



# L-type $\text{Ca}^{2+}$ Channels at Low External Calcium Differentially Regulate Neurotransmitter Release in Proximal–Distal Compartments of the Frog Neuromuscular Junction

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## Abstract

L-type  $\text{Ca}^{2+}$  channels (LTCCs) are key elements in electromechanical coupling in striated muscles and formation of neuromuscular junctions (NMJs). However, the significance of LTCCs in regulation of neurotransmitter release is still far from understanding. Here, we found that LTCCs can increase evoked neurotransmitter release (especially asynchronous component) and spontaneous exocytosis in two functionally different compartment of the frog NMJ, namely distal and proximal parts. The effects of LTCC blockage on evoked and spontaneous release as well as timing of exocytotic events were prevented by inhibition of either protein kinase C (PKC) or P2Y receptors (P2Y-Rs). Hence, endogenous signaling via P2Y-R/PKC axis can sustain LTCC activity. Application of ATP, a co-neurotransmitter able to activate P2Y-Rs, suppressed both evoked and spontaneous exocytosis in distal and proximal parts. Surprisingly, inhibition of LTCCs (but not PKC) decreased the negative action of exogenous ATP on evoked (only in distal part) and spontaneous exocytosis. Lipid raft disruption suppressed (1) action of LTCC antagonist on neurotransmitter release selectively in distal region and (2) contribution of LTCCs in depressant effect of ATP on evoked and spontaneous release. Thus, LTCCs can enhance and desynchronize neurotransmitter release at basal conditions (without ATP addition), but contribute to ATP-mediated decrease in the exocytosis. The former action of LTCCs relies on P2Y-R/PKC axis, whereas the latter is triggered by exogenous ATP and PKC-independent. Furthermore, relevance of lipid rafts for LTCC function as well as LTCCs for ATP effects is different in distal and proximal part of the NMJ.

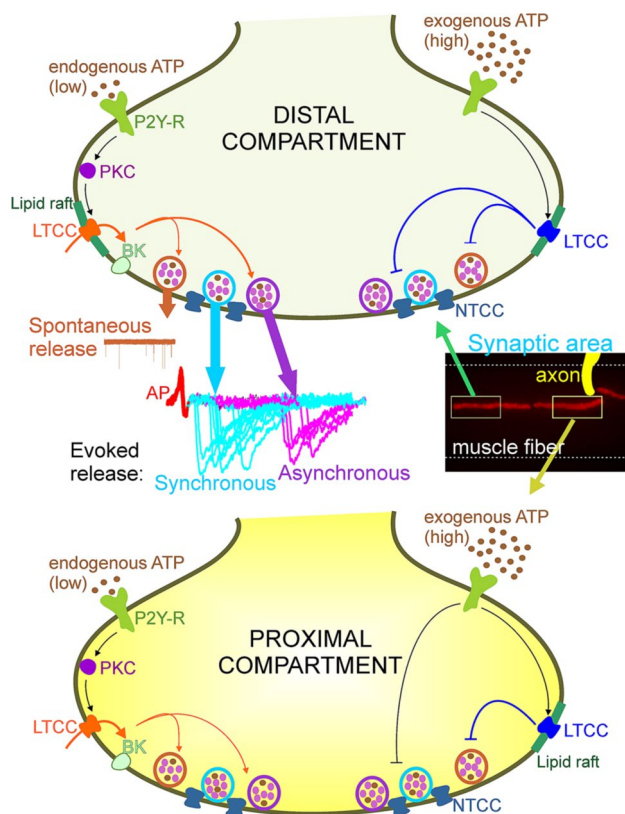
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## Graphic Abstract



**Keywords** Asynchronous exocytosis · Neurotransmitter release · L-type  $\text{Ca}^{2+}$  channels · ATP · Lipid rafts · Protein kinase C

### Abbreviations

ACh	Acetylcholine
AP	Action potential
CgTx	Omega-conotoxin GVIA
CTxB	Cholera toxin B subunit
EPP	End-plate potential
MEPP	Miniature EPP
LTCC	L-type $\text{Ca}^{2+}$ channel
NMJ	Neuromuscular junction
NTCC	N-type $\text{Ca}^{2+}$ channel
PKC	Protein kinase C
P2Y-R	P2Y-receptor
SV	Synaptic vesicle
VGCC	Voltage-gated $\text{Ca}^{2+}$ channel

### Introduction

Neurotransmission relies on exocytotic neurotransmitter release due to fusion of synaptic vesicles (SVs) with presynaptic membrane. Neurotransmitter release is triggered by

$\text{Ca}^{2+}$  influx through voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs) anchored in sites of SV exocytosis, active zones. Some VGCCs are located relatively far from active zones and  $\text{Ca}^{2+}$  influx from these channels can modulate neurotransmitter release via action on resting  $\text{Ca}^{2+}$  levels as well as  $\text{Ca}^{2+}$ -dependent enzymes and channels.

L-type  $\text{Ca}^{2+}$  channels (LTCCs), also known as  $\text{Ca}_v1$ , are expressed in skeletal muscle, heart, retina, and brain. LTCCs (Cav1.3 and Cav1.4), characterized by slow inactivation, operate in triggering SV exocytosis in ribbon synapses in retina and inner ear. In contrast to conventional phasic synapses, these sensory synapses are designed for analogue neurotransmission when neurotransmitter release is proportional to the changes in membrane depolarization (Dolphin and Lee 2020). Additionally, LTCCs participate in catecholamine release from chromaffin cells of adrenal glands in response to splanchnic nerve stimulation or nicotinic acetylcholine (ACh) receptor activation (Marcantoni et al. 2007). Evidently, that in most synapses, LTCCs have a modulatory role in control of neurotransmitter release. Particularly, activity of LTCCs can contribute to enhancement of SV

exocytosis after learning and induction of long-term potentiation in cortical neurons (Subramanian et al. 2013). In the mammalian neuromuscular junctions (NMJs), LTCCs can participate in maintenance of exocytosis at high-frequency activity via promoting formation of new SVs by endocytosis (Perissinotti et al. 2008). Furthermore, there are silent LTCCs whose participation in ACh release in response to intense motor nerve terminal depolarization is unmasked under the conditions of  $K_{Ca}$  channel inhibition (Flink and Atchison 2003). In addition, 4-beta-phorbol ester, an activator of protein kinase C (PKC), can increase quantal ACh release due to opening of quiescent LTCCs at resting potential without apparent depolarization in motor nerve terminals (Arenson and Evans 2001). Some important presynaptic receptors can affect neurotransmitter release partially via modulation of LTCC activity. LTCCs are involved in inhibitory effect of adenosine metabolite inosine on spontaneous and evoked ACh release (Cinalli et al. 2013) as well as in excitatory effects of adenosine A2A receptor activation and muscarinic M1 receptor agonist on neurotransmitter release in NMJs (Palma et al. 2011; Oliveira and Correia-de-Sa 2005). Importantly,  $Ca^{2+}$  influx through LTCCs is critical for clustering of ACh receptors in the center of muscle fiber at the onset of NMJ development (Kaplan et al. 2018) as well as formation cluster of SVs and prevention of neurotransmission in immature and regenerating presynaptic nerve terminals (Chipman et al. 2014). Loss of amyloid precursor protein, important for structure and function of developing NMJs, can facilitate aberrant activity of LTCCs contributing to changes in neurotransmitter release during repetitive stimulation in mouse neuromuscular junctions (Yang et al. 2007).

The complex character of LTCC involvement in neurotransmission can be related with location of the channels in presynaptic, postsynaptic, and glial cell membranes as well as changes in the channel functioning during ontogenesis (Robitaille et al. 1996; Dolphin and Lee 2020; Kaplan et al. 2018). Additionally, distribution of LTCCs in membrane microdomains (lipid rafts) (Park and Kim 2009) as well as inclusion of the channels into different complexes within one cell (Calin-Jageman and Lee 2008) can create functional diversity of LTCCs. Important unresolved aspects related to LTCCs are about existence of the channel functional heterogeneity along the nerve terminals and possible engagement of LTCCs in control of timing of the neurotransmitter release. In addition, the implication of lipid rafts in LTCC function as well as LTCC contribution to ATP-dependent regulation of spontaneous and evoked neurotransmitter release in different regions of NMJs are open questions. To address these issues, in the present study, the changes in quantum content, asynchrony of evoked ACh release as well as alteration in spontaneous release were detected in proximal and distal parts of the frog NMJs. These parts of the

motor nerve terminal are characterized by higher and low neurotransmitter release probabilities, respectively, as well as have specific features in electrogenesis, SV exo-endocytosis and regulation of neurotransmission (Tsentsevitsky et al. 2020a, 2011; Ginebaugh et al. 2020; Zefirov et al. 2003). These properties make the long frog motor nerve terminals are suitable for investigation of LTCC compartmentalization-related processes in synapses.

