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Inhibition of protein kinase C affects on mode of synaptic vesicle exocytosis due to cholesterol depletion

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ABSTRACT

Previous studies demonstrated that depletion of membrane cholesterol by 10 mM methyl-beta-cyclodextrin (MCD) results in increased spontaneous exocytosis at both peripheral and central synapses. Here, we investigated the role of protein kinase C in the enhancement of spontaneous exocytosis at frog motor nerve terminals after cholesterol depletion using electrophysiological and optical methods. Inhibition of the protein kinase C by myristoylated peptide and chelerythrine chloride prevented MCD-induced increases in FM1-43 unloading, whereas the frequency of spontaneous postsynaptic events remained enhanced. The increase in FM1-43 unloading still could be observed if sulforhodamine 101 (the water soluble FM1-43 quencher that can pass through the fusion pore) was added to the extracellular solution. This suggests a possibility that exocytosis of synaptic vesicles under these conditions could occur through the kiss-and-run mechanism with the formation of a transient fusion pore. Inhibition of phospholipase C did not lead to similar change in MCD-induced exocytosis.

1. Introduction

Presynaptic nerve terminals release neurotransmitters by synaptic vesicle exocytosis. When an action potential depolarizes the presynaptic membrane, Ca^{2+}channels open, causing a local increase in the intracellular Ca^{2+} concentration at the active zone thus triggering a fusion event. After the fusion pore opens, synaptic vesicle membrane is recovered through clathrin-mediated endocytosis. Then, synaptic vesicles are filled with neurotransmitters and supply the synaptic vesicle pool. This mechanism of synaptic vesicle recycling could be observed at the majority of synapses [1]. However, in some cases, synaptic vesicles may recycle via "kiss-and-run" mechanism, where the fusion pore opens and closes and synaptic vesicle undergoes undocking [2].

Membrane cholesterol is an essential component that is involved in key steps of the synaptic vesicular cycle [3]. Cholesterol in presynaptic membrane is implicated in exocytosis, whereas vesicular cholesterol is required for endocytosis [4–7]. Cholesterol depletion by extracellular sequestration with methyl-β-cyclodextrin or acute statin-mediated inhibition of de novo cholesterol biosynthesis dramatically reduces evoked neurotransmitter release but at the same time augments spontaneous fusion rates at the neuromuscular junction, synaptosomes, cerebellar and hippocampal synapses [7–12]. The precise mechanism by which spontaneous synaptic vesicle fusion might be increased remains elusive It is assumed that the activity of presynaptic signaling enzymes, regulating spontaneous neurotransmitter release, may be sensitive to changes in the membrane cholesterol content [7,8,13]. So the depletion of cholesterol could lead to activation of several protein kinases and phosphatase PP2B, resulting in enhanced spontaneous exocytosis [8,11,13].

The protein kinase C (PKC) family of enzymes is a known regulator of synaptic function, including release of neurotransmitter at cholinergic, γ-aminobutyric acid (GABA)-ergic, dopaminergic, and glutamatergic synapses [14]. Specifically, several key exocytotic proteins (synaptotagmin, Munc18, SNAP-25, NSF) can be modulated via PKC–mediated phosphorylation [15]. The activation PKC or conversely the lack of its activation may be observed following membrane cholesterol depletion in different cell types. Depletion of cholesterol resulted in activation PKC and the PKC-θ isoform being translocated to plasma membrane at T cells [16]. PKCδ was activated during statin (cholesterol lowering drug) treatment of acute promyelocytic leukemia cells [17]. On the other hand,

Abbreviations: MCD, methyl-β-cyclodextrin; MEPP, miniature postsynaptic endplate potentials; PKC, protein kinase C; SR101, sulforhodamine 101.

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cholesterol enrichment-induced inhibition of M currents through KCNQ channels in superior cervical ganglion neurons was mediated by PKC phosphorylation. This effect was prevented when membrane cholesterol level was restored [18]. Perhaps, cholesterol may increase PKC activity by modulating physicochemical membrane properties [19].

Our data demonstrate that the specific type of synaptic vesicle exocytosis (full or kiss-and-run modes), induced by cholesterol depletion, may be dependent on protein kinase C activity, not requiring activation of phospholipase C.

