Porosome in astrocytes

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Abstract

Secretion is a universal cellular process occurring in bakers yeast, to the complex multicellular organisms, to humans beings. Neurotransmission, digestion, immune response or the release of hormones occur as a result of cell secretion. Secretory defects result in numerous diseases and hence a molecular understanding of the process is critical. Cell secretion involves the transport of vesicular products from within cells to the outside. Porosomes are permanent cup-shaped supramolecular structures at the cell plasma membrane, where secretory vesicles transiently dock and transiently fuse to release intravesicular contents to the outside. In the past decade, porosomes have been determined to be the universal secretory machinery in cells, present in the exocrine pancreas, endocrine and neuroendocrine cells, and in neurons. In this study, we report for the first time the presence of porosomes in rat brain astrocytes. Using atomic force microscopy on live astrocytes, cup-shaped porosomes measuring 10–15 nm are observed at the cell plasma membrane. Further studies using electron microscopy confirm the presence of porosomes in astrocytes. Analogous to neuronal porosomes, there is a central plug in the astrocyte porosome complex. Immunoisolation and reconstitution of the astrocyte porosome in lipid membrane, demonstrates a structure similar to what is observed in live cells. These studies demonstrate that in astrocytes, the secretory apparatus at the cell plasma membrane is similar to what is found in neurons.

Keywords: cell secretion • porosomes/fusion pores • secretion machinery

Introduction

Cellular cargo destined for secretion are packaged and stored in membranous sacs or vesicles, which on demand dock and fuse at specialized plasma membrane structures. In the past decade, specialized supramolecular structures called ‘porosomes’ have been identified at the cell plasma membrane in neurons, exocrine pancreas and in endocrine and neuroendocrine cells, where membrane-bound secretory vesicles transiently dock and fuse to expel their contents to the outside during cell secretion [1–8]. These earlier findings demonstrate porosome to be the universal secretory machinery in cells [9, 10]. The overall structure, composition and reconstitution of this secretory machinery in the exocrine pancreas and in neurons have been well documented [1, 2, 5–8], however the presence of such a secretory machinery in astrocytes had not been previously reported. Since astrocytes are secretory cells, the presence of porosomes at specific plasma membrane locations was investigated in these cells.

Part of the cellular secretion machinery is composed of cholesterol, and specialized proteins involved in the fusion of the membrane-bound secretory vesicles at the porosome. A primary understanding of membrane fusion in cells was made possible following discovery of an N-ethylmaleimide-sensitive factor (NSF) [11] and SNAP proteins [12–14], and their involvement in membrane fusion [15, 16]. Target membrane proteins, SNAP-25 and syntaxin (t-SNARE) and secretory vesicle-associated membrane protein (VAMP or v-SNARE), are part of the conserved protein complex involved in fusion of opposing bilayers [16]. VAMP and syntaxin are both integral membrane proteins, with the soluble SNAP-25 associated with syntaxin. At the nerve terminal, for instance, target membrane proteins SNAP-25 and syntaxin (t-SNAREs) associated at the base of porosomes [6], and synaptotagmin-associated protein VAMP or v-SNARE, interact in a circular array [17, 18] to form conducting channels in the presence of calcium [19].
During neurotransmission, for instance, in the presence of calcium, 40–50 nm in diameter membrane-bound synaptic vesicles containing neurotransmitters dock and fuse at the base of 12–17 nm in diameter cup-shaped neuronal porosomes via interaction of secretory vesicle-associated v-SNARE protein with t-SNAREs at the porosome base [5–8]. Similarly, in exocrine pancreas and neuroendocrine cells such as growth hormone-secreting cells of the pituitary gland, porosomes range in size from 100–180 nm, where 200–1200 nm in diameter secretory vesicles transiently dock and fuse to release intravesicular contents [1, 2, 4, 6, 7]. In the present study, we report for the first time, the presence of porosomes in rat brain astrocytes. Using atomic force microscopy (AFM) to image live astrocytes in physiological buffered solution, cup-shaped porosomes measuring 10–15 nm were observed at certain locations of the cell plasma membrane. Further studies using electron microscopy confirm the presence of porosomes in astrocytes. Immunoisolation and reconstitution of the astrocyte porosome in lipid membrane, demonstrates a structure similar to what is observed in the live cell. Similar to neuronal porosomes, there is a central plug in the astrocyte porosome, when observed at high resolution using AFM. Immunoanalysis of isolated astrocyte porosomes further demonstrates the presence of actin, L-type calcium channel and SNAREs, similar to in neurons. These studies demonstrate that the secretory apparatus in astrocyte is similar, if not identical to what is present in neurons.

