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Differential efficacies of somatostatin receptor agonists for G-protein activation and desensitization of somatostatin receptor subtype 4-mediated responses*

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Abbreviations: CHO, Chinese hamster ovary; CST-17, cortistatin-17; EAR, extracellular acidification rate; GPCR, G-protein-coupled receptor; SRIF, somatotropin release inhibiting factor; LTT-SRIF-28, (Leu^8, D-Trp^{22}, Tyr^{25})-SRIF-28; h sst, human sst; [^{35}S]GTPγS, [^{35}S]guanosine-5´-O-(3-thio)triphosphate

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ABSTRACT

Although desensitization represents an important physiological feedback mechanism that protects against over-stimulation, 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 sst_4)-mediated extracellular acidification rate (EAR) response in intact CHO cells. We have compared this propensity to the efficacies of the agonists as measured in a [^{35}S]guanosine-5´-*O*-(3-thio)triphosphate binding assay with membranes of the same CHO-h sst_4 cell line. We observed that J-2156, a superagonist at the h sst_4 with higher efficacy 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, which we also found to be an h sst_4 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 sst_4-mediated EAR responses. The finding that on the h sst_4, J-2156 is not only a superagonist, but does also show 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 sst_4-mediated responses.

Introduction

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 sst_1 through sst_5, 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 and 1999). Nevertheless, these initially 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 is the result of three principal modes of regulation: (i) desensitization, in which the receptors become refractory to continued stimuli; (ii) internalization, whereby the receptors are removed from the cell surface; and (iii) 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 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

cyclic AMP accumulation and adrenocorticotropic hormone release (Reisine and Axelrod, 1983). Further, 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_2, h sst_3, h sst_4, and h sst_5 displayed rapid (within minutes) agonist-dependent internalization of [^{125}I]LTT-SRIF-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_5 expressing cells displayed the highest degree of internalization (78 % and 66 %, respectively), followed by h sst_4 (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 [^{35}S]guanosine-5´-*O*-(3-thio)triphosphate ([^{35}S]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, (Leu^8, D-Trp^{22}, Tyr^{25})-SRIF-28 (LTT-SRIF-28) and cortistatin-17 (CST-17) were purchased from Bachem (Bubendorf, Switzerland). Methyl (2S)-5-{[amino(imino)methyl]amino}-2-{[4-[5-7-difluoro-2-phenyl-1H-indol-3-yl)butanoyl]amino}pentanoate (Rohrer et al., 1998; herein referred to as compound A) and the heptapeptide somatostatin analogue TT-232 (D-Phe-Cys-Tyr-D-Thr-Lys-Cys-Thr-NH_2; Kéri 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 previously described (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 F12 medium (Life Technologies, Carlsbad, CA) containing 5% fetal calf serum and 200 µg/ml G418 (Calbiochem, 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% CO_2. Confluent cells were harvested in phosphate-buffered saline containing 0.6 mM EDTA, pH 7.4. Harvested cells were centrifuged (500 x g, 5 min) and the pellets 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 PBS (37°C) and incubated in serum-free Ham's F12 medium for 1 h at 37°C in an atmosphere of 5% CO_2. Cells were then incubated for 4 h at 37°C in Ham's F12 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

PBS, and membranes were prepared from harvested cells as previously described (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 [^{35}S]GTPγS (Amersham Biosciences UK Limited, Buckinghamshire, UK; specific activity 1,100-1,200 Ci/mmol) to G-proteins in membranes of CHO-h sst_4 cells. The assay was essentially conduced as described earlier (Engström et al., 2005). Briefly, 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 $MgCl_2$, 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 [^{35}S]GTPγS (0.07-0.16 nM). After a 45-min incubation at 30°C (15-min pre-incubation 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 $CaCl_2$, 5 mM $MgCl_2$, 500 mM NaCl, pH 7.4), dried and impregnated with scintillate, and their radioactivity was measured by scintillation counting. Each experiment was repeated at least three times and experimental results were calculated by non-linear least square curve fitting.

Measurement of extracellular acidification rate. A four-channel Cytosensor® microphysiometer instrument (Molecular Devices, Menlo Park, CA) was used to measure the EAR of CHO-h sst_4 cells before, during and after the application of SRIF receptor agonists. Cultured CHO-h sst_4 cells were seeded into 12-mm capsule cups at a density of 3 x 10^5 cells per cup and cultured in an atmosphere of 5% CO_2 at 37°C for 18 h. The capsule cups were then loaded into the sensor chambers of the instrument and the chambers were perfused with running medium (bicarbonate-free αMEM supplemented with 2 mM glutamine, 26 mM NaCl, 50 U/ml penicillin and 50

µg/ml streptomycin) at a flow rate of 100 µl/min. Agonists were diluted into running medium and perfused through a second fluid path. Valves directed the flow from either fluid path to the sensor chamber. During a 2-min pump cycle, the pump was on for 1 min 20 s and then switched off for the remaining 40 s. The pH of the running medium was recorded from 1:25 to 1:55 min in every cycle. At 2 min the pump was started to initiate the next cycle. Cells were exposed to agonists for 10 or 50 s before the flow was stopped and during the 40 s when there was no flow. Repeat exposures to compounds were conducted with 30-min intervals, *i.e.* there was a 30-min agonist wash-out period between subsequent exposures. The propensity of agonists to cause desensitization of the EAR response was assessed using a two-application protocol. The concentrations of the ligands were chosen to represent approximately 1000-fold the K_i value (except TT-232, which was tested at 30 µM, *i.e.* at a 150x higher concentration than its K_i value), as determined in receptor ligand-binding assays (Engström et al., 2005 and unpublished data). The effects of agonists were calculated as the difference between the peak values after agonist addition and the average of the three measurements taken immediately before the addition of agonists. Non-linear least square curve fitting was used to analyze dose-dependent increases of the EAR (in case of bell-shaped curves, only the ascending part of the curve was used in the analysis). Each experiment was repeated at least three times, and statistical significance was determined using Student's two-tailed unpaired *t* test.

