Neuropeptides xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

Nonsulfated cholecystokinins in cerebral neurons

Mikkel Agersnap [a], Ming-Dong Zhang [b,c], Tibor Harkany [b,d], Tomas Hökfelt [b], Jens F. Rehfeld [a,*]

[a] Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark
[b] Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden
[c] Division of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden
[d] Department of Molecular Neurosciences, Center for Brain Research, Medical University of Vienna, Vienna, Austria

ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form 5 August 2016
Accepted 8 August 2016
Available online xxxx

Keywords:
Cholecystokinin (CCK)
Neuropeptides
O-sulfation
Posttranslational processing
proCCK
Peptidergic neurotransmission

ABSTRACT

Cholecystokinin (CCK) is a widely expressed neuropeptide system originally discovered in the gut. Both cerebral and peripheral neurons as well as endocrine I-cells in the small intestine process proCCK to tyrosyl-O-sulfated and α-carboxyamidated peptides. Recently, we reported that gut endocrine I-cells also synthetize nonsulfated CCK in significant amounts. Accordingly, we have now examined whether porcine and rat cerebral tissues (four cortical regions, hypothalamus and cerebellum) also synthesize nonsulfated CCK. A new, specific radioimmunoassay showed that all brain samples from pigs (n = 15) and rats (n = 6) contained nonsulfated CCK. The highest concentrations were measured in the neocortex; 4.7 ± 0.25 pmol/g (7.4%) in the rat and 4.3 ± 1.88 pmol/g (2.3%) in the pig. Chromatography of porcine cortical extracts revealed that 96.4% of the CCK was O-sulfated CCK-8. A higher fraction of the larger peptides (CCK-58 and CCK-33) was nonsulfated in comparison with the shorter forms (CCK-22 and CCK-8), i.e., 8.1% and 4.3% versus 0.9% and 1.5%. Immunohistochemical analysis of the rat brain showed an overall similar distribution pattern in selected regions when comparing the antibody specific for nonsulfated CCK-8 with an antibody recognizing both sulfated and nonsulfated CCK. However, nonsulfated CCK immunoreactivity was stronger than that of sulfated CCK in cell bodies and weaker in nerve terminals. We conclude that only a small fraction of neuronal CCK is nonsulfated. The intracellular distribution of nonsulfated CCK in neurons suggests that they contribute only modestly to the CCK transmitter activity.

1. Introduction

Cholecystokinin (CCK) is a classic gut hormone, but also a widespread peptide transmitter in the central and peripheral nervous systems. In the digestive tract hormonal CCK has important effects on gallbladder-contraction, satiety signaling, pancreatic growth and enzyme secretion as well as on gastric emptying (for review, see Rehfeld et al., 2007). In the brain CCK peptides modulate pain responses and affect memory and emotions. Especially the anxiogenic effect of CCK has been in focus (Rehfeld, 1992; Biró et al., 1997; Noble and Roques, 2002).

Originally CCK was identified by its ability to stimulate gallbladder-contraction in a bioassay (Mutt and Jorpes, 1968), hence the name. Later this activity was shown to be due to binding to the CCK-A (or CCK_1) receptor expressed on gallbladder myocytes. O-sulfation of Tyr_{77} in proCCK (Wank et al., 1992) is essential for CCK-A receptor activation and consequently, it has been assumed that bioactive CCK peptides per definition would have to be O-sulfated.

The gastrin receptor, now termed CCK-B (or CCK_2) receptor (Noble and Roques, 1999; Dufresne et al., 2006), binds sulfated as well as nonsulfated CCK peptides, in addition to gastrin (Saito et al., 1980; Kopin et al., 1992). In other words, sulfation does not influence the binding to the CCK-B receptor. The CCK-B receptor is the predominant cerebral CCK receptor (the "brain receptor"). Moreover, reports on occasional in vivo occurrence of nonsulfated CCK-8 peptides in the brain and the thyroid gland as well as presence of nonsulfated CCK-58 in the gut have subsequently surfaced (Frey, 1983; Rehfeld and Hansen, 1986; Laurberg and Rehfeld, 1987; Rehfeld et al., 1990; Bonetto et al., 1999; Reeve et al., 2004).

