A Regulated Secretory Pathway in Cultured Hippocampal Astrocytes

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Glia cells have been reported to express molecules originally discovered in neuronal and neuroendocrine cells, such as neuropeptides, neuropeptide processing enzymes, and ionic channels. To verify whether astrocytes may have regulated secretory vesicles, the primary cultures prepared from hippocampi of embryonic and neonatal rats were used to investigate the subcellular localization and secretory pathway followed by secretogranin II, a well known marker for dense-core granules. By indirect immunofluorescence, SgII was detected in a large number of cultured hippocampal astrocytes. Immunoreactivity for the granin was detected in the Golgi complex and in a population of dense-core vesicles stored in the cells. Subcellular fractionation experiments revealed that SgII was stored in a vesicle population with a density identical to that of the dense-core secretory granules present in rat pheochromocytoma cells. In line with these data, biochemical results indicated that 40–50% of secretogranin II synthesized during 18-h labeling was retained intracellularly over a 4-h chase period and released after treatment with different secretagogues. The most effective stimulus appeared to be phorbol ester in combination with ionomycin in the presence of extracellular Ca2+, a treatment that was found to produce a large and sustained increase in intracellular calcium [Ca2+]i transients. Our findings indicate that a regulated secretory pathway characterized by (i) the expression and stimulated exocytosis of a typical marker for regulated secretory granules, (ii) the presence of dense-core vesicles, and (iii) the ability to undergo [Ca2+]i increase upon specific stimuli is present in cultured hippocampal astrocytes.

Astrocytes make up a large percentage of the cell composition of the central nervous system (CNS) and are thought to be involved in many important brain functions. Increasing evidence indicates that these cells can interact with the surrounding neurons and exhibit the equipment to receive, integrate, and transmit signals. It has been reported that astrocytes express a number of membrane ionic channels, transporters, and receptors linked to the most important signal transduction pathways and are capable of intracellular propagation of slow calcium waves (1–6). Furthermore, astrocytes may secrete different regulatory molecules, neurotrophic factors, and neuropeptides. In situ hybridization and immunohistochemical studies have revealed the presence of prenekephalin in cultured as well as in rat brain astrocytes (7–10). Other regulatory (poly)peptides (somatostatin, cholecystokinin) and neuropeptide processing enzymes (carboxypeptidase E and peptidylglycine-amidating mono-oxygenase), which are known to be stored in secretory granules (11, 12), have been detected in astrocytes (10, 13, 14). In addition, it has been shown that cultured astrocytes and Bergmann glial cells express secretogranin II (SgII) and chromogranin A (CgA), respectively (15, 16), two well characterized members of the granin family (for reviews, see Refs. 17–19). Besides the discovery of their presence, little information is available, however, on the subcellular localization and the role of SgII and CgA in glial cells.

Like the well established neuropeptides and hormones, granins are known to be stored in the dense-core secretory granules of many neuroendocrine cells (18, 19). However, the distribution of the individual members of the granin family is more widespread than that known for any other neuropeptide or polypeptide hormone. Furthermore, the granins were detected in dense-core vesicles of endocrinologically silent neuroendocrine tumors such as the nonfunctioning pituitary adenomas (20), thus suggesting that they may be sufficient for the formation of the dense-matrix of secretory granules. Given their widespread distribution, the granins have been used as the most useful markers to investigate the presence of dense-core granules (also referred to as large dense-core vesicles) in neurons of different areas of the mammalian CNS (18, 19, 21).

Consistent with their subcellular localization, the granins are widely investigated in vivo and in vitro with the aim of studying the mechanisms of secretory granule biogenesis (22–24). This process can be divided into two major steps: (i) the selective sorting of regulated secretory proteins from other proteins destined for different pathways and (ii) the budding from the trans-Golgi network of the dense-core vesicles (12, 25, 26). These vesicles are then stored intracellularly until a specific signal evokes their exocytosis. The regulated release of neurotransmitters and regulatory polypeptides from neuronal and neuroendocrine cells has been shown to depend on molecular interactions between integral membrane proteins of the neurosecretory vesicles (v-SNARE) and the plasma membrane (t-SNARE) (for reviews see Refs. 27–29). Interestingly, it has recently been found that some of these proteins are also expressed in glial cells (30, 31). These findings, in addition to recent data demonstrating that bradykinin and prostaglandins...
stimulate a Ca\textsuperscript{2+}-dependent release of glutamate from astrocytes (32, 33), suggest the presence of a regulated secretory mechanism in at least certain types of glial cells. However, the presence of classical synaptic-like vesicles or dense-core granules in glial cells has never been reported.