## Methods

### Animals, Preparations, and Chemicals

Frogs (*Rana ridibunda*) were obtained from lakes and then placed into a pool with dechlorinated flowing water (4 °C). The pool was located in the dark room under temperature- and humidity-stable conditions. All experiments were performed within autumn–winter period. After euthanasia, the cutaneous pectoris muscles were excised.

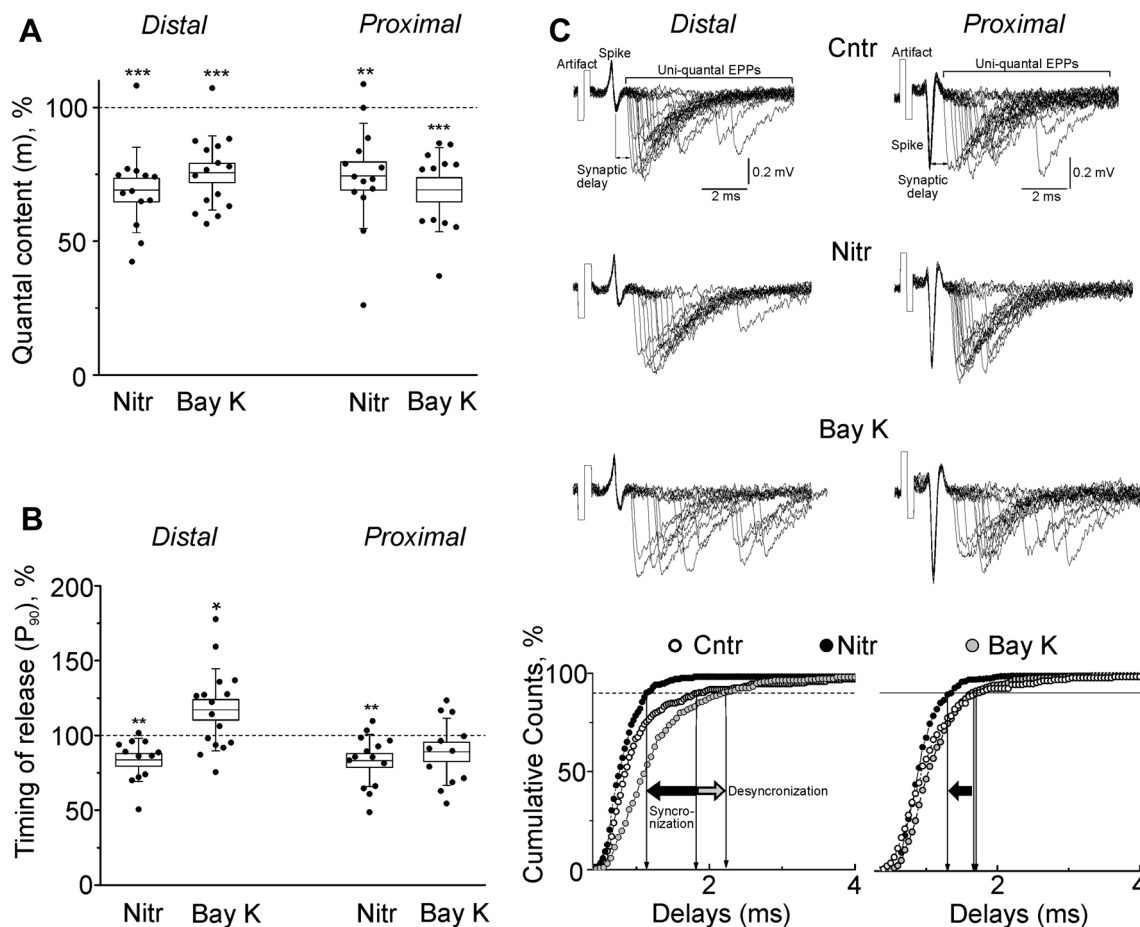
Isolated cutaneous pectoris muscle with nerve stub was pinned into a Sylgard-coated thermostatically controlled chamber (total volume–5 ml), which was continuously perfused (5 ml\*min<sup>-1</sup>) with the Ringer's solution: 113.0 mM NaCl, 2.5 mM KCl, 4.0 mM MgCl<sub>2</sub>, 0.3 mM CaCl<sub>2</sub>, 1.5 mM NaHCO<sub>3</sub>, and 5 mM HEPES; pH was adjusted to 7.3. The bathing temperature was kept at 20 ± 0.3 °C. The following drugs were used: 5 μM nitrendipine (LTCC blocker) (Palma et al. 2011; Katz et al. 1996), 1 μM (–)-Bay K 8644 (agonist of LTCCs) (Flink and Atchison 2003; Gaydukov et al. 2009), 10 nM omega-conotoxin GVIA/CgTx (selective antagonist of N-type Ca<sup>2+</sup> channel/NTCC) (Tsentsevitsky et al. 2020a), 100 μM ATP (De Lorenzo et al. 2006; Giniatullin et al. 2015, 2005; Sokolova et al. 2003; Tsentsevitsky et al. 2011), 100 μM suramin (P2Y receptor antagonist) (De Lorenzo et al. 2006; Giniatullin et al. 2005; Sokolova et al. 2003), 2 μM chelerythrine (PKC inhibitor) (Sokolova et al. 2003; Petrov et al. 2015; Zakyrganova et al. 2021), 100 nM iberiotoxin (selective antagonist of Ca<sup>2+</sup>-dependent K<sup>+</sup> channels/BK channels) (Gaydukov et al. 2009), 1 mM N-acetyl-L-cysteine/NAC (antioxidant) (Giniatullin et al. 2005; Tsentsevitsky et al. 2011, 2020a), 0.5 μM CdCl<sub>2</sub> (Tsentsevitsky et al. 2020a). The concentrations of used chemicals were selected based on previous data (including our) obtained from studies on the NMJs. All reagents were purchased from Sigma. Application of the reagents lasted for 20–40 min. In the case of evaluation of effect of one “X” drug on a background of other “Y” reagent application, the “Y” reagent was added to the bathing saline 20–40 min prior to exposure to “X” drug and remained in the working solution throughout the experiment. In some experiments, muscles were pretreated with 0.1 mM methyl-β-cyclodextrin (MβCD, lipid raft disrupting agent) for 15 min (Zakyrganova et al. 2021), and then MβCD

was removed out of the bathing solution and the muscle were rinsed for 10 min before drug application.

## Electrophysiological Assays

Recording of postsynaptic electrical events, namely endplate potentials (EPPs) and miniature EPPs (MEPPs), was carried out extracellularly as described in (Tsentsevitsky et al. 2011). Nerve stimulation at 0.5 Hz (via a suction electrode) was used to evoke EPPs. Two Ringer's

saline-filled glass microelectrodes (with a resistance of 2–3 M $\Omega$ ) were placed in proximal (~3 to 5  $\mu$ m from the end of the last myelinated segment; 1st electrode) and distal region (~90 to 120  $\mu$ m; 2nd electrode) of the nerve terminal. In the proximal regions, an action potential (AP) had a characteristic shape (Mallart 1984), which is distinguishable from that of AP in the distal region (Fig. 1). This was another hallmark of the correct microelectrode position. The consistency of microelectrode position was



