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Lack of evidence for cross-competition between vasoactive intestinal peptide and somatostatin at their respective receptors

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Abstract

A possible cross-competition between vasoactive intestinal peptide (VIP) and somatostatin (somatotropin release inhibiting factor; SRIF) and their respective receptors, was investigated at native or recombinant SRIF and VIP/pituitary adenylate cyclase-activating polypeptide (PACAP) receptors. The activity of VIP was examined in radioligand binding assays at mouse sst_{1-5}, rat sst_{1-2} and human sst_{1-5} receptors; or at human tumours preferentially expressing each of the five SRIF receptors. Moreover, SRIF was investigated at human tumoral tissues known to exclusively express specific VIP/PACAP receptor(s). VIP had no significant effect on any of the radioligand binding sites of the SRIF receptor family of rat, mouse or human origin tested. Conversely, SRIF did not interfere with the human VIP/PACAP binding sites tested. Taken together, the results cast reservation on the claimed cross-competition between VIP and SRIF at, specifically human sst_3 receptors, or any of the cloned SRIF or VIP/PACAP receptors recognised to date.

Keywords: VIP (vasoactive intestinal peptide); SRIF (somatotropin release inhibiting factor); (Mouse); (Rat); (Human); Somatostatin receptor, recombinant; CCL-39 cell; Tumour

1. Introduction

1.1. Somatostatin (somatotropin release inhibiting factor, SRIF)

Somatostatin or somatotropin release inhibiting factor (SRIF) was first identified as a low molecular weight peptide from hypothalamic extracts capable of inhibiting growth hormone (GH) secretion from cultured anterior pituitary cells (Brazeau et al., 1973). Two bioactive forms of SRIF are known to exist in mammal, i.e. $SRIF_{14}$ and $SRIF_{28}$, which act as neurotransmitters/modulators or hormones, depending on the site of action and/or the target cell type. There are currently five SRIF receptors, all of which have been cloned from various species including humans, termed sst_1–sst_5, with the sst_2 receptor demonstrating a splice variant in certain species. These receptors can be subdivided into two main classes, which demonstrate differential structural and pharmacological features (Hoyer et al., 1995a). $SRIF_1$ receptors ($sst_{2A,\ 2B}$, sst_3, sst_5) with high affinity for octreotide and $SRIF_2$ (sst_1, sst_4) with very low affinity for octreotide. All belong to the superfamily of G-protein coupled receptors with seven α-helical transmembrane spanning domains (Bruns et al., 1994; Hoyer et al., 1994). The sst_{1-5} receptors couple negatively to adenylyl cyclase via G-proteins and regulate other signalling pathways including ion channels (K^+, Ca^{2+}), protein serine/threonine and tyrosine phosphatases as well as phospholipase A_2 (Patel, 1997). Hormonal effects of SRIF include the suppression of release of growth hormone releasing factor; in addition to the inhibition of release of other pituitary, pancreatic and gastrointestinal hormones/secretory proteins. Prominently, SRIF analogues inhibit the growth and proliferation of various tumour cells demonstrated to express SRIF receptors (see Lamberts et al., 1991; Buscail et al., 1995).

1.2. Vasoactive intestinal peptide (VIP)

In 1969, a peptide demonstrating smooth-muscle relaxing properties in pulmonary tissue was described (Said and

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Mutt, 1969); it was subsequently isolated from porcine intestine and thereafter named vasoactive intestinal peptide (VIP; Said and Mutt, 1970). In 1974, the sequence of the 28 amino acids, comprising this porcine vasoactive peptide, was established (Mutt and Said, 1974). VIP is formed by cleavage of a 170 amino acid precursor, prepro-VIP, released from secretory vesicles. It is structurally related to several other peptides which display varying degrees of homology to VIP, including peptide histidine methionine, peptide histidine valine, pituitary adenylate cyclase-activating polypeptide (PACAP), helospectin, secretin, glucagon and gonadotropin-releasing hormone. VIP is widely distributed in both the central and peripheral nervous systems, in addition to the circulatory system, gastrointestinal and respiratory tracts.

The effects of VIP are elicited via interaction with specific membrane-bound VIP receptors; three subtypes of which exist and were originally termed VIP_1, VIP_2 and PACAP receptors, based on their relative affinities for VIP and PACAP. However, a recent alteration by the NC-IUPHAR subcommittee on VIP receptors recommended these former names be changed to $VPAC_1$, $VPAC_2$ and PAC_1, respectively (Harmar et al., 1998). These receptors couple positively with adenylyl cyclase via G-proteins and regulate other signalling pathways including cAMP-dependent protein kinases or cAMP-dependent ion channels, resulting in reduction of intracellular Ca^{2+} via Ca^{2+} sequestration and extrusion and activation of myosin light chain phosphatases (Vaudry et al., 2000).

$VPAC_1$ receptors are predominantly expressed in many of the most frequently occurring human tumours, including breast, prostate, pancreas, lung, colon, stomach, liver, and urinary bladder carcinomas as well as lymphomas and meningiomas (Reubi et al., 2000a). $VPAC_2$ receptors are found mainly in smooth muscle, vessels and stroma, and are often expressed in leiomyomas (Reubi et al., 2000a). Preferential expression of PAC_1 receptors is found in glial tumours, pituitary adenomas, paragangliomas, pheochromocytomas and endometrial carcinomas, in the adrenal medulla and in some uterine glands (Robberecht et al., 1993, 1994; Reubi et al., 2000a).

