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Rat Somatostatin Receptor Type 1 Couples to G Proteins and Inhibition of Cyclic AMP Accumulation

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SUMMARY

The pharmacology, signal transduction, and coupling to G proteins of the rat somatostatin (SRIF) receptor (SSTR)1 have been characterized in transfected Chinese hamster ovary (CHO) (K1 strain) cells. The expressed receptor exhibited saturable, high affinity binding of several radioiodinated SRIF analogues. Three different radioligands were used to determine the pharmacological properties of this SSTR subtype. [^{125}I-Tyr^{11}]SRIF-14 (^{125}I-S-14), [Leu^{8},D-Trp^{22},^{125}I-Tyr^{25}]SRIF-28 (^{125}I-S-28), and cyclo(D-Trp-Lys-Abu-Phe-MeAla-^{125}I-Tyr) (^{125}I-peptide C) displayed the following rank order of affinity (K_d) for the SSTR1 subtype: ^{125}I-S-14 ≥ ^{125}I-S-28 > ^{125}I-peptide C. Competition of ^{125}I-S-14 with S-14, S-28, or peptide C displayed the same rank order of potency. Chemical cross-linking of specifically bound ^{125}I-S-28 to membranes from CHO cells expressing the receptor indicated that the molecular weight of the SSTR1 expressed in CHO cells is ~70,000, suggesting that it is heavily glycosylated. Previous reports have suggested that the human SSTR1 [*Mol. Pharmacol.* **42**:28–34 (1992)] couples poorly to G proteins. The coupling of the rat SSTR1 to G proteins was demonstrated by three independent methods. (a) Binding of ^{125}I-S-14 to the SSTR1 subtype was inhibited in a dose-dependent fashion by incubation of membranes with guanosine-5′-*O*-(3-thio)triphosphate. (b) Treatment of cells with pertussis toxin decreased binding by 80%. (c) Immunoprecipitation of ^{125}I-S-14 binding was observed with antiserum specific for $G_{i\alpha1,2}$, but not with antiserum specific for $G_{s\alpha}$, in membranes from transfected cells. In CHO cells transfected with the SSTR1 cDNA, SRIF inhibited forskolin-stimulated cAMP accumulation by up to 50%, in a dose-dependent fashion (ED_{50} = 1.1 nM). Pertussis toxin treatment decreased both the efficacy and the potency of the SRIF-mediated inhibition of cAMP accumulation (from 50% to 22%), compared with control untreated cells. These data suggest that the rat SSTR1 inhibits cAMP accumulation by coupling to pertussis toxin-sensitive G proteins.

Most if not all of the biological effects mediated by SRIF are via the interaction of the hormone with specific high affinity cell surface receptors that are coupled to heterotrimeric G proteins (1). The biological responses include inhibition of adenylyl cyclase and Ca^{2+} influx and stimulation of K^+ efflux and tyrosine phosphatase activity. Most of the effects of SRIF in cells are blocked by pertussis toxin treatment (2). The high affinity binding of radiolabeled SRIF agonists to membranes from many tissues (3) and cell lines (4, 5) is inhibited by GTP, a common characteristic of G protein-linked receptors.

To date, five different SSTR subtypes (SSTR1–5) have been cloned and expressed (6–12). SSTR2, SSTR3, and SSTR5 all mediate inhibition of adenylyl cyclase and/or cAMP accumulation (10, 12, 13). One subtype, the human SSTR1, has been reported to couple poorly to G proteins and not to mediate inhibition of adenylyl cyclase or cAMP accumulation (14). Very little is known about the pharmacological and biochemical properties of this SSTR subtype. The SSTR1 subtype appears to have a dramatic species difference in its distribution (6, 7, 9). In humans SSTR1 is expressed predominantly in the gut. In rats this subtype is expressed primarily in the brain. Thus, this subtype (both rat and human) has been difficult to characterize pharmacologically in tissues because of the distinct species tissue distribution and multiple subtype expression in the brain. In the present work we report that, in contrast to the human SSTR1, the rat SSTR1 subtype couples predominantly to pertussis toxin-sensitive G proteins. In addition, we report that the rat SSTR1 is coupled to inhibition of cAMP accumulation, a characteristic effect mediated by SRIF in many tissues.

Experimental Procedures

Isolation of the full-length SSTR1 subtype. Based on the sequence reported by Meyerhof *et al.* (7), the full-length coding region of the rat SSTR1 was cloned using a polymerase chain reaction strategy. A 5′ end sense-strand primer with a unique *Hind*III site (CCACTAAGCTTGGGATGTTCCCCAATGGCACCGCC) and an internal 3′ antisense primer for positions 486–505 (GGTAGCGCGGTGCCTT-

ABBREVIATIONS: SRIF, somatostatin (somatotropin release-inhibitory factor); SSTR, somatostatin receptor; CHO, Chinese hamster ovary; ^{125}I-S-14, [^{125}I-Tyr^{11}]somatostatin-14; ^{125}I-S-28, [Leu^{8},D-Trp^{22},^{125}I-Tyr^{25}]somatostatin-28; ^{125}I-peptide C, cyclo(D-Trp-Lys-Abu-Phe-MeAla-^{125}I-Tyr); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CMV, cytomegalovirus; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

GATC) were used to amplify the 5′ end of the gene. A 3′ end antisense-strand primer (GTTAGTCTAGAGCCTCAAAGCGTGCTGATCCTGGA) with a unique *Xba*I site and an internal 5′ primer for positions 451–470 (CTTAGTGTGGACCGCTATGT) were used to amplify the 3′ end of the receptor cDNA. The conditions for polymerase chain reaction with 100 ng of rat genomic DNA were as follows: melting at 94° for 1 min, annealing at 60° for 1 min, and extension at 72° for 1 min. The fragments were isolated on gels and digested at a unique *Apa*LI site. The two fragments were ligated and cloned into pBluescript. Both strands were sequenced using an Applied Biosystems automated DNA sequencer and are identical to previously published sequences of the rat SSTR1 (7, 9).

