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Identification of the receptors for somatostatin (SST) and cortistatin (CST) in chickens and investigation of the roles of cSST28, cSST14, and cCST14 in inhibiting $cGHRH_{1-27NH2}$-induced growth hormone secretion in cultured chicken pituitary cells

Fengyan Meng, Guian Huang, Shunyu Gao, Juan Li, Zhenxin Yan, Yajun Wang *

Key Laboratory of Bio-Resources and Eco-Environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610065, PR China

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ABSTRACT

Somatostatin receptors (SSTRs) are proposed to mediate the actions of somatostatin (SST) and its related peptide, cortistatin (CST), in vertebrates. However, the identity, functionality, and tissue expression of these receptors remain largely unknown in most non-mammalian vertebrates including birds. In this study, five *SSTRs* (named *cSSTR1*, *cSSTR2*, *cSSTR3*, *cSSTR4*, *cSSTR5*) were cloned from chicken brain by RT-PCR. Using a pGL3-CRE-luciferase reporter system, we demonstrated that activation of each cSSTR expressed in CHO cells by cSST28, cSST14 and cCST14 treatment could inhibit forskolin-induced luciferase activity of CHO cells, indicating the functional coupling of all cSSTRs to Gi protein(s). Interestingly, cSSTR1-4 expressed in CHO cells could be activated by cSST28, cSST14 and cCST14 with high potencies, suggesting that they may function as the receptors common for these peptides. In contrast, cSSTR5 could be potently activated by cSST28 only, indicating that it is a cSST28-specific receptor. Using RT-PCR, wide expression of c*SSTRs* was detected in chicken tissues including pituitary. In accordance with their expression in pituitary, cSST28, cSST14, and cCST14 were demonstrated to inhibit basal and novel $cGHRH_{1-27NH2}$-induced GH secretion in cultured chicken pituitary cells dose-dependently (0–10 nM) by Western blot analysis, suggesting the involvement of cSSTR(s) common for these peptides in mediating their inhibitory actions. Collectively, our study establishes a molecular basis to elucidate the roles of SST/CST in birds and provide insights into the roles of SST/CST in vertebrates, such as their conserved actions on pituitary.

1. Introduction

Somatostatin (SST) was originally isolated from ovine hypothalamus and identified as an inhibitory factor of pituitary growth hormone (GH) secretion (Brazeau et al., 1973). Two biologically active SST peptides – SST14 (14 amino acids) and its N-terminally extended form, SST28 (28 amino acids) – have been identified in mammals (Esch et al., 1980; Pradayrol et al., 1980; Schally et al., 1980). Both peptides are encoded by a single somatostatin (*SST*) gene and processed from the same large somatostatin precursor. It is becoming clear that SST is widely distributed in the central nervous system (CNS) and peripheral tissues and plays diverse roles in many tissues of mammals, including neurotransmission, neuromodulation, regulation of endocrine and exocrine secretions, as well as inhibition of cell proliferation (Patel, 1999).

In mammals, the biological actions of SST14 and SST28 are reported to be mediated by 5 structurally related G protein-coupled receptors (GPCR), namely SSTR1, SSTR2, SSTR3, SSTR4, and SSTR5, respectively (Patel, 1999). SSTR1-4 have been shown to bind both SST14 and SST28 with comparable nanomolar affinity, while SSTR5 can also bind SST28 and SST14 with high affinity, but display a weak selectivity for SST28 (Patel and Srikant, 1994). Activation of these receptors upon ligand binding can initiate multiple signaling cascades, including inhibition of adenylate cyclase (AC) activity. Although all 5 SSTRs share high degree of structural and functional similarity, they still have non-identical signaling properties and tissue/cell-specific expression patterns (Kumar et al., 1999; Patel,

Abbreviations: c, chicken; h, human; SST, somatostatin peptide; CST, cortistatin peptide; SSTR, somatostatin receptor; GH, growth hormone; GHRH, growth hormone-releasing hormone; GHRHR1, GHRH type I receptor; GHRHR2, GHRH type II receptor; GHRH-LP, GHRH-like peptide; CHO, Chinese hamster ovary; *SS1*, somatostatin gene; *SS2*, cortistatin/somatostatin 2 gene; RT-PCR, reverse transcription and polymerase chain reaction; CNS, central nervous system; AC, adenylate cyclase.

* Corresponding author. Tel.: +86 8541 2650; fax: +86 8541 4886.
E-mail address: cdwyjhk@gmail.com (Y. Wang).

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1999; Ben-Shlomo and Melmed, 2010), thus being capable of mediating diverse actions of SST14/SST28 in various tissues.

In addition to SST, a CST peptide, which is encoded by cortistatin (*CST*) gene and shares high structural similarity with SST peptide, has also been identified in mammals (de Lecea et al., 1996). Like SST, CST peptides of different lengths with an identical C-terminus have also been isolated in mammals, such as CST14 (14 amino acids) and CST29 (29 amino acids) identified in rats (Puebla et al., 1999). Interestingly, CST has been shown to be capable of binding all SSTRs with high affinity (Fukusumi et al., 1997), thus it has also been viewed as an SST analogue in mammals. Both *in vitro* and *in vivo* evidences suggest that CST can mimics the actions of SST in most mammalian tissues via interaction with SSTRs (Gahete et al., 2008). There is also evidence showing that CST may play roles in the CNS and pituitary different from those of SST (Broglio et al., 2007; Cordoba-Chacon et al., 2011), though it remains controversial whether these CST actions are mediated by SSTR(s), or by other receptors, such as ghrelin receptor 1A (GHSR-1A) and Mrgx2 receptor (Broglio et al., 2007; Cordoba-Chacon et al., 2011).

