Pyroglutamate-Amyloid- β and Glutaminyl Cyclase Are Colocalized with Amyloid- β in Secretory Vesicles and Undergo Activity-Dependent, Regulated Secretion

Holger Cynis^a Lydiane Funkelstein^{b, c} Thomas Toneff^{b, c} Charles Mosier^{b, c}
Michael Ziegler^{b, c} Birgit Koch^a Hans-Ulrich Demuth^{a, d} Vivian Hook^{b, c}

^aFraunhofer IZJ-MWT, Halle, Germany; ^bSkaggs School of Pharmacy and Pharmaceutical Sciences and ^cSchool of Medicine, University of California, San Diego, La Jolla, Calif., USA; ^dProbiobdrug AG, Halle, Germany

Key Words

Pyroglutamate · Glutaminyl cyclase · Amyloid- β · Secretory vesicles

Abstract

Background and Aims: N-truncated pyroglutamate (pGlu)-amyloid- β [A β (3–40/42)] peptides are key components that promote A β peptide accumulation, leading to neurodegeneration and memory loss in Alzheimer's disease. Because A β deposition in the brain occurs in an activity-dependent manner, it is important to define the subcellular organelle for pGlu-A β (3–40/42) production by glutaminyl cyclase (QC) and their colocalization with full-length A β (1–40/42) peptides for activity-dependent, regulated secretion. Therefore, the objective of this study was to investigate the hypothesis that pGlu-A β and QC are colocalized with A β in dense-core secretory vesicles (DCSV) for activity-dependent secretion with neurotransmitters. **Methods:** Purified DCSV were assessed for pGlu-A β (3–40/42), A β (1–40/42), QC, and neurotransmitter secretion. Neuron-like chromaffin cells were analyzed for cosecretion of pGlu-A β , QC, A β , and neuropeptides. The cells were treated with a QC inhibitor, and pGlu-A β production was measured. Human neuroblastoma cells were also exam-

ined for pGlu-A β and QC secretion. **Results:** Isolated DCSV contain pGlu-A β (3–40/42), QC, and A β (1–40/42) with neuropeptide and catecholamine neurotransmitters. Cellular pGlu-A β and QC undergo activity-dependent cosecretion with A β and enkephalin and galanin neurotransmitters. The QC inhibitor decreased the level of secreted pGlu-A β . The human neuroblastoma cells displayed regulated secretion of pGlu-A β that was colocalized with QC. **Conclusions:** pGlu-A β and QC are present with A β in DCSV and undergo activity-dependent, regulated cosecretion with neurotransmitters.

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Introduction

Accumulation of neurotoxic β -amyloid (A β) peptides in the brain represents a key factor in the development of memory deficits in Alzheimer's disease (AD) [1–4]. A β peptides of multiple forms are present in AD brains. Notably, affected AD brains contain the N-terminally trun-

Holger Cynis and Lydiane Funkelstein contributed equally to this study.

cated pyroglutamate (pGlu) forms of A β (3–40/42) (pGlu-A β) as a major portion of the total A β peptides, which includes the known full-length A β (1–40/42) peptides [5–7]. The accumulation of pGlu-A β (3–40/42) in the brain occurs before that of the A β (1–40/42) peptides [5]. Significantly, pGlu-A β facilitates the seeding of A β peptides into neurotoxic oligomers [8] that are thought to participate in the development of memory deficits in AD [9]. Increasing the brain levels of pGlu-A β (3–42) resulted in aggravated memory deficits and amyloid plaque accumulation in the 5XFAD mouse model of AD [10], illustrating the key role of N-truncated pGlu-modified forms of A β in AD.

The pGlu-A β (3–40/42) peptides start with N-terminal glutamate, the third amino-terminal residue of A β (1–40/42). Notably, this residue is converted to pGlu by the enzyme glutamyl cyclase (QC) [8, 11, 12]. QC is elevated in AD brains [5–7]. Moreover, inhibition of QC [13, 14] results in decreased brain pGlu-A β levels, and QC gene knockout results in decreased brain pGlu-A β levels with improved behavioral deficits in 5XFAD mice [12]. These findings indicate that QC is involved in the development of AD via formation of pGlu-A β .

