Effector Coupling of Somatostatin Receptor Subtypes on Human Endocrine Tumors

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Effector coupling of somatostatin receptor subtypes sst_1 and sst_2 was examined in a reconstituted system. Forskolin-stimulated cyclic adenosine monophosphate (cAMP) formation was inhibited 66% by somatostatin (SRIF-14) in CHO cells expressing somatostatin receptor 1 (sst_1) (CHO-SR1), but not sst_2, in a dose-dependent manner with an ED_{50} of 1×10^{-9} mol/L SRIF-14. The inhibition was blocked by pertussis toxin (PTX), indicating that sst_1 is coupled to adenylyl cyclase via PTX-sensitive G_i protein. In CHO cells, $G_i\alpha 2$ and $G_i\alpha 3$ mRNAs were detected. In adenylyl cyclase assays, 1 μmol/L SRIF-14 caused a 16% inhibition of forskolin-stimulated adenyly cyclase activity. Preincubation with $G_i\alpha 3$, but not $G_i\alpha 1$ / $G_i\alpha 2$, antiserum blocked this inhibition. By contrast, sst_2 is coupled to adenylyl cyclase via $G_i\alpha 1$. In cells expressing sst_2 with $G_i\alpha 1$(CHO-SR2G1), SRIF-14 significantly inhibited forskolin-stimulated cAMP formation by 53% and with an ED_{50} at 4×10^{-9} mmol/L SRIF-14, which was completely blocked by PTX; ED_{50} values for sst_1 and sst_2 agree with the IC_{50} values in binding assays. In CHO-SR1, the rank of potency of agonists affecting adenyl cyclase was SRIF-14 = SRIF-28 > RC 160 > SMS 201-995. In CHO-SR2G1, the rank was RC-160 > SRIF-14 = SRIF-28 > SMS201-995.

SOMATOSTATIN has diverse biological functions, including inhibition of secretory and proliferative processes in many target organs.[1-3] Based on these actions of somatostatin, various somatostatin analogs have been developed for clinical applications.[4,5] One of the analogs, octreotide (SMS 201-995), has been approved for the treatment of endocrine tumors such as pituitary adenoma, pancreatic endocrine and carcinoid tumors,[3] as well as for in vivo autoradiography, which has made it possible to localize somatostatin receptor-positive tumors and their metastases.[3] These effects of somatostatin are mediated by its specific receptors. We have recently cloned five human somatostatin receptor subtypes (sst_{1-5}), which have characteristic features of G-protein–coupled receptors.[6-8] In a previous report, we revealed the expression of ssts in glucagonomas, insulinomas, pheochromocytomas, and carcinoid tumors by the reverse-transcription polymerase chain reaction (RT-PCR) method, and demonstrated that the expression of sst_2 in endocrine tumors may determine the efficacy of octreotide.[9] We have demonstrated in a previous report that sst_3 is coupled to adenylyl cyclase.[7] Here, we report the effector coupling of two somatostatin receptor subtypes, sst_1, which shows a wide distribution in human endocrine tumors, and sst_2, which has the highest affinity for octreotide, in a reconstituted system.

MATERIALS AND METHODS

Establishment of CHO Cells Stably Expressing sst_1 and sst_2

Establishment of CHO cells stably expressing sst_1 and sst_2 was performed as described previously.[10,11] Briefly, mammalian expression vector pCMV6b[6] containing human sst_1 or sst_2 gene was transfected into the CHO cells, and stable transfectants were selected. To establish CHO cells stably expressing sst_2 with $G_i\alpha 1$, expression vector pMT3DSV2 containing rat $G_i\alpha 1$ cDNA was transfected into the CHO cells expressing sst_2 and stable transfectants were selected.

