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EFFECT OF FULLERENE C_{60} ON ATPase ACTIVITY AND SUPERPRECIPITATION OF SKELETAL MUSCLE ACTOMYOSIN

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Creation of new biocompatible nanomaterials, which can exhibit the specific biological effects, is an important complex problem that requires the use of last accomplishments of biotechnology. The effect of pristine water-soluble fullerene C_{60} on ATPase activity and superprecipitation reaction of rabbit skeletal muscle natural actomyosin has been revealed, namely an increase of actomyosin superprecipitation and Mg^{2+}, Ca^{2+}- and K^{+}-ATPase activity by fullerene was investigated. We conclude that this finding offers a real possibility for the regulation of contraction-relaxation of skeletal muscle with fullerene C_{60}.

Key words: fullerene C_{60}, skeletal muscle natural actomyosin, ATPase activity, superprecipitation, atomic force microscopy, molecular docking.

The purposeful application of biocompatible low toxic nanomaterials (no more than 100 nm in dimensions) is a relevant problem in modern nanobiotechnologies. It is assumed that the use of nanoparticles will help to solve the problems of early disease diagnostics and targeted delivery of drugs into tissues and cells, as well as new methods of selective therapies. Among other possible effective therapeutic agents a prominent position belongs to the fullerene C_{60} [1]. We know that it normalizes cellular metabolism and neural processes, increasing resistance to stress, exhibits antiviral properties, has a pronounced anti-inflammatory and anti-allergic action, enhances the activity of enzymes and regenerative capacity of tissues [2]. Finally, fullerene C_{60} and its derivatives can be an aid in the treatment of cancer by improving the functions of the immune and antioxidant systems [3–5].

Fullerene C_{60} is hydrophobic – it is insoluble in polar solvents, which limit its bioavailability [6]. In order to increase its hydrophilic properties the C_{60} molecule was widely modified (chemical functionalization) [7]. Aqueous solution of pristine (unmodified) fullerene C_{60} (C_{60}FAS) has been received by the transferring of molecules C_{60} from toluene in water with following sonication [8]. It was found that the dark brown solution is a typical colloidal system containing a single molecule C_{60} and spherical clusters with a diameter of 2-3 nm or more (depending on the concentration of fullerenes C_{60} in water) in hydrated state [9]. The most energetically favorable structure in this aqueous solution is clathrate $C_{60}(H_2O)_{60}$ [8].

Due to the nanodimension the pristine water-soluble fullerene C_{60} can penetrate the cell membrane [10].

Toxicity is a major concern in using fullerene-containing materials for biomedical applications since fullerenes can undoubtedly have toxic effects on human cells and animals [11]. It is important to note that used C_{60}FAS does not show a toxic effect with respect to rat erythrocyte and thymocyte cells at concentrations below 0.1 mg/ml within 24 h of incubation [12, 13].

Despite the large number of the studies on the interaction of fullerene C_{60} with biological objects *in vitro* and *in vivo*, almost no information on their impact on the functional properties of muscles were reported. Only one investigation shows changes in physiological and biochemical processes in the muscles under the influence of fullerene C_{60} and its derivatives. So, it was found that the fullerene derivative monomalonic acid C_{60} (10^{-5} M) inhibited the endothelium-dependent relaxation induced by acetylcholine but did not affect the agonist-induced contractile response of aortic smooth muscle of rabbit [14]. Fullerenol (polyhydroxylated derivative of fullerene C_{60}) inhibits the proliferative responses in a number of cells, including rat

aortic and human coronary artery smooth muscle cells in a concentration dependent manner [15]. Fullerene-based nanocationic particles (porphyrin adducts of cyclohexyl fullerene C_{60}) were described to treat hypoxia-induced mitochondrial dysfunction in the mammalian heart muscle [16]. Electron microscopy demonstrated the ability of complexes of C_{60} with polyvinylpyrrolidone not to prevent the formation of actin filaments, and not to destroy them *in vitro* [17]. Finally, fullerenol nanoparticles have been validated as potential candidates for the creation of artificial muscles because of their excellent proton conductivity, hydrophilicity and biocompatibility [18]. But the mechanisms of nanostructure's action on muscle are still not clear.