2. Materials and methods

2.1. Ethical approval

All animal experiments were performed in accordance with the guidelines for the use of laboratory animals of Kazan State Medical University. The experimental protocol met the requirements of the European Communities Council Directive 2010/63/EU and was approved by the Ethical Committee of Kazan Medical University. Frogs (Rana ridibunda) were killed by decapitation and destruction of the brain and the spinal cord. Muscles were then quickly excised.

2.2. Preparation, solution and treatments

Experiments were performed on the isolated cutaneous pectoris muscles of the frog. Muscle with a nerve were attached to the bottom of a glass sylgard lined chamber, which was superfused during the entire course of the experiment with the frog Ringer's solution, containing (in mM): NaCl 113, KCl 2.5, CaCl2 1.8, NaHCO3 1, Hepes 5. pH was maintained at 7.2–7.4 at 22–24 °C. Methyl-β-cyclodextrin (MCD, 10 mM) was used to deplete cell membranes of cholesterol. In all experiments tetrodotoxin (1 μM) was added to prevent generation of action potential during application of MCD. Myristoylated PKC inhibitor peptide (75 μM, Myr-RFARKGALRQKNV) and chelerythrine chloride (1 μM) were used to specifically block the PKC. Compound 48/80 (100 μM) and U-73122 (10 μM) were used to inhibit phospholipase C. Myristoylated PKC inhibitor peptide, chelerythrine chloride, compound 48/80 and U-73122 were added to the external solution 40–50 min before MCD treatment and remained in the bath throughout the experiment. But in some experiments, the preparation was pretreated with myristoylated PKC inhibitor peptide which then was washed out prior to the MCD treatment. Sulforhodamine 101 (SR101, 7 μM) was used for quenching the fluorescence of FM1-43 in some experiments. All reagents were from Sigma except for FM1-43 and ADVASEP-7, which were purchased from Biotium.

2.3. Electrophysiology

Recording of miniature postsynaptic end-plate potentials (MEPP) was performed using the standard intracellular electrode (3–5 MΩ resistance; filled with 2.5 M KCl). The recorded MEPPs were digitized at 50 kHz, stored in a PC and analyzed off-line to calculate the frequency of MEPPs. Recording instrumentation consisted of an amplifier, Model 1600 (A-M System) and a LA II digital I/O board (Pushino, Russia) under the control of original software.

2.4. Fluorescence microscopy

Fluorescent dye FM1-43 (5 μM) was used to measure the rate of synaptic vesicle exocytosis. This dye reversibly binds to the presynaptic membrane and becomes locked in the recycled vesicles within the cytoplasm of nerve terminals during endocytosis. This process will be referred to as a "dye loading". The motor nerve was stimulated with a suction electrode and DS3 stimulator (Digitimer Ltd) by electrical pulses with duration of 0.1 ms and subthreshold amplitudes. Trains of 20 Hz stimulation (comprising of 3600 action potentials) for 3 min's were used to load FM1-43 into the nerve terminals. The dye was present in the bath during and 5 min after stimulation. Following the loading process, the preparations were washed with the physiological saline with 3 μM ADVASEP-7 (sulfonated β-cyclodextrin) for 30 min to decrease background fluorescence. This reagent accelerates dissociation of FM1-43 from the surface membranes and decrease nonspecific fluorescence. Tetrodotoxin (1 μM) was added to perfusion after the dye loading. To estimate the influence on synaptic vesicles exocytosis, the dye-loaded nerve terminals were treated by 10 mM MCD (10 min), but without dye present in the bath. MCD itself can bind FM1-43 and in this scenario MCD acts as reagent which accelerating dissociation of FM1-43 from surface membranes. So for preventing any influence of these on the unloading curves we tried to minimize of nonspecific fluorescence by applying ADVASEP-7 [8].

Fluorescence images were acquired using an Olympus BX51 microscope with a confocal attachment Disk Speed Unit and LumPlanPF 100xw objectives. Images were captured with color DP71 CCD camera (Olympus). Image analysis was performed using Cell^P (Olympus) and ImagePro software (Media Cybernetics, Bethesda, MD, USA). Background fluorescence was estimated as mean fluorescence intensity in the 50 × 50 pixels area outside of the nerve terminal. Fluorescence intensity was expressed in arbitrary units [20].

