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Effects of Mitochondrial Calcium Regulation on Post-tetanic Potentiation at the Crayfish Neuromuscular Junction

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ABSTRACT

Synaptic plasticity is a fundamental property of the vertebrate central nervous system and is thought to be involved in learning and memory processes. In the presynaptic terminal, mitochondria regulate synaptic plasticity by influencing neurotransmitter release. To elucidate the effect of mitochondrial calcium release on synaptic transmission and post-tetanic potentiation (PTP), a form of synaptic plasticity that has been observed at both excitatory and inhibitory synapses, we adopted the crayfish neuromuscular junction (NMJ) preparation as our model system. We employed a specific pharmacological inhibitor for mitochondria and monitored neurotransmitter release by postsynaptic electrophysiological recording. Our results demonstrated that PTP was reduced when mitochondrial Ca^{2+} release was inhibited, which suggested that PTP would be a result of the slow Ca^{2+} release from mitochondria, and that Ca^{2+} alone was able to cause substantial changes in postsynaptic PTP.

INTRODUCTION

Activity-dependent modulation of synaptic transmission is a fundamental mechanism for the development and functioning of the nervous system. The processing of neuronal information, coupled tightly with firing of action potentials at different frequencies, often leads to changes in the efficacy of synaptic transmission, a phenomenon known as synaptic plasticity. Neuronal plasticity is defined as adaptive changes in the structure and function of neurons in response to a changing environment, and it was thought to be responsible for learning and memory formation (Larkman and Jack 1995; Bear and Malenka 1994).

When applying a repetitive high-frequency stimulation to presynaptic neurons, many synapses display a gradual potentiation of synaptic transmission. After the presynaptic activity subsides, individual action potentials continue to evoke potentiated postsynaptic potentials and to enhance neurotransmitter release for several minutes, a process termed post-tetanic potentiation (PTP). This short-term enhancement of synaptic plasticity and efficacy was primarily due to changes in presynaptic neurotransmitter secretion (Zucker and Regehr 2002). PTP reflected an increase in the number of quanta of neurotransmitter released by trains of repetitive action potentials (Wojtowicz and Atwood 1986) that were caused by the persistent, residual elevation in the pre-synaptic calcium concentration, which was also believed to contribute to presynaptic plasticity (Delaney *et al*. 1989).

Mitochondria play a central role in a variety of cellular processes, including ATP production by oxidative phosphorylation, cellular respiration through the citric acid cycle, regulation of calcium homeostasis, and initiation of apoptosis through interactions with the Bcl-2 protein family (Morris and Hollenbeck 1993). Many critical processes involved in synaptic plasticity require ATP as their primary energy source.

Previous studies have shed light on the role of mitochondria in synapses, and have hinted that mitochondria worked to regulate the supply of ATP and buffer cytoplasm Ca^{2+} ions. Sustained elevations in presynaptic Ca^{2+} following rapid, repetitive neuronal firing were correlated with enhancement of synaptic transmission (Zenisek and Matthews 2000). Mitochondria were able to remove Ca^{2+} from the cytoplasm, in response to Ca^{2+} influx or efflux from endoplasmic reticulum (ER). In addition, interactions mediated by Ca^{2+} between mitochondria and ER have been shown in neurons and might play important roles in regulating plasticity and cell survival (Griffiths and Rutter 2009). The synchronization between mitochondrial conductivity and calcium dynamics in the pre-synaptic terminal suggested an active role of mitochondria in synaptic plasticity (Ly and Verstreken 2006).

Mitochondira have also been shown to release Ca^{2+} into the cytoplasm. As Yang *et al.* (2003) suggested, mitochondria were partially loaded with Ca^{2+} before incoming trains of synaptic impulses. Na^{+} influx triggered by the opening of voltage-gated Na^{+} channels would then induce Ca^{2+} release. This

mitochondria-dependent, protein kinase C dependent Ca^{2+} release was, in turn, responsible for the post-tetanic potentiation of neurotransmitter release from the nerve terminal. As a result, mitochondria act as a rapidly mobilizable Ca^{2+} pool activated by the ionic change responsible for the membrane depolarization. Nevertheless, the specific molecular mechanisms that define the role of mitochondria in calcium and metabolite management during high frequency pre-synaptic activity are not yet known.