Receptor expression constructs. The full-length rat SSTR1 was inserted into the *Hind*III and *Xba*I sites of the expression vector pRC/CMV (Invitrogen). Transfections of CHO-K1 cells were performed using a Stratagene kit for calcium phosphate transfections. Six individual clones were selected for stable expression of the SSTR1 cDNA, using G-418 (300 μg/ml; GIBCO/BRL). The rat SSTR2 has been characterized previously (13).

Membrane preparation. Membranes were prepared from cells as described by Eppler *et al.* (15).

Radioligands. Three radioligands were used in the present study, ^{125}I-S-14, ^{125}I-S-28, and ^{125}I-peptide C. Peptide C was a gift from David Coy (Tulane University). All other peptides were purchased from Bachem. All were radioiodinated using the chloramine T method and were purified by high performance liquid chromatography.

Radioligand binding assays. Radioligand binding assays were performed as described by Eppler *et al* (15), with bound ligand being separated from free radioligand by centrifugation or by rapid filtration using an Inotech cell harvester. The two methods yielded equivalent results, except that ^{125}I-S-28 displayed very high nonspecific binding to filters (>75%). All competition curves were obtained using ^{125}I-S-14 only (250 pM, in a 200-μl final volume), in 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.25% bovine serum albumin (binding buffer), with the following protease inhibitors: 5 μg/ml leupeptin, 5 μg/ml aprotinin, 100 μg/ml bacitracin, and 100 μg/ml benzamidine. These four protease inhibitors were used in all membrane preparations.

Chemical cross-linking of ^{125}I-S-28 to SSTR1. Chemical cross-linking of ^{125}I-S-28 to SSTR1 and SSTR2 in CHO cell membranes was performed as described by Eppler *et al* (15).

cAMP accumulation. SRIF-mediated inhibition of cAMP accumulation was measured in intact cells coincubated with the diterpene forskolin (5 μM). Cells were detached from plates and suspended in Krebs' Ringer phosphate buffer containing 2 mM $CaCl_2$ and 100 μM isobutylmethylxanthine. Reactions were allowed to proceed for 15 min at 37° and terminated by the addition of 1 N HCl, followed by heating of the samples for 3 min at 100°. The samples were then neutralized at 37° and terminated by the addition of 1 N HCl, followed by heating of the samples for 3 min at 100°. The samples were then neutralized with 1 N NaOH. Measurements of cAMP accumulations were performed as described by Hadcock *et al* (16).

Pertussis toxin treatment of cells. CHO cells transfected with the SSTR1 cDNA were treated for 24 hr with pertussis toxin (holotoxin, 50 ng/ml) or vehicle. Radioligand binding in membranes prepared from cells and cAMP accumulation in intact cells were then assayed as described above.

Preparation of anti-G protein antisera for immunoprecipitations. Two different antisera were used for immunoprecipitation studies. Antisera corresponding to residues 346–355 of rat $G_{i\alpha1,2}$ (SB-04) and residues 384–394 of $G_{s\alpha}$ (SB-07) (17) were a generous gift of Dr. Suleiman Bahouth (University of Tennessee, Memphis, TN). Anti-G protein antisera were prebound to goat anti-rabbit IgG-Sepharose at a ratio of 1:10, as described by Hadcock *et al* (18). The equivalent of 2.5 μl of each antiserum were used for each immunoprecipitation.

Immunoprecipititation of ^{125}I-S-14 binding with anti-G protein antisera. Immunoprecipitation of ^{125}I-S-14/receptor/G protein complexes was performed as described by Law *et al* (19), with several modifications. Based on a strategy used for the purification of the GH_4C_1 cell SSTR, we chose to prebind the receptor with ^{125}I-S-14 before a solubilization step (15). This procedure has been shown to greatly enhance the stability of receptor/G protein complexes, compared with solubilization followed by a ligand-binding step (15). Membranes (50 μg) from CHO cells transfected with SSTR1 or wild-type cells were suspended in binding buffer containing 500 pM ^{125}I-S-14, in the presence and absence of 100 nM unlabeled S-14 (final volume, 150 μl). After prebinding of ^{125}I-S-14 to membranes for 1 hr, the samples were pelleted and then solubilized in binding buffer containing 0.15% deoxycholate/lysophosphatidylcholine. The insoluble fraction was removed by centrifugation, and 25 μl of anti-G protein antiserum prebound to goat anti-rabbit IgG-Sepharose were added. The samples were incubated overnight, washed three times, and quantified by γ counting in an LKB γ counter. The concentration of deoxycholate/lysophosphatidylcholine used has been shown to solubilize ~50% of the ^{125}I-S-14/receptor/G protein complex, with a half-life for the complex of 18 hr (15). Therefore, approximately 25% of the original specific binding (^{125}I-S-14/receptor/G protein complex) remains intact after the solubilization and incubation steps. In a typical assay this represents a decline from 50,000 cpm specifically bound to 12,500 cpm bound.

