British Journal of Pharmacology (1997) 122, 663–670

Somatostatin sst_5 inhibition of receptor mediated regeneration of rat aortic vascular smooth muscle cells

[1,*,#]H. Lauder, *L.A. Sellers, #T.-P.D. Fan, *W. Feniuk & *P.P.A. Humphrey

*Glaxo Institute of Applied Pharmacology and #Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ

1 The aim of the present study was to determine the effect of somatostatin (SRIF) on mitogen-induced regeneration of rat aortic vascular smooth muscle cells (VSMC) and for comparison Chinese hamster ovary (CHO)-K1 cells expressing human recombinant sst_5 receptors ($CHOsst_5$), following partial denudation of a confluent cell monolayer. Regeneration was assessed by measuring areas of recovery into the denuded area and by counting total cell numbers.

2 In VSMC, SRIF (0.1 nM – 1 μM) had no effect on the basal levels of regeneration but caused a concentration-dependent inhibition (pIC_{50} 8.0 – 8.6) of the stimulated regeneration induced by sub-maximal concentrations of basic fibroblast growth factor (bFGF, 10 ng ml^{-1}), platelet-derived growth factor-BB (PDGF, 5 ng ml^{-1}) or endothelin-1 (ET-1, 100 nM). SRIF (pIC_{50} 8.8) also inhibited bFGF-induced regeneration of $CHOsst_5$ cells.

3 In VSMC, the inhibitory action of SRIF on the regeneration induced by bFGF (10 ng ml^{-1}) was due to an anti-proliferative effect, rather than an effect on cell migration, as SRIF (0.1 nM – 1 μM) abolished bFGF-induced increases in total cell numbers. The bFGF-induced increase in cell numbers was also abolished by actinomycin D (0.1 μg ml^{-1}).

4 The sst_5 receptor-selective agonist, L-362,855 (pIC_{50} 10.5), was about 100 times more potent than SRIF at inhibiting bFGF-induced regeneration of both VSMC and $CHOsst_5$ cells whilst the sst_2 receptor-selective agonist, BIM-23027 (pIC_{50} 6.8), was approximately 20 times weaker than SRIF.

5 The sst_5 receptor antagonist, BIM-23056 (100 nM), antagonized SRIF-induced inhibition of bFGF-induced regeneration in both VSMC and $CHOsst_5$ cells (estimated pK_B values 8.8 and 8.3, respectively).

6 SRIF-induced inhibition of bFGF-induced regeneration of VSMC and $CHOsst_5$ cells was abolished by pretreating cells with pertussis toxin (100 ng ml^{-1}) for 20 h.

7 These findings suggest that SRIF-induced inhibition of the proliferation of rat aortic VSMC is mediated via activation of receptors which are similar to human sst_5 receptors. Furthermore this inhibitory effect is transduced via pertussis toxin-sensitive G_i/G_o proteins.

Keywords: Somatostatin; sst receptors; BIM-23056; L-362,855; rat aortic vascular smooth muscle cells; CHO-K1 cells; proliferation

Introduction

Somatostatin (SRIF) is a tetradecapeptide which was originally isolated from the hypothalamus as a growth hormone-release inhibitory factor and is distributed widely throughout the central nervous system and peripheral tissues (Brazeau *et al*., 1973; Reichlin, 1983). SRIF has diverse physiological properties including the inhibition of secretory and proliferative processes (Reichlin, 1983; Lamberts *et al*., 1986; Brevinin *et al*., 1993). The *in vitro* action of SRIF on cell proliferation was first investigated in 1982, where indirect immunofluorescence microscopy showed that nanomolar concentrations of SRIF inhibited epidermal growth factor-induced centrosomal separation, in early G1 phase, in both gerbil fibroma and HeLa cell lines. At similar concentrations SRIF also inhibited DNA synthesis, as determined by incorporation of [^{3}H]-methylthymidine, and decreased cell number (Mascardo & Sherline, 1982). Since this first observation, there has been increasing evidence to support the view that SRIF and some of its stable analogues have anti-proliferative effects. For example, the SRIF analogue, octreotide, effectively inhibits proliferation of various kinds of tumour cells cultured *in vitro*, such as the human breast adenocarcinoma cell line MCF7 (Pagliacci *et al*., 1991), the rat pancreatic tumour cell line AR42J (Viguerie *et al*., 1989) and the human prostatic cancer cell line LNCaP (Brevinin *et al*., 1993). Furthermore, SRIF and its analogues have been shown to inhibit tumour growth in animal models for cancers of the pancreas (Paz-Bouza *et al*., 1987; Rivard *et al*., 1991), gastrointestinal tract (Smith & Solomon, 1988), prostate (Schally & Redding, 1987; Zalatnai *et al*., 1988) and pituitary (Lamberts *et al*., 1986).

There is now accumulating data to suggest that SRIF may have a role in inhibiting vascular smooth muscle cell proliferation. Indeed, angiopeptin, a somatostatin analogue, has been demonstrated to inhibit myointimal formation both *in vivo* (Foegh *et al*., 1989) and *in vitro* by use of blood vessel explants (Vargas *et al*., 1989). Furthermore, rat coronary artery smooth muscle cells express SRIF receptors which are involved in the regulation of cell proliferation (Leszczynski *et al*., 1993). As a result of these studies angiopeptin is in clinical trials for the treatment of myointimal hyperplasia in restenosis after angioplasty and accelerated atherosclerosis of transplanted organs (Emanuelsson *et al*., 1995; Eriksen *et al*., 1995).

Since the cloning of five distinct SRIF receptor genes ($sst_1 - sst_5$) encoding a family of G protein-coupled receptors (for review see Hoyer *et al*., 1994; 1995) much research is being carried out to determine which SRIF receptor subtypes mediate the diverse biological actions of this peptide (e.g. Feniuk *et al*., 1993; McKeen *et al*., 1994; Wyatt *et al*., 1996). Indeed, Buscail *et al*. (1995) have recently demonstrated that in Chinese hamster ovary (CHO) cells expressing the five individual somatostatin receptor subtypes sst_1 to sst_5, only sst_2 and sst_5 receptors mediate the inhibition of cell

[1] Author for correspondence.

proliferation induced by the somatostatin analogue RC-160. However, the nature of the receptors mediating inhibition of VSMC re-growth is unknown.

Therefore, the main aim of this investigation was to determine the effect of SRIF and some receptor selective analogues on vascular smooth muscle cell growth, by use of an *in vitro* model of vascular smooth muscle regeneration (Fan & Frost, 1990), in order to determine which SRIF receptor subtype may be important in regulating growth.

Methods

Cell culture

Rat aortic smooth muscle cells (VSMC) were isolated by a modification of the enzyme dispersion method described by Gimbrone & Cotran (1975), as cells obtained by this method have been proposed to be more differentiated and more closely resemble their *in vivo* counterparts. 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% foetal calf serum (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 (Voyta *et al*., 1984) and by the presence of α-smooth muscle actin (Skalli *et al*., 1986). CHO-K1 cells expressing human recombinant sst_5 receptors (Wilkinson *et al*., 1996) were grown in DMEM/Ham's F-12 nutrient (1:1) mix supplemented with Glutamax I, 10% FCS and 500 $\mu g\ ml^{-1}$ G418 sulphate. In all experiments, cells were used between passages 15 and 30 from the original dilution clone. Both cell types were maintained at 37°C in humidified air/5% CO_2.