A β peptides have been demonstrated to be released from brain neurons in an electrical activity-dependent manner [15–19]. Neural activity modulates the formation and secretion of A β [20, 21]. Recent evidence indicates that endogenous neuronal activity regulates regional brain vulnerability to A β deposition [22]. Significantly, neuronal activity is fundamental for the regulated secretion of neurotransmitters [23, 24]. These features of A β peptides raise the question of whether pGlu-A β and its biosynthetic enzyme QC are cosecreted with A β and neurotransmitters in a regulated, activity-dependent manner. Therefore, this study investigated the hypothesis that cosecretion of truncated pGlu-A β and full-length A β occurs with neurotransmitters stored and released from secretory vesicles, resulting in extracellular A β peptide forms. These studies utilized neuronal chromaffin cells that display activity-dependent secretion of neurotransmitters and also produce A β [17, 19], as well as human IMR32 neuroblastoma cells.

Our results show that secretory vesicles of the dense-core secretory vesicle (DCSV) type contain pGlu-A β with QC and A β combined with catecholamine and peptide neurotransmitters. Cellular pGlu-A β and QC undergo cosecretion from the regulated secretory pathway of neuron-like chromaffin cells stimulated by nicotine or KCl depolarization. pGlu-A β also undergoes regulated cosecretion with A β and the peptide neurotransmitters me-

thionine Met-enkephalin and galanin peptide. Treatment of cells with a QC inhibitor resulted in decreased levels of pGlu-A β released from the regulated secretory pathway. Furthermore, human neuroblastoma cells displayed regulated cosecretion of pGlu-A β (3–40) with A β (1–40/42) combined with colocalization of pGlu-A β and QC. These findings support the hypothesis that pGlu-A β and QC undergo cosecretion with A β and peptide neurotransmitters from the activity-dependent, regulated secretory pathway.

Experimental Procedures

Content of pGlu-A β , QC, A β Peptides, and Neurotransmitters in DCSV

Secretory vesicles were purified from fresh bovine adrenal medulla to evaluate the content of QC, pGlu-A β , A β peptides, Met-enkephalin and galanin peptide neurotransmitters, and the catecholamine neurotransmitters dopamine, norepinephrine, and epinephrine. These DCSV, also known as chromaffin granules, were isolated by differential sucrose density gradient centrifugation, as previously described [25–29]. The density gradient isolation procedure has been established to yield secretory vesicles of high purity based on the assessment of organelle markers and on electron microscopy [25–29]. The procedure results in purified secretory vesicles that lack biochemical markers for other subcellular organelles of lysosomes (acid phosphatase marker), cytoplasm (lactate dehydrogenase marker), mitochondria (fumarate and glutamate dehydrogenase markers), and the endoplasmic reticulum (glucose-6-phosphatase marker). Enzyme markers in the purified secretory vesicle preparation represent less than 1% of the total amount of homogenate markers. Furthermore, electron microscopy and enzyme markers have confirmed the integrity and purity of isolated DCSV [25–29].

The presence of QC in isolated DCSV was assessed by monitoring QC enzymatic activity as previously described [30–32]. QC in chromaffin granules was subjected to immunoprecipitation with anti-QC – conducted as previously described [33] – followed by Western blotting with anti-QC sera (rabbit CPC1301, from Pro-Biodrug), using Western blot procedures as described [25, 33–35].

For the measurement of A β peptides and neurotransmitters, purified secretory vesicles were lysed by freeze-thawing in buffer (50 mM Na acetate, 50 mM NaCl, 1 mM EDTA) containing a cocktail of protease inhibitors (pepstatin A, leupeptin, chymostatin, and E64c at 10 μ M each, and PMSF at 500 μ M). An acid extract (0.1 N acetic acid) was prepared from the lysed secretory vesicles (as described previously [25, 26, 34, 35]) for the measurement of pGlu-A β (3–40), pGlu-A β (3–42), A β (1–40), A β (1–42), Met-enkephalin, and galanin, and of the catecholamines dopamine, norepinephrine, and epinephrine. DCSV samples were subjected to ELISA measurements of pGlu-A β (3–40) (No. 27418; IBL International, Toronto, Ont., Canada), pGlu-A β (3–42) (No. 27716; IBL), A β (1–40) (No. 27718; IBL), and A β (1–42) (No. 27712; IBL), and RIA for Met-enkephalin and galanin (the RIAs were conducted as previously reported [28, 33, 35]). The well-established ELISA kits have been characterized to demonstrate their specificities for the

particular peptides (by the manufacturer IBL). The ELISAs for A β (1–40) and A β (1–42) do not cross-react with each other, nor with the pGlu-A β peptides; the ELISA for pGlu-A β (3–40) does not detect pGlu-A β (3–42) or A β (1–40/42), and the ELISA for pGlu-A β (3–42) specifically detects this N-truncated peptide rather than pGlu-A β (3–40) and does not detect A β (1–40/42). QC activity was measured in the media, as previously described [30–32], using H-Gln- β NA as the substrate in a fluorometric assay. Dopamine, norepinephrine, and epinephrine catecholamines were measured by radioenzymatic assays, as previously described [36, 37]. The protein content of the purified DCSV was measured by the Bio-Rad DC Protein Assay (Lowry) kit (Bio-Rad, Hercules, Calif., USA). The contents of A β peptides and neurotransmitters were expressed as picograms per milligram protein.

