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Phenylarsine oxide is able to dissipate synaptic vesicle acidic pool

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Abstract

Phenylarsine oxide (PAO) has a number of targets in the neurons, one of them is exocytotic process. In this study, we have focused on the mechanisms of phenylarsine oxide action on Ca^{2+}-dependent and Ca^{2+}-independent neurotransmitter release from rat brain synaptosomes. We investigated the influence of phenylarsine oxide on: (i) L-[^{14}C]glutamate and [^{3}H]GABA release and uptake; (ii) plasma membrane potential using a potential-sensitive fluorescent probe rhodamine 6G; (iii) exo/endocytotic process using a pH-sensitive fluorescent probe acridine orange (AO). It has been found that phenylarsine oxide induced deacidification of synaptic vesicles. This effect was completely abolished by preliminary treatment of synaptosomes with a protonophore FCCP indicating that both reagents injured a proton electrochemical gradient. Dissipation of the proton gradient by low concentrations of phenylarsine oxide (not exceed 1 μM) did not prevent KCl-triggered exocytotic response, but essentially modified endocytotic one. At higher concentrations of phenylarsine oxide (up to 10 μM), the proton gradient dissipation was intensified and the exocytotic response was fully abolished. The reagent did not change plasma membrane potential, but depolarized mitochondria. It also caused potent inhibition of the Ca^{2+}-stimulated L-[^{14}C]glutamate and [^{3}H]GABA release and increase the Ca^{2+}-independent release of L-[^{14}C]glutamate, but not of [^{3}H]GABA. Disulfide-reducing reagents (dithiothreitol and β-mercaptoethanol) completely prevented phenylarsine oxide-evoked injuries. They could also restore the initial levels of the mitochondrial potential, the exocytotic response to KCl and the release and uptake of neurotransmitters.

Our data provide the evidence that phenylarsine oxide causes dissipation of synaptic vesicle acidic pool resulting in the reduction of vesicle filling and as consequence in attenuation of Ca^{2+}-stimulated neurotransmitter release.

Keywords: Acridine orange; Exo/endocytosis; Neurotransmitter release; Phenylarsine oxide; Rat brain synaptosomes; Rhodamine 6G; SH-reagents

1. Introduction

Synaptic transmission is achieved through the regulated release of neurotransmitters by exocytosis. Vesicular trafficking in presynaptic nerve terminals, docking and fusion of synaptic vesicles with plasma membrane are the result of assembly and disassembly of multiple specialized vesicle and target membrane proteins. Interactions of these proteins can be modulated by a number of drugs. The studies on mechanisms of their actions are aimed at better understanding of the molecular biology of synaptic transmission. One of widely used tools for investigations of exo- and endocytotic processes is phenylarsine oxide (PAO).

Hydrophobic sulfhydryl group reagent PAO is known to react with two thiol groups of closely spaced protein cysteine residues to form stable dithioarsine rings (Van Iwaarden et al., 1992). It has been shown that in nerve cells it reduces calcium entry through N-type calcium channels (Searl and Silinsky, 2000), abolishes syntaxin 1A modifying action on voltage-gated calcium channels (Arien et al., 2003), inhibits the activity of protein tyrosine phosphatases (Lohmann et al., 2004) and phosphatidylinositol 4-kinase (Wiedemann et al., 1998; Micheva et al., 2001). The last enzyme has an important regulatory function in vesicular trafficking as a key part of the pathway for the synthesis of phosphatidylinositol 4,5-

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biphosphate (PIP2) by phosphorylation of PI to PI(4)P (Toker, 1998).

Our recent work has demonstrated the essential inhibitory effect of PAO on both Ca^{2+}-dependent 4-aminopyridine-evoked [^{3}H]GABA release and Ca^{2+}-independent release stimulated by α-latrotoxin (Linetska et al., 2003). These results are consistent with the data of Wiedemann et al. (1998) concerning PAO-evoked inhibition of Ca^{2+}-dependent glutamate release stimulated by different depolarizing agents. They have also established that the suppression of Ca^{2+}-dependent glutamate release occurred concomitantly with inhibition of phosphatidylinositol 4-kinase localized to small synapic vesicles. Recently, Zheng et al. (2004) studying the connection between phosphoinositide metabolism and the exocytotic phase of the vesicle cycle have found that inhibition of PI(4)P and PIP2 synthesis by PAO impairs exocytosis of both types of vesicles—synaptic and dense core vesicles. However, the conclusions about the injury of exocytotic process were based on observed attenuation in the release of labeled neurotransmitters.

