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Somatostatin Receptor Expression and Biological Functions in Endocrine Pancreatic Cells

Review Based on a Doctoral Thesis

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Abstract

Type 1 diabetes is resulting from the selective destruction of insulin-producing beta-cells within the pancreatic islets. Somatostatin acts as an inhibitor of hormone secretion through specific receptors ($sst_{1–5}$).

All ssts were expressed in normal rat and mouse pancreatic islets, although the expression intensity and the co-expression pattern varied between ssts as well as between species. This may reflect a difference in response to somatostatin in islet cells of the two species.

The Non-Obese Diabetic (NOD) mouse model is an experimental model of type 1 diabetes, with insulitis accompanied by spontaneous hyperglycaemia. Pancreatic specimens from NOD mice at different age and stage of disease were stained for ssts. The islet cells of diabetic NOD mice showed increased islet expression of $sst_{2–5}$ compared to normoglycemic NOD mice. The increase in $sst_{2–5}$ expression in the islets cells may suggest either a contributing factor in the process leading to diabetes, or a defense response against ongoing beta-cell destruction.

Somatostatin analogues were tested on a human endocrine pancreatic tumour cell line and cultured pancreatic islets. Somatostatin analogues had an effect on cAMP accumulation, chromogranin A secretion and MAP kinase activity in the cell line. Treatment of rat pancreatic islets with somatostatin analogues with selective receptor affinity was not sufficient to induce an inhibition of insulin and glucagon secretion. However, a combination of selective analogues or non-selective analogues via co-stimulation of receptors can cause inhibition of hormone production. For insulin and glucagon, combinations of sst_2 + sst_5 and sst_1 + sst_2, respectively, showed a biological effect.

In summary, knowledge of islet cell ssts expression and the effect of somatostatin analogues with high affinity to ssts may be valuable in the future attempts to influence beta-cell function in type 1 diabetes mellitus, since down-regulation of beta-cell function may promote survival of these cells during the autoimmune attack.

Introduction

The rodent pancreas consists of approximately 4000–5000 islets of Langerhans (1,2). Islets are composed of hormone-producing cells: insulin-secreting beta-cells (60–70%); glucagon-secreting alpha-cells (25%), somatostatin-secreting delta-cells (10%) and pancreatic polypeptide-secreting PP cells (1–5%). In recent years a new islet cell type called epsilon-cells has been identified that secretes grehlin (3). The islet core contains mainly beta-cells, while the other cell types are located in the periphery. In addition, the islets contain nerves, fibroblasts, macrophages, den-

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dritic cells and endothelial cells. The function of the endocrine cells is to produce hormones, which regulate especially carbohydrate metabolism.

Diabetes mellitus is one of the most common endocrine disorders. The disease is normally classified into two major groups, type 1 diabetes (insulin-dependent) and type 2 diabetes (non-insulin dependent). Type 1 diabetes mellitus is caused by a destruction of the pancreatic beta-cells, rendering the pancreas unable to synthesize and secrete sufficient amounts of insulin. This form of the disease has two distinct phases: insulitis, characterized by the infiltration of autoimmune cells within the islets, and diabetes, when most of the beta-cells have been destroyed and there is no longer sufficient insulin production to regulate blood glucose levels, resulting in hyperglycaemia. Type 2 diabetes is primarily caused by impairment of the ability to secrete sufficient amounts of insulin together with insulin resistance in the target tissues. Type 1 diabetes results in a lifelong requirement of insulin therapy and usually affects younger people. Type 2 diabetes is more frequently seen in older people, and is associated with obesity. However, today the disease is seen also in younger age groups. For both diabetes groups, inadequate control of blood glucose levels induces severe complications such as retinopathy, neuropathy and nephropathy.

The aim of the present study was to investigate the expression and biological roles of somatostatin receptor subtypes (ssts) in pancreatic endocrine cells. For this purpose we used somatostatin analogues and antibodies specific for each receptor. Furthermore, we wanted to study if the pancreatic islet expression of different ssts is affected during the development of autoimmune diabetes in NOD mice.

Background

Somatostatin

In 1972, Brazeau et al isolated a peptide, somatostatin, from the hypothalamus of sheep (4). Somatostatin was found to be a cyclic polypeptide with two biologically active isoforms, somatostatin-14 (Fig. 1) and the N-terminal extended version somatostatin-28. Somatostatin-14 has subsequently been found with the same amino acid sequence in almost all vertebrates groups, and seem to be the most conserved peptide identified so far in vertebrates (5). The primary structure of the N-terminal domain of somatostatin-28 has also been conserved, although to a lesser extent than somatostatin-14. The peptides' first known function was to inhibit growth hormone secretion, but today somatostatin is known to be a multifunctional peptide, located in most brain regions as well as in peripheral organs (reviewed in (6–8)). In the periphery somatostatin is produced by the delta-cells of the pancreas, where it plays an important role in the control of both insulin, glucagon, somatostatin and pancreatic polypeptide (PP) secretion (5,9). Somatostatin is also produced in the gastrointestinal tract where it controls the release of hormones, such as VIP and cholecystokinin, and influences gastric acid secretion (8). Other actions of somatostatin also include modulation of other neuropeptides and neurotransmitter system and effects on gastrointestinal motility (5).