Neuron-Like Chromaffin Cells in Primary Culture

Neuron-like chromaffin cells in primary culture were obtained from the adrenal medulla of the sympathetic nervous system. In our study, they were prepared from fresh adrenal medulla tissue (bovine), as previously described [25, 35, 38]. Briefly, chromaffin cells were dissected from fresh adrenal glands, dissociated in a collagenase/DNase solution at 37°C, filtered, and centrifuged. The cells were plated onto fibronectin-coated dishes (EMD Chemicals, Gibbstown, N.J., USA) in medium containing DMEM (Cellgro, Manassas, Va., USA), 10% FBS (Invitrogen, Carlsbad, Calif., USA), and penicillin/streptomycin. The cells were maintained at 37°C and 6% CO₂.

Regulated Secretion of pGlu-A β , QC, A β , and Peptide Neurotransmitters from Chromaffin Cells

After 6 days in culture, the chromaffin cells were subjected to KCl depolarization or treatment with nicotine to stimulate regulated, activity-dependent secretion. They were incubated in standard release medium (as described by Bark et al. [28]) with 0.25 μ g/ml BSA for 90 min at 37°C, followed by removal of the medium, which represents basal secretion. Then they were stimulated for 90 min (at 37°C) in standard release medium containing KCl (50 mM) or nicotine (10 μ M), followed by collection of the medium, which represents regulated secretion. The secretion medium was collected with addition of a cocktail of protease inhibitors (1 mM EDTA, 500 μ M AEBSF, and 10 μ M each of E64c, leupeptin, pepstatin A, and chymostatin). Then it was concentrated by ultrafiltration through a 2-kDa cut-off membrane (Vivaspin 2 2K MWCO Hydrosart; Sartorius, Göttingen, Germany), which retains A β . The concentrated sample was subjected to measurements of pGlu-A β (3–40), pGlu-A β (3–42), A β (1–40), A β (1–42), Met-enkephalin, and galanin, as described above for DCSV. QC activity was measured in the medium, as previously described [30, 31], using H-Gln- β NA as the substrate in a fluorometric assay.

Inhibition of Endogenous QC in Chromaffin Cells and Levels of Secreted pGlu-A β (3–40)

The chromaffin cells were incubated with the QC inhibitor PQ529 (Probiobdrug AG, Halle, Germany) at 50 μ M for 18 h. They were then subjected to regulated secretion induced by treating cells with KCl (50 mM) for 90 min. Control incubation without KCl was also included. The cell culture medium was then collected for the measurement of pGlu-A β (3–40) by ELISA (as described above).

Cellular Immunofluorescence Localization of pGlu-A β and QC with Met-Enkephalin Neurotransmitter Present in Secretory Vesicles

Immunofluorescence microscopy of the chromaffin cells was conducted to assess the subcellular localization of pGlu-A β and QC with Met-enkephalin in the DCSV that undergo regulated secretion. The chromaffin cells were fixed for immunofluorescence deconvolution microscopy, conducted as previously described [33–35]. The primary antibodies used for immunocytochemistry were rabbit anti-QC 1301 (1:250; Probiobdrug AG), mouse anti-Met-enkephalin (1:100; Abcam No. 23503; Abcam, Cambridge, Mass., USA), mouse anti-pGlu-A β (1:50; Probiobdrug AG) detecting the N-terminus of both pGlu-A β (3–40) and pGlu-A β (3–42), rabbit anti-Met-enkephalin (1:50; Millipore, Billerica, Mass., USA), and mouse anti-amyloid precursor protein (APP) clone 6E10 (1:100; Covance, Princeton, N.J., USA). The primary antibodies were detected with the secondary antibodies anti-rabbit IgG-Alexa Fluor 594 (goat, 1:200 dilution, red fluorescence; Molecular Probes, Eugene, Oreg., USA) and anti-mouse IgG-Alexa Fluor 488 (goat, 1:200 dilution, green fluorescence; Molecular Probes). The immunofluorescent images were analyzed by DeltaVision Spectris Image Deconvolution Systems on an Olympus IX70 epifluorescence microscope using the software softWoRx Explorer by Applied Precision (GE Healthcare Company, Issaquah, Wash., USA), as previously described [33, 39]. As a control procedure, immunostaining with only secondary antibody was performed, and the controls obtained demonstrated a lack of immunofluorescence, indicating the specificity of the primary immunoreactivity observed.