Measurement of regeneration

Cell lesioning Cells were seeded, at an appropriate cell density of 30,000 and 200,000 for VSMC and CHO-K1 cells, respectively, onto 13 mm diameter Thermanox (Nunc, Life Technologies) coverslips in 24-well plates containing 0.5 ml medium per well and incubated at 37°C until they reached confluence. By use of a mechanical wounder (Fan & Frost, 1990) 11 parallel lesions, 400 μm wide, were produced across the cell monolayer. Coverslips were rinsed in phosphate buffered saline (PBS), to remove dislodged cells and cellular debris, and placed into a well containing drug or vehicle in medium containing no FCS. At 18 h post-lesioning, the experiment was stopped by washing the coverslips three times in PBS, fixing in 100% ethanol for 5 min and allowing them to air dry, before image analysis.

Visual analysis The regeneration of cells into the denuded area was quantified by use of a Seescan (Cambridge) semi-automated, computerized, image analysis system. Each field of view covered approximately 2% of the total coverslip area. For each coverslip 5 fields of view were selected at random. The lesion area in each field of view was measured and by use of the data for time zero (T_0), the lesion area was then converted to give % regeneration (%R), for each individual coverslip, from the expression:

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

where T_t is the number of hours post-lesion and T_0 is immediately post-lesion.

Cell counting

When direct cell counts were measured, cells were harvested by washing the coverslip three times with PBS and then adding 100 μl 0.05% trypsin/0.02% EDTA for 2–5 min. The reaction was stopped by adding complete medium and the single cell suspension was counted with a haemocytometer. Cell viability was assessed by use of Trypan blue exclusion.

Analysis of concentration-effect curves

The IC_{50} values were calculated, by interpolation from the concentration–effect curves, as the concentration of drug which was required to produce half its maximum possible inhibition for each concentration-effect curve and then the individual values were meaned. The data were then transposed as the negative logarithm of the IC_{50} value, denoted as pIC_{50}. The potency of the antagonism by BIM-23056 was expressed as the pK_B value which was calculated by use of the Gaddum equation:

$$pK_B = \log(\text{conc. ratio} - 1) - \log(\text{antagonist conc.})$$

Where the concentration ratio was defined as the concentration of agonist producing 50% of the maximum in the presence of an antagonist, divided by the concentration of agonist producing the same effect in the absence of antagonist.

Statistical analysis

All data shown were calculated from a minimum of 3 experiments with 4 replicates per test group in each experiment. Values are expressed as the arithmetic mean ± s.e.mean, with the exception of concentration ratio values which are expressed as geometric mean (95% confidence limits). Statistical analysis was by analysis of variance followed by Fisher's partial least-squares difference test, taking $P < 0.05$ as the level of significance.

Materials

Unless otherwise stated, all reagents were purchased from Sigma. Dulbecco's modified Eagle's medium/Hams F-12 nutrient, Hank's balanced salt solutions, M199 foetal calf serum, Glutamax I and G418 were obtained from Life Technologies (Paisley, Scotland). Dil-Ac-LDL were purchased from Biogenesis (Bournemouth). Thermanox coverslips were obtained from NUNC (Life Technologies). Endothelin-1 was obtained through Neosystem (Surrey) and SRIF from Peninsula Laboratories Europe Ltd (St Helens, Merseyside). BIM-23027 (c[N-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe]), BIM-23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2) were custom synthesized by Peptide and Protein Research consultants (University of Exeter, U.K.) and L-362,855 (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe]) was synthesized by Dr J. Murray's team (GlaxoWellcome Chemistry Unit, Department of Chemistry, University of Cambridge). All of the peptides, with the exception of basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF), endothelin-1 (ET-1) and BIM-23056, were initially dissolved in distilled water to produce a concentration of 1 mM. bFGF and PDGF were initially dissolved in 1% serum buffered saline, ET-1 was dissolved in 0.1% acetic acid to give 100 μM stock and BIM-23056 was initially dissolved in 10% dimethylsulphoxide (DMSO). All peptides were then divided into aliquots and

stored at −20°C. Fresh aliquots were used on each experimental day.

Results

Effect of somatostatin on VSMC regeneration

The effect of SRIF (100 nM) on bFGF-induced regeneration of rat aortic VSMC is illustrated in Figure 1. The basal rate of regeneration of VSMC at 18 h following partial denudation of confluent cell monolayer was 5.6±0.8%. SRIF (100 nM) had no effect on this basal rate of regeneration (Figure 2) in any experiment. Submaximal concentrations (data not shown) of bFGF (10 ng ml^{-1}), ET-1 (100 nM) and PDGF (5 ng ml^{-1}) increased regeneration of VSMC by 15.6±0.7%, 17.7±0.4% and 22.1±1.0% respectively. In contrast to the lack of effect observed on basal regeneration, SRIF (0.1 nM–1 μM) caused a concentration-dependent inhibition of bFGF and ET-1-induced regeneration (Figure 2) with respective pIC_{50} values of 8.0±0.1 and 8.6±0.1. Similar concentrations of SRIF also inhibited PDGF-induced regeneration (Figure 2) but the pIC_{50} value could not be calculated since a maximal inhibition was not obtained at the highest concentration tested. There were also slight differences in the apparent SRIF maxima. Thus, SRIF maximally inhibited ET-1-induced re-growth to a level observed in untreated cells, whilst when bFGF was used as the mitogen the maximum inhibitory effect of SRIF was lower than that observed in the untreated cells (Figure 2).