**Fig. 1** Effect of LTCC modulators on evoked neurotransmitter release. Change in quantum content,  $m$  (a) and  $P_{90}$  parameter, an indicator of synchrony of neurotransmitter release (b) after treatment with LTCC inhibitor (nitrendipine; Nitr) or activator (Bay K). Shown the changes in distal and proximal parts of the NMJs. Data are represented as circles—values in individual animals, box range—SEM, whiskers—SD. Horizontal dash line is value (100%) before inhibitor/activator application.  $N=12-16$  for each group.  $***P<0.001$ ,  $**P<0.01$ ,  $*P<0.05$ —compared to value prior to LTCC modulator addition. **c** Superimposed uni-quantal EPPs recorded extracellularly (in response to 70 stimuli) in distal and proximal region of the same nerve terminal. Stimulus artifact, presynaptic AP (spike), and synaptic delay are denoted (for Control/"Cntr" traces). In distal and proximal parts, there is different shape of the spike. Quantal content was calculated from logarithmic ratio between the number of total

stimuli and number of failures (the method of "failures"). Decrease in quantal content manifests as a decrease in number of uni-quantal EPPs in response to the same number of impulses (compare the number of EPP traces in Cntr, Nitr, and Bay K). EPPs appear with different synaptic delays after spikes. Synchronous exocytotic events are characterized by a close coupling to spike (short delay), whereas asynchronous release occurs with longer and variable delay. Bottom, shown calculation of  $P_{90}$  (for the individual experiments) from cumulative histogram of synaptic delays of the EPPs; vertical arrowed lines denote the 90% percentile (in ms). The decrease in  $P_{90}$  due to LTCC inhibition indicates on synchronization of exocytotic events with action potential, i.e., suppression of asynchronous component of the release. Agonist of LTCCs has opposite effect (desynchronization of the release/increase in asynchronous exocytosis)

monitored throughout the experiment [see in Tsentsevitsky et al. (2011)].

The timing of the single exocytotic event (degree of synchronicity with presynaptic AP) can be precisely assessed by analysis of real synaptic delays of the uni-quantal EPPs (Katz and Miledi 1965). Therefore, experiments were carried out in the presence of 0.3 mM  $\text{Ca}^{2+}$  and 4.0 mM  $\text{Mg}^{2+}$ ; and quantal content ( $m$ ) was determined using the equation (“method of failures”):  $m = \ln N/N_0$ , where  $N$  is the total number of stimuli and  $N_0$  is the number of stimuli unaccompanied by EPP (synaptic failures) (Tsentsevitsky et al. 2011, 2020b; Del Castillo and Katz 1954; Katz and Miledi 1965). To receive reliable measurements, in individual experiment, 1500–2500 stimuli were applied to motor nerve prior to and after drug application. The real synaptic delays were analyzed as previously described in detail (Katz and Miledi 1965; Tsentsevitsky et al. 2011). Initially, the synaptic delay, as time from the peak of the presynaptic sodium current spike to the point corresponding to 20% rise phase of the uni-quantal EPP, was estimated (Katz and Miledi 1965; Tsentsevitsky et al. 2011). Then, indicative value (90% percentile, in ms) for dispersion of the real synaptic delay ( $P_{90}$ ) was estimated from cumulative distribution of synaptic delays [described in detail (Tsentsevitsky et al. 2011; Bukcharaeva et al. 1999)].  $P_{90}$  is the interval between the minimal synaptic delay and the point at which 90% of all uni-quantal EPPs had occurred (Fig. 1). Decrease and increase in value of  $P_{90}$  corresponds to a synchronization and desynchronization of the exocytotic event, respectively (Bukcharaeva et al. 1999).

### Lipid Raft Labeling

Cholera toxin B subunit (CTxB) conjugated to fluorescent dye AlexaFluor 488 (ThermoFisher) was used to lipid raft visualization. Fluorescent-labeled CTxB specifically interacts with GM1 ganglioside clusters, predominantly resided into the lipid rafts (Margheri et al. 2014). The muscles were exposed to CTxB (1  $\mu\text{g}/\text{ml}$ ) for 15 min and then rinsed out for 30 min, and after that the fluorescence was detected (Kasimov et al. 2016). Additionally, tetramethylrhodamine-labeled  $\alpha\text{Btx}$  (a marker for NMJ) was added simultaneously with CTxB to the bathing saline. Fluorescence of CTxB/ $\alpha\text{Btx}$  was excited at a 488/555 nm and the emission was recorded with bandpass filter of a 505–545/610–650 nm. Fluorescent images were captured from 15 to 20 different NMJs in individual muscle. The values from the NMJs were pooled to calculate mean value in each muscle. BX51WI microscope (Olympus) equipped with spinning disk confocal unit (Olympus), DIC-optics, and LumPlanPF 100xw objective were used. Images were captured by DP71 camera (Olympus) under control of cellSens software (Olympus).

### Statistics

Statistical analysis was carried out using OriginPro software. Data represent the mean  $\pm$  SD, where sample size ( $n$ ) is the number of experiments on individual animals. The sample size is indicated in figure legends. Statistical significance was assessed by Kolmogorov–Smirnov test ( $P_{90}$ ) or paired (for values before and after drug application) two-tail  $t$ -test and Mann–Whitney  $U$  test (for comparison of two unpaired groups); in case of multiple comparisons, one-way ANOVA was used. Values of  $P \leq 0.05$ ,  $P \leq 0.01$ ,  $P \leq 0.001$  were considered as significant.

### Results

#### Effects of Modulation of L-Type $\text{Ca}^{2+}$ Channel Activity on Neurotransmitter Release

We recorded EPPs in two functionally specific compartments of the same nerve terminal, in the distal and proximal parts (Fig. 1). Inhibitor of LTCCs (nitrendipine, 5  $\mu\text{M}$ ) decreased quantum content ( $m$ ) of EPPs in both parts to the similar degree (Fig. 1a). Surprisingly, agonist of LTCCs (Bay K, 1  $\mu\text{M}$ ) had the same action (Fig. 1a). In contrast to the same direction of action of antagonist and agonist on the quantum content, nitrendipine decreased  $P_{90}$  parameter (an indicator for dispersion of synaptic delays and asynchrony of neurotransmitter release) in distal and proximal parts, but Bay K increased  $P_{90}$  in the distal part of the nerve terminal (Fig. 1b). In the proximal part, there were no significant changes in  $P_{90}$  in response to Bay K administration.

Accordingly, activation or inhibition of LTCCs could reduce quantum content. This suggest that “optimal” LTCC activity is required to set up the level of neurotransmitter release at the endogenous content of ATP/ADP (denoted in the text as basal conditions). At the same time, asynchrony of the neurotransmitter release (in the distal part) is increased or decreased due to activation or inhibition of LTCCs, respectively. Since, LTCC is a positive regulator of asynchronous exocytosis.

The suppression of asynchronous neurotransmitter release is a typical response to inhibition of VGCCs that control neurotransmitter release. Likely under these conditions, only “most active” SVs docked closely to the VGCC cluster preferably undergo exocytosis upon arriving AP (Tsentsevitsky et al. 2020a). Indeed, inhibition of main N-type of VGCCs (with 10 nM  $\text{CgTx}$ ) in active zone decreased  $P_{90}$  parameter as well as quantum content of EPPs in both distal and proximal parts of the nerve terminal (S. Fig. 1A). Theoretically, the changes in both quantal content and timing of the neurotransmitter release in response to modulation of LTCCs can be directly  $\text{Ca}^{2+}$ -dependent or rely on

stimulation of  $\text{Ca}^{2+}$ -dependent enzymes or channels. For example, BK channels can be activated by  $\text{Ca}^{2+}$  influx via VGCCs (including L-type) and regulate bidirectionally neurotransmitter release in synapses, including NMJs (Wang et al. 2020; Xu and Slaughter 2005; Flink and Atchison 2003; Liu et al. 2007). To test a possible link of LTCCs and BK channels, the latter were blocked with iberiotoxin (100 nM). Preliminary inhibition of BK channels with iberiotoxin suppressed the effects of nitrendipine on quantum content and  $P_{90}$  in both distal and proximal part of NMJs. At the same time, iberiotoxin itself had no significant influence on quantum content and neurotransmitter release timing (S. Fig. 1B, C). Hence, LTCC-dependent BK channel activation can contribute to facilitation of neurotransmitter release and its desynchronization.

These results focus on dual role of LTCCs in regulation of neurotransmitter release in the NMJs. Probably, activity of LTCCs might increase or decrease evoked quantum secretion in context-dependent manner and there are different population of LTCCs in NMJs.

