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Superagonism at the Human Somatostatin Receptor Subtype 4

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ABSTRACT

We have discovered a novel compound, J-2156 [(1′*S*, 2*S*)-4-amino-*N*-(1′-carbamoyl-2′-phenylethyl)-2-(4″-methyl-1″-naphthalenesulfonylamino)butanamide], that belongs to a new class of somatostatin receptor ligands. J-2156 binds with nanomolar affinity to the human somatostatin receptor subtype 4 and is over 400-fold subtype-selective against the other somatostatin receptors. When evaluated in a [^{35}S]guanosine-5′-*O*-(3-thio) triphosphate binding assay, J-2156 elicited a response 2 to 3 times as large as that of somatostatin-28 and somatostatin-14. That somatostatin-14 is clearly not a maximally efficacious agonist could be verified by demonstrating that it displays the typical behavior of a partial agonist when tested against J-2156. Increasing concentrations of somatostatin-14 cause a concentration-dependent rightward shift of the dose-response curves for J-2156, without affecting its maximal response. This lack of reduction of the maximal response and the fact that the superior efficacy of J-2156 is detected in membranes argue against desensitization and internalization as possible explanations for the superior efficacy of J-2156. More likely is that somatostatin-14 and J-2156 stabilize distinct receptor conformations that differ in their ability to interact with G-proteins. In a cyclic AMP assay, J-2156, somatostatin-28, and somatostatin-14 all act as full agonists. However, this outcome is most likely due to the presence of a receptor reserve in the cyclic AMP assay since there is a large gain of apparent potency in the cyclic AMP assay and the gain is larger for J-2156 than for somatostatin. We conclude that the endogenous ligands somatostatin-14 and somatostatin-28 do not define maximal agonism on the human somatostatin receptor subtype 4 and that J-2156 represents a so-called superagonist.

Somatostatin, also known as somatotropin release-inhibiting factor (SRIF), was first identified as a peptide that inhibits growth hormone release from the anterior pituitary (Krulich et al., 1968; Brazeau et al., 1973). Later, SRIF has been shown to also have other physiological functions, including the inhibition of both endocrine secretion, e.g., thyroid-stimulating hormone, prolactin, insulin, and glucagon, and exocrine secretion, e.g., gastric acid, intestinal fluid, and pancreatic enzymes (for review, see Patel, 1999). In addition, SRIF functions as a modulator of neuronal activity and may act as a neurotransmitter (Patel, 1999). The wide range of physiological actions of SRIF are mediated by a family of G-protein-coupled receptors (GPCRs), which comprises five distinct receptor subtypes (Reisine and Bell, 1995) termed sst_1 through sst_5. In addition to the initially identified 14-amino acid peptide (SRIF-14), there is also a second form of somatostatin in humans, which consists of 28 amino acids (SRIF-28) (Pradayrol et al., 1980). Both endogenous SRIF forms bind to the five receptor subtypes with approximately equal affinity, even though SRIF-28 has been reported to show some preference for the sst_5 (Reisine and Bell, 1995).

Somatostatin receptors are widely expressed in many tissues. Among the broad spectrum of SRIF effects, several responses have been identified as being mediated through distinct receptor subtypes. The inhibition of gastric acid secretion, for example, is considered to occur through the sst_2, whereas the inhibition of insulin release appears to be mediated by the sst_2 and sst_5 (Weckbecker et al., 2003).

Although SRIF has attracted considerable attention due to its wide range of biological functions, it is one of the least exploited hormones in medical therapeutics. One reason is the rather short biological half-lives of the endogenous forms of SRIF. More stable analogs, such as the octapeptides octreotide and lanreotide, have been synthesized to overcome this problem and are in clinical use for the treatment of acromegaly and neuroendocrine tumors (Lamberts, 1988; Weckbecker et al., 1993; Chaudhry and Kvols, 1996). How-

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ABBREVIATIONS: SRIF, somatotropin release-inhibiting factor; GPCR, G-protein-coupled receptor; $sst_{1–5}$, somatostatin receptor subtypes 1 through 5; h $sst_{1–5}$, human $sst_{1–5}$; J-2156, (1′*S*, 2*S*)-4-amino-*N*-(1′-carbamoyl-2′-phenylethyl)-2-(4″-methyl-1″-naphthalenesulfonylamino)butanamide; DMF, *N,N*-dimethylformamide; DCM, dichloromethane; Fmoc, 9-fluorenylmethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; CHO, Chinese hamster ovary; ^{125}I-LTT-SRIF-28, (^{125}I-Tyr)-[Leu^8,DTrp^{22}]-somatostatin-28; [^{35}S]GTPγS, [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate.

ever, these shortened peptides do not interact equally well with all the five subtypes but show distinct preferences for the subtypes sst_2 and sst_5, and to a somewhat lesser degree, sst_3. Since this pattern of subtype preference has been a general feature of octapeptide or hexapeptide SRIF analogs, it has given rise to an operational subdivision of the five somatostatin receptors into two subfamilies, one consisting of sst_2, sst_3, and sst_5 and the other of sst_1 and sst_4 (Hoyer et al., 1995).

