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RESEARCH PAPER

Targeting V_{1A}-vasopressin receptors with [Arg^6, D-Trp^{7,9}, N^{me}Phe^8]-substance P (6-11) identifies a strategy to develop novel anti-cancer therapies

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Background and purpose: The anti-cancer agent [Arg^6, D-Trp^{7,9}, N^{me}Phe^8]-substance P (6-11) (SP-G) modulates gastrin releasing peptide (GRP) and arginine vasopressin signalling in small cell lung cancer cells leading to growth arrest and apoptosis. We have shown that SP-G acts as a biased agonist at GRP receptors. This work examines the hypothesis that SP-G acts as a biased agonist at the V_{1A} vasopressin receptor.
Experimental approach: The human V_{1A} receptor was expressed in CHO-K1 cells. Extracellular regulated kinase (ERK) activation and intracellular Ca^{2+} were measured using activation state-specific antibodies and Fura-2-AM respectively. The effect of SP-G on tumourigenicity was assessed by colony assay.
Key results: In V_{1A} receptor expressing cells, SP-G caused a sustained activation of ERK via a stimulation of V_{1A} receptor coupling to G_i. Inhibition of G_i with *Pertussis* toxin attenuated the inhibition by SP-G of the growth of CHO-K1 cells stably expressing the V_{1A} receptor. Chimeric V_{1A} receptors containing the second or third intracellular loop of the V_2 receptor were capable of binding vasopressin and SP-G but had altered ability to activate phospholipase C (PLC) and ERK. The second intracellular loop of the V_{1A} receptor was essential for vasopressin-stimulated PLC and ERK activation but not for SP-G-induced ERK activation.
Conclusions and implications: This work provides mechanistic insight, for biased agonists at V_{1A} receptors and highlights a potential role for such agents as anti-cancer agents.
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Keywords: [Arg^6, D-Trp^{7,9}, N^{me}Phe^8]-substance P (6-11); vasopressin; V_{1A} receptor; cancer; directed signalling; biased agonist

Abbreviations: SP-G, [Arg^6, D-Trp^{7,9}, N^{me}Phe^8]-substance P (6-11); PLC, phospholipase C; PKC, protein kinase C; GPCR, G-protein coupled receptor; ERK, extracellular signal regulated kinase; JNK, c-jun-N-terminal kinase; Fura-2-AM, Fura-2-tetraacetoxymethylester; PBS, phosphate buffered saline; PMSF, phenyl methyl sulphonyl fluoride; AVP, arginine vasopressin; GRP, gastrin releasing peptide; BSA, bovine serum albumin; PTX, *Pertussis* toxin

Introduction

Studies on neuroendocrine tumours such as small cell lung cancer (SCLC) have shown that these tumours show altered expression and sensitivity to neuropeptides which act as mitogens and promote SCLC cell growth via their G-protein coupled receptors (GPCRs) (Sethi and Rozengurt, 1991; Sethi *et al.*, 1992). Gastrin releasing peptide (GRP) receptors are frequently aberrantly expressed in human neuroendocrine lung tumours (Cuttitta *et al.*, 1985; Moody *et al.*, 2003) and 2A11 a monoclonal antibody that binds GRP, thus preventing receptor interaction, has been shown to inhibit the growth of SCLC *in vitro* and as xenografts in nude mice (Cuttitta *et al.*, 1985). SCLC cells also secrete arginine vasopressin (AVP) and express V_{1A} receptors implying the existence of an autocrine growth loop (North *et al.*, 1998a,b). SCLC patients frequently display symptoms of inappropriate AVP secretion such as hyponatremia and urinary hyperosmolality (Johnson *et al.*, 1997). Independent studies have shown expression of V_{1A} receptors in 5/5 SCLC lines and 0/4 non-small cell lung cancer (NSCLC) lines (Ocejo-Garcia *et al.*, 2001) while we showed expression of V_{1A} receptors in 4/4 SCLC lines (Waters *et al.*, 2003). Expression of V_{1A} receptors and AVP is the most useful diagnostic tool for differentiating SCLC from NSCLC and other cancers (Coulson *et al.*, 2003). SCLC cells can also express the AVP gene as

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pro-vasopressin, which remains attached to the cell membrane and could possibly contribute to the autocrine-driven mitogenesis (Friedmann *et al.*, 1994). Antibodies recognising this cell surface antigen have been developed as a potential diagnostic and therapeutic tool that targets SCLC tumours *in vivo* (Keegan *et al.*, 2002). Taken together, these findings demonstrate that the AVP/V_{1A} axis represents a novel target for new therapies for SCLC.

Gastrin releasing peptide and V_{1A} receptors couple predominantly to G_q causing an increase in phospholipase C (PLC) activation, generation of inositol trisphosphates (IP_3) and an increase of intracellular free calcium (Woll and Rozengurt, 1989; Sethi and Rozengurt, 1991) and increase activation of the extracellular signal regulated kinase (ERK) cascade through both protein kinase C (PKC) dependent and independent mechanisms (Ghosh *et al.*, 2004; Sinnett-Smith *et al.*, 2004). Stimulation of these pathways by GRP and AVP leads to an enhanced overall transcriptional activity, which controls cell proliferation and increases cell survival (Gutkind *et al.*, 1997; Rozengurt, 1998).

