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MOLECULAR BIOPHYSICS

Isoform-Specific Functions of Na,K-ATPase in Skeletal Muscle

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Abstract—The published data and the results of the author's own research in the field of the molecular and functional diversity of Na,K-ATPases are reviewed. Na,K-ATPase is an integral membrane protein that maintains the concentration gradients of Na^+ and K^+ that are essential for electrogenesis, excitability, and several other processes of cellular transport. Most of the Na,K-ATPase of vertebrates is found in the skeletal muscle tissue, which co-expresses the α1 and α2 isoforms of the catalytic and transport α-subunit of Na,K-ATPase. The activity of Na,K-ATPase is crucial for the contractile function and prolonged activity of skeletal muscle. The data that have accumulated indicate that the α1 isoform of Na,K-ATPase fulfills the major pumping function. The α2 isoform fulfills additional functions related to the specific membrane localization of the protein, the functional interactions with the proteins and lipids of the environment, and fine-tuned regulation by a variety of factors, including motor activity.

Keywords: Na,K-ATPase, isoforms, nicotinic acetylcholine receptor, safety factor, neuromuscular transmission, cardiotonic steroids, motor activity

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THE Na,K-ATPase AND ITS MAIN FUNCTIONS

The contractile activity of skeletal muscle is an extremely important factor in the functioning of mammals, including humans. The weight of skeletal muscle amounts to 40% of the total body weight; up to 75% of all K^+ in the body is found in the skeletal muscle, whereas the fraction of extracellular K^+ accounts for only 2%. The normal K^+ concentration in muscle cells is approximately 160 mM, whereas the value for blood plasma and the extracellular fluid ranges from 3.5 to 5.3 mM [1, 2]. The generation of endplate potentials and muscle action potentials required for contractile activity is accompanied by the accumulation of Na^+ in the myoplasm, whereas K^+ is accumulated in the external medium [1, 3–5]. The concentration of K^+ in blood that flows out of active muscle can be as high as 8 mM, whereas the concentration in the extracellular medium may exceed 10 mM, while that in the narrowest parts of the network of T-tubules can be tens of mM [1–3, 5, 6]. The decrease of the gradient of K^+, which is the main potential-forming ion, leads to depolarization of the sarcolemma, inactivation of voltage-gated Na^+ ($Na_v1.4$)-channels, a decrease in excitability, and disturbances in the generation of action potentials and the functioning of the excitation–contraction coupling system [1, 3, 4]. Moreover, the accumulation of K^+ in the synaptic clefts may suppress the induced quantum secretion of acetylcholine from motor-nerve endings [7]. The presynaptic and muscle disorders listed above lead to impaired working capacity, fatigue, and sometimes even blockade of neuromuscular transmission [3, 5, 8, 9].

The Na,K-ATPase plays a key role in the physiological processes that prevent the disturbances in Na^+ and K^+ gradients and maintain the normal concentration of extracellular K^+ in the skeletal muscle [9]. This transport system, which was discovered by J. Skou [10] (Nobel Prize in Chemistry 1997), is formed by an integral plasma-membrane protein that acts as an Na,K-pump and is found in almost all animal cells. The main function of Na,K-ATPase is the maintenance of transmembrane gradients of Na^+ and K^+ by means of ATP-dependent active transport of these ions. These gradients provide the necessary level of membrane potential and cell excitability and play an important role in the regulation of the cell volume and intracellular pH. The Na^+ gradient formed by the Na,K-ATPase provides the functioning of the conjugate transporters for Ca^{2+} and H^+, glucose, amino acids, neuromediators, and vitamins [1, 3, 4, 11–14].

The major pool of Na,K-ATPase is accumulated in skeletal muscle. The density of Na,K-ATPase in the sarcolemma is extremely high, ranging from 1000 to 3350/μm^2 in different types of muscle tissue [8]. Intracellular Na^+ and extracellular K^+ are the most important regulators of the functioning of Na,K-ATPase; the concentrations of these ions depend on motor activity. This form of regulation is rapid and short acting; it is mediated by changes in the catalytic activity of the enzyme. Moreover, the activity and biosynthesis of

Abbreviations: nAChR, nicotinic acetylcholine receptors; CTSs, cardiotonic steroids.

Na,K-ATPase may be regulated by various neurotransmitters and hormones, such as catecholamines, insulin, and insulin-like growth factor 1, as well as thyroid and corticosteroid hormones. These factors may be involved in short-term regulation mediated by the appropriate receptors and intracellular messenger systems. However, long-term regulation of the enzyme activity occurs as well. The changes of the number of Na,K-ATPase molecules in the sarcolemma that underlie this type of regulation may be due to changes in the rate of protein synthesis and degradation [3, 8] or in the rate of translocation (exchange with the intracellular pool of Na,K-ATPase) [15].

The transport of ions by Na,K-ATPase is mediated by ATP-dependent cyclic transitions between the two major conformational states, E1 and E2, that enable selective binding of three intracellular Na^+ ions and two extracellular K^+ ions, respectively [16, 17]. The energy of a single ATP molecule is used to transport three Na^+ ions from the cell; two K^+ ions are transported into the cell at the same time; thus, a single positive charge is removed from the cell within one cycle. Therefore, the factors that determine the value of the resting membrane potential (V_m) of a cell are not limited to ion gradients. In fact, the direct effect of the electrogenic Na,K-pump leads to additional polarization of the membrane due to the lack of compensation between the flows of Na^+ and K^+ across the membrane (the so-called electrogenic component of the V_m). The registration of this component of the potential (abolished by ouabain) is used to evaluate the electrogenic activity of Na,K-ATPase. This activity is rather high in skeletal-muscle fibers: its contribution can amount to 12–20 mV depending on the muscle type and the specific area of the sarcolemma [18–22].

