2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents/chemicals

APS	Roche, (Mannhein, Germany)
β-mercaptoethanol	Merck (Darmstadt, Germany)
Blotting Grade Blocker Non-Fat Dry Milk	BioRad Laboratories Inc., (Hercules, CA)
Chloroform	Merck (Darmstadt, Germany)
DEPC-treated Water	Ambion (Huntingdon, UK)
D-Glucose	Sigma Aldrich (St. Louis, MO)
Dispase II	Roche, (Mannhein, Germany)
DMSO	Sigma Aldrich (St. Louis, MO)
DMEM	Sigma Aldrich (St. Louis, MO)
DMEM without Phenol Red	Sigma Aldrich (St. Louis, MO)
EDTA	Sigma Aldrich (St. Louis, MO)
Exendin-3 (9-39)	BACHEM Inc. (Budendorf, Switzerland).
Exendin 4	Sigma-Aldrich (St. Louis, MO)
High-fat-diet 60 kcal% fat	Research Diets, Inc., Denmark
Tween-20	Sigma Aldrich (St. Louis, MO)
MgSO4	Sigma Aldrich (St. Louis, MO)
Somatostatin-14	BioTrend, Chemikalien GmBH (Cologne, Germany)
SDS	SERVA Electrophoresis, (Heidelberg, Germany)
Forskolin	BioTrend, Chemikalien GmBH (Cologne, Germany)
Liberase RI	Roche Boehringer-Mannheim (Indianapolis, IN)
Tolbutamide	Sigma Aldrich (St. Louis, MO)

Tris-HCl	Carl Roth (Karlsruhe, Germany)
TEMED	BioRad Laboratories Inc., (Hercules, CA)
Trizol reagent	Invitrogen, Carlsbad, CA)
Poly-L-Lysine Hydrobromide	Sigma Aldrich (St. Louis, MO)
L-Arginine HCL	Sigma Aldrich (St. Louis, MO)
Protease K	Roche, (Mannhein, Germany)
HBSS ’	Invitrogen (Karlsruhe, Germany)
HEPES Buffer 1M	Invitrogen (Karlsruhe, Germany)
RPMI 1640 with HEPES	PAA laboratories (Pasching, Austria)
Trypsin-EDTA	PAA laboratories (Pasching, Austria)
L-Glutamine 200 mM	Invitrogen (Karlsruhe, Germany)
Na-Pyruvate MEM (100 mM)	Invitrogen (Karlsruhe, Germany)
Tris/Glycine/SDS 10X Buffer	BioRad Laboratories Inc., (Hercules, CA)
Laemmli Sample Buffer	BioRad Laboratories Inc., (Hercules, CA)
Complete, EDTA-free Protease Inhibitor Tablets	Roche (Mannhein, Germany)
PMSF	Roche (Mannhein, Germany)
SNX-482	ICS- (München, Germany)
FURA 2-AM	Sigma-Aldrich (St. Louis, MO)
Methanol	Merck (Darmstadt, Germany)
Water, Double Processed Cell Culture	Sigma-Aldrich (St. Louis, MO)
FBS South American (CE)	Invitrogen (Karlsruhe, Germany)
PBS (10X, without Ca^{++} and Mg^{++})	Invitrogen (Karlsruhe, Germany)
Glucagon (AA 1-29) [Des-His1,Des-Phe6,Glu9]	BioTrend, Chemikalien GmBH (Cologne, Germany)
DPX	Sigma-Aldrich (St. Louis, MO)

Agarose (electrophoresis grade)	SERVA Electrophoresis, (Heidelberg, Germany)
Ethanol	CARL Roth, (Karlsruhe, Germany)
Ethidium bromide solution (10mg/ml)	Carl Roth (Karlsruhe, Germany)
Ethylenediamine tetra-acetic acid	Sigma-Aldrich (St. Louis, MO)
Fetal calf serum (FCS)	Gibco (Karlsruhe, Germany)
NaCl	Merck (Darmstadt, Germany)
NaOH	Merck (Darmstadt, Germany)
KOH	Merck (Darmstadt, Germany)
$NaHPO4.2H_2O$	Merck (Darmstadt, Germany)
NaHCO3	Merck (Darmstadt, Germany)
Nitrocellulose membrane	(Amersham Biosciences, Freiburg, Germany)
RPMI 1640 medium	PAA laboratories (Pasching, Austria)
Sodium citrate	Merck (Darmstadt, Germany)
Sodium DL-lactate	(Sigma, Aldrich) (St. Louis, MO)
Sudan IV solution	Waldeck GmbH, (Münster, Germany)

2.1.2. PCR reagents and molecular weight markers

10X PCR buffer (with 15 mM $MgCl_2$)	Applied Biosystems (Darmstadt, Germany)
50 bp DNA ladder	Invitrogen (Karlsruhe, Germany)
100 bp DNA step ladder	Invitrogen (Karlsruhe, Germany)
Superscript	Invitrogen (Karlsruhe, Germany)
IQ Syber Green Supermix	Bio Rad Laboratories (München, Germany)
DNase I	(Ambion Inc., Austin,TX)
DNase set	(Sigma, Aldrich) (St. Louis, MO)
SUPERase-In	Ambion (Huntingdon, UK)

PCR clean-up columns	(Qiagen Inc., Valencia CA)
HotStarTaq DNA Polymease	Qiagen (Düsseldorf, Germany)
TaqMan Universal MasterMix	Applied Biosystems, (Darmstadt, Germany)
ABi-Prism Optical Tubes	Applied Biosystems, (Darmstadt, Germany)
FG,Optical Caps	Applied Biosystems, (Darmstadt, Germany)
Ampli *Taq* DNA polymerase	Applied Biosystems (Darmstadt, Germany)
Taq DNA Polymerase	Qiagen (Düsseldorf, Germany)
dNTP-Set 1	Carl Roth (Karlsruhe, Germany)
Dithiothreitol (DTT)	Invitrogen (Karlsruhe, Germany)
Gel loading dye	Sigma (Deisenhofen, Germany)
Low mass DNA ladder	Invitrogen (Karlsruhe, Germany)
ECL western markers	Amersham Biosciences (Freiburg, Germany)

2.1.3. Primary Antibodies

Akt	New England Biolabs GmbH, (Frankfurt am Main, Germany)
pAKT Ser 473	New England Biolabs GmbH, (Frankfurt am Main, Germany)
pAkt Thr 308	New England Biolabs GmbH, (Frankfurt am Main, Germany)
CREB	New England Biolabs GmbH, (Frankfurt am Main, Germany
p-CREB(Ser133)	New England Biolabs GmbH, (Frankfurt am Main, Germany)

FKHR	New England Biolabs GmbH, (Frankfurt am Main, Germany)
p-FoxO1 (Ser 256)	New England Biolabs GmbH, (Frankfurt am Main, Germany)
p-FoxO1 (Thr24) /FoxO3a (Thr32)	New England Biolabs GmbH, (Frankfurt am Main, Germany)
GAPDH (14C10)	New England Biolabs GmbH, (Frankfurt am Main, Germany)
Glycogen Synthase (p-S640)	Abcam Abcam Inc. (Cambridge, MA)
GP (Glycogen phosphorylae)	(Gift from Prof. Dr. B. Hamprecht, University of Tübingen, Germany)
GSK-3	New England Biolabs GmbH, Frankfurt am Main, Germany
PDK1	New England Biolabs GmbH, (Frankfurt am Main, Germany)
p-PDK1(Ser241)	New England Biolabs GmbH, (Frankfurt am Main, Germany)
PHKA2	ABNOVA GmbH (Heidelberg, Germany)

