Acta Histochemica xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Acta Histochemica

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

Elements of molecular machinery of GABAergic signaling in the vertebrate cholinergic neuromuscular junction

Leniz F. Nurullin[a,b,c], Evgeny E. Nikolsky[a,b,c], Artem I. Malomouzh[a,c,*]

[a] Kazan Institute of Biochemistry and Biophysics, Subdivision of the Federal State Budgetary Institution of Science "Kazan Scientific Center of the Russian Academy of Sciences", P.O. Box 30, Kazan 420111, Russia
[b] Kazan Medical University, 49 Butlerova St., Kazan 420012, Russia
[c] Kazan Federal University, 18 Kremlyovskaya St., Kazan 420008, Russia

ARTICLE INFO

Keywords:
Neuromuscular transmission
Acetylcholine
GABA
GAD
GABA transporter

ABSTRACT

It is generally accepted that gamma-aminobutyric acid (GABA) is a signaling molecule abundant in central synapses. In a number of studies though, it has been shown that GABA signaling functions in the peripheral nervous system as well, in particular, in the synapses of sympathetic ganglia. However, there exists no firm evidence on the presence of GABAergic signaling cascade in the intercellular junctions of the somatic nerve system.

By the use of immunohistochemistry methods, in the synaptic area of cholinergic neuromuscular contact in rat diaphragm, we have detected glutamate decarboxylase, the enzyme involved in synthesis of GABA, molecules of GABA, and also GAT-2, a protein responsible for transmembrane transport of GABA. Earlier we have also shown that metabotropic $GABA_B$ receptors have overlapping localization in the same compartment. Moreover, activation of $GABA_B$ receptors affects the intensity of acetylcholine release. These data taken together, allows us to suggest that in the mammalian cholinergic neuromuscular junction, GABA is synthesized and performs certain synaptic signaling function.

1. Introduction

γ-Aminobutyric acid (GABA) is generally considered to be a major inhibitory neurotransmitter in synapses of central nervous system, where it plays an important role in development, maturation and functioning of adult brain (Watanabe et al., 2002; Obata, 2013). GABA molecules are formed by decarboxylation of glutamate which is catalyzed by the enzyme L-glutamic acid decarboxylase (GAD) (EC 4.1.1.15) and subsequently transported into synaptic vesicles by vesicular GABA transporter (VGAT) (Omote and Moriyama, 2013). The synaptic action of GABA occurs via activation of ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors (Bowery et al., 2002; Olsen and Sieghart, 2008) and is terminated by uptake of GABA into neurons and glial cells. At present, the following proteins have been identified that are capable of transporting GABA molecules through membrane and thus enabling the uptake of neurotransmitter: GABA transporters 1–3 (GAT1, GAT2, GAT3) and betaine-GABA transporter (BGT1) (Zhou and Danbolt, 2013). Noteworthy, that under certain conditions, transportation process can be reversed and GABA can be released to extracellular space (Attwell et al., 1993).

Based on numerous studies carried out in the end of the 20th century, data were collected indicating that GABA may perform signaling function in peripheral nervous system as well (Watanabe et al., 2002). In particular, various elements of GABAergic signaling cascade (GABA itself, GAD, GABA receptors and transporters) were found in gastrointestinal tract, glands (thyroid, adrenal, salivary) and sympathetic ganglia, and in some preparations Ca^{2+}-dependent and tetrodotoxin sensitive mechanism of stimulus-evoked GABA release was identified in neuronal components of the tissues (Jessen et al., 1983; Tanaka, 1985). By the methods of molecular biology and genetics it has been discovered that γ2 and α1-α5 subunits of $GABA_A$ receptors are expressed in neurons of both myenteric and submucosal plexuses (Seifi et al., 2014). It has been also shown that α4 и β2/3 subunits of $GABA_A$ receptors are present in cholinergic neurons of sympathetic ganglia (Park et al., 2006; Elinos et al., 2016). Also, the neurons of the myenteric plexus were found to express both subunits, $GABA_{B1}$ and $GABA_{B2}$, of metabotropic receptor (Torashima et al., 2009). All these findings relate to the vegetative division of the peripheral nervous system, while the data on the presence of GABAergic signaling system in the somatic division are scarce. Immunoreactivity to GAD has been found in human

