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Effects of Oxidation of Membrane Cholesterol on the Vesicle Cycle in Motor Nerve Terminals in the Frog *Rana Ridibunda*

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Experiments on neuromuscular preparations from the frog *Rana ridibunda* assessed the effects of oxidation of membrane cholesterol on the presynaptic vesicle cycle. Application of cholesterol oxidase (1 activity unit) for 30 min oxidized about 0.007 mg of cholesterol per g of preparation and decreased the stability of lipid rafts in nerve terminals. Electrophysiological studies demonstrated that oxidation of cholesterol decreased evoked transmitter release. Experiments using fluorescent FM dyes demonstrated suppression of synaptic vesicle exo- and endocytosis processes, along with dispersal of clusters of synaptic vesicles. Comparative analysis of electrophysiological and optical data, along with experiments using a dye quencher, demonstrated that transmitter could be released from some synaptic vesicles through transient fusion pores (the kiss-and-run mechanism). It is suggested that oxidation of cholesterol suppresses evoked exocytosis and delivery of synaptic vesicles of the reserve pool to the exocytosis site, thus interfering with their clustering. Vesicles of the recycling pool release transmitter by the "kiss-and-run" mechanism.

Keywords: transmitter release, exocytosis, kiss and run, synaptic vesicle pools, cholesterol oxidase, lipid rafts.

Transmitter release from synapses occurs via exocytosis of transmitter-filled synaptic vesicles at specific sites on the nerve terminal, i.e., active zones. The vesicle cycle is constantly occurring in nerve terminals, and includes "complete" exocytosis, with insertion of the vesicle membrane into the presynaptic membrane, and endocytosis, which is the formation of new vesicles and their transport to the active zone for reuse in transmitter secretion [5]. Although "complete" exocytosis is regarded as the main mechanism for transmitter release [5, 17], transmitter secretion in some experimental conditions can occur through pores forming transiently between the synaptic vesicle and the nerve terminal membrane in the active zone, followed by closure. This mechanism of secretion is termed kiss and run [2, 35].

Synaptic vesicles form a number of pools: the recyclable pool consists of a small portion of docked vesicles located close to the active zone; the mobilization pool, which tops up the recyclable pool as it is depleted; and the large reserve pool, dispersed through the cytoplasm of the nerve terminal. Synaptic vesicles in the first two of these pools are replenished quite quickly by endocytosis, so they are often combined into a single pool termed the recycling pool [1, 5, 33].

It is now known that exo- and endocytosis of synaptic vesicles occur as a result of the complex interaction of large numbers of presynaptic membrane and vesicle membrane proteins [5, 19]. At the same time, the lipid components of the membrane are significant in these processes. It has been suggested that the cholesterol molecule is one of the key regulators of the synaptic vesicle exo/endocytosis cycle [6, 20, 39]. In the nerve terminal membrane, cholesterol determines the activities of ion channels and proteins of the exocytosis mechanism [22, 39], stabilizes fusion pores [37], and facilitates membrane deformation during endocytosis [7, 13]. It is possible that many of the synaptic functions of cholesterol are linked with its ability to form, along with sphingomyelin, membrane microdomains, i.e., lipid rafts, which are seen in the membranes of active zones and vesicles [8, 20]. It has been suggested that lipid rafts in active

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zones are involved in coordinating the process of exocytosis [6, 27], while rafts in synaptic vesicles prevent the dispersal of vesicle lipids and proteins inserted into the superficial membrane of the nerve terminal after vesicle exocytosis, facilitating endocytosis [7, 8, 13, 30].

Cholesterol has a long "lifetime" in synaptic membranes (for example, from five to 12 months in mice) [11], during which it can undergo oxidative modification under the influence of reactive oxygen species and amyloid peptide complexed with Cu^{2+} ions [31]. Membrane cholesterol can be oxidized using the microbial enzyme cholesterol oxidase (ChO), which oxidizes cholesterol to 4-cholesten-3-one [21]. Although mammalian cells lack ChO, about 1.5% of the cholesterol in mouse and human brains is present as 4-cholesten-3-one. The brain level of this cholesterol derivative increases in conditions of moderate hypoxia and inflammation, as well as in a number of neurodegenerative diseases (Alzheimer's disease, cerebrotendinous xanthomatosis) [21].

Many studies addressing the role of cholesterol in the functioning of the presynaptic apparatus are based on the use of methyl-β-cyclodextrin – a polysaccharide able to extract cholesterol from membranes [7, 9, 13, 20, 22, 30, 34, 36, 37, 39]. We present here an analysis of the results obtained by enzymatic oxidation of cholesterol on the processes forming the presynaptic vesicle cycle.

Methods

Experiments were performed on isolated neuromuscular preparations of the sartorius muscle and cutaneous-pectoris muscle preparations from frogs (*Rana ridibunda*) in the autumn-winter period. Muscles were fixed in glass baths and were perfused with Ringer's solution (pH 7.2–7.4, 20–22°C) for cold-blooded animals throughout the experiments; solution contained 115.0 mM NaCl, 2.5 mM KCl, 1.8 mm $CaCl_2$, and 2.4 mM $NaHCO_3$. Motor nerves were stimulated with square-wave electrical impulses of duration 0.1 msec, at suprathreshold amplitude. Superficial membrane cholesterol was oxidized with ChO (1 activity unit). All substances were obtained from Sigma, except for reagents used for estimation of oxidized cholesterol, identification of lipid rafts, and analysis of the processes of exo- and endocytosis, which were from Molecular Probes.

Electrophysiology. Endplate currents (EPC) and miniature EPC (mEPC) were recorded by two-electrode muscle fiber membrane potential clamping with glass microelectrodes with resistance 3–5 MΩ filled with 2.5 M KCl. The method has been described in detail elsewhere [15, 16]. Contraction was prevented by incising the muscle. EPC were recorded by stimulating the motor nerve either with single impulses (one stimulus every 20 sec) or high-frequency trains of impulses (20 Hz).

