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Somatostatin$_5$ Receptor-Mediated [^{35}S]Guanosine-5′-*O*-(3-thio)triphosphate Binding: Agonist Potencies and the Influence of Sodium Chloride on Intrinsic Activity

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SUMMARY

We studied the activation of the human somatostatin$_5$ receptor recombinantly expressed in CHO-K1 cells by using some newly available agonists and antagonists. Somatostatin-28 bound to this receptor with a higher affinity than somatostatin-14 and was more potent in increasing [^{35}S]guanosine-5′-*O*-(3-thio)-triphosphate ([^{35}S]GTPγS) binding. Somatostatin-14-induced [^{35}S]GTPγS binding to membranes from this cell line was decreased in a concentration-related manner by increasing concentrations of GDP and sodium chloride. At 50 mM (low) sodium, agonist EC_{50} values for stimulating [^{35}S]GTPγS binding were lower than those at 150 mM (high) sodium and were closer to their respective affinity estimates (dissociation equilibrium constants) for binding to the receptor in the absence of sodium. Both agonist binding to the high affinity state of the receptor and agonist-induced [^{35}S]GTPγS binding were abolished by pertussis toxin pretreatment. The putative somatostatin$_5$ receptor-selective ligand L-362,855, unlike somatostatin-14 and somatostatin-28, showed differential intrinsic activity for stimulation of [^{35}S]GTPγS binding, behaving as a partial agonist in high sodium and a full agonist in low sodium. In contrast, BIM-23056 did not behave as an agonist under any conditions studied but was able to antagonize somatostatin-14-induced [^{35}S]GTPγS binding. We conclude that measurement of [^{35}S]GTPγS binding mediated by somatostatin receptor activation in the presence of different concentrations of sodium chloride provides a useful functional assay for assessing the relative agonist efficacies of novel ligands identified from radioligand binding studies.

Somatostatin-14 is a biologically active tetradecapeptide (1–3). To date, five distinct members of the somatostatin receptor family have been identified, and all are putative seven-transmembrane G protein-coupled receptors (4–8). The human and rat isoforms of sst$_5$ are the only somatostatin receptors claimed to show preferential affinity for somatostatin-28 compared with somatostatin-14 (8, 9). Somatostatin receptors have been demonstrated to couple to a multitude of transduction mechanisms, such as activation of potassium currents, inhibition of calcium currents, stimulation of phosphoinositide turnover, and interaction with protein phosphatase cascades, in addition to the inhibition of adenylate cyclase (10–14). The sst$_5$ receptor, like the other recombinant somatostatin receptor types, negatively couples to adenylate cyclase when recombinantly expressed in cell lines (8, 14–17). The human sst$_5$ receptor has also been shown to mediate activation of phosphoinositide metabolism and the accumulation of intracellular calcium (12, 18). Furthermore, there is evidence that this somatostatin receptor type can couple to both pertussis toxin-sensitive and -insensitive G proteins (12, 19, 20).

The aim of this work was to study the ability of a number of agonists to stimulate the binding of [^{35}S]GTPγS to G proteins, mediated by the activation of the human recombinant sst$_5$ expressed in CHO-K1 cells. This technique relies on the receptor-stimulated exchange of GDP for GTP (or [^{35}S]GTPγS) and therefore provides a quantitative measure of signal transduction close to the level of receptor activation. [^{35}S]GTPγS binding has already been used in the study of other G protein-coupled receptors, such as human muscarinic receptors (21, 22) and adenosine A_1 receptors (23), to provide quantitative profiles of receptor/G protein interactions. Although sst$_5$ receptor-mediated decreases in cAMP levels and increases in inositol-1,4,5-trisphosphate production are both pertussis toxin sensitive, they are not necessarily mediated by the same pertussis toxin-sensitive G protein (12). The study of the initial step of the transduction pathway should provide a gross measure of receptor/G protein activation, independent of whether the activated G proteins form a heterogeneous or homogenous population. This approach has provided an opportunity to explore the pharmacology of the newly available, reportedly sst$_5$ receptor-selective ligands, L-362,855 and BIM-23056 (18, 19).

ABBREVIATIONS: sst$_5$, somatostatin$_5$; CHO, Chinese hamster ovary; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Experimental Procedures

Materials. [^{35}S]GTPγS was purchased from DuPont (1000–1500 Ci/mmol; Bad Homburg, Germany). [^{125}I]-Tyr^{11}-somatostatin-14 (2000 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). *Bordetella pertussis* toxin was purchased from Calbiochem (San Diego, CA). Bacitracin and phenylmethylsulfonyl fluoride were supplied by Sigma Chemical (Poole, Dorset, UK). Cell culture products were purchased from Life Technologies (Paisley, UK). Somatostatin-14 and somatostatin-28 were purchased from Peninsula Laboratories Europe (Merseyside, UK). BIM-23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH$_2$), BIM-23027 (c[*N*-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe]), and L-362,855 (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe], where Nal is β-(2-naphthyl)alanine, Abu is aminobutyric acid, and Aha is 7-amino-heptanoic acid) were custom-synthesized by Peptide and Protein Research Consultants (University of Exeter, Exeter, UK). All of the peptides, with the exception of BIM-23056, were initially dissolved in distilled water to produce a concentration of 1 mM and then divided into aliquots and stored frozen. BIM-23056 was initially dissolved in 10% dimethylsulfoxide.

Cell culture and membrane preparation. Production and culturing conditions of a CHO-K1 cell line stably expressing the human sst$_5$ receptor (CHOsst$_5$) have been previously described (18). To prepare a membrane fraction, cells were scraped into ice-cold phosphate-buffered saline and centrifuged for 20 min at 4° at 250 × *g*. The pellet was resuspended in a 10 mM HEPES buffer, pH 7.4, at 4° containing 0.1 mM EDTA, 0.1 mg/ml bacitracin, and 0.1 mM phenylmethylsulfonyl fluoride and homogenized in a Dounce ground glass homogenizer (20 strokes). After centrifugation for 30 min at 4° at 10,000 × *g*, the resultant membrane pellet was resuspended in the above buffer and stored at −80°.

[^{125}I]-Tyr^{11}-somatostatin-14 binding assays. To determine ligand affinities, ~2 μg of membrane protein was incubated with 0.03 nM [^{125}I]-Tyr^{11}-somatostatin-14 and a range of competing ligand concentrations in a 10 mM HEPES buffer, pH 7.4, containing 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 mg/ml bacitracin for 120 min at 21°. Reactions were terminated by vacuum filtration onto 0.5% polyethylenimine-pretreated filters using a Brandell cell harvester, and radioactivity bound was quantified using a Cobra II γ-counter. Total [^{125}I]-Tyr^{11}-somatostatin-14 bound to the membranes was ~3000 dpm, with nonspecific binding of ~500 dpm, as defined with 1 μM somatostatin-14.