Results

Coumpound A, which has previously been described as an agonist in a cyclic AMP-based assay (Rohrer et al., 1998), and TT-232, which is a purported sst_4 agonist (Pintér et al., 2002), were tested for their ability to stimulate [^{35}S]GTPγS binding in membranes of CHO-h sst_4 cells. SRIF-14 was used as reference compound. CST-17, SRIF-28 and LTT-SRIF-28 were included in the experiments as prototype compounds for full agonism (Siehler and Hoyer, 1999), while 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 sst_4 was J-2156 > compound A > LTT-SRIF-28 = SRIF-28 ≥ SRIF-14 = CST-17. A complete dose-response curve could not be obtained for TT-232 under the routine testing conditions (100 mM NaCl, 10 µM GDP; Fig. 1A). GDP and Na^+, which are important ingredients in [^{35}S]GTPγS 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., 1999; Selley et al., 2000). We therefore tested the SRIF receptor ligands also under lower sodium and GDP concentrations (20 mM NaCl, 3 µM GDP; Fig. 1B). These conditions did cause elevated levels of basal [^{35}S]GTPγS 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 sst_4 cells was determined using a four-channel Cytosensor® 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 ± 0.03 nM, 0.49 ± 0.06 nM and 0.31 ± 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 ± 0.005 nM and 36 ± 3 nM, respectively (Fig. 2C). The observed increases in acidification rates upon agonist exposure appear to be due to the coupling of the h sst_4 to pertussis toxin-sensitive G-proteins of the $G_{i/o}$ family, since 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-pretreatment (Fig. 3A). Further, CHO cells lacking the h sst_4, but instead expressing the human α_{2A}-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 α_2-adrenoceptor agonist epinephrine.

It has previously been reported that SRIF induces desensitization of h sst_4-mediated EAR responses, whereas the synthetic peptide agonist L-362855 does not (Smalley et al., 1998). We have continued the examination of sst_4 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 wash-out 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, while 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 µM SRIF-14 resulted in a much smaller increase in the acidification rate, confirming that SRIF-14 does cause desensitization of the h sst_4-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 µM TT-232 or 1 µM 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 sst_4-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 ≥ SRIF-14 ≥ SRIF-28 > CST-17 > LTT-SRIF-28 > J-2156 ≥ TT-232.

In a second, crossover type of agonist exposure experiment, CHO-h sst_4 cells were first challenged with 3 µM SRIF-14, 3 µM compound A or 1 µM J-2156 and then, after the 30 min wash-out interval, with a second agonist (Fig. 4B). This led to a >70 % reduction in the EAR response towards J-2156 compared to the effect of J-2156 when the cells had not been pre-challenged with either SRIF-14 or compound A (Fig. 4B). In contrast, a challenge with 1 µM J-2156 during the first pulse did not affect the subsequent response to 3 µM SRIF-14 or compound A in the second pulse compared to the first pulse response towards SRIF-14 or compound A (Fig. 4B). The results thus indicate that desensitization of the h sst_4-mediated EAR response is agonist-specific.

In an attempt to determine at which level of the signaling cascade the desensitization was occurring, agonist-stimulated [^{35}S]GTPγS binding was measured

in membranes of SRIF-14-pretreated CHO-h sst_4 cells. The cells had been pretreated for 4 h with 3 μM SRIF-14 in serum-free Ham's F12 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 [^{35}S]GTPγS binding.

Discussion

In this report we demonstrated that desensitization of h sst_4-mediated responses is not directly linked to agonist efficacy, as determined at the G-protein level. Although the ability to regulate their responsiveness to continued agonist exposure is a common property of many GPCRs, the loss of receptor responsiveness, *i.e.* desensitization, may limit the therapeutic usefulness of drugs. For example, acute administration of SRIF produces a large number of inhibitory effects, but the initial response diminishes with continued exposure to the peptide (Lamberts et al., 1996; Patel, 1999). The identification of compounds, that do not cause desensitization, could therefore provide a method for prolonging the biological effects mediated by the receptors.

We have determined the ability of h sst_4 receptor ligands to cause G-protein activation in a [^{35}S]GTPγS binding assay and have compared it to the propensity of the compounds to induce desensitization of EAR responses in a microphysiometry assay. While the [^{35}S]GTPγS binding assay measures the most proximal event in GPCR signaling after receptor activation, the Cytosensor® microphysiometer measures EAR as a means of monitoring overall cell metabolism. We ascertained that the EAR responses observed in the CHO-h sst_4 cells were mediated via h sst_4 and involved most likely the participation of G-proteins belonging to the $G_{i/o}$ family, by demonstrating that the same host cells (CHO-K1) transfected with the α_{2A}-adrenoceptor rather than the h sst_4, did not show any EAR response when exposed to SRIF-14 or J-2156 and that pretreatment of the CHO-h sst_4 cells with pertussis toxin abolished any response to subsequent exposure to sst_4 agonists.

In cumulative EAR dose-response experiments we found ordinary sigmoidal dose response curves for some agonists (J-2156, TT-232) while 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 EC_{50} values in the EAR assay compared to the [^{35}S]GTPγS binding assay. [^{35}S]GTPγS binding assays are known to underestimate agonist potencies (Williams et al., 1997), a tendency which appears 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 to the [^{35}S]GTPγS binding assay, since this assumption could explain why the EAR assay did not reproduce the efficacy differences seen in the [^{35}S]GTPγS assay and why the agonist potencies in the EAR assay were found to be higher than the reported K_i values from the competition binding assays (Rohrer et al., 1998; Engström et al., 2005)[1]