Fluorescence microscopy. Recording of fluorescence. Fluorescence was detected using the CCD of a DP71 video camera (Olympus, Germany) and an Olympus BX51 (Japan) fitted with an Olympus LUMPLFL60^{xw} objective and a DSU confocal system. Fluorescence intensity was assessed in relative pixel brightness units (rel. units) (using the programs Cell^P and ImagePro).

Estimation of ChO-oxidized cholesterol. The method was based on estimation of the hydrogen peroxide (H_2O_2) concentration in the extracellular medium, this being a product of the reaction catalyzed by ChO. ChO oxidizes cholesterol to 4-cholesten-3-one, forming an equivalent quantity of H_2O_2 [21]. H_2O_2 is utilized by horseradish peroxidase in the oxidation of Amplex® Red to form the stable fluorescent product resorufin. Resorufin molecules were excited using light with a wavelength of 535 nm, with emission being recorded at 590/40 nm [24, 30]. Baseline H_2O_2 production was evaluated by incubating whole neuromuscular preparations in controls for 30 min in Ringer's solution containing Amplex® Red (400 ∝M) and HRP (2 activity units). This solution was then collected and baseline fluorescence was measured. Preparations were then perfused with normal physiological saline for 30 min. Neuromuscular preparations were then placed in physiological saline containing not only Amplex® Red (400 ∝M) and horseradish peroxidase (2 activity units), but also ChO (1 activity unit) for 30 min. After 30 min, solution was collected for analysis. Fluorescence was calibrated by measuring emission using solutions containing Amplex® Red, horseradish peroxidase, and defined H_2O_2 concentrations, and in media containing Amplex® Red, horseradish peroxidase, ChO, and defined concentrations of cholesterol in DMSO.

Identification of lipid rafts. Lipid rafts contain ganglioside GM1, which is a marker molecule for rafts. Rafts were identified using the cholera toxin B subunit (CT-B) conjugated with a fluorescence label (Alex Fluor 555), which irreversibly binds several ganglioside GM1 pentasaccharide chains simultaneously [8]. Neuromuscular preparations were placed in cold physiological saline containing CT-B (1 ∝g/ml) for 15 min, followed by incubation in cold physiological saline containing antibodies interacting with membrane-bound CT-B molecules for 20 min. Preparations were then illuminated at an excitation wavelength of 535 nm and red fluorescence was measured (emission filter 600–640 nm).

Studies of the processes of exo- and endocytosis of synaptic vesicles and use of fluorescent dyes. Experiments used fluorescent dyes FM1-43 and FM2-10 at concentrations of 3 and 20 ∝M, respectively. These dyes bind reversibly with the presynaptic membrane and, during endocytosis, appear within newly forming synaptic vesicles (they are "loaded" into nerve terminals) [3, 12, 18]. In this case, nerve terminals form a bright spot reflecting groups (clusters) of synaptic vesicles which have taken up the dye in the active zone. Naturally, fluorescence intensity is determined both by the number of vesicles undergoing exocytosis and the intensity of endocytosis processes. Dye "loading" experiments used long (3 min) and short periods (20 sec) of high-frequency stimulation (20 Hz). With the aim of decreasing baseline fluorescence, dye loading was followed by washing of

preparations for 15–20 min with Ringer's solution. When nerve terminals were initially loaded with dye, stimulation (20 Hz for 5 min or 2 Hz for 10 min) of the motor nerve led to release of dye into the synaptic cleft, along with the transmitter released by vesicle exocytosis. In this case, dye is "unloaded" from the nerve terminal and fluorescence intensity decreases. Baseline fluorescence was determined as the mean fluorescence intensity in a square of 50 · 50 pixels in a part of the image lacking nerve terminals. Fluorescence of nerve terminals was determined after subtraction of baseline fluorescence [29, 40]. Fluorescence intensity was evaluated as the mean fluorescence of all the pixels within the outline of an individual nerve terminal (nerve terminal fluorescence) or the mean fluorescence of all the pixels within the outline of individual spots (spot fluorescence). FM dye fluorescence was quenched in some experiments using sulforhodamine 101 (5–7 ∝M) [32].

Data were analyzed statistically using Origin Pro. Quantitative results are presented as mean ± standard error; *n* is the number of independent experiments. Significant differences were identified using Student's *t* test and ANOVA.

Results

ChO effectively oxidizes membrane cholesterol in frog neuromuscular preparations. Incubation of preparations for 30 min in medium containing horseradish peroxidase and Amplex® Red reagent led to the appearance of fluorescence in the test solution at a level of 25.7 ± 11.3 rel. units ($n = 5$), corresponding to baseline hydrogen peroxide production of ~13 nM. Exposure of preparations to ChO (1 activity unit) for 30 min in the presence of horseradish peroxidase and Amplex® Red reagent was followed by detection of brighter solution fluorescence – 140.8 ± 10.7 rel. units ($n = 5$, $p < 0.01$), corresponding to the formation of ~105 nM H_2O_2 (i.e., ChO led to the synthesis of 92 nM H_2O_2 in addition to the 13 nM H_2O_2 due to 30 min of endogenous production). Oxidation of cholesterol by ChO forms equivalent quantities of H_2O_2 and 4-cholesten-3-one [21], such that 30-min exposure to ChO led to the oxidation of about 92 nM cholesterol (0.007 mg of cholesterol per g of neuromuscular preparation). This conclusion was verified using another approach. Thus, in vitro studies using solution containing not only ChO, horseradish peroxidase, and Amplex® Red, but also 92 nM cholesterol, yielded fluorescence of 113.2 ± 5.3 rel. units ($n = 4$), which is close to the level of fluorescence recorded after exposure of preparations to ChO and subtraction of baseline fluorescence (140.8 – 25.7 = 115.1). These data provide evidence that ChO effectively oxidizes cholesterol in frog motor nerve terminals, though only a small fraction of the cholesterol undergoes oxidative modification.

Membrane cholesterol oxidation affects the stability of lipid rafts in nerve terminal membranes. In control preparations, the fluorescence brightness of nerve terminals stained with CT-B was 23.3 ± 1.2 rel. units and was about 45% ($n = 5$, $p < 0.05$) greater than the fluorescence of muscle fibers (Fig. 1, *A*). When the lipid raft visualization procedure was performed after 30-min exposure of preparations to ChO, there was no change in muscle fiber fluorescence, while the level of nerve terminal fluorescence was 17.5 ± 1.0 rel. units ($n = 5$, $p < 0.05$), which was 25% lower than that control level (Fig. 1, *B*). Thus, these data point to impairment of lipid raft stability in motor nerve terminals on exposure to ChO.

