Neurochemistry International 63 (2013) 576–582

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Etomidate evokes synaptic vesicle exocytosis without increasing miniature endplate potentials frequency at the mice neuromuscular junction

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ARTICLE INFO

Article history:
Received 1 April 2013
Received in revised form 19 August 2013
Accepted 8 September 2013
Available online 15 September 2013

Keywords:
Etomidate
Neuromuscular junction
FM1-43
Synaptic vesicle
Nicotinic receptors

ABSTRACT

Etomidate is an intravenous anesthetic used during anesthesia induction. This agent induces spontaneous movements, especially myoclonus after its administration suggesting a putative primary effect at the central nervous system or the periphery. Therefore, the aim of this study was to investigate the presynaptic and postsynaptic effects of etomidate at the mouse neuromuscular junction (NMJ). Diaphragm nerve muscle preparations were isolated and stained with the styryl dye FM1-43, a fluorescent tool that tracks synaptic vesicles exo-endocytosis that are key steps for neurotransmission. We observed that etomidate induced synaptic vesicle exocytosis in a dose-dependent fashion, an effect that was independent of voltage-gated Na^+ channels. By contrast, etomidate-evoked exocytosis was dependent on extracellular Ca^{2+} because its effect was abolished in Ca^{2+}-free medium and also inhibited by omega-Agatoxin IVA (30 and 200 nM) suggesting the participation of P/Q-subtype Ca^{2+} channels. Interestingly, even though etomidate induced synaptic vesicle exocytosis, we did not observe any significant difference in the frequency and amplitude of miniature end-plate potentials (MEPPs) in the presence of the anesthetic. We therefore investigated whether etomidate could act on nicotinic acetylcholine receptors labeled with α-bungarotoxin-Alexa 594 and we observed less fluorescence in preparations exposed to the anesthetic. In conclusion, our results suggest that etomidate exerts a presynaptic effect at the NMJ inducing synaptic vesicle exocytosis, likely through the activation of P-subtype voltage gated Ca^{2+} channels without interfering with MEPPs frequency. The present data contribute to a better understanding about the effect of etomidate at the neuromuscular synapse and may help to explain some clinical effects of this agent.

1. Introduction

Etomidate is an intravenous agent used in clinical practice to induce unconsciousness with a good cardiovascular protection. Therefore, it is less likely to cause a significant depression in blood pressure when compared to other induction agents (Zed et al., 2006). It also produces less apnea than propofol, does not induce histamine release and is rarely involved in allergic reactions (Bovill, 2006). In addition, there are studies emphasizing that this anesthetic has cerebral protective effects (Liang et al., 2013; Yao et al., 2012).

It is well known that neurotransmission mediates neuronal communication and therefore it seems plausible that anesthetics such as etomidate could have molecular targets in neurons. For example, previous studies have shown that the action of etomidate in the central nervous system (CNS) is mediated by $GABA_A$ receptors since clinically relevant concentrations of etomidate (1–8 μM) modulate the receptor activation (Giese and Stanley, 1983). Therefore, in the presence of etomidate $GABA_A$ receptors are activated by lower concentrations of γ-amino butyric acid (GABA) than that required in the absence of the anesthetic (Belelli et al., 1999; Giese and Stanley, 1983; Yang and Uchida, 1996). On the other hand, at supra-clinical concentrations, etomidate can act directly on $GABA_A$ receptors by activating them in the absence of the neurotransmitter, characterizing a direct action also known as GABA-mimetic effect or allosteric agonist (Rusch et al., 2004; Yang and Uchida, 1996).

There are few studies about the effects of etomidate on basic steps of other neurotransmission systems. Pashkov and Hemmings

Abbreviations: α-BGT, alpha-bungarotoxin; ACh, acetylcholine; EGTA, etilenoglicol-bis-β-aminoetil éster; GABA, γ-amino butyric acid; MEPP, miniature end plate potential; TTX, tetrodotoxin; NMJ, neuromuscular junction.

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http://dx.doi.org/10.1016/j.neuint.2013.09.008

(2002) observed that clinical and supra-clinical concentrations of etomidate did not affect basal or stimulus-evoked [^{3}H] norepinephrine release from isolated rat cortical nerve terminals. However, another study conducted by Xie et al. (2004), demonstrated that clinically relevant concentrations of etomidate were able to stimulate the release of catecholamines in chromaffin cells demonstrating that etomidate at clinical concentrations can affect the release of neurotransmitters by distinct ways.

It is known that involuntary myoclonic movements are common during the use of etomidate that could be related to an effect of this agent at the NMJ level. Therefore, in this study we investigated the effect of subclinical, clinical and supra-clinical concentrations of etomidate in one of the main steps of neuromuscular transmission: the synaptic vesicles life cycle, using the diaphragm NMJ as an experimental model. To address this issue, we used fluorescence microscopy and electrophysiology techniques and we provide evidences that etomidate exerts presynaptic effects on the NMJ, probably through activation of voltage-gated Ca^{2+} channels. Furthermore, we demonstrate that etomidate can act in postsynaptic receptors by changing the pattern of distribution of nicotinic receptors as well as the response mediated by them. Thus, these findings may help to understand some of the clinical effects of this agent on neuromuscular function.