Materials and methods

Preparation of astrocyte culture

Neuron-glia cultures from neonatal rat cerebral cortex were established as described. Cortex from three newborn pups (1–3 days old) were dissected, and the tissue was enzymatically treated in 2 ml of papain solution [1.54 mg/ml of Earl’s Balanced Salt Solution (EBSS); 40 min. at 37°C], followed by a 5 min. wash using EBSS-trypsin inhibitor (stock of soybean trypsin inhibitor 7000 U/mg in 3 ml EBSS, final concentration 300 μl of the stock/5 ml EBSS). The tissue in Dulbecco’s Modified Eagle’s Medium (DMEM) containing high glucose, 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin (1 ml/100 ml DMEM) was then mechanically dissociated using pipettes. Cells were plated in the same media (DMEM high glucose+ 10% FBS + penicillin/streptomycin) in culture flasks (25 mm flasks) and maintained at 37°C in a humidified 5% CO2/95% air atmosphere. The cells were maintained by changing the medium every 2–3 days. After mixed cultures reach confluency in 9–12 days, the flasks were ‘pre-shaken’ (260 rpm) for 90 min. to remove microglia and the dividing type I astroglia. The cultures were agitated overnight (12–18 hrs) at 260 rpm at 37°C. Cultures enriched in type I astroglia were obtained by trypsinizing (0.25%) the attached cells for 3 min. Trypsin was inactivated by adding 3 ml DMEM (the same media as above) supplemented with 10% heat-inactivated FBS (serum contains protease inhibitors). Astrocytes were plated on poly-L-lysine (100 mg/ml; MW 100,000) coated glass coverslips, and experiments performed on cells that had been in culture for 1–3 days.

Atomic force microscopy

Astrocytes cultured on glass coverslips were immersed in PBS, pH 7.5, and imaged using the AFM (BioScope III, Digital Instruments, Santa Barbara, CA, USA). AFM imaging was performed using the ‘tapping’ mode, using silicon nitride tips with a spring constant of 0.06 N/m, and an imaging force of <200 pN. Images were obtained at line frequencies of 1.98 Hz, with 512 lines per image, and constant image gains. Tip velocity 11.4 mm/s; tip spring constant 0.06 N/m; sample/line 512; integral gain 2.0; proportional gain 1.0; amplitude set point 0.12–0.28V; drive frequency 7.76–8.12 KHz and drive amplitude 150–400 mV were used. Topographical dimensions of both native and lipid-reconstituted porosomes were analysed using the NanoScope IIIa version 4.43r8 software, supplied by Digital Instruments.

Transmission electron microscopy

Isolated rat astrocytes were fixed in 2.5% buffered paraformaldehyde for 30 min., and the pellets were embedded in Unicryl resin and were sectioned at 40–70 nm. Thin sections were transferred to coated specimen TEM grids, dried in the presence of uranyl acetate and methylcellulose, and examined using a transmission electron microscope. For negative-staining electron microscopy, purified protein suspensions in Phosphate Buffered Saline (PBS) were adsorbed onto hydrophilic carbon support films that were mounted onto formvar coated, metal specimen grids (EMS, Ft. Washington, PA, USA). The adsorbed protein was washed in double-distilled water and negatively stained using 1% aqueous uranyl acetate. After the grids were dried in the presence of the uranyl acetate solution, they were examined by transmission electron microscopy. To prevent bleaching by the electron beam, micrographs were obtained on portions of the grid not previously examined.

