Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes

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Abstract **Astrocytes, a sub-type of glial cell in the central nervous system, can release the excitatory transmitters glutamate and aspartate in response to elevated levels of internal calcium. To investigate potential release mechanisms that may be present in these cells we have determined whether protein components of the neuronal secretory apparatus are expressed in astrocytes. Western blots, immunocytochemistry and RT PCR demonstrate that astrocytes express cellubrevin, synaptobrevin II and syntaxin, proteins known to form a macromolecular fusion complex. However, SNAP-25 which is another neuronal protein of the fusion complex, was not detected. Astrocyte cellubrevin and synaptobrevin II were also shown to be sensitive to the proteolytic activity of tetanus toxin. Together these data indicate that astrocytes express some proteins that are known to form a fusion complex indicating that regulated exocytosis might mediate calcium-regulated transmitter release from these cells.**

Key words: Astrocyte; Exocytosis; Vesicle; SNAP-25; Synaptobrevin; Cellubrevin; Syntaxin; Synapse; Secretion

1. Introduction

Recent studies on astrocytes indicate that they have the potential to play many roles in the nervous system in addition to simply being a supportive matrix for neurons. For example, glia possess many of the voltage-gated channels that are present in neurons [1] and express neurotransmitter gated ion channels, several of which lead to changes in calcium homeostasis [2]. A question that arises is what is the physiological role for calcium elevations in astrocytes? Diverse stimuli which elevate astrocyte or Schwann cell calcium cause the release of the excitatory amino acids glutamate and aspartate, and when neurons are co-cultured with astrocytes, a subsequent glutamate-dependent neuronal calcium elevation is produced [3,4].

There are at least two signaling mechanisms implicated in astrocyte-neuron signaling – gap junctions [5] and the release of neurotransmitter [3]. Our previous work has demonstrated that the neuroligand bradykinin causes a calcium-dependent release of the excitatory amino acids glutamate and aspartate from cultured astrocytes [3]. When applied to mixed astrocyte-neuron cultures, bradykinin evokes the release of glutamate from astrocytes which in turn causes an elevation of neuronal internal calcium concentration. This action is mediated through the *N*-methyl-D-aspartate receptor. Additionally, contact-induced calcium waves in astrocytes cause a D-2-amino-5-phosphonopentanoic acid (D-AP5)-sensitive elevation of neuronal calcium [3,6]. Although these data point to a new type of intercellular signaling in the central nervous system, the underlying mechanism of calcium-dependent glutamate release is unknown.

A priori there are three mechanisms that might mediate neurotransmitter release from astrocytes [7]: (i) release through a cell swelling dependent mechanism [8,9], (ii) the reversal of glutamate transporters [7,10], or (iii) a calcium-dependent vesicular release process. We have shown that bradykinin does not cause cell swelling [11]. Furthermore, bradykinin-induced glutamate release is not blocked by addition of glutamate transport inhibitors [3,11]. However, α-latrotoxin, which stimulates quantal release of neurotransmitter from nerve terminals, causes calcium-independent glutamate release from astrocytes suggesting that astrocytes might release glutamate using a mechanism resembling the neuronal secretory process [11].

The exocytotic release of neurotransmitter from nerve terminals critically relies on a macromolecular fusion complex formed between the vesicle protein synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25 [12,13]. In order to determine whether astrocytes contain secretory apparatus similar to that of nerve terminals we investigated the presence of these synaptic proteins in purified astrocytes.

2. Materials and methods

2.1. Cell culture

Mixed astrocyte-neuron cultures from visual cortices or hippocampii were prepared as previously described [3]. Briefly, cortices were freshly dissected from 1–3-day-old Sprague-Dawley rat pups and treated with papain (20 IU/ml) for 1 h at 37°C. After subsequent treatment with trypsin inhibitor (10 mg/ml, type II-O), cortices were mechanically dispersed through a fire-polished glass pipette. Cells were plated into poly(L-lysine) (1 mg/ml, MW 100 000)-coated glass-bottomed dishes. Cultures were kept at 37°C in a humidified 5% CO_2/95% air atmosphere for 12–14 days. Culture medium consisted of Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum and containing 40 mM glucose, 1 mM pyruvate, 2 mM glutamine, 14 mM sodium bicarbonate, 100 IU/ml penicilin and 100 μg/ml streptomycin (pH 7.4). Arabinosylcytosine (ARA-C; 5 μM) was added after 2–3 days in culture.

