A somatostatin receptor 1 selective ligand inhibits Ca^{2+} currents in rat insulinoma 1046-38 cells

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Abstract Rat insulinoma 1046-38 cells represent a model system to study β-cell function. The mRNAs for sst1 and sst2, two of the five somatostatin receptors, were detected by reverse transcription polymerase chain reaction amplification in these cells. Displacement binding analysis suggested that sst1 represents the major somatostatin receptor subtype. The sst1 selective compound CH-275 did not inhibit adenylyl cyclases while compounds that activated sst2 did. In contrast, CH-275 caused a marked inhibition of voltage-operated Ca^{2+} channels while the sst2 specific analog octreotide elicited a less pronounced effect suggesting that in rat insulinoma 1046-38 cells sst1 preferably mediates the inhibition of Ca^{2+} channels.

Key words: Neuropeptide; Ca^{2+} channel; Adenylyl cyclase; Insulinoma; Receptor; Somatostatin

1. Introduction

The inhibitory neuropeptide, somatostatin (SST), occurs in at least two biologically active forms, SST-14 and the N-terminally elongated SST-28, and displays a broad anatomical distribution [1]. Both SST peptides mediate similar physiological actions such as the inhibition of hormone release from a variety of different neuroendocrine cells, of exocrine secretion and gut motility and they function as neuromodulators in the central nervous system [2,3]. SST acts on at least five somatostatin receptor subtypes (ssts) that are products from distinct genes [4]. Moreover, the sst2 subtype occurs in two variants that differ in their carboxy-terminal tails, sst2A and sst2B, that result from alternative splicing [5]. Sequence relationship and pharmacology indicated that the somatostatin receptor family is divided into two subfamilies comprising sst1, sst4 and sst2, and sst3 and sst5, respectively [6]. All ssts bind SST-14 and -28 with high affinity and little selectivity although moderate preferential binding of SST-28 has been reported for rat and human sst5 [4,6,7]. The ssts differ, however, in their affinity for synthetic peptides. For instance, octreotide represents a key ligand with clear selectivity for sst2 [6] and CH-275 the only analog with selectivity for sst1 [8]. No sst3 and sst4 selective compounds are available to date [9]. When expressed in cell lines ssts are coupled to a variety of cellular effectors. In the case of sst1, sst2 and sst4 coupling to adenylyl cyclases (AC) has been observed or not while sst3 and sst5 have consistently been shown to lower cAMP levels [4]. The observed differential coupling to AC of sst2A and sst2B [10] and different cellular environments for sst1 and sst4 may account for the discrepancies. Moreover, all ssts activate phospholipase C-β and involvement in serine/threonine or phosphotyrosine dephosphorylation has been observed for sst1, sst2 and sst5 [4,11]. sst2 and sst3 have been reported to modulate ion channels. In particular, sst2 has been demonstrated to inhibit Ca^{2+} currents, highlighting its importance in endocrine regulation [4,6]. In contrast, inhibition of Ca^{2+} channels by sst1 has not been observed so far.

Here we report that neuroendocrine RIN1046-38 cells possess sst1 and sst2 somatostatin receptors and that sst2 selective peptides cause strong inhibition of AC while the sst1 preferring compound CH-275 did almost not inhibit AC. Quite in contrast, this compound caused stronger inhibition of voltage-activated Ca^{2+} channels than sst2 selective peptides. These results suggest that in RIN1046-38 cells the somatostatin receptor subtype 1 causes inhibition of Ca^{2+} channels and hence may play a role in endocrine regulation.

2. Materials and methods

2.1. Reverse transcriptase polymerase chain reaction amplification

RIN1046-38 cells were grown in RPMI 1640 medium supplemented with 25 mM HEPES, 5% fetal calf serum and 5% newborn serum. Cells (50–100 mg) were collected by low speed centrifugation and homogenized in RNAzol (AGS, Heidelberg, Germany) and isolation of RNA, cDNA synthesis, and PCR of sst1–sst5 cDNA fragments, blotting and hybridization to ^{32}P-labeled sst subtype specific oligonucleotides were performed essentially as described previously [12].