Our results confirm a suggestion made by Smalley et al (1998) based on the compound pair SRIF-14 and L-362855, namely that desensitization of the h sst_4 is an agonist-specific process. We can add to this suggestion the finding that the propensity of agonists to cause desensitization is not connected to their agonist efficacy, at least not to their agonist efficacy as detected in the [^{35}S]GTPγS binding assay (Fig. 6). We have recently described a novel, highly sst_4-selective peptidomimetic, coded J-2156, which exhibits much greater agonist efficacy in the [^{35}S]GTPγS 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 to SRIF-14 and CST-17. On the other hand, the peptidomimetic compound A (Rohrer et al., 1998), which we also found in our [^{35}S]GTPγS assay to be a sst_4 superagonist, albeit to a somewhat lesser degree than J-2156, did display a pronounced propensity to desensitize the EAR response. While

[1] The K_i of TT-232 for h sst_4 was found to be 200 ± 10 nM (unpublished observation)

the propensity of agonists to cause desensitization obviously must be encoded in their structure, the set of sst_4 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 non-peptidic nature: among the desensitizing agonists, SRIF-14, SRIF-28 and CST-17 are peptides, while compound A is a much smaller peptidomimetic; and among the non-desensitizing agonists TT-232 is a peptide, while 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 μ-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 sst_4. 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}S]GTPγS binding. Our results thus suggest that one or more among these activated receptor conformations couple strongly to the activation of $G_{i/o}$, but not to another pathway that leads to desensitization. The mechanistic details how this 'signal trafficking' is brought about obviously remains 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 sst_4, as receptor phosphorylation appears 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 and

2001) have reported that the desensitization of h sst_4-mediated EAR responses is not likely to involve receptor internalization, but appears to take place upstream of mitogen-activated protein kinase (Smalley et al., 1999).

Sst_4-transfected CHO cells obviously represent an artificial system. Even though rat recombinant sst_4 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 sst_4 are 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 to synthetic sst_4 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 tumour 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 anti-fibroproliferative response, while an analogue 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 caotid denudation assay may therefore 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 sst_4-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 sst_4-mediated responses.

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Footnotes

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Legends for Figures

Fig. 1. Stimulation of [^{35}S]GTPγS binding in membranes of CHO cells expressing h sst_4 (CHO-h sst_4). Trace amounts of [^{35}S]GTPγS were added to membranes of CHO-h sst_4 (about 10 μg/sample) that had been preincubated with the indicated concentration of SRIF-14 (■), CST-17 (□), J-2156 (O), TT-232 (△) or compound A (▽) in the presence of 10 mM $MgCl_2$, 10 μM (in A) or 3 μM GDP (in B), 100 mM (in A) or 20 mM NaCl (in B), and 10 μg/ml saponin. Basal [^{35}S]GTPγS binding to membranes of CHO-h sst_4 cells were 74 ± 3 fmol/mg (in A) and 349 ± 36 fmol/mg (in B), while maximal [^{35}S]GTPγS binding in response to saturating concentrations of SRIF-14 was 154 ± 9 fmol/mg (in A) and 495 ± 44 fmol/mg (in B). The percentage of [^{35}S]GTPγS 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.

Fig. 2. The extracellular acidification rate (EAR) response of CHO cells expressing h sst_4 (CHO-h sst_4) upon exposure to progressively increasing concentrations (six concentrations, one log unit difference between each subsequent concentration) of SRIF-14 (0.03 nM-3 μM), CST-17 (0.03 nM-3 μM), J-2156 (0.01 nM-1 μM), TT-232 (0.3 nM-30 μM) and compound A (0.03 nM-3 μM). The cells were challenged with SRIF receptor agonists for 50-s pulses separated by wash-out periods of 30-min duration. A) A representative microphysiometer recording of agonist-induced increases in the EAR of CHO-h sst_4 cells. The addition of test substance is indicated by an arrow. B-C) Dose-response curves for SRIF-14 (■), CST-17 (□), compound A (▽), TT-232 (△) and J-2156 (O), were plotted from the pooled data of three separate experiments, one of them exemplified in Fig. 2A. In B, the curves were bell-shaped

and only the ascending parts were included in the analysis (dashed lines). The increases in the EAR are given as % stimulation over basal.

Fig. 3. The extracellular acidification rate (EAR) response of CHO cells expressing h sst_4 (CHO-h sst_4) that had been pretreated with 100 ng/ml pertussis toxin for 16 h (in A) or of CHO cells expressing the human α_{2A}-adrenergic receptor (CHO-C10) (in B). A) Pertussis toxin pretreated CHO-h sst_4 cells were exposed to two 10-s pulses of 1 μM J-2156 or 1 μM ATP. B) CHO-C10 cells were exposed to two 10-s pulses of 3 μM SRIF-14 or 1 μM J-2156 and then one 10-s pulse with 100 μM epinephrine. The addition of test substance is indicated by an arrow. The increases in the EAR are given as % stimulation over basal and a representative microphysiometer recording of three different experiments is shown.

Fig. 4. Differential efficacies of SRIF receptor agonists to induce desensitization of the extracellular acidification rate (EAR) response. A) CHO-h sst_4 cells were challenged with 3 μM SRIF-14, 3 μM CST-17, 3 μM compound A, 30 μM TT-232, 1 μM J-2156, 3 μM SRIF-28 or 3 μM LTT-SRIF-28 for 10-s pulses (1^{st} exposure, control) followed by a 30-min wash-out period before a 2^{nd} exposure with the same agonist for 10 s. B) CHO-h sst_4 cells were challenged for 10 s with 1 μM J-2156, 3 μM SRIF-14 or 3 μM compound A (1^{st} exposure, control). Pretreated cells (pretreatment as indicated by the vertical labels inside the graph) were challenged again after a 30-min wash-out period for another 10 s with a second SRIF receptor agonist (1 μM J-2156, 3 μM SRIF-14 or 3 μM compound A; 2^{nd} exposure). The response to the 2^{nd} exposure is significantly different from the response to the 1^{st} exposure (**, $p < 0.01$ and ***, $p < 0.001$; Student's unpaired *t* test).