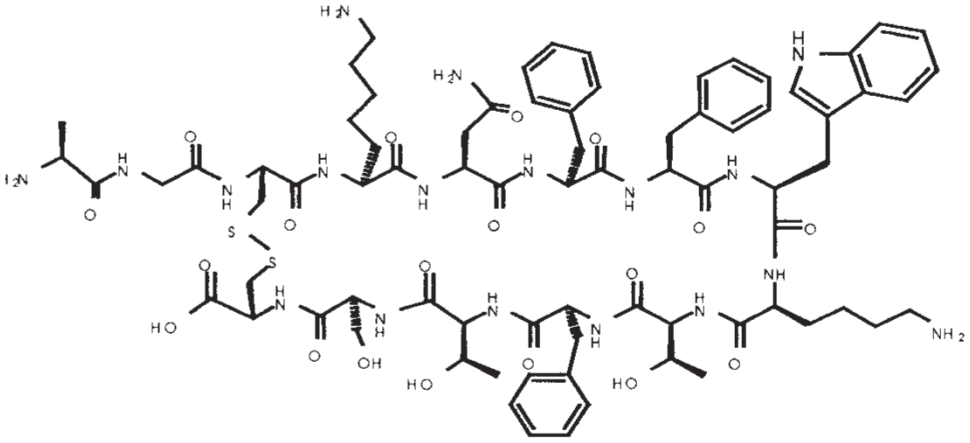

Figure 1. Structure of somatostatin-14 (48).

Somatostatin and beta-cells

Insulin secretion from the beta-cells is subject to stimulatory, modulatory and inhibitory influences. Beta-cell secretion is reduced or blocked by a variety of inhibitors, including galanin, somatostatin and noradrenaline, which reach the cells either via the islet vascular system or are released locally from sympathetic and peptidergic nerves terminating in the pancreas or by paracrine action (5,6,10). Somatostatin is considered to be an important regulator of secretion of insulin, glucagon, and PP by beta-, alpha- and PP cells, respectively (5). The pancreatic islets receive a rich vascular supply and they get up to 10% of the total pancreatic blood flow although they only constitute 1% of the tissue (11–13). Afferent blood vessels penetrate close to the centre of the islet before branching out and returning to the periphery of the islet. Based on this hypothesis, the innermost cells of the islet therefore would receive arterial blood, which upon the further blood efflux may contain insulin secretion that could affect peripheral islet cells (14–16). This implies that released somatostatin must pass through the systemic circulation before acting upon beta-cells. Other studies have shown that immunoneutralisation of islet somatostatin resulted in a stimulation of insulin secretion, suggesting that intraislet somatostatin is a direct inhibitory regulator of beta-cell secretion (11,17,18). Another explanation to the diversity of data may be the species differences in islet anatomy. In human pancreatic islets delta-cells are not only located in the periphery of the islet but also in the core. Situated close to the beta-cells the secreted somatostatin from the delta-cells may directly affect insulin release (17). In the rodents, there is a more stringent localisation of the islet cells, with beta-cells located in the centre of the islets and the alpha-, delta- and PP cells in the mantle zone (15,16). However, it is well known that hormones may act in a paracrine fashion and it is also feasible that somatostatin can be secreted and acts directly on islets cells in this manner.

Table 1. Summary of different properties for individual sst subtype. + = effect; – = no effect (5,6,22)

Properties	sst_1	sst_2	sst_3	sst_4	sst_5
Inhibition of hormones secreted from endocrine cells	–	+	–	–	+
Transfer a growth anti-proliferative signal	+	+	–	–	+
Inhibition of adenylyl cyclase activation	+	+	+	+	+
Induction of apoptosis	–	+	+	–	–
Coupling to MAP kinase activity	+	+	–	+	+
Coupling to Ca^{2+} channels	–	+	–	–	+

Somatostatin receptors

The wide spectra of cell or tissue responses to stimulation with somatostatin and the variety of physiological activities of the hormones indicated the existence of different somatostatin receptor subtypes (ssts). These ssts would be able to activate a variety of intracellular signalling mechanisms, leading to different cell responses. In 1992 the first receptor subtype was discovered (19) and so far five ssts have been cloned ($sst_{1–5}$) and named according to the order of identification (19–21). The five ssts belong to a family of G-protein coupled receptors with seven transmembrane domains. The amino acid sequences for the ssts range in size from 364 (sst_5) to 418 (sst_2) amino acids and their sequence identity varies from 39–57% between receptor subtypes (5). Greater sequence identity is seen in the transmembrane domains than in the extracellular N- and intracellular C-terminal domains. The ssts can be divided in two groups according to sequence identity, sst_1 and sst_4, and sst_2, sst_3 and sst_5, respectively. The individual ssts also display a remarkable degree of structural conservation across species. Somatostatin-14 and somatostatin-28 bind with high affinity to all five ssts. The somatostatin receptor subtypes are distinguished by their pharmacological specificity for different somatostatin analogues, their tissue distribution, their regulation, and their intracellular signalling pathways (5,6,22). Table 1 summarizes some of the properties for individual sst.

As previously mentioned, ssts are widely distributed throughout many tissues and organs. The cloning of ssts genes made subtype specific probes available, which allowed the investigation of ssts expression at the mRNA level (23–25). Although some receptors show a higher expression in some particular areas, often multiple ssts are present. Recently developed antibodies specific for the different ssts have been raised to investigate the more exact cellular distribution of ssts in various tissues by immunohistochemical methods (26–29).