The V_m of the muscle-fiber membrane at rest is close to –60 mV if the cell membrane is depolarized due to the blocking of Na,K-ATPase activity by ouabain or other means (cooling or immersion in a potassium-free solution) [18–23]. Ion gradients apparently play a major role in maintaining this V_m value in the absence of ion transport. In contrast, activation of Na,K-ATPase causes hyperpolarization of the cell membrane. Activation of the Na,K-ATPase of the mammalian skeletal muscle by pharmacological agents or muscle stimulation leads to muscle-membrane hyperpolarization; the value of hyperpolarization can reach 10 mV [18–21, 24–27].

Activation of Na,K-ATPase and the increase of the electrogenic component of the V_m are of fundamental importance for the maintenance of the contractile function and efficiency of the neuromuscular system exposed to various modifying factors. Numerous studies have shown that activation of Na,K-ATPase by means of tetanic stimulation of muscle or the application of pharmacological agents contributes to the recovery of muscle contractility [3, 4, 8].

Importantly, Na,K-ATPase also plays a key role in the maintenance of the balance of K^+ in the central nervous system where the extracellular K^+ concentration can increase significantly due to neuronal activity [28].

THE MOLECULAR FORMS OF THE Na,K-ATPase AND ITS LOCALIZATION

Na,K-ATPase is a dimer that consists of α- and β-subunits, and certain tissue-specific isoforms of the enzyme contain a third subunit, one of the proteins of the FXYD family [11, 12, 14, 29–31]. The α-subunit is a relatively large (~110 kDa) integral protein that includes ten transmembrane domains; both ends of the peptide chain extend into the cytoplasm. This subunit contains binding sites for Na^+, K^+, and ATP and is responsible for the catalytic and transport properties of the Na,K-ATPase. The presence of a binding site (receptor) for cardiac glycosides that serve as specific ligands (inhibitors) of Na,K-ATPase is an important feature of the extracellular part of the α-subunit of the enzyme [14, 32–35]. The β-subunit is a single-pass membrane glycoprotein (~ 31.5 kDa) with a short N-terminal fragment exposed to the cytoplasm and a large C-terminal fragment located on the external side of the cell membrane. This subunit plays an important role in the localization and stabilization of the α/β functional complex in the plasma membrane and is involved in the modulation of Na,K-ATPase activity and in cell adhesion [36].

Proteins of the FXYD family (~7 kDa) have a transmembrane domain as well, but differ from the β-subunit with regard to being a non-essential structural and functional component of Na,K-ATPase. The FXYD subunit is involved in the modulation of the affinity of Na,K-ATPase to Na^+, K^+, and ATP; it also affects the kinetic and transport characteristics of the enzyme [37, 38]. The non-phosphorylated FXYD subunit is assumed to inhibit the catalytic activity of Na,K-ATPase in muscle cells. Phosphorylation of FXYD involves protein kinases A and C and promotes an increase in the affinity of the intracellular sites for Na^+, as well as a general activation of Na,K-ATPase [38, 39].

The existence of several molecular forms is characteristic of Na,K-ATPase, as well as of other functional proteins [40–43]. Four isoforms of the α-subunit (α1–α4), three isoforms of the β-subunit, and seven proteins of the FXYD family have been discovered in mammals; all these proteins are encoded by individual genes. Isoforms of the α-subunit differ with regard to sensitivity to Na^+, K^+, and ATP and have some other distinguishing features. Combination of the different isoforms of α- and β-subunits with specific FXYD proteins provides the high level of molecular and functional diversity of Na,K-ATPase [11, 12, 29, 31, 44].

The α1 isoform is found in all animal cells and is the only isoform expressed in certain tissues (such as the kidneys, the liver, and erythrocytes). The cells of most tissues express one of the α2–α4 isoforms in addition to the α1-isoform. The α2-isoform is found in skeletal, cardiac, and smooth muscle, adipocytes, and glial cells; the α3-isoform predominates in the

nervous tissue, and the expression of the α4-isoform is restricted to the testis and sperm [11–13, 29, 31].

The α1-isoform of Na,K-ATPase usually has a relatively uniform distribution in the plasma membrane. Restricted localization in microdomains of a specific organization formed by the plasma membrane and intracellular organelles (sarco-(endo-)plasmic reticulum or mitochondria) is a characteristic feature of α2–α4-isoforms expressed in smooth and cardiac muscle, astrocytes, and spermatozoa. Localization to these compartments allows the interaction of these isoforms of Na,K-ATPase with ion transporters that are dependent on the gradient of Na^+ (Na^+,Ca^{2+}, and Na^+,H^+ exchangers), ion channels, receptors, and other functional proteins [45–49]. A fragment that is necessary for the capacity of clustering to the microdomains described above was detected in the N-termini of the α2- and α3-isoforms of Na,K-ATPase [50]. Moreover, cytoskeletal proteins, such as spectrin and ankyrin, are involved in the formation of such microdomains [51–53].

The membrane proteins are also known to interact with the lipid environment, which exerts considerable effects on the functioning of Na,K-ATPase. A distinct pool of Na,K-ATPase is located in specialized microdomains of lipid membranes (lipid rafts and caveolae). The interaction of Na,K-ATPase with the environment in these microdomains enables the formation of regulatory multimolecular complexes and the fulfillment of novel functions, especially those related to signaling [33, 47, 53–56].

