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Molecular Pharmacology of Somatostatin Receptors

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By:

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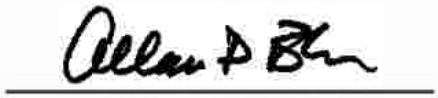

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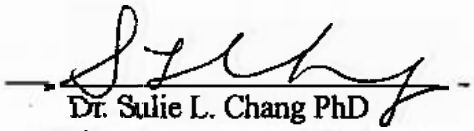

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Abstract:

Somatostatin (somatotropin release inhibitory factor, SRIF), inhibits animal cell secretion and proliferation. SRIF is transcribed from a single gene as a 116 amino acid precursor that is cleaved in a cell specific manner into two distinct peptides, SRIF-14 and SRIF-28. Each binds with high affinity to all five known SRIF-receptor subtypes (sst_1 – sst_5) and several of these receptor subtypes are endogenously expressed in the AtT-20 cell, a murine pituitary corticotroph. SRIF receptors belong to the G protein-coupled family of integral membrane receptors. Within the SRIF receptor family, it has been shown that the sst_2 receptor subtype exists as splice-variants (sst_{2A} and sst_{2B}) that exhibit over-lapping tissue expression. The sst_{2A} and sst_{2B} splice-variants occur within the carboxyl-terminus of the receptor, an area of the protein, which is not involved in ligand binding, thereby making a pharmacologic distinction of these two subtypes extremely difficult. Indeed, the function of these receptor splice-variants, as well as the significance of their cellular co-expression is unknown. To delineate the function of these closely related receptors, we have employed double-stranded RNA interference (dsRNAi), a highly selective post-transcriptional gene silencing method. dsRNAi allows sst_{2A} mRNA to be specifically targeted and inhibited while testing the functional capabilities of the remaining receptor population. When used in conjunction with receptor subtype selective agonists, dsRNAi provides a powerful genetic tool to explore receptor function. In the current study, we inhibit the expression of the sst_{2A} receptor with dsRNAi, and examine the functional consequences of this inhibition by monitoring receptor protein expression with subtype selective antibodies, as well as assessing the functional consequences of this inhibition on intracellular cAMP accumulation. Our results demonstrate that RNA silencing oligonucleotides against the sst_{2A} carboxyl terminus can be incorporated into

AtT-20 cells in a time and concentration-dependent manner. Furthermore, the uptake of these sst_{2A} inactivating oligonucleotides, suppresses sst_{2A} receptor expression and inhibits receptor cyclic nucleotide effects. Taken together, these results suggest that RNA interference is a promising approach to defining the function of highly homologous receptors that cannot be distinguished through conventional pharmacologic approaches.

Introduction :

Somatostatin (SRIF) is an important modulator of cellular physiology, exhibiting a wide spectrum of biological functions such as inhibiting cellular excitability, proliferation, endocrine and exocrine secretion, as well as possessing immunomodulatory properties. Cellular mechanisms that are induced by SRIF include inhibition of adenylyl cyclase, modulation of K^+ and Ca^+ channels, and protein dephosphorylation (Puente et al., 2001). SRIF is transcribed from a single gene as a 116 amino acid precursor, preprosomatostatin (Gillies, 1997). Two distinct forms of SRIF are derived from the SRIF-prepropeptide, SRIF-14 and SRIF-28, which differ in amino acid composition and cellular origin. SRIF is widely distributed throughout the human body, however, SRIF-28 is predominately found in the gastrointestinal tract, while SRIF-14 is localized to the nervous system (Reisine et al., 1997).

The biological effects of SRIF are mediated through its specific binding to a highly homologous family of G protein-coupled receptors that are designated sst_1-sst_5 (Hoyer et al., 1995). SRIF receptors couple to heterotrimeric guanine nucleotide binding proteins (G proteins) that are predominately inhibitory G_i and G_o proteins (Law et al., 1991). All somatostatin receptors bind SRIF-14 with high affinity and serve to reduce cellular responsiveness by inhibiting cAMP production, regulating ionic conductances and controlling protein phosphorylation states (Pfeiffer et al., 2000).

Of the five currently identified SRIF receptors only one exists as a splice-variant. Interestingly, the sst_2 receptor exists as two splice-variants, sst_{2A} and sst_{2B}, which are cleaved from the carboxyl terminus of the same mRNA transcript (Vanetti et al., 1992). In rodents, there appears to be greater expression of the sst_{2A} receptor in comparison to the sst_{2B} (Sarret et al., 1998). The SRIF splice-variants (sst_{2A} and sst_{2B}) as well as the

five SRIF-receptor subtypes (sst_1-sst_5), exhibit a broad and overlapping tissue distribution, (Breder et al., 1992) making for the assignment of functional responses to an individual SRIF receptor subtype extremely difficult.