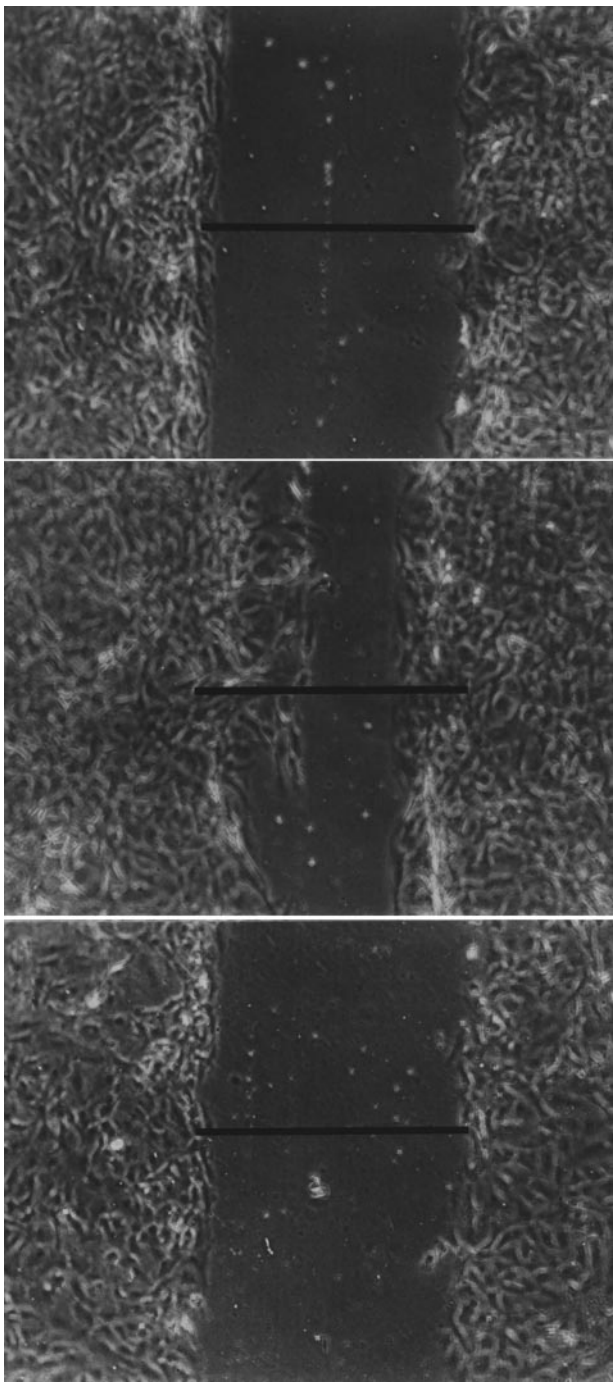

Figure 1 Phase contrast photographs of the regeneration at 18 h of a lesioned VSMC monolayer, at magnification × 160. (a) Control regeneration in the absence of growth factor, (b) regeneration induced by bFGF (10 ng ml^{-1}) and (c) regeneration induced by bFGF (10 ng ml^{-1}) in the presence of SRIF (100 nM). Calibration bar represents 400 μm.

Effect of somatostatin on VSMC proliferation

The effect of SRIF on the proliferation of VSMC monolayers 18 h following partial denudation of a confluent monolayer of cells was also directly determined by counting total cell numbers. SRIF (100 nM) had no effect on the number of VSMC, 18 h following denudation (Figure 3). However, bFGF (10 ng ml^{-1}) increased the cell numbers by 87±2.9%. SRIF (0.1 nM–1 μM) caused a concentration-dependent inhibition of bFGF-induced increase in cell numbers (Figure 3). At the highest concentration of SRIF (1 μM), the mitogenic effect of bFGF was abolished and total cell numbers were similar to those observed in untreated cells. The potency of SRIF (pIC_{50} 8.2±0.1) was similar to that observed in the regeneration studies (see above). The bFGF-induced increase in cell numbers was also abolished by actinomycin D (0.1 μg ml^{-1}) (Figure 3).

Effect of SRIF on regeneration and proliferation of CHO-K1 cells

SRIF (0.1 nM–1 μM) had no effect on basal or bFGF-induced regeneration of wild type CHO-K1 cells (data not shown). SRIF also had no effect on the basal rate of regeneration of CHO-K1 cells recombinantly expressing human sst_5 receptors ($CHOsst_5$ cells) but caused a concentration-dependent inhibition (Figure 4) of bFGF-induced regeneration (pIC_{50} 8.8±0.5). At the highest concentration studied the effect of bFGF was abolished. Despite the fact that SRIF had no effect on the basal rate of regeneration of $CHOsst_5$ cells, bFGF-induced responses were inhibited to a level below that observed under basal conditions. When proliferation was assessed by total cell counting (data not shown), the mitogenic effect of bFGF was abolished by SRIF (100 nM).

Effect of SRIF analogues on bFGF-induced re-growth of VSMC and $CHOsst_5$ cells

In order to characterize the SRIF receptors mediating inhibition of bFGF-induced regeneration of VSMC and $CHOsst_5$ cells, the effects of a number of SRIF-receptor selective peptides have been studied (Figure 4). In both VSMC and $CHOsst_5$ cells, the sst_5 receptor-selective agonist, L-362,855, was the most potent agonist examined at inhibiting bFGF-induced regeneration, being some 30–100 times more potent than SRIF (Figure 4 and Table 1). In marked contrast the sst_2 receptor selective peptide, BIM-23027, was approximately 10 times weaker than SRIF (Table 1). SRIF and SRIF-28 were of similar potency at inhibiting bFGF-induced regeneration (Table 1), but the maximum response to SRIF-28 appeared lower than that of SRIF in VSMC (Figure 4). BIM-23056 (in concentrations up to 10 μM) had no significant effect on bFGF-induced regeneration in either cell type.

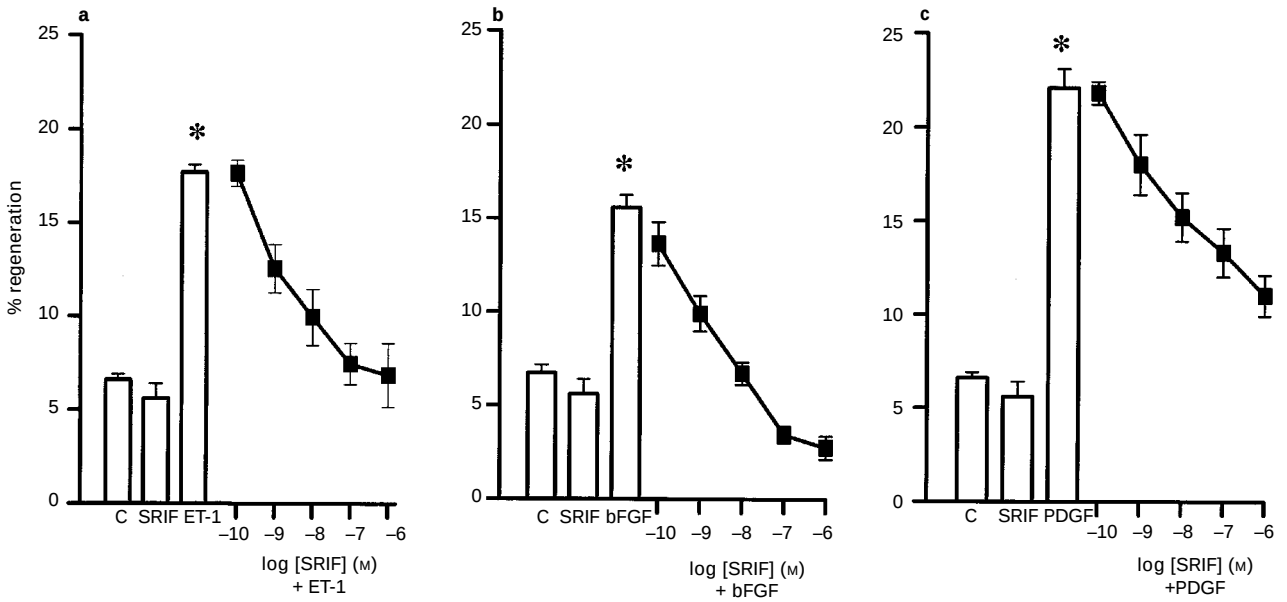

Figure 2 The effect of SRIF (100 nM) on basal regeneration and on the regeneration induced by either (a) ET-1 (100 nM), (b) bFGF (10 ng ml^{-1}) or (c) PDGF (5 ng ml^{-1}) in VSMC monolayers. Values are expressed as the mean % regeneration at 18 h for each test group or for various log concentrations of SRIF ($n=3$, 4 replicates). The vertical lines represent s.e.mean. *Represents $P<0.001$ significant difference from control (C). The effects of SRIF at the concentration range of 1 nM – 1 μM were significantly ($P<0.01$) different from the effects of growth factor treatment alone.

