Biochimica et Biophysica Acta 1861 (2016) 606–616

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Similar oxysterols may lead to opposite effects on synaptic transmission: Olesoxime versus 5α-cholestan-3-one at the frog neuromuscular junction

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ARTICLE INFO

Article history:
Received 18 December 2015
Received in revised form 17 March 2016
Accepted 15 April 2016
Available online 18 April 2016

Keywords:
Olesoxime
5α-Cholestan-3-one
Synaptic vesicle cycle
Neuromuscular junction
Neurotransmitter release

ABSTRACT

Cholesterol oxidation products frequently have a high biological activity. In the present study, we have used microelectrode recording of end plate currents and FM-based optical detection of synaptic vesicle exo-endocytosis to investigate the effects of two structurally similar oxysterols, olesoxime (cholest-4-en-3-one, oxime) and 5α-cholestan-3-one (5αCh3), on neurotransmission at the frog neuromuscular junction. Olesoxime is an exogenous, potentially neuroprotective, substance and 5αCh3 is an intermediate product in cholesterol metabolism, which is elevated in the case of cerebrotendinous xanthomatosis. We found that olesoxime slightly increased evoked neurotransmitter release in response to a single stimulus and significantly reduced synaptic depression during high frequency activity. The last effect was due to an increase in both the number of synaptic vesicles involved in exo-endocytosis and the rate of synaptic vesicle recycling. In contrast, 5αCh3 reduced evoked neurotransmitter release during the low- and high frequency synaptic activities. The depressant action of 5αCh3 was associated with a reduction in the number of synaptic vesicles participating in exo- and endocytosis during high frequency stimulation, without a change in rate of the synaptic vesicle recycling. Of note, olesoxime increased the staining of synaptic membranes with the B-subunit of cholera toxin and the formation of fluorescent ganglioside GM1 clusters, and decreased the fluorescence of 22-NBD-cholesterol, while 5αCh3 had the opposite effects, suggesting that the two oxysterols have different effects on lipid raft stability. Taken together, these data show that these two structurally similar oxysterols induce marked different changes in neuromuscular transmission which are related with the alteration in synaptic vesicle cycle.

1. Introduction

Chemical synaptic transmission results from the release from the nerve terminal of neurotransmitter that is packaged in synaptic vesicles. During neurotransmission, synaptic vesicles fuse at the active zone and their membranes become incorporated into the presynaptic membrane. These vesicle components must be captured by endocytosis to maintain the number of vesicles and the molecular identity of the presynaptic membrane. The newly formed vesicles are then filled with neurotransmitter and transported to the vesicle pool. Thus, as a consequence of synaptic activity, vesicles undergo a membrane-trafficking cycle in the nerve terminals [1]. This synaptic cycle is a tightly regulated process which is closely influenced by the availability of cholesterol. Cholesterol depletion may alter the balance between evoked and spontaneous exocytosis, and the distribution of proteins and ion channels involved in exocytosis. It may also lead to severe disturbances of endocytosis [2–8]. Neuronal membrane cholesterol has a very long half-life and can be oxidized by specific enzymes and reactive oxygen species [9,10]. In addition, oxidized cholesterol-like molecules produced in extraneuronal tissues can act on neurons. Some forms of oxysterol show high biological activity such as regulating cell survival and apoptosis [9,11]. Recent studies indicate that 24(S)-hydroxycholesterol may function as an endogenous modulator of NMDA receptors in the hippocampus [12] and enzymatic cholesterol oxidation can influence on synaptic vesicle cycle at the frog neuromuscular junction [13]. However, the details of how these cholesterol derivatives influence synaptic transmission are still poorly understood.