Thus, the wide distribution of VIP/PACAP receptors throughout the human body suggests an important role of these receptors in human physiology. Their high expression in a number of tumours (as outlined above) has been suggested to represent a clinical application for VIP/PACAP in in vivo scintigraphy and radiotherapy of tumours as well as VIP/PACAP analogue treatments for tumour growth inhibition.

1.3. Cross-competition between VIP and SRIF receptors

Both VIP and SRIF receptors are over-expressed in certain tumour cells and in vivo SRIF receptor scintigraphy is a valuable method for the visualisation of primary tumours and metastases in diseased patients. It has recently been suggested that specific SRIF binding to primary tumour cells and cell lines was inhibited by VIP, leading the authors to propose the existence of a common receptor-acceptor site for both VIP and SRIF; or alternatively that VIP interacts with SRIF receptors (Virgolini et al., 1994). Moreover, in vitro ligand receptor interactions demonstrated that a variety of tumours co-express receptors for VIP and SRIF and cross-competition was suggested to take place at the cell surface (Virgolini et al., 1994; Virgolini, 1997). It was concluded that the SRIF receptor at which cross-competition with VIP took place on tumour cell membranes/cell lines was the $hsst_3$ receptor, with displaceable binding by VIP in the low nanomolar range (Virgolini, 1997; Virgolini et al., 1998a,b; Raderer et al., 1999).

With regard to the important implications of a potential cross-competition for physiological and pathological conditions, the aim of the present study was to evaluate the aforementioned, by estimating the affinity of VIP on native rat and mouse $sst_{1,2}$ receptors expressed in cerebral cortex, native $hsst_{1-5}$ receptors expressed in human tumours and recombinant $hsst_{1-5}$ and $msst_{1,\,3-5}$ receptors expressed in CCL-39 cells. Conversely, to clarify the possibility of such cross-competition, the effects of SRIF on human tumoral ($VPAC_{1-2}$ and PAC_1) or normal ($VPAC_{1-2}$) tissues, exclusively or co-expressing specific receptor subtype(s) of the VIP/PACAP or both the SRIF and VIP/PACAP receptor families, respectively, were also established.

2. Methods

2.1. Somatostatin receptor binding to recombinant cells, normal and tumoral tissues

2.1.1. Mouse or rat cerebral cortex preparation

Male OF1 mice (25–30 g) were obtained from ICO. Animals were killed by exposure to CO_2, decapitated and the brains quickly removed and placed in ice-cold HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 10 mM, pH 7.5). The cerebral cortex was dissected free, on ice, and the tissue homogenised in HEPES (10 mM, pH 7.5) using a Polytron tissue homogeniser at full speed for 45 s. The homogenate was centrifuged (22,000 rpm, 20 min, 4 °C; Kontron ultracentrifuge, rotor TFT 50.38). The resulting supernatant was discarded, the pellet resuspended in HEPES (10 mM; pH 7.5), homogenised (45 s) and centrifuged as before (22,000 rpm, 20 min, 4 °C). The resulting final pellet was resuspended in HEPES (10 mM, pH 7.5) aliquoted into 2.5 ml batches (0.1 mg original tissue ml^{-1}) and frozen at −80 °C. Rat cerebral cortex samples were purchased directly from ANAWA (Wangen, Switzerland).

2.1.2. Cells expressing recombinant SRIF receptors

Somatostatin receptors ($hsst_{1-5}$, $msst_{1,\,3-5}$) were stably transfected into CCL-39 cells (established line of Chinese

hamster lung fibroblasts; American Type Culture Collection; Siehler et al., 1998, 1999; Feuerbach et al., 2000) and cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM; 3.7 g l^{-1} $NaHCO_3$, 1.0 g l^{-1} D-glucose, with stable glutamine) and Ham's F-12 Nutrient Mixture (1.176 g l^{-1} $NaHCO_3$, with stable glutamine), supplemented with 10% (v v^{-1}) foetal bovine serum, and the antibiotic(s) G418 sulphate (100 μg ml^{-1}) and/or Hygromycin B (100 μg ml^{-1}) at 37 °C, 5% CO_2 and 95% relative humidity. For storage, the cells were resuspended in medium containing dimethyl sulfoxide (DMSO, 10% final concentration) and 20% foetal bovine serum, and frozen in liquid nitrogen. For preparation, stocks were thawed rapidly in a 37 °C water bath, and the contents transferred to a tube containing DMEM/Ham's F12 nutrient mix. The cells were centrifuged (2000 rpm, 3 min, 4 °C; Omnifuge 2.0), and the supernatant aspirated. The pellet was resuspended in culture medium corresponding to the respective cell-line, i.e. $hsst_1$ $msst_1$, 3–5 were resuspended in DMEM/Ham's F12 mix, 10% FCS, G418 (100 μg ml^{-1}) and Hygromycin B (100 μg ml^{-1}); while $hsst_{2-5}$ were resuspended in DMEM/Ham's F12 mix, 10% foetal bovine serum and G418 (100 μg ml^{-1}) and the cells transferred to an appropriate cell culture flask (generally NUNC 175 cm^2 cell culture plates, but for mass propagation NUNC 530 cm^2 Bioassay plates were utilised). For passaging, the cells were detached from the cell culture flask by washing with phosphate-buffered saline (PBS) followed by a brief (3–5 min) incubation with trypsin (0.5 mg ml^{-1})/ethylenediaminetetraacetate (EDTA; 0.2 mg ml^{-1}) every 2–3 days. On the experimental day, cells were resuspended at varying densities (see below) in HEPES (10 mM, containing 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) and homogenised (Polytron) for 45 s.

