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Presynaptic effects of grayanotoxin III on excitatory and inhibitory nerve terminals in rat ventromedial hypothalamic neurons

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ABSTRACT

Mad honey poisoning caused by grayanotoxin (GTX) is associated with autonomic nervous system symptoms, such as excessive perspiration, hypersalivation, vomiting, and bradycardia. Neurons in the ventromedial hypothalamus (VMH) play an important role in body homeostasis and in the activity of the autonomic nervous system. Among the 18 isoforms of GTX found in mad honey, GTX I–IV are a unique class of toxic diterpenoids; GTX III is the principal toxic isomer. In the present study, we determined the effects of GTX III on synaptic transmission in VMH neurons. Both spontaneous and evoked GABA-ergic and glutamate-ergic postsynaptic currents were measured using patch clamp recordings in single VMH neurons which had been mechanically dissociated. GTX III increased the frequency of spontaneous GABA-ergic and glutamate-ergic postsynaptic currents (sIPSCs and sEPSCs, respectively) in a dose-dependent manner without affecting their amplitude, demonstrating that GTX III enhances transmitter release from both inhibitory and excitatory nerve terminals synapsing onto VMH neurons. GTX III significantly enhanced the amplitude and the success rate (Rsuc) of both evoked inhibitory and excitatory postsynaptic currents (eIPSCs and eEPSCs, respectively), suggesting that GTX III increases the probability of transmitter release from these terminals, and also the amount of transmitter released from a single nerve terminal. The action of GTX III on sIPSC frequency was absent in a Na^+-free solution and in the presence of tetrodotoxin (TTX; 300 nM) or cadmium (Cd^{2+}; 100 μM). The present study indicates that GTX increases Ca^{2+} influx through voltage-dependent Ca^{2+} channels secondary to activation of voltage-dependent Na^+ channels in inhibitory and excitatory nerve terminals synapsing on VMH neurons, and the subsequent increased release of GABA and glutamate from these terminals may be responsible for the autonomic symptoms of GTX intoxication.

1. Introduction

Mad honey poisoning occurs predominantly in the Black Sea region of Turkey, but cases of mad-honey intoxication have been reported worldwide (Gunduz et al., 2007; Choo et al., 2008). Mad honey poisoning was first described in 401 BC by Xenophon, an Athenian author and military commander, and used as a weapon by King Mithradates IV of northeast Anatolia (Turkey) against Pompey in 67 BC. Roman troops who ate mad-honey were incapacitated and easily overcome by their enemies (Leach, 1972).

The symptoms of mad honey poisoning range from mild-to-severe, and include dizziness, weakness, excessive perspiration, hypersalivation, vomiting, and paresthesias. More severe intoxication may lead to life-threatening cardiac complications, such as complete atrioventricular block (Ergun et al., 2005). The severity of poisoning generally depends on the amount ingested, and the concentration of toxin ingested may differ from case-to-case (Gunduz et al., 2006). The cause of mad honey poisoning is the toxin, grayanotoxin (GTX), which is found in the honey produced from the nectar of rhododendron species (Gunduz et al., 2007; Choo et al., 2008). Rhododendron species are commonly used as garden plants worldwide, and some species are widely used as an analgesic for the treatment of rheumatic or dental pain, common colds, and edema (Tasdemir et al., 2003). Among the 18 isoforms of GTX found in mad honey, GTX I-IV are a unique class of toxic diterpenoids, which are polyhydroxylated cyclic hydrocarbons that do not contain nitrogen (Kan et al., 1994; Gunduz et al., 2006). GTX I and GTX II are present in mad honey in small amounts, while GTX III is the principal toxic isomer (Koca and Koca, 2007).

The toxic effects of GTX are known to be due to its interaction with voltage-dependent sodium channels (Na^+ channels). GTX binds directly to Na^+ channels in the cell membrane, resulting in an

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increase in membrane Na^+ permeability and inhibits inactivation of Na^+ channels. Consequently, GTX results in a prolonged depolarization of the cell membrane (Seyama et al., 1985). These membrane effects by GTX are observed in skeletal and cardiac muscles, and also in peripheral and central nerve cells (Gunduz et al., 2006).

Neurons in the ventromedial hypothalamus (VMH) play an important role in regulating body homeostasis and behavior (Canteras et al., 1994). In particular, the VMH neurons control the activity of the autonomic nervous system (Itoi et al., 1991; Narita et al., 1994). The VMH neurons receive both γ-aminobutyric acid (GABA)-ergic and glutamate-ergic innervation, and these synaptic inputs regulate the activity of VMH neurons (McCarthy et al., 1992; Priestley, 1992; Takenaka et al., 1995). Activation of VMH neurons is responsible for suppression of the circulatory system in rats, including decreased blood pressure and heart rate (Takenaka et al., 1996; Hirasawa et al., 1998).

It is well-known that GTX intoxication is associated with persistent activation of Na^+ channels and that the symptoms of GTX intoxication are closely related to dysfunction of the autonomic nervous system; however, the modulatory effects of GTX on neurotransmitter release from presynaptic terminals projecting onto the VMH neurons have not been fully investigated. In the present study, we therefore examined the effects of GTX III on both spontaneous and evoked release of glutamate and GABA from excitatory and inhibitory presynaptic nerve terminals in VMH neurons.