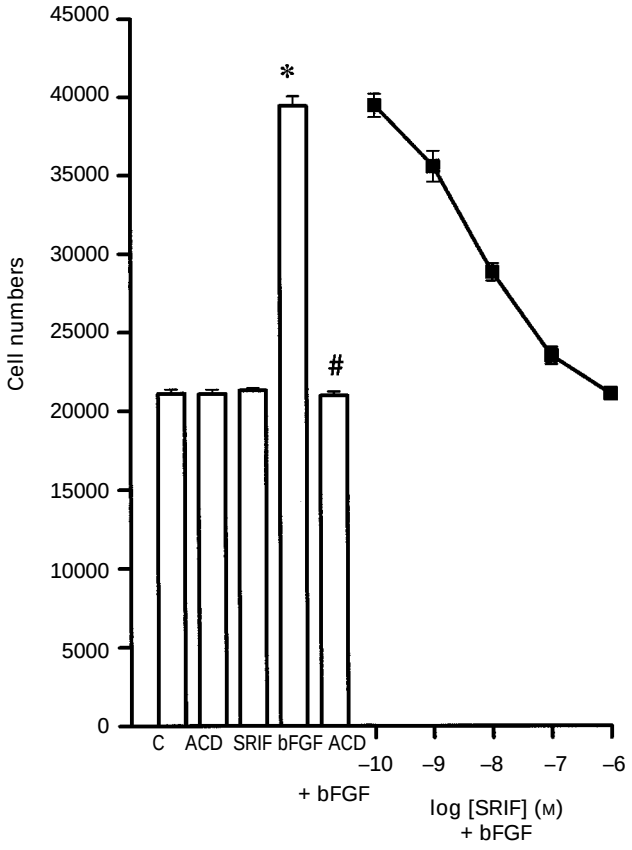

Figure 3 The effect of SRIF on cell proliferation in the presence of bFGF (10 ng ml^{-1}) in VSMC. Values are expressed as the mean cell numbers at 18 h for each test group or against log concentration of SRIF ($n=3$, 4 replicates). The vertical lines represent the s.e.mean. * and # represent $P<0.001$ significant difference compared to control (C) and bFGF treatment, respectively. The effects of SRIF at the concentration range 1 nM – 1 μM were significantly ($P<0.01$) different from the effects of growth factor treatment alone. ACD represents actinomycin D (0.1 μg ml^{-1}).

Antagonistic effect of BIM-23056

Since BIM-23056 was devoid of agonistic activity, it was tested as an antagonist of SRIF-induced inhibition of bFGF-stimulated regeneration in both VSMC and $CHOsst_5$ cells. BIM-23056 (100 nM) caused a rightward displacement of the concentration-effect curves to SRIF in VSMC and $CHOsst_5$ cells (concentration ratios 20 (10 – 30) and 60 (39 – 80), respectively) (Figure 5). The estimated pK_B values were 8.3 ± 0.3 and 8.9 ± 0.4 for VSMC and $CHOsst_5$ cells, respectively.

Effect of pertussis toxin treatment

Pretreatment with pertussis toxin (100 ng ml^{-1} for 20 h) had no effect on basal regeneration of VSMC and $CHOsst_5$ cells or on bFGF-induced regeneration of either cell type. However, the inhibitory effect of SRIF on bFGF-induced regeneration was abolished following pertussis toxin treatment (Figure 6).

Discussion

The ability of SRIF or some of its more stable peptide analogues such as octreotide and angiopeptin to inhibit the growth of various cell types, including tumour cells and vascular smooth muscle cells has long been known (Foegh *et al*., 1989; Lundergan *et al*., 1989; Viguerie *et al*., 1989; Pagliacci *et al*., 1991). This anti-proliferative effect has been thought to be due to a direct effect on DNA synthesis, but the SRIF receptor subtypes involved in these actions have not been identified. In this study we sought to characterize the SRIF receptor subtype mediating the anti-proliferative effect of SRIF in vascular tissue by investigating the actions of some SRIF receptor selective peptides on the proliferation of rat aortic vascular smooth muscle cells in culture. Similar studies were made for comparison on CHO-K1 cells expressing human recombinant sst_5 receptors.

Proliferation was studied in two ways. The first method involved measuring the regeneration of cells into a denuded area of a previously confluent monolayer of cells. However, this method cannot distinguish between an effect of drugs on

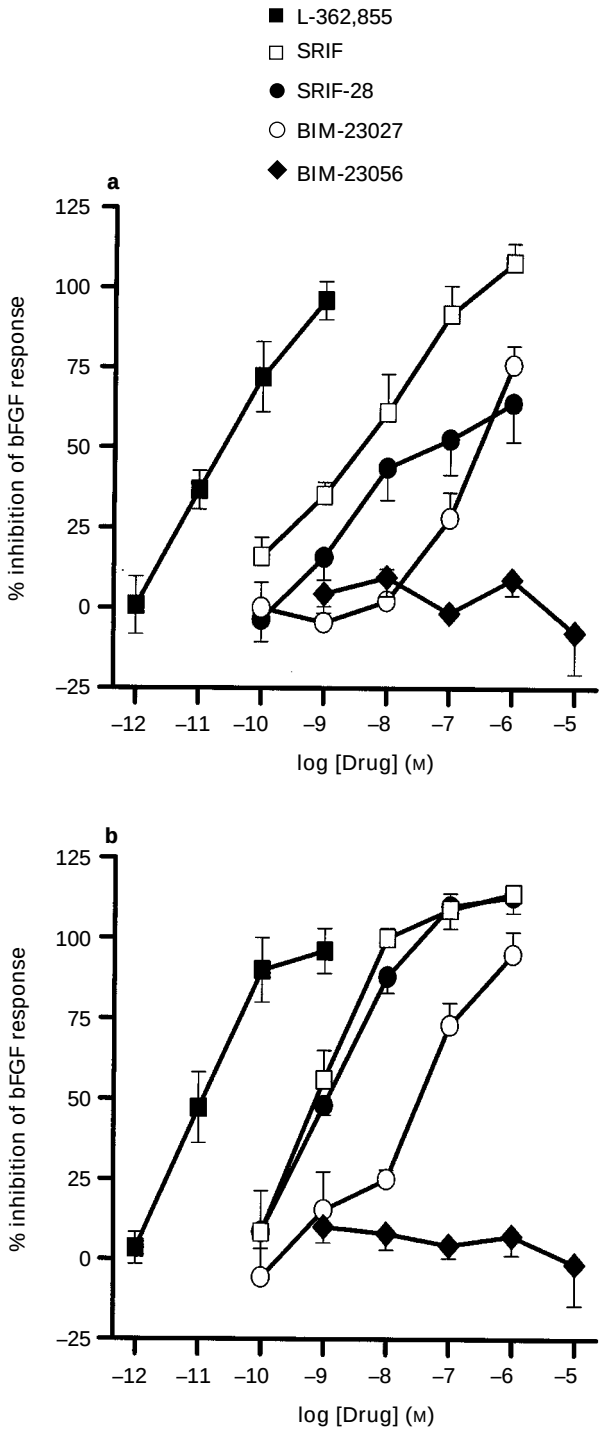

Figure 4 The inhibitory effects of L-362,855, SRIF, SRIF-28, BIM-23027 and BIM-23056 on the growth response induced by bFGF (10 ng ml^{-1}) in lesioned VSMC (a) and in lesioned CHO-K1 cells expressing human sst_5 receptors (b). Values are expressed as the mean % inhibition of bFGF-induced response at 18 h against log concentration of somatostatin analogue ($n=3$, 4 replicates). The vertical lines represent s.e.mean. Where no error bar is shown, the s.e.mean lies within the symbol.

cell migration and/or proliferation. Therefore a second method was also used which involved counting total cell numbers. Essentially similar results were obtained with either technique. The inhibitor of DNA synthesis, actinomycin D, had no effect on the levels of basal regeneration in the absence of growth factors but abolished the bFGF-induced increase in cell numbers (Figure 3). This suggests that in the absence of growth factors the regeneration observed during the first 18 h is predominantly a reflection of cell migration. However, in the presence of growth factor the increase in regeneration is mediated by an increased cellular proliferation.

