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Cortistatin modulates calcitonin gene-related peptide release from neuronal tissues of rat. Comparison with somatostatin

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ABSTRACT

Cortistatin (CST) is an endogenous neuropeptide bearing strong structural and functional analogies with somatostatin (SST). Gene expression of CST and its putative receptor MrgX2 in dorsal root ganglia (DRG) neurons in man suggests the involvement of CST in pain transmission. In this study we have investigated the effects of CST and SST on calcitonin gene-related peptide (CGRP, the main neuropeptide mediator of pain transmission) from primary cultures of rat trigeminal neurons. Moreover, here for the first time we used organotypic cultures of rat brainstem to investigate the release of CGRP form nucleus caudalis as a model of pre-synaptic peptide release. In both experimental paradigm CGRP release was evaluated in the presence of CST or SST, with or without the addition of known secretagogues (namely high KCl concentrations, veratridine and capsaicin). We found that CST and SST do not modify basal CGRP secretion from trigeminal neurons, but both peptides were able to inhibit in a concentration-dependent manner the release of CGRP stimulated by KCl, veratridine or capsaicin. Likewise, in brainstem organotypic cultures CST and SST did not modify baseline CGRP secretion. Of the secretagogues used, capsaicin proved to be most effective compared to KCl and veratridine (8-fold *vs* 2-fold increase, respectively). Thereafter, CST and SST were tested on capsaicin-stimulated CGPR release only. Under these conditions, CST but not SST was able to inhibit in a significant manner pre-synaptic CGRP release from the brainstem, providing further evidence in support of a role for CST in pain transmission.

1. Introduction

Cortistatin (CST) is a cyclic neuropeptide cloned from human, rat and mouse tissues [30]. CST expression was initially reported to be restricted to the cerebral cortex and hippocampus, whereas it is currently known to have wide distribution in many organs [14]. In the rat, CST is synthesized as a prepropeptide (preproCST) form that yields two biologically active peptides: CST-29 and CST-14 (thereafter referred to as CST), the latter preferentially released via the regulated secretory pathway [22,12]. CST exhibits a remarkable structural and functional resemblance to somatostatin (SST), although it is the product of a different gene [10,11,30]. CST shares with SST the ability to bind and activate all five cloned SST receptors (SSTR1-5), with similar efficacy and potency [29]. This finding may well explain the considerable overlapping between CST and SST on several biological actions, including the depression of neuronal activity and the inhibition of cell proliferation [10,7].

However, the profile of CST biological activities is not simply redundant with respect to SST; in fact, recent studies showed that CST has certain proprieties distinct from, and even opposite to those of SST [4,15,13]. Indeed, evidence exists that CST might signal through receptors distinct from those of SST, namely the putative CST receptor MrgX2 and the GHSR-1a ghrelin receptor [1,9,25]. Moreover, four truncated but functional SST5 receptor variants (SST5TMD4, SST5TMD2 and SST5TMD1 in the mouse; SST5TMD1 in the rat) have been described; these variants show distinct ligand-selective signaling profiles for CST and SST, respectively [8].

Pain perception involves several anatomical structures within both the central and peripheral nervous system, as well as various agents such as neurotransmitters, neuropeptides and cytokines. CST and SST show different activities on the modulation of pain perception. In fact, intracerebroventricular injections of CST elicit a significant increase in pain threshold, assessed through the hot-plate test in the rat; in this paradigm, SST displays no significant activity [20]. Thus, the effect of CTS on pain threshold may not involve SST receptors. MrgX2 receptors, which bind with high affinity CTS but not SST, are expressed in human principally in the small sensory neurons of the dorsal root ganglia (DRG), which include primary sensory fibers associated with acute and neuropatich pain [25]. Human DRG also express CST mRNA. Therefore, a role for this

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ligand-receptor pair can be postulated in the control of nociception [25].

Within this framework, in the present study we have investigated the effects of CTS on the release of calcitonin gene-related peptide (CGRP, the main neuromediator of trigeminal signaling) from primary cultures of rat trigeminal ganglia neurons as well as from rat brainstem explants containing trigeminal nucleus caudalis. Moreover the effects of CST and SST in this experimental paradigm were also compared. CGRP secretagogues were 56 mM KCl solutions, veratridine and capsaicin. The first two should be considered as non-specific depolarizing agents, with veratridine acting primarily through the opening of Na^+ channels, whereas 56 mM KCl solutions elicit directly Ca^{2+} ion influx within the cells. In contrast, the C-fiber stimulating agent capsaicin is considered a specific stimulus, acting via binding to and activation of vanilloid receptors [5].