To study whether astrocytes contain regulated secretory vesicles, we endeavored to determine the intracellular localization and the secretory pathway taken by SgII in cultured hippocampal astrocytes. In this study, dense-core vesicles containing SgII were identified for the first time in astrocytes. Moreover, treatments with ionomycin or ionomycin plus PMA were found to produce large [Ca\textsuperscript{2+}], increases in hippocampal astrocytes.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The antibodies against rat SgII and chromogranin B (CgB) were raised in rabbits, purified by affinity chromatography, and characterized as described previously (34). New antisera against the granins were prepared starting from rat pheochromocytoma cells (PC12). Briefly, the heat-stable protein fraction of PC12 cells, enriched in SgII and CgB, was subjected to DEAE-cellulose chromatography and preparative two-dimensional polyacrylamide gel electrophoresis (PAGE), and transferred onto nitrocellulose filters. The filters were stained with Ponceau S (Serva Finebiochemica, Heidelberg, Germany), and the pieces containing SgII and CgB were excised, cruised to a fine powder using a glass potter in liquid N\textsubscript{2}, and resuspended in phosphate-buffered saline, pH 7.4. The filter homogenates were emulsified with complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for the booster injections. For each injection, 100 or 50 \mu g of purified protein were used. The anti-SgII and -CgB antibodies were purified by affinity chromatography as described previously (22). The specificity of the antibodies was tested by immunoblotting, immunoprecipitation, and immunofluorescence. The monoclonal antibodies against tubulin, glial fibrillary acidic protein (GFAP), microtubule-associated protein-2, anti-synaptophysin, and anti-synaptain 1A (HPC-1) were obtained from Roche Molecular Biochemicals. The antibodies against galanin, met-enkephalin, substance P, and cholecystokinin (HPC-1) were obtained from Peninsula Laboratories Inc. (Belmont, CA). The antibodies against nestin, mannosidase II, and the \alpha-subunit of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase were kind gifts of Dr. W. B. Huttner (University of Heidelberg, Germany) and Dr. M. G. Farquhar (University of California at San Diego, La Jolla, CA) and G. Pietrini (University of Milan), respectively. Secondary antibodies conjugated to fluorescein isothiocyanate, Texas Red, 10- or 5-nm gold particles, peroxidase, and protein A were also obtained from Research Laboratories (West Grove, PA).

**Cell Cultures**—The PC12 cells were grown as described previously (35). Primary astrocyte cultures were prepared from embryonic day 18 (E18) or post-natal (day 2) rat hippocampi, cerebral cortex, and cerebellum, as described by McCarthy and De Vellis (36) and Banker and Goslin (37). After dissection, tissues were incubated for 15 min at 37°C in 0.25% trypsin and then gently dissociated by trituration with a fire-polished Pasteur pipette. The cells were plated onto glass coverslips (Zeus Super, Italy) or Petri dishes (Falcon, Becton Dickinson, Meylan Cedex, France) at a density of 0.5 \times 10\textsuperscript{5} cells/ml. The cultures were grown in minimal essential medium supplemented with 20% fetal bovine serum (Life Technologies, Inc.) and glucose at a final concentration of 5.5 \text{gl}iter.

**Subcellular Fractionation and Immunoblotting**—To compare the relative density of SgII-containing vesicles from astrocytes and PC12 cells, homogenates prepared from both types of cells were separated on sucrose equilibrium gradients as described (38) with the following modifications. PC12 cells and astrocytes, grown on Petri dishes until near confluence, were scraped from the dishes, washed, and resuspended 1:4 in homogenization buffer (10 \text{mM} Hepes-KOH, pH 7.4, 250 \text{mM} sucrose, 1 \text{mM} magnesium acetate, 0.5 \text{mM} phenylmethylsulfonyl fluoride, 2 \mu M pepstatin, 10 \mu M aprotinin). The cells were homogenized using a cell cracker (European Molecular Biology Laboratory, Heidelberg, Germany) and centrifuged at 1,000 \times g for 10 min to prepare the post-nuclear supernatants (PNS). The PNS was loaded onto a 0.4–1.8 m sucrose gradient and spun in a 41 SW rotor (Beckman Instruments) at 25,000 rpm for 18 h. Fractions (1 ml) were collected and analyzed by SDS-PAGE followed by Western blotting as described (22, 39).