2.1.4. Antibodies used for immunostaining of tissues

Polyclonal rabbit Anti-human glucagon Ab	DakoCytomation, (Hamburg, Germany)
Mouse monoclonal anti-human insulin Ab	Biotrend, (Kölln, Germany)
Cy3-labeled goat anti-rabbit IgG Ab	Dianova, (Hamburg, Germany)
POD-labeled goat anti-mouse IgG Ab	Vector Labs, (Burlingame, CA)
SSTR2 and SSTR3 Ab	Gift from Dr. Stefan Schulz, Magdeburg, Germany

Table 4. Dilution representation of primary and secondary antibodies

Antibody	**Source**	**Dilution of primary Ab.**	**Dilution of secondary Ab.**	**Blocking solution**	**Size of specific signal (kda)**
Akt	Rabbit (pAb)	1:1000	1:5000	2.5% milk	60
p-Akt Thr 308	Rabbit (pAb)	1:1000	1:5000	2.5% milk	60
p-Akt Ser 473	Rabbit (pAb)	1:1000	1:5000	2.5% milk	60
CREB	Rabbit (pAb)	1:1000	1:2000	5% milk	43
p-CREB	Rabbit (pAb)	1:1000	1:2000	5% milk	43
FKHR	Rabbit (pAb)	1:1000	1:5000	2.5% milk	78-82
p-FOXO1(Ser)	Rabbit (pAb)	1:1000	1:5000	2.5% milk	82
p-FOXO1(Thr24)/FoxO3a(Thr32)	Rabbit (pAb)	1:1000	1:5000	2.5% milk	78 to 82, 95
GAPDH	Rabbit (mAb)	1:1000	1:5000	2.5% milk	37
GSK3β	Rabbit (pAb)	1:1000	1:5000	2.5% milk	46
GS	Rabbit (pAb)	1:1000	1:2500	2.5% milk	93
PhK	Mouse (pAb)	1:5000	1:5000	5% BSA	136
PDK1	Rabbit (pAb)	1:1000	1:5000	2.5% milk	58-68
p-PDK1	Rabbit (pAb)	1:1000	1:5000	2.5% milk	58-68
GP	Rabbit	1:500	1:5000	2.5% milk	97

Table 5. Description of secondary antibodies

Secondary Antibodies	Source
Anti-rabbit IgG HRP-linked Ab	New England Biolabs GmbH, Frankfurt am Main, Germany
anti-mouse IgG HRP-linked Ab	New England Biolabs GmbH, Frankfurt am Main, Germany

Table 6. Primer sequence used in the real-time PCR and qualitative RT-PCR

All the primers have been ordered from TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany

Gene/transcript (abbreviation)	Forward	Reverse
h-SSTR1	cgaaatgcgtcccagaacgg	ggtttactaccttggccacg
h-SSTR2	tgacagtcatgagcatcgac	gcaaagacagatgatggtga
h-SSTR3	tcatctgcctctgctacctg	gagcccaaagaaggcaggct
h- SSTR4	atcttcgcagacaccagacc	atcaaggctggtcacgacga
h-SSTR5	cgtcttcatcatctacacgg	ggccaggttgacgatgttga
h-Glut1	catgtccttccagtatgtgg	gtcaggtttggaagtctcat
h-Glut2	gtacaatgacagaagataag	tgctactaacatggctttga
h-Glucokinase (GK)	tcactgtgggcgtggatgg	accgaaaaactgagggaagagg
h-Acetyl-CoA carboxylase (ACC)	ctgctcgtggatgaaccagac	tgtcagccatcgcccgagc
h-G6-Pase	ttcagccacatccacagcatc	ggggtttcaaggagtcaaagacg
h-Fatty acid synthase (FAS)	acagggacaacctggagttct	ctgtggtcccacttgatgagt

r-SSTR2	gggagccaagtgtggatacct	accgcgttgcttgtcatgt
r-SSTR3	atggccgctgttacctatcct	gctagtgccagcagatgcatt
r-Insulin	tgtggggaacgtggtttctt	ggtgcagcactgatccacaat
r-Actin	tctgtgtggattggtggctcta	ctgcttgctgatccacatctg
r- Foxo	acgtgcattccctggtgtat	tcattgtggggaggagagtc
m-SSTR1	tctggatctcaccttccacct	ccatcagtttcctgttgctgt
m-SSTR2	gaggcctttcccctagagtt	caccgtaacgcttgtcctt
m-SSTR3	tccaagaagccaccagctaa	aacatcgaagggagcattga
m-SSTR4	tctgcatcgtcctggcttt	cttggccagttcctgtttcc
m-SSTR5	tggtctttgggaaggtgaaag	tgtccacagtcggaaatggt
m-Lipoprotein lipase (LPL)	agtagactggttgtatcggg	agcgtcatcaggagaaagg
m-Glycogenphosphorylase (GP)	tgaacactatgcgcctctgg	ccgacattaaagtcttgaaggttaaa
m-SREBP	gcggttggcacagagctt	ggacttgctcctgccatcag
m- PEPCK	ggcggagcatatgctgatcc	ccacaggcactagggaaggc
m-G6-Pase	tcaacctcgtcttcaagtggatt	gctgtagtagtcggtgtccagga
m-Actin	aggtcatcactattggcaacga	cacttcatgatggaattgaatgtagtt
Hamster-proglucagon	tccaggatgttgataagataacaactt	cacagaagaataatctcgctgctac
Hamster-Actin	tgctgaccggatgcagaa	tcaggaggagcaatgatcttga
Myco-1	gggagcaaacaggattagataccct	tgcaccatctgtcactctgttaacctc

H = human; m = mouse; r = rat

2.1.5. Buffers

Buffer A for *in vitro* experiments with isolated human hepatocytes (pH 7.4)

117.6 mmol/l NaCl

5.4 mmol/l KCl

0.82 mmol/l Mg_2SO_4

1.5 mmol/l KH_2PO_4

20 mmol/l HEPES

9 mmol/l $NaHCO_3$

0.1% BSA

2.25mM $CaCl_2$

TNES Buffer

10 mM Tris, (pH = 7.5)

400 mM NaCl

100 mM EDTA

0.6 % SDS

TNA Buffer

50 mM Tris-Cl (pH = 7.0)

150 mM NaCl

0.1 % Natriumazide

0.1 % NP-40

3x Detergent mix

50 mM Tris Cl (pH = 7.0)

150 mM NaCl

0.1 % Natriumazide

3 % NP-40

1.5 % Natriumdesoxycholate

0.3 % SDS

KRB-Buffer (pH 7.4): For 1000 ml

6 8ml of 2 M NaCl

4.7 ml of 1 M KCl

10 ml of 100 mM $CaCl_2$

60 ml of 20 mM $MgSO_4$

1.2 ml ofl MKH_2PO_4

5 ml of 1 $MNaHCO_3$

10 µl of 1 M HEPES

5 g/l BSA

Cell lysis buffer: For 1 ml

900 µl Loading buffer

50 µl β- mercaptoethanol

10 µl100 mM PMSF (serine protease inhibitor such as chymotrypsin, trypsin, and thrombin, and the cysteine protease papain

40 µl25 x protease inhibitor (PI) Mixture of several protease inhibitors for the inhibition of serine, cysteine.