Measurements of Cyclic Adenosine Monophosphate Formation in Whole Cells

The cells were incubated with the buffer containing 50 mmol/L tris (pH 7.4), 200 mmol/L sucrose, 5 mmol/L $MgCl_2$, 1 mg/mL bacitracin, 10 mg/mL bovine serum albumin, and 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX) with or without test reagents at 37°C for 30 minutes. The reaction was stopped by adding 30% trichloroacetic acid and cyclic adenosine monophosphate (cAMP) levels were determined by radioimmunoassay.

Adenylyl Cyclase Assays Using Cell Membrane Preparations

Membrane preparations were performed as described previously.[10] Membranes were incubated with normal rabbit serum, or with G_i antisera (each 1/100 dilution) at 4°C for 1 hour and the reaction was started by adding the preincubated membranes (45 μg per tube) to the buffer containing 50 mmol/L tris (pH 7.4), 100 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L IBMX, 0.3 mmol/L adenosine triphosphate 5 mmol/L phosphocreatine, 30 U/mL creatine phosphokinase, and 10 mmol/L guanosine triphosphate. After incubation for 5 minutes at 30°C with or without test reagents, the reaction was stopped and the cAMP product analyzed by radioimmunoassay.

RESULTS AND DISCUSSION

Effector Coupling of sst_1 and sst_2

Signal transduction pathways and effectors that are mediated by ssts have been studied using mainly tissues and cell lines. However, these studies have been limited in their characterization of the properties of ssts because of the possible existence of multiple receptor subtypes in tissue or cell preparations. In this study, we established CHO cell lines stably expressing cloned ssts. Among the ssts expressed in human endocrine tumors, we have investigated the effector coupling of sst_1, which shows a wide distribution in endocrine tumors, and of sst_2, which has the highest affinity for octreotide.[9] Figure 1 shows the effects of somatostatin on forskolin-stimulated cAMP formation in nontransfected CHO cells and CHO cells expressing sst_1 (CHO-SR1). In nontransfected CHO cells, SRIF-14 did not affect forskolin-stimulated cAMP formation. In CHO-

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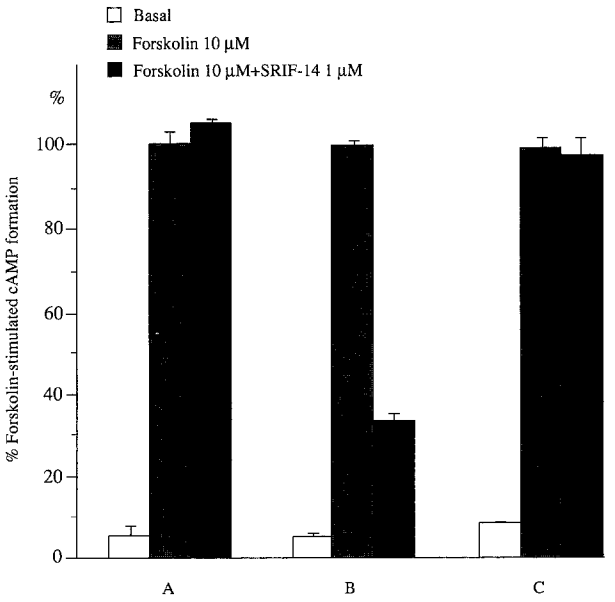


Fig 1. Effects of SRIF-14 on forskolin-stimulated cAMP formation. Nontransfected (A) or sst_1-expressing (B,C) CHO cells were incubated with 10 mmol/L forskolin and with or without 1 mmol/L SRIF-14 for 30 minutes. (C) The sst_1-expressing CHO cells were incubated with 100 ng/mL of PTX for 24 hours before the assay. Values are means ± SE of triplicate determinations.

SR1, SRIF-14 caused a 66% inhibition of forskolin-stimulated cAMP formation. SRIF-14 inhibited forskolin-stimulated cAMP formation in a dose-dependent manner and half-maximal inhibition (ED_{50}) of forskolin-stimulated cAMP formation occurred at 1×10^{-9} mmol/L SRIF-14 (Fig 2). The inhibition of forskolin-stimulated cAMP formation by SRIF-14 was blocked by PTX, indicating that sst_1 is coupled to adenylyl cyclase via PTX-sensitive G_i protein.