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Fig. 5. Effect of pretreatment with 3 μM SRIF-14 on [^{35}S]GTPγS binding induced by SRIF-14. CHO-h sst_4 cells were incubated at 37°C in Ham's F12 medium in the absence (control cells; □) or presence of 3 μM SRIF-14 (pretreated cells; ■) for 4 h. After the incubation, the cells were washed extensively and membranes were prepared. The increase in [^{35}S]GTPγS binding is given as % stimulation over basal. The combined data of four different experiments performed in duplicate are shown.

Fig. 6. The propensity of agonists to cause desensitization was plotted against the agonist efficacy. For each agonist, the desensitization (%) as determined in the microphysiometry assay is shown on the *x*-axis, whereas the efficacy (%) as determined in the [^{35}S]GTPγS binding assay is plotted on the *y*-axis. The % desensitization was calculated from the difference between the extracellular acidification rate (EAR) response due to the first compared to the second agonist exposure (EAR responses are shown in Fig. 4). The efficacy (%) was normalized against SRIF-14 (set as 100 %; efficacies are given in Table 1).

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Table 1

Agonist activity (EC_{50} and efficacy relative to SRIF-14) was assessed as the ability of the compounds to stimulate [^{35}S]GTPγS binding in membranes of CHO cells expressing the human sst_4 (CHO-h sst_4). Under routine testing conditions (100 mM NaCl and 10 µM GDP), SRIF-14 concentration-dependently increased [^{35}S]GTPγS binding to give fitted curve maxima of ~110 % over basal, while the corresponding value under lower NaCl and GDP conditions (20 mM NaCl and 3 µM GDP) was ~45 % over basal. Data represent means ± S.E.M. from at least three experiments. Representative curves for J-2156, compound A, SRIF-14 and CST-17 are shown in Fig. 1.

	100 mM NaCl, 10 µM GDP		20 mM NaCl, 3 µM GDP	
	EC_{50} (nM)	Efficacy (normalized against SRIF-14)	EC_{50} (nM)	Efficacy (normalized against SRIF-14)
J-2156	17 ± 1	340 ± 20	3.9 ± 1.0	300 ± 20
compound A	15 ± 4	210 ± 10	3.4 ± 1.7	270 ± 40
LTT-SRIF-28	80 ± 15	120 ± 10	n.d.	n.d.
SRIF-28	7.8 ± 1.9	110 ± 10	n.d.	n.d.
SRIF-14	6.5 ± 0.7	100	4.4 ± 0.7	100
CST-17	46 ± 6	93 ± 7	27 ± 11	120 ± 20

Agonist activities of J-2156, SRIF-14 and LTT-SRIF-28 as determined in the presence of 100 mM NaCl and 10 µM GDP have been reported by us earlier (Engström et al., 2005).

