-long space flight onboard the Russian spacecraft Foton-M3.

MATERIALS AND METHODS

Twelve Mongolian gerbils were selected (4.0-4.5-month-old, mean mass 51.6 g) and divided to two groups, "flight" and "control". The "flight" group gerbils ($n = 6$) underwent real weightlessness for 12 days onboard the Russian spacecraft Foton-M3. Simultaneously gerbils of the control group ($n = 6$) were kept under conditions of terrestrial gravitation. Animals of both groups were placed in a specially equipped module "Contour-L" consisting of a hermetic container-cage for animals and of their survival system. The cage is equipped with a feeder to provide for free access to food in the form of pellets with 18-20% content of water, which corresponds to the moisture content in plant food eaten by gerbils in nature. All procedures connected with maintenance and slaughtering of animals were carried out in the Institute of Medical-Biological Problems of the Russian Academy of Sciences and were approved by Commission on Biomedical Ethics. Experimental material from the flight and control animal groups was taken a day after landing of the spacecraft. Specimens of gerbil left ventricle were frozen in liquid nitrogen immediately after sacrifice and then stored in a Sanyo freezer (Japan) at –80°C.

A macroporous gel with 2.0-2.3% polyacrylamide and 0.5-0.6% agarose content, prepared as described in [27], was used for electrophoretic separation of high molecular mass titin forms (molecular mass 2000-3700 kDa) in the presence of SDS. Electrophoresis was carried out in apparatus with vertical gel arrangement (Helicon, Russia; C.B.S. Scientific Co., USA) in 8 × 10-cm gel plates. Gel densitometry was carried out using the Total Lab v.1.11 computer program. Titin content was estimated relative the content of heavy myosin chains. Table 1 shows arithmetic means of ratios of integral densities of corresponding protein bands in gels and their errors. Statistical data processing was carried out using the nonparametric Mann–Whitney U-criterion. Differences with confidence level $p < 0.05$ were considered as significant.

Titin was Western blotted according to a published procedure [28] with our modifications [17]. Protein transfer from polyacrylamide gel to PVDF membrane (Millipore, USA) after electrophoresis was performed in Tris-glycine/methanol buffer with SDS at current 0.8-1.0 mA/cm^2 for 48-72 h with continuous cooling. Monoclonal antibodies AB5 to the titin molecule region in the A-disc localized near the sarcomere M-line [29] were used as primary antibodies (courtesy of L. Tskhovrebova and J. Trinick, Institute for Molecular and Cellular Biology and Astbury Centre for Structural Molecular Biology, University of Leeds, Great Britain). Antibodies to mouse IgG conjugated with horseradish peroxidase (Sigma, USA) were used as secondary antibodies. Protein bands were identified using 3,3′-diaminobenzidine.

Purity of titin preparations isolated from left ventricle (LV) of *M. unguiculatus* according to Trinick et al. technique [30] was tested by electrophoresis in 7% polyacrylamide gel in the presence of SDS [31].

Straightened separate titin molecules were obtained as described by Tskhovrebova and Trinick [32] with only slight modifications. A drop of solution containing 50% glycerol, 0.5 M KCl, 15 mM phosphate buffer, pH 7.0, and titin at concentration 0.05 mg/ml was sprayed on mica fixed horizontally on a disc (radius 4.8 cm) placed on a spindle of a CM-50 centrifuge (ELMI, Latvia) rotor. After centrifuging for 20-30 sec at 10,000 rpm, the mica with specimen was dried a in vacuum device constructed in the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, and spray coated with platinum at the angle of 30° with rotation, then with carbon at the angle of 90°. The replica was transferred on bidistilled water and placed on copper grids. Electronmicroscopic investigations were carried out in a JEM-100B microscope (Japan) at accelerating voltage 80 kV and magnification ×20,000-40,000. The length of straightened titin molecules was calculated using the Axio Vision LE 4.8.2 software package.