Analysis of data. Data analyses were performed using the INPLOT program (GraphPAD). The data presented are means ± standard errors unless noted otherwise.

Results

The functional expression of SSTR1 was examined in CHO cells (K1 strain) transfected with SSTR1 cDNA under the control of the CMV promoter (pRC/CMV vector; Invitrogen). There was no detectable binding of ^{125}I-S-14 (<2 fmol/mg of protein) in membranes prepared from wild-type CHO cells. Six individual colonies of CHO cells transfected with the SSTR1 subtype were chosen after selection with 300 μg/ml G418. Membranes prepared from all six G418-resistant colonies displayed binding of ^{125}I-S-14 (ranging from 40 to 1600 fmol/mg). One of these, clone 3, was chosen for further evaluation (Fig. 1; Table 1). As shown in Fig. 1, membranes prepared from CHO cells expressing the SSTR1 subtype displayed high affinity saturable binding of three different radioiodinated SRIF analogues, with the following rank order of potency: S-28 ≥ S-14 > peptide C. B_{max} values were equivalent for all of the radioligands (Fig. 1; Table 1).

Further analysis was performed by competition of ^{125}I-S-14 with S-14, S-28, and peptide C (Fig. 2; Table 1). In close agreement with the K_d values determined by saturation binding, the IC_{50} values for the rat SSTR1 subtype were calculated to be 141 p WITHTHEK_d values determined by saturation binding, the IC_{50} values for the rat SSTR1 subtype were calculated to be 141 pM for S-14 and 102 pM for S-28. For S-14 displacement, 91% of the sites appeared to be of high affinity. The IC_{50} for the low affinity site was calculated to be 9 nM (data not shown). S-28 and peptide C displacement curves also displayed a small percentage of sites (<10%) in a low affinity state. Surprisingly, the IC_{50} value for peptide C was found to be 2.5 nM, an almost 10-fold lower affinity than the K_d observed for the iodinated analogue. MK-678, a short SRIF analogue reported not to bind to human or rat SSTR1 (9, 14), did not displace ^{125}I-S-14 (data not shown).

The sizes of the rat SSTR1 and SSTR2 subtypes were examined by chemical cross-linking of specifically bound ^{125}I-S-28 to membranes from CHO cells expressing each of the receptor subtypes (Fig. 3). CHO cells expressing SSTR2 displayed a B_{max} of ~1.5 pmol/mg of protein (13). To date, the size of the SSTR1 subtype has been unknown, because at least four SSTR subtypes are expressed in rat brain (6, 7, 10, 11). No specific cross-linking of ^{125}I-S-28 was observed in membranes prepared from wild-type CHO cells (13). The size (60–