Porosome isolation and reconstitution

The porosome complex from astrocyte was immunoisolated using SNAP-23 specific antibody conjugated to protein A-sepharose beads. Fifty micrograms of astrocyte homogenate, prepared by solubilizing astrocytes in Triton/Lubrol solubilization buffer (0.5% Lubrol; 1 mM benzamidine; 5 mM Mg-adenosine-5’-triphosphate (ATP); 5 mM ethylenediaminetraacetic acid (EDTA); 0.5% Triton X-100, in PBS) containing a protease inhibitor cocktail (Sigma, St. Louis, MO, USA), was used. SNAP-23 antibody conjugated to the protein A-sepharose was incubated with the solubilized homogenate for 1 hr at room temperature followed by washing with wash buffer (500 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.5). The immunoprecipitated sample attached to the immunosepharose beads was eluted using low pH buffer to obtain the porosome complex. To prepare lipid membrane on mica for AFM studies, freshly cleaved mica disks were placed in a fluid chamber. Two hundred microlitres of the bilayer bath solution containing 140 mM NaCl, 10 mM Hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and 1 mM CaCl2 was placed at the centre of the cleaved mica disk. Ten microliters of the brain lipid vesicles were added to the above bath solution. The mixture was then allowed to incubate for 60 min. at room temperature, before washing (×10), using 100 μl bath solution/wash. The lipid membrane on mica was imaged by the AFM before and after the addition of immunoisolated porosomes from astrocytes.
Western blot analysis

Immunoblot analysis was performed on isolated porosome complex, and on total rat brain and astrocyte homogenates. Protein in the fractions was estimated by the Bradford method [20]. All fractions were boiled in Laemmli reducing sample preparation Laemmli buffer [21] for 5 min., cooled, and used for SDS-PAGE. Proteins were resolved in a 12.5% SDS-PAGE and electrotransferred to 0.2 mm thick nitrocellulose sheets for immunoblot analysis using specific antibodies. Immunoblot analysis was performed using specific antibodies to VAMP-2 and syntaxin-1 (Alomone Labs, Jerusalem, Israel), NSF (Exalpha Biologicals, Boston, MA, USA) and SNAP-23 and -25 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The nitrocellulose membrane was incubated for 1 hr at room temperature in blocking buffer (5% non-fat milk in PBS containing 0.1% Triton X-100 and 0.02% NaNO3), and immunoblotted for 2 hrs at room temperature with the primary antibody. The immunoblotted nitrocellulose sheets were washed in PBS containing 0.1% Triton X-100 and 0.02% NaN3 and were incubated for 1 hr at room temperature in horseradish peroxidase-conjugated secondary antibody at a dilution of 1:2000 in blocking buffer. The immunoblots were then washed in the PBS buffer, processed for enhanced chemiluminescence and photographed using a Kodak Image Station 414.

Immunocytochemistry

Astrocytes were fixed in ice-cold 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH 7.4), washed twice in 0.01 M PBS (pH 7.4) and incubated for 30 min. at room temperature in blocking buffer (5% non-fat milk in PBS containing 0.1% Triton X-100 and 0.02% NaN3). Cells were then washed with PBS which contained 0.2% Triton X-100 and 0.02% NaN3, stained with 30 nM 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Inc.) for 5 min. followed by washing in PBS buffer, and examined by a Zeiss Laser Scanning Confocal System (LSM-310) at 40×.

Results and discussion

Live astrocytes (Fig. 1A) growing on glass coverslips, when incubated in PBS pH 7.5, and imaged at higher force but low resolution using the AFM, clearly demonstrate the outline of the tightly adhering cells, and the intact cytoskeletal network within (Fig. 1B). When such cultured astrocytes were imaged at lower force but at higher resolution, as expected, only certain regions of the cell surface exhibited the presence of approximately 12–15 nm in diameter structures (Fig. 1C–F) resembling neuronal porosomes both in size and morphology [5, 8]. As previously observed in neuronal porosomes [5], a central plug measuring approximately 20–30 Å was also found in the porosome complex of astrocytes (Fig. 1F, red arrowhead). These AFM studies on live astrocytes, strongly suggested the presence of porosomes in these cells.

To confirm the AFM findings of porosomes in astrocytes, electron microscopy was carried out on fixed astrocyte cultures. Careful examination of the astrocyte cell membrane in the electron micrographs, demonstrated the presence of 12–15 nm cup-shaped porosomes with 40–50 nm in diameter membrane-bond secretory vesicles docked at its base (Fig. 2A–E). Although frequently a single secretory vesicle was found docked to the base of a porosome complex, on rare occasion, a lone vesicle was found docked at the base of two porosomes (Fig. 2A). These porosomes in astrocytes were very similar to the neuronal porosomes previously observed in electron micrographs [5, 8]. Toward the cytoplasmic compartment, fibrillar structures originating from the porosome surface made contact with the cell plasma membrane. These fibrillar elements may provide stability and support to the porosome complex in astrocytes (Fig. 2E). Depending on the angle of the cut through the structure, the porosome opening in electron micrographs appears to be approximately 8–10 nm (Fig. 2D), with fibrillar elements originating from the base of the structure (Fig. 2B). Hence both AFM studies on live astrocytes, and EM studies on fixed cells, demonstrate the presence of porosomes involved in secretion from astrocytes.

