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Somatostatin receptor subtype 1 modulates basal inhibition of growth hormone release in somatotrophs

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Abstract Somatostatin (SST) inhibits the secretion of many peptide hormones including growth hormone (GH). The various functions of SST are mediated through at least five different receptor subtypes (SSTR1–5), their precise physiological roles have not been solved yet. Here we report on studies concerning the functional role of SSTR1 in the modulation of GH release from somatotrophs. Primary cell cultures from pituitaries of wild-type SSTR1 mice exposed to the SSTR1 selective somatostatin analog CH-275 show reduction of basal levels of GH secretion whereas somatotrophs isolated from SSTR1 null mutant mice did not respond to the agonist-mediated effect. This suggests that SSTR1 is involved in modulating basal GH levels in primary pituitary cell cultures and, together with SSTR2, may control the secretion of GH in the body.

Key words: SSTR1 null mutation; Growth hormone release

1. Introduction

The neuropeptide somatostatin regulates the release of many hormones and neurotransmitters in multiple tissues such as the brain, pituitary and the pancreas. The actions of somatostatin are mediated by five different seven transmembrane somatostatin receptors (SSTR1–5) which are all coupled to pertussis-toxin sensitive G-proteins of the Gi/Go type [1]. Due to the overlapping tissue distribution of SSTRs [2] and the limited availability of subtype-specific agonists or antagonists, it has been unclear so far which of the effects of somatostatin and its recently discovered homolog cortistatin are mediated by which receptor subtype. The only receptor of which the function is understood in some detail is SSTR2, because several subtype specific agonists as well as antagonists are available [3]; in addition a knock out (ko) mouse for this subtype has been generated [4,5]. Taken together, the data from these experiments indicate that SSTR2 is involved in the regulation of gastric acid secretion, some aspects of pancreatic hormone release and the hypothalamic control of growth hormone (GH) release.

The situation is far less clear for the other subtypes including SSTR1. This subtype is widely expressed in brain, but occurs also in peripheral tissues such as the gut [6]. Its possible contribution in the release inhibition actions of somatostatin in the pituitary has been proposed [7]. Here we show by comparing somatotrophs derived from SSTR1 (+/+) and SSTR1 (−/−) mice that the basal level of GH release is under the control of SSTR1.

2. Materials and methods

2.1. Analysis of GH release from primary cultured mouse pituitary cells

The pituitaries of 15 mice (C57BL/6) were prepared, sliced and incubated in 30 ml of dispersion buffer (3 mg/ml collagenase II (200 U/mg, Worthington, St. Katharinen, Germany) in 1×Hanks' balanced salt solution (Seromed, Berlin, Germany, 10 mM D-glucose, 10 mM HEPES, pH 7.4, 0.5 mg/ml bovine serum albumin (BSA) fraction V) at 37°C for 45 min with vigorous shaking by hand every 15 min. The resulting tissue pieces were pelleted by centrifugation (5 min, 200×*g*) and dispersed in 3 ml of a trypsin solution (0.05% trypsin, 0.5 mM EDTA in phosphate buffered saline (PBS)) by up and down pipetting using a Pasteur pipette. The incubation with trypsin was stopped by addition of 3 ml of Dulbecco's modified Eagle medium (DMEM) medium (3.7 mg/ml $NaHCO_3$, 1 mg/ml D-glucose, 15 μg/ml phenol red, Seromed, Berlin, Germany) containing 0.1% BSA fraction V. Cells were pelleted by centrifugation (200×*g*), resuspended in 2 ml of DMEM/BSA and 10^5 cells were seeded per well of a polylysine (Sigma, Deisenhofen, Germany) coated 24 well plate. 1.5–2×10^6 cells were routinely obtained. When the cells were settled the DMEM/BSA medium was replaced by DMEM medium containing 10% fetal bovine serum, 2 mM glutamine, 50 U/ml Penicillin and 50 ng/ml Streptomycin. The cells were maintained 4 days at 37°C in a humidified 5% CO_2/95% air atmosphere and the medium was exchanged every 2 days. Before testing various compounds for their ability to inhibit GH release, cells were washed twice, 2 h and immediately before the experiment with the culture medium containing 25 mM HEPES, pH 7.4. Experiments were conducted in triplicates. For the analysis of inhibition of basal GH release the somatostatin peptides were applied in 1 ml of fresh medium to each well. After incubation for 3 h under cell culture conditions the medium was collected and analyzed for the concentration of GH using a double antibody radioimmunoassay protocol (Amersham-Pharmacia, Freiburg, Germany). For analysis of inhibition of forskolin-stimulated GH release cells were washed twice with culture medium lacking phenol red and incubated with forskolin (1 μM) for 3 h in the absence or presence of peptides.