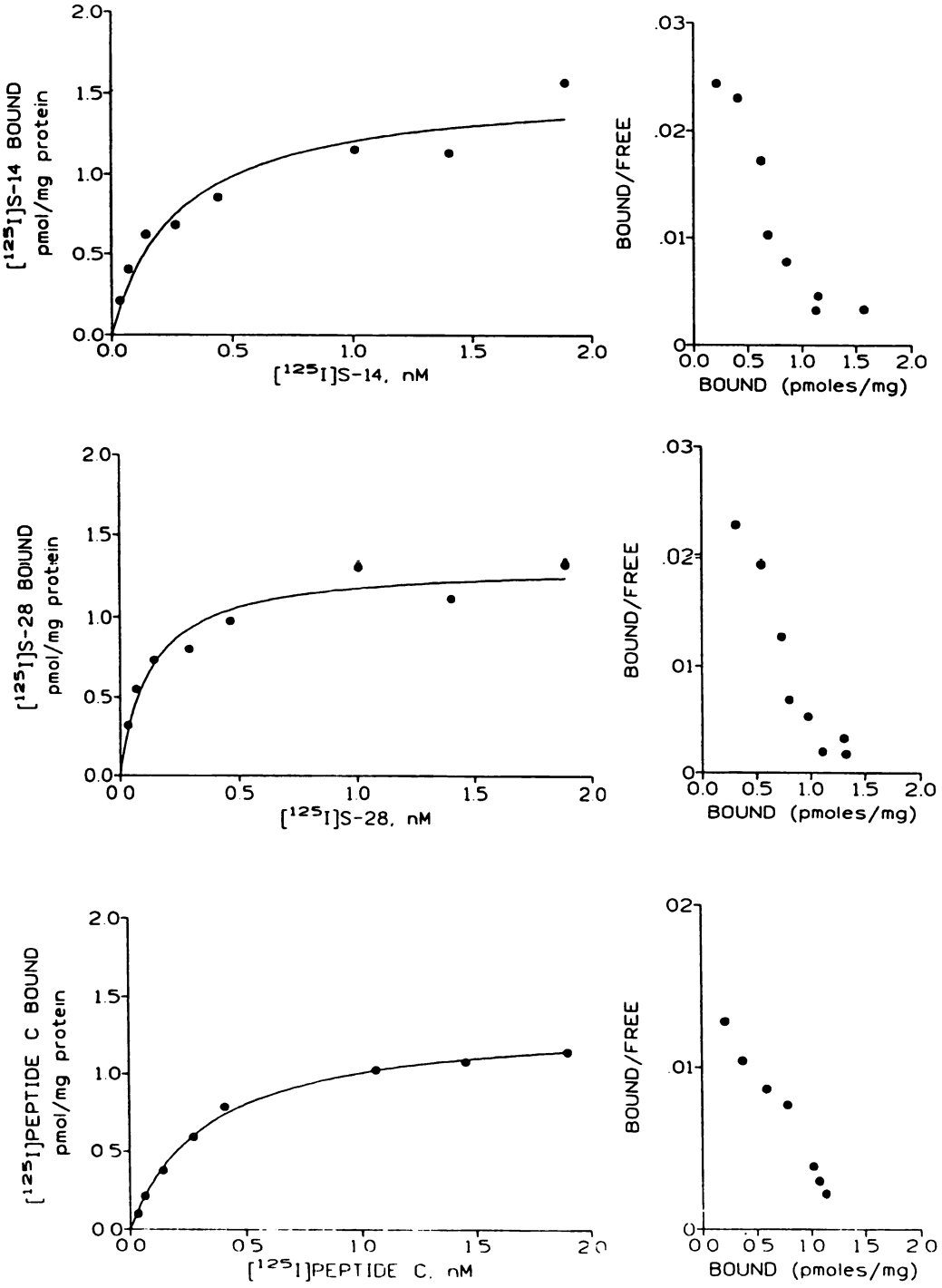

Fig. 1. Saturation binding of ^{125}I-SRIF analogues to membranes of CHO cells transfected with the SSTR1 subtype. Membranes from CHO cells expressing the SSTR1 subtype were prepared as described in Experimental Procedures. *Left*, Saturation binding was performed with 0–2000 pM ^{125}I-S-14 (*top*), ^{125}I-S-28 (*middle*), or ^{125}I-peptide C (*bottom*). Nonspecific binding for each *point*, measured as cpm bound in the presence of 5 μM unlabeled S-14, ranged from 10 to 40%. For each point 3.5 μg of protein/tube were used. Displayed is a representative experiment for each radioligand, performed in triplicate. *Right*, Scatchard transformation for each of the radioligands.

70 kDa) of SSTR1 was found to be more consistent in membrane preparations from transfected CHO cells (Fig. 3). In contrast, the size (60–90 kDa) of the SSTR2 subtype was found to be variable when different membrane preparations of the same clone were compared (Fig. 3) (13). The predicted molecular mass of the rat SSTR1 subtype without glycosylation is 43 kDa, whereas SSTR2 has a predicted molecular mass of 42 kDa. The SSTR2 has four putative glycosylation sites in the amino terminus and the rat SSTR1 subtype has three putative glycosylation sites (9). As predicted from the primary sequences of the two subtypes, both subtypes appear to be glycosylated.

The ability of the rat SSTR1 to couple to G proteins was examined by three different methods, i.e., determination of sensitivity of radioligand binding to guanine nucleotides, determination of sensitivity to pertussis toxin treatment, and immunoprecipitation of the ^{125}I-S-14/SSTR1/G protein complex with anti-G protein α subunit antisera. ^{125}I-S-14 binding to the rat SSTR1 subtype was reduced 75% when membranes were coincubated with 100 μM GTPγS (Fig. 4A). The ED_{50} for GTPγS-dependent inhibition of ^{125}I-S-14 binding to SSTR1 was calculated to be 10 nM. In agreement with previous reports on the GH_4C_1 cell SSTR (5), GTPγS decreased the percentage of high affinity sites from 91% to 20% for S-14. The K_i values for S-14 displacement of ^{125}I-S-14 in the presence of GTPγS (100 μM) were found to be 100 pM (20%) and 11 nM (80%) for the high and low affinity sites, respectively. Surprisingly, a

TABLE 1

Ligand-binding affinities of SRIF analogues for the rat SSTR1 subtype

Membranes were prepared from CHO cells expressing the rat SSTR1. Saturation binding isotherms and competition binding assays were performed. The results displayed are the means ± standard deviations of either two (saturation binding) or three (competition binding) individual experiments.

Compound	Radioiodinated peptide saturation binding		Unlabeled peptide competition binding, IC_{50}[a]
	K_d	B_{max}	
	pM	fmol/mg	pM
S-14	205 ± 110	1600 ± 77	141 ± 94
S-28	108 ± 27	1479 ± 205	102 ± 39
Peptide C	348 ± 12	1443 ± 142	2650 ± 494

[a] Determined in competition binding versus ^{125}I-S-14 (250 pM).