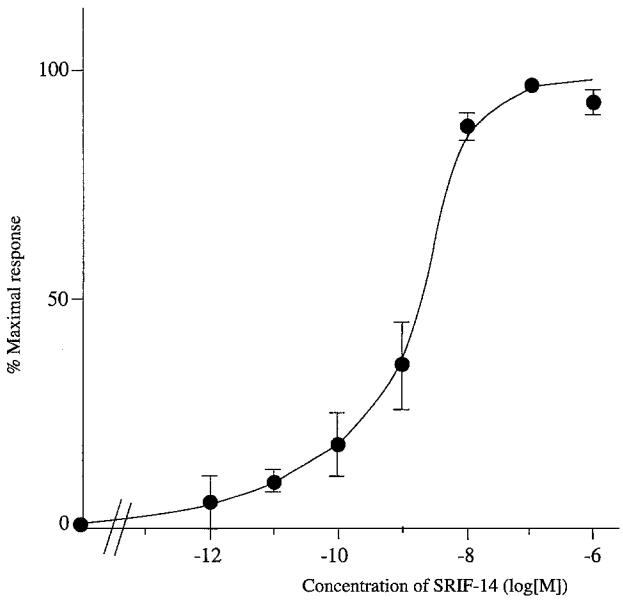


Fig 2. Dose-response analysis of somatostatin-induced cAMP inhibition in CHO-SR1. Values are means ± SE of triplicate determinations.

There are three known subtypes of the α subunit of G_i protein, which are termed $G_i\alpha1$, $G_i\alpha2$, and $G_i\alpha3$. In CHO cells, $G_i\alpha2$ and $G_i\alpha3$ mRNAs were detected, but $G_i\alpha1$ mRNA was not.[10] To determine which subtype of $G_i\alpha$ is coupled to sst_1, we have examined the effects of antisera directed against $G_i\alpha$ subtypes on the actions of somatostatin (Fig 3). AS/7 and EC/2 are the antisera directed against $G_i\alpha1/G_i\alpha2$ and $G_i\alpha3$, respectively. In adenylyl cyclase assays using membrane preparations preincubated with normal rabbit serum, 1 μmol/L SRIF-14 caused a 16% inhibition of forskolin-stimulated adenyly cyclase activity. Preincubation of the membranes with $G_i\alpha1/G_i\alpha2$ antiserum had no effect on the ability of SRIF-14 to inhibit forskolin-stimulated adenylyl cyclase activity, but that with $G_i\alpha3$ antiserum blocked the inhibitory effect of SRIF-14, indicating that $G_i\alpha3$ dominantly mediates the signal transduction from sst_1 to adenylyl cyclase. In contrast to sst_1, SRIF-14 did not affect forskolin-stimulated cAMP formation in CHO cells expressing sst_2 (Fig 4). Since CHO cells lack the expression of $G_i\alpha1$, we transfected $G_i\alpha1$ cDNA-containing expression vector into CHO cells expressing sst_2 and established the cell line expressing sst_2 with $G_i\alpha1$ (CHO-SR2G1). In CHO-SR2G1 cells, SRIF-14 significantly inhibited the forskolin-stimulated cAMP formation with maximal inhibition of 53% (Fig 4). This inhibitory effect of somatostatin on cAMP formation was completely blocked by PTX. The ED_{50} of forskolin-stimulated cAMP formation oc-