Analogues of substance P such as [Arg^6, D-$Trp^{7,9}$, $N^{me}Phe^8$]-substance P (6-11) (SP-G), [D-Arg^1, D-Phe^5, $DTrp^{7,9}$, Leu^{11}-]-substance P (SP-D) and [D-Arg^1, $DTrp^{5,7,9}$, Leu^{11}-]-substance P (SP-A) while being relatively poor tachykinin antagonists, block the mitogenic effects of AVP and GRP in fibroblasts and SCLC cells (Woll and Rozengurt, 1988; Langdon *et al.*, 1992; Sethi *et al.*, 1992). These analogues have been termed 'broad spectrum neuropeptide antagonists' and block the proliferation of SCLC cells in liquid culture and *in vivo* as xenografts in nude mice (Langdon *et al.*, 1992; Sethi *et al.*, 1992). SP-A has also been demonstrated to block angiogenesis in pancreatic cancer xenografts *in vivo* (Guha *et al.*, 2005). SP-G has been taken into phase I clinical studies for SCLC where therapeutic plasma levels were achieved without dose-limiting toxicity (Clive *et al.*, 2001).

Our work has focused on the mechanism of action of these analogues. SP-G and SP-D block GRP and AVP-induced calcium elevations but can also activate the receptors independently leading to a protracted increase in ERK and c-jun-N-terminal kinase (JNK) activation leading to apoptosis (Jarpe *et al.*, 1998; MacKinnon *et al.*, 2001; Waters *et al.*, 2003). The results suggest that substance P analogues induce ligand-specific receptor conformations which result in opposing efficacy for two sets of responses; a property called 'biased agonism' (Jarpe *et al.*, 1998; MacKinnon *et al.*, 2001). Given that this pharmacological activity may have a clinical utility for cancer therapy, the molecular mechanisms of this biased agonism required more rigorous investigation.

Using CHO-K1 cells expressing V_{1A} receptors we sought to dissect the mechanisms of SP-G biased agonism at V_{1A} receptors by examining the effects of AVP and SP-G on intracellular Ca^{2+} and ERK activation and the subsequent effects on cell growth. We show that SP-G activates a G_i-dependent pathway to stimulate sustained ERK activation, but blocks AVP/G_q-stimulated increase in intracellular Ca^{2+}. Blocking G_i with *Pertussis* toxin (PTX) inhibits SP-G-induced inhibition of cell growth in transfected CHO-K1 cells and SCLC cells. Using V_{1A}/V_2 receptor chimeras we show that the second intracellular loop of $V_{1A}R$ is essential for PLC activation and increased intracellular Ca^{2+} but not for SP-G-induced ERK activation. This study provides experimental evidence for agonist selective V_{1A} receptor conformations and gives mechanistic insight into the therapeutic utility for V_{1A} receptor biased agonists as anti-cancer agents in SCLC.

Methods

Cell culture and transfections

H69-SCLC cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum (FCS) 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin and 5 μg mL^{-1} L-glutamine. For experimental purposes, H69-SCLC cells were cultured in SITA medium consisting of RPMI-1640 medium supplemented with 30 nmol·L^{-1} selenium, 5 μg mL^{-1} insulin, 10 μg mL^{-1} transferrin and 0.25% (w/v) bovine serum albumin (BSA). CHO-K1 cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) FCS, 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin and 5 μg mL^{-1} L-glutamine in a humidified atmosphere of 5% CO_2/95% air at 37°C. CHO-K1 cells were transfected with full-length V_{1A} receptor or V_{1A} receptor chimeras using lipofectamine plus (Invitrogen) according to the manufacturer's instructions. Stable cell cultures were maintained in the presence of 400 μg mL^{-1} G418-sulphate.

Liquid growth

Exponentially growing H69-SCLC or CHO-K1 cells were suspended in SITA medium (H69-SCLC cells) or DMEM with 5% FCS (CHO-K1 cells) at a density of 5×10^4 cells per plate in the presence or absence of mediators in triplicate. Cells were grown for 1–9 days and cell number determined using a Coulter Counter (model Z1, Coulter).

MTT assay

In some assays MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) formazan production (Sigma) was used to measure proliferation according to the manufacturer's instructions.

Clonogenic assay

H69-SCLC cells or CHO-K1 (2×10^4) cells were suspended in SITA (H69-SCLC) or DMEM with 5% FCS (CHO-K1) containing 0.3% agarose in the presence or absence of mediators and layered over a solid base of 0.5% agarose in 35 mm plastic dishes. The cultures were incubated at 37°C for 1–10 days, and then stained with 1 mg mL^{-1} MTT overnight at 37°C. Colonies from 10 separate fields were counted using a microscope with a ×4 objective. Cloning efficiency was calculated as the % of original number of seeded cells forming colonies of >6 cells.

Aggregation assay

CHO-K1 cells (2×10^4) were suspended in DMEM in the presence of 5% FCS and seeded into low adhesion tissue culture plates on top of a layer (1 mL) of 0.5% agar. Under these

conditions the cells did not adhere. Cells were maintained in culture for 7 days briefly trypsinized to disaggregate clusters and viable cells counted.

