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Differential agonist activity of somatostatin and L-362855 at human recombinant sst_4 receptors

[1,2]K.S.M. Smalley, [1]W. Feniuk & [1]P.P.A. Humphrey

[1]Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 The operational characteristics of somatostatin (SRIF) sst_4 receptors are poorly understood. In this study, we have characterized human recombinant sst_4 receptors expressed in CHO cells ($CHOsst_4$) by radioligand binding and microphysiometry.

2 Increasing concentrations SRIF or other SRIF receptor ligands inhibited specific [^{125}I]-Tyr^{11}-SRIF binding in $CHOsst_4$ cell membranes with respective pIC_{50} values of SRIF (8.82), L-362855 (7.40), BIM-23027 (<5.5) and MK-678 (<5.5).

3 These ligands displayed agonist activity, producing concentration-dependent increases in rates of extracellular acidification (EAR) with pEC_{50} values of SRIF (9.6) and L-362855 (8.0), respectively. BIM-23027 and MK-678 were at least 1000 times weaker than SRIF. The SRIF maximum was about 40% of that observed with L-362855.

4 In the presence of SRIF (0.1–1 nM), concentration-effect curves to L-362855 were displaced to the right with a progressive reduction in the L-362855 maximum.

5 When cells were only exposed to a single maximally effective concentration of SRIF or L-362855, there was no difference in the magnitude of the agonist-induced increase in EAR. However, a second agonist challenge, 30 min later showed that responses to SRIF but not L-362855 were markedly desensitized.

6 When concentration-effect curves to SRIF and L-362855 were obtained by combining data from cells exposed to only a single agonist concentration, SRIF (pEC_{50} 9.2) was approximately 20 times more potent than L-362855 (pEC_{50} 8.0) but the maxima were the same. Responses to both SRIF and L-362855 were abolished by pertussis toxin.

7 SRIF and L-362855-induced increases in EAR were inhibited by N-ethyl isopropyl amiloride (10 μM) but were not modified by inhibitors of PKC (Go-6976), MAP kinase (PD-98059), tyrosine kinase (genistein) or tyrosine phosphatase (sodium orthovanadate).

8 The results suggest that SRIF-induced increases in EAR in $CHOsst_4$ cells involved activation of the Na^+/H^+ antiporter and were mediated *via* Gi/Go G proteins. Responses to SRIF, but not L-362855, were subject to marked desensitization which may be a consequence of differential activation of receptor-effector coupling pathways.

Keywords: Somatostatin; sst_4 receptors; SRIF; L-362855; CHO cells; microphysiometry; desensitization

Introduction

Somatostatin (Somatotropin Release Inhibitory Factor, SRIF) is a 14-amino acid peptide with a wide range of effects in both the peripheral and central nervous systems (Hoyer *et al*., 1994; Schindler *et al*., 1996). SRIF, the N-terminally extended form, SRIF-28, and a possible third endogenous ligand, cortistatin (Fukusumi *et al*., 1997), have been shown to interact with a family of five heptahelical G-protein linked receptors named sst_1–sst_5 (Hoyer *et al*., 1995) which can be subdivided into two groups, $SRIF_1$ and $SRIF_2$, on the basis of amino acid sequence homology as well as operational characteristics. To date, more investigative work has focused upon receptors belonging to the $SRIF_1$ group (sst_2, sst_3 and sst_5). Selective agonists and antagonists for some of these receptors have now been identified which has allowed the study of these receptors in both heterologous expression systems as well as tissues (Wilkinson *et al*., 1996; 1997; Bass *et al*., 1996; Lauder *et al*., 1997; Hicks *et al*., 1998). In contrast, little is known about the $SRIF_2$ receptor group (sst_1 and sst_4), with most experimental work being confined to radioligand binding studies on cell membranes expressing recombinant receptors, which show that the cyclic hexapeptides, such as BIM-23027 and MK-678 have a low affinity for these receptors (Raynor *et al*., 1993; Patel & Srikant, 1994). When expressed in CHO-K1 cells, the rat sst_4 receptor has been shown to mediate stimulation of MAP kinase, the release of arachidonic acid and inhibition of adenylyl cyclase in a pertussis toxin-sensitive manner (Bito *et al*., 1994). Until very recently (see Liapakis *et al*., 1996; Ankersen *et al*., 1998), no selective ligands for sst_1 or sst_4 receptors had been described and as a consequence, the operational characteristics of these receptors have largely been defined on the basis of exclusion criteria.

The functional significance of endogenously expressed sst_4 receptors is at present unknown. Receptor localisation studies using Northern blotting, *in situ* hybridization and RT–PCR analyses have revealed the presence of the sst_4 receptor type in both the brain, particularly the cortex and hippocampus, as well as the lung (Bruno *et al*., 1992; Rohrer *et al*., 1993; Harrington *et al*., 1995; Schloos *et al*., 1997). In a recent study in *Xenopus* oocytes co-expressing rat recombinant sst_4 receptors and a G-protein coupled inwardly rectifying potassium channel, SRIF-induced activation of an inward K^+ current did not desensitize (Kreienkamp *et al*., 1997). However, SRIF-induced internalization of sst_4 receptors has

[2]Author for correspondence.

been reported in CHO-K1 cells (Hukovic *et al*., 1996) whilst the same receptor expressed in HEK cells reportedly did not internalize (Roth *et al*., 1997).