**Immunocytochemistry**—For double immunofluorescence, the cells were immersed in 120 mM phosphate buffer, pH 7.2, containing 4% formaldehyde and 4% sucrose. After fixation, cells were immunostained using primary antibodies followed by the appropriate secondary antibodies, as described previously (22). In some experiments, the cells were treated with 10 \mu g/ml cycloheximide (Sigma) before fixation. Specificity
of SgII immunostaining was demonstrated by antisera preadsorption with the purified antigen. Briefly, 3 μg of affinity-purified anti-SgII-antibody were preadsorbed with 15 μg of SgII purified by two-dimensional PAGE and transferred onto nitrocellulose filters. The filter pieces containing the desired amount of SgII were preincubated in 20 mM phosphate buffer, pH 7.3, 500 mM NaCl, 0.2% gelatin (immunofluorescence buffer) for 3 h at room temperature and then incubated with affinity-purified anti-SgII antibodies for 18 h at 4 °C. Electron or immunoelectron microscopy was performed as described previously (20).

After fixation, the astrocytes were scraped from the dishes and pelleted in an Eppendorf centrifuge. The pellets were either dehydrated and infiltrated in Epon 812 for preparation of plastic sections or infiltrated in 2.1 M sucrose for the preparation of cryosections. The ultra-thin frozen sections were immunolabeled using anti-rat SgII antibodies revealed with protein A conjugated to 5-nm gold particles. Double labeling with anti-SgII and anti-GFAP was revealed using anti-rabbit IgG conjugated to 10-nm gold particles and anti-mouse IgG antibodies conjugated to 5-nm gold particles.

**Fura-2 Videomicroscopy—**Astrocytes were loaded for 1 h at 37 °C with 3–4 μM Fura-2 pentacetoxymethyl ester in Krebs-Ringer solution buffered with Hepes (150 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM glucose, 10 mM Hepes/NaOH, pH 7.4), washed in the same solution to allow deesterification of the dye, and transferred to the recording chamber of an inverted microscope (100 TV; Zeiss) equipped with a calcium imaging unit. For the assays, a modified CAM-230 dual wavelength microfluorometer (Jasco, Tokyo, Japan) was used as a light source. Fluorescence images were collected with an intensified CCD camera (Hamamatsu Photonics), digitized, and integrated in real time in an image processor developed in the laboratory (40). Image files were processed off-line to convert fluorescence data into [Ca2+]i maps according to the 340/380-nm excitation wavelength ratio method (41). Mean ratio values in discrete areas of interest were calculated from sequences of images to obtain quantitative temporal analyses.

**RESULTS**

**Cellular Distribution of SgII in Cultured Astrocytes—**To investigate the possible presence of regulated secretory vesicles in astrocytes, we decided to investigate the intracellular distribution of SgII, a diffuse marker of dense-core granules (17–19), in glia primary cultures. The cell cultures were prepared from brains isolated from 18-day-old embryos or 2–5-day post-natal rats as described (37). After dissection, the cells were maintained in culture in the presence of fetal bovine serum without supplements to promote survival and proliferation of astrocytes. After 3 weeks, the majority of the cells showed a polygonal shape and expressed high levels of the intermediate filament protein GFAP, which is usually expressed by differentiated astrocytes (42) (Figs. 1–4 and data not shown). Cells expressing markers of the neuronal phenotype (i.e. neurofilament proteins and microtubule-associated protein-2) were not detected (data not shown). In 3-week old cultures prepared from embryonic hippocampi, only a small amount of the cells (10%, n = 2) were found to synthesize the intermediate filament protein marker of neuronal stem cells, nestin (43), thus suggesting that the majority of the astrocytes present in the primary culture prepared were largely differentiated.

