Differential Efficacies of Somatostatin Receptor Agonists for G-Protein Activation and Desensitization of Somatostatin Receptor Subtype 4-Mediated Responses

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ABSTRACT
Although desensitization represents an important physiological feedback mechanism that protects against overstimulation, it can significantly limit the therapeutic usefulness of drugs. In the current investigation, we have employed Cytosensor microphysiometry for the purpose of determining the propensity of somatostatin receptor agonists to induce desensitization of the human somatostatin receptor subtype 4 (h sst4)-mediated extracellular acidification rate (EAR) response in intact Chinese hamster ovary (CHO) cells. We have compared this propensity with the efficacies of the agonists as measured in a [35S]guanosine-5′-O-(3-thio)triphosphate binding assay with membranes of the same CHO-h sst4 cell line. We observed that (1′S,2S)-4-amino-N-(1′-carbamoyl-2′-phenylethyl)-2′-(4′′-methyl-1′′-naphthaenesulfonfylamino)butanamide (J-2156), a superagonist at the h sst4 with higher efficiency than somatostatin-14 itself (Engström et al., 2005), was considerably less prone to cause desensitization of the EAR response than somatostatin-14, somatostatin-28, and cortistatin-17. In contrast, compound A (methyl (2S)-5-[(amino(imino)methyl)amino]-2-[[4-[[5–7-difluoro-2-phenyl-1H-indol-3-yl]butanoyl]amino]pentanoate), which we also found to be an h sst4 superagonist, albeit to a lesser degree than J-2156, demonstrated a high propensity to cause desensitization. Our results indicate that there is no relationship between the efficacy of the agonists to cause G-protein activation and their ability to induce desensitization of the h sst4-mediated EAR responses. The finding that on the h sst4, J-2156 is not only a superagonist but also shows a low propensity to cause desensitization, might offer therapeutic advantages. At a minimum, the compound will be a powerful tool to study the mechanisms connected to efficacy and desensitization of h sst4-mediated responses.

Somatostatin or somatotropin release-inhibiting factor (SRIF) is a widely distributed peptide occurring in two endogenous forms in humans, SRIF-14 and SRIF-28. In addition to playing an important regulatory role in neurotransmission and secretion (Patel, 1999), the peptides may control cellular proliferation in normal and tumorous tissues (Schally, 1988; Lamberts et al., 1991). The biological responses to SRIF are mediated via high-affinity G-protein-coupled receptors (GPCRs), of which five different subtypes, termed sst1 through sst5, have been cloned and characterized in humans (Reisine and Bell, 1995; Patel, 1999).

In accordance with the widespread distribution of SRIF receptors throughout the brain and peripheral tissues, administration of SRIF or its analogs of it induces a variety of biological effects (Patel, 1997, 1999). Nevertheless, these initial potent responses often diminish with continued exposure (Lamberts et al., 1996; Patel, 1999). The adaptive processes connected to the decreased cellular responsiveness of GPCRs upon continuous exposure to an agonist are the result of three principal modes of regulation: desensitization, in which the receptors become refractory to continued stimuli; internalization, whereby the receptors are removed from the cell surface; and down-regulation, where the total cellular receptor amount is decreased. However, even though it appears that the processes connected to the attenuation of the cellular responses predominantly occur at the receptor level, it should be borne in mind that there are probably also other steps that affect signaling pathway components further downstream (Clark et al., 1999). There are a number of


