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OPPOSITE MODULATION OF TIME COURSE OF QUANTAL RELEASE IN TWO PARTS OF THE SAME SYNAPSE BY REACTIVE OXYGEN SPECIES

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Abstract—Reactive oxygen species (ROS) are potent regulators of transmitter release in chemical synapses, but the mechanism of this action remains almost unknown. Presynaptic modulation can change either the release probability or the time course of quantal release, which was recently recognized as an efficient mechanism determining synaptic efficiency. The nonuniform structure and a big size of the frog neuromuscular junction make it a useful model to study the action of ROS in compartments different in release probability and in time course of transmitter release. The time course (or kinetics) of quantal release could be estimated by measuring the dispersion of the synaptic delays for evoked uniquantal endplate currents (EPCs) under low release probability. Using two-electrode recording technique, the action of ROS on kinetics and release probabilities were studied at the proximal and distal parts within the same neuromuscular junction. The stable ROS hydrogen peroxide (H_2O_2) increased the dispersion of synaptic delays of EPCs (i.e. desynchronized quantal release) within the distal part but decreased delay dispersion (synchronized quantal release) within the proximal part of the same synapse. Unlike the opposite modulation of kinetics, H_2O_2 reduced release probability in both distal and proximal parts. Since ATP is released from motor nerve terminals together with acetylcholine and can be involved in ROS signaling, we tested the presynaptic action of ATP. In the presence of the pro-oxidant Fe^{2+}, extracellular ATP, similarly to H_2O_2, induced significant desynchronization of release in the distal regions. The antioxidant N-acetyl-cysteine attenuated the inhibitory action of ATP on release probability and abolished the action of H_2O_2 and ATP in the presence of Fe^{2+}, on release kinetics. Our data suggest that ROS induced during muscle activity could change the time course of transmitter release along the motor nerve terminal to provide fine tuning of synaptic efficacy.

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Abbreviations: EPCs, end-plate currents; H_2O_2, hydrogen peroxide; *m*, quantal content; MEPCs, miniature end-plate currents; NAC, N-acetylcysteine; NSF, N-ethylmaleimide-sensitive factor; ROS, reactive oxygen species; SNARE, Souble NSF attachment receptor.

Key words: reactive oxygen species, neuromuscular junction, transmitter release, ATP, acetylcholine.

Reactive oxygen species (ROS) are currently recognized as important signaling molecules both in the central and peripheral nervous systems (Kovacic and Hall, 2010). ROS could be generated by mitochondrial respiration or via multiple oxidases, for instance, by the NADPH oxidase (Michaelson et al., 2010). In chemical synapses, the presynaptic transmitter releasing machinery represents the most redox-sensitive part (Giniatullin et al., 2006). While superoxide is the primary ROS, hydrogen peroxide (H_2O_2), which is generated from the superoxide by superoxide dismutase (SOD) appears to be the most stable and diffusible form of ROS that can easily cross cell membranes (Bienert et al., 2006). H_2O_2 was shown to reduce the level of spontaneous and evoked transmitter release both in amphibian (Giniatullin and Giniatullin, 2003) and mammalian synapses (Giniatullin et al., 2006). In the presence of divalent iron cations such as Fe^{2+}, H_2O_2 is transformed into the strongest oxidant, hydroxyl radical (Klebanoff et al., 1989).

Independent of release probability, the kinetics of vesicle release has emerged recently as an efficient mechanism to control the amplitude of postsynaptic currents and the efficiency of synaptic transmission (Lin and Faber, 2002). Thus, catecholamines or cholinergic agents, change the amplitude of end-plate currents via modulation of the transmitter release kinetics (Bukcharaeva et al., 1999; Bukharaeva et al., 2002; Samigullin et al., 2003a). In a large synapse, like the neuromuscular junction, which is composed of up to 500 active zones, different compartments have their own functional properties (Robitaille and Tremblay, 1987). The distributions of synaptic delays of quantal release that reflect the kinetics or the time course of quantal secretion, differ between the proximal and distal regions of the frog motor nerve terminal (Bukharaeva et al., 2002; Nikolsky et al., 2004; Samigullin et al., 2003b). In the proximal region the dispersion of quantal release is more pronounced than in the distal region (Samigullin et al., 2003b). Effect of various drugs on a synapse may depend on the initial degree of synchronization of quantal release. It has been found that noradrenaline synchronizes the release in the proximal regions in which the release is originally the most dispersed, but is much less effective in the distal regions, which are characterized by synchronous release (Bukharaeva et al., 2002). Thus, the long and linear structure of the frog end-plate provides a possibility

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of comparing the effects of exogenous or endogenous modulators in the distinct parts of the same synapse.

Factors which determine kinetics of transmitter release are largely unknown. However, it has been shown that the inactivation of the presynaptic fusion protein Snap25 with the botulinum toxin type A is associated with a reduced release rate (Sakaba et al., 2005). Snap25, unlike other soluble NSF attachment receptor (SNARE) proteins, demonstrated selective sensitivity to ROS (Giniatullin et al., 2006). We showed previously that inhibition of evoked multiquantal currents by H_2O_2 was accompanied by prolongation of their decays, suggesting redox modulation of release kinetics (Giniatullin and Giniatullin, 2003). In this study, we directly address this issue by measuring synaptic delays of uni-quantal end-plate currents (EPCs) in the distal and proximal parts of the same synapse. We found that, while H_2O_2 indeed desynchronized quantal release in distal parts, secretion of individual quanta in the proximal parts of the very same junction was synchronized. In the presence of the pro-oxidant Fe^{2+}, the ubiquitous neuromodulator ATP induced significant desynchronization of quantal release in distal regions. The antioxidant N-acetylcysteine (NAC) abolished the effect of ATP in the presence of divalent iron on release kinetics.

