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Original Article

Cerebellar Giant Synaptosomes: a Model to Study Basal and Stimulated Release of [^{3}H]gamma-Aminobutyric Acid

S.M. Hosseini,[1] A. Asgari,[1] H.A. Mehrani,[2] A. Khoshbaten[1,3]

Abstract

Background: Neurotransmitter release is an essential link in cell communication of the nervous system. Many investigations have focused on gamma amino butyric acid (GABA)-ergic neurotransmission, because it has been implicated in the pathophysiology of several central nervous system disorders. To bypass complications related to homo- and heterosynaptic modulation and to avoid indirect interpretations of data, we herein describe a simple approach for direct measurement of GABA release.

Material and Methods: Giant synaptosomes originated from nerve terminals of rat cerebellum mossy fibers were prepared for the study. Electron micrographs as well as lactate dehydrogenase assay are used for morphological and biochemical verifications. Giant synaptosomes were preloaded with labeled [^{3}H]GABA. Spontaneous and stimulated release of [^{3}H]GABA was measured using a superfusion apparatus. Stimulation was evoked by increasing exteracellular concentration of K^+ ions.

Results: Spontaneous [^{3}H]GABA release had a constant and nearly linear kinetics. [^{3}H]GABA outflow evoked by depolarizing solution containing 15 mM of K^+ showed 2-3 fold increases over the basal release. The same effect was also reproducible after several minutes.

Conclusion: The present findings indicate that this preparation could be used as a suitable and versatile *in vitro* model to study GABA release from axon terminals under basal and evoked conditions.
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Keywords ● Synaptosome ● Neurotransmitter release ● Cerebellum ● GABA

Departments of [2]Biochemistry,
[1]Physiology & Biophysics and
[3]Chemical Injuries,
Baqhiyatallah University of Medical Sciences,
Tehran, Iran.

Correspondence:
Alireza Asgari PhD,
Department of Physiology & Biophysics,
Baqiyatallah University of Medical Sciences,
Tehran, Iran.
Tel/Fax: +98 21 2281561
E-mail: Asgari@bmsu.ac.ir

Introduction

Synapses serve as the site for anterograde information transfer from presynaptic to postsynaptic neurons or other effectors. The release of neurotransmitter from nerve terminals is regulated and modulated by a variety of mechanisms. This modulation is important for a number of nervous system functions. In addition, these mechanisms may be targets for a number of inherited and acquired diseases as well as the development of new drugs.[1-4] Different methods and models have been used to study chemical neurotransmission

and their presynaptic regulatory elements in great details (e.g., receptors, channels, uptake systems, and etc). Although electrophysiological approach (clamp technique) has led to much progress in this filed, it provides only an indirect measure of transmitter release, because it is mainly based on the recording of postsynaptic receptor currents.[5] Indeed, a number of variables, such as possible saturation of postsynaptic receptors, rapid desensitization of the receptors, and the occurrence of activity-dependent changes in postsynaptic receptor density may play a role.[5] Hence, the interpretation of these results in terms of presynaptic transmitter release is rather complex.

Synaptosomes (detached synapses) are resealed presynaptic nerve terminals isolated from brain tissue by homogenization.[6] Under metabolizing conditions, synaptosomes respire, utilize glucose, extrude Na^+, accumulate K^+, maintain a normal membrane potential, and upon depolarization release neurotransmitter.[7] They are the simplest brain-tissue preparations that preserve the functional activity of presynapsis and are proved to be useful in studying different synaptic events such as uptake, storage, synthesis and release of neurotransmitters.[8] Cerebellum is one of the best-known regions of the central nervous system both in terms of simplicity of neuronal circuitry and neurotransmitters involved in specific synapses.[9] All inhibitory neurons in the cerebellum, Golgi, basket, stellate, and Purkinje cells seem to utilize GABA as their neurotransmitter.[10] Hence, the aforementioned considerations prompted us to prepare cerebellar synaptosomes as a simple model to study GABA release.

Materials and Methods

All experimental procedures involving animals are in accordance with the protocols established in the NIH/NRC Guide for Care and Use of Laboratory Animals, reviewed and authorized by the Ethical Committee of the University. Adult male Sprague-Dawley rats (180-240 g) were kept in a temperature-controlled room (22±1°C) with a 12-hour light-dark illumination cycle and having free access to rat chow (Pars Dam Co. Iran) and water.

