



Acetylcholine-Induced Inhibition of Presynaptic Calcium Signals and Transmitter Release in the Frog Neuromuscular Junction

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Acetylcholine (ACh), released from axonal terminals of motor neurons in neuromuscular junctions regulates the efficacy of neurotransmission through activation of presynaptic nicotinic and muscarinic autoreceptors. Receptor-mediated presynaptic regulation could reflect either direct action on exocytotic machinery or modulation of Ca²⁺ entry and resulting intra-terminal Ca²⁺ dynamics. We have measured free intra-terminal cytosolic Ca²⁺ ([Ca²⁺]_i) using Oregon-Green 488 microfluorimetry, in parallel with voltage-clamp recordings of spontaneous (mEPC) and evoked (EPC) postsynaptic currents in post-junctional skeletal muscle fiber. Activation of presynaptic muscarinic and nicotinic receptors with exogenous acetylcholine and its non-hydrolyzed analog carbachol reduced amplitude of the intra-terminal [Ca²⁺]_i transients and decreased quantal content (calculated by dividing the area under EPC curve by the area under mEPC curve). Pharmacological analysis revealed the role of muscarinic receptors of M₂ subtype as well as d-tubocurarine-sensitive nicotinic receptor in presynaptic modulation of [Ca²⁺]_i transients. Modulation of synaptic transmission efficacy by ACh receptors was completely eliminated by pharmacological inhibition of N-type Ca²⁺ channels. We conclude that ACh receptor-mediated reduction of Ca²⁺ entry into the nerve terminal through N-type Ca²⁺ channels represents one of possible mechanism of presynaptic modulation in frog neuromuscular junction.

Keywords: neuromuscular synapse, calcium transient, presynaptic acetylcholine receptors, muscarinic receptors, nicotinic receptors, N-type Ca channels, quantum secretion of acetylcholine

INTRODUCTION

Acetylcholine (ACh) when released from nerve endings upon action potential-driven depolarization, not only triggers postsynaptic response in muscle cell, but also regulates its own secretion from presynaptic terminal (Ciani and Edwards, 1963; Parnas et al., 2000; Tomas et al., 2014). This presynaptic regulation has an important for reliability of synaptic transmission. It has been shown that various subtypes of muscarinic and nicotinic acetylcholine receptors are expressed

in motor nerve endings and they modulate release of ACh into the synaptic cleft (Bowman et al., 1990; Miller, 1990; Tomas et al., 2014). The direct and indirect Ca^{2+} influx resulting from the activation of neuronal nicotinic acetylcholine receptors can modulate presynaptic neurotransmitter release (Shen and Yakel, 2009). Two subtypes of muscarinic receptors are involved in the ACh release modulation: the M_1 receptor mediates enhancement of ACh release, while the M_2 is involved in inhibition of release (Slutsky et al., 1999). Muscarinic agonists have been shown to decrease the number of quanta released in response to stimulation of motor nerve (Arenson, 1989; Slutsky et al., 1999; Samigullin et al., 2014). Muscarinic receptors can also be activated in the absence of stimulation during spontaneous neurotransmitter release (Kupchik et al., 2008).

Although this phenomenon has received a significant research attention in the past (Nikol'skii and Giniatullin, 1979; Wessler, 1989; Macleod et al., 1994; Van der Kloot et al., 1997; Nikolsky et al., 2004), fine details of ACh-dependent presynaptic regulation remain obscure. Both direct action of ACh on exocytotic machinery (Linial et al., 1997) and inhibition of presynaptic Ca^{2+} entry (Wu and Saggau, 1997; Parnas et al., 2000; Khaziev et al., 2012) have been suggested. The latter possibility, being seemingly logical, has not been analyzed in detail. The present study was designed to investigate the role of presynaptic calcium influx in autoregulation of ACh secretion by presynaptic receptors in the frog neuromuscular junction.

MATERIALS AND METHODS

Experimental Preparation and Ethical Approval

Experiments were performed on isolated nerve-muscle preparations of *musculus cutaneous pectoris* from the frog *Rana ridibunda*. The experimental procedures were performed in accordance with the guidelines for use of laboratory animals of Kazan Federal University and Kazan Medical University, in compliance with the NIH Guide for Care and Use of Laboratory Animals. Experimental protocols met the requirements of the European Communities Council Directive 86/609/EEC and were approved by the Ethical Committee of Kazan Medical University.

Intracellular Ca^{2+} monitoring

Cytosolic concentration of ionized Ca^{2+} ($[\text{Ca}^{2+}]_i$) in nerve endings was monitored using fluorescent microfluorimetry (Tsien, 1989). Each nerve-muscle preparation was loaded with Ca^{2+} -sensitive dye by soaking nerve stump in 50 mM solution of fluorescent Ca^{2+} -indicator Oregon Green 488 BAPTA-1 Hexapotassium Salt (Molecular Probes, Eugene, Oregon, USA); for details of loading technique see (Peng and Zucker, 1993; Wu and Betz, 1996; Tsang et al., 2000; Samigullin et al., 2015). At the end of loading protocol, all terminals in the proximal part of the nerve trunk had sufficient levels of fluorescence. It has been estimated that the intra-terminal concentration of the probe varied between 40 and 150 μM (Suzuki et al., 2000).

Abbreviations: Ach, Acetylcholine; EPC, evoked postsynaptic currents; mEPC, spontaneous postsynaptic currents.

The fluorescent probe by definition binds Ca^{2+} and hence may affect both $[\text{Ca}^{2+}]_i$ dynamics and physiology of the neuromuscular junction. Our own observations (Samigullin et al., 2015) as well as observations of others (Wu and Betz, 1996) failed to detect any appreciable influence of loaded Ca^{2+} probe on the amplitude of the postsynaptic response or on the frequency of the miniature end-plate potentials. Nonetheless we additionally performed control experiments to compare spontaneous endplate currents (mEPC) and quantal release before and after Ca^{2+} probe loading. It appeared that Ca^{2+} probe affects neither; the mEPC frequency was 0.32 ± 0.03 Hz ($n = 4$, $P < 0.05$) before and 0.32 ± 0.15 Hz ($n = 4$, $P < 0.05$) after loading of the probe; similarly the quantal content was 1.06 ± 0.09 ($n = 3$, $P < 0.05$) and 0.99 ± 0.07 ($n = 3$, $P < 0.05$). We may conclude, therefore adding Ca^{2+} probe into the cytosol of the terminal does not affect physiological parameters of neurotransmission.