Expression of the α1- and α2-isoforms of Na,K-ATPase is characteristic of the skeletal muscle of rodents [11, 44, 57], whereas both these isoforms and the α3-isoform are expressed in human skeletal muscle [58–61]. Both the α1- and α2-isoform may be associated with β1 or β2 subunits [62, 63], as well as with the FXYD1 subunit (phospholemman) [20, 21].

There are numerous reports of isoform-specific functions of Na,K-ATPase in skeletal muscle [2, 64–66]. The induction and the regulation of activity by the thyroid hormone, insulin, glucocorticoids, extracellular K^+, cholinergic ligands, endogenous digitalin-like factors, and physical activity are more pronounced in case of the α2-isoform of the skeletal muscle fibers [2, 19, 22, 62, 67–70]. The relative abundance of the α2-isoform in various types of muscle ranges from 60 to 87% of the total Na,K-ATPase [44, 57, 62]. However, the α1-isoform fulfills the basic pumping function related to ion gradient maintenance in the resting state and the contribution of the α2-isoform to V_m in skeletal-muscle fibers is relatively small [19, 20, 71].

Substantial heterogeneity of the distribution of α1- and α2-isoforms of Na,K-ATPase in the plasma membrane and membrane compartments, such as the T-tubules and caveolae, has been reported. The α1 isoform in the surface plasma membrane is distributed uniformly and the content of this isoform in the caveolae is negligible. The α2-isoform is mostly located in the T-tubules and is much less abundant in the caveolae [9, 63, 72]. The activity of the α2-isoform, which is located in the T-tubules, has been shown to play an important role in the maintenance of the functional capacity of the skeletal muscle under heavy loading accompanied by a significant increase in the concentration of K^+ in the narrow parts of the T-tubules characterized by low efficiency of diffusion [64, 65]. The α1-isoform is assumed to fulfill the major pumping function in the resting state and during weak contractile activity, since the affinity of this isoform for K^+ is relatively high. The activity of the α2-isoform is low under these conditions; however, it increases quickly in the case of intensive functioning of the muscle accompanied by the accumulation of K^+ in the T-tubules and thus plays a critical role in maintaining the contractility of skeletal muscle [65].

The existence of a distinct pool of the α2-isoform of Na,K-ATPase in the end plate [20, 22] is worth mentioning. The functional role of this pool will be considered in the next section of this review.

THE Na,K-ATPase AND THE SAFETY FACTOR OF NEUROMUSCULAR TRANSMISSION

The maintenance of the V_m of the muscle fibers at a certain level is a prerequisite for the proper functioning of skeletal muscle. Membrane depolarization leads to the inactivation of Na^+-channels, reduction of excitability, deceleration of the development and propagation of action potentials, and impairment of the functioning of the excitation–contraction coupling system [73, 74]. Disturbances of electrogenesis in the parasynaptic region of the sarcolemma have the most dramatic negative consequences, since the local potential of the end plate is transformed into the propagating action potential in this area. The contractile capacity of a muscle fiber depends on the so-called safety factor of neuromuscular transmission, which is defined as the ratio of the amplitude of the endplate potential to the threshold of action potential generation [73, 75]. The contraction of each individual muscle fiber can occur if the value of the safety factor is higher than one. The safety factor for different muscle types ranges from 2 to 5; importantly, this value decreases during exhausting rhythmic activity [73].

Prolonged depolarization of the endplate membrane leads to slow inactivation of a considerable number of sodium channels in this area, although the degree of depolarization may be relatively low. This leads to an increase in the threshold of the generation of action potential in the muscle and to a decrease of the safety factor of neuromuscular transmission, whereas the hyperpolarization of the membrane has an opposite effect [75]. In light of this, the hyperpolarization of the endplate membrane of the skeletal muscle relative to the extrasynaptic area of the sarcolemma (by 2–4 mV) is of special importance. This local hyperpolarization is considered an important factor for the structural and functional organization of the

neuromuscular synapse. A range of studies have indicated the constant presence of nanomolar concentrations of non-hydrolyzed acetylcholine (regardless of acetylcholinesterase activity) in the synaptic cleft as the reason for an increase of the electrogenic activity of Na,K-ATPase and the resulting hyperpolarization in the parasynaptic area [25, 27, 76]. Non-quantal release of the neuromediator from the motor nerve ending and the muscle fibers presumably is the major source of acetylcholine, although individual molecules of acetylcholine may persist in the synaptic cleft after neuronal discharge.

Later studies pointed at the functional interaction between the nicotinic acetylcholine receptors (nAChRs) and Na,K-ATPase as the basis of the hyperpolarizing effect of acetylcholine. Two models of this interaction have been proposed.

The first model points to the entry of Na^+ through the open nAChR channels as the process that mediates the functional interaction between nAChRs and Na,K-ATPase. The universally accepted concept of nAChR functioning implies conformational transitions between the closed, open, and desensitized states; the transitions are assumed to obey the laws of probability [77–79]. Activation of the nAChR and opening of the ion channel of the receptor requires micromolar concentrations of acetylcholine. The local concentration of acetylcholine in the active zones of individual quanta may amount to 10^{-2}–10^{-4} M, so that acetylcholine exerts its effects at a saturating concentration and the highest efficiency of the interaction of the neurotransmitter and the nAChRs is attained. Activation of nAChRs leads to the opening of the ion channel of the receptor and depolarization of the cell membrane. However, cholinergic agonists can cause the opposite effect (membrane hyperpolarization) due to the functional interaction of nAChRs with Na,K-ATPase. As an example, trace hyperpolarization of the membrane may develop in neurons after spike activity or exposure to acetylcholine. Experiments on rat ganglion neurons showed that the stable hyperpolarization (that may persist for several minutes) that occurs after exposure to acetylcholine in micromolar concentrations was mainly due to activation of Na,K-ATPase by sodium ions that enter through the nAChR channels. Hyperpolarization of this type is assumed to serve as a mechanism of internal regulation of membrane excitability and the intensity and frequency of postsynaptic neuron discharges [80].