Figure 1

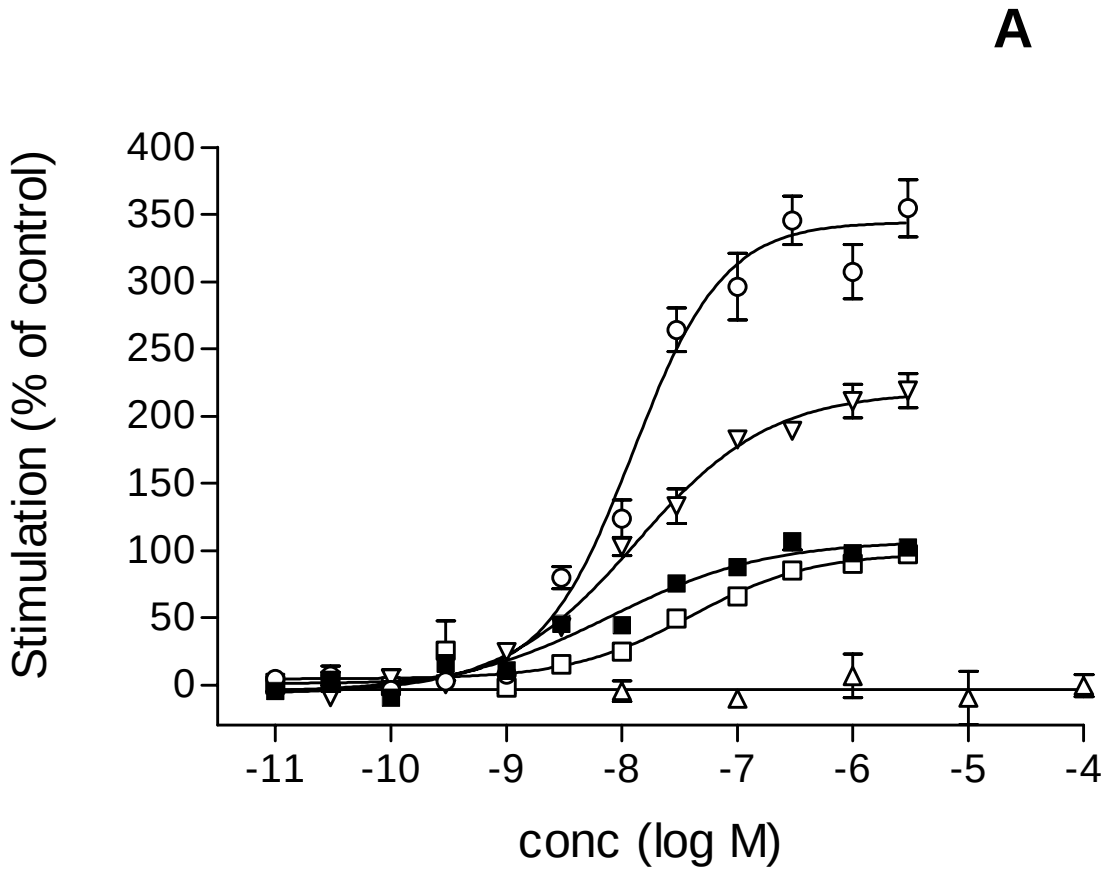


Figure 1

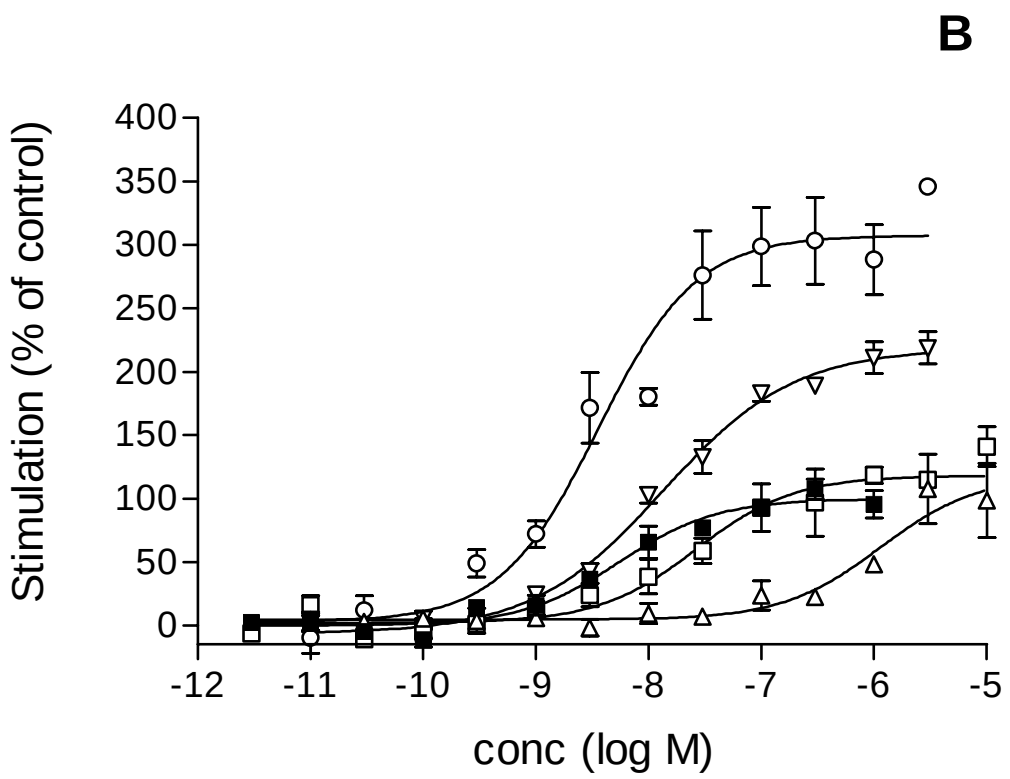
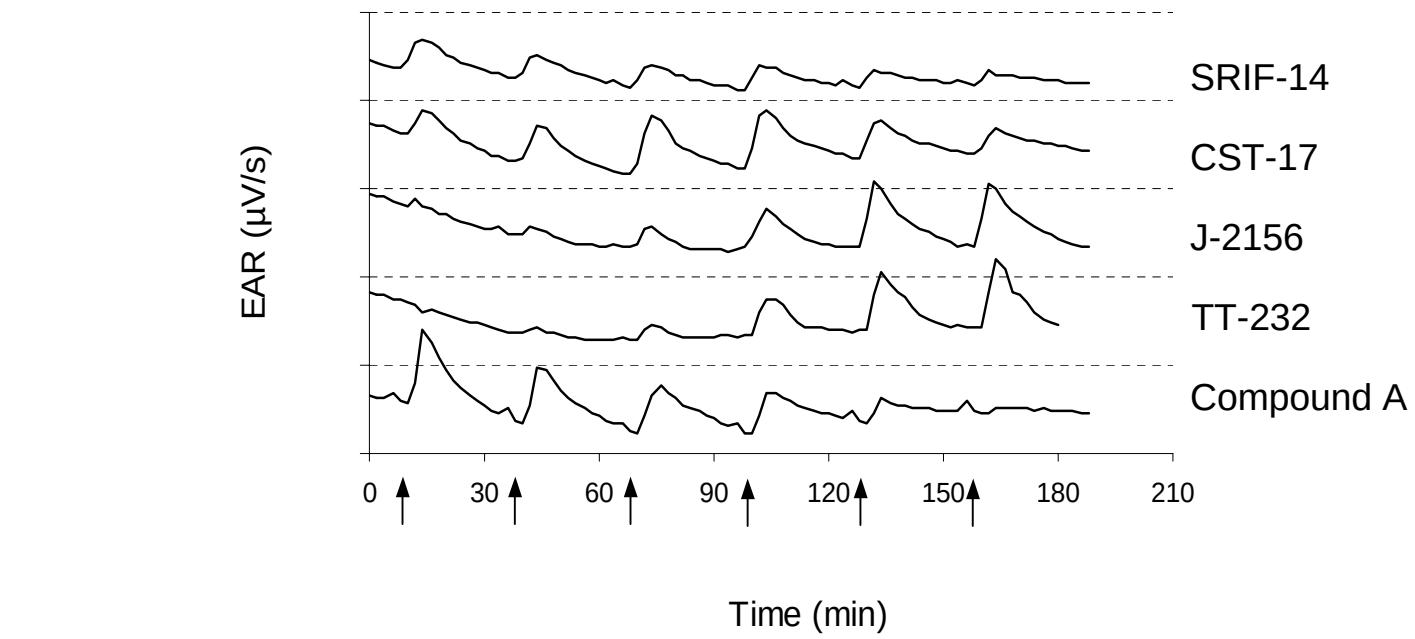