2.5. Statistics

The results of the experiments are presented as Mean ± SEM, n – number of experiments with statistical significance assessed by the Student's paired or ANOVA. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Influence of PKC inhibitors on MCD-mediated increase in spontaneous neurotransmitter release

Concentration of MCD (10 mM) is widely used for cholesterol depletion and it was applied because the low concentrations (1 mM and less) do not vastly change spontaneous release at the frog neuromuscular junctions [9,10]. In order to explore the role of PKC in the effects of MCD treatment, we performed the experiments with the membrane permeable peptide (75 μM) - an inhibitor of PKC (which specifically inhibits calcium and phospholipid dependent PKC in concentration range 10–100 μM [21]) and chelerythrine chloride (1 μM).

We found that inhibition of PKC resulted in reduced MEPP frequency (to 0.7 ± 0.2 Hz, $n = 7$, inhibitor peptide, paired t test, $p < 0.01$; to 1.1 ± 0.1 Hz, $n = 7$, chelerythrine chloride, paired t test, $p < 0.05$) under control conditions (1.6 ± 0.2 Hz, $n = 7$) (Fig. 1A), but did not change dramatically the MCD effects on spontaneous release (Fig. 1B). MCD treatment causes a rapid increase in the MEPP frequency, which is persistent even after the end of MCD application (Fig. 1B). If complex MCD-cholesterol is applied immediately after MCD application, then the MEPP frequency relatively fast decreases to pre-MCD treatment baseline [8]. When PKC was blocked by inhibitor peptide/chelerythrine chloride, MCD treatment increased the MEPP frequency to 39.6 ± 3.6 ($p < 0.001$, versus pre-MCD baseline, $p < 0.05$ versus MCD-control)/32.0 ± 3.1