5X TBE electrophoresis buffer: For 1000 ml

54 g Tris base

27.5 g Boric acid

20 ml of 0.5 M EDTA (pH 8.0)

2.1.6. Kits

BCA protein assay kit	(Pierce, Rockford, Illinois, USA)
cAMP, Biotrack EIA System	Amersham Biosciences (Freiburg, Germany)
Onestep RT-PCR kit	Qiagen (Düsseldorf, Germany)
RNeasy mini kit	Qiagen (Düsseldorf, Germany)
Glucagon RIA kit	DPC Biermann (Bad Nauheim, Germany)
Insulin (Human) ELISA kit	DRG Instruments GmBH (Marburg, Germany)

Insulin (Rat) ELISA kit	DRG Instruments GmBH (Marburg, Germany)
LINCOplex kit	Linco Res. Inc., (St. Charles, MI)
Free fatty acids colorimetric assay kit	Roche Diagnostics, (Manheim, Germany)
Plasma Triglycerides colorimetric assay kit	Roche, (Nutley, NJ)
Hepatic triglyceride colorimetric assay kit	(Cypress Diagnostics, Langdorp, Belgium)
Glucose hexokinase assay kit	Sigma Aldrich (St. Louis, MO)
Glcogen PAS-staining kit	Sigma Aldrich (St. Louis, MO)
ECL detection kit	GE Healthcare Amersham, Piscataway, NJ)

2.1.7. Instruments

Stereomicroscope	(Zeiss, Jena, Geramny)
UltraTurrax homogenizer	(IKA, Stauffen, Germany)
Axiophot microscope with Axiocam camera system	(Zeiss, Jena, Germany)
iCycler PCR machine	(Bio-Rad Laboratories)
SPECTRAmax PLUS ^{384} Microplate Spectrophotometer	Molecular Devices Corporation, California
One Touch^{TM} glucometer	(LifeScan, Inc., Milpitas, CA)
Electrophoresis apparatus	BioRad Laboratories GmbH, (München, Germany)
Gene Amp PCR System 9700	Applied Biosystems, (Darmstadt, Germany)

2.2. Methods

2.2.1. Human Islets

2.2.1.1. Isolation and culture of isolated human islets

Islet isolation procedure was performed at the Giessen Islet Isolation and Transplantation Center, according to institutional standards (University of Giessen, Germany).

Isolated islets were then shipped by train (duration of up to 5 h) at RT to our laboratory.

Briefly, human pancreata were surgically harvested from healthy cadavers, fulfilling criteria of multiorgan donors. The legal consent was obtained either by the organ donor registry or living relatives, in accordance with the Euro transplant International Foundation.

Fig. 12 demonstrates the islet isolation procedure. The pancreata were preserved by hypothermic perfusion with 3,000 to 4,000 ml of University of Wisconsin Solution. Each pancreatic segment was intraductally distended with cold (8 °C) HBSS with 1.7 ml/g pancreatic tissue containing 2000 PZ-U collagenase NB 1 and 40 PZ-U neutral protease. Distended organs were placed in a stainless steel chamber. While recirculating the solution, the digestion chamber was set in vertical motion. Digestion temperature was 28-32 °C, depending on the amount and dissociation of the tissue. The digested tissue was collected in pre-cooled (4 °C) 250 ml conical centrifugation tubes and, subsequent to washing, was dissolved in 400 ml of iso-osmolar ficoll-sodium-diatrizoate (density, 1082 g/ liter) and loaded into a precooled Cobe 2991 cell separator at 4 °C. Islets were separated and purified by automated procedure using a continuous digestion-filtration device.

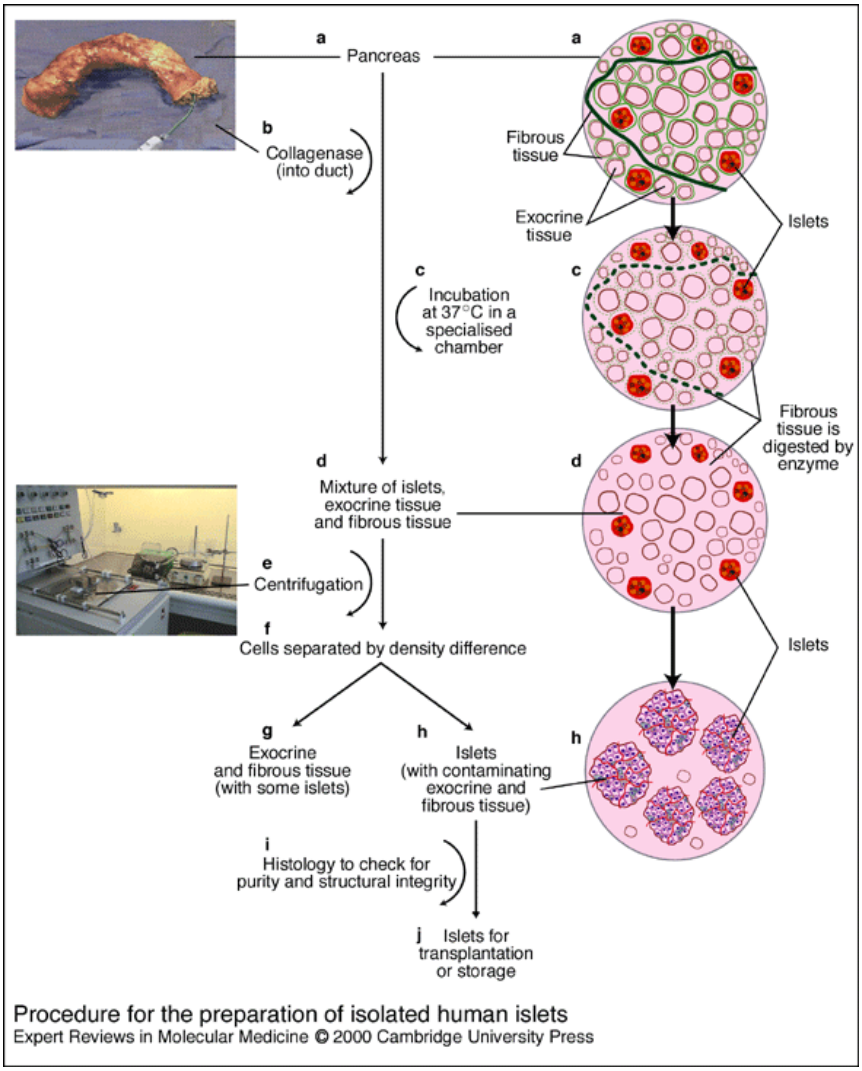

Fig. 12. Schematic representation of the steps involved in the procedure for the preparation of isolated human pancreatic islets

Adapted from Expert reviews in Molecular Medicine 2000 Cambridge University Press

2.2.1.2. Incubation experiments

Islets were allowed to recover for 72 – 96 h in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C prior to experiments. Islets were centrifuged at 800 g for 10 min at RT and resuspended in RPMI 1640 medium containing 0.1 % BSA and 3.3 mM glucose. Islets were handpicked under stereo-microscope and washed twice with serum-free RPMI 1640 and then incubated in the same medium for 1 h at 37 °C. Then, islets were washed twice and pre-treated with SST, SSTR-selective agonists or vehicle for 30 min 5 islets in a total volume of 20 µl were transferred into individual wells of a 24-well plate containing 480 µl of RPMI 1640 without additives. The secretion of insulin was stimulated for 1 h using 20 mM glucose, whereas glucagon secretion was induced by exposure of islets to 20 mM L-arginine. The reaction was terminated by aspiration of the incubation medium, without damaging the islets. Medium was immediately centrifuged to remove contaminating cellular debris and stored at -80 °C for further analysis. Islet purity and viability was monitored by a membrane integrity test (trypan blue exclusion).

2.2.1.3. Human hepatocytes

Hepatocytes were isolated using a modified two-step collagenase NB8 (Serva, Heidelberg, Germany) perfusion protocol. After purification via percoll (Biochrom AG, Berlin, Germany) density gradient centrifugation, hepatocytes were suspended in Williams medium E (10 % FCS, 50 U/ml penicillin, 50 µg/ml streptomycin), transferred into 2 % collagen-coated 6-well plates and allowed to recover for 24 h at 37 °C, 5 % CO_2.

