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Differential effects of somatostatin and angiopeptin on cell proliferation

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1 Somatostatin (SRIF) exerts antiproliferative effects, and angiopeptin (an sst_2/sst_5 receptor-selective analogue) has recently been evaluated in clinical trials for the prophylaxis of restenosis following coronary angioplasty. Using an *in vitro* model of cell growth we have examined the effects of SRIF and angiopeptin on cell proliferation in CHO-K1 cells stably transfected with the human or rat recombinant sst_2 or sst_5 receptor and compared these with their effects on rat aortic vascular smooth muscle cells (VSMC) expressing endogenous somatostatin receptors.

2 In CHO-K1 cells, expressing either human or rat recombinant sst_2 or sst_5 receptors, or in rat aortic VSMC, SRIF and angiopeptin (0.1–1000 nM) had no effect on basal re-growth of cells into a denuded area of a previously confluent monolayer. In contrast, basic fibroblast growth factor (bFGF, 10 ng ml^{-1}) stimulated re-growth of these cells.

3 SRIF (0.1–1000 nM) caused a concentration-dependent inhibition of the bFGF-stimulated re-growth in CHO-K1 cells expressing human sst_2 (h sst_2) or sst_5 (h sst_5) receptors ($pIC_{50} = 8.05 \pm 0.03$ and 8.56 ± 0.12, respectively). In contrast, angiopeptin (0.1–1000 nM) acted as a partial agonist at the h sst_2 receptor ($44.6 \pm 2.7\%$ inhibition of the bFGF-stimulated re-growth at 100 nM; $pIC_{50} = 8.69 \pm 0.25$) but was devoid of any agonist activity at the h sst_5 receptor.

4 In CHO-K1 cells stably expressing rat recombinant sst_2 (r sst_2) or sst_5 (r sst_5) receptors, SRIF (0.1–1000 nM) was able to inhibit the bFGF-stimulated re-growth ($pIC_{50} = 7.98 \pm 24$ and 8.50 ± 0.12, respectively). Angiopeptin (0.1–1000 nM) caused a concentration-dependent inhibition of bFGF-stimulated re-growth at the r sst_2 receptor ($pIC_{50} = 8.08 \pm 0.24$) but acted as a partial agonist at the r sst_5 receptor (maximum response $= 57.7 \pm 3.6\%$ inhibition of bFGF-stimulated re-growth at 100 nM; $pIC_{50} = 8.60 \pm 0.16$).

5 Although angiopeptin was inactive as an agonist at the h sst_5 receptor, 100 nM angiopeptin potently antagonized the SRIF-induced inhibition of proliferation in CHO h sst_5 (estimated $pK_B = 10.4 \pm 0.3$). 5-Hydroxytryptamine (0.1 nM–10 μM) also inhibited bFGF-stimulated re-growth ($pIC_{50} = 8.36 \pm 0.11$) and angiopeptin had no effect on this response ($pK_B < 7$).

6 SRIF (0.1–1000 nM) caused a concentration-dependent ($pIC_{50} = 8.04 \pm 0.08$) inhibition of bFGF-stimulated re-growth in VSMC, whereas angiopeptin displayed weak agonist activity, only inhibiting bFGF-stimulated re-growth at concentrations greater than 100 nM. Angiopeptin (100 nM) caused a rightward displacement of the concentration-effect curve to SRIF with an estimated pK_B value of 7.70 ± 0.12.

7 These findings suggest that the low intrinsic activity of angiopeptin at the h sst_2 receptor, combined with its lack of agonist activity at the h sst_5 receptor, may explain the poor clinical efficacy of angiopeptin in trials for coronary artery restenosis, which contrasts with encouraging data found in equivalent *in vivo* animal studies.

Keywords: Somatostatin; sst_2 receptors; sst_5 receptors; angiopeptin; CHO-K1 cells; rat aortic vascular smooth muscle cells; proliferation

Introduction

Somatostatin (SRIF) was initially discovered as an inhibitor of growth hormone release from rat anterior pituitary cells (Brazeau *et al*., 1973) and is known to exert potent inhibitory effects on the growth of a wide variety of cells types, (see Mascardo *et al*., 1984; Payan *et al*., 1984; Lewin, 1992) including tumour cells (see Lamberts *et al*., 1987; Schally & Redding, 1997 for reviews). More recently, a number of groups have demonstrated that SRIF, and some of its analogues, can inhibit vascular smooth muscle cell proliferation and migration (Grant *et al*., 1994; Mooradian *et al*., 1995; Lauder *et al*., 1997a). One such analogue is the sst_2/sst_5 receptor-selective analogue angiopeptin (Foegh, 1992; Patel & Srikant, 1994) which in animal models has been shown to prevent the proliferation of smooth muscle cells in the rat carotid artery both *in vitro* (Vargas *et al*., 1989) and *in vivo* (Mennander *et al*., 1993; Hayry *et al*., 1993) after aortic allograft transplants. The smooth muscle cell proliferation stimulated by 'air-drying' of the endothelium can also be inhibited by angiopeptin and the somatostatin analogue BIM23034 (Lundergan *et al*., 1989). In addition to studies in rat animal models, the antiproliferative effects of SRIF and angiopeptin have been demonstrated in both rabbit (Foegh *et al*., 1989; Light *et al*., 1993; Howell *et al*., 1993; Foegh *et al*., 1994) and pig (Foegh, 1992) models of vascular smooth muscle cell proliferation.

Five distinct SRIF receptor genes ($sst_1–sst_5$) have been cloned (see Hoyer *et al*., 1994; 1995) and it has recently been shown that when individually expressed in CHO cells only recombinant sst_2 and sst_5 receptors are involved in mediating the antiproliferative effects of SRIF (Buscail *et al*., 1995).