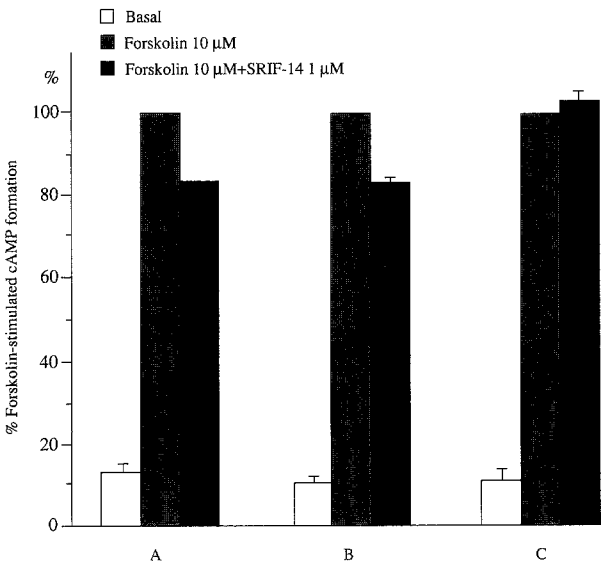


Fig 3. Effect of $G_i\alpha1/G_i\alpha2$ and $G_i\alpha3$ antisera on the inhibitory action of SRIF-14 on forskolin-stimulated adenylyl cyclase activity. Membrane preparations from CHO-SR1 cells were preincubated with normal rabbit serum (A), $G_i\alpha1/G_i\pm$ antiserum (B), or G_i α3 antiserum (C), and the effect of 1 μmol/L SRIF-14 on forskolin-stimulated cAMP formation was examined. SRIF-14, 1 μmol/L, inhibited forskolin-stimulated adenylyl cyclase activity by 16.4% ± 2.8% when the membranes were preincubated with normal serum (in a representative experiment with normal rabbit serum, adenylyl cyclase activity was as follows: basal, 18.4 ± 2.3; forskolin-stimulated, 153 ± 4.5; and forskolin with SRIF-14, 129.4 ± 5.4 pmol/mg/min). Data are expressed as percent of forskolin-stimulated levels. Data are means ± SE of 3 independent experiments performed in triplicate or quadruplicate determinations.

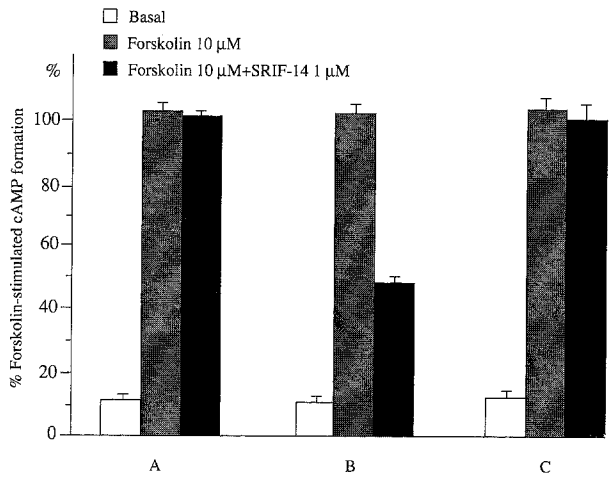


Fig 4. Effects of SRIF-14 on forskolin-stimulated cAMP formation in (A) CHO-SR2 cells, (B) CHO-SR2G1 cells, and (C) CHO-SR2G1 cells preincubated with PTX. Values are means ± SE of triplicate determinations.

curred at 4×10^{-9} mmol/L SRIF-14 (Fig 5). In summary, both sst_1 and sst_2 are coupled to adenylyl cyclase in CHO cells, and ED_{50} values are 1×10^{-9} mmol/L SRIF-14 for sst_1 and 4×10^{-9} mmol/L for sst_2, each of which corresponds well with the inhibitory concentration of half maximal response (IC_{50}) values in the binding assays, 1.5×10^{-9} mmol/L for sst_1 and 1.6×10^{-9} mmol/L for sst_2.[6] The sst_1 and sst_2 are coupled to adenylyl cyclase via PTX-sensitive G protein, but the subtypes of the α subunit of G protein are different between two receptors, sst_1 via $G_i\alpha 3$ and sst_2 via $G_i\alpha 1$.

