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Breakdown of phospholipids and the elevated nitric oxide are involved in M3 muscarinic regulation of acetylcholine secretion in the frog motor synapse

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ABSTRACT

Previously, we found that muscarine downregulates the acetylcholine release at the frog neuromuscular junction acting via M3 muscarinic receptors. Here, the molecular mechanisms underlying the inhibitory effect of muscarine on the quantal secretion of acetylcholine were studied. Inhibition of phospholipase C (with U-73122) prevented the reduction of evoked neurotransmitter release induced by muscarine. Interruption of synthesis of phosphatidylinositol 3-phosphate by the inhibitor of phosphoinositide-3-kinase (wortmannin) did not affect the depressant action of muscarine but precluded the restoration of secretion after removal of muscarine from the bathing solution. The effect of muscarine was not significantly modified by the blockade of endocannabinoid receptors (with AM 281), but it was abolished by the inhibitor of nitric oxide synthase (L-NAME) as well as extracellular nitric oxide (NO) chelator (hemoglobin). Moreover, muscarine increased NO-sensitive dye fluorescence in junctional region, which was prevented by the M3 receptor antagonist 4-DAMP. The data obtained indicate that the attenuation of acetylcholine release mediated by muscarine is associated with a change in the activity of both lipid-metabolizing enzymes and NO synthases.

1. Introduction

Muscarinic cholinoreceptors are known to be involved into the modulation of acetylcholine (ACh) secretion in the vertebrate neuromuscular junctions [1–5]. However, the precise molecular pathways of muscarinic regulation are not established so far, and seem to be dependent on a number of experimental conditions such as animal species, age of animals, extracellular Ca^{2+} level, temperature etc. [1,4–8].

Newman et al. [9] have investigated molecular mechanisms of ACh release regulation mediated by activation of M3 receptors in lizard neuromuscular synapses. It was shown that the activation of phospholipase C (PLC) followed by the rise in the production of endogenous cannabinoids and the activation of presynaptic cannabinoid receptors type 1 (CB1) are the necessary links of this chain. Second essential factor is nitric oxide (NO) which production seems to be enhanced upon M3 receptor activation.

Earlier we have shown that in frog neuromuscular junction the process of ACh secretion is modulated by muscarinic cholinergic receptors of M1, M2/M4 and M3 subtypes [10]. Application of cholinomimetic muscarine caused a significant decrease in the ACh quantal release. Depressing effect of muscarine persisted in the presence of non-selective muscarinic antagonists atropine and gallamine (unpublished data), and selective inhibitors of M1, M2 and M4 cholinergic receptors, but was completely prevented by pre-incubation of nerve-muscle preparation with M3 blockers 4-DAMP and J-104129.

During the past few decades, drugs targeting the muscarinic acetylcholine receptor family, including M3 type receptors, are considered as potential therapeutic tools [11,12]. Therefore, molecular mechanisms underlying M3 receptor dependent signaling is

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Abbreviations

ACh	acetylcholine
CB1	cannabinoid receptor type 1
DAG	diacylglycerol
EPP	endplate potential
IP_3	inositol 1,4,5-trisphosphate
NE	nerve ending
NO	nitric oxide
NOS	nitric oxide synthase
PLC	phospholipase C
PI3K	phosphoinositide-3-kinase
PIP_3	phosphatidylinositol 3, 4, 5 trisphosphate

of importance.

Here we studied the molecular mechanisms underlying the action of muscarine on the evoked ACh secretion using EPPs recorded under conditions of low Ca^{2+}/high Mg^{2+} in bathing solution. This approach allows to analyze not only the EPP quantal content but also the timing of the release by the analysis of real synaptic delay distribution [13].

It was shown that unlike lizard neuromuscular endplates, in frog, the inhibition of ACh secretion in presence of muscarine is associated for the greater part with an increased activity of important lipid-metabolizing enzymes – PLC and phosphoinositide-3-kinases (PI3K) as well as with a change in the activity of NO-synthase (NOS). Optical experiments provided direct evidence that muscarine increases NO production in junctional region, which is associated with the activation of M3 cholinoreceptors.