2. Materials and methods

2.1. Trigeminal neuronal cultures

Trigeminal neuronal cultures were prepared from 6- to 7-day-old Wistar rats as previously described [5]. The use of animals for this experimental work has been approved by the Italian Ministry of Health (licensed authorization to P. Navarra). In brief, animals were decapitated and, after removal of the brain, basal skulls were exposed and trigeminal ganglia from both sides were aseptically removed. Tissues were collected in a Petri dish containing 3–5 ml of ice-cold phosphate buffer saline without Ca^{2+} and Mg^{2+} (PBS w/o; Sigma Chemicals Co., St. Louis, MO, USA), supplemented with antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin; Sigma Chemicals Co., St. Louis, MO, USA) and D-glucose (6 g/l). Tissues were then digested in 5 mg/ml collagenase (Biospa, Milan, Italy) for 20 min at 37 °C, followed by 5 min incubation in 0.125% trypsin (EuroClone, Pero, Milan, Italy). Ten μl of DNAse I 1 mg/ml (2320 Kunitz/ml) was added in the last 5 min of incubation. At this point, digested tissues were resuspended in 5 ml Ham's F12 medium (Biospa, Milan, Italy), containing 10% heat-inactivated endotoxin-free fetal calf serum (Gibco, Milan, Italy) and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin), and cells were mechanically dissociated using a Pasteur pipette. Cell suspension was plated on a 25 cm^2 flask (Corning, Turin, Italy) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 2–3 h (pre-plating). This additional step allowed us to separate neurons from non-neuronal cells on the basis of differential adhesion. At the end of pre-plating incubation, neurons were harvested from the flask and plated in 24-well tissue culture plates, previously coated with poly-D-lysine (40 mg/ml; MW 70,000–130,000, Sigma Chemicals Co., St. Louis, MO, USA) at a density of 100,000 cells/well. The incubation volume was 1 ml/well of complete culture medium (see above), enriched with 50 ng/ml of 2.5 S murine Nerve Growth Factor (Alexis-Vinci Biochem, Vinci, Florence, Italy). The culture medium was changed within 24 h from seeding and 10 mM cytosine arabinoside was added to further reduce non-neuronal cell growth. All experiments were carried out 6–7 days after dissection, when cells reached complete maturation. At the beginning of experiments, the incubation medium was replaced and cells were incubated with 300 μl of complete Ham's F12 medium (containing 10% FCS, 1 μM bacitracin, 10 mg/ml aprotinin, without NGF) at 37 °C. Basal and stimulated CGRP release was assessed incubating cultures with medium alone or medium containing test drugs. In the experiments in which KCl was used as depolarizing agent, 56 mM KCl solution was prepared. The solution consisted of 56 mM KCl, 67 mM NaCl and other ions at the same concentrations as in the incubation medium, plus aprotinin, bacitracin and 0.1% bovine serum albumin. At the end of the experiments, media were collected and stored at −35 °C until the assays were performed.

2.2. Brainstem organotypic cultures

Male Wistar rats, weighting 200–300 g, were used. They were kept four per cage and maintained at a temperature of 23 ± 1.5 °C, with a relative humidity of 65 ± 2%. The animals were exposed to 12 h of light (06.00 am–06.00 pm) followed by 12 h of dark, and they had free accessed to food and water. The use of animals for this experimental work has been approved by the Italian Ministry of Health (licensed authorization to prof. P. Navarra). On day of the experiment the animals were decapitated and the brains were rapidly removed. After removal of the cerebellum, brainstem was dissected within the following limits: coronal sections approximately from −9 to −15 mm posterior to bregma, 4 mm lateral to bregma (Paxinos' The Rat Brain in Stereotaxic Coordinates), with a depth of about 2–3 mm. This CNS area contains the second order neurons of the nucleus of the spinal tract of the trigeminal nerve (Sp5; Sp5C; Sp5I and Sp5O), where CGRP is released from C-fibers arising from trigeminal ganglia. Total dissection time was <2 min from decapitation.

The brainstem were incubated in a 24-well plate (one brainstem per well), at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% O_2 in a 300-μl incubation medium, Minimum Essential Medium (MEM) with Earle's salts (Gibco, Milan, Italy), supplemented with 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 60 μg/ml ascorbic acid (Sigma Chemical Co., St. Louis, MO) and 20 IU/ml aprotinin (Sigma Chemical Co., St. Louis, MO), pH 7.4. In this experimental model, brainstems remain viable and functional during the time frame of the experiments, as assessed by the lactate dehydrogenase (LDH) assay for cellular toxicity, which showed no statistical difference between control and treated hypothalami (data not shown). Thus, variation in CGRP release did not appear to be correlated with toxic damage to the tissues.

After 1 h pre-incubation, the explants were subjected to a 30-min control incubation in plain medium to assess basal CGRP release. This was followed by a second 30-min incubation in medium containing test substances or, for the control group, in medium alone. Whenever KCl was used, MEM was replaced by a medium consisting of 56 mM KCl and 67 mM NaCl, with the same concentration of the other ions as found in MEM. Medium samples were stored at −35 °C until assayed for CGRP immunoreactivity.

2.3. CGRP radioimmunoassay (RIA)

CGRP release was measured by a radioimmunoassay technique validated in our laboratory and previously described in detail [5]. Briefly, a buffer containing 10 mM sodium phosphate, 154 mM NaCl, 25 mM ethylene diamine tetraacetic acid (EDTA), 0.01% thimerosal, 0.5% BSA and 0.03% Tween 20, pH 7.2 was used. The RIA was performed as follows: 100 μl of sample or standard solution was diluted 3-fold into RIA buffer containing anti-hα-CGRP at a final dilution of 1:120,000. This antiserum was previously tested for cross-reactivity against calcitonin, human and rat amylin, human β-CGRP, rat α- and β-CGRP, rat α-CGRP (8–37), rat α-CGRP (29–39), cholecystokinin-8, neurokinin A and B, physalaemin, somatostatin, substance P and vasoactive intestinal peptide [5]. After 24 h at 4 °C, 100 μl of [^{125}I]-hα-CGRP (6000 cpm/tube) was added and the incubation continued at 4 °C for 48 h. Separation of free from bound α-CGRP was achieved by adding anti-rabbit goat serum (at final dilution 1:200) and 500 μl of 6.6% polyethylene glycol solution in 5 mM phosphate buffer, pH 7.4. After 2 h at 4 °C, the tubes were centrifuged at 3000 × *g* for 30 min at 4 °C, and the pellet counted in a γ-counter. The standard curve ranged