Human IMR32 Neuroblastoma Cells and Regulated Secretion of pGlu-A β with A β Peptides

Human IMR32 neuroblastoma cells were obtained from ATCC (American Type Culture Collection, Crystal City, Va., USA) and cultured according to the manufacturer's instructions. Activity-dependent secretion was achieved by stimulating secretion in the medium by incubation with KCl (50 mM) for 90 min. Control secretion for 90 min without KCl was included. The secretion media were collected, concentrated, and subjected to measurements of pGlu-A β (3–40), A β (1–40), and A β (1–42) (ELISA kits from IBL). Cellular localization of pGlu-A β and QC was conducted by immunofluorescence confocal microscopy, as described above for chromaffin cells.

Statistical Analysis of Data

The secretion of A β peptides and neurotransmitters was conducted in triplicate in each experiment, and the experiments were repeated 3 times. Data were considered statistically significant at $p < 0.05$ by Student's t test.

Results

Secretory Vesicles Contain pGlu-A β and QC with A β and Neurotransmitters

Several studies in the field have demonstrated that A β undergoes activity-dependent, regulated secretion from neurons [15–18], implicating release from secretory ves-

Fig. 1. a Neuronal secretion of neurotransmitters. Secretory vesicles of neurons provide synthesis, storage, and regulated secretion of neuropeptides, catecholamines, and bioactive neurotransmitter molecules that are critical for cell-cell communication in the nervous system. **b** DCSV observed by electron microscopy. Secretory vesicles of the DCSV type were isolated from chromaffin cells of bovine adrenal medulla tissue by differential sucrose gradient centrifugation, which results in a highly purified preparation of homogeneous DCSV [25, 28, 29]. The integrity of the purified DCSV is illustrated here.

Table 1. A β peptides and neurotransmitters in purified DCSV

	Content, pg/mg protein
A β peptides	
pGlu-A β (3–40)	0.022 $\pm$ 0.004
pGlu-A β (3–42)	0.070 $\pm$ 0.009
A β (1–40)	0.220 $\pm$ 0.010
A β (1–42)	0.085 $\pm$ 0.004
Peptide neurotransmitters	
Met-enkephalin	215,000 $\pm$ 14.4
Galanin	408 $\pm$ 0.015
Catecholamine neurotransmitters	
Dopamine	1,150 $\pm$ 0.13
Norepinephrine	4,560 $\pm$ 0.34
Epinephrine	6,490 $\pm$ 1.02

The concentrations of A β peptides and neurotransmitters in purified chromaffin DCSV were measured by assays as described in the Experimental Procedures section.

icles that store and release neurotransmitters (fig. 1a). Therefore, our study investigated the hypothesis that pGlu-A β and its biosynthetic enzyme QC may be present with A β and neurotransmitters in secretory vesicles for extracellular release.

DCSV of neuron-like chromaffin cells store neurotransmitter contents for activity-dependent, regulated secretion; thus, they were isolated for this study. The DCSV were purified by sucrose density gradient centrifugation and the integrity of the isolated DCSV was illustrated by electron microscopy (fig. 1b). Previous reports have documented the high purity of these isolated DCSV by assessing enzyme markers for subcellular organelles [25, 28, 29].

The isolated DCSV contained pGlu-A β (3–40), pGlu-A β (3–42), A β (1–40), and A β (1–42), quantitated by ELISA assays (table 1). The concentrations of pGlu-A β (3–40) and pGlu-A β (3–42) (at approx. 0.022 and 0.070 pg/mg protein) represented 10 and 82% of the concentrations of A β (1–40) and A β (1–42), respectively. Moreover, the DCSV contained enkephalin and galanin peptide neurotransmitters, as well as the catecholamines dopamine, norepinephrine, and epinephrine (table 1). These data show that the DCSV contained pGlu-A β and A β peptides with peptide and catecholamine neurotransmitters.