### Contribution of LTCCs to Depressant Effect of Exogenous ATP on Neurotransmitter Release

ATP is a main co-neurotransmitter in vertebrate NMJs. Application of ATP (100  $\mu\text{M}$ ) decreased quantum content of EPP in distal (less) and proximal (more profound) regions of the NMJ (Fig. 2a). To test a dependence of this effect on LTCCs, ATP was added into the bathing solution after preliminary inhibition or activation of LTCCs with nitrendipine or Bay K, respectively. Inhibition of LTCCs attenuated the depressant effect of ATP, whereas agonist of LTCCs potentiated the ATP-induced suppression of neurotransmitter release in the distal part (Fig. 2a). At the same time, modulation of LTCCs had no marked influence on ATP-mediated decrease in quantum content in the proximal part. ATP itself and under the conditions of preliminary LTCC activity modulation did not affect the timing of neurotransmitter release (Fig. 2b). Hence, LTCCs can markedly augment ATP-mediated depression of neurotransmitter release specifically in distal part. Probably, ATP-driven LTCC activation can suppress quantum release, without affecting timing of the release.

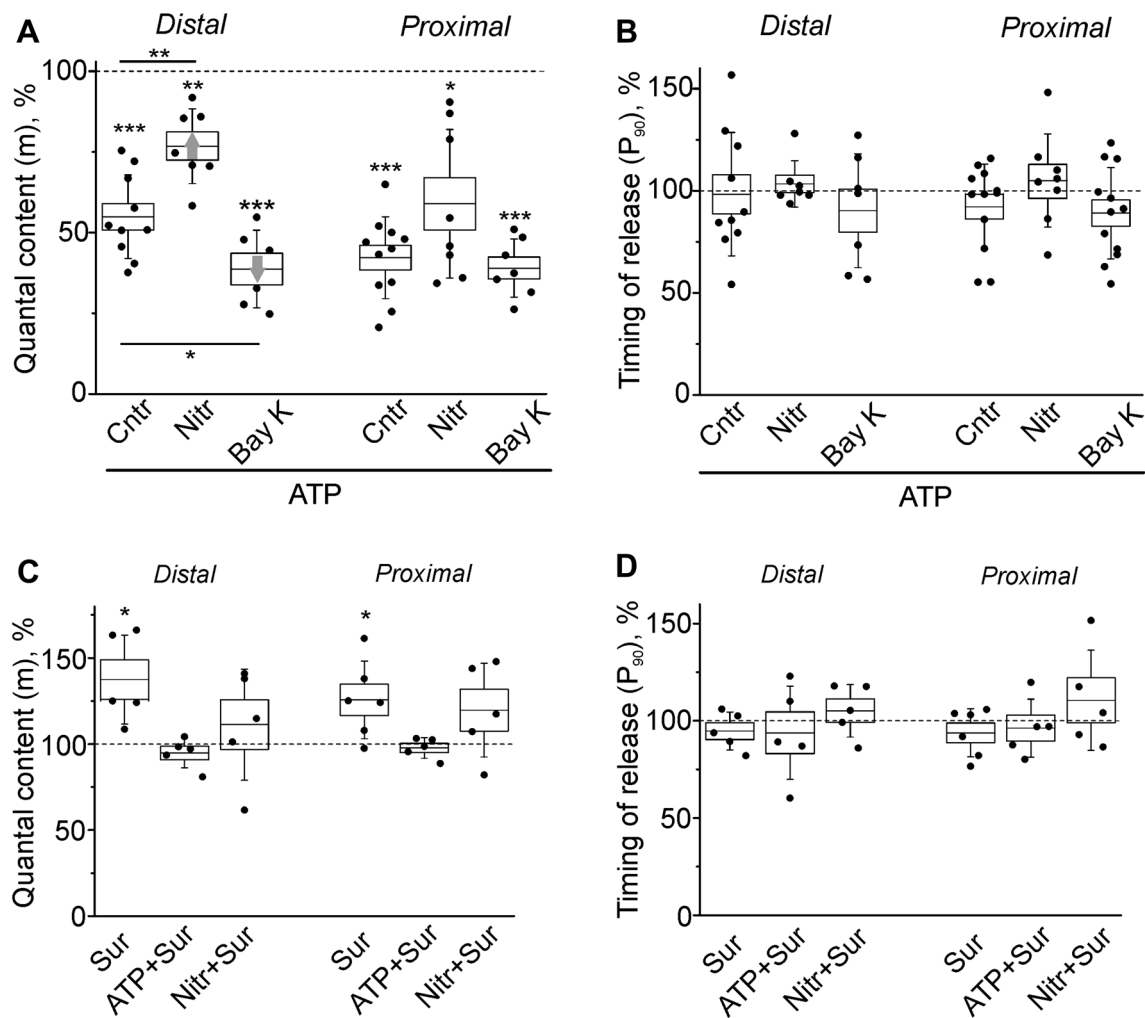
Notably, that selective blockage of N-type of VGCCs (a main type accountable for triggering evoked exocytosis) as well as pretreatment with  $\text{Cd}^{2+}$  at very low concentration (0.5  $\mu\text{M}$ ), in which it seems to affect the main VGCC subtype, did not modulate the depressant action of ATP in proximal part of NMJ and facilitated ATP effect in the distal region (S. Fig. 2a). This points to different regulation of neurotransmitter release in the distal and proximal regions as well as opposite roles of NTCCs and LTCCs in ATP action in the distal region of nerve terminal.

In the NMJs, endogenous and exogenous ATP act mainly via P2Y metabotropic receptors, particularly P2Y<sub>12/13</sub> (Giniatullin et al. 2015; Guarracino et al. 2016). Indeed, an inhibitor of P2Y-R, suramin (100  $\mu\text{M}$ ), increased quantum content of EPPs and blocked the effect of ATP administration in both distal and proximal parts (Fig. 2c). This suggests occurrence of depressant action of endogenous ATP on evoked neurotransmitter release. Importantly, when P2Y-Rs were blocked with suramin, inhibitor of LTCCs lost ability to affect the quantum content of EPP in the distal and proximal parts (Fig. 2c). At the same time, when purine receptors were preliminarily stimulated by exogenous ATP, nitrendipine still suppressed quantum release in both proximal and distal part of NMJs (S. Fig. 2b). These data indicate that stimulation of P2Y-Rs is required for maintenance of LTCC activity, and on background of P2Y-R inactivation, LTCCs do not contribute to neurotransmitter release significantly. Consistent with this possibility is that P2Y-R inhibition prevented the effect of LTCC antagonist on timing of neurotransmitter release (Fig. 2d). It should be noted that like ATP, suramin alone or in combination with ATP had no effect on timing of the neurotransmitter release (Fig. 2d).

The complexity of LTCC function manifests as opposite influence of these channel modulation on neurotransmitter release in different contexts. Obtained data suggest that in the basal conditions, stimulation of P2Y-Rs with endogenous ligands supports the overall activity of LTCCs, and the latter can maintain the certain level of neurotransmitter release along the entire nerve terminal as well as increase asynchronous release. At the same time, suppression of the neurotransmitter release by exogenous ATP partially relies on LTCC activation in the distal part of NMJ. In the latter case, LTCCs are negative regulator of neurosecretion, but they cannot modulate a timing of the neurotransmitter release under these conditions. Probably that different functional populations of LTCCs as well as receptors for ATP exist in the distal part of NMJs.

### Protein Kinase C (PKC) in Effects of LTCC Blocker

Presynaptic effects of ATP and adenosine as well as ACh in NMJs can be partially related with PKC (Galkin et al. 2001; Santafe et al. 2006; Branisteanu et al. 1989). Moreover, this protein kinase can positively modulate LTCCs in NMJs (Fu and Huang 1994; Urbano et al. 2001). Hence, we compared the effects of PKC and LTCC inhibition. Antagonist of PKC (chelerythrine, 2  $\mu\text{M}$ ) had the same effect on quantum content of EPPs as LTCC blocker (Fig. 3a). Furthermore, preliminary blockage of PKC completely prevented effect of nitrendipine in both proximal and distal parts of NMJs (Fig. 3a). Importantly, chelerythrine synchronized neurotransmitter release (but only in the distal part of the NMJs) and abolished effects of