In the present study, we have focused our attention on the mechanisms by which exocytosis could be affected by PAO. We explored the method with a pH-sensitive dye acridine orange (AO) (Zoccarato et al., 1999) that permits to investigate exocytotic response, vesicle recycling, and also alterations in synaptic vesicle proton gradient. So we could determine what caused the inhibition of neurotransmitter release—low release probability or release of incompletely filled or depleted vesicles. We also used fluorescent dye rhodamine 6G to monitor plasma membrane potential and labeled neurotransmitters, L-[^{14}C]glutamate and [^{3}H]GABA, for the measurements of Ca^{2+}-dependent and Ca^{2+}-independent neurotransmitter release. Our results indicate that PAO-induced impairment of Ca^{2+}-dependent neurotransmitter release is, at least partially, due to ability of PAO to dissipate vesicle acidic pool and thereby to eliminate the driving force for vesicle filling.

2. Materials and methods

2.1. Isolation of rat brain synaptosomes

Male Wistar rats (100–120 g body weight) were housed and handled in accordance with the European Guidelines and International Laws and Policies. The animal was decapitated, the brain was rapidly removed and homogenized in ice-cold 0.32 M sucrose, 5 mM HEPES-NaOH, pH 7.4 and 0.2 mM EDTA. 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 (Linetska et al., 2004). All manipulations were performed at 0–4 °C, all buffers and synaptosomal suspension were constantly oxygenated. The synaptosomal suspensions were used in experiments during 2–4 h after isolation. The standard salt solution contained (in mM): NaCl—126, KCl—5, $MgCl_2$—1.4, NaH_2PO_4—1.0, HEPES—20, pH 7.4 and D-glucose—10. The Ca^{2+}-supplemented medium contained 2 mM $CaCl_2$. The Ca^{2+}-free medium contained 1 mM EGTA and no added Ca^{2+}.

Protein concentration was measured as described by Larson (1986).

2.2. Membrane potential measurements

Membrane potential measurements were performed using the potentiometric optical dye rhodamine 6G (Rh 6G) (Aiuchi et al., 1982) as previously described by Linetska et al. (2004). Briefly, Rh 6G (final concentration 0.5 μM) was added to synaptosomal suspension (~0.15 mg/ml final protein concentration) and kinetics of dye uptake was recorded for 3-5 min until the plateau level of Rh 6G fluorescence (F_t) has been reached. After application of a depolarizing agent the new steady state level was monitored.

To estimate changes of synaptosomal plasma membrane potential the ratio (F) as index of membrane potential was applied:

$$F = \frac{F_t}{F_0},$$

where F_0 and F_t—fluorescence intensities of Rh 6G in the absence and in the presence of synaptosomes, respectively. F_0 was calculated by extrapolation of exponential decay function to $t = 0$. Fluorescence in the steady state (F_t) was monitored before and after application of the membrane depolarizing factor. To calibrate F as function of the membrane potential, external K^+ and Na^+ concentrations (with maintenance $[K^+]_{out} + [Na^+]_{out} = 131$ mM) were varied and membrane potential was calculated by the Goldman equation (Mandala et al., 1999).

Rh 6G fluorescence measurements were carried out using Hitachi MPF-4 spectrofluorimeter at 528 nm (excitation) and 551 nm (emission) wavelengths (slit bands 5 nm each) in a stirred cuvette thermostatted at 30 °C.

2.3. Vesicle acidification measurement

Fluorescent dye acridine orange, metachromatic weak base, that selectively accumulates in synaptic vesicles was used for monitoring exo/endocytosis and vesicle acidification (Zoccarato et al., 1999; Melnik et al., 2001). Fluorescence changes were measured using Hitachi MPF-4 spectrofluorimeter at excitation/emission wavelengths of 490/530 nm, respectively (slit bands 5 nm each). The reaction was started by addition of 100 μl of synaptosomal suspension (0.2 mg/ml final protein concentration) to stirred cuvette thermostatted at 30 °C that contained the standard salt solution with added AO (5 μM). The equilibrium level of dye fluorescence has been achieved for 15 min. Fluorescence (F) was defined as

$$F = \frac{F_t}{F_0},$$