2.2. Membrane preparation and ligand binding

Cells were harvested and membranes prepared as described [12]. The membrane fraction was suspended in 1 ml of the same buffer per 175 cm^2 cell culture flask, the protein concentration determined using a protein assay kit (BioRad, Munich, Germany) and frozen in 10-μl aliquots in liquid nitrogen. Ligand binding was performed [12] using 7.5 μg membrane protein per assay. Bound radiolabeled peptide was measured in a γ-counter (Canberra Packard, Dreieich, Germany). Background binding was determined in the presence of 10 μM SST-14. Calculations were carried out using SigmaPlot.

2.3. cAMP assays

For the determination of intracellular cAMP levels cells were plated the day before into 24-well microtiter plates and incubated in serum-

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Abbreviations: SST, somatostatin; sst, somatostatin receptor subtype

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free medium with 0.5 mM 3-isobutyl 1-methylxanthine. Ten minutes later 25 μM forskolin was added together with or without peptide agonists at the indicated concentrations. After 45 min, incubations were terminated by the addition of ethanol (−20°C) to 70%. Following extraction at −20°C for 14 h the supernatant was removed from the cells and the solvent evaporated. The remainder was dissolved in cAMP assay buffer and subjected to cAMP determinations as recommended by the manufacturer (Amersham, Braunschweig, Germany).

2.4. Electrophysiological recordings

Voltage-clamp recordings were obtained using the whole-cell mode of the patch-clamp technique [13]. Small coverslips with adherent cells were placed into a recording chamber (0.3 ml) mounted on an inverted microscope (Axiovert 100, Zeiss, Jena, Germany). Cells were continuously perfused at room temperature (22–24°C) with a standard external solution which comprised: 130 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.3). Patch pipettes were pulled from borosilicate capillary glass (Hilgenberg, Malsfeld, Germany). The pipettes had an initial resistance of 7 to 10 MΩ when filled with a solution containing 130 mM CsCl, 4 mM $MgCl_2$, 3 mM ATP, 10 mM EGTA, 10 mM HEPES (pH 7.3). Seal resistances >10 GΩ were routinely obtained by applying gentle suction to the pipettes. Membrane rupture was monitored electrically as an increase in capacitance. Pipette capacitance, membrane capacitance, and series resistance were electronically compensated to achieve minimal capacitive currents [14]. Cell responses were recorded by an Axopatch 200 A amplifier (Axon Instruments, Inc., Foster City, CA, USA). A 486 computer in combination with the ISO 2 patch-clamp software (MFK, Niedernhausen, Germany) was used for data acquisition and analysis. Whole-cell currents were filtered at 1 kHz. Currents were corrected for linear leakage and capacitive components before being digitized and stored by the computer at a sampling rate of 5 kHz. To isolate inward currents through voltage-gated calcium channels, barium was used as charge carrier in a sodium- and potassium-free solution consisting of: 120 mM *N*-methyl-D-glucosamine, 10.8 mM $BaCl_2$, 5.4 mM CsCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.3).

3. Results and discussion

3.1. Identification of ssts in RIN1046-38 cells

Polymerase chain reaction amplification experiments, DNA blotting and hybridization of the amplified fragments with receptor subtype specific probes [12] were carried out in order to determine which particular ssts might be present in RIN1046-38 cells. The observed size of the detected fragments of about 320 bp and 330 bp using sst1 and sst2 specific oligonucleotides corresponded to the predicted fragment sizes of 318 and 332 bp. It is therefore concluded that these cells contain mRNAs for sst1 and sst2 (Fig. 1). However, the band corresponding to sst1 mRNA appears to be much more intense than that corresponding to sst2 mRNA. Other bands have not been detected. This observation suggests that RIN1046-38 cells likely contain a dual somatostatin receptor

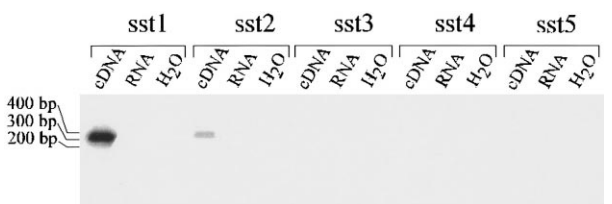

Fig. 1. DNA blot analysis of somatostatin receptor cDNA fragments following RT-PCR amplification, agarose gel electrophoresis and transfer to nylon membranes. Amplification reactions were carried out with sst specific primers using either cDNA (cDNA) or, for control of contamination with genomic or other DNA, with RNA (RNA) or in the absence of a DNA template (H_20). The fragment lengths of a size standard run in parallel are indicated on the left.