Recently, we have reported that 5α-cholestan-3-one (5αCh3), in low concentration (0.2 μM), reduces the number of vesicles which are actively recruited during synaptic transmission and alters membrane properties at the mouse neuromuscular junction [14]. The production of this oxysterol is up-regulated in the rare genetic disease, cerebrotendinous xanthomatosis, which is associated with central–peripheral distal axonopathy. The level of 5αCh3 in the blood from sterol 27-hydroxylase gene-deficient mice (model of cerebrotendinous

Abbreviations: α-Btx, α-bungarotoxin; 5αCh3, 5α-cholestan-3-one; bodipy-ganglioside GM1, BODIPY® FL C5-ganglioside GM1; MEPC, miniature postsynaptic end-plate currents; EPC, postsynaptic end-plate current; 22-NBD-cholesterol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol; CTxB, subunit B from cholera toxin.

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http://dx.doi.org/10.1016/j.bbalip.2016.04.010
1388-1981/

xanthomatosis) is about 0.2 μM [15]. On the other hand a structurally related compound, olesoxime (cholest-4-en-3-one, oxime), has beneficial effects in disease models of amyotrophic lateral sclerosis, peripheral neuropathies and neurodegenerative pathologies. Recently, clinical trials (phases II and III) have shown that treatment with olesoxime was associated with maintenance of motor function and decrease in frequency of complications in patients with spinal muscle atrophy [16]. Note that olesoxime shows the potent protective properties in the submicromolar range [11,17–19]. However, nothing is known about the influence of olesoxime on synaptic transmission. In the current study we have investigated how two similar oxysterols (olesoxime and 5α-cholestan-3-one) change neurotransmitter release and the synaptic vesicle cycle at the frog neuromuscular junction.

2. Material and methods

2.1. Ethical approval

The investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Frogs (*Rana ridibunda*) were collected from lakes during early autumn, and were kept in the dark at 4 °C in a humidity- and temperature-controlled environment. Frogs were provided with a pool of dechlorinated flowing water. Experiments were carried out in the autumn–winter period. Frogs were killed by decapitation and destruction of the brain and the spinal cord, and then the muscles were quickly excised. All efforts were made to minimize suffering. The experimental protocol met the requirements of the European Communities Council Directive 86/609/EEC and was approved by the Ethical Committee of Kazan Medical University.

2.2. Solution and chemicals

Isolated cutaneous pectoris muscles with a nerve were pinned to the bottom of a glass chamber lined with Sylgard, and were superfused during the experiment with frog Ringer's saline containing (in mM): NaCl — 113.0, KCl — 2.5, $CaCl_2$ — 1.8, and $NaHCO_3$ — 2.4. pH was adjusted to 7.3 with NaOH/HCl and the temperature was kept at 23–24 °C. The muscle fibers (MF) were cut transversely to prevent muscle contractions while maintaining the physiological level of quantal release of acetylcholine at the neuromuscular junction [20]. Experiments were started after washing the muscle for 40 min with Ringer's solution. 5αCh3 and olesoxime were dissolved in DMSO (dimethylsulfoxide) such that the final concentration of DMSO in the working solution did not exceed 0.001%. In concentration 0.001% DMSO in bathing solution did not change the parameters of both evoked and spontaneous postsynaptic signals, labelings with FM1–43 dye and membrane-markers used here. We therefore used these data as the additional control.

Applications of 5αCh3 (200 nM) or olesoxime (200 nM) lasted 20 min. After the treatments, these reagents were washed out for 10 min. The concentration was chosen based on (1) our previous study, where 5αCh3 in this concentration had profound presynaptic effects [14], (2) data about the level of 5αCh3 in the blood from the mice with disrupted sterol 27-hydroxylase gene [15] and (3) observations that olesoxime could effectively protect cells from oxidative stress and death even in the submicromolar range [11,17,18]. The incubation period was 20 min because this time is necessary for the changes in postsynaptic currents, induced by these drugs, to reach a steady state level.

All reagents were from Sigma, except for fluorescent dyes, which were purchased from Molecular Probes.