Figure 2A



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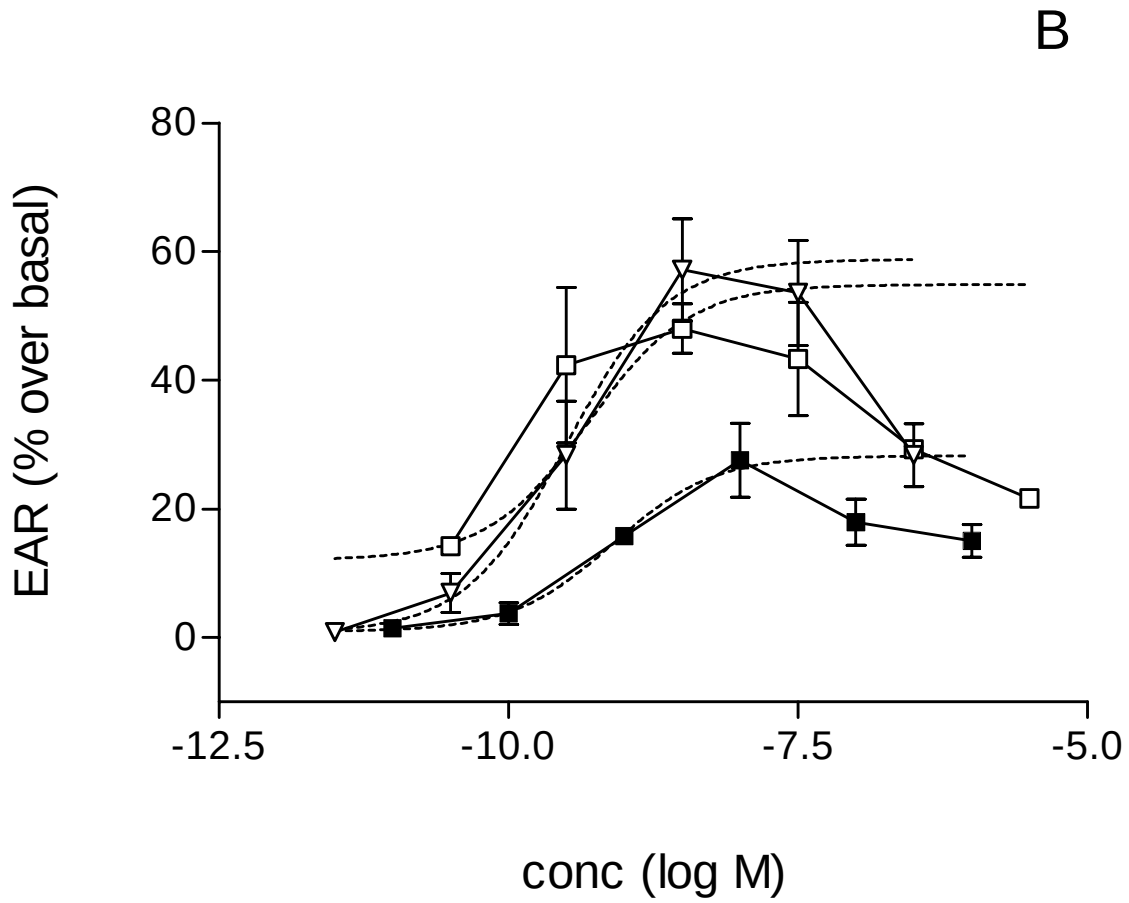

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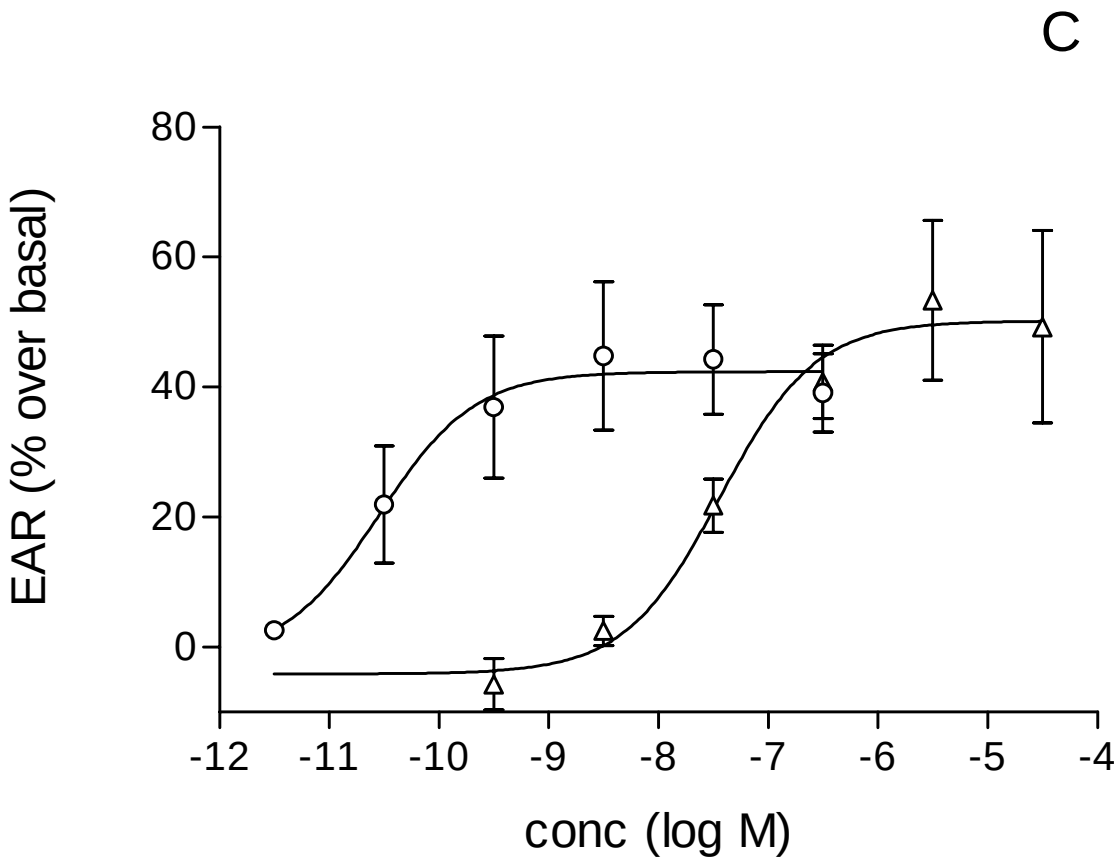