The pancreas is an important target for the action of somatostatin and immunohistochemical studies on rodent pancreas have demonstrated expression of sst_2 on alpha-cells and sst_5 on beta-cells, suggesting that sst_2 regulates the glucagon secretion and sst_5 the insulin secretion (27,30). This has also been confirmed by in vitro studies using isolated mouse and rat pancreatic islets (31–34).

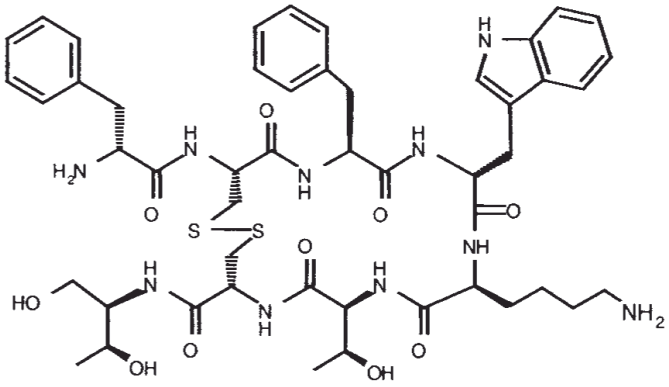

Figure 2. Structure of octreotide (48)

Somatostatin analogues

Extensive efforts have been directed toward identification of different physiological roles of ssts and intracellular transducing mechanisms coupled to each sst subtype. Somatostatin was shown to reduce the hormone related symptoms in patients with carcinoid tumours, such as flushes and diarrhoea. The natural somatostatin binds to all five ssts with high affinity, but has unfortunately a very short half-life (about 90 sec), which makes somatostatin impractical for clinical use. To avoid the problem with the short half-life, more metabolically stable somatostatin analogues have been developed. Functional studies of the structure have shown that the amino acid residues phenylalanine (Phe), tryptophan (Trp), lysine (Lys) and threonine (Thr) form a β turn and are necessary for biological activity (35,36). In this way synthetic peptides have been designed which show greater metabolical stability and, for some, pharmacological selectivity to different ssts compared to natural somatostatin.

One of the most frequently used analogues in the clinic today is octreotide (Fig. 2), first described in 1982 (37). This analogue binds to sst_2 and sst_5 with high

Table 2. Relative affinity of $sst_{1–5}$ of somatostatin and analogues; +++ denotes strong binding affinity; ++ denotes moderate binding affinity; + denotes weak binding affinity; – denotes no binding affinity to $sst_{1–5}$. Data adopted from (48,76)

Somatostatin analogues	sst_1	sst_2	sst_3	sst_4	sst_5
SST-14	+++	+++	+++	+++	+++
Octreotide	–	+++	+	–	++
SOM-230	+++	+++	+++	–	+++
BIM-23926	+++	–	–	–	–
BIM-23120	–	+++	–	–	–
BIM-23206	–	–	–	–	+++

affinity and sst_3 with moderate affinity (see Table 2). Octreotide is used to treat patients with the carcinoid syndrome, VIPomas and glucagonomas (38–41). The half-life of octreotide is about 116 minutes after subcutaneous injection and the side effects are very few.

In order to investigate the regulatory role of individual sst receptors selective somatostatin analogues have been developed and studied (42,43). However, several studies revealed that a co-stimulation of several ssts were needed in order to get the maximal hormone inhibition (42,44). This has further been supported by the observation that ssts can homo- and heterodimerize. It was shown that sst_5 dimers display different properties, such as binding affinity, ligand-induced internalization or up-regulation compared to monomers (45). Sst_5 was also observed to heterodimerize with sst_1, but not sst_4, as well with the longer form of the dopaminergic D2 receptor (45,46). Sst_2 and sst_3 can also form heterodimers when co-expressed in HEK 293 cells and this results in an inactivation of sst_3 function (47). The development of non-selective somatostatin analogues with high binding affinity for several of ssts has lead to SOM-230 that exhibit high affinity to all ssts except sst_4 (see Table 2). The SOM-230 has a half-life about five times longer than octreotide and shows prolonged inhibitory effects on growth hormone and insulin growth factor 1 (IGF-1) release (48). In similarity to octreotide, SOM-230 was found to be a well tolerated drug with the same kinds of mild side effects (48).

Somatostatin and diabetes mellitus

A new interesting field for somatostatin analogue treatment includes both obesity and the complications of diabetes mellitus, such as retinopathy, neuropathy and nephropathy.

Diabetic retinopathy is a specific microvascular complication, resulting in new vessel formation and retinal thickening. This represents the leading cause of blindness among individuals of working age (49). Ssts are expressed in the retina (50) and clinical studies have shown that octreotide is highly effective and the need for surgery was reduced in treated patients as compared to the control group (51–53). Octreotide is believed to have an effect on IGF-1 and vascular endothelial growth factor (VEGF) on retinal endothelial cells (53).

Octreotide has also been shown to reduce the weight of obese people without changes in life-style, diet or exercise. This effect was suggested to be due to the fact that octreotide could control insulin hypersecretion in obese people (54). The study could also conclude that the higher beta-cell activity was before treatment, the more weight was lost.