The second model based on the assumption of direct molecular interaction of nAChRs and Na,K-ATPase in the skeletal muscle as the basis of the functional interaction of these proteins [20, 81, 82]. The facts used to develop this model are listed below. The ion currents through the open nAChR channel (especially incoming Na^+) apparently do not act as a decisive factor in the functional interaction between nAChRs and Na,K-ATPase [20, 81, 83]. The binding of acetylcholine to nonconductive desensitized nAChRs has been reported. The affinity of a desensitized receptor to acetylcholine is elevated by 2–4 orders of magnitude and lies in the nanomolar range [84–86]; therefore, it is comparable to the concentration of non-hydrolyzed acetylcholine in the synaptic cleft. The latter observation is in good agreement with reports of the hyperpolarizing action of nanomolar concentrations of acetylcholine [24, 25, 27] and nicotine [83] on skeletal muscle. The effect is reportedly mediated by activation of the α2-isoform of Na,K-ATPase [20, 81, 83, 87]. Moreover, pharmacological agents that shift the balance between the conformational states of rest and desensitization of the nAChRs are reportedly capable of modulating the hyperpolarizing action of acetylcholine [20]. Experiments with membrane preparations from the *Torpedo* electric organ (cell-free system) revealed reciprocal molecular interactions between nAChRs and Na,K-ATPase present in the membranes. Conformational changes that followed the specific binding of the respective ligands were shown to mediate the interaction [20, 81]. Finally, the co-localization of nAChRs and the α2-Na,K-ATPase isoform in the end plate and co-immunoprecipitation of nAChRs, Na,K-ATPase, phospholemman (FXYD1 protein), and caveolin-3 has been demonstrated, this is indicative of the putative localization of this multimolecular complex in the caveolae [20, 21].

These data provided the foundations for the hypothesis of the existence of a functional molecular complex of nAChR and α2Na,K-ATPase in the caveolae. The desensitized state of the nAChR and the conformational state E2 of the Na,K-ATPase were thought to be the essential prerequisites for the interaction of these proteins [20, 66, 81, 82]. Local hyperpolarization of the endplate membrane is the result of this interaction; hyperpolarization is regarded as a physiological mechanism that contributes to the prevention of the inactivation of Na^+-channels and to the maintenance of the safety factor of neuromuscular transmission [20, 66, 82].

Importantly, the functional and molecular reciprocal relationship of this type is not limited to Na,K-ATPase and the nAChRs of the muscle type in skeletal muscle: a similar interaction was observed in case of neuronal-type nAChRs of insects [88]. Additional evidence of a new functional role of the nAChRs as modulators of Na,K-ATPase has been obtained; this function of the receptors is not related to the functioning of the nAChRs as ligand-gated ion channels.

The putative role of other components of the molecular environment (except for FXYD1 and caveolin-3) in the organization of the nAChR/α2Na,K-ATPase complex remains uncharacterized. As an example, very little is known of the contribution of cytoskeletal proteins. The cytoskeletal protein dystrophin is involved in the organization of the post-synaptic scaffold and in nAChR clustering [89, 90]. Experiments on *mdx* mice with defects in dystrophin synthesis point to the involvement of this protein in the

molecular processes that underlie the stable local hyperpolarization of the endplate membrane related to the functional interaction of nAChRs and the α2-isoform of Na,K-ATPase [91, 92]. Spectrins and ankyrins, which determine the specific localization of Na,K-ATPase [51, 52, 72, 93], may mediate the functional interaction between this enzyme and nAChRs as well; however, this issue requires additional analysis.

The lipid environment exerts a considerable influence on the functioning of membrane proteins [94]. As an example, the interaction of cholesterol with nAChRs is important for the normal functioning of these receptors and for their clustering in the end plate [95–97]. These data, as well as the documented role of cholesterol in the compartmentalization, stabilization, and regulation of Na,K-ATPase [56, 98–101] point to the putative involvement of cholesterol in the formation of functional interaction between nAChRs and the α2-isoform of Na,K-ATPase. As an example, the redistribution of cholesterol of the lipid rafts upon the application of methyl-β-cyclodextrin is reportedly accompanied by a decrease of the electrogenic activity of the α2-isoform of Na,K-ATPase and the extinction of the local hyperpolarization of the end plate membrane of the rat diaphragm [102].

The role of the actin cytoskeleton involved in the modulation, clustering, and diffusion of membrane proteins, along with lipid raft stabilization, should be taken into account as well [103–105].

THE Na,K-ATPase AND CIRCULATING CHOLINERGIC LIGANDS

The effects of tobacco are due to the physiological effects of the alkaloid nicotine, a specific exogenous ligand of nAChRs, which mediate the variety of effects of the neuromediator acetylcholine. These receptors are involved in many physiological and behavioral functions of the central nervous system (cognitive processes, memory and learning, neuronal development, cerebral blood flow and metabolism, formation of nicotine addiction, and others) [106, 107], whereas the nAChRs of the skeletal muscle mediate the synaptic transmission of the signal from the motor nerve to the muscle fiber.