The 3-week-old astrocyte cultures prepared from post-natal or E18 rats were then probed for the presence of SgII using two different anti-rat SgII antibodies. In the hippocampal cultures, immunoreactivity for the granin was observed in a large number (58.8 ± 3.7%; n = 5) of GFAP-positive cells (see Fig. 1). The SgII-immunoreactive cells were frequently clustered to form groups scattered throughout the culture. Although expressed to different extents, a high level of immunoreactivity for the granin was localized in a perinuclear region corresponding to the Golgi complex as demonstrated (i) by immunofluorescence of SgII and mannosidase II staining patterns (compare Fig. 1 panels A–'C' with panel D) and (ii) by co-localization of SgII and wheat germ-agglutinin stainings (not shown). In addition, SgII immunoreactivity was found in puncta dispersed throughout the cell cytoplasm (Fig. 1A–'C'). Quantitative analysis by immunoblotting of SgII expressed by astrocytes or PC12 cells revealed that at the steady state, astrocytes expressed a 4- to 6-fold lower amount of the granin than PC12 cells. SgII-immunostaining was also immunodetected in hippocampal astrocytes analyzed 18 h after plating or co-cultured with hippocampal neurons (Fig. 2 and data not shown). On the other hand, astrocytes prepared from other regions of the CNS, such as the cerebral cortex or cerebellum isolated from E18 and neonatal rats, were found to be negative for SgII by immunofluorescence, suggesting that glial cells isolated from these regions synthesized low amounts, if any, of the granin (data not shown). The specificity of the immunostaining observed with the anti-SgII antibodies was verified by abolition of the labeling after preadsorption of the antibodies with the purified SgII (data not shown). Immunoreactivity for another member of the granin family, CgB, was not detected in the hippocampal astrocytes (data not shown). Taken together, these data indicate that differentiated hippocampal astrocytes in culture specifically express detectable level of SgII.

**Intracellular Storage of SgII in Dense-core Granules—**The punctate appearance of SgII immunoreactivity observed in astrocytes by immunofluorescence suggested an accumulation of the protein in vesicles. To test whether the SgII-positive vesicles were stored intracellularly, cultured hippocampal astrocytes were incubated with cycloheximide to prevent protein synthesis (Fig. 3). After 2 h of drug treatment, no SgII immunoreactivity was detected in the Golgi complex as expected, since the newly synthesized protein was chased out from the biosynthetic pathway. On the other hand, after 4 h of drug treatment, SgII-immunolabeling was still present in the puncta dispersed throughout the cytoplasm (Fig. 3A'). Even

![Fig. 1. Immunolocalization of SgII in astrocytes.](image)
18 h after cycloheximide treatment, cells, although few, containing SgII-positive vesicles were observed (Fig. 3B9). Altogether these data suggest the intracellular storage of the SgII-containing vesicles in astrocytes.

To define the morphology of this storage compartment, we analyzed the distribution of SgII in cultured hippocampal astrocytes by electron microscopy. As shown in Fig. 4A, when immunolabeling was performed using antibodies against SgII, immunoreactivity for the granin was found in the Golgi complex and in dense-core vesicles with a diameter of about 114 ± 6 nm (Fig. 4, B–G). These vesicles were also found near the tubular structure of the trans-Golgi network where biogenesis of secretory granules is known to take place (Ref. 12 and references therein) (Fig. 4C). Double immunolabeling performed using anti-SgII and anti-GFAP antibodies confirmed the presence of dense-core SgII-positive vesicles in astrocytes (Fig. 4, F and G). In addition, few smaller and less-dense SgII labeled vesicles were also detected in the astrocytes (Fig. 4, D and E). These vesicles could represent sections through tubular structures (e.g. of the Golgi complex) or the edge of the dense-core vesicles. On the other hand, the biochemical data showing a fast release of a portion of SgII immediately after synthesis (see above) suggest that some of these structures may represent constitutive secretory vesicles also containing SgII.

To further characterize the intracellular organelles where SgII is accumulated in hippocampal astrocytes, we performed...
Regulated Secretion in Astrocytes

To analyze the metabolism of SgII in astrocytes, 18-day-old cultures were labeled for 60 min with [35S]methionine/cysteine and then chased for consecutive 60 and 180 min (Fig. 6). The labeled SgII was then detected in the cell lysates and media by immunoprecipitation using specific antibodies. As shown in Fig. 6A, a major band corresponding to SgII was detected in each sample. Quantitative analysis of labeled SgII (Fig. 6B) indicated that a large fraction of the newly synthesized granin (~48%, n = 2) was released in the medium in the first 60 min of chase with minor further release (23%) during the following 120 min. At the end of the chase, 28% (n = 2) of the total labeled SgII was still found in the cells extracts. We had previously found that CgB (a member of the grgin family with a kinetics of transport along the secretory pathway identical to that of SgII (23, 35)) was rapidly released via the constitutive pathway when expressed in cells devoid of the regulated secretion. In these cells, 90–95% of the protein synthesized during the 60-min pulse was found in the medium at the end of a 3–4-h chase period (Ref. 47 and data not shown). Altogether these results indicate that although a fraction of the labeled SgII is released with a rapid kinetics via the constitutive pathway, a substantial amount of the granin is stored intracellularly.