Clinical studies have indicated that inhibitors of insulin release will be of benefit in treatment or prevention of diabetes. Administration of octreotide to newly diagnosed type 1 diabetes patients showed that the patients go earlier and more frequently into remission and the duration was longer compared to control group. The authors suggest that the beneficial effect of octreotide can be explained by the

ability of octreotide to block insulin secretion, induce beta-cell rest and thus contribute to the recovery of beta-cells (55).

In conclusion, somatostatin analogues seems to be interesting tools for use in attempts to influence hormone producing cells during development of e.g. type 1 diabetes in which beta-cell rest could be beneficial.

NOD mouse model

As the symptoms of diabetes appear late in the process of beta-cell destruction, it is difficult to study the early phases of disease in humans and therefore animal models have been developed. In 1974, the Non-Obese Diabetic (NOD) mouse was discovered and today these animals are used frequently as an experimental model of type 1 diabetes to explore the many features of type 1 diabetes in this model that are shared with the human form. This includes a gradual development of pancreatic insulitis accompanied with hyperglycaemia (56–60). The inflammatory process in the pancreatic islets of Langerhans in the NOD mouse starts around 3–4 weeks after birth. Antigen presenting cells such as macrophages and dendritic cells appear early, followed by CD4+ and CD8+ T- and B-cells (61,62). The T-cells are likely to produce Th1-type cytokines, mainly IFN-γ, which may also be involved in the beta-cell destruction (63). Although insulitis is evenly distributed between sexes, the incidence of diabetes is much higher in females (~70%) than in males (<10%) in our colony, which is in line with incidence figures in other colonies (64). This pronounced female gender bias is not observed in humans. At an early stage, the insulitis is usually located around the islets (peri-islet), and at about 10 weeks of age, extensive infiltration of the pancreatic islets can be observed (intra-islet).

General methods

Animals

Pancreatic specimens from adult male C57BL/6J mice, adult male Sprague-Dawley rats and NOD mice were examined. Altogether 142 pancreatic glands were collected from NOD mice (95 females/47 males), ranging from 4 to 66 weeks of age. A NOD mouse was considered diabetic when the non-fasting blood glucose concentration exceeded 10 mmol/l. Moreover, the mice were collected at different occasions and among the females 31 out of 95 (33%) were diabetic on the particular days of organ collection. None of the males were diabetic. The NOD mouse strain is derived from the ICR mouse, and therefore 5 + 5 ICR mice, aged 11 and 20 weeks, were studied as non-diabetic prone controls.

All experiments were approved by the local animal ethics committee at Uppsala University, Sweden.

Cell culture

BON-1, a human neuroendocrine pancreatic tumour cell line was a kind gift from C.M. Townsend, University of Texas Medical Branch, Galveston, TX USA. BON-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) and F12K Nutrient Mixture supplemented with 10% fetal calf serum, 1% penicillin + streptomycinsulphate and 1% L-glutamine until approx 75% confluence. The cell line was kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

Islet isolation and culture

The animals were anesthetized (Mebumal 60 mg/kg) before they were killed by cervical dislocation. The abdomen was immediately opened by a transverse incision, followed by resection of the pancreas. Pancreatic islets were isolated from male Sprague-Dawley rats by collagenase digestion (65) and islets were hand-picked with a braking pipette under stereomicroscope. Islets were precultured 5–7 days before experiments in 5 ml RPMI 1640 medium containing 11.1 mM glucose, supplemented with 10% FCS, benzylpenicillin (100 U/ml) and streptomycin (0.1 mg/ml), in an atmosphere of humidified air + 5% CO_2. Cell culture medium was changed every second day.

Somatostatin analogues

The somatostatin analogues octreotide and SOM-230 were kind gifts from Novartis Pharma AG, Basel, Switzerland and SST-14 purchased from ICN Biomedicals Inc. The selective somatostatin analogues BIM-23926, BIM-23120 and BIM-23206 were synthesised and provided by Biomeasure Inc, Milford, MA, USA. The relative binding affinities for the compounds used in our experiments are shown in Table 2.

Development of antibodies against different sst subtypes

The selective ssts antibodies were originally raised against human sst receptor sequences (29). However, these sequences are similar to the corresponding sequences in mouse and rat. The peptide sequence used for sst_1 is identical in humans, rats, and mice, whereas the sequences for sst_{2-5} differ in only a few amino acids located in parts of the peptide that are considered not to interfere with the cross-reactivity of the developed antibodies for the species used in. To verify this, the specificity of the human sst antibodies on mouse and rat tissues was investigated by preincubating the sst subtype selective antibodies with the peptides used for immunisation. We were thereby able to completely block the staining for all receptors in both mice and rats.

Immunohistochemistry

Single staining with $sst_{1–5}$ antibodies

The pancreatic sections of normal mice, rats and NOD mice were stained for all five ssts. The sections were pre-treated in the microwave oven for 10 min in 50 mM Tris-HCl (pH 8.0). Non-specific binding of secondary antibody was blocked by incubation for 30 min with normal goat serum. Incubations of the primary antibodies against sst_1, sst_2, sst_3, sst_4 and sst_5, respectively, were performed at 4°C in a humidified chamber. The secondary antibody, biotinylated goat $f(ab')_2$ anti-rabbit IgG, was applied to the tissues for 45 min. The immune reaction was amplified by an avidin-biotin complex coupled to alkaline phosphatase and visualised with Vector Red as substrate. Finally, the tissues were counterstained with Mayer's hematoxylin and permanently mounted with Mountex and coverslips.