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Furthermore, *in vitro* studies with rat aortic smooth muscle cells have shown that SRIF can inhibit bFGF-stimulated cell proliferation; data with receptor selective peptide ligands indicated that this effect was predominantly mediated via the sst_5 receptor (Lauder *et al*., 1997a).

The proliferation of smooth muscle cells is believed to be the principal cause of restenosis, a re-narrowing of the coronary arteries occurring in 30–50% of all patients who undergo balloon angioplasty treatment for atherosclerosis (Faxon & Currier, 1995). The known antiproliferative effect of SRIF on vascular smooth muscle cells has generated interest in the possible development of somatostatin analogues as clinical agents for the prophylaxis of restenosis and angiopeptin is currently in clinical trials for this effect (Eriksen *et al*., 1995). However, to date results obtained from these studies have proved disappointing (Emanuelsson *et al*., 1995).

The pharmacology of angiopeptin, acting through different somatostatin receptor types to inhibit cell proliferation, remains to be examined. In the guinea-pig spontaneously beating right-atrium, angiopeptin has weak agonist activity but can antagonize the SRIF-14 and SRIF-28 induced inhibition of this spontaneous activity (Feniuk *et al*., 1993) which suggests it may be a partial agonist at some somatostatin receptor types. In the present study we have used an *in vitro* model of cell proliferation and re-growth (Lauder *et al*., 1998) to elucidate the effects of angiopeptin and SRIF on cell proliferation at the recombinant human and rat sst_2 and sst_5 receptors, when expressed in Chinese hamster ovary (CHO)-K1 cells, and also the effect of angiopeptin in rat vascular smooth muscle cells (VSMC).

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

Methods

Cell culture

Chinese hamster ovary cells (CHO-K1 cells) stably expressing either the recombinant human sst_2 (CHO h sst_2) or human sst_5 (CHO h sst_5) receptors were produced by transfection of the respective full length cDNA sequence, after subcloning into the vector pCIN4, a derivative of the high expression vector pCIC1 (see Wilkinson *et al*., 1996). CHO-K1 cells transfected with a haemagglutinin (HA) epitope-tagged version of the rat sst_2 (CHO r sst_2) and rat sst_5 (CHO r sst_5) receptor were provided by Affymax (Palo Alto, California, U.S.A.). The HA insert (9 amino acids in length) is located two residues in from the end of the N-terminus. Levels of receptor expression in all the CHO-K1 cells were high and of the same order (mean B_{max} estimates ($n=2-3$) in pmol mg^{-1} protein: h sst_2 12.5; r sst_2 12.0; h sst_5 4.4; r sst_5 30.2). All CHO-K1 cell types were maintained in Dulbecco's modified Eagles medium/Hams F-12 nutrient (1 : 1) mix supplemented with Glutamax I, 10% foetal calf serum (FCS) and 0.5 mg ml^{-1} G418 sulphate (Geneticin) as a selection agent for CHO-K1 cells containing the transfected somatostatin receptor. The cells were grown in 75 cm^2 tissue culture flasks (Costar Ltd) and maintained in the appropriate medium at 37°C in humidified air containing 5% carbon dioxide. Cells were routinely passaged every 3–4 days upon reaching 95% confluence, as assessed by eye and in all experiments, cells were used between passages 8 and 50 from the original dilution clone.

Rat aortic smooth muscle cells (VSMC) were isolated as previously described (Lauder *et al*., 1997a). Briefly, thoracic aortae were dissected from eight freshly killed adult (250 g) male Sprague-Dawley rats and placed in Hank's balanced salt solution supplemented with 10% FCS. The tunica media were separated from the adventitia and endothelium by fine dissection after treatment with 0.3% collagenase solution in Medium 199 (M199) with Hank's salts for 30 min at 37°C. Cells from the remaining tunica media were dispersed by incubation in M199 with 0.1% elastase solution for 30 min at 37°C, followed by addition of collagenase (0.3% solution) for 2 h at 37°C. The dispersed cells were centrifuged at 100 *g* for 5 min and resuspended in Dulbecco's modified Eagles medium (DMEM) supplemented with Glutamax I and 10% FCS and plated into a 75 cm^2 flask; in all experiments cells were used between passages 4 and 10. The cells were characterized by the absence of Dil acetylated low-density lipoprotein (Dil-Ac-LDL) uptake and by the presence of smooth muscle α-actin.

Experimental procedure

Cells were seeded at a density of 2×10^5 onto Thermanox (NUNC, Life Technologies) plastic coverslips in 24-multiwell plates containing 0.5 ml medium per well and grown to confluence. Cells on coverslips not reaching 95% confluence by three days were discarded. Once 95% confluence was achieved, coverslips were removed from the multiwell plates and a perspex comb drawn across the coverslip to produce eleven parallel areas (400 μm wide) denuded of cells (Lauder *et al*., 1998). Coverslips were washed three times in phosphate buffer saline (PBS) to remove any cellular debris, and placed into a well containing either the drug or vehicle. Cells post-denudation were grown in serum-free medium. Experiments were stopped 24 h post-denudation by washing coverslips three times in PBS and fixing in 100% ethanol for five minutes. The ethanol was then aspirated and the cells left to air dry before image analysis.

