

Fibre type distribution of the muscle surface initiator of excitation-contraction coupling, the nicotinic acetylcholine receptor

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Abstract

The first critical step of excitation-contraction coupling in the skeletal muscle periphery is represented by the activation of the nicotinic acetylcholine receptor. In order to determine potential differences in the muscle fibre type distribution of the receptor complex, we have performed nicotine binding and immunoblotting experiments. A greater abundance of the receptor and a higher density of nicotine binding sites were found in slow versus fast fibres, and an increase in nicotine binding was shown to occur during fibre type shifting. This indicates a molecular modification of the central functional unit of the post-synaptic muscle surface during fast-to-slow fibre transitions.

Keywords: nicotine, nicotinic acetylcholine receptor, excitation-contraction coupling, muscle fibre types, neuromuscular junction.

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Introduction

The first critical step of excitation-contraction coupling in the skeletal muscle periphery is represented by the activation of the nicotinic acetylcholine receptor complex [18]. Once sarcolemmal depolarization is triggered and sufficiently propagated, distinct changes in ion fluxes mediate the signal transduction mechanism at the triad junctions [1, 4, 9]. Many subtypes of nicotinic acetylcholine receptors exist, each defined by a different combination of subunits [6, 8]. The skeletal muscle receptor is a typical ionotropic protein complex involved in fast synaptic transmission at the neuromuscular junction and a different subunit arrangement is found in fetal versus mature fibres [5-7]. The neurotransmitter-gated ion channel is made up of five subunits (α - α - β - γ - δ/ϵ), which form an ion channel cluster with a central transmembrane pore [7]. Sophisticated electron microscopical studies have revealed subunit interactions and changes in receptor structure following activation [16]. The two ligand binding sites in the extracellular portion of the nicotinic acetylcholine receptor are positioned at the interface between the principle α -subunit and its adjoining γ - and δ/ϵ -subunits [7]. According to a recent review by Unwin [17], neurotransmitter interactions initiate rotations of the protein chains on opposite sides of the pore entrance, thereby triggering the opening of the ion gate. Since distinct molecular and cellular differences

exist between slow and fast twitching skeletal muscles [14], it was of importance to determine whether variations in the abundance of the nicotinic acetylcholine receptor complex exist in different muscle types. A recent immunoblot analysis of chronic low-frequency stimulated fast muscle has shown an increased expression of the neuromuscular junction receptor in transformed fibres [12]. Based on this finding, this report has determined potential differences in the abundance of nicotine binding sites in different muscle types and electro-stimulated fast fibres.

Methods

In order to determine potential differences in nicotine binding to predominantly slow- and fast-twitching muscle fibres, skeletal muscle microsomes were isolated from extensor digitorum longus (EDL), gastrocnemius (GA) and soleus (SO) muscles from New Zealand white rabbits by an established subcellular fractionation protocol [3]. For studying potential changes during fibre type shifting, low-frequency stimulated muscle samples were also analysed. Electro-stimulated tibialis anterior specimens [13] were supplied by Prof. Dr. Dirk Pette (Department of Biology, University of Konstanz, Germany) and normal muscle samples by the Biomedical Facility of the National University of Ireland, Dublin. Nicotine binding to muscle membrane preparations was performed according to the protocol of Sundman et al. [15]. Initial optimisation experiments for

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determining the ideal concentration of ^3H -nicotine (1.2 mCi/ml; Sigma) in the muscle membrane assay system were carried out within a concentration range of 0.05 to 3.5 nM of ^3H -nicotine. A radioactive nicotine concentration of 0.35 nM resulted in the highest amount of specific binding without increasing non-specific values and was therefore chosen for the in-depth analysis of different fibre types. Solubilised muscle membrane preparations, with a final concentration of 0.5 mg/ml protein, were prepared in 5 to 10 duplicate pairs and incubated with and without a 100-fold excess of unlabelled cold nicotine for 10 min at 37°C with gentle agitation in a final volume of 1 ml [2]. Receptor-bound and free nicotine was separated by rapid vacuum filtration through Whatman GF/B fibre glass filter paper and washing with ice-cold 50 mM Tris-Cl, pH 7.4, 100 mM NaCl and 1% (v/v) Triton X-100. Washed filters were placed in 4 ml of scintillation liquid and nicotine binding determined by radioactive counting. The gel electrophoretic separation of muscle proteins and immunoblotting was carried out by standard procedures [3, 12]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out with 7% resolving gels and 20 µg protein per lane using a Mini-MP3 electrophoresis system from Bio-Rad Laboratories (Hempel Hempstead, Herts., UK). Following electrophoretic separation, gels were transferred onto Immobilon NC membranes in a Bio-Rad mini blotting cell system [3]. Blocking and incubation of nitrocellulose replicas with antibodies was performed by standard techniques [12]. The enhanced chemiluminescence detection method was used to visualize decorated protein bands. A Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) and ImageQuant V3.0 software was employed for the densitometric scanning of enhanced chemiluminescence blots.