We performed a dilution series for each ssts antibody to find the most suitable concentration. However, it should be noted that a negative staining does not exclude the possibility of expression of the receptor below the detection level of the staining protocol.

Double fluorescence staining of $sst_{1–5}$ and islet cells

The pancreatic sections were simultaneously stained for each of the five ssts together with insulin, glucagon, somatostatin, or PP. The sections were pre-treated in the microwave oven for 10 min in 50 mM Tris-HCl (pH 8.0). Non-specific binding of secondary antibody was blocked by incubation for 30 min with normal donkey serum. Each of the rabbit anti-$sst_{1–5}$ antibodies was mixed with chicken anti-insulin, chicken anti-glucagon, sheep anti-somatostatin or sheep anti-PP antibodies. The sections were incubated over night at 4°C in a humidified chamber. The pancreatic specimens were incubated a cocktail consisting of the secondary antibodies Cy3-conjugated anti-rabbit IgG + Cy2-conjugated donkey anti-sheep IgG or Cy2-conjugated donkey anti-chicken IgG for 45 min. The tissues were mounted with mounting medium containing TBS and glycerol and coverslips.

Light microscope evaluation

Intensity of the single ssts staining was scored semiquantitatively: – = negative staining; + = weak positive staining; ++ = positive staining; +++ = strong positive staining. Also the location of the positive staining of ssts were scored (centre/periphery), referring to whether the ssts was distributed at the centre of the islet or only in the periphery.

The double immunofluorescence sections were examined in a Leica Leitz DMR fluoroscence microscope equipped with filters of 492 nm to 510 nm for Cy2 (green) and 550 nm to 570 nm for Cy3 (red). Pictures from a Zeiss Axiocam camera of each pancreatic islet, using both filters, were merged together with Adobe Photoshop 7.0 software, in which a yellow colour indicated co-expression of ssts with any of the four islet hormones tested. The results were expressed as a percentage of ssts-positive cells in relation to the total number of the respective islet cell type in a specific pancreatic islet.

Cyclic AMP content in BON-1 cells

BON-1 cells were plated in 48 well dishes until sub confluence at a density of 7.5 x 10^4 cells per well. The cells were incubated at 37°C for 30 min with 45 mM isobutylmethylxanthine before adding 10 mM forskolin in the absence or presence of somatostatin analogues in increasing concentrations for 30 min. The cells were washed twice with ice-cold PBS. After isopropanol extraction, intracellular cAMP levels were quantified by a commercial radioimmunoassay kit according to the instructions of the manufacturer.

CgA secretion in BON-1 cells

BON-1 cells were plated in 35 mm diameter dishes at a density of 7.5 x 10^5 cells per dish and incubated at 37°C until sub confluence. The cells were serum deprived for 1 h at 37°C before adding somatostatin analogues in increasing concentrations for 24 h. CgA measurements on the cell supernatant were performed with a RIA as previously described (66).

MAP kinase activity in BON-1 cells

BON-1 cells were plated in 60 mm diameter dishes at a density of 7.5 x 10^5 cells per dish and incubated at 37°C until sub confluence. After 18 h of serum deprivation the cells were incubated at 37°C with 0.01 mM cholecystokinin-8 (CCK-8) in the absence or presence of somatostatin analogues in increasing concentrations for 10 min. The cells were washed twice with ice-cold PBS and lysed with lysis buffer (50 mM TrisHCl, 150 mM NaCl, 10% glycerol and 0.5% Triton X-100, pH 8.0) with protease inhibitors tablets. After a 15 min incubation at 4°C, the lysate was collected and centrifuged at 13 000*g for 10 min at 4°C to remove insoluble material. The samples were separated by Western Blotting technique with a primary antibody against phospho-p42/p44 MAP kinase. A secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG was used followed by ECL detection. Estimation of relative molecular size was performed using a Rainbow coloured protein molecular weight marker (14.3–220 kDa). The Western blots were analysed by BioRads GS-710 and the data were generated using BioRads computer programme Quantity One.

Insulin and glucagon accumulation and secretion in pancreatic islets

After the pre-culture, pancreatic islets in groups of 50 were transferred to flat bottom multi-well plates containing 2 ml RPMI 1640 supplemented with 11.1 mM glucose and incubated with or without addition of the six somatostatin analogues alone or in combination (10^{-9}–10^{-6} M). After 48 h, samples were taken from the culture medium for insulin and glucagon determinations by ELISA and RIA, respectively. After culture, the islets were incubated short-term in the absence of somatostatin analogues. Triplicates of ten islets from each experimental group were transferred to glass vials containing 250 µl KRBH buffer supplemented with 16.7 mM glucose and 2 mg/ml bovine serum albumin. After 1 h the medium was removed, the islets retrieved, homogenised in water and insulin extracted in acid ethanol for assess-

ments of islet insulin content. The insulin and glucagon levels were determined as described above.

In a separate study, experiments were designed to examine if putative stimulatory or inhibitory effects could be reversible. Islets in groups of 50 were cultured with or without addition of the six somatostatin analogues in a concentration of 10^{-7} M. After 48 h, samples were taken from the culture medium for insulin and glucagon determinations. Islets were then incubated for 1 h as described above at 16.7 mM glucose, or cultured further for another 3, 6, 24 or 48 h in the absence of somatostatin analogues, and subsequently tested during 1 h incubations.

