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Tetrodotoxin abruptly blocks excitatory neurotransmission in mammalian CNS

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ABSTRACT

The present study utilised a 'synaptic bouton' preparation of mechanically isolated rat hippocampal CA3 pyramidal neurons, which permits direct physiological and pharmacological quantitative analyses at the excitatory and inhibitory single synapse level. Evoked excitatory and inhibitory postsynaptic currents (eEPSCs and eIPSCs) were generated by focal paired-pulse electrical stimulation of single boutons. The sensitivity of eEPSC to tetrodotoxin (TTX) was higher than that of the voltage-dependent Na^+ channel whole-cell current (I_{Na}) in the postsynaptic CA3 soma membrane. The synaptic transmission was strongly inhibited by 3 nM TTX, at which concentration the I_{Na} was hardly suppressed. The IC_{50} values of eEPSC and I_{Na} for TTX were 2.8 and 37.9 nM, respectively, and complete inhibition was 3–10 nM for eEPSC and 1000 nM for I_{Na}. However, both eEPSC and eIPSC were equally and gradually inhibited by decreasing the external Na^+ concentration ($[Na]_o$), which decreases the Na^+gradient across the cell membrane. The results indicate that TTX at 3–10 nM could block most of voltage-dependent Na^+ channels on presynaptic nerve terminal, resulting in abruptly inhibition of action potential dependent excitatory neurotransmission.

1. Introduction

Synaptosomes (pinched-off nerve endings) prepared by centrifugation of dissociated mammalian brain cells are a good model system for studying membrane potential (Blaustein and Goldring, 1975), voltage-dependent Na^+ and Ca^{2+} influx (Blaustein, 1975), and neurotransmitter release (Blaustein and Goldring, 1975; Cotman et al., 1976). Membrane potential and Ca^{2+} uptake were measured with a fluorescent probe and Na^+ uptake, with the ^{22}Na isotope (Blaustein and Goldring, 1975; Krueger and Blaustein, 1980). Pharmacological studies using voltage-dependent Na^+ channel (Na_V) activators (batrachotoxin, veratridine) and blockers (saxitoxin and tetrodotoxin [TTX]) proved the existence of TTX-sensitive Na_V in the synaptosomes of rat forebrains (Krueger and Blaustein, 1980).

The intra-terminal Na^+ concentration and [^{3}H] glutamate (Glu) release in the rat hippocampus synaptosomes were reported by using fluorescence and radioactivity (Aldana and Sitges, 2012). According to Engel and Jonas (2005), presynaptic Na_V in the hippocampal mossy fibre boutons are abundant and have faster inactivation kinetics than the somatic Na_V. They suggested that the Na_V between the subcellular compartments of the same cell have different functional characteristics.

Since majority of the excitatory and inhibitory nerve endings (boutons) of the mammalian central nervous system (CNS) have very small diameters (φ0.5–3 μm), it is difficult to directly apply conventional whole-cell patch recording techniques on them. Fortunately, we have successfully developed a 'synaptic bouton' preparation where single boutons adhere to mechanically dissociated CNS neurons. This preparation enables selective electrical stimulation of single boutons on the mechanically isolated neuronal cell body and its dendrite, using 'focal electrical stimulation' technique (Akaike and

Abbreviations: ATP-Mg, adenosine 5′-triphosphate magnesium salt; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Cont, control; d-AP5, D-(-)-2-amino-5-phosphonovaleric acid; EGTA, ethylene glycol teraacetic acid; eEPSC(s), evoked excitatory postsynaptic current(s); eIPSC(s), evoked inhibitory postsynaptic current(s); $GABA_A$, γ-aminobutyric acid type A; I_{Na}, voltage-dependent Na^+ channel whole cell currents; $[Na]_o$, external Na^+ concentration; Na_V, voltage-dependent Na^+ channel; PPR, paired-pulse ratio; Rf, failure rate; TEA, tetraethylammonium; V_H, holding potential; CNS, central nervous system.

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Moorhouse, 2003; Akaike et al., 2002; Koyama et al. 2002; Murakami et al. 2002). In order to determine the physiological functions and pharmacological properties of presynaptic Na_V that trigger transmitter release, we investigated the current amplitude, failure rate (Rf), and paired-pulse ratio (PPR) (P_2/P_1) of evoked excitatory glutamatergic and inhibitory GABAergic postsynaptic currents (eEPSCs and eIPSCs) using a combination of the rat hippocampal CA3 'synaptic bouton' preparation and focal stimulation technique (Wakita et al. 2014, 2012).

2. Materials and methods

2.1. The 'synaptic-bouton' preparation

The use of experimental animals was approved by The Ethics Committee of Kumamoto Kinoh Hospital. All experiments were performed in accordance with The Guiding Principles for Care and Use of Animals in The Field of Physiological Sciences of The Physiological Society of Japan. All efforts were made to minimise animal suffering, to reduce the number or animals used.

Details of the 'synaptic-bouton' preparation have been described previously (Akaike and Moorhouse, 2003; Akaike et al. 2002). Briefly, Wistar rats (10–19 days old, either sex) were decapitated after pentobarbital anaesthesia [50 mg/kg, intraperitoneal (i.p.)]. The brain was quickly removed, immersed in ice-cold incubation medium, and oxygenated with 95% O_2 and 5% CO_2. Hippocampal slices, 400-μm thick, were prepared using a vibrating microtome (VR 1200S; Leica, Nussloch, Germany), and then incubated in oxygenated medium at room temperature (21–24 °C) fonr at least 1 h before mechanical dissociation via a fire-polished glass pipette coupled to a vibration device (S1-10 cell isolator; K.T. Labs, Tokyo). The tip of the glass pipette was lightly placed on the surface of the hippocampal CA3 region, and vibrated horizontally (0.2–2.0 mm displacement) at about 50 Hz. After dissociation, the slices were removed from the dish and the mechanically dissociated neurons were left to settle and adhere to the bottom of the dish for at least 15 min.