As in mammals, SST and CST peptides have also been reported to exist in non-mammalian vertebrates, including birds, amphibians, and fish (Lin et al., 1999b; Tostivint et al., 1996; Trabucchi et al., 2003). SST14 has been found to be almost fully conserved across vertebrates, while CST14 also shares high degree of structural conservation (Gahete et al., 2010). Moreover, there is growing evidence supporting the notion that SST14 and CST14 may play similar roles in non-mammalian vertebrates, such as modulation of pituitary GH secretion (Canosa et al., 2007; Jeandel et al., 1998; Lin et al., 1999b; Yunker et al., 2003) and pancreatic functions (Eilertson and Sheridan, 1993; Sheridan and Kittilson, 2004). However, the mechanisms underlying SST and CST actions remain unclear in most non-mammalian vertebrate groups including birds, in spite of several, but not all, SSTR subtypes being functionally characterized in teleost fish (Lin et al., 1999a, 2000, 2002; Lin and Peter, 2003; Ocampo Daza et al., 2012).

In chickens, SST14 was reported to be capable of inhibiting pituitary hormone secretion (Harvey et al., 1978; Perez et al., 1987; Donoghue and Scanes, 1991), pancreatic insulin and glucagon secretion (Honey et al., 1980, 1981), lipolysis (Strosser et al., 1983), thyroid function, as well as stimulating food intake (Tachibana et al., 2009, 2011). In contrast, the information regarding the roles of cCST14 in chickens is rather scarce. Moreover, the identity and functionality of the receptors for cSST and cCST peptides remains unclear. Although SST inhibits heterogeneous (human) GHRH-induced GH secretion in chicken pituitary (Taylor et al., 1986; Perez et al., 1987; Scanes and Harvey, 1989; Donoghue and Scanes, 1991; Piper and Porter, 1997; Anderson and Scanes, 2012), whether SST can inhibit pituitary GH secretion induced by the novel 'authentic chicken GHRH peptide', which was identified in our recent studies, remains unknown (Wang et al., 2007a, 2010; Huang et al., 2012). Therefore, using chicken as an experimental model, our present study aims to: (1) identify all the functional receptors for SST14, SST28 and CST14; (2) investigate the inhibitory effects of SST, CST, and their receptor(s) on the novel cGHRH-induced pituitary GH secretion. Considering the fact that the full array of receptors for SST and CST peptides have not yet been functionally characterized in non-mammalian vertebrate including birds and amphibians (Ocampo Daza et al., 2012), and birds occupy a unique evolutionary position in vertebrates, the results from our present study will not only establish an important molecular basis to uncover the roles of the SST/CST in birds, but also contribute significantly to our better understanding of the physiological roles of SSTRs and their ligands played in vertebrates, such as their conserved actions on pituitary hormone secretion.

2. Materials and methods

2.1. Chemicals and hormones

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO), and restriction enzymes were purchased from TaKaRa (TaKaRa, Dalian, China) unless stated otherwise. Chicken SST14 (cSST14), SST28 (cSST28), CST14 (cCST14) and GHRH (cGHRH$_{1-27NH2}$) (Huang et al., 2012) were synthesized with solid-phase Fmoc chemistry (GL Biochem, Shanghai, China). The purity of synthesized chicken peptides is >95% (analyzed by HPLC), and their structure was verified by mass spectrometry (GL Biochem).

2.2. Total RNA extraction

Adult chickens (or chicks) (Lohmann Sandy strain) were purchased from local supplier. Chickens were killed and different tissues including brain, heart, duodenum, kidney, liver, lung, muscle, ovary, testis, anterior pituitary, spleen, and pancreas were collected and frozen into liquid nitrogen. All samples were stored in −80 °C before use. Total RNA was extracted from chicken tissues with RNAzol reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions and resuspended in H_2O treated with diethyl pyrocarbonate (DEPC). All experimental chickens or chicks used in this study were treated in compliance with the national laws and the guidelines provided by the Animal Ethics Committee of Sichuan University.

2.3. Cloning of the cDNA containing open reading frame of cSSTR

According to the genomic sequences of 5 chicken *SSTRs* (http://www.ensembl.org/Gallus_gallus), gene-specific primers were designed to amplify the cDNA covering an open reading frame of each *SSTR* from chicken brain with the use of high-fidelity Taq DNA polymerase (TOYOBO) (Table 1). The amplified PCR products were cloned into pTA2 vector and sequenced by ABI3100 Genetic Analyzer (BGI, Shanghai, China). Finally, the cDNA sequence of each *cSSTR* was determined by sequencing three independent clones.

2.4. Rapid amplification of 5′-cDNA ends (5′-RACE) of cSSTRs

To determine 5′-untranslated region (5′-UTR) of chicken *SSTRs*, gene-specific primers were used to amplify the 5′-UTR of *cSSTRs* from adult chicken brain using SMART-RACE cDNA amplification Kit (Clontech, Palo Alto, CA). The amplified PCR products were cloned into pTA2 vector (TOYOBO, Japan) and sequenced by ABI3100 Genetic Analyzer (BGI).

2.5. Reverse transcription and polymerase chain reaction (RT-PCR)

Reverse transcription (RT) was performed at 42 °C for 1.5 h in a total volume of 10 μL consisting of 2 μg total RNA from different tissues, 5 × MMLV buffer, 0.5 mM each deoxynucleotide triphosphate (dNTP), 0.5 μg oligo-deoxythymidine, and 100 U MMLV reverse transcriptase (TaKaRa). All negative controls were carried out under the same condition without reverse transcriptase added in the 10 μL of reaction mix. Since *cSSTR1* gene is intronless, thus, total RNA samples were subjected to DNase I treatment to remove the genomic DNA contamination before being used for RT-PCR assay of *SSTR1* expression, as described in our recent study (Wang et al., 2012).