[^{35}S]GTPγS binding assays. For studies of [^{35}S]GTPγS binding, 2–6 μg of membrane protein was incubated for 120 min at 21° in a 10 mM HEPES buffer, pH 7.4, containing 50 or 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.3 μM GDP with ligand, unless indicated otherwise. The protein concentration was the same within each set of experiments. To identify optimal conditions, experiments were performed to evaluate the effects of NaCl and GDP on [^{35}S]GTPγS binding. Condition optimization studies were performed in 100 mM NaCl, 10 mM $MgCl_2$, and 1 μM GDP, except where one of these was under analysis, as indicated in the figure legend for the particular experiment. Steady state receptor occupation was achieved after 80–120 min in studies of [^{125}I]-Tyr^{11}-somatostatin-14 binding to the receptor. For this reason, in studies of [^{35}S]GTPγS binding, ligands were preincubated with membranes for 90 min before the addition of 0.2 nM [^{35}S]GTPγS to ensure the maximal possible agonist activation of the receptor. Under optimized conditions (0.3 μM GDP and 5 mM $MgCl_2$) and 150 mM NaCl and after preincubation, somatostatin-14-induced [^{35}S]GTPγS binding was linear over the initial 50 min. A 30-min incubation period was routinely used to quantify the agonist-activated [^{35}S]GTPγS binding. Reactions were terminated by vacuum filtration using a Packard Filtermate harvester. The filters were dried, and the amount of radioactivity bound was determined after the addition of 50 μl of Microscint-O (Packard) scintillation fluid by counting with a Canberra Packard Topcount Scintillation Counter. In 150 mM NaCl, this protocol resulted in basal [^{35}S]GTPγS binding of ~2000 dpm, rising to 4000 dpm after incubation with 10 μM somatostatin-14. Nonspecific binding of [^{35}S]GTPγS defined with 10 μM GTPγS was ~100 dpm. This represented 3.8 ± 0.6% (n = 4) and 2.2 ± 0.2% (n = 4) of the total binding observed in the presence of 10 μM somatostatin-14 in 150 and 50 mM NaCl, respectively. Nonspecific binding was not subtracted from experimental data as it represented such a relatively small percentage of the overall binding, so all values represent the total levels of [^{35}S]GTPγS binding that were observed.

Pertussis toxin treatment. Cells were pretreated with 100 ng/ml pertussis toxin for 18 hr before the cells were harvested for the production of a membrane fraction.

Data analysis. Competition binding data were analyzed by nonlinear least-squares regression using Prism (GraphPAD Software, San Diego, CA). The fitting of competition curves for [^{125}I]-Tyr^{11}-somatostatin-14 binding to membranes from CHOsst$_5$ cells by somatostatin-14 and the other ligands generated half-maximal inhibitory concentrations (IC_{50} values). Hill slopes were close to unity, in most cases not significantly different from 1, and were constrained to unity for calculation purposes. To calculate K_D and B_{max} values from competition studies, the following equations were used: $K_D = IC_{50} - [A]$, where K_D is the equilibrium dissociation constant of the radioligand, IC_{50} is the half-maximal inhibitory concentration of somatostatin-14, and [A] is the concentration of [^{125}I]-Tyr^{11}-somatostatin-14 present in the assay medium (0.03 nM); and $B_{max} = ([B] \times IC_{50})/[A]$, where B_{max} is the receptor density, and [B] is the concentration of specific [^{125}I]-Tyr^{11}-somatostatin-14 bound to the receptor in the absence of competing ligand.

For the other ligands, each dissociation constant (K_i) was calculated by substitution directly into the Cheng-Prusoff equation: $K_i = (IC_{50})/\{1 + [B]/K_D)\}$, where IC_{50} is the half-maximal concentration of the ligand for displacement of [^{125}I]-Tyr^{11}-somatostatin-14 binding to the receptor.

The pEC_{50} values for increasing [^{35}S]GTPγS binding were calculated as the negative $\log_{10}$ of the molar concentration of the agonist producing 50% of the maximal response for that agonist. Similarly, pIC_{50} values were calculated for the inhibitory effects of various components of the GTPγS assay incubation medium on [^{125}I]-Tyr^{11}-somatostatin-14 binding. The pK_B estimates (negative logarithm of the estimated dissociation equilibrium constant) were calculated from agonist concentration ratios determined at each antagonist concentration from the Gaddum-Schild equation (24). If such estimates are not significantly different for different antagonist concentrations, the antagonism is consistent with, but not definitive proof of, competitive antagonism (24).

Estimates of the equilibrium dissociation constant (K_P) for the partial agonist, L-362,855, were determined using the Black-Leff operational model (25). Each pair of concentration-effect curves for somatostatin-14, one in the absence and one in the presence of a given concentration of L-362,855, was fitted simultaneously to the following logistic equation:

$$E = \frac{E_m([A]K_P + \tau_P[P][EC_{50}])^n}{[EC_{50}]^n(K_P + [P])^n + ([A]K_P + \tau_P[P][EC_{50}])^n}$$

where [A] is the concentration of somatostatin-14, and [P] is the concentration of L-362,855, used to calculate the shared parameters of K_P, EC_{50}, E_m, n, and τ_P, an efficacy parameter for L-362,855. The EC_{50} value is a fitted estimate of the concentration of somatostatin-14 required to produce 50% of its own maximum effect in the absence of the partial agonist. The theoretical maximum achievable effect, E_m, is also obtained from the computer-generated fit. The slope of the receptor occupancy-effect relationship is defined by n, and τ_p is defined as the total number of receptors divided by the concentration of agonist/receptor complex necessary to produce a half-maximal response. The data were fitted using the program Uridian (Torac, Harlow, Essex, UK). The derived estimates for each parameter were mean values for all data, and the K_P and EC_{50}

values were expressed as their negative logarithms (pK_P and pEC_{50}, respectively).

Values are given as mean ± standard error from n experiments, and the differences were tested at the 5% level of significance using the Student's t test. Absolute levels of [^{35}S]GTPγS binding are also expressed in pmol of [^{35}S]GTPγS bound/mg of protein (pmol/mg). Where appropriate, the 95% confidence limits are given.

Results

Binding of somatostatin-14 and its analogues to the sst$_5$ receptor. Somatostatin-14 displacement of [^{125}I]-Tyr^{11}-somatostatin-14 from its binding sites in membranes prepared from the CHOsst$_5$ cells gave an IC_{50} value for somatostatin-14 of 0.21 ± 0.037 nM with an estimated B_{max} value of 3.01 ± 0.3 pmol/mg (n = 3). The receptor density remained constant over the time course of the study, with the corresponding B_{max} value being 2.91 ± 0.93 pmol/mg (n = 3) at the end of the series of experiments. No specific binding was detected in untransfected CHO-K1 cells (data not shown). Affinity estimates for a number of somatostatin-14 analogues in membranes from CHOsst$_5$ cells were determined from their abilities to compete with [^{125}I]-Tyr^{11}-somatostatin-14 for binding to the receptor. Their binding profiles are illustrated in Fig. 1, and the calculated parameter values are presented in Table 1. The data were fitted in each case to a one-site sigmoidal binding curve, although individual curves of L-362,855, BIM-23027, and BIM-23056 competition for [^{125}I]-Tyr^{11}-somatostatin-14 binding fitted better to a two-site model in a few cases. Somatostatin-28, somatostatin-14, and L-362,855 all bound with high affinity, in the subnanomolar range, with somatostatin-28 displaying an ~4-fold higher affinity than somatostatin-14 (K_i = 0.046 and 0.18 nM, respectively; Table 1). The following rank order of ligand affinity estimates (pK_i values) was observed: somatostatin-28 (10.34) ≥ L-362,855 (10.09) ≥ somatostatin-14 (9.74) > BIM-23056 (8.61) > BIM-23027 (7.89).