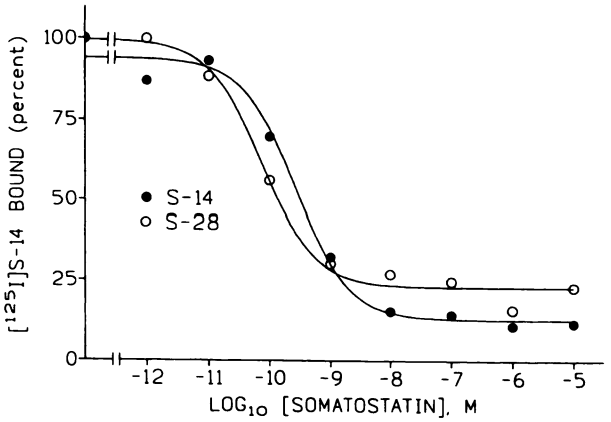

Fig. 2. Competition binding of ^{125}I-S-14 with S-14 and S-28. The ability of increasing concentrations (10^{-12} M to 10^{-5} M) of S-14 and S-28 to displace ^{125}I-S-14 (50 fmol, 250 pM) was examined in membranes prepared from CHO cells expressing the SSTR1 subtype. A representative experiment performed in triplicate is displayed.

small percentage of binding sites were of high affinity in the presence of 100 μM GTPγS. These data suggest that GTPγS promotes uncoupling of most but not all of the SSTR1/G protein complexes. In an analogous fashion, pertussis toxin treatment (50 ng/ml, 24 hr) of CHO cells transfected with the SSTR1 subtype abolished 80% of the specific binding of ^{125}I-S-14 to membranes prepared from these cells (Fig. 4B). These observations are consistent with a shifting of the receptor to a low affinity state by uncoupling of G_i and/or G_o from the receptor.

The ability of anti-G protein antisera to immunoprecipitate ^{125}I-S-14/receptor/G protein complexes was examined in solubilized membranes from transfected cells (Table 2). Membranes were preincubated with ^{125}I-S-14, solubilized, and incubated with anti-G protein α subunit antisera precoupled to an IgG-Sepharose resin. This precoupling of ligand followed by solubilization has been used to copurify the GH_4C_1 cell SSTR and its associated G proteins, with a half-life of ~18 hr (15, 20). Law *et al.* (19) showed previously that antipeptide antiserum (antiserum 8730) generated against the carboxyl terminus of $G_{i\alpha1,2}$ can immunoprecipitate SSTR/$G_{i\alpha}$ complexes. The ability of antisera generated against the carboxyl-terminal sequence of $G_{i\alpha1,2}$ (identical antigen to that of antiserum 8730) and $G_{s\alpha}$ to immunopreciptate ^{125}I-S-14/SSTR1/G protein complexes was assessed. As reported previously for brain SSTR subtypes (19), this antiserum generated against $G_{i\alpha1,2}$ was able to immunoprecipitate the ^{125}I-S-14/SSTR1/G protein complex. In

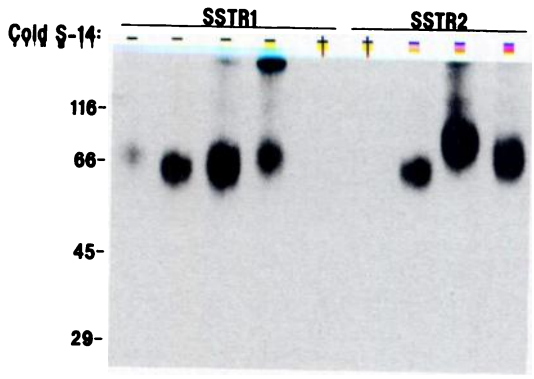

Fig. 3. Chemical cross-linking of ^{125}I-S-28 to membranes of wild-type CHO cells and CHO cells transfected with the SSTR1 and SSTR2. Membranes were prepared from wild-type CHO cells and CHO cells expressing the SSTR1 (clone 3) and SSTR2 (clone 2) subtypes. Each *lane* represents a different membrane preparation. Conditions used were identical to those described by Eppler *et al* (15). Briefly, after a radioligand binding step (^{125}I-S-28, 150,000 cpm/sample) samples were resuspended in ice-cold binding buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$) and 1 μl of 10 mM *N*-azido-2-nitrobenzoyloxysuccinimide in dimethylsulfoxide was added to each sample (80 μg/lane). The samples were mixed and UV irradiated. The reactions were stopped and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For each set of membranes, ^{125}I-S-28 was incubated in the presence (+) or absence (−) of 1 μM unlabeled S-14. Because no cross-linked product was observed from any samples incubated with 1 μM unlabeled S-14, only one of these controls is shown for each receptor subtype. No cross-linking of ^{125}I-S-28 was observed in wild-type CHO cells (13). The characterization of the rat SSTR2 has been described previously (13).