Recent approaches used to study SRIF receptor subtypes include the development of subtype-selective, non-peptidyl SRIF agonists (Rohrer et al., 1998). This pharmacological technique relies on the high affinity and selectivity of the synthetic agonists for given receptor subtypes. With high affinity interactions that demonstrate a high degree of specificity, biological responses can be attributed to individual receptor subtypes within a mixed population of homologous receptors. The availability of highly selective non-peptidyl agonists for each of the SRIF-receptors has greatly facilitated the study of subtype-selective coupling of SRIF receptors and the physiological consequences of receptor function (Rohrer et al., 1998; Parmar et al., 1999; Strowski et al., 2000; Blake, 2001; Cervia et al., 2002; Strowski et al., 2002; Cervia et al., 2003). However, this technique currently lacks the ability required to delineate between receptor subtype splice-variants when the receptor regions are not involved in ligand binding.

An alternate approach used in the study of SRIF receptor subtypes is genetic, requiring the generation of knock-out organisms that lack the gene for the receptor of interest (Zhang et al., 1997; Strowski et al, 2003). By genetically inactivating the gene of interest, the remaining phenotypic consequences can be monitored. Although this method is capable of distinguishing between receptor subtypes, it is expensive, time-consuming and lacks the ability to delineate between receptor subtype splice-variants, as they are transcribed from the same gene.

Recently, a powerful technique termed double-stranded RNA interference (RNAi) has been developed in mammalian cells that enables for the study of highly homologous proteins, including receptor splice-variants. RNAi has been shown to be remarkably effective at suppressing specific gene expression in *Caenorhabditis elegans*, *Dropsophila mealanogaster*, *Trypanosoma brucci*, and plants by a pathway involving sequence-specific posttranscriptional gene silencing (Sharp, 2002). It has also been demonstrated that RNAi functions in cultured mammalian neurons, where introduction of low concentrations of dsRNA into rat hippocampus and forebrain cultures can be effective in suppressing endogenous and heterologous genes (Krichevsky et al., 2002). Numerous genes have been successfully knocked-down in mammalian somatic and embryonic cell lines as well, including HeLa, HEK293, and P19 (Harborth et al., 2002). A detailed understanding of RNAi mechanisms remains unclear. However, there is evidence that suggests that dsRNAs are cleaved by ribonuclease III into 21-22 nucleotide RNA duplexes termed small interfering-RNAs or siRNAs (Zamore et al., 2000). These molecules, containing 2 to 3 nt 3' overhanging ends, a 5' phosphate, and 3' hydroxyl termini (Elbashir et al., 2001), have been shown to assemble with a multi-component enzymatic complex referred to as the RNA-induced silencing complex, RISC (Hammond et al., 2000). The single-stranded antisense siRNAs, when paired with RISC, serve to guide the enzyme to target mRNA transcripts for cleavage (Martinez et al., 2002). It is in this manner that RNAi holds great promise for exploring the functional significance of receptor splice-variants, where it can be used to selectively target and degrade complimentary mRNA sequences.

Establishing a reliable methodology for studying SRIF receptor subtype splice-variants is critical to understanding the role of these proteins in the cell. In the present study we use RNAi to examine the functional significance of SRIF receptor splice-variants in the AtT-20 cell model. AtT-20 cells functionally co-express $sst_{2A, 2B}$, sst_5, but do not appear to express $sst_{1, 3,4}$ (Strowski et al., 2002; Cervia et al., 2003). Through the ability of RNAi to selectively inhibit sst_{2A} mRNA and therefore receptor expression, we determine the function of the sst_{2A} receptor by correlating inhibition of receptor expression with loss of function. The effects of RNAi on the production of intracellular cyclic nucleotides such as cAMP were examined as SRIF receptor subtypes have been shown to inhibit cAMP accumulation (Strowski et al., 2002). Our results indicate that inhibition of sst_{2A} expression in RNAi treated cells results in a corresponding supression of sst_{2A} receptor protein production, as well as a loss in the ability of the sst_{2A} receptor to inhibit forskolin-stimulated cAMP production. These findings suggest that the sst_{2A} receptor splice-variant serves to modulate intracellular cyclic nucleotide production, and provide evidence that RNAi is a powerful gene-silencing tool capable of distinguishing between co-expressed receptor subtype splice-variants.