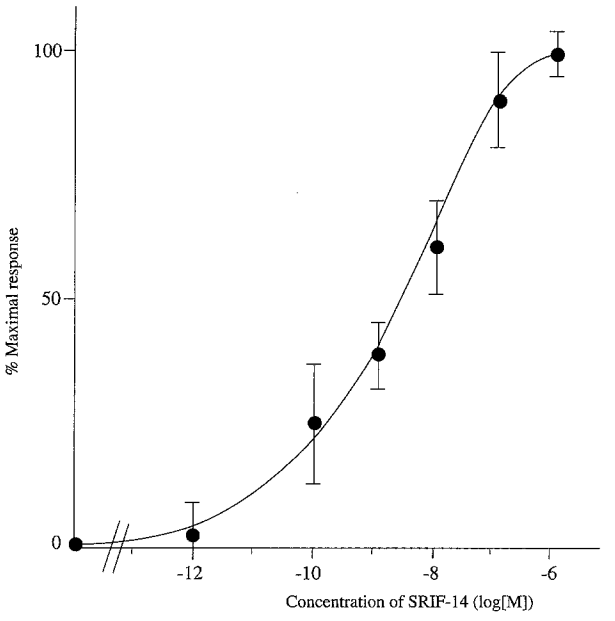
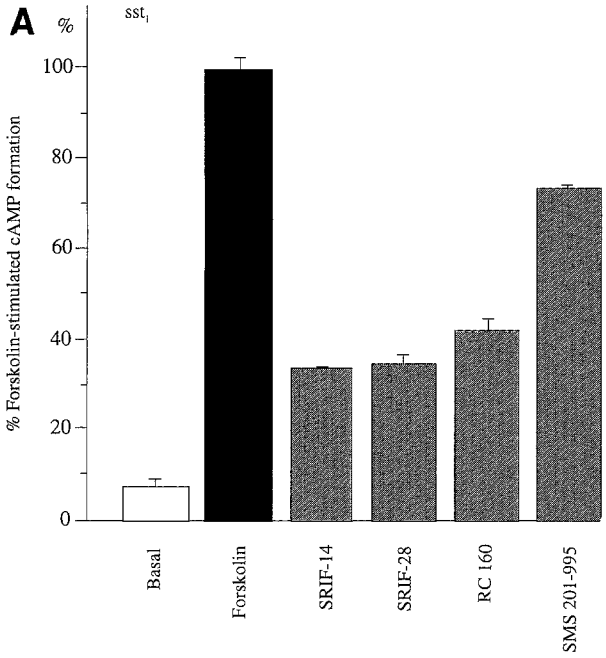


Fig 5. Dose-response analysis of somatostatin-induced cAMP inhibition in CHO-SR2G1. Values are means ± SE of triplicate determinations.



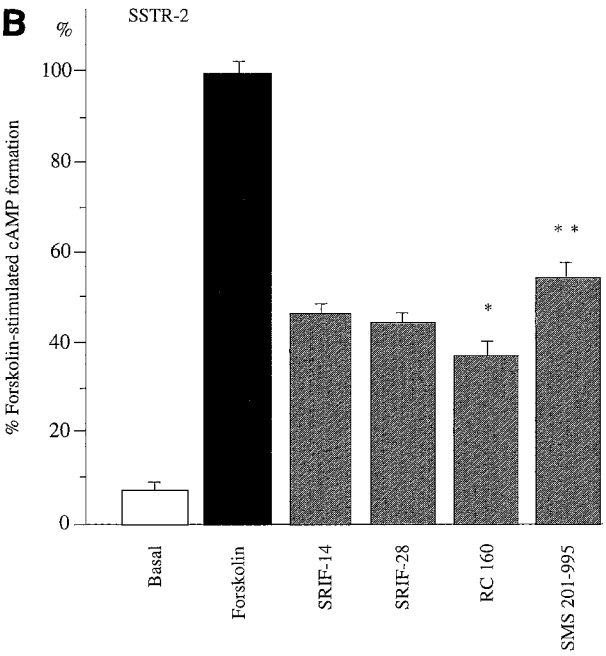


Fig 6. Effects of various agonists on forskolin-stimulated cAMP formation in CHO-SR1 (A) and CHO-SR2G1 (B). Values are means ± SE of triplicate determinations. (*$P < .05$, **$P < .01$ *v* SRIF-14 and SRIF-28.)