The ATPase reaction is the main driving force behind the process of muscle contraction as its chemical stage [19]. The activity in the presence of small concentrations (mM) of Mg^{2+} and enhanced by concentrations of Ca^{2+} of several mM is referred to as Mg^{2+},Ca^{2+}-ATPase. This activity is important considering the physiological conditions in muscle cells. The activity in the presence of EGTA (absence of divalent cations) and high concentrations of KCl is referred to as K^+(EGTA)-ATPase.

It was found that fullerenol influences the membrane ATPase, namely, decreases the activity of Na^+,K^+-ATPase as well as Mg^{2+},Ca^{2+}-ATPase and, thus, may modulate ion transport across membranes [20]. However, the effect of fullerene C_{60} on the actomyosin ATPase activity, which is crucial to the contractile mechanism, is not established.

There is no uncertainty that the ATPase activity underlies the contractile processes of the actomyosin system, but it is noticed that not all the actomyosin ATPase activity is expressed as contraction [21]. The superprecipitation of actomyosin is generally accepted to be basically the phenomenon of skeletal muscle contraction *in vitro* [22]. The superprecipitation reaction can be considered as a simplified model of muscle contraction.

Thus, the aim of this investigation was to measure the effect of fullerene C_{60} in different concentrations on ATPase activity and superprecipitation of rabbit skeletal muscle actomyosin.

Materials and Methods

Material preparation and characterization. The highly stable C_{60}FAS with a maximum concentration of 0.1 mg/ml was prepared according to the procedure [8].

The state of fullerene C_{60} in aqueous solution was monitored using an atomic force microscopy (AFM) technique («Solver Pro M» system; NT-MDT, Russia). Samples were deposited onto cleaved mica substrates by precipitation from an aqueous solution droplet. AFM measurements were performed after complete evaporation of the solvent. Sample visualization was carried out in a semicontact (tapping) mode. NSG10 (NT-MDT) probes were used.

The RCSB PDB database was used as a template to construct a three-dimensional model of skeletal muscle actomyosine complex (actin-1atn [23]; myosin-1mmg [24]). The actin and myosin were «stitched» into a single complex using a Chimera software package (NIGMS, USA). Molecular docking (MD) on the affinity of actomyosin+fullerene C_{60} system was performed with software package flo+ [25].

Contraction of skeletal muscle is based on the interaction of the myosin head (S1) with actin, and is powered by the coupled hydrolysis of ATP. Natural actomyosin was prepared from rabbit (*Soviet Chinchilla*) skeletal muscle using the method suggested by Perry and Corsi [26]. Protein concentration was determined by the biuret reaction, bovine serum albumin being taken as a standard. Natural actomyosin contains the principal protein components such as actin, myosin, and troponin/tropomyosin for the muscle contractile system. Composition of proteins was controlled by electrophoresis [27].

The animals used in this study were treated in accordance with international principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986).

Measurement of ATPase activity. To evaluate the effect of fullerene C_{60} on actomyosin complex the activity of ATPase of native actomyosin (as a control) in the presence of magnesium and calcium (Mg^{2+},Ca^{2+}-ATPase activity or actin-activating ATPase) and in the presence of chelator of Ca^{2+} ions – EGTA (K^+-ATPase activity or actin-relaxing ATPase) was measured. Changes of ATPase activity of protein complexes in the presence of various fullerene C_{60} concentrations (in the range from 10^{-3} to 10^{-7} mg/ml) were measured.