The presence of QC activity in the isolated DCSV was demonstrated by an activity assay using H-Gln- β NA as substrate. The time-dependent formation of the QC product is illustrated in figure 2a. Heat inactivation as well as omitting the auxiliary enzyme of the assay underlined the specificity of the test. Furthermore, QC protein is present in DCSV, as observed by anti-QC Western blots (fig. 2b).

pGlu-A β and QC Undergo Activity-Dependent, Regulated Secretion from Neuron-Like Chromaffin Cells with A β and Peptide Neurotransmitters

The activity-dependent secretion of neurotransmitters from neuron-like chromaffin cells was stimulated by KCl depolarization, which represents regulated secretion, and by nicotine, which activates the nicotinic cholinergic receptor [38, 40–42]. KCl depolarization and nicotine activation of the cholinergic receptor of chromaffin cells are utilized in the field to assess activity-dependent, regulated secretion [35, 40, 43–50]. KCl depolarization and nicotine stimulated the secretion of pGlu-A β (3–40) (fig. 3a). Stimulated secretion (with KCl) was severalfold greater than basal secretion (no KCl). High KCl and nicotine stimulated the release of approximately 3–4% of the total cellular A β (3–40) (data not shown), but KCl and nicotine

Fig. 2. a QC activity in DCSV. QC activity (black symbols) in purified DCSV of chromaffin cells (also known as chromaffin granules) was measured in time-course assays. The secretory vesicles showed no activity after heat inactivation (red symbols; colors in online version only) and after omission of the auxiliary enzyme of the test (green symbols). **b** QC protein in DCSV. Purified DCSV were subjected to anti-QC Western blots. Endogenous QC immunoreactive bands of approximately 60 and 48 kDa were observed. The selectivity of anti-QC for detecting these immunoreactive

bands was demonstrated by conducting a Western blot with only the secondary anti-rabbit serum (omitting the primary anti-QC serum), which resulted in the absence of immunoreactivity (unpublished data). It is noted that, on SDS-PAGE, endogenous bovine pituitary QC has been observed with an apparent molecular weight of approximately 40–45 kDa [101], and recombinant non-glycosylated murine QC at a molecular weight of 37–40 kDa [31]. Glycosylation of endogenous QC is known to modify its apparent molecular weight [102].

Fig. 3. Activity-dependent cosecretion of pGlu-A β and QC from neuron-like chromaffin cells. * Statistically significant for comparison of stimulated cells (by KCl or nicotine) with unstimulated control cells ($p < 0.05$, Student's t test). **a** Regulated secretion of pGlu-A β . This was induced by KCl depolarization and nicotine treatment of neuron-like chromaffin cells (90-min incubation time) with the inclusion of unstimulated controls. The secretion medium was collected for measurement of pGlu-A β (3–40). **b** Regulated secretion of QC activity. The secretion media from the experiments of **a** were collected and measured for QC activity.

stimulated the predominate portion of secreted A β (3–40) compared with the condition of basal constitutive secretion. Notably, QC, the biosynthetic enzyme for pGlu-A β , undergoes activity-dependent cosecretion with pGlu-A β (3–40) (fig. 3b).

Furthermore, KCl and nicotine stimulated the secretion of A β (1–40) and A β (1–42) (fig. 4). Because regulated, activity-dependent secretion is fundamental for the release of neurotransmitters, the peptide neurotransmitters Met-enkephalin and galanin were evaluated in these

Fig. 4. Activity-dependent, regulated secretion of A β and peptide neurotransmitters. * Statistically significant for comparison of stimulated cells (by KCl or nicotine) with unstimulated control cells ($p < 0.05$, Student's t test). **a, b** Regulated secretion of A β (1–40) (**a**) and A β (1–42) (**b**) from chromaffin cells was stimulated by KCl depolarization and by nicotine, as conducted in figure 3. A β (1–40) and A β (1–42) were measured in the secretion media. **c, d** Regulated secretion of Met-enkephalin (**c**) and galanin peptide (**d**) neurotransmitters from chromaffin cells was stimulated by KCl depolarization and by nicotine, as conducted in figure 3, and the neuropeptides Met-enkephalin and galanin were measured in the secretion media.

Fig. 5. Inhibitor of QC reduced the release of pGlu-A β from the regulated secretory pathway. Chromaffin cells were either incubated with the QC inhibitor PQ529 for 18 h or not. Cells were then subjected to regulated secretion induced by KCl depolarization (for 90 min), whereas control cells remained unstimulated. The secretion media were collected and measured for concentrations of pGlu-A β (3–40). The data show that the inhibitor substantially reduced the amount of pGlu-A β (3–40) released from the regulated secretory pathway, representing activity-dependent secretion. # Statistically significant comparison of PQ529-treated with control cells (without PQ529) undergoing KCl depolarization-induced secretion of pGlu-A β (3–40) ($p < 0.05$, Student's t test). * Statistically significant comparison of KCl with control cells (without inhibitor treatment) with respect to secretion of pGlu-A β (3–40) ($p < 0.05$, Student's t test).

experiments. Met-enkephalin and galanin were secreted in an activity-dependent manner, stimulated by KCl depolarization or nicotine (fig. 4). These data illustrate the cosecretion of pGlu-A β (3–40) and QC with A β (1–40/42) and the neurotransmitters Met-enkephalin and galanin.

