Available online at www.sciencedirect.com
ScienceDirect
Neuropharmacology 51 (2006) 671–680
NEURO PHARMACOLOGY
www.elsevier.com/locate/neuropharm

Mechanism of β-bungarotoxin in facilitating spontaneous transmitter release at neuromuscular synapse

Jau-Cheng Liou [a,*], Kai-Hsiang Kang [a], Long-Sen Chang [b], Shih-Yin Ho [a]

[a] *Department of Biological Sciences, National Sun Yat-sen University, No 70, Lein-Hai Rd., Kaohsiung 804, Taiwan*
[b] *Institute of Biomedical Sciences, National Sun Yat-sen University, No 70, Lein-Hai Rd., Kaohsiung 804, Taiwan*

Received 30 December 2005; received in revised form 12 May 2006; accepted 15 May 2006

Abstract

The mechanism of the action of β-bungarotoxin (β-BuTx) in the facilitation of spontaneous transmitter release at neuromuscular synapse was investigated in *Xenopus* cell culture using whole-cell patch clamp recording. Exposure of the culture to β-BuTx dose-dependently enhances the frequency of spontaneous synaptic currents (SSCs). Buffering the rise of intracellular Ca^{2+} with BAPTA-AM hampered the facilitation of SSC frequency induced by β-BuTx. The β-BuTx-enhanced SSC frequency was reduced when the pharmacological Ca^{2+}-ATPase inhibitor thapsigargin was used to deplete intracellular Ca^{2+} store. Application of membrane-permeable inhibitors of inositol 1,4,5-trisphosphate (IP_3) but not ryanodine receptors effectively occluded the increase of SSC frequency elicited by β-BuTx. Treating cells with either wortmannin or LY294002, two structurally different inhibitors of phosphatidylinositol 3-kinase (PI3K) and with phospholipase C (PLC) inhibitor U73122, abolished the β-BuTx-induced facilitation of synaptic transmission. The β-BuTx-induced synaptic facilitation was completely abolished while there was presynaptic loading of the motoneuron with $GDP_{\beta}S$, a non-hydrolyzable GDP analogue and inhibitor of G protein. Taken collectively, these results suggest that β-BuTx elicits Ca^{2+} release from the IP_3 sensitive intracellular Ca^{2+} stores of the presynaptic nerve terminal. This is done via PI3K/PLC signaling cascades and G protein activation, leading to an enhancement of spontaneous transmitter release.

Keywords: β-bungarotoxin; Synaptic transmitter; Motoneuron; Neuromuscular junction; Transmitter release; Calcium

1. Introduction

β-Bungarotoxin (β-BuTx), the presynaptic neurotoxin was first purified from the venom of the elapid snake *Bungarus multicinctus* (Taiwan banded krait) and pharmacologically characterized as a 21.8 kDa phospholipase A_2 (PLA_2) neurotroxin (Chang and Lee, 1963). A rich history of research dating back to the time of Lee and Chang suggests that this toxin consists of two dissimilar polypeptide subunits, a structurally homologous PLA_2 subunit named A chain, and a non-PLA_2 subunit B chain which is long thought to act as an affinity probe to guide the neurotoxin to its target on nerve terminal (Chang, 1985). Both the A chain and B chains are covalently linked by one disulfide bridge, and modern chromatographic techniques and amino acid analysis have revealed that they consist of three main isoforms of β-BuTx which share a common PLA_2 subunit but possess distinct B subunit (Chu et al., 1995). Envenoming bites by kraits are associated with an acute onset of neuromuscular paralysis followed by a prolonged, widespread neuronal cell death throughout the mammalian and avian CNS (Rowan, 2001). Results from several studies performed on frog sartorius and mouse phrenic nerve-diaphragm preparation have suggested that upon exposure isolated nerve-muscle preparations to β-BuTx, the development of neuromuscular transmission failure undergoes a triphasic change. An initial phase of weak reduction of spontaneous acetylcholine release is followed by a second prolonged phase of facilitated release, and then by a third phase of progressive decline of spontaneous

* Corresponding author. Tel.: +886 7 525 3628; fax: +886 7 525 3609.
E-mail address: netliou@mail.nsysu.edu.tw (J.-C. Liou).

0028-3908/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2006.05.016

neurotransmission (Su and Chang, 1984). It has been shown that β-BuTx causes massive Ca^{2+} influx through NMDA and L-type Ca^{2+} channels that induced the production of reactive oxygen species and disturb mitochondrial function, resulting in collapse of mitochondrial membrane potential and ATP depletion (Shakhman et al., 2003; Tseng and Lin-Shiau, 2003). Furthermore, results from recent studies using cultured cerebellar granule neurons and rat phrenic nerve-diaphragm preparation have indicated that β-BuTx-induced depletion of synaptic vesicles result in synaptic blockade (Prasarnpun et al., 2004; Rigoni et al., 2004).