Statistical analysis

The statistical analysis on the immunohistochemistry studies were either performed by comparing groups of data with Student's t-test or performed by comparing groups of data by the Fisher's exact test. It is important to note that the number of animals varied considerably in different groups. The somatostatin analogues SST-14, octreotide and SOM-230 and the three selective BIM-analogues the statistical analysis was performed by either the non-parametric Mann-Whitney test or Student's *t*-test. A P–value < 0.05 was regarded significant.

Results and discussion

Staining with $sst_{1–5}$

Pancreatic islets of normal mice and rats

By single-staining with selective ssts antibodies we found that all ssts were expressed in the pancreatic islets of normal mice and rats (67). However, the staining intensity and distribution varied among ssts and species. By performing double fluorescence staining on pancreatic islets of the same animals we were able to observe the co-expression of the five ssts in beta-, alpha-, delta- and PP-cells. Sst_1, sst_2, and sst_5 were expressed in a majority of all beta-cells in the mouse, while only half of beta-cells co-expressed the same receptor subtypes within the rat pancreas. The fraction of cells displaying co-localisation of sst_3 and sst_4 in beta-cells did not significantly differ between species. The co-expression of ssts and glucagon in alpha-cells were found to be similar in mice and rats, with sst_2 and sst_5 being expressed in 70%, while sst_3 and sst_4 were only expressed in 20%. The expression of sst_1, sst_2 and sst_5 in of delta-cells were about the same in both species. However, the rat pancreas had a somewhat stronger co-expression of sst_3 and sst_4 than the mouse. PP cells stained together with $sst_{2–5}$ revealed a nearly 60% co-expression in islets of the mice, while only 15% of the PP-cells in the rat expressed ssts.

When comparing the degree of co-localization of ssts with the various cell types, we found intriguing differences between the two species. Several reports have demonstrated that inhibition of insulin secretion is mediated via sst_5 in rodents (32,68), which is in line with its high expression. It has been shown that sst_1 and sst_5 het-

erodimerize in cells upon stimulation (45), this may explain why sst_1 and sst_5 are expressed in mouse islets to almost the same degree. An interesting possibility in this context is that receptor-positive and receptor-negative cells may represent two different functional populations of beta-cells. Therefore, the former would be responsive to somatostatin-induced inhibition of insulin secretion, whereas the latter cells would not be affected by the hormone.

We found that sst_4 was the receptor subtype showing the most extensive expression in delta-cells in mouse and rat pancreatic islets. The significance of this finding is unclear because this receptor is the least studied and very little is known about its function in the pancreas. The role of the highly expressed sst_3 in delta-cells in rat pancreas is also unknown. Furthermore, only about 35% of the delta-positive cells expressed sst_5. It may be suggested that sst_2 and/or sst_1, rather than sst_5, are involved in the inhibition of somatostatin secretion in the pancreas.

The most "conserved" islet cell type was the alpha-cell, with a fairly similar co-expression pattern in the two species for all five ssts. It has been reported that sst_2 is the main inhibitor of glucagon secretion in mouse and rat (31,69), but the results from the present study may suggest that the sst_2 and sst_5 act together to contribute to the inhibitory effect. Using sst_2- and sst_5-selective analogues in an in vitro study of glucagon secretion may elucidate this possibility.

Pancreatic islets of normoglycemic and diabetic NOD mice

In this study we investigated the distribution and expression of $sst_{1–5}$ in NOD mouse islets in relation to age, sex and rank of insulitis (70).

With increasing age and presence of diabetes an increased fraction of mice showed positive sst_3, sst_4 and sst_5 immunostaining. To rule out that this was merely an affect of age we also studied age matched non-diabetic prone ICR controls, which the NOD mouse is derived from. Staining for $sst_{1–5}$ in the pancreatic islets of ICR of different ages revealed no differences in the expression pattern, suggesting that the observed alterations in ssts depend on the progression of disease and/or extent of insulitis rather than increasing age.

Since the female NOD mice have a higher risk of developing diabetes than males, we wanted to compare normoglycemic female and male NOD mice to see if there were any differences in ssts expression. No such alteration was found, suggesting that the difference in diabetic animals is due to pathogenesis and/or symptoms of type 1 diabetes and not due to a gender difference.

The sst_1 receptor was observed to be expressed mainly in the centre of the islet. Most beta-cells in C57BL/6 mice were shown to be sst_1 positive, but in the NOD mice only 35% of the normal females and 5% of the diabetic mice co-expressed sst_1 on beta-cells. Moreover, the occurrence of insulitis led to a lower ssts expression on beta-cells compared to animals without invading immune cells. This finding suggests that the physiological regulation of sst_1 in beta-cells is more influenced by the autoimmune inflammation of the pancreatic islets than by the hyperglycaemia/diabetes.

About half of the normoglycemic females and males in this study expressed sst_2

in their islets, whereas more than 80% of the diabetic animals displayed sst_2 expression. We observed that some of the normal mice expressing sst_2 showed a more pronounced peripheral immunostaining of the islet. As mentioned earlier, glucagon secretion may be regulated by sst_2 in mice (31), which may explain a higher expression of sst_2 in glucagon positive cells compared to other islet cells. This is in line with our observations showing that expression of sst_2 in alpha-cells was close to 100% in diabetic animals and 73% in normoglycemic NOD mice. A peripheral islet cell expression pattern, however, was not seen in the diabetic NOD mice, which might be due to the fact that alpha-cells were more centred within the islets in the diabetic NOD mice.