Receptor binding

Confluent cultures of CHO-K1 cells expressing V_{1A} receptor were washed twice in ice-cold phosphate buffered saline (PBS). The cells were lysed in ice-cold lysis buffer (10 mmol·L^{-1} Tris HCl pH 7.4, 5 mmol·L^{-1} EDTA, 5 mmol·L^{-1} EGTA, 1 mmol·L^{-1} phenyl methyl sulphonyl fluoride) and briefly homogenised using a Polytron tissue homogeniser. After centrifugation at 500× *g* for 4 min, the supernatant was centrifuged at 49 000× *g* for 15 min at 4°C and the pellet washed twice by repeated homogenisation and centrifugation in lysis buffer. The final pellet was suspended in 50 mmol·L^{-1} Tris HCl (pH 7.4), adjusted to 1 mg mL^{-1} protein, and stored at –80°C. Protein was determined using Pierce BCA protein assay reagent (Pierce UK). Membranes (150–250 μg protein) were incubated with 1 nmol·L^{-1} [^{3}H]-AVP and test agents for 30 min at 37°C in 50 mmol·L^{-1} Tris HCl (pH 7.4), 0.5% BSA, 3 mmol·L^{-1} $MgCl_2$. The assay was terminated by immediate filtration over GFB glass fibre filters using a Tomtec 96 cell harvester. The filters were washed with 2 × 3 mL binding buffer, dried and counted for ^{3}H in a Wallac Betaplate scintillation counter. Non-specific binding was determined with 1 μmol·L^{-1} AVP. The binding parameters K_d and B_{max} were calculated from competition binding isotherms with unlabelled ligand (DeBlasi *et al*., 1989). The IC_{50} (concentration of drug displacing 50% specific binding) was converted to the inhibitory constant (*K*i), where $Ki = IC_{50}/(1 + [ligand]/K_d)$ (Cheng and Prusoff, 1973).

PLC activation

[^{3}H]-inositol phosphate formation was measured by labelling cells overnight in serum-free and inositol-free medium containing 1 μCi mL^{-1} myo-[2-^{3}H]-inositol. Cells (2×10^6 per assay point) were washed and incubated in Hank's balanced salt solution containing 20 mmol·L^{-1} HEPES, 1.8% glucose, 0.2% BSA and 20 mmol·L^{-1} LiCl, for 30 min at 37°C prior to addition of agonist for a further 30 min. Reactions were extracted by removal of assay buffer and addition of ice-cold 10 mmol·L^{-1} formic acid for 60 min on ice. [^{3}H]-inositol phosphates were separated by anion exchange chromatography on Dowex (200–400 mesh, formate form) columns, and [^{3}H] determined by scintillation counting.

[^{35}S]-GTPγS binding and immunoprecipitation of G_α subunits

[^{35}S]-Guanosine 5′-O-(γ-thio)triphosphate ([^{35}S]-GTPγS) binding was carried out essentially as previously described (Weiland and Jakobs, 1994). Briefly, cell membranes (~10 μg protein) were incubated in a final volume of 100 μL binding buffer (20 mmol·L^{-1} HEPES, pH 7.4, 100 mmol·L^{-1} NaCl, 3 mmol·L^{-1} $MgCl_2$, 10 μmol·L^{-1} GDP, 0.2 mmol·L^{-1} ascorbic acid) with 0.2 nmol·L^{-1} [^{35}S]-GTPγS for 60 min at 4°C in the presence or absence of test compounds. Bound radioactivity was determined by filtration onto GF-B glass fibre filters and scintillation counting. Non-specific binding was determined in the presence of 100 μmol·L^{-1} unlabelled GTPγS. [^{35}S]-GTPγS binding to $G_{\alpha i}$ or $G_{\alpha q/11}$ was determined as above and membrane pellets solubilised in 50 μL 1.5% Triton, 0.2% SDS. Pellets were diluted in 1 mL immunoprecipitation buffer [50 mmol·L^{-1} Tris HCl pH 7.4, 150 mmol·L^{-1} NaCl. 2 mmol·L^{-1} KCl, 1 mmol·L^{-1} EDTA, 20 mmol·L^{-1} $MgCl_2$, 1% Triton and protease inhibitors (Complete inhibitor tablet, Roche Belgium)] and subunits immunoprecipitated overnight at 4°C with 1 μg rabbit anti-$G_{\alpha i}$ or $G_{\alpha q/11}$. Complexes were captured with protein A agarose and washed ×4 with immunoprecipitation buffer. Bound [^{35}S]-GTPγS was eluted in SDS-PAGE buffer and counted by scintillation counting.

Determination of intracellular Ca^{2+} concentration

CHO-K1 cells stably expressing the V_{1A} receptor chimeras were grown to confluence on 10 cm plates and rested overnight in DMEM containing 0.1% FCS. Cells (5×10^6 cells per data point) were trypsinised and loaded with FURA-2-tetraacetoxymethylester (Fura-2-AME 1 μmol·L^{-1}) in calcium-free Hank's balanced salt solution for 10 min at 37°C. The cells were pelleted and resuspended in 2 mL of Hank's balanced salt solution containing 1.8 mmol·L^{-1} $CaCl_2$. Fluorescence was recorded in a Model F2000 fluorescence spectrophotometer (Hitachi). Alternate dual wavelength excitation at 380 nm and 410 nm allowed ratiometric analysis of bound and unbound Fura-2 when measured at 505 nm. [Ca^{2+}] was calculated according to the equation $[Ca^{2+}] = K(F - F_{min})/(F_{max} - F)$, where F is the ratio of the unknown sample, F_{max} is the ratio after the addition of 0.1% triton X-100 and F_{min} is the ratio after Ca^{2+} chelation with 10 mmol·L^{-1} EGTA. K is the dissociation constant for Fura-2, which is 224 nmol·L^{-1}.