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In accordance with the widespread distribution of SRIF
reports in the literature on the desensitization and internalization of the SRIF receptors (Reisine and Axelrod, 1983; Koper et al., 1990; Vanetti et al., 1993; Beaumont et al., 1998; Mazza and Blake, 2004). For example, after long-term (16-h) pretreatment of mouse anterior pituitary AtT-20 cells with SRIF, the ligand shows reduced potency to inhibit corticotropin release factor- and forskolin-stimulated cAMP accumulation and adrenocorticotropic hormone release (Reisine and Axelrod, 1983). Furthermore, loss of sensitivity with respect to both prolactin secretion and cell growth has been observed in cells prepared from the transplantable rat prolactin-secreting rat pituitary tumor 7315b, and this loss of sensitivity was accompanied by a complete down-regulation of SRIF receptors on the tumor cells (Koper et al., 1990). In Chinese hamster ovary (CHO) cells stably expressing one of the five human sst (h sst) subtypes, h sst 1, h sst 2, h sst 3, and h sst 4 displayed rapid (within minutes) agonist-dependent internalization of (Leu 6,Tyr 3)-SRIF-28 ((125I)J-LTTSRIF-28) in a time- and temperature-dependent manner (Hukovic et al., 1996). Maximum internalization of the radioligand occurred within 60 min. The h sst 3 and h sst 4-expressing cells displayed the highest degree of internalization (78 and 66%, respectively), followed by h sst 2 (29%) and h sst 2 (20%). In contrast, h sst 1 displayed almost no (4%) internalization.

We have recently reported that the novel peptidomimetic J-2156 produces a much greater response than SRIF-14, when tested for its ability to stimulate [35S]GTPγS binding to membranes of CHO cells stably expressing the h sst 4 (Engström et al., 2005). To gain further insight into the regulation of the h sst 4, we have now examined the effects of different ligands on the desensitization of h sst 4-mediated responses. The desensitization was evaluated with Cytosensor microphysiometry by looking at changes in the extracellular acidification rate (EAR) of CHO-h sst 4 cells, a method already successfully employed for this purpose by Smalley et al. (1998).

Materials and Methods

Drugs. SRIF-14, SRIF-28, LTT-SRIF-28, and cortistatin-17 (CST-17) were purchased from Bachem (Bubendorf, Switzerland). Compound A (Rohrer et al., 1998) and the heptapeptide somatostatin analog J-Phe-Cys-Tyr-γ-Thr-Lys-Cys-Thr-NH2 (TT-232; Keri et al., 1993) were kind gifts from Merck Research Laboratories (Rahway, NJ) and Prof. János Szolcsányi (Department of Pharmacology and Pharmacotherapy, University of Pécs, Pécs, Hungary), respectively. J-2156 was synthesized as described previously (Engström et al., 2005).

Cell Culture. Recombinant CHO-K1 cells expressing the h sst 4 (CHO-h sst 4 ) (Engström et al., 2005) were grown in Ham’s F-12 medium (Life Technologies, Carlsbad, CA) containing 5% fetal calf serum and 200 µg/ml G418 (Calbiochem, San Diego, CA). The estimated receptor density as determined in membranes prepared for the current investigation was 3.3 pmol/mg membrane protein. Cells were grown at 37°C in an atmosphere of 5% CO2. Confluent cells were harvested in phosphate-buffered saline containing 0.6 mM EDTA, pH 7.4. Harvested cells were centrifuged (500g, 5 min), and the pellets were stored at −70°C for membrane preparation. Alternatively, the harvested cells were resuspended and seeded into microcapsules for use in the Cytosensor microphysiometry assay.

Pretreatment of CHO-h sst 4 Cells with SRIF-14. Confluent CHO-h sst 4 cells were washed once with phosphate-buffered saline (37°C) and incubated in serum-free Ham’s F-12 medium for 1 h at 37°C in an atmosphere of 5% CO2. Cells were then incubated for 4 h at 37°C in Ham’s F-12 medium in the absence (control cells) or presence of 3 µM SRIF-14 (pretreated cells). After the incubation, the cells were washed four times with 20 ml of phosphate-buffered saline, and membranes were prepared from harvested cells as described previously (Engström et al., 2003).