What is the signaling mechanism responsible for β-BuTx-induced change on synaptic transmission? Most studies have conducted by employing in vitro twitch tension, central neuronal cell culture, or synaptic membranes from brain tissue and biochemical techniques to investigate the effects of β-BuTx. Although several experimental investigations have been carried out to reveal the molecular basis of presynaptic neurotoxicity of β-BuTx, we still do not know the exact underlying molecular mechanisms for the early changes in transmitter release and the subsequent block of transmitter release induced by β-BuTx. Therefore, searching for an ideal in vitro model that is more amenable to the direct evaluation the β-BuTx-induced activation of signaling cascades related to the change of synaptic transmission is required. We had previously shown that in *Xenopus* motoneuron-muscle cell co-culture, β-BuTx caused a dramatic change in the frequency of spontaneous neurotransmitter release (Liou et al., 2004). In this study, we try to understand how β-BuTx facilitates the spontaneous release of neurotransmitter. Based on our data, we show for the first time that the facilitation on spontaneous synaptic transmission induced by β-BuTx is resulted from triggering the liberation of Ca^{2+} from internal store, which is the result of pleiotropic convergent signaling pathways involving PLC, phosphatidylinositol 3-kinase (PI3K) and G protein activation. Moreover, our results also indicate that *Xenopus* nerve-muscle cultures, by virtue of its simplicity and easy accessibility, can be used as a model system for further investigation in the structure-toxicity relationship and underlying signal transduction pathway of presynaptic neurotoxins.

2. Methods

2.1. Cell culture

Xenopus laevis nerve-muscle culture was prepared as previously reported (Liou et al., 2003). Briefly, the neural tube and the associated myotomal tissue of 1-day-old (stage 20–22) *Xenopus* embryos were dissected and dissociated in the Ca^{2+} and Mg^{2+}-free Ringer's solution supplemented with 0.15 mM EDTA. The dissociated cells were plated and used for experiments after incubation at room temperature (20–25 °C) for 1 day. The culture medium consisted of 50% (vol/vol) Ringer solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES [pH 7.6]), 49% L-15 Leibovitz medium (Sigma, St Louis, MO, USA), and 1% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin sulfate). β-BuTx and various inhibitors were applied directly to the culture media at the time of recording.

One day after cell plating, functional synapses are rapidly established between cultured spinal neurons and embryonic muscle cells. The present study utilized synapses of myocytes innervated by single co-cultured spinal neurons. The frequency of spontaneous synaptic events during the first day of synaptogenesis was found to vary greatly from cell to cell, over two orders of magnitude, and the frequency of SSC events increased with time of synapse development (Evers et al., 1989). To test the facilitation effect induced by β-BuTx treatment in simpler conditions, our analyses were performed mostly in low-activity synapses (<1.5 Hz).

2.2. Electrophysiology and data analysis

Gigaohm-seal whole-cell recording methods followed those described previously (Hamill et al., 1981). Patch pipettes (Hilgenberg, Malsfeld, Germany) were pulled with a two-stage electrode puller (PP-830, Narishige, Tokyo, Japan), and the tips were polished immediately before the experiment using a microforge (MF-830, Narishige, Tokyo, Japan). SSCs were detected from innervated myocytes by whole-cell recording in the voltage-clamp mode. Recordings were made at room temperature in Ringer's solution, and the solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.2). Data were collected using a patch-clamp amplifier WPC-100 (E.S.F. electronic, Göttingen, Germany) and Axoscope 8.0 (Axon Instruments, Union City, CA, USA). Signals were filtered at 10 KHz (Digidata 1322; Axon Instruments, Union City, CA, USA). SSCs were detected and analyzed using the Mini Analysis Program 5.0 (Synaptosoft Inc., Decatur, GA, USA).

The drug's effect on SSC frequency was evaluated as those described previously (He et al., 2000). To quantitatively measure the changes in neurotransmitter release, a time course of SSC frequency was first constructed on a minute-to-minute basis. The SSC frequencies for a 6 min period right before drug application was averaged as a control. The changes in SSC frequency were measured by averaging a 6-min period recording starting from the highest number after drug application, and the results were expressed as Mean ± S.E.M. The time needed for the SSC frequency to reach its plateau after bath application of β-BuTx could be varied in different experiments, with a lag period from 12 to 23 min in general. In those inhibitor-pretreated experiments with no obviously change in SSC frequency after 25 min bath application of β-BuTx, the effect of β-BuTx on SSC frequency was calculated by averaging a 6 min period starting from the 20th min. The statistical significance was evaluated by Student's paired *t*-test. For comparison of SSC amplitude distribution, the composite graph of cumulative frequency of all SSC events was constructed, and only the synapse with a total number of events exceeding 180 was used for analysis. The statistical difference between these graphs was tested by the Kolmogorov–Smirnov test (Liao et al., 2004).

2.3. Presynaptic loading of drug and fluorescent dye

Presynaptic spinal neurons were dialyzed using a patch pipette containing 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM $GDP_\beta S$ and 1 mg/ml of fluorescent dye Lucifer Yellow while the whole-cell recordings were being made at the cell body (Liou et al., 2005). The dialysis of the drug and Lucifer yellow could be visualized directly in the inverted microscope under fluorescence mode. After a 10-min dialysis, the patch pipette was pulled off after damaging the seal by a large hyperpolarizing current injection.

2.4. Chemicals

The following chemicals were used: β-BuTx was a generous gift from L-S Chang. 8-(dethylamino) octyl 3, 4, 5-trimethoxybenzoate (TMB-8), Lucifer yellow, ruthenium red, $GDP_\beta S$, genistein, thapsigargin, aristolochic acid, glycyrrhizin, 1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxy-methyl) ester (BAPTA-AM), indomethacin, pancreatic PLA_2, and nordihydroguaiaretic acid (NDGA) were purchased from Sigma (St Louis, MO, USA), whereas wortmannin, U73122, and LY294002 were obtained from Tocris Cookson (Bristol, UK), and Xestospongin C (XeC) and 2-APB were purchased from Calbiochem (San Diego, CA, USA). All drugs were applied directly to the culture media at the times indicated.