The reaction mixture for Mg^{2+},Ca^{2+}-ATPase as well as for K^+-ATPase at 37 °C consisted of 0.28 mg/ml of actomyosin, 50 mM KCl, 20 mM imidasole buffer (pH 7.5), 2.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Moreover, 1 mM EGTA was added under the K^+-ATPase activity of actomyosin study. Under these conditions ATPase activity of actomyosin was determined by measuring hydrolyzed P_i. The reaction was started by the addition of 1 mM ATP and stopped by the addition of an equal volume of cold 10% TCA. The amount of P_i liberated during the 5 min incubation was determined according to the method suggested by Fiske

and Subbarow [26]. ATPase activity was expressed in nmol P_i $min^{-1} \cdot mg^{-1}$ protein.

All chemicals used in this study were purchased from Sigma (Germany).

Measurement of superprecipitation reaction. The superprecipitation was examined using the method suggested by Ohizumi [26]. The superprecipitation was carried out in a medium which contained 0.2 mg/ml of natural actomyosin, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM KCl, 0.1 mM EGTA and 20 mM Tris·HCl at pH 7.5 and 25 °C, and the change in the absorbance at 450 nm was measured. Fullerene C_{60} was added to incubation medium before 1 mM ATP, which initiates a superprecipitation reaction.

Registration of kinetic curves of superprecipitation of actomyosin was set out on spectrophotometer (SPECORD M40, Germany). From the obtained kinetic curves, the value of superprecipitation was calculated by the formula ($A_{max(450)} - A_0$), where A_0 and $A_{max(450)}$ are the values of the initial and maximum absorbtion of actomyosin, respectively, during the superprecipitation reaction. Absolute error in the measurement of the absorbtion was ± 0.004.

The kinetic Burdyga-Kosterin method [28] was used for the analysis of the results. It allows one to calculate the normalized maximum speed (V_n) of superprecipitation reaction

$$V_n = \frac{1}{A_{max}} \frac{dA}{dt} = \left| -\frac{(n-1)^{\frac{n-1}{n}} \cdot (n+1)^{\frac{n+1}{n}}}{4n\tau} \right|, \qquad (1)$$

where n is a slope (steepness) coefficient of superprecipitation curve; τ is a characteristic time that is necessary to achieve the half of the maximum value of superprecipitation - $1/2(A_{max(450)} - A_0)$. These kinetic parameters are from the experiment.

Changes of superprecipitation reaction value in the presence of fullerene C_{60} at concentrations from 10^{-3} to 10^{-7} mg/ml were measured.

Statistics. Statistical analysis was performed by conventional methods of variation statistics [29]. Validity of the difference between the control and experimental measurements was estimated within the Student's *t*-test using Origin 8.0 software (OriginLab Corporation, USA). The difference between the compared values was considered valid at $P < 0.05$.

Results and Discussion

The AFM picture in Fig. 1, which corresponds to the initial concentration of fullerene C_{60} in aqueous solution (0.1 mg/ml), demonstrates both the individual molecules of C_{60} with diameter

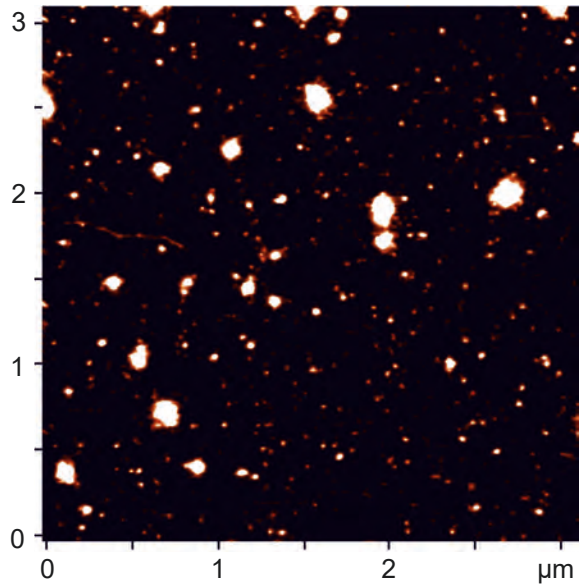

Fig. 1. AFM image of fullerene C_{60} aqueous solution with concentration 0.1 mg/ml on mica (tapping mode)

0.8 ± 0.2 nm as well as their clusters with size 1.5-2 nm, that is in a good agreement with our theoretical calculations and neutron spectroscopic data [8, 9]. In AFM image the presence of individual fullerene C_{60} aggregates with size 10-100 nm was seen too.