The technique of microphysiometry allows continuous monitoring of cellular metabolic activity by measuring extracellular acidification rates (EAR) (McConnell *et al*., 1992) and has the advantage of being non-invasive. Using this technique, the characteristics of recombinant sst_2 receptor expressed in Ltk^- cells (Castro *et al*., 1996) or CHO-K1 cells (Taylor *et al*., 1996) have been shown to be similar to those seen in native tissues (Chessell *et al*., 1996; Wyatt *et al*., 1996; Koenig *et al*., 1997). In the present study we have investigated the effect of SRIF, BIM-23027, MK-678 and L-362855 on the extracellular acidification rate (EAR) of human sst_4 receptors recombinantly expressed in CHO-K1 cells. L-362855 was chosen as part of this analysis since its affinity at sst_4 receptors lie between the affinity of SRIF and the cyclic hexapeptides, BIM-23027 and MK-678 (Raynor *et al*., 1993). The mechanism of SRIF-induced increases in EAR and the susceptibility of these responses to desensitization have also been investigated.

Preliminary accounts of some of the findings have been presented to the British Pharmacological Society (Smalley *et al*., 1997; 1998).

Methods

Cell culture

The cDNA encoding the human sst_4 (GlaxoWellcome, Stevenage, U.K.) was subcloned into CHO-K1 cells using the mammalian expression vector pCIN4 harbouring a neomycin resistant gene as a selection marker. Transfection was achieved using 10 μg of sst_4-pCIN4/0.5×10^6 cells using a cationic liposome formulation-mediated transfer (LipofectAMINE™, Life technologies). Clonal cell lines expressing the cDNA were isolated by single cell cloning and receptor expression assessed by binding of [^{125}I]-[Tyr^{11}]-SRIF. Cells were grown in monolayer culture in Dulbecco's modified Eagles medium (DMEM)/Hams F-12 (1:1) mix supplemented with Glutamax 1 (1 mM), 10% foetal calf serum and G418 (0.5 mg ml^{-1}). Cultures were maintained at 37°C in a 5% CO_2/humidified air atmosphere and were used between passage 10–40.

Cell membrane preparation

CHO sst_4 cells were homogenized in assay buffer [50 mM Tris-HCl (pH 7.4) 5 mM $MgCl_2$, 10 μg ml^{-1} leupeptin, 1 μg ml^{-1} soyabean trypsin inhibitor, and 0.2 mg ml^{-1} bacitracin] in a Dounce glass homogenizer (50 strokes, 4°C). The homogenate was centrifuged at $500 \times g$ for 10 min at 4°C and the supernatant spun at $20{,}000 \times g$ for 30 min at 4°C. The resultant pellet was re-suspended in cold assay buffer and stored in 400 μL aliquots at −70°C.

Radioligand binding assays

Cell membranes were incubated with 0.03 nM [^{125}I]-[Tyr^{11}]-SRIF (Amersham, U.K.) and increasing concentrations of competing ligand for 90 min at room temperature. Non-specific binding was defined with 1 μM cold SRIF. The assay was terminated by rapid filtration through Whatman GF/C glass fibre filters soaked in 0.5% polyethylenimine (PEI), followed by 7×3 ml washes of 50 mM Tris-HCl. Membrane radioactivity was determined using a Canberra Packard Cobra II auto-γ counter. To calculate K_d and B_{max} values from competition studies with SRIF, the following 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 SRIF and [A] is the concentration of [^{125}I]-[Tyr^{11}]-SRIF present: $B_{max} = ([B].\ IC_{50})/[A]$, where B_{max} is the receptor density and [B] is the concentration of specific [^{125}I]-[Tyr^{11}]-SRIF bound in the absence of competing ligand.

Extracellular acidification

Cells were seeded out into disposable polycarbonate membrane cell capsules, at a density of 5×10^5 cells per cup, and were maintained in serum containing DMEM/Hams F-12 (1:1) mix supplemented with Glutamax and G418 (0.5 mg ml^{-1}) at 37°C in a 5% CO_2/humidified air atmosphere for 18 h until the start of the experiment. To measure the rate of acidification, cells were loaded into the chambers of the Cytosensor® microphysiometer (Molecular Devices). The chambers were perfused with a bicarbonate-free medium DMEM supplemented with 1 mg ml^{-1} bovine serum albumin (BSA). Cells were perfused with media for 43 s, after which the pump was stopped for 10 s, allowing the accumulation of metabolic by products. When the flow was resumed the acid was washed out. Agonists remained in contact with the cells for a period of 3 min 40 s which was sufficient time for responses to plateau. In some experiments cells were treated with pertussis toxin (100 ng ml^{-1}) for 18 h before use.

After an initial 30 min period of equilibration and a UTP challenge (3 μM), cells were allowed to equilibrate for 90 min. Sequential concentration-effect curves were constructed to the SRIF agonists with dosing at 30 min intervals. In some experiments responses to L-362855 were obtained in the continuous presence of different concentrations of SRIF (0.1–1 nM).

Concentration-effect curves to SRIF and L-362855 were also constructed by exposing cells to only a single agonist challenge and responses normalized to the initial response to UTP. In order to study the decay time of the SRIF and L-362855 induced increases in EAR response, single challenges of either SRIF (30 nM) or L-362855 (300 nM) were applied continuously for 30 min. The decay in response was calculated in terms of the initial peak response to either SRIF or L-362855 and the $t_{1/2}$ determined by fitting the data to a single site exponential decay curve (Graph Pad Prism).