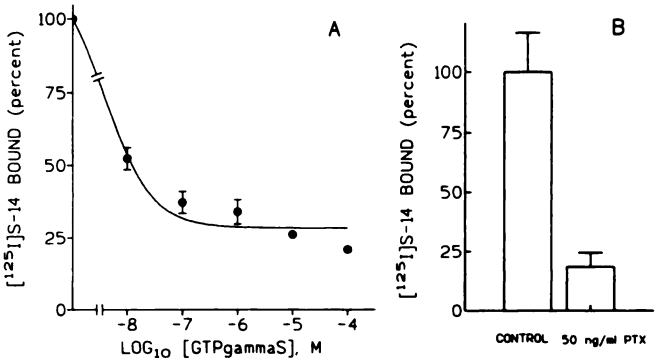

Fig. 4. Effects of GTPγS and pertussis toxin on binding of ^{125}I-S-14 to the SSTR1 subtype. A, Radioligand binding to SSTR1 in membranes prepared from CHO cells was performed in the absence and presence of increasing amounts of GTPγS (0–100 μM). Binding of ^{125}I-S-14 to membranes was measured. B, Transfected CHO cells were treated with 50 ng/ml pertussis toxin (*PTX*) (holotoxin) or vehicle for 24 hr and membranes were prepared. Binding of ^{125}I-S-14 to membranes was measured as described in Experimental Procedures. Data are expressed as the percentage of binding observed in membranes from CHO cells treated with vehicle only. The results are means ± standard deviations of two independent experiments performed in triplicate.

agreement with the GTPγS-dependent loss of binding, we observed a substantial decrease in the amount of ^{125}I-S-14 immunoprecipitated with the anti-$G_{i\alpha1,2}$ antiserum. Incubation of the complex with 10 μM GTPγS decreased the specific counts in the immunoprecipitate by 80%. In contrast, the antiserum raised against the carboxyl terminus of $G_{s\alpha}$ did not specifically immunoprecipitate the complex. No immunoprecipitation of specific ^{125}I-S-14 binding was observed in wild-type CHO cell membranes. Immunoprecipitation of the ^{125}I-S-14/SSTR1/G protein complex accounted for 20–30% of the bound ^{125}I-S-14 remaining after solubilization and overnight incubation. Ini-

TABLE 2

Immunoprecipitation of SSTR1/G protein complexes with anti-G protein antisera

Membranes were prepared from CHO cells expressing the SSTR1. Immunoprecipitation of ^{125}I-S-14/receptor/G protein complexes with antisera selective for $G_{i\alpha1,2}$ or $G_{o\alpha}$ was performed as described in Experimental Procedures.

CHO cell type	Antiserum	^{125}I-S-14 binding			n^a
		−S-14	+S-14[b]	Specific binding	
			cpm		
CHO wild-type	$G_{i\alpha1,2}$	1060 ± 185	999 ± 213	61 ± 269	4
CHO SSTR1	$G_{i\alpha1,2}$	3530 ± 471	1148 ± 246	2382 ± 401	6
CHO SSTR1/GTPγS[c]	$G_{i\alpha1,2}$	1463 ± 69	998 ± 97	465 ± 69	4
CHO SSTR1	$G_{o\alpha}$	1305 ± 679	1273 ± 751	32 ± 137	6

[a] The data are the means ± standard deviations of four to six independent determinations (*n*) in two separate experiments.
[b] 100 nM s-14.
[c] The ^{125}I-S-14/receptor/G protein complex was immunoprecipitated with antiserum selective for $G_{i\alpha1,2}$ as described in Experimental Procedures. Then, the complex was incubated with 10 μM GTPγS for 1 hr before washing and quantification.

tially, the amount of ^{125}I-S-14 specifically bound to membranes was 50,000 cpm. After solubilization (50% efficiency) and dissociation of the ligand/receptor complex (half-life, 18 hr) (15), the amount remaining bound was 12,000–15,000 cpm, of which ~3000 cpm could be specifically immunoprecipitated with the anti-$G_{i\alpha1,2}$ antiserum. Thus, three independent lines of evidence, i.e., determination of sensitivity of radioligand binding to guanine nucleotides and pertussis toxin and immunoprecipitation of ^{125}I-S-14 binding, indicate that the SSTR1 subtype is coupled to the G_i/G_o family of G proteins.

Next, the ability of the SSTR1 subtype to couple to inhibition of cAMP accumulation was examined in intact CHO cells expressing SSTR1 (Fig. 5). No inhibition of forskolin-stimulated cAMP accumulation by S-14 (1 μM) was detected in wild-type CHO cells (13). In cells transfected with the SSTR1 subtype, a dose-dependent inhibition (ED_{50} = 1.1 ± 0.35 nM, four experiments) of forskolin-stimulated cAMP accumulation was observed in response to S-14. S-14 inhibited forskolin-stimulated cAMP accumulation by 50%. At comparable expression levels, the rat SSTR2 was found to inhibit up to 70% (ED_{50} = 350 pM) of the forskolin stimulation of cAMP accumulation (13). Inhibition of adenylyl cyclase and cAMP accumulation is mediated by the G_i family of G proteins. Concentrations of pertussis toxin as low as 30 ng/ml have been shown to completely catalyze the ADP-ribosylation of G_i/G_o in various cell lines and *in vivo* (21, 22). The effect of pertussis toxin (50 ng/ml, 24 hr) on the ability of S-14 to inhibit cAMP accumulation in intact cells was examined. In agreement with the pertussis toxin-mediated loss of binding, maximal inhibition of cAMP accumulation by 1 μM SRIF declined to only 20% in pertussis toxin-treated cells and toxin treatment shifted the dose-response curve to the right by 40-fold (Fig. 5). The ED_{50} for SRIF-mediated inhibition of cAMP accumulation in pertussis toxin-treated cells was calculated to be 41 ± 5 nM (four experiments), a 40-fold decrease in potency. These data suggest that SSTR1 inhibits forskolin-stimulated cAMP accumulation primarily via pertussis toxin-sensitive G proteins.

