3. RESULTS

3.1. Expression of SSTRs

Our *in vitro* (human islets and SSTR2$^{-/-}$ mice islets) and *in vivo* results suggest that SSTR2 has an important role in inhibiting both insulin and glucagon secretion. To characterize the effects of SSTR2 on intracellular signalling in insulin and glucagon producing cells we used permanent cell lines: INS-1 cells (rat insulinoma) and InR1-G9 cells (hamster glucagonoma).

3.1.1. Expression of SSTRs in INS-1 cells

To identify the SSTRs expressed in INS-1 cells, we performed RT-PCR analysis using rat specific primers. PCR products of the predicted size of 78 and 87 base pairs, corresponding to the SSTR2 and SSTR3 mRNA amplicons were generated by RT-PCR (**Fig 15 A**), whereas SSTR1, SSTR4 and SSTR5 were not expressed.

The expression of SSTRs was confirmed by immunofluorescence. SSTR2 transfected RIN cells **{Fig. 15 B (ii)}** (provided by Dr. Stefan Schulz, Magdeburg) were used as a positive control and CHO cells **{Fig 15 B (iii)}** were used as a negative control **Fig 15 B** shows the expression of SSTR2 receptor in INS-1 cells **{Fig 15 B (i)}**

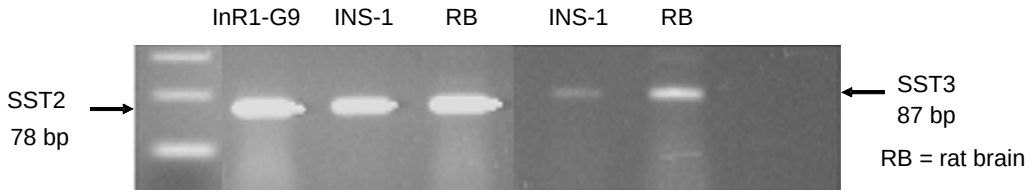
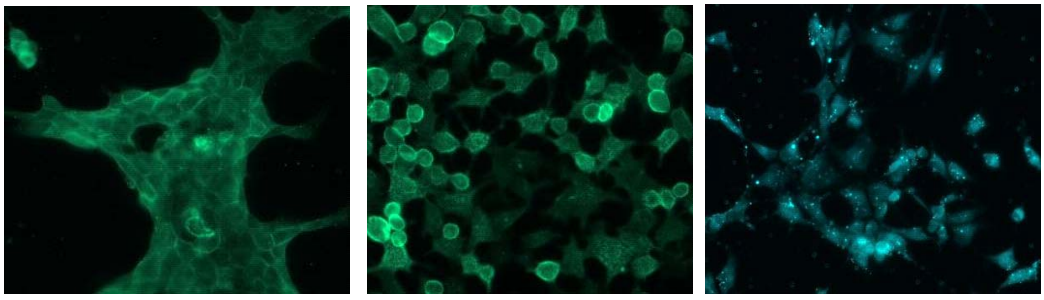


Fig. 15 (A). Identification of SSTRs expression by RT-PCR in INS-1 and InR1-G9 cells. Amplification of SSTR2 and SSTR3 in the given cell models by using rat primers. No signal for SST1, 4 and 5. Positive controls for RT-PCR: rat brain (RB) for all SSTRs.



(i) **(ii)** **(iii)**

Fig. 15 (B). Identification of SSTR2 by immunofluorescence. (i) represents INS-1 cells, (ii) is the positive control for SSTR2 (SSTR2 transfected RIN cells), and (iii) is the negative control(CHO cells).