2.2. Null mutation

By screening a 129sv genomic library (λFixIIDash, Stratagene, La Jolla, CA, USA) a genomic clone containing the mouse sstr1 gene was obtained containing 8 kb of genomic DNA encompassing 2.5 kb upstream and 4.0 kb downstream of the coding sequence. From this region, a targeting vector was constructed in which the entire

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Abbreviations: SSTR, somatostatin receptor; SST, somatostatin; ES cells, embryonic stem cells; CH-275, [des-Ala[1], des-Gly[2], des-Asn[5], D-Trp8, IAmp[9]] somatostatin-14; GH, growth hormone; BSA, bovine serum albumin; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle medium; SST-14, somatostatin-14; GAPDH, glycerine aldehyde phospho dehydrogenase; ko, knock out; wt, wild-type

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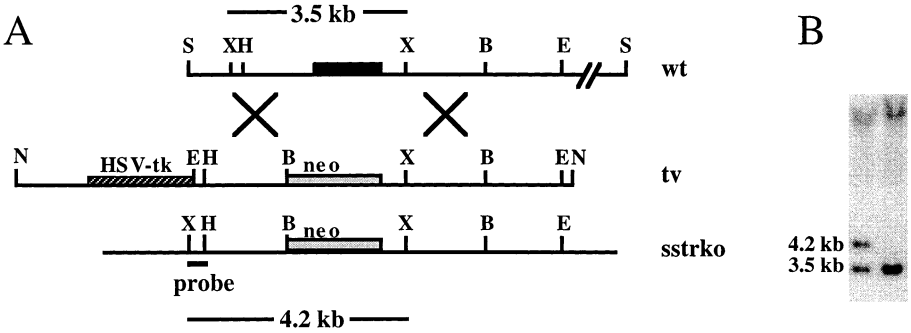

Fig. 1. Strategy for targeting the SSTR1 gene. A: The upper row shows the mouse wt SSTR1 locus used for construction of the targeting vector (tv) in which the SSTR1 coding region was replaced with a neomycin cassette in reverse orientation (middle row). The herpes simplex virus thymidine kinase (HSV tk) gene was inserted for negative selection. The bottom line shows the final orientation of the targeting cassette in the mutant sstr1 locus. Restriction sites are depicted as B (*Bam*HI); E (*Eco*RI), H (*Hin*dIII), S (*Sal*), X (*Xho*I), and N (*Not*I). B: Southern blot analysis using genomic DNA derived from ES cell clones. DNA was digested with *Xho*I, and the *Xho*I–*Hin*dIII fragment depicted in A was used as a probe for hybridization. According to (A) the wt allele yields a band of 3.5 kb and the targeted allele of 4.2 kb in size. Left lane, positive ES cell clone showing both alleles; right lane, control ES cells.

SSTR1 coding sequence was replaced by the neomycin resistance gene (Fig. 1A). Fragments containing either 5′ or 3′ flanking regions of the SSTR1 gene were subcloned into pBluescript; the identity of the inserts was confirmed by DNA sequencing. The 5′ and 3′ flanking regions of the SSTR1 gene were then cloned into the vector pPNT to obtain the SSTR1ko targeting vector. The vector was linearized using the *Not*I restriction enzyme, and transfected into J1 embryonic stem (ES) cells by electroporation (pPNT vector and stem cells were kindly provided by Dr. R. Jaenisch, Boston, MA, USA). ES cells were positively selected for the neomycin resistance gene with G418 and negatively with Gancyclovir. Individual clones were isolated, expanded and duplicated. ES cells were grown on mitotically inactivated primary fibroblasts. 500 clones were selected and screened for correct insertion of the transgene by Southern blot analysis of genomic DNA digested with *Xho*I (Fig. 1B). Filter hybridization was performed with a α-^{32}P-labelled probe consisting of 5′-flanking sequence of 500 bp (*Xho*I/*Hin*dIII fragment; see Fig. 1) yielding a 3.5 kb fragment wild-type (wt) or 4.2 kb fragment (ko) by using standard protocols [8]. A total of 15 clones with a deletion of one SSTR1 allele could be obtained. Two clones, #5 and #9 were used for injection into blastocysts of C57BL/6 mice. Custom injections were performed at BRL Biological Research Laboratories, Füllinsdorf, Switzerland. These animals were crossed back into a C57BL/6 background. Only one of these animals gave rise to germ line transmission of the desired transgene as analyzed by coat color and Southern hybridization analysis of tail biopsies.