CD spectra were measured on a J815 CD spectrometer (Jasco, Japan) at 4°C in a 1-mm pathlength quartz cell in buffer containing 0.6 M KCl, 30 mM KH_2PO_4, 1 mM DTT, pH 7.0. The volume of the solution was 200 μl, and the titin preparations had a concentration of 0.1 mg/ml. Secondary structure of proteins was calculated using the CONTINLL module of the CDPro program [33].

To study *in vitro* effect of titin from cardiac muscle of the flight and control group gerbils on actin-activated ATPase activity of myosin at pCa 7.5 and 4.6, myosin of rabbit cardiac muscle obtained as described previously [34] was used along with actin of skeletal muscle of the same animal obtained as described in [35]. ATPase activity of reconstructed rabbit actomyosin in the presence or absence of titin of cardiac muscle of the control or flight group gerbils was measured by the yield of inorganic phosphate using a colorimetric method with the dye Malachite green [36]. Myosin filaments were formed in advance by dialysis in the presence or absence of titin in

solution containing 0.12 M KCl, 2 mM $MgCl_2$, 10 mM imidazole-HCl buffer, pH 7.0. The reaction was initiated by addition of preformed myosin filaments to solution containing necessary amounts of actin and ATP (Sigma) and proceeded at 27°C. The reaction mixture contained 0.05 mg/ml myosin, 0.05 mg/ml actin, 0.0125 mg/ml titin, 2 mM ATP, 0.12 M KCl, 2 mM $MgCl_2$, and 10 mM imidazole-HCl buffer, pH 7.0. The necessary Ca^{2+} concentration in the medium was achieved by addition of 0.1 mM $CaCl_2$ or 1 mM EGTA (pCa 4.6 and 7.5, respectively).

The bound phosphate content in titin isolated from cardiac muscle of control and flight group gerbils was estimated as described in [37].

RESULTS AND DISCUSSION

Change in titin isoform composition in *M. unguiculatus* myocardium after 12-day-long space flight. Since our previous data were indicative of decrease of the known N2A titin isoform content (approximately by half) and of a new higher molecular mass form of this protein (3-4 times) in human and rat atrophied muscle soleus after 7- or 14-day-long gravitational relief [6-9, 16, 17], we expected to find a similar decrease of titin content in cardiac muscle of the flight group of *M. unguiculatus*. However, results of electrophoretic investigations did not reveal the decrease in total content of known N2BA and N2B titin isoforms and of a higher molecular mass form of this protein in left ventricle (LV) of the flight group gerbils (Fig. 1 (a and b) and Table 1). However, in this case almost twofold increase in the content of long N2BA titin isoform relative that of short N2B isoform was observed (Fig. 1 (a and b) and Table 1) as confirmed by Western blotting of titin (Fig. 1c).

What is physiological importance of the discovered rearrangement of titin isoform composition in gerbil hearts under conditions of real weightlessness?

It is known that high content of titin N2BA isoform having a longer sequence among immunoglobulin-like (IgC2) domains in the sarcomere I disc, compared to its N2B isoform, correlates with higher elasticity and, as a result, with higher extensibility of cardiac muscle which, according to the Franck–Starling law, results in increased power of heart contractions [39]. It has been shown that humans and animals (rat, cat, dog) staying under conditions of modeled or real weightlessness show transfer of blood and interstitial liquid into the cranium. This results in activation of volume atrial natriuretic reflex causing enhanced water release from the organism via the kidneys [25, 40]. It was also found that in rats after 14-day-long modeling of microgravitation these alterations are accompanied by increase in the blood viscosity due to increase in the total protein content [40]. Increase in blood viscosity was also observed in the flight group gerbils (Prof. E. A. Il'in, personal communication).