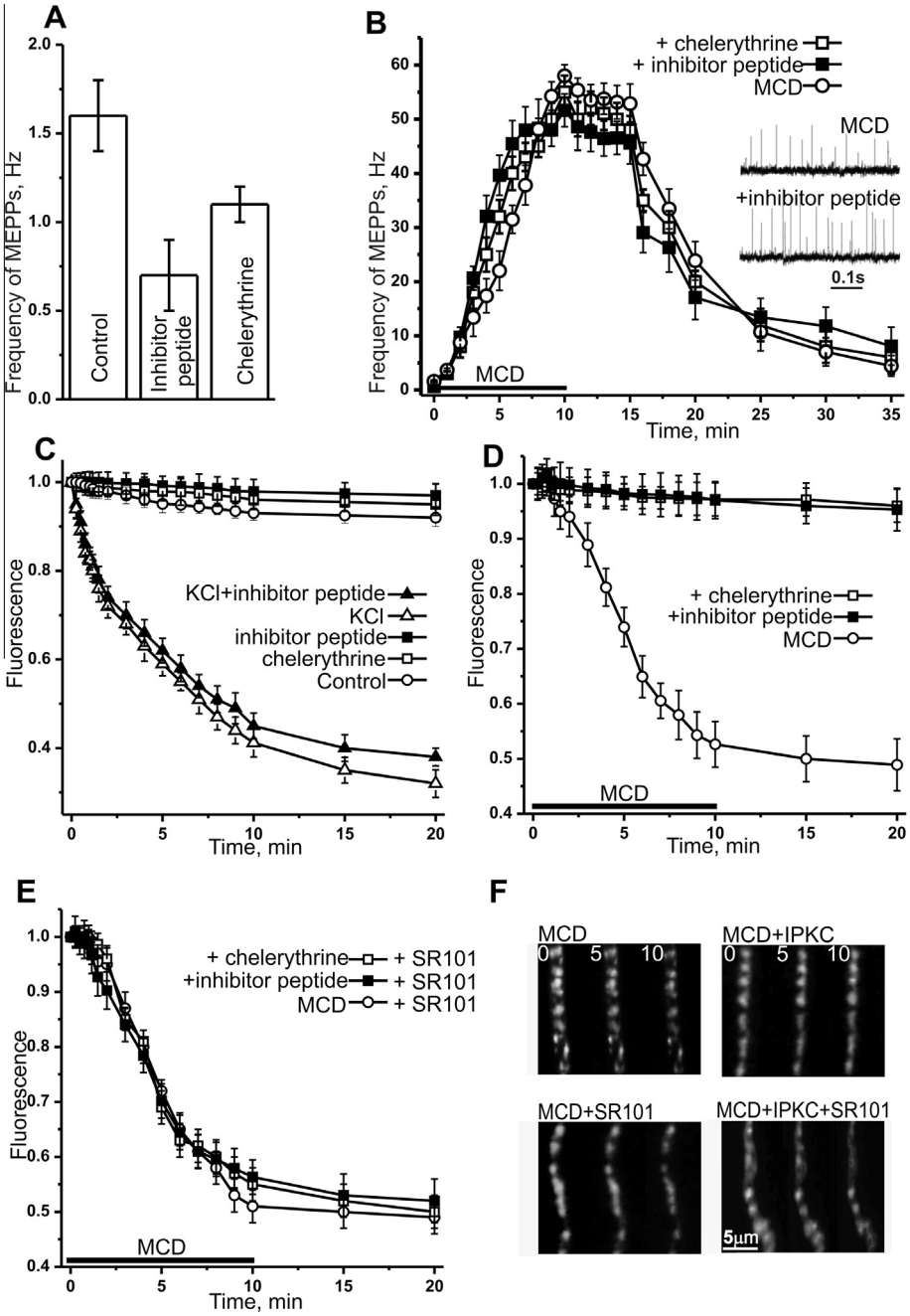

Fig. 1. Effects of cholesterol depletion on MEPP frequency and FM1-43 unloading. Implication of PKC. (A) The MEPP frequency in rest conditions and after exposure with PKC inhibitors (myristoylated inhibitor peptide and chelerythrine chloride). (B) The MEPP frequency changes in response on MCD application (light circles) and under the action of protein kinase C inhibitors (peptide – dark squares and chelerythrine – light squares). Time of MCD application is shown by a black horizontal bar. On the inset it is displayed samples traces of MEPPs from the experiments shown in B for 5 min treatment with MCD and under the action of protein kinase C inhibitor peptide. (C–E) The decrease in fluorescence of preloaded by FM1-43 nerve terminals: (C) at rest conditions (light circles), during the incubation with PKC inhibitors (peptide – dark squares, and chelerythrine – light squares), upon KCl (40 mM) application alone (light triangles) and when PKC was blocked (dark triangles); (D) due to MCD treatment alone (light circles), and under conditions of PKC blockade (with peptide – dark squares, or chelerythrine – light squares); (E) indicates MCD-induced FM1-43 destaining (light circles – in control, dark and light squares – in conditions of PKC inhibition by peptide or chelerythrine respectively) in experiments where the sulforhodamine 101 (SR101) was in extracellular solution. (F) Fluorescent images of nerve terminal segments at various time points (0, 5 and 10 min) during the MCD treatment alone and under conditions of action the PKC inhibitor peptide. Lower panel – in presence of SR101 in perfusion solution.

($p < 0.001$, versus pre-MCD baseline, $p < 0.05$ versus MCD-control) and 51.6 ± 3.0Hz ($n = 7$, $p < 0.001$, versus pre-MCD baseline, $p > 0.05$ versus MCD-control)/55.0 ± 3.0 ($n = 7$, $p < 0.001$ versus pre-MCD baseline, $p > 0.05$ versus MCD-control) by the 5th and 10th min respectively. We tested different concentrations (from 10 to 150 μM) of PKC inhibitor peptide and in all cases the MCD effect was manifested as a significant increase in the MEPP frequency (data not shown).