Conditions for [^{35}S]GTPγS binding. Suitable conditions for somatostatin-14-induced increases in [^{35}S]GTPγS binding to CHOsst$_5$ membranes were sought by attempting to maximize specific stimulation while reducing basal [^{35}S]GTPγS binding. When GDP concentrations were increased from 0.1 nM to 0.3 mM, basal levels of binding were decreased with little effect on stimulated binding (Fig. 2). Optimal increases in somatostatin-14-induced binding of [^{35}S]GTPγS were observed with 0.3 μM GDP due to the markedly decreased levels of basal binding. Hence, this concentration was used in further studies unless otherwise stated. In the presence of 0.3 μM GDP, basal [^{35}S]GTPγS binding was 41.3 ± 4.9%, and somatostatin-14-stimulated (1 μM) levels were 59.6 ± 7.3% of that in the absence of GDP.

Sodium chloride decreased specific [^{35}S]GTPγS binding with an IC_{50} value of 130 mM (95% confidence limits, 105–160 mM; n = 3) and a Hill slope of 3.8 ± 1.2; at sodium concentrations of >200 mM, somatostatin-14-stimulated G protein activation was not evident (Fig. 3). At 150 mM NaCl, the inhibition of somatostatin-14-stimulated (1 μM) [^{35}S]GTPγS binding was approximately half-maximal, whereas at 50 mM NaCl, maximal levels of specific [^{35}S]GTPγS binding were observed (Fig. 3B). Because sodium also reduced basal [^{35}S]GTPγS binding (Fig. 3A), somatostatin-14-stimulated (1 μM) binding did not differ greatly between 150 and 50 mM sodium chloride when expressed as a percentage of basal levels (130.0 ± 2% compared with 149.6 ± 4.4% of the different basal values; n = 3).

Optimal agonist-stimulated [^{35}S]GTPγS binding was observed at 5 mM magnesium chloride (data not shown), and this concentration was subsequently used in all agonist-dependent experiments.

Agonist potencies for stimulation of [^{35}S]GTPγS binding in low and high sodium. The potency estimates and relative maximal effects of the peptide analogues studied are summarized in Table 1. All data were normalized to the maximal binding observed in 150 mM NaCl, which was produced by 10 μM somatostatin-28 because experiments in 50 and 150 mM NaCl were carried out simultaneously (Fig. 4). [^{35}S]GTPγS binding in 150 mM NaCl and in the absence of any ligand was 47.8 ± 7.3% (n = 4), and in 50 mM NaCl, basal

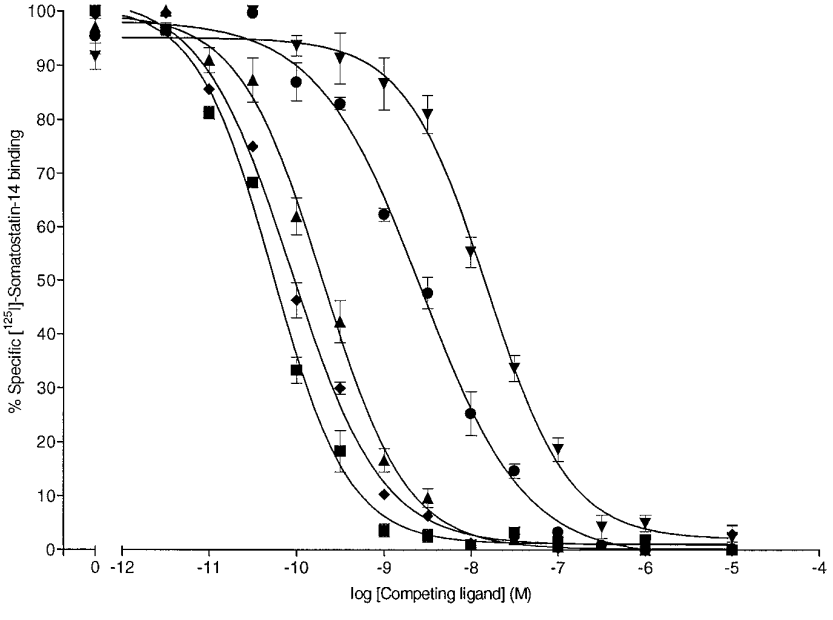

Fig. 1. Displacement of binding of [^{125}I]-Tyr^{11}-somatostatin-14 (0.03 nM) from membranes of CHOsst$_5$ cells by somatostatin-14 (▲), somatostatin-28 (■), L-362,855 (♦), BIM-23056 (●), and BIM-23027 (▼). Assays were incubated at 21° until equilibrium was attained (2 hr) and were performed in the absence of sodium chloride (see Experimental Procedures for details regarding the buffer). Data are the mean ± standard error from three separate experiments performed in duplicate and are expressed as a percentage of specific [^{125}I]-Tyr^{11}-somatostatin-14 binding.

TABLE 1

Estimates of binding affinities and agonist potencies for stimulating [^{35}S]GTPγS binding by somatostatin sst$_5$ receptor activation

Ligand affinity estimates (dissociation equilibrium constants, K_i values) are compared for membranes prepared from CHOsst$_5$ cells with potencies (EC_{50}) and maximal effects for stimulating [^{35}S]GTPγS binding in the presence of 5 mM magnesium chloride, 0.3 μM GDP, and 50 or 150 mM sodium chloride. Hill slopes (n_H) are also shown. The maximal [^{35}S]GTPγS binding response is expressed as a percentage of the response observed with somatostatin-28 (10 μM) in 150 mM NaCl. Values are mean ± standard error of nonlinear regression curves fitted to the experimental data (n = three to five). SRIF-28 and SRIF-14 refer to somatostatin-28 and somatostatin-14, respectively.

	K_i	n_H	GTPγS (50 mM NaCl)			GTPγS (150 mM NaCl)		
			EC_{50}	n_H	Maximal response	EC_{50}	n_H	Maximal response
	nM		nM			nM		
SRIF-28	0.046 ± 0.01	1.00 ± 0.06	5.9 ± 2.8	0.96 ± 0.4	161 ± 4	52.7 ± 14	0.76 ± 0.2	102 ± 4
SRIF-14	0.18 ± 0.04	0.91 ± 0.06	29.6 ± 19	0.91 ± 0.4	158 ± 5	125.7 ± 34	0.89 ± 0.3	91 ± 5
L-362,855	0.082 ± 0.0	0.84 ± 0.04	9.4 ± 4.7	0.96 ± 0.4	152 ± 4	16.9 ± 8.7	1.32 ± 1.5	62 ± 3[a]
BIM-23056	2.4 ± 0.4	0.73 ± 0.05						
BIM-23027	12.9 ± 1.0	0.92 ± 0.09	>1000			>1000		

[a] Statistically different from the equivalent values for somatostatin-28 and somatostatin-14 using Student's unpaired t test (p = 0.05).