Taking into account the above-mentioned data, it seems that the revealed by us increase in fraction of long N2BA titin isoform in left ventricle of the flight group gerbils is aimed at enhancement of the myocardium contractile activity for more viscous blood release from the heart under conditions of space flight. It is interesting to note that similar increase in the fraction of long N2BA titin isoform was earlier registered in different compartments of cardiac muscle of hibernating ground squirrels during hibernation [41], which, most probably, contributes to enhancement of the myocardium contractile activity of these animals during hibernation [42]. The authors explained enhancement of cardiac muscle contractile response in hibernating ground squirrel by increased blood viscosity and increased peripheral resistance of vessels under conditions of hypothermia [41, 42]. A similar adaptation response was also found in *M. unguiculatus* under conditions of real weightlessness. Note that staying of the flight group gerbils under conditions of terrestrial gravitation during the first day after flight could result in alteration of ratio between content of N2BA and N2B titin isoforms (Fig. 1 and Table 1). However, taking into account that the titin half-life is three days [43], it seems that first day re-adaptation of gerbils to terrestrial gravitation could not significantly contribute to alteration of titin isoform composition in cardiac muscle of these animals.

Alteration of secondary structure, phosphorylation extent, and functional properties of titin of *M. unguiculatus* cardiac muscle after 12-day-long space flight. To elucidate possible changes of structure–functional properties of titin of cardiac muscle of the flight group gerbils, in particular of its *in vitro* effect on actin-activated ATPase activity of myosin, titin was isolated from the heart LV of control and flight group gerbils as described by Trinick et

Table 1. Titin content in left ventricle of gerbil heart of control and flight groups

Variant	NT/MHC	N2BA/MHC	N2B/MHC	N2BA/N2B	T2/MHC
Control ($n = 6$)	0.016 ± 0.003	0.027 ± 0.003	0.078 ± 0.006	0.352 ± 0.037	0.032 ± 0.003
Flight ($n = 6$)	0.017 ± 0.002	0.048 ± 0.004*	0.070 ± 0.007	0.682 ± 0.049*	0.038 ± 0.005

* Significance level $p < 0.05$ (comparison with control group).