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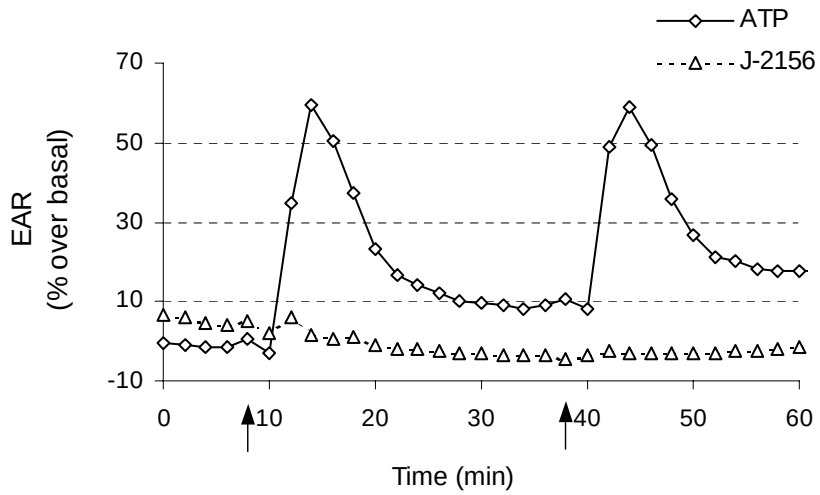

Figure 3B

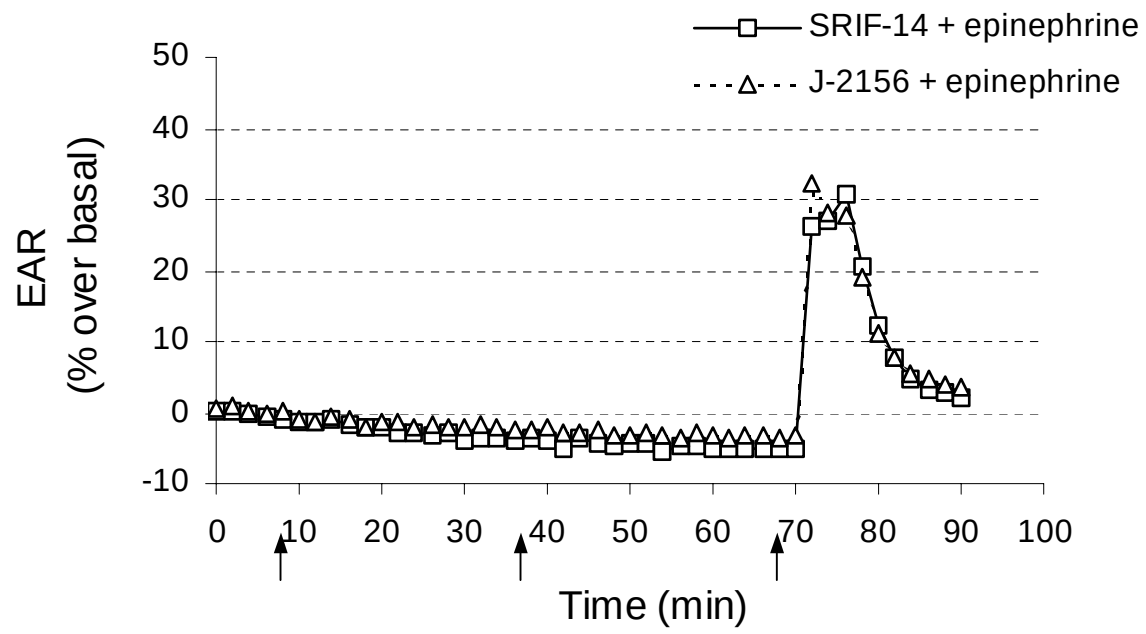

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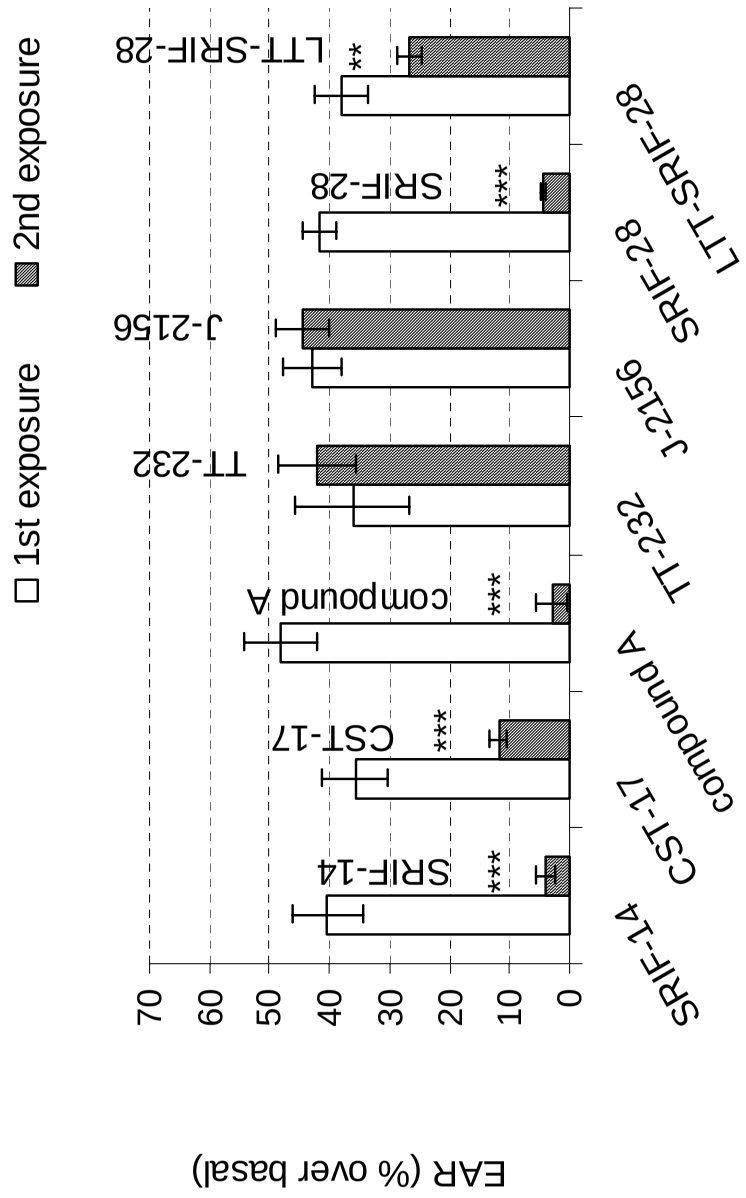
Figure 4A