The increases in EAR responses were internally controlled by expressing them as a percentage of the initial challenge to UTP in the same cells (3 μM).

Desensitization of SRIF and L-362855-induced increase in EAR response

After equilibration, cells were challenged with a single concentration of SRIF (30 nM) or L-362855 (300 nM) for 3 min 40 s and re-challenged at either a 30, 60, 120 or 180 min time-point. In some studies, these experiments were repeated in the presence of various inhibitors or pertussis toxin.

Statistical analysis

Unless otherwise stated, all values are means ± s.e.mean from at least four experiments. All pEC_{50} values were determined from individual experiments by non-linear regression, using a four parameter logistic equation (GraphPad Prism). Statistical comparisons of responses were made by means of unpaired *t*-tests and statistically significant differences were noted when $P < 0.05$.

Drugs and reagents

Unless otherwise stated, all reagents were purchased from Sigma. Tissue culture media were from Life Technologies, Paisley, U.K. and tissue culture ware from Costar. SRIF was obtained from Peninsular Laboratories Europe Ltd (St. Helens, Merseyside, U.K.) MK-678 (c[N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe]) and BIM-23027 (c[N-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe]) were synthesized by Dr J. Murray's team (GlaxoWellcome Chemistry Unit, University of Cambridge, U.K.). L-362855 (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe]) was custom synthesized by Protein Research Consultants (University of Exeter, U.K.). PD-98059 [2′-amino-3′-methoxyflavone], Gö-6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-indolo(2-3-a) pyrrolo(3,4-c) carbazole]; EIPA, ethylisopropyl amiloride, sodium orthovanadate, genistein and pertussis toxin were from Calbiochem, U.K.

Results

Radioligand binding assays

In CHOsst$_4$ cell membranes, SRIF (pIC$_{50}$, 8.82±0.02; nH 0.97±0.13) caused a concentration-dependent inhibition of specific [^{125}I]-[Tyr^{11}]-SRIF binding. It was calculated that [^{125}I]-[Tyr^{11}]-SRIF had an estimated K$_d$ value of 1.60±0.18 nM and that the density of specific binding sites (B$_{max}$) was 9.38±0.46 pmol mg^{-1} protein.

Competition studies (Figure 1) were also carried out with a number of SRIF-analogues. Although SRIF displayed the highest affinity, L-362855 (pIC$_{50}$, 7.40±0.06; nH 0.74±0.11) also inhibited specific [^{125}I]-[Tyr^{11}]-SRIF binding. The sst$_2$ receptor selective peptides, MK-678 and BIM-23027 (pIC$_{50}$ < 5.5) were weak displacers.

Effects of SRIF and analogues upon EAR

After equilibration, basal EAR rates were 100–300 μV s^{-1} (0.10–0.30 pH units min^{-1}) and the mean increase in EAR in response to the initial challenge with UTP (3 μM) was 128.6±14.7 μV s^{-1}. Progressively increasing concentrations of SRIF (0.01–30 nM) caused concentration-dependent increases in EAR (pEC$_{50}$ 9.69±0.23), with a maximal EAR response of 19.6±0.90% of the UTP response. The concentration-effect curve was found to be bell-shaped, with supramaximal SRIF concentrations causing smaller increases in EAR (Figure 2). L-362855 (pEC$_{50}$ 8.01±0.06) also caused increases in EAR (Figure 2) but surprisingly the maximum was approximately 250% of the SRIF maximum (55.9±7.60% of the UTP response) (Figure 3). Both BIM-23027 and MK-678 were weak at stimulating increases in EAR and clearly defined maxima were not obtained in concentrations up to 1 μM (Figure 3). Neither SRIF nor L-362855 (in concentrations up to 300 nM) had any effect on extracellular acidification rates in non-transfected CHO-K1 cells (data not shown).

Effect of SRIF on L-362855-induced increases in EAR

Continuous exposure to CHOsst$_4$ cells to SRIF (0.1–1 nM) caused a concentration-dependent inhibition of L-362855-induced increases in EAR. The L-362855 concentration-effect curve was displaced to the right with a progressive reduction in the maximum response (Figure 4). The lowest concentration of SRIF examined (0.1 nM) caused an approximate 20 fold rightward displacement in the L-362855 concentration-effect curve and in the presence of a higher concentration of SRIF (1 nM) responses to L-362855 were abolished. SRIF (0.3 nM) had no effect on the increase in EAR produced by UTP (3 μM) (190±14 μV s^{-1} and 196±14 μV s^{-1} in the absence and presence of SRIF, respectively).

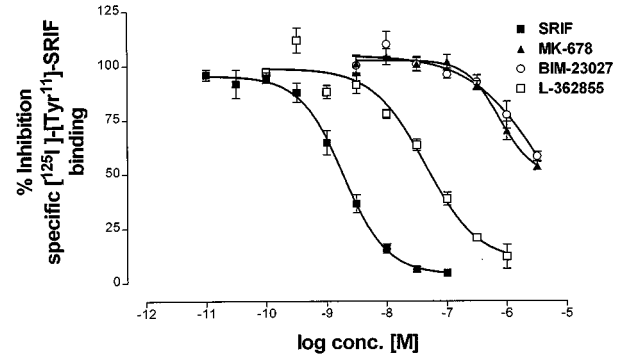

Figure 1 Inhibition of specific [^{125}I]-[Tyr^{11}]-SRIF binding in CHO-K1 cell membranes expressing human recombinant sst$_4$ receptors. All values shown are means±s.e.mean from three experiments.