On the day of experiment for gluconeogenesis, cells (2 x 10^6) were washed with PBS and incubated with 10 nM glucagon (Sigma, Aldrich) with or without 100 nM SST-14 or an SSTR2-selective agonist in 2 ml glucose-free DMEM medium (Sigma, Aldrich,St. Louis, MO)), containing 0.2 % (w/v) BSA, 50 U/ml penicillin, 50 µg/ml streptomycin, 4 mmol/l pyruvate (Sigma, Aldrich) and 16 mmol/l sodium DL-lactate (Sigma, Aldrich). Aliquots were taken, centrifuged at different time points (2, 4, 8 and 24 hours) and glucose concentration was determined by glucose (hexokinase) assay kit (Sigma-Aldrich).

To study glycogenolysis, after the initial 24 h, the medium was replaced for 20 h with basal medium supplemented with 10 mmol/l glucose and 10 nM insulin to build up

glycogen reserves. Hepatocytes were washed with buffer A (117.6 mmol/l NaCl, 5.4 mmol/l KCl, 0.82 mmol/l Mg_2SO_4, 1.5 mmol/l KH_2PO_4, 20 mmol/l Hepes, 9 mmol/l $NaHCO_3$, 0.1 % BSA, 2.25 mM $CaCl_2$, pH 7.4), pre incubated for 4 h in buffer A, washed and then treated with 10nM glucagon and test agents 100 nM SST-14 or an SSTR2-selective agonist. The amount of glucose released into buffer A reflected glycogenolysis. Aliquots were taken, centrifuged at different time points (2, 4, 8 and 24 hours) and glucose concentration was determined by glucose (hexokinase) assay kit (Sigma-Aldrich).

2.2.2. Animals

All experimental animal procedures were approved by the Institutional Review Board for the Care of Animal Subjects (protocol G 0107/03) and performed in accordance with the German legislation on the protection of animals. SSTR2-/- animal colony was continuously maintained by breeding. Off springs were genotyped by PCR.

For the high fat diet (HFD) study, all animals were individually caged and maintained under controlled conditions of 25 °C and 12 h cycle (light 7 AM/dark 7 PM) with food and water available *ad libitum*.

2.2.2.1. Maintenance of the animal colony

Pairs of males and females homozygous SSTR2-/- mice were bred. At three weeks of age DNA from the tail biopsies was isolated. Tail pieces of approximately 0.5 to 1.0 cm were used for the isolation of genomic DNA. The genotype of the off springs was confirmed by PCR.

2.2.2.2. DNA isolation from the tail biopsies

Tail biopsies were digested overnight in 600 µl of TNES and 35 µl of proteinase K (10 mg/ml) at 55 ºC rotor by vigorous shaking. On the next day 166.7 µl of 6 MNaCl was added and shaken vigorously for 15 sec and centrifuged at (12,000 to 14,000 xg) for 5 min at room temperature (RT). Supernatant was removed to the new tube and one volume of ice cold 90 % ethanol) was added. Tubes were inverted slowly up and down several times. Precipitated DNA was collected by the blunt capillary and rinsed once in 70 %

ethanol (dipping DNA in a tube filled with 70 %). DNA was allowed to air dry for 5 -10 min Finally the precipitate was resuspended in 100 – 500 µl of TE buffer. The suspension was heated to 65 °C for 10 min to dissolve DNA. DNA concentration was quantified by spectrophotometer at the wavelength A_{260}:A_{280} and was stored at 4 °C. A_{260} is the wavelength of light which is absorbed by the nucleic acids and A_{280} is used in the ratio A_{260}:A_{280} which determines the purity of the DNA. The ratio is taken adequate for the purity if the ratio of A_{260}:A_{280} lies between 1.9 to 2.0. DNA is calculated by the formula A_{280}* 50 * dilution factor, where 50 is the concentration in ng/µl.

2.2.2.3. Genotyping of mice by PCR

Genotyping of both parent and offspring mice was done using genomic DNA by standard polymerase chain reaction (PCR). In the first cycle, the dsDNA template was denatured at 94 °C for 45 sec, in the second step (annealing at 54 °C for 45 sec was performed. In the third and final step, extension reaction for 45 sec at 72 °C was performed. Genomic DNA (100ng) was amplified by 35 cycles of PCR and the amplified PCR products were run on a 1.0 % agarose gel for verification of the expected amplicon size. The composition of master mix for PCR genotyping of mouse genomic DNA and the cycling parameters used in PCR are listed below.

The amplified SST2 transgene amplicon size = 0.45 kb, forward (5'-cat aag cgc atg ctc cag ac-3') and reverse primer (5'–cag gat gtg aat gtc ttc cag-3') pairs were used for the amplification of the null allele (ko = 0.45 kb)

To cross check the SSTR2-/- mice primer pairs, (HW 72) forward: (5'-ggc ctc cgg agc aac cag tgg ggc-3') and (HW 76) reverse: (5'-ccg ctc cgg att gtg aat tgt atg-3') were used to identify the wild type mice, the knock out mice had no band whereas the $SST2^{+/+}$ and $SST2^{+/-}$ (WT = 0.9 kb) had the amplification of WT. This fragment gave the amplification of the endogenous SST gene.

Composition of master mix for genotyping of mice PCR

Template DNA	100 ng
Forward Primer	10 µmol
Reverse Primer	10 µmol
dNTP mix	10 mM
10 X PCR buffer with 15mM $MgCl_2$ 2.5µl	
Taq DNA polymerase	0.5 µl
H_2O	to the total volume of 25 µl

Cycling parameters for genotyping of mouse tail snips by PCR

Activation of Taq polymerase	94 °C (5 min)
Amplification for 35 cycles	
a) Denaturation	94 °C (45 sec)
b) Annealing	54 °C (45 sec)
c) Extension	72 ºC (45 sec)
d) Final Extension	72 ºC (7 min)

2.2.2.4. Agarose gel electrophoresis of genomic DNA

Ethidium bromide stained agarose gel electrophoresis was performed to visualize the PCR products. DNA samples (total volume of 25 µl) were mixed with 5 µl of DNA-loading dye (DNA loading dye increases the density of the DNA samples and gives it color, the dye also migrates in the same direction as the nucleic acids and acts as a marker of the progress of the electrophoresis). 10 µl of this mixture was loaded into the wells. Electrophoresis was carried out at a constant voltage of 100 V for 1 h in 1.0 % agarose gel containing ethidium bromide (final concentration of 0.5 µg/ml) in 1X TBE buffer, pH

8.0. Gel was exposed to UV-light. Size of the DNA was compared to a standard DNA molecular weight marker.

2.2.2.5. High fat diet (HFD) experimental set up

2.2.2.5.1. Induction of obesity and insulin resistance in mice

SSTR2-/- deficient male mice (C57BL/6J, 10-14 weeks of age) and corresponding wild type (WT) controls were switched from chow diet (7012) (Harlan Teklad, Madison, WI) to high fat diet (HFD) (36 % fat content (w/w), 60 kcal %) (Research Diets Inc., New Brunswick, NJ) and fed *ad libitum* for up to 14 weeks. Food intake, changes of body weights and fasting and non fasting blood glucose levels were recorded weekly.