The concentration of nicotine in the blood via smoking is approximately 100 nM [108, 109]. Research on the effects of chronic exposure to nicotine at such low concentrations on the function of nAChRs in various systems of the body (the central nervous system, the peripheral nervous system, and skeletal muscle) makes an important contribution to the understanding of the mechanisms that underlie nicotine intoxication. The role of the nAChRs as regulators of neuronal activity in brain structures related to the reward system responsible for the formation of addiction to a range of drugs (cocaine, amphetamine, morphine, and others) other than nicotine [110] motivates researchers to focus on the role of nicotine in the central nervous system. As an example, the problem of nAChR desensitization upon prolonged exposure to nicotine is given much attention, since the desensitization of receptors is an important factor of functional changes in a number of structures of the central nervous system [111].

The effects of smoking and chronic exposure to nicotine on skeletal muscle have received very little attention [112–115], in spite of the large pool of nAChRs (putative targets of circulating nicotine) in the skeletal muscle. The data on the functional and molecular interaction between the nAChRs and the α2-isoform of Na,K-ATPase presented above point at the possibility of modulation of Na,K-ATPase of the skeletal muscle by nicotine. Desensitization of a part of the nAChRs upon chronic exposure to nicotine at low concentrations may serve as a modulatory signal that causes a compensatory response, which is manifested as changes in the expression and activity of Na,K-ATPase.

Chronic administration of nicotine to rats by subcutaneous injection for 2 or 3 weeks caused a decrease in the electrogenic contribution of the α2-isoform of Na,K-ATPase and membrane depolarization in the diaphragm muscle fibers [116]. Similar depolarization was observed in the rat m. soleus upon short-term (3-day) local application of higher concentrations of nicotine to the muscle using silicone implants [117]. The addition of nicotine to the drinking water of the rats during 3 to 4 weeks caused a decrease in the amounts of the α2-isoform of Na,K-ATPase in the sarcolemma of muscle fibers of the diaphragm. Membrane depolarization was observed as well, in spite of the decrease of the electrogenic activity of this isoform [21]. Notably, a specific decrease of the activity and expression of the α2-isoform of Na,K-ATPase occurred in the vessels of the blood–brain barrier, as well as in the rat brain, upon chronic administration of nicotine (for 14 days) via osmotic mini-pumps [118].

The mechanism that underlies the effect of long-term exposure to nicotine on the function of Na,K-ATPase remains uncharacterized. The data point to the complex character of this mechanism and the contribution of the activation of protein kinase C and enhanced phosphorylation of phospholemman (FXYD1) [21]. Importantly, effects of the same type can be expected in the case of other cholinergic ligands. As an example, the clinical use of anticholinesterase drugs (for memory stimulation in a number of neurodegenerative disorders and in the treatment of myasthenia and other diseases) is accompanied by an increase in the levels of non-hydrolyzed endogenous acetylcholine in the circulation. Therefore, the significance of the studies described above is not limited to the elucidation of the novel mechanisms that underlie nicotine intoxication, but rather contributes to a better understanding of the side effects of anticholinesterase drugs used in clinics and the mechanisms that underlie the toxic effects of the substances of this type.

Interestingly, the chronic effects of morphine, another psychoactive substance, also involve the

depolarization of smooth-muscle cells and myenteric neurons due to decreased electrogenic activity of Na,K-ATPase; the decrease in activity is due to the decrease in the levels of the neuronal α3-isoform of the enzyme [119]. The mechanism that underlies this change is not clear, but the data that indicate the colocalization and the putative functional and molecular interaction between the Na,K-ATPase and opioid receptors of different types is increasing [120, 121].

THE BINDING SITE FOR CARDIOTONIC STEROIDS

The extracellular portions of the α-subunit of Na,K-ATPase form a specific receptor for digitalis-like cardiac glycosides, which are widely used in the treatment of cardiovascular diseases. These glycosides of plant or animal origins share the ability to inhibit Na,K-ATPase and a steroid structure; they are collectively termed cardiotonic steroids (CTSs) [14, 33, 122].

The isoforms of the α-subunit of Na,K-ATPase differ with regard to sensitivity to ouabain, which is a specific inhibitor of Na,K-ATPase, and other CTSs. The variation in the affinity for ouabain is the highest for the isoforms of the rodent α-subunit: the constant of blocking of the α1-isoform ranges from ~ 50 to 450 μM, whereas the respective values for the α2- and α3-isoforms are two to four orders of magnitude lower and equal tens and hundreds of nM [11, 13, 33]. The sensitivity of the α4-isoform of Na,K-ATPase is the highest at less than 10 nM [11]. However, the α1-isoforms from other mammals (human, pig, dog, sheep, rabbit, and others) are relatively sensitive to ouabain [11, 13, 14, 33]. The values of the affinity of the human α1-, α2- and α3-isoforms to ouabain are in the nanomolar concentration range [58–61], whereas the affinity of these enzymes to other CTSs is lower (tens or hundreds of nM) [60, 61].

The specific CTS receptor is highly conserved. The extracellular regions between transmembrane domains M1–M2, M5–M6 and M7–M8 of the α-subunit form a major part of this receptor, although certain amino-acid residues from the M4, M6, and M10 fragments are involved in the binding as well [14, 32, 33, 123]. The nature of the two amino-acid residues at positions 111 and 122 between the transmembrane domains M1 and M2 determines the sensitivity to ouabain. Amino-acid substitutions at these positions were used to produce transgenic mice with a varying ratio of ouabain sensitivities of the α1- and α2-isoforms for the analysis of the physiological role of the specific CTS binding site [33].