Cholesterol oxidation has no effect on spontaneous and suppresses evoked transmitter secretion. The effects of ChO (1 activity unit) on spontaneous transmitter secretion were assessed by recording mEPC. Exposure to ChO for 30 min had no significant effect on mEPC frequency. Exposure of neuromuscular preparations to ChO had no effect on the amplitude and rise time, but increased the half-decay time of mEPC to 133.0 ± 8.1% ($n = 7$, $p < 0.05$). Changes in the mEPC half-decay time may indicate that ChO has postsynaptic effects.

Evoked transmitter secretion was assessed by recording EPC in response to stimulation of the motor nerve. In this case, 30-min exposure of preparations to ChO led to a significant decrease in EPC amplitude to 89.0 ± 4.1% ($n = 7$, $p < 0.05$) of the initial level, and there was also an increase in the half-decay time, to 110.2 ± 3.1% ($p < 0.05$) (Fig. 2, *A*).

Our previous studies of frog neuromuscular synapses showed that low concentrations of exogenous H_2O_2 (less than 300 nM) did not affect transmitter secretion or the amplitude-time parameters of mEPC or EPC [15, 16]. This suggests that the effects of ChO (1 activity unit) described below are due to oxidation of cholesterol and not to the actions of H_2O_2.

Oxidation of cholesterol enhances depression of transmitter secretion with high-frequency stimulation. In control experiments, prolonged (3 min) high-frequency (20 Hz) stimulation of the motor nerve was accompanied by a characteristic change in EPC amplitude [1, 4, 29]. There was a gradual and almost linear decrease in EPC amplitude. EPC amplitude after 20 sec of stimulation was 89.5 ± 1.5% ($n = 6$) of initial, while values at 1 and 3 min were 62.5 ± 1.6% and 16.1 ± 4.0%, respectively (Fig. 2, *B*).

Following initial exposure of neuromuscular preparations to enzyme solution for 30 min, the dynamics of EPC amplitude in conditions of high-frequency stimulation changed significantly. As in controls, preparations incubated in ChO responded to high-frequency stimulation with a slight suppression of transmitter secretion in the first 20 sec – EPC amplitude decreased to 87–92% of initial. This was followed by a rapid decrease, and amplitude depression was more marked than in controls (Fig. 2, *B*). EPC amplitude decreased to 28.0 ± 3.5% and 4.1 ± 1.0% (n = 7) at 1 and 3 min, respectively.

The decrease in EPC amplitude during high-frequency stimulation is linked with a decrease in the number of transmitter quanta released in response to each sequential action

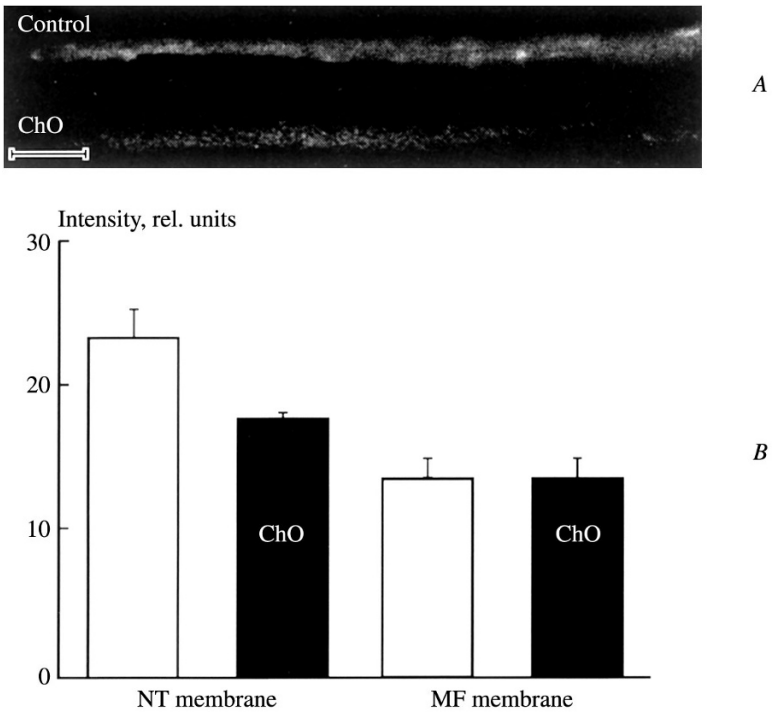

Fig. 1. Lipid rafts in a neuromuscular preparation. *A*) Fluorescence of areas of nerve terminals stained with cholera toxin B subunit (CT-B) conjugated with fluorescent label in controls and after 30-min incubation with ChO (muscle fiber fluorescence subtracted). The scale bar is 5 ∝m; *B*) nerve terminal (NT) and muscle fiber (MF) membrane fluorescence intensity in controls (white columns) and after exposure to ChO (black columns).