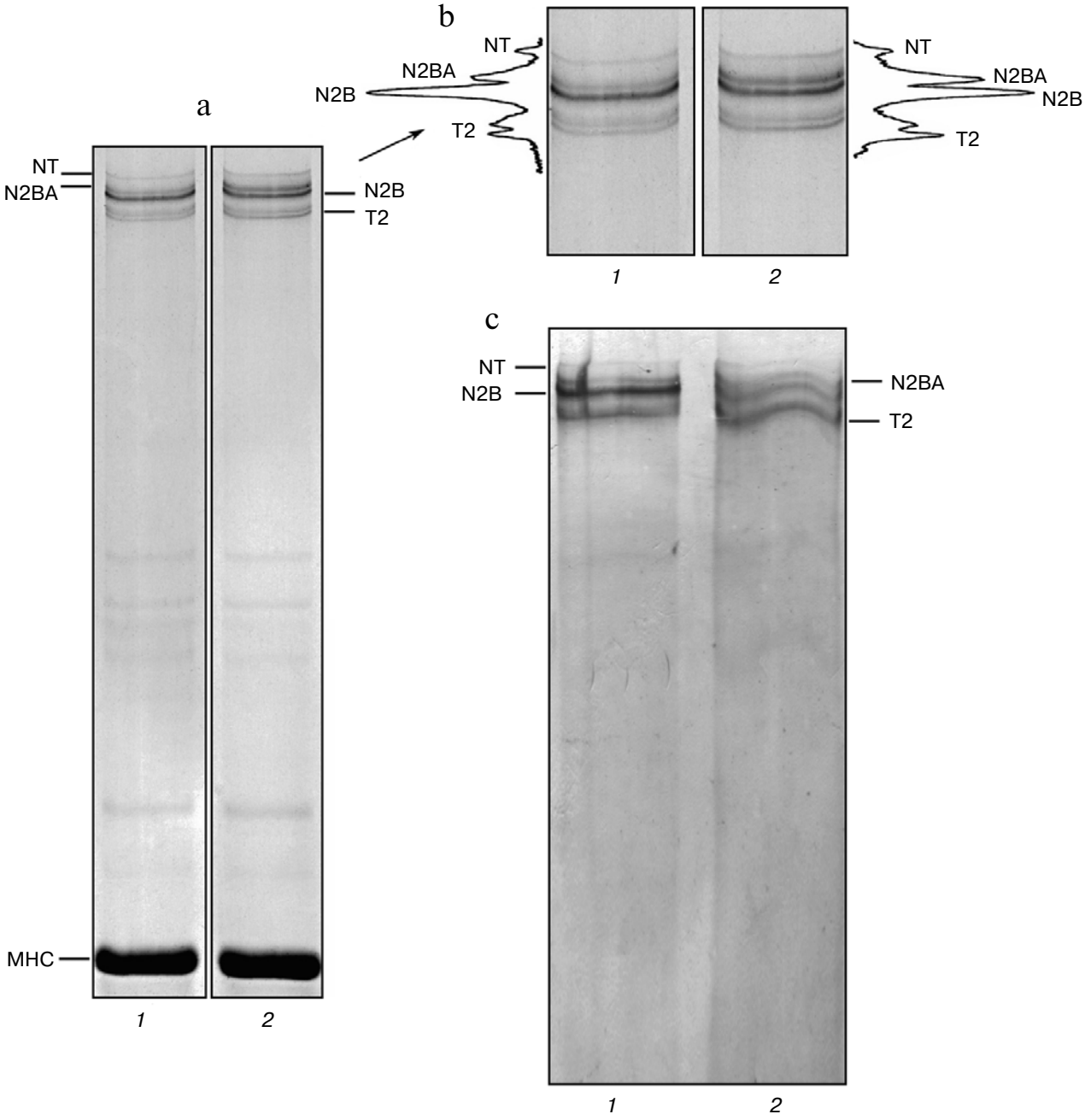

Fig. 1. Changes in titin isoform composition in left ventricle (LV) of gerbil heart after 12-day-long space flight: 1) control group; 2) flight group. a) Electrophoregram of gerbil heart LV in gel containing 2.1% polyacrylamide and 0.55% agarose; b) enlarged upper part of electrophoregram with densitograms; c) Western blotting of titin with AB5 antibodies. Protein bands of myosin heavy chains (MHC), titin T2 fragments, known N2BA and N2B titin isoforms, and of new higher molecular mass intact titin isoforms (NT), whose existence was predicted during investigation of titin gene sequence and expression [38], are designated.

al. [30]. They believe that this method allows isolation of intact protein molecules (titin-1, T1) with mean molecule length 800-900 nm.

Figure 2 shows electron microphotographs of straightened separate titin molecules from gerbil cardiac muscle. The titin molecule looks like a long flexible filament with a globular head at one end, which agrees with data from the literature for titin from rabbit muscle [32]. Most titin molecules from cardiac muscle of both control and flight gerbil groups were of 810-980 nm in length, but the molecule length varied from 630 to 1100 nm (Fig. 2), which also correlates with published data for intact T1 isolated from cardiac and skeletal rabbit muscle [32]. Length scattering of separate straightened titin molecules