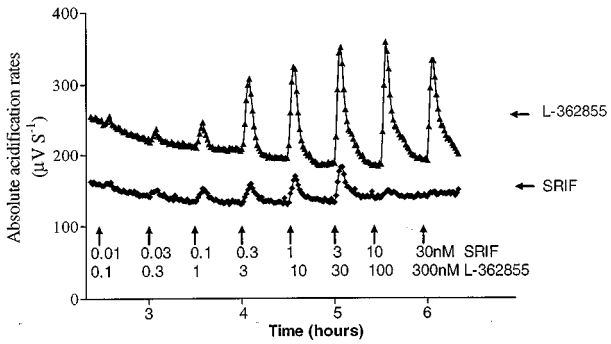

Figure 2 Representative microphysiometer recording of SRIF (0.01–30 nM) and L-362855 (0.1–300 nM)-induced increases in extracellular acidification rates in CHO-K1 cells expressing human recombinant sst$_4$ receptors. Note the diminished responses with high SRIF concentrations and the greater maximal effects of L-362855.

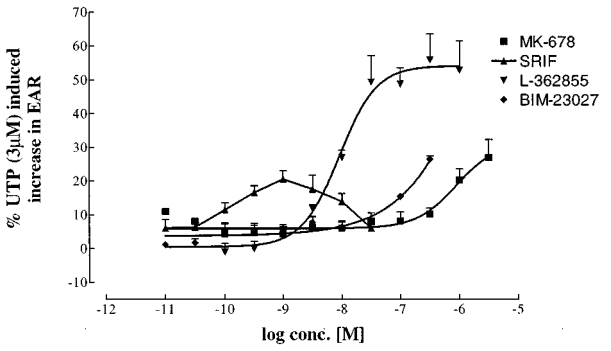

Figure 3 Effect of increasing concentrations of SRIF ligands on extracellular acidification rates of CHO-K1 cells expressing human recombinant sst$_4$ receptors. Agonists were applied for 3 min 40 s at 30 min intervals in progressively increasing concentrations. Data are expressed as a percentage of the initial increase in acidification produced by a single UTP challenge at the start of the experiment. All values are means±s.e.mean from at least four experiments.

Concentration-effect curves to SRIF and L-362855 constructed from single challenges

Concentration-effect curves were also constructed to SRIF and L-362855 after cells were challenged with a single agonist concentration. SRIF (pEC_{50} 9.2 ± 0.1) was approximately 20 times more potent than L-362855 (pEC_{50} 8.0 ± 0.3). However, in contrast to the data obtained by sequentially increasing agonist concentrations (see Figure 3), there was no significant difference in the maxima for SRIF ($89.4 \pm 12.5\%$ of UTP response) and L-362855 ($78.8 \pm 17.2\%$ of UTP response) (Figure 5). Pre-treatment of cells with pertussis toxin (100 ng ml^{-1}) abolished the responses to both SRIF and L-362855 (Figure 5).

Desensitization of SRIF and L-362855-induced increases in EAR

SRIF-induced increases in EAR in $CHOsst_4$ cells were highly susceptible to desensitization. Following an initial challenge with a just maximally effective concentration of SRIF (30 nM), responses to a second challenge, 30 min later, were markedly decreased. Indeed, responses had not recovered even when the interval between successive challenges was increased to 180 min (Figure 6a). In marked contrast, L-362855-induced increases in EAR were highly reproducible when cells were re-challenged at 30 min intervals although re-challenging at 10 min resulted in a significant reduction in the agonist response (Figure 6b).

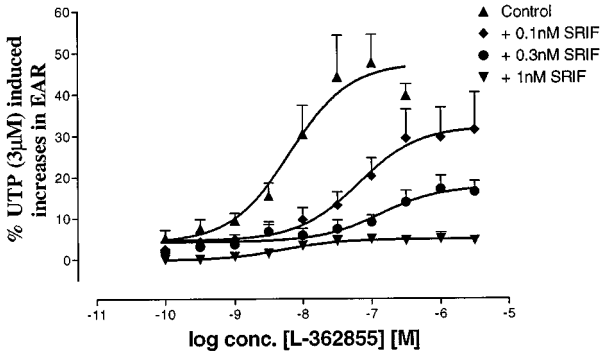

Figure 4 L-362855-induced increases in extracellular acidification rates in CHO-K1 cells expressing human recombinant sst_4 receptors in the absence and continual presence of SRIF (0.1–1.0 nM) L-362855 was given 3 min 40 s at 30 min intervals in progressively increasing concentrations. Data are expressed as percentage of the initial increase in acidification produced by a single UTP challenge at the start of the experiment. All values are means ± s.e.mean from at least four experiments.