2.1.3. sst_1 Receptors

2.1.3.1. Rat or mouse brain (cerebral cortex) sst_1 receptor binding. Membranes were resuspended in binding assay buffer (HEPES 10 mM, containing 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) to 0.01 mg ml^{-1} (original protein weight), and 150 μl of homogenised membranes incubated with 50 μl [^{125}I]$SRIF_{14}$ (Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-(^{125}I-Tyr)-Ser-Cys]OH; 2175 Ci $mmol^{-1}$; final concentration 29–34 pM, in binding assay buffer containing NaCl (120 mM) and the protease inhibitor, bacitracin, 5 μg ml^{-1}), and either 50 μl binding assay buffer (total binding) or 50 μl of various concentrations of VIP for 1 h at 22 °C (see Hoyer et al., 1995b).

The following protocol is valid for all SRIF receptor binding assays; i.e. non-specific binding was determined in the presence of $SRIF_{14}$ (1 μM) and incubations were terminated by vacuum filtration through glass fibre filters (Packard Unifilter-96, GF/C plates), pre-soaked for 60 min in 2.5% (w v^{-1}) polyethyleneimine. The filters were washed three times with 300 μl ice-cold 10 mM Tris–HCl buffer containing 154 mM NaCl, pH 7.5 and dried for a minimum of 2 h. Filter-bound radioactivity was determined using scintillation fluid (Microscint 40, 40 μl) and counting for 2 min in a γ-counter (Packard TopCount). All experiments were conducted in triplicate.

2.1.3.2. Human or mouse recombinant sst_1 receptor binding. Cells were resuspended in binding assay buffer (HEPES 10 mM, containing 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) to densities of 50,000 (human) and 75,000–150,000 (mouse) cells $well^{-1}$. One hundred and fifty microliters of cell homogenate was incubated with 50 μl of [^{125}I]CGP 23996 (c[Lys-Asu-Phe-Phe-Trp-Lys-Thr-(^{125}I-Tyr)-Thr-Ser]) or [^{125}I]LTT-$SRIF_{28}$ ([Leu^8,DTrp^{22}, ^{125}I-Tyr^{25}]$SRIF_{28}$ (Ser-Ala-Asn-Ser-Asn-Pro-Ala-Leu-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-DTrp-Lys-Thr-(^{125}I-Tyr)-Thr-Ser-Cys]OH); 2175 Ci $mmol^{-1}$; final concentrations 56–60 and 28–31 pM, respectively, in binding assay buffer containing $MgCl_2$ (5 mM), and the protease inhibitor, bacitracin, 5 μg ml^{-1}), and either 50 μl binding assay buffer (total binding) or 50 μl of various concentrations of VIP for 1 h at 22 °C (see Siehler et al., 1999).

2.1.4. sst_2 Receptors

2.1.4.1. Rat or mouse brain (cerebral cortex) sst_2 receptor binding. Membranes were resuspended in binding assay buffer (HEPES 10 mM, containing 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) to 0.01 mg ml^{-1} (original protein weight), and 150 μl of homogenised membranes incubated with 50 μl [^{125}I]Tyr^3-octreotide (DPhe-c[Cys-(^{125}I-Tyr)-DTrp-Lys-Thr-Cys]Thr-OH); 2175 Ci $mmol^{-1}$; final concentration 35–37 pM, in binding assay buffer containing $MgCl_2$ (5 mM) and the protease inhibitor, bacitracin, 5 μg ml^{-1}), and either 50 μl binding assay buffer (total binding) or 50 μl of various concentrations of VIP for 1 h at 22 °C (see Schoeffter et al., 1995).

2.1.4.2. Human recombinant sst_2 receptor binding. Cells were resuspended in binding assay buffer (HEPES 10 mM, containing 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) to a density of 100,000 cells $well^{-1}$. One hundred and fifty microliters of cell homogenate was incubated with 50 μl of [^{125}I]CGP 23996, [^{125}I]LTT-$SRIF_{28}$ or [^{125}I]Tyr^3-octreotide (2175 Ci $mmol^{-1}$; final concentrations 56–60, 28–31 and 35–37 pM, respectively, in binding assay buffer containing $MgCl_2$ (5 mM), and the protease inhibitor, bacitracin, 5 μg ml^{-1}), and either 50 μl binding assay buffer (total binding) or 50 μl of various concentrations of VIP for 1 h at 22 °C (see Siehler et al., 1999).

2.1.5. sst_3 Receptors

2.1.5.1. Human or mouse recombinant sst_3 receptor binding. Cells were resuspended in binding assay buffer (HEPES 10 mM, containing 0.5% (w v^{-1}) bovine serum

albumin, pH 7.5) to densities of 50,000 (human) and 20,000–40,000 (mouse) cells well^{-1}. One hundred and fifty microliters of cell homogenate was incubated with 50 μl of [^{125}I]CGP 23996 or [^{125}I]LTT-SRIF$_{28}$ (2175 Ci mmol^{-1}; final concentrations 56–60 and 28–31 pM, respectively, in binding assay buffer containing $MgCl_2$ (5 mM), and the protease inhibitor, bacitracin, 5 μg ml^{-1}), and either 50 μl binding assay buffer (total binding) or 50 μl of various concentrations of VIP for 1 h at 22 °C (see Siehler et al., 1999).