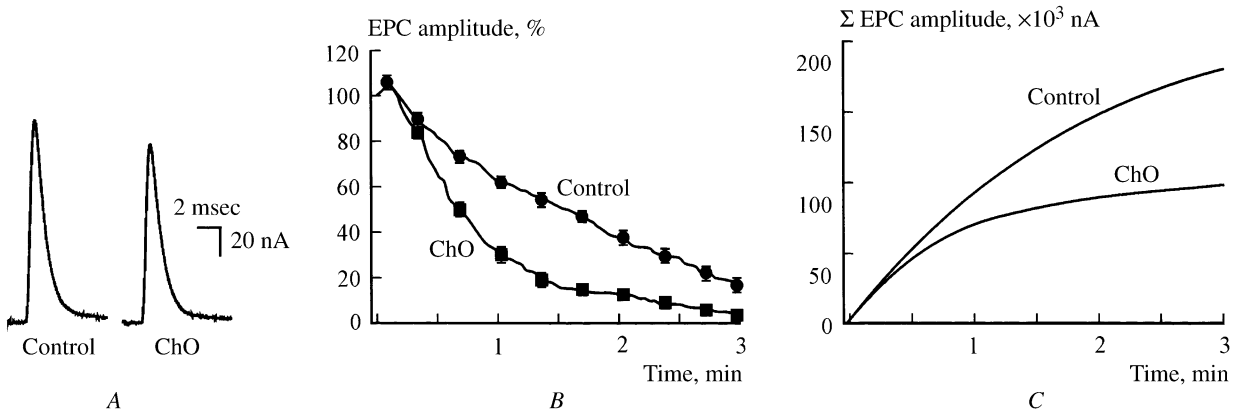

Fig. 2. Effects of cholesterol oxidation on evoked transmitter secretion. *A*) Native EPC in low-frequency stimulation (0.05 Hz) in controls and after 30-min incubation in ChO; *B*) dynamics of EPC amplitude during 3 min of stimulation at 20 Hz (squares show mean values for each 360th signal); *C*) cumulative curves of EPC amplitudes plotted on the basis of the data presented in *B*. The ordinates show EPC amplitude, % of the amplitude of the first EPC in the train (*B*) or the cumulative EPC amplitude (*C*), nA; the abscissa shows stimulation time, min.

potential. The value obtained by summing the EPC amplitude is therefore directly proportional to the number of transmitter quanta released during the stimulation period [4, 29]. The cumulative EPC amplitude curve (Fig. 2, *C*) shows an approximately twofold (by ~46%) decrease in the number of transmitter quanta released during high-frequency stimulation in preparations exposed to ChO as compared with controls.

Thus, oxidation of cholesterol in the presynaptic membrane decreases the probability of exocytosis in response to

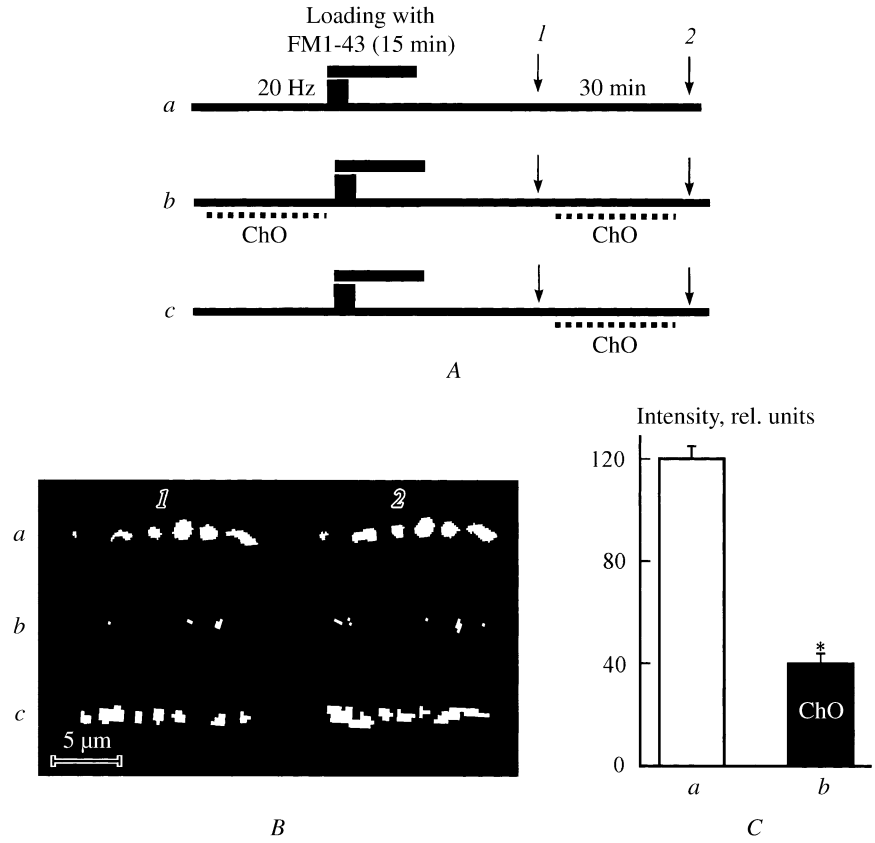

Fig. 3. Effects of cholesterol oxidation on FM1-43 loading into nerve terminals. *A*) Diagram of experiments. Black rectangles show stimulation for 3 min at 20 Hz; thick lines (above) show application of FM1-43; dotted lines (beneath) show the period of incubation with ChO; arrows labeled *1* and *2* show the moments at which fluorescence was recorded; *B*) images of parts of nerve terminals taken at time points *1* and *2*; *C*) fluorescence intensity of the nerve terminals loaded with FM1-43 using the protocols imaged in *a*, *b*, and *c*.

single stimuli and enhances depression of transmitter secretion during high-frequency activity.

Oxidation of cholesterol decreases FM1-43 dye loading in nerve terminals and induces dispersal of synaptic vesicle clusters. In control conditions (Fig. 3, *A*, *a*, *C*, *a*), the fluorescence intensity of dye-loaded nerve terminals (20 Hz – 3 min) was 120.1 ± 5.1 rel. units (n = 7), nerve terminal diameter was 1.3 ± 0.15 ∝M, and fluorescent spots (fluorescence brightness was 145.2 ± 4.1 rel. units) were relatively uniformly distributed across terminals at distances of 1–3 ∝M from each other and usually had a characteristic oval shape [3] (Fig. 3, *B*). After preliminary (30 min) exposure to ChO, FM1-43 loading was significantly less, such that nerve terminal fluorescence intensity was 40.0 ± 4.1 rel. units (n = 6) (Fig. 3, *B*, *b*, *C*, *b*). Nerve terminal diameter was no different from that in controls. These data provide evidence of a decrease in the number of newly formed (by endocytosis) synaptic vesicles in conditions of membrane cholesterol oxidation. In this case, it is likely that weakening of endocytosis induced by prolonged high-frequency stimulation is one of the causes of suppression of endocytosis (Fig. 2, *C*).

