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GE-25-like immunoreactivity in the rat eye

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ABSTRACT

This study aimed to investigate the presence and distribution of the chromogranin A-derived peptide GE-25 in the rat eye. The molecular form detected by the GE-25 antiserum was evaluated in the rat trigeminal ganglion, retina and remaining tissues of the rat eye by means of Western blots and the distribution pattern of GE-25-like immunoreactivity was studied in the rat eye and rat trigeminal ganglion by immunofluorescence. One single band of approximately 70 kDa was stained in the trigeminal ganglion and retina which represents the uncleaved intact chromogranin A indicating that the proteolytic processing of chromogranin A to GE-25 is limited in these tissues. Sparse GE-25-like immunoreactive nerve fibers were visualized in the corneal stroma, at the limbus around blood vessels, in the sphincter and dilator muscle and stroma of the iris, in the stroma of the ciliary body and ciliary processes and in the stroma and around blood vessels in the choroid. This distribution pattern is characteristic for neuropeptides whereas the presence of immunoreactivity in the corneal endothelium and in Müller glia in the retina is atypical. GE-25-like immunoreactivity was found in small to medium-sized ganglion cells in the rat trigeminal ganglion clearly indicating that the nerve fibers in the rat eye are of sensory origin. The colocalization of GE-25-immunoreactivity with SP-immunoreactivity in the rat ciliary body is in agreement with the presumption of the sensory nature of the innervation of the anterior segment of the eye by GE-25.

1. Introduction

Chromogranin (Cg) A is a member of the chromogranin family which comprise CgA, CgB (review, see [35]), secretogranin (Sg) II (review, see [8]) and the neuroendocrine secretory protein NESP55 [12]. The chromogranins are water-soluble glycoproteins which are widely distributed in the secretory granules of neuroendocrine tissues and are stored in large dense core vesicles in the nervous system. Several single and paired basic residues are present within the primary amino acid sequence of each chromogranin which represent cleavage sites for prohormone convertases (PCs). The processing of CgA is accomplished primarily by PC2 [2] whereas CgB and SgII are processed both by PC1 and PC2 [4,10,17]. Proteolytic processing of CgA leads to the generation of functionally active neuropeptides such as pancreastatin which inhibits glucose-stimulated insulin release from perfused rat pancreas or isolated acini [7], or vasostatin which exerts effects on vascular smooth muscle [1], or catestatin which is an effective inhibitor of catecholamine secretion from chromaffin cells [23], or WE-14 which stimulates histamine release from mast cells [9]. Two further CgA-derived peptides are EL35 [25] and GE-25 [14] but for both of these peptides no biological effects have been described so far. Proteolytic processing of CgB leads to the generation of secretolytin which was found to display antibacterial properties [29] or PE-11 which seems to be inert [16] whereas secretoneurin (SN) represents a SgII-derived neuropeptide with potent angiogenic and chemotactic effects (review, see [34]).

GE-25 is generated from CgA throughout the whole neuroendocrine system. The highest concentrations were found in the adrenal medulla (AM), but significant amounts are also present in the three lobes of the pituitary gland, the endocrine pancreas and the intestinal mucosa [14]. The proteolytic processing of CgA to GE-25 varies from tissue to tissue. In particular, in the AM most of the GE-25-immunoreactivity (IR) was found to be present as intact CgA whereas in anterior pituitary and intestine a shift to smaller peptides was seen [14]. By contrast, in the posterior and intermediate pituitary and pancreas the predominant immunoreactive material was represented by the free peptide GE-25 [14]. Furthermore, CgA is processed to GE-25 during axonal transport in the pig splenic

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nerve [22], endoproteolytic processing of CgA decreases in brains of mice lacking 7B2 when compared with controls [18] and in contrast to mock-transfected control SH-SY5Y human neuroblastoma cells, PC2-transfected cells show extensive processing of CgA resulting in high levels of the free peptide GE-25 [5]. In human and bovine cerebrospinal fluid, the concentrations of chromogranins are much higher than those of classical neuropeptides and CgA-IR is present as intermediate-sized peptides with little intact CgA and free GE-25 peptide [13]. Furthermore, GE-25 is elevated and especially the GE-25/SN ratio is significantly higher in lumbar cerebrospinal fluid of schizophrenic patients when compared with controls [24]. And finally, there are significant amounts of CgA in human neuroblastomas and ganglioneuromas and this protein is processed in these tumors to a great extent to GE-25 [6].

With respect to the eye, the presence and distribution of the CgA-derived neuropeptide WE-14 has been extensively explored in porcine ocular tissues previously [3]. Thus, sparse nerve fibers have been visualized throughout the eye, in particular in the cornea, limbus, iris, ciliary body, choroid and sclera and in the retina, cells in the inner nuclear and ganglion cell layer were labeled as well as laminae in the inner plexiform layer [3]. The aim of the present study was to investigate the presence and distribution of the CgA-derived peptide GE-25 in the rat eye using a specific antibody raised against rat GE-25.

2. Materials and methods

2.1. Antiserum production

Rat GE-25 (GWRPSSREDVEARGDFEE) was synthesized by standard t-BOC chemistry and purified by reversed phase HPLC. The peptide was coupled to maleimide activated keyhole hemocyanin via an additional N-terminal Cystein residue. The conjugate was used to generate polyclonal peptide antisera in Chinchilla Bastard rabbits following a standard immunization protocol. The antiserum recognizes both full-length CgA and the small peptide GE-25 [18].