2. Materials and methods

2.1. Ethics statement

Experiments were performed in accordance with the guidelines for the use of laboratory animals of Kazan Medical University, which are in compliance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol met the requirements of the EU Directive 2010/63/EU for animal experiments and was approved by the Ethical Committee of Kazan State Medical University. The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.2. Electrophysiological studies

The isolated neuromuscular preparation of m. cutaneous pectoris of frogs Rana Ridibunda of both genders were used in the experiments. The experimental protocol of extracellular recordings is described in details in Ref. [10] and in Supplemental Materials. Briefly, the nerve-muscle preparation was placed in a 3-ml translucent chamber and superfused with the following solution: NaCl, 113.0 mM; KCl, 2.5 mM; HEPES, 5 mM; $NaHCO_3$, 1.5 mM; $MgCl_2$, 4.0 mM; and $CaCl_2$, 0.3 mM (the pH was adjusted with NaOH to 7.3, temperature 20.0 ± 0.3 °C). The nerve ending (NE) potentials and focal endplate potentials (EPPs) were recorded extracellularly (Fig. 1) with micropipettes filled with bathing solution with a resistance of 2–4 MΩ. 250–400 NE potentials followed by successive EPPs were recorded in control and in 25–30 min after drug application. The mean quantal content of the EPPs (m) was determined using the "method of failures" [13]: $m = \log_e N/N_0$, where N is the total number of stimuli and N_0 represents the number of synaptic failures. To analyze the timing of ACh release, uni-quantal EPPs were selected according to the first peak of the histogram of EPP amplitudes (Supplemental Fig. S1 C).

In experiments with wortmannin, nerve-muscle preparations were pre-incubated with the drug during 1 h followed by 30 min washout with physiological solution.

2.3. NO measurements using fluorescence dyes

NO oxidation products were measured using a cell-permeable fluorescent dye DAF-FM-diacetate as described previously [14]. Briefly, the isolated muscles were exposed to the dye (4 μM) for 20 min at room temperature. Then, the muscles were washed during 60 min with physiological solution and measurements of the fluorescence in the junctional and extrajunctional regions were performed. The regions of interest were recognized using DIC-optics. 480/15 nm and 505–560 nm filters were used for excitation and emission, respectively.

To identify junctional and extrajunctional regions, postsynaptic nAChR were stained with rhodamine-conjugated alpha-bungarotoxin (Btx, 20 ng/ml; ThermoFisher). Btx was added simultaneously with DAF-FM diacetate. Red fluorescence of Btx was visualized at 555/15 nm excitation and 610–650 nm emission. The calculation of junctional and extrajunctional fluorescence was made in surface Btx-positive regions and area (10 × 20 mkm^2) outside Btx-positive neuromuscular junctions, respectively. Fluorescence images were captured using an Olympus BX51WI microscope with a semi-confocal attachment Disk Speed Unit, 40x objective and DP71 CCD camera (Olympus). Image analysis was performed using Cell^P (Olympus) and ImagePro software (Media Cybernetics, Bethesda, MD, USA). Analysis of intensity of fluorescence was made in regions of interest in arbitrary units, which were then converted into percentages, according to calculation of (F–F0)/F0, where F0 is baseline fluorescence before muscarine administration.

2.4. Drugs

The following drugs were used: (±)-muscarine chloride hydrate, AM281 (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-

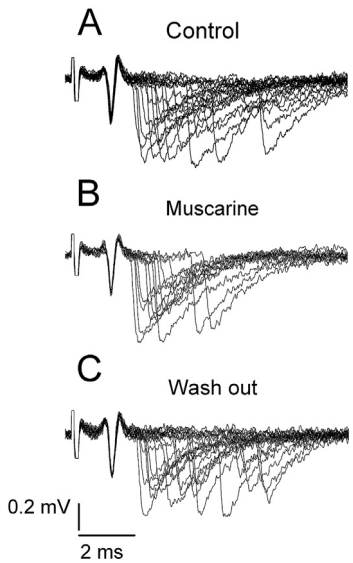

Fig. 1. Depressing effect of muscarine on evoked ACh secretion. Representative EPPs acquired extracellularly in one typical experiment in control (A), after muscarine (10 μM) application (B) and its removal from bathing solution (C) in response to 55 stimuli in each case.

morpholinyl-1H-pyrazole-3-carboxamide, hemoglobin from bovine blood, 4-DAMP (1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide, Sigma-Aldrich, USA); U-73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione), wortmannin (Santa-Cruz, USA); L-NAME (NG-Nitro-L-arginine methyl ester hydrochloride, Tocris, UK), and DAF-FM-diacetate (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate, Molecular Probes, USA).