Inhibition of Endogenous QC Reduces pGlu-A β in the Regulated Secretory Pathway

The role of QC in the production of pGlu-A β was evaluated with the QC inhibitor PQ529 [51]. Chromaffin cells

were treated with PQ529 (50 μ M) for 18 h, and then subjected to regulated secretion stimulated by KCl depolarization. The results demonstrated that PQ529 significantly reduced the amount of pGlu-A β (3–40) released via the regulated secretory pathway (fig. 5). Basal, constitutive levels of secreted pGlu-A β (3–40) were not reduced by PQ529, although a modest, insignificant decrease was observed with PQ529. These findings support the role of QC in producing pGlu-A β in DCSV via the regulated secretory pathway that provides activity-dependent secretion.

Fig. 6. In secretory vesicles, cellular pGlu-A β and QC are colocalized with the enkephalin neurotransmitter, as analyzed by immunofluorescence deconvolution microscopy. **a** pGlu-A β (green; colors in on-line version only) and enkephalin localization (red). Merged image: areas of colocalization of pGlu-A β with enkephalin (yellow, arrows). **b** QC (green) and enkephalin localization (red). Merged image: areas of colocalization of QC with enkephalin (yellow, arrows). **c** QC (green) and 6E10 APP/A β localization (red). Merged image: areas of colocalization of QC with 6E10 APP/A β (yellow, arrows). Controls that were not treated with the primary antisera but only with the secondary fluorescence-labelled antisera resulted in an absence of immunofluorescence signals. The 6E10 antibody, generated to antigen A β (1–16), recognizes various A β - and APP-related forms [103, 104].

Cellular Localization of pGlu-A β and QC with Enkephalin in Secretory Vesicles

Neurotransmitter and neurosecretory components that undergo regulated secretion are stored in secretory vesicles. Therefore, the colocalization of pGlu-A β and QC with the Met-enkephalin neurotransmitter was demonstrated by immunofluorescence confocal microscopy (fig. 6). The data show the cellular colocalization of pGlu-A β with Met-enkephalin in chromaffin cells (fig. 6a). The punctate pattern of subcellular pGlu-A β localization coincides with that of Met-enkephalin which is present in secretory vesicles, shown by their merged yellow immunofluorescence (fig. 6a). Furthermore, QC is also colocalized with enkephalin (fig. 6b), indicating the presence of both QC and pGlu-A β in enkephalin neurotransmitter-containing secretory vesicles. These data are consistent with the presence of pGlu-A β and QC in secretory vesicles that contain neurotransmitters (table 1; fig. 2).

The colocalization of QC with A β - and APP-related forms was assessed by the 6E10 antibody, which detects A β - and APP-related immunoreactivity. The data show a punctate localization of QC and 6E10 immunoreactivity, showing colocalization (fig. 6c). These results support the hypothesis on the combined presence of pGlu-A β and QC with APP- and A β -related peptide forms in secretory vesicles.

Human Neuroblastoma Cells Display Activity-Dependent, Regulated Secretion of pGlu-A β with A β Peptides

An evaluation in a human neuroblastoma cell line (IMR32) was conducted to assess the regulated secretion of pGlu-A β in a human neuronal model. The secretion of pGlu-A β (3–40) was stimulated by KCl and compared with basal control cells (no KCl; fig. 7a). In addition, regulated cosecretion of A β (1–40) and A β (1–42) was stimu-

Fig. 7. Activity-dependent cosecretion of pGlu-Aβ with Aβ peptides in IMR32 neuroblastoma cells. **a** pGlu-Aβ(3-40) secretion stimulated by KCl depolarization. Human IMR32 neuroblastoma cells were subjected to secretion induced by KCl depolarization (90 min), and pGlu-Aβ(3-40) was measured in the secretion media by ELISA. **b** Aβ(1-40) secretion induced by KCl. Aβ(1-40) was measured in the secretion media, as described above. **c** Aβ(1-42) secretion induced by KCl. Aβ(1-42) was measured in the secretion media, as described above. **a-c** * Statistically significant for KCl

compared with control (unstimulated; $p < 0.05$, Student's *t* test). **d** Colocalization of pGlu-Aβ (red; colors in online version only) with QC (green). The subcellular localizations in the human IMR32 neuroblastoma cells were assessed by immunofluorescence microscopy. Merged image: areas of colocalization of pGlu-Aβ with QC (yellow, arrows). Controls that were not treated with the primary antisera but only with the secondary fluorescence-labelled antisera resulted in an absence of immunofluorescence signals for pGlu-Aβ and QC.