To obtain direct evidence for a stimulated release of SgII, astrocytes were metabolically labeled for longer time. Since SgII is post-translationally modified in the trans-Golgi network by means of sulfation of tyrosine residues (35), 3-week-old cultures of hippocampal astrocytes isolated from E18 rats were labeled for 18 h with [35S]sulfate. The pattern of the total proteins revealed the presence of several [35S]sulfate-labeled molecules in astrocyte cell lysates and media (Fig. 7A, left panels). To identify SgII, samples were analyzed by immunoprecipitation. As shown in Fig. 7A (right panels), a band corresponding to [35SO4]2–labeled SgII was detected in the cell lysates and media after overnight labeling. Moreover, a considerable amount of the labeled granin (40–50%) that detected in the cells after the long labeling period (n = 2) was still found in the cell lysates after 4 h chase. With this in mind it is interesting to

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**FIG. 5.** Sucrose equilibrium gradient analysis of relative densities of the vesicles containing SgII in astrocytes and PC12 cells. The PNS from PC12 cells (panel A) and astrocytes (panel B) were centrifuged through a 0.4–1.8 M sucrose gradients. Twelve fractions were collected from each gradient and pelleted in 80% acetone at ~20°C. Aliquots of each fractions were analyzed by immunoblotting using antibodies against the α-subunit of Na+/K+-ATPase (diluted 1:50000), synaptophysin (Syn, diluted 1:2000), SgII (diluted 1:5000), mannosidase II (Man II, diluted 1:10000), or syntaxin 1A (Syx, diluted 1:4000). Panel C reports the molar concentration of sucrose in each fraction. Blots representative of two independent experiments are shown.

Subcellular fractionation on sucrose gradients and compared the density of SgII-containing vesicles present in astrocytes with those of SgII-positive dense-core granules present in PC12 cells (24). Following described procedures (38, 39) the PNS prepared from astrocytes or PC12 cells was subjected to a sucrose equilibrium gradient. Aliquots of each collected fractions were processed by Western blotting using antibodies against SgII or various protein markers (Fig. 5). When a PNS prepared from PC12 cells was separated on the gradient, SgII immunoreactivity was found mainly in the bottom fractions (fractions 7–12, Fig. 5A, third line), where dense-core secretory granules are known to migrate, whereas the synaptic vesicles (immunoreactive for synaptophysin) were found to peak in fractions 5 to 9 (Fig. 5A, middle line). On the other hand the α-subunit of the Na+/K+-ATPase, a marker for the plasma membrane (44), peaked in fractions 7–10. When a PNS from

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**FIG. 6.** A time course of total [35S]sulfate-labeling in astrocytes. To obtain direct evidence for a stimulated release of SgII, 18-day-old cultures were labeled for 18 h with [35S]sulfate. The pattern of the total proteins revealed the presence of several [35S]sulfate-labeled molecules in astrocyte cell lysates and media (Fig. 7A, left panels). To identify SgII, samples were analyzed by immunoprecipitation. As shown in Fig. 7A (right panels), a band corresponding to [35SO4]2–labeled SgII was detected in the cell lysates and media after overnight labeling. Moreover, a considerable amount of the labeled granin (40–50%) detected in the cells after the long labeling period (n = 2) was still found in the cell lysates after 4 h chase. With this in mind it is interesting to...
were labeled with \([35S]\)methionine/cysteine for 60 min (chase 0) and then chased for 60 and 180 min. SgII was immunoprecipitated from two independent experiments.

Note that in very similar experimental conditions, proteins secreted via the constitutive secretory pathway are found almost completely (∼90%) in the chase medium (48).