2. Materials and methods

FM1-43, FM1-43 fx and α-bungarotoxin (α-BGT) were purchased from Molecular Probes (Eugene, OR, USA); D-tubocurarine, tetrodotoxin (TTX), omega-conotoxins were purchased from Sigma–Aldrich (St. Louis, MO, USA). Etomidate was obtained from Cristália (São Paulo, Brazil). All other chemicals and reagents were of analytical grade. All the following procedures were approved by the local animal care committee (CETEA-UFMG protocol# 82/2008).

2.1. Staining and destaining with FM1-43

Diaphragm nerve-muscle preparation was dissected from Swiss female adult mice (30–40 g) and pinned flat in a sylgard-line perfusion chamber containing mouse Ringer solution (134 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM D-glucose) gassed with 95% O_2–5% CO_2. To stain the recycling pool of synaptic vesicles we used the fluorescent probe FM1-43 (4 μM) (Betz et al., 1992). The structure of this dye presents a hydrophobic tail that allows reversibly bind to biological membranes and a polar head that impairs its fully permeation to the plasma membrane (Betz and Bewick, 1993). Therefore, FM1-43 binds to synaptic membrane and after submitting the nerve terminal to a stimulus that causes exocytosis of synaptic vesicles followed by compensatory endocytosis, the fluorescent dye is internalized resulting in a typical pattern of staining (Betz et al., 1992). When the nerve terminals are submitted to a new round of stimulation, in the absence of FM1-43 in the external medium, the dye is released to the hydrophilic medium, resulting in a decrease of fluorescence intensity which reflects the exocytosis of synaptic vesicles (Betz and Bewick, 1993; Rizzoli and Betz, 2005).

In our experiments, the muscles were incubated with D-tubocurarine (16 μM) to avoid contractions during the stimulation protocol. The preparations were stimulated for 10 min with modified Ringer solution containing a high concentration of KCl (80 mM NaCl, 60 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM D-glucose) in the presence of FM1-43 (4 μM). Thereafter, the preparation was kept resting in mouse Ringer solution for 10 min to guarantee maximum FM1-43 uptake. The excess of FM1-43 adhered to the muscle membranes were removed during a 20 min washing period in gassed mouse Ringer solution. Images were acquired during 7 min time frame with intervals of 2 min.

After labeling the recycling pool of synaptic vesicles, neuromuscular preparations were exposed to different concentrations of etomidate (1, 2, 8, 10 and 40 μM) during 7 min to evaluate its effect on spontaneous exocytosis. The destaining at the absence of stimulus due to photobleaching of FM1-43 (about 10% of decrease in fluorescence) was used as a control.

Experiments investigating the role of extracellular Ca^{2+} ions in exocytosis induced by etomidate were performed in modified Ringer solutions with equimolar substitution of $CaCl_2$ for $MgCl_2$ plus etilenoglicol-bis-β-aminoetil éster EGTA (1 mM). In addition we also used the channel blockers omega-Conotoxin MVIIC (50 μM) and omega-Agatoxin IVA (30 and 200 nM). The preparations were pre-incubated in normal Ringer containing the toxins for 10 min before adding etomidate. To investigate the effect of etomidate on voltage-gated Na^+ channels we used tetrodotoxin TTX (1 μM), a toxin that blocks these channels.

For staining with FM1-43 fx (fixable analogue of FM1-43), hemidiaphragms were stimulated with a high-KCl solution (60 mM KCl) for 10 min or were stimulated with etomidate (40 μM) for 10 min in the presence of the dye. After stimulation, the preparations were maintained in normal Ringer solution for 10 min to guarantee maximal FM1-43 fx uptake and compensatory endocytosis. Excess of FM1-43 fx adhered to the muscle plasma membrane was removed during a 15 min washing period in mouse Ringer solution containing Advasep-7 (1 mM) to reduce background fluorescence. Preparations were then fixed with 4% paraformaldehyde for 40 min. After fixation, the muscles were washed with glycine (40 mM) in PBS (0.1 M) to reduce excess fixative then mounted onto slides using ProLong® Gold (Invitrogen, SP, Brazil) mounting medium.

Nicotinic receptors were identified by labeling with α-BGT. A hemidiaphragm was treated with α-BGT (12 μM) conjugated with Alexa Fluor 594 for 7 min at room temperature (24 °C). The other hemidiaphragm was submitted to the same treatment, but in the presence of etomidate (1 μM).

2.2. Optical imaging

All images were acquired using a fluorescence microscope (Leica DM2500) coupled to a CCD camera (Micromax) and visualized in a computer. The microscope was equipped with a water immersion objective (63×, 0.95NA). Excitation light came from a 100 W Hg lamp and passed through filters to select the fluorescein spectrum for the FM1-43 experiments and the rhodamine spectrum for α-BGT experiments. All image variables like exposure time and binning were kept the same for pairs of hemidiaphragms.

2.3. Image analysis

Images were analyzed using the softwares Image J and Microsoft Excel. The mean fluorescence intensity was determined for each cluster of spots and plotted against the time as percentage of its mean initial fluorescence using the software GraphPad Prisma 4.0. Statistical analysis was performed using unpaired Student's *t*-test. $P < 0.05$ values were considered statistically significant.

2.4. Electrophysiology

Hemidiaphragms were isolated and pinned to a Sylgard pad in a 5 ml acrylic chamber containing the following Ringer solution: 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM D-glicose, pH 7.4, gassed with 95% O_2–5% CO_2 through a perfusion system. Microelectrodes were made from borosilicate glass and had resistances of 5–15 Ω when