Enriched astrocyte type-1 cultures were prepared as previously described [3,14]. Following dissection and dissociation of rat cortices, cells were plated into culture flasks. After 8–14 days in culture, cells were shaken twice, first for 1.5 h and then for 18 h on an orbital shaker at 260 rpm. The population of adherent cells were returned to the incubator for 4–16 days. At that time, they were either removed from the substratum for Western analysis, or were detached using trypsin (0.1%). Detached cells were spun at 100 × *g* for 10 min. Cells were resuspended

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and plated into poly-L-lysine-coated glass-bottomed dishes for immunocytochemistry.

2.2. Immunocytochemistry

The presence of glial fibrillary acidic protein (GFAP), the microtubule associated protein, MAP-2 and localization of synaptic proteins were determined by indirect immunocytochemistry. Immunocytochemistry was performed using monoclonal antibodies raised against GFAP (ICN Immunobiological), MAP-2 (Boehringer) and synaptotagmin (cl 41.1 [15]). The monoclonal antibody against syntaxin I (HPC-1) was generously provided by Dr. C.J. Barnstable [16], and a polyclonal antibody against cellubrevin (MC-16) was generously supplied by Dr. P. DeCamilli. A monoclonal antibody (cl. 69.1) was used to probe for synaptobrevin II [17].

2.3. Preparation of subcellular fractions

Synaptosomal membrane enriched (LP-1) and small synaptic vesicle (LP-2) preparations were prepared according to standard procedures (Huttner et al., 1983). To obtain a postnuclear membrane and vesicle extract from purified astrocytic culture, cells were detached from the substratum using a cell scraper. Cells were spun at $100 \times g$ for 10 min, and the pellet was resuspended in buffer containing: 320 mM sucrose, 1 mM EDTA, 0.2–0.4 mM phenylmethylsulphonyl fluoride, 2–10 μg/ml pepstatin A and 10 mM HEPES (pH 7.4). In some experiments, buffer additionally included 10 μg/ml leupeptin and 10 μg/ml aprotinin. The cell suspension was passed (about 60 times) through a 25 gauge needle. Nuclei and unbroken cells were removed by centrifugation at $600 \times g$ for 10 min at 4°C. Membranes were then collected by centrifugation at $150\,000 \times g$ for 30 min at 4°C. Membranes were stored at −70°C or immediately subjected to SDS-PAGE and immunoblot analysis. In addition to the antibodies used in immunocytochemistry, we probed membranes with three antibodies generated against SNAP-25 (MC-21: generously supplied by Dr. P. DeCamilli and clones 71.1 and 71.2 [18]). Immunoreactive bands were detected using enhanced chemiluminesence (ECL, Amersham).

In experiments using tetanus toxin, 25 ng/μl of the holotoxin (Alomone Labs) were reduced with 10 mM DTT in a solution containing 50 mM NaCl, 10 mM HEPES (pH 7.2) at 37°C for 30 min. 3.3 ng/μl of reduced tetanus toxin was incubated with 1.5 μg/μl of protein from astrocyte membrane preparation in a solution containing 70 mM NaCl, 250 μM $ZnCl_2$, 1.66 mM DTT, 10 mM HEPES (pH 7.2) at 37°C for 2 h whereupon the reaction was stopped by adding 3 × SDS gel sample buffer.

2.4. RT PCR

Astrocyte mRNA was isolated using a QuickPrep Micro mRNA purification kit (Pharmacia). First strand cDNA was synthesised with specific primers complementary to mRNA using a First Strand cDNA Synthesis Kit (Pharmacia). Two pairs of nested primers were used to amplify cDNA using the the polymerase chain reaction. For synaptobrevin II, primers corresponding to nucleotides at positions 9–27 and 56–78 (start codon corresponds to position 1) were used as upstream primers. Positions 750–771 and 872–895 were used as downstream primers. For syntaxin Ib primers at positions −70 to −52 and −34 to −16 were used as upstream primers (start codon as 1), and 552–570 and 531–549 as downstream primers.

Fig. 1. Astrocyte cultures are immunopositive for GFAP. Scale bar: 20 μm.

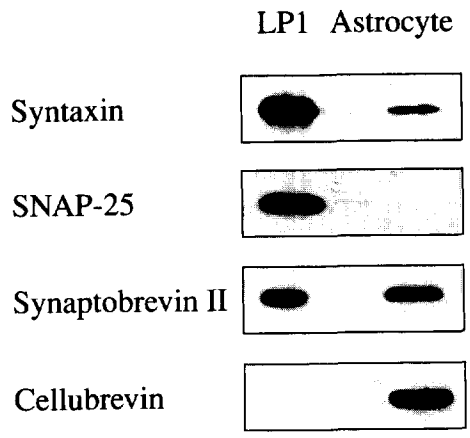

Fig. 2. Astrocytes in culture express specific synaptic proteins. Following SDS-PAGE (1 μg per lane for LP-1 and 10–15 μg per lane for post-nuclear astrocytic cell membrane extract) immunoblots indicate the presence of cellubrevin, synaptobrevin II and syntaxin I, but not SNAP-25, in astrocytes. In the LP-1 preparation synaptobrevin II, syntaxin I and SNAP-25 are detected.