3.2. Effect of PKC inhibitors on MCD-induced FM-41 unloading

Under the resting conditions, the decrease in fluorescence occur very slightly at the nerve terminals preloaded with FM1-43, but it was significantly accelerated upon KCl (40 mM) application (Fig. 1C). It indicates a weak release of the fluorescent dye by spontaneous exocytosis at the rest. Exposure of preparation with PKC inhibitors slightly decreased a basal trend to the reduction of fluo-

rescence in the preloaded nerve terminals (Fig. 1C). As shown previously, in response to the addition of MCD to the perfusion solution, the marked decrease in fluorescence is observed [8]. Unexpectedly, MCD-induced decreases in fluorescence were suppressed when PKC was blocked (Fig. 1D and F). Thus, fluorescence was 0.98 ± 0.03 ($p > 0.05$)/0.98 ± 0.02 ($p > 0.05$) and 0.97 ± 0.03 ($n = 7$, $p > 0.05$, versus pre-MCD baseline, inhibitor peptide)/0.97 ± 0.03 ($n = 7$, $p > 0.05$, versus pre-MCD baseline, chelerythrine chloride) by the 5th and 10th min, respectively. If the preparation was pretreated with peptide PKC inhibitor only prior to the MCD exposure period then MCD induced unloading was also blocked (data are not shown).

The effect of PKC inhibitor peptide on MCD induced dye unloading was dose-dependent and the inhibitor in the lower concentrations (10 and 50 μM) had a less prominent influence on the decrease in fluorescence upon MCD exposure (data not shown). It should be noted that PKC inhibitor peptide did not change the KCl (40 mM) induced dye unloading (Fig. 1C, in these experiments tetrodotoxin was not added to perfusion). It indicates that the PKC inhibitor selectively interferes the MCD induced destaining.

3.3. Use of sulforhodamine 101 as FM1-43 quencher

The addition of FM1-43 quencher, sulforhodamine 101 (SR101), to the extracellular solution together with MCD prevented the effect of PKC blockade on FM1-43 unloading (Fig. 1E and F). In the presence of SR101 alone, the treatment with MCD led to the decrease in fluorescence to 0.72 ± 0.02 ($p < 0.001$) and 0.51 ± 0.03 ($n = 7$, $p < 0.001$ versus pre-MCD baseline) by the 5th and 10th min, respectively (it had no difference from the control MCD treatment, $p > 0.05$). Under conditions of PKC blockade with inhibitor peptide/chelerythrine chloride, the fluorescence was 0.70 ± 0.03 ($p < 0.001$)/0.69 ± 0.03 ($p < 0.001$) and 0.56 ± 0.03 ($n = 7$, $p < 0.001$, versus pre-MCD baseline)/0.55 ± 0.03 ($n = 7$, $p < 0.001$, versus pre-MCD baseline) by the same time points (it had no difference from the control action of MCD, $p > 0.05$). Thus, SR101 "disclosed" the dye unloading at the preparation than was treated by PKC inhibitor but by SR101, itself, had no effect on the MCD-induced unloading of FM1-43.

3.4. Influence of phospholipase C inhibitors on MCD-induced exocytosis

Cholesterol depletion-related activation of PKC may depend on phospholipase C activity [14]. It should be noted that inhibitors of phospholipase C often have several side effects. We, therefore, tested the effects of compound 48/80 (100 μM, that is known to inhibit both phospholipase C and calmodulin) and U-73122 (10 μM, which more specific inhibits the hydrolysis of phosphoinositides to inositol-3-phosphate and the coupling of G protein-