* Corresponding author at: Kazan Institute of Biochemistry and Biophysics, Subdivision of the Federal State Budgetary Institution of Science "Kazan Scientific Center of the Russian Academy of Sciences", P.O. Box 30, Kazan, 420111, Russia.
E-mail address: artur57@gmail.com (A.I. Malomouzh).

https://doi.org/10.1016/j.acthis.2018.02.003
Received 13 November 2017; Received in revised form 19 January 2018; Accepted 14 February 2018

Table 1
Characterization of the antibodies used.

Antigen	Immunogen	Manufacturer, species, antibody type	Dilution used
GABA	GABA conjugated to BSA	Sigma-Aldrich (A2052, Lot. 112M4768), rabbit, polyclonal	1:200
GAD-65/67	epitope mapping at the C-terminus of GAD-67 of human origin	Santa Cruz Biotechnology (sc-7513, Lot. G0913), goat, polyclonal	1:200
VGAT	epitope mapping near the N-terminus of VGAT of human origin	Santa Cruz Biotechnology (sc-49574, Lot. K1212), goat, polyclonal	1:200
GAT-1	an extracellular epitope of rat GABA Transporter 1	Alamone Labs (AGT-001, Lot. AGT001AN0102), rabbit, polyclonal	1:200
GAT-2	an epitope of rat GABA Transporter 2	Alamone Labs (AGT-002, Lot. AGT002AN0102), rabbit, polyclonal	1:200

Abbreviations: BSA, Bovine serum albumin; GABA, γ-aminobutyric acid; GAD, glutamate acid decarboxylase; GAT, GABA transporter; VGAD, vesicular GABA transporter.

and primate neuromuscular junctions, as well as in axons of motor neurons within the nerve bundle in human muscles (Chan-Palay et al., 1982). Recently, by means of immunohistochemistry, we have detected $GABA_B$ receptors in cholinergic neuromuscular junctions of rat (Malomuzh et al., 2015; Malomouzh et al., 2015). We have also shown that activation of these receptors results in inhibition of acetylcholine release from motor nerve endings (Malomouzh et al., 2015). These data allowed us to suggest that GABA could play certain modulatory role in the neuromuscular transmission. To test this hypothesis we have conducted the current study in an effort to identify key GABAergic signaling molecules (GABA, GAD and GABA transporters) in peripheral cholinergic synapse.

2. Materials and methods

2.1. Animals and tissue preparation

Eight male Wistar rats (body weight 150–200 g) from the Laboratory of Animal Breeding Facility (Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Puschino, Moscow Region, Russia) were allowed to acclimate to in the vivarium for at least 1 week before experiments. Animals were kept in sawdust-lined plastic cages in a well-ventilated, 12-h light/dark cycle room at 20–26 °C and given *ad libitum* access to food and water. Experiments were performed in accordance with the guidelines for the use of laboratory animals of the Kazan Federal University and the Kazan Medical University, in compliance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol met the requirements of the European Communities Council Directive 86/609/EEC and was approved by the Ethical Committee of Kazan Medical University.

Animals were deeply anesthetized by isoflurane inhalation until the tail-pinch reflex disappeared and were then quickly decapitated. All experiments were performed on isolated phrenic nerve-diaphragm preparations. The diaphragm preparations from rats were excised and cut longitudinally into the strips of parallel intact muscle fibers. Samples were fixed in 4% paraformaldehyde solution for 5 min.