3.1.2. Inhibition of intracellular cAMP levels by SST-14, SSTR2 and SSTR3 selective agonists in INS-1 cells

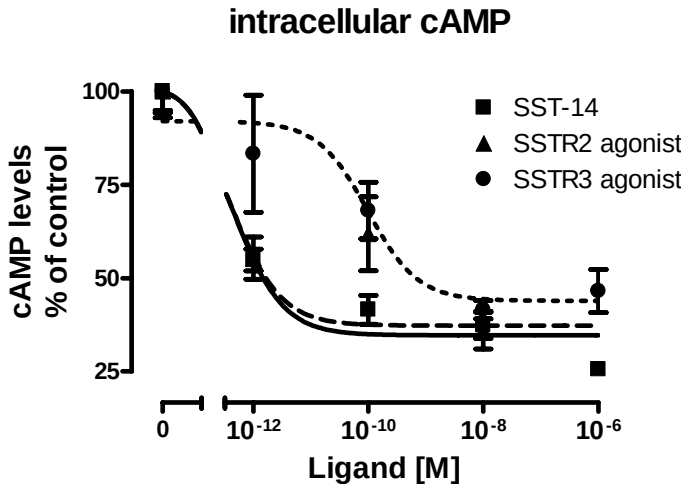


Fig. 16. Effect of SST-14, SSTR2 and SSTR3-selective agonists (10^{-6} M – 10^{-12} M) on forskolin-induced intracellular cAMP accumulation in INS-1 cells. (n=4)

To characterize the SSTRs in respect to their functionality, we tested the effects of SST-14, SSTR2 and SSTR3 selective agonists on intracellular cAMP accumulation in INS-1 cells. The intracellular cAMP production was stimulated with forskolin (10 μM) for 5 min SST-14 and SSTR2 selective agonist potently inhibited forskolin-induced cAMP accumulation in a dose-dependent manner (EC_{50} values in nM: SST-14: 4.4 x 10^{-13}, SSTR2 selective agonist: 4.1 x 10^{-13}) (**Fig. 16**). SSTR3 selective agonist less potently reduced forskolin-stimulated intracelullar cAMP formation (EC_{50}: 9.8 x 10^{-11} nM; n = 3; $p < 0.05$ vs SST-14 and vs SSTR2 selective agonist) (**Fig. 16)**

SSTR2 selective agonist is a more potent inhibitor of stimulated cAMP production in INS-1 cells as compared to SSTR3 selective agonist.

3.1.3. Inhibition of insulin secretion by SST-14, SSTR2 and SSTR3 selective agonist in INS-1 cells

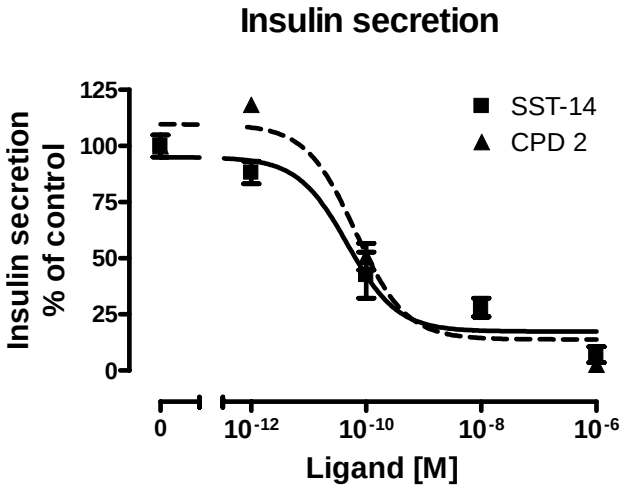


Fig. 17. Effect of SST-14 and SSTR2 selective agonist (10^{-6} M – 10^{-12} M) on 20 mM glucose + 10 nM exendin-4 (GLP-1 agonist) induced insulin secretion in INS-1 cells. Cells were incubated for 1 h with SST-14 and SSTR2 selective agonist. The concentration of released insulin in the buffer was determined. Basal secretion was determined at 3.3 mM glucose (n = 4)

Cells were stimulated with 20 mM glucose + 10 nM exendin-4 (GLP-1 agonist) for one hour. Basal insulin secretion rate was defined as the concentration of insulin in supernatant after 1 h of incubation with 3.3 mM glucose. SST-14 and SSTR2 selective agonist equipotently inhibited 20 mM glucose + 10 nM exendin-4 stimulated insulin secretion, **(Fig. 17)** while SSTR3 selective agonist was less effective. The calculated EC_{50} values in nM were for: SST-14: 4.698e-011 and 6.511e-011 for SSTR2 selective agonist.

SST-14 inhibits 20 mM glucose + 10 nM exendin-4 stimulated insulin secretion in the given cell model mainly via SSTR2 receptor.

3.1.4. Effect of SNX 482 (R-type Ca^{2+} channel blocker) on SST-14, SSTR2 and SSTR3 agonists dependent inhibition on insulin secretion

Next, we evaluated the role of R-type VOCC in conferring the effects of SST-14, SSTR2 and SSTR3 selective agonists on insulin secretion. Since SST-14 failed to influence basal insulin secretion, we stimulated insulin secretion by incubating INS-1 cells with the mixture of 20 mM glucose and 10 nM exendin-4.