Cultured astrocytes express at the cell surface a variety of ionotropic or metabotropic receptors including the bradykinin receptors and respond to external stimuli such as neurotransmitters or hormones, which generate changes in the cytoplasmic Ca\(^{2+}\) concentration (5, 32). To investigate whether the release of SgII could be evoked by secretagogues, after long labeling with \([35S]\)sulfate followed by 4-h chase to deplete the constitutive secretory pathway, astrocytes were incubated with various stimuli (Fig. 7B). Depolarization (55 mM KCl) did not change the rate of SgII secretion (data not shown), which is in line with the observations indicating the lack of voltage-activated calcium channels in the plasma membrane of astrocytes cultured under our experimental conditions (2). On the other hand, bradykinin (1 μM), dibutyryl-cAMP (5 mM), ionomycin (1 μM), PMA (100 nM), and ionomycin in combination with PMA induced the granin release with different levels of efficiency. We found that ionomycin plus PMA, applied in the presence of extracellular Ca\(^{2+}\), was the most effective stimulus (the level of SgII secreted was 4-fold greater than control). More modest but still significant effects were observed after treatment with bradykinin, dibutyryl-cAMP, PMA, and ionomycin (Fig. 7B). Moreover, the SgII release evoked by ionomycin and ionomycin plus PMA was largely reduced in the absence of extracellular Ca\(^{2+}\).

Given the Ca\(^{2+}\) dependence of the ionomycin plus PMA stimulation in inducing SgII release, we investigated whether the applied stimuli gave rise to \([Ca^{2+}]_i\) increases. FURA 2/AM-loaded cultured hippocampal astrocytes were characterized by the occurrence of spontaneous \([Ca^{2+}]_i\) rises (Fig. 8), which may in part account for the high levels of SgII in the medium in the absence of secretagogues. When astrocytes were challenged with the different secretagogues, ionomycin and ionomycin in combination with PMA were found to be the most effective in producing long and sustained \([Ca^{2+}]_i\) elevations. Bradykinin produced transient increases in cytosolic free Ca\(^{2+}\). A smaller increase in \([Ca^{2+}]_i\) was found to be induced by the application of ionomycin plus PMA in the absence of extracellular Ca\(^{2+}\).

**DISCUSSION**

The data presented here demonstrate that cultured hippocampal astrocytes express a regulated secretory pathway. Several experimental observations support this conclusion. First, a widespread marker of the regulated secretory pathway SgII, is shown to be synthesized in hippocampal astrocytes.
Second, immunocytochemical data clearly show that the gramin is packaged in dense-core vesicles. Third, these vesicles are stored intracellularly as demonstrated by cycloheximide treatment. Fourth, and most importantly, the release of intracellular-stored SgII is evoked by treatment with various secretagogues in a calcium-dependent manner. To our knowledge, this is the first report showing the presence of dense-core vesicles and the regulated release of their content in hippocampal astrocytes. These vesicles appear by morphology and density similar to the dense-core granules found in neuroendocrine cells (Ref. 49 and references therein), and like these vesicles, they contain a well known marker of the regulated secretory pathway, SgII. Finally, the expression of the gramin observed in hippocampal astrocytes seems to be independent of the culturing conditions since SgII-immunostaining is detected in GFAP-positive cells after only 18 h of plating and also in hippocampal astrocytes co-cultured with neurons.

Regulated Secretory Mechanisms in Astrocytes—Our data arguing for the existence of a regulated secretory mechanism in cultured hippocampal astrocytes are in accordance with other recent results. It has been reported that both cultured astrocytes and astrocytes present in hippocampal slices may secrete glutamate in a calcium-dependent manner in response to bradykinin or prostaglandin stimulations and after treatment with α-latrotoxin (32, 33, 50). However, vesicles for glutamate storage have not yet been identified in astrocytes, and the biological mechanisms that link [Ca^{2+}], rise to neurotransmitter release await further studies.

We now show that hippocampal astrocytes are capable of secreting SgII in response to secretagogues that may also increase the intracellular levels of Ca^{2+}. Calcium rises have been demonstrated in different astrocytes in vitro as well as in vivo systems (5, 6). Furthermore, increases in cytosolic free calcium can be propagated via gap junctions between adjacent astrocytes, resulting in Ca^{2+} waves traveling in the astrocyte network (1, 51). [Ca^{2+}], transients have been thought to play an essential role in glial cell functions, being involved in the induction of regulated release of biologically active molecules (32, 33). Moreover, it has recently been reported that Ca^{2+} waves may stimulate the release of a not yet identified extracellular signal that may play an important role in the wave propagation itself (52). In our experimental conditions the maximal induction of SgII secretion is achieved by stimuli elevating [Ca^{2+}] when applied in combination with activators of protein kinase C in the presence of extracellular Ca^{2+}. The synergistic effect of ionomycin and PMA suggests that protein kinase C activation and Ca^{2+} mobilization are required for the most efficient regulated release in astrocytes, as is known to occur in neuroendocrine cells.