Materials and Methods :

Materials:

The AtT-20 cell line used in this study was a gift from Dr Terry Reisine (Los Angeles, CA) and cell culture dishes and flasks were purchased from Corning (Corning, NY). Cell culture, western blotting, and protein gel reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA). Western blotting detection reagents were acquired from Amersham (Piscataway, NJ). Blue sensitive X-ray film was purchased from Denville Scientific (Metuchen, NJ) and was developed using an automatic Futura 2000E X-ray film processor received from Fischer Industries (Geneva, IL). All RNAi reagents were obtained from Gene Therapy Systems, Inc. (San Diego, CA). SRIF-14 was purchased from Peninsula Labs (Behnont, CA) while the non-peptidyl sst_{2A}-selective analog L-779,976 was obtained from Merck and Co, Inc. (Rahway, NJ). The cAMP radioimmunoassay kits (RIAs) were purchased from Amersham (Piscataway, NJ) and the results were quantified with the Wallac Wizard 1740 Automatic gamma scintillation counter from Wallac Inc. (Gaithersburg, MD).

Cell Culture :

AtT-20 cells were cultured in Dulbecco's modified Eagle's medium, DMEM, (with GlutaMAXTH, high glucose, 110mg/L sodium pyruvate and pyridoxine-HCL, Cat. No. 10569-010) containing 100 U ml^{-1} of penicillin and 100 µg ml^{-1} streptomycin and 10 % fetal calf serum in 5 % CO_2 at 37°C. Cell monolayers were grown in T75-cm^2 flasks and passaged when the monolayers achieved 70 % confluence. Cells were passaged by washing with 5.0 ml of phosphate buffered saline (PBS) (without CaCl and MgCl, Cat. No. 14190-136) incubating with 0.5 ml trypsin/EDTA (with 5.0 g trypsin, 2.0 g EDTA-

Na and 8.5 g NaCl/L, Cat. No. 15400-054) and 5.0 ml PBS for 3 min, and resuspended in 5.0 ml of DMEM. Cell suspensions were transferred at a 1:10 dilution to new T75-cm^2 flasks and incubated at 37° C in a humidified atmosphere of 5% CO_2, 95% air.

RNA Interference Studies :

AtT-20 cells were subcultured in 24-well plates to achieve 60-70 % confluence prior to RNAi experiments. The media was then aspirated and cells were washed twice with 1.0 ml of the reduced serum medium, Opti-MEM, (with GlutaMAX™, HEPES buffer and 2,400 mg/L sodium bicarbonate, Cat. No. 51985-034). The RNA interference reagents were prepared by first placing dsRNA into a 90°C heating block for 1 min followed by a 37° C water bath for 30 min. The GeneSilencer™ dsRNA Transfection Reagent was prepared in three stages: (1) GeneSilencer™ (GTS) reagent was diluted with 25 µl of Opti-MEM for a final volume of 3.5 µl/well. (2) The dsRNA solution was prepared by mixing 1.0–10.0 µg of dsRNA, 10.0 µl of dsRNA-Dilutent and 15.0 µl of Opti-MEM per well, followed by a 5 min incubation at room temperature. Final concentrations of dsRNA ranged from 0.3 –3.0 µM/well. (3) The diluted GeneSilencer™ (GTS) reagent from (1) was added to the dsRNA solution from (2) and incubated an additional 5 min at room temperature to allow for the formation of dsRNA/lipid complexes. Various amounts of the complexed GTS/dsRNA-solution were mixed with 200 µl of Opti-MEM, added to the corresponding wells, and incubated at 37° C for 48 hours. Following incubation, cells were placed in a fluorescent plate reader and intracellular fluorescence was recorded using a Cytofluor 4000 fluorescence plate reader (Excitation= 485 nm, emission= 530 nm). Cells were then washed twice in 1.0 ml/well

PBS (containing CaCl and MgCl), mixed with 1.0 ml of Opti-MEM/well and fluorescence was measured a second time. The recorded results were analyzed using GrapPad Prism 3.0 (Blake, 2001).