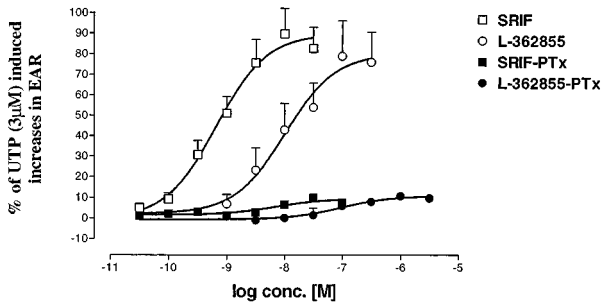

Figure 5 Effect of increasing concentrations of SRIF and L-362855 on extracellular acidification rates of CHO-K1 cells expressing human recombinant sst_4 receptors in untreated cells and cells pretreated with pertussis toxin (100 ng ml^{-1}). Data are expressed as percentage of the initial increase in acidification produced by a single UTP challenge at the start of the experiment. Concentration-effect curves were obtained by pooling the data from cells exposed to only a single agonist challenge with either SRIF or L-362855. All values are means ± s.e.mean from at least four experiments.

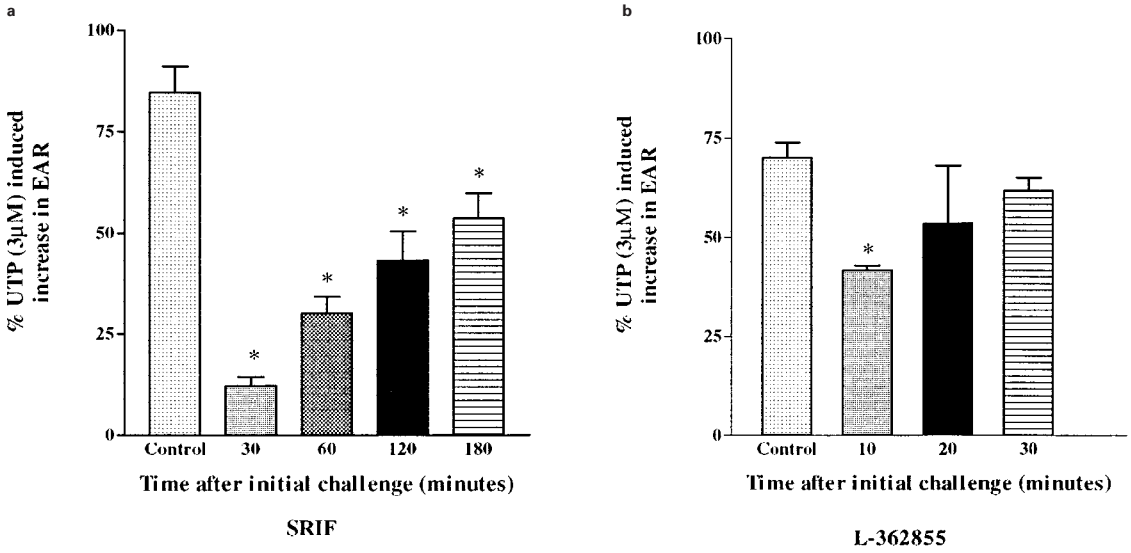

Figure 6 SRIF (30 nM) and L-362855 (300 nM) induced increases in extracellular acidification rates of CHO-K1 cells expressing human recombinant sst_4 receptors. Cells were given an initial single agonist challenge which was then repeated on a second occasion at either 30, 60, 120 or 180 min after the first challenge in the case of SRIF (a) or 10, 20 or 30 min in the case of L-362855 (b). Data are expressed as percentage of the initial increase in acidification produced by a single UTP challenge at the start of the experiment. All values are means ± s.e.mean from at least four experiments. *Significantly different from control ($P < 0.05$).

In order to study the fade of the EAR response, cells were exposed to a single prolonged (30 min) supramaximal challenge of either SRIF (30 nM) or L-362855 (300 nM). The magnitude of EAR response reached was similar for both SRIF and L-362855 (98.5±4.4 and 118.2±12.0% of the UTP response, respectively). Continuous application of SRIF and L-362855 was associated with a time-dependent fade from the peak of the agonist-induced increase in EAR which was similar for both SRIF and L-362855 ($t_{1/2}$ 332±94 and 282±98 s, respectively).

The effect of inhibitors upon SRIF and L-362855 induced changes in EAR

In order to determine the possible mechanism of SRIF and L-362855-induced increases in EAR, a range of inhibitors were perfused over the cells for 30 min before challenge with either a single concentration of SRIF (30 nM) or L-362855 (300 nM). The protein tyrosine kinase inhibitor, genistein (50 μM) and the Na^+/H^+ exchanger inhibitor, EIPA (10 μM), led to decreases in basal acidification rate of 47.9±3.4% and 16.6±1.4%, respectively, whilst the protein kinase C inhibitor, Go-6976 (100 nM), led to increases in basal acidification rate of 34.6±2.8%. Neither the MAP kinase inhibitor, PD-98059 (10 μM), nor the tyrosine phosphatase inhibitor, orthovanadate (5 μM), affected basal EAR.

Responses to SRIF and L-362855 were significantly ($P<0.05$) reduced by EIPA (Table 1) but were unaltered by any of the other inhibitors (Table 1).

The reduced EAR response following a second challenge with SRIF (30 nM) at 30 min intervals was not altered when cells were pre-incubated with Go-6976, orthovanadate, genistein or PD-98059 (Figure 7).