In this regard it is necessary to note the effect of ionic strength of the medium on the structural organization of fullerene C_{60} that is important for the clarification of the mechanisms of their specific biological action. So, in paper [30] it was shown that fullerene C_{60} in various physiological solutions forms close-packed islands in the thickness of a monolayer and significantly affects the processes of growth of salt crystals.

MD demonstrates the binding of fullerene C_{60} with actomyosin complex in the active site of myosin (Fig. 2). The calculated energy parameters for the actomyosin+fullerene C_{60} system are shown in Table. Compared with the model of actomyosin binding with ATP, the studied model of fullerene C_{60} binding does not presume the formation of hydrogen bonds. The calculations of the contact energy indicate that fullerene C_{60} well closes the active site of actomyosin complex, filling the pocket and holding there. However, the energy of van der Waals interactions between the ligand and the protein shows a significant steric perturbation, which can neutralize positive contribution to energy of ligand retention. Therefore, the MD results show the possibility of fullerene C_{60} binding in the active center of actomyosin, with a temperate strength.

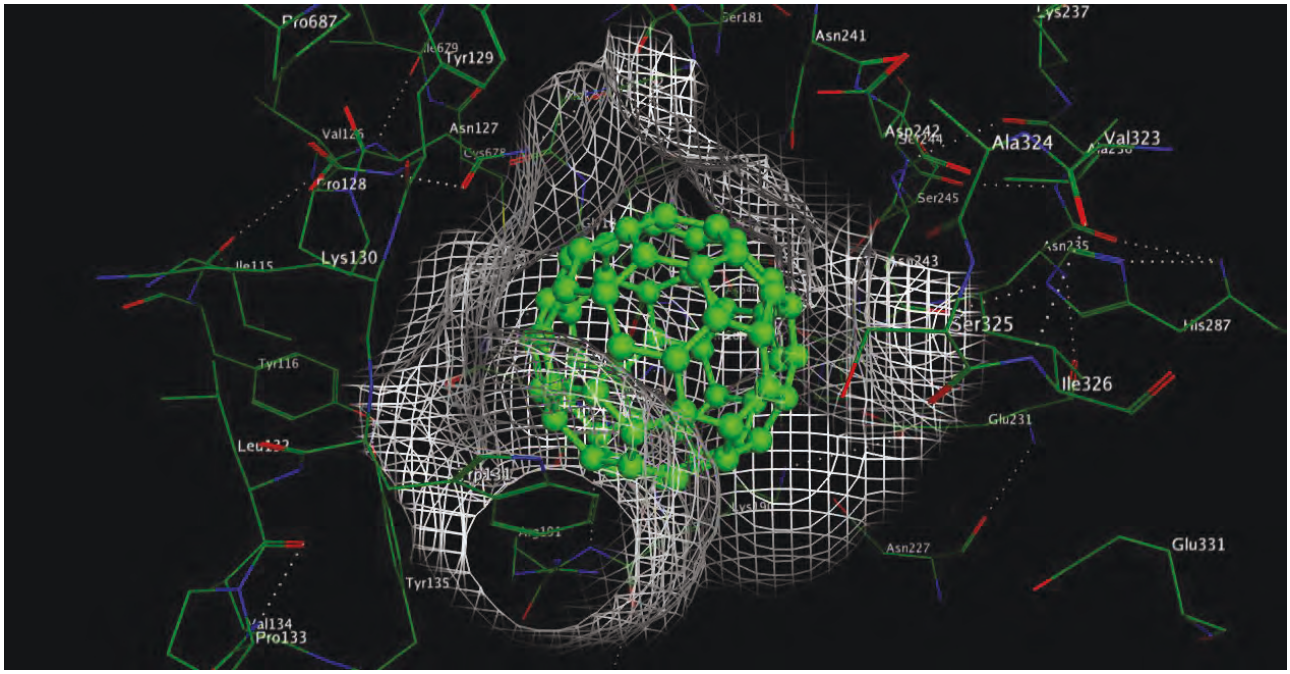

Fig. 2. MD calculated binding model of fullerene C_{60} with active site of myosin

