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Beta-amyloid peptides undergo regulated co-secretion with neuropeptide and catecholamine neurotransmitters

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ABSTRACT

Beta-amyloid (Aβ) peptides are secreted from neurons, resulting in extracellular accumulation of Aβ and neurodegeneration of Alzheimer's disease. Because neuronal secretion is fundamental for the release of neurotransmitters, this study assessed the hypothesis that Aβ undergoes co-release with neurotransmitters. Model neuronal-like chromaffin cells were investigated, and results illustrate regulated, co-secretion of Aβ(1–40) and Aβ(1–42) with peptide neurotransmitters (galanin, enkephalin, and NPY) and catecholamine neurotransmitters (dopamine, norepinephrine, and epinephrine). Regulated secretion from chromaffin cells was stimulated by KCl depolarization and nicotine. Forskolin, stimulating cAMP, also induced co-secretion of Aβ peptides with peptide and catecholamine neurotransmitters. These data suggested the co-localization of Aβ with neurotransmitters in dense core secretory vesicles (DCSV) that store and secrete such chemical messengers. Indeed, Aβ was demonstrated to be present in DCSV with neuropeptide and catecholamine transmitters. Furthermore, the DCSV organelle contains APP and its processing proteases, β- and γ-secretases, that are necessary for production of Aβ. Thus, Aβ can be generated in neurotransmitter-containing DCSV. Human IMR32 neuroblastoma cells also displayed regulated secretion of Aβ(1–40) and Aβ(1–42) with the galanin neurotransmitter. These findings illustrate that Aβ peptides are present in neurotransmitter-containing DCSV, and undergo co-secretion with neuropeptide and catecholamine neurotransmitters that regulate brain functions.

1. Introduction

Beta-amyloid peptides (Aβ) are a major factor involved in the development of neurodegeneration of Alzheimer's disease (AD), resulting in severe loss of memory and cognitive functions [6,13,18,51,53]. Secretion of Aβ peptides from brain neurons provides accumulation of extracellular Aβ peptides to result in loss of neurons in hippocampal and cortical brain regions that are responsible for memory function.

Studies in the field have illustrated regulated secretion of Aβ that represents electrical activity-dependent neuronal secretion [9,19,20,22,26,37,38]. More recently, evidence has emerged indicating that brain neuronal activity is related to Aβ deposition [3]. However, the secretory organelle mechanisms for regulated Aβ secretion have not yet been examined in detail.

Brain function requires regulated secretion of neurotransmitters from secretory vesicles [52,55], the fundamental mechanism for synaptic neurotransmission. The property of regulated secretion of neurotransmitters from neurons, combined with evidence for regulated activity-dependent secretion of Aβ, suggests the hypothesis that Aβ and neurotransmitters undergo co-secretion. To test this hypothesis, the goal of this study was to assess regulated, co-secretion of Aβ with neuropeptide and catecholamine neurotransmitters from model neuronal-like chromaffin cells of the sympathetic nervous system. Chromaffin cells have been utilized extensively for studies of regulated secretion of neurotransmitters from dense core secretory vesicles (DCSV), and have advanced knowledge of enzymes that produce such neurotransmitters in brain [5,39].

Results of this study show that Aβ(1–40) and Aβ(1–42) peptides undergo regulated secretion with neuropeptide and catecholamine neurotransmitters upon stimulation of regulated secretion by KCl

Abbreviations: Aβ, β-amyloid; APP, amyloid precursor protein; BSA, bovine serum albumin; DCSV, dense core secretory vesicles; NPY, neuropeptide Y; PBS, phosphate-buffered saline.

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depolarization and nicotine. Regulated co-secretion of Aβ and neurotransmitters was also stimulated by forskolin that stimulates cAMP production and secretion [11,49,50]. The co-localization of Aβ with enkephalin and NPY neurotransmitters in secretory vesicles of chromaffin cells was demonstrated. Because these neurotransmitters are secreted from DCSV of neurons, the content of Aβ peptides with such neurotransmitters in isolated DCSV was quantitated. Notably, the DCSV contain β- and γ-secretases that convert APP, present in DCSV, into neurotoxic Aβ peptides. Thus, the DCSV organelle contains the APP processing components that produce Aβ peptides, which are co-secreted with neuropeptide and catecholamine neurotransmitters. In addition, human IMR32 neuroblastoma cells display regulated co-secretion of Aβ with the galanin neurotransmitter. These findings illustrate the co-secretion of Aβ peptides with neuropeptides and catecholamines, suggesting that Aβ functions with multiple neurotransmitter systems.