Neuromuscular preparations were continuously perfused with the Ringer solution of the following content (in mM): NaCl–113, KCl–2.5, NaHCO_3 –3, MgCl_2 –6, CaCl_2 –0.9; pH was adjusted to 7.4. Low extracellular Ca^{2+} (0.9 mM) and high level of external MgCl_2 (6 mM) were used to block muscle contraction. All experiments were performed under these conditions except the serie with decreasing extracellular Ca^{2+} . Experiments were performed at $20.0 \pm 0.3^\circ\text{C}$.

Fluorescent signal was recorded using photometric setup on the base of Olympus BX-51 microscope with x60 water-immersion objective connected to photodiode S1087 (Hamamatsu, Japan) as described in Sinha and Saggau (1999) and Samigullin et al. (2010, 2015). The region for recording was selected by optical viewfinder (Till Photonics, Munich, Germany). Excitation light (488 nm) was generated by Polychrome V (Till Photonics, Munich, Germany). To minimize bleaching of the dye and decrease background fluorescence, the recording area of nerve terminal was restricted by an iris diaphragm. Illumination was controlled by a shutter with a typical exposure time of 400 ms and a delivery rate of 0.5 Hz. Motor nerve was electrically stimulated by rectangular voltage pulses of 0.2 ms in duration and supra-threshold amplitude at a frequency of 0.5 Hz using the “suction” electrode described earlier (Kazakov et al., 2015). The photodiode signal was digitized by the ADC Digidata 1440A (Molecular Devices, USA) with sampling rate 10.256 kHz. Fluorescence recordings, illumination and electrical stimulation were all controlled by WinWCP software (Strathclyde University, Glasgow, UK). The peak amplitude of $[\text{Ca}^{2+}]_i$ transients was measured and changes in fluorescence are represented as $\Delta F/F_0$ (the change in fluorescence intensity relative to the background fluorescence as a percentage). For each experiment, about 60 fluorescence responses were averaged.

Electrophysiological Recordings

Spontaneous and evoked endplate currents (mEPC and EPC, respectively) were recorded with a two-electrode voltage clamp technique at a holding potential of -60 mV. Intracellular microelectrodes (5–10 $\text{M}\Omega$ in resistance) were filled with 2.5 M KCl. Currents were recorded using Axoclamp 900A amplifier and

digitized by Digidata 1440A (Molecular Devices, USA) under control of Clampex software v. 10.5. Quantum content of EPCs was calculated by dividing the area under EPC curve by the area under mEPC curve.

Chemicals

All reagents were obtained from Sigma (Saint Louis, Missouri, USA). Drugs were diluted in extracellular solution to get the following final concentrations: carbachol (10 μ M), acetylcholine (100 μ M), neostigmine (1 μ M), atropine (1 μ M), d-tubocurarine (10 μ M), muscarine (10 μ M), nicotine (10 μ M), pirenzepine (100 nM), methoctramine (10 nM), mecamlamine (640 nM–6.9 μ M), metillicakonitin (10 nM), ω -conotoxin GVIA (300 nM).

Data Analysis

Experimentally measured relative amplitudes were tested for normal distribution. Statistical significance of relative amplitudes we assessed by Student's *t*-test for pairwise variant. Then data are presented as mean (%) \pm SEM. Values of *P* < 0.05 were considered significant.

RESULTS

Acetylcholine and Carbachol Reduce $[Ca^{2+}]_i$ Transients and Quantal Content of EPC

Application of 10 μ M of carbachol decreased amplitudes of mEPC and EPC by $26 \pm 5\%$ ($n = 4$, $P < 0.05$) and $61 \pm 8\%$ ($n = 4$, $P < 0.05$), respectively (Figure 1A). Carbachol also reduced quantal content by $46 \pm 10\%$ ($n = 4$, $P < 0.05$) as compared to control (Figure 1B). 100 μ M ACh attenuated quantal content by $49 \pm 7\%$ ($n = 18$, $P < 0.05$, Figure 1B). Both carbachol and ACh also attenuated amplitude of $[Ca^{2+}]_i$ transients by $11 \pm 1\%$ ($n = 5$, $P < 0.05$) and $13 \pm 4\%$ ($n = 6$, $P < 0.05$), respectively (Figures 1C,D). Since inhibitory effects of exogenous acetylcholine and carbachol were similar, we used carbachol in all subsequent experiments to activate nicotinic and muscarinic receptors simultaneously. Nicotine and muscarine, however, were used to selectively activate respective receptors types. Also, carbachol is not hydrolysed by acetylcholine esterase, and hence its concentration remains constant over the entire experiment duration.

$[Ca^{2+}]_i$ Transients and Quantal Release after Changing Driving Force for Ca^{2+} Entry into Nerve Terminal

We were interested to elucidate whether a 11% decrease in the peak magnitude of $[Ca^{2+}]_i$ transient is sufficient to account for a 46% decline in neurotransmitter release observed with carbachol (Figures 1A–C). For this purpose, we have reduced the external concentration of Ca^{2+} from 0.6 to 0.3 mM and found that it decreased the evoked $[Ca^{2+}]_i$ transient by $23 \pm 4\%$ ($n = 3$) (Figure 2A). In parallel with this, quantal content dropped by $80 \pm 4\%$ (Figure 2B). Thus, carbachol-induced decrease in the size of evoked $[Ca^{2+}]_i$ transients is more than sufficient to produce inhibition of neurotransmitter release observed in our

experiments earlier. Similar observations were made previously while studying quantal content and $[Ca^{2+}]_i$ transients under activation of cannabinoid receptors (Newman et al., 2007).

$[Ca^{2+}]_i$ Transient Modulation by Muscarine and Nicotine

Exposure to 10 μ M nicotine reduced the amplitude of $[Ca^{2+}]_i$ transient by $15 \pm 3\%$ ($n = 6$, $P < 0.05$, Figure 3B). Application of 10 μ M muscarine decreased the amplitude of $[Ca^{2+}]_i$ transient by $8 \pm 2\%$ ($n = 5$, $P < 0.05$, Figure 3B). Nicotinic antagonist d-tubocurarine (10 μ M) partially but significantly reduced nicotine effect on the $[Ca^{2+}]_i$ transient, while incubation with muscarinic blocker atropine (1 μ M) completely eliminated presynaptic effect of muscarine (Figure 3B). In the presence of atropine (1 μ M), carbachol (10 μ M) reduced $[Ca^{2+}]_i$ transient by $9 \pm 5\%$ ($n = 6$, $P < 0.05$, Figure 3B), while in the presence of d-tubocurarine (10 μ M)—by $18 \pm 9\%$ ($n = 4$, $P < 0.05$, Figure 3B). Pre-incubation with the mixture of d-tubocurarine (10 μ M) and atropine (1 μ M), completely eliminated carbachol effects on $[Ca^{2+}]_i$ transients (Figures 3A,B).