2.1.6. sst_4 Receptors

2.1.6.1. Human or mouse recombinant sst_4 receptor binding. Cells were resuspended in binding assay buffer (HEPES 10 mM, containing 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) to densities of 50,000 (human) and 50,000–100,000 (mouse) cells well^{-1}. One hundred and fifty microliters of cell homogenate was incubated with 50 μl of [^{125}I]CGP 23996 or [^{125}I]LTT-SRIF$_{28}$ (2175 Ci mmol^{-1}; final concentration 56–60 and 28–31 pM, respectively, in binding assay buffer containing $MgCl_2$ (5 mM), and the protease inhibitor, bacitracin, 5 μg ml^{-1}), and either 50 μl binding assay buffer (total binding) or 50 μl of various concentrations of VIP for 1 h at 22 °C (see Siehler et al., 1999).

2.1.7. sst_5 Receptors

2.1.7.1. Human or mouse recombinant sst_5 receptor binding. Cells were resuspended in binding assay buffer (HEPES 10 mM, containing 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) to densities of 50,000 (human) and 25,000–350,000 (mouse) cells well^{-1}. One hundred and fifty microliters of cell homogenate was incubated with 50 μl of [^{125}I]CGP 23996, [^{125}I]LTT-SRIF$_{28}$or [^{125}I]Tyr^3-octreotide (2175 Ci mmol^{-1}; final concentrations 34–109 pM, respectively, in binding assay buffer containing $MgCl_2$ (5 mM), and the protease inhibitor, bacitracin, 5 μg ml^{-1}), and either 50 μl binding assay buffer (total binding) or 50 μl of various concentrations of VIP for 1 h at 22 °C (see Siehler et al., 1999).

2.1.8. [^{125}I]LTT-SRIF$_{28}$ binding to human tumours

Human tumour samples, specifically expressing one of the five SRIF receptor subtypes, as reported previously using in situ hybridisation and/or subtype-selective binding assays (Schaer et al., 1997), were used to perform displacement binding experiments with [^{125}I]LTT-SRIF$_{28}$ and increasing concentrations of either SRIF$_{14}$ or VIP. In

Table 1
Inhibition of [^{125}I]CGP 23996, [^{125}I]LTT-SRIF$_{28}$, [^{125}I]Tyr^3-octreotide and [^{125}I]SRIF$_{14}$ binding at mouse and rat SRIF receptors by vasoactive intestinal peptide (1–10 μM)

		[^{125}I]CGP 23996	[^{125}I]LTT-SRIF$_{28}$	[^{125}I]SRIF$_{14}$	[^{125}I]Tyr^3-octreotide
msst$_1$	VIP 10^{-6}	−1.0±2.8	9.5±3.2	−17.0±2.3	
	VIP 10^{-5}	10.5±3.9	22.0±1.4	−38.0±7.7	
msst$_2$	VIP 10^{-6}				7.7±1.2
	VIP 10^{-5}				16.3±1.2
msst$_3$	VIP 10^{-6}	2.0±3.5	5.5±2.5		
	VIP 10^{-5}	2.0±0.0	22.5±1.1		
msst$_4$	VIP 10^{-6}	4.5±3.9	−0.5±1.8		
	VIP 10^{-5}	16.5±1.8	18.0±1.4		
msst$_5$	VIP 10^{-6}	−6.0±0.0	−2.5±1.1		−11.0±6.4
	VIP 10^{-5}	27.5±1.8	8.5±1.1		12.5±2.5
rsst$_1$	VIP 10^{-6}			−5.7±1.4	
	VIP 10^{-5}			−1.7±1.2	
rsst$_2$	VIP 10^{-6}				−1.5±3.2
	VIP 10^{-5}				15.7±4.0

Results are expressed as mean percentage decrease in total binding± S.E.M. of two to three individual experiments performed in triplicate.

Table 2
Inhibition of [^{125}I]CGP 23996, [^{125}I]LTT-SRIF$_{28}$ and [^{125}I]Tyr^3-octreotide binding at human SRIF receptors expressed in CCL-39 cells, by vasoactive intestinal peptide (VIP, 1–10 μM)

		[^{125}I]CGP 23996	[^{125}I]LTT-SRIF$_{28}$	[^{125}I]Tyr^3-octreotide
hsst$_1$	VIP 10^{-6}	1.5±1.8	−1.0±0.7	
	VIP 10^{-5}	3.0±0.0	10.0±1.4	
hsst$_2$	VIP 10^{-6}	−11.0±0.0	−4.5±1.8	0.0±1.4
	VIP 10^{-5}	6.5±5.6	−2.0±2.8	5.5±0.4
hsst$_3$	VIP 10^{-6}	−0.5±1.8	4.5±0.4	
	VIP 10^{-5}	10.5±1.8	15.0±0.0	
hsst$_4$	VIP 10^{-6}	0.8±0.5	0.8±0.5	
	VIP 10^{-5}	6.0±0.0	3.0±1.4	
hsst$_5$	VIP 10^{-6}	−2.0±0.0	−1.0±3.5	−2.0±2.8
	VIP 10^{-5}	8.5±0.4	1.0±1.4	14.0±2.8

Results are expressed as mean percentage decrease in specific binding± S.E.M. of two to three individual experiments performed in triplicate.