2.2.2.6. Determination of metabolic parameters

Blood was collected from non-anesthetized animals either by a tail nick or from the retro orbital vein plexus (within 20 sec) using heparin-coated capillaries. Collected blood was centrifuged at 6000 rpm for 15 min Serum was collected and immediately stored at -80 °C for glucagon and insulin measurement. Plasma levels of insulin, leptin and glucagon-like peptide 1 (GLP-1) were measured by ELISA (Alpco, Windham, NH) or by mouse LINCO*plex* kit (Linco Res. Inc., St. Charles, MI). Glucagon concentration was determined by a rat glucagon RIA (DPC Biermann, Bad Nauheim, Germany). The specificity for glucagon was 95 %, the limit of sensitivity 13 pg/ml. The intra- and interassay coefficients of variation (CV) were below 6.5 % and 12 %, respectively. Plasma levels of non-esterified fatty acids were quantified by the colorimetric method (half-micro test, Roche Diagnostics, Mannheim, Germany). Blood glucose concentration was detected by One Touch™ glucometer (Life Scan, Inc., Milpitas, CA).

2.2.2.7. Tolerance tests

Unless otherwise stated, test agents (1.5 g/kg BW of glucose, 0.5 IU/kg BW of insulin, 100 µg/kg BW of SST-14) were injected i.p. (200 µl/40 g BW) into mice. Blood was drawn by tail incisions for the measurement of blood glucose levels. For the measurement of hormones in serum, blood was collected from the retro orbital plexus in heparin coated capillaries and serum was collected and stored at -80 °C after centrifugation for 15 min at

6000 rpm. 35 µl of serum was taken for glucagon measurement and 5 µl for insulin and 10 µl for determination of Leptin, GLP-1, glucagon and insulin by LINCOplex ELISA kit.

2.2.2.7.1. Glucose tolerance test

For glucose tolerance test, animals were fasted for 8 h and glucose (1.5 g/kg BW) was injected intraperitonially. Blood glucose was measured at the given time points by One Touch™ glucometer (Life Scan, Inc., Milpitas, CA) from both SSTR2-/- and wild type mice. For oral glucose tolerance test, animals were fasted overnight and 1.5 g/kg BW) glucose was given by the feeding (gavage) and blood was measured as previously described.

2.2.2.7.2. Somatostatin- and insulin tolerance tests

Mice were fasted for 2 h and injected with SST-14 (100 µg/kg BW). Blood glucose concentration was measured One Touch™ glucometer (Life Scan, Inc., Milpitas, CA).
Human insulin (0.5 IU/kg BW) was injected i.p. into 2 h fasted animals to investigate the effect of exogenous insulin on blood glucose concentrations in wild type and SSTR2-/- mice. Blood glucose levels were measured at the given time points by One Touch™ glucometer (Life Scan, Inc., Milpitas, CA).

2.2.2.8. Isolation of murine pancreatic islets

For the isolation of pancreatic islets, animals were killed by cervical dislocation and the abdomen was opened by transverse incision to expose the pancreas. Clamping was done at the common bile duct (CBD) and 3 ml liberase ^{TM} RI (25 mg/ml in Hank's buffered saline solution (HBSS / DNase I / HEPES) was injected into the main pancreatic duct under stereomicroscope **(Fig. 13)**. The perfused pancreas **(Fig. 14)** was excised immediately transferred into a dish and digested for 15 min in 37 ºC water bath shaker. The digestion was stopped by the addition of ice cooled quenching buffer (HBSS buffer with 100 mg DNase and 25 ml of 1 M HEPES + 110 ml of FCS). The suspension was centrifuged at 800 g for 10 min at 4 ºC. Supernatant was discarded and the islets were washed three more times in ice cooled quenching buffer. Finally, the islets were

suspended in 15 ml of ice cooled quenching buffer for manual handpicking under stereo-microscope. After purification, islets were left overnight in RPMI 1640 medium with 10 % FCS and 5 % streptomycin/penicillin at 37 ºC.

On the day of experiment, islets were washed twice in KRB buffer containing 3.3 mM glucose + 0.1 % BSA and incubated for 45 min in KRB buffer containing 3.3 mM glucose + 0.1 % BSA. Islets were washed once in KRB buffer and then treated with the different concentrations of SST and SSTR-selective analogues for 45 min. Islets were washed again and 10 islets/well were handpicked and transferred into a new dish. The secretion was stimulated either with 20 mM glucose (for insulin secretion) or with 20 mM L-arginine (for glucagon secretion). Islets were co-incubated with SST and SSTR-selective agonists for 2 h. After the incubation supernatant was collected carefully and stored at -80 ºC. The concentration of insulin and glucagon was measured by rat insulin ELISA or rat glucagon RIA respectively. The islets were stored at -80 ºC for the analysis of intra-islet insulin and glucagon concentration.