3. Results and discussion

Phase-contrast microscopy of astrocyte-enriched cultures indicated that these cultures were neuron-free. This was confirmed by immunocytochemistry which showed that cultures were not immunoreactive for synaptotagmin I or for microtubule associated protein (MAP-2), but were immunopositive for glial fibrillary acidic protein (GFAP; Fig. 1) a characteristic marker of astrocytes. In parallel cultures which contained neurons, synaptotagmin and MAP-2 (data not shown) immunoreactivity were reliably detected. Thus, cultures used in this study are devoid of neurons and contain GFAP-positive polygonal astrocytes.

To test for the presence of synaptic proteins in astrocytes, we prepared a post-nuclear membrane extract from cultured astrocytes which was subjected to SDS-PAGE (Fig. 2). Synaptosomal membrane enriched preparation (LP-1) was used as a positive control. Immunoblot analysis revealed the presence of cellubrevin, a non-neuronal homolog of synaptobrevin [19], in astrocytes, but not in the LP-1 preparation (Fig. 1). Additionally, astrocyte cultures were found to contain synaptobrevin II and syntaxin I. We could not detect expression of SNAP-25 in astrocytes using three different antibodies, although SNAP-25 was reliably detected with these antibodies in LP-1 preparations.

We performed RT-PCR using mRNA isolated from rat brain and from astrocyte cultures. PCR products of the correct predicted size of about 600 and 700 base pairs were obtained from astroycte mRNA for synaptobrevin II and syntaxin Ib, respectively. PCR product was not detected for SNAP-25 from astrocyte mRNA. In parallel positive controls SNAP-25 PCR prod-

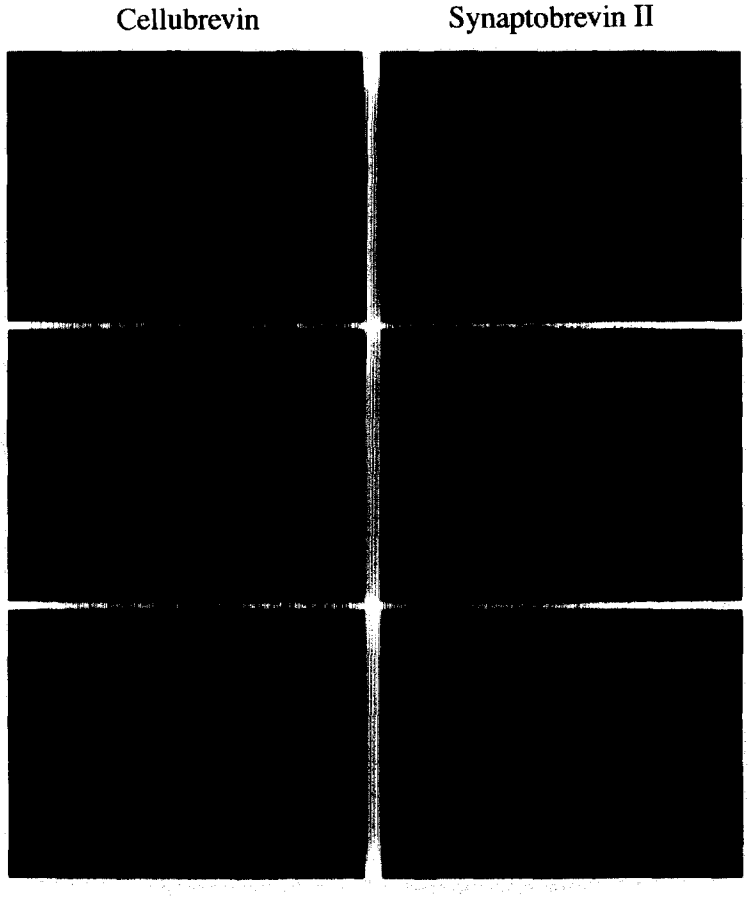

Fig. 3. Subcellular localization of cellubrevin and synaptobrevin II. Astrocyte cultures were immunopositive for cellubrevin (left) and synaptobrevin II (right). Scale bar: 20 μm.

uct was obtained from whole brain mRNA. Sequence analysis demonstrated that the astrocyte PCR products for synaptobrevin II and syntaxin Ib had identical sequence to the previously published neuronal sequence. The PCR product for synaptobrevin II spanned the tetanus toxin cleavage site. Because the astrocyte and neuronal synaptobrevin II sequences are identical, astrocyte synaptobrevin II is likely to be subject to proteolysis by tetanus toxin. Taken together, RT-PCR and Western blots demonstrate that cultured astrocytes express synaptobrevin II and syntaxin Ib, but not SNAP-25.