Until recently, the lack of sst_4-selective ligands has made it difficult to connect sst_4 more closely to particular (patho-)physiological functions. However, the expression of both sst_1 and sst_4 has been reported to be associated with smooth muscle cell proliferation and vascular intimal thickening (Aavik et al., 2002). Pharmacological agents acting on sst_1 and/or sst_4 may therefore be advantageous for the treatment of diseases involving pathological vascular proliferation, e.g., in connection with pathological angiogenesis and restenosis (Curtis et al., 2000; Häyry and Patel, 2003). Compounds acting on sst_4 have also been suggested to be useful as anti-glaucoma agents (Mori et al., 1997). Furthermore, based on the high sst_4 expression levels in cerebral cortex, hippocampus, amygdala, and hypothalamus, sst_4-selective ligands may have therapeutic potential in the treatment of various central nervous system disorders (Bito et al., 1994; Harrington et al., 1995; Perez and Hoyer, 1995; Liu et al., 1999).

Considerable progress has been made in recent years in the identification of nonpeptide ligands for somatostatin receptor. Most prominently, a research group at Merck has been able to identify subtype-selective, nonpeptide agonists for each of the five somatostatin receptor subtypes by using a combinatorial chemistry approach (Rohrer et al., 1998).

With the promises of subtype-selective somatostatin receptor ligands in mind, we initiated a chemistry program to identify new, nonpeptide chemical entities selective for human sst_4 (h sst_4). In the current communication, we report that a member of a novel class of somatostatin receptor ligands, i.e., sulfonamido-peptidomimetics, displays the unique property of superagonism at the h sst_4.

Materials and Methods

Ligands. J-2156 (Fig. 1) was synthesized as follows. H-Phe-NH_2 hydrochloride (0.57 mmol; Advanced Chemtech, Louisville, KY) was dissolved in 2 ml of dry *N,N*-dimethylformamide (DMF)/dichloromethane (DCM) (1/1) and triethylamine (0.68 mmol; Fluka, Buchs, Switzerland) was added. After 30 min, a DMF/DCM (1/1.4 ml) solution containing 4-(*tert*-butyloxycarbonyl)amino-2-(9-fluorenylmethoxycarbonyl)aminobutanoic acid (Fmoc-Dbu(Boc)-OH) (0.57 mmol; Advanced Chemtech), *N,N*-diisopropylcarbodiimide (0.57 mmol; Acros, Tournai, Belgium), and 1-hydroxybenzotriazole (0.57 mmol, Acros) was added. After 16 h, the solvent was evaporated, and DCM was added. The organic phase was washed three times with water, once with brine, and subsequently evaporated. The Fmoc protection was removed by treating (1′*S*,2*S*)-4-(*N*-Boc-amino)-*N*′-(1′-carbamoyl-2′-phenylethyl)-2-(*N*′′-Fmoc-amino)butanamide with 4.5 ml of 20% (v/v) piperidine in DMF for 45 min. After solvent evaporation, the residue was dissolved in 9 ml of dry tetrahydrofuran/DMF (1/1) and 4-methyl-1-naphthalenesulfonyl chloride (1.5 Eq; Maybridge Chemicals, Trevillet, UK) plus triethylamine (0.85 mmol; Fluka) were added. After 16 h, the solvent was evaporated, and the residue was purified via silica column chromatography (mobile phase from 5% MeOH in DCM up to 20% MeOH in DCM). As a final step, the Boc protection was removed by treating the product with 2.5 ml of 25% (v/v) trifluoroacetic acid in DCM for 1 h. Then the solvent was evaporated, and the residue was purified via reverse-phase high-performance liquid chromatography to give 52.5 mg of (1′*S*, 2*S*)-4-amino-*N*-(1′-carbamoyl-2′-phenylethyl)-2-(*N*′-(4″-methyl-1″-naphthalenesulfonyl)amino)butanamide. The structure was verified by ^{1}H NMR and liquid chromatography-tandem mass spectroscopy analysis.

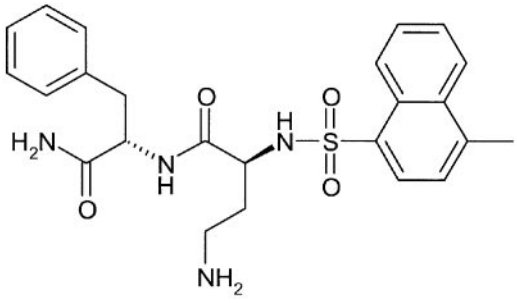

Fig. 1. Structure of J-2156.