The presence of regulated secretory pathway(s) in astrocytes is not surprising. These cells, which for a long time were thought to play a mechanical role in the CNS, are capable of physiologically interacting with neurons and express functions until now unexpected. In addition, regulated secretion processes appear more widely present than previously thought. Regulated secretory vesicles that are similar to secretory granules in several aspects (e.g. Ca^{2+} dependence of secretion and slow turnover in the absence of secretagogues) have been found in constitutive secretory cells (53). Although we were unable to detect SgII-positive vesicles in astrocytes prepared from other brain regions, we could not exclude the presence of dense-core vesicles in these cells, containing other regulated secretory proteins. Further analysis using different markers is required for solving this issue. The function(s) of regulated secretory

![Image of regulated secretion in astrocytes](https://example.com/regulated-secretion-astrocytes)
pathways present in nonneurosecretory cells are unknown, although they may be involved in intracellular communication and autocrine or paracrine secretion.

Although cultured hippocampal astrocytes release SgII in a regulated manner, it is not known whether they express all proteins of the neuronal machinery for regulated exocytosis, including the neuronal isoforms of t- and v-SNAREs (27–29). Although SNAP25 and synaptotagmin I have not been found in astrocytes, synaptobrevin II and syntaxin 1 have been recently detected in glial cells (30, 31) by Western blotting and reverse transcription-polymerase chain reaction. In line with these data, our data demonstrate that syntaxin 1A is expressed also in hippocampal astrocytes where it is mainly distributed at the plasmalemma as in neuronal and neuroendocrine cells. Besides syntaxin 1A and synaptobrevin II, the recently discovered SNARE isoforms TI-VAMP (54, 55) and SNAP-23 (54, 56) appear to be present in cultured astrocytes.3 It cannot be excluded that these or other still unidentified SNARE proteins may be implicated in the regulated exocytosis present in astrocytes, and further studies are needed to completely dissect the molecular mechanisms implicated in this process.

Possible Roles of SgII in Hippocampal Astrocytes—In this study we show that SgII is specifically expressed by a population of hippocampal astrocytes. The absence of SgII in cortical and cerebellar astrocytes is in line with the results of previous studies showing that the expression of regulatory peptides varies in relation to the region of the brain from which the astrocytes are isolated. On the other hand, we were unable to immunodetect other neuropeptides, (such as enkephalin, substance P, galanin, cholecystokinin) or other members of the granin family by immunofluorescence in hippocampal astrocytes.2 We could not exclude that other regulatory molecules or neurotrophic factors are stored with SgII in the dense-core vesicles of astrocytes.

Despite the fact that granins have been found throughout the CNS and in endocrine glands, their physiological functions have not yet been clarified. Like many prohormones, they are processed by the endopeptidases of the kex-2-like family (57) into smaller peptides (58, 59), which may have regulatory functions (Ref. 60 and references therein). It has been discovered that the 33-amino acid neuropeptide secretoneurin, which is derived from SgII by enzymatic processing, may stimulate the release of dopamine from rat striatum (61) and may play a role in neurogenic inflammation as suggested by its stimulated release by capsaicin and its chemotactic effects on fibroblasts (62). Although SgII does not seem to undergo any evident proteolysis when synthesized and stored in cultured astrocytes (no smaller peptides were detected after immunoprecipitation), we cannot exclude that SgII is processed after secretion in vivo. On the other hand SgII or SgII fragments may play a role in the modulation of neurotransmitters and/or in the modulation of their cell-substrate adhesion. It has been reported that CgA fragments and CgB may locally mediate cell-substrate adhesion although via different mechanisms (63, 64). Finally, we cannot exclude a possible function of SgII in astrocytes before secretion, for example in the packaging of neuropeptides and or neurotrophic factors. It has recently been reported that CgB, a member of the granin family, may help packaging of propionolactoncortin-derived peptides into secretory granules (65).