Preparation of synaptosomes

Rats were anesthetized with diethyl ether and sacrificed by decapitation. Cerebellum was rapidly removed and placed in ice-cold medium. Dissection, homogenization and all fractionations were conducted at 0-4°C and synaptosomes were prepared according to Maura's method.[11] In brief, cerebellum was quickly removed and homogenized in 40 volumes of ice cold medium A (0.32 M sucrose buffered with 0.1 M disodium phosphate at pH 7.4), in a glass potter homogenizer adjusted at 90 rounds/min and 25 up and down strokes. The homogenate was then centrifuged for 5 min at 1000g. The pellet (P_1; crude nuclear fraction) was re-suspended in an equal volume of medium A, and thence filtered through a double gauze layer, and re-centrifuged for another 5 min. The pellet containing the synaptosomes was resuspended in medium B, with the composition of (all in mM): 125 NaCl (125), KCl (3), $MgSO_4$ (1.2) $CaCl_2$ (1.2), NaH_2PO_4 (1mM), $NaHCO_3$ (22), glucose (10) and amino-oxy-acetic acid (0.1), a GABA transaminase inhibitor retarding GABA deamination. The medium B was aerated at room temperature for a minimum of 30 min with 5% CO_2 in 95% O_2 in adjusting the pH of the solution to 7.2-7.4. All solutions were made fresh from stock solutions.

Electron microscopy

Electron micrographs were taken from a few samples for morphological verification of synaptosomes, as described elsewhere.[11] Briefly, the suspension of giant synaptosomal fractions were fixed with 1% glutaraldehyde, then mixed with agarose and placed in plastic mold. Synaptosome-containing agarose films were post-fixed in osmium tetroxide, suctioned by ultramicrotome, stained with uranyl acetate and lead citrate, and then studied with Siezz EM900 electron microscope.

Protein and Enzyme assay

Protein concentration was determined by Bradford's method using bovine serum albumin as the standard.[12] The changes in activity of the marker enzyme, lactate dehyrogenase (EC1.1.1.27; LDH) were measured before and after membrane disruption by Triton X-100 (Sigma; USA), according to Moss and Henderson.[13]

Incorporation of [^{3}H]GABA into synaptosomes

The synaptosomal pellets were re-suspended to a final protein concentration of 5 mg/ml in medium B. Tritiated-gamma amino butyric acid ([^{3}H]GABA; S.A 99Ci/mmol; Amersham, UK) was added to a final concentration of 0.04 µM. Then synaptosomes were incubated in a bath-shaker for 15 min (loading period) at 37°C. At the end of this period incorporation of [^{3}H]GABA was stopped by extended dilution using aerated medium B.

Neurotransmitter release

The release of [^{3}H]GABA from synaptosomes was measured in a continuous superfusion system.[14] Aliquots of the synaptosomal suspension containing about 0.2 mg protein were distributed on filters (0.65 µm pore; Millipore) placed at the

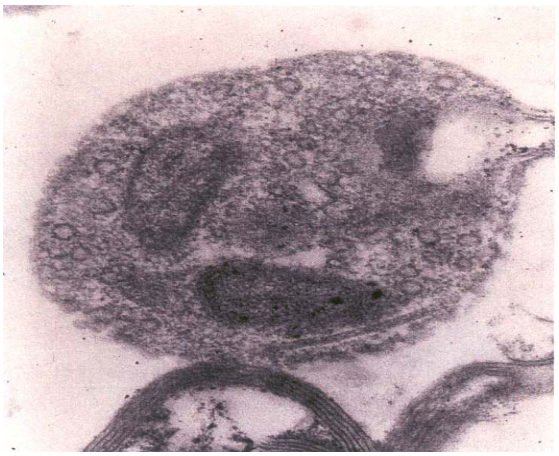


Fig 1: Electron micrograph of rat cerebellar giant synaptosomes. The synaptoplasm contains synaptic vesicles and two mitochondria. (Uranyl acetate-lead citrate staining, X50000).

bottom of the superfusion chambers maintained at 37°C. A peristaltic pump was connected to the bottom of the superfusion chambers and its flow adjusted to 0.5 ml/min/chamber. A washout period of 40 min (equilibration time) was allowed to obtain a stable flow,[8] and then [^{3}H]GABA samples were collected every 1 or 2 minutes (t=0). Then synaptosomes were stimulated for 2 minutes by 15 mM K^+ solution at 3-min (S1) followed by 18-min rest. The second stimulation started at 23-min (S2), the period at which the first evoked release of [^{3}H]GABA was completely returned to pre-stimulation baseline level.[7] The perfusion medium was replaced every 10 min with a continuously aerated fresh medium maintained at 37°C. At the end of sample collection, 400 µl of aliquots of each sample was thoroughly mixed with 4 ml of scintillation liquid (ASCII; Amersham, UK); the filters containing the remnant of synaptosomes were also completely covered by scintillated liquid. The presence of radioactivity in various samples filtrates was quantified by liquid-scintillation counting (1414 Win Spectral LSC, Wallac, Finland). Since the aminooxyacetic acid (GABA transaminase inhibitor, Sigma; USA) prevents GABA metabolism, any tritium outflow from preloaded synaptosomes represents [^{3}H]GABA release and it was shown that the metabolic changes of [^{3}H]-neurotransmitter content following incubation of synaptosomes with [^{3}H]GABA in the presence of this inhibitor is negligible.[15]