It is interesting to note that initially FM1-43-loaded nerve terminals in neuromuscular preparations exposed to ChO underwent a change in spot morphology (Fig. 3, *A*). Fluorescing spots became wider (from 1.3 ± 0.3 to 3.1 ± 0.5 ∝m in diameter), with the result that adjacent fluorescing groups of spots coalesced (Fig. 3, *B*). The mean fluorescence in spots showed some decrease (from 145.1 ± 4.2 to 128.2 ± 4.1 rel. units), while fluorescence within nerve terminals remained unaltered (values before and after exposure to ChO were 120.1 ± 5.1 and 118.4 ± 5.1 rel. units, n = 10). Thus, ChO led to "diffuse" fluorescence, arising from FM1-43-containing vesicles, indicating dispersal of clusters of synaptic vesicles.

Oxidation of cholesterol slows FM1-43 unloading, especially at the beginning of high-frequency stimulation. In controls, long-term high-frequency stimulation of the motor nerve in pre-loaded nerve terminals (Fig. 4, *A*) decreased fluorescence intensity in two phases – an initially rapid phase (by 18–21% at 15 sec of stimulation), followed by a slower phase. At 1 and 3 min of stimulation, fluorescence intensity dropped to 59.1 ± 2.1% and 42.0 ± 2.2%

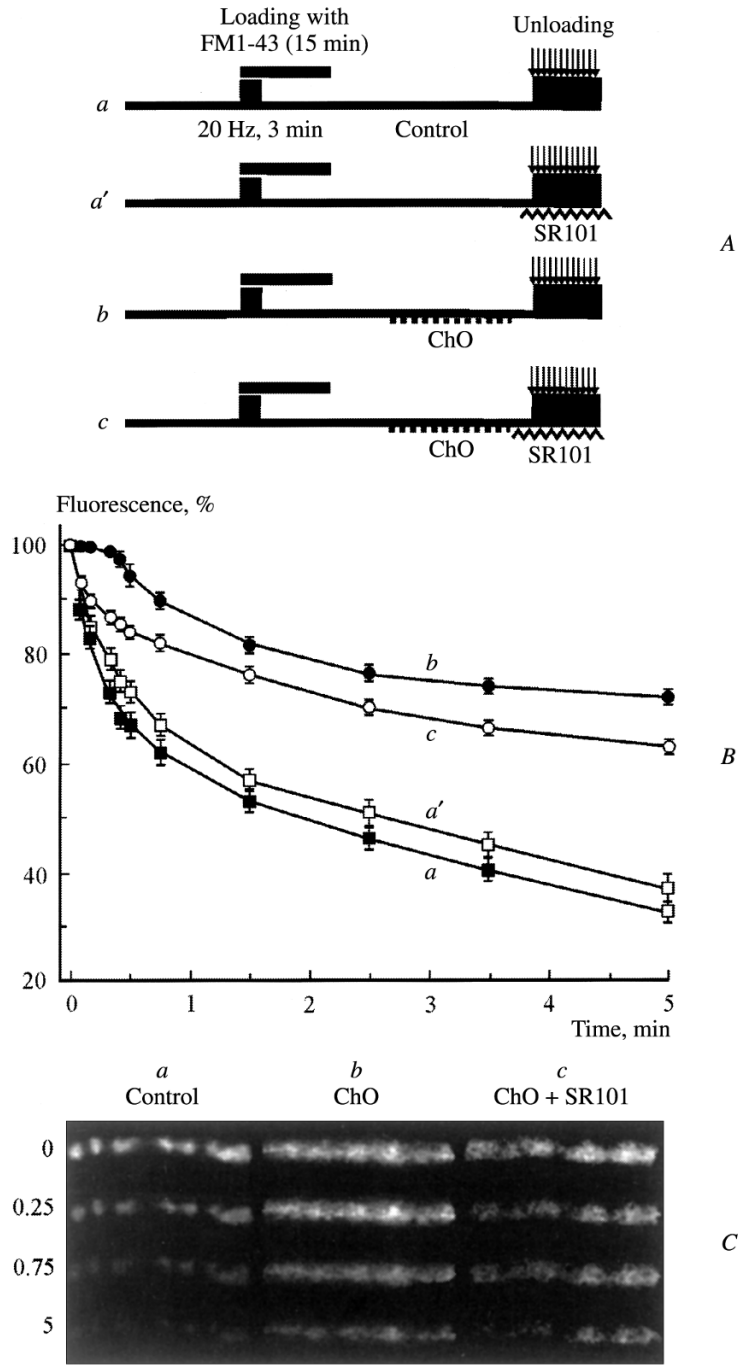

Fig. 4. Effects of cholesterol oxidation on FM1-43 unloading from motor nerve terminals. *A*) Diagrams of experiments, details as Fig. 3, *A*; the zigzag line show application of sulforhodamine 101 (SR101); *B*) dynamics of the decrease in neuron fluorescence (dye unloading) on stimulation (20 Hz) of nerve terminals preloaded with FM1-43. The ordinates show fluorescence intensity, % (fluorescence before stimulation was taken as 100%);); the abscissa shows stimulation time, min; *C*) fluorescence images of nerve terminals. The vertical axis shows stimulation time, min.

(n = 10), respectively (Fig. 4, *B*, *C*). Exposure of neuromuscular preparations to ChO after loading of terminals with dye (Fig. 4, *A*) completely prevented dye unloading at the beginning of high-frequency stimulation: fluorescence at 15 sec of stimulation was 99.1 ± 1.0% (Fig. 4, *B*, *C*). However, at 20–30 sec from the beginning of stimulation, there was a decrease in fluorescence brightness, though this was less marked than in controls. Thus, at 1 and 3 min of stimulation, fluorescence brightness decreased to 87.1 ± 1.5% and 76.0 ± 1.7% (n = 10), respectively. Thus, after exposure to