Western Blotting and Immunodetection :

AtT-20 cells were cultured in 24-well plates, subjected to RNAi and subsequently analyzed by western blotting. Growth medium was aspirated and cells were lysed with (1X) NuPage™ Sample Buffer. Protein samples were collected and stored at –20° C. Prior to loading, protein samples were allowed to thaw at room temperature, sonicated, and placed in a 70° C heating block for 10 min. Samples were loaded at 15.0 µl/well and electrophoriesed on 10% Bis-Tris Gels for 2 hours. Samples were transferred from the gel to PVDF membranes for 1 hour, and immersed in 5 % non-fat dried milk solution, on a shaker, for 45 min to block non-specific binding. Membranes were rinsed in TBST (with 20 mM Tris-HCL, 150 mM NaCl and 0.5 ml Tween 20) and incubated overnight in a diluted (1:1,000 in TBST) rabbit polyclonal anti-sst_{2A} (R2-88-B6) antibody. Membranes were washed 4 times at 15 min intervals in TBST and bound primary antibody was detected with a diluted (1:2,000 in TBST) horseradish anti-rabbit secondary antibody during a 45 min incubation. The membranes were incubated at room temperature for 5 min with a 40:1 ratio of chemiluminescence reagent (3.0 ml solution A, 75.0 µl solution B), dried, and placed protein side up into an X-ray film cassette. Films were exposed for 1, 5 and 10 min intervals, developed with an automatic X-ray film processor, and the results were analyzed with the Scion Software version of NIH Image.

cAMP Accumulation Studies :

AtT-20 cell monolayers were subcultured in 24-well plates to 70 % confluence and exposed to dsRNA as previously described. Cells were incubated in growth medium plus 0.5 mM isobutylmethylxanthine (IBMX) for 30 min at 37° C. To stimulate intracellular cAMP accumulation, the culture medium was removed and replaced with medium with or without 10 μM forskolin plus somatostatin analogs (SRIF-14, L-779,976), and transferred to 37° C for 10 minutes. The reaction was terminated upon aspiration of experimental conditions, cells were lysed and cAMP levels were stabilized with addition of 0.5 ml/well of 1 N HCL. Plates were stored at –20° C until cAMP accumulation was determined by radioimmunoassay (Amersham). Data obtained was analyzed by nonlinear regression analysis with GrapPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

A.

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp
|
Cys-Ser-Thr-Phe-Thr-Lys

B.

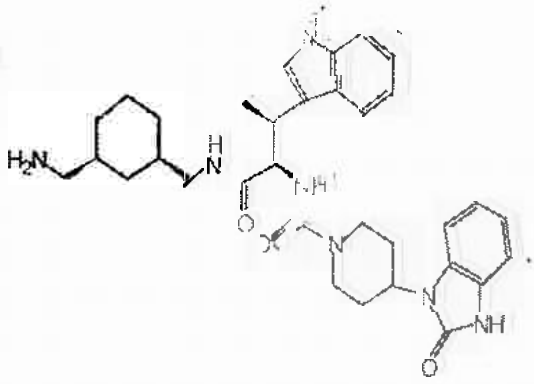

Figure 1 : A. Amino acid sequence of SRIF-14. B. Structure of the sst_{2A}-selective analog L-779,976 (Rohrer et al., 1998).

Results:

Determination of dsRNA uptake

We have used the gene-silencing tool, RNA interference, to inhibit the expression of the sst_{2A} receptor to determine if there was a corresponding loss of receptor function. In order for RNAi to be effective, dsRNA must be synthesized and packaged into liposomes that can then fuse with cellular membranes to release their oligonucleotide contents within the cell. In order to determine if uptake of dsRNA occurs and whether it is saturable, an AtT-20 cell population was subjected to various concentrations of synthetic oligonucleotide incorporated with a fluorescien isothiocynate marker (FITC) and GTS reagent, the cationic lipid solution that forms dsRNA vesicular complexes. According to the data obtained (Figure 1), dsRNA concentrations of 0.5 – 1.5 μM /well produced maximal uptake prior to saturation with 1.5 – 3.0 μM/well concentrations. Conversely, when AtT-20 cells were subjected to various volumes of GTS reagent, with a constant 2.5 μg/well of dsRNA, a linear uptake of oligonucleotide occurred with 10.0 μl/well GTS reagent samples producing maximal fluorescence and no marked saturation (Figure 2). This data set the guidelines for dsRNAi experiments, where 2.5 μg of dsRNA and 10.0 μl of GTS reagent per well were used in these studies.