Table 3
Effects of vasoactive intestinal peptide (VIP; 1 μM) on [^{125}I]LTT-SRIF$_{28}$ binding in human tumours preferentially expressing one of the five SRIF receptor subtypes

Tumour type	Displacement by 1 μM VIP
sst$_1$-Expressing tumours $n=5$ (leiomyoma, leiomyosarcoma, ependymoma, leiomyoblastoma, prostate cancer)	No displacement
sst$_2$-Expressing tumours $n=5$ (two breast cancers, neuroblastoma, gastroenteropancreatic tumour, meningioma)	No displacement
sst$_3$-Expressing tumours $n=5$ (inactive pituitary adenomas)	No displacement
sst$_4$-Expressing tumours $n=1$ (leiomyoma)	No displacement
sst$_5$-Expressing tumours $n=3$ (pituitary adenoma, gastric carcinoma, ependymoma)	No displacement

Table 4
Effects of vasoactive intestinal peptide (VIP; 1 μM) on [^{125}I]LTT-SRIF$_{28}$ binding in human tumours co-expressing SRIF receptors and a specific member of the VIP/PACAP receptor family

Tumour type	Displacement by 1 μM VIP
Tumours expressing VPAC$_1$ together with somatostatin receptors	No displacement
two prostate cancers co-expressing sst$_1$; one breast cancer co-expressing sst$_2$	
Tumours expressing VPAC$_2$ together with somatostatin receptors	No displacement
two leiomyomas co-expressing sst$_1$	No displacement
Tumours expressing PAC$_1$ together with somatostatin receptors	
three pheochromocytomas co-expressing sst$_2$	

separate experiments, tumours co-expressing both SRIF and a specific receptor subtype of the VIP/PACAP receptor family (VPAC$_1$, VPAC$_2$ or PAC$_1$) were also analysed to establish the effect of VIP on [^{125}I]LTT-SRIF$_{28}$ binding.

2.2. VIP binding to cell lines and human tissues

2.2.1. [^{125}I]VIP binding to sst$_1$–sst$_5$ transfected cells

Using cell pellets preferentially expressing one of the sst$_1$–sst$_5$ receptors, as previously described (Reubi et al., 2000b,c), the binding of monoiodo [^{125}I]VIP was evaluated and compared to that of [^{125}I]LTT-SRIF$_{28}$.

2.2.2. Effects of SRIF$_{14}$ on [^{125}I]VIP, [^{125}I]RO 25-1553 and [^{125}I]PACAP binding to human tumoral and normal tissues

Human tumours, selected on the basis of their preferential expression of either VPAC$_1$, VPAC$_2$ or PAC$_1$, as previously reported (Reubi et al., 2000a), were used in displacement experiments using [^{125}I]VIP, [^{125}I]Ro 25-1553 or [^{125}I]PACAP, respectively, and increasing concentrations of VIP, Ro 25-1553, PACAP and SRIF$_{14}$. Breast tumours co-expreasing VPAC$_1$ and sst$_2$ receptors were also tested. Moreover, normal tissues expressing VPAC$_1$ or VPAC$_2$ receptors were also utilised to assess the binding affinity of SRIF$_{14}$ for the aforementioned VIP/PACAP receptor subtype(s).

2.3. Materials

Vasoactive intestinal peptide (porcine), polyethyleneimine, bovine serum albumin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Trizma pre-set crystals (Tris [Hydroxymethyl] aminomethane and Tris–HCl), sodium chloride (NaCl) and magnesium chloride hexahydrate ($MgCl_2$ $6H_2O$) were purchased from Sigma Aldrich (Schnelldorf, Germany). Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture were purchased from Seromed (Biochrom, Berlin, Germany). Trypsin/EDTA and Foetal bovine serum were from Gibco, while the antibiotics G418 sulphate and Hygromycin B were purchased from Promega (Madison, WI, USA) and Calbiochem (La Jolla, CA, USA), respectively. SRIF$_{14}$ (Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Tyr-Ser-Cys]OH) was purchased from Bachem

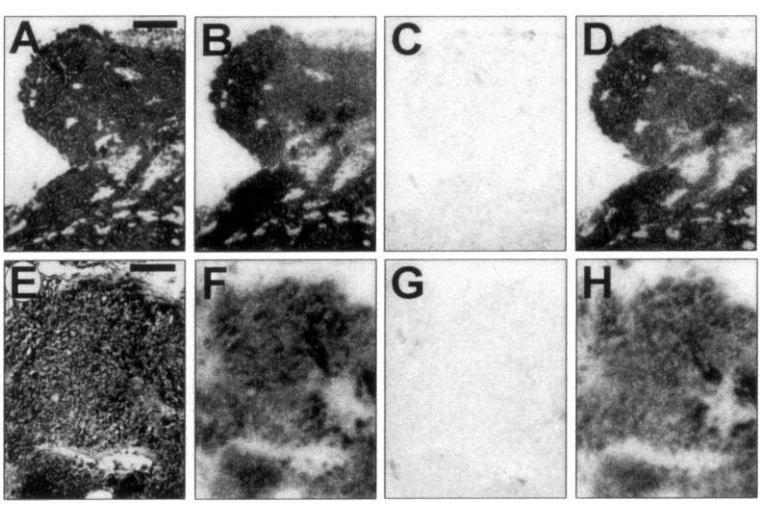

Fig. 1. Representative experimental autoradiograms demonstrating the lack of effect of VIP (1 μM) on [^{125}I]LTT-SRIF$_{28}$ binding to an sst$_3$-expressing inactive pituitary adenoma (panels A–D) and an sst$_2$- and VPAC$_1$-expressing breast carcinoma (panels E–H). Panels A and E: Hematoxylin-eosin stained sections; scale bar represents 1 mm. Panels B and F: Autoradiograms showing total binding of [^{125}I]LTT-SRIF$_{28}$. Panels C and G: Autoradiograms showing non-specific binding of [^{125}I]LTT-SRIF$_{28}$ (in the presence of 10^{-7} M SRIF$_{28}$). Panels D and H: Autoradiograms showing binding of [^{125}I]LTT-SRIF$_{28}$ (in the presence of 10^{-6} M VIP).

