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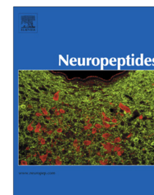

Catestatin-like immunoreactivity in the rat eye

Oliver W. Gramlich [a], Katrin Lorenz [a], Franz H. Grus [a], Maren Kriechbaum [a], Daniela Ehrlich [b], Christian Humpel [b], Reiner Fischer-Colbrie [c], Nikolaos E. Bechrakis [d], Josef Troger [d,*]

[a] *Experimental Ophthalmology, Department of Ophthalmology, University Medical Center, Johannes Gutenberg-University Mainz, Langenbeckstraße 1, 55131 Mainz, Germany*
[b] *Laboratory of Psychiatry and Experimental Alzheimers Research, Department of Psychiatry and Psychotherapy, Anichstraße 35, 6020 Innsbruck, Austria*
[c] *Department of Pharmacology, Medical University of Innsbruck, Peter Mayrstraße 1a, 6020 Innsbruck, Austria*
[d] *Department of Ophthalmology, Medical University of Innsbruck, Austria*

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ABSTRACT

The aim of the study was to investigate the presence and distribution of the chromogranin A-derived peptide catestatin in the rat eye and trigeminal ganglion by immunofluorescence using an antibody which recognizes not only free catestatin but also larger fragments containing the sequence of catestatin. Western blots were performed in an attempt to characterize the immunoreactivities detected by the catestatin antiserum. Sparse immunoreactive nerve fibers were visualized in the corneal stroma, in the chamber angle, in the sphincter muscle but also in association with the dilator muscle, in the stroma of the ciliary body and processes, but dense in the irideal stroma, around blood vessels at the limbus and in the choroid and in cells of the innermost retina representing amacrine cells as identified by colocalization with substance P. Furthermore, catestatin-immunoreactivity was detected in the trigeminal ganglion in small to medium-sized cells and there were abundant catestatin-positive nerve fibers stained throughout the stroma of the ganglion. Double immunofluorescence of catestatin with substance P revealed colocalization both in cells of the trigeminal ganglion as well as in nerve fibers in the choroid. The immunoreactivities are present obviously as free catestatin and/or small-sized catestatin-containing fragments in the retina and ocular nerves but as large processed fragments as well, weak in the retina and more prominent in remaining ocular tissues, possibly in endothelial cells. This indicates that this peptide is a constituent of sensory neurons innervating the rat eye and the presence in amacrine cells in the retina is typical for neuropeptides. Catestatin is biologically highly active and might be of significance in the pathophysiology of the eye.

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1. Introduction

Catestatin (CST) is a neuropeptide which is generated *in vivo* by proteolytic processing of chromogranin (Cg) A (Mahata et al., 1997). CgA is a member of the granin family, the acidic proteins of secretory granules of endocrine and neuroendocrine cells which comprise CgA, CgB (review, see Winkler and Fischer-Colbrie, 1992) and secretogranin (Sg) II (review, see Fischer-Colbrie et al., 1995) as main constituents. The granins are widely distributed throughout the neuroendocrine system, are stored in large dense core vesicles in the nervous system and are coreleased with neurotransmitters from these vesicles by Ca^{2+}-dependent exocytosis. The primary amino acid sequence of the granins features numerous pairs of basic amino acids which represent cleavage sites for protein convertases. At these sites the chromogranins are proteolytically processed to smaller peptides at a varying extent. In particular, proteolytic processing of SgII leads to the generation of secretoneurin (SN), processing of CgB to secretolytin and PE-11, and processing of CgA to pancreastatin, vasostatin, serpinin, WE-14, GE-25 and CST (reviews, see Helle, 2010; Loh et al., 2012).

CST is a biologically highly active peptide (previous reviews, see Loh et al., 2012; Mahata et al., 2010). It was originally found to be a non-competitive nicotinic cholinergic antagonist which inhibits catecholamine release from noradrenergic neurons, PC12 and bovine chromaffin cells by an autocrine negative feedback loop (Mahata et al., 1997, 2000) and nicotine-induced desensitization of catecholamine release (Mahata et al., 1999). Furthermore, CST causes release of histamine from peritoneal and pleural mast cells (Kruger et al., 2003), vasodilation in rats (Kennedy et al., 1998) and humans (Fung et al., 2010), induces chemotaxis of human monocytes (Egger et al., 2008) and acts as an antibacterial peptide (Briolat et al., 2005; Radek et al., 2008). CST also exerts effects on the cardiovascular system. In particular, it abolishes isoproterenol-in-

* Corresponding author. Address: Department of Ophthalmology, Anichstraße 35, 6020 Innsbruck, Austria. Tel.: +43 512 504 27343; fax: +43 512 504 23722.
E-mail address: josef.troger@i-med.ac.at (J. Troger).

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duced positive inotropic and lusitropic effects in the rat heart (Angelone et al., 2008) and regulates blood pressure by acting as an inhibitor of peripheral (Fung et al., 2010; Mahapatra et al., 2005) as well as central (Gaede et al., 2009; Gaede and Pilowsky, 2010) nicotinic-cholinergic receptors and β-adrenoceptors (Angelone et al., 2008; Gaede et al., 2009). The significance of CST in the regulation of blood pressure should be emphasized by the facts that lower plasma levels of CST are a risk factor for the development of hypertension in humans (O'Connor et al., 2002), that a naturally occurring human variant of CST alters autonomic function and blood pressure (Rao et al., 2007) and that arterial hypertension of chga knockout mouse is rescued by exogenous injection of CST (Mahapatra et al., 2005). Furthermore, CST acts as an insulin-sensitizing peptide, inhibits gluconeogenesis and lipogenesis and stimulates fatty acid oxidation (Loh et al., 2012). And finally, CST is a potent angiogenic cytokine which acts via a basic fibroblast growth factor-dependent mechanism (Theurl et al., 2010).