EXPERIMENTAL PROCEDURES

Preparation and solutions

Experiments were carried out *in vitro* at the room temperature on the cutaneous pectoris neuromuscular preparations isolated from the frog *Rana ridibunda* (for details see Bukcharaeva et al., 1999; Nikolsky et al., 2004). Briefly, animals were anesthetized with ether and sacrificed by pithing and decapitation. All procedures were performed in accordance with the European Communities Council Directive (24th November 1986; 86/609/EEC). The muscle preparation was pinned to the bottom of the flow chamber (volume 3.5 ml) and superfused (3 ml/min) with the Ringer solution containing (in mM): NaCl 113.0, KCl 2.5, $CaCl_2$ 0.3, $NaHCO_3$ 3.0, and $MgCl_2$ 4.0, pH was adjusted to 7.3 with HCl. Drugs (all from Sigma-Aldrich, St. Louis, MO, USA) were applied via the superfusing solution and the measurements started 20 min after drug application. ATP and $FeSO_4$ were used at concentrations of 100 μM while the standard concentration of H_2O_2 was 300 μM.

Electrophysiology

Nerve action potentials and EPCs were elicited by application of supramaximal stimuli (0.1 ms duration, every 2 s) to the motor nerve through a pair of platinum electrodes located in a small adjacent moist compartment which allowed us to minimize the stimulus artifact. Two Ringer-filled extracellular glass micropipettes (1–3 MΩ resistance) were positioned under visual control (magnification 256×) in the proximal part (~3–5 μm from the end of the myelinated segment, Electrode 1 in Fig. 1) and in the distal part (~90–120 μm, Electrode 2 in Fig. 1) of the nerve terminal. In the proximal part the nerve action potential had a typical shape (Mallart, 1984, Fig. 1A), different from the action potential in the distal part which served as an additional criterion for electrode positioning. The recorded responses were filtered (bandpass 0.03–10 kHz) and digitized at 3-μs intervals. Estimating the time course of the release of individual quanta from the value of the dispersions of the synaptic delays required measuring the uni-quantal endplate currents (Katz and Miledi, 1965). Therefore, experiments were carried out in the presence of 0.3 mM Ca^{2+} and

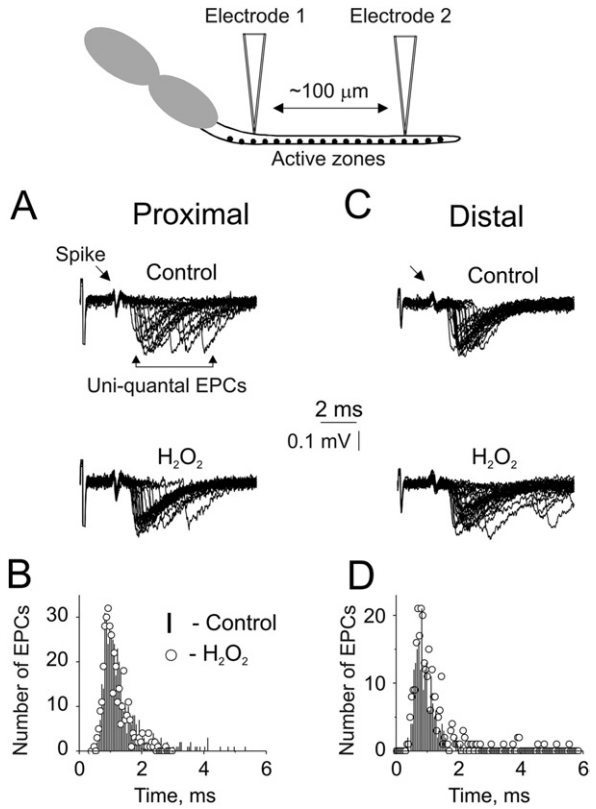

Fig. 1. Position of electrodes and effects of H_2O_2 (300 μM) in the proximal and distal parts of the nerve terminal. Top: a schematic presentation of electrode positions along the nerve terminal (Electrode 1—in the proximal, Electrode 2—in the distal part). Black spots indicate active zones for transmitter release. (A, C) Motor nerve spikes (arrows) and uni-quantal end-plate currents (EPCs), in control and in the presence of H_2O_2 in the proximal (A) and distal (C) parts of the nerve terminal. Superposition of 27–30 signals. (B, D) The distribution of synaptic delays in control (black lines) and after application of H_2O_2 (open circles) in the proximal (B) and distal (D) parts of the nerve terminal recorded from a single synapse. The bin width is 0.05 ms.

4.0 mM Mg^{2+}. The mean quantal content (m) was determined from the equation: $m=\ln N/n_o$, where N is the total number of stimuli and n_o is the number of synaptic failures (Del Castillo and Katz, 1954). To obtain reliable EPC measurements, in each experiment up to 2500 stimuli were applied to the motor nerve before and after treatment. From 60 to 100 miniature end-plate currents (MEPCs) were recorded before stimulation (control) and after drug applications to calculate the mean frequency of spontaneous release.

Synaptic delays measurements and analysis

Synaptic delays of uni-quantal EPCs were estimated as the time between the peak of the presynaptic sodium spike and the time point corresponding to the 20% rise phase of the EPC (Katz and Miledi, 1965). Because amplitudes of the sodium currents decrease along the nerve terminals (Mallart, 1984), the distal recording sites were selected so that the sodium spikes, from which the delays were measured, were clearly distinguished from the noise. With low Ca^{2+}/high Mg^{2+} solution the mean quantal content was 0.44 ± 0.07 predicting (according to Poisson distribution) the number of two-quantal EPCs $<8\%$ of all responses. In cases of double release, synaptic delays for both quanta were calculated. The stability of electrode position is crucial during long-lasting extra-