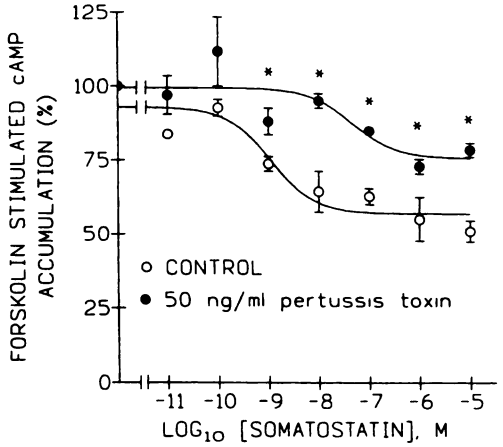

Fig. 5. SRIF-mediated inhibition of cAMP accumulation in CHO cells expressing the SSTR1 subtype and effect of pertussis toxin treatment. CHO cells expressing the SSTR1 subtype were treated with vehicle (○) or 50 ng/ml pertussis toxin (●) for 24 hr. The ability of S-14 to inhibit forskolin-stimulated (10 μM) cAMP accumulation in intact CHO cells expressing the SSTR1 subtype was examined. cAMP accumulations were performed as described in Experimental Procedures. Displayed are the means ± standard errors of four separate experiments. Forskolin-stimulated cAMP accumulation was 59 ± 10 pmol/10^6 cells in control cells and 53 ± 17 pmol/10^6 cells in pertussis toxin-treated cells. *, Significantly different from control cells ($p < 0.05$).

Discussion

In the present report we describe the pharmacological characterization of the rat SSTR1 subtype expressed in CHO cells and assign a functional response to this subtype. We demonstrate that the rat SSTR1 subtype is coupled to inhibition of cAMP accumulation by a pertussis toxin-sensitive G protein. The rat SSTR1 also displayed a second hallmark of many G protein-linked receptors, i.e., loss of high affinity agonist binding upon addition of GTPγS. In this regard, it appears that, unlike the human SSTR1 as described by Rens-Damiano *et al.* (14), the rat SSTR1 subtype is not an atypical SSTR subtype. The human SSTR1 subtype has been reported to lack many of the characteristic hallmarks of G protein-coupled receptors (14). Rens-Damiano *et al.* (14) reported that the human SSTR1 subtype does not couple to G proteins or inhibition of adenylyl cyclase. This group reported that radioligand binding of ^{125}I-S-14 to the human SSTR1 subtype is not sensitive to guanine nucleotides or to treatment of cells with pertussis toxin. In contrast, Kaupmann *et al.* (23) recently reported that both the human SSTR1 and SSTR2 subtypes do couple to inhibition of adenylyl cyclase activity in HEK-293 cells transfected with either subtype. In addition to coupling of rat SSTR1 to inhibition of cAMP accumulation, we observed that binding of ^{125}I-S-14 to the SSTR1 subtype is very sensitive to both GTPγS treatment and pertussis toxin treatment, two characteristics attributed to receptors coupled to the G_i/G_o family. The ED_{50} for GTPγS-mediated loss of binding was calculated to be 10 nM. In addition, we have found that most of the binding (80%) of ^{125}I-S-14 to the SSTR1 subtype is abolished by pretreatment

of cells with pertussis toxin. It is possible that coupling of the SSTR1 to pertussis toxin-insensitive G proteins may be responsible for the residual binding (20%) remaining after pertussis toxin treatment. Immunoprecipitation of ^{125}I-S-14 binding with anti-$G_{i\alpha 1,2}$ antisera confirms that this receptor subtype forms a complex with a pertussis toxin-sensitive G protein, although association with other, pertussis toxin-insensitive, G proteins cannot be ruled out. Thus, in contrast to previous reports with the human SSTR1 subtype (14), the rat SSTR1 subtype does appear to be coupled predominantly to the G_i/G_o family of G proteins.

Although the rat and human SSTR1 subtypes share 98% identity with each other, the minor species differences may account for the apparent discrepancies between the two studies. The differences between the species homologues are in the amino-terminal region (six amino acids) and in the carboxyl-terminal region (two amino acids) (9). The amino terminus of G protein-linked receptors has not been implicated in receptor-G protein coupling. The two amino acid differences (positions 385 and 389) in the carboxyl terminus may permit coupling of the rat receptor but not the human homologue to G proteins. The intracellular loops are the major determinants for receptor-G protein coupling (24). The amino acid sequence of the species homologues are identical in these regions (9). In a recent report, Kaupmann *et al* (23) demonstrated the coupling of the human SSTR1 to inhibition of adenylyl cyclase in transfected HEK-293 cells. Thus, differences in amino acid sequence probably contribute little, if at all, to the discrepancies between this report and the previous report (14). Differences in coupling efficiency can also arise from alternative splicing of genes. Alternatively spliced G protein-linked receptor subtypes have been shown to exhibit different biochemical and pharmacological characteristics. Examples include the mouse SSTR2 (25), dopamine D2 receptor (26), prostaglandin E EP3 receptor (27, 28), and pituitary adenylate cyclase activating protein receptor (29). Splice variants that display altered coupling and/or signal transduction have been observed in two domains, i.e., the third intracellular loop and the carboxyl terminus (25–29). Both third intracellular loop and carboxyl terminus splice variants can differ in their capacity to transduce a signal or can even couple to specific G proteins. For example, the short forms of SSTR2 and dopamine D2 receptors have been reported to couple more efficiently to inhibition of adenylyl cyclase (25, 26), although the corresponding long forms also mediate inhibition (13, 23, 25, 26). Splice varaints of either the prostaglandin E EP3 receptor or the pituitary adenylate cyclase activating protein receptor clearly display preferential coupling to various effectors. A possible explanation for the apparent discrepancies between the work of Rens-Damiano *et al.* (14) and others (present work) (23) is the possible existence of a splice variant of SSTR1. To date, no splice variants of SSTR1 have been reported. Two consensus 5′ splice donor sites exist in the carboxyl-terminal region of SSTR1. Little information exists on the genomic structure and possible 3′ acceptor sites (if any) of SSTR1.