To further characterize the astrocyte porosome, it was immunoisolated and reconstituted into artificial lipid membrane, followed by AFM imaging (Fig. 3). In Fig. 3A, a low-magnification image of porosome-reconstituted lipid membrane is shown, with yellow arrowheads pointing to a few of the reconstituted porosome complexes. Figures 3A–D are examples of micrographs of three representative membrane-reconstituted porosome complexes at high resolution. Results from this study demonstrate that isolated porosomes when reconstituted in lipid membrane exhibit structural features similar to porosomes in live astrocytes, and in neurons. The central plug too (red arrowhead), is clearly visible in the reconstituted porosome complex of astrocytes, as is observed in its native state at the cell membrane. Since earlier studies [5–7] demonstrate among other proteins, the presence of actin, calcium channels, NSF and SNAREs, in the prosome complex of the exocrine pancreas and neurons, the presence of these proteins in the astrocyte porosome was investigated. Immunoisolated astrocyte porosomes resolved using SDS-PAGE, when subjected to immunoblot analysis, demonstrate the presence of actin and L-type calcium channel (Fig. 4A). Significant SNAP-23 immunoreactivity was demonstrated in the astrocyte porosome complex, however, this immunoreactivity was found as a 68 kDa protein, possibly as a SDS-resistant SNARE complex (Fig. 4A). No detectable level of the NSF protein was found associated with the astrocyte porosome complex (data not shown). To further determine if NSF immunoreactivity was present in astrocytes, but absent from the porosome complex, immunoblot analysis were performed using equal amounts of homogenerate protein, both from astrocyte and whole rat brain tissue (Fig. 4B). Our studies demonstrate that astrocytes have much less NSF and SNARE immunoreactivity (Syntaxin 1, SNAP-23 and VAMP 2), when
compared to whole brain tissue. Although SNAP-25 was present at much higher concentrations than SNAP-23 in brain tissue, SNAP-25 was undetectable in immunoblots of the astrocyte homogenate (Fig. 4). Nonetheless, these studies demonstrated that similar to neurons, in astrocyte the membrane fusion machinery (t-SNAREs, v-SNARE and NSF) is present.

It was puzzling however, that although such high concentration of SNAP-25 was detected in immunoblots of total brain homogenates, no detectable amounts were observed in immunoblots of the astrocyte homogenate. Either astrocytes have no SNAP-25, or the protein may be undergoing rapid degradation in that cell, compared to neuronal tissue. The use of greater concentration of protease inhibitors in the homogenizing buffer however, failed to yield any detectable SNAP-25 immunoreactivity in astrocytes (data not shown). To further confirm our immunoblot analysis, immunocytochemistry was performed on fixed astrocyte cultures using both SNAP-23 and SNAP-25 specific antibodies (Fig. 5). Surprisingly, although SNAP-23 immunofluorescence was found to be greater, both SNARE proteins were demonstrated to be present in astrocytes [22, 23]. Both SNAP-23 and SNAP-25 immunoreactivity exhibit a punctate distribution, localizing primarily to the cell plasma membrane (Fig. 5). Hence, as previously demonstrated in the exocrine pancreas, neuroendocrine cells and neurons [1–8], the porosome in astrocytes was demonstrated for the first time. As in other secretory cells, the presence of porosomes at the astrocyte plasma membrane would allow
Porosomes in astrocytes would allow similar to what synaptic vesicles in neurons undergo during neurotransmission, or what secretory vesicles in exocrine pancreas experience, without loss of vesicle identity [24, 25]. Indeed in agreement, a recent study [26] demonstrates transient fusion, or the so-called ‘Kiss-and run’ form of glutamate secretion occurring in cultured and freshly isolated rat hippocampal astrocytes. It has been demonstrated that porosomes in pancreatic acinar or growth hormone-secreting cells are permanent structures at the cell plasma membrane, with a 100–180 nm diameter opening [9, 10]. Membrane-bound secretory vesicles ranging in size from 0.2–1.2 μm in diameter dock and fuse at porosomes to release vesicular contents. Following fusion of secretory vesicles at porosomes, only a 20–35% increase in porosome diameter is demonstrated. It is therefore concluded that secretory vesicles ‘transiently’ dock and fuse at the base of porosomes, instead of complete merger of the secretory vesicle membrane at the site. In contrast to previously accepted belief, if secretory vesicles were to completely incorporate at porosomes, the plasma membrane structure would distend much greater than what is observed. Furthermore, if secretory vesicles were to completely merge at the cell plasma membrane, there would be a loss in vesicle number following cell secretion. Examination of secretory vesicles within cells before and after secretion demonstrates that the total number of secretory vesicles remains constant following cell secretion [9, 10]. In agreement, it has been demonstrated that secretory granules are re-captured largely intact following stimulated exocytosis in cultured endocrine cells. Similarly in neurons, 10–15 nm porosomes at the presynaptic membrane allow for the transient docking and fusion of synaptic vesicles, during neurotransmission. Studies report that single synaptic vesicles fuse transiently and successively without loss of vesicle identity [9, 10].