Detailed analysis of the structure of the specific CTS binding site pointed to the putative existence of two sites, one with a high affinity and the other with a low affinity to ouabain [32, 34, 35]. The model postulates that both sites are located near the sites that bind the ions transported by Na,K-ATPase. The external site with a low affinity is located closer to the extracellular medium, whereas the site that has high affinity for ouabain is located closer to the intracellular medium [34]. However, many aspects of the process of CTS binding to the α-subunit of Na,K-ATPase remain uncharacterized.

The α2-isoform of Na,K-ATPase was shown to play a major role in the implementation of the positive inotropic effect of the CTSs in cardiac and smooth muscle [124, 125]. The regulation of contractile activity is presumably related to a local increase in the level of Ca^{2+} at the sites where the plasma membrane is very close to the sarcoplasmic reticulum and the α2-isoform of Na,K-ATPase is clustered with the Na^+,Ca^{2+} exchanger, a variety of Ca^{2+} channels, and Ca^{2+}-ATPases (the so-called PlasmERosome model). As follows from this model, inhibition of Na,K-ATPase upon CTS binding leads to the accumulation of Na^+ and the associated accumulation of Ca^{2+} in the narrow spaces near the membrane due to changes in the functioning of the Na^+,Ca^{2+} exchanger and to restricted diffusion. Potential-dependent and depot-dependent Ca^{2+} channels of the plasma membrane, SERCA, and ryanodine and IP_3 receptors of the sarcoplasmic reticulum are involved in the regulation of the local concentration of Ca^{2+} as well [46–48].

The positive inotropic effect of the CTSs in skeletal muscle has been demonstrated [57, 126, 127], but the underlying mechanism remains unknown. Certain data point to the increase of the intracellular Ca^{2+} concentration mediated by the α2-isoform of Na,K-ATPase as the putative reason for this effect [57, 66], similarly to the phenomenon observed in heart muscle. The triad combination of the T-tubules and terminal cisternae of the sarcoplasmic reticulum can be considered a PlasmERosome analog in skeletal muscle; the dihydropyridine receptor, the Na^+,Ca^{2+}-exchanger, Ca^{2+}-ATPase, ryanodine receptors, and SERCA [128–130] are colocalized in this area. The α2-isoform is found in the T-tubules of skeletal muscle fibers, and even close to the triads, as reported by some authors [9, 63, 72], and therefore a role in the regulation of the local intracellular concentration of Ca^{2+} according to the PLasmERosome mechanism can be predicted for this isoform [66].

The function of the α-subunit of Na,K-ATPase as a specific receptor for the CTSs has motivated the search for the putative endogenous ligands for this receptor. Ouabain, digoxin, marinobufagenin, telocinobufagenin, and other Na,K-ATPase inhibitors have been isolated from various tissues and biological fluids of animals and humans [14, 33, 47, 123, 131–133]. These ligands belong to the structural class of steroids as well; they are presumably synthesized in the adrenal cortex, the hypothalamus and the pituitary gland. A range of studies have shown the important role of these compounds in various physiological responses and pathophysiological processes. Reciprocal changes in the levels of endogenous CTSs and Na,K-ATPase activity are commonly observed in various pathologies of the cardiovascular system and the central nervous

system, as well as in the case of considerable physical loads and adaptation to extreme conditions (such as hypoxia). The endogenous analogs of ouabain and marinobufagenin have been characterized in most detail. The concentration of these endogenous inhibitors of Na,K-ATPase in the blood plasma and the cerebrospinal fluid is normally in the subnanomolar concentration range, but can increase rapidly under the conditions mentioned above [13, 47, 123, 133, 134].

A range of studies provided evidence of the regulatory role of endogenous CTSs in various tissues [13, 47, 123, 133, 134], as well as of the putative neuroprotective effects of these compounds [135–137]. However, very little is known about the functions of endogenous CTSs in skeletal muscle [2, 19, 127]. Ouabain and marinobufagenin reportedly increase the strength of contractions of isolated rat diaphragm when applied at subnanomolar concentrations [127] that fall within the concentration range for the circulating endogenous analogs of these inhibitors of Na,K-ATPase. The effect is though to be mediated by the α2-isoform of Na,K-ATPase [66]. This assumption is confirmed by the results of Digibind (antibodies that bind circulating CTSs) application to the skeletal muscle of mice with a genetically modified ouabain-resistant α2-isoform of Na,K-ATPase. The regulation of the α2-isoform by endogenous CTSs has been demonstrated, and a physiological role in the dynamic adaptation of skeletal muscle to physical activity has been proposed for this enzyme [2].

THE Na,K-ATPase AND MOTOR ACTIVITY

The activity of Na,K-ATPase is crucial for the electrogenesis, excitability, and contractile function of skeletal muscle that is essential for a high quality of life and for proper functioning of the central nervous system [138]. On the other hand, the functioning of Na,K-ATPase in skeletal muscle is subject to regulation by motor activity. Various forms of motor-activity enhancement are known to promote an increase in the amount of Na,K-ATPase activity, and certain studies point to the dissimilar effects of physical activity on the α1- and α2-isoforms of the enzyme [3, 4, 139–144]. Elevated physical activity is accompanied by increases of the intracellular Na^+ level [141], the number of phospholemman molecules (FXYD1 protein, interaction partner of the α-subunit of Na,K-ATPase) in the sarcolemma [142, 145], and the level of phospholemman phosphorylation [144].