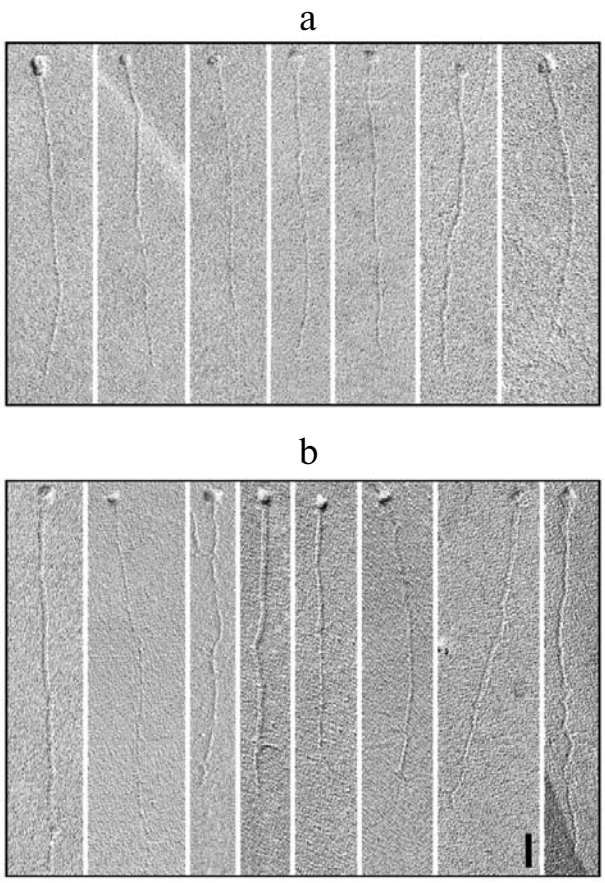

Fig. 2. Electron microphotographs of individual straightened titin molecules from cardiac muscle of control (a) and flight group (b) gerbils in solution containing 0.5 M KCl, 15 mM phosphate buffer, and 50% glycerol, pH 7.0, obtained by circular shadowing with platinum. Scale, 100 nm.

can be explained both by probability of terminal parts of titin molecules getting dusted with metal during preparation of electron microscopic specimens and by obtaining different length molecules of this protein during its isolation.

It was shown by electrophoresis in 7% SDS-polyacrylamide gel according to [31] that electrophoretic mobility of titin isolated from myocardium of *M. unguiculatus* corresponds to that of intact T1 (Fig. 3). These results agree with data of different authors who used the above-mentioned electrophoretic technique and showed that titin isolated from rabbit skeletal muscle [30] and bovine skeletal muscle [44], the mean length of which according to electron microscopy was 800-900 nm, is intact T1.

However, since 7% polyacrylamide gel prepared as described in [31] is not suitable for separation of high molecular mass isoforms of intact titin (in particular, of N2B, N2BA, and NT), electrophoretic analysis of purified titin preparation from gerbil cardiac muscle was carried out in 2.2% polyacrylamide gel with addition of agarose (Fig. 4). The results were unexpected. It was found that electrophoretic mobility of titin isolated from gerbil cardiac muscle corresponds to that of titin T2 fragment but not of its intact isoforms (Fig. 4). These data supported the words of titin's (connectin) discoverer Maruyama [45] who noted in 1986: "At present we have not yet been successful in the isolation of the mother molecule, α-connectin. What has been isolated independently in Japan, the United States, and the United Kingdom is its proteolytic product, β-connectin..." Results of this work show that so far attempts to isolate intact titin isoforms by known methods of preparative biochemistry are also unsuccessful. Therefore, a new method for isolation of intact titin molecules is needed.