Effects of Various Agonists on Adenyl Cyclase in CHO-SR1 and CHO-SR2G1

To characterize the potencies and relative efficacies of the agonists, we determined the effects of 1 μmol/L SRIF-14, SRIF-28, RC 160, and SMS 201-995 of forskolin-stimulated cAMP formation in both CHO-SR1 and CHO-

SR2G1 (Fig 6). In CHO-SR1, the rank of the potency of these agonists was SRIF-14 = SRIF-28 > RC 160 > SMS 201-995, which was in reasonable agreement with that of the agonists in inhibiting the binding of radiolabeled SRIF-14.[10] In CHO-SR2G1, the rank of the potency of these agonists was RC-160 > SRIF-14 = SRIF-28 > SMS201-995. Considering that the affinity of RC-160 is significantly less than SRIF-14,[11] it is interesting that RC-160 inhibits cAMP formation better than SRIF-14. This may be attributed to the stability of the peptides during the experiments.

CONCLUSION

We elucidated that sst_1 and sst_2 are coupled to adenylyl cyclase via different G proteins, and revealed the relative efficacies of somatostatin analogs to inhibit intracellular cAMP levels through sst_1 and sst_2. Although it remains to be determined whether these signal transduction systems in a reconstituted system operate similarly in cells natively expressing ssts, these results provide a basis for the understanding of the mechanism of somatostatin action, as well as for the development of selective analogs for sst subtypes.

REFERENCES

1. Reichlin S: Somatostatin. N Engl J Med 309:1495-1501, 1556-1563, 1983

2. Lamberts SWJ, Krenning EP, Reubi JC: The role of somatostatin and its analogues in the diagnosis and treatment of tumours. Endocr Rev 12:450-482, 1991

3. Evers BM, Parekh D, Townsend CM Jr, et al: Somatostatin and analogues in the treatment of cancer. Ann Surg 213:190-198, 1991

4. Bauer W, Briner U, Doepfner W, et al: SMS-201-995: A very potent and selective octapeptide analogue of somatostatin with prolonged action. Life Sci 31:1133-1140, 1982

5. Cai RZ, Szoke B, Lu R, et al: Synthesis and biological activity of highly potent octapeptide analogues of somatostatin. Proc Natl Acad Sci USA 83:1896-1900, 1986

6. Yamada Y, Post SR, Wang K, et al: Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract and kidney. Proc Natl Acad Sci USA 89:251-255, 1992

7. Yamada Y, Reisine T, Law SF, et al: Somatostatin receptors, an expanding gene family: cloning and functional characterization of human SSTR-3, a protein coupled to adenylyl cyclase. Mol Endocrinol 6:2136-2142, 1992

8. Yamada Y, Kagimoto S, Kubota A, et al: Cloning, functional expression and pharmacological characterization of a fourth (hSSTR-4) and a fifth (hSSTR-5) human somatostatin receptor subtype. Biochem Biophys Res Commun 195:844-852, 1993

9. Kubota A, Yamada Y, Kagimoto S, et al: Identification of somatostatin receptor subtypes and an implication for the effecacy of SMS-201-995 in treatment of human endocrine tumours. J Clin Invest 93:1321-1325, 1994

10. Kubota A, Yamada Y, Kagimoto S, et al: Multiple effector coupling of somatostatin receptor subtype SSTR-1. Biochem Biophys Res Commun 204:176-186, 1994

11. Kagimoto S, Yamada Y, Kubota A, et al: Human somatostatin receptor, SSTR-2, is coupled to adenylyl cyclase in the presence of Giα1 protein. Biochem Biophys Res Commun 202:1188-1195, 1994