SSTR1 null mutant mice were reared in the animal facility up to an age of 18 months without any apparent abnormalities. They reproduced normally with litter sizes of 6 to 10 animals. When homozygous (−/−) animals were compared with their wt littermates, no obvious abnormalities were detected; i.e. no significant differences in the body weight of adult animals were observed. Upon dissection of adult animals internal organs were found in normal size and shape.

Table 1
Growth hormone release

Somatotrophs	SSTR1 mediated basal GH release (%)	
	forskolin	
	−	+
Somatotrophs	100	240
+SST-14	49	65
+CH-275	75	275
+Octreotide	53	70

Primary pituitary cells were prepared from mice and incubated in the absence (−) or presence of 1 μM forskolin (+) and with 0.5 μM of SST-14, CH-275 (Neosystem, Strasbourg, France) or octreotide. The basal GH release of 4.85 ± 0.65 ng GH per 100 μl medium and 1×10^5 cells was set as 100%.

3. Results and discussion

The major action of somatostatin, inhibition of GH release from pituitary somatotrophs has been attributed to SSTR2 [9] although all SSTR subtypes are present in the hypophysis and even in individual pituitary cells [2]. Recently, it has been shown that the SSTR1 specific somatostatin-14 (SST-14) analogue [des-Ala^1, des-Gly^2, des-Asn^5, D-Trp8, IAmp^9] SST-14 (CH-275) mediates the inhibition of voltage-operated calcium channels suggesting that SSTR1 may well be engaged in the inhibition of hormone release [7]. In accordance with this observation Table 1 shows that the SSTR1 selective agonist CH-275 mediates the inhibition of basal GH release (25 ± 5%) although at a smaller extent than SST-14 (51 ± 7%) and the SSTR2/SSTR5 selective agonist octreotide (47 ± 4%). This may be due to different levels of binding sites for each ligand present on somatotrophs. The SSTR1 selective compound CH-275 does not affect the forskolin-stimulated GH release while the SSTR2/SSTR5 selective agonist octreotide and SST-14 are very effective.

In order to study the precise contribution of SSTR1 in the inhibitory process of basal GH release somatotrophs were cultured from pituitaries of SSTR1 null mutant mice (Fig. 2). Homozygous SSTR1 (−/−) mice were obtained by cross-

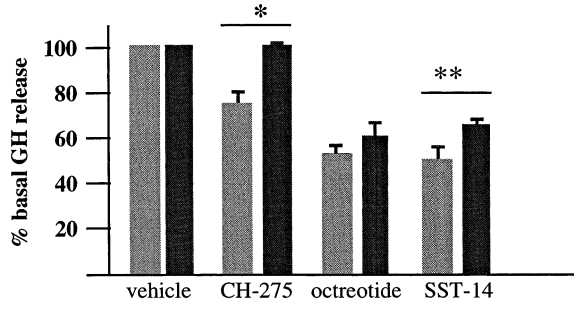

Fig. 2. SSTR1 mediates inhibition of basal GH release. Primary pituitary cells were prepared from SSTR1 (−/−) (black bars) and (+/+) (gray bars) mice and incubated in the presence of 0.5 μM of CH-275, (Neosystem, Strasbourg, France), octreotide or SST-14. Each experiment was performed twice in triplicate. The basal GH release of 4.85 ± 0.65 and 7.85 ± 0.35 ng GH per 100 μl medium and 1×10^5 cells was set as 100% for pituitary cells from SSTR1 (+/+) and SSTR1 (−/−) mice, respectively. *; $P<0.01$, **; $P<0.02$.