The intracellular location of the proteins was investigated by indirect immunocytochemistry. Staining with anti-cellubrevin showed a punctate pattern of immunoreactivity (Fig. 3). Even though puncta were present throughout the entire cell body (left bottom), there were particularly concentrated in subcellular region corresponding to the Golgi apparatus as well as at the leading edge of cells (left, top and middle). A similar pattern of immunoreactivity was observed using anti-synaptobrevin II (Fig. 3). However, synaptobrevin II was located primarily at the leading edge of cells. We could not determine the subcellular locatization of syntaxin. This is probably due to a very low level of expression of syntaxin in astrocytes as compared to neurons since this antibody readily revealed punctate immunoreactivity in neuronal cultures.

We determined whether cellubrevin and synaptobrevin II were sensitive to proteolysis by tetanus toxin (Fig. 4). Since astrocytes do not contain a receptor for the holotoxin we added reduced tetanus toxin to membrane preparations. Tetanus toxin decreased the levels of synaptobrevin II in neuronal and astrocyte preparations. In addition, tetanus toxin also cleaved

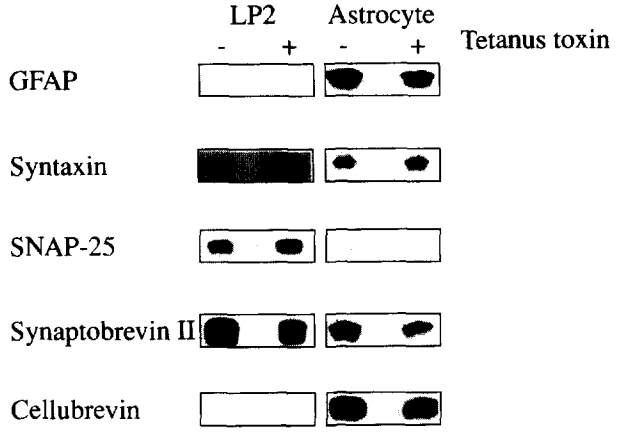

Fig. 4. Tetanus toxin selectively cleaves synaptobrevin II and cellubrevin. LP-2 and astrocyte membrane preparations were incubated with reduced tetanus toxin in the presence of zinc. Immunoblots demonstrate that astrocyte synaptobrevin II and cellubrevin are sensitive to tetanus toxin, while the astrocyte protein GFAP and syntaxin are unaffected by this toxin.

astrocyte cellubrevin as expected from previous data [19]. This cleavage of synaptobrevin II and cellubrevin was specific since GFAP, syntaxin and SNAP-25 were unaffected by tetanus toxin treatment.

Taken together these data support physiological studies and assert that astrocytes express some of the components of the neuronal secretory apparatus. Synaptobrevin II and syntaxin I are critical proteins in the exocytotic fusion apparatus of the synapse. Cleavage of syntaxin by botulinum toxin C1 [20] and of synaptobrevin by tetanus toxin [21], and botulinum toxins B, D, G and F [22–26] prevent synchronized exocytosis and reduce the basal frequency of spontaneous quantal exocytosis at the synapse. The expression of these proteins in astrocytes suggest that a secretory mechanism similar to the neuronal pathway is expressed in these non-neuronal cells.

Immunocytochemistry demonstrated a rather diffuse pattern of immunoreactivity for cellubrevin and synaptobrevin II in astrocytes. Unlike neurons we did not detect distinct regions of heightened immunoreactivity characterstic of the synapse. The pattern seen in astrocytes is more consistent with a distributed exocytotic release pathway devoid of dense accumulations of vesicles.

Synaptic proteins have been detected in other non-neuronal cells. Adrenal chromaffin cells contain synaptobrevin, syntaxin and SNAP-25 in addition to synaptotagmin [27]. Rat skeletal muscle contains synaptobrevin II and cellubrevin, but neither SNAP-25 nor syntaxin [28]. However, it is entirely possible that a non-neuronal homolog of SNAP-25 is present in astrocytes and muscle given that a yeast protein, SEC9p with homology to SNAP-25 is essential for fusion of post-Golgi membrane with the plasma membrane [29]. Since chromaffin cells, muscle and astrocytes all express some synaptic proteins and have regulated transmitter release properties it is tempting to speculate that these proteins play a role in secretion in these cells.

Having identified cellubrevin and synaptobrevin II in astrocytes it should be possible using tetanus toxin to determine whether calcium-dependent glutamate release from these cells is mediated by these proteins in a process similar to the neuronal secretory mechanism.

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