**Fig. 2** Crosstalk between LTCCs and ATP-sensitive P2Y-Rs in control of evoked neurotransmitter release in distal and proximal parts of NMJ. **a, b** The effects of exogenous ATP on quantum content (**a**) and timing of neurotransmitter release,  $P_{90}$  (**b**) in control (Cntr) and after pre-treatment with LTCC antagonist (Nitr) or agonist (Bay K). 100% is value before ATP application (dash line). Data pointing to inhibitory effect of LTCC activity on evoked secretion are highlighted with

gray arrows (**a, c, d** Changes in quantum content (**c**) and  $P_{90}$  (**d**) due to P2Y-R inhibition with suramine (Sur) and in response to ATP or nitrendipine application on background of preliminary P2Y-R inhibition (ATP + Sur or Nitr + Sur). Data are expressed as circles—values in individual animals, box range—SEM, whiskers—SD.  $N=5-12$  for each group. \*\*\* $P<0.001$ , \*\* $P<0.01$ , \* $P<0.05$ —compared to value prior to drug application or between two groups

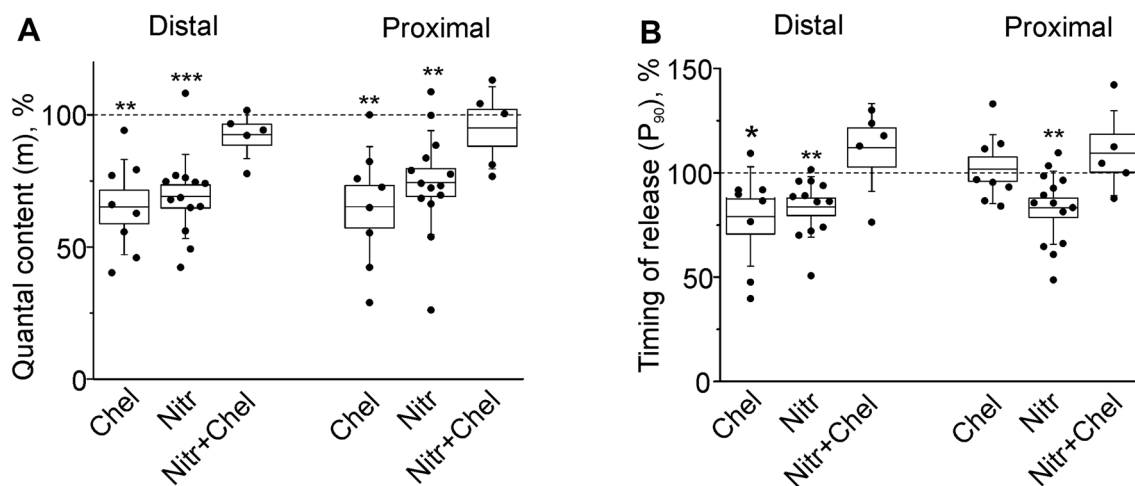
nitrendipine on timing of the neurotransmitter release (Fig. 3b). Thus, PKC activity can be required for maintaining LTCC activity, which is important for regulation of the quantum content and timing of the neurotransmitter release.

Surprisingly, that in contrast to LTCC blockage, inhibition of PKC with chelerythrine did not significantly affect the reduction of neurotransmitter release due to exogenous ATP application in both the distal and proximal parts of NMJ (S. Fig. 3). At the same time, blockage of LTCCs on background of PKC inhibition attenuated the depressant effect of exogenous ATP. This suggests that exogenous

ATP can decrease quantum content via PKC-independent and LTCC-dependent pathway (S. Fig. 3).

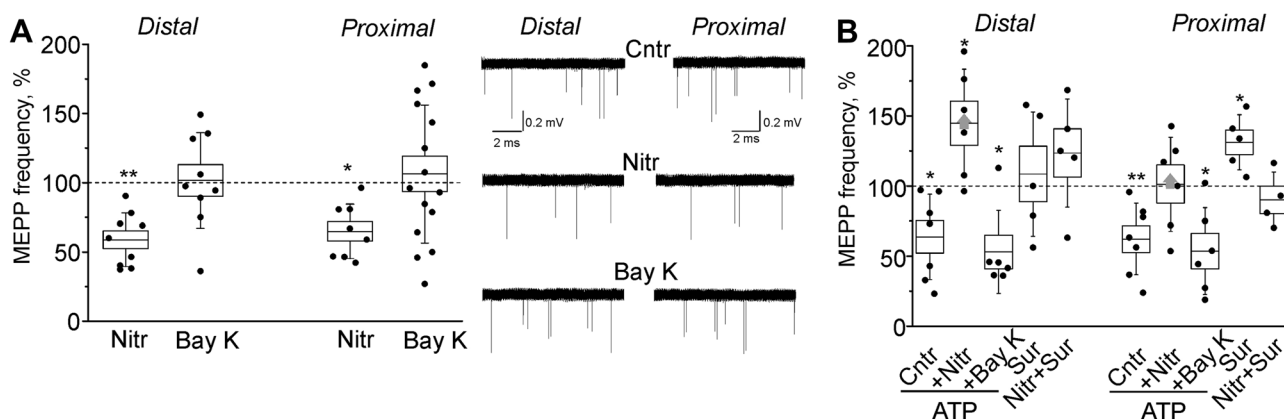
### LTCCs in Spontaneous Neurotransmitter Release

Spontaneous and evoked modes of neurotransmitter release can be regulated by different mechanisms in NMJs (Petrov et al. 2014; Liu et al. 2019; Rodrigues et al. 2013). Particularly, spontaneous release can be  $Ca^{2+}$ -independent or rely on bulk intracellular  $Ca^{2+}$  levels (Kaeser and Regehr 2014). Antagonist of LTCCs nitrendipine decreased frequency of MEPPs (indicator of spontaneous neurotransmitter release) in both proximal and distant part of the NMJs (Fig. 4a). At



**Fig. 3** PKC in maintenance of LTCC activity in distal and proximal parts of nerve terminal. **a, b** Changes in quantal content,  $m$  (**a**) and synchronicity of the release,  $P_{90}$  (**b**) due to PKC inhibition with chelerythrine (Chel) or in response to LTCC blockage after preliminary inhibition of PKC (Nitr+Chel). The effect of LTCC antagonist itself

(Nitr; from Fig. 1) is shown on **a** and **b**. Data are expressed as circles—values in individual animals, box range—SEM, whiskers—SD.  $N=5-14$  for each group. \*\*\* $P<0.001$ , \*\* $P<0.01$ , \* $P<0.05$ —compared to value (100%) prior to drug application



**Fig. 4** LTCC-dependent regulation of spontaneous release in distal and proximal part. **a** Effect of LTCC antagonist (Nitr) and agonist (Bay K) on frequency of MEPPs (in %). Right, typical MEPPs recorded extracellularly. **b** LTCC contribution in ATP and P2Y-R-driven control of spontaneous release. Shown changes in MEPP frequency in response to ATP administration in control (Cntr) and after preliminary inhibition (+Nitr) or activation (+Bay) of LTCCs.

Also, influence of P2Y-R inhibition with suramine (Sur) and LTCC inhibition on background of pre-treatment with P2Y-R antagonist (Nitr+Sur) are displayed. Data pointing to inhibitory effect of LTCC activity on spontaneous secretion are highlighted with gray arrows (**b**). Data are expressed as circles—values in individual animals, box range—SEM, whiskers—SD.  $N=4-15$  for each group. \*\* $P<0.01$ , \* $P<0.05$ —compared to value before drug application

the same time, agonist of LTCCs had no influence on MEPP frequency (due to high variability in data). Notably, inhibitor of PKC decreased MEPP frequency (in distal part) and completely prevented additional action of the LTCC blocker on this parameter in both distal and proximal parts (S. Fig. 4A). Thus, like in case with evoked exocytosis, a tonic activity of PKC–LTCC axis may be required for maintenance of spontaneous release in the NMJs.

Exogenous ATP suppressed spontaneous release in both parts of NMJs (Fig. 4b). If LTCCs were inhibited, ATP lost

ability to suppress spontaneous neurotransmitter release, whereas the effect of ATP was completely preserved in conditions of LTCC agonist action (Fig. 4b). Furthermore, ATP increased spontaneous release in the distal part if LTCCs were blocked (Fig. 4b). This suggests that activity of LTCCs is engaged in ATP-driven suppression of spontaneous neurotransmitter release. It should be noted that inhibition of NTCCs or  $Cd^{2+}$  at low concentration decreased frequency of MEPPs (S. Fig. 4B), but did not prevent the depressant action of ATP on spontaneous release (S. Fig. 4C). The



similar situation was observed on background of PKC inhibition (S. Fig. 4C). These results suggest that NTCCs as well as PKC can enhance spontaneous release, but, in contrast to LTCCs, they were not involved in the depressant action of ATP. This points to PKC-independent action of ATP on spontaneous exocytosis in the NMJ.