Following incubation with 20 mM glucose + 10 nM exendin-4 for 1 hour, insulin secretion clearly increased as compared to basal (determined at 3.3 mM glucose). These values were set to 100 %. SST-14, SSTR2 and SSTR3 selective agonist inhibited the 20 mM glucose + 10 nM exendin-4 stimulated insulin secretion by 89 ± 24 %, 103 ± 18 %, and 73 ± 6 % of control ($n = 4$; $p < 0.01$), respectively. The inhibition of insulin secretion by SST-14 was clearly reduced in the presence of SNX-482 at different concentrations (1 – 20 nM). For example, SNX-482 (10 nM) significantly reduced SSTR2-dependent inhibition of insulin secretion by 1673 ± 967 % of maximal inhibition (set to 100 %) ($n = 4$; $p < 0.05$). Similar results were obtained using SSTR3 selective agonist, however, at lower levels (e.g. 5 nM SNX-482: 122 ± 28 %; $n = 4$). SNX-482 alone also clearly reduced insulin secretion (e.g. 10 nM SNX-482: 195 ± 92 % of control (stimulated insulin secretion); $n = 4$).

In summary, blockade of the R-type channel activity by the specific blocker SNX-482 attenuated SST-14 and SSTR2 and SSTR3 selective agonists dependent inhibition of insulin secretion from INS-1 cells. These data suggests that the inhibition of insulin secretion from INS-1 cells requires an intact R-type VOCC.