Table 5
Lack of binding of [^{125}I]VIP to stably transfected cells expressing sst_1–sst_5 receptors

	Specific binding [^{125}I]LTT-$SRIF_{28}$	Specific binding [^{125}I]VIP
sst_1 Cells	+	∅
sst_2 Cells	+	∅
sst_3 Cells	+	∅
sst_4 Cells	+	∅
sst_5 Cells	+	∅

(Bubendorf, Switzerland). [^{125}I]CGP 23996: (c[Lys-Asu-Phe-Phe-Trp-Lys-Thr-(^{125}I-Tyr)-Thr-Ser]); [^{125}I]Tyr^3-octreotide: (DPhe-c[Cys-(^{125}I-Tyr)-DTrp-Lys-Thr-Cys]Thr-OH); [Leu^8,$DTrp^{22}$,^{125}I-Tyr^{25}]$SRIF_{28}$ ([^{125}I]LTT-$SRIF_{28}$; Ser-Ala-Asn-Ser-Asn-Pro-Ala-Leu-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-DTrp-Lys-Thr-(^{125}I-Tyr)-Thr-Ser-Cys]OH), [^{125}I]Tyr^{11}-$SRIF_{14}$ (Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-(^{125}I-Tyr)-Ser-Cys]OH), [^{125}I]VIP, [^{125}I]Ro 25-1553 and [^{125}I]PACAP (2000–2200 Ci $mmol^{-1}$) were custom labelled, and rat cerebral cortex samples supplied, by ANAWA. ^{3}H-Hyperfilms and autoradiographic [^{125}I]microscales were from Amersham (Aylesbury, UK).

3. Results

3.1. Effects of VIP on native or recombinant SRIF receptors

As illustrated in Table 1, VIP (1–10 μM) had no significant effects on specific binding in either CCL-39 cells expressing $msst_{1,\ 3-5}$ or native $msst_{1-2}$ receptors in cerebral cortex. In the same experiments, $SRIF_{14}$ (1 μM) induced marked inhibition of the total binding of [^{125}I]CGP 23996, [^{125}I]LTT-$SRIF_{28}$, [^{125}I]Tyr^3-octreotide and [^{125}I]$SRIF_{14}$, at the aforementioned receptors, by 56–94%. Moreover, VIP (1–10 μM) was without effect on specific binding at native $rsst_{1-2}$ receptors in cerebral cortex (Table 1). In parallel experiments, $SRIF_{14}$ (1 μM) induced marked inhibition of total [^{125}I]Tyr^3-octreotide and [^{125}I]$SRIF_{14}$ binding at these receptors by 94% and 73%, respectively.

Similarly, VIP (1–10 μM) was without effect on specific binding in CCL-39 cells expressing $hsst_{1-5}$ receptors (Table 2), whereas in parallel experiments, $SRIF_{14}$ (1 μM) induced marked inhibition of [^{125}I]CGP 23996, [^{125}I]LTT-$SRIF_{28}$, [^{125}I]Tyr^3-octreotide and [^{125}I]$SRIF_{14}$ binding at these sites by 70–98%.

In competition, binding experiments with sst_1-, sst_2-, sst_3-, sst_4- and sst_5-receptor expressing tumours, VIP, up

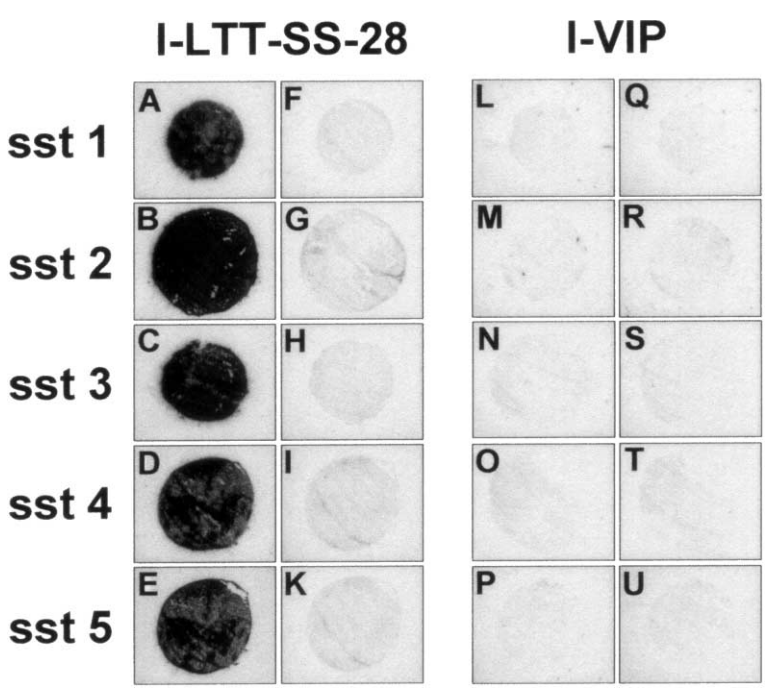

Fig. 2. Representative experimental autoradiograms comparing [^{125}I]LTT-$SRIF_{28}$ (I-LTT-SS-28) and [^{125}I]VIP (I-VIP) binding to stably transfected cells expressing sst_1–sst_5 receptors. Horizontal lanes demonstrate total (panels A–E) and non-specific (panels F–K; in the presence of 10^{-7} M $SRIF_{28}$) binding of [^{125}I]LTT-$SRIF_{28}$; in addition, total (panels L–P) and non-specific (panels Q–U; in the presence of 10^{-6} M VIP) binding of [^{125}I]VIP to cell pellets preferentially expressing each of the five SRIF receptor subtypes are shown. In each case, [^{125}I]VIP binding is absent whereas marked [^{125}I]LTT-$SRIF_{28}$ is demonstrated.