[^{35}S]GTPγS binding was 98.94 ± 11.86% (n = 4). In high and low sodium, both somatostatin-14 and somatostatin-28 concentration-dependently increased [^{35}S]GTPγS binding to give fitted curve maxima of 91 ± 4.5% and 102 ± 4.1% (high sodium) and 158 ± 5.04% and 161 ± 3.66% (low sodium), respectively (Fig. 4). Thus, somatostatin-28 (10 μM) increased [^{35}S]GTPγS binding by 0.228 ± 0.027 pmol/mg in 150 mM NaCl and 0.204 ± 0.055 pmol/mg in 50 mM NaCl. The potency estimates (pEC_{50} values) for somatostatin-14 were 6.90 in high sodium and 7.52 in low sodium, reflecting a 5-fold greater potency with reduced sodium; somatostatin-28 was 9-fold more potent in low sodium than in higher sodium (Table 1). BIM-23027 was relatively weak at stimulating [^{35}S]GTPγS binding, causing an observable effect only at concentrations of >1 μM, in both low and high sodium (Table 1).

Antagonist effects of L-362,855 and BIM-23056. In both 50 and 150 mM NaCl, L-362,855 stimulated [^{35}S]GTPγS binding with high potency (pEC_{50} = 8.03 and 7.77, n = 4, respectively; for corresponding EC_{50} values, see Table 1). Although potent at stimulating [^{35}S]GTPγS binding in the presence of 150 mM NaCl, L-362,855 stimulation of [^{35}S]GTPγS binding reached a plateau at 30 nM, with a significantly lower curve maximum than those of somatostatin-14 and somatostatin-28 (unpaired t test, n = 4; see Table 1). When L-362,855, at concentrations of >30 nM, was coincubated with somatostatin-14, it was found to surmountably antagonize the responses to somatostatin-14 in a concentration-dependent manner (Fig. 5A). Fitting the data to the Black-Leff model provided an estimated pK_P value for L-362,855 of 7.44 ± 0.11, computed E_m value of 101.9 ± 0.8, n value of 0.75 ± 0.03, and τ_P value of 0.81 ± 0.1 (n = 11). In low sodium, the maximum of L-362,855 increased so it no longer significantly differed from those of somatostatin-14 and somatostatin-28 (unpaired t test, n = 4; see Table 1). In contrast to somatostatin-14 and somatostatin-28, there was no difference between the pEC_{50} values for L-362,855 in low and high sodium (unpaired t test, n = 4; see Table 1).

BIM-23056 was inactive in stimulating [^{35}S]GTPγS binding at concentrations of ≤1 μM, but at 10 μM in both high and low sodium, it stimulated [^{35}S]GTPγS binding in excess of that of any other compound tested (286.07 ± 50.8% of the maximal 10 μM SRIF-28 response in 150 mM sodium, n = 4). This effect was not seen with the vehicle but was observed in the absence of membranes. For this reason, the BIM-23056 responses at 10 μM have been omitted from the graphs, and BIM-23056 was not used as an antagonist at concentrations of >1 μM. The ability of BIM-23056 to antagonize responses to somatostatin-14 was investigated at 150 mM sodium chloride (Fig. 5B). The somatostatin-14 concentration-effect curve was shifted to the right with increasing concentrations of BIM-23056 (0.057, 0.24, and 1 μM), with agonist concentration ratios of 2.7 ± 0.6, 6.1 ± 0.7, and 33.5 ± 22.2, n = 4 for each, respectively. Analysis of these data using the Gaddum-Schild equation gave mean antagonist pK_B estimates of 7.29 ± 0.27, 7.30 ± 0.06, and 7.26 ± 0.25, respectively, which were not significantly different from each other. This allowed an average mean pK_B value for BIM-23056 of 7.28 ± 0.11 (n = 12) to be calculated.

Comparison of agonist affinity estimates with potencies for stimulating [^{35}S]GTPγS binding. The different conditions used to determine agonist binding and functional measurements would be expected to influence the affinity estimates of the various ligands. To address this issue, attempts were made to perform both assays under similar conditions. Reducing the sodium content of [^{35}S]GTPγS binding assays to only 5 mM resulted in very high basal [^{35}S]GTPγS binding, above which agonist-stimulated [^{35}S]GTPγS binding was not readily discernible (data not shown, n = 5). Therefore, the effects were examined of varying components of the GTPγS assay on specific [^{125}I]-Tyr^{11}-somatostatin-14 binding to the sst$_5$ receptor. The addition of GTPγS, GDP, or NaCl reduced specific binding of [^{125}I]-Tyr^{11}-somatostatin-14 (0.03 nM) to CHOsst$_5$ membranes: 150 mM NaCl by 59.5 ± 1.5%, 0.1 nM GTPγS by 15.4 ± 0.8%, and 0.3 μM GDP by 41.4 ± 10.9% (n = 3). All three components together reduced specific [^{125}I]-Tyr^{11}-somatostatin-14 binding by 83.2 ± 3.5% (n = 3). The effects of GTPγS and GDP were concentration dependent, with pEC_{50} values of 9.00 ± 0.09 and 6.92 ± 0.40 and Hill slopes of 0.60 ± 0.07 and 0.51 ± 0.21, respectively (n = 3). Thus, under the conditions used for measurement of agonist-stimulated [^{35}S]GTPγS binding, it was not possible to conduct filtration binding assays using [^{125}I]-Tyr^{11}-somatostatin-14.

Pertussis toxin sensitivity. An 18-hr pretreatment with 100 ng/ml pertussis toxin abolished [^{125}I]-Tyr^{11}-somatostatin-14 high affinity binding to the receptor. Somatostatin-14 (1 μM) stimulation of [^{35}S]GTPγS binding was also profoundly reduced to only 3.4 ± 2% and 4.2 ± 1% of the stimulated binding in non-pertussis toxin-pretreated mem-