2.2. Western blots

For the immunoblots, the AMs, the trigeminal ganglia (TGs), retinae and remaining tissues of the eye of two rats were dissected. Western blot was performed as described previously [27,33]. Briefly, the tissues were dissolved in 200 μl ice-cold PBS with a protease inhibitor cocktail (P-8340, Sigma), homogenized using an ultrasonic device (Branson sonifier 250, Darnburry) and centrifuged at 16,000 × *g* at 4 °C for 10 min. Protein was determined by Bradford analysis. The supernatants (20–100 μg) were loaded onto a 10% Bis–Tris polyacrylamide gel (Invitrogen) and electrophoresed for 25 min at 200 V. Samples were electrotransferred to nylon PVDF Immobilon-PSQ membranes (Millipore) for 90 min at 30 V with 40% methanol blotting buffer (Invitrogen). For detection, the Western Breeze Chemiluminescent System (Invitrogen) was used. Blots were blocked for 30 min with blocking buffer, then incubated over night at 4 °C with the primary antibody rabbit anti-GE-25 (1:500) or rabbit anti-actin antibody (1:1000, Sigma–Aldrich). Blots were washed and incubated with alkaline phosphatase-conjugated anti-rabbit antibodies for 30 min at room temperature. After being washed, blots were incubated (free-floating) in CDP-Star chemiluminiscent substrate solution (Invitrogen) for 10 min and the signal was visualized (exposure time 1200 s) with cooled CCD camera (SearchLight, Thermoscience).

2.3. Immunofluorescence studies

Nerve fibers in the rat eye were visualized by immunofluorescence. The eyes were enucleated and under a dissecting microscope, an incision was made in the cornea. Then, the eyeball was immersed in 4% paraformaldehyde for 1 h at room temperature followed by preparation of particular tissues inclusive removal of the lens and vitreous. Subsequently, the eyes were washed with PBS for a few times and then cryoprotection was performed. In particular, the eyecup was incubated in 10% succrose solution (1.5 ml/well) at room temperature for approximately 30 min until the eye was saturated and fell down to the bottom of the well. Then, the 10% succrose solution was replaced by 20% succrose solution and incubated at room temperature for approximately 2 h. Finally, the 20% succrose solution was replaced by 30% succrose solution and the eyecup was incubated over night at 4 °C. Then, the eyes were embedded with O.C.T. compound freezing medium (Tissue Tek), frozen in liquid nitrogen and stored at −70 °C. Five to 15 μm thick sections were cut from the specimens on a cryostat (Reichert Jung; Leica-Reichert, Vienna, Austria) and transfered onto Superfrost Plus slides (Thermo Scientific, Braunschweig, Germany).

The slides were dried for 30 min in an incubator and rinsed in PBS. For antigen retrival, the slides were cooked in Tris–EDTA buffer (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) at 90 °C for 10 min. Cooling down was performed at room temperature for 20 min. The sections were then preincubated for 30 min with 0.25% normal goat serum/0.5% bovine serum albumin in PBS with 0.1% Triton X-100, afterwards rinsed in PBS and subsequently incubated over night in disposable immunostaining chambers at room temperature with anti-rat GE-25 at a dilution of 1:500 in PBS containing 0.1% Triton X-100. After a few washes with washing buffer (Dako, Hamburg, Germany) and PBS, sections were incubated with the secondary antibody (Cy3-conjugated goat anti-rabbit IgG; Linaris, Dossenheim, Germany) diluted 1:500 with PBS for 3 h in immunostaining chambers at room temperature. Stained sections were rinsed with washing buffer and washed three times in PBS, mounted with Vectashield (Vector, Burlingame, CA, USA) containing diamidin-2-phenylindol (DAPI) and coverslipped. Sections were visualized with a Nikon TE 2000 microscope (Nikon, Düsseldorf, Germany) equipped with a high sensitive cooled black and white CCD camera and Nikon's Lucia G/F software. For double immunofluorescence sections were incubated in some experiments with rabbit anti rat GE-25- and mouse antibodies against substance P (SP) or glia fibrillary acidic protein (GFAP). SP or GFAP was immunostained by adding mouse anti-SP antibody (1:250, Abcam, Cambridge, UK) or mouse anti-GFAP antibody (1:400, Linaris, Dossenheim, Germany) to the primary GE-25 incubations solution. Mouse antibodies were detected via fluorescein-isothiocyanate labeled goat anti-mouse IgG (1:400, Abnova, Heidelberg, Germany) diluted in the secondary antibody solution of Cy3-conjugated goat anti-rabbit IgG. Specificity of the staining was evaluated by a further procedure, in particular by incubating sections without the primary GE-25 antiserum, SP or GFAP.

To detect GE-25-IR in the rat trigeminal ganglion (TG) two male rats (200 g) were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg) and perfused transcardially with saline followed by 4% paraformaldehyde. The TGs were removed and paraffinized to a paraffine tissue block. 10 μm sections were cut on a microtome and mounted on Superfrost Plus slides. The slides were dried in an incubator at 60 °C for 30 min and then deparaffinized/rehydrated. For this purpose, sections were incubated in three washes of xylene for 5 min each, incubated in two washes of 100% ethanol for 10 min each, incubated in two washes of 95% ethanol for 10 min each, incubated in one wash of 70% ethanol for 5 min and rinsed twice in dH_2O for 5 min each. The next steps inclusive antigen unmasking, blocking, incubating with the primary and secondary antiserum and visualizing were the same as described above.