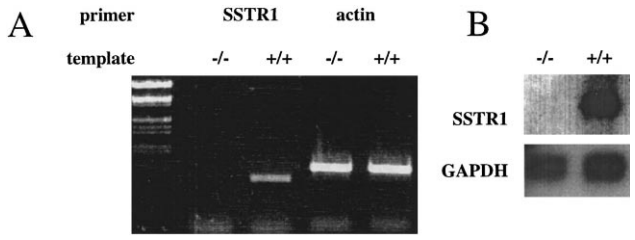

Fig. 3. RT-PCR and Northern blot analysis of SSTR1 gene expression. A: Total RNA was isolated from adult mouse brains, and used for RT-PCR analysis using primers recognizing a cDNA fragment encompassing the SSTR1 N-terminus [2]: forward primer, 5′-GCCGCACCGGCCCACTTC (bp 63–80 of the mouse SSTR1 gene; accession number M81831, GenBank database); reverse primer, 5′-GTAGCGCGCCGAAGGGCC (bp 472–455 of the mouse SSTR1 gene). Actin specific primers were used in a control reaction. Predicted fragment sizes are 450 bp for the SSTR1 reaction and 600 bp for actin. A similar result was obtained with primer sets specifically recognizing the SSTR1 C-terminus (not shown). Left lane, marker (λ*Eco*RI/*Hin*dIII). B: Northern blot analysis of poly $(A)^+$ RNA from brains of SSTR1 (+/+) and SSTR1 (−/−) mice. 5 μg poly $(A)^+$ RNA was separated on 1% agarose gels in 40 mM morpholinopropane sulfonic acid, 10 mM sodium acetate, 6.6% formaldehyde. After electrophoresis RNA was transferred to nylon filters (Hybond N, Amersham, Braunschweig, Germany) and UV crosslinked to the membrane. Filters were hybridized at 50°C in 5×SSC, 10× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS, 50% formamide for 16 h. Blots were washed twice in 2×SSC/0.1% SDS for 5 min at room temperature and twice at 55°C for 15 min. Exposure of the filters to X-ray films was for 96 h at −80°C. Hybridization with a glycerine aldehyde phospho dehydrogenase (GAPDH) probe was performed as a control experiment.

ing heterozygous SSTR1 (±) mice yielding off-springs with SSTR1 (+/+), SSTR1 (±) and SSTR1 (−/−) mice at the expected Mendelian ratios. Absence of the transgene in SSTR1 null mutants was confirmed by RT-PCR (Fig. 3A) and Northern blot analysis (Fig. 3B) of RNA isolated from the brains of SSTR1 (−/−) and SSTR1 (+/+) mice using primers directed at either the 5′- or the 3′-part of the coding region. The possibility that the lack of the SSTR1 gene could be compensated by an upregulated expression of one of the other SSTRs was excluded by Northern blot analysis (data not shown).

Somatotrophs from SSTR1 (−/−) mice were not sensitive to CH-275 suggesting that SSTR1 needs to be present to mediate the inhibition of GH release by CH-275 (Fig. 2). The contribution of SSTR1 to the inhibition of basal GH release is also reflected by an increased basal concentration of GH in the culture medium of pituitary cells from SSTR1 (−/−) mice. Moreover, a smaller effect of SST-14 to reduce basal GH release from somatotrophs of SSTR1 (−/−) mice compared to wt animals was also observed. In contrast, the effect of octreotide is not diminished. As expected, CH-275 does not affect the forskolin-stimulated GH release neither in pituitary cells derived from SSTR1 (+/+) nor from SSTR1 (−/−) mice (data not shown) while the SSTR2/SSTR5 selective agonist octreotide and SST-14 are very effective (Table 1).

In conclusion, the data reported here strongly support the notion that expression of the SSTR1 gene is essential in the regulation of basal levels of GH in mice. Mice lacking a functional SSTR1 gene are unable to modulate these basal GH levels in primary pituitary cell cultures. This may point to a more complex role of the SST receptor subtypes in growth control which may include not only SSTR2 but also SSTR1.

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