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Kinetics of neurotransmitter release in neuromuscular synapses of newborn and adult rats

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ABSTRACT

The kinetics of the phasic synchronous and delayed asynchronous release of acetylcholine quanta was studied at the neuromuscular junctions of aging rats from infant to mature animals at various frequencies of rhythmic stimulation of the motor nerve. We found that in infants 6 (P6) and 10 (P10) days after birth a strongly asynchronous phase of quantal release was observed, along with a reduced number of quanta compared to the synapses of adults. The rise time and decay of uni-quantal end-plate currents were significantly longer in infant synapses. The presynaptic immunostaining revealed that the area of the synapses in infants was significantly (up to six times) smaller than in mature junctions. The intensity of delayed asynchronous release in infants increased with the frequency of stimulation more than in adults. A blockade of the ryanodine receptors, which can contribute to the formation of delayed asynchronous release, had no effect on the kinetics of delayed secretion in the infants unlike synapses of adults. Therefore, high degree of asynchrony of quantal release in infants is not associated with the activity of ryanodine receptors and with the liberation of calcium ions from intracellular calcium stores.

1. Introduction

During postnatal neuromuscular development, the plasticity of synaptic transmission at the pre-and postsynaptic level is mostly realized by modifications of postsynaptic acetylcholine receptors (Fournier et al., 1991; Zucker and Regehr, 2002; Shi et al., 2012), changing the size and number of acetylcholine (ACh) quanta released by the nerve stimulation (Van der Kloot and Molgó, 1994; Slater, 2008) and also by massive non-quantal ACh release during the synapse elimination period (Vyskočil et al., 2009). Another mechanism for regulating synaptic transmission is the time delay between the presynaptic spike and the release of individual quanta, which accumulate over time to form the final postsynaptic end-plate current (EPC) (Katz and Miledi, 1965; Lin and Faber, 2002). The kinetics of quantal release at the neuromuscular synapses can be regulated by various physiologically active compounds and their receptors (Samigullin et al., 2012), including cholinergic (Nikolsky et al., 2004), adrenergic (Bukcharaeva et al., 1999; Bukharaeva et al., 2002) and purinergic ones (Tsentsevitsky et al., 2011). Also the frequency and number of nerve stimulation might influence the synaptic latency of a single quantum release (Kovyazina et al., 2010). These changes in the kinetics of quanta secretion (or synaptic latencies) affect the amplitude and time course of the integral EPC and modulate the efficiency of synaptic transmission (Slater, 2008; Kovyazina et al., 2010).

Presynaptic action potential evokes at least two kinetically distinguishable modes of neurotransmitter release (Hagler and Goda, 2001). One is the phasic release of many quanta, which results in an almost synchronous activation of the postsynaptic receptors, and this is followed by an asynchronous release that occurs up to hundreds of ms after nerve stimulation (Katz and Miledi, 1968; Goda and Stevens, 1994; Hestrin and Galarreta, 2005).

The initial phasic release is fast but not absolutely synchronous. At room temperature, the quantal release usually begins 0.2–0.8 ms after the peak of the action potential. This shortest 5% of all latencies is called the minimal synaptic latency. Latency distribution then rapidly reaches a maximum at about 3 ms and decays over the next several milliseconds (Barrett and Stevens, 1972; Van der

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Kloot, 1988). In addition to this phasic, relative synchronous neurotransmitter secretion, there is a delayed asynchronous release of not more than 0.01–1% of the total number of secreted quanta (Rahamimoff and Yaari, 1973; Atluri and Regehr, 1998). There is also the intermediate component of quantal release which is observed 3–8 ms after the action potential (Chen and Regehr, 1999). This component is referred to as the late phasic release (Barrett and Stevens, 1972; Bukharaeva et al., 2007) since these quanta can still take part in the formation of the integral EPC (Van der Kloot, 1988).

The physiological significance of delayed asynchronous release is not completely clear. It has been shown in central synapses that it can participate in ensuring the plasticity of the synaptic contacts which form the neuronal network (Hagler and Goda, 2001; Otsu et al., 2004). Asynchronous release might maintain an excitatory or inhibitory tone during high-frequency synaptic transmission (Lu and Trussell, 2000; Iremonger and Bains, 2007; Daw et al., 2009) and also might provide a bidirectional activity-dependent modification of synaptic efficacy (Ali and Todorova, 2010; Chang and Mennerick, 2010).

Both phasic and asynchronous delayed release are Ca^{2+}-dependent processes, although driven by different mechanisms (Kirischuk and Grantyn, 2003; Hui et al., 2005; Dudel, 2009; Vasin et al., 2010; Yoshihara et al., 2010). Systems that control the intracellular Ca^{2+} concentration, including Ca^{2+}-induced Ca^{2+} release from intracellular stores mediated by the ryanodine receptor are involved in the modulation of delayed asynchronous release (Nishimura et al., 1990; Narita et al., 1998; Bouchard et al., 2003; Smith et al., 2012). The vast number of studies on the kinetics of secretion were performed either at the synapses of adult animals (Barrett and Stevens, 1972; Bukharaeva et al., 2002, 2007; Dudel, 2009) or at the synapses of the central nervous system, which are mainly used in tissue cultures (Chang and Mennerick, 2010) or in brain slices of newborn animals (Atluri and Regehr, 1998; Daw et al., 2009; Ali and Todorova, 2010).