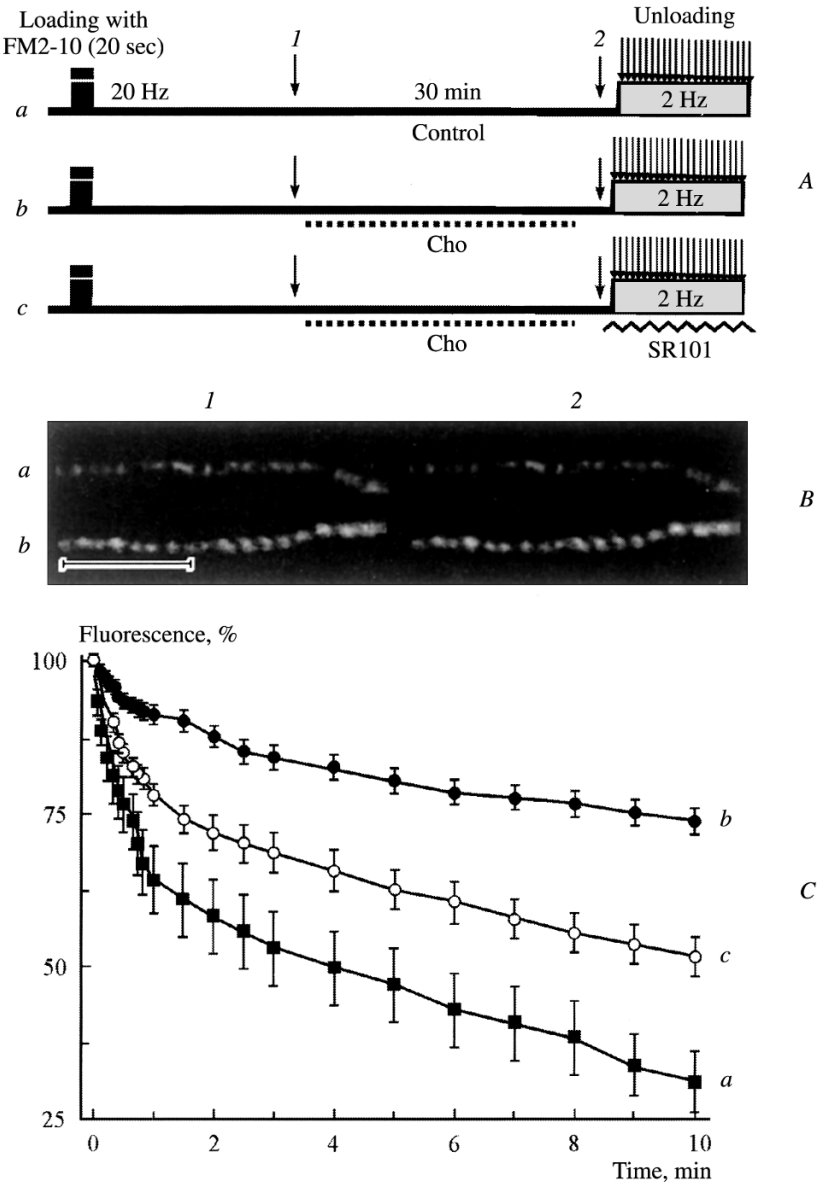

Fig. 5. Effects of cholesterol oxidation on the functioning of synaptic vesicles of the recycling pool. *A*) Diagrams of experiments, details as in Fig. 3, *A*. The zigzag line shows application of sulforhodamine 101 (SR101). Gray rectangles show stimulation at 2 Hz; *B*) images of parts of nerve terminals before (*1*) and after 30-min incubation (*2*) in physiological saline (*a*) or solution containing ChO (*b*, *c*). Scale bar – 10 ∝m; *C*) dynamics of decreases in nerve terminal fluorescence brightness (FM2-10 unloading) during stimulation at 2 Hz. The ordinate shows fluorescence intensity, % (fluorescence before stimulation was taken as 100%); the abscissa shows stimulation time, min.

ChO, stimulation at a frequency of 20 Hz for 3 min released about 60% less dye than in controls. Thus, oxidation of cholesterol suppresses synaptic vesicle exocytosis in conditions of prolonged high-frequency stimulation.

Sulforhodamine 101 (an FM1-43 quencher) eliminates slowing of the reduction in nerve terminal fluorescence at the beginning of high-frequency stimulation on oxidation of cholesterol. Sulforhodamine 101 is a small (about 1 nm in diameter) water-soluble molecule which can interact with FM1-43 inserted into a membrane, instantly quenching the fluorescence of the latter [32, 38]. In controls, the presence of sulforhodamine 101 in the extracellular medium (Fig. 4, *A*) had no significant effect on the dynamics of the decrease in FM1-43 fluorescence. Thus, fluorescence decreased by 15–20% in the first 15 sec of high-frequency stimulation, and then to 65.0 ± 2.1% and 47 ± 2.3% at 1 and 3 min ($n = 7$) respectively. (Fig. 4, *B*). When previously loaded nerve terminals were exposed to ChO for 30 min, followed by initiation

of perfusion with solution containing sulforhodamine 101 (Fig. 4, *A*), the nature of the drop in FM1-43 fluorescence on stimulation changed significantly (Fig. 4, *B*, *C*). A sharp decrease in the green fluorescence of FM1-43 was seen at the beginning of high-frequency stimulation: fluorescence at 15 sec decreased to 88.1 ± 1.0% ($n = 7$). The decrease in fluorescence then slowed, such that FM1-43 fluorescence at 1 and 3 min was 80.0 ± 1.2% and 68.1 ± 1.5%, respectively.

The recycling vesicle pool and the effects of cholesterol oxidation. The recycling synaptic vesicle pool in frog neuromuscular preparations accounts for 10–20% of the total number of vesicles and can support neurotransmission for long periods of stimulation at 1–2 Hz but is quickly depleted over a period of 10–20 sec in response to high-frequency stimulation (20–30 Hz) [4, 29, 33].

This series of experiments addressed the effects of ChO on the processes of exo- and endocytosis of vesicles of the recycling pool. The endocytosis marker FM2-10 was used – this is less hydrophobic than FM1-43 and therefore dissociates from superficial membranes more quickly [5, 33]. Selective dye was loaded into recycling pool vesicles by inducing exocytosis with a 20-sec burst of stimulation at 20 Hz, during which the external solution contained FM2-10 (Fig. 5, *A*). When stimulation was complete, the neuromuscular preparation was perfused with physiological saline. In controls, the fluorescence of nerve terminals loaded as described was 20.2 ± 2.0 rel. units ($n = 12$) (Fig. 5, *B*). Prior 30-min exposure of preparations to ChO almost completely prevented loading with FM2-10; in this case, fluorescence intensity was 3.2 ± 2.0 rel. units ($n = 6$, $p < 0.001$).