Western blotting/ERK phosphorylation

Quiescent cell cultures (1×10^6 cells) were treated as described in figure legends and lysed at 4°C in lysis buffer containing; 25 mmol·L^{-1} HEPES pH 7.4, 0.3 M NaCl, 1.5 mmol·L^{-1} $MgCl_2$, 0.2 mmol·L^{-1} EDTA, 0.5% Triton X-100, 20 mmol·L^{-1} β-glycerophosphate, 0.5 mmol·L^{-1} dithiothreitol, 1 mmol·L^{-1} sodium orthovanadate and protease inhibitors [protease inhibitor cocktail (Boehringer Mannheim, Sussex, UK)]. Lysates were clarified by centrifugation, equalised for protein content using Pierce BCA protein assay reagent and denatured by boiling (5 min) in SDS-PAGE loading buffer. A total of 20 μg lysate per lane was resolved on 12% SDS-PAGE gels and electroblotted onto nitrocellulose membranes. Membranes were blocked in 3% BSA in PBS containing 0.05% tween-20. Blots were probed with primary antibody followed by the appropriate horseradish peroxidase-labelled goat IgG (DAKO, UK). Bands were visualised using enhanced chemiluminescence (ECL plus, Amersham) and quantified by Image J.

Statistical analysis

Results are presented as means ± SEM. Significance of the differences between means was assessed using Student's *t*-test or by ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, Dunnet's *post hoc* test was used to compare individual means. Differences were considered statistically significant at $P < 0.05$. Unless

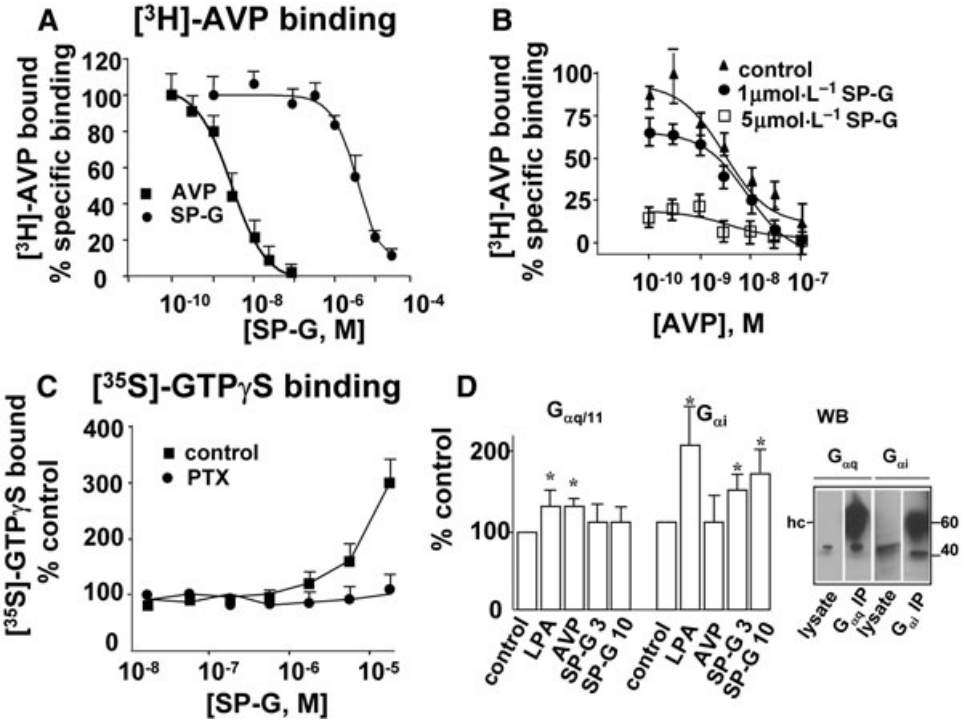

Figure 1 Receptor binding and [^{35}S]-GTPγS binding assay. A. Membranes of CHO-K1 cells stably expressing the V_{1A} receptor were incubated with [^{3}H]-AVP and increasing concentrations of SP-G or unlabelled AVP. Binding isotherms for K_d and B_{max} were calculated from AVP competition curves as described in the *Methods*. B. SP-G inhibition is non-competitive. Membranes were incubated with increasing concentrations of [^{3}H]-AVP in the presence of 1 or 5 μmol·L^{-1} SP-G as indicated. C. [^{35}S]-GTPγS binding. [^{35}S]-GTPγS binding to the whole membrane fraction was determined as described in the *Methods*. Membranes were incubated with SP-G in the presence or absence of 100 ng mL^{-1} PTX for 60 min at 4°C. D. [^{35}S]-GTPγS binding to specific G_α subunits. Membranes were incubated with [^{35}S]-GTPγS in the presence of LPA (1 μmol·L^{-1}), AVP 30 nmol·L^{-1}, or SP-G at 3 or 10 μmol·L^{-1} as indicated. Solubilised pellets were immunoprecipitated with anti-$G_{\alpha q/11}$ or anti-$G_{\alpha i}$ as described in the *Methods*. Results represent the mean ± SEM of four independent experiments. (*$P < 0.05$ vs. untreated control membranes ANOVA with Dunnets's post-test.) Representative Western blots (WB) of whole-cell lysate and immunoprecipitated $G_{\alpha q/11}$ or $G_{\alpha i}$ confirm expression of these α subunits in V_{1A} receptor transfected CHO-K1 cells. Immunoprecipitating antibody heavy chain (hc).

stated otherwise, studies were performed on 3–6 independent occasions.