To address the effect of mitochondrial Ca^{2+} release on pre- and postsynaptic functions and plasticity, we adopted the crayfish neuromuscular junction (NMJ) as our model preparation due to its feasibility and ease of access to the terminal tissues for electrophysiological recording and fluorescent imaging, as well as for computational assessments and modeling. We investigated the mitochondrial regulation of synaptic plasticity by examining the effect of mitochondrial Ca^{2+} release on PTP in the crayfish NMJ. We hypothesized that the application of a mitochondrial Ca^{2+} release inhibitor would effectively reduce postsynaptic PTP and synaptic transmission, suggesting that mitochondrial Ca^{2+} release was essential for synaptic potentiation.

METHODS

Crayfish Dissection and Preparation

The Crayfish *Procambarus clarkii* (Carolina Biologicals, NC) was anaesthetized in an ice bath for 15 minutes before dissection. Dissection was performed on the crayfish tail muscle. The abdomen part of crayfish was separated from the cephalothorax and surrounding tissues to expose only the exoskeleton of dorsal surface and superficial extensor muscles along the dorsal surface. The autotomized tail part was then pinned in a dish and submerged in a low-calcium Ringer's solution (195 mM NaCl, 1.9 mM $CaCl_2$, 5.4 mM KCl, 2.6 mM $MgCl_2$, 10 mM HEPES, pH = 7.4) with continuous perfusion at room temperature. All procedures were conducted according to the Grinnell College Institutional Animal Care and Use Committee Protocol.

Electrophysiological Recording

Standard intracellular and extracellular recording techniques were used. Recording electrodes were prepared by pulling 1.2 mm glass capillaries (PUL-1 apparatus, World Precision Instruments) and filled with 3.0 M KCl (resistance 4-8 MΩ). Suction electrodes were filled with Ringer's solution. Excitatory junction potentials (EJPs) were recorded from central-proximal or proximal muscle fibers. Baseline EJPs were collected by repeatedly stimulating the nerve at low frequency 0.3 Hz (with 3 ms delay and 0.2 ms duration). Post-tetanic potentiations were elicited by stimulating the nerve for 1 minute at high frequency 30 Hz (Grass SD9 stimulator). EJPs were recorded every 5 seconds (*Scope* data acquisition system v4.1.2, Powerlab, Analog Digital Instruments) throughout the tetanus and post-tetanic processes. PTP was revealed by the post-tetanic decay in EJP amplitude back to pre-tetanic levels.

Pharmacological Agent

A lipophilic tetraphenylphosphonium choride (TPP^+ Cl, 375 g/mol, Sigma Aldrich) was first diluted to a stock concentration of 50 mM in DMSO, and then to a final working concentration of 50 µM in standard Ringer's solution (total volume 20 mL) in order to block Na^+-dependent and Na^+-independent mitochondrial Ca^{2+} efflux with minimal effect on ATP production. After the addition of TPP^+ to the bathing medium, EJP amplitudes were measured as stated above for approximately 5 minutes. Baseline PTP in the absence of TPP^+ was also recorded. After TPP^+ treatment, the preparation was washed for 15 minutes with Ringer's solution before stimulated again to measure the PTP recovery. Data represented the average of two biological replicates. Statistical significance was assessed by Student's *t* test.

RESULTS

PTP required mitochondrial Ca^{2+} release.

To investigate the role of mitochondrial calcium on synaptic plasticity through post-tetanic potentiation, we utilized a pharmacological agent, TPP^+ chloride, to block mitochondrial Ca^{2+} release and measured the strength of post-synaptic responses and potentiation through electrophysiological recording. While TPP^+ should have no effects on pre-tetanic EJP strength, ruling out any non-specific post-synaptic effects, we observed a significant lower level of EJP amplitudes during PTP in the presence of TPP^+ than during the control, which confirmed our initial hypothesis. We used the Ringer's solution with lower calcium concentration in order to reduce the difficulties produced by muscle contraction.