Table 1 A comparison of the potencies for some SRIF ligands on the inhibition of regeneration of either vascular smooth muscle cells (VSMC) or CHO-K1 cells expressing recombinant sst_5 receptors

Compound	*Rat aortic vascular smooth muscle cells* pIC_{50}	*CHO-K1 cells expressing recombinant* sst_5 *receptors* pIC_{50}
SRIF-14	8.0±0.2	8.8±0.5
SRIF-28	8.3±0.3	8.7±0.7
BIM-23027	6.8±0.4	7.7±0.6
BIM-23056	>1000	>1000
L-362,855	10.5±0.4	11.0±0.7

Values are expressed as the mean pIC_{50} for each test group ($n=3,4$ replicates). The rank orders of agonist potency in VSMC and the transfected CHO-K1 cells were similar, therefore suggesting that the functional response in VSMC is mediated by an sst_5 receptor.

Whilst SRIF had no effect on the basal rate of regeneration of VSMC, it caused a concentration-dependent inhibition of the re-growth induced by bFGF, ET-1 and PDGF. The potency of SRIF was similar irrespective of the mitogen used to stimulate regeneration but there were small differences in the apparent maxima. Thus, at the highest concentration tested, SRIF (1 μM) abolished the mitogenic effect of bFGF and ET-1 but incompletely inhibited (approximately 75%) the mitogenic effect of PDGF. This difference could possibly be accounted for by a difference in the mechanism of the mitogenic action of PDGF compared with bFGF or ET-1. Alternatively, since the mitogenic effect of PDGF was slightly larger than that produced by either bFGF or ET-1, the magnitude of the inhibitory action of SRIF may have been proportionately smaller when PDGF was used as the mitogen. Although SRIF had no effect on basal regeneration, it was also noted that SRIF maximally inhibited bFGF-induced re-growth in VSMC to a level below that observed in the untreated cells. The reason for this is unknown since such an effect was not observed when proliferation was directly measured by cell counting. It may be that an additional inhibitory effect of SRIF on cell migration is revealed when bFGF but not ET-1 or PDGF is used as the mitogenic stimulus. Few studies have addressed the question as to whether SRIF or its analogues can affect cell migration. However, Mooradian *et al.* (1995) showed that angiopeptin was a more potent inhibitor of vascular smooth muscle cell migration than of proliferation.

SRIF also caused a concentration-dependent inhibition of the regeneration of CHO-K1 cells expressing human recombinant sst_5 receptors. The potency of SRIF was similar to that observed in rat aortic VSMC and similar results were obtained with both experimental techniques employed. As was seen in the VSMC, SRIF inhibited bFGF-induced regeneration of CHOsst_5 cells to a level below that observed in the controls. This was not observed with the direct cell counting method. As already mentioned it is possible that SRIF may additionally influence cell migration when bFGF is used as the mitogenic stimulus.

The effect of some SRIF-receptor selective agonists on bFGF-induced regeneration of rat aortic VSMC were examined in order to characterize the SRIF receptors which mediate the inhibitory effect on cell growth. The sst_5 receptor selective agonist, L-362,855 (Raynor *et al.*, 1993a,b; Wilkinson *et al.*, 1997; Williams *et al.*, 1997), was by far the most potent SRIF-receptor agonist studied, being some 30–100 times more potent than SRIF. In contrast the sst_2 receptor selective agonist, BIM-23027 (Raynor *et al.*, 1993a,b; Castro *et al.*, 1996), was approximately twenty times weaker than SRIF. The overall agonist potency order was L-362,855 >> SRIF = SRIF-28 > BIM-23027 suggesting that the antiproliferative effect of

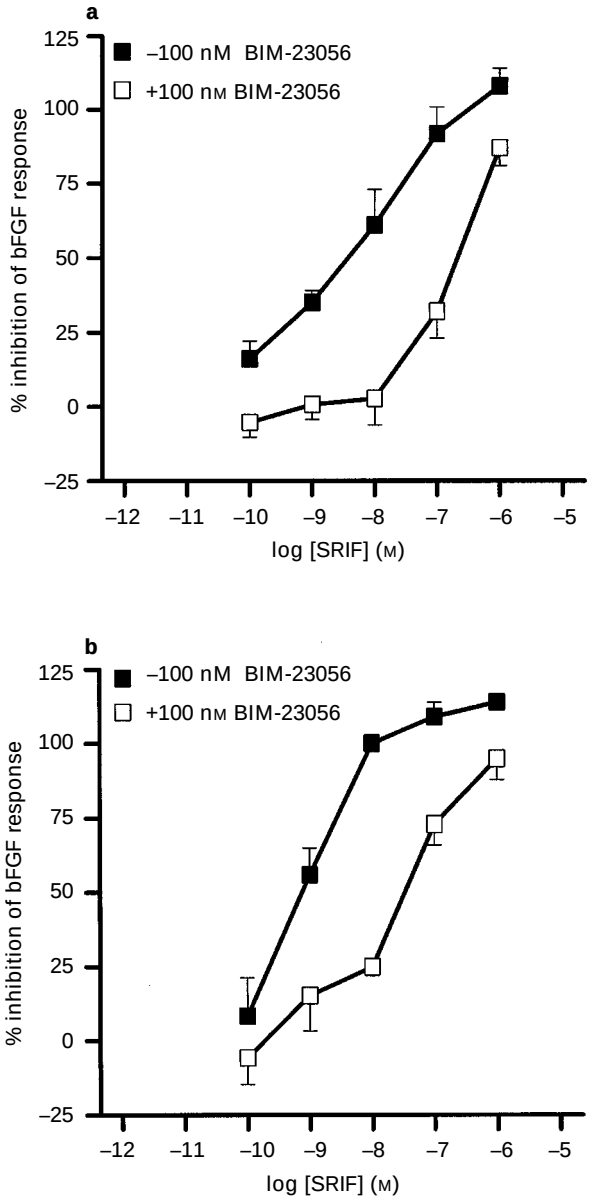

Figure 5 Antagonism with BIM-23056 (100 nM) of the inhibition by SRIF of the regeneration induced by bFGF (10 ng ml^{-1}) in lesioned VSMC (a) and CHO-K1 cells expressing human sst_5 receptors (b). Values are expressed as the mean % inhibition of bFGF-induced response at 18 h against log concentration of SRIF ($n=3$, 4 replicates). The vertical lines represent s.e.mean. Where no error bar is shown, the s.e.mean lies within the symbol.