2. Materials and methods

2.1. Preparation of single VMH neurons

Wistar rats (10–17 days old) were decapitated under pentobarbital anesthesia (50 mg/kg ip). The brain was quickly removed and immersed in an ice-cold incubation medium, saturated with 95% O_2 and 5% CO_2. Coronal slices at a thickness of 400 μm containing the VMH region were prepared with a vibrating microtome (VT 1200S; Leica, Nussloch, Germany). The brain slices were then incubated in a medium oxygenated with 95% O_2 and 5% CO_2 at room temperature (21–24 °C) for at least 1 h before mechanical dissociation. For mechanical dissociation, slices were transferred into a 35-mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ) containing the standard external solution, and the region of the VMH was identified under a binocular microscope (SMZ645; Nikon, Tokyo, Japan). The mechanical dissociation procedure was described in detail previously (Rhee et al., 1999; Akaike and Moorhouse, 2003). Briefly, the VMH neurons were mechanically dissociated using a fire-polished glass pipette oscillating at 50–60 Hz; the tip was lightly placed on the VMH region on the surface of the slice and vibrated horizontally (0.1–0.2 mm displacement) for about 2 min. The slices were then 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 before recordings commenced. All experiments conformed to the guidelines of National Institutes of Health (NIH) and guiding principles for the care and use of animals approved by the Council of the Physiological Society of Japan.

2.2. Current measurements

All electrical measurements used the conventional whole-cell patch recording mode under current- or voltage-clamp conditions. GABA-ergic inhibitory postsynaptic currents (IPSCs) were isolated pharmacologically using 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 30 μM dl-2-amino-5-phosphonovaleric acid (AP-5) for blocking glutamate-ergic AMPA/KA and NMDA responses, respectively. For recording excitatory postsynaptic currents (EPSCs), external solutions contained 3 μM bicuculline to block GABA-ergic responses. Patch pipettes were made from borosilicate capillary glass (1.5 mm outer diameter, 0.9 mm inner diameter; G-1.5; Narishige, Tokyo, Japan) in two stages on a vertical pipette puller (PP-830; Narishige). The resistance of the recording pipettes filled with internal solution was 3–5 MΩ. The isolated neurons were observed under phase contrast on an inverted microscope (Diapot; Nikon). To record GABA- or glutamate-induced currents, neurons were voltage clamped at holding potentials (V_Hs) of 0 and −60 mV, respectively (CEZ-2300; Nihon Kohden, Tokyo, Japan) at room temperature (21–24 °C). The current and voltage were continuously monitored on an oscilloscope (VC-6023; Hitachi, Tokyo, Japan) and a pen recorder (RECTI-HORIT-8K; Sanei, Tokyo, Japan). The membrane currents were filtered at 3 kHz (E-3201A Decade Filter; NF Electronic Instruments, Tokyo, Japan) and stored on a computer using pCLAMP 10.2 (Axon Instruments, Whipple Road Union, CA). Ten millivolt hyperpolarizing step pulses (30 ms) were used to monitor the access resistance, which ranged from 5 to 12 MΩ, and recordings were discontinued if access resistance changed by >20%.

2.3. Focal stimulation of a single bouton

We previously described the method of focal electrical stimulation on a single bouton adherent to mechanically isolated neurons (Akaike et al., 2002; Akaike and Moorhouse, 2003). Briefly, 100 μs-voltage pulses were applied to a glass stimulating pipette (diameter, 0.5–0.6 mm) at a frequency of 0.3 Hz using a stimulus isolation unit (SS-403J; Nihon Koden). The stimulating pipette was filled with standard external solution and was placed close to the surface of a VMH neuron from which a whole-cell recording was being made. The stimulating pipette was then carefully moved along the surface of the soma and proximal dendrites until an inward current appeared in response to focal stimulation, indicating that the stimulating pipette was positioned just above a bouton. The fact that the evoked current arises from a single bouton was confirmed by the observation that the elicited synaptic responses were evoked in an all-or-nothing fashion and that they disappeared when the stimulus strength was reduced or when the stimulation pipette was moved (Akaike et al., 2002; Akaike and Moorhouse, 2003). The currents evoked by focal stimulation were analyzed using pCLAMP 10.2 (Axon Instruments).

2.4. Solutions

The ionic composition of the incubation medium consisted of 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 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 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES). The Na^+-free external solution was 150 mM choline Cl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. These external solutions were adjusted to a pH of 7.5 with Tris-base. For recording GABA-ergic sIPSCs, the external solutions contained 10 μM CNQX and 30 μM AP-5, while glutamate-ergic sEPSCs were recorded in external solution containing 3 μM bicuculline. The ionic composition of the internal (patch pipette) solution was 145 mM Cs–methanesulfonate, 5 mM TEA Cl, 5 mM CsCl, 2 mM EGTA, and 10 mM HEPES with or without 4 ATP-Mg. Except where indicated, the internal solutions routinely contained 500 μM lidocaine N-ethyl bromide (QX-314) to block the