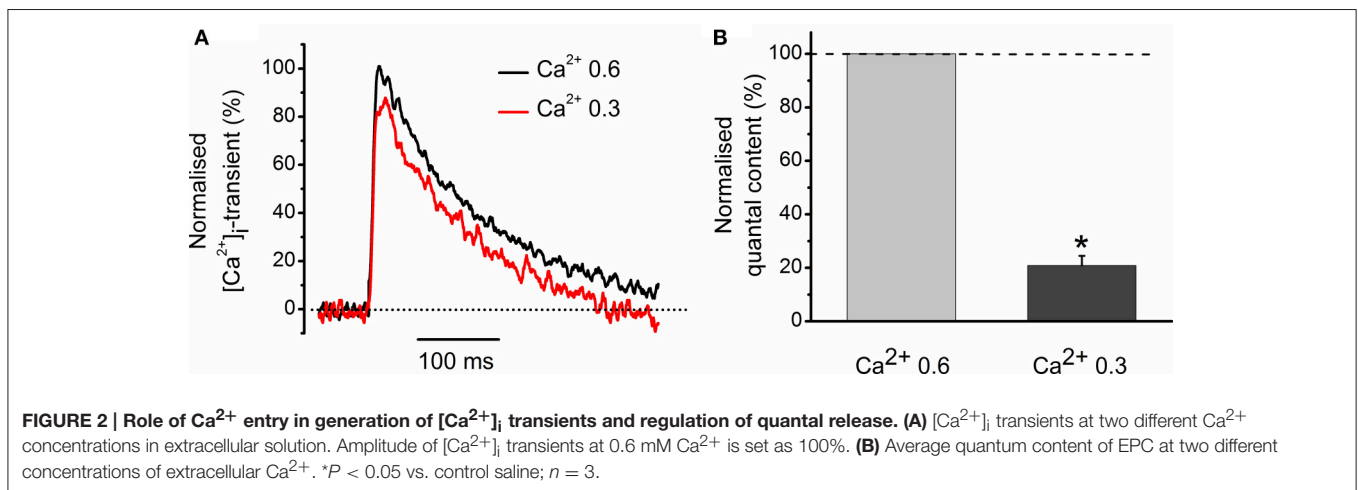
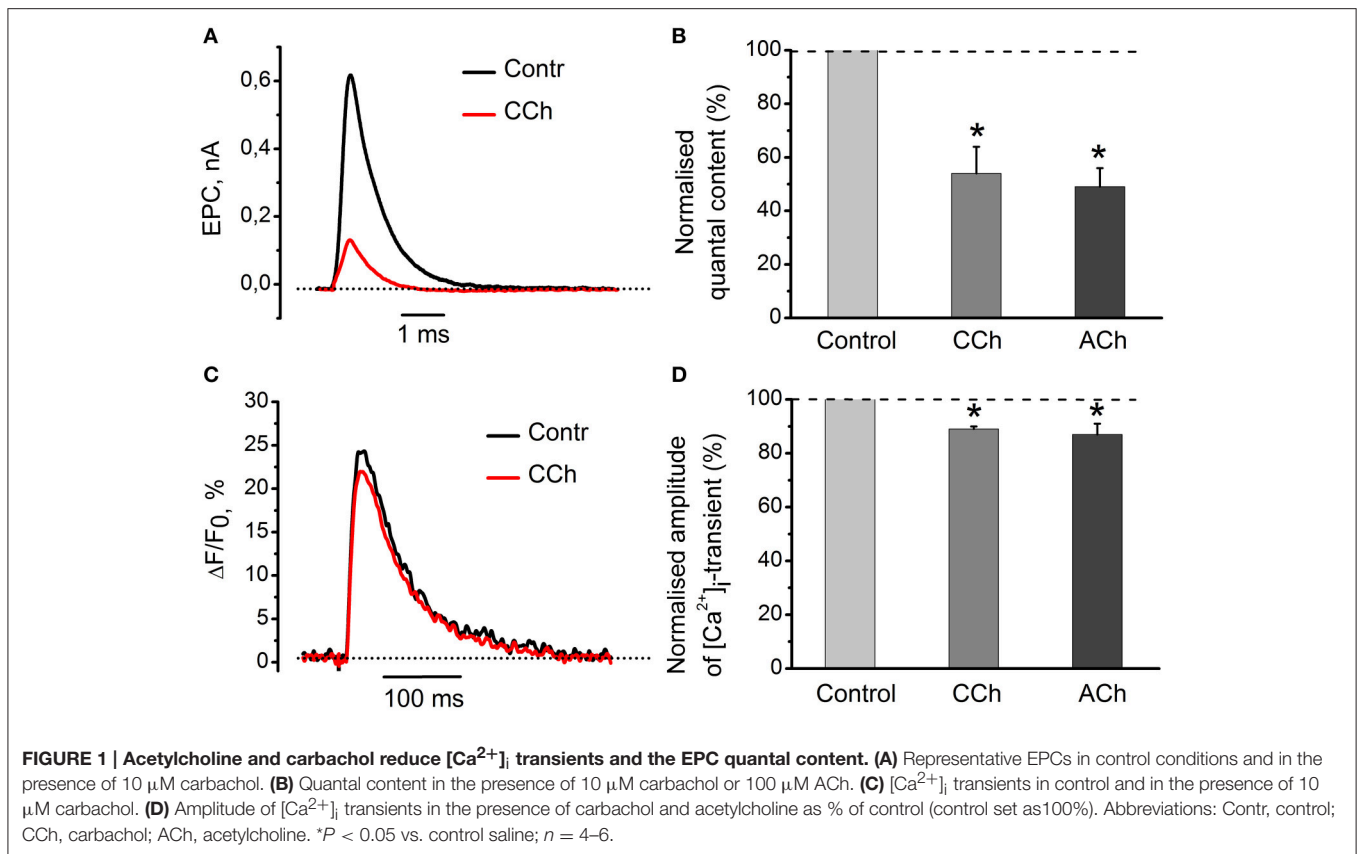
Nicotinic blocker d-tubocurarine did not change the inhibitory effect of muscarine alone—it reduced $[Ca^{2+}]_i$ transients by $8 \pm 2\%$ ($n = 7$, $P < 0.05$, Figures 4A,B). Similarly, blockade of muscarinic receptors by atropine did not significantly affect the inhibitory action of nicotine— $[Ca^{2+}]_i$ transients were reduced by $8 \pm 3\%$ ($n = 7$, $P < 0.05$, Figures 4C,D).