Image analysis

The re-growth of cells into the denuded area was quantified with a Seescan semi-automated, computerized, image analysis system (Seescan, Cambridge, U.K.). Each field of view measured approximately 2% of the total area of the coverslip. For each coverslip five fields of view were measured from random positions on the coverslip. The lesion area in each field of view was measured and with the data for time zero (T_0), the lesion area was converted to give the % re-growth into the denuded area (%R) for each individual coverslip. This was calculated from the equation:

$$\%R = [1 - (\text{lesion area at } T_t / \text{lesion area at } T_0)] \times 100\%$$

where T_t is the time post-wounding and T_0 is immediately post-lesion. Image analysis was carried out with the operator "blind" and only after analysis was the code broken.

Direct cell counting

Cells were harvested from the whole surface of the coverslip by washing the coverslip with 0.5 ml PBS three times to remove excess medium and then adding 0.1 ml 0.05% trypsin/0.02% EDTA for 2–5 min. The reaction was stopped by adding 0.4 ml complete medium to the well and the single cell suspension was then counted with a Coulter Counter (Coulter Euro Diagnostics GmbH). The wells were examined under a microscope after cell harvesting to ensure that all cells had been removed.

Data analysis

All % re-growth data are expressed as the mean ± s.e.mean, calculated from a minimum of three experiments containing four replicates per test group and five measurements made per replicate. For each set of experiments separate basal and mitogen-stimulated values were obtained. Statistical significance was determined by either Fisher's partial least squares difference test or Student's t test with $P<0.05$ as the level of significance. For concentration-effect curves, pIC_{50} values (negative log of the concentration of each agonist to produce half its own maximum inhibition) were calculated following non-linear regression analysis of the data (Graphpad Prism).

The potency of the antagonism by angiopeptin was expressed as the pK_B value which was calculated from the Gaddum-Schild equation:

$$pK_B = \log(\text{concentration ratio} - 1) - \log(\text{antagonist molar concentration})$$

where the concentration ratio was defined as the concentration of agonist producing 50% of the maximum response in the presence of an antagonist, divided by the concentration producing the same response in the absence of antagonist (see Jenkinson *et al*., 1995).

Materials

Dulbecco's modification of Eagle's medium (DMEM)\Ham's F-12 nutrient mix medium, Hanks balanced salt solution, Medium 199 (M199), trypsin/EDTA solution, Dulbecco's phosphate buffer saline (PBS), foetal calf serum (FCS) and G418 sulphate were obtained from Life Technologies (Paisley, Scotland). Dil-Ac-LDL was purchased from Biogenesis (Bournemouth). Thermanox coverslips were purchased from NUNC (Life Technologies). All tissue culture plastic was purchased from Costar Ltd (High Wycomb, Bucks). SRIF-14 was obtained from Peninsula Laboratories Europe Ltd (St. Helens, Merseyside). Angiopeptin (BIM23014), 5-hydroxytryptamine (5-HT) and basic fibroblast growth factor (bFGF) were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset).

Stock solutions of SRIF-14 and angiopeptin were initially dissolved in distilled water and bFGF was reconstituted with a 0.2 μm filtered solution containing 1% foetal calf serum in buffered saline solution. All the drugs were aliquoted and stored at -20°C. Fresh aliquots were used on each day of the experiment and none of the samples was thawed and subsequently refrozen. Further dilutions of all drugs were made in Dulbecco's modified Eagle's medium/Hams F-12 nutrient (1 : 1) mix supplemented with Glutamax but without FCS.

Results

The effects of SRIF and angiopeptin upon re-growth in CHO h sst_2 and CHO h sst_5 cells

The basal rate of re-growth in CHO h sst_2 and CHO h sst_5 cells at 24 h following partial denudation of a confluent monolayer (7.5 ± 0.2% and 7.4 ± 0.2%, respectively) was unaffected by either somatostatin or angiopeptin at all concentrations tested (0.1 – 1000 nM; Figure 1). However, in the presence of a sub-maximal concentration of bFGF (10 ng ml^{-1}) the percentage re-growth into the denuded area increased to 21.8 ± 1.0% and 19.9 ± 0.8% in CHO h sst_2 and CHO h sst_5 cells, respectively (Figure 1). The effect of SRIF and angiopeptin (0.1 – 1000 nM) upon bFGF-stimulated re-growth after partial denudation was examined in the CHO h sst_2 and CHO h sst_5 cells. SRIF caused complete and concentration-dependent inhibition of the bFGF-stimulated re-growth in CHO h sst_2 and CHO h sst_5 cells with pIC_{50} values of 8.05 ± 0.03 and 8.56 ± 0.12, respectively (Figure 2). In CHO h sst_2 cells, angiopeptin (0.1 – 1000 nM) was also able to inhibit bFGF-stimulated re-growth but with a maximum response of only 44.6 ± 2.7% at 100 nM and a pIC_{50} value of 8.69 ± 0.25 (Figure 2a). In CHO h sst_5 cells angiopeptin was unable to inhibit the bFGF-stimulated re-growth (Figure 2b).

The effects of SRIF and angiopeptin upon re-growth in CHO r sst_2 and CHO r sst_5 cells

Concentration-effect curves were constructed to SRIF (0.1 – 1000 nM) and angiopeptin (0.1 – 1000 nM) upon basal and bFGF-stimulated re-growth in CHO-K1 cells expressing recombinant rat sst_2 or sst_5 receptors. In the presence of bFGF (10 ng ml^{-1}) re-growth into the denuded area increased from 12.0 ± 0.9% to 23.2 ± 1.4% and 10.2 ± 0.6% to 23.0 ± 1.0% in CHO r sst_2 and CHO r sst_5 cells, respectively (Figure 3). At all concentrations tested (0.1 – 1000 nM) somatostatin and angiopeptin had no effect upon basal re-