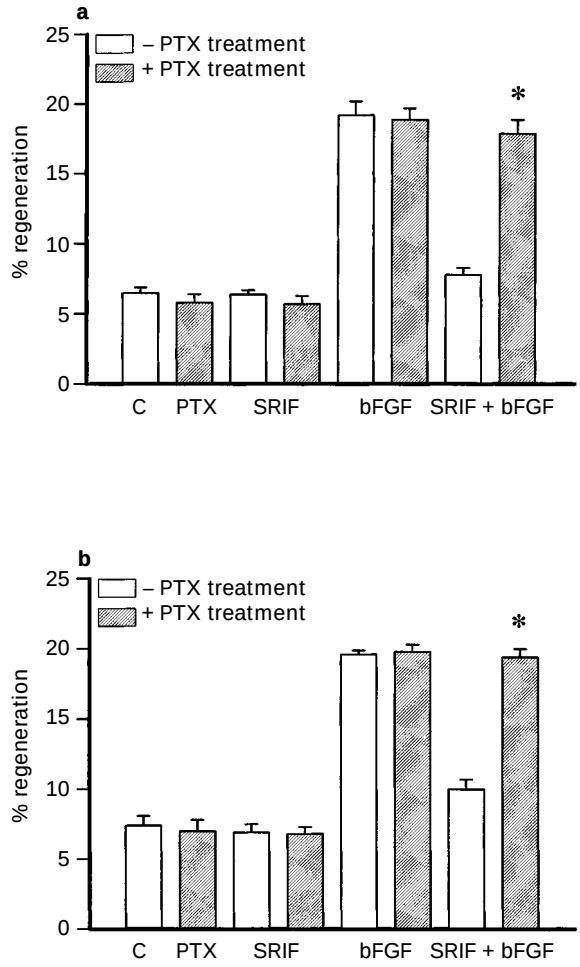

Figure 6 The effect of 20 h pretreatment with pertussis toxin (100 ng ml^{-1}, PTX) on the regeneration of lesioned VSMC (a) and CHO-K1 cells expressing human sst_5 receptors (b) following treatment with either SRIF (100 nM) or bFGF (10 ng ml^{-1}) in the absence and presence of SRIF (100 nM). Values are expressed as mean % regeneration at 18 h for each test group in the presence and absence of pertussis toxin ($n=3$, 4 replicates). The vertical lines represent s.e.mean. *Represents $P<0.001$ significant difference from the effect of SRIF with bFGF in the absence of PTX pretreatment. C represents control.

SRIF in rat aortic VSMC was predominantly mediated by receptors which are similar to the sst_5 receptor. This was supported by the data in CHO-K1 cells expressing the human recombinant sst_5 receptor, where an identical agonist potency order was obtained. However there were discrepancies in the absolute values of potency. Thus BIM-23027 was approximately 10 times more potent at inhibiting bFGF-induced regeneration in $CHOsst_5$ cells (pIC_{50}, 7.7) than in rat aortic VSMC (pIC_{50}, 6.8). This was surprising since radioligand binding studies on membranes from CHO-K1 cells expressing either rat or human recombinant sst_5 receptors (O'Carroll *et al*., 1994) have shown that the apparent affinity of BIM-23027 for rat sst_5 receptors is higher than for human sst_5 receptors. Whilst we have no obvious explanation for this reversal in potency, it is evident that species differences do exist.

The order of agonist potencies in both cell lines was broadly consistent with other functional data which we have described in the $CHOsst_5$ cells. Such studies have included measurements of agonist-induced increases in extracellular acidification rates (Thurlow *et al*., 1996), inhibition of adenylyl cyclase (Williams *et al*., 1996), stimulation of GTPγS binding (Williams *et al*., 1997) and stimulation of phosphoinositide metabolism (Wilkinson *et al*., 1997). However the extremely high potency of L-362,855 at inhibiting bFGF-induced regeneration of both rat aortic VSMC and $CHOsst_5$ cells was surprising and might suggest that the receptors are very efficiently coupled to the transduction process involved in inhibition of cell proliferation, since in other systems it has been shown to behave as a partial agonist (Wilkinson *et al*., 1997; Williams *et al*., 1997). This is all the more significant in the case of the rat aortic VSMC where the density of the SRIF receptors is so low that we were unable to detect any specific [I^{125}]-[Tyr^{11}]-SRIF binding in membranes prepared from these cells (unpublished observations). L-362,855 has been best characterized as a partial agonist in sst_5-receptor coupled systems, such as stimulation of GTPγS binding and activation of phosphoinositide metabolism, where SRIF itself is relatively weak (Williams *et al*., 1997; Wilkinson *et al*., 1997). In contrast when SRIF itself is a potent agonist, as it is for sst_5 receptor mediated inhibition of adenyl cyclase, L-362,855 behaves as a full agonist (Williams *et al*., 1996; 1997). Since SRIF was a potent agonist in the present study, the high intrinsic activity exhibited by L-362,855 is to be

expected. However, the additional finding that it is about 100 times more potent than SRIF seems somewhat incongruent with classical concepts of drug receptor theory and partial agonism, since the affinity of L-362,855 appears no more than 3–10 greater than that of SRIF at the sst_5 receptor (see Williams *et al*., 1997). It remains to be determined whether or not the high relative potency of L-362,855 in this study reflects the putative phenomenon of agonist trafficking of receptor signalling, whereby different agonists will have variable potencies according to the transduction system invoked (see Kenakin, 1995). Certainly, the selective metabolism of SRIF would seem unlikely in view of its high potency.

Characterization of the functional properties of SRIF receptors has always been hindered by the absence of potent and specific receptor blocking drugs. We have recently shown that BIM-23056 is devoid of agonist activity and potently and specifically antagonizes sst_5 receptor mediated increases in intracellular Ca^{2+} and inositol phosphate accumulation in CHOsst_5 cells (Wilkinson *et al*., 1996; 1997). In the present study, BIM-23056 had no effect on bFGF-induced regeneration of rat aortic VSMC and CHOsst_5 cells and further studies were thus undertaken to investigate its effect on the inhibitory action of SRIF. BIM-23056 (100 nM) antagonized the inhibitory effect of SRIF on bFGF-induced regeneration in both rat aortic VSMC and CHOsst_5 cells with apparent pK_B estimates of 8.3 and 8.9, respectively. This provides further evidence that the SRIF receptor mediating the anti-proliferative effect of SRIF in rat aortic VSMC is similar to the human sst_5, receptor expressed in CHO-K1 cells. Whilst the estimated pK_B values for BIM-23056 at antagonizing the inhibitory effect of SRIF on bFGF-induced re-growth were similar, they were somewhat higher than those obtained in other functional studies in CHOsst_5 cells (Wilkinson *et al*., 1996). It is possible that the much longer BIM-23056 contact period in this study (18 h) is responsible for this difference but this requires investigation.