Energy parameters of actomyosin + fullerene C_{60} and actomyosin + ATP (for comparison) systems

Energy/Investigated system	Actomyosin + fullerene C_{60}, kJ	Actomyosin + ATP, kJ
Free energy	50.0	-17.3
Contact energy	-94.0	-97.0
Hydrogen bonds	0	17.0
Energy of van der Waals interactions between ligand and protein	63.5	11.1
Internal stress in the ligand	34.9	23.7

The above MD calculation assumed that actomyosin mainly interacts with a single fullerene C_{60}, and interaction of the fullerene cluster (aggregate) with an active center of actomyosin is unlikely.

The obtained results of impacting fullerene C_{60} on the actomyosin ATPase activity are presented in Fig. 3. Fullerene C_{60} in a low concentration of 10^{-7} mg/ml does not affect the Mg^{2+},Ca^{2+}-ATPase activity of actomyosin, but inhibit K^+-ATPase activity by 29%. Fullerene C_{60} in concentration of 10^{-5} mg/ml caused the increasing in Mg^{2+},Ca^{2+}-ATPase activity by 19% compared with the control value. K^+-ATPase activity of actomyosin also was elevated by 15% under above concentration of fullerene C_{60}. The influence of fullerene C_{60} in concentration of 10^{-3} mg/ml on Mg^{2+},Ca^{2+}-ATPase and K^+-ATPase activity was of the similar character: Mg^{2+},Ca^{2+}-ATPase activity increased by 29%, and K^+-ATPase activity – by 35%. Note that Mg^{2+},Ca^{2+}-ATPase and K^+-ATPase activities were 152.98 and 64.21 nmol $P_i \cdot min^{-1} \cdot mg^{-1}$, respectively, in the control.

In this context, one has to mention that if actomyosin in a dilute solution (10^{-7} mg/ml) preferentially interacts with a single fullerene C_{60}, with the concentration increasing to 10^{-3} mg/ml the interaction with a small fullerene C_{60} aggregate may be predominant, that leads to a more pronounced (see Fig. 3, *a*), or even opposite (see Fig. 3, *b*) effect of fullerene C_{60} on the ATPase activity of actomyosin.

Fig. 4 shows the actomyosin superprecipitation reaction recorded under the fullerene C_{60} action. The results show that the characteristics of superprecipitation reaction were increased depending on the used concentration of fullerene C_{60}: in the presence of 10^{-7} mg/ml of fullerene C_{60} the value