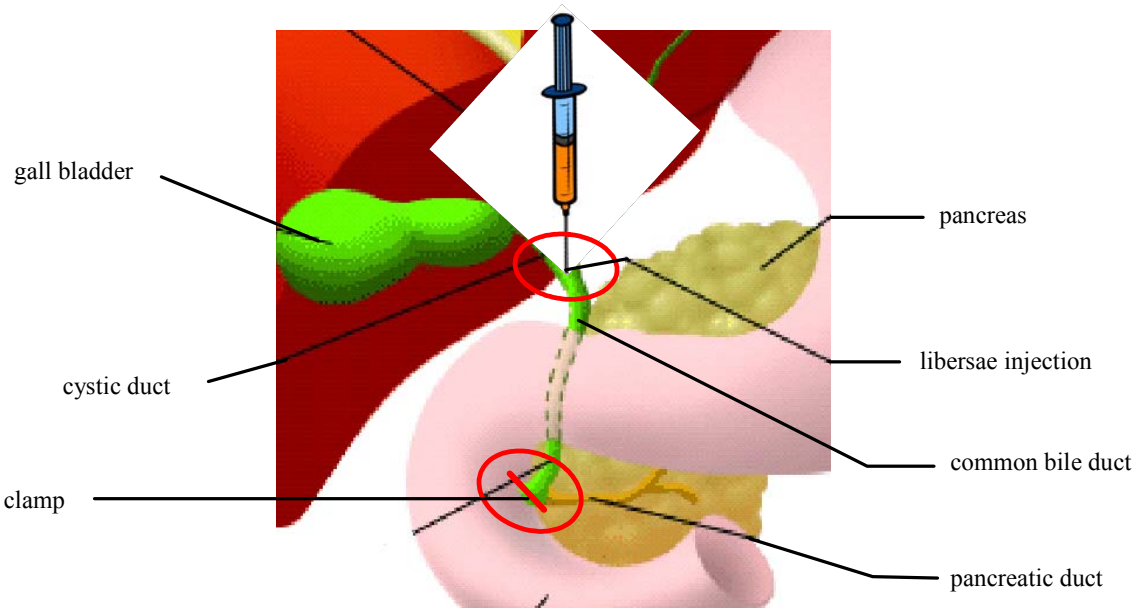

Fig. 13. Schematic representation of perfusion of mice pancreas

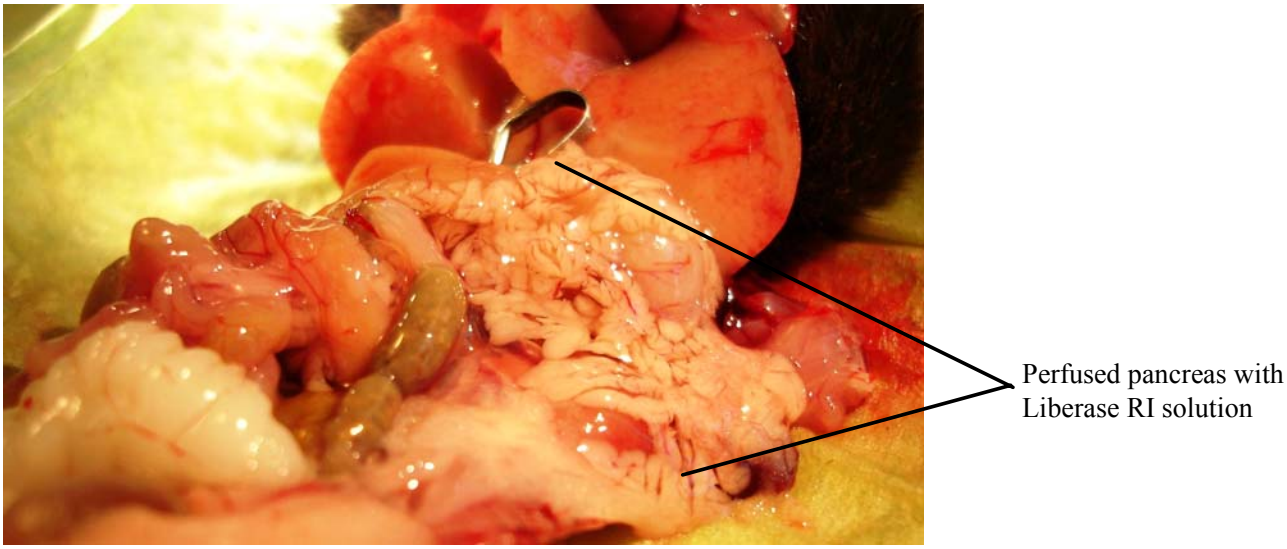

Fig. 14. Murine pancreas in situ injected with a solution containing liberase RI

2.2.2.8.1. Incubation experiments

Islets were allowed to recover for 72 – 96 h in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C prior to experiments. Islets were centrifuged at 800 g for 10 min at RT and resuspended in RPMI 1640 medium containing 0.1 % BSA and 3.3 mM glucose. Islets were handpicked under stereo-microscope and washed twice with serum-free RPMI 1640 and then incubated in the same medium for 1 h at 37°C. Then, islets were washed twice and pre-treated with SST, SSTRs-selective agonists or vehicle for 30 min 5 islets in a total volume of 20 µl were transferred into individual wells of a 24-well plate containing 480 µl of RPMI 1640 without additives. The secretion of insulin was stimulated for 1 h using 20 mM glucose/10 nM exendin-4, whereas glucagon secretion was induced by exposure of islets to 20 mM L-arginine at 3.3 mM glucose. The reaction was terminated by aspiration of the incubation medium, without damaging the islets. Medium was immediately centrifuged to remove contaminating cellular debris and stored at -80 °C for further analysis. Islet purity and viability was monitored by a membrane integrity test (trypan blue exclusion).

2.2.2.9. Excision of mouse organs

Animals were weighed and epididymal white adipose tissue (eWAT), liver, interscapular brown adipose tissue (iBAT), skeletal muscle and pancreas were dissected out. Wet weights of tissues were measured. Tissues were then quickly frozen in liquid nitrogen and stored in -80 °C for further processing.

2.2.2.9.1. Determination of glycogen content in murine livers

For the determination of liver glycogen content, tissues (150 mg) were treated with 1 ml of KOH (30 % w/v), and heated for 30 min at 95 °C. Lysed tissues were cooled to RT and precipitated over night at 4 °C in 1.5 ml of 95 % ethanol. Next day precipitated glycogen was recovered by centrifugation at 3000 g for 20 min and the pellet was resuspended in 0.6 M NaOH. This suspension was heated to 100 °C for 2 h and neutralized with 0.6 M HCl. The neutralized suspension was centrifuged to remove debris. 100 µl of the supernatant were analyzed for glycogen content using glucose hexokinase assay kit (Sigma-Aldrich Inc.).

2.2.2.9.2. Determination of triglyceride content in mouse livers

To determine the intrahepatic triglyceride content was, approximately 100 mg of tissue was weighed and homogenized in a mixture of chloroform/methanol/water for 2 min. The homogenate was then collected in 15 ml falcon tubes and 1 ml of water + 2 ml of chloroform was added and shaked vigorously with hands. The mixture was then centrifuged at 4000 x g for 10 min at RT to separate organic and aqueous phases. The organic phase was collected and evaporated at 70 °C and was then redissolved in 500µl of isopropanol. Triglyceride concentration was determined in 96-well plate using a colorimetric triglyceride kit (Cypress Diagnostics, Langdorp, Belgium) at the wavelength of 505 nm.

2.2.2.9.3. Immunofluorescence of cryosectioned pancreas

The tissue sections were fixed in Carnoy solution (60 % ethanol, 30 % chloroform, 10 % glacial acetic acid) for 20 min at RT. Afterwards sections were washed in PBST (PBS with 0.1 % Tween 20) for 5 min and tissue sections were blocked in 2 % fat-free milk solution prepared in PBST for 30 min at RT to prevent the non-specific binding of the antibodies. Blocking step was followed by two times washing for 5 min in PBST. Incubation with primary antibodies was done overnight at 4 °C in blocking solution. Next day sections were washed three times in PBST followed by incubation with secondary antibody (1:400) for an h at RT. Sections were again washed three times for 5 min in PBST. Afterwards sections were treated with aqua bidest for 10 sec and in ethanol for 5 min and mounted in glycerol/gelatine mixture.

2.2.3. Histochemical analysis of tissue sections

2.2.3.1. Histochemical detection of hepatic glycogen content

2.2.3.1.1. PAS (Periodic Acid Schiff) staining for hepatic glycogen

Carbohydrate content of tissues is detected by PAS staining. PAS staining is a histochemical reaction in which the periodic acid oxidizes the carbon to carbon bond aldehydes, which react to the fuchsin-sulfurous acid (present in Schiff reagent) and gives the red/purple color to the glycogen. Glycogen PAS-staining was performed using the solutions and protocol from kit Sigma Aldrich (#395b-1KT). The tissue sections were

fixed in Carnoy solution (60 % ethanol, 30 % chloroform, 10 % glacial acetic acid) for 20 min at RT. After fixation sections were kept in 96 % ethanol solution for 10 min and washed with distilled water for 5 min. Afterwards tissue sections were treated with periodic acid for 5 min and washed in running distilled water for 5 min. After washing tissue sections were treated with Schiff reagent for 15 min at RT and washed with distilled water for 5 min and treated with hematoxylin for 90 sec and washed in running water. After washing sections were treated in 70 % ethanol for 2 min and twice in 96 % ethanol for 2 min afterwards sections were dehydrated in Rotihistol (Roth, Karlsruhe, Germany) and mounted using Faramount (DAKO, Hamburg, Germany).

2.2.3.1.2. Histochemical detection of hepatic triglyceride content by Sudan dye method

Sudan dyes have high affinity to fats such as triglycerides, lipids etc. In this study lipids were detected by Sudan stain method. Tissue sections were fixed in 4 % PFA (paraformaldehyde) for 20 min, washed twice in distil water for 5 min and incubated in 50 % ethanol for 2 min. Afterwards, Sudan IV solution (#2C282, Waldeck GmbH, Münster, Germany) was added for 15 min. Sections were then washed three times for 2 min in 50 % ethanol solution followed by two times washing in distil water for 5 min. Sections were counterstained with hemealaun, washed in running water and mounted using glycerol gelatin (Merck, Darmstadt, Germany).