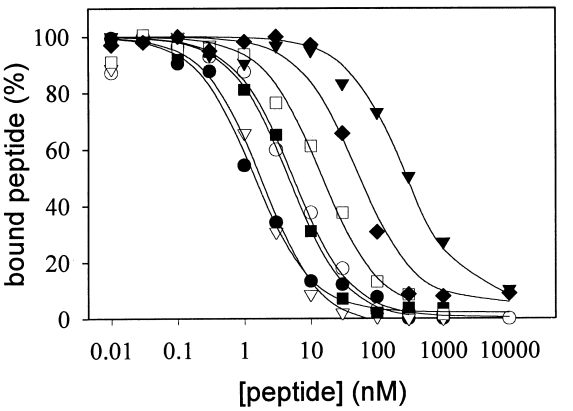

Fig. 2. Displacement of ^{125}I-Tyr^{11}-SST-14 from RIN1046-38 cell membranes by various SST analogs. The data represent one typical of three independent experiments performed in duplicate. The data were fitted by non-linear regression using the SigmaPlot program. Observed IC_{50} values were 1.4 ± 0.2 nM for SST-28 (filled circles), 1.8 ± 0.3 nM for SST-14 (open triangles), 4.7 ± 0.5 nM for BIM23295 (filled squares), 5.4 ± 0.7 nM for BIM23268 (open circles), 14 ± 2 nM for BIM23052 (open squares), 51 ± 10 nM for CH-275 (diamonds) and 238 ± 34 nM for octreotide (filled triangles).

population consisting primarily of sst1 and to a lesser extent of sst2. The sst mRNAs appear to be translated into functional receptors since RIN1046-38 cells contain high affinity binding sites for ^{125}I-Tyr^{11}-somatostatin-14 [15]. Displacement binding experiments have been used to identify which sst subtypes contribute to the population of somatostatin receptors (Fig. 2). The two naturally occurring peptides, SST-14 and SST-28, were about equally potent to displace the radioligand. Since sst5 shows slightly higher affinity for SST-28 [4,6,7] it appears unlikely that RIN1046-38 cells contain high levels of this subtype. Octreotide which has high affinity for rat sst2 (K_i = 2 nM) and moderate affinity for rat sst3 and sst5 (14 and 17 nM, respectively) [9] displays an IC_{50} value of 300 nM. No partial displacement of the radioligand was observed at lower concentrations of the competitor. These results exclude the possibility that the sst2, sst3 and sst5 subtypes represent the major somatostatin receptor in RIN1046-38 cells. However, the sst1 preferring analog CH-275 [8] which displays an IC_{50} value of 46 nM in human embryonic kidney cells transfected with sst1 cDNA completely displaced the radioligand from RIN1046-38 cell membranes with a similar IC_{50} value (51 ± 10 nM). CH-275 has been demonstrated to be of at least 5-fold or 10-fold lower affinity at rat sst5 or sst4, and of even lower affinity at the other rat sst subtypes [12]. Therefore, sst1 represents most likely the major somatostatin receptor in RIN1046-38 cells. The reasonable correlation of the IC_{50} values of the somatostatin analogs BIM23052, BIM23268 and BIM23295 (Fig. 2) observed in RIN1046-38 cells with their IC_{50} values obtained using cell lines transfected with sst1 cDNA [9] (51, 5.7 and 4.7 nM vs. 97, 12 and 5.4 nM) also supports this conclusion. To date there is no sst4 selective analog available [4,6,9] which can be used to directly rule out the possibility that sst4 receptors are present in RIN1046-38 cells. However, the observed lack of sst4 mRNA is indicative of the absence of the sst4 subtype. Taken together these findings suggest that the vast majority of somatostatin receptors in the membranes of RIN1046-38 cells are of the sst1 subtype.