Identification of Muscarinic Receptor Subtypes That Mediate the Effects of Cholinomimetics

M_1 -receptor blocker pirenzepine (100 nM) by itself decreased $[Ca^{2+}]_i$ transients by $14 \pm 7\%$ ($n = 5$, $P < 0.05$, Figures 5A,B). Addition of muscarine further decreased the amplitude of $[Ca^{2+}]_i$ transient (Figures 5A,B). Exposure to 10 nM methoctramine (specific blocker of muscarinic M_2 receptor subtypes), did not affect $[Ca^{2+}]_i$ transients by itself. In the presence of methoctramine, however, the inhibitory action of muscarine on $[Ca^{2+}]_i$ transient was completely eliminated (Figures 5C,D).

Identification of Nicotinic Receptor Subtypes That Mediate the Effects of Cholinomimetics

In these experiments nicotine was used as a specific agonist of nicotinic acetylcholine receptors. Mecamlamine is able to block various subtypes of nicotinic receptors depending on concentration within the range from 640 nM to 6.9 μ M (Papke et al., 2001; Rabenstein et al., 2006; Ostroumov et al., 2008). Next, we tested the effects of different concentrations of mecamlamine. Application of 640 nM mecamlamine increased $[Ca^{2+}]_i$ transient amplitude by $18 \pm 3\%$ ($n = 5$, $P < 0.05$, Figure 6). When 640 nM mecamlamine was applied with nicotine $[Ca^{2+}]_i$ transient amplitude decreased by $9 \pm 4\%$ ($n = 5$, $P < 0.05$, Figure 6). At 2.5 μ M, mecamlamine increased $[Ca^{2+}]_i$ transient amplitude by $16 \pm 2\%$ ($n = 3$, $P < 0.05$, Figure 6), while subsequent addition of nicotine reduced $[Ca^{2+}]_i$ transient

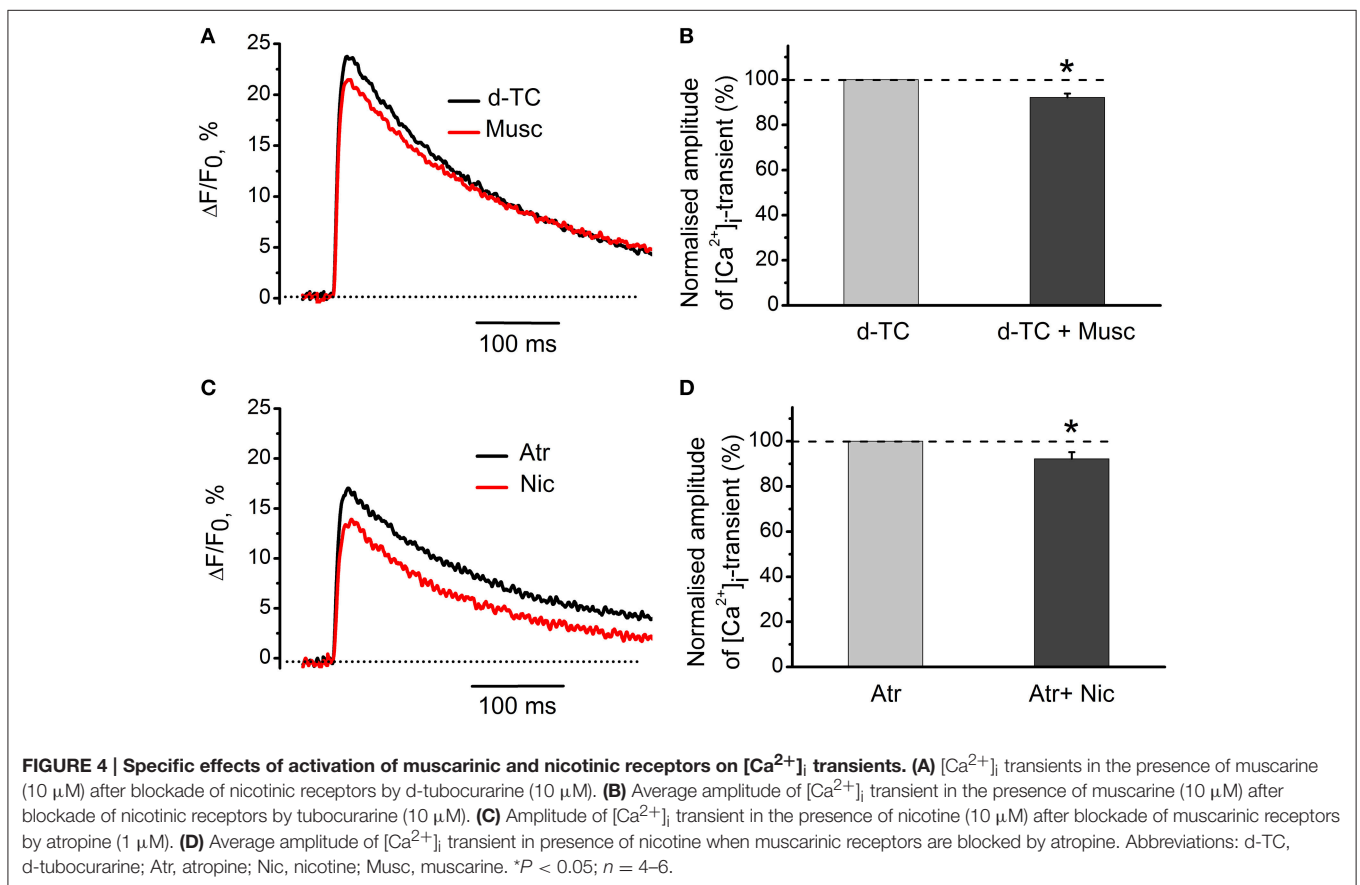
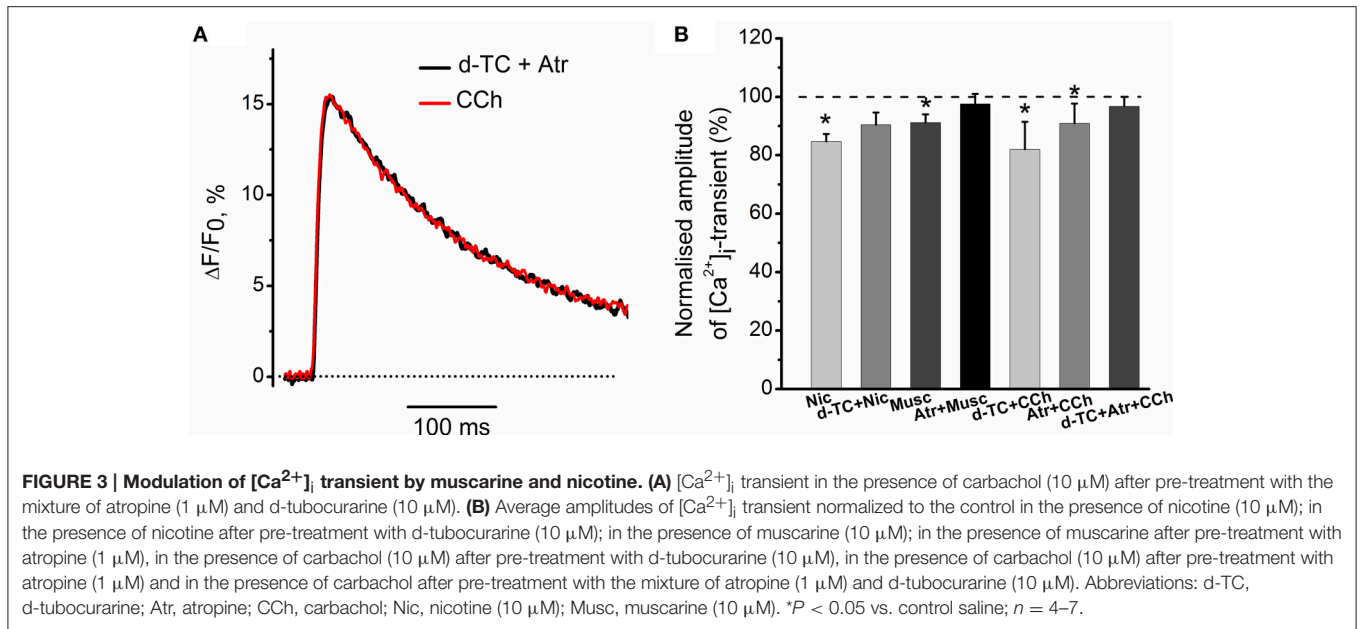