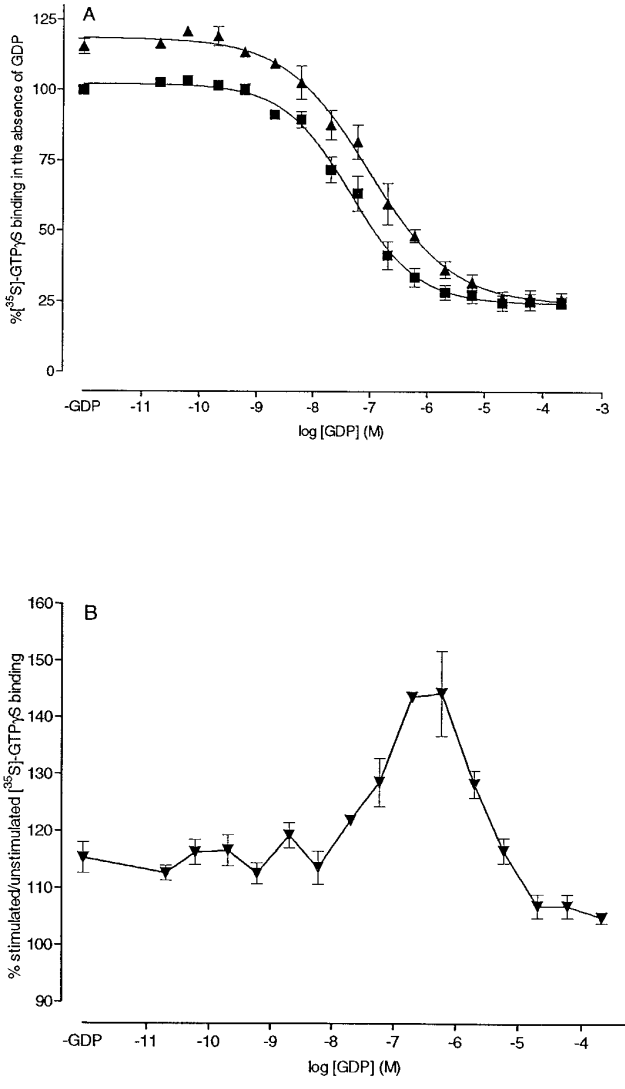

Fig. 2. A, Effect of GDP concentration on binding of [^{35}S]GTPγS (0.2 nM) to membranes from CHOsst$_5$ cells in the absence (■) and presence (▲) of 1 μM somatostatin-14. Data are the mean ± standard error of the percentage of unstimulated [^{35}S]GTPγS binding in the absence of GDP. B, Effect of GDP on specific somatostatin-14-stimulated [^{35}S]GTPγS binding (▼). Data are the mean ± standard error and are expressed as a percentage of basal [^{35}S]GTPγS binding at each GDP concentration. Assays were carried out in triplicate in three separate experiments in 100 mM NaCl and 10 mM $MgCl_2$. Basal levels of 1.51 ± 0.25 pmol/mg were observed in the absence of GDP, rising to 1.71 ± 0.27 pmol/mg with somatostatin-14.

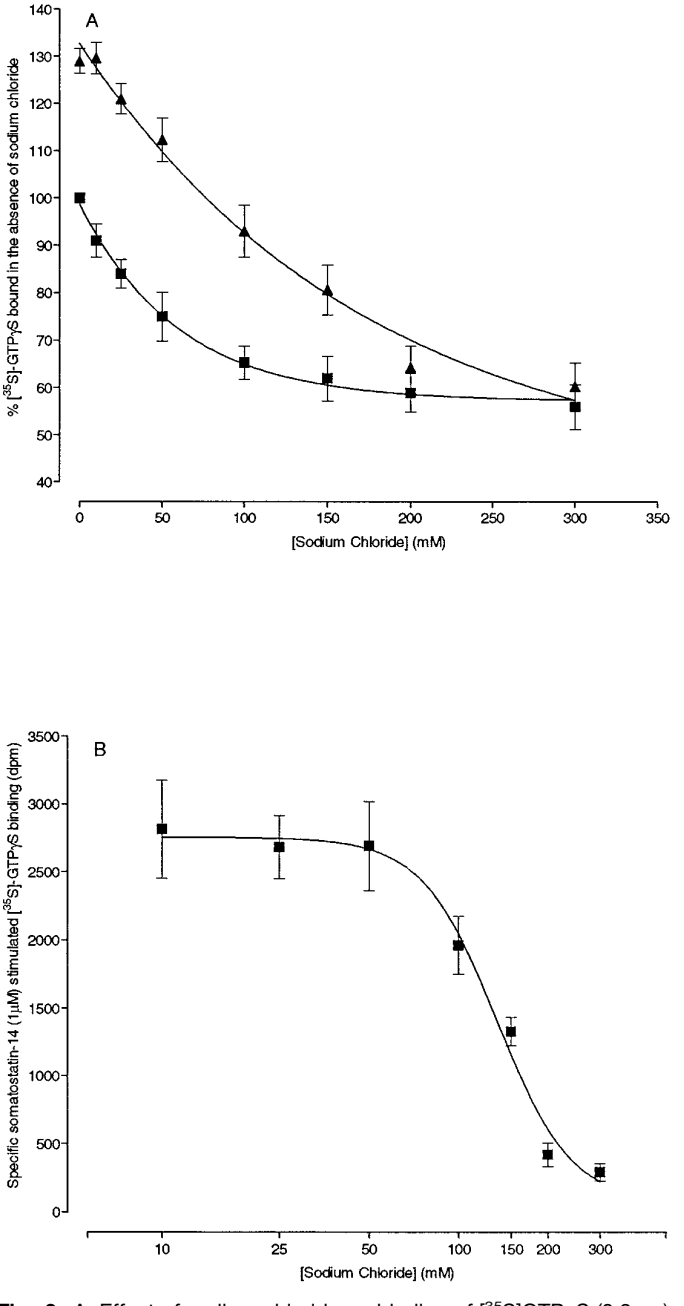

Fig. 3. A, Effect of sodium chloride on binding of [^{35}S]GTPγS (0.2 nM) to membranes from CHOsst$_5$ cells in the absence (■) and presence (▲) of 1 μM somatostatin-14. Data are the mean ± standard error and are expressed as a percentage of [^{35}S]GTPγS binding in the absence of sodium chloride. B, Effect of sodium chloride on specific 1 μM somatostatin-14 stimulated [^{35}S]GTPγS binding. Data are the mean ± standard error. Assays were carried out in triplicate in three separate experiments in 1 μM GDP and 10 mM $MgCl_2$. In the absence of sodium chloride, basal levels of 0.72 pmol/mg were measured, increasing to 1.02 ± 0.36 pmol/mg in the presence of somatostatin-14.

branes in 50 and 150 mM NaCl, respectively. In comparison with untreated controls, reductions in the basal levels of [^{35}S]GTPγS binding were also observed in membranes prepared from both pertussis toxin-pretreated CHOsst$_5$ and pertussis toxin-pretreated wild-type CHO-K1 cells. Basal [^{35}S]GTPγS binding was reduced by the same extent by pertussis toxin pretreatment in CHOsst$_5$ and wild-type cells (62.8±5.3% and 75.3 ± 3.7%, and 20.0 ± 14.8% and 22.8 ± 12.1%) in the absence and presence of 150 mM NaCl, respectively (n = 4–5).