In the diabetic animals no beta-cells expressing sst_2 were found, which could suggest that a somatostatin-induced inhibition of insulin release may not be possible to achieve in the hyperglycaemic mice. This would facilitate insulin secretion when insulin is lacking. Furthermore, this notion is corroborated by the observation that beta-cell expression of sst_5 decreased in the diabetic females, since the latter receptor subtype is important for inhibition of insulin secretion. The importance of increased expression of sst_2 in delta- and PP-cells of diabetic animals is unclear. Insulitis itself did not affect alpha- and beta-cell expression of sst_2, whereas it reduced the expression of this receptor in delta- and PP-cells.

We found a higher frequency of animals expressing sst_2 in pancreatic islet cells with infiltration of immune cells. Since somatostatin is able to inhibit alpha- and beta-cell function this might down-regulate glucagon secretion during a condition of increasing hyperglycaemia and render the remaining beta-cells less vulnerable to cytokine induced destruction during the development of insulitis. Thus, the increased expression of islet sst_2 receptors may reflect a defence mechanism by the islet cells against the secreted cytokines (71).

The expression pattern for sst_3 was similar to sst_1, but was only detected in about 25% of the pancreatic islets of the normal female NOD mice. However, the expression of sst_3 was observed in about 60% of the islets of the diabetic animals. In comparison, normal mouse islets have been reported to only express sst_3 in a minority of the islet cells (67). The expression seemed to be higher in diabetic animals compared to normal females in pancreatic sections with less islet inflammation i.e. rank B. The expression of sst_3 on alpha-cells was found to be about 60% in diabetic NOD mice compared to 10% in normoglycemic. However, animals without insulitis had also a high expression of sst_3 on alpha-cells. In delta-cells almost half of the cells expressed sst_3, while the PP cells in diabetic animals did not express sst_3 at all in diabetic animals. This may suggest that sst_3 expression is affected by the diabetes progression, but not by the influence of infiltrating immune cells. The function of sst_3 in pancreatic islet cells is presently unknown and needs further investigation.

About 10% of the normal female NOD mice were found positive for islet sst_4 expression. More diabetic animals compared to normoglycemic NOD mice showed expression of sst_4. The distribution of the receptor was similar to sst_1 and sst_3. Normal mouse islet cells have been reported to express sst_4 to a lower degree (67).

Interestingly, the receptor was co-expressed in 50–80% in alpha-, delta-, and PP-cells in diabetic NOD mice, and not at all in beta-cells. However, sst_4 is the receptor subtype, that has been studied the least and the function of sst_4 in islets cells is essentially unknown.

About 50% of the normal females and 85% of diabetic NOD mice were found immunoreactive for sst_5 in their islets. This receptor expression correlated to increasing age and insulitis score B. A more pronounced peripheral immunoreactivity in the islets, as for sst_2, was also observed in a majority of normal females. However, unlike sst_2, this staining pattern was detected in a third of the diabetic females. Sst_5 was found, together with sst_2, to be expressed in 100% of alpha-cells in diabetic animals. The immunofluorescence study on diabetic animals showed 11% expression of sst_5 on beta-cells compared to 61% in normoglycemic animals in the same age group. Our results may indicate that sst_5 is involved in several other functions besides inhibition of insulin secretion in the pancreatic islets, for example inhibition of glucagon release.

In conclusion, the changed $sst_{2–5}$ expression in the islets cells of diabetic mice may suggest either that ssts are a contributing factor to, or a defense response against, ongoing beta-cell destruction and hyperglycemia. We also found that the islet architecture and co-expression of ssts with islet hormones were altered in diabetic animals. The present investigation of sst subtype expression in islet cells of NOD mice at different stages of disease may provide useful information for understanding islet function, and if attempts are performed to modulate progression of type 1 diabetes by somatostatin analogues.

Cyclic AMP content, CgA secretion and MAP kinase activity in BON-1 cells

The forskolin induced cAMP accumulation in BON-1 cells in the presence of somatostatin analogues was measured (43). It was found that the selective sst_2 an sst_5 agonists inhibited cAMP accumulation in a dose-dependent manner. This was also seen for the natural ligand SST-14, which was the drug that inhibited cAMP the most, by about 80% at 10^{-6} M. Octreotide and the analogue selective for sst_1 failed to inhibit cAMP accumulation.

Previously, the effect of the somatostatin analogue PTR-3173, which binds to sst_2, sst_4, and sst_5 was studied in BON-1 cells (72). In accordance to our results PTR-3173 inhibited cAMP accumulation. Moreover, the incubation of BON-1 cells with the selective sst_2 and sst_5 also revealed inhibition of cAMP in a dose-dependent manner, but not as strong as for SST-14. We may hereby conclude the inhibition of cAMP is mainly regulated by sst_2 and sst_5 and that a co-stimulation of different ssts may be needed.

Secretion of CgA from BON-1 cells was inhibited at some concentrations by all five tested somatostatin analogues (43). Again, sst_2 and sst_5 selective agonists and SST-14 inhibited the secretion in a dose-dependent manner. Since the selective agonists also inhibited cAMP accumulation at the same concentrations, this may indicate that for these receptors, the adenylyl cyclase-cAMP pathway is coupled to the hormone secretion pathway. Thus, these results support the notion that both sst_2

and sst_5 are important receptor subtypes in inhibiting secretion of hormones from endocrine cells.