amplitude by $19 \pm 4\%$ ($n = 3$, $P < 0.05$, **Figure 6**). At 3.6 μ M mecamylamine increased $[Ca^{2+}]_i$ transient by $23 \pm 7\%$ ($n = 6$, $P < 0.05$, **Figure 6**), whereas its combined application with nicotine decreased $[Ca^{2+}]_i$ transient by $14 \pm 2\%$ ($n = 6$, $P < 0.05$, **Figure 6**). Finally, at 6.9 μ M, mecamylamine increased $[Ca^{2+}]_i$ transient by $15 \pm 8\%$ ($n = 3$, $P < 0.05$, **Figure 6**), while combined application with nicotine decreased $[Ca^{2+}]_i$ transient amplitude by $8 \pm 2\%$ ($n = 3$, $P < 0.05$, **Figure 6**). Metilikkakonitin is a specific blocker of the $\alpha 7$ -nicotinic receptor subunit. Adding metilikkakonitin at 10 nM increased the amplitude of $[Ca^{2+}]_i$

transient by $13 \pm 11\%$ ($n = 5$, $P < 0.05$, **Figure 6**), while subsequent addition of nicotine decreased $[Ca^{2+}]_i$ transients by $11 \pm 5\%$ ($n = 6$, $P < 0.05$, **Figure 6**).

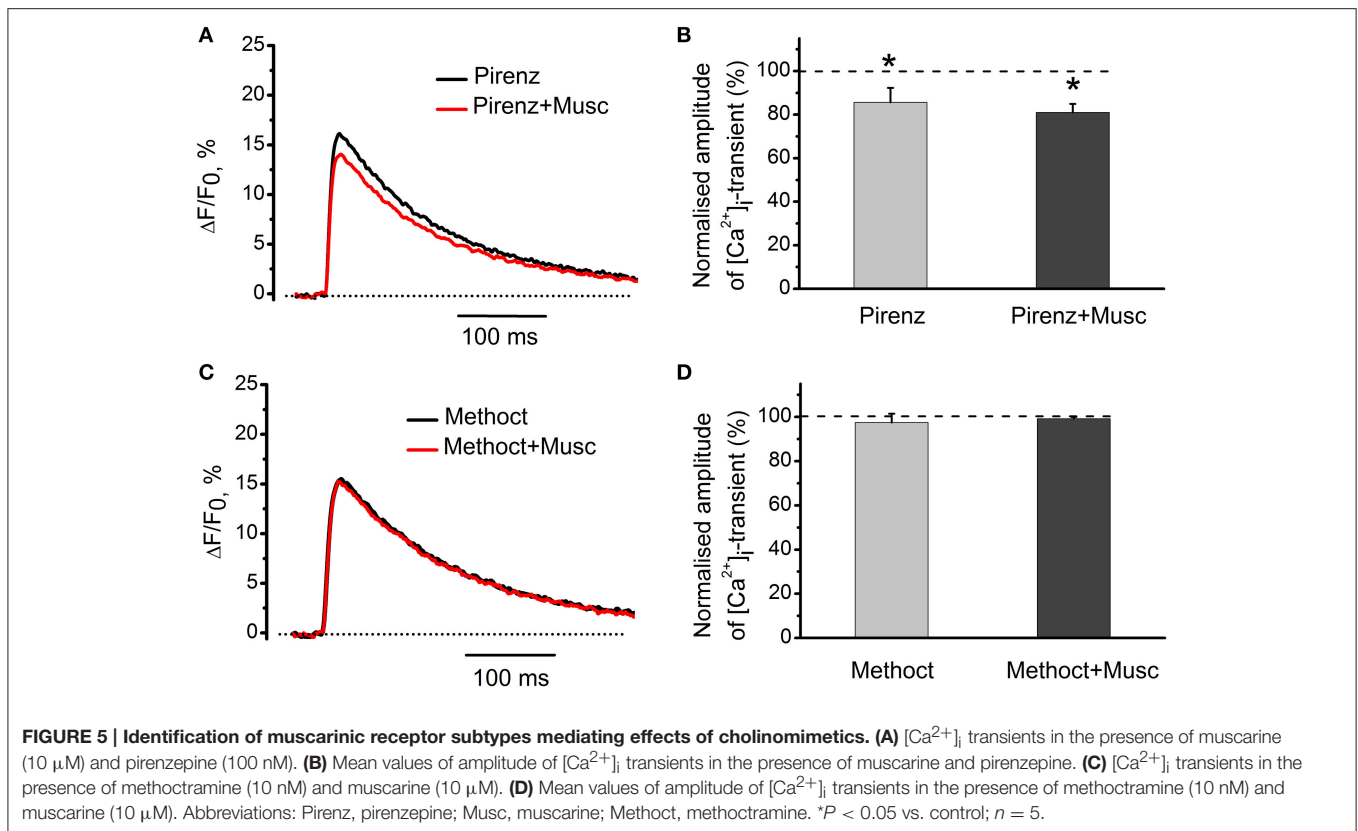
Voltage-Gated Ca^{2+} Channels Involved in Carbachol Modulation of $[Ca^{2+}]_i$ dynamics

Since both acetylcholine and carbachol reduce $[Ca^{2+}]_i$ transient and quantum content of endplate currents, we suggested that cholinergic modulation of neurotransmitter release could result from attenuated Ca^{2+} entry into motor nerve endings (Khaziev



et al., 2012). In this work, we found that application of specific N-type Ca^{2+} channel blocker, ω -conotoxin GVIA (300 nM) reduced $[Ca^{2+}]_i$ transient by $35 \pm 7\%$ ($n = 5$, $P < 0.05$,

Figures 7A,B). In the presence of ω -conotoxin GVIA carbachol became ineffective (**Figures 7C,D**), indicating that N-type Ca^{2+} -channels play role in presynaptic modulation observed.