Discussion

The purpose of this study was to investigate agonist-stimulated [^{35}S]GTPγS binding mediated by the human sst$_5$ receptor and compare the functional characteristics of some newly available, purportedly receptor-selective ligands. In addition, determination of agonist affinity estimates for the receptor allows comparison with equivalent estimates from functional studies. Because the literature contains some discrepancies in the absolute potencies of ligands for binding to the recombinant sst$_5$ receptor (see below), it was necessary to determine agonist affinity estimates for the receptor by ra-

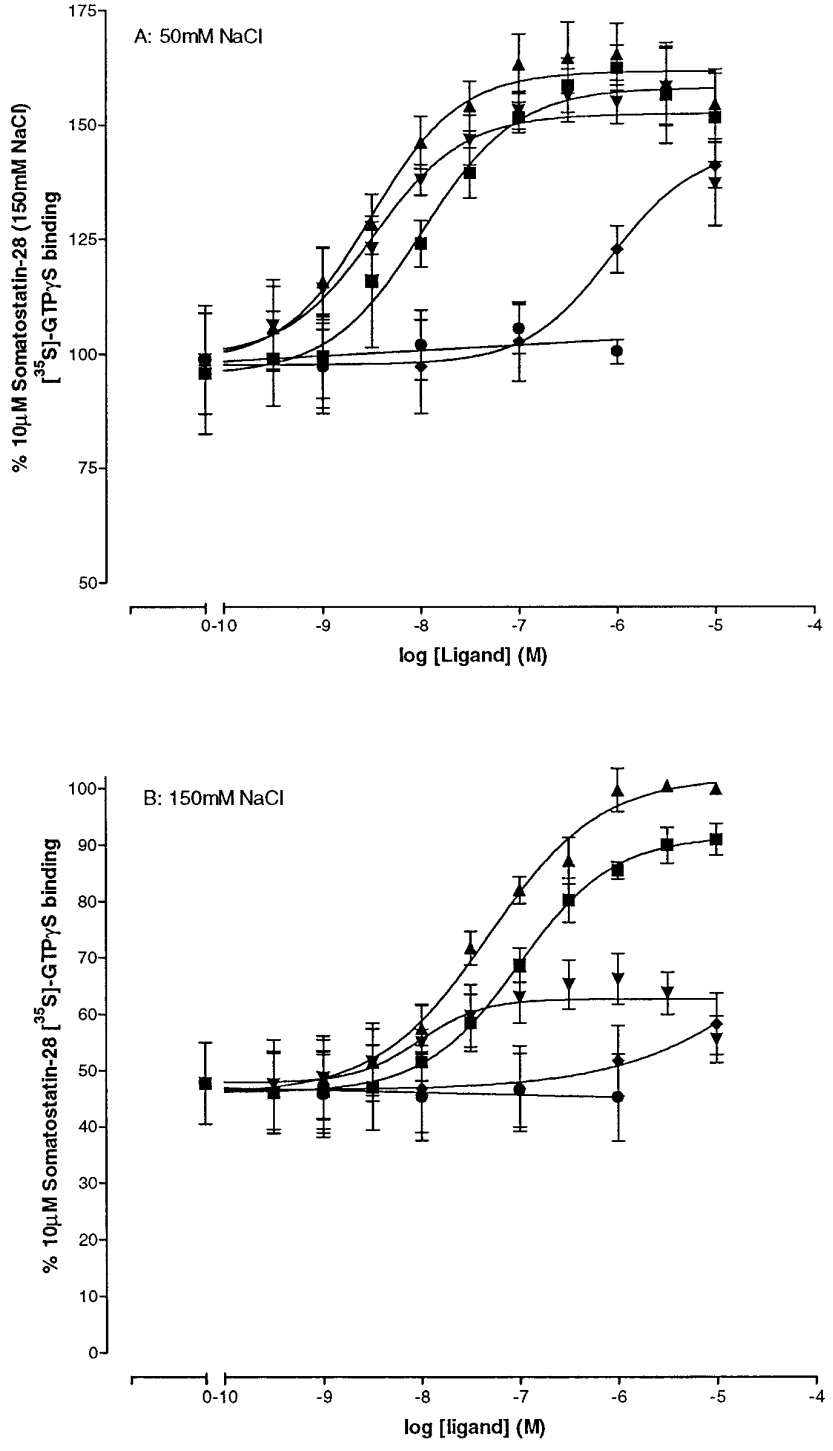

Fig. 4. Stimulation of [^{35}S]GTPγS binding in membranes from CHOsst$_5$ cells by various concentrations of somatostatin-14 (■), somatostatin-28 (▲), L-362,855 (▼), BIM-23056 (●), and BIM-23027 (♦) in the presence of 5 mM magnesium chloride, 0.3 μM GDP, and 50 mM sodium chloride (A) or 150 mM sodium chloride (B). Data are the mean ± standard error from four separate experiments performed in duplicate, in which 100% [^{35}S]GTPγS binding was the maximum response observed with 10 μM somatostatin-28 at 150 mM sodium chloride. For potency estimates, see Table 1. At 50 mM NaCl, basal binding was 0.51 ± 0.07 pmol/mg, and the net agonist effects at 10 μM were 0.20 ± 0.07, 0.20 ± 0.06, and 0.12 ± 0.09 pmol/mg for somatostatin-14, somatostatin-28, and L-362,855, respectively. At 150 mM NaCl, basal binding was 0.23 ± 0.04 pmol/mg, and 10 μM somatostatin-14, somatostatin-28, or L-362,855 produced 0.19 ± 0.04, 0.23 ± 0.03, or 0.03 ± 0.03 pmol/mg increases above basal levels, respectively.

dioligand binding in the same CHOsst$_5$ cells used in this study.

Somatostatin-28 bound to the sst$_5$ receptor with a greater affinity than somatostatin-14, with K_i values of 0.046 and 0.182 nM, respectively, confirming the preferential affinity of this receptor for somatostatin-28 (see introduction). Somatostatin-28 and somatostatin-14 bound to the human sst$_5$ with affinities very similar to those observed by O'Carroll *et al.* (17), who reported IC$_{50}$ values of 0.05 and 0.16 nM for somatostatin-28 and somatostatin-14, respectively. Similarly, the estimated dissociation constant for somatostatin-28 reported by Patel and Srikant (26) at the human sst$_5$ receptor (K_i = 0.07 nM) is very close to our finding. However, these authors (26), like Panetta *et al.* (16), found the affinity of somatostatin-14 to be lower, with K_D values of 0.9 and 2.24 nM, respectively (16). With regard to the somatostatin analogues, our results show the affinity of L-362,855, the putative sst$_5$-selective compound for the human sst$_5$ receptor, to be high (K_i = 0.082 nM), although somewhat less than previously reported (IC$_{50}$ = 0.016 nM; Ref. 17). The reportedly sst$_2$ and sst$_3$ receptor-selective compounds, BIM-23027 and BIM-23056, respectively, bound with lower affinity to this