Cell Culture and Transfections. Chinese hamster ovary (CHO) cells (K1 strain) were cultured in Ham's F-12 medium (Nutrient Mixture Ham's F-12; Invitrogen, Carlsbad, CA) containing 10% fetal calf serum. The cDNA encoding the h sst_1 or h sst_4 (received from Dr. Einari Aavik, University of Helsinki, Finland) was subcloned from a construct in pBluescript (Stratagene, La Jolla, CA) into a bicistronic mammalian expression construct pIRESNeo2 (BD Biosciences Clontech, Palo Alto, CA) with an amino-terminal hemagglutinin tag (BD Biosciences Clontech) and EF1 promoter (Invitrogen, Groningen, Netherlands) in h sst_4 cloning or CMV promoter (BD Biosciences Clontech) in h sst_1 cloning. The cloned construct was then transfected into CHO cells using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Stable transfectants were selected in Ham's F-12 containing the neomycin analog G418 (Calbiochem, San Diego, CA) at 500 μg/ml. After selection, transfected CHO-h sst_4 cells were sorted and the highest expressing cells collected by fluorescence-activated cell sorting (Flow Cytometry Core Facility, Turku, Finland). Hereafter, both the sorted CHO-h sst_4 and the unsorted CHO-h sst_1 cells were cloned by dilution-plating. The highest expressing clone was isolated in SRIF binding assays using (^{125}I-Tyr)-[Leu^8,DTrp^{22}]-SRIF-28 (^{125}I-LTT-SRIF-28) (Anawa, Zurich, Switzerland; specific activity, 2000 Ci/mmol) as a tracer. CHO cells expressing the h sst_2, h sst_3, and h sst_5 subtypes were a kind gift from Prof. Kjell Öberg (Uppsala University Hospital, Uppsala, Sweden). The recombinant CHO cells expressing the somatostatin receptor subtypes were grown in Ham's F-12 medium supplemented with 5% fetal bovine calf serum and 200 to 250 μg/ml G418.

The receptor densities (B_{max}) and radioligand K_d values for the CHO-h sst_4 clones were determined in saturation binding experiments using ^{125}I-LTT-SRIF-28 as radioligand (Siehler et al., 1999). A CHO-h sst_4 cell clone having a receptor density of 5.2 pmol/mg membrane protein and a K_d of 0.48 nM for ^{125}I-LTT-SRIF-28 (average of two experiments performed with triplicate concentrations) was chosen for further pharmacological studies.

Receptor Ligand-Binding Assays. Recombinant CHO cells expressing 1 of the 5 human somatostatin receptor subtypes were harvested in phosphate-buffered saline and frozen at −70°C. Membranes were prepared as previously described (Engström et al., 2003). In competition binding assays membranes (3–20 μg of total protein per sample, depending on the expression level of individual subtypes) were incubated in assay buffer (10 mM Hepes, 1 mM EDTA, 5 mM $MgCl_2$, 5 mg/ml bovine serum albumin, and 30 μg/ml bacitracin, pH 7.6) with 20 to 40 pM ^{125}I-LTT-SRIF-28 and six concentrations of the test ligand. Each concentration was tested in duplicate. Nonspecific binding was defined with 1 μM SRIF-14 and corresponded to 5 to 25% of total binding. After 60 min at room temperature, incubations were terminated by rapid vacuum filtra-

tion through GF/B glass fiber filter mats (presoaked at 4°C in 200 ml of 10 mM Hepes, 1 mM EDTA, and 5 mM $MgCl_2$, pH 7.6) and three 5-ml washes with ice-cold wash buffer (20 mM Tris, 1 mM EDTA, and 5 mM $MgCl_2$, pH 7.4). The filters were then dried and impregnated with scintillate, and their radioactivity was measured by scintillation counting.

The affinity profiling on a number of GPCRs other than sst_{1-5} was conducted by Cerep (Le Bois L'Evêque, Celle L'Evescault, France) using documented standard procedures.

G-Protein Activation. The ability of ligands to stimulate the receptor-mediated binding of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) (PerkinElmer Life and Analytical Sciences, Boston, MA; specific activity, 1250 Ci/mmol) to G-proteins in membranes of CHO cells expressing the h sst_4 was measured. Membranes (about 10 μg of protein per sample), prepared as previously described (Engström et al., 2003), were incubated in 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 μM GDP, 100 mM NaCl, and 10 μg/ml saponin (incubation buffer) with different concentrations of test compound. Each concentration was tested in duplicate. After a 45-min incubation period at 30°C (15-min preincubation without label followed by a 30 min-stimulation period past the addition of the radiolabel), the reaction was terminated by rapid vacuum filtration through GF/B glass fiber filter mats. Filters were then washed four times with 5 ml of ice-cold wash buffer (20 mM Tris, 1 mM EDTA, and 5 mM $MgCl_2$, pH 7.4), dried, and counted for radioactivity in a scintillation counter. To ascertain that the stimulatory effect of J-2156 on [^{35}S]GTPγS binding was mediated exclusively via the h sst_4, control experiments with membranes of CHO cells transfected with the α_{2A}-adrenoceptor (Pohjanoksa et al., 1997) were conducted under identical conditions as the experiments with membranes of CHO-h sst_4 cells.

The effects of SRIF-14 on the J-2156-induced stimulation of [^{35}S]GTPγS binding in membranes of CHO cells expressing the h sst_4 were determined by establishing dose-response curves for J-2156 in the presence of fixed concentrations of SRIF-14 (100 nM, 300 nM, and 1 μM). The assays were run as described above for the determination of agonist activities, except that the total incubation time was 75 min (15-min preincubation without label followed by a 60-min stimulation after the addition of the radiolabel).