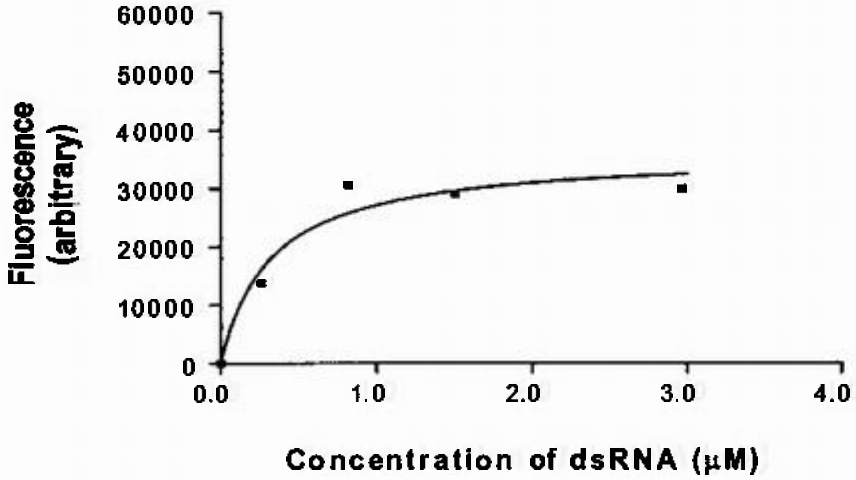

Figure 1 : Fluorescent-labeled synthetic oligonucleotide uptake study with AtT-20 cells. Cell monolayers at 70 % confluence were treated with various concentrations of dsRNA while fluorescence was measured and quantified with a Cytofluor 4000 fluorescent plate reader. The results were analyzed using Graphpad Prism 3.0.

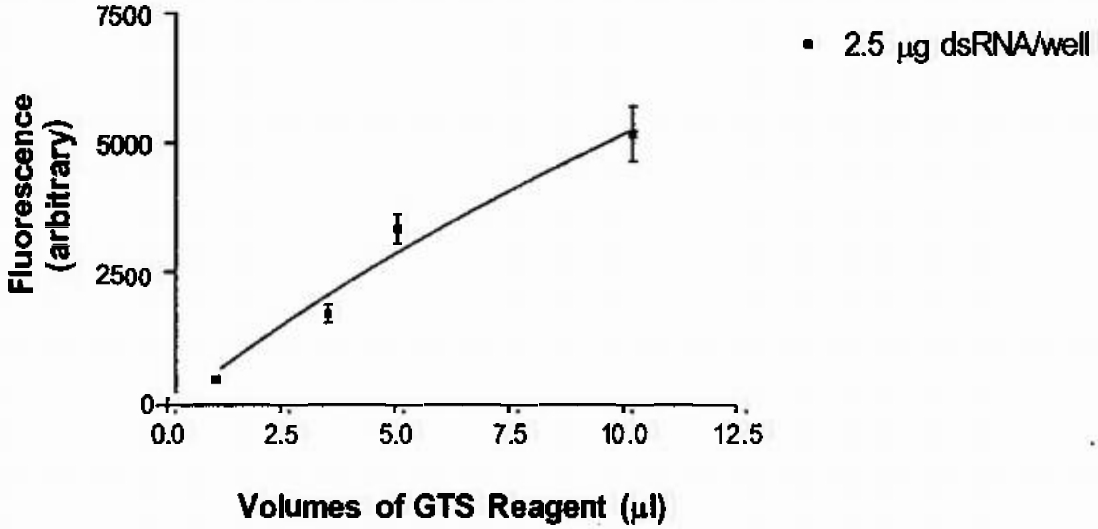

Figure 2 : Fluorescent-labeled synthetic oligonucleotide uptake study with increasing volumes of GTS reagent. AtT-20 cell monolayers were treated with increasing volumes of GTS reagent and a constant 2.5 μg of dsRNA/well. Fluorescence was measured with a Cytofluor 4000 fluorescence plate reader and the results were analyzed using Graphpad Prism 3.0.

RNA Interference Inhibits sst_{2A} Receptor Expression

After determining the concentrations of dsRNA and volumes of GTS reagent that yielded optimal uptake of oligonucleotide, (Fig 1 and Fig 2) the ability of RNAi to inhibit sst_{2A} receptor protein expression was determined. Cell lysates were prepared and proteins separated by SDS-PAGE. Western Blot analysis was performed to detect for changes in the expression of the sst_{2A} receptor on AtT-20 cell populations that were exposed to various volumes of GTS reagent with a constant 2.5 μg of dsRNA.

