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IMMUNOHISTOCHEMICAL EXPRESSION AND COLOCALIZATION OF SOMATOSTATIN, CARBOXYPEPTIDASE-E AND PROHORMONE CONVERTASES 1 AND 2 IN RAT BRAIN

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Abstract—The processing of many peptides for their maturation in target tissue depends upon the presence of sorting receptor. Several previous studies have predicted that carboxypeptidase-E (CPE), prohormone convertase 1 (PC1) and prohormone convertase 2 (PC2) may function as sorting elements for somatostatin (SST) for its maturation and processing to appropriate targets. However, nothing is currently known about whether brain, neuronal culture or even endocrine cells express SST, CPE, PC1 and PC2 and exhibit colocalization. Accordingly, in the present study using peroxidase immunohistochemistry, double-labeled indirect immunofluorescence immunohistochemistry and Western blot analysis, we mapped the distributional pattern of SST, CPE, PC1 and PC2 in different rat brain regions. Additionally, we also determined the colocalization of SST with CPE, PC1 and PC2 as well as colocalization of CPE with PC1 and PC2. The localization of SST, CPE, PC1 and PC2 reveals a distinct and region specific distribution pattern in the rat brain. Using an indirect double-label immunofluorescence method we observed selective neuron specific colocalization in a region specific manner in cortex, striatum and hippocampus. These studies provide the first evidence for colocalization between SST, CPE, PC1 and PC2 as well as CPE with PC1 and PC2. SST in cerebral cortex colocalized in pyramidal and non-pyramidal neurons with CPE, PC1 and PC2. Most importantly, in striatum and hippocampus colocalization was mostly observed selectively and preferentially in interneurons. CPE is also colocalized with PC1 and PC2 in a region specific manner. The data presented here provide a new insight into the distribution and colocalization of SST, CPE, PC1 and PC2 in rat brain. Taken together, our data anticipate the possibility that CPE, PC1 and PC2 might be potential target for the maturation of SST.

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Abbreviations: CPE, carboxypeptidase-E; NGS, normal goat serum; PC1, prohormone convertase 1; PC2, prohormone convertase 2; PSST, prosomatostatin; RSP, regulated secretory pathways; SST, somatostatin; TBS, Tris-buffered saline; TBST, Tris-buffered saline Tween.

Key words: carboxypeptidase-E, prosomatostatin, prohormone convertase, regulatory secretory pathway, sorting receptor.

Somatostatin (SST) is a multifunctional peptide with variety of functions in different target tissues. In several pathophysiological conditions a gradual decrease in SST has been associated with disease progression, such as neurological disorders and cancer of different origin. SST like many other neuropeptides is synthesized as an inactive prohormone (prosomatostatin, PSST) and targeted into the regulated secretory pathway (RSP). This sorting process is dependent partially on the structure of the prosegment of PSST. Secretory cells, such as endocrine cells, contain two distinct pathways for protein secretion, a RSP that releases protein from a granular storage pool in response to specific stimuli and a constitutive secretory pathway. To define the underlying traffic control mechanisms, three models have been proposed to explain how proteins are sorted to the RSP. The first proposes that regulated secretory proteins have an intrinsic ability to form aggregates leading to packaging of condensed products into secretory granules. Support for this model comes from the tendency of a number of secretory granule proteins such as prolactin, growth hormone, the chromogranins, carboxypeptidase-E (CPE), and prohormone convertase 2 (PC2) to aggregate at the mildly acidic pH in the trans-Golgi network (Chanat and Huttner, 1991; Shennan et al., 1994; Song and Fricker, 1995b; Yoo, 1995, 1996; Lee et al., 2001). The second model suggests that regulated secretory proteins contain a sorting signal within a specific motif, allowing proteins to be sorted in a receptor-dependent mechanism in trans-Golgi network/immature secretory granules (Gumbiner and Kelly, 1982; Burgess and Kelly, 1987; Kuliawat and Arvan, 1994). The third model is a hybrid of the first two.