2.5. Statistical analysis

Data are presented as the mean ± SEM, excluding experiments with DAF-FM, where are the mean ± SD. n represents the number of endplates. A comparison of the mean quantal contents estimated in individual synapses before and after drug application was performed using a paired Student's t-test. All statistical analyses were performed using Origin 8.1 software. $P < 0.05$ was considered significant.

3. Results

As it was reported previously [10], under conditions of reduced extracellular Ca^{2+}, 10 μM muscarine reversibly reduced the quantal content of EPPs by 41.5 ± 3.9% (from 0.33 ± 0.04 in control to 0.18 ± 0.03, n = 12, $P < 0.001$) and synchronized the secretion process (Fig. 1 A, B, C). These muscarinic effects were prevented by the application of M3 blockers, 4-DAMP and J-104129. In this study, we explored possible pathways of realization of muscarinic regulation of ACh secretion triggered by M3 receptors activation.

3.1. Effects of muscarinic agents on ACh secretion are associated with stimulation of PLC

The most studied signaling enzyme associated with M1/M3 receptors activation is PLC, responsible for the breakdown of membrane phospholipids (phosphatidylinositol 3, 4, 5 trisphosphate, PIP_3) and the release of secondary messengers inositol 3-phosphate (IP_3) and DAG.

In our experiments the blockade of PLC by the pre-incubation of muscle for 1 h with 5 μM U-73122 completely prevented the depressing effect of muscarine on the quantal release of acetylcholine (Fig. 2).

3.2. Inhibition of PI3K affects the ACh secretion

Another important signaling enzyme, which could be activated by G-proteins, particularly βγ dimer, and regulates synthesis of 3-phosphorylated inositol phospholipids and ACh release in neuromuscular junction is a PI3K [15,16].

Application of 100 nM wortmannin, a non-selective PI3K inhibitor [17], did not cause any significant changes in the secretion intensity: the average EPP quantal content after incubation with wortmannin was 0.34 ± 0.05 as compared to 0.33 ± 0.03 (n = 6) in control. The timing of ACh secretion after wortmannin treatment also did not differ significantly from one in control (data are not shown). After pre-incubation with wortmannin, muscarine reduced the EPP quantal content to the same extent as with intact phospholipids re-synthesis: to 60.1 ± 10.9% (n = 6) vs 58.5 ± 3.9% in intact preparations (n = 7, Fig. 2). However, after wortmannin pretreatment, the inhibitory effect of muscarine on ACh release became irreversible. So, EPP quantal content in intact preparations recovered to 88.5 ± 11.1% after muscarine washout (n = 5, P = 0.34, Fig. 2), and at impaired re-synthesis of phospholipids, EPP quantal content after muscarine removal remained at the level of 56.0 ± 11.9% (n = 6, % calculated from the value before muscarine application, Fig. 2).

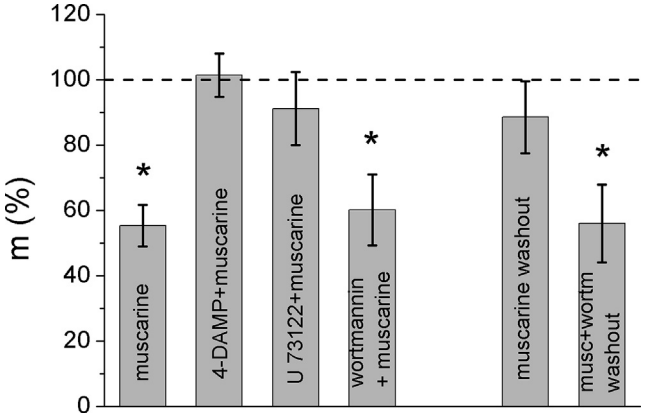

Fig. 2. Depressing effect of muscarine on evoked ACh secretion is associated with M3 receptors and PLC activation. Relative changes in EPP quantal content (m) in the presence of 10 μM muscarine (musc), after M3 receptor blockade by 4-DAMP (1 μM) and inactivation of phospholipase C (U17122, 5 μM), after muscarine washout, application of muscarine against the background of wortmannin (wortm, 100 nM) and its washing out. In case of 4-DAMP + muscarine, U17122+muscarine, and wortmannin + muscarine, m prior to muscarine application was taken as 100%. * - $P < 0.05$. n = 8.