2.2. Electrophysiological measurements

All recordings were obtained from the soma of the CA3 pyramidal neurons using conventional whole-cell patch clamp recordings in the voltage-clamp mode. Voltage-dependent Na^+ channel whole cell currents (I_{Na}) on CA3 soma membrane were recorded at a holding potential (V_H) of −70 mV (Multiclamp 700B; Molecular Devices, Sunnyvale, CA). Action potential-dependent eEPSCs were recorded at a V_H of −65 mV. All experiments were performed at room temperature (21–24 °C) (Wakita et al. 2013, 2012).

Patch pipettes were made from borosilicate capillary glass (1.6 mm outer diameter, 0.9 mm inner diameter, G-1.5, Narishige, Tokyo, Japan) in two stages on a vertical pipette puller (PC-10, Narishige, Tokyo, Japan). The resistance of the recording patch pipettes filled with the internal pipette solution (see below) was 3–6 MΩ. Current and voltage were continuously monitored using an oscilloscope (DCS-7040, Kenwood, Tokyo, Japan). All membrane currents were filtered at 3 kHz (E-3201A Decade Filter; NF Electronic Instruments, Tokyo, Japan), and their values were stored in a computer using pCLAMP 10.2 (Axon Instruments, Union City, CA, USA). Hyperpolarizing step pulses of 5 mV (30 ms duration) were used to monitor the access resistance. If the resistance changed by more than 20%, the recordings were rejected.

2.3. Paired-pulse focal electrical stimulation of single glutamatergic or GABAergic boutons using glass pipettes

Focal electrical stimuli can be employed to activate a single nerve terminal, to measure eEPSCs and eIPSCs resulting from a single glutamatergic and GABAergic presynaptic nerve ending rather than a fused event from multiple boutons (Jang et al., 2005), respectively. This technique offers a unique evaluation of how a drug acts on the pre- and post-synaptic transmission mechanisms at the single synapse level.

Focal electrical stimulation of a single bouton adherent to the mechanically dissociated hippocampal CA3 neurons has been described previously (Akaike and Moorhouse, 2003; Akaike et al. 2002). The stimulating pipette was made from a pulling θ glass tube (tip diameter ~ 0.5 μm) and filled with the standard external solution (see below). The stimulating electrode was placed as close as possible to the soma of a single CA3 neuron from which the whole-cell recording was successfully obtained. Responses were evoked using a paired-pulse stimulation protocol, with each stimulus pulse of 100 μs duration and of same intensity, applied at a frequency of 0.2 Hz, using a stimulus isolator (SS-202 J, Nihon Koden, Tokyo, Japan). The stimulus intensity was 0.05–0.09 mA, and the inter-stimulus interval was 20–30 ms for eEPSCs and 30–70 ms for eIPSCs.

2.4. Drugs and application

The solution reagents were obtained from Wako Pure Chemicals (Osaka, Japan). Other pharmacological reagents such as tetraethylammonium (TEA)-Cl, tetrodotoxin (TTX), ethyleneglycol-bis-(α-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), adenosine 5′-triphosphate magnesium salt (ATP-Mg), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 2-[(2,6-dimethylphenyl)amino]-*N*,*N*,*N*-triethyl-2-oxoethanaminium (QX-314) were obtained from Sigma Chemicals (St. Louis, MO, USA). D(-)-2-amino-5-phosphonovaleric acid (d-AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from Tocris Cookson (Ellisville, MO, USA). All test solutions containing drugs were applied using a 'Y-tube system,' which allowed solution exchange around the cells within 20 ms (Murase et al. 1989).

2.5. Solutions

The ionic composition of the incubation medium was 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM glucose, and was well saturated with 95% O_2 and 5% CO_2. The pH was adjusted to 7.45. The standard external solution used for recordings contained 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. In the experiments using various external Na^+ concentrations ($[Na]_o$), NaCl in the standard external solution was replaced with an equimolar choline chloride (choline-Cl). The modified external solution for recording I_{Na} contained 150 mM NaCl, 10 mM choline-Cl, 10 mM CsCl, 10 mM glucose, 0.01 mM $LaCl_3$, 5 mM TEA-Cl, and 10 mM HEPES. All external solutions were adjusted to pH 7.4 using Tris base.

The composition of the internal pipette solution for glutamatergic EPSCs recording was 5 mM CsCl, 130 mM CsF, 5 mM TEA-Cl, 2 mM EGTA, 10 mM HEPES, and 5 mM QX-314 bromide, and for GABAergic IPSCs was 5 mM CsCl, 130 mM Cs-methanesulfonate, 5 mM TEA-Cl, 10 mM EGTA, 10 mM HEPES, and 4 mM ATP-Mg. The internal pipette solution for I_{Na} measurements was 120 mM CsF, 15 mM NaF, 5 mM CsCl, 5 mM TEA-Cl, 2 mM EGTA, 10 mM HEPES, and 2 mM ATP-Mg. All pipette (internal) solutions were adjusted to pH of 7.2 with Tris base. ATP-Mg was dissolved in the internal solution just before use. Glutamatergic eEPSCs were isolated from GABAergic evoked inhibitory postsynaptic current (eIPSCs) by recording at a V_H of −65 mV, close to the Cl^- equilibrium potential (E_{Cl}). GABAergic IPSCs were isolated from the EPSCs using CNQX and d-AP5, and by recording at a V_H of 0 mV, close to the reversal potential of the glutamate response. (Wakita et al. 2013, 2012).

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