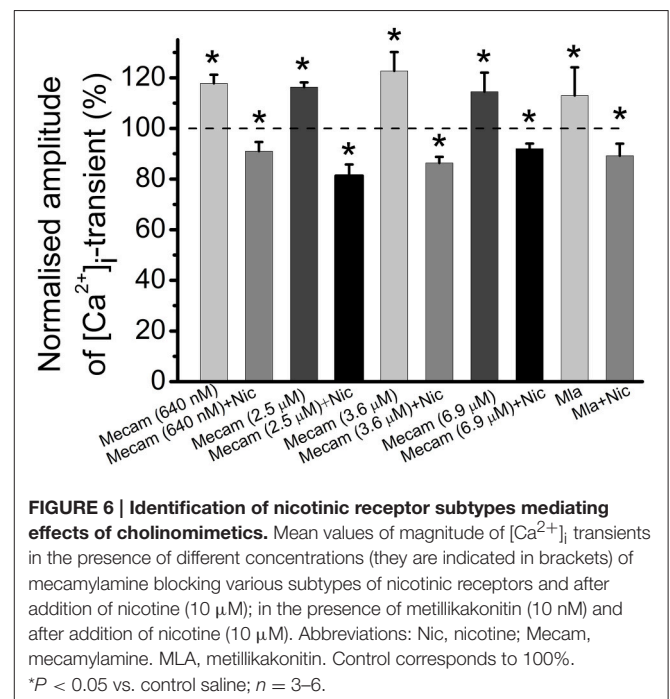


Action of Antagonists of Nicotinic and Muscarinic Acetylcholine Receptors on $[Ca^{2+}]_i$ Dynamics in the Absence of Exogenous Cholinomimetics

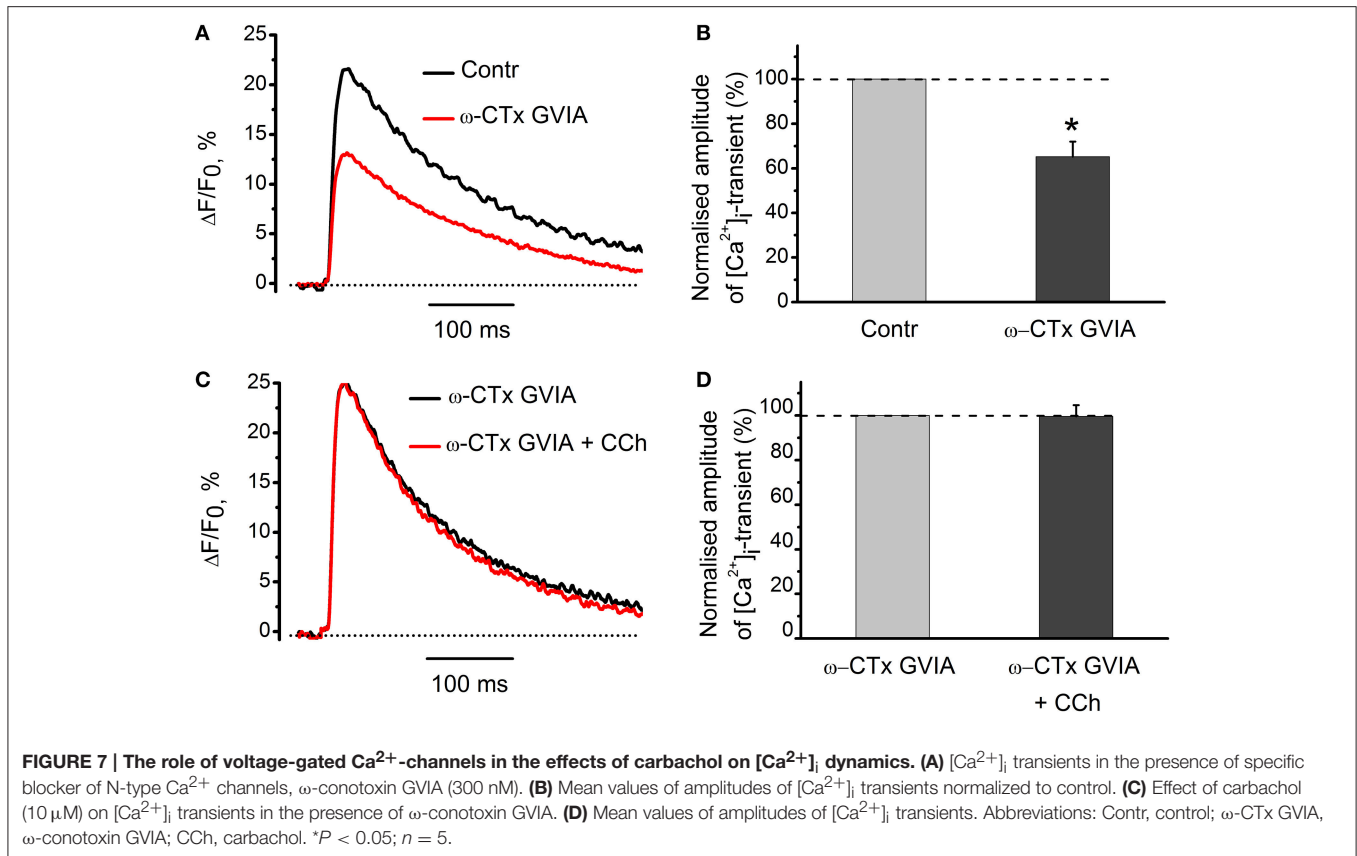
Non-specific blocker of all types of nicotinic receptors, d-tubocurarine (10 μ M) increased the amplitude of $[Ca^{2+}]_i$ transients by $11 \pm 3\%$ ($n = 15$, $P < 0.05$, Figures 8A,B). Non-specific blocker of muscarinic receptors, atropine (1 μ M) also increased $[Ca^{2+}]_i$ transient by $9 \pm 2\%$ ($n = 19$, $P < 0.05$, Figures 8C,D). When applied together, d-tubocurarine and atropine increased the amplitude of $[Ca^{2+}]_i$ transients by $7 \pm 2\%$ ($n = 7$, $P < 0.05$), indicating the absence of additive facilitating effects on $[Ca^{2+}]_i$ transient. An increase in Ca^{2+} -response in the presence of nicotinic or muscarinic acetylcholine receptor blockers suggests the presence of some tonic concentration of endogenous acetylcholine in the synaptic cleft. This acetylcholine interacts with nicotinic and muscarinic receptors and causes inhibition of Ca^{2+} -ions entry into nerve terminal.