Measurement of G-Protein Activation. The functional properties of different SRIF receptor ligands were determined as their ability to stimulate the receptor-mediated binding of [35S]GTPγS (Amersham Biosciences UK Limited, Buckinghamshire, UK; specific activity 1100–1200 Ci/mmol) to G-proteins in membranes of CHO-h sst 4 cells. The assay was essentially conducted as described earlier (Engström et al., 2005). In brief, membranes (approximately 10 µg of protein per sample), prepared as described previously (Engström et al., 2003), were incubated in 20 mM HEPES, pH 7.4, 10 mM MgCl2, 3 or 10 µM GDP, 20 or 100 mM NaCl, and 10 µg/ml saponin (incubation buffer) with different concentrations of test compounds, and trace amounts of [35S]GTPγS (0.07–0.16 nM). After a 45-min incubation at 30°C (15-min preincubation without label followed by a 30-min stimulation period after addition of the radiolabel), the reaction was terminated by rapid vacuum filtration through glass fiber filters. Filters were then washed three times with 5 ml of ice-cold wash buffer (25 mM HEPES, 1 mM CaCl2, 5 mM MgCl2, and 500 mM NaCl, pH 7.4), dried, and impregnated with scintillant, and their radioactivity was measured by scintillation counting. Each experiment was repeated at least three times, and experimental results were calculated by nonlinear least-squares curve fitting.
TT-232, which is a purported sst4 agonist (Pinté et al., 2002), were tested for their ability to stimulate \[35S\]GTP\(_S\) binding in membranes of CHO-h sst4 cells. SRIF-14 was used as reference compound. CST-17, SRIF-28, and LTT-SRIF-28 were included in the experiments as prototypes for full agonism (Siehler and Hoyer, 1999), whereas J-2156 was included as a prototype compound for superagonism (Engström et al., 2005). As can be deduced from Table 1, the rank order of agonist efficacy at the h sst4 was J-2156 > compound A > LTT-SRIF-28 \(\geq\) SRIF-28 = CST-17. A complete dose-response curve could not be obtained for TT-232 under the routine testing conditions (100 mM NaCl, 10 \(\mu\)M GDP, Fig. 1A). GDP and Na\(^+\), which are important ingredients in \[35S\]GTP\(_S\) binding experiments to reduce the otherwise high background, are known to influence apparent agonist potencies and efficacies (Williams et al., 1997; Wurster et al., 1998; Costa et al., 1990; Selley et al., 2000). We therefore tested the SRIF receptor ligands also under lower sodium and GDP concentrations (20 mM NaCl, 3 \(\mu\)M GDP, Fig. 1B). These conditions did cause elevated levels of basal \[35S\]GTP\(_S\) binding but still allowed for the detection of agonist-mediated responses. However, although TT-232 was able to elicit an agonist effect under these conditions at the highest tested concentrations, the dose-response curve was still incomplete, and the apparent agonist results for the compound have been omitted from Table 1.

The ability of the set of SRIF receptor agonists with varying efficacies for G-protein activation to stimulate the EAR of CHO-h sst4 cells was determined using a four-channel Cyto-sensor microphysiometer, which measures changes in the rate at which cells acidify the only lightly buffered flow medium. In line with a previous report (Smalley et al., 1998), the cumulative concentration-effect curve for SRIF-14 was bell-shaped with high SRIF-14 concentrations causing smaller increases in EAR. The same outcome was also seen for CST-17 and compound A (Fig. 2A). The analysis of the ascending parts of the bell-shaped curves yielded EC\(_{50}\) estimates of 0.80 \(\pm\) 0.03, 0.49 \(\pm\) 0.06, and 0.11 \(\pm\) 0.02 nM for SRIF-14, CST-17, and compound A (Fig. 2B). In contrast, the dose-response curves for J-2156 and TT-232 were sigmoidal and yielded EC\(_{50}\) values of 0.029 \(\pm\) 0.005 and 36 \(\pm\) 3 nM, respectively (Fig. 2C). The observed increases in acidification rates upon agonist exposure seem to be due to the coupling of the h sst4 to pertussis toxin-sensitive G-proteins of the G\(_i/o\) family because it was abolished after the cells had been pretreated for 18 h with pertussis toxin (Fig. 3A). To confirm the viability of the pertussis toxin-pretreated cells and to demonstrate their ability to respond to receptor-mediated stimuli, we used ATP, which acts through an endogenous G\(_q\)-linked purinergic receptor to increase the EAR (Garnovskaya et al., 1997), as a positive control. The EAR response to ATP was not abolished by the pertussis toxin pre-