Discussion

Somatostatin modifies cellular function by activating specific receptors belonging to the seven transmembrane spanning super-family of G protein-coupled receptors. Five receptor types exist and on the basis of structural and operational characteristics, these receptors can be divided into two groups. Thus the $SRIF_1$ groups which comprises sst_2, sst_3 and sst_5 is clearly distinct from the $SRIF_2$ group and comprises the sst_1 and sst_4 receptor types. At the outset of this study, no selective tools were available to study sst_4 receptors. Since SRIF receptors show a widespread and in many instances an overlapping distribution both in the brain and periphery (Schindler *et al.*, 1996), little is known about their function. The objective therefore of the current study was to study the characteristics of the human sst_4 receptor recombinantly expressed in CHO-K1 cells ($CHOsst_4$ cells), using the technique of microphysiometry which monitors cellular metabolic activity by measuring changes in the rate of extracellular acidification (EAR). This technique of microphysiometry has been successfully employed to characterize human recombinant sst_2 receptors expressed in both CHO-K1 cells and mouse fibroblast Ltk^- cells (Castro *et al.*, 1996; Taylor *et al.*, 1996).

Increasing concentrations of SRIF, L-362855 or the cyclic hexapeptides, BIM-23027 and MK-678, perfused over $CHOsst_4$ cells at 30 min intervals, caused concentration-dependent increases in EAR. SRIF was the most potent agonist studied being approximately 50 times more potent than L-362855 and at least a 1000 times more potent than the cyclic hexapeptides, BIM-23027 and MK-678. The relative potencies of these peptides to cause increases in EAR were closely paralleled by their ability to inhibit specific [^{125}I]-Tyr^{11}-SRIF binding from $CHOsst_4$ cell membranes. Thus SRIF displayed the highest binding affinity whilst the cyclic hexapeptides, BIM-23027 and MK-678 were weak, confirming data obtained from previously published studies on human as well as rat sst_4 receptors (Bruno *et al.*, 1992; Xu *et al.*, 1993a; Patel & Srikant, 1994; Bruns *et al.*, 1996). As was seen in the microphysiometry studies, L-3628855 was approximately 50 times weaker than SRIF as an inhibitor of specific [^{125}I]-[Tyr^{11}]-SRIF binding to $hsst_4$ receptors. The similarity in the affinity estimates from the ligand binding studies on $CHOsst_4$ cell membranes and their

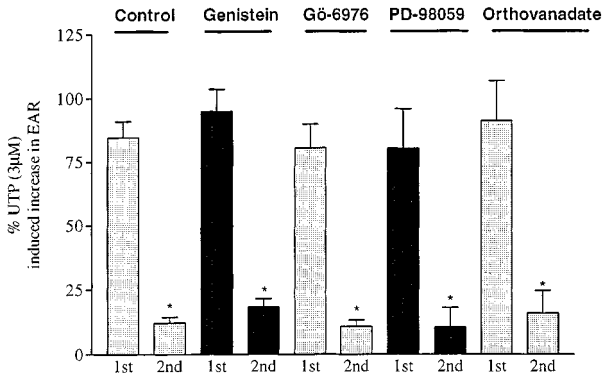

Figure 7 SRIF(30 nM)-induced desensitization of EAR responses in the absence (control) and presence of inhibitors of PKC (Go-6976, 100 nM), tyrosine kinase (genistein 50 μM), or MAPkinase (PD-98059, 10 μM or sodium orthovanadate (5 μM). All values are means±s.e.mean from at least four experiments. *Significantly different from control ($P<0.05$), unpaired *t*-test.

Table 1 SRIF and L-362855-induced increases in extracellular acidification rates in CHO-K1 cell expressing human recombinant sst_4 receptors in the absence (control) and presence of various inhibitors

		% UTP (3 μM) induced increase in EAR		*% UTP (3 μM) induced increase in EAR*	
Treatment	*UTP* ($\mu V\ s^{-1}$)	*SRIF control*	*SRIF treatment*	*L-362855 control*	*L-362855 treatment*
Go-6976 (100 nM)	145.0±8.2	75.0±7.5	80.5±9.4	78.8±9.0	74.2±11.1
PD-98059 (10 μM)	147.4±11.6	77.7±18.8	80.3±15.5	60.7±11.9	66.3±9.4
EIPA (10 μM)	178.2±14.4	66.0±4.8	15.4±3.3*	102.1±14.2	25.2±7.5*
Genistein (50 μM)	147.1±19.2	92.5±15.3	95.0±8.6	71.8±4.1	93.3±16.1
Orthovanadate (5 μM)	117.6±10.8	95.0±22.0	91.0±15.8	85.6±11.0	71.7±6.1

Responses were measured as a percentage of an initial UTP (3 μM) challenge. All values are means±s.e.mean from at least four experiments. Values marked* are significantly different from control value at $P<0.05$ (unpaired *t*-test).