Materials

CHO-K1 cells and H69-SCLC cells were purchased for the European Cell Culture Collection; DMEM, RPMI-1640, AVP, d(CH$_2$)-5-TyrMe-AVP and monoclonal antibody to diphosphorylated ERK 1 and 2 (M 8159) were from Sigma (Poole, UK); polyclonal antibodies to $G_{\alpha i}$ (sc-26761) and $G_{\alpha q/11}$ (sc-392) and ERK2 were from Insight Biotechnology; Rabbit anti-caspase-3 (Asp-175) from Cell Signalling Technology; [Arg^6, D-Trp^{7,9}, N^{me}Phe^8]-substance P (6-11) (SP-G) was synthesized by Cancer Research UK (London, UK). PTX was from Alexis Biochemicals (Nottingham, UK). The human V_{1A} receptor construct in pcDNA3.1 was a kind gift from M. Thibonnier (Case Western Reserve University School of Medicine, OH, USA). The V_{1A} receptor mutants V_{1i2} and V_{1i3} were kindly provided by Dr Wess (National Institute of Health, Bethesda, MD, USA). The following sequences were exchanged between the rat V_{1A} and the human V_2 receptor: V_{1i2} (V_{1A} 152–172 for V_2 140–161), V_{1i3} (V_{1A} 237–303 for V_2 225–277). [^{3}H]-AVP (60 Ci/mmol) was from New England Nuclear (Perkin Elmer).

Results

V_{1A} receptor expression in CHO cell line

Previous studies have shown that V_{1A} receptors can activate G_q and G_i linked signalling pathways in response to different agonists (Thibonnier *et al.*, 1993; Abel *et al.*, 2000; Chiu *et al.*, 2002). To examine SP-G-induced biased agonism we stably expressed V_{1A} receptors in CHO-K1 cells. In membrane preparations from these cells, [^{3}H]-AVP bound with high affinity, $K_d = 3.3 \pm 1.6$ nmol·L^{-1}, $B_{max} = 383 \pm 74$ fmoles mg protein^{-1} ($n = 4$, Fig. 1A). SP-G inhibited [^{3}H]-AVP binding in CHO-V_{1A} cells with a $K_i = 3.1 \pm 0.6$ μmol·L^{-1} and $n_H = 1.25$ ($n = 6$). The inhibition of [^{3}H]-AVP binding by SP-G was non-competitive as it significantly decreased saturable binding (B_{max}) from 383 ± 74 fmoles mg protein^{-1} in control cells to 285 ± 25 and 82 ± 12 fmoles mg protein^{-1} for 1 and 5 μmol·L^{-1} SP-G ($P < 0.05$ and $P < 0.01$, respectively, by Student's *t*-test), while having no appreciable effect on affinity ($K_d = 3.3 \pm 1.6$, 3.8 ± 1.38 and 4.8 ± 1.6 nmol·L^{-1} for control, 1 and 5 μmol·L^{-1} SP-G respectively, $P > 0.05$, Fig. 1B). Moreover, in kinetic experiments, SP-G at 5 μmol·L^{-1} significantly accelerated the dissociation of [^{3}H]-AVP from receptor expressing cells (data not shown).

We next sought to determine whether SP-G could promote receptor coupling to G-proteins by measuring [^{35}S]-GTPγS binding to V_{1A} receptor transfected cells. This assay measures GTP/GDP exchange on G-proteins and is a measure of G_α activation. As shown in Figure 1C SP-G increased [^{35}S]GTPγS binding to V_{1A}-CHO-K1 membranes. PTX catalyses ADP-ribosylation of the α subunits of G_i-like proteins thus blocking their coupling and activation. Treatment with PTX completely abolished [^{35}S]GTPγS binding induced by SP-G suggesting that SP-G induced coupling to G_i-like proteins. Activation of G_i proteins by SP-G was confirmed by increased [^{35}S]GTPγS binding to G_i proteins immunoprecipitated from solubilised cell membranes. SP-G increased binding to $G_{\alpha i}$ subunits by 45 ± 10 and 68 ± 9% at 3 and 10 μmol·L^{-1} respectively ($n = 4$, Fig. 1D). AVP did not significantly increase activation of G_i suggesting that the natural agonist does not induce V_{1A} receptor coupling to G_i in these cells. Receptor-mediated activation of G_i was confirmed in cells stimulated with lysophosphatidic acid (LPA; 1 μmol·L^{-1}, 102% increase over basal). LPA and AVP but not SP-G stimulated [^{35}S]GTPγS binding to G_q (35 ± 7 and 34 ± 9% increase over basal, respectively, Fig. 1D). These data establish that SP-G selectively activated G_i in V_{1A} receptor transfected cells.

SP-G induced growth inhibition of V_{1A} expressing CHO-K1 cells

We have shown previously that AVP stimulates SCLC cell growth and colony formation (Sethi and Rozengurt, 1991; Sethi *et al.*, 1992). To examine the ability of substance P analogues to inhibit V_{1A} receptor-mediated growth we compared proliferation of V_{1A} receptor transfected CHO-K1 cells with the V_{1A} receptor expressing SCLC cell line H69. As shown in Figure 2A SP-G significantly inhibited growth of H69-SCLC cells in liquid culture ($IC_{50} = 5.1 \pm 0.5$ μmol·L^{-1}, $n = 4$) and inhibited colony formation in semi-solid agarose (Fig. 2B, $IC_{50} = 2.5 \pm 0.4$ μmol·L^{-1}, $n = 4$). CHO-K1 cells transfected with the human V_{1A} receptor showed a significant increase in proliferation compared with vector transfected cells (Fig. 2C) and formed significantly more colonies in soft agar (Fig. 2D). The expression of the V_{1A} receptor also increased the number of viable cell aggregates formed after 72 h culture in low adhesion culture plates (Fig. 2E). This is in agreement with our previous observations suggesting that V_{1A} receptor expression allows for anchorage- and serum-independent growth consistent with transformation (MacKinnon *et al.*, 2005). Moreover, it suggests that these receptors show some constitutive activity, as wild-type receptor expression alone was sufficient to increase transformation in the absence of