In the neuromuscular synapse, the delayed asynchronous release during rhythmic stimulation and the role of ryanodine- sensitive Ca^{2+}-dependent Ca^{2+} release over the course of maturation had not yet been studied. This was the aim of this investigation, in which we analyzed the kinetics of the phasic and delayed asynchronous release of ACh quanta at various frequencies of rhythmic stimulation of the motor nerve in rats at different stages of postnatal ontogenesis. We found that in infants 6 (P6) and 10 (P10) days after birth there is a marked asynchronous phase of quantal release, along with a reduced number of quanta compared to the synapses of adults. The intensity of delayed asynchronous release in infants increased with the frequency of stimulation more than in adults. A blockade of the ryanodine receptor in the endoplasmic reticulum, which can contribute to the formation of delayed asynchronous release in adults, had no effect on the kinetics of delayed secretion in the infants. Therefore a Ca^{2+}-induced Ca^{2+} release system (CICR) is not taking part in the attenuation of Ca^{2+}-dependent quantal release at least up to 10 days after birth.

2. Materials and methods

2.1. Experimental animals and drugs

Experiments were performed on isolated phrenic nerve–diaphragm preparations from Wistar rats of both sexes 6 days (P6), 10 days (P10) after birth and adult ones at the age of 10–12 weeks. Animals were anesthetized with ether before being decapitated in accordance with the European Communities Council Directive (November 24, 1986; 86/609/EEC). The choice of 6- and 10-day animals was due to the fact that in the earlier postnatal periods (1–5 days) it was very difficult to accumulate the necessary number of uniquantal responses for the correct analysis of synaptic latencies. In animals at later stages (15–20 days), there was already no significant difference in the release pattern compared to older animals, according to several pilot experiments (data not shown).

The preparations were pinned to the bottom of a 3.5 ml translucent chamber, and superfused with the following low-Ca^{2+} high-Mg^{2+} solution (mM): NaCl 120.0, KCl 5.0, $CaCl_2$ 0.4, $NaHCO_3$ 11.0, $NaHPO_4$ 1.0, $MgCl_2$ 5.0, glucose 11, and pH 7.3–7.4. The solution was bubbled with 95% O_2 and 5% CO_2. The solution flowed through the muscle chamber at a rate of 5 ml/min. Monitoring the bath solution throughout the experiment did not reveal any changes in pH after passing through the muscle chamber. The temperature was controlled with a Peltier semiconductor device (Thermoelectric module TM-127, SCTB, Moscow, Russia). The experiments were performed at 20.0 ± 0.3 °C. Ryanodine and 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) (Sigma, St. Louis, MO, USA) were dissolved before use in 0.4% dimethylsulfoxide and added to the bath saline.

2.2. Immunostaining

Immune histochemical technique was used for the estimation of the size of synaptic contacts. Isolated neuromuscular diaphragm preparations were fixed in 2% paraformaldehyde solution for 5 min and washed three times in phosphate buffered saline (PBS), every 10 min over a period of 30 min. Muscles were then incubated in 0.5% Triton X-100 solution for 30 min and then kept for 15 min in blocking solution (prepared in PBS) of the following composition: 1% BSA (bovine serum albumin), 5% NGS (normal goat serum), 0.5% Triton X-100. Then preparations were incubated for 12 h with primary monoclonal antibodies against synaptophysin in PBS containing 1% BSA, 0.5% Triton X-100 at 4 °C. Afterwards they were washed for 30 min in PBS and incubated for 1 h at room temperature with secondary antibodies conjugated with Alexa350, followed by washing for 30 min in PBS. Incubation for 30 min with alpha-bungarotoxin conjugated with tetramethylrhodamine (TMR) was carried out for the localization of postsynaptic nicotinic acetylcholine receptors. After washing off with PBS, preparation was placed in a 1:1 glycerol/PBS on a slide under cover glass. Measurements were performed using laser scanning confocal microscope Leica TCS SP5 MP (Leica Microsystems, Germany) with oil-immersion lenses 63×. Endplates were identified by the binding of fluorescent alpha-bungarotoxin to acetylcholine receptors. Laser with a wavelength of 350 nm for Alexa350 and 557 nm for TMR was used for image acquisition. Images were processed using the software ImageJ (NIH, Bethesda, MD, USA). To measure the area of endplates (μm^2) the three-dimensional confocal image was transformed to planar by averaging a series of confocal slices. Statistical significance was estimated by the Student's *t*-test ($p < 0.05$).

2.3. Electrophysiology

Suprathreshold stimuli with durations of 0.1 ms were applied to the phrenic nerve at frequencies of 0.5, 4, 10 and 15 Hz *via* a suction electrode filled with extracellular solution. Nerve action potentials and extracellular endplate currents (EPCs) were recorded using heat-polished Ringer-filled extracellular pipettes with tip diameters of 2–3 μm and resistances of 1–3 MΩ. The extracellular pipette was positioned under visual control (magnification 250×) near the nerve ending at a site where a triphasic nerve action potential could be recorded (Brigant and Mallart, 1982). The recorded signals were filtered between 0.03 Hz and 10 kHz, digitized at 3 μs intervals with a 9-bit analog–digital converter, sampled, stored in a computer and processed using our application package (Bukcharaeva et al.,