The prohormone convertases are the major endoproteolytic processing enzymes of the secretory pathway (Seidah and Chretien, 1994; Steiner, 1998). There are seven known prohormone basic amino acid-specific PCs of mammalian origin, among which only prohormone convertase 1 (PC1) and PC2 act primarily in the regulatory pathway as they are exclusively expressed in neural and endocrine cells of the CNS and in endocrine organs (Seidah and Chretien, 1994; Seidah et al., 1996). Previously, using cell lines constitutively expressing furin, but not PC1 and PC2, displayed a significant conversion of PSST into SST28 (Sevarino et al., 1989; Stoller and Shields, 1989; Sevarino and Stork, 1991; Galanopoulou et al., 1993; Song and Fricker, 1995a; Cool et al., 1997; Zhang et al., 1999; Mouchantaf et al., 2001; Nillni et al.,

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2002; Ramirez et al., 2002). These observations suggested that widely expressed furin or furin-like enzymes are candidate monobasic converting enzymes. Previously, it has been shown that both PC1 and PC2 can process PSST at its dibasic site to generate SST-14 (Galanopoulou et al., 1995a; Patel et al., 1997). While PC1 is more active in regulated versus constitutive secretory cells, PC2 absolutely requires a RSP for activity (Galanopoulou et al., 1993, 1995a; Seidah et al., 1999).

CPE is an exopeptidase, and exists in two forms: one is an ~53 kDa soluble protein and the other is an ~55 kDa membrane-associated form via a C-terminal amphipathic sequence (Hook, 1985; Dhanvantari et al., 2002). Several previous studies have shown that CPE is involved in pro-opiomelanocortin, proinsulin, opioid, thyroid releasing prohormone and pro-enkephalin biosynthesis (Cool et al., 1997; Loh et al., 2002; Zhang et al., 1999; Boudarine et al., 2002; Nillni et al., 2002; Normant and Loh, 1998). CPE knockout mice are characterized by multiple disorders resulting from impaired processing of a number of neuroendocrine and endocrine prohormones. CPE exhibits an optimal acidic pH allowing it to best function within secretory granules of the RSP (Fricker, 1988). While CPE has been proposed to function as a candidate receptor for the RSP (Cool et al., 1997; Loh et al., 2002), other proposed mechanisms suggest that the presence of a dibasic motif and PC1 is required for this sorting process (Jutras et al., 1997, 2000; Garcia et al., 2005; Mulcahy et al., 2005).

Previous ex vivo data suggested that PC1, PC2 and CPE might play key roles in PSST processing (Galanopoulou et al., 1993, 1995a; Brakch et al., 1995; Patel and Galanopoulou, 1995). However, it is not known whether SST producing cells also colocalize with PC1, PC2 and CPE in brain. Accordingly, in the present study using a combination of immunohistochemistry and biochemistry, we determined the expression profiles of SST, CPE, PC1 and PC2 in rat brain. Since SST plays important role in neurological disorders such as Alzheimer's disease, Huntington's disease and epileptic seizure involving cortex, striatum and hippocampus, accordingly, we confined our immunohistochemical mapping only to these brain regions. In the present study we have demonstrated a distinct and overlapping expression pattern of these proteins in a region specific manner in rat brain.