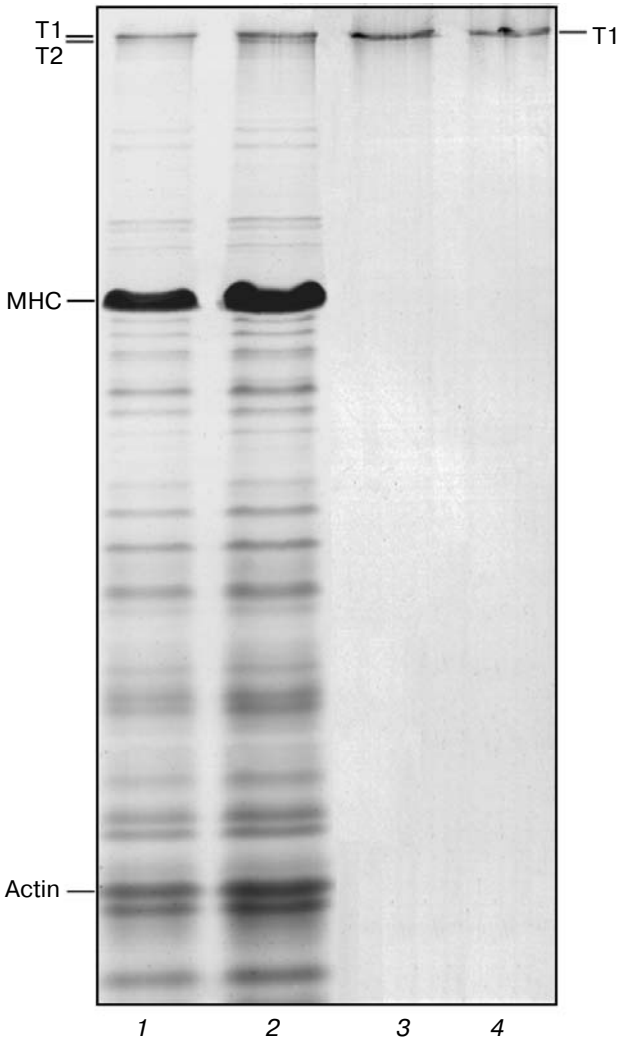

Fig. 3. Electrophoretic testing of the purified titin preparations from gerbil cardiac muscle in 7% polyacrylamide gel. *1*) LV of control group gerbil heart; *2*) LV of flight group gerbil heart; *3*) purified preparation of LV titin from control group; *4*) purified preparation of LV titin from flight group. MHC, myosin heavy chains; T1, intact titin; T2, proteolytic fragment of intact T1.

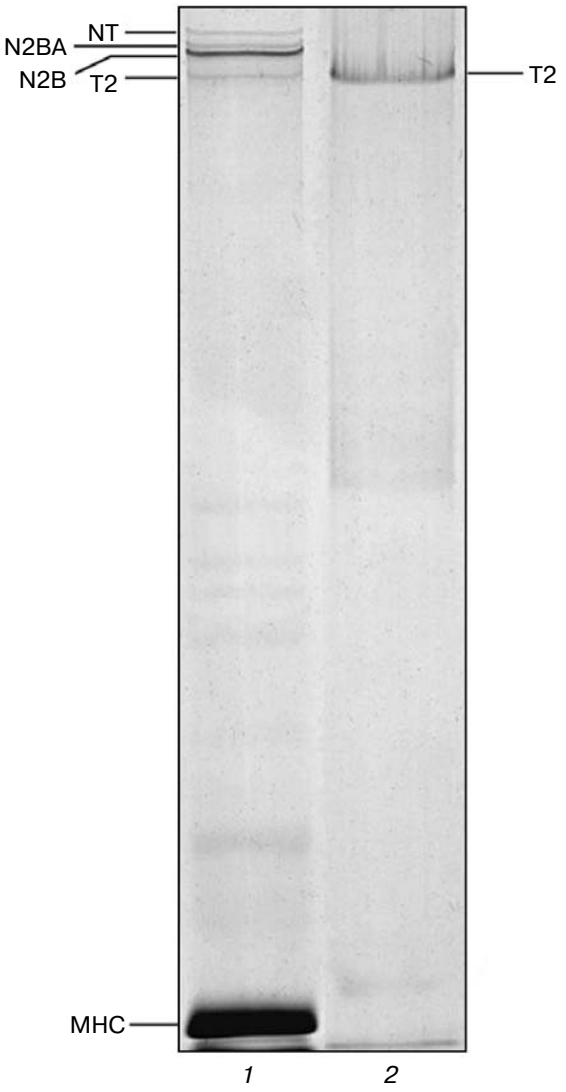

Fig. 4. Electrophoretic testing the purified titin preparation from gerbil cardiac muscle in 2.2% polyacrylamide gel containing agarose. 1) LV of control group; 2) purified preparation of LV titin from control group. MHC, myosin heavy chains; T2, proteolytic fragment of intact N2B, N2BA, and NT titin isoforms.