2.2. Immunohistochemistry

After fixation procedure, the nerve-muscle preparations were washed three times in phosphate buffered saline every 10 min for a period of 30 min. Next, the muscles were incubated in 0.5% Triton X-100 solution for 30 min and then, for another 15 min, in the blocking solution (prepared in phosphate buffered saline) of the following composition: 1% bovine serum albumin, 5% normal goat serum, and 0.5% Triton X-100. The preparations were then incubated for 15 h at 4 °C with primary antibodies against GABA (Sigma-Aldrich, St. Louis, MO, USA), GAD-65/67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), VGAT (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAT-1 (Alamone Labs, Jerusalem, Israel), and GAT-2 (Alamone Labs, Jerusalem, Israel). After this step, the muscles were rinsed three times in Triton X-100 for 30 min and incubated for 1 h with secondary donkey, anti-goat, or anti-rabbit antibodies conjugated to Alexa-488 or Alexa-647 (1:800, Invitrogen, CA, USA) at 20 °C. After wash-off, the preparations were incubated with tetramethylrhodamine-α-bungarotoxin (TMR-α-bungarotoxin) (20 μg/mL; Sigma, St Louis, MO, USA) for 30 min in the dark at 20 °C, to enable visualization of postsynaptic nicotinic acetylcholine receptors (Krause and Wernig, 1985).

The final step was to mount the preparations in a special solution of anti-fading reagent (polyvinyl alcohol mounting medium with DABCO; Sigma-Aldrich, St. Louis, MO, USA) on glass slides for microscopic observation using a Zeiss LSM 510 Meta laser-scanning confocal microscope (Carl Zeiss, Jena, Germany) with 63× oil-immersed objective lens (Carl Zeiss, Oberkochen, Germany). Excitation laser light with a wavelength of 488 nm for Alexa-488, 647 nm for Alexa-647, and 543 nm for TMR-a-bungarotoxin was used for image acquisition, and the images were processed using ImageJ software (NIH, Bethesda, MD, USA).

The presence of each antigen was tested in neuromuscular junctions in three distinct animals (*n* indicates the number of observed synapses). The size of each preparation permitted visualization of 14–25 superficially located synaptic contacts.

2.3. Antibody characterization

Characterization of antibodies used in the present study is shown in Table 1. The proper dilution factor for each antibody was determined by testing serial dilutions of the antibody concentrations. To evaluate the immunohistochemical reactivity specificities of primary antibodies, the latter were pre-incubated with corresponding blocking peptides (sc-7513P for antibodies against GAD-65/67; sc-49754P for antibodies against VGAT; peptide (C)ERNMHQMTDGLDK, corresponding to amino acids residues 194–206 of rat GABA Transporter 1, for antibodies against GAT-1; peptide (C)KVEEDGTLEREQWTNK, corresponding to amino acids residues 23–38 of rat GABA Transporter 2, for antibodies against GAT-2). Negative control experiments were performed by omitting the primary antibodies.

3. Results

In all neuromuscular preparations studied, immunohistochemical labeling of GABA molecules revealed a distinct immunopositive reaction, which was expressed in somewhat non-uniform patterns with areas of very high intensity of staining. Double staining of acetylcholine receptors (by TMR-α-bungarotoxin) and GABA showed that the latter are located in the synaptic area and are absent outside neuromuscular junction (Fig. 1). GABA immunoreactivity was detected at all synapses ($n = 56$).

In addition to GABA immunoreactivity in synaptic region, we detected immunoreactivity to GAD enzyme (Fig. 1), considered to be a major enzyme involved in GABA synthesis in living organism. The pattern of GAD staining overlaps and extends in places (by several micrometers) beyond staining region of acetylcholine receptors at all neuromuscular junctions ($n = 47$).

Finally, we carried out studies to identify transporters of GABA. With antibodies used, we detected no immunoreactivity to VGAT and GAT-1 transporters (Fig. 1) at all visualized synapses ($n = 42$ and $n = 49$, respectively). At the same time, a pronounced immune