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Reactive oxygen species induced by presynaptic glutamate receptor activation is involved in [^{3}H]GABA release from rat brain cortical nerve terminals

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ABSTRACT

We investigated the production of reactive oxygen species (ROS) as a response to presynaptic glutamate receptor activation, and the role of ROS in neurotransmitter (GABA) release. Experiments were performed with rat brain cortical synaptosomes using glutamate, NMDA and kainate as agonists of glutamate receptors. ROS production was evaluated with the fluorogenic compound dichlorodihydrofluorescein diacetate (H_2DCF-DA), and GABA release was studied using synaptosomes loaded with [^{3}H]GABA. All agonists were found to stimulate ROS production, and specific antagonists of NMDA and kainate/AMPA receptors, dizocilpine hydrogen maleate (MK-801) and 6-cyano-7-nitroquinoxaline-2,3-done (CNQX), significantly inhibited the ROS increase. Spontaneous as well as agonist-evoked ROS production was effectively attenuated by diphenyleneiodonium (DPI), a commonly used potent inhibitor of NADPH oxidase activity, that suggests a high contribution of NADPH-oxidase to this process. The replacement of glucose with pyruvate or the simultaneous presence of both substrates in the medium led to the decrease in spontaneous and NMDA-evoked ROS production, but to the increase in ROS production induced by kainate. Scavenging of agonist-evoked ROS production by a potent antioxidant N-acetylcysteine was tightly correlated with the inhibition of agonist-evoked GABA release. Together, these findings show that the activation of presynaptic glutamate receptors induces an increase in ROS production, and there is a tight correlation between ROS production and GABA secretion. The pivotal role of kainate/AMPA receptors in ROS production is under discussion.

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1. Introduction

The efficacy of communication between brain cells is crucial for the successful functioning of neuronal networks, and molecular mechanisms that are involved in intracellular signaling pathways are in focus of intensive research. One of the main mechanisms underlying the regulation of neurons' communication is the modulation of neurotransmitter release from nerve terminals by presynaptic receptors, which seem to play the role of principal modulators (Contractor et al., 2001; Duguid and Smart, 2004; Engelman and MacDermott, 2004; Glitsch, 2008; Ruiz et al., 2010). In this respect, presynaptic glutamate receptors are of significant interest for both basic and applied neuroscience research (Rodriguez-Moreno and Lerma, 1998; Cunha et al., 2000; Cossart et al., 2001; Pinheiro et al., 2005; Campbell et al., 2007; Nakamura et al., 2010). There is a great body of evidence that, depending on location of glutamate receptors, synaptic or extrasynaptic, their activation, in particular of NMDA receptor activation, can trigger processes as diverse as cell survival or neuronal death (Hardingham and Bading, 2010). According to recent data, the neuroprotective effect of synaptic receptor activation is attributed to the changes in gene expression linked with an enhancement of mitochondrial health, boosting of antioxidant defenses and suppression of caspase activation (Papadia et al., 2005; Zhang et al., 2007; Milnerwood et al., 2010). NMDA receptor activation is also associated with the increased production of superoxide anion that, in turn, causes redox modification of ryanodine- and IP3 receptors (Hidalgo et al., 2005; Mathew and Hablitz, 2008), which can contribute to calcium-induced activation of signaling cascades required for synaptic plasticity.

Our recent studies have shown that the application of glutamate to cortical and hippocampal axon terminals triggered exocytotic process, which appeared to be, at least partially, mediated by the efflux of Ca^{2+} ions from internal stores (Tarasenko et al., 2011). In light of the latest findings showing a close relationship between spontaneous transmitter release, Ca^{2+} efflux from internal stores and reactive oxygen species (ROS) generation (Emptage et al., 2001; Kemmerling et al., 2007), it is reasonable to ask whether ROS are responsible for triggering the events observed in nerve terminals during activation of presynaptic glutamate receptors. This question is of great interest because, despite the extensive studies of ROS generation triggered by the activation of postsynaptic and extrasynaptic glutamate receptors (Reynolds and Hastings, 1995;

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Vesce et al., 2004; Brennan et al., 2009; Ha et al., 2010), there is a little evidence concerning ROS production in axon terminals under presynaptic glutamate receptor activation.

The present study focuses on elucidating the questions of (i) whether the activation of presynaptic glutamate receptors triggers ROS production in axon terminals; (ii) how ROS production is correlated with stimulation of GABA release from nerve terminals; (iii) what is the contribution of NADPH oxidase in ROS generation during glutamate receptor activation. The latter is of particular interest given a recent finding of Brennan et al. (2009) about a dominant role for NADPH oxidase in NMDA receptor-mediated superoxide production in neuron cultures. NADPH oxidase is suggested to play multiple roles in neuronal signaling processes in physiological as well as pathophysiological conditions (Demaurex and Scorrano, 2009), and findings that NADPH oxidase subunits $p67^{phox}$ and $gp91^{phox}$ are closely associated with the synaptic markers synaptophysin and synaptotagmin (Tejada-Simon et al., 2005; Infanger et al., 2006) strongly support the presynaptic location of the enzyme.