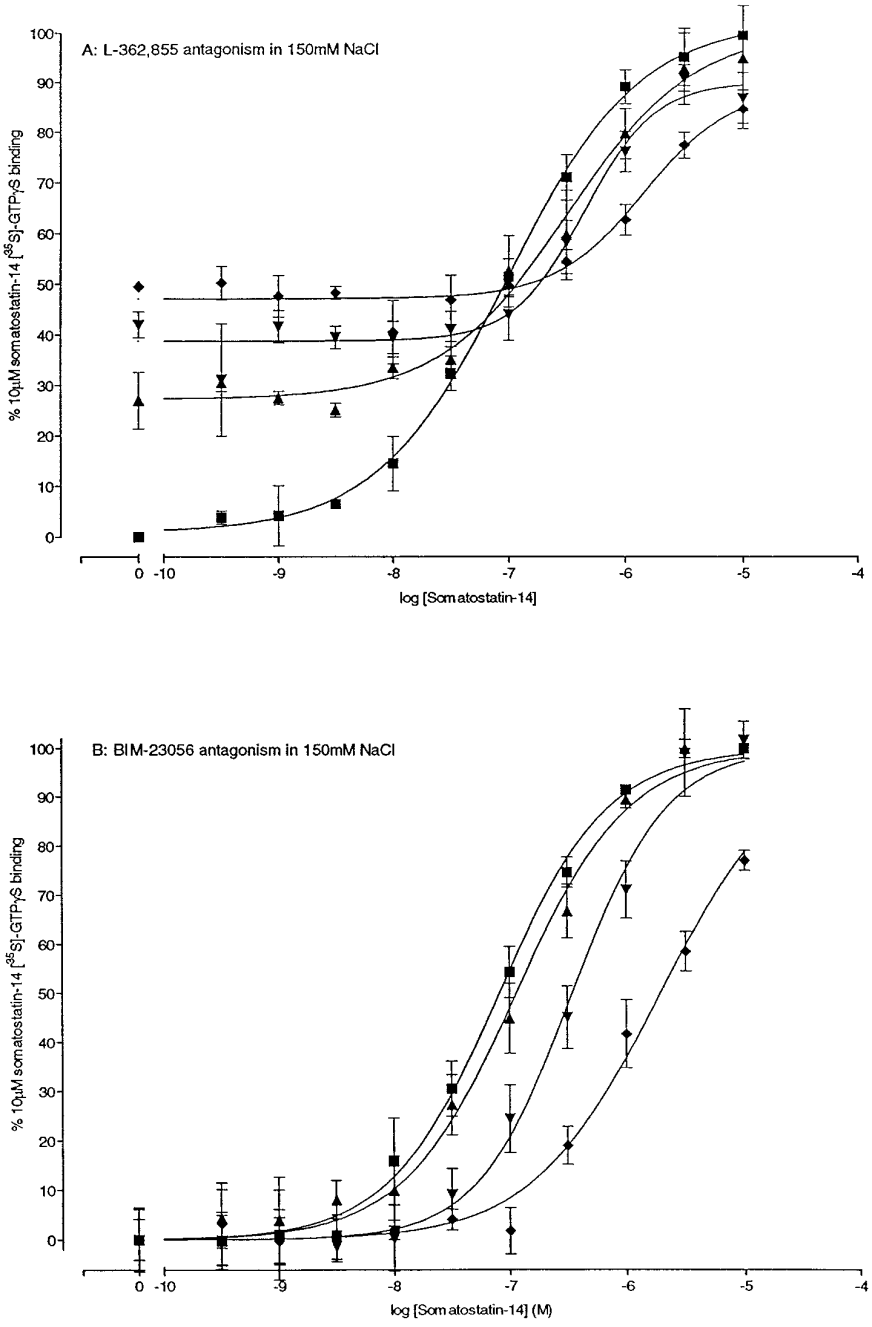

Fig. 5. A, Inhibition of somatostatin-14 stimulated [^{35}S]GTPγS binding was measured in the absence (■) or presence of increasing concentrations of L-362,855, 0.03 μM (▲), 0.1 μM (▼), and 0.3 μM (♦), in 150 mM sodium chloride. B, Inhibition of somatostatin-14 stimulated [^{35}S]GTPγS binding was measured in the absence (■) or presence of increasing concentrations of BIM-23056, 0.057 μM (▲), 0.24 μM (▼), and 1 μM (♦), in 150 mM sodium chloride. Data are the mean ± standard error of four separate experiments performed in duplicate and are expressed as percentage of the 10 μM somatostatin-14 response in each case. Basal levels of [^{35}S]GTPγS were 0.176 ± 0.016 and 0.179 ± 0.404 pmol/mg, and 10 μM somatostatin-14 raised this by 0.236 ± 0.019 and 0.225 ± 0.018 pmol/mg in A and B, respectively (n = 4).

receptor. Patel and Srikant (26) have published an affinity estimate (K_D) for BIM-23056 at this receptor of 5.7 nM, which is in agreement with our observations, whereas BIM-23027 has been shown to be ~10-fold weaker than the value we determined (IC_{50} = 176 nM; Ref. 17). Collectively, these values demonstrate a considerably lower affinity for BIM-23027 at the human sst_5 compared with the human sst_2 receptor for which BIM-23027 is selective (e.g., IC_{50} = 0.04 nM; Ref. 27). Thus, our radioligand binding data provide a rank order of ligand affinities for the human sst_5 receptor of somatostatin-28 ≥ L-362,855 ≥ somatostatin-14 > BIM-23056 > BIM-23027, which is in broad agreement with those of other researchers.

In common with studies on other seven-transmembrane receptors, [^{35}S]GTPγS binding was dependent on a number of variables, such as the concentrations of GDP, sodium chloride, and magnesium chloride (28). GDP reduced basal [^{35}S]GTPγS binding, as did sodium chloride. In fact, sodium chloride was found to be obligatory to reduce basal binding to observe an agonist-dependent effect. Reduction of basal [^{35}S]GTPγS binding by GDP is probably a consequence of its competition for the nucleotide binding site on all classes of G proteins. At concentrations of <1 μM, GDP preferentially reduced basal [^{35}S]GTPγS binding compared with somatostatin-14-stimulated binding and resulted in an optimal increase in specific binding at 0.3 μM. At higher concentrations,

GDP decreased the specific somatostatin-14 stimulation, presumably by competing with [^{35}S]GTPγS for the G proteins that couple to sst$_5$ receptors. The GDP dependence of somatostatin-14-activated [^{35}S]GTPγS binding is similar to that seen for other receptor systems (29). Interestingly, the rate of hydrolysis of GTP by G_o has previously been shown to correlate with the rate of release of GDP from G_o (Ref. 30 and references therein). From such studies, it has been suggested that GDP dissociation from the α subunit of G_o is rate limiting for receptor-mediated G protein activation. Thus, exogenously applied GDP may reduce the rate of GDP release and, therefore, the rate of G protein activation (31). Traynor and Nahorski (29) suggested that GDP-dependent [^{35}S]GTPγS binding is a characteristic of receptors negatively coupled to adenylyl cyclase, such as the adenosine A_1 receptor and the μ-opioid receptor, and our observations are consistent with this proposal (25, 29). It should, however, be noted that norepinephrine-stimulated [^{35}S]GTPγS binding to α_{2D}-adrenoceptors in PC-12 cells has been shown to be GDP independent (32).

In 50 and 150 mM sodium chloride, both somatostatin-28 and L-362,855 were more potent than somatostatin-14 at stimulating [^{35}S]GTPγS binding to the sst$_5$ receptor. The preferential potency of somatostatin-28 over somatostatin-14 for the human sst$_5$ receptor, evident in the functional measurements, correlated well with their relative affinity estimates for the receptor from binding studies. Indeed, the agonist profile of receptor activation was very similar to the profile of ligand binding to the receptor, showing the same rank order of agonist potencies, except that the potency estimates were 115–159-fold (low sodium) and 206-1130-fold (high sodium) weaker for stimulation of [^{35}S]GTPγS binding. The low potency of somatostatin-14 to stimulate [^{35}S]GTPγS binding compared with its affinity in [^{125}I]-Tyr^{11}-somatostatin-14 displacement binding studies suggests that in the former, the sst$_5$ receptors are primarily in a low affinity conformational state. Being necessarily restricted at present to the use of agonist radioligands it is not theoretically possible to detect binding to the receptor in its low affinity state. Indeed, it was not possible in practice to detect binding of [^{125}I]-Tyr^{11}-somatostatin-14 to the receptor under conditions identical to those used for the [^{35}S]GTPγS assay (see Results).