Table 6
Lack of effect of $SRIF_{14}$ (1 μM) on [^{125}I]VIP, [^{125}I]Ro 25-1553 and [^{125}I]PACAP binding in human tumours preferentially expressing $VPAC_1$, $VPAC_2$ or PAC_1 receptors; [^{125}I]VIP binding to tumours co-expressing $VPAC_1$ and sst_2 receptors, and [^{125}I]VIP binding in normal tissues expressing either $VPAC_1$ or $VPAC_2$ receptors

	Displacement by 1 μM $SRIF_{14}$
Tumour type	
$VPAC_1$-expressing tumours	[^{125}I]VIP
$n = 15$ (six breast; four colon; five prostate cancers)	No displacement
$VPAC_2$-expressing tumours	[^{125}I]Ro 25-1553
$n = 2$ (leiomyomas)	No displacement
PAC_1-expressing tumours	[^{125}I]PACAP
$n = 9$ (three pheochromocytomas; one paraganglioma; three pituitary adenomas; two astrocytomas)	No displacement
Tumours co-expressing $VPAC_1$ and sst_2	[^{125}I]VIP
$n = 3$ (breast cancers)	No displacement
Normal tissues	
$VPAC_1$-expressing tissues	[^{125}I]VIP
$n = 4$ (breast; liver; prostate)	No displacement
$VPAC_2$-expressing tissues	[^{125}I]Ro 25-1553
$n = 3$ (vessels and smooth muscles)	No displacement

to concentrations of 1 μM, was unable to affect specific [^{125}I]LTT-$SRIF_{28}$ binding (Table 3). Furthermore, in tumours co-expressing SRIF and individual VIP receptors ($VPAC_1$, $VPAC_2$ or PAC_1), VIP was also devoid of any effect on [^{125}I]LTT-$SRIF_{28}$ binding (Table 4). Representative autoradiograms, from the aforementioned experiments, are shown in Fig. 1, and demonstrate marked [^{125}I]LTT-$SRIF_{28}$ binding in both an sst_3-expressing inactive pituitary adenoma (panel B) and an sst_2- and $VPAC_1$-expressing breast carcinoma (panel F). In both instances, displacement of [^{125}I]LTT-$SRIF_{28}$ binding was achieved in the presence of $SRIF_{28}$ (100 nM; panels C and G), yet was unaffected by VIP (1 μM; panels D and H). Conversely, no specific [^{125}I]VIP binding could be established in any of the sst_1–sst_5 receptor transfected cells, while specific binding of [^{125}I]LTT-$SRIF_{28}$ was obviously present (Table 5 and Fig. 2).

3.2. Effects of $SRIF_{14}$ on [^{125}I]VIP, [^{125}I]Ro 25-1553 and [^{125}I]PACAP binding to human tissues

In binding experiments with $VPAC_1$-, $VPAC_2$- or PAC_1-expressing tumours, $SRIF_{14}$, up to concentrations of 1 μM, was without effect on [^{125}I]VIP, [^{125}I]Ro 25-1553 or [^{125}I]PACAP binding, respectively (Table 6). Furthermore, $SRIF_{14}$ was without effect on [^{125}I]VIP binding in breast carcinomas co-expressing SRIF and VIP/PACAP receptors. Representative autoradiograms are shown in Fig. 3, and demonstrate marked [^{125}I]VIP binding to a $VPAC_1$-expressing breast carcinoma (panel B); [^{125}I]Ro 25-1553 binding to a $VPAC_2$-expressing leiomyoma tumour (panel F) and [^{125}I]PACAP binding to a PAC_1-expressing pheochromocytoma (panel K). In all instances, marked displacement of [^{125}I]VIP, [^{125}I]Ro 25-1553 and [^{125}I]PACAP binding was achieved in the presence of VIP (1 μM; panel C), Ro 251553 (100 nM; panel G) and PACAP (1 μM;