lated by KCl (fig. 7b, c). Immunofluorescence microscopy illustrated the colocalization of pGlu-Aβ with QC (fig. 7d). pGlu-Aβ and QC were each observed in discrete, punctate patterns of subcellular distribution. Merging of their images displayed their colocalization (shown by the yellow immunofluorescence). These data demonstrate the regulated cosecretion of pGlu-Aβ with Aβ peptides, and the colocalization of pGlu-Aβ with QC in human neuroblastoma.

Discussion

This study demonstrates an activity-dependent, regulated cosecretion of pGlu-Aβ and QC with Aβ and peptide neurotransmitters from secretory vesicles. The isolated secretory vesicles (DCSV type) used in our study contained pGlu-Aβ, QC, full-length Aβ peptides, as well as peptide and catecholamine neurotransmitters. The

data show that release of DCSV contents results in the cosecretion of pGlu-Aβ with QC, Aβ, and peptide neurotransmitters from the regulated secretory pathway of neuron-like chromaffin cells. Cellular immunofluorescence microscopy also illustrated the colocalization of pGlu-Aβ with QC and enkephalin, and of QC with APP- and Aβ-related immunoreactivity. Treatment of cells with a QC inhibitor resulted in reduced levels of pGlu-Aβ released from the regulated secretory pathway, indicating the role of QC in the production of pGlu-Aβ. Furthermore, human neuroblastoma cells displayed regulated secretion of pGlu-Aβ and Aβ, and pGlu-Aβ was colocalized with QC. These data demonstrate that pGlu-Aβ and QC undergo regulated cosecretion with Aβ from neurotransmitter secretory vesicles to provide extracellular pGlu-Aβ with Aβ that accumulates in AD brains.

The combined presence of pGlu-Aβ and QC with Aβ in neurotransmitter secretory vesicles raises the question of how Aβ and pGlu-Aβ are produced in this organelle.

Fig. 8. pGlu-Aβ and Aβ peptides with QC and the APP-processing machinery in secretory vesicles containing neurotransmitters. In this study, the secretory vesicles (DCSV type) isolated from model neuron-like chromaffin cells were demonstrated to contain pGlu-Aβ(3-40/42) and QC combined with Aβ(1-40/42). Prior studies have indicated the presence of β-, γ-, and α-secretases in DCSV [58, 59, 62]. DCSV contain cathepsin B, which has been identified as a new alternative β-secretase [59-61], and the well-known β-secretase BACE1 [58, 62], an aspartyl protease [54-57]. The components of the γ-secretase complex are also present in DCSV [58], i.e. presenilins 1 and 2, nicastrin, Aph-1, and Pen-2, which together function as γ-secretase [63, 64]. The α-secretase ADAM10 protease is also present in DCSV [58]. These findings illustrate the presence of the APP-processing machinery in the production of pGlu-Aβ and Aβ peptides in neurotransmitter secretory vesicles containing neuropeptides and catecholamines.

The reason for this question is that it may be hypothesized that APP and its processing secretases may be present in these secretory vesicles for the production of Aβ, and for the production of pGlu-Aβ by QC (fig. 8). Our previous studies have shown that these secretory vesicles contain APP [17, 52, 53] and Aβ peptides, indicating the presence of secretases that convert APP to Aβ. Indeed, β- and γ-secretases are present in the secretory vesicles with APP. The β-secretase BACE1 [54-57] is present in DCSV [19, 58]. A wild-type β-secretase was recently identified as cathepsin B [59-61] and is present in DCSV [58, 59]. The presenilin 1 γ-secretase component is present in secretory vesicles [58, 62], as well as the nicastrin, Aph-1, and Pen-2 components [58] of the γ-secretase complex

[63, 64]. Subsequent to the secretases, N-terminal truncation of full-length Aβ is predicted to occur in order to generate Aβ(3-40/42), which serves as a substrate for QC to produce pGlu-Aβ(3-40/42) [65-68]. In future studies, it will be of interest to determine the relative levels of the N-truncated Aβ peptides Aβ(2-40/42) and Aβ(3-40/42) compared with pGlu-Aβ(3-40/42), utilizing mass spectrometry to distinguish these peptide species. Thus, secretory vesicles contain the APP-processing proteases that produce Aβ peptides combined with QC for the production of pGlu-Aβ(3-40/42) (fig. 8). The range of pH conditions necessary for the activities of these secretases [69-74] coincides with the internal pH conditions of these secretory vesicles [75-77]. The secretory vesicle organelle, thus, contains the APP-processing machinery for producing Aβ and pGlu-Aβ peptide forms.