The nature of the receptor mediated transduction cascade leading to the anti-proliferative effect of SRIF in rat aortic VSMC and CHOsst_5 cells is unknown. The effects were clearly mediated via pertussis-toxin sensitive G proteins, since preincubation with pertussis toxin abolished the inhibitory effect of SRIF. Numerous studies (e.g. Buscail *et al*., 1994) have implicated the importance of reversible tyrosine phosphorylation as well as activation of phospholipase C (Ashkenazi *et al*., 1989; Gupta *et al*., 1992) in growth promotion. Indeed, Buscail *et al*. (1995) have argued that an inhibitory effect on intracellular Ca^{2+} mobilization may be responsible for the ability of the SRIF analogue, RC-160, to inhibit serum and cholecystokinin-induced growth of CHO cells expressing human recombinant sst_5 receptors. In our view such a mechanism would seem unlikely since we and others have shown that SRIF activates phospholipase C and increases intracellular Ca^{2+} in CHO-K1 cells expressing human sst_5 receptors (Akbar *et al*., 1994; Wilkinson *et al*., 1996; 1997). The effects of SRIF on tyrosine phosphorylation in rat aortic VSMC is currently under investigation.

Whilst this *in vitro* study clearly implicates the importance of the sst_5 receptor in mediating the anti-proliferative effect of SRIF in rat aortic vascular smooth muscle cells, we do not know whether a similar mechanism can be demonstrated *in vivo* or is of importance clinically. Recent clinical studies with the SRIF analogue, angiopeptin, have provided equivocal data with respect to its effectiveness at preventing coronary arterial restenosis following coronary angioplasty (Emanuelsson *et al*., 1995). Furthermore, we have recently found (unpublished observation) that immunoblots of whole cell extracts of rat aortic VSMC probed with specific anti-sst receptor antisera (Helboe *et al*., 1997), revealed the expression of all five SRIF receptor subtypes cloned to date. The identification of more selective, ideally non-peptidic agonists, is needed not only to determine the importance of these receptors in the control of vascular smooth muscle regeneration but also in other aspects of vascular function.

H.L. gratefully acknowledges the receipt of a studentship from the Medical Research Council.

References

AKBAR, M., OKAJIMA, F., TOMURA, H., MAFID, M.A., YAMADA, Y., SEINO, S. & KONDO, Y. (1994). Phospholipase C activation and Ca^{2+} mobilization by cloned human somatostatin receptor subtypes 1-5, in transfected COS-7 cells. *FEBS Lett*., **348,** 192–196.

ASHKENAZI, A., PERALTA, E.G., WINSLOW, J.W., RAMACHANDRAN, J. & CAPON, D.J. (1989). Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. *Cell*, **56,** 487–493.

BRAZEAU, P., VALE, W., BURGUS, R., LONG, N., BUTCHER, M., RURIER, J. & GUILLENIU, R. (1973). Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science*, **179,** 77–79.

BREVININ, T.A.L., BIANCHI, R. & MOTTA, M. (1993). Direct inhibitory effect of somatostatin on the growth of the human prostatic cancer cell line LNCaP: possible mechanism of action. *J. Clin. Endocrinol. Metab*., **77,** 626–631.

BUSCAIL, L., ESTEVE, J., SAINT-LAURENT, N., BERTRAND, V., REISINE, T., O'CARROLL, A., BELL, G.I., SCHALLY, A.V., VAYSSE, N. & SUSINI, C. (1995). Inhibition of cell proliferation by the somatostatin analogue RC-160 is mediated by somatostatin receptor subtypes SSTR2 and SSTR5 through different mechanisms. *Proc. Natl. Acad. Sci. U.S.A*., **92,** 1580–1584.

CASTRO, S.W., BUELL, G., FENIUK, W. & HUMPHREY, P.P.A. (1996). Differences in the operational characteristics of the human recombinant somatostatin receptor types, sst_1 and sst_2, in mouse fibroblast (Ltk) cells. *Br. J. Pharmacol*., **117,** 639–646.

EMANUELSSON, H., BEATT, K.J., BAGGER, J.P., BALCON, R., HEIKKILA, J., PIESSENS, J., SCHAEFFER, M., SURYAPRANATA, H. & FOEGH, M. (1995). Long-term effects of angiopeptin treatment in coronary angioplasty. Reduction of clinical events but not angiographic restenosis. European Angiopeptin Study Group. *Circulation*, **91,** 1689–1696.

ERIKSEN, U.H., AMTORP, O., BAGGER, J.P., EMANUELSSON, H., FOEGH, M., HENNINGSEN, P., SAUNAMAKI, K., SCHAEFFER, M., THAYSSEN, P., ORSKOV, H., KUNTZ, R.E. & POPMA, J.J. (1995). Randomized double-blind Scandinavian trial of angiopeptin versus placebo for the prevention of clinical events and restenosis after coronary balloon angioplasty. *Am. Heart J*., **130,** 1–8.

FAN, T.P.D. & FROST, E. (1990). A new *in vitro* assay for the study of endothelial cell wound repair. *Eur. J. Pharmacol*., **183,** 1809.

FENIUK, W., DIMECH, J. & HUMPHREY, P.P.A. (1993). Characterisation of somatostatin receptors in the guinea-pig isolated ileum, vas deferens and atrium. *Br. J. Pharmacol*., **110,** 1156–1164.

FOEGH, M.L., KHIRABADI, B.S., CHAMBERS, E., AMAMOO, S. & RAMWELL, P.W. (1989). Inhibition of coronary artery transplant atherosclerosis in rabbits with angiopeptin, an octapeptide. *Atherosclerosis*, **78,** 229–236.

GIMBRONE, M.A. & COTRAN, R.S. (1975). Human vascular smooth muscle cells in culture. *Lab. Invest*., **33,** 16–27.

GUPTA, S.K., GALEEGO, C. & JOHNSON, G.L. (1992). Mitogenic pathways regulated by G protein oncogenes. *Mol. Biol. Cell*, **3,** 123–128.

HELBOE, L., MOLLER, M., NORREGAARD, L., SCHIODT, M. & STIDSEN, C.E. (1997). Development of selective antibodies against the human somatostatin receptor subtypes sst1-sst5. *Mol. Brain Res*., (in press).

HOYER, D., BELL, G.I., BERELOWITZ, M., EPELBAUM, J., FENIUK, W., HUMPHREY, P.P.A., O'CARROLL, A.M., PATEL, Y.C., SCHONBRUNN, A., TAYLOR, J.E. & REISINE, T. (1995). Classification and nomenclature of somatostatin receptors. *Trends Pharmacol. Sci*., **16,** 86–88.

HOYER, D., LUBBERT, H. & BRUNS, C. (1994). Molecular pharmacology of somatostatin receptors. *Naunyn Schmiedeberg's Arch. Pharmacol*., **350,** 441–453.

KENAKIN, T. (1995). Agonist receptor efficacy II: agonist trafficking of receptor signals. *Trends Pharmacol. Sci.*, **16,** 232–238.

LAMBERTS, S.W.J., REUBI, J.-C., UITERLINDEN, P., ZUIDERWIJK, J.V.P. & VAN HAL, P. (1986). Studies on the mechanism of action of the inhibitory effect of the somatostatin analog SMS 201-995 on the growth of the prolactin/adrenocorticotropin-secreting pituitary tumor 7315a. *Endocrinology*, **118,** 2188–2194.

LESZCZYNSKI, D., ZHAO, Y., CATHAPERMAL, S.S., NILSSON, J. & FOEGH, M.L. (1993). Rat heart smooth muscle cells express high and low affinity receptors for somatostatin-14, which are involved in regulation of cell proliferation. *Life Sci.*, **53,** 1663–1674.