Figure 3 : Autoradiographic results showing inhibition of sst_{2A} protein upon treatment with 10.0 μl/well of GTS reagent and 2.5 μg/well of dsRNA. AtT-20 cell monolayers were cultured in serum free basal medium and incubated in the absence or presence of varying volumes of GTS reagent. Cell lysates were resolved on Nu-Page 10 % Bis-Tris gels, electroblotted and incubated with an anti-sst_{2A} (R2-88-B6) primary antibody. Immunoreactivity was detected using a horseradish anti-rabbit secondary antibody in conjunction with chemiluminescence. Lanes 1 and 2 represent controls not treated with dsRNA. Lane 3 and Lane 4 represent individual populations that were treated with 5.0 and 10.0 μl/well of GTS reagent respectively.

A comparison between lanes 3 and 4 (Figure 3), showed a significant decrease in expression of sst_{2A} protein levels following treatment with a greater concentration of GTS reagent as compared to control samples (Figure 3, lanes 1 /2). However, 5.0 μl/well GTS reagent samples (lane 3, Fig 3), did not result in loss of receptor expression. This suggests that increased levels of GTS reagent corresponded with an increase in dsRNA uptake, resulting in greater inhibition of the sst_{2A} receptor. These findings correlate with our previous results which indicated that 10.0 μl/well of GTS reagent produced maximal uptake of synthetic oligonucleotide and furthermore, provided evidence that RNAi was an effective gene-silencing tool.

Determination of sst_{2A} Receptor Function with RNAi

We next examined sst_{2A} receptor function. It is important to establish whether the reduction in sst_{2A} receptor protein that we observed correlated with a decrease in SRIF activity in the AtT-20 cell. In untreated cells, SRIF-14 demonstrated a dose-dependent decrease in forskolin-stimulated cAMP production (Figure 4).

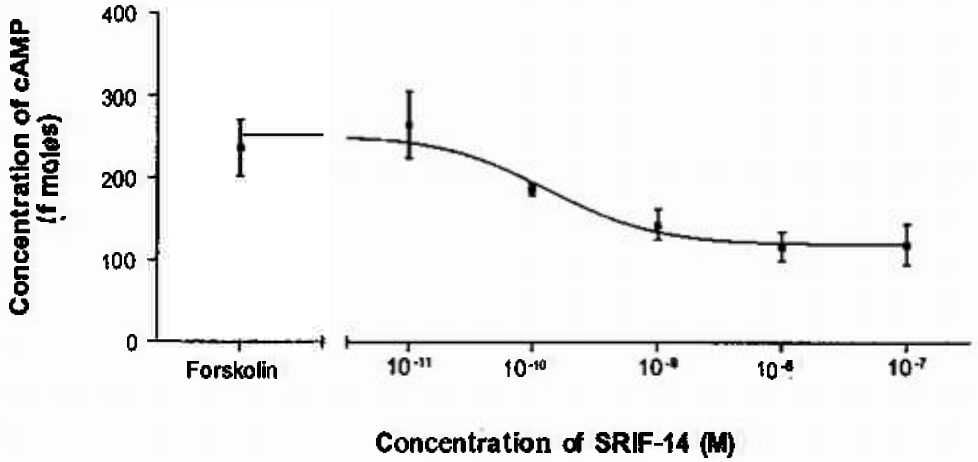

Figure 4 : SRIF-14 inhibition of forskolin-stimulated cAMP accumulation in AtT-20 cells. Intracellular cAMP accumulation was measured in the presence of 10 μM forskolin plus SRIF-14 at various molar concentrations. Cell monolayers were incubated with 0.5 mM IBMX for 30 mins and were treated with experimental conditions for 10 mins. Following cell lysis, intracellular cAMP levels were measured by radioimmunoassay and the data was analyzed using Graphpad Prism 3.0 (Blake, 2001).