These data support the hypothesis that pGlu-Aβ is produced by QC in secretory vesicles that release neurotransmitter components in a regulated, activity-dependent manner. QC converts the N-terminal glutamate of Aβ(3-40/42) peptides to the pyroglutamate of pGlu-Aβ(3-40/42). The QC substrates Aβ(3-40/42) are presumably produced by N-terminal truncation of Aβ(1-40/42). The presence of full-length Aβ(1-40/42) in secretory vesicles [19, 58, 59] is compatible with biosynthesis of pGlu-Aβ from Aβ in these secretory vesicles.

The DCSV is a key organelle for the regulated, activity-dependent release of peptide and catecholamine neurotransmitters. Indeed, regulated secretion of pGlu-Aβ and Aβ occurs with the peptide neurotransmitters Met-enkephalin and galanin. Evidence for cosecretion of Aβ peptides with neurotransmitters supports involvement of activity-dependent deposition of Aβ peptides in AD brains, as demonstrated in vivo [21, 22]. It will be of interest in future studies to examine the variety of secretagogues known to induce neurotransmitter secretion [78-83] for effects on the stimulation of the regulated secretion of pGlu-Aβ peptides in order to gain further understanding of factors controlling pGlu-Aβ release. It is noted that the DCSV isolated from the peripheral sympathetic nervous system (from adrenal medulla) contain pGlu-Aβ, whereas this is not detectable in normal aged human brains [84]. Thus, the DCSV utilized in this study provide a model for understanding the neurobiology of pGlu-Aβ.

Further, it is noted that it is not yet known whether pGlu-Aβ is located in other subcellular organelles that contain full-length Aβ(1-40/42). Full-length Aβ is present in endosomes [85-89], lysosomes [90-92], autophagosomes [91, 93-95], exosomes [96, 97], and other

related subcellular organelles. It will be important in future studies to compare the organelle locations of pGlu-A β and A β peptides, which will provide an understanding of the neuronal trafficking of pGlu-A β compared with A β .

The neurobiology of the regulated, activity-dependent secretion of pGlu-A β and A β is significant with respect to the functional role of pGlu-A β in neurodegeneration and memory loss in AD. pGlu-A β comprises a major portion of the A β species in AD compared with A β (1–40) and A β (1–42) [5–7]. Quantification indicates the presence of pGlu-A β peptides at similar and greater levels than A β (1–40) and A β (1–42) in AD brains. Also, pGlu-A β is present as oligomeric complexes in AD brains [98]. In vitro studies have shown that pGlu-A β displays a higher aggregation propensity as well as a stronger tendency to seed the aggregation of other A β species [8, 9, 99]. Moreover, pGlu-A β is present in AD brains but not in normal aged brains [84].

In vivo studies have illustrated the neurotoxicity of pGlu-A β in memory deficits. Overexpression of pGlu-A β in mice correlates with A β and behavioral deficits [10, 12] as well as neuronal loss and impaired long-term potentiation [100]. These studies also showed that pGlu-A β formation is dependent on QC, since knockout of QC in

transgenic mice resulted in reduced levels of brain pGlu-A β . These findings support a role of pGlu-A β in amyloid plaque formation and memory loss in AD.

Reduction of pGlu-A β formation may provide a therapeutic strategy for AD, since inhibition of QC results in decreased brain pGlu-A β [13, 14] and QC gene knockout decreases brain pGlu-A β with improved behavior [12]. These findings indicate that QC is involved in the development of AD via the formation of pGlu-A β .

Based on the results of this study, inhibition of QC reduces the amount of pGlu-A β released from the regulated secretory pathway. In future studies, it will be of interest to assess the effects of QC inhibition on the regulated, activity-dependent deposition of amyloid in animal AD model brains. The production of pGlu-A β by QC in secretory vesicles that produce and secrete A β peptides and neurotransmitters in an activity-dependent manner suggests a close association of pGlu-A β and A β neurotoxicity with neurotransmitter functions.

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