In order to accurately quantitate the expression of nonsulfated CCK, we have developed an assay that requires preanalytic tryptic cleavage of the tissue extract followed by radioimmunoassay (RIA) measurement using an antiserum (no. 94,179) that is entirely specific for unsulfated CCK peptides (Agersnap and Rehfeld, 2014). The assay has shown that the endocrine I-cells in the porcine and rat small intestinal mucosa also synthetize nonsulfated CCK peptides with amino acid sequences corresponding to those of the known sulfated peptides (CCK-58, CCK-33, CCK-22 and CCK-8). The nonsulfated peptides constituted up to 20% of the α-amidated CCK-peptides in the gut (Agersnap and Rehfeld, 2015). Since the brain expresses at least as much CCK as the

* Corresponding author.
E-mail address: jens.f.rehfeld@regionh.dk (J.F. Rehfeld).

http://dx.doi.org/10.1016/j.npep.2016.08.003
0143-4179/

gut (Rehfeld, 1978; Crawley, 1985), we have now examined the occurrence and molecular pattern of nonsulfated CCK peptides in extracts of porcine and rat brain biopsies. Moreover, using immunohistochemistry (IHC) we have compared the neuronal localization of CCK immunoreactivity in the rat brain using antiserum no. 94,179 specific for nonsulfated CCK and antiserum no. 18 recognizing both sulfated and nonsulfated CCK (Frey, 1983).

2. Materials and methods

2.1. Tissues

Samples (0.15–2 g) of the cerebral cortex, the hypothalamus and the cerebellum were obtained from six healthy 4-month old pigs (The Panum Institute, University of Copenhagen, Denmark). The pigs had regularly access to food and water, but were fasted overnight prior to surgery. The animals were anesthetized and used for minor experiments before termination. Immediately after termination, the brain was removed and the dissected samples immersed in liquid nitrogen until storage at −80 °C. Samples from nine additional porcine frontal cortices were collected for further characterization of CCK by chromatography.

Samples of the cerebral cortex, the hypothalamus and the cerebellum (0.05–0.1 g) were acquired from six healthy 6-month-old wistar rats (Rigshospitalet, The Panum Institute, University of Copenhagen, Denmark) using the same methods for collection. The rats were kept with free access to water and food until they were anesthetized and sacrificed. For the extraction, neuronal tissue was preferably gathered from the cortical mantle, excluding the underlying corpus callosum, in order to maximize the concentration of CCK peptides (Rehfeld, 1978; Marley et al., 1984; Rehfeld and Hansen, 1986).

2.2. Extraction

Samples of 0.05 to 0.5 g were collected into disposable tissueTUBEs (Covaris, Woburn, MA), and stored on dry ice. The tissueTUBEs were subsequently immersed in liquid nitrogen and pulverized by a cryoPREP impactor (Covaris, Woburn, MA). This step was repeated in order to maximize the pulverization. The pulverized tissues were transferred to glass tubes, boiled in 10 mL distilled water/1 g tissue (or at least 1 mL) for 20 min, and then homogenized. The tubes were subsequently centrifuged at 10,000 rpm at 4 °C for 30 min and the supernatant collected as “neutral extract”. The pellet was resuspended in 10 mL 0.5 M acetic acid/g tissue (or at least 1 mL), homogenized, incubated for 20 min and subsequently centrifuged at 10,000 rpm at 4 °C for 30 min. The supernatant was assigned the “acid extract”.

2.3. Radioimmunoassays

Two sequence-specific radioimmunoassays (RIAs) were used to measure sulfated and nonsulfated CCK separately in extracts and chromatographic fractions (Rehfeld, 1998; Agersnap and Rehfeld, 2014). Antiserum no. 92,128 binds only CCK peptides that are both O-sulfated and α-carboxyamidated (Fig. 1) and was used to measure all sulfated CCKs (CCK-8, -22, -33, and -58) (Rehfeld, 1998). Antiserum no. 94,179 is specifically directed against the N-terminal sequence of nonsulfated CCK-8 (Fig. 1). Measurement of the longer, nonsulfated N-terminally extended CCKs (CCK-22, -33 and -58), therefore required tryptic cleavage of the binding between Arg_9 and Asp_8 (as numbered from the C-terminus) and subsequently measurement by the RIA (Agersnap and Rehfeld, 2014).