These results suggest that muscarine application can lead to a significant imbalance between of membrane phospholipids generation and breakdown, which in turn can affect the state of signaling proteins involved in the regulation of ACh secretion.

3.3. Role of endogenous cannabinoids and NO in the effect of muscarine on ACh release

Next, we examined the participation of endocannabinoid signaling pathway in the realization of depressing action of muscarine.

Inactivation of CB1 receptors by selective blocker AM281 (5 μM) led to a significant decrease in the EPP quantal content (by 33 ± 5.6%, n = 8, P = 0.003, Fig. 3A). The time course of ACh secretion remained unchanged. However, muscarine continued to exert its depressing effect after CB1 receptors blockade: EPP quantal content decreased to 67.4 ± 4.2% (n = 7, P = 0.001).

The inhibition of NO production (inactivation of NOS by 100 μM L-NAME) prevented the inhibitory effect of muscarine on ACh release (Fig. 3B). L-NAME *per se* did not exert a statistically significant effect on ACh secretion: the EPP quantal content was 88.3 ± 9.8% of control values (n = 6, P = 0.53, Fig. 3A).

Application of 20 μM hemoglobin, the chelator of extracellular NO [14], also prevented the depressing action of muscarine on ACh release: EPP quantal content under muscarine in the presence of hemoglobin was 79.5 ± 10.9% (P = 0.13, n = 5, Fig. 3B) of its value in hemoglobin alone. It should be noted, that hemoglobin *per se* diminished significantly the EPP quantal content (by 40.7 ± 4.1%, n = 7, P = 0.03, Fig. 3A).

3.4. Optical detection of NO production upon muscarine application

To estimate the NO synthesis in junctional region we used a fluorescent dye, DAF-FM (Fig. 4). Application of muscarine led to a slight but statistically significant increase in DAF-FM fluorescence by 4.0 ± 0.7% (*vs* pre muscarine-baseline, P = 0.03, n = 6) in junctional region. This enhancement in fluorescence was prevented by inhibitor of M3 cholinergic receptors (4-DAMP, n = 6). In the extrajunctional region, muscarine did not modify DAF-FM fluorescence in control (n = 6) and in the presence of 4-DAMP (n = 6). Note that baseline fluorescence in both junctional and extrajunctional

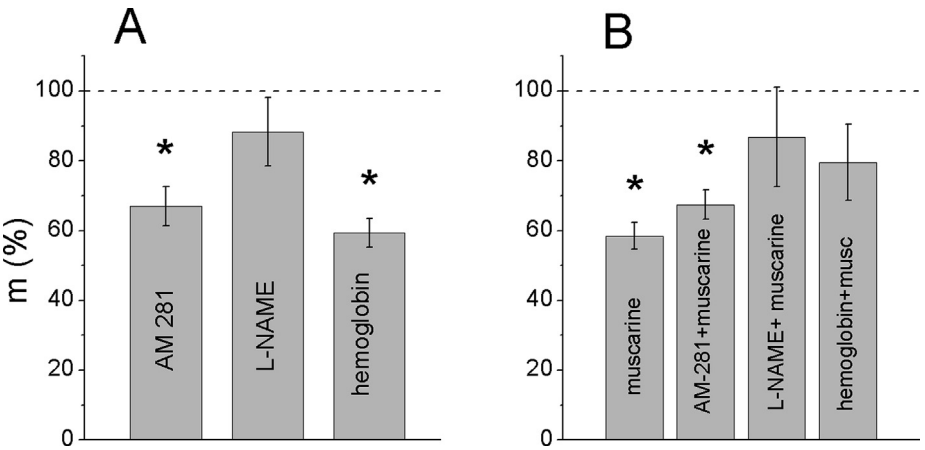

Fig. 3. Depressing effect of muscarine on evoked ACh secretion is not associated with CB1 receptor activation but requires active NO-synthase. A. Relative changes in EPP quantal content (m) after the blockade of cannabinoid receptors (AM 281, 5 μM), inhibition of NO-synthase (L-NAME, 100 μM), and application of NO chelator hemoglobin (20 μM). m before drugs application was taken as 100%. B. Relative changes in EPP quantal content (m) in the presence of muscarine alone and under conditions of cannabinoid receptors blockade (AM 281), inhibition of NO-synthase (L-NAME), and in the presence of hemoglobin. In case of AM281+muscarine, hemoglobin + muscarine, and L-NAME + muscarine, m prior to muscarine application was taken as 100%. n = 7. * - P < 0.05.

regions was stable for 20 min in control and with 4-DAMP (data not shown). These results suggest that muscarine can increase NO production mainly via stimulation of M3 receptors.