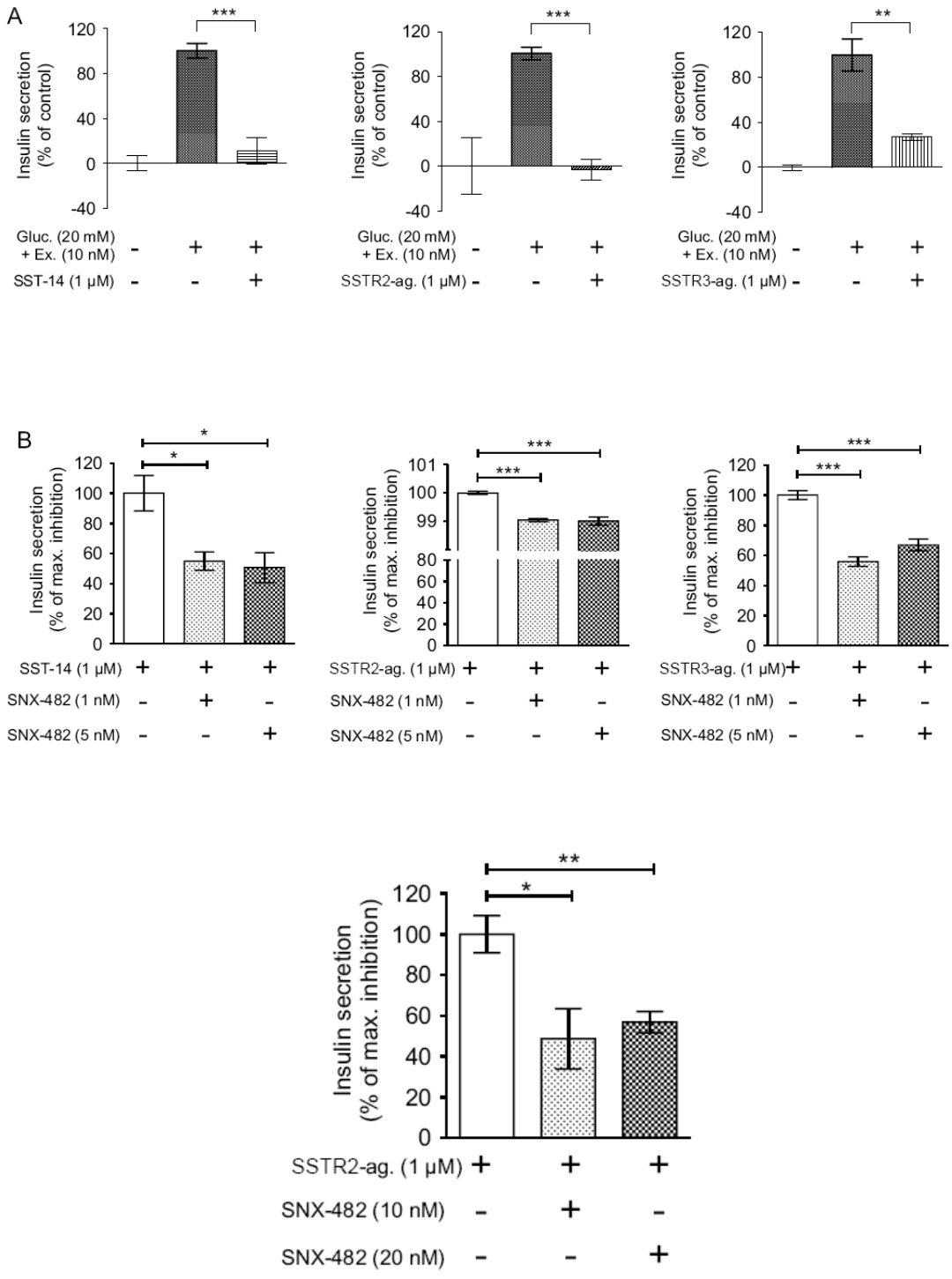


Fig. 18. Consequence of the of R-type Ca^{2+} channel blockade on SST-14 and SSTR2 and SSTR3 selective agonists induced inhibition of 20 mM glucose + 10 nM exendin-4

(GLP-1 agonist) stimulated insulin secretion from INS-1 cells. INS-1 cells were incubated for 1 h with SST-14, SSTR2, and SSTR3 selective agonists (at the indicated concentrations). The concentration of secreted insulin in the medium was determined by ELISA. The data are expressed in percent of maximal secretion, defined as the amount of secreted insulin in the presence of 20 mM D-glucose + 10 nM exendin-4, only. Basal insulin secretion was determined at 3.3 mM glucose. n= 4, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. stimulated secretion (1 h-secretion at 20 mM glucose + 10 nM exendin-4). **A:** The inhibitory effect of SST-14, SSTR2 and SSTR3 selective agonists on 20 mM glucose + 10 nM exendin-4 induced insulin secretion. The weakest inhibition of insulin secretion was observed in the presence of SSTR3 selective agonist (right panel). **B:** The inhibitory effect of SST-14, SSTR2 and SSTR3 selective agonists on 20 mM glucose + 10 nM exendin-4 induced insulin secretion was reduced by the R-type Ca^{2+} channel blocker SNX-482 (at the indicated concentrations). The maximal inhibition by SST-14, SSTR2 and SSTR3 selective agonist effect was set to 100 %. The weakest reduction of the inhibition of insulin by SNX-482 was observed in SSTR3-treated cells (right panel).