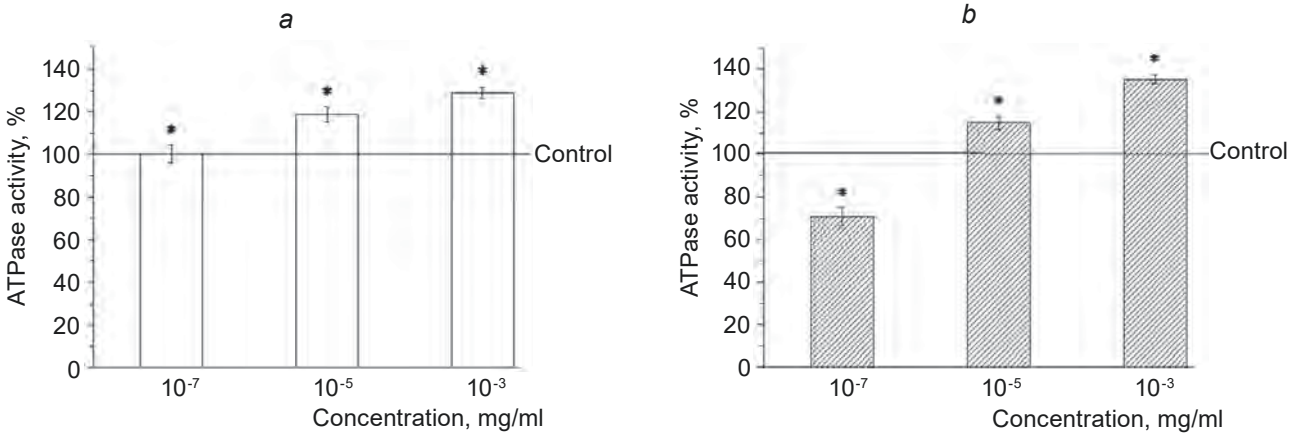

*Fig. 3. Mg^{2+},Ca^{2+}-ATPase (a) and K^{+}-ATPase (b) activity of actomyosin under the different concentration of fullerene C_{60} ($M \pm m$, $n = 9$; *significant differences compared with a control (ATPase reaction without fullerene C_{60}) at $P < 0.05$)*

of superprecipitation enhanced from 0.115 ± 0.002 unities in the control to 0.135 ± 0.002 unities. The time that is necessary to achieve the half maximum value of superprecipitation did not change relative to the control value and was 5.6 min. For the fullerene C_{60} concentrations of 10^{-5} and 10^{-3} mg/ml this parameter increased and reached 6.0 and 7.0 min, respectively. For a concentration of 10^{-5} mg/ml the value of superprecipitation also increased relative to the control and was 0.144 ± 0.002 unities. The greatest value of superprecipitation of 0.180 ± 0.001 unities was observed in the presence of 10^{-3} mg/ml of fullerene C_{60}.

For more information the maximum normalized rate of superprecipitation reaction was calculated by the formula (1). As shown in Fig. 5, at the fullerene C_{60} concentration of 10^{-7} mg/ml, there was a slight increase in normalized maximum rate by 4.7% compared with the control, the value of which was taken as 100%. For higher concentrations of fullerene C_{60}, 10^{-5} and 10^{-3} mg/ml, the value of the normalized maximum rate decreased by 5.5% and 12.2%, respectively, relative to the control.

Thus, fullerene C_{60} showed dose-dependent effect, which may be due to the formation of aggregates (clusters) with increasing concentration in the sample [8-9].

It is known [31–33] that a common feature of the most muscles diseases (progressive muscu-

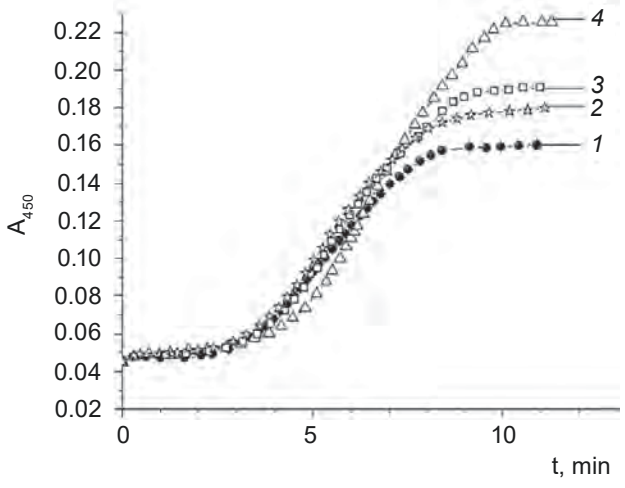

Fig. 4. Typical kinetic curves of rabbit skeletal muscle actomyosin superprecipitation under the different concentration of fullerene C_{60}: 1 – native actomyosin (control); 2 – 10^{-7}; 3 – 10^{-5} and 4 – 10^{-3} mg/ml