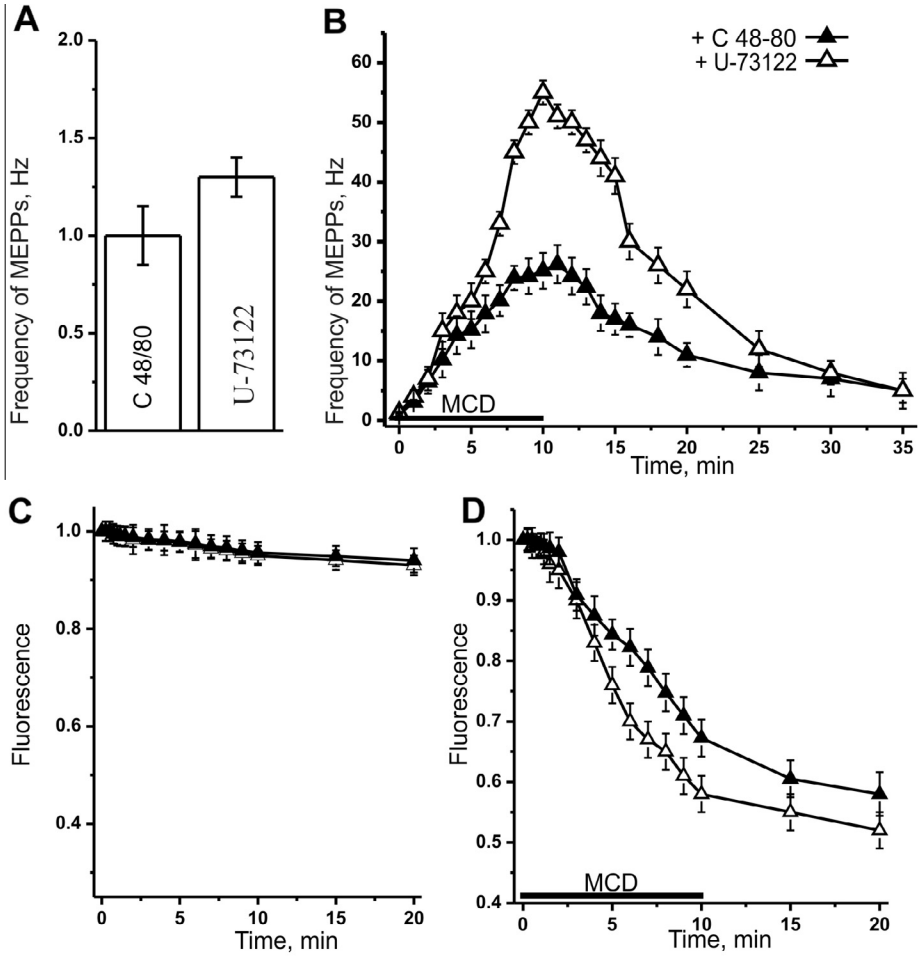

Fig. 2. Involvement of phospholipase C in effects of cholesterol depletion. (A) The MEPP frequency after exposure to phospholipase C inhibitors (compound 48/80 and U-73122). (B) The MEPP frequency changes in response on MCD application, when phospholipase C was inhibited by compound 48/80 (dark triangles) and U-73122 (light triangles). (C and D) The decrease in fluorescence of preloaded by FM1-43 nerve terminals: (C) during period of perfusion with phospholipase C inhibitors (compound 48/80 – dark triangles and U-73122 - light triangles); (D) due to MCD application in conditions of phospholipase C inhibition by compound 48/80 (dark triangles) and U-73122 (light triangles).

phospholipase C activation). However, we found that inhibition of phospholipase C was not associated with the effects analogous to those observed with the PKC inhibitor (Fig. 2).

Application of compound 48/80 led to lowering the MEPP frequency (until to 1.0 ± 0.15 Hz, $p < 0.05$, Fig. 2A) and did not significantly change a trend to the decrease of FM1-43 (Fig. 2C). The effects of MCD on MEPP frequency and FM1-43 unloading were proportionally attenuated when phospholipase C was blocked by compound 48/80 (Fig. 2B and D). Specifically, the MEPP frequency increased to 15.2 ± 3.1 Hz ($p < 0.01$ versus pre-MCD baseline, $p < 0.05$ versus MCD control) and 25.1 ± 3 Hz ($n = 7$, $p < 0.001$ versus pre-MCD baseline, $p < 0.001$ versus MCD control) by 5th and 10th min of MCD application, respectively; whereas the fluorescence decreased to 0.84 ± 0.02 ($p < 0.01$, versus pre-MCD baseline, $p < 0.05$, versus MCD control) and 0.67 ± 0.03 ($n = 7$, $p < 0.001$ versus pre-MCD baseline, $p < 0.01$ versus MCD control) by the same time intervals.