3.1.5. Effect of SST-14 and SSTR2 selective agonist on PI3K pathway

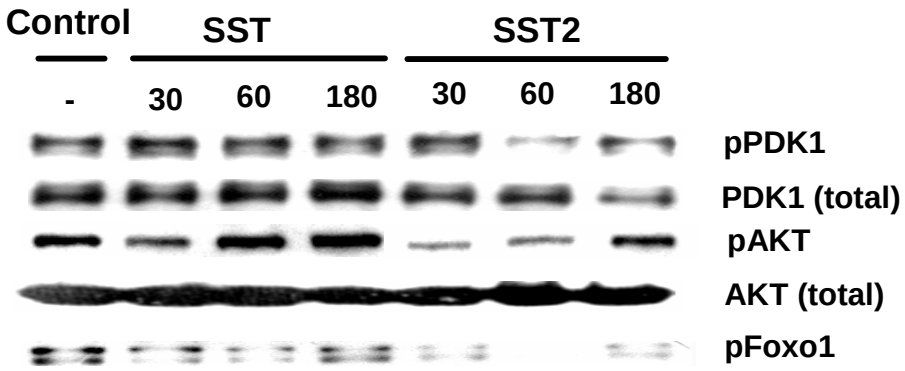


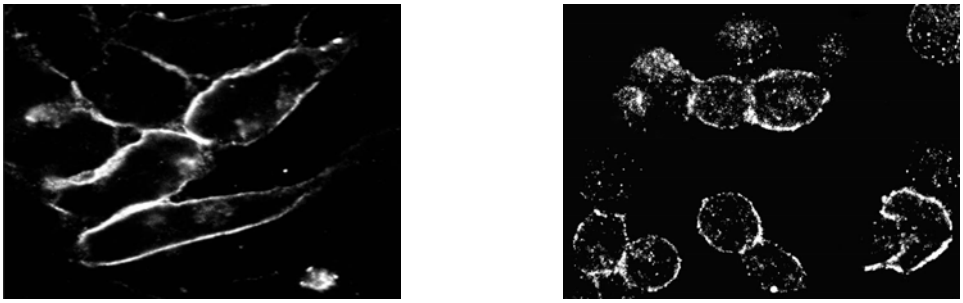
Fig. 19. Effect of SST-14 and SSTR2 selective agonist on PI3K dependent pathway by Western blot. Decrease of PDK1 and Akt phosphorylation levels, no effect on total PDK1 and Akt protein in INS-1 cells.

The PI3K signaling pathway plays an important role in regulating β-cell function such as insulin synthesis and secretion. SST-14 and SSTR2 selective agonist decreased PDK1 and Akt phosphorylation in INS-1 cells, without influencing total PDK1 and Akt protein expression (**Fig. 19)**. Akt transduces its signals by phosphorylating and activating or inactivating a number of substrates. The forkhead transcription factor Foxo1 is phosphorylated by Akt and accumulates in the cytoplasm and therefore becomes inactivated. The SSTR2 analogue decreased Foxo1 phosphorylation, (**Fig. 19)**. Therefore, Foxo1 is possibly involved in the suppression of insulin secretion. It is known that Foxo1 suppresses Foxa2 mediated transcription and subsequently decreases Pdx1 gene expression (downstream target molecule of PI3K pathway) and inhibits insulin secretion. Therefore, we can suggest that SST-14 and SSTR2 selective agonist suppress insulin secretion by inhibiting members of the PI3K pathway and subsequently Foxo1 phosphorylation. Our secretion data **(Fig. 17)** shows that SST-14 and SSTR2 selective agonist inhibits insulin secretion in INS-1 cells.

3.2. InR1-G9 cells (hamster glucagonoma cell line)

3.2.1. Expression of SSTRs in InR1-G9 cells:

RT-PCR revealed that InR1-G9 cells express only SSTR2 (**Fig. 15 A**). The expression of SSTRs was confirmed by immunofluorescence. SSTR2 transfected (human embryonic kidney cells) HEK 293 cells (**Fig. 20)** (ii) (provided by Dr. Stefan Schulz, Magdeburg, Germany) were used as a positive control. InR1-G9 cells showed a strong expression of SSTR2, (**Fig. 20**)



(i) (ii)

Fig. 20. Identification of SSTR2 by immunofluorescence. (i) represents InR1-G9 cells, (ii) represents HEK 293 (positive control) for SSTR2.

3.2.2. Inhibition of intracellular cAMP levels by SST-14 and SSTR2 selective agonist in InR1-G9 cells

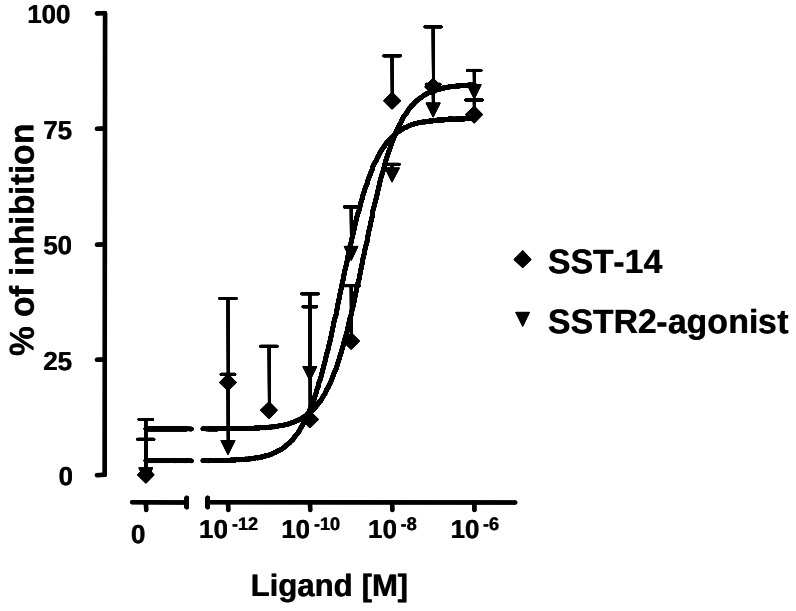


Fig. 21. Effect of SST-14 and SSTR2-selective agonists (10^{-6} M – 10^{-12} M) on forskolin-induced intracellular cAMP accumulation in InR1-G9 cells. (n = 4)