Despite the impossibility of isolation of intact titin, the effect of T2 fragment of titin from cardiac muscle of control and flight group gerbils on the myosin actin-activated ATPase activity at pCa 7.5 and 4.6 was studied. Considering the fact that *in vivo* T2 (β-connectin) binds myosin filaments along their full length in the sarcomere A disc [46], it can be claimed that studying the *in vitro* effect of this fragment on myosin interaction with actin can be useful for better understanding of actin–myosin interaction and the contribution of titin to this process. It is known from the literature that at μ = 0.08, titin from chicken skeletal muscle exhibited an insignificant activating effect on actomyosin (AM) ATPase [47]. Our previous experiments showed that at ionic strength close to physiological (μ = 0.12), titin of rabbit [48] and ground squirrel [49] skeletal muscle significantly activated actin-activated myosin ATPase and increased its Ca^{2+} sensitivity (i.e. the ability of actin-activated myosin ATPase to increase *in vitro* its activity upon changing pCa from 7.5 to 4.6) [50]. Investigations in our laboratory showed that the ATPase- as well as structural Ca^{2+}-sensitivity of myosin (the latter is expressed in removal of the myosin bridge from the stem of reconstructed myosin filament upon pCa change from 7.5 to 4.6, which results in distortion of axial periodicity in arrangement of myosin bridge layers on the filament surface, i.e. the order–disorder transition is observed) are determined by preservation of the light myosin chains [51-53].

To study the effect of the gerbil cardiac muscle titin on actin-activated ATPase activity of myosin, myosin filaments preformed by dialysis in the presence of titin were used. It was supposed that combined dialysis of these proteins tightly bound within sarcomere [45] will result in formation of myosin filaments with more native structure. Then actin filaments were added to these filaments to study the effect of titin on actin-activated myosin ATPase. Table 2 shows the results of investigation of the effect of titin from cardiac muscle of control and flight group gerbils on actin-activated ATPase activity of rabbit myosin at pCa 7.5 and 4.6. In the presence of the gerbil cardiac muscle titin an increase in the actomyosin ATPase activity was found (1.4-1.6 times). It should be noted that in the case of addition of linear titin aggregates, formed at μ = 0.12, to myosin and actin filaments preformed at this ionic strength [54], inhibition of actin-activated myosin ATPase was observed. This result can be explained by the fact that bundles of linear titin fibrils, which are several microns long, prevent the actin–myosin interaction *in vitro*.

On one side, the titin activating effect on the myosin actin-activating ATPase, revealed in the case of preliminary combined myosin and titin dialysis, can be explained by formation of myosin filaments with more native structure, and on the other side, by titin ability to bind not only myosin filaments [45, 47], but actin filaments as well [55]. In this case, actin–myosin interaction will be maintained by two types of bonds: by ATP-sensitive interaction of myosin subfragment 1 (S1) with actin and by actin–titin–myosin interaction. The latter will contribute to keeping actin filaments close to myosin filaments during the ATP hydrolysis cycle despite periodic dissociation of S1–actin complex, which will result in increased level of actomyosin enzyme activity. Note that the system used in our experiments models the actin–myosin interaction *in vivo*, thus preventing the actin and myosin filaments from moving away from each other, like in a sarcomere where the position of actin and myosin filaments is fixed.

Comparison with the effect of control group titin on actin-activated myosin ATPase showed that titin of the flight group exhibited lower activating effect on AM ATPase both at pCa 7.5 and at pCa 4.6 (Table 2). These changes in functional properties of the flight group titin

Table 2. Effect of titin from gerbil cardiac muscle on actin-activated myosin ATPase activity at pCa 7.5 and 4.6 (μ = 0.12)

Activity of actin-activated myosin ATPase, nmol P_i/min per mg		Ca^{2+} sensitivity of AM, %	Activity of actin-activated myosin ATPase in presence of control group heart muscle titin, nmol P_i/min per mg		Ca^{2+} sensitivity of AM + $T_{control}$, %	Activity of actin-activated myosin ATPase in presence of flight group cardiac muscle titin, nmol P_i/min per mg		Ca^{2+} sensitivity of AM + T_{flight}, %
pCa 7.5	pCa 4.6		pCa 7.5	pCa 4.6		pCa 7.5	pCa 4.6	
51.7 ± 1.5	60.4 ± 1.8	~16.8	83.6 ± 2.4 (↑~161%)	98.1 ± 2.5 (↑~162%)	~17.3	74.2 ± 2.4 (↑~143%)	81.3 ± 3.1 (↑~135%)	~9.5

Note: Symbol ↑ indicates increase (in %) of actin-activated myosin ATPase activity in presence of titin (T) under conditions with low (pCa 7.5) or high (pCa 4.6) calcium content in the medium. In this case levels of AM ATPase activity both at pCa 7.5 and 4.6 are taken as 100%.