Effects of Acetylcholinesterase Inhibitor Neostigmine on $[Ca^{2+}]_i$ Dynamics

It has been shown earlier that anticholinesterase drugs lead to accumulation of endogenous acetylcholine in synaptic cleft (Loewi and Hellauer, 1938; Fatt and Katz, 1952; Fedorov, 1976). At low frequencies of motor nerve stimulation, acetylcholinesterase inhibitor, neostigmine (1 μ M), decreased $[Ca^{2+}]_i$ transients by $13 \pm 5\%$ ($n = 5$, $P < 0.05$, Figures 9A,B).



This inhibitory effect of neostigmine was fully reversed in the presence of blockers of nicotinic and muscarinic receptors (Figure 9B). We can therefore suggest that endogenous



acetylcholine modulates intra-terminal $[\text{Ca}^{2+}]_i$ transients by acting on presynaptic nicotinic and muscarinic acetylcholine receptors.

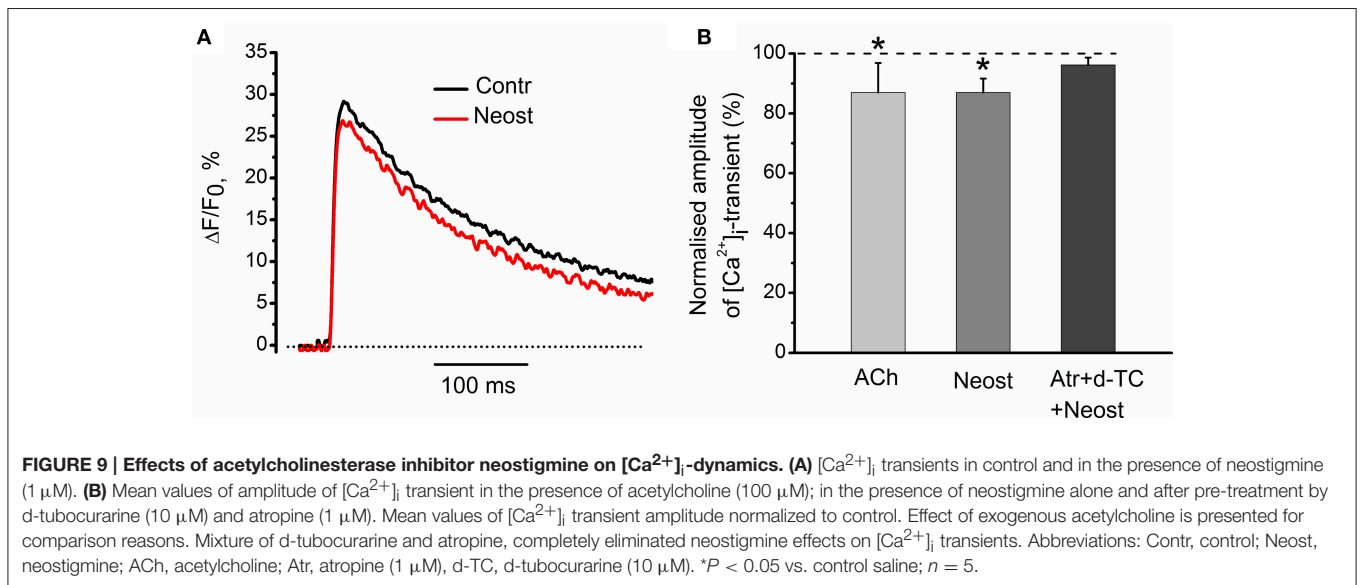
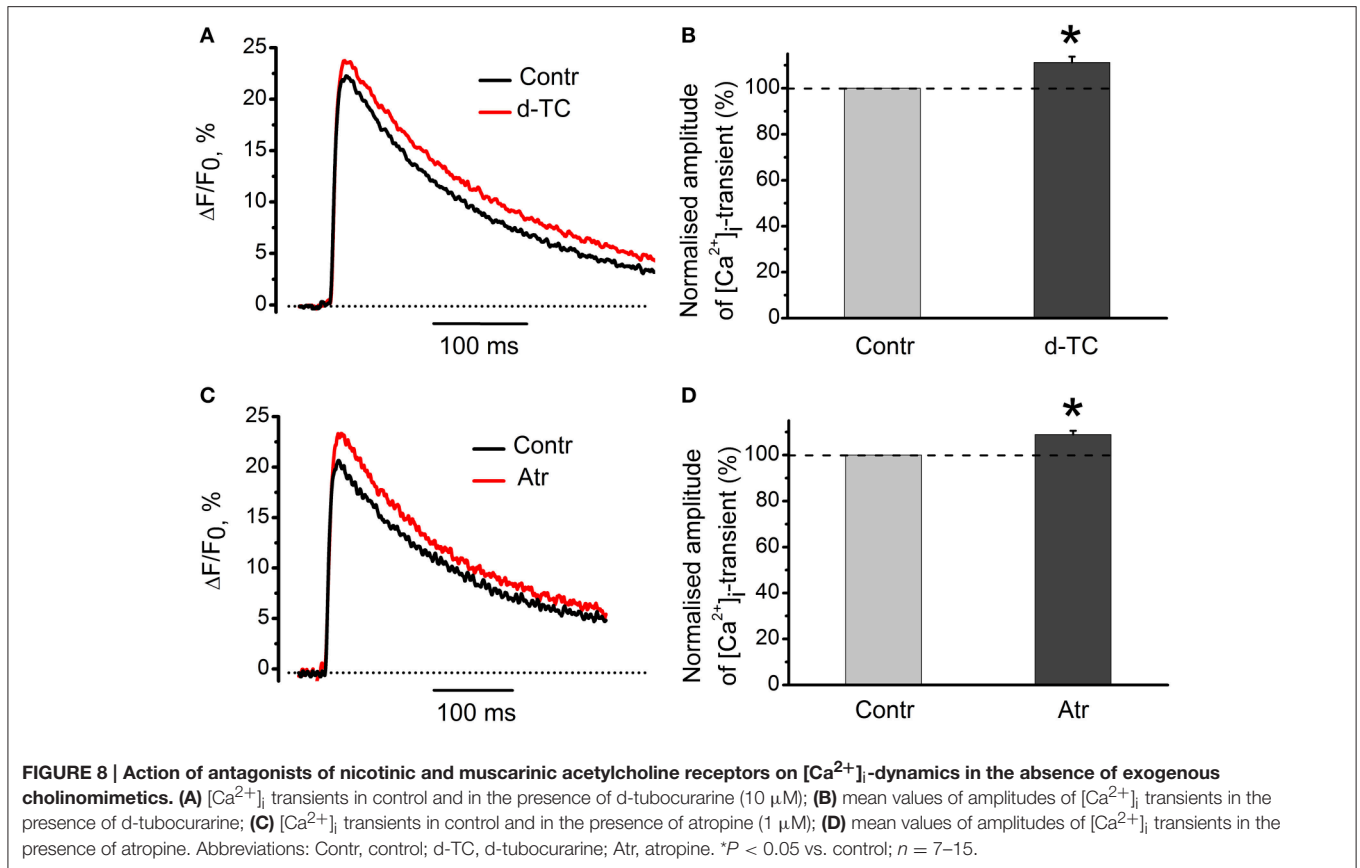
DISCUSSION