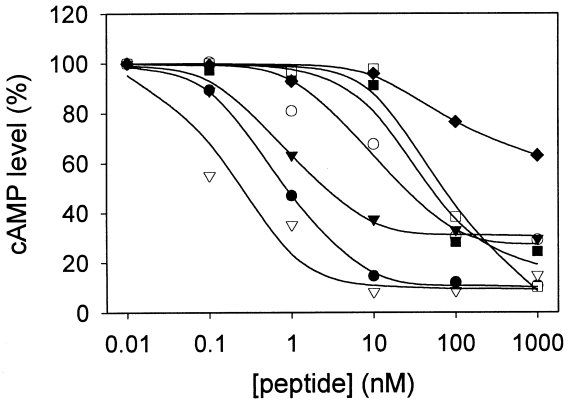

Fig. 3. Dose–response relationship for the inhibition of forskolin-stimulated adenylyl cyclase by various SST analogs. The data are the mean of three independent experiments that have been carried out in triplicate. The data were fitted by non-linear regression using the SigmaPlot program. SST14 (EC_{50} = 0.3 nM; open triangles), SST-28 (EC_{50} = 0.7 nM; filled circles), octreotide (EC_{50} = 1 nM, filled triangles), BIM23268 (EC_{50} = 30 nM; open circles), BIM23295 (EC_{50} = 100 nM; filled squares), BIM23052 (EC_{50} = 80 nM; open squares) and CH-275 (EC_{50} cannot be determined due to incomplete inhibition at very high peptide concentrations; diamonds).

3.2. Coupling of sst2 but not sst1 to the inhibition of AC

Application of SST-14 and SST-28 caused a severe inhibition of forskolin-stimulated AC (Fig. 3). Surprisingly a similar response was also seen when the cells were stimulated with octreotide. This observation is in line with the observed low abundance of sst2 mRNA and suggests that RIN1046-38 cells contain a marginal population of sst2 receptors. Apparently, this is too low to be detected in the displacement binding experiments. The small number of sst2, however, suffices to induce a strong inhibition of AC. Consistent with this assumption is the observation that the compounds BIM23052, BIM23268 and BIM23295 inhibited AC at concentrations (80 nM, 30 nM, 100 nM, respectively) that correspond reasonably to their affinities at sst2 (69 nM, 101 nM, 163 nM, respectively) [9,12]. The high IC_{50} values of these compounds also indicate that the inhibition of AC is not mediated by sst3 or sst5 which have 10- to 100-fold higher affinity for these analogs [9]. The sst1 preferring analog CH-275 caused only a small inhibition of AC at very high concentrations. In this high concentration range the sst2 subtype may bind and hence be activated by CH-275. Thus, it is concluded that the inhibition of AC in RIN1046-38 cells is mediated by sst2 and not by sst1 somatostatin receptors.

3.3. sst1 and sst2 mediate inhibition of voltage-activated Ca^{2+} channels

Electrophysiological recordings (n = 12 cells) have been carried out to analyze whether SST or its analogs can cause inhibition of voltage-activated Ca^{2+} channels and which sst subtype may be involved. Fig. 4 shows that 10 nM SST-14 reduced inward Ba^{2+} currents through voltage-activated Ca^{2+} channels by 12–53% (n = 8; Fig. 4A). Six of these cells responded to application of CH-275 with a 18–34% inhibition of Ca^{2+} channels (Fig. 4B). Three of these cells that were responsive to SST-14 and CH-275 were also tested with octreotide. This analog reduced inward Ba^{2+} currents through voltage-activated Ca^{2+} channels similar to SST-14 and CH-275 in all three cells. Four other cells that responded to CH-275 challenge with the inhibition of Ca^{2+} channels (15–48%) but were not tested with SST-14 were also exposed to octreotide. This compound induced a weaker response in these cells (10–18%; Fig. 4C). Thus the sst1 preferring compound CH-275 caused a marked inhibition of voltage-activated Ca^{2+} channels in RIN1046-38 cells that exceeded that induced by octreotide. Therefore, the results suggest that in addition to sst2 and sst5 also the sst1 subtype can functionally couple to Ca^{2+} channels.

