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Diverging effects of cortistatin and somatostatin on the production and release of prostanoids from rat cortical microglia and astrocytes

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ABSTRACT

Here we compared the effects of cortistatin and somatostatin on the production of prostanoids from primary cultures of rat cortical microglia and astrocytes. We found that both cortistatin and somatostatin do not modify basal PGE_2 release from cultured astrocytes in 24-h experiments. Somatostatin further enhanced the increase in PGE_2 release induced by IL-1β, whereas cortistatin inhibited such increase. Experiments on microglia showed that somatostatin has no effect on basal and IL-1β-stimulated PGE_2 release, whereas cortistatin reduced baseline prostanoids production and abolished stimulation elicited by IL-1β. The latter effect was associated to the inhibition of COX-2 gene over-expression induced by the cytokine.

1. Introduction

Cortistatin (CST) is a 14-aa peptide belonging to the family of somatostatin(SS)-like Cys–Cys loop peptides. Cortistatin displays an amino acid sequence identical to SS in the portion of the molecule interacting with SS receptors; such identity accounts for the fact that CST binds all the SS receptor subtypes with affinity similar to that of SS, and that several physiological effects of CST are mediated via the activation of these receptors (Spier and de Lecea, 2000; Siehler et al., 2008). However, the profile of CST biological activities is not simply redundant with respect to SS; in fact, CST gene expression is not up-regulated in SS knockout mice, despite the fact that SS accounts for 97% of total brain SS/CST immuno-reactivity (Ramírez et al., 2002). Moreover, CST has been shown to signal through receptors distinct from those for SS, namely the putative CST receptor MrgX2 (Robas et al., 2003) and the GHSR-1a ghrelin receptor (Broglio et al., 2007), and to exert biological effects different or even opposite to SS, such as the induction of slow-wave sleep and the reduction of locomotor activity (Spier and de Lecea, 2000).

Cortistatin was originally described in scattered neurons of the rat cerebral cortex and hippocampus (Spier and de Lecea, 2000), where the peptide co-localizes with GABA (de Lecea et al., 1997). Co-localization with SS is partial; less than half CST-expressing neurons also express SS, and about one quarter of SS-containing neurons express CST (de Lecea et al., 1997). Similar to the CNS, systemic CST localization is also restricted to relatively few peripheral tissues, both in rats and humans (de Lecea et al., 1996; Dalm et al., 2004). Interestingly, CST—but not SS—was shown to be expressed and produced by different human cell types of the immune-inflammatory lineage (Dalm et al., 2003; van Hagen et al., 2008). These findings suggest a possible role for CST in the regulation of immune-inflammatory responses. In fact, CST exerts protective, anti-inflammatory activities in mouse models of endotoxemia and inflammatory bowel disease, such effects being associated to a decreased production of inflammatory mediators by activated macrophages *in vitro* (Gonzalez-Rey et al., 2006a,b). This evidence from *in vivo* and *in vitro* models of immune-inflammatory systemic disorders candidates CST as an endogenous immuno-modulatory agent with potential therapeutic applications (Gonzalez-Rey and Delgado, 2007). However, the role of CST in the control of immune-inflammatory responses within the CNS compartment remained so far unexplored.

In this work we compared the effects of CST and SS on the production and release of prostanoids from primary cultures of rat cortical microglia and astrocytes. We have previously characterized these *in vitro* models (Vairano et al., 2002), and found that they are suitable for investigations on the role of drugs and endogenous factors in neuroinflammatory processes (Tringali et al., 2004, 2005). Putative effects of SS and/or CST in these model might have pathophysiological relevance, since both peptides are normally present in the extra-cellular *milieu* of the brain cortex (de Lecea et al., 1997). The levels of PGE_2 immuno-reactivity measured in the incubation medium of cultured cells were taken as a marker of cyclooxygenase (COX) activity; whether any inhibitory effect was observed, it was further analyzed investigating the effect of test substances on inducible COX gene expression by real-time polymerase chain reaction (RT-PCR).

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2. Materials and methods

2.1. Cell cultures

Primary cultures of rat cortical astrocytes and microglia were prepared as previously described (Vairano et al., 2002). In brief, 1- to 2-day-old Wistar rats were sacrificed. The brains were removed under aseptic conditions and placed in phosphate buffer saline with Ca^{++} and Mg^{++} (PBS-w), containing antibiotics (100 IU/ml of penicillin plus 100 μg/ml streptomycin). Under a stereomicroscope, the meninges were carefully removed and the cortex was dissected. The tissue was cut into small fragments, digested with trypsin in PBS without Ca^{++} and Mg^{++} (PBS-wo), for 25 min at 37 °C and for further 5 min in the presence of 65 UI/ml DNase I. This step was followed by mechanically dissociation in Dulbecco's MEM with Glutamax-I containing 10% fetal calf serum (FCS) and antibiotics as above, to obtain single cells. Viability was roughly 45%.

2.1.1. Astrocytes

The cells thus obtained were seeded in 75-cm^2 flasks at a density of 1×10^7 cells/10 ml and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed after 24 h and then twice a week. At confluence, the cultures were vigorously shaken to remove non-adherent cells (oligodendrocytes and microglia) and re-plated. Astrocytes were grown to confluence in 24-well plates. All the experiments were carried out in 1% FCS DMEM with antibiotics, and the incubation time was 24 h. At the end of each experiment, the medium was collected under sterile conditions and stored at −35 °C until PGE_2 assays were performed.