The amount of radioactivity released in each fraction was expressed as the fractional release (F_n), i.e., the [^{3}H]GABA content in the collected fraction expressed as a percentage of the total synaptosomal [^{3}H]GABA at the time the sample was taken according to the following equation:
Where: F_n = fractional release calculated for the fraction collected at the n position;

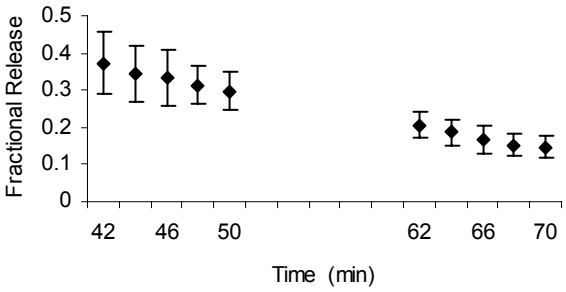


Fig 2: Kinetic of spontaneous [^{3}H]GABA release from synaptosomes after 40-min stabilization period. Data a70 min.re given as mean± SEM and displayed as fractional release observed for each 2-min fraction from five independent experiments performed in duplicates. Superfused fractions were collected every 2-min within two intervals between 40 to 50 min and 60 to.

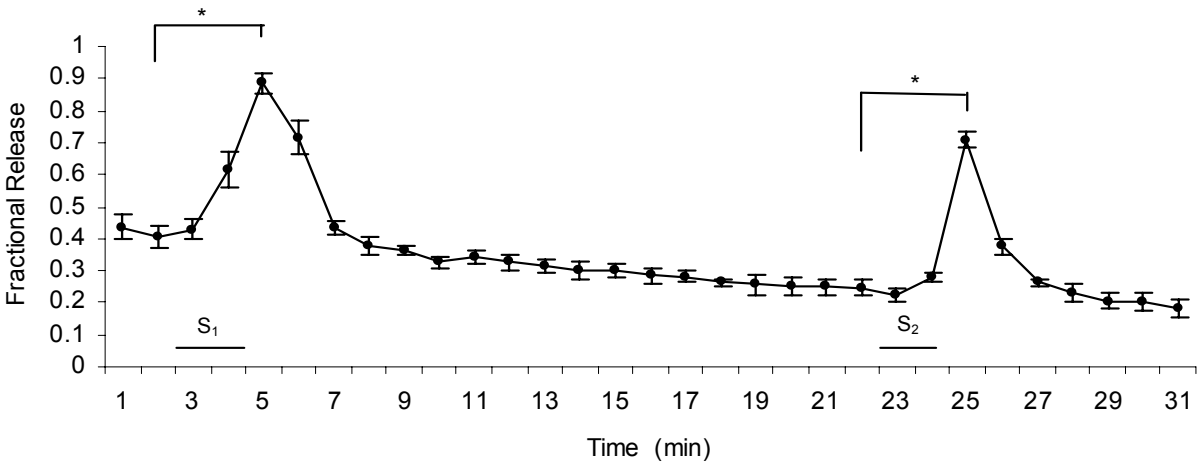
$$F_n = (R_n) / [(R_t - (R_1 + \ldots + R_{n-1})]$$

R_n = [^{3}H]GABA content of the fraction collected at the n position;
R_t = sum of [^{3}H]GABA content in all fractions collected and the filter containing synaptosomes; and n =1st, 2nd, 3rd, … fraction.[8]

Statistical analysis
All data are presented as mean±SEM. Statistical analysis was carried out using unpaired Student's *t* test and the level of significance considered as $P<0.05$.