2.4. Chromatography

The neutral and acid extracts of nine porcine frontal cortices were applied to calibrated Sephadex G50 superfine columns to examine the molecular pattern of the sulfated versus nonsulfated CCK peptides. Elution was performed with 0.02 mol/L barbital buffer, pH 8.4, containing 1 g/L bovine albumin (Ortho) and 0.1 M NaCl at a flow rate of 4 mL/h. The fractions were collected in volumes of 1.3 mL and subsequently subjected to quantitation by the immunoassays.

2.5. Calculation of the tissue concentrations of α-amidated CCK peptides

The total amount of sulfated and nonsulfated CCK was calculated by summing the concentrations in the neutral and acid extracts up. For the determination of each molecular form of CCK the chromatographic elutions were analysed. In the chromatographic elutions of both neutral and acid tissue extracts, the Area Under the Curve (AUC) of each molecular form was calculated in accordance with the known elution positions in calibrated columns. Subsequently the AUC of each form was divided by the total AUC of all CCK immunoreactivity, and then multiplied with the total concentration of sulfated or nonsulfated concentration, respectively, in the extract. The concentration of each molecular form of sulfated and nonsulfated CCK was hence measured separately in the neutral and acidic extracts and subsequently summed up.

2.6. Immunohistochemistry and imaging

Sprague Dawley rats (adult males, 300–350 g) used for immunohistochemistry were obtained from SCANBUR AB (Stockholm, Sweden). To retain CCK peptides in neuronal cell bodies, some rats were treated with an intracerebroventricular injection of an axoplasmic transport inhibitor, colchicine, as previously described (Bergman et al., 2014). Colchicine treated rats and controls were deeply anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde in 0.16 M phosphate buffer (PB) supplemented with picric acid (0.2%) as described (Shi et al., 2012). Brains were dissected out, post-fixed in the same fixative at 4 °C for 90 min, and rinsed with 10% sucrose in 0.1 M phosphate buffer containing 0.01% sodium azide (VWR International, Leuven, Belgium) and 0.02% bacitracin (Sigma, St. Louis, MO). Brains were kept in 10% sucrose solution at 4 °C for at least 48 h, frozen

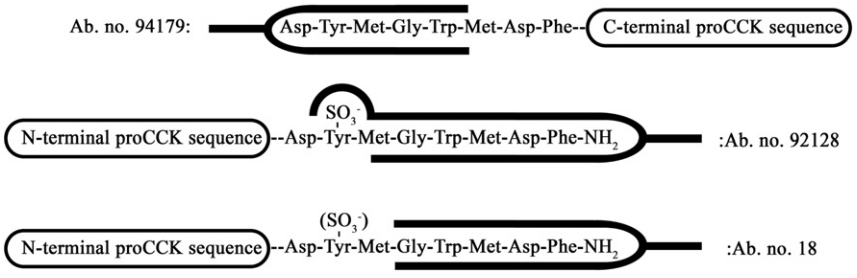

Fig. 1. Schematic illustration of the antibody binding epitopes on CCK-8: Ab no. 94,179 binds the N-terminus of nonsulfated CCK-8 irrespective of C-terminal extention. Ab no. 92,128 binds the alpha amidated C-terminus of all sulfated CCKs irrespective of N-terminal extention. Ab no. 18 (PF) binds all sulfated and nonsulfated alpha amidated CCK peptides irrespective of N-terminal extention.

Please cite this article as: Agersnap, M., et al., Nonsulfated cholecystokinins in cerebral neurons, Neuropeptides (2016), http://dx.doi.org/10.1016/j.npep.2016.08.003