Adenylyl Cyclase Activity. CHO cells expressing h sst_4 (2.5×10^4 cells per well in 96-well plates) were cultured for 20 h at 37°C in Ham's F-12 medium containing 5% fetal calf serum and thereafter in serum-free medium for 1 h. Cells were further incubated for 10 min with 0.03 mM indomethacine and 0.03 mM rolipram in the cell incubator before the addition of various concentrations of agonists and/or 10 μM forskolin. After 30 min, incubations were terminated by removing the medium and adding 20 μl of lysis buffer (PerkinElmer Wallac, Turku, Finland) per well. Cyclic AMP was measured using a DELFIA cyclic AMP kit as described by the manufacturer (PerkinElmer Wallac).

Data Analysis. Results from individual experiments were determined via nonlinear least-squares curve fitting procedures. Affinity constants (K_i values), as determined in competition binding assays, were calculated from the IC_{50} values according to the Cheng-Prusoff's equation (Cheng and Prusoff, 1973). Apparent pA_2 values and their 95% confidence limits were estimated by Schild analysis (Arunlakshana and Schild, 1959). When the 95% confidence interval for the regression line of the Schild plot accommodated a straight line with the slope of −1, pA_2 values were calculated with the slope constrained to unity. Data are given as the average of at least three experiments and statistical significance was determined using Student's two-tailed paired *t* test.

Results

Compound J-2156 belongs to a new class of somatostatin ligands, i.e., sulfonamido-peptidomimetics (Fig. 1). We found that this compound possesses an affinity of 1.2 nM for the h sst_4 (Table 1). This provides J-2156 with equal or even a slightly higher h sst_4 affinity compared with the endogenous ligands SRIF-14 and SRIF-28 or cortistatin-17, which has also been suggested to act via somatostatin receptors (Fukusumi et al., 1997). Since the affinity of J-2156 on the four other human somatostatin receptor subtypes is 500 nM or lower, the compound is endowed with a minimum selectivity of about 400-fold for the h sst_4. The profiling of J-2156 at a concentration of 1 μM against a number of other peptide GPCRs shows very little interaction (Table 2), suggesting the affinity value of the compound on these receptors is well above 1 μM.

When tested for its ability to stimulate the binding of [^{35}S]GTPγS to membranes of CHO cells stably expressing the h sst_4, J-2156 surprisingly produced a much higher response than the endogenous ligands SRIF-14 and SRIF-28 (Fig. 2A; Table 3). This enhanced response toward J-2156 is clearly mediated through pertussis toxin-sensitive G-proteins since the stimulatory effect of J-2156, as well as that of SRIF-14, is completely ablated in membranes from h sst_4-expressing CHO cells that have been pretreated with pertussis toxin, an agent known to uncouple $G_{i/o}$ proteins from GPCRs (Fig. 2A). Neither J-2156 nor SRIF-14 at micromolar concentrations produce any significant increase in [^{35}S]-GTPγS binding with membranes from CHO cells that lack the h sst_4 but which instead stably express the human α_{2A} adrenergic receptors (Fig. 2B). In contrast, the α_2-adrenoceptor agonist epinephrine produces the expected dose-dependent increase in [^{35}S]GTPγS binding in these membranes.

Increasing amounts of SRIF-14 cause a concentration-dependent right-shift of the dose-response curve for J-2156 without affecting its maximal response (Fig. 3A). For the six analyses conducted, the linear regression analysis yielded a slope of 1.3 ± 0.2 (r^2 = 0.8119). The confidence limits of the Schild regression comfortably accommodate a slope of unity and a linear regression computed with the slope constrained to such a value yielded a pA_2 value of 8.00 (Fig. 3B).

To exclude the possibility that the higher efficacy of J-2156 at the h sst_4 might be due to metabolic instability of SRIF-14,

TABLE 1

Binding affinity (K_i) of ligands for human recombinant SRIF receptor subtypes (h sst_{1-5}) stably expressed in CHO cells

The data are given as the mean ± S.E.M. of at least three experiments. If the competition curve was incomplete, the K_i is given as greater than the highest concentration tested.

	K_i				
	h sst_1	h sst_2	h sst_3	h sst_4	h sst_5
			nM		
SRIF-14	1.4 ± 0.2	0.62 ± 0.15	1.6 ± 0.2	4.1 ± 0.6	4.1 ± 0.4
SRIF-28	0.57 ± 0.09	0.34 ± 0.14	0.28 ± 0.11	1.9 ± 0.3	0.30 ± 0.12
Cortistatin-17	0.77 ± 0.32	1.9 ± 0.4	1.9 ± 0.4	3.5 ± 0.3	3.3 ± 0.3
J-2156	500 ± 150	>5000	1400 ± 100	1.2 ± 0.4	540 ± 80

TABLE 2
The effect of 1 μM J-2156 on specific radioligand binding at various receptor targets
The data are given as a percentage of inhibition of specific binding (control set as 0%).

Receptor	Inhibition
	%
ANP	0
Bombesin[a]	0
Bradykinin-1	4
Bradykinin-2	14
Glucagon	8
MCH	0
Melanocortin-1	0
Melanocortin-3	0
Melanocortin-4	3
Melanocortin-5	0
Motilin	0
Neuropeptide Y	8
Opioid-δ_2	0
Opioid-κ	0
Opioid-μ	5
Urotensin II	12
VIP-1	5
VIP-2	5
Vasopressin-1a	0
Vasopressin-1b	0
Vasopressin-2	1

ANP, atrial natriuretic peptide; MCH, melanin-concentrating hormone; VIP, vasoactive intestinal peptide.
[a] Nonselective, tissue source rat brain.