Table 3. Secondary structure of titin isolated from cardiac muscle of control ($T_{control}$) and flight (T_{flight}) group gerbils (error ±5-8%)

Specimen	α-Helices, %	β-Rods, %	Disordered regions, %
$T_{control}$	14.8	33.8	51.4
T_{flight}	9.1	23.3	67.6

can be due to changes in the secondary structure of the protein (Fig. 5 and Table 3). In particular, an increase in the content of disordered structure due to lowered content of α and β structures in the flight group titin was found compared to these parameters in control group (Table 3). The differences in secondary structure can be explained by prevalence of T2 fragments of long N2BA titin isoform in the protein preparations isolated from the flight group myocardium (Fig. 1).

The change in the protein phosphorylation extent could also contribute to lowering the flight group titin activating effect. It is known that a part of titin T2 fragment localized in the sarcomere M-line contains phosphorylated sites [56]. The *in vivo* phosphorylation of titin from mouse striated muscle was shown [37]. Our previous investigations showed that the increase of phosphorylation extent of skeletal muscle titin of hibernating ground squirrels is accompanied by decrease of its activating effect on AM ATPase *in vitro* [49]. Taking these data into consideration, we supposed there may be an increase in the titin phosphorylation extent *in vivo* in the flight group. Our results confirmed this supposition. It was found that the flight group titin contains 1.30-1.35 times more bound phosphate than titin of the control group.

The decrease of activating effect of the flight group titin on AM ATPase both at pCa 7.5 and at pCa 4.6 may be explained by the change of titin total charge due to changes in secondary structure and phosphorylation extent of the protein. On one side, these changes could result in the decrease of titin affinity to actin and/or myosin in the *in vitro* system and thus to lowering the amounts of actin–myosin interactions and AM ATPase activity. On the other side, the observed changes could result in enhancement of binding of FnIII-like domains of titin T2 fragments to the myosin S1 [57], which could result in increased amount of myosin bridges pressed to the stem of the thick filament and due to this, the decrease of AM ATPase activity could be observed. Note the decrease of activating effect of the flight group titin on actin-activating myosin ATPase at pCa 4.6 (Table 2) is aimed to increase the diastole time, which together with increase in content of more elastic N2BA titin isoform (Fig. 1 and Table 1) will contribute to the extent of the heart ventricle extension. Most probably, the adaptation significance of these changes is the enhancement of contractile response of gerbil myocardium for more viscous blood flow through the blood vasculature under conditions of authentic microgravitation.

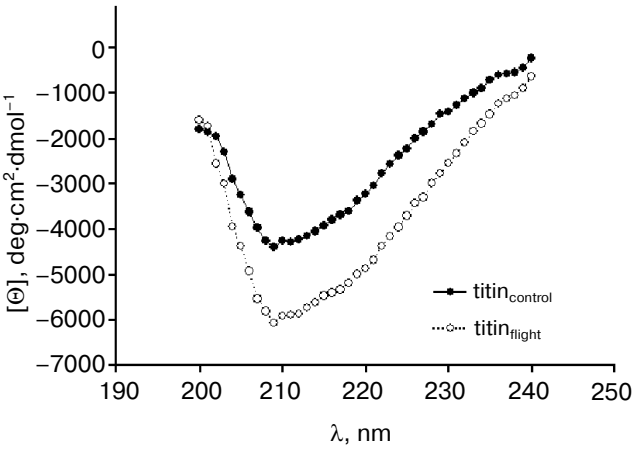

Fig. 5. CD spectrum of titin from control and flight groups.

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