2.3. Electrophysiology

Recording of the postsynaptic end-plate currents (EPCs) and miniature EPC (MEPCs) was performed using standard two-electrode voltage clamp technique with intracellular glass microelectrodes (tip diameter ~ 1 μm, resistance 3–5 MΩ, filled with 2.5 M KCl). The synaptic zone was located between two electrodes separated by a distance of ~200–300 μm. The holding potential for the cut MFs was kept at −40 mV (leak current in the range of 10–30 nA, ≤10% of EPC amplitudes). EPCs were elicited by supra-threshold stimulation (0.1 ms duration) of the motor nerve via a suction electrode connected to an extracellular stimulator (DS3 Digitimer Ltd., UK). The motor nerve was stimulated by a single stimulus (1 stimulus in 20 s, 0.05 Hz) or by high-frequency trains (20 stimuli per 1 s, 20 Hz, for 3 min). The recorded signals were digitized at 50 kHz and analyzed off-line using PC software [3]. No changes in the muscle cable properties occur after cutting the muscle fibers and the voltage clamp technique enables long-lasting stable recording of postsynaptic currents [21]. Recording instrumentation consisted of an Axoclamp 900 A (Molecular devices, USA) amplifier and LA II digital I/O board (Pushchino, Russia) under the control of locally written software.

2.4. Fluorescence microscopy

An Olympus BX51WI microscope with a confocal Disk Speed Unit attachment and a focus stepper (for multiple z axis optical sections; ECO-MOT, Marzhauser Wetzlar GmbH & Co. KG, Germany) were used for image acquisition. Images were captured with UPLANSapo 60xw or LumPlanPF 100xw objectives and an Orca R2 (Hamamatsu, UK) CCD camera under control of Cell^P (Olympus) software. Image analysis in regions of interest was performed using ImagePro software (Media Cybernetics, Bethesda, MD, USA) and the fluorescence was calculated in arbitrary unit (a.u.), and in some cases then converted to percentages. Only surface-lying nerve terminals were studied.

2.4.1. Loading and unloading of FM1–43

The fluorescent probe FM1–43 (3 μM, Molecular Probes) was used for imaging of synaptic vesicle exo- and endocytosis [22]. To load the dye into the nerve terminals, we electrically stimulated the nerve for 3 min at 20 Hz. The FM1–43 was in the bath during the stimulation period and for 7 min afterwards. The muscles were then perfused for at least 30 min with dye-free physiological saline with ADVASEP-7 (3 μM, Sigma) to decrease background fluorescence [23]. Electrical stimulation of the nerve at 20 Hz evoked unloading of the dye (due to synaptic vesicle exocytosis) from the pre-loaded nerve terminals. To estimate the impact on endocytosis, the muscles were exposed to 5αCh3 or olesoxime before dye loading. The oxysterols were added after washing with ADVASEP-7 but prior to the start of the unloading stimulation.

The dye FM2–10 (24 μM, Molecular Probes) was used to label synaptic vesicles in the recycling pool. The motor nerves were stimulated at 2 Hz for 5 min in the presence of FM2–10, the muscles were then washed in normal physiological saline with ADVASEP-7 for the first 30 min and with oxysterol (or without in the control) for the next 20 min. After this unloading was performed using 2 Hz stimulation.

The dye FM1–43 (or FM2–10) was excited by light of 480/10 nm wavelength and was recorded using a 515–555 nm band pass emission filter. Nerve terminal fluorescence was defined as the mean intensity per pixel in the regions of interest after subtracting background FM-fluorescence, measured in a 20 μm^2 area outside of the nerve terminal [23]. For plotting of the dye-unloading curves, values of the initial nerve terminal fluorescence (a.u. before the stimulation) were set to 1.0.

2.4.2. Labeling of lipid rafts

Ganglioside GM1, a well-established lipid raft component, was visualized using Alexa Fluor 488-labeled cholera toxin subunit B (CTxB) (Molecular Probes), which is pentavalent for ganglioside GM1 and preferentially interacts with GM1-molecules from lipid rafts [24]. The muscles were exposed to CTxB (1 μg/ml, diluted in Ringer's saline) for 15 min at 23–24 °C and were then perfused for 30 min prior to image