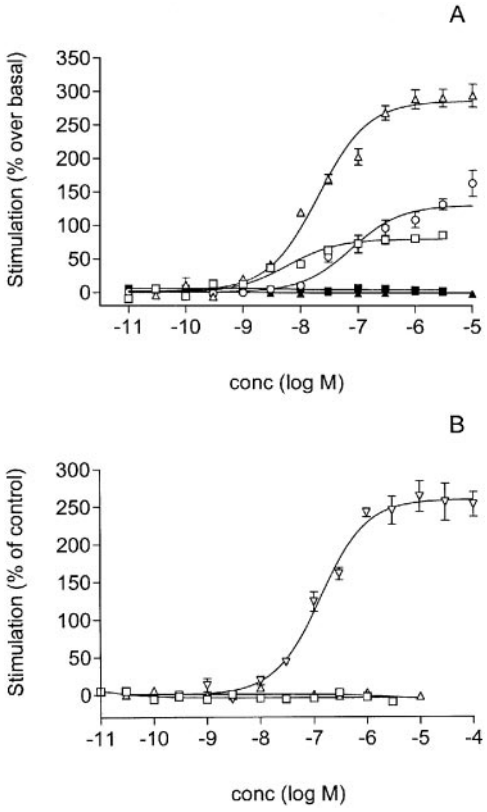

Fig. 2. Stimulation of [^{35}S]GTPγS binding in membranes of CHO cells expressing h sst$_4$ (A) or human α_{2A}-adrenergic receptor (CHO-C10; B). A, CHO-h sst$_4$ cells either nonpretreated or pretreated with pertussis toxin. Trace amounts of [^{35}S]GTPγS were added to membrane CHO-h sst$_4$ cells (about 10 μg/sample) that had been preincubated with the indicated concentration of SRIF-14 (□, ■), SRIF-28 (○), or J-2156 (△, ▲) in the presence of 10 mM $MgCl_2$, 10 μM GDP, 100 mM NaCl, and 10 μg/ml saponin. The membranes were prepared from nonpretreated cells (open symbols) or from cells that had been pretreated with 100 ng/ml pertussis toxin for 16 h (closed symbols). B, trace amounts of [^{35}S]GTPγS added to membranes of transfected cells (about 10 μg/sample) that had been preincubated with the indicated concentration of SRIF-14 (□), J-2156 (△), or epinephrine (▽) in the presence of 10 mM $MgCl_2$, 10 μM GDP, 100 mM NaCl, and 10 μg/ml saponin. Basal [^{35}S]GTPγS binding to membranes of the nonpretreated and pretreated CHO-h sst$_4$ cells were 103 ± 9 and 29 ± 3 fmol/mg protein, respectively, whereas the corresponding value in membranes of CHO-C10 cells was 185 ± 7 fmol/mg protein. Maximal [^{35}S]GTPγS binding to membranes of CHO-h sst$_4$ cells was 396 ± 13 fmol/mg protein (stimulated by J-2156; A); maximal binding to membranes of CHO-C10 cells was 661 ± 21 (stimulated by epinephrine; B). Stimulation is given as a percentage over basal. The combined data of at least three different experiments with each concentration run in duplicate are shown.

we measured the stimulation kinetics of saturating concentrations of both compounds at various time points up to 60 min. As can be seen from Fig. 4, the extent of binding elicited by both compounds, as well as the basal binding of [^{35}S]GTPγS, is linear over the whole time period with the stimulation of J-2156 possessing a clearly steeper slope. Thus, the higher efficacy J-2156 is observable already at the earliest time points measured, and the response toward SRIF-14 does not show signs of compound depletion. Furthermore, a stability analysis by high-performance liquid chromatography revealed that maximally 25% of SRIF-14 is being degraded during the 1-h incubation (data not shown).

To further characterize the functional activity of J-2156 at the h sst$_4$, we evaluated its effect on forskolin-stimulated cyclic AMP production in the same CHO-h sst$_4$ cell line, from which membranes were prepared for use in the [^{35}S]GTPγS binding studies. The addition of 10 μM forskolin increases cellular cyclic AMP accumulation by about 10-fold in the CHO-h sst$_4$ cells. SRIF-14, SRIF-28, and J-2156 act as apparent full agonists by completely inhibiting the forskolin-stimulated adenylyl cyclase activity (Fig. 5; Table 3). However, in the cyclic AMP assay, the compounds are considerably more potent than in the [^{35}S]GTPγS binding assay (Table 3). In control CHO cells, expressing the human α_{2A} adrenergic receptors, there is no effect of J-2156 on forskolin-stimulated adenylyl cyclase activity (data not shown).

