Neuroscience 138 (2006) 25–35

PRESYNAPTIC $GABA_A$ RECEPTORS FACILITATE SPONTANEOUS GLUTAMATE RELEASE FROM PRESYNAPTIC TERMINALS ON MECHANICALLY DISSOCIATED RAT CA3 PYRAMIDAL NEURONS

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Abstract—Mossy fiber-derived giant spontaneous miniature excitatory postsynaptic currents have been suggested to be large enough to generate action potentials in postsynaptic CA3 pyramidal neurons. Here we report on the functional roles of presynaptic $GABA_A$ receptors on excitatory terminals in contributing to spontaneous glutamatergic transmission to CA3 neurons. In mechanically dissociated rat hippocampal CA3 neurons with adherent presynaptic nerve terminals, spontaneous excitatory postsynaptic currents were recorded using conventional whole-cell patch clamp recordings. In most recordings, unusually large spontaneous excitatory postsynaptic currents up to 500 pA were observed. These large spontaneous excitatory postsynaptic currents were highly sensitive to group II metabotropic glutamate receptor activation, and were still observed even after the blockade of voltage-dependent Na^+ or Ca^{2+} channels. Exogenously applied muscimol (0.1–3 μM) significantly increased the frequency of spontaneous excitatory postsynaptic currents including the large ones. This facilitatory effect of muscimol was completely inhibited in the presence of 10 μM 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid HBr, a specific $GABA_A$ receptor antagonist. Pharmacological data suggest that activation of presynaptic $GABA_A$ receptors directly depolarizes glutamatergic terminals resulting in the facilitation of spontaneous glutamate release. In the current-clamp condition, a subset of large spontaneous excitatory postsynaptic potentials triggered action potentials, and muscimol greatly increased the frequency of spontaneous excitatory postsynaptic potential-triggered action potentials in postsynaptic CA3 pyramidal neurons. The results suggest that presynaptic $GABA_A$ receptors on glutamatergic terminals play an important role in the excitability of CA3 neurons as well as in the presynaptic modulation of glutamatergic transmission onto hippocampal CA3 neurons. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: ω-AgTx, ω-agatoxin IVA; AP-5, DL-2-amino-5-phosphonovaleric acid; ω-CgTx, ω-conotoxin GVIA; CNQX, 6-cyano-7-nitroquinozaline-2,3-dione disodium; DCG-IV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine; K-S, Kolmogorov-Smirnov; mEPSC, spontaneous miniature excitatory postsynaptic current; mGluR, metabotropic glutamate receptor; QX-314, lidocaine *N*-ethyl bromide; sEPSC, spontaneous excitatory postsynaptic current; sEPSP, spontaneous excitatory postsynaptic potential; SR95531, 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid HBr; TTX, tetrodotoxin; VDCC, voltage-dependent Ca^{2+} channel.

0306-4522/06$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.11.001

Key words: presynaptic $GABA_A$ receptors, presynaptic facilitation, CA3 pyramidal neurons.

Hippocampal CA3 pyramidal neurons receive at least three excitatory glutamatergic afferents; axon collaterals from CA3 pyramidal neurons, perforant path from the entorhinal cortex, and mossy fibers arising from the dentate gyrus granule cells (Johnston and Amaral, 1990). Of these excitatory inputs, the mossy fibers have some striking anatomical features (Chicurel and Harris, 1992; Acsady et al., 1998). For example, mossy fiber terminals innervating the proximal apical dendrites of CA3 pyramidal neurons can be large, with multiple release sites containing up to several tens of active zones, and they contain not only smaller traditional vesicles (diameters ~40 nm) but also large, clear vesicles with diameters ranging up to 200 nm (Chicurel and Harris, 1992; Henze et al., 2002a). In addition to these anatomical properties, mossy fiber terminals are known to give rise to unusually large, "giant" miniature excitatory postsynaptic currents (mEPSPs) (Henze et al., 1997; 2002a). These large mEPSCs seem to arise from Ca^{2+}-independent release of glutamate from giant vesicles, which then diffuses to activate all the AMPA receptors localized beneath a large bouton, rather than from synchronized release from the multiple release sites within these large boutons (Henze et al., 2002a). The strong depolarization due to a single giant mEPSPs (of 3 or more mV), and the proximal location of the mossy fiber synapses on to CA3 neurons, mean that a single giant mEPSP has the potential to elicit action potentials in CA3 pyramidal neurons in the absence of action potentials in the parent granule cells (McNaughton and Morris, 1987; Henze et al., 2000).