It has been shown, when measuring the rate of extracellular acidification in CHOsst$_5$ cells, that L-362,855 produced a significantly lower maximal response compared with somatostatin-14, although it was more potent (EC_{50} = 0.09 nM versus 0.54 nM, respectively; Ref. 19). In agreement with data from this study in high sodium, we have shown that L-362,855 potently increased [^{35}S]GTPγS binding with a lower maximal response than either somatostatin-14, or somatostatin-28, suggesting that it behaved as a highly potent agonist with low efficacy. Consistent with its partial agonist nature, L-362,855 produced rightward shifts of concentration-effect curves to somatostatin-14, yielding an estimated dissociation equilibrium constant (pK_P) of 7.44. As would be expected for an agonist with low efficacy, which theoretically is required to occupy all the available receptors for a maximum response, this value was close to the pEC_{50} value of 7.77 for L-362,855 for activation of the receptor in high sodium. Tallent *et al*. (33) recently reported the partial agonist nature of L-362,855 at murine sst$_5$ receptors for reducing calcium ion influx into an anterior pituitary cell line (AtT-20) and for inhibiting adenylate cyclase through the recombinant human sst$_5$ receptor expressed in CHO cells, although they provided no quantitative estimates of potency.

BIM-23056 exhibited a relatively high affinity for the sst$_5$ receptor in competition binding studies but was not able to stimulate [^{35}S]GTPγS binding and was therefore tested as an antagonist. In high sodium, BIM-23056 concentration-dependently shifted the concentration-effect curve for somatostatin-14 rightward along the abscissa. BIM-23056 did not seem to decrease the curve maxima, which suggested it was acting in a competitive, or at least a surmountable, manner. Furthermore, the Gaddum-Schild analysis was consistent with competitive antagonism allowing calculation of the dissociation equilibrium constant for BIM-23056 in antagonizing somatostatin-14-induced [^{35}S]GTPγS binding studies. Its pK_B value of 7.28 was lower than its estimated affinity for the sst$_5$ receptor from the binding data in the absence of sodium (pK_B = 8.61). An intermediate pK_B value was recently reported for BIM-23056 in antagonizing the effects of somatostatin-14-induced increases in intracellular calcium ion mobilization (8.0; Ref. 18). It is apparent that the estimates of the dissociation constant for BIM-23056 are dependent on the experimental conditions and the agonist response measured.

Costa *et al*. (34, 35) studied opioid receptor-stimulated GTPase activity and the effects of sodium on both ligand binding to the receptor and ligand-dependent GTPase activity. They showed that their experimental data could be fitted to the ternary complex model if they assumed that sodium increased the equilibrium dissociation constant for the receptor/G protein interaction. They also observed ligands with negative intrinsic activity, which was taken as evidence for the existence of constitutive receptor activation. In the absence of any known inverse agonist for the sst$_5$ receptor, evidence for constitutive receptor activity can only be provided by the ability of other agents, which disrupt receptor/G protein interaction, to reduce the apparent basal level of [^{35}S]GTPγS binding. Increasing concentrations of GDP and sodium reduced basal and somatostatin-14-stimulated [^{35}S]GTPγS binding with a profile close to that observed in other studies (25, 32). However, we have also shown that pertussis toxin pretreatment, which prevents [^{35}S]GTPγS binding to activated G proteins, reduced basal [^{35}S]GTPγS binding in membranes from wild-type and CHOsst$_5$ cells to a similar extent in a sodium-dependent manner. Thus, the presence of the sst$_5$ receptor does not seem to modify the amount of agonist-independent constitutive activity measured in these CHO-K1 cells, so it is unlikely that the sst$_5$ receptor possesses constitutive activity under these conditions. The cause of the constitutive activity observed here is unknown but has been demonstrated to be sodium sensitive and may be due to endogenously expressed G protein-coupled receptors in these cells.

According to the model used by Costa *et al*. (34,35), the efficacy of a ligand depends on its ability to induce coupling of the receptor to its G protein. Thus, factors such as the GDP or sodium chloride concentration, which alter the position of the binding equilibrium between the receptor and G protein, will affect the efficacy of a ligand in a manner inversely related to the efficacy of that ligand. A reduction in the sodium chloride concentration would be expected to increase the efficacy of a ligand. This prediction of their model seems

to be borne out when studying the agonist activation of the sst_5 receptor in CHO-K1 cell membranes by quantification of [^{35}S]GTPγS binding. At low sodium compared with high sodium, the partial agonist L-362,855 became a full agonist with no significant increase in EC_{50} value, whereas somatostatin-14 and somatostatin-28 displayed the same maximal intrinsic activity while their EC_{50} values decreased. Such profiles are consistent with the concept of differential behavior of partial and full agonists in the presence of changing receptor occupancy and/or receptor-effector coupling efficiency (e.g., Ref. 36).

The exact location of the site of sodium sensitivity remains to be determined; however, many sodium-sensitive G protein-coupled receptors possess a conserved aspartate residue in their second membrane-spanning domain (37–39). Mutation of this residue can lead to sodium-insensitive receptors and the perturbation of receptor/G protein interactions (38, 39). Therefore, the site of sodium sensitivity seems to be located on the receptor rather than the G protein. Somatostatin receptors also possess a conserved aspartate residue (amino acid 86 of the human sst_5 receptor), suggestive of a similar sodium-sensitive site. Agonist binding to the murine sst_2 receptor has been shown to be sensitive to sodium ions. whereas mutation of Asp89 to Asn89 rendered the receptor insensitive to sodium, but surprisingly it retained its sensitivity to pertussis toxin and GTPγS (40). Further studies are needed to investigate the effects of sodium ions on the function of the different somatostatin receptor subtypes and the precise mechanism(s) involved.

In conclusion, we measured [^{35}S]GTPγS binding to G proteins to directly study the activation of the recombinant human sst_5 receptor when stably expressed in CHO-K1 cells. The endogenous ligand somatostatin-14 is capable of stimulating human sst_5 receptor-mediated [^{35}S]GTPγS binding in a concentration-dependent and pertussis toxin-sensitive manner. We also observed the negative influence of sodium on ligand potency and intrinsic activity such that the potencies of the agonists somatostatin-14, somatostatin-28, and BIM-23027 were increased as the sodium concentration decreased, whereas L-362,855, a partial agonist, exhibited full agonist activity when the sodium concentration was lowered. The modulation of intrinsic activity in this system by sodium chloride seems to provide a robust system *in vitro* to discriminate between full and partial agonists at human recombinant receptors, thereby generating information of potential clinical importance. However, the quantitative relationship of such data with that in more intact functional systems requires further study.

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