To establish whether the dose-dependent inhibition of forskolin stimulated cAMP accumulation was due to activation of sst_{2A}, AtT-20 cells were treated with RNAi prior to the experiment. Forskolin-stimulated cAMP accumulation was then performed in the presence or absence of SRIF-14 or the sst_{2A} selective ligand, L-779,976. A cAMP radioimmunoassay was then performed on cell supernatants and the ability of SRIF-14 or L-779,976 to decrease forskolin-stimulated cAMP accumulation was assessed. As shown in Figure 5, the ability of forskolin-stimulated cAMP levels to decrease upon addition of

an sst_{2A} selective-ligand (L-779,976) was markedly attenuated in RNAi pre-treated cells. When compared to the SRIF-14 controls, L-779,976 inhibition was reduced. L-779,976 is known to be a potent inhibitor of forskolin-stimulated cAMP accumulation in AtT-20 cells with a potency and maximal inhibition of cyclic nucleotide levels that is equivalent to SRIF-14 (Strowski et al., 2002). The reduction in L-779,976 function appears to correlate with the reduction of sst_{2A} expression observed in the western blot analysis (Figure 3) suggesting that RNAi inhibited both receptor expression and attenuated receptor function. Furthermore, this data supports the notion that sst_{2A} plays a key role in the AtT-20 cell's ability to regulate intracellular nucleotide levels.

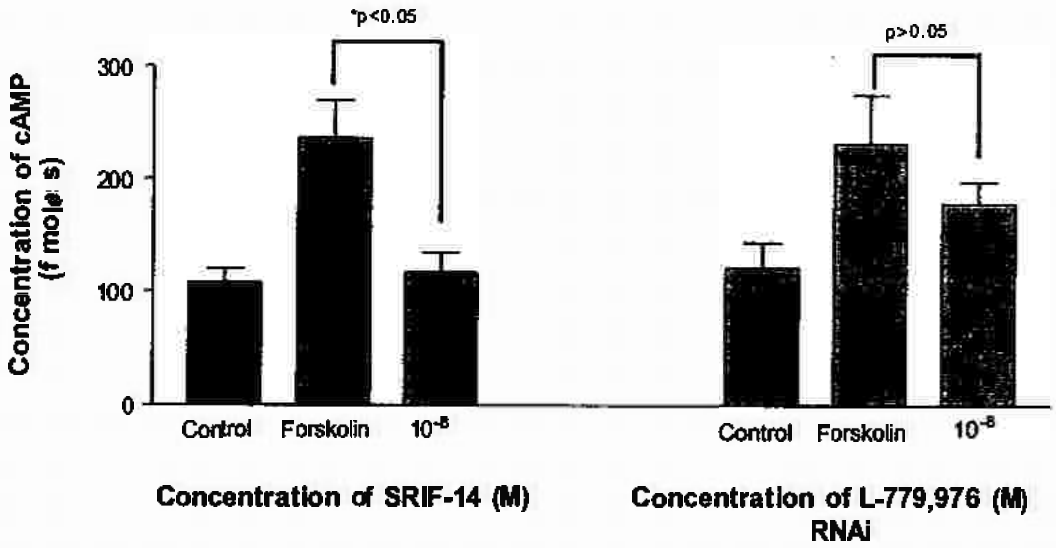

Figure 5 : RNAi prevents sst_{2A} receptor inhibition of forskolin-stimulated cAMP accumulation in the AtT-20 cellular model. Cell monolayers in the absence or presence of 2.5 µg dsRNA were incubated with 0.5 mM IBMX for 30 mins and then treated with 10 µM forskolin ± SRIF-14 or L-779,976 for 10 mins. Following cell lysis, intracellular cAMP levels were measured by radioimmunoassay and the data was analyzed using Graphpad. Statistical significance (*$p < 0.05$) was determined, relative to forskolin control, by GraphPad Prism 3.0 (Blake, 2001).

Discussion :

The present study demonstrated that the sst_{2A} receptor regulates intracellular cyclic nucleotide production in the murine corticotropic model. This is an important finding since the functional significance of receptor subtype slice-variants are difficult to characterize given the current pharmacological methods implemented in their study. Here it was shown that a relatively new gene-silencing technique, RNA interference, was effective in selectively targeting and inhibiting the sst_{2A} receptor, allowing for the functional consequences of the ablation of the receptor to be determined. RNAi was shown to inhibit sst_{2A} receptor expression as seen in western blot analysis when 2.5 μg of dsRNA was diluted in 10.0 μl/well of GTS reagent. Upon addition of SRIF-14 there was a dose-dependent decrease in forskolin-stimulated cAMP production that was inhibited by pre-treatment with dsRNA.

In choosing both optimal concentrations and volumes of dsRNA and GTS reagent required for RNAi experiments, it was important to achieve maximal uptake of oligonucleotide while minimizing any non-specific effects. Although the mechanism of RNAi is highly specific, excessive amounts of dsRNA could possibly alter the physiological homeostasis of the cell, producing inaccurate results or lead to non-specific effects (Parrish, et al., 2001). For this reason, concentrations of both dsRNA and GTS reagent were chosen prior to saturation of intracellular fluorescence. Our initial studies indicated that 2.5 μg/well of dsRNA and 10.0 μl/well of GTS reagent yielded optimal internal fluorescence that corresponded to maximal uptake of FITC-labeled oligonucleotide (Figure 1, 2).