Results and Discussion

The synaptic transmission process at the neuromuscular junction is initiated by the release of acetylcholine from neuronal vesicles, followed by the diffusion of the neurotransmitter along a concentration gradient within the sub-synaptic cleft and finally results in ligand binding to the two α -subunit associated binding sites on the nicotinic acetylcholine receptor [6, 7]. A ligand-triggered switch in the conformation of the α -subunit activates the post-synaptic receptor complex [17] resulting in the efficient transmission of an action potential from the innervating motoneuron into depolarisation of the muscle sarcolemma. Here, we determined whether differences exist in the relative abundance of this key element of the neuromuscular junction in fast versus slow fibres. Prior to the nicotine binding survey, a comparative immunoblot analysis was performed with antibodies to the α -subunit of the nicotinic acetylcholine receptor, members of the utrophin-glycoprotein complex and various fibre type markers in membrane preparations derived from

extensor digitorum longus, gastrocnemius and soleus muscles.

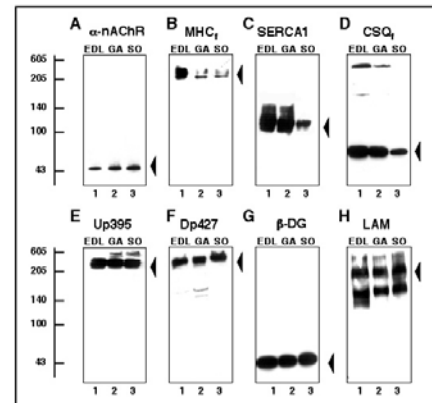


Figure 1. Immunoblot analysis of the expression profile of the nicotinic acetylcholine receptor in slow versus fast muscle. Shown are identical immunoblots labeled with antibodies to the α -subunit of the nicotinic acetylcholine receptor (α -nAChR) (A), the fast isoform of the myosin heavy chain (MHC_f) (B), the fast isoform of the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA1) (C), the fast isoform of the Ca^{2+} -binding protein calsequestrin (CSQ_f) (D), the full-length 395 kDa isoform of utrophin (Up395) (E), the full-length 427 kDa isoform of dystrophin (Dp427) (F), the surface glycoprotein β -dystroglycan (β -DG) (G), and the extracellular matrix protein laminin (LAM) (H). Lanes 1 to 3 represent microsomal membranes derived from extensor digitorum longus (EDL), gastrocnemius (GA) and soleus (SO) muscle homogenates, respectively. The position of molecular mass standards (in kDa) is indicated on the left and immuno-decorated protein bands are marked by arrowheads.

As illustrated in the immunoblotting survey of Fig. 1, the relative abundance of the nicotinic acetylcholine receptor is higher in slow muscles as compared to faster fibres. As internal standards of fibre type distribution, membranes were immuno-decorated with antibodies to the fast isoforms of the myosin heavy chain, the sarcoplasmic reticulum Ca^{2+} -ATPase and the Ca^{2+} -binding protein calsequestrin (Fig. 1B-D). The isoforms MHC_f , SERCA1 and CSQ_f were all found to be of higher density in extensor digitorum longus microsomes as compared to soleus vesicles. In contrast, the α -subunit of the nicotinic acetylcholine receptor was shown to be enriched in microsomes from soleus muscles (Fig. 1A). The densitometric analysis of enhanced chemiluminescence blots revealed clear differences of receptor labeling between fast and slow fibres (Fig. 2B). No major differences in the expression were found for members of the dystrophin/utrophin-

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complex, representing markers of the membrane cytoskeleton, sarcolemma and the extracellular matrix. The relative density of the full-length utrophin isoform Up395, dystrophin of apparent 427 kDa, the integral glycoprotein β -dystroglycan and laminin were relatively comparable between predominantly slow and fast fibres (Fig. 1E-H).