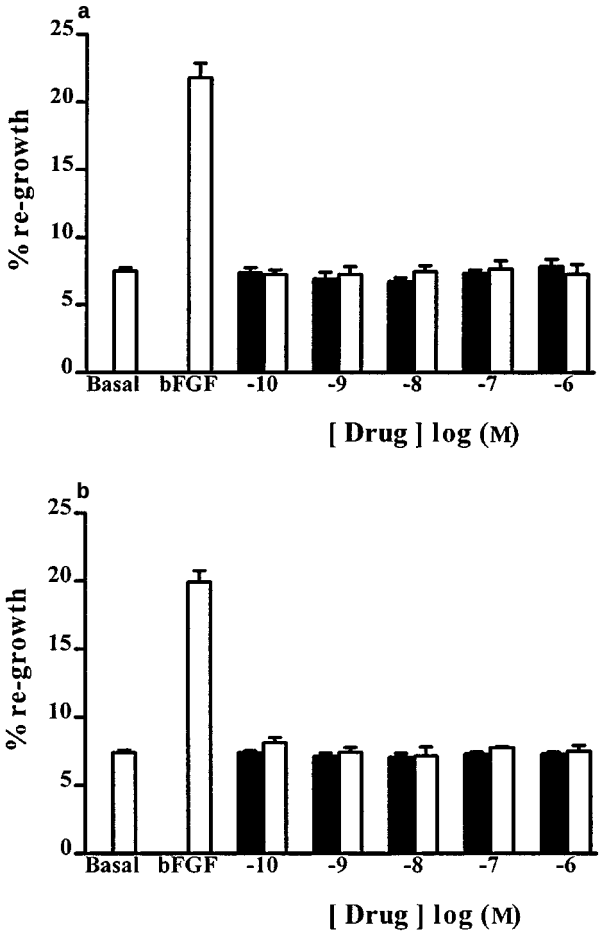

Figure 1 The effects of bFGF (10 ng ml^{-1}), SRIF (0.1 – 1000 nM; solid columns) and angiopeptin (0.1 – 1000 nM; open columns) on basal re-growth after partial denudation of (a) CHO hsst_2 and (b) CHO hsst_5 cells. Values are expressed as the mean % re-growth at 24 h from 3 experiments performed in quadruplicate. Vertical lines represent the s.e.mean ($n=3$).

growth in CHO r sst_2 and CHO r sst_5 cells (Figure 3). Similar to the results obtained in cells expressing human somatostatin receptors, SRIF caused a complete and concentration-dependent inhibition of the bFGF-stimulated re-growth in CHO r sst_2 and CHO r sst_5 cells with pIC_{50} values of 7.98 ± 0.24 and 8.50 ± 0.12, respectively (Figure 4). Angiopeptin was able to inhibit maximally bFGF-stimulated re-growth in CHO r sst_2 cells with a pIC_{50} value of 8.08 ± 0.24 (Figure 4a), but acted as a partial agonist in the CHO r sst_5 cells (maximum response $= 57.7 \pm 3.6\%$ inhibition of bFGF-stimulated re-growth at 100 nM; $pIC_{50} = 8.60 \pm 0.16$; Figure 4b).

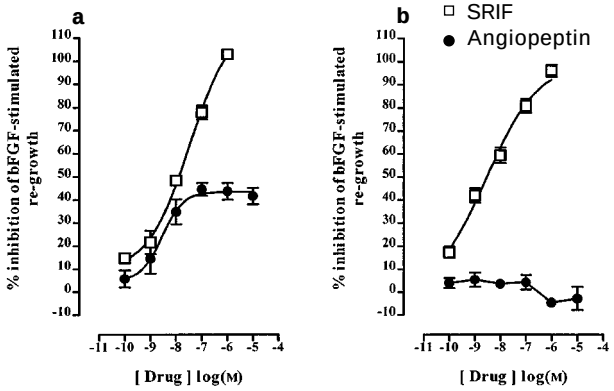

Figure 2 Concentration-effect curves were constructed for SRIF and angiopeptin against bFGF (10 ng ml^{-1})-stimulated re-growth in (a) CHO h sst_2 and (b) CHO h sst_5 cells after partial denudation. Values are expressed as the mean % inhibition at 24 h of bFGF-stimulated re-growth. All values are the mean from 3 experiments performed in quadruplicate. Vertical lines represent the s.e.mean ($n = 3$).