Results

An electron micrograph of a giant synaptosome prepared from rat cerebellum is presented in Fig 1. Most of the synaptosomes were densely packed with synaptic vesicles containing well preserved intra synaptosomal mitochondria.
Changes of occluded LDH activity (a cytosomal marker enzyme), was chosen as a biochemical indicator of the integrity of synaptosomes.[16] Disruption of particles with Triton X-100 increased enzyme activity significantly such that the ratio of this activity relative to samples obtained from the same intact synaptosomes was 9±1.8 (n=5).
The time-course of synaptosomal basal [^{3}H]GABA release, after incubation, is shown in Fig 2. Multiple 2-min fractions were collected after the equilibrium period. As indicated in this figure, during sample collection, [^{3}H]GABA release decayed constantly with time. Fig 3 shows the kinetics of [^{3}H]GABA release from synaptosomes. After application of 15 mM K^+ solution for 120 seconds (composition of the depolarizing medium was similar to medium B, except for NaCl, which was adjusted to maintain osmolarity), the trends of [^{3}H]GABA release increases gradually reaching to its maximum, and then decreases to recovering basal levels (Fig 3).



*= prestimulation in comparison with the peak of stimulation as shown by lines (P<0.05)

Fig 3: The kinetics of spontaneous and evoked [^{3}H]GABA release from synaptosomes following a 40-min stabilization period (t=0). Bars indicate duration of synaptosome depolarization for 120 seconds using 15 mM K^+ at 3 min (S1) and again at 23 min (S2). Points represent mean± SEM values of five different duplicate experiments.

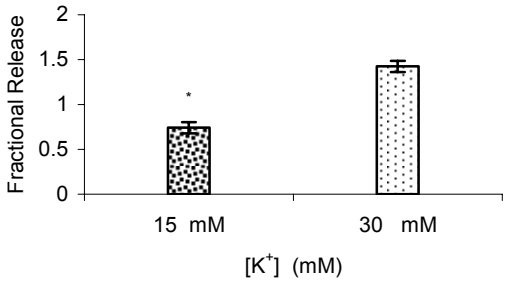
This response was very similar in both depolarization pulses. Stimulation with 30 mM [K^+] proportionally increased the peak response (Fig 4).

Discussion

Synaptosomes are extensively used as a model system for investigating presynaptic phenomena, especially uptake and release of neurotransmitters. It is shown that cerebellar giant synaptosomes release endogenous glutamate.[11,17] Since they are prepared rapidly and in a simple way, we tested the ability of this preparation as a GABA releasing model.

From interpretation of micrographs obtained from subcellular fraction it is concluded that synaptosomes are considered as membrane-bound bodies that contain synaptic vesicles. LDH assay and almost linear and constant spontaneous outflow of [^{3}H]GABA for several minutes provided further support regarding their intact membranes. K^+-induced overflow clearly demonstrated a physiologic response and [^{3}H]GABA release was increased immediately following stimulation. After application of a standard medium, which terminates this fixed and clamped membrane depolarization, prestimulation basal kinetic was once again restored.

The regulation of release through presynaptic elements is a general mechanism that involves all the neurotransmitters known so far. It is mentioned that superfused synaptosomes represent the preparation of choice in studying presynaptic auto- and heteroreceptors, because the released transmitters are removed by the superfusion fluid before they get a chance to activate receptors. Furthermore, no interaction, due to spill over of transmitter molecules, will occur from neighboring nerve terminals[14]. Thus a thin layer of synaptosomes having their membrane targets (transporters, receptors and channels) make it possible to selectively activate each of them. In this way an observed effect can be interrelated without being confused with homo- and heterosynaptic interactions.[8] Although, *in vivo* techniques have the advantage of studying the neurotransmitter release and preserving the intact neural circuitry loops, they do not provide a direct measurement of the released neurotransmitter. Experimenters use the concentration of extracellular neurotransmitter as an indirect measure of release.[5] *In vitro* electrophysiological approach, records currents or potentials in cell culture, tissue slices, and membrane patches very sensitively. Nevertheless, they can only provide indirect clues regarding transmitter release. If a drug has both pre- and postsynaptic effects, results can be confusing.



*=Values are significantly different from each other at P<0.05.

Fig 4: Effects of two concentration of K ion [K^+] on the evoked release of [^{3}H]-GABA. Data obtained as described in Fig 3.

Two types of synaptosomes, standard and giant synaptosomes are produced during ho-

mogenization of cerebellar tissue. The latters are more likely originate from the endings of mossy fibers, can be loaded with GABA and upon depolarization releasing it in a well preserved manner,[11,17] and their release is well-preserved as shown in Fig 3. Hence, cerebellar giant synaptosomes can be used as a simple *in vitro* model in providing model that provides direct assessment of challenges regarding GABA release (carrier-mediated or exocytotic) and different presynaptic regulatory mechanisms such as transporters and receptors.
In conclusion it seems that cerebellar synaptosomes can be used for GABA release studies.

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