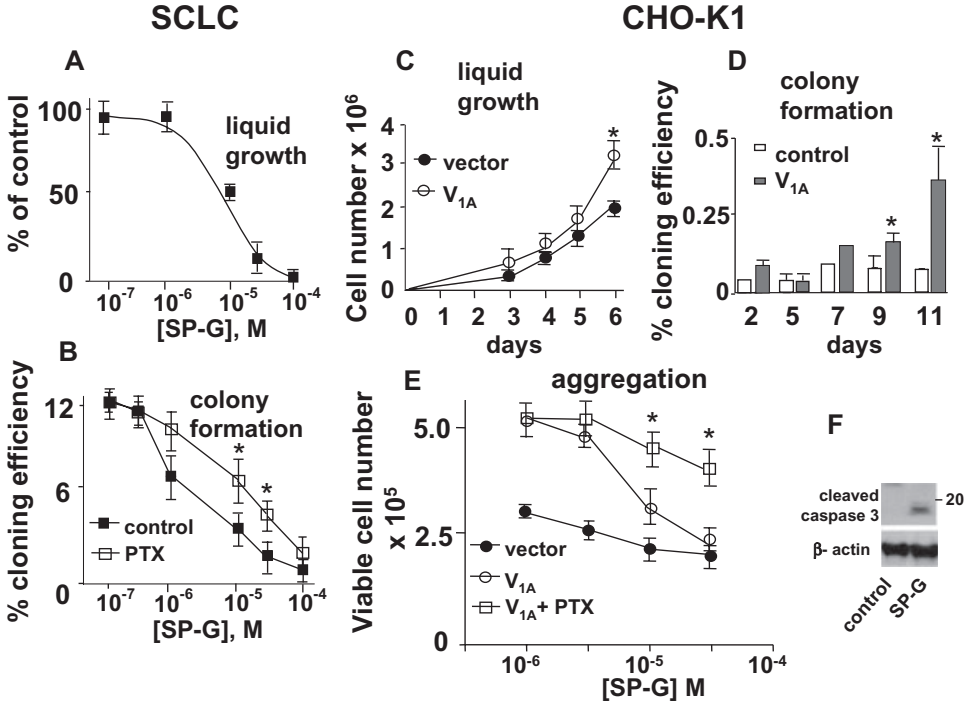

Figure 2 Effect of SP-G on proliferation of SCLC cells and V_{1A} expressing CHO-K1 cells. A, B. H69-SCLC cell growth. The effect of SP-G on liquid growth over 7 days (A) and on colony formation in soft agarose (B) was carried out in the presence or absence of 100 ng mL^{-1} PTX, as indicated. The results represent the mean ± SEM or four independent experiments. (*$P < 0.05$, Student's *t*-test compared with SP-G in non-PTX treated.) C. CHO-K1 cells stably expressing the V_{1A} receptor or empty vector were grown in liquid culture and viable cells counted at the time points indicated. Results represent the mean ± SEM of four experiments. (*$P < 0.05$ Student's *t*-test compared with vector transfected cells.) D. Colony formation. Control or V_{1A} receptor expressing CHO-K1 cells were cultured in 0.3% agarose for up to 11 days and colony formation calculated as % cloning efficiency. Results represent the mean ± SEM of four experiments. (*$P < 0.05$ Student's *t*-test compared with vector transfected cells.) E. Effect of PTX. CHO-K1 cells were cultured in low adhesion plates in the presence or absence of 100 ng mL^{-1} PTX for 7 days. Viable cells were counted by propidium iodide exclusion and Coulter counting. Results represent the mean ± SEM of four experiments. (*$P < 0.05$ Student's *t*-test compared with untreated V_{1A} receptor expressing cells.) F. SP-G induces cleavage of caspase-3. V_{1A}-CHO-K1 cells were incubated with SP-G for 48 h. Cells were lysed and Western blots probed for cleaved caspase-3.