In contrast to ATP, inhibition of P2Y-Rs with suramin increased spontaneous neurotransmitter release in proximal part of the NMJs (Fig. 4b). Also, on background of P2Y-R inhibition, nitrendipine did not affect frequency of MEPPs in both distal and proximal parts (Fig. 4b). This also points to dependence of LTCC inhibitor effect on P2Y-R activation and the requirement of endogenous ATP/ADP-driven (basal) P2Y-R activity for maintenance of LTCC function.

Hence, as in case with evoked quantum secretion, LTCCs can have opposite influence on spontaneous release at basal conditions and in response to exogenous ATP. At endogenous levels of ATP, LTCCs could positively regulate spontaneous release, but ATP-driven activation of LTCCs can be engaged in attenuation of spontaneous exocytosis. Indeed, if spontaneous release was preliminarily suppressed with ATP, subsequent application of nitrendipine was able to further decrease the frequency of MEPPs, indicating that separate set of LTCCs can support spontaneous release under these conditions (S. Fig. 4C).

Previously, we have found that increase in reactive oxygen species production can partially mediate depressant effect of ATP on evoked release in the frog NMJs (Giniatullin et al. 2005). However, an antioxidant NAC (1 mM) was not able to attenuate effect of ATP on the spontaneous release in both proximal and distal parts of the NMJs (S. Fig. 4C). This makes LTCCs as a potential key element in ATP-mediated suppression of spontaneous release at the NMJs.

### Role of Lipid Rafts in LTCC Function

A number of ion channels and receptors, including P2Y<sub>12</sub>, can reside in lipid rafts in synapses, including NMJs (Krivoi and Petrov 2019; Giniatullin et al. 2015; Petrov et al. 2011a). Relocation of channels or receptors from raft to non-raft membranes can markedly affect their functions. Different role of LTCCs (under basal condition vs exogenous ATP administration) in neurotransmitter release can be linked with distribution of the channel as well as P2Y-Rs into lipid rafts. To test lipid raft-dependence of the effects of LTCC inhibition by its own and in combination with ATP, pretreatment with methyl- $\beta$ -cyclodextrin (M $\beta$ CD) at low concentration (0.1 mM) was used to lipid raft disruption. This approach is effective in vertebrate NMJs (Giniatullin et al. 2015; Kasimov et al. 2015; Mukhutdinova et al. 2018; Petrov et al. 2011b). Indeed, staining with a lipid raft marker CTxB was markedly decreased by 15-min exposure to 0.1 mM M $\beta$ CD (S. Fig. 5A).

After M $\beta$ CD-pretreatment, inhibition of LTCCs lost the ability to suppress quantum content selectively in the distal part of NMJs, whereas effect of nitrendipine was preserved in the proximal region (Fig. 5a). Surprisingly, synchronization of neurotransmitter release due to LTCC blockage was unaffected by the lipid raft disruption (Fig. 5a). Also, nitrendipine still suppressed spontaneous release in proximal part in M $\beta$ CD-preexposed muscles (Fig. 5a). There was a trend to decrease in the depressant action of LTCC blocker on spontaneous release in distal part after lipid raft disruption. Accordingly, raft disruption can attenuate activity of LTCC specifically in distal part of nerve terminals. Along the same lines, pretreatment with raft disrupting agent prevented effect of LTCC blocker on quantum content on background of preliminary ATP administration, which normally might facilitate LTCC activity (S. Fig. 5B).

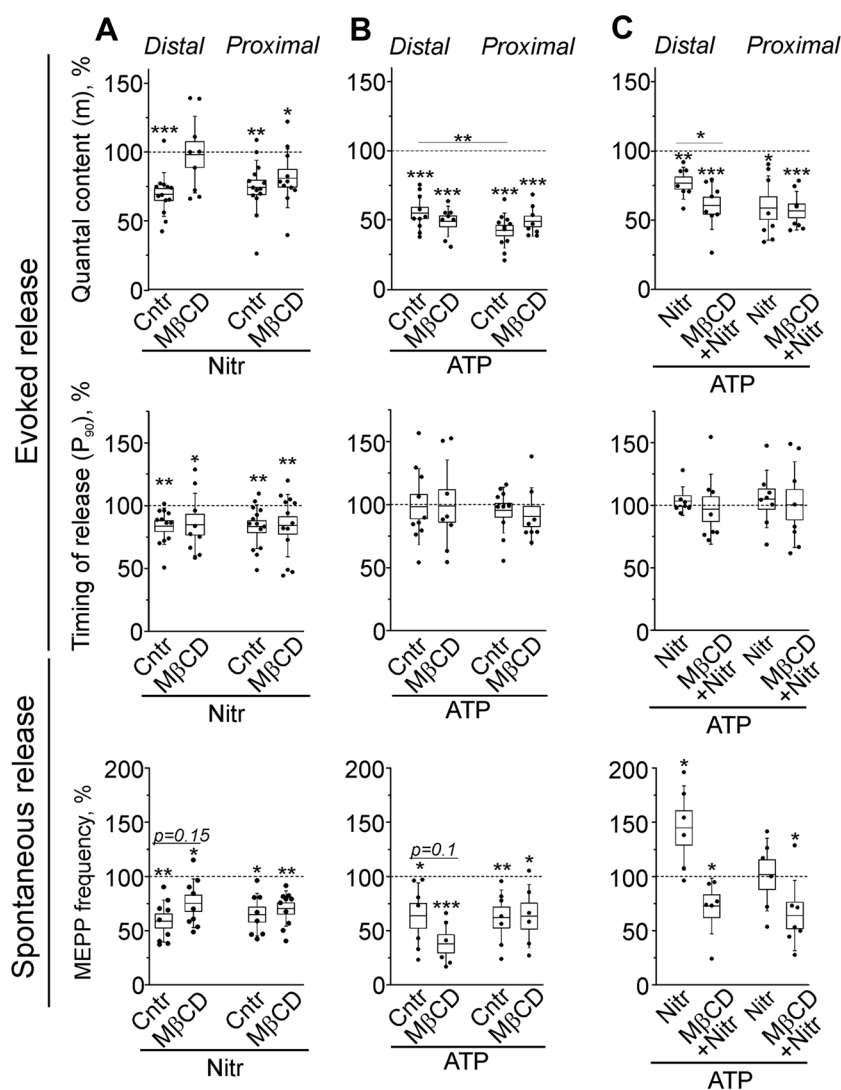
Lipid raft disruption did not markedly modify the effects of ATP on evoked and spontaneous release (Fig. 5b). Only tendency to enhancement of ATP-mediated depression of both evoked and spontaneous release was observed in distal part of M $\beta$ CD-treated NMJs. As a result, the differences in expression of ATP effect on evoked release in distal vs proximal part disappeared in M $\beta$ CD-pretreated muscles. Indeed, in control, ATP depressed quantum content more significantly in proximal vs distal part, whereas after lipid raft disruption, ATP reduced it to similar degree in both parts of the NMJ (Fig. 5b).

After lipid raft disruption, the LTCC blockage lost the ability to decrease the depressant effect of ATP on evoked release in distal part of the NMJs (Fig. 5c). Lipid raft disruption and LTCC inhibition, both individually and in combination, did not change the effect of ATP on quantum content in proximal part of the NMJs (Fig. 5c). So, raft integrity in distal part of the nerve terminal can be required for participation of LTCCs in the effect of purinoreceptor activation with ATP. Probably, if lipid rafts are disorganized, ATP can suppress neurotransmitter release via LTCC-independent (alternative) pathway.

In addition, when lipid raft integrity was perturbed, LTCC antagonist lost the ability to prevent the depressant action of exogenous ATP on spontaneous release in both distal and proximal parts. Indeed, in M $\beta$ CD + nitrendipine-treated NMJs, ATP decreased MEPP frequency to the similar levels as in control samples, whereas ATP did not change and even increased MEPP frequency in proximal and distal parts, respectively, of nitrendipine-treated NMJs (Fig. 5c). These data indicate that lipid rafts are required for realization of ATP effect on spontaneous release via LTCC-dependent pathway.