2.2.3.2. Preparation of RNA from tissues

Total RNA was isolated using a Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Briefly, 100 mg of tissue was taken for the RNA isolation. To the tissue 0.5 ml of Trizol ™ and 0.1 ml of chloroform was added and shaken by hand for 2-3 min. Tissue was then homogenized by homogenizer (Ultra-Turrax T8 IKA Labortechnik) and centrifuged at 12,000 g for 10 min. Clear supernatant was carefully collected and transferred into a new tube. 0.25 ml of isopropanol was added and the precipitation was carried out at RT for 10 min. Precipitate was recovered by centrifugation at 12,000 xg for 10 min at 4 °C. The supernatant was discarded and 0.5 ml of 70 % ethanol was added and centrifuged at 12,000 xg for 10 min at 4 °C. Supernatant was discarded and the pellet was

air dried under the laminar flow. The pellet was dissolved in 20 µl of sterile H_2O and RNA was quantified by spectrophotometer and stored at -80 °C for cDNA preparation.
The concentration and purity of RNA was determined by the absorption at 260 nm (260 wavelength is the maximum absorption by the nucleic acids) while the value at 280 gives the protein contamination reading in the RNA. The ratio A260/A280 purity of the RNA isolated. The optimal value for the clean RNA free from protein and phenol contamination lies between 1.9 to 2.0. RNA concentration is calculated by the formula A_{260}* 40* dilution factor (100), where 40 is the concentration in ng/µl. (1 µl of RNA + 99 µl of DEPC water).

2.2.3.3. Preparation of proteins from tissue

100 – 200 mg of frozen tissues were homogenized with a polytron homogenizer (Fisher, Pittsburgh, PA) in TNA-buffer (50 mM Tris-Cl pH 7.0, 150 mM NaCl, 0.1 % sodium azide, 0.1 % NP-40) containing 1 x proteinase-inhibitor cocktail (Complete®, Roche). 500 µl of a solubilization buffer (50 mM Tris-Cl pH 7.0, 150 mM NaCl, 0.1 % sodium azide, 3 % NP-40, 1.5 % Na-desoxycholate, 0.3 % SDS) was added to the lysates. After solubilization on ice (20 min), samples were centrifuged (20,000 xg, 45 min, 4 °C), supernatants were aspirated, quantified by BCA protein assay kit (Pierce, Rockford, Illinois, USA) and stored at -80 °C.

2.2.4. Cell culture

Rat insulinoma (INS-1) and hamster glucagonoma (InR1-G9) cell lines were obtained from the ATCC (American Type Culture Collection, Rockville, Maryland). INS-1 cells were grown in RPMI 1640 medium with HEPES and 2 g/l of glucose, medium for INS-1 cells was supplemented with 2 mM L-glutamine, 1 mM NaPyruvate, 50 µM B-mercaptoethanol, 5 ml of penicillin/streptomycin and 10 % (w/v) FCS. InR1-G9 cells were grown in RPMI-1640 medium containing 2 g/l of glucose, 10 % (w/v) FCS, and 5 ml penicillin/streptomycin. The cells were grown in a humidified atmosphere containing 5 % CO_2 in air at 37 °C incubator.

2.2.4.1. Immunofluorescence study in INS-1 and InR1-G9 cells

Human embryonic kidney 293 HEK293 InR1-G9 and INS-1 cells were plated on poly-l-lysine (50 µg/ml) precoated coverslips (15/18 mm diameter) in 12 well plate and left for 80 % confluency in 37 °C incubator with 95 % O_2 and 5 % CO_2 conditions. Next day cells were washed with PBS carefully and fixed in Zamboni solution for 30 min at RT. Afterwards cells were washed in Tris-PBS followed by 3 min incubation in 50 % methanol and 3 min incubation in 100 % ice cold methanol. After these incubations cells were washed three times in Tris-PBS solution for 3 min each. 3 min incubation was done in 1 % normal goat serum (NGS) prepared in Tris-PBS followed by 1 h incubation in 3 % normal goat serum in Tris-PBS solution at RT. Primary antibody for SSTR2 and SSTR3 (gift from Dr. Stefan Schulz, Magdeburg) with 1:1250 dilution in TPBS containing 1 % goat serum was added and incubated overnight. Afterwards, cells were washed twice for 3 min each in Tris-PBS buffer. Treatment with fluorochrom antibody Cy3- conjugated goat anti rabbit IgG (Jackson Immuno Research Laboratories) with 1:500 dilution was done for 2 h at RT in dark. Washing was performed three times for 3 min each in Tris-PBS buffer, further washing was performed as follows:

70 % ethanol for 3 min

80 % ethanol for 3 min

95 % ethanol for 3 min

100 % ethanol for 3 min

After the washing steps, object slide were coated with gelatine-chromalaun for 5 min at RT and were dried overnight at 37 ºC followed by dehydration step in Rotihistol and mounted using DPX dry 1 h under the laminar flow.

2.2.4.2. Insulin secretion assay using INS-1 cells

Cells ($2.5x10^5$) were plated in 24 well-plate and grown for three days in RPMI 1640 medium. On the day of experiment, cells were washed three times and preincubated for 30 min in KRB buffer containing 0.5 % BSA and 3.3 mM glucose. Then cells were washed once with PBS. Afterwards, test substances (SST and SSTR-selective agonists (10^{-6}M – 10^{-12}M) were added and cells were incubated for 30 min. Cells were washed with PBS once and the secretion was stimulated with 20 mM Glucose + 10 nM exendin-

4. SST and SSTR-selective agonists were also present in the incubation medium along with stimuli of insulin secretion. After 2 h, supernatant was collected, centrifuged and stored at -80 °C for determination of insulin concentration by rat insulin ELISA.

For the characterization of the effects of R-type Ca^{2+} blocker on SST-dependent inhibition of insulin secretion INS-1 cells were treated with R-type Ca^{2+} blocker [SNX-482 (1 nM – 20 nM) for 10 min. Afterwards SST or SSTR-selective agonists (1 µM) were added into the appropriate wells and incubated for 30 min. Cells were then washed with PBS once and the secretion was stimulated with 20 mM Glucose + 10 nM exendin-4. SNX-482, SST and SSTR-selective agonists were also present in the incubation medium along with stimuli of insulin secretion. After 2 h, supernatant was collected, centrifuged and stored at -80 °C for determination of insulin concentration by rat insulin ELISA.

For the determination of intracellular insulin content, cells were treated overnight at 4 °C in 1ml acidified ethanol containing 75 % (v/v) ethanol + 0.5 % HCl. The extract was diluted to 1:5000 for insulin ELISA in 0.2 M glycine/NaOH buffer (pH=8.8) supplemented with 0.25 % BSA.

2.2.4.3. Glucagon secretion assay using InR1-G9 cells

0.2 x 10^5cells/well were plated in 24 well-plate in RPMI 1640 medium supplemented with 10 % FCS or FBS and maintained for 3 days. 12 h before treatment with test agents medium was substituted by a serum-free medium. Cells were preincubated in KRB buffer containing 0.5 % BSA and 2mM glucose for 30 min. Then the cells were washed once with PBS and incubated with test substances (SST and SSTR2-selective agonist (10^{-6} M – 10^{-12} M) for 30 min. Cells were washed with PBS and stimulus (20 mM L-arginine or 20 mM KCl) was added along with the test substances. Incubation was carried out for 1 h. Then, supernatant was collected, centrifuged and stored at -80 °C for determination of glucagon using a rat RIA kit. (DPC, Salzburg, Austria).

2.2.4.4. cAMP determination

2 x 10^5 INS-1, or 0.2 x 10^5 InR1-G9 cells/well were plated in a 96 well-plate in RPMI 1640 medium supplemented with 10 % FBS or FCS and allowed to attach to the bottom. On the next day, cells were washed once with PBS and serum-free medium with (1:5 dilution of penicillin/streptomycin) was added to the cells for over night. On the next day cells were incubated in a cAMP incubation medium (serum-free RPMI 1640 medium supplemented with 1 % BSA and 500 µM of IBMX (an inhibitor of phosphodiesterase activity) for 30 min at 37 °C in a cell incubator with 95 % CO_2 and 95 % O_2. Cells were washed once with PBS and RPMI 1640 medium containing test substances. Afterwards, SST or SSTR-selecitve agonist (10^{-6} M – 10^{-12} M) were added to the appropriate wells. Incubation was carried out for 15 min. Then, 10 µM forskolin (postreceptor activator of adenylyl cyclase) was added and incubation was continued for additional 5 min at 37 °C. Reaction was then stopped by aspirating the medium. The intracellular cAMP content was determined by cAMP Biotrack Enzyme Iimmunoassay (EIA) kit and the absorption was detected at 630 nm using an ELISA reader.