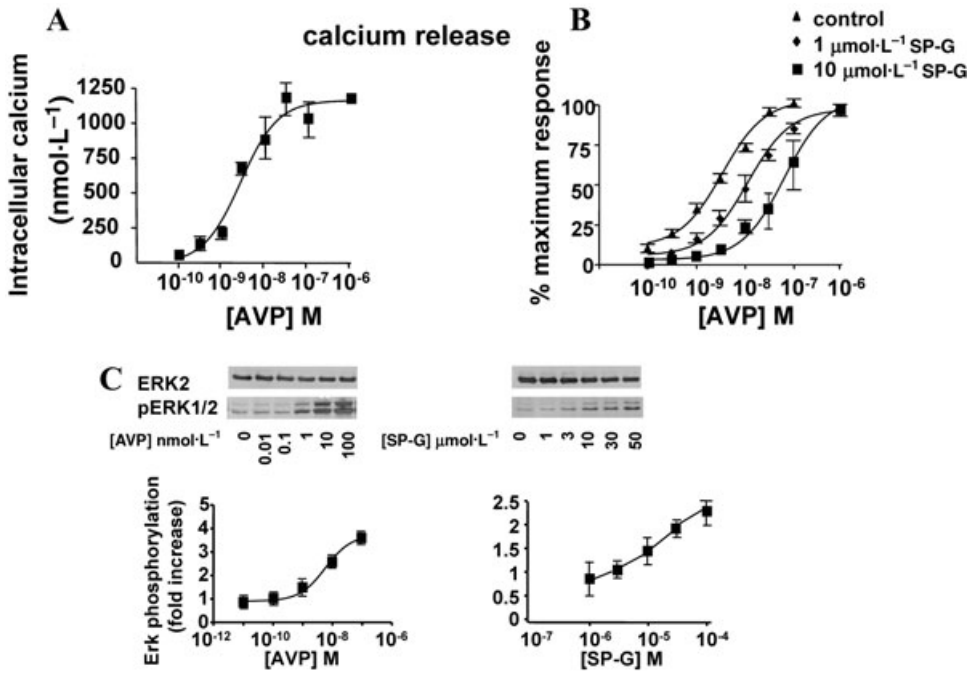

Figure 3 Signalling pathways activated by SP-G. A. Intracellular calcium. Quiescent CHO-K1 cells expressing V_{1A} receptors were incubated with FURA-2-AM for 15 min at 37°C before stimulation with the indicated concentrations of AVP. Ratiometric fluorescence was monitored at 37°C following addition of AVP at the indicated concentrations. Results represent the mean ± SEM of four experiments. B. SP-G inhibits AVP-induced calcium elevation. CHO-K1 cells expressing V_{1A} receptors were incubated with FURA-2AM for 15 min at 37°C and ratiometric fluorescence monitored at 37°C. Concentration response curves to AVP were carried out in control cells and in cells pretreated for 2 min with 1 or 10 μmol·L^{-1} SP-G. Results are expressed as % maximum control response to AVP and represent the mean ± SEM of four experiments. C. ERK activation. CHO-K1 cells were incubated with AVP (left) or SP-G (right) at the indicated concentrations for 10 min at 37°C. Cells were lysed and Western blots probed for pERK1/2 or total ERK2. Representative blots from four separate experiments are shown, with quantitative summary data below (band densities were quantitated by Image J).

exogenously added neuropeptide. This increase in proliferation by V_{1A} receptor expression was significantly blocked by co-incubation with SP-G (IC_{50} = 5.1 ± 0.9 μmol·L^{-1}, Fig. 2E). Figure 2E shows that PTX (100 ng mL^{-1}) attenuated SP-G-induced inhibition of cell growth in V_{1A} expressing CHO-K1 cells suggesting that SP-G can inhibit growth in part by inducing V_{1A} receptor-mediated G_i activation.

As has been shown previously, PTX did not affect basal growth of SCLC cells in culture (Codignola *et al.*, 1998). However, PTX attenuated SP-G-induced inhibition of SCLC colony formation (Fig. 2B) suggesting that SP-G-induced growth inhibition is in part mediated via G_i. Our previous work has shown that SP-G induces apoptosis in SCLC cells (MacKinnon *et al.*, 1999; MacKinnon and Sethi, 2003). In Figure 2F we show that SP-G induced cleavage of caspase-3 in V_{1A} receptor expressing CHO-K1 cells suggesting that the anti-proliferative activity, induced by SP-G through the V_{1A} receptor, may be in part due to an induction of apoptosis.

Signalling pathways activated by AVP and SP-G

Arginine vasopressin has been shown to produce a G_q-dependent elevation of intracellular calcium in CHO-K1 cells and other cell types (Laszlo *et al.*, 1991; Thibonnier *et al.*, 1993; Liu and Wess, 1996; Hawtin *et al.*, 2006). In this study, AVP produced an elevation of intracellular calcium in V_{1A}-CHO cells (EC_{50} = 2.4 ± 0.64 nmol·L^{-1}, Fig. 3A). SP-G had no effect on calcium elevation alone suggesting that SP-G does not stimulate G_q-mediated PLC activation which is in agreement with our previous studies (Waters *et al.*, 2003). SP-G did however inhibit AVP-stimulated increase in intracellular calcium with pA_2 = 6.28 ± 0.21 (Fig. 3B). These data suggest that SP-G acts as a competitive antagonist of AVP-mediated intracellular calcium elevation which is mediated by G_q-stimulated PLC activation in V_{1A}-receptor expressing CHO-K1 cells (Thibonnier *et al.*, 1993).

ERK activation

Arginine vasopressin also caused a concentration-dependent stimulation of ERK in cells expressing V_{1A} receptors (Fig. 3C). AVP gave a 3.6-fold stimulation of ERK activity with an EC_{50} value of 2.8 ± 0.3 nmol·L^{-1}. Although SP-G was an antagonist for calcium elevation, it was also an agonist for ERK stimulation in V_{1A} receptor expressing cells (Fig. 3C) producing a 2.2-fold stimulation at 100 μmol·L^{-1} and an EC_{50} of 11.1 ± 1.9 μmol·L^{-1}. SP-G-induced activation of ERK was rapid and sustained. Figure 4 shows that AVP-induced ERK phosphorylation was maximal at 5 min and declined thereafter to baseline levels after 30 min. SP-G-induced ERK phosphorylation was maximal after 10 min and showed sustained activation after 60 min stimulation. We investigated whether ERK activation by SP-G was mediated via G_i and show that SP-G-induced, but not AVP-induced, ERK activation was inhibited by pretreatment with PTX in V_{1A} receptor cells (Fig. 4B). These data confirm that SP-G promotes V_{1A} coupling