Taken together, these results suggest that lipid rafts are important for LTCC-dependent regulation of evoked release under both basal conditions and ATP administration in distal (but not proximal) part of the NMJs. In addition, lipid

**Fig. 5** Role of lipid rafts in LTCC-dependent regulation of neurotransmitter release. **a** Effect of LTCC antagonist (Nitr) on parameters of evoked (m and  $P_{90}$ ) and spontaneous (MEPP frequency) release in control (Cntr; from Figs. 1 and 4a) and M $\beta$ CD-pretreated muscles. **b, c** Influence of lipid raft disruption with M $\beta$ CD on the effects of exogenous ATP in native sample (**b**) and muscle pretreated with LTCC antagonist (**c**). The effects of ATP under control conditions and on background of LTCC inhibition (from Figs. 2a, b, 4b) are denoted on **b** and **c**, respectively. Shown changes in quantal content (m), timing of the release ( $P_{90}$ ), and frequency of spontaneous exocytotic events. Data are expressed as circles—values in individual animals, box range—SEM, whiskers—SD.  $N=6-14$  for each group. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ —compared to value prior to Nitr (**a**) or ATP (**b, c**) application or between two groups



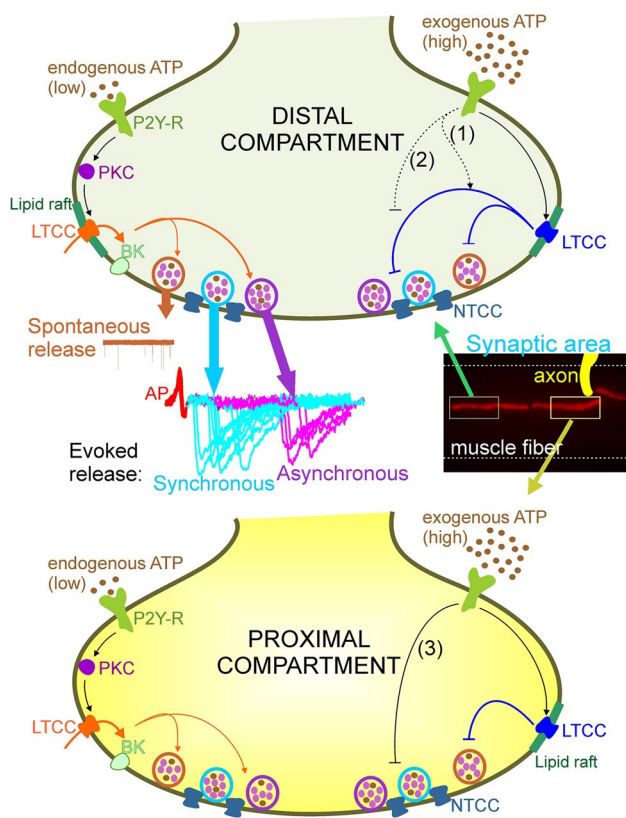
rafts seem to participate in exogenous ATP/LTCC-mediated control of spontaneous release in both distal and proximal parts of the NMJs.

## Discussion

The main findings of the present study (Fig. 6) are as follows: (1) expression of stimulatory effect of LTCCs on spontaneous and evoked (especially, asynchronous) release is dependent on basal (endogenous) activity of P2Y-Rs and PKC; (2) however, LTCC (in PKC-independent manner) can contribute to suppression of neurotransmitter release under condition of exogenous ATP administration; (3) engagement of LTCCs in neurotransmitter release as well as its timing have specific features in distal and proximal compartment of the nerve terminal. Specifically, (3a) depressant effect of exogenous ATP on evoked exocytosis partially relies on

LTCC activity in distal part, whereas negative action of ATP on spontaneous release is linked with LTCCs both in distal and proximal parts; (3b) lipid rafts are required for involvement of LTCCs in basal and ATP-driven control of evoked neurotransmitter release and synchrony of exocytotic events specifically in the distal region, and at the same time, ATP-induced suppression of spontaneous release via LTCCs is lipid raft-dependent process in both distal and proximal compartments.

SV exocytosis occurring in active zone is triggered by  $Ca^{2+}$  influx through NTCCs in frog NMJs (Dittrich et al. 2018). LTCCs can play a modulatory role in neurotransmission, including in NMJs (Dolphin and Lee 2020). Indeed, pharmacological activation of LTCCs with BAY K 8644 increased quantal content of EPPs at low-frequency stimulation in the rat NMJs (Atchison and O'Leary 1987). Generally, in mammalian NMJs, LTCCs contribute to neurotransmission under special conditions,



**Fig. 6** Putative role of LTCCs in regulation of neurotransmitter release in distal and proximal compartments of NMJs. Left side of cartoon: endogenous ATP released at basal conditions as co-neurotransmitter or from glial cell (not shown) stimulates P2Y-Rs which can maintain activity of LTCCs (in orange) via PKC-dependent signaling. In turn, LTCCs increase spontaneous exocytosis and evoked release (especially, asynchronous component). One possibility is that  $\text{Ca}^{2+}$  influx through LTCCs can simulate  $\text{Ca}^{2+}$ -dependent enzymes and channels (e.g.,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, BK), thereby affecting the neurotransmitter release in both distal and proximal parts. This LTCC-driven regulation of neurotransmitter release is dependent on lipid raft integrity specifically in distal (but not proximal) compartment. Right side of cartoon: LTCC (in blue) can negatively regulate neurotransmitter release under certain conditions. Indeed, stimulation of P2Y-Rs with exogenous ATP at high concentration may suppress neurotransmitter release partially via activation of LTCCs. Under this context, LTCCs attenuate both evoked exocytosis (in distal region) and spontaneous release (in distal and proximal compartments). ATP-triggered depressant action of LTCCs is dependent on lipid rafts. Note that if: (1) LTCCs are blocked, ATP can even increase in spontaneous release in distal compartment; (2) lipid raft are disrupted, then ATP may reduce evoked release in LTCC-independent manner in distal region. In proximal region, ATP attenuates evoked exocytosis in LTCCs and lipid rafts independent way (3). Also, exogenous ATP-mediated activation of LTCCs had no effect on timing of neurotransmitter release. Since, different functional population of LTCCs can operate in basal conditions (left side) and in response to exogenous ATP (right side). Distal compartment is characterized by more complex mechanisms which are engaged LTCCs, P2Y-Rs, and lipid rafts for bidirectional control of neurotransmitter release. AP, action potential; NTCC, N-type  $\text{Ca}^{2+}$  channel

including high-frequency stimulation (Perissinotti et al. 2008), intense nerve terminal depolarization (Flink and Atchison 2003), action of 4-beta-Phorbol 12-myristate 13-acetate, an activator of PKC and exocytotic protein Munc-13 (Arenson and Evans 2001), genetic deficit of amyloid precursor protein (Yang et al. 2007). Under all these conditions, activation of LTCC can increase neurotransmitter release. Here, we found that under lower level of  $[\text{Ca}^{2+}]_{\text{out}}$ , either inhibitor or activator of LTCCs can decrease evoked exocytosis, but spontaneous release is sensitive only to LTCC blockage. The appearance of marked effects of LTCC inhibition in low  $[\text{Ca}^{2+}]_{\text{out}}$  solution indicates a possible disinhibition of LTCCs by the decrease in external  $\text{Ca}^{2+}$ . In addition, paradoxical depressant effect of pharmacological LTCC stimulation can be related with decreased extracellular  $\text{Ca}^{2+}$  levels and, as a consequence, a lesser influx of  $\text{Ca}^{2+}$ , which can activate another signaling enzyme(s) able to depress ACh release. For example,  $\text{Ca}^{2+}$ -activated phosphatase PP2B can depress quantal neurotransmitter release in mice NMJs (Gaydukov et al. 2013). In addition, increase in  $[\text{Ca}^{2+}]_{\text{in}}$  in terminal Schwann cells, which also express LTCCs (Robitaille et al. 1996), can negatively regulate neurotransmitter release in NMJs (Ko and Robitaille 2015). Importantly, inhibition of LTCCs decreased asynchronous release, whereas activation had reverse (in distal part) or no effect (in proximal part) on timing of the exocytotic events. Accordingly, optimal LTCC activity can maintain evoked neurotransmitter release at certain level and positively regulate asynchronous component of AP-induced exocytosis as well as spontaneous release.