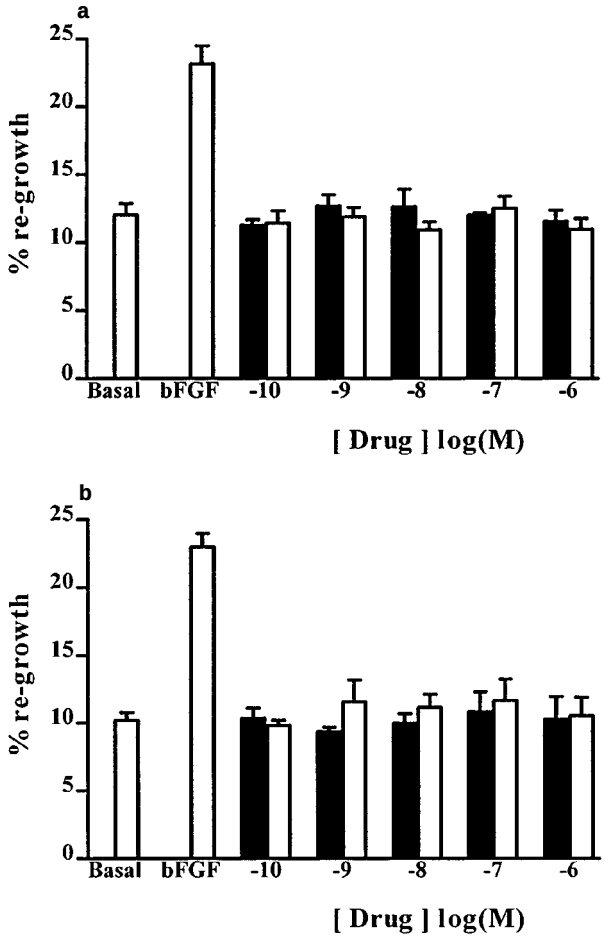

Figure 3 The effects of bFGF (10 ng ml^{-1}), SRIF (0.1–1000 nM; solid columns) and angiopeptin (0.1–1000 nM; open columns) on basal re-growth after partial denudation of (a) CHO r sst_2 and (b) CHO r sst_5 cells. Values are expressed as the mean % re-growth at 24 h from 3 experiments performed in quadruplicate. Vertical lines represent the s.e.mean ($n = 3$).

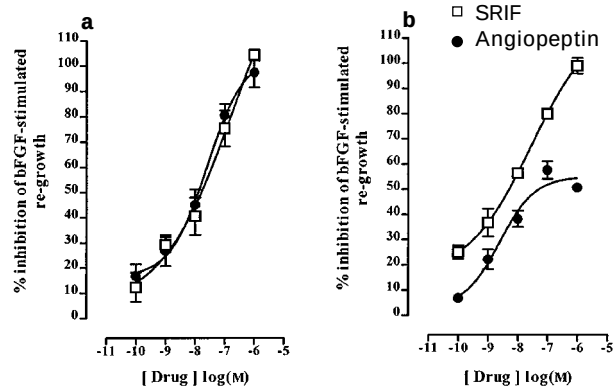

Figure 4 Concentration-effect curves were constructed for SRIF and angiopeptin against bFGF (10 ng ml^{-1})-stimulated re-growth in (a) CHO r sst_2 and (b) CHO r sst_5 cells after partial denudation. Values are expressed as the mean % inhibition at 24 h of bFGF-stimulated re-growth. All values are the mean from 3 experiments performed in quadruplicate. Vertical lines represent the s.e.mean ($n = 3$).

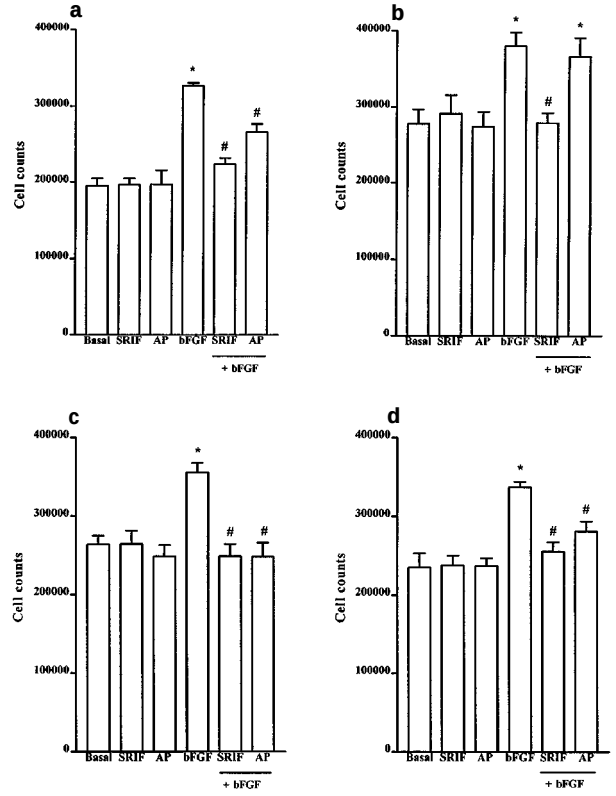

Figure 5 The effect of SRIF (100 nM) and angiopeptin (AP, 100 nM) on basal and bFGF (10 ng ml^{-1})-stimulated cell proliferation in (a) CHO h sst_2, (b) CHO h sst_5, (c) CHO r sst_2 and (d) CHO r sst_5 cells after partial denudation. Values are expressed as mean cell count at 24 h. * and # represent $P < 0.05$ significance to basal and bFGF treatment alone, respectively. All values are the mean from 4 experiments performed in triplicate. Vertical lines represent the s.e.mean ($n = 4$).

The effect of SRIF and angiopeptin upon cell proliferation

The effects of SRIF and angiopeptin on cell numbers in each of the four cell types were also determined by direct cell counts. Somatostatin and angiopeptin (both 100 nM) had no effect upon basal cell numbers in CHO h sst_2 (Figure 5a) and CHO h sst_5 cells (Figure 5b) or CHO r sst_2 (Figure 5c) and CHO r sst_5 cells (Figure 5d). In all the cell types bFGF (10 ng ml^{-1}) stimulated a significant increase in cell numbers which was abolished by SRIF (100 nM). Angiopeptin (100 nM) abolished the bFGF-stimulated increase in cell numbers in CHO r sst_2 cells (Figure 5c) but only partially inhibited bFGF-stimulated cell proliferation in CHO h sst_2 and CHO r sst_5 cells (Figure 5a and d). Angiopeptin had no effect upon cell proliferation stimulated by bFGF in CHO h sst_5 cells (Figure 5b).