4. Discussion

The concept of autoregulation of neuromuscular transmission involving muscarinic receptors has been attracting the attention of researchers for several decades [1–5,18–23]. Recently, some evidences appeared that cholinoreceptors of M3 type are present in the vertebrate neuromuscular junctions and can be functionally significant [9,10,21–23]. A number of studies [9,21] reported the existence of the negative autoregulation in the lizard neuromuscular junction, an increase in NO production and simultaneous release of endocannabinoids from the muscle associated with postsynaptic M3 cholinergic receptors activation. To check whether similar mechanisms are involved into the regulation of neurosecretion in frog synapses, we conducted the experiments with blockade of the cannabinoid receptors of CB1 type and the application of muscarine against its background. The presence of cannabinoid receptors of the CB1 subtype in the neuromuscular junction of amphibians has been shown previously [24–26]. In the present study, it was shown that the blockade of CB1 receptors led to a decrease of evoked ACh secretion, at least at reduced ambient [Ca^{2+}]. This fact could be explained by described earlier coupling of CB1 receptors to $Gq/_{11}$ G proteins aimed to increase the concentration of intracellular calcium [27].

Application of muscarine in the presence of AM281 led to the same decrease of EPP quantal content as at active CB1 receptors. At the same time, the blockade of PLC and of NOS completely prevented the depressing effect of muscarine on the quantal ACh release. The same effect was observed in the presence of extracellular hemoglobin, a non-specific NO chelator. It should be noted that in our experimental condition hemoglobin *per se* significantly depressed ACh release. This could be off-target effect of hemoglobin consistent with previous reports [28,29] or with its ability to chelate other reactive compounds as ROS, H_2S, CO. Fluorescence studies with NO dyes confirmed the assumption that the level of NO is enhanced upon muscarine application. At this, NO fluorescence was elevated in the presence of muscarine in the synaptic region solely and this elevation was fully prevented by incubation with M3 blocker 4-DAMP suggesting the involvement of muscarinic receptor of M3 type. Here, we observed only small alterations in the fluorescent signal, indicating slight changes in NO levels. However,

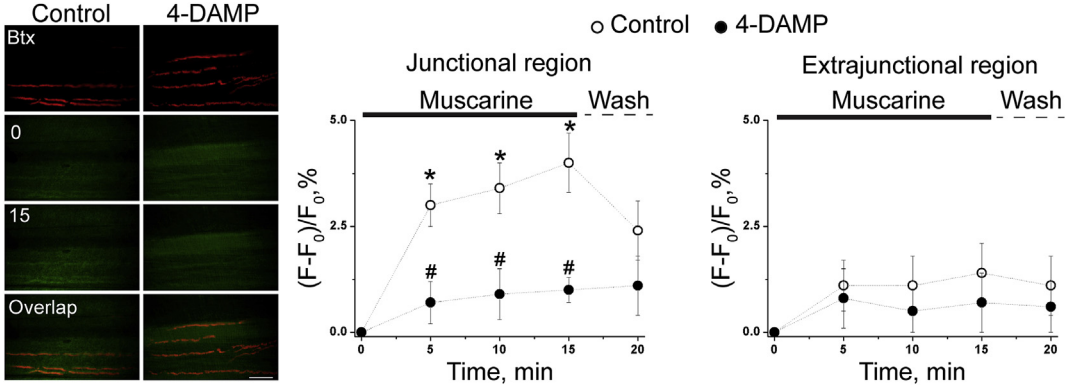

Fig. 4. M3 receptors activation causes the increase of intensity of DAF-FM fluorescence. Left, representative images of Btx and DAF-FM fluorescence prior (0) and during (15 min) muscarine application. Right, time course of DAF-FM fluorescence during and after application of muscarine in control and in the presence of 4-DAMP. X-axis – time (min) from onset of muscarine application. Y-axis - fluorescence intensity, (F–F0)/F0. Value of fluorescence intensity prior to muscarine application was taken as 100%. * - P < 0.05 compared to pre-muscarine baseline, # - P < 0.05 between control and 4-DAMP. n = 6.

even small enhancement in NO production may markedly affect neuromuscular transmission [14,30], probably through activation of cGMP cascade, which regulates ACh release in frog neuromuscular junctions [31,32]. Considering the short distance between the Schwann cell, nerve ending, and the postsynaptic membrane, we can only speculate about localization of the source of NO synthesis, and future studies are necessary for the precise localization of M3-muscarinic receptor sensitive NO synthases at the neuromuscular junction.