The cholecystokinin-8 induced MAP kinase activity in BON-1 cells in the presence of somatostatin analogues was measured (43). It was found that the selective analogue for sst_1 activated the MAP kinase, while sst_2 selective agonist inhibited the same. The analogue selective for sst_5 occasionally inhibited MAP kinase, and no effect was observed in BON-1 cells incubated with octreotide or SST-14.

The activation of MAP kinase by BIM-23026 (sst_1-selective) and inhibition of the same by sst_2 and sst_5 were in line with other studies (42,73,74). The incubation of BON-1 cells with SST-14 revealed no effect on MAP kinase activity. This may be explained by a conflict of interest since SST-14 binds to all five ssts and sst_1 was found to activate MAP kinase and both sst_2 and sst_5 inhibited MAP kinase. Furthermore, we also observed a U-shaped curve in the MAP kinase activity assay when the BON-1 cells were incubated with BIM-23206 (sst_5-selective). This may indicate that there is a concentration optimum for the analogue and that a higher concentration is not always more efficient.

In summary, somatostatin analogues selective for sst_2 and sst_5 had an inhibitory effect on cAMP accumulation, CgA secretion and MAP kinase activity in BON-1 cells, while the analogue with high affinity for sst_1 inhibited the CgA secretion but stimulated the MAP kinase activity. SST-14 inhibited cAMP production and CgA secretion, while octreotide had an inhibitory effect on CgA secretion.

Insulin and glucagon accumulation and secretion in pancreatic islets

Effects of long-term exposure to somatostatin analogues

When the rat pancreatic islets were cultured with somatostatin analogues for 48 h, the insulin and glucagon accumulation into the culture medium was measured (75). It was observed that SST-14 and octreotide inhibited insulin accumulation in a dose-dependent manner and SOM-230 had the strongest inhibitory effect. On the other hand, the selective sst_2 analogue mediated an increased medium insulin accumulation, while the other selective analogues tested failed to affect insulin accumulation. After the removal of the analogues and the following 1 h incubation at 16.7 mM glucose, islets pre-incubated with octreotide and SST-14 were found to show stimulated insulin secretion, while islets pre-incubated with SOM-230 continued to have an inhibition on the release. The removal of the selective somatostatin analogues showed no differences compared to control islets, although in many groups the mean values increased. The insulin content was elevated in islets treated with octreotide, SST-14 and SOM-230, while the content was inhibited by BIM-23926 (sst_1-selective). No consistent effects of any of the analogues tested were seen concerning the glucagon accumulation in the culture medium.

Effects of long-term exposure with combinations of selective analogues

Since we observed that the analogues with binding affinity to several ssts inhibited insulin secretion, while the selective somatostatin analogues had a tendency

to stimulate the hormone release, combinations of the selective analogues were studied (75). It was found that the combination the analogues selective for sst_2 + sst_5, respectively, inhibited the medium insulin accumulation. The combination of sst_1 + sst_2 selective analogues had a tendency to inhibit insulin accumulation. After removal of the analogues, no differences were observed in insulin secretion when comparing the treated islets to control islets. The insulin content in the islets was higher in many of the groups of pancreatic islets treated with the two analogue combinations. The combination of sst_1 + sst_2 analogues caused a dose-dependent decrease in medium glucagon accumulation.

Comparing our present data it appears that in order to obtain inhibitory effects more than one of the sst receptor subtypes must be activated. This is achieved by SST-14, octreotide and SOM-230. However, when the selective analogues were used alone, if anything, a stimulatory action on hormone accumulation was frequently observed for the sst_2 (BIM-23120) and sst_5 (BIM-23206) analogues. The results using combinations of the ssts agonists suggest that a combination sst_2 + sst_5 agonists mediates inhibition of insulin secretion, whereas a combination of sst_1 + sst_2 agonists mediates inhibition of glucagon secretion.

Reversible effects after long-term exposure to SST-14, octreotide or SOM-230

Since we observed signs of a rebound effect on insulin secretion of the pancreatic islets after removal of the somatostatin analogues we aimed to investigate if such an effect was reversible (75). It was shown that the insulin secretion was elevated up to 6 h after withdrawal of SST-14 and after that the insulin levels returned back to control levels. SOM-230 inhibited the insulin release for about 3 h after drug removal and returned to control values afterwards. The effects of octreotide were already reversed at time point 0 h. Insulin content in the islets remained higher compared to control islets for 6–24 h after culture with SST-14 and SOM-230. The glucagon secretion showed an initial decline after culture with octreotide, while the other substances did not exert any changes during the 48 h follow-up.

It remains to be investigated which intracellular signalling pathways are affected by prolonged exposure to octreotide and SST-14 leading to an enhanced insulin response. In contrast to octreotide and SST-14, an inhibition of glucose-stimulated insulin release remained after withdrawal of SOM-230. Based on these findings we investigated whether the alterations in insulin secretion were reversible or not after the culture with octreotide, SST-14 and SOM-230 had been discontinued. It can be envisaged that during this recovery period various adaptations in gene expression and protein translations take place in the islet cells of importance for both regulation of insulin synthesis and secretion.

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