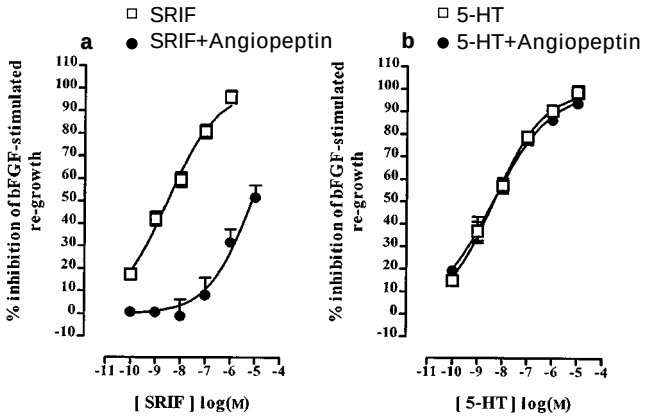

Figure 6 The effects of angiopeptin on SRIF and 5-HT-mediated inhibition of bFGF-stimulated (10 ng ml^{-1}) re-growth at 24 h in CHO h sst_5 cells. Concentration-effect curves to (a) SRIF and (b) 5-HT were constructed in the absence and presence of 100 nM angiopeptin. Values are the mean from 3 experiments performed in quadruplicate. Vertical lines represent the s.e.mean ($n=3$).

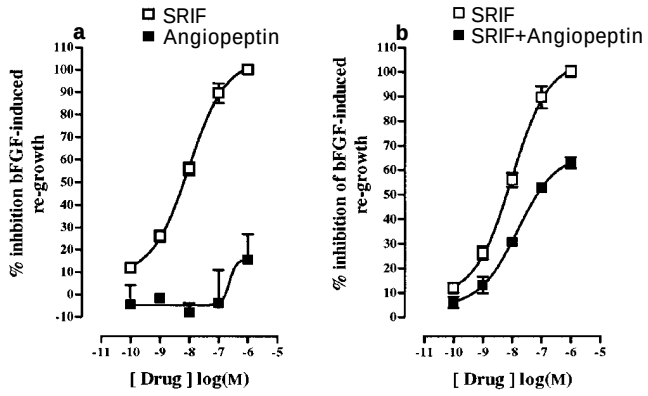

Figure 7 The effects of SRIF and angiopeptin upon bFGF-stimulated re-growth in rat aortic VSMC at 24 h. (a) Concentration-effect curves to SRIF and angiopeptin were constructed against bFGF (10 ng ml^{-1})-stimulated re-growth. The effect of angiopeptin was significantly different from control values at 1 μM. (b) The effect of angiopeptin as an antagonist of SRIF was examined. Concentration effect curves to SRIF were constructed in the absence and presence of angiopeptin (100 nM). Values are the mean from 3 experiments performed in quadruplicate. Vertical lines represent the s.e.mean ($n=3$).

Antagonist effect of angiopeptin in CHO h sst_5 cells

Although angiopeptin was inactive as an agonist at the h sst_5 receptor, 100 nM angiopeptin potently antagonized the SRIF-induced inhibition of proliferation (estimated $pK_B = 10.4 \pm 0.3$) in CHO h sst_5 (Figure 6). 5-Hydroxytryptamine (0.1 nM – 10 μM) also inhibited bFGF-induced re-growth in CHO h sst_5 cells ($pIC_{50} = 8.36 \pm 0.11$) and angiopeptin had no effect on this response ($pK_B < 7$; see Figure 6).

Effects of SRIF and angiopeptin in rat aortic vascular smooth muscle cells

SRIF and angiopeptin (0.1 – 1000 nM) had no effect on the basal re-growth ($6.4 \pm 0.8\%$) in rat aortic VSMC (data not shown). Somatostatin was able to inhibit completely bFGF (10 ng ml^{-1})-stimulated re-growth ($20.5 \pm 0.7\%$) with a pIC_{50} of 8.04 ± 0.08 (Figure 7a). In contrast, angiopeptin only inhibited bFGF-stimulated re-growth at concentrations greater than 100 nM. Since angiopeptin had weak agonist activity, it was examined as an antagonist of SRIF-induced inhibition of bFGF-stimulated re-growth in the VSMC. Angiopeptin (100 nM) caused a rightward displacement of the concentration-effect curve to SRIF with an estimated pK_B of 7.70 ± 0.12 (Figure 7b).

Discussion

In addition to having an inhibitory action against a number of hormones generally involved in cell growth and development (Gerich, 1981), somatostatin can directly inhibit the proliferation of vascular smooth muscle cells by acting upon high affinity membrane bound receptors (Leszczynski *et al*., 1993). There is now growing evidence for the receptor subtype that mediates the inhibition of proliferation belonging to the $SRIF_1$ group (Vidal *et al*., 1994) and in particular the sst_2 and sst_5 receptor types. SRIF mediates its antiproliferative effect in rat smooth muscle cells through the sst_5 receptor (Lauder *et al*., 1997a) and another recent study, mimicking balloon angioplasty in rats, suggests that after injury there is an upregulation of sst_2, but not the sst_3 or sst_5, receptors on endothelial cells (Chen *et al*., 1997). It is possible, therefore, that both the sst_2 and sst_5 receptors play separate roles in wound repair with each receptor inhibiting proliferation of a particular cell type. Although angiopeptin has been shown to bind with high affinity to the sst_2 and sst_5 receptors (Patel & Srikant, 1994), the role that both of these receptors may play in its antiproliferative effect is still unclear.

Using a previously described *in vitro* model of cell re-growth and proliferation (Lauder *et al*., 1998), we have characterized the antiproliferative effects of SRIF and angiopeptin in CHO-K1 cells individually expressing the human or rat recombinant sst_2 or sst_5 receptors. In addition, we have also examined the effect of SRIF and angiopeptin in rat aortic VSMC. In this study we have quantified the re-growth into a denuded area of a previously confluent monolayer and also measured direct cell counts from the whole coverslip to confirm that the effects observed were on cell proliferation.