equivalent agonist potency estimates in whole cells in the microphysiometry studies suggests that the receptors were reasonably well coupled to the transduction processes leading to increases in extracellular acidification. Surprisingly however, the response maximum to SRIF was approximately 40% of that produced by L-362855. One possible interpretation of this data was that SRIF was acting as a partial agonist or alternatively L-362855-induced increases in EAR in the $CHOsst_4$ cells were a consequence of activation of additional receptors, endogenously expressed in the $CHOsst_4$ cells. The suggestion that a natural ligand could be acting as a partial agonist is not unprecedented. Thus 5-HT has been claimed to act as a partial agonist at $5\text{-}HT_2$ receptors mediating contraction of rabbit isolated aorta (Barrett *et al*., 1986). In an attempt to obtain evidence that SRIF and L-362855 were acting at a common site, concentration-effect curves to L-362855 were obtained in the continuous presence of different concentrations of SRIF. In these studies, SRIF (0.1 – 1.0 nM) caused a concentration-dependent blockade of L-362855-induced increases in EAR. Concentration-effect curves to L-362855 were displaced to the right with a progressive reduction in the L-362855 maximum. At the highest concentration tested, responses to L-362855 were abolished. The antagonism was specific in that UTP-induced increases in EAR were unaltered when cells were continuously exposed to SRIF. The results from these studies suggest that SRIF and L-362855 were interacting with a common receptor site, however the potency of SRIF at blocking L-362855-induced increases in EAR and the non-surmountable characteristics of the blockade are difficult to reconcile with the simple interaction between a partial and full agonist interacting at a single receptor site (Kenakin, 1993) suggesting that alternative mechanisms were responsible. One possibility was that responses to SRIF, but not L-362855, were more susceptible to agonist-induced receptor desensitization and that the reduction of L-362855-induced increases in EAR was a consequence of a progressive SRIF-induced receptor desensitization. Selective agonist-induced desensitization has been demonstrated at mu, kappa and delta opioid receptors (Sternini *et al*., 1996; Blake *et al*., 1997; Cvejic & Devi, 1997).

Further studies were therefore carried out to compare the reactivity of the $CHOsst_4$ cells to repeat challenges of maximally effective concentrations of SRIF and L-362855. When cells were repeatedly exposed to a maximally effective concentration of either SRIF or L-362855 at 30 min intervals, the second response to SRIF, but not L-362855, was markedly attenuated suggesting that SRIF but not L-362855 had induced sst_4 receptor desensitization. Responses to SRIF did not fully recover even when the interval between successive challenges was increased to 3 h. Construction of concentration-effect curves by combining the data from cells exposed to only a single agonist concentration again showed that SRIF was approximately 20 times more potent than L-362855 but the agonist maxima were now similar. These results suggest that when concentration-effect curves were obtained by progressively increasing the concentration of SRIF at 30 min intervals the receptors underwent a progressive desensitization. Although the responses to L-362855 showed no evidence that desensitization had occurred when repeat challenges were made at 30 min intervals, it is possible that when L-362855 was used as the agonist, recovery from desensitization had occurred before the second L-362855 challenge. The decline of peak responsiveness in the continuous presence of agonist had been attributed to desensitization of a component of the receptor/G protein/effector complex and waning to basal levels of such responses is indicative of full desensitization (Wojcikiewicz *et al*., 1993; Willars & Nahorski, 1995; Wilkinson *et al*., 1997). Experiments were therefore carried out to determine the kinetics of both the SRIF- and L-362855-induced increases in EAR when cells were continuously exposed to a maximally effective concentration of either agonist for 30 min. In these studies, the decline of the peak increase in EAR to both SRIF and L-362855 was similar, suggesting that either the 'fade' was not indicative of desensitization, or that when cells were challenged with brief single concentrations of L-362855 at 30 min intervals, sufficient time elapsed for resensitization to occur. When $CHOsst_4$ cells were re-challenged with L-362855 at 10 min intervals, the response to the second challenge was significantly reduced, although this was still less than that observed when SRIF was re-challenged at 30 min intervals. Since reproducible SRIF-induced increases in EAR could not be achieved, even when the interval between successive challenges was increased to 3 h, the mechanism of SRIF and L-362855-induced increases in EAR were subsequently studied by comparing the responsiveness of control (untreated) cells, exposed to only a single agonist challenge with cells which had been pre-treated with various drugs. Such a protocol also allowed the effect of these drugs on SRIF-induced receptor desensitization to be studied since the cells could be exposed to a second SRIF challenge 30 min later under conditions when SRIF receptor desensitization was apparent.

Both SRIF and L-362855 induced increases in EAR were prevented when cells were pre-incubated with pertussis toxin 18 h earlier, suggesting that the agonist-induced increases in EAR were exclusively mediated by G proteins of the Gi or Go types. This contrasts with data obtained in CHO-K1 cells expressing human recombinant sst_5 receptors where both pertussis toxin-sensitive and -insensitive responses have been identified (Thurlow *et al*., 1996; Williams *et al*., 1996). The SRIF- and L-362855-induced increases in EAR were markedly attenuated when cells were incubated with EIPA suggesting that the increased extrusion of protons into the extracellular medium was associated with increased cellular activity and subsequent activation of the Na^+/H^+ antiporter. Activation of sst_4 receptors expressed in CHO-K1 cells has been shown to be coupled to a number of different transduction pathways. In these cells SRIF mediates adenylate cyclase inhibition and activation of MAP kinase, and these effects have been shown to be abolished by pertussis toxin pre-treatment (Xu *et al*., 1993a; Bito *et al*., 1994; Sakanaka *et al*., 1994 and our own unpublished observations). Whilst pertussis toxin also abolished the increases in EAR-induced by both SRIF and L-362855, the MAP kinase inhibitor (PD98059) had little or no effect on SRIF- or L-362855-induced increases in EAR. The SRIF- and L-362855-induced increases in EAR also did not involve activation of PKC, tyrosine kinase or tyrosine phosphatase since responses were not modified by Go-6976, genistein or orthovanadate, respectively. The inability of the MAP kinase inhibitor to modify EAR responses to either SRIF or L-362855 is likely to result from the fact that these responses reflect the total increase in metabolic demand of the cell which results from the summation of the many diverse processes occurring as a consequence of receptor activation (McConnell *et al*., 1992).