Although several biological effects have been described so far, the presence and distribution of CST in neuroendocrine tissues have not yet been explored sufficiently enough. Mass spectrometry of bovine adrenal chromaffin granules revealed the presence of one major catestatin form, in particular of bovine $CgA_{332-364}$, and two additional forms, namely of bovine $CgA_{333-364}$ and $CgA_{343-362}$ (Taylor et al., 2000). In human pheochromocytoma chromaffin granules, $CgA_{340-372}$ predominated (Taylor et al., 2000). By means of histochemistry, this CST was found in high amounts in carcinoid tumors of the appendix (Prommegger et al., 2004) and in all ECLomas in gastric human mucosa (Tartaglia et al., 2006). Furthermore, CST-IR was found to be abundant in the rostral ventrolateral medulla, in particular frequently in TH-immunoreactive neurons (Gaede and Pilowsky, 2010) and the distribution has been examined in the human auditory system where it is present in the inner ear in spiral ganglion cells and also in related nuclei in the brainstem (Bitsche et al., 2003).

With respect to the eye, several CgA-derived peptides might be present there, in particular pancreastatin, vasostatin, serpinin, WE-14, GE-25 and CST. But so far only the distribution of WE-14 (Curry et al., 2003) and GE-25 (Lorenz et al., 2012) has been investigated in detail in porcine ocular tissues and the rat eye, respectively. Apart from stimulating histamine release from rat mast cells by WE-14 (Forsythe et al., 1997), no biological effects have been described for these peptides and thus, the relevance of WE-14 and GE-25 is of limited interest in the pathophysiology of the eye. In contrast, the other CgA-derived peptides are biologically much more active which makes them more important. The present study aimed (i) to investigate the presence and distribution of another CgA-derived peptide in the rat eye, namely of CST, (ii) to demonstrate the expression of CST in cells of the trigeminal ganglion (TG) as potential source of the nerve fibers, and (iii) to evaluate the molecular forms detected by the CST antiserum in the rat eye and TG.

2. Methods

2.1. Western blot

Western blots were performed as described by us previously (Lorenz et al., 2012). Briefly, the tissue was dissolved in 200 μl ice-cold PBS with a protease inhibitor cocktail (P8340, Sigma), homogenized by using an ultrasonic device (Branson sonifier 250, Danburry) and centrifuged at 16,000*g* at 4 °C for 10 min. Protein was determined by the Bradford assay. The denaturated (70 °C 10 min) supernatants (25 μg) were loaded onto a 10% Bis-Tris polyacrylamide gel (Invitrogen) and electrophoresed for 25 min at 200 V. Catestatin standards (10–1000 ng/lane) were run as a control. Samples were electrotransferred to nylon PVDF (Immobilon-PSQ membranes (Millipore) for 90 min at 30 V with 40% methanol blotting buffer (Invitrogen) was used. Blots were blocked for 30 min with blocking buffer, then incubated overnight at 4 °C with the primary antibody rabbit catestatin (1:3000) or for 90 min with a 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 washing, blots were incubated (free-floating) in CDP-Star chemiluminescent substrate solution (Invitrogen) for 10 min and the signal was visualized (exposure time 1200 s) with a cooled CCD camera (SearchLight, Thermoscience).

2.2. Immunofluorescence studies

To detect CST-IR in the rat eye and the trigeminal ganglion (TG) Lewis rats ($n = 6$, 200 g) were sacrificed by CO_2 anesthesia followed by cardiac puncture and decapitation for eye examinations or were deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg) and perfused transcardially with saline followed by 4% paraformaldehyde for investigations of the TG. The whole eyes were immersions-fixed in 4% phosphate-buffered paraformaldehyde for 2 h, paraffinized along with the TGs in Sakura's Tissue Tec using a standard protocol and finally casted as paraffin tissue block. 5, 7 and 10 μm sections were cut on a microtome (Leica-Reichert, Vienna, Austria) and mounted on Superfrost Plus slides. The slides were dried at 37 °C over night and dewaxed in an incubator at 60 °C for 2 h 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.

For antigen retrieval, the slides were cooked in DAKO's Target Retrieval Solution (TRS, Dako, Hamburg, Germany) at 80 °C for 15 min. Cooling down was performed at room temperature for 15 min and shortly rinsed in PBS. The sections were then preincubated for 30 min with 0.25% normal goat serum/0.5% bovine serum albumin in PBS with 0.1% Tween-20 (blocking buffer), afterwards rinsed in PBS and subsequently incubated over night in disposable immunostaining chambers at room temperature with rabbit anti-rat CST (Phoenix Pharmaceuticals, Berlingame CA 94010, USA) at a dilution of 1:300 in the blocking buffer. This antibody was used both in western blots and immunofluorescence and recognizes free CST but small and larger fragments containing the sequence of CST as well including intact CgA.

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 and dH_2O, 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 CST- and mouse antibodies against substance P (SP). SP was immunostained by adding mouse anti-SP antibody (1:250, Abcam, Cambridge, UK) to the primary CST incubation 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 further procedures, in particular by

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