EXPERIMENTAL PROCEDURES

Materials

Rabbit polyclonal antibodies directed against the carboxy-terminal segments 629–726 of mPC1 (Seidah et al., 1991) and 529–637 of mPC2 (Seidah et al., 1990) have been previously characterized for specificity (Benjannet et al., 1992, 1993; Marcinkiewicz et al., 1993). Mouse anti-CPE monoclonal antibody was purchased from BD Biosciences (Pharmingen, San Diego, CA, USA). Rabbit polyclonal antibody R149 directed against the central segment of SST-14 with equal affinity for SST-14 and amino terminally extended forms of SST-14 was used (Galanopoulou et al., 1995a; Puebla et al., 1999). Sheep anti-SST antiserum against SST-14 was used for colocalization as previously characterized (Patel et al., 1985). The peroxidase Vectastain ABC systems were purchased from Vector Laboratories (Burlingame, CA, USA). Chemiluminescent ECL detection system was purchased from Amersham Ltd. (Oakdale, ON, Canada). FITC-conjugated goat anti-rabbit, FITC-conjugated donkey anti-sheep, Cy3-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit affinity-purified IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All other reagents were of analytical grade and were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada).

Western blot analysis

Brain tissues obtained from adult male Sprague–Dawley rats were homogenized on ice in lysis buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 0.5% sodium desoxycholate and supplemented with freshly prepared protease inhibitor cocktail (Sigma-Aldrich). After centrifugation at 14,000 r.p.m. for 30 min at 4 °C the total protein content in the supernatant was determined by Bradford method. Fifteen micrograms of protein was fractionated by electrophoresis on 8% SDS polyacrylamide gel and then transferred onto 0.2 μm nitrocellulose membrane (GE Healthcare, Mississauga, ON, Canada) at 4 °C overnight. The membrane was blocked with 5% non-fat dried milk in Tris-buffered saline Tween (TBST; 20 mM Tris base, 137 mM NaCl and 0.1% Tween 20, pH 7.6) at room temperature for 1 h and subsequently incubated overnight at 4 °C with specific primary antibodies. PC1 and PC2 antibodies were diluted 1:2000, CPE and SST antibodies were diluted 1:1000 in 5% non-fat milk. After washing with TBST buffer the membrane was incubated at room temperature for 1 h with secondary anti-rabbit peroxidase conjugated antibody to detect PC1, PC2 and SST, and secondary anti-mouse peroxidase conjugated antibody to detect CPE. The proteins were detected by enhanced chemiluminescence (GE Healthcare) according to the manufacturer's instruction.

Immunohistochemistry

All experiments conformed to international guidelines on the ethical use of animals, and every effort was made to minimize the number of animals used and their suffering. Four adult male Sprague–Dawley rats (200–250 g) were anesthetized with ketamine/xylazine in accordance with the animal care guidelines of McGill University, Montreal, and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were then removed and postfixed for 4 h in the same fixative at 4 °C, and cryoprotected in 30% sucrose at 4 °C. Forty micrometer thick sections were cut on a vibratome and collected in Tris buffered saline (TBS) containing 50 mM Tris–HCl and 0.9% NaCl, pH 7.4. Immunostaining was performed on free-floating rat brain sections with the avidin–biotin–peroxidase complex method using the Vectastain ABC kit (Vector Laboratories) as previously described (Kumar, 2005). Briefly, sections were incubated in 0.3% H_2O_2 in TBS to block endogenous peroxidase followed by incubation with 5% normal goat serum (NGS) and 0.1% Triton X-100 in TBS for 1 h at room temperature. Sections were then incubated overnight in a humid atmosphere at 4 °C with rabbit anti-SST (1:500), mouse anti-CPE (1:500), rabbit anti-PC1 or anti-PC2 (1:1000 and 1:2000) specific antibodies diluted in TBS containing 1% NGS. After three washes in TBS, sections were incubated with biotinylated goat anti-rabbit and goat anti-mouse IgGs (1:100) for 60 min at room temperature. Following three subsequent washes in PBS sections were incubated in ABC complex (1:100) for 30 min at room temperature and the final color was developed in the presence of 0.05% 3,3-diaminobenzidine in 0.1 M Tris buffer (pH 7.6) containing 0.01% H_2O_2. Subsequently, sections were washed three times in TBS and mounted onto slides. Photographs were taken on Leica DML microscope attached to a Cool Snap CCD camera. Controls used to validate the specificity of the immunoreactivity included omission of primary antibody in incubation medium.