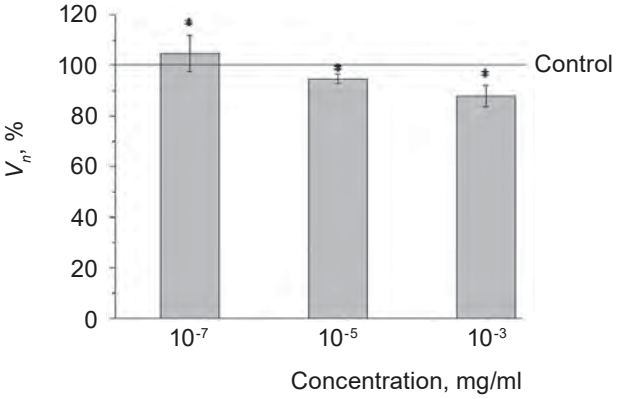

*Fig. 5. Normalized maximum speed (V_n) of superprecipitation reaction (in % compared with control taken as 100%) of rabbit skeletal muscle natural actomyosin under the different concentration of fullerene C_{60} ($M \pm m$, $n = 10$; *significant differences compared with a control (superprecipitation reaction without fullerene C_{60}) at $P < 0.05$)*

lar dystrophy, muscle atrophy, inflammatory myopathy including myositis and polymyositis, etc.), which are accompanied by muscle weakness (e.g., chronic muscle fatigue), is a decline of muscle myofibril protein content. The fall in the ATPase activity of myosin can be also observed in the muscle pathology. On this basis the fact of superprecipitation reaction and ATPase activity of actomyosin promotion by fullerene C_{60} can be promising for further research.

Thus, we conclude that pristine water-soluble fullerene C_{60} effects the function of the actomyosin complex, namely, with increasing fullerene concentration (10^{-5}—10^{-3} mg/ml) the fullerene C_{60} reliably activates the ATPase activity and the superprecipitation reaction of rabbit skeletal muscle actomyosin. This may be due to the binding of fullerene C_{60} with aminoacid residues of the active site of myosin. The obtained data are of great interest with respect to further application of this nanosystem in the regulation of muscle contraction, especially for pathology condition.

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ВПЛИВ ФУЛЕРЕНУ C_{60} НА АТРазну АКТИВНІСТЬ ТА СУПЕРПРЕЦИПІТАЦІЮ АКТОМІОЗИНУ СКЕЛЕТНИХ М'ЯЗІВ

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Створення нових біосумісних наноматеріалів, здатних виявляти специфічну біологічну дію, є важливою комплексною проблемою, яка потребує використання останніх досягнень біотехнології. У роботі досліджено вплив немодифікованого водорозчинного фулерену C_{60} на АТРазну активність і реакцію суперпреципітації натурального актоміозину скелетних м'язів кроля. Встановлено дозозалежний характер його дії на Mg^{2+},Ca^{2+}- та K^+-АТРазну активність та суперпреципітацію актоміозину. Це відкриває перспективи регуляції процесу скорочення—розслаблення скелетних м'язів за допомогою досліджуваного C_{60}.

Ключові слова: фулерен C_{60}, актоміозин скелетних м'язів, АТРазна активність, суперпреципітація, атомна силова мікроскопія, молекулярний докінг.

ВЛИЯНИЕ ФУЛЛЕРЕНА C_{60} НА АТРазную АКТИВНОСТЬ И СУПЕРПРЕЦИПИТАЦИЮ АКТОМИОЗИНА СКЕЛЕТНЫХ МЫШЦ

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Создание новых биосовместимых наноматериалов, способных проявлять специфическое биологическое действие, является важной комплексной проблемой, которая требует использования последних достижений биотехнологии. В работе исследовано влияние немодифицированного водорастворимого фуллерена C_{60} на АТРазную активность и реакцию суперпреципитации натурального актомиозина скелетных мышц кролика. Установлен дозозависимый характер его действия на Mg^{2+},Ca^{2+}- и K^+-АТРазную активность и суперпреципитацию актомиозина. Это открывает перспективы регуляции процесса сокращения—расслабления скелетных мышц с помощью исследованного фуллерена C_{60}.

Ключевые слова: фуллерен C_{60}, актомиозин скелетных мышц, АТРазная активность, суперпреципитация, атомная силовая микроскопия, молекулярный докинг.

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