LTCC can affect neurotransmitter release through multiple  $\text{Ca}^{2+}$ -dependent mechanisms. One possibility is that LTCC-dependent  $\text{Ca}^{2+}$  influx could activate BK channels (Sun et al. 2003). The latter might bidirectionally control neurotransmitters release (Wang et al. 2020; Xu and Slaughter 2005; Flink and Atchison 2003; Liu et al. 2007; Gaydukov et al. 2009). Inhibition of BK channels precluded the effects of LTCC blockage on evoked neurotransmitter release (inducing asynchronous component) in both distal and proximal parts. This suggest that BK channels can be downstream component in LTCC-dependent regulation of the neurotransmitter release in the frog NMJs. According to this scenario, BK channel can be positive regulator of neurotransmitter release. Local increase in  $[\text{K}]_{\text{out}}$  due to activity of BK channels in active zone can increase  $\text{Ca}^{2+}$  currents and neurotransmitter release (Xu and Slaughter 2005). Similarly, knockout of BK channels suppressed neurotransmitter release likely via interfering with  $\text{Ca}^{2+}$ -induced release in mice NMJs (Wang et al. 2020). It should be noted that inhibition of BK channels itself had no effect on evoked neurotransmitter release in our experiments. This can reflect a silence of BK channels when extracellular  $\text{Ca}^{2+}$  level is

low. In contrast, at 2.0 mM  $[Ca^{2+}]_{out}$  in bathing solution, BK channel antagonists increased quantum content of EPPs, which was dependent on subsequent LTCC activation in NMJs of mice diaphragm (Gaydukov et al. 2009; Flink and Atchison 2003). Although, under normal  $[Ca^{2+}]_{out}$  conditions, three BK channel blockers had no effect on neurotransmitter release in tibialis anterior muscle of adult mice (Wang et al. 2020).

Importantly, the effects of LTCC inhibition were completely prevented by preliminary inhibition of either P2Y-Rs or PKC. Taking into account that activation of P2Y-Rs by ATP and ADP can stimulate PKC in NMJs (Sokolova et al. 2003; De Lorenzo et al. 2006), and PKC can open quiescent LTCCs in motor nerve terminal (Arenson and Evans 2001), endogenous ATP/ADP can support activation of LTCCs via stimulation of P2Y-R/PKC signaling. Note that the ability of LTCCs to enhance asynchronous release suggests that these channels can be an important source of  $Ca^{2+}$  for triggering this type of exocytotic event, which play role in synaptic plasticity and coincidence detection (Kaeser and Regehr 2014). Furthermore, shift from synchronous to asynchronous exocytosis can occur under the pathological conditions, including models of amyotrophic lateral sclerosis and spinal muscular atrophy (Ruiz et al. 2010; Pagani et al. 2006).

$Ca^{2+}$  influx via LTCCs is mainly considered as positive modulator of neurotransmitter release (Dolphin and Lee 2020; Perissinotti et al. 2008; Flink and Atchison 2003; Arenson and Evans 2001; Yang et al. 2007). Although,  $Ca^{2+}$ -dependent enzymes and channels (e.g., protease calpain,  $Ca^{2+}$ /calmodulin-dependent protein kinase II and BK channels) can also suppress neurotransmitter release (Ando et al. 2005; Liu et al. 2007). We found that in distal part of motor nerve terminal, depressant effect of exogenous ATP on evoked neurotransmitter release can be enhanced by LTCC agonist and suppressed by LTCC antagonist. This points to the ability of ATP to attenuate evoked neurotransmitter release via activation of some population of LTCCs specifically in distal part. In this scenario, LTCCs can serve as negative regulator of AP-induced exocytosis. Although agonist of LTCCs did not significantly modulate ATP-mediated decrease in spontaneous release, inhibitor of LTCC prevent and even invert this effect of ATP in proximal and distal parts, respectively. Hence, LTCC activation can suppress both spontaneous and evoked neurotransmitter release in the context of purino-receptor stimulation with exogenous ATP. Moreover, the contribution of LTCCs in the depressant action of ATP seems more in distal compartment of the nerve terminal. This compartment is characterized by specific mechanism of regulation as well as lower probability of neurotransmitter release (Tsentsevitsky et al. 2011, 2020a; Robitaille and Tremblay 1991; Tsentsevitsky and Petrov 2021). Furthermore, this part of nerve terminals is a key site for remodeling and growth. Accordingly, specific

function of LTCCs in the distal part can be linked with an importance of LTCCs in early NMJ formation and axon branching (Kaplan et al. 2018) as well as suppression of neurotransmission in newly sprouted NMJs (Chipman et al. 2014). Indeed, LTCCs can suppress neurotransmitter release in newly formed frog and rodent NMJs (Balezina et al. 2007; Sugiura and Ko 1997). Interestingly, BK channel inhibition did not prevent LTCC blocker-mediated increase in quantal release in regenerating frog NMJs (Sugiura and Ko 1997).

Some portion of P2Y-Rs for ATP as well as LTCCs can reside in lipid rafts in synapses (Giniatullin et al. 2015; Mercer et al. 2011). In additional, lipid rafts and membrane cholesterol are essential for axonal growth and regeneration; these processes are dependent on signaling in axonal distal region (Rosello-Busquets et al. 2019; Petrov and Zefirov 2013). Accordingly, specific functioning of LTCCs in the distal vs proximal compartment as well as different role of LTCCs under basal conditions and in response to exogenous ATP can be partially linked with cholesterol-rich membrane microdomains, lipid rafts. Indeed, in distal compartment, lipid raft disruption precluded participation of LTCCs in regulation evoked release (including its timing) either under basal condition or ATP application. At the same time, there was no effect of lipid raft disruption on LTCC-related control of evoked release in proximal part of the same nerve terminal. This suggests that lipid rafts of the distal parts are required for LTCC functioning as well as their coupling to ATP receptors. Probably some specific lipid components, cytoskeletal, or scaffold proteins can be responsible to more close interactions between LTCCs, lipid rafts, and sites of evoked exocytosis in distal part of the motor nerve terminal. For example, scaffold-like protein MARCKS is essential for axonal development and enriched in tip of axonal branches (Sosa et al. 2016). In chromaffin cells, MARCKCs along with LTCC and PKC $\epsilon$  can form a lipid raft-associated complex which selectively regulates exocytosis of large dense-core vesicles (Park and Kim 2009). Interestingly, participation of LTCCs in effect of exogenous ATP on spontaneous exocytosis was sensitive to lipid raft disruption in both distal and proximal parts of the nerve terminal. This indicates that there are different mechanisms of LTCC-dependent regulation of spontaneous and evoked release.

## Conclusion

In the present study, using low  $Ca^{2+}$  external solution allowed to reveal that LTCCs can bidirectionally control the neurotransmitter release and this LTCC-mediated regulation occurs in compartment, lipid raft, and purinoreceptor-dependent manner in the NMJs. Endogenous activity of P2Y-R-PKC axis can sustain LTCC activity, which is required for maintenance of evoked release and enhancement

of asynchronous and spontaneous release. At the same time, overstimulation of the purinoreceptors with exogenous ATP can suppress evoked and spontaneous release partially via activation of LTCCs. This role of LTCCs in depressant effects of ATP is more pronounced in distal part of the nerve terminal. Also, in the distal compartment, LTCC-dependent regulation of evoked release as well as engagement of LTCCs in the action of exogenous ATP on evoked exocytosis specifically depend on lipid raft integrity. Hence, distal region of the axon represents a unique presynaptic compartment, where activity of LTCCs can be regulated by specific mechanism. Physiological significance of such complex LTCC-mediated regulation of presynaptic function as well as whether this regulation operates in normocalcium external solution are topics for further studies. The main limitations of the current work are use of pharmacological approaches to interfere with the channel function, so genetic and molecular studies are needed to understand the precise role of LTCCs in control of presynaptic processes in NMJs.

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**Author Contributions** ANT produced and analyzed data. A.N.T. and A.M.P. interpreted results of experiments, designed, and supervised the research. A.M.P. wrote the manuscript. All the authors approved the final version of the manuscript.

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**Data Availability** All mentioned data are represented in the main manuscript figures and supplementary figures. Other additional data will be made available on reasonable request.

## Declarations

**Conflict of interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. All authors consent to publish the article.

**Ethical Approval** The present study was conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the requirements of the EU Directive 2010/63/EU. The protocol of experiments was approved by the Ethical Committee of Kazan Medical University.

**Informed Consent** This is not applicable. This manuscript does not contain any studies with human participants.

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