Thus, the mechanism of muscarine action on the evoked ACh secretion mediated via M3 receptors in the frog neuromuscular synapses is different from that reported for the lizard endplates [9,21]. In amphibians, it is associated with the activation of M3 cholinoreceptors, PLC, and, apparently, an increase in the production of NO, which, as shown earlier, depresses the ACh secretion from the nerve endings of amphibians [31,33,34]. However, it does not require additional link with activation of CB1 receptors as in lizard synapses.

The inhibition of PI3K by wortmannin did not enhance the depressing effect of muscarine on the EPP quantal content, but completely prevented the restoration of initial level of secretion after removal of muscarine from the bathing solution. That is, some disbalance between membrane phospholipids synthesis and breakdown occurs in the presence of exogenous cholinomimetics muscarine which can influence the properties of numerous signaling proteins involved into exocytosis.

The results of our studies indicate that at least two components are involved in the depressing action of muscarine – an increased PIP_3 breakdown by PLC (which is compensated by PI3K activity after muscarine washout) and the level of activity of NOS. The activity of NOS is upregulated by intracellular Ca^{2+}, which concentration may increase upon the activation of Gq-protein bound to metabotropic receptors, in particular, of M3 subtype. Phosphatidylinositol-3-phosphate generated by PI3K could activate Akt kinase, which also stimulates eNOS activity [35]. In addition, it is known that neuronal NOS can be localized near the cell membrane and form complexes with transmembrane proteins [36,37], and, consequently, its activity may depend on the properties of plasmalemma. One can suggest that the breakdown of membrane phospholipids caused by muscarine application can change the activity of NOS. However, verification of this hypothesis requires further investigations.

Over the last years, drugs targeting the muscarinic acetylcholine receptor family including M3 type receptors are considered as potential therapeutic tools and actively developing partly owing to molecular docking approach and structure-based design [11,12]. Therefore, molecular mechanisms underlying M3 receptor activation and possible effects associated with free radical production accompanying these processes should be considered when proposing muscarinic agents as medications.

Author contributions

I.K. developed the initial concept. I.K, A.T and A.P wrote the paper. A.T., G.Z., and A.P. performed the experiments and analyzed the data.

Declaration of competing interest

The authors declare that there is no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.01.112.