Nerve terminals with loaded recycling pools were subjected to low-frequency stimulation (2 Hz), and there was a decrease in the intensity of fluorescence, characterizing the exocytosis of loaded vesicles and loss of dye from them. In controls, stimulation at 2 Hz led initially to a rapid reduction in fluorescence of spots: brightness at 1 min of stimulation decreased to 64.0 ± 5.5% ($n = 9$) (Fig. 5, *C*). The drop in fluorescence intensity then decreased; intensity by 10 min of stimulation decreased to 30.9 ± 5%. Exposure to FM2-10-preloaded preparations to ChO significantly slowed dye unloading. Fluorescence brightness over 1 min of stimulation decreased only to 91.2 ± 1.4% ($n = 10$) and fluorescence intensity at 10 min was 73.8 ± 2.1% (Fig. 5, *C*). It is interesting to note that exposure to ChO was not followed by any increase in the area of spots, indicating the absence of dispersal of vesicles of the recycling pool (Fig. 5, *B*).

When enzymatic oxidation of cholesterol was followed by dye unloading and stimulation in the presence of sulforhodamine 101 in the extracellular solution (Fig. 5, *A*), the decrease in nerve terminal fluorescence brightness occurred significantly more quickly. Thus, fluorescence decreased to 78.1 ± 1.7% at 1 min of stimulation ($n = 8$) and to 51.5 ± 2.0% at 10 min (Fig. 5, *C*).

Thus, oxidation of cholesterol acted on the mechanism of transmitter release by vesicles of the recycling pool. The vesicles of this pool did not disperse within the nerve terminal after exposure to ChO.

Discussion

Effects of cholesterol oxidation on the processes of exocytosis and delivery of vesicles to exocytosis sites. Cholesterol in the presynaptic membrane takes part in the functioning of protein complexes supporting the docking and priming of synaptic vesicles [5, 6]. On the one hand, cholesterol can bind directly with exocytosis proteins, such as synaptobrevin and syntaxin [20, 27, 36, 37]. On the other hand, cholesterol, along with sphingomyelin, forms lipid rafts at exocytosis sites, and these function as platforms for the concentration and activation of proteins of the SNARE complex, which are involved in the fusion of vesicles with the presynaptic membrane [6, 8]. Partial displacement of cholesterol by 1 mM methyl-β-cyclodextrin from nerve terminal membranes leads to suppression of evoked transmitter secretion and enhancement of depression during high-frequency activity [7, 9, 22, 30, 39]. The appearance of oxidized derivatives of cholesterol (4-cholesten-3-one) in model membranes affects the ability of cholesterol to interact with proteins and lipids. 4-Cholesten-3-one may be able to alter the phase state of the membrane [10]. In our experiments, staining of preparations for accumulations of ganglioside GM1 revealed selective decreases in the numbers of lipid rafts in nerve terminals after exposure to ChO (Fig. 1). This may be the cause of the suppression of transmitter secretion and exocytosis of synaptic vesicles in response to single stimuli in conditions of plasma membrane cholesterol oxidation (Fig. 2, *A*). Suppression of exocytosis was more dramatic in conditions of high-frequency stimulation, starting from 20–30 sec of high-frequency stimulation (Fig. 2, *B*, *C*). Considering that in frogs, release of transmitter after 20–30 sec of stimulation at 20 Hz mainly involves vesicles of the reserve pool [1, 4, 29, 33], it can be suggested that there is an impairment to the delivery of reserve pool vesicles to exocytosis sites after exposure to ChO. The slowing of dye loading after 20–30 sec of high-frequency stimulation is evidence for weakening of transport of synaptic vesicles to the release-ready pool (Fig. 4, *B*).

Lipid rafts include many signal molecules, so damage to rafts induces changes in intracellular signaling (activation of a number of protein kinases) and consequent rearrangement of the cytoskeleton [14, 34]. It is possible that impairment of vesicle transport is associated with impairment of the cytoskeleton, which in natural conditions does not allow vesicles to "scatter" to the edges and supports the formation of clusters of reserve pool vesicles. This is supported by the enlargement of synaptic vesicle clusters after exposure to ChO (Fig. 3, *B*), pointing to movement of a portion of vesicles away from exocytosis sites in the active zone. Analogous impairments to vesicle clustering in frog neuromuscular synapses and hippocampal synapses is seen in response to the phosphatase inhibitor okadaic acid, which also leads to widening of FM1-43 fluorescence areas and suppression of transmitter release in response to high-fre-

quency stimulation [12, 14, 18]. Increases in the sizes of fluorescing spots, reflecting dispersal of vesicle clusters, may occur as a result of excessive activation of various protein kinases. Thus, dispersal of clusters of synaptic vesicles is seen on the background of phosphorylation of synapsins by MAP- and cyclin-dependent kinases, synapsins being proteins involved in the organization of intervesicular interactions [25]. It is interesting to note that the "blurring" of spots after incubation in ChO was not seen if the selectively recycling pool of vesicles in nerve terminals was labeled with dye FM2-10 (Fig. 5, *B*). This suggests impairment to vesicle clustering affecting only the reserve pool after oxidation of membrane cholesterol.