2. Experimental procedures

2.1. Drugs and solutions

The following drugs were used: glutamate, N-methyl-D-aspartic acid (NMDA), kainic acid, 6-cyano-7-nitroquinoxaline-2,3-done (CNQX), dizocilpine hydrogen maleate (MK-801), diphenyleneiodonium chloride (DPI), N-acetylcysteine (NAC), sodium pyruvate, D-glucose, HEPES, DMSO were purchased from Sigma (USA). Ficoll 400 was from Pharmacia LKB Biotechnology Inc. (Sweden), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was from Molecular Probes (USA), chloro[[2,2′-[1,2-ethanediylbis[(nitrilo-κN) methylidyne]]bis[6-methoxyphenolato-]]]-manganese (EUK-134) was purchased from Cayman Chemicals (USA), 4-aminopyridine (4-AP) was from RBI (USA), [^{3}H]GABA (94 Ci/mmol) and Aqueous Counting Scintillant (ACS) were from Amersham (UK). Analytical grade salts were from Reachim (Ukraine).

All glutamate receptor agonists, NAC and CNQX were dissolved in bidistilled water to form stock solutions. Stock solutions of EUK-134, MK-801 and DPI were made with DMSO, aliquoted and frozen at −20 °C until analysis. H_2DCF-DA (10 mM) was freshly prepared in DMSO. Final concentration of DMSO in all experiments was ⩽0.07%, except Experiment 6 (⩽1.5%), where the effect of DPI was studied.

Experiments were performed in the standard salt solution containing 126 mM NaCl, 5 mM KCl, 2.0 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 20 mM HEPES (pH 7.4) and 10 mM D-glucose. In some experiments, the incubation medium contained 4 mM pyruvate instead of glucose or both energy substrates simultaneously. Ca^{2+}-free medium contained 1 mM EGTA and no added $CaCl_2$. To prevent the possible blockade of NMDA receptor activation, experiments were performed in Mg^{2+}-free medium containing 0.1 mM glycine.

2.2. Isolation of rat brain synaptosomes

Experiments were carried out on male Wistar rats (3–4 weeks old) in accordance with European Community Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering. The number of animals was dictated by the necessity to obtain valid results. The animals were instantly decapitated by guillotine, and the cortex was rapidly removed and placed in ice-cold solution containing 0.32 M sucrose, 0.2 mM EDTA and 5 mM HEPES (pH adjusted to 7.4 with NaOH). The tissue was minced and homogenized using the ratio 10:1 volume/tissue weight. Synaptosomes were prepared by differential and Ficoll-400 density gradient centrifugation of rat brain homogenate according to the method of Cotman (1974) with slight modifications as described by Linetska et al. (2004). All manipulations were performed at 0–4 °C, and all buffers and synaptosomal suspensions were constantly oxygenated. Protein concentration was measured as described by Larson et al. (1986).

2.3. Detection of ROS generation

Reactive oxygen species (ROS) production was assessed spectrofluorometrically using 2′,7′-dichlorodihydro fluorescein diacetate (H_2DCF-DA), a cell-permeable non-fluorescent probe, which, after intracellular de-esterification, becomes highly fluorescent upon oxidation. H_2-DCFDA was added to synaptosomal suspension (0.3 mg/ml final protein concentration) to a final concentration of 5 μM, and then synaptosomes were incubated for 5 min in stirred cuvette thermostatted at 35 °C. DCF fluorescence was detected using a Hitachi MPF-4 spectrofluorometer (Tokyo, Japan) at excitation/emission wavelengths of 502/525 nm (slit bands 5 and 10 nm, respectively). The intensity of fluorescence, spontaneous as well as evoked by glutamate receptor agonists, was measured over a 20-min period. The fluorescence signal was normalized to the intensity registered at time point $t = 25$ s, when agonists were added. Data were presented for each test condition as the change of normalized fluorescence over time.

2.4. GABA release experiments

Synaptosomes were diluted with standard salt solution to 2 mg of protein/ml and, after pre-incubation for 5 min at 37 °C, were loaded with [^{3}H]GABA (50 nM, 4.7 μM/ml) in Ca^{2+}-supplemented oxygenated standard salt solution for 10 min. Amino-oxyacetic acid, a GABA transaminase inhibitor, was present at 100 μM throughout all experiments involving [^{3}H]GABA loading and release. After loading, the suspension was washed with 10 volumes of ice-cold oxygenated standard salt solution, resuspended in the same solution to obtain a protein concentration of 1 mg/ml and immediately used for release experiments. [^{3}H]GABA release from synaptosomes was estimated in all experiments with the following procedure: the investigated samples were pre-incubated with/without NAC, a potent antioxidant, for 10 min at 37 °C and then different agents were applied. Aliquots of the samples (120 μl of suspension) were taken out at 2 min for glutamate, 3 min for kainate, 5 min for H_2O_2/4-aminopyridine and rapidly sedimented in a microcentrifuge (15 s at 10000*g*). The amount of [^{3}H]GABA released in each collection period was measured in aliquots of supernatants (90 μl) by liquid scintillation counting with ACS (1.5 ml) as scintillation cocktail and was expressed as a percentage of total neurotransmitter incorporated.

Neurotransmitter release from synaptosomes incubated without any stimulating agents was defined as the basal release. [^{3}H]GABA release stimulated by 4-AP, H_2O_2, glutamate or glutamate receptor agonists was calculated by subtracting the basal value from the value in the presence of stimulants. The synaptosomal suspensions were used in experiments during 2–4 h after isolation.

2.5. Statistics

Statistical analyses were carried out using 'Origin Pro 8.6.0, b70' (OriginLab Corporation, Northampton, MA, USA). Student's *t*-test was used to compare the data for two experimental groups. Statistical differences among more than two groups were tested using one-way ANOVA followed by Bonferroni multiple comparison test. All data are presented as the mean ± standard error of the mean (SEM). Results were considered statistically significant at *p*-values ⩽0.05.

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