References

[1] M.S. Arenson, Muscarinic inhibition of quantal transmitter release from the magnesium-paralyzed frog sartorius muscle, Neuroscience 30 (1989) 827–836.
[2] L. Re, Modulation of acetylcholine release by presynaptic muscarinic autoreceptors, Acta Physiol. Pharmacol. Ther. Latinoam. 49 (1999) 215–223.
[3] I. Slutsky, H. Parnas, I. Parnas, Presynaptic effects of muscarine on ACh release at the frog neuromuscular junction, J. Physiol. 514 (1999) 769–782.
[4] J. Minic, J. Molgó, E. Karlsson, E. Krejci, Regulation of acetylcholine release by muscarinic receptors at the mouse neuromuscular junction depends on the activity of acetylcholinesterase, Eur. J. Neurosci. 15 (2002) 439–448.
[5] M.M. Santafé, M.A. Lanuza, N. Garcia, M. Tomàs, J. Tomàs, Coupling of presynaptic muscarinic autoreceptors to serine kinases in low and high release conditions on the rat motor nerve terminal, Neuroscience 148 (2007) 432–440, https://doi.org/10.1016/j.neuroscience.2007.06.017.
[6] R. Robitaille, B.S. Jahromi, M.P. Charlton, Muscarinic Ca^{2+} responses resistant to muscarinic antagonists at perisynaptic Schwann cells of the frog neuromuscular junction, J. Physiol. 504 (1997) 337–347.
[7] L. Oliveira, M.A. Timóteo, P. Correia-de-Sá, Modulation by adenosine of both muscarinic M1-facilitation and M2-inhibition of [3H]-acetylcholine release from the rat motor nerve terminals, Eur. J. Neurosci. 11 (2002) 1728–1736.
[8] J. Tomàs, M.M. Santafé, N. Garcia, M.A. Lanuza, M. Tomàs, N. Besalduch, T. Obis, M. Priego, et al., Presynaptic membrane receptors in acetylcholine release modulation in the neuromuscular synapse, J. Neurosci. Res. 92 (2014) 543–554, https://doi.org/10.1002/jnr.23346.
[9] Z. Newman, P. Malik, T.Y. Wu, C. Ochoa, N. Watsa, C. Lindgren, Endocannabinoids mediate muscarine-induced synaptic depression at the vertebrate neuromuscular junction, Eur. J. Neurosci. 6 (2007) 1619–1630, https://doi.org/10.1111/j.1460-9568.2007.05422.x.
[10] A.N. Tsentsevitsky, I.V. Kovyazina, L.F. Nurullin, E.E. Nikolsky, Muscarinic cholinoreceptors (M1-, M2-, M3- and M4-type) modulate the acetylcholine secretion in the frog neuromuscular junction, Neurosci. Lett. 649 (2017) 62–69, https://doi.org/10.1016/j.neulet.2017.04.015.
[11] A.C. Kruse, J. Li, J. Hu, B.K. Kobilka, J. Wess, Novel insights into M3 muscarinic acetylcholine receptor physiology and structure, J. Mol. Neurosci. 53 (3) (2014) 316–323, https://doi.org/10.1007/s12031-013-0127-0.
[12] H. Liu, J. Hofmann, I. Fish, B. Schaake, K. Eitel, A. Bartuschat, J. Kaindl, H. Rampp, et al., Structure-guided development of selective M3 muscarinic acetylcholine receptor antagonists, Proc. Natl. Acad. Sci. U.S.A. 115 (47) (2018) 12046–12050, https://doi.org/10.1073/pnas.1813988115.
[13] B. Katz, R. Miledi, The measurement of synaptic delay and the time course of acetylcholine release at the neuromuscular junction, Proc. R. Soc. Lond. B Biol. Sci. 161 (1965) 483–495.
[14] M.R. Kasimov, M.R. Fatkhrakhmanova, K.A. Mukhutdinova, A.M. Petrov, 24S-Hydroxycholesterol enhances synaptic vesicle cycling in the mouse neuromuscular junction: implication of glutamate NMDA receptors and nitric oxide, Neuropharmacology 117 (2017) 61–73, https://doi.org/10.1016/j.neuropharm.2017.01.030.
[15] S.O. Rizzoli, W.J. Betz, Effects of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one on synaptic vesicle cycling at the frog neuromuscular junction, J. Neurosci. 22 (24) (2002) 10680–10689.
[16] A.R. Bresnick, J.M. Backer, PI3Kβ - a versatile transducer for GPCR, RTK and small GTPase signaling, Endocrinology (2019), https://doi.org/10.1210/en.2018-00843 [Epub ahead of print].
[17] B. Thyagarajan, J.G. Potian, P. Baskaran, J.J. McArdle, Capsaicin modulates acetylcholine release at the myoneural junction, Eur. J. Pharmacol. 744 (2014) 211–219, https://doi.org/10.1016/j.ejphar.2014.09.044.
[18] S. Ciani, C. Edwards, The effect of acetylcholine on neuromuscular transmission in the frog, J. Pharmacol. Exp. Therapeut. 142 (1963) 21–23.
[19] Y.M. Kupchik, O. Barchad-Avitzur, J. Wess, Y. Ben-Chaim, I. Parnas, H. Parnas, A novel fast mechanism for GPCR-mediated signal transduction – control of neurotransmitter release, J. Cell Biol. 192 (1) (2011) 137–151, https://doi.org/10.1083/jcb.201007053.
[20] M.P. Caulfield, Muscarinic receptors characterization, coupling and function, Pharmacol. Ther. 58 (1993) 319–379.
[21] A.R. Graves, K.A. Lewin, C.A. Lindgren, Nitric oxide, cAMP and the biphasic muscarinic modulation of ACh release at the lizard neuromuscular junction, J. Physiol. 559 (2004) 423–432, https://doi.org/10.1113/jphysiol.2004.064469.
[22] N. Garcia, M.M. Santafé, I. Salon, M.A. Lanuza, J. Tomàs, Expression of muscarinic acetylcholine receptors (M1-, M2-, M3- and M4-type) in the neuromuscular junction of the newborn and adult rat, Histol. Histopathol. 20 (3) (2005) 733–743, https://doi.org/10.14670/hh-20.733.
[23] I.V. Kovyazina, A.N. Tsentsevitsky, E.E. Nikolsky, Identification of the