Previous work in our laboratories with both VSMC (Lauder *et al*., 1997a) and CHO-K1 cells (unpublished observation) has demonstrated that the basal re-growth is mainly due to cell migration. In the present study SRIF and angiopeptin were unable to inhibit the basal re-growth in rat aortic VSMC or in CHO-K1 cells expressing the human or rat recombinant sst_2 or sst_5 receptors. This is in keeping with the finding that the SRIF

analogues octreotide and angiopeptin, alone or combined, are unable to inhibit migration in human coronary smooth muscle cells when triggered by IGF-1, bFGF or PDGF (Grant *et al*., 1993). However, in contradiction, angiopeptin has been shown to inhibit potently migration stimulated by type I collagen in rat aortic smooth muscle cells when examined in the Boyden chamber (Mooradian *et al*., 1995). There is no obvious explanation for the discrepancies observed with the migration studies, although the chemotactic agent used to trigger migration in each of these studies was different, possibly indicating that SRIF can only inhibit migration when stimulated by certain agents. Previous studies examining the antiproliferative effect of SRIF have shown that SRIF and octreotide can only inhibit cell proliferation when growth is stimulated (Bjork *et al*., 1993), causing little effect upon basal level of growth in pancreatic tumour cells (Vidal *et al*., 1994) and smooth muscle cells (Grant *et al*., 1994). This is also consistent with our finding that SRIF and angiopeptin had no effect on basal re-growth and cell proliferation.

Somatostatin, acting through both the human and rat sst_2 and sst_5 receptors, was able to abolish potently bFGF-stimulated re-growth in a concentration-dependent manner. Direct measurement of cell counts revealed that this effect of SRIF was through the inhibition of bFGF-stimulated cell proliferation. A comparison of the pIC_{50} values obtained for both human and rat receptor types indicates that SRIF is slightly more potent (3–5 fold) at inhibiting bFGF-stimulated proliferation through the sst_5 receptor compared to the sst_2 receptor.

At the h sst_2 receptor angiopeptin acts as a partial agonist to inhibit cell proliferation and re-growth and has a low intrinsic activity compared with SRIF. This partial agonist activity of angiopeptin is similar to that found in the guinea-pig ileum, where the sst_2 receptor mediates inhibition of neurogenic contraction (Feniuk *et al*., 1993). However, this is in contrast to the rat recombinant sst_2 receptor, where angiopeptin in this study acted as a full agonist to inhibit cell proliferation. Although angiopeptin binds with moderate affinity to the sst_3 receptor (Patel & Srikant, 1994), SRIF and angiopeptin have no effect on either basal or bFGF stimulated re-growth in CHO-K1 cells transfected with the human recombinant sst_3 receptor (unpublished observation).

Surprisingly, angiopeptin was unable to inhibit mitogen-stimulated re-growth at the h sst_5 receptor at concentrations of up to 1 μM, even though SRIF could potently inhibit re-growth through this receptor. Angiopeptin acted as a partial agonist at the r sst_5 receptor when expressed either exogenously in CHO-K1 cells or endogenously in rat aortic VSMC, although angiopeptin was considerably weaker in the VSMC. This weaker activity of angiopeptin is probably a reflection of lower receptor density in the VSMC compared to CHO-K1 cells recombinantly expressing the r sst_5 receptor. Indeed, we have been unable to demonstrate any binding of [^{125}I]-[Tyr^{11}]-SRIF in VSMC (unpublished observation), although we have previously demonstrated the presence of all five SRIF receptor types, with receptor-specific anti-sera (Lauder *et al*., 1997a). Interestingly, in the guinea-pig vas deferens, which contains a putative sst_5 receptor on its sympathetic innervation (Feniuk *et al*., 1995), angiopeptin acts as a partial agonist with low intrinsic activity (unpublished observation). Similarly, the guinea-pig right-atrium is thought to contain an sst_5 receptor, which can mediate inhibition of spontaneous beating and in this preparation angiopeptin also has weak agonist activity (Feniuk *et al*., 1993). Therefore, there appear to be differences in the action of angiopeptin between the human sst_5 receptor and the guinea-pig and rat sst_5 receptor, with angiopeptin being either inactive or acting as a partial agonist, respectively. Since the recombinant systems used in this study all expressed high levels of receptor protein, the partial agonism observed with angiopeptin in the CHO h sst_2 and CHO r sst_5 cells is unlikely to be a function of differing levels of receptor protein in these cell systems and presumably reflects its low agonist efficacy.

When examined as an antagonist, angiopeptin potently and specifically (see below) antagonized the effects of SRIF at the h sst_5 receptor and also antagonized the inhibition of bFGF-stimulated proliferation by SRIF in rat aortic VSMC, although the pK_B obtained for antiopeptin in VSMC (7.7) was considerably lower than that obtained in CHO h sst_5 cells (10.4). Angiopeptin has been shown to bind to the h sst_5 receptor with a moderate affinity (estimated pIC_{50} value of 8.5; O'Carroll *et al*., 1994 and our own unpublished observations) which is incongruent with the high pK_B value obtained for the antagonism of SRIF by angiopeptin at the h sst_5 receptor in this study. However, since the binding studies involved the use of an agonist radioligand ([^{125}I]-[Tyr^{11}]-SRIF), it may be that the affinity of angiopeptin for the agonist high affinity conformation is markedly different to its affinity at the G-protein-independent low affinity conformation predominating in the functional model, where guanosine nucleotides are present (see Birdsall & Lazareno, 1997). Interestingly, it has previously been found that angiopeptin, which behaves as a partial agonist, can functionally antagonize the SRIF-mediated inhibition of spontaneous beating in the guinea-pig right atrium, via an sst_5 receptor (Feniuk *et al*., 1993; 1995), with a similar pK_B estimate (7.4) to that obtained for the proliferation studies in VSMC. The differences between the pK_B value found in the human sst_5 receptor, compared with those at the rat and guinea-pig sst_5 receptor in these functional studies, may reflect species differences as well as the conformational state(s) of each receptor, considering that angiopeptin appears to have more intrinsic efficacy at rat and guinea-pig than human receptors.