![Fig. 1. Stimulation of \[35S\]GTP\(_S\) binding in membranes of CHO cells expressing h sst4 (CHO-h sst4). Trace amounts of \[35S\]GTP\(_S\) were added to membranes of CHO-h sst4 (approximately 10 \(\mu\)g/sample) that had been preincubated with the indicated concentration of SRIF-14 (\(\bullet\)), CST-17 (\(\square\)), J-2156 (\(\triangle\)), TT-232 (\(\diamondsuit\)), or compound A (\(\bigcirc\)) in the presence of 10 mM MgCl\(_2\), 10 \(\mu\)M GDP, 100 mM NaCl, and 10 \(\mu\)g/ml saponin. Basal \[35S\]GTP\(_S\) binding to membranes of CHO-h sst4 cells were 74 \(\pm\) 3 (A) and 349 \(\pm\) 36 (B) fmol/mg, whereas maximal \[35S\]GTP\(_S\) binding in response to saturating concentrations of SRIF-14 was 154 \(\pm\) 9 (A) and 495 \(\pm\) 44 (B) fmol/mg. The percentage of \[35S\]GTP\(_S\) binding was normalized against the maximal effect of the reference compound SRIF-14, which was set as 100%. The combined data of three different experiments performed in duplicate are shown.

### Table 1

<table>
<thead>
<tr>
<th>compound</th>
<th>100 mM NaCl, 10 (\mu)M GDP</th>
<th>20 mM NaCl, 3 (\mu)M GDP</th>
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<tbody>
<tr>
<td></td>
<td>EC(_{50}) (nM)</td>
<td>Efficacy (normalized against SRIF-14)</td>
</tr>
<tr>
<td>J-2156</td>
<td>17 (\pm) 1.0</td>
<td>340 (\pm) 20</td>
</tr>
<tr>
<td>Compound A</td>
<td>15 (\pm) 4</td>
<td>210 (\pm) 10</td>
</tr>
<tr>
<td>LTT-SRIF-28</td>
<td>80 (\pm) 15</td>
<td>120 (\pm) 10</td>
</tr>
<tr>
<td>SRIF-28</td>
<td>7.3 (\pm) 1.9</td>
<td>110 (\pm) 10</td>
</tr>
<tr>
<td>SRIF-14</td>
<td>6.5 (\pm) 0.7</td>
<td>100</td>
</tr>
<tr>
<td>CST-17</td>
<td>46 (\pm) 6</td>
<td>93 (\pm) 7</td>
</tr>
</tbody>
</table>

N.D., not determined.
Furthermore, CHO cells lacking the h sst4, but instead expressing the human \(\alpha_2\)-adrenergic receptor (Pohjanoksa et al., 1997), failed to give rise to any increases of the EAR after SRIF-14 and J-2156 challenges (Fig. 3B). However, in accordance with a previous study (Pihlavisto and Scheinin, 1999), a clear increase in the acidification rate (~30% over basal) was observed in these cells in response to the \(\alpha_2\)-adrenoceptor agonist epinephrine.

It has been reported previously that SRIF induces desensitization of h sst4-mediated EAR responses, whereas the synthetic peptide agonist L-362855 does not (Smalley et al., 1998). We have continued the examination of sst4 agonists for their propensity to cause desensitization of the EAR response by employing an experimental paradigm consisting of two consecutive agonist challenges separated by a 30-min washout interval. The purpose of the initial 10-s exposure to a saturating concentration (see Materials and Methods) of the test compound of interest was to induce desensitization where applicable, whereas the second agonist pulse 30 min later served to probe for the occurrence of such desensitization. The results for SRIF-14, CST-17, compound A, TT-232, J-2156, SRIF-28, and LTT-SRIF-28 in this experimental set-up are shown in Fig. 4A. The acidification rates in response to the first exposure of agonists increased by ~40% in all cases. In accordance with the report by Smalley et al. (1998), the repeat exposure to 3 \(\mu\)M SRIF-14 resulted in a much smaller increase in the acidification rate, confirming that SRIF-14 does cause desensitization of the h sst4-mediated EAR response. The same outcome as for SRIF-14 was also seen for CST-17, compound A, and SRIF-28. In contrast, repeated exposure to 30 \(\mu\)M TT-232 or 1 \(\mu\)M J-2156 did not significantly affect the EAR increase produced by the first agonist pulse, arguing that these two compounds do not desensitize the h sst4-mediated EAR response. LTT-SRIF-28 caused an intermediate degree of desensitization. The relative efficacies of the agonists for promoting desensitization of the EAR response were, thus, compound A \(\geq\) SRIF-14 \(\geq\) SRIF-28 > CST-17 > LTT-SRIF-28 > J-2156 \(\geq\) TT-232.