In this study, as a result of new technological developments resulting in improved instrumentation and refined experimental procedures, we were able for the first time, to identify and study porosomes in live astrocytes at 8–10 Å resolution. Similar to neuronal porosomes, the porosome in astrocytes measuring 10–15 nm, are an order of magnitude smaller than those in the exocrine pancreas or in neuroendocrine cells (100–180 nm). However, in contrast to the neuronal porosome, which possess eight globular proteins at the outer rim of the complex, the porosome complex appears to possess 12 such globular structures. Nature has designed the porosome as the universal secretory machinery, but has fine-tuned its use to suite secretion from different cell types. Hence, the size of porosomes in different cells may represent a form of such fine-tuning. It is well established that smaller vesicles fuse more efficiently than larger ones [27, 28], and hence curvature of both secretory vesicles and the porosome base, would dictate the efficacy and potency of vesicle fusion at the cell membrane.
plasma membrane. In this regard, astrocytes and neurons being fast secretory cells possess small 40–50 nm secretory vesicles and porosome bases 2–4 nm, for rapid and efficient fusion. In contrast, a slow secretory cell like the exocrine pancreas possesses larger secretory vesicles ranging in size from 0.2–1.2 μm in diameter that fuse at porosome bases measuring approximately 20–30 nm. A further understanding of the astrocyte porosome complex will require studies to determine the distribution of the various constituent proteins within the complex. This is no small task, since the current state of technology limits this membrane-associated supramolecular complex to be examined using either Nuclear Magnetic Resonance (NMR) or X-ray crystallography. One needs to appreciate that unlike membrane receptors and channels, the porosome complex is a much larger membrane structure, composed of many more proteins. There is, however, an effort to further resolve the porosome structure, using ultra...
high-resolution AFM of 2D porosome crystals, and 3D topology mapping using cryoelectron microscopy. Toward this goal, a recent study in the laboratory (unpublished data), has successfully utilized AFM, electron microscopy and electron density measurements followed by 3D topography mapping of the isolated neuronal porosome complex, providing for the first time, structural details of the porosome complex at the nanoscale. Results from this study demonstrate a set of eight protein units lining the porosome cup, each connected via spoke-like elements to a central plug region within the structure. This finding sets the stage for the isolation of intact porosome complex for near atomic resolution using cryoelectron diffraction measurements.