GABA is the primary inhibitory neurotransmitter in the mammalian CNS. In general, $GABA_A$ receptor-mediated inhibitory actions are due to an increase in the Cl^- conductance leading to direct membrane hyperpolarization. In developing neurons, however, either synaptically released or exogenously applied GABA induces membrane depolarization, which leads to an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) secondary to the activation of voltage-dependent Ca^{2+} channels (VDCCs) and/or the generation of action potentials (Hales et al., 1994; Leinekugel et al., 1995; Obrietan and van den Pol, 1995; Owens et al., 1996). Depolarizing and excitatory actions of $GABA_A$ receptor activation is also observed at some adult synapses (Gulledge and Stuart, 2003 and references therein). In addition to $GABA_A$ receptor-mediated postsyn-

aptic depolarization, growing evidence has revealed that presynaptic $GABA_A$ receptor-mediated depolarization can modulate neurotransmitter release from nerve terminals (Eccles et al., 1963; Segev, 1990; Cattaert and El Manira, 1999; Jang et al., 2001, 2002, 2005; Turecek and Trussell, 2002). Presynaptic $GABA_A$ receptors have been recently reported to regulate the excitability of mossy fibers in the hippocampal CA3 region (Ruiz et al., 2003). In that study, activation of $GABA_A$ receptors depolarized mossy fibers, causing an increase in the intraterminal Ca^{2+} concentration and a change in axonal excitability (Ruiz et al., 2003). However, the functional consequences of activation of these presynaptic $GABA_A$ receptors on glutamate release and synaptic responses were not directly addressed.

In the present study, therefore, we have directly investigated whether presynaptic $GABA_A$ receptors exist on excitatory nerve terminals, whether their activation can directly regulate glutamatergic release from these terminals and whether presynaptic $GABA_A$ receptor activation can simultaneously change the excitability of CA3 pyramidal neurons. To test this, we used mechanically dissociated rat CA3 pyramidal neurons in which functional presynaptic nerve terminals are isolated adherent to the CA3 neurons. This preparation, the so-called 'synaptic bouton preparation' (Rhee et al., 1999; for review see Akaike and Moorhouse, 2003), allows us to investigate the presence and functional roles of presynaptic receptors without complications arising from activity of the granule cell soma, from altered activity of the surrounding neuronal elements and microenvironment, or from postsynaptic effects in poorly clamped dendritic regions of CA3 neurons.

EXPERIMENTAL PROCEDURES

Preparation

All experiments confirmed the guiding principles for the care and use of animals approved by the Council of the Physiological Society of Japan and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Wistar rats (12–15 days old) were decapitated under pentobarbital anesthesia (50 mg/kg, i.p.). The brain was dissected and transversely sliced at a thickness of 370 μm using a microslicer (VT1000S; Leica, Nussloch, Germany). Slices containing the hippocampus were kept in an incubation medium (see below) saturated with 95% O_2 and 5% CO_2 at room temperature (22–24 °C) for at least 1 h before the mechanical dissociation. For dissociation, slices were transferred into a 35 mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) containing a standard external solution (see below), and the CA3 region of the hippocampus was identified under a binocular microscope (SMZ-1; Nikon, Tokyo, Japan). Details of the mechanical dissociation have been described previously (Rhee et al., 1999). Briefly, mechanical dissociation was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at about 50–60 Hz (0.3–0.5 mm) on the surface of the hippocampal CA3 region. Slices were removed and the mechanically dissociated neurons left for 15 min to allow the neurons to adhere to the bottom of the culture dish. These dissociated neurons retain a short portion (~100 μm in length) of their thick proximal dendrites (Fig. 1A). As discussed below, it should be noted that these experiments were carried out in a more reduced preparation as compared with a slice preparation.