In a second crossover type of agonist exposure experiment, CHO-h sst4 cells were first challenged with 3 \(\mu\)M SRIF-14, 3
The pulse did not affect the subsequent response to SRIF-14-pretreated CHO-h sst4 cells. The cells had been washed out interval, with a second agonist (Fig. 4B). This led to a diated EAR response is agonist-specific. The cells were challenged with SRIF-14 did not lead to desensitization of the EAR response toward SRIF-14 or compound A (Fig. 4B). The results, thus, indicate that desensitization of the h sst4-mediated EAR response is agonist-specific.

In an attempt to determine at which level of the signaling cascade the desensitization was occurring, agonist-stimulated [35S]GTPγS binding was measured in membranes of SRIF-14-pretreated CHO-h sst4 cells. The cells had been pretreated for 4 h with 3 μM SRIF-14 in serum-free Ham's F-12 medium and then washed four times before the preparation of membranes. As shown in Fig. 5, pretreatment of cells with SRIF-14 did not lead to desensitization of the SRIF-14-induced [35S]GTPγS binding.}

### Discussion

In this report, we demonstrated that desensitization of h sst4-mediated responses is not directly linked to agonist ef-

In cumulative EAR dose-response experiments, we found ordinary sigmoidal dose response curves for some agonists (J-2156, TT-232), whereas other compounds (SRIF-14, CST-17, and compound A) produced bell-shaped curves, indicative of desensitizing responses. When the sigmoid curves or the ascending parts of the bell-shaped dose-response curves were analyzed for agonist potencies, we obtained considerably lower EC50 values in the EAR assay compared with the [35S]GTPγS binding assay. [35S]GTPγS binding assays are known to underestimate agonist potencies (Williams et al., 1997), a tendency that seems to be a consequence of the need to suppress otherwise high levels of “basal” turnover by sufficiently large concentrations of GDP and sodium ions in the assay medium. However, the presence of a receptor reserve might also contribute to the higher agonist potencies observed in the EAR assay compared with the [35S]GTPγS binding assay because this assumption could explain why the
against SRIF-14 (set as 100%; efficacies are given in Table 1). Responses are shown in Fig. 4). The efficacy (percentage) was normalized due to the first compared with the second agonist exposure (EAR reinstitution was calculated from the difference between the EAR response (Fig. 6). We have described recently a novel, highly sst4-selective peptidomimetic, coded J-2156, which exhibits much greater agonist efficacy in the \[^{35}\text{S}]\text{GTP}\gamma\text{S} assay than SRIF-14 (Engström et al., 2005). In the current study, we demonstrate that this compound is considerably less prone to cause desensitization of the EAR response compared with SRIF-14 and CST-17. On the other hand, the peptidomimetic compound A (Rohrer et al., 1998), which we also found in our \[^{35}\text{S}]\text{GTP}\gamma\text{S} assay to be a sst4 superagonist, albeit to a somewhat lesser degree than J-2156, did display a pronounced propensity to desensitize the EAR response. Although the propensity of agonists to cause desensitization obviously must be encoded in their structure, the set of sst4 agonists used for the current investigation suggests that this signal is not connected to an overt structural feature such as molecular size of the ligands or their peptidic versus nonpeptidic nature; among the desensitizing agonists, SRIF-14, SRIF-28, and CST-17 are peptides, whereas compound A is a much smaller peptidomimetic. Among the nondesensitizing agonists, TT-232 is a peptide, whereas J-2156 is not. The lack of correlation between agonist efficacy and the propensity to cause desensitization is in marked contrast to what has generally been observed for GPCRs (Clark et al., 1999), but it is not unprecedented. For example, Zhang et al. (1998) have reported that of etorphine and morphine, two \(\mu\)-opioid agonists with comparable ability to activate the opioid receptor, only etorphine elicits robust receptor phosphorylation and internalization.