2. Materials and methods

2.1. Secretion of Aβ with peptide and catecholamine neurotransmitters from neuronal-like chromaffin cells in primary culture

Primary cultures of chromaffin cells were prepared from fresh bovine adrenal medulla (from Sierra Medical Sciences, Whittier, CA), as we have described previously [40,66]. Cells were plated in 6-well plates at 1.5×10^6 cells/well. Cells were subjected to regulated secretion achieved by stimulation by depolarization with high KCl (50 mM) in the medium for 15 min at 37 °C. Controls included incubation of cells without KCl for basal, constitutive secretion. The media was collected for measurements of Aβ(1–40) and Aβ(1–42) by ELISA assays (ELISA kits #27718 and #27711, respectively, IBL International, Toronto, Canada). The peptide neurotransmitters of galanin, NPY, and (Met)enkephalin were measured by radioimmunoassays (Phoenix Pharmaceuticals, Burlingame, CA). The catecholamines dopamine, norepinephrine, and epinephrine were also measured in the media, conducted as described [67]. In addition, nicotine stimulation of regulated secretion from chromaffin cells was also conducted by incubating cells with nicotine (10 μM for 15 min.), and without nicotine as control; the secretion media was subjected to measurements of Aβ(1–40) and Aβ(1–42), peptides, and catecholamines as described for media from KCl secretion experiments. Furthermore, regulated secretion was induced by treating cells with forskolin (50 μM, 2 hrs incubation) which elevates cAMP [49] that induces regulated secretion [11,50]. Each secretion experiment (with control, KCl, nicotine, or forskolin conditions) was conducted in triplicate, and each experiment was repeated at least three times. Statistical analyses of Aβ peptides, neuropeptides, and catecholamine levels in secretion media from stimulated and unstimulated control cells were conducted by the Student's *t*-test, with significance level of $p < 0.05$. Results are expressed as the mean ± s.e.m. (standard error of the mean).

2.2. Immunofluorescence deconvolution microscopy of Aβ-related forms and peptide neurotransmitters

Immunofluorescence histochemistry of Aβ and peptide neurotransmitters was assessed by deconvolution microscopy, as we have described [12]. Briefly, chromaffin cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, and incubated with anti-APP 6E10 (1:100, mouse, Covance, San Diego, CA), anti-galanin (1:200, rabbit, Bachem, Torrance, CA), anti-NPY (1:300, rabbit, Chemicon/Bioscience Research Reagents/Millipore, Temecula, CA), or anti-(Met)enkephalin (1:50, mouse, Abcam, Cambridge, MA) in PBS containing 3% bovine serum albumin (PBS-BSA 3%) for 2 h at room temperature. After washing with PBS, cells were then incubated with secondary goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (green and red fluorescence, respectively, 1:300, Molecular Probes/Life Technologies, Grand Island, NY), respectively, in PBS-3% BSA. Immunofluorescent co-localization of Aβ-related forms (anti-APP 6E10 detects Aβ and its APP precursor forms) with peptide neurotransmitters was examined with the Delta Vision Spectris Image Deconvolution System on an Olympus IX70 microscope using Softworx Explorer software from Applied Precision. As control, incubation with only secondary antibodies (no primary antibodies) was performed, resulting in a lack of immunofluorescence thus indicating specific immunofluorescence signals resulting from the primary antisera.

2.3. Purification of dense core secretory vesicles (DCSV) and analyses of Aβ and neurotransmitters

Secretory vesicles from fresh bovine adrenal medulla tissue, specifically the dense core secretory vesicles (DCSV), was purified by sucrose density gradient centrifugation as we have described previously [63]. The high purity of the isolated secretory vesicles has been established by enzyme markers of subcellular organelles [1,54,62,63,65]. The homogeneity and integrity of the purified DCSV was confirmed by electron microscopy, conducted as we have described previously [66].

The purified DCSV were lysed by freeze–thawing in buffer (50 mM Na-acetate, pH 6.0, 150 mM NaCl, 1 mM EDTA) containing a cocktail of protease inhibitors consisting of 10 μM pepstatin A, leupeptin, and chymostatin, and E64c, and 500 μM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride). An acid extract was prepared from the lysed DCSV by bringing the sample to 1.0 N acetic acid, heating at 95 °C for 10 min, centrifuging at 13,000 × *g* for 10 min, and collecting the supernatant for measurement of Aβ(1–40), Aβ(1–42), galanin, NPY, (Met)enkephalin, and the catecholamines dopamine, norepinephrine, and epinephrine. Assays for these components by ELISAs and RIAs are described above for secretion media in this methods section. VIP and somatostatin neuropeptides were also measured in DCSV by radioimmunoassays (from Phoenix Pharmaceuticals, Burlingame, CA and Bachem, Torrance, CA). Protein content of the purified DCSV was measured by the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Aβ and neurotransmitter contents in DCSV were expressed as pg per μg protein.

2.4. Western blot analyses of DCSV for β- and γ-secretase components with APP and Aβ

Purified DCSV were subjected to western blots analyses to assess the presence of β- and γ-secretase components utilized for processing APP, as well as APP and Aβ in DCSV [45,48]. Western blots were conducted as we have described previously [57,66]. Antibodies to peptide domains of Aβ and APP were used in western blots to assess the presence of Aβ, APP, as well as APP-derived fragments in DCSV. Custom-generated antisera were produced to peptide antigens consisting of Aβ(17–28), and the N-terminus of APP of residues 1–18 (APP1-18) (these antisera were produced by Immuno-Dynamics, La Jolla, CA). The R7 and R1 antisera to APP were gifts from the laboratory of Professor Nikolaos Robakis (Mt. Sinai Univ., New York [57]). The 22C11 antibody detecting an N-domain region of APP (APP66-81) was obtained from Millipore Chemicals (Billerica, MA).

Antisera utilized for western blots were directed to the BACE1 β-secretase (Sigma, St. Louis, MO) and the wild-type β-secretase recently identified as cathepsin B [13,17,25] (Athens Research &

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