Electrical measurements

All electrical measurements were performed using conventional whole-cell patch recordings and a standard patch-clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo, Japan). Neurons were voltage clamped at a holding potential (V_H) of −60 mV, which is close to experimentally measured E_{GABA} (−62 to −66 mV), except where indicated. 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 (PB-7; Narishige). The resistance of the recording pipettes filled with internal solution was 4–6 MΩ. Membrane potentials were corrected for the liquid junction potential (~−11 mV, measured by exchanging bath solution from internal solution to standard external solution) and pipette capacitance was compensated for. Neurons were viewed under phase contrast on an inverted microscope (Diapot; Nikon). Current and voltage were continuously monitored on an oscilloscope (VC-6023; Hitachi), a pen recorder (RECTI-HORIT-8K; Sanei, Tokyo, Japan) and recorded on a digital-audiotape recorder (RD-120TE; TEAC). Membrane currents were filtered at 3 kHz (E-3201A Decade Filter; NF Electronic Instruments, Tokyo, Japan), digitized at 10 kHz, and stored on a computer equipped with pCLAMP 8.02 (Axon Instruments, Union City, CA, USA). During recordings, 10 mV hyperpolarizing step pulses (30 ms in duration) were periodically applied to monitor the access resistance. All experiments were performed at room temperature (22–24 °C).

Data analysis

Spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous excitatory postsynaptic potentials (sEPSPs) were counted and analyzed using the MiniAnalysis program (Synaptosoft, Inc., Decatur, GA, USA) as described previously (Jang et al., 2001, 2002). Briefly, sEPSCs and sEPSPs were detected automatically using an amplitude threshold of 10 pA and 1 mV, respectively, and then visually accepted or rejected based upon the rise and decay times. Basal noise levels during voltage- and current-clamp recordings were typically less than 8 pA and 0.5 mV, respectively. The average values of both the frequency and amplitude of sEPSCs and sEPSPs during the control period (5–10 min) were calculated for each recording, and the frequency and amplitude of all the events during different experimental conditions were normalized to these values. The inter-event intervals and amplitudes of a large number of synaptic events obtained from the same neuron were examined by constructing cumulative probability distributions and compared using the Kolmogorov-Smirnov (K-S) test with Stat View software (SAS Institute, Inc.). The rise time (10–90%) of sEPSCs was measured using the MiniAnalysis program. The decay time constant was derived from fitting a single exponential function to the sEPSCs using Clampfit (Axon Instruments). Numerical values are provided as the mean±standard error of the mean (S.E.M.) using values normalized to the control, except where indicated. Significant differences in the mean amplitude and frequency were tested using Student's paired two-tailed *t*-test, using absolute values rather than normalized ones. Values of $P<0.05$ were considered significantly different.

Solutions

The ionic composition of the incubation medium consisted of (in mM) 124 NaCl, 3 KCl, 1.5 KH_2PO_4, 24 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgSO_4$ and 10 glucose saturated with 95% O_2 and 5% CO_2. The pH was about 7.45. The standard external solution was (in mM) 150 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES, and was adjusted to a pH of 7.4 with Tris-base. For recording sEPSCs, these standard external solutions routinely contained 50 μM DL-2-amino-5-phosphonovaleric acid (AP-5) to block NMDA receptors. The ionic composition of the internal (pipette) solution for voltage clamp studies consisted of (in mM) 135 CsF,