CHO-K1 cells endogenously express 5-HT_{1B} receptors (Giles *et al*., 1996) and 5-HT inhibited mitogen-stimulated proliferation in this model of cell re-growth and proliferation. This important observation was surprising, since 5-HT is classically considered to have mitogenic activity (Watts, 1996; Fanburg & Lee, 1997), and certainly deserves further investigation (see below). The antiproliferative effect of 5-HT was unaffected by angiopeptin, thus demonstrating that the antagonist effect of angiopeptin against SRIF at the sst_5 receptor was specific.

Regardless, there are clear differences between the action of angiopeptin and its ability to modulate cell growth at each of the SRIF receptors examined when expressed in CHO-K1 cells. It remains to be seen whether the functional antagonism presently demonstrated can be observed in terms of intracellular second messenger signalling. The novel anti-proliferative effect of 5-HT observed in this study may provide further insights into the biochemical pathways involved in the equivalent SRIF-mediated effect. There are a number of similarities between the transduction mechanism for 5-HT_{1B} receptors and sst_2 and sst_5 receptors. All of these receptor types couple negatively to adenylyl cyclase through a pertussis toxin-sensitive mechanism (Akbar *et al*., 1994; Dickenson & Hill, 1995). Furthermore, the 5-HT_{1B}, sst_2 and sst_5 receptors can also mediate increases in phospholipase C (PLC) and intracellular Ca^{2+} through G_i/G_o proteins, presumably via the associated $\beta\gamma$ subunits (Van Obberghen-Schilling *et al*., 1991; Akbar *et al*., 1994; Wilkinson *et al*., 1997). However, in the case of 5-HT, these increases in $[Ca^{2+}]_i$ are usually associated

with a proliferative effect (Durham & Walton, 1982; Abdel-Baset *et al*., 1992; Montero *et al*., 1995). The ability of 5-HT to stimulate potently cell proliferation has been studied in a variety of cell types, including vascular smooth muscle cells (Lee *et al*., 1991; 1994; Pakala *et al*., 1994) and endothelial cells (Cirillo *et al*., 1996). Furthermore, it has been shown by Seuwen *et al*. (1988) that the proliferative action of 5-HT in fibroblasts correlates well with the ability of 5-HT_{1B} receptors to couple to adenylyl cyclase. However, 5-HT can also inhibit cell proliferation in lymphocytes (Ferriere *et al*., 1996) and smooth muscle cells (Lee *et al*., 1991). In fact, Lee *et al*. (1991) showed that 5-HT can exert a dual effect upon smooth muscle cell (SMC) growth, stimulating proliferation through an intracellular action and inhibiting proliferation through a cell-surface action. The antiproliferative effect of 5-HT was only observed in the presence of isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor, and was associated with an increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Lee *et al*., 1994). Proliferative as well as antiproliferative effects of SRIF have also been observed (Ruiz-Torres *et al*., 1993; Lauder *et al*., 1997b; Wyatt *et al*., 1997). These diametrically opposed effects of both SRIF and 5-HT upon cell proliferation, and the respective signal transduction pathways mediating them, are as yet poorly understood. However, there is evidence to suggest that the antiproliferative effect of the SRIF analogue RC-160, at least through the sst_2 receptor, involves activation of a protein phosphatase (Buscail *et al*., 1995).

There have been several studies examining the effect of angiopeptin in various *in vivo* animal models of balloon angioplasty, including myointimal thickening caused by air-drying in the rat carotid artery (Lundergan *et al*., 1989), rat aortic allograft (Mennender *et al*., 1993), rabbit balloon injury (Foegh, 1992, 1994; Howell *et al*., 1993), and pig coronary artery transplant atherosclerosis (Foegh *et al*., 1992). In all of these animal models angiopeptin can inhibit transplant atherosclerosis and myointimal thickening, the major cause of restenosis and clinical events following angioplasty. Although the receptor subtype(s) mediating this effect *in vivo* is uncertain, results from this study have shown that angiopeptin is able to act through both the rat sst_2 and the rat sst_5 receptor to inhibit cell proliferation. Admitedly angiopeptin behaved as an antagonist rather than an agonist in rat isolated VSMC, but the SRIF receptor density was found to be very low. Nevertheless angiopeptin displayed sufficient intrinsic activity as an agonist on the recombinant rat sst_5 receptor to expect that it would behave as an agonist in the whole animal, where the receptor density might be greater and/or the receptor-effector coupling efficiency higher.

In contrast to studies in animals, clinical trials have demonstrated a relatively weak effect of angiopeptin in preventing restenosis (Emanuelson *et al*., 1995). The small beneficial effect noted by some investigators may be a consequence of the partial agonist activity of angiopeptin at the h sst_2 receptor. However, more importantly, the lack of agonist activity of angiopeptin at the h sst_5 which we have observed may explain the paradoxical clinical finding that angiopeptin is ineffective in preventing human coronary artery restenosis, in contrast to its encouraging effects in equivalent *in vivo* rat models.

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