Nicotine binding experiments agreed with the immunoblot analysis. Binding sites for nicotine were approximately 2-fold and 3.5-fold more abundant in preparations of gastrocnemius and soleus muscle, respectively, as compared to vesicles derived from extensor digitorum longus homogenates (Fig. 2A). Since the α -subunit of the nicotinic acetylcholine receptor contains the major binding domains of nicotine in skeletal muscle microsomes, this finding clearly suggests that this key element of the neuromuscular junction exhibits a fibre type specific distribution. To further evaluate this expression profile, muscles undergoing a fibre transition process were assayed. Nicotine binding increased drastically to membrane preparations isolated from low-frequency stimulated tibialis anterior muscle. As shown in Fig. 2C, during the period of 5 to 20 days of chronic electro-stimulation, nicotine binding increased by over 3-fold. A slight decrease in nicotine binding occurred after 6 weeks of muscle conditioning. Successful fibre type transformation in this experimental system has previously been demonstrated by isoform shifting of contractile and sarcoplasmic reticulum proteins [10-12]. Hence, the stimulation-induced phenotypic change to slower twitch characteristics is accompanied by a drastic increase in the expression of the nicotinic acetylcholine receptor. This finding confirms the immunoblotting data that this receptor complex exhibits a higher density in slow versus fast fibers (Fig. 1A, 2B).

Besides the central neurotransmitter receptor, other components are essential for the formation and proper maintenance of the connection between the innervating nerve and its target fibres. This includes the utrophin-glycoprotein complex, the muscle-specific kinase MuSK, rapsyn and agrin [19]. In contrast to the nicotinic acetylcholine receptor, the relative concentration of utrophin and its associated glycoproteins does not change during fibre type shifting [12] and is very comparable between different fibre types, as shown in this report. We could show previously that the α - and δ -subunits of the acetylcholine receptor were increased in 10 to 78 day stimulated muscle specimens using comparative immunoblotting [12]. This fully agrees with the nicotine binding data presented here and demonstrates that the expression profile of the neuromuscular junction receptor is influenced by enhanced neuromuscular activity under conditions of maximum activation. It has previously been established that chronic electro-stimulation protocols trigger the degeneration of fast-twitching glycolytic fibres, induce a trans-

differentiation process leading to a fast-to-slow fibre transformation and cause the activation of muscle precursor cells [13]. This study confirms the cellular transition mechanism following low-frequency stimulation. Increased nicotine binding of electro-stimulated fibres suggests that a molecular modification of the acetylcholine receptor complex occurs during the remodeling of nerve-muscle contacts or within existing neuromuscular junctions.

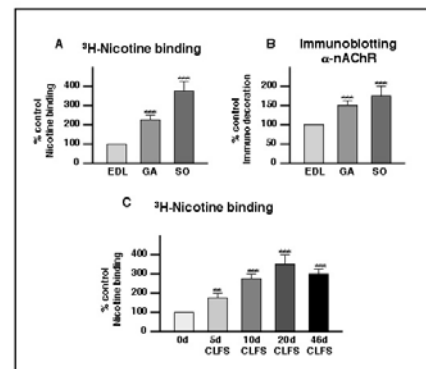


Figure 2. Analysis of nicotine binding to fibre type specific membrane preparations and electro-stimulated muscle samples. Shown is the graphical presentation of the specific nicotine binding profile to microsomal membranes derived from extensor digitorum longus (EDL), gastrocnemius (GA) and soleus (SO) muscle homogenates ($n=10$). Values are expressed as a percentage of control binding (A). For comparison, the densitometric analysis of immuno-decoration with antibodies to the α -subunit of the nicotinic acetylcholine receptor (α -nAChR) is shown in panel (B) ($n=5$). Increased nicotine binding to 5 to 46 days (d) chronic low-frequency stimulated (CLFS) tibialis anterior specimens is shown in panel (C) ($n=5$). (SD; ** $P<0.05$; *** $P<0.01$).

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