2.1.2. Microglia

Primary enriched cultures of rat microglia were prepared from primary mixed cultures of cortical glial cells (Dello Russo et al., 2004). In brief, after 13 days from dissection, microglial cells were detached from the astrocyte monolayer by gentle shaking (see above). The cells were plated in 96-well plates at a density of 3×10^5 cells/cm^2 for PGE_2 release assessment, or in 48-well plates for RT-PCR analysis, using 100 μl/well or 300 μl/well, respectively, of DMEM-F12 containing 10% FCS and antibiotics. Under these conditions, the cultures were 95–98% CD11b positive. Experiments were performed within 24 h from the last plating; at that time, culture medium was replaced with fresh medium containing or not the drugs under study. All the experiments were carried out in 10% FCS medium to reduce microglial death which normally occurs after separation from astrocytes (Vairano et al., 2002). At the end of each experiment, media were collected and stored at −35 °C until assays for PGE_2 immuno-reactivity.

2.2. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total cytoplasmic RNA of glia cells was extracted using the RNeasy®Micro kit (Qiagen, Hilden, Germany), which included a 15 min DNAse treatment. RNA concentration was measured using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen). A standard curve in the range of 0–100 ng was run in each assay using 16S and 23S ribosomal RNA (rRNA) from *E. coli* as standard. Aliquots (0.1 μg) of RNA were converted to cDNA using random hexamer primers and the ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA). Quantitative changes in mRNA levels were estimated by real-time PCR (Q-PCR) using the following cycling conditions: 35 cycles of denaturation at 95 °C for 20 s; annealing at 59 °C for 30 s; and extension at 72 °C for 30 s; Brilliant SYBR Green QPCR Master Mix 2X (Stratagene, La Jolla, CA, USA) was used. PCR reactions were carried out in a 20 μl reaction volume in a MX3000P real-time PCR machine (Stratagene, La Jolla, CA, USA). The primers used for COX-2 detection were 677 forward (5′-GCA TTC TTT GCC CAG CAC TTC ACT-3′), corresponding to bases 677–700; and 774 reverse (5′-TTT AAG TCC ACT CCA TGG CCC AGT-3′), complementary to bases 751–774 of the rat COX2 mRNA sequence, which yield a 98 bp product; the primers used for COX-1 detection were 154 forward (5′-CCT CAC CAG TCA ATC CCT GT-3′), corresponding to bases 154–173; and 384 reverse (5′-AGG TGG CAT TCA CAA ACT CC-3′) complementary to bases 365–384 of the rat COX1 mRNA sequence, which yield a 231 bp product. The primers used for GAPDH were: 732 forward (5′-TCC CAG AGC TGA ACG GGA AGC TCA GTG-3′), and 1070 reverse (5′-TGG AGG CCA TGT AGG CCA TGA GGT CCA-3′), which yield a 339 bp product. Relative mRNA concentrations were calculated from the take-off point of reactions (threshold cycle, Ct) using the comparative quantization method and the software included in the unit. For this analysis, controls were used as calibrators and the Ct values for GAPDH expression as normalizers. Thus, using the $-\Delta\Delta Ct$ method, we calculated the differences (fold changes) in the expression of COX_2 or COX_1 target gene after a specific treatment vs. its respective control (Livak and Schmittgen, 2001). Moreover, in each run we calculated the PCR efficiency using serial dilution of one experimental sample; efficiency values were found between 94 and 98% for each primer set. At the end of Q-PCR, the products were separated by electrophoresis through 2% agarose gels containing 0.1 μg/ml ethidium bromide to ensure production of correct sized product.

2.3. PGE_2 radioimmunoassay (RIA)

PGE_2 was measured by radioimmunoassay (RIA). Incubation mixtures of 1.5 ml were prepared in disposable plastic tube. Aliquots of medium collected either from astrocytes or microglial cells were diluted to 250 μl with 0.025 M phosphate buffer, pH 7.5. The remainder 1.25 ml was prepared by adding together 3000–3300 cpm of [^{3}H] PGE_2 and antiserum at a final dilution of 1:115,000. A duplicate standard curve (ranging from 1 to 400 pg/tube) was run with each assay. The detection limit of the assay was 1 pg/tube and the EC_{50} of 28 pg/tube. The intra- and inter-assay variability was 5% and 10%, respectively. Separation of free from antibody-bound PGE_2 was obtained with charcoal, which absorbs 95–98% of free PGE_2. After centrifugation for 10 min at 4 °C, supernatants were decanted directly into 10 ml of liquid scintillation fluid. Radioactivity was measured by liquid scintillation counting.

2.4. Chemicals

CST and SST were purchased from Phoenix Pharmaceutical Inc. (Burlingame, CA, U.S.A.). Neuropeptides were dissolved in deionized water, and further diluted to working concentrations in incubation medium. Recombinant human interleukin-1β (IL-1β) was obtained from Endogen (Pierce Biotechnology, Rockford, IL, USA). The cytokine was dissolved in deionized water and further diluted in incubation medium. All drugs tested did not interfere with the PGE_2 assay. Cell culture reagents [Dulbecco's modified Eagle's medium (DMEM), and antibiotics] were from Biochrom AG (Berlin, Germany). Fetal calf serum (FCS) and DMEM-F12 were from GIBCO, Invitrogen Corporation (Paisley, Scotland).

2.5. Statistical analysis

Each experiment was repeated two or three times, according to a randomized block design. Data were analyzed by two-way ANOVA; since no significant difference was found between different experiments conducted with the same experimental protocol, integrated pooled analysis of the data was justified, and all results were presented as the mean ± SEM of n replicates per experimental group. A two-way ANOVA for the factors time and treatment was also used to analyze each time-course experiment. Subsequent to ANOVA, *post-hoc* Newman–Keuls or Dunnet tests for comparisons between group means, were carried out when appropriate. All descriptive statistics