Treatment with U-73122 slightly decreased MEPP frequency (until 1.3 ± 0.1 Hz, $n = 7$, $p = 0.05$) and had no significant influence on MCD driven enhancement of MEPP frequency (Fig. 2A and B). So, 5 and 10 min after MCD exposure the MEPP frequency achieved 20.0 ± 3.1 ($p < 0.01$, versus pre-MCD baseline, $p > 0.05$ versus MCD control) and 55 ± 2 ($n = 7$, $p < 0.001$, versus pre-MCD baseline, $p > 0.05$ versus MCD control). Also dynamic of FM1-43 basal fluorescence and MCD induced FM1-43 unloading were the same in U-73122-treated and control preparations (Fig. 2C and D). The FM1-43 fluorescence reduced to 0.76 ± 0.03 ($p < 0.01$, versus pre-MCD baseline, $p > 0.05$ versus MCD control) and 0.58 ± 0.03 ($n = 7$, $p < 0.001$ versus pre-MCD baseline, $p > 0.05$ versus MCD control) by 5 and 10 min of MCD application.

4. Discussion

It has been demonstrated that PKC is involved in the enhancement of spontaneous exocytosis caused by cholesterol depletion from the surface membranes of cerebellar synapses and synaptosomes [11] and [13]. However, in our experiments, blocking PKC did not influence dramatically the MCD effects on MEPP frequency. Only in the initial period of MCD action (until 7–8 min) the MEPP frequency was increasing more sharply, when PKC was blocked. Paradoxically, we found that the dye unloading did not occur despite increased MEPP frequency. It could be explained by neurotransmitter release through a transient fusion pore ("kiss-and-run" mechanism), allowing the neurotransmitter to be released but is impermeable for the dye. To test this hypothesis, we used SR101, which has a small size and hydrophilicity and can penetrate through the fusion pore and quench the FM1-43 fluorescence in vesicles [22]. Indeed, in the presence of SR101 in external medium, MCD induced decrease in fluorescence occurred identically both under control conditions and when PKC was inhibited. As SR101 itself did not affect the FM1-43 unloading caused by MCD treatment, it can be assumed that PKC activity is required for the redirection MCD induced exocytosis to the full fusion mechanism. Hypothetically, kiss-and-run pathway may provide a more rapid rise in spontaneous exocytosis after MCD application, which we observe in case of protein kinase inhibition. It is possible that PKC is activated by the cholesterol depletion and promotes full exocytosis through the phosphorylation of exocytosis machinery proteins (e.g., SNAP-25 and Munc18) or direct interaction with these proteins [15]. There is evidence indicating the tight relationship between cholesterol and the type of exocytosis. We have recently suggested that "kiss-and-run" pathway of neurotransmitter release can be used by vesicles belonging to the recycling pool after enzymatic oxidation of membrane cholesterol [23].

PKC can be activated by DAG (a product of phospholipid cleavage by phospholipase C), calcium, reactive oxygen species and other factors [14]. In order to test the role of phospholipase C in MCD effects, we performed the experiments with compound 48/80, an inhibitor of phospholipase C and calmodulin, and U-73122. The MCD effects on MEPPs frequency and dye unloading were proportionately attenuated by compound 48/80 and were not changed by U-73122. In the case of compound 48/80 treatment, it might be linked with blockage of calmodulin, which participated in the effects MCD on spontaneous exocytosis. Similar results were obtained under conditions of calcium chelation or inhibition of Ca-calmodulin dependent phosphatase 2B, calcineurin [8].

Therefore, the type of MCD-induced exocytosis (full or "kiss-and-run" modes) is dependent on calcium- and phospholipase C-independent effects of PKC activation. Cholesterol depletion could lead to an increase in "kiss-and-run" exocytosis, but the simultaneous activation of PKC may switch the exocytosis to the full mode. Further experiments will be needed to identify detailed mechanism of this phenomenon and the specific PKC isoform, involved in this.

Acknowledgments

All experiments were performed in laboratory of normal physiology department at Kazan Medical University. A.M.P. and Z.G.F., A.A.Y. performed all experiments, data collection and analysis. A.M.P. was responsible for the conception and design of the study. A.M.P. and A.L.Z. were involved in the interpretation of data and drafting the article. All the authors discussed the results and commented on the manuscript. All the authors approved the final version of the manuscript.

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