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References

1. Schneider SW, Sritharan KC, Geibel JP, Oberleithner H, Jena BP. Surface dynamics in living acinar cells imaged by atomic force microscopy: identification of plasma membrane structures involved in exocytosis. Proc Natl Acad Sci USA. 1997; 94: 316–21.
2. Cho SJ, Quinn AS, Stromer MH, Dash S, Cho J, Taatjes DJ, Jena BP. Structure and dynamics of the fusion pore in live cells. Cell Biol Int. 2002; 26: 35–42.
3. Cho SJ, Wakade A, Pappas GD, Jena BP. New structure involved in transient membrane fusion and exocytosis. Ann NY Acad Sci. 2002; 971: 254–6.
4. Cho SJ, Jeftinija K, Glavaski A, Jeftinija S, Jena BP, Anderson LL. Structure and dynamics of the fusion pores in live GH-secreting cells revealed using atomic force microscopy. Endocrinology. 2002; 143: 1144–8.
5. Cho WJ, Jeremic A, Rognlien KT, Zhvania MG, Lazrishvili I, Tamar B, Jena BP. Structure, isolation, composition and reconstitution of the neuronal fusion pore. Cell Biol Int. 2004; 28: 699–708.
6. Jena BP, Cho SJ, Jeremic A, Stromer MH, Abu-Hamdah R. Structure and composition of the fusion pore. Biophys J. 2003; 84: 1–7.
7. Jeremic A, Kelly M, Cho SJ, Stromer MH, Jena BP. Reconstituted fusion pore. Biophys J. 2003; 85: 2035–43.
8. Cho WJ, Jeremic A, Jin H, Ren G, Jena BP. Neuronal fusion pore assembly requires membrane cholesterol. Cell Biol Int. 2007; 31: 1301–8.
9. Jena BP. Secretion machinery at the cell plasma membrane. Curr Opin Struct Biol. 2007; 17: 437–43.
10. Jena BP. Molecular Machinery and Mechanism of Cell Secretion. Exp Biol Med. 2005; 230: 307–19.
11. Block MR, Glick BS, Wilcox CA, Wieland FT, Rothman JE. Purification of an N
ethylmaleimide-sensitive protein catalyzing vesicular transport. Proc Natl Acad Sci USA. 1988; 85: 7852–6.
12. Trimble WS, Cowam DM, Scheller RH. VAMP-1: A synaptic vesicle-associated integral membrane protein. Proc Natl Acad Sci USA. 1988; 85: 4538–42.
13. Oyler GA, Higgins GA, Hart RA, Battenbarg M, Bloom FE, Wilson MC. The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol. 1989; 109: 3039–52.
14. Bennett K, Calakos N, Scheller RH. Syntaxin: A synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science. 1992; 257: 255–9.
15. Wilson DW, Whiteheart SW, Wiedmann M, Brunner M, Rothman JE. A multisubunit particle implicated in membrane fusion. J Cell Biol. 1992; 117: 531–8.
16. Weber T, Zemelman BV, McNew JA, Westerman B, Gmachi M, Parlati F, Sollner TH, Rothman JE. SNAREpins: minimal machinery for membrane fusion. Cell. 1998; 92: 759–72.
17. Cho SJ, Kelly M, Rognlien KT, Cho J, Hoerber JKH, Jena BP. SNAREs in opposing bilayers interact in a circular array to form conducting pores. Biophys J. 2002; 83: 2522–7.
18. Cho WJ, Jeremic A, Jena BP. Size of supramolecular SNARE complex: membrane-directed self-assembly. J Am Chem Soc. 2005; 127: 10156–7.
19. Jeremic A, Kelly M, Cho WJ, Cho SJ, Horber JKH, Jena BP. Calcium drives fusion of SNARE-apposed bilayers. Cell Biol Int. 2004; 28: 19–31.
20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem. 1976; 72: 248–54.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227: 680–5.
22. Jettinja SD, Jettinja KV, Stefanovic G. Cultured astrocytes express proteins involved in vesicular glutamate release. Brain Res. 1997; 750: 41–7.
23. Wilhelm A, Volknandt W, Langer D, Nolte C, Kattenmann H, Zimmerman H. Localization of SNARE proteins and secretory organelle proteins in astrocytes in vitro and in situ. Neurosci Res. 2004; 48: 249–57.
24. Aravanis AM, Pyle JL, Tsien RW. Single synaptic vesicles fusing transiently and successively without loss of identity. Nature. 2003; 423: 643–7.
25. Thorn P, Fogarty KE, Parker I. Zymogen granule exocytosis is characterized by long fusion pore openings and preservation of vesicle lipid identity. Proc Natl Acad Sci USA. 2004; 101: 6774–9.
26. Chen X, Wang L, Zhou Y, Zheng L-H, Zhou Z. “Kiss-and run” glutamate secretion in cultured and freshly isolated rat hippocampal astrocytes. J Neuro Sci. 2005; 20: 3126–33.
27. Wilschut J, Duzgunes N, Papahadjopoulos D. Calcium/magnesium specificity in membrane fusion: kinetics of aggregation and fusion of phosphatidylserine vesicles and the role of bilayers curvature. Biochemistry. 1981; 20: 9236–43.
28. Ohki S. Effects of divalent cations, temperature, osmotic pressure gradient, and vesicle curvature on phosphatidylserine vesicle fusion. J Membr Biol. 1984; 77: 265–75.