In contrast, reduction of physical activity is accompanied by a decrease in the concentration of Na,K-ATPase in tissues [3, 4]. Prolonged functional idleness (disuse) of a skeletal muscle leads to loss of muscle mass, impairment of the contractile function, and ultimately to atrophy [146, 147]; therefore, elucidation of the putative role of Na,K-ATPase in muscle adaptation to lack of loading is a relevant research task. The relatively scarce data point to isoform-specific disturbances of Na,K-ATPase functioning in the case of prolonged absence or restriction of motor activity. The amounts of both the α1- and α2-isoforms of Na,K-ATPase decrease and the expression of FXYD1 [148] is downregulated in humans with spinal cord injuries. Knee injury is accompanied by a specific decrease of the content of the α2-isoform in limb muscles [149]. The lower amounts of the α2-isoform in the skeletal muscle of elderly people may be related to lower physical activity in this age group [150].

Antiorthostatic suspension of rodents by the tail is widely used as a model of functional and gravitational load reduction. This treatment causes atrophy of postural skeletal muscle and allows analysis of the molecular mechanisms of these disorders [151]. Intensive atrophic processes in the rat m. soleus begin within 3 to 7 days after the onset of load reduction, concomitantly to changes in the expression of certain isoforms of contractile and regulatory proteins [146, 152, 153]. Atrophy is accompanied by depolarization of the membrane of muscle fibers [154–156] due to a decrease in the electrogenic activity of the α2-isoform of Na,K-ATPase [157]. However, disturbances of the function of the α2-isoform are observed prior to the emergence of symptoms of atrophic changes as soon as 24 h after load reduction [70].

Recent studies have shown that even short-term (6–12 h) antiorthostatic suspension has specific effects on the electrogenic activity, protein level, and the synthesis of mRNA of the α2-isoform of Na,K-ATPase in the rat m. soleus [22]. Suppression of the activity of the extrasynaptic pool of the α2-isoform has been shown to stem from the absence of motor loading and shown to persist upon an increase in the protein and mRNA levels observed after 12 h of load reduction. In contrast, the postsynaptic pool of the α2-isoform is regulated by additional (possibly circulating) factors and can revert to a normal level of activity regardless of loading reduction. Importantly, even infrequent and short-term stimulation of the motor nerve enables the recovery of electrogenic activity of both pools of the α2-isoform. These data reveal the tight regulation of the functioning of the α2-isoform of Na,K-ATPase in skeletal muscle by motor activity and the different pathways of regulation of the activities of the postsynaptic and extrasynaptic pools of the enzyme. The absence of a correlation between the decrease of the activity of the α2-isoform of Na,K-ATPase and the changes in protein levels, mRNA expression, localization in the membrane, or interaction with nAChRs has been demonstrated, while specific suppression of the functional activity of this isoform was revealed. The suppression of enzyme activity may be due to the increase in the number of phospholemman (FXYD1 protein) molecules and enhanced association of this protein with the α2-subunit of Na,K-ATPase [22].

An increase in the mRNA expression level for the α2-isoform upon a decrease in enzyme activity was observed under skeletal muscle fatigue as well. Increased expression may represent a compensatory

mechanism for the maintenance of muscle function [158]. However, the molecular mechanisms that underlie the regulation of the activity of the α2-isoform of Na,K-ATPase in the absence of a motor load remain largely uncharacterized. Factors other than FXYD1 may be involved in the regulation.

As an example, the role of the incoming Na^+ is not entirely clear, although the intracellular concentration of sodium ions plays an important role in the regulation of the activity and expression of Na,K-ATPase [8]. The activation of neuronal Na,K-ATPase manifested as membrane hyperpolarization after exposure to high concentrations of acetylcholine may be due to the entry of Na^+ through the open nAChR channels of the neuronal type [80]. Experiments with cells of various types have shown that the activation of the entry of Na^+ promotes an increase of the protein level and the level of Na,K-ATPase mRNA [139, 159–161]; an opposite effect was observed when the entry of Na^+ was blocked [161]. The enhancement of the entry of Na^+ in skeletal muscle cells selectively promoted an increase in the amount of the α2-isoform of Na,K-ATPase [161]. This adaptive reaction might contribute to the prevention of the accumulation of Na^+ under intensive contractile activity. Reduction of the motor activity during the initial period of hind limb suspension in rats is accompanied by a sharp reduction of cell activity registered by electromyography and neurography in the rat m. soleus [162, 163]; therefore, a decrease in the concentration of intracellular Na^+ is likely to occur under these conditions. On the one hand, a decrease in the activity of Na,K-ATPase should ensue. On the other hand, short-term suspension (6 to 12 hours) leads to increased phosphorylation of FXYD1 in the rat m. soleus [22], and thus the affinity of the intracellular sites of Na,K-ATPase to Na^+ may increase, as well as the enzyme activity [38, 39].

Other Na,K-ATPase regulators dependent on the motor activity of skeletal muscle, such as AMP-activated protein kinase (AMPK), are known. This metabolic sensor is activated in case of contractile activity due to an increase in energy consumption and the intracellular AMP/ATP ratio. The activation of AMP-activated protein kinase during exercise has been shown to stimulate Na,K-ATPase and the stimulation is regarded as an important factor in the maintenance of ion homeostasis and the prevention of fatigue [164]. A large body of data on the changes of the nitric oxide (NO) level in skeletal muscle subjected to functional unloading [165] and on the role of NO in the regulation of Na,K-ATPase [166, 167] has recently become available. The roles of these regulators of Na,K-ATPase under the conditions of reduced motor activity remain to be characterized.