After demonstrating the expression of SSTRs in InR1-G9 cells, we investigated the effect of SST-14 and SSTR2 selective agonist on intracellular cAMP levels. cAMP accumulation was stimulated with forskolin (10 μM). SST-14 and SSTR2 selective agonist potently inhibited forskolin-induced cAMP accumulation in a dose-dependent fashion (EC_{50} values in nM: SST-14: 2.1, SSTR2 selective agonist: 0.98) **(Fig. 21)**. Data are expressed in % of inhibition.

3.2.3. Inhibition of glucagon secretion by SST-14 and SSTR2 selective analogue in InR1-G9 cells

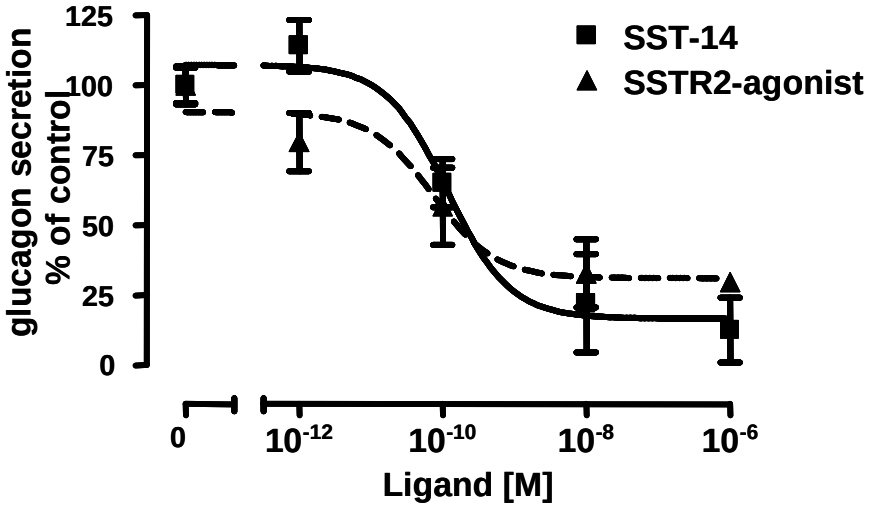


Fig. 22. Effect of SST-14 and SSTR2 (10^{-6} M – 10^{-12} M) selective agonist on glucagon secretion from InR1-G9 cells at 5mM glucose. Cells were incubated in KRB buffer containing 20 mM L-arginine+ 100 µM IBMX for 1 h with SST-14 and SSTR2 selective agonist. The concentration of released glucagon in the buffer was determined. The data are expressed in percent of maximal secretion (± SEM), defined as secreted glucagon in the presence of 20 mM L-arginine+ 100 µM IBMX. (n = 4).

Furthermore, we evaluated the effect of SST-14 and SSTR2 selective agonist on glucagon secretion. Cells were stimulated with 20 mM L-arginine + 100 µM IBMX for 1 h in the presence of 5mM glucose. Basal glucagon secretion rate was defined as the concentration of the secreted hormone in supernatant after 1 h incubation of cells in 5 mM glucose concentration. Data represents the inhibition of stimulated glucagon secretion (% of control). Both SST-14 and SSTR2 selective agonist inhibited 20 mM L-arginine + 100 µM IBMX stimulated glucagon secretion **(Fig. 22).** The calculated EC_{50}

values in nM: SST-14: 3.584e-011 to 3.819e-010 and for SSTR2 selective agonist: 1.347e-011 to 4.000e-010.

Taken together these results demonstrate that SSTR2 selective agonist inhibits intracellular cAMP levels and glucagon secretion with similar potency as SST-14 in InR1-G9 cells.

Die restlichen Seiten des Kapitels 3 „Results“ werden elektronisch nicht veröffentlicht, da es sich um Original-Zeitschriftenartikel handelt.