Discussion

In this report, we describe a novel compound, J-2156, that belongs to a new class of somatostatin receptor ligands, i.e., sulfonamido-peptidomimetics. J-2156 possesses nanomolar affinity for the h sst$_4$ and is at least 400-fold selective for that receptor over any of the other four human somatostatin receptor subtypes. Although the affinity of J-2156 on the h sst$_4$ is comparable with that of SRIF-14 and SRIF-28, its functional properties are uniquely different by demonstrating superagonism at the h sst$_4$.

We used two functional assays to measure agonist-induced responses at the h sst$_4$: first, the binding of [^{35}S]GTPγS in membranes containing the h sst$_4$ and, second, the agonist-mediated inhibition of cyclic AMP accumulation in forskolin-stimulated cells expressing the receptor. The [^{35}S]GTPγS binding assay measures the most proximal event after receptor activation, whereas the inhibition of cyclic AMP accumulation represents a more distal step at the level of second messenger production. In the [^{35}S]GTPγS binding assay, J-2156 produces a significantly greater response compared with SRIF. This stimulation of [^{35}S]GTPγS binding by J-2156 is fully dependent on pertussis toxin-sensitive G-proteins

TABLE 3

Agonist effects of ligands at the human sst_4

Agonist activity (EC_{50} and efficacy) was assessed as the ability of the ligand to stimulate [^{35}S]GTPγS binding in membranes of CHO cells expressing human sst_4 (CHO-h sst_4) or as the ability of the compound to inhibit forskolin-stimulated cyclic AMP accumulation in CHO-h sst_4 cells (see Figs. 2A and 5). The data are given as the mean ± S.E.M. of at least three experiments.

	[^{35}S]GTPγS Binding Assay		Cyclic AMP Assay	
	EC_{50}	Efficacy (Stimulation over Basal)	EC_{50}	Efficacy (Inhibition of Cyclic AMP Accumulation)
	nM	%	*nM*	%
SRIF-14	4.9 ± 0.4	80 ± 10	0.42 ± 0.12	98 ± 3
SRIF-28	81 ± 24	130 ± 10*	0.73 ± 0.18	93 ± 5
J-2156	17 ± 2	290 ± 20**	0.07 ± 0.03	102 ± 2

* Efficacy significantly different from SRIF-14 ($p < 0.05$, Student's paired t test).
** Efficacy significantly different from SRIF-14 ($p < 0.01$, Student's paired t test).

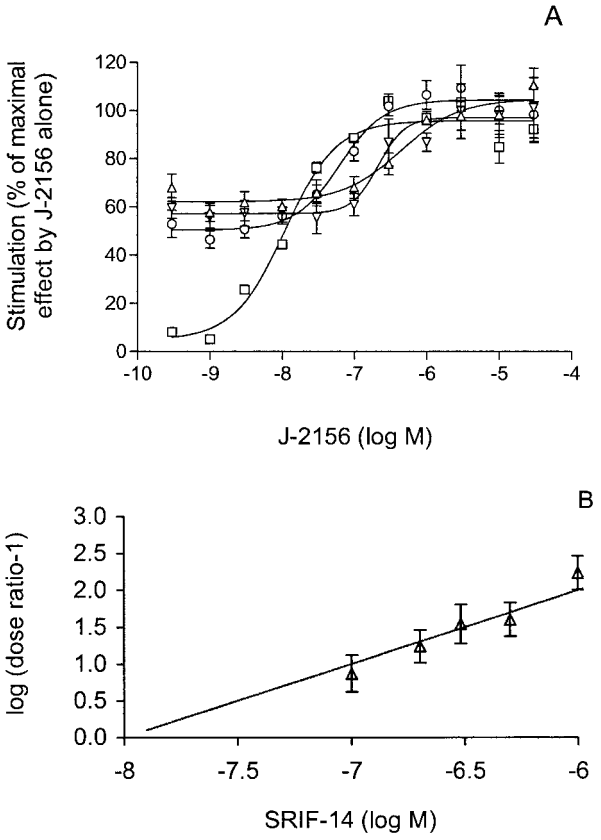

Fig. 3. Inhibition of J-2156-induced stimulation of [^{35}S]GTPγS binding in membranes of CHO cells expressing the h sst_4. Trace amounts of [^{35}S]GTPγS were added to membranes of transfected cells (about 10 μg/sample) that had been preincubated without (□) or with increasing concentrations of SRIF-14, 100 nM (○), 300 nM (▽), and 1 μM (△), in the presence of 10 mM $MgCl_2$, 10 μM GDP, 100 mM NaCl, and 10 μg/ml saponin (A). B, Schild analysis of antagonism by SRIF-14 of J-2156-induced stimulation of [^{35}S]GTPγS binding. Basal [^{35}S]GTPγS binding to membranes of CHO-h sst_4 cells that had been preincubated without or with increasing concentrations of SRIF-14 were 57 ± 4 (w/o SRIF), 123 ± 5 (100 nM SRIF-14), 132 ± 8 (300 nM SRIF-14), and 139 ± 9 (1 μM SRIF-14) fmol/mg protein. Maximal [^{35}S]GTPγS binding after preincubation (without or with SRIF-14) and stimulation with J-2156 were 188 ± 13 (without SRIF-14), 207 ± 9 (100 nM SRIF-14), 206 ± 20 (300 nM SRIF-14), and 203 ± 11 (1 μM SRIF-14) fmol/mg protein. To calculate the dose ratios, EC_{50} values were taken as the equiactive agonist concentrations. In A, the combined data of three different experiments with each concentration run in duplicate are shown, and stimulation is given as a percentage of the maximal effect produced by J-2156 in the absence of SRIF-14 (control, set as 100%). In B, a Schild plot was generated from the data of six experiments of the type shown in A. The 95% confidence interval for the regression line in the Schild analysis easily accommodated a slope of unity; therefore, the slope was constrained to that value. Under these circumstances, the apparent pA_2 was 8.00.