The finding that sst1 mediates inhibition of voltage-gated Ca^{2+} channels is rather surprising. Firstly, because it has been shown that in another insulin-secreting rat insulinoma cell line, RINm5F cells, the sst2 subtype mediated inhibition of voltage-activated Ca^{2+} currents [16]. Secondly, because transfection of sst1 cDNA in RINm5F cells did not lead to SST-induced inhibition of voltage-gated Ca^{2+} channels while transfection of sst2 cDNA did [17]. To rule out that an as yet unidentified receptor displays a pharmacology similar to sst1 and mediates the inhibition of Ca^{2+} currents, the cDNA fragment amplified with the sst1 specific primers was subcloned and sequenced (results not shown). Six from seven sequences differed in nine nucleotide positions from the published DNA sequence [18] without changing the amino acid sequence. The seventh sequence displayed in addition an A to C transversion in codon 306 resulting in the exchange of glutamine for proline. Whether this sequence corresponds to an allelic variant of sst1 or represents a PCR artifact remains unknown. In the

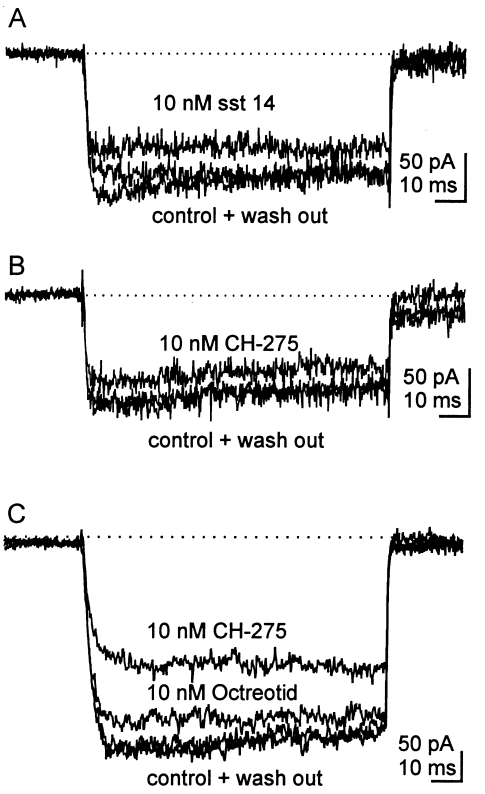

Fig. 4. Inhibition of voltage-activated inward Ba^{2+} currents through voltage-activated Ca^{2+} currents by SST peptides. Cells were voltage-clamped at −80 mV and depolarized to 0 mV for 100 ms before and during peptide application as well as after washing out of the peptide. Representative current traces are depicted. Current traces obtained from one cell are shown superimposed. A: response to SST-14; B: response to CH-275; C: response to CH-275 or octreotide.

former case the mutation would be localized in the third extracellular loop. It has been demonstrated that in sst1 this region contains important determinants that are responsible for hexapeptide and octapeptide binding while binding of SST-14 and CH-275 depends on determinants present in the second extracellular loop [19,20]. Therefore, it seems highly unlikely that a mutant sst1 receptor with an altered pharmacology accounts for our observations. It is also unlikely that another unidentified receptor that would not be amplified with sst1 specific primers mediates the inhibition of calcium channels. The closest relatives of somatostatin receptors are the opioid receptors. It is known that these receptors do bind SST analogs. They are, however, cyclic octapeptides [21]. CH-275 comprises eleven amino acids [8]. In RINm5F cells a specific G protein trimer, $G\alpha_{o2}/\beta_1/\gamma_3$, couples SST via the sst2 to the inhibition of voltage-activated Ca^{2+} currents [16]. It could be that in RIN1046-38 cells another G protein trimer couples the sst1 to this cellular effector. Alternatively, sst1-mediated inhibition of Ca^{2+} channels could proceed through a completely different pathway. Nonetheless, the inhibition of voltage-activated Ca^{2+} currents by sst1 observed in the present report points to an importance of this receptor subtype in endocrine regulation.

It has been reported [22] that in neocortical membranes at least two types of somatostatin binding sites are present: one, termed SS-1 or SRIF-1, with high affinity for SST-14 and the synthetic ligand MK-678; and another one, SS-2 or SRIF-2, with high affinity for SST-14 and the analog CGP23996 [23,24]. Both analogs, MK-678 and CGP23996, mediated the inhibition of voltage-activated Ca^{2+} channels in these cells while only the former additionally potentiated a potassium conductance [22]. Later on the pharmacological identity between somatostatin SS-2 binding sites and the sst1 somatostatin receptor subtype has been demonstrated [25]. These findings suggest that in addition to the sst1-mediated inhibition of voltage-activated Ca^{2+} channels in RIN1046-38 cells the same subtype may also elicit the inhibition of voltage-activated Ca^{2+} channels in rat neocortical neurons.

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