An alternative possibility for the apparent discrepancies between the two studies involves the different patterns of G protein expression in the cell lines used to express the receptors. The two studies were performed with two different CHO strains, with different G proteins being expressed in the two strains (14, 30). Rens-Damiano *et al.* (14) used the CHO-DG44 strain. The strain used for the present study is the CHO-K1 strain. The CHO-K1 strain expresses detectable amounts of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ (30). $G_{i\alpha 1}$ and $G_{o\alpha}$ were not detected in the K1 strain (30). Rens-Damiano *et al.* (14) could detect $G_{i\alpha 3}$ but not $G_{i\alpha 1}$, $G_{i\alpha 2}$, or $G_{o\alpha}$ in the CHO-DG44 strain. The expression of $G_{i\alpha 2}$ in the CHO-K1 strain may allow for the observed coupling of the rat SSTR1 to G proteins and adenylyl cyclase. $G_{i\alpha 2}$ and $G_{i\alpha 1}$ have both been implicated in the coupling of SSTRs and other G protein-linked receptors to inhibition of adenylyl cyclase activity (18, 31–34).

In agreement with previous reports, we could not detect binding of the SSTR2-selective SRIF analogue MK-678 (up to 1 μM) to the SSTR1 subtype (9, 14). The ligand-binding characteristics, in terms of rank order of potency, are similar between the rat and human SSTR1 (9, 14) (present study). The affinity of S-14 in the present study (saturation and competition binding) and in the report by Li *et al.* (9) is 10-fold higher than that previously reported for the human SSTR1 (10, 14) and might also reflect a species difference. It is possible that the human and rat SSTR1 subtypes display pharmacologically distinct characteristics. Alternatively, the intracellular environment (patterns of G protein expression) could also alter agonist binding. Differences in coupling efficiencies would affect the observed ligand binding affinities, coupling to G proteins, and the ability of S-14 to transduce a signal (inhibition of cAMP accumulation) through this receptor subtype. Differences in ligand binding affinities across species have been reported for both SSTR3 and SSTR4 (10, 11, 35).

Barber *et al.* (36) have reported that β-adrenergic receptors and SSTRs expressed in S49 cells couple to a Na^+/H^+ exchanger. The actions of these two G protein-linked receptors on the Na^+/H^+ exchanger appear not to be mediated via cholera or pertussis toxin-sensitive G proteins (36). At least for β-adrenergic receptors, activation of the Na^+/H^+ exchanger may be mediated by G proteins other than G_s (37). Based on the inability to observe coupling of G proteins to the human SSTR1, one report (14) suggested that the SSTR1 mediates these actions in S49 cells. It is clear from the present study that the rat SSTR1 can and does couple to pertussis toxin-sensitive G proteins. This does not eliminate the possibility of SSTR1 coupling to pertussis toxin-insensitive G proteins. Additional work will be required to determine which particular SSTR subtypes couple to the Na^+/H^+ exchanger.

Earlier studies (1, 14) reported that the human SSTR1 and SSTR2 subtypes do not couple to inhibition of adenylyl cyclase or cAMP accumulation. Based on the partial amino acid sequence of purified SSTRs from GH_4C_1 cells (15, 38), SSTR2 is the predominant SSTR subtype in GH_4C_1 cells. The GH_4C_1 cell SSTR does couple to inhibition of cAMP accumulation in these cells (5), as does recombinant rat SSTR2 (both long and short forms) in transfected CHO cells (13, 25). The levels of expression of human SSTR1 (14) were equivalent to the levels of expression we report for the rat SSTR1 (990 and 1500 fmol/mg of protein, respectively). Interestingly, pertussis toxin abolished 80% of ^{125}I-S-14 binding but only 50% of the inhibition of cAMP accumulation. These data suggest that there is a large receptor reserve in these cells. In CHO cells expressing the rat SSTR1 at levels as low as 40 fmol/mg of protein (40-fold lower levels), SRIF inhibited cAMP accumulation (data not shown). In summary, we demonstrate that the rat SSTR1 subtype not only couples to pertussis toxin-sensitive G proteins but also

inhibits cAMP accumulation in transfected CHO-K1 cells. In addition, these data suggest that the rat SSTR1-mediated inhibition of cAMP accumulation is transduced, for the most part, by pertussis toxin-sensitive G proteins.

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