The exact genetic mechanisms by which RNA interference inhibits the expression of certain proteins is unknown, although there is evidence that suggests multiple molecular hypotheses. One such mechanism relies on the enzymatic degradation of dsRNA into smaller, single-stranded fragments, that serve to guide multi-component enzymatic complexes to complementary mRNA for ablation (Hammond et al., 2000). Interestingly, RNA interference has been shown to inhibit the expression of genes at the post-transcriptional level in mammalian cells (Krichevsky et al., 2002), however, since this study was one of the first to utilize this technique to inhibit the expression of splice-variants, it was necessary to evaluate the ability of RNAi to inhibit the expression of the sst_{2A} receptor. Western blot analysis using subtype-selective antisera, performed on AtT-20 cells treated with various amounts of GTS reagent, confirmed the ability of RNAi to inhibit the expression of the sst_{2A} receptor. Figure 3 clearly depicts a significant decrease in the percent-expression of the sst_{2A} receptor that resulted from treatment with 10.0 µl/well of GTS reagent and 2.5 µg/well of dsRNA. These findings are significant in that they illustrate the effective use of RNAi as a pharmacological tool that can distinguish between receptor subtype splice-variants.

In answering the experimental hypothesis, we attempted to determine if inhibition of sst_{2A} receptor expression with RNAi, correlated with any cellular consequences that could be attributed as the function of the sst_{2A} receptor. Since there is evidence that suggests SRIF-receptor subtypes as inhibitors of cell proliferation, excitability and secretion (Puente et al., 2001), we focused our study on an important regulator of signal transduction cascades, cAMP, and the effects that the sst_{2A} receptor has on the production of this cyclic nucleotide. As illustrated in Figure 4, SRIF-14

decreases forskolin-stimulated cAMP accumulation in the AtT-20 cellular model. SRIF-14 is known to have a high degree of affinity for the sst_{2A} receptor and has been shown to have similar effects on forskolin-stimulated cAMP production as that of the sst_{2A} selective-agonist, L-779,976 (Strowski et al., 2002). In order to determine if this cellular response was mediated by the sst_{2A} receptor, we examined the ability of AtT-20 cell populations that were pre-treated with dsRNA to inhibit forskolin-stimulated cAMP production. The cells that were pre-treated with dsRNA and hence exhibited decreased expression of the sst_{2A} receptor, showed a significant decrease in their ability to inhibit forskolin-stimulated cAMP production upon the addition of an sst_{2A}-specific agonist (Figure 5). From these findings we can conclude that the sst_{2A} receptor serves to decrease cyclic nucleotide production in the AtT-20 cellular model. Overall, these data indicate that RNAi was a vital tool in understanding the functional significance of receptor subtype splice-variants, where through it's use we determined that the sst_{2A} receptor functions to regulate the production of intracellular cyclic nucleotides in murine corticotrophs.

Conclusion :

The results presented here demonstrate that the sst_{2A} receptor splice-variant appears to regulate the production of intracellular cyclic nucleotide levels in the AtT-20 cellular model. Through the use of dsRNA interference, SRIF-14 and the sst_{2A}-selective agonist, L-779,976, we were able to characterize the function of the sst_{2A} receptor in inhibiting forskolin-stimulated cAMP production.

Our results show that 0.5 - 1.5 μM of dsRNA diluted in 10.0 μl/well of GTS reagent are required to yield maximal uptake of synthetic oligonucleotide. In addition, dsRNA interference proved to be an effective post-transcriptional gene-silencing tool in inhibiting sst_{2A} receptor expression as seen in the ablation of sst_{2A} protein levels in western blot analysis. Furthermore, dsRNAi revealed loss of sst_{2A} receptor function corresponding to inhibition of receptor expression, suggesting that the function of the sst_{2A} receptor in the AtT-20 cell line is to inhibit forskolin-stimulated cAMP production.

In conclusion, the present study determined the functional significance of the sst_{2A} receptor subtype splice-variant through the use of a receptor subtype-selective technique, RNA interference. The ability to silence genes in this manner allows for furthering the understanding of intracellular pathways involved in cell signaling and may have future therapeutic implications.

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