LUNDERGAN, C., FOEGH, M.L., VARGAS, R., EUFEMIO, M., BORMES, G.W., KOT, P.A. & RAMWELL, P.W. (1989). Inhibition of myointimal proliferation of the rat carotid artery by the peptides, angiopeptin and BIM 23034. *Atherosclerosis*, **80,** 49–55.

MASCARDO, R.N. & SHERLINE, P. (1982). Somatostatin inhibits rapid centrosomal separation and cell proliferation induced by epidermal growth factor. *Endocrinology*, **111,** 1394–1396.

MCKEEN, E.S., FENIUK, W.F. & HUMPHREY, P.P.A. (1994). SRIF14 receptors mediate the contractile action of somatostatin in the rat isolated distal colon; studies using some novel SRIF analogues. *Br. J. Pharmacol.*, **113,** 628–634.

MOORADIAN, D.L., FERNANDES, B., DIGLIO, C.A. & LESTER, B.R. (1995). Angiopeptin (BIM23014C) inhibits vascular smooth muscle cell migration in vitro through a G-protein-mediated pathway and is associated with inhibition of adenyl cyclase and cyclic AMP accumulation. *J. Cardiovasc. Pharmacol.*, **25,** 611–618.

O'CARROLL, A., RAYNOR, K., LOLAIT, S.J. & REISINE, T. (1994). Characterization of cloned human somatostatin receptor SSTR5. *Mol. Pharmacol.*, **46,** 291–298.

PAGLIACCI, M.C., TOGNELLINI, R., GRIGNANI, F. & NICOLETTI, I. (1991). Inhibition of human breast cancer cell (MCF-7) growth in vitro by the somatostatin analogue SMS 201-995: effects on cell cycle parameters and apopotic cell death. *Endocrinology*, **129,** 2555–2562.

PAZ-BOUA, J.I., REDDING, T.W. & SCHALLY, A.V. (1987). Treatment of nitrosamine-induced pancreatic tumours in hamsters with analogues of somatostatin and luteinizing hormone-releasing hormone. *Proc. Natl. Acad. Sci. U.S.A.*, **84,** 112–116.

RAYNOR, K., MURPHY, W.A., COY, D.H., TAYLOR, J.E., MOREAU, J., YASUDA, K., BELL, G.I. & REISINE, T. (1993a). Cloned somatostatin receptors: identification of subtype-selective peptides and demonstration of high affinity binding of linear peptides. *Mol. Pharmacol.*, **43,** 838–844.

RAYNOR, K., O'CARROLL, A., KONG, H., YASUDA, K., MAHAN, L.C., BELL, G.I. & REISINE, T. (1993b). Characterization of cloned somatostatin receptor subtypes 4 and 5. *Mol. Pharmacol.*, **44,** 358–362.

REICHLIN, S. (1983). Somatostatin. *New Engl. J. Med.*, **309,** 1495–1501.

RIVARD, N., GUAN, D., TURKELSON, C.M., PETITCLERC, D., SOLOMON, T.E. & MORISSET, J. (1991). Negative control by sandostatin on pancreatic and duodenal growth: a possible implication of insulin-like growth factor I. *Regul. Pept.*, **34,** 13–23.

SCHALLY, A.V. & REDDING, T.W. (1987). Somatostatin analogues as adjuncts to agonists of luteinizing hormone in the treatment of experimental prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.*, **84,** 7275–7279.

SKALLI, O., ROPRAZ, P., TRZECIAK, A., BENZONANA, G., GILLESSEN, D. & GABBIANI, G. (1986). A monoclonal antibody against α-smooth muscle actin: A new probe for smooth muscle cell differentiation. *J. Cell Biol.*, **106,** 403–413.

SMITH, J.P. & SOLOMON, T.E. (1988). Effect of gastrin, proglumide and somatostatin on growth of human colon cancer. *Gastroenterology*, **95,** 1541–1548.

THURLOW, R.J., SELLERS, L., COOTE, J.E., FENIUK, W. & HUMPHREY, P.P.A. (1996). Human recombinant sst_5 receptors expressed in CHO-K1 cells mediate increases in extracellular acidification by pertussis toxin-sensitive and -insensitive pathways. *Br. J. Pharmacol.*, **117,** 9P.

VARGAS, R., BORMES, G.W., WROBLEWSKA, B., FOEGH, M.L., KOT, P.A. & RAMWELL, P.W. (1989). Angiopeptin inhibits thymidine incorporation in rat carotid artery. *Transplant. Proc.*, **21,** 3702–3704.

VIGUERIE, N., TAHIRI-JOUTI, N., AYRAL, A.M., CAMBILLAU, C., SCEMAMA, J.L., BASTIE, M.J., KNUHTSEN, S., ESTEVE, J.P., PRADAYROL, L., SUSINI, C. & VAYSSE, N. (1989). Direct inhibitory effects of a somatostatin analog, SMS 201-995, on AR4-2J cell proliferation via pertussis toxin-sensitive guanosine triphosphate-binding protein-binding protein-independent mechanism. *Endocrinology*, **124,** 1017–1925.

VOYTA, J.C., VIA, D.P., BUTTERFIELD, C.E. & ZETTER, B.R. (1984). Identification and isolation of endothelial cells based on their increased uptake of aceylated low density lipoprotein. *J. Cell Biol.*, **99,** 2034–2040.

WILKINSON, G.F, FENIUK, W. & HUMPHREY, P.P.A. (1997). Characterization of human recombinant somatostatin sst_5 receptors mediating activation of phosphoinositide metabolism. *Br. J. Pharmacol.*, **121,** 91–96.

WILKINSON, G.F., THURLOW, R.J., SELLERS, L.A., COOTE, J.E., FENIUK, W. & HUMPHREY, P.P.A. (1996). Potent antagonism by BIM-23056 at the human recombinant somatostatin sst_5 receptor. *Br. J. Pharmacol.*, **118,** 445–447.

WILLIAMS, A.J., MICHEL, A.D., FENIUK, W. & HUMPHREY, P.P.A. (1996). The human recombinant somatostatin sst_5 receptor couples to pertusssis toxin-sensitive and insensitive G proteins. *Br. J. Pharmacol.*, **119,** 11P.

WILLIAMS, A.J., MICHEL, A.D., FENIUK, W. & HUMPHREY, P.P.A. (1997). Somatostatin sst_5 receptor-mediated guanosine-5′-O-(3-[^{35}]thio)triphosphate binding: agonist potencies and the influence of sodium chloride on intrinsic activity. *Mol. Pharmacol.*, (in press).

WYATT, M.A., JARVIE, E., FENIUK, W. & HUMPHREY, P.P.A. (1996). Somatostatin sst_2 receptor-mediated inhibition of parietal cell function in rat isolated gastric mucosa. *Br. J. Pharmacol.*, **119,** 905–910.

ZALATNAI, A., PAZ-BOUZA, J.I., REDDING, T.W. & SCHALLY, A.V. (1988). Histologic changes in the rat prostate cancer model after treatment with somatostatin analogs and D-Trp-6-LH-RH. *Prostate*, **12,** 85–98.

(Received July 4, 1997
Accepted July 17, 1997)