PERSPECTIVES

The data presented above suggest that experimental models of locomotor activity disorders manifested as various forms of myopathy can be used for further research on the specific functions of Na,K-ATPase isoform in skeletal muscle.

Mice of the *mdx* line are among the disease models that are worth mentioning in this regard. The dystrophin glycoprotein complex is known to play an important role in maintaining the structural integrity of the membrane, postsynaptic scaffold organization, and nAChR clustering in the endplate [89, 90]. The cytoskeletal protein dystrophin is the main component of this complex. Mutations in the dystrophin gene and defects in the components of the complex cause muscular dystrophy, including one of the most severe forms of the disorder, X-linked Duchenne muscular dystrophy. The *mdx* mutant mice that lack dystrophin synthesis due to a point mutation in the X chromosome represent the most popular experimental model of the disorder [168]. Impairment of the integrity of the sarcolemma, degenerative changes in the neuromuscular junctions [89, 169], disturbances of the ion balance [74], and many other defects are characteristic of the skeletal muscle in these animals.

Depolarization of the membrane of the muscle fibers is an additional grave defect observed in the *mdx* mice [74, 170, 171]. This depolarization is primarily due to sarcolemmal damage caused by the lack of the cytoskeletal protein dystrophin. Concomitant factors, such as enhanced Na^+ entry due to the disruption of voltage-gated sodium channels of the $Na_V1.4$ type [172] and the accumulation of intracellular Ca^{2+} [173], may play an important role as well. The decrease in electrogenic activity of Na,K-ATPase can probably affect the V_m value. The early studies reported an increase in the activity and the amount of Na,K-ATPase [174, 175], but subsequent studies demonstrated a decrease in the electrogenic activity of this protein in the skeletal muscle of *mdx* mice [74, 171]. Importantly, no signs of functional impairment of the α1-isoform were observed in [74]; this is indirect evidence of a specific disorder related to the α2-isoform of Na,K-ATPase. The latter hypothesis is confirmed by the absence of local hyperpolarization of the endplate membrane in the diaphragm of *mdx* mice (in addition to V_m decrease), a sign of the impairment of the functional interaction between the nAChRs and the α2-isoform of Na,K-ATPase [91, 92].

Mice with impaired function of the dysferlin protein might serve as a promising model as well. The mechanisms that underlie the maintenance of plasma-membrane integrity are among the most important features of a cell. This physiological mechanism is fundamental for the maintenance of the functioning of skeletal muscle cells throughout their lifetime; disturbances of this mechanism lead to the development of different forms of muscular dystrophy. Thus, intense physical activity can cause microdamage to the integrity of the sarcolemma with subsequent entry of external Ca^{2+} into these portions of the fiber, impairment of the electromechanical coupling, and activation of a number of Ca^{2+}-dependent processes, including

Ca^{2+}-dependent proteolysis and oxidative stress. The combined action of these factors promotes further damage to muscle fibers, myopathy, inflammation, and necrosis [176–178]. The modern concept of the recovery of plasma membrane integrity implies Ca^{2+}-dependent fusion of membrane-bound intracellular vesicles with the sarcolemma at the sites of damage during the natural contractile activity. The protein dysferlin is assumed to play a key role in the control of sarcolemma recovery.

The physiological functions of dysferlin are still poorly characterized. This protein has a single transmembrane domain at the C-terminus and seven consecutive intracellular C2 domains at the N-terminus; these domains are sensitive to Ca^{2+} and phospholipids. This protein is located in the sarcolemma, but it is also found in intracellular vesicles of the T-tubules of skeletal muscle [177, 178]. Dysferlin forms a molecular complex with various proteins, with caveolin-3 and annexins being the most important components, and is presumably involved in the processes of vesicle traffic and fusion. The mechanism that underlies the recovery of plasma membrane integrity implies a complex Ca^{2+}-dependent process. This process involves several successive stages of intracellular vesicle fusion with the sarcolemma at the damaged site and the formation of a highly organized scaffold that acts as a "patch" for the membrane [176, 179].

Mice with disturbances of dysferlin synthesis show progressive muscular dystrophy, whereas humans that carry mutations in the dysferlin gene have dysferlinopathies [176–178]. Therefore, the molecular mechanism that underlies this specific form of myopathy is fundamentally different from the one that underlies the disorders caused by the loss of connections between the cytoskeleton, the dystrophinoglycan complex, and the proteins of the extracellular matrix in Duchenne muscular dystrophy.

The possible effects of dysferlin mutations on muscle electrogenesis and the functioning of Na,K-ATPase have not been reported as yet. However, there is indirect evidence of the role of dysferlin in the regulation of cholinergic transmission in muscle tissue of *C. elegans* and mouse, as well as of the ability of acetylcholinesterase inhibitors to compensate for the defects in the functioning of this protein [180]. The localization of dysferlin in lipid rafts and abnormal lipid metabolism in dysferlin-deficient Bla/J mice has been reported [181]. Taken together, these facts (the association of dysferlin and caveolin-3, localization in lipid rafts, and a role in the processes of trafficking and vesicle fusion) point to a possible role of dysferlin in the regulatory effects that the lipid environment exerts on the molecular complex of the α2-isoform of Na,K-ATPase.

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