Human sst_4 receptors have multiple potential serine threonine phosphorylation sites in the third intracellular loop and carboxyl terminus (Xu *et al*., 1993b; Rohrer *et al*., 1993) which may be important in regulating SRIF-induced desensitization as a consequence of either receptor phosphorylation and or internalization (Mayor *et al*., 1987; Hofland *et al*., 1995; Hipkin *et al*., 1997; Roth *et*

al., 1997). In the case of β_2-adrenoceptors, both mechanisms appear important since overexpression of receptor kinases can restore agonist-dependent sequestration of an otherwise internalization-defective receptor mutant (Ferguson *et al*., 1995). Receptor phosphorylation can occur *via* at least three distinct kinase activities, PKA, PKC and as already mentioned *via* members of the G protein-linked receptor kinase family (Shih & Malbon, 1996). As was observed with SRIF in the present study, nociceptin/orphanin FQ-induced increases in EAR in CHO cells expressing human recombinant ORL-1 receptors, is also susceptible to agonist-induced desensitization. Since this desensitization could be prevented by the PKC inhibitor, Go 6976 (Pei *et al*., 1997) we examined the effect of Go 6976 on SRIF-induced desensitization in CHOsst_4 cells. In our study, Go 6976 had no effect on SRIF-induced desensitization, suggesting that PKC was not involved in this effect. In addition, the SRIF-induced desensitization was not modified by genistein, orthovanadate or PD-98059.

The mechanism of SRIF-induced desensitization in CHOsst_4 cells observed in the present study is unknown. The desensitization was agonist specific in that the lower affinity agonist L-362855 was far less able to cause desensitization. A similar phenomenon has been described for other G protein-coupled receptors. Thus sst_5 receptors expressed in HEK cells are only internalized in the presence of SRIF-28, but not SRIF (Roth *et al*., 1997), and μ-opioid receptors are internalized by DAMGO but not by morphine (Arden *et al*., 1995), suggesting that in both cases the two agonists induce different conformations in the activated receptor and subsequent differential receptor signalling. Although rat recombinant sst_4 receptors are not internalized in HEK cells (Roth *et al*., 1997) or rat insulinoma 1046-38 cells (Roosterman *et al*., 1997), we do not know whether a similar situation exists with respect to the human counterpart expressed in CHO-K1 cells. It is possible that differential agonist desensitization seen in these studies, is a consequence of SRIF but not L-362855 induced internalization of human sst_4 receptors expressed in CHO cells. However given the very high receptor density in these cells and the marked desensitization which occurs with very low concentrations of SRIF, such a mechanism would seem unlikely to predominate.

One of the surprising findings from the present study was the inhibition of the L-362855-induced increases in EAR by concentrations of SRIF as low as 0.1 nM which caused only small increases in EAR. As has been mentioned the degree of antagonism of the L-362855-induced increases in EAR was not consistent with the simple interaction of a full and partial agonist interacting at a single site. It appears that at least in the case of SRIF, the ability to induce desensitization is greater than its ability to increase EAR. Similar findings have been observed for neurotensin-induced increases in EAR of HT-29 cells (Richards *et al*., 1997), indeed, receptor desensitization could be achieved by concentrations of agonist which themselves did not cause increases in EAR. This suggests that diverse transduction pathways can be differentially activated and that there can be preferential G protein coupling to those intracellular signalling cascades resulting in agonist desensitization.

Whilst transcripts for sst_4 receptors have been identified in the brain and peripheral organs such as the lung, eye and placenta (Mori *et al*., 1997; Caron *et al*., 1997; Schloos *et al*., 1997) the functional role of these receptors is still unknown. Kreinkamp *et al*. (1997) have shown that SRIF-induced activation of rat sst_4 receptors expressed in *Xenopus* oocytes injected with cRNA for the mouse GIRK1 subunit causes activation of an inward potassium current which is not susceptible to desensitization. We do not know whether similar results to those obtained in our studies, can be demonstrated in other mammalian cells expressing either endogenous or recombinant sst_4 receptors but the data clearly has important implications for experimental design. Ankersen *et al*. (1998) have recently identified NNC-26-9100 as a non-peptidic and selective sst_4 receptor selective agonist. We have confirmed (unpublished observations) its selectivity and affinity (pIC_{50} 8.0) at sst_4 receptors. Furthermore in microphysiometry studies, following administration of sequentially increasing concentrations, NNC-26-9100 caused similar increases in EAR and maximum responses as L-362855, but was approximately two times more potent (pEC_{50} 8.3). In view of this selectivity and apparent lower ability to cause desensitization (compared with SRIF), NNC-26-9-100 should be a useful tool to investigate the functional significance of endogenously expressed sst_4 receptors.

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