muscarinic receptor subtypes involved in autoregulation of acetylcholine quantal release from frog motor nerve endings, Dokl. Biol. Sci. 460 (2015) 5–7, https://doi.org/10.1134/s0012496615010081.
[24] P.E. Silveira, N.A. Silveira, C. Morini Vde, C. Kushmerick, L.A. Naves, Opposing effects of cannabinoids and vanilloids on evoked quantal release at the frog neuromuscular junction, Neurosci. Lett. 473 (2) (2010) 97–101, https://doi.org/10.1016/j.neulet.2010.02.026.
[25] X. Trujillo, E. Sánchez-Pastor, F. Andrade, M. Huerta, Presence and colocalization of type-1 cannabinoid receptors with acetylcholine receptors in the motor end plate of twitch skeletal muscle fibers in the frog, J. Membr. Biol. 247 (11) (2014) 1199–1205, https://doi.org/10.1007/s00232-014-9721-5.
[26] E. Sánchez-Pastor, X. Trujillo, M. Huerta, F. Andrade, Effects of cannabinoids on synaptic transmission in the frog neuromuscular junction, J. Pharmacol. Exp. Therapeut. 321 (2) (2007) 439–445.
[27] J.E. Lauckner, B. Hille, K. Mackie, The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to $Gq/_{11}$ G proteins, Proc. Natl. Acad. Sci. U.S.A. 102 (52) (2005) 19144–19149, https://doi.org/10.1073/pnas.0509588102.
[28] S. Yip, B.R. Sastry, Effects of hemoglobin and its breakdown products on synaptic transmission in rat hippocampal CA1 neurons, Brain Res. 864 (1) (2000) 1–12.
[29] S. Yip, J.K. Ip, B.R. Sastry, Electrophysiological actions of hemoglobin on rat hippocampal CA1 pyramidal neurons, Brain Res. 713 (1–2) (1996) 134–142.
[30] K.A. Mukhutdinova, M.R. Kasimov, A.R. Giniatullin, G.F. Zakyrjanova, A.M. Petrov, 24S-hydroxycholesterol suppresses neuromuscular transmission in SOD1 (G93A) mice: a possible role of NO and lipid rafts, Mol. Cell. Neurosci. 88 (2018) 308–318, https://doi.org/10.1016/j.mcn.2018.03.006.
[31] S. Thomas, R. Robitaille, Differential frequency-dependent regulation of transmitter release by endogenous nitric oxide at the amphibian neuromuscular synapse, J. Neurosci. 21 (4) (2001) 1087–1095.
[32] A.M. Petrov, A.R. Giniatullin, G.F. Sitdikova, A.L. Zefirov, The role of cGMP-dependent signaling pathway in synaptic vesicle cycle at the frog motor nerve terminals, J. Neurosci. 28 (49) (2008) 13216–13222, https://doi.org/10.1523/jneurosci.2947-08.2008.
[33] A.L. Zefirov, R.R. Khaliullina, A.A. Anuchin, A.V. Yakovlev, The effects of exogenous nitric oxide on the function of neuromuscular synapses, Neurosci. Behav. Physiol. 32 (6) (2002) 583–588.
[34] S.J. Etherington, A.W. Everett, Postsynaptic production of nitric oxide implicated in long-term depression at the mature amphibian (Bufo marinus) neuromuscular junction, J. Physiol. 559 (2) (2004) 507–517, https://doi.org/10.1113/jphysiol.2004.066498.
[35] D.S. Bredt, Nitric oxide signaling specificity - the heart of the problem, J. Cell Sci. 116 (1) (2003) 9–15.
[36] L.M. Descarries, S. Cai, R. Robitaille, E.M. Josephson, D.K. Morest, Localization and characterization of nitric oxide synthase at the frog neuromuscular junction, J. Neurocytol. 27 (11) (1998) 829–840.
[37] B. Chanrion, C. Mannoury la Cour, F. Bertaso, M. Lerner-Natoli, M. Freissmuth, M.J. Millan, J. Bockaert, P. Marin, Physical interaction between the serotonin transporter and neuronal nitric oxide synthase underlies reciprocal modulation of their activity, Proc. Natl. Acad. Sci. U. S. A. 104 (19) (2007) 8119–8124, https://doi.org/10.1073/pnas.0610964104.