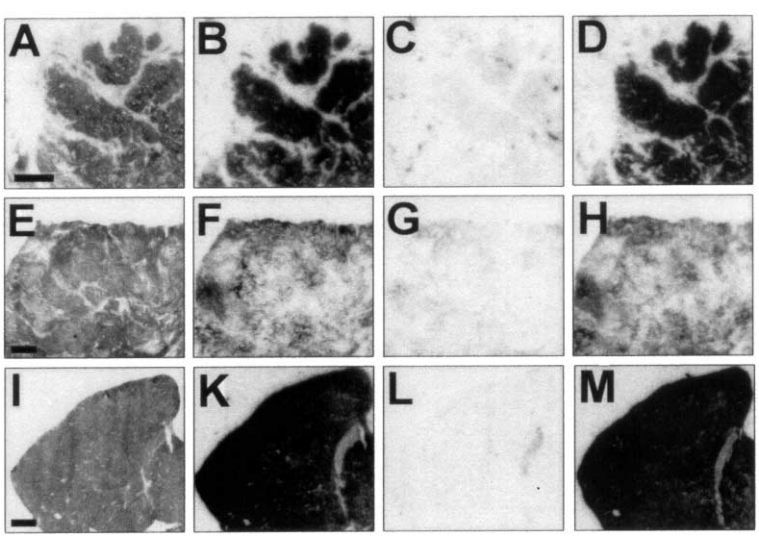

Fig. 3. Representative experimental autoradiograms demonstrating the lack of effect of 100 and/or 1000 nM $SRIF_{14}$ at VIP/PACAP receptors in a $VPAC_1$-expressing breast carcinoma (panels A–D), a $VPAC_2$-expressing leiomyoma (panels E–H) and a PAC_1-expressing pheochromocytoma (panels I–M). Panels A, E and I: Hematoxylin-eosin stained sections; scale bar represents 1 mm. Panels B, F and K: Autoradiograms showing total binding of [^{125}I]VIP (panel B), [^{125}I]Ro 25-1553 (panel F) and [^{125}I]PACAP (panel K). Panels C, G and L: Autoradiograms showing non-specific binding of [^{125}I]VIP (panel C; in presence of 10^{-6} M VIP), of [^{125}I]Ro 25-1553 (panel G; in presence of 10^{-7} M Ro 25-1553) and of [^{125}I]PACAP (panel L; in presence of 10^{-6} M PACAP). Panels D, H and M: Autoradiograms showing the lack of displacement by 100 nM (panel H) and 1000 nM (panels D and M) $SRIF_{14}$ of [^{125}I]VIP (panel D), [^{125}I]Ro 25-1553 (panel H) or [^{125}I]PACAP (panel M) binding.

panel L), respectively, yet binding remained unaffected by $SRIF_{14}$ (0.1–1 μM; panels D, H and M). Similarly, in $VPAC_1$- and $VPAC_2$-expressing normal tissues, $SRIF_{14}$ was without effect on [^{125}I]VIP and [^{125}I]Ro 25-1553 binding, respectively (Table 6).

4. Discussion

The present study attempted to establish the affinity of VIP to SRIF receptors: $hsst_{1-5}$, $msst_{1-5}$ and $rsst_{1-2}$ receptors either stably expressed in CCL-39 cells, or native receptors from rat or mouse cerebral cortex, or SRIF receptors expressed in human tumours. Furthermore, radioligands known to bind to VIP/PACAP receptors were tested in cells expressing SRIF receptors. In addition, attempts were made to establish the effect of VIP on SRIF binding in human tissues expressing both SRIF and VIP/PACAP receptors. Finally, experiments were performed to determine possible effects of SRIF in tissues known to express receptors of the VIP/PACAP family.

VIP, up to concentrations of 10 μM, had no relevant effect on any of the SRIF receptor subtypes analysed in this study, whether recombinant mouse or human receptors, or native receptors expressed either in mouse or rat brain. Similarly, VIP had no effects on SRIF binding in various human tumours expressing each of the five SRIF receptor subtypes alone or in combination. These results are inconsistent with those obtained by Virgolini et al. (1998b) who reported cross-competition binding between VIP and SRIF at human SRIF receptors. In particular, no evidence for VIP binding to either human or mouse recombinant or native sst_3 receptors could be established in the present experiments. Thus, our results are incompatible with the reported low nM affinity values achieved in the aforementioned studies. Furthermore, evidence to corroborate binding of both $SRIF_{14}$ and VIP at the 'VIP acceptor site', or sst_3 receptor, the suspected receptor at which such cross-competition was reported to take place (Virgolini, 1997; Raderer et al., 1999) and which possesses higher affinity for VIP than octreotide was not forthcoming and is at serious odds with the results determined in this study.

Equally, verification of cross-competition binding between SRIF and VIP, at sites labelled with radioligands selective for members of the VIP/PACAP receptor family, was not forthcoming, therefore providing no evidence to substantiate claims for cross-competition between SRIF and VIP receptors.

There are a number of possible scenarios that may account for these discrepancies, which are primarily related to our (still) incomplete knowledge of the SRIF and VIP/PACAP receptor systems, and which could not be addressed in this study. Firstly, the results do not preclude the possible existence of a novel SRIF receptor subtype(s) awaiting identification/cloning (i.e. in addition to sst_{1-5}), at which VIP may interact with high affinity. Both cloned and native SRIF receptors display high affinity for both the somatostatin and cortistatin peptide families; thus, such a putative novel SRIF receptor may well be a specific cortistatin receptor that shares high affinity for cortistatin and SRIF (DeLecea et al., 1996; Siehler et al., 1998) and, conceivably, for VIP. Such circumstance however, remains equivocal to date. Secondly, the possibility remains that one or another of the putative SRIF receptor heterodimers which have been, or remain to be, described may share high affinity for both SRIF and VIP (Rocheville et al., 2000a,b). Alternatively, it may be the case that VIP and SRIF receptors heterodimerise; however, the latter would seem unlikely, given that human tissues, known to co-express both SRIF and VIP/PACAP receptors, were also analysed in the present study, and again no evidence for cross-competition between these receptor families was established.

Therefore, the aim of this present study to establish the affinity of VIP for native and/or recombinant SRIF receptors from various species (mouse, rat and human), expressed in normal or tumoral tissue, was not conclusive. Finally, confirmation of the identity of the 'VIP acceptor site' which binds both $SRIF_{14}$ and VIP with higher affinity than octreotide was not forthcoming at the presently cloned/identified SRIF receptor subtypes.

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