Revealing of T-Type Low-Voltage Activated Calcium Channels (Ca\textsubscript{V}3) in Frog Neuromuscular Junctions

L. F. Nurullin\textsuperscript{a}, A. N. Tsentsevitsky\textsuperscript{a}, A. I. Malomouzh\textsuperscript{a}, and Academician E. E. Nikolsky\textsuperscript{a, b}

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Increase in the intracellular level of Ca\textsuperscript{2+} concentration leads to the initiation of a wide range of molecular processes, including the activation of Ca\textsuperscript{2+}-dependent enzymes, gene expression, neurotransmitter release, etc. [1]. In spite of the variety of channels and pumps involved in the regulation of the intracellular Ca\textsuperscript{2+} metabolism, the main role in Ca\textsuperscript{2+} signaling is attributed to voltage-activated Ca\textsuperscript{2+} channels [2]. According to the modern classification based on the structural features of the pore-forming subunit \( \alpha \), all voltage-activated Ca\textsuperscript{2+} channels (Ca\textsubscript{V}) are divided into three families. The first family (Ca\textsubscript{V}1.1, Ca\textsubscript{V}1.2, Ca\textsubscript{V}1.3, Ca\textsubscript{V}1.4) includes high-voltage activated channels containing the subunits \( \alpha_{1A}, \alpha_{1C}, \alpha_{1D}, \alpha_{1F} \), respectively, which mediate L-type Ca\textsuperscript{2+} currents. The second family (Ca\textsubscript{V}2.1, Ca\textsubscript{V}2.2, Ca\textsubscript{V}2.3) includes high-voltage activated channels with subunits \( \alpha_{2A}, \alpha_{2B}, \alpha_{2E} \), respectively, which mediate the P/Q-, N-, and R-types of Ca\textsuperscript{2+} currents. The third family (Ca\textsubscript{V}3.1, Ca\textsubscript{V}3.2, Ca\textsubscript{V}3.3) includes low-voltage activated channels with subunits \( \alpha_{3A}, \alpha_{3H}, \alpha_{3I} \), respectively, which mediate T-type Ca\textsuperscript{2+} channels [1].

It was established that in synapses of the central nervous system Ca\textsuperscript{2+} channels of different types [3] may be involved in the process of neuromodulation, whereas in synapses of peripheral nervous system this process is mediated mainly by one type of channels. For example, in mammalian neuromuscular junctions, these are Ca\textsubscript{V}2.1 (P/Q-type) channels, and in amphibian synapses, these are Ca\textsubscript{V}2.2 (N-type). However, it was shown that, in addition to the main type of channels, blocking of which leads to the blockage of evoked quantal mediator release, other types of high-voltage activated Ca\textsuperscript{2+} channels are also found in motor endplates, and their role in neurotransmission is not entirely clear yet [4–9]. At the same time, the existence and functioning of low-voltage activated Ca\textsuperscript{2+} channels (of the T type) at neuromuscular junction remains an open question, which was the reason for our study.

The purpose of the study was to detect low-voltage activated Ca\textsuperscript{2+} channels (Ca\textsubscript{V}3) in neuromuscular synapses and to reveal their potential role in neurotransmission to example of the frog neuromuscular junctions.

Experiments were carried out on neuromuscular preparations of \textit{m. cutaneous pectoris} of the frog \textit{Rana ridibunda} with the use of immunocytochemical and electrophysiological methods.

For the immunocytochemical detection and location of Ca\textsuperscript{2+} channels of the Ca\textsubscript{V}3 type, the neuromuscular preparations were fixed in 3% formaldehyde (Sigma, USA), sequentially incubated in 0.3% Triton X-100 (15 min), and then, the neuromuscular preparations were incubated during 15 h at a temperature of 4°C with rabbit polyclonal antibodies against \( \alpha_{3A}, \alpha_{3H}, \alpha_{3I} \) subunits (Santa Cruz Biotechnologies, USA) corresponding to the channels Ca\textsubscript{V}3.1, Ca\textsubscript{V}3.2, Ca\textsubscript{V}3.3 (at a dilution of 1:200). After “washing,” the samples were incubated for 1 h at room temperature with secondary (anti-rabbit) antibodies conjugated with Alexa 488 (Invitrogen, USA) at a dilution of 1:500. Visualization of synaptic areas was conducted by staining postsynaptic nicotinic acetylcholine (ACh) receptors with tetramethylrhodamine-\( \alpha \)-bungarotoxin (Sigma, 20 \( \mu \)g/ml; 30 min) [10].

The images were obtained using a Leica TCS SP5 MP laser confocal microscope (Leica Microsystems, Germany).

To evaluate the role of Ca\textsuperscript{2+} channels of the Ca\textsubscript{V}3 type in neuromuscular junctions, the parameters of spontaneous and evoked ACh secretion in the presence of the blocker of these channels mibebradil.
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(Sigma, 2 μM) were estimated by electrophysiological methods. For this purpose, a neuromuscular preparation was placed into a 3-ml chamber containing Ringer’s solution of the following composition (in mM): NaCl, 113; KCl, 2.5; CaCl₂, 0.3; NaHCO₃, 3; MgCl₂, 4 (pH 7.3, t = 20.0 ± 0.3°C). The nerve ending currents and endplate currents were recorded from the proximal part synapse by the extracellular microelectrodes filled with Ringer’s solution (with a resistance of 2.0–4.0 MΩ).

The intensity of spontaneous quantal ACh secretion was evaluated by the mean frequency of miniature endplate currents (MEPCs), which in each experiment was calculated after recording of more than 120 signals.

The quantal content of evoked endplate currents (EPCs) was estimated by the “failure” method [11]. The motor nerve was stimulated by 0.1-ms rectangular impulses of supramaximal strength with a frequency of 0.5 impulses/s. 250–400 single-quantal postsynaptic responses and nerve ending currents were recorded; they were digitized with a sampling rate of 5 μs and analyzed using a software developed in our laboratory.

In addition to the quantal content of EPCs, the degree of the neurotransmitter quanta release synchrony in response to electrical stimuli was evaluated by analyzing the variance of true synaptic delays (the interval from peak of the sodium component of the nerve ending current to the beginning of the EPC at the 20% level of its maximal amplitude) [12]. For this purpose, distribution histograms and cumulative curves of synaptic delays were plotted. Cumulative curves were used to estimate the parameter P₉₀ (the interval where 90% of all synaptic delays fell), which characterizes the synchrony of the neurotransmitter quantum release [12].

Fig. 1. Immunocytochemical staining of the frog cutaneous pectoris muscle with antibodies against α₁G, α₁H, and α₁I subunits of potential-activated Ca²⁺ channels of the Caᵥ3.1, Caᵥ3.2, and Caᵥ3.3 types, respectively. Synaptic location of Ca²⁺ channels is confirmed by staining of postsynaptic ACh receptors with tetramethylrhodamine-α-bungarotoxin (α-BTX). Scale bar, 10 μm.

Fig. 2. Effect of the T-type Ca²⁺ channels (Caᵥ3) blocker mibebradil (2 μM) on the intensity of spontaneous and evoked quantal ACh secretion in a frog neuromuscular junction. The control values are taken to be 100% (the dotted line). Gray columns show the values after 30 min of mibebradil treatment; light columns, 45 min after washing off of the blocker. * Significant difference from the control (p < 0.05).