As shown in Figure 1, 50 µM TPP^+ effectively blocked the post-tetanic EJP amplitudes, suggesting that mitochondrial Ca^{2+} sequestration and release were also prevented. We observed a significant reduction in post-tetanic EJP amplitudes in TPP^+, compared to control experiments in normal Ringer's solution ($P<0.001$). Subsequent washes after TPP^+ treatment effectively restored the post-tetanic EJP amplitudes back to control levels. We also observed

an increased rate of decay of post-tetanic EJPs in the presence of TPP^+ treatment, and an increase in peak EJP amplitude during the high-frequency tetanic stimulation.

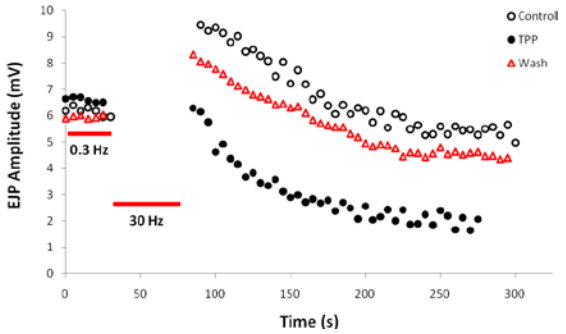

Figure 1. Effects of TPP^+ on PTP. Controls showing normal EJP amplitudes in response to tetanus (30 Hz for 1 min); running averages of two trials of post-tetanic EJP amplitudes in the presence of 50 µM TPP^+ and in subsequent washes in Ringer's solution. TPP^+ significantly reduced EJP amplitudes from control ($p<0.001$).

DISCUSSION

Our results indicated that mitochondria played a critical role in the generation of PTP. By blocking the mitochondrial Ca^{2+} efflux, TPP^+ was able to successfully reduce postsynaptic EJP amplitudes compared to control level, suggesting that PTP strength and synaptic transmission were reduced due to the inability of mitochondrial Ca^{2+} release. Our results, therefore, were consistent with PTP being produced by a mechanism involving mitochondrial Ca^{2+} sequestration during tetanic stimulation and subsequent post-tetanic Ca^{2+} efflux into cytoplasm. This slow mitochondrial efflux of Ca^{2+}, balanced with plasma membrane extrusion pumps, then leads to a minutes-lasting plateau of Ca^{2+} concentration, which in turn act at a site distinct from those causing exocytosis and facilitation to induce PTP (Delaney *et al.* 1989).

It is worth noting that in the wash experiments the EJP amplitudes were still lower than control experiments. Since the wash experiment was done on the same preparation as the TPP^+ treatment, it is possible that the preparation age and/or incomplete removal of the inhibitor might contribute to this disparity. We selected TPP^+ as our preferred blocker of mitochondrial Ca^{2+} transport since it did not share some major defects present in other mitochondrial inhibitors, such as affecting ATP production and requiring presynaptic injection.

In addition to loading mitochondria with Ca^{2+}, tetanic stimulation has been shown to fill terminals with Na^+, reducing transmembrane Na^+ gradients and slowing the efflux of cytoplasmic Ca^{2+} dependent on Na^+/Ca^{2+} exchange. This process also contributes to the prolongation of residual Ca^{2+} responsible for PTP (Mulkey and Zucker, 1992). Further study on the involvement of mitochondria and/or ER in presynaptic Ca^{2+} regulation is necessary to determine whether mitochondria or ER play essential roles in synaptic plasticity. Additional biochemical studies of synaptic events that cause the synapse to fail to function during neurodegenerative diseases or acute injury are also required.

Regulation of the synaptic activities is crucial for determining whether a neuronal pathway will become strengthened during processes like learning and memory formation, which involve the effects of long-term synaptic plasticity on forming new synaptic connections during neural development. This project will likely benefit the scientific community of neurobiology by establishing a fundamental understanding of mitochondrial modulation of synaptic transmission, and providing valuable insights for developing clinical treatment for impaired memory and learning systems and various neurological disorders.

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