Neurotransmitter release from frog nerve endings is modulated by cholinomimetics: by acting through nicotinic receptors, they change kinetics of secretion and its quantum content, while by activation of muscarinic receptors they contribute only to regulation of quantum content (Ciani and Edwards, 1963; Nikolsky et al., 2004). Both quantum content and kinetics of neurotransmitter secretion directly depend on the level of $[\text{Ca}^{2+}]_i$ in presynaptic terminal (Katz and Miledi, 1965; Nikol'skii et al., 2000; Samigullin et al., 2005). Hence, modulation of Ca^{2+} entry could be a plausible mechanism accounting for inhibitory effects of cholinomimetics on quantum secretion. To test this hypothesis, we monitored presynaptic $[\text{Ca}^{2+}]_i$ transients while pharmacologically manipulating acetylcholine receptors and Ca^{2+} channels. In our experiments the Ca^{2+} probe was loaded through the nerve stump, as described by Peng and Zucker (1993) and Wu and Betz (1996). At the end of loading protocol, all terminals in the proximal part of the nerve trunk had sufficient levels of fluorescence to allow $[\text{Ca}^{2+}]_i$ recordings. We (in this paper), as well as others have shown that the loading of the fluorescent dye in the nerve ending does not significantly

alter the physiological parameters of secretion, such as quantal content and frequency of mEPC (Wu and Betz, 1996; Samigullin et al., 2015).

We found that carbachol and acetylcholine decreased both Ca^{2+} response and quantum content of EPCs, indicating that cholinergic modulation of neurotransmitter release can be related to changes in Ca^{2+} entry (Khaziev et al., 2012). We further showed that a relatively small decrease in the driving force for Ca^{2+} influx into nerve terminal causes significant inhibition of neurotransmitter release. This was achieved by lowering extracellular Ca^{2+} concentration from 0.6 to 0.3 mM (Figures 1, 2). This observation is in a good agreement with strong non-linear dependence of quantum content from Ca^{2+} entry into the presynaptic terminal (Dodge and Rahamimoff, 1967).

Inhibition of synaptic acetylcholinesterase by neostigmine decreased $[\text{Ca}^{2+}]_i$ transients, indicating that endogenous acetylcholine is able to modulate Ca^{2+} entry into the nerve ending. Exposure to d-tubocurarine and specific antagonists of nicotinic receptors (in the absence of exogenous cholinomimetics) also augmented $[\text{Ca}^{2+}]_i$ transient, thus indicating that tonically released ACh can block Ca^{2+} entry into the presynaptic terminal. Increase of the amplitude of $[\text{Ca}^{2+}]_i$ transients by atropine is in a qualitative agreement with atropine-induced increase in the secretion at frog synapses due to elimination of tonic action of endogenous acetylcholine as described earlier (Slutsky et al., 1999). We suggest that



release of endogenous ACh during stimulation of the motor nerve and spontaneous quantal and non-quantal release under physiological conditions results in modulation of presynaptic Ca^{2+} entry and provides a physiologically important negative feedback (Katz and Miledi, 1965; Vyskocil et al., 2009). Presynaptic acetylcholine receptors of both muscarinic and

nicotinic nature are involved in this regulation. Since blockade of M_1 -muscarinic receptor by pirenzepine reduced of $[Ca^{2+}]_i$ transient, we may presume that activation of these receptors could enhance Ca^{2+} entry. These results are in good agreement with previous observations indicating that activation of M_1 receptors enhances, whereas activation of M_2 receptors inhibits

ACh release (Slutsky et al., 1999). According to our results M_2 subtype is involved in modulation of presynaptic Ca^{2+} entry, because specific M_2 blocker methoctramine eliminates inhibitory effect of muscarine, whereas M_1 antagonist pirenzepine has no such effect.

Mecamylamine and metillikakonitin did not prevent the inhibitory effect of nicotine while d-tubocurarine did. This indicates that some d-tubocurarine-sensitive nicotinic receptors are involved in the presynaptic inhibitory action of endogenous acetylcholine, and inhibition of this receptor in the absence of exogenous cholinomimetics enhances $[Ca^{2+}]_i$ transients. Specific N-type Ca^{2+} -channel antagonist conotoxin GVIA removes inhibition of $[Ca^{2+}]_i$ transients by carbachol, pointing to a primary role of N-type channels in providing Ca^{2+} entry into the nerve terminal. Furthermore, our data about the role for N-type of Ca^{2+} channels are fully compatible with earlier results of Van der Kloot et al. (1997) who demonstrated that ω -conotoxin eliminated effects of cholinomimetics on quantal content in frog synapses. We conclude that in the frog neuromuscular synapse activation of presynaptic M_2 muscarinic and d-tubocurarine-sensitive nicotinic acetylcholine receptors by exogenous cholinomimetics and/or endogenous acetylcholine decreases quantal content of mediator secretion by reducing the entry of Ca^{2+} ions into the nerve endings due to inhibition of voltage-gated N-type Ca^{2+} channels.

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AUTHOR CONTRIBUTIONS

EK, DS, NZ, NF, EB, and EN contributed to the study design and acquirement of ethical approval. EK, DS, NZ, NF, and EB contributed to data collection. DS, EN, and AV analyzed the data, interpreted the data, and drafted the initial manuscript. The remaining authors critically revised the manuscript. All authors approved the final version of the manuscript. DS, EB, and EN are guarantors of the manuscript and take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

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