Cholesterol oxidation and the kiss-and-run mechanism of transmitter release. After exposure to ChO, the dynamics of transmitter secretion during the first 20 sec of high-frequency activity were no different from controls (Fig. 2, *B*). However, there was no FM1-43 dye unloading in the first 20 sec of high-frequency stimulation of ChO-treated preparations. (Fig. 4, *B*). In other words, after oxidation of cholesterol (at the beginning of high-frequency stimulation), transmitter release from synaptic vesicles occurs when molecules of the fluorescent dye, with a membrane dissociation time of the order of tens of seconds, are absent. This phenomenon can be explained if we suggest that recycling pool vesicles operating at the beginning of high-frequency stimulation release transmitter through short-lived protein pores (the kiss-and-run mechanism), which prevents lateral diffusion of lipophilic FM1-43 molecules. Thus, the suggestion that the kiss-and-run mechanism operates in motor nerve terminals and hippocampal terminals was based on the fact that hyperosmotic sucrose solutions increase spontaneous transmitter release not accompanied by FM1-43 unloading [2, 35]. Possible candidates for the role of pore-forming proteins are syntaxin, synaptobrevin, and the V0 part of the proton pump [5, 19, 26]. These results are supported by experiments using sulforhodamine 101. This substance, because of its small size, can pass through pores of diameter greater than 1.5 nm. In all probability, sulforhodamine 101 molecules, entering vesicle cavities through short-lived pores, bind with F1-43 molecules inserted into the membranes of synaptic vesicles and quench the green fluorescence of the dye [38]. This accelerates the decrease in the level of green FM1-43 fluorescence at the beginning of high-frequency stimulation (Fig. 4, *B*, *C*).

Studies to verify this suggestion consisted of additional experiments with the more hydrophilic dye FM2-10, which was used to load the recycling pool [33] (Fig. 5, *A*). Prior exposure to ChO almost completely prevented the uptake of FM2-10 into nerve terminals. This indicates release of transmitter over a period of 20 sec of high-frequency stimulation through pores impermeable to FM2-10 molecules. When preparations preloaded with FM2-10 were exposed to ChO, subsequent dye unloading induced by low-frequency stimulation (2 Hz), which selectively stimulates the exocytosis of vesicles of the recycling pool, occurred much more slowly than in controls. On the background of sulforhodamine 101, the decrease in FM2-10 fluorescence was significantly accelerated (Fig. 5, *C*). This also points to quenching of emissions from FM2-10 molecules inserted into vesicle membranes by sulforhodamine 101 molecules passing through the pore into the vesicle cavity on exocytosis.

Discussion of the existence of the kiss-and-run mechanism for transmitter release in presynaptic nerve terminals in physiological conditions continues [2, 5, 17, 19, 35]. Changes in the mechanism of transmitter release on exposure to ChO may be associated with changes in intracellular signaling. It has been suggested that phosphorylation (for example by protein kinase C) of chaperone NSF, which supports the disassembly of the SNARE complex, or complexins, which stabilize the SNARE complex, may regulate the mechanism of exocytosis [5, 23] associated with the transient formation of fusion pores. In addition, myosin II, which is involved in enlarging fusion pores in chromaffin cells, also has several sites for phosphorylation by protein kinases [28]. The cholesterol molecule is important for stabilizing fusion pores and interacts directly with the transmembrane domain of synaptobrevin, which forms the pore [37]. It may be that oxidation of cholesterol sharply decreases the rate of enlargement of fusion pores, increases their lifetime and the probability of closure, and triggers the kiss-and-run mechanism. On oxidation of membrane cholesterol, the kiss-and-run mechanism uses only some of the vesicles. This may be associated with the fact that vesicles of the recycling pool have a unique protein-lipid composition, such that in certain conditions they can release transmitter through transiently existing pores.

It should be noted that FM1-43 and FM2-10 unloading from ChO-treated nerve terminals is partial, and that a significant portion of the dye remains in the nerve terminals, while the decrease in the fluorescence of both dyes is more marked on the background of sulforhodamine 101 (Fig. 4, *B*; Fig. 5, *C*). Recycling pool vesicles may, after releasing transmitter, continue to occupy exocytosis sites for a prolonged period at the beginning of high-frequency stimulation, while reserve pool vesicles are delivered to their own exocytosis sites. This supports the hypothesis that there are separate exocytosis sites and recycling pathways for vesicles of the recycling and reserve pools [1].

Oxidation of cholesterol and the endocytosis of synaptic vesicles. Prior enzymatic oxidation of membrane cholesterol led to an almost threefold decrease in uptake of dye FM1-43 induced by prolonged high-frequency stimulation (Fig. 3, *C*). However, this decrease in the intensity of endocytosis processes can be completely explained by changes in intensity and mechanism of synaptic vesicle exocytosis. Firstly, suppression of the process of exocytosis on long-lasting high-frequency stimulation after exposure to ChO (by about 46%) should lead to an equivalent decrease in label loading (Fig. 2, *C*). In addition, if vesicles of the recy-

cling pool (accounting for 10–20% of the total population of synaptic vesicles [1, 29, 33]) release transmitter via short-lived fusion pores (the kiss-and-run mechanism, Fig. 4, *B*) and do not take up FM1-43, dye loading into nerve terminals in high-frequency activity will be even smaller [2, 35]. This leads to the suggestion that oxidation of presynaptic membrane cholesterol has no significant direct effect on the mechanism of endocytosis of synaptic vesicles. This is consistent with previous data from our laboratory demonstrating the minor effects of displacement of cholesterol from the presynaptic membrane on synaptic vesicle endocytosis, the occurrence of which depends on vesicular cholesterol [7, 13, 30].

Thus, oxidation of a small amount of cholesterol destabilizes lipid rafts in motor nerve terminals and induces significant changes in synaptic transmission. The probability of synaptic vesicle exocytosis decreases and the transportation of reserve pool vesicles to exocytosis sites is suppressed, probably because of impairment to their clustering. It has also been suggested that recycling pool vesicles start to use the kiss-and-run mechanism for transmitter release. Thus, not only the presence of cholesterol [6, 7, 9, 13, 22, 30, 34, 36, 37, 39], but also its state, affect exocytosis processes and the delivery of vesicles to the active zone. The data obtained here provide the first evidence of changes in the presynaptic vesicle cycle induced by the appearance of oxidized forms of cholesterol in the membrane. Considering that cholesterol oxidation in natural conditions is constantly occurring in synaptic membranes [31], 4-cholesten-3-one can be regarded as a new potential endogenous regulator of the synaptic vesicle cycle. The molecular mechanisms of these effects of oxidation of membrane cholesterol on the presynaptic vesicle cycle require further study.

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