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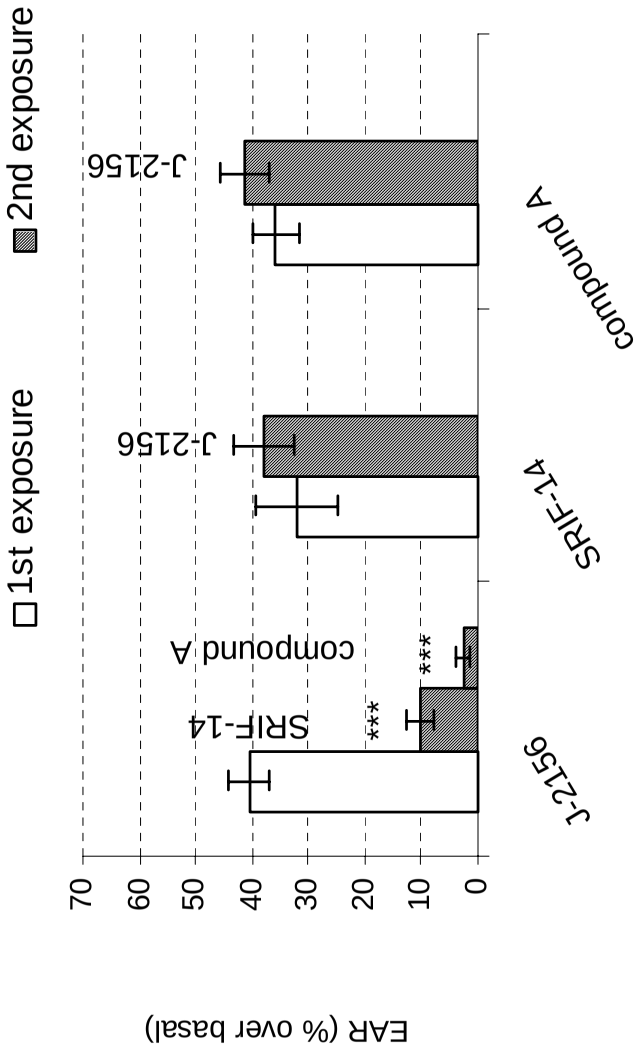

Figure 4B

Figure 5

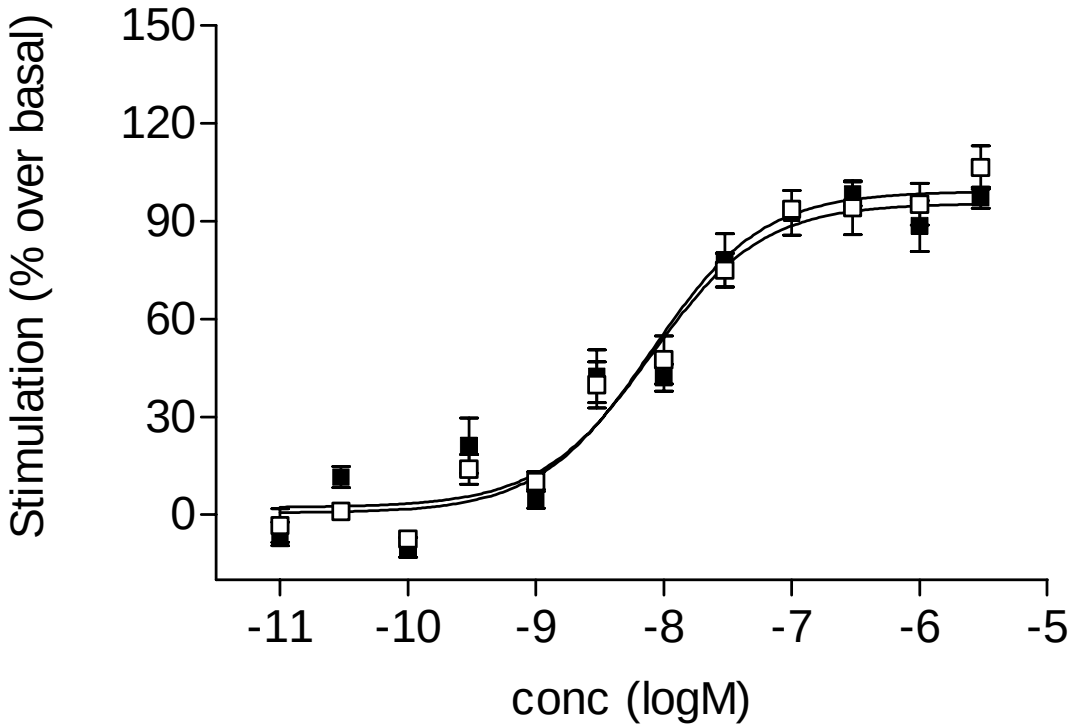

Figure 6