The lack of correlation between the two different ligand-induced processes is not compatible with the presence of a single activated conformation of the h sst4. However, it is explainable within the framework of the ensemble hypothesis (Kenakin, 2002), which assumes that a receptor can exist in a variety of conformations, the distribution of which is differentially affected by ligands due to their different affinities for them. Pretreatment with SRIF-14 did not lead to a reduction in the magnitude of the increase in \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding. Our results, thus, suggest that one or more among these activated receptor conformations couple strongly to the activation of G\text{\(\alpha\)}\ass, but not to another pathway that leads to desensitization. The mechanistic details on how this “signal trafficking” is brought about obviously remain to be resolved and will be a topic of future investigations. An effect to look at in this regard will be the possible agonist-dependent phosphorylation of the h sst4 because receptor phosphorylation seems to be a primary means of implementing desensitization. However, there are possibly also other types of desensitization that affect components downstream of the receptors; examples include agonist-induced phosphorylation of G-proteins, phospholipase C, and adenylyl cyclase (Clark et al., 1999). Smalley et al. (1998, 2001) have reported that the desensitization of h sst4-mediated EAR responses is not likely to involve receptor internalization but seems to take place upstream of mitogen-activated protein kinase (Smalley et al., 1999).

Sst4-transfected CHO cells obviously represent an artificial system. Even though rat recombinant sst4 does not seem to be subject to agonist-dependent receptor phosphorylation or internalization when expressed in human embryonic kidney 293 cells (Roth et al., 1997; Tulipano et al., 2004), we do not know whether the receptor would undergo, like its human counterpart, agonist-dependent desensitization when expressed in CHO cells. Another important point that remains to be clarified is whether endogenously expressed sst4 is desensitized. If this were to be the case, the data collected so far would suggest the fascinating possibility that compounds like J-2156 or TT-232 may have distinctly different in vivo profiles compared with synthetic sst4 agonists like compound A or the endogenous SRIF receptor agonists SRIF-14, SRIF-28, and CST-17. TT-232 has already been shown to be very efficacious in various tumor models (Kéri et al., 1996; Tejeda et al., 2003), as well as in models of acute and chronic pain (Szolcsányi et al., 2004). Interestingly, in an in vivo rat carotid denudation assay, compound A did cause an antifibroproliferative response, whereas an analog of J-2156, which shares its properties of superagonism and lack of desensitization, caused pronounced thickening of the vascular wall (Häyry and Aavik, personal communication). As reviewed by Lu et al. (2001), the mitogen-activated protein kinase pathway may play a positive or negative role in the regulation of cell proliferation depending on the intensity and duration of SRIF receptor activation. The opposite effects on cellular proliferation in the rat carotid denudation assay, therefore, may be a consequence of differential activation patterns in regulatory kinase cascades. However, clearly a lot of work still needs to be done before it can be established whether ligands with in vitro properties of superagonism and/or a low propensity to cause desensitization will indeed be agonists with superior effects in vivo.

To conclude, we did not find a relationship between the
ability of SRIF receptor agonists to cause G-protein activation and desensitization of the h sst4-mediated EAR response. We demonstrated that J-2156, despite its high efficacy, shows a much lower propensity than SRIF-14 to cause desensitization of the EAR response. This combination of properties in J-2156 might offer therapeutic advantages, but at a minimum, the compound will be a useful tool when studying the intracellular mechanisms connected to efficacy and desensitization of sst4-mediated responses.

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References


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