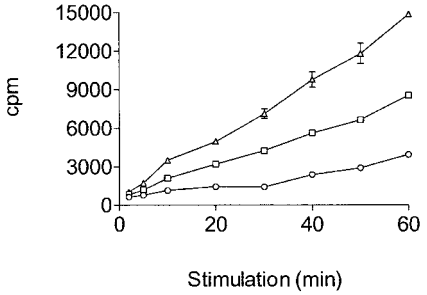

Fig. 4. [^{35}S]GTPγS binding in membranes of CHO cells expressing the h sst_4 after different stimulation times. Trace amounts of [^{35}S]GTPγS, membranes of transfected cells (about 10 μg/sample), and buffer (basal; ○) or saturating concentrations of SRIF-14 (3 μM; □) or J-2156 (10 μM; △) were incubated for the indicated times in the presence of 10 mM $MgCl_2$, 10 μM GDP, 100 mM NaCl, and 10 μg/ml saponin. One representative example of three different experiments performed in triplicate is shown.

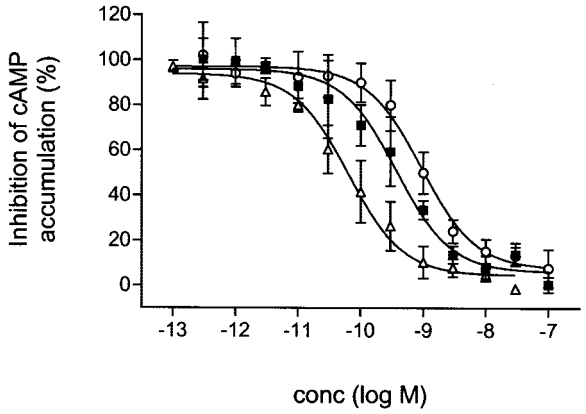

Fig. 5. Inhibition of forskolin-stimulated cyclic AMP accumulation. Cyclic AMP accumulation in forskolin-stimulated CHO cells stably transfected with h sst_4 was measured in the presence of increasing concentrations of SRIF-14 (■), SRIF-28 (○), or J-2156 (△). The signal in the assay was 95 ± 15 pmol/well, the readouts for blank (measured in the absence of cells) and total forskolin-stimulated cyclic AMP accumulation (measured in CHO-h sst_4 cells in absence of ligand) being 7.3 ± 1.4 and 102 ± 16 pmol/well, respectively. The readout for maximal inhibition by SRIF-14 was 11 ± 5 pmol/well, by SRIF-28 10 ± 3 pmol/well, and by J-2156 6.0 ± 2.4 pmol/well. The data are normalized against the forskolin-induced cyclic AMP concentration in the absence of agonist (control, set as 100%). The combined data of three different experiments with each concentration run in triplicate are shown.

and on the presence of the h sst_4 in the CHO cell membranes. The latter observation excludes a possible contribution from other GPCRs in the background of the CHO cells toward the response caused by J-2156. The amplitude of the J-2156

dose-response curve implies that the endogenous ligands SRIF-14 and SRIF-28 are not able to cause the maximal possible degree of receptor activation on the h sst_4. Consistent with this hypothesis, SRIF-14 was found to behave like a typical partial agonist when tested against J-2156. That is to say, increasing concentrations of SRIF-14 cause a rightward shift of J-2156 dose-response curves, without affecting its maximal response. Therefore, J-2156 can be classified as a superagonist at h sst_4.

In the cyclic AMP assay, for which the same CHO-h sst_4 cell line was used that served as the source for the membranes of the [^{35}S]GTPγS binding assay, J-2156 and SRIF cause a complete inhibition of forskolin-stimulated adenylyl cyclase activity. In many cases of heterologously expressed receptors, G-protein activation assays offer an advantage over second messenger-based assays with respect to the discrimination of partial versus full agonists. Cyclic AMP-based assays in particular appear to be prone to the receptor reserve phenomenon, which can mask partial agonist activity (Adham et al., 1992; Umland et al., 2001). Umland et al. (2001), for example, have reported for the human α_{2C}-adrenergic receptor that a 20-fold greater receptor occupancy was required for half-maximal agonist-induced [^{35}S]GTPγS binding to membranes compared with the inhibition of cyclic AMP formation. The reason for this susceptibility of cyclic AMP-based assays to receptor reserves appears to lie in the relative amounts of receptor, G-proteins and adenylyl cyclase, with the cyclase representing the stoichiometrically limiting component (Kim et al., 1994). In our experiments, the potency of J-2156 is 240-fold higher for the inhibition of adenylyl cyclase than for the stimulation of [^{35}S]GTPγS binding. We also found higher potencies for SRIF-14 and SRIF-28 in the cyclic AMP assay compared with the [^{35}S]GTPγS binding assay, the fold shift being greater for the more efficacious SRIF form (110- and 12-fold for SRIF-28 and SRIF-14, respectively). These agonist potency shifts are consistent with the existence of a receptor reserve in the cyclic AMP assay or a greater receptor reserve in the cyclic AMP assay compared with the [^{35}S]GTPγS binding assay. The presence of a receptor reserve is predicted to produce a greater gain in apparent potency for highly efficacious agonists compared with partial agonists because lower levels of receptor occupancy are required to elicit the maximal response at the level of the effector enzyme. Our interpretation of the results implies that an efficacy readout in the form of a cyclic AMP assay may provide a misleading picture in terms of agonist efficacies when using recombinant cell lines with large receptor expression levels.