2.2.4.5. RNA isolation from cells

Cells were washed once with PBS and lysed with cell lysis buffer and the total RNA was extracted by using RNeasy Mini Kit (Qiagen, Hilden, Germany). DNA contamination was removed by RNase-Free DNase Set (Sigma) as described in section **2.2.4.5.1 of Materials and Methods**

2.2.4.5.1. Nuclease digestion to remove DNA and ssRNA

The DNase/RNase treatment digests template DNA and any ssRNA remaining in the dsRNA. RNase digestion reaction mixture was assembled on ice with following quantities of reagents.

RNA	1 µg
(1 U/µl, Sigma) DNase I	1 µl
10X DNaseI Reaction buffer	1 µl

Total volume to 10 µl in DEPC-treated water/RNase-free water

The digestion was performed for 15 min at 25 °C. The reaction was stopped by the addition of a solution containing 25 mM EDTA (pH 8.0). The reaction was extended for 10 min at 65 °C.

RNA (5 µg) was reversely transcribed to cDNA with oligo-dT primers and SuperScript II (Invitrogen, Karlsruhe, Germany).

The reverse transcription was performed in presence of 0.1 M DTT, 10mM dNTP, 0.5 µl/µl of oligo-dT and 40 U/µl of RNAaseout. The reaction was performed for 1 h at 42 ºC. 1 µl of cDNA was used for PCR amplification,

After this dNTP and oligo-dt was added

10 mM dNTP	1 µl
0.5 µg/µl of oligo-dt	1 µl was added at 65 °C for 5 min

Samples were cooled on ice and master mix (see below for the ingredients) was added:

2 µl of 10X PCR buffer

4 µl of 25 mM $MgCl_2$

2 µl of 0.1 M DTT

1µl of (40 U/µl) RNase Out

The reaction was performed for 60 min at 42 °C followed by 15 min at 72 °C.

After 2 min 1 µl of (50 U/µl) Superscript II (Invitrogen Life Technologies) was added to all the tubes. After completion of the reaction, samples were immediately cooled on ice and 30 µl of molecular grade water was added to the cDNA and the mixture was stored at -20 °C for real-time PCR.

2.2.5. (Quantitative) real-time RT-PCR

For real time PCR, we used a double-stranded DNA binding dye (SYBR-green I), which quantifies the amplicon production. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA.

Some selected genes that were found to be differentially regulated were validated by means of conventional RT-PCR. Synthesis of complementary DNA was done as previously described in section **2.2.4.5.1** of Materials and Methods. 1 µg of total RNA

was reverse transcribed into cDNA. The reaction was quickly chilled on ice, and stored at -20 °C till further use.

Real-time PCR analysis of selected genes was done by using iCycler PCR machine (Bio-Rad Laboratories) in three step RT-PCR kit employing IQ SYBR Green Supermix (Bio-Rad Laboratories) for the detection of the double stranded DNA product. Six standards (serial dilutions) and a negative control without template were included in each run. Each PCR reaction was carried out in a 25 µl reaction using the following master mix as shown below.

Composition of master mix for real Time RT-PCR

Diluted cDNA template	1 µl
Forward Primer	10 µmol
Reverse Primer	10 µmol
2X Syber Green master mix	12.5 µl
H_2O	to make the volume to 25 µl

Melting curve analysis and agarose gel electrophoresis confirmed the exclusive amplification of the expected PCR products. Expression levels of all genes were normalized to beta actin (house-keeping gene) mRNA.

Cycling parameters for quantification of gene expression by real time RT-PCR

Activation of Taq Polymerase	95 °C (3 min)
Amplification for 40 cycles	
a) Denaturation	95 °C (2 sec)
b) Annealing	60 °C (1 sec)
c) Extension	72 °C (1 sec)
and final extension	72 °C (2min)

2.2.6. Protein preparation from cells

For preparation of total cell lysates, the cells were harvested by scraping in ice-cold lysis buffer (25 mM Tris-HCL (pH 6.8), 1.25 % β-mercaptoethanol, 1 % sodiumduodecyl-sulfate, 5 % glycerol, 0.0125 % bromphenol-blue and protease inhibitor cocktail (Roche, Mannheim, Germany). The protein content was measured by BCA (bicinchoninic acid) protein assay reagent kit. This kit (BCA protein assay kit (Pierce, Rockford, Illinois, USA)) is a detergent-compatible formulation based on bicinchoninic acid for the colorimetric detection and quantification of total protein. The principle of method is based on a reduction of Cu^{2+} to Cu^{1+} by protein in the biuret reaction (the alkaline medium). After the quantification of protein 75µg of protein, was loaded onto the Tris-glycine SDS-Polyacrylamide gel electrophoresis.

2.2.6.1. SDS-Page and western blotting

Proteins were denatured by heating at 100 ºC for 5 min in SDS loading buffer, BioRad). 12 % Tris/glycine/SDS -polyacrylamide electrophoresis separating gel was casted using 3.3 ml of water, 4 ml of 30 % acrylamide solution containing 0.8 % bisacrylamide (BioRad Inc.) 2.5 ml Tris-HCl pH 8.8 (Merck) 100 µl of 10 % SDS, 100 µl of 10 % ammonium persulphate (APS) and polymerized by 4 µl of N,N,N',N' - tetramethylethylenediamine (TEMED). The electrophoresis was performed using a mini gel apparatus (BioRad Inc.) in a 1x Tris/glycine/SDS running buffer (prepared from 10x Tris/glycine/SDS stock buffer, BioRad Inc.) at 120 Volts for approximately 2.5 -3 h at RT. Proteins were then transferred onto a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany) in transfer buffer Tris/Glycine/SDS + 200 ml of methanol) at 300 mA current for 1 h. Proteins transferred on nitrocellulose membrane were detected by staining the membrane in 0.1 Ponceau S (sodium salt), (Sigma, Aldrich) in 1 % acetic acid (Merck, Darmstadt, Germany) for 5 min. After the detection of transferred proteins, nonspecific binding of antibodies was blocked for 1 h in 5 % fat-free milk powder in 0.1 % Tween-20. Incubation with primary antibody was done overnight at 4 ºC in 2.5 % fat-free milk powder or 5 % BSA solution prepared in 0.1 % Tween-20. Next day, blots were washed three times for 5 min in PBST (PBS with 0.1 % Tween-20)

and treated for 1 h with appropriate secondary antibody (**Table 4**) anti-rabbit-HRP or anti-mouse-HRP (New England Biolabs GmbH, Frankfurt am Main, Germany) (**Table 5**) with dilutions (**Table 4**) at RT. After incubation with the secondary antibody, blots were washed three times for 5 min in PBST. For detection of proteins of interest, blots were processed with enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Freiburg, Germany). The antigen-antibody complex was visualized by exposure to a light-sensitive film for 1-30 min.

2.2.6.2. Statistical Methods

The results presented in figures and tables are representative for at least three experiments with comparable results. EC_{50} and maximal response are reported as the mean ± SEM of individual experiments. Unless otherwise stated all other data are expressed as mean ± SEM. The data were analyzed by two-way ANOVA with Bonferroni post tests or Student's t test.

Values of $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered as statistically significant. The computer programme used for the statistical analysis was Graph Pad Prism (San Diego, CA).