In an attempt to obtain evidence that SRIF-14 and J-2156 act at a common site on the h sst_4, we established dose-response curves for J-2156 in the presence of different concentrations of SRIF-14. The presence of SRIF-14 has two effects on the dose-response curves of J-2156. It causes a larger extent of [^{35}S]GTPγS binding at ineffective concentrations of the compound, and it concentration dependently shifts the dose-response curves of J-2156 to the right. Importantly, however, there are no significant differences in the maximal response toward J-2156 in the absence or presence of SRIF-14. In other words, SRIF-14 displays the typical behavior of a partial agonist in its effects on the dose-response curve of J-2156. A Schild analysis of the data results in a regression line with a slope of 1.3 $\pm$ 0.2. The 95% confidence interval for the regression line easily accommodates a straight line with a slope of unity. It is, therefore, not likely that J-2156 acts as an allosteric ligand on the h sst_4. Such an allosteric interaction has been proposed for a compound designated ASWL, which has been described as a superagonist of the chemokine receptor CXCR4 (Sachpatzidis et al., 2003). Sachpatzidis et al. (2003) have suggested that the superagonism of ASWL might stem from its inability to induce receptor internalization, which may lead to continuous receptor activation. Such a differential propensity of J-2156 and SRIF to cause receptor internalization does not appear likely as an explanation for the superagonism of J-2156 since a recent publication has reported that the extent of h sst_4 internalization in CHO cells upon stimulation with SRIF-14 was so small that it could not be observed by confocal microscopy (Smalley et al., 2001). Furthermore, the superagonism of J-2156 is detected in membrane preparations, in which receptor internalization is not expected to play a role. This same reason also argues against a lack of desensitization by J-2156 compared with SRIF-14. Although SRIF-14 has been described to desensitize the h sst_4 receptor in intact CHO cells (Smalley et al., 1998), we did not observe a reduction in the maximal response of J-2156 in the presence of increasing concentrations of SRIF-14. This, however, is what would be expected to occur if SRIF-14 were to desensitize the h sst_4 in membranes. Maybe SRIF-14 and J-2156 stabilize distinct receptor conformations of the h sst_4 that differ significantly in their ability to interact productively with G-proteins. This has been suggested as a possible mechanistic difference between partial and full GPCR agonists (Kenakin, 1996a,b; Tucek, 1997; Seifert et al., 2001). The use of subtype-specific G-protein antisera would allow one to study which G-protein α-subunits physically associates with sst_4 and whether the extent of interaction between the receptor and a specific G-protein is agonist-dependent. In previous studies, it has been shown by immunoblotting that $G\alpha_{i1}$, $G\alpha_o$, and $G\alpha_z$ are not present at detectable levels in CHO-K1 membranes and that $G\alpha_{i2}$ and $G\alpha_{q/11}$ are present at much higher levels than $G\alpha_{i3}$ (Dell'Acqua et al., 1993; Gettys et al., 1994; Gu and Schonbrunn, 1997). Pertussis toxin completely abolishes the [^{35}S]GTPγS binding stimulated by SRIF-14 or J-2156, indicating that sst_4 couples to the $G\alpha_{i/o}$ class of G proteins in CHO-K1 cells. Taken into account the differential expression profiles of the G-protein α-subunits in CHO-K1 cells, the agonist-mediated increase of [^{35}S]GTPγS binding in membranes of CHO-h sst_4 cells most likely involves $G\alpha_{i2}$ and/or $G\alpha_{I3}$. Furthermore, one could hypothesize that the binding domains of peptides and small molecule agonists may differ to a sufficiently large degree as to give rise to the observed differences in agonist efficacies. To explore this possibility further, site-directed mutagenesis and molecular modeling will be needed to pinpoint the critical residues in the h sst_4 involved in the binding of the endogenous peptides and J-2156.

In conclusion, we describe a novel compound, J-2156, that belongs to a new class of somatostatin receptor ligands, i.e., sulfonamido-peptidomimetics. J-2156 possesses high affinity and selectivity for the h sst_4 over any of the other four human somatostatin receptor subtypes. Although the affinity of J-2156 at the h sst_4 is comparable with that of SRIF-14 and SRIF-28, its functional properties are unique in demonstrating superagonism at the h sst_4.

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