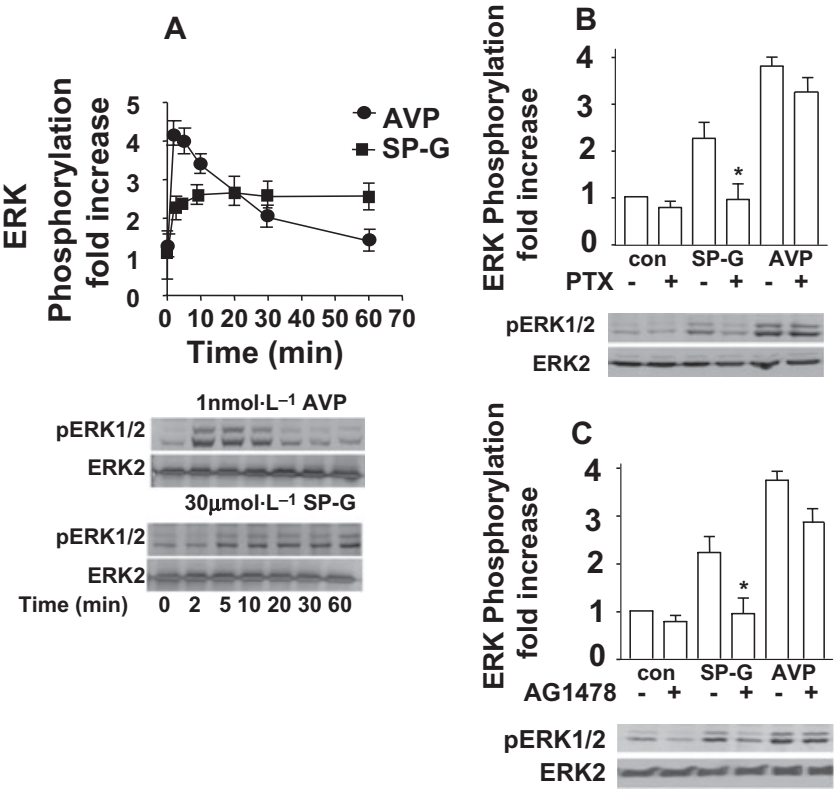

Figure 4 Mechanisms of ERK activation. A. SP-G-induced ERK activation is sustained. CHO-K1 cells expressing V_{1A} receptor were stimulated with 30 μmol·L^{-1} SP-G or 1 nmol·L^{-1} AVP for various times prior to lysis. Cells were lysed and immunoblotted for pERK1/2 or total ERK. Representative blots from four separate experiments are shown, below the summary data in the graph [band densities were quantitated by Image J]. B. Effect of PTX. Cells were pretreated for 18 h with 50 ng mL^{-1} PTX and stimulated with 30 μmol·L^{-1} SP-G or 1 nmol·L^{-1} AVP for 5 min. (*$P < 0.05$ compared with SP-G alone.) C. Effect of AG1478. Cells were incubated with AG1478 (1 μmol·L^{-1}) for 30 min at 37°C prior to stimulation for 5 min with 10 μmol·L^{-1} SP-G or 1 nmol·L^{-1} AVP as indicated. Aliquots of cell lysate were resolved by SDS-PAGE and Western blots probed with monoclonal anti-pERK1/2 antibody. Representative Western blots are shown.

to G_i proteins which mediate an activation of ERK. Figure 4C shows that the EGF receptor tyrosine kinase inhibitor AG1478 (1 μmol·L^{-1}) inhibited SP-G-induced ERK activation but only partially inhibited AVP-stimulated ERK activation, suggesting different mechanisms of ERK activation by AVP and SP-G in CHO cells.

V_{1A} receptor chimeras

It has been previously shown that the second intracellular loop of the V_{1A} receptor is essential for V_{1A} receptor coupling to G_q (Liu and Wess, 1996). However, it is not known what region of the receptor may be involved in coupling to G_i. To address this, V_{1A} receptor mutants expressing the second (V_{1i2}) or third (V_{1i3}) intracellular loop of the V_2 receptor were transiently transfected into CHO-K1 cells. Table 1 shows that both chimeric receptors bind [^{3}H]-AVP with similar affinity (wild-type K_d 5.71 ± 2.54 nmol·L^{-1}, V_{1i2} K_d 2.64 ± 0.65 nmol·L^{-1}, V_{1i3} K_d 1.14 ± 0.57 nmol·L^{-1}, $n = 3$); however, there was a marked difference in the ability of the chimeras to activate PLC. AVP at concentrations up to 100 nmol·L^{-1} was unable to increase generation of total inositol phosphates in V_{1i2} receptor expressing cells, whereas in V_{1i3} cells a fourfold increase in inositol phosphates was observed which was comparable to the activation observed in wild-type receptor expressing cells. (EC_{50} for AVP was 0.89 ± 0.10 and 1.04 ± 0.12 nmol·L^{-1} in V_{1A} and V_{1i3} receptor expressing cells respectively, Fig. 5A.) SP-G inhibited AVP-induced inositol phosphate production in V_{1A} and V_{1i3} cells (Table 1). This suggests that the second intracellular loop of the V_{1A} receptor is essential for V_{1A} receptor activation of PLC.

Table 1 AVP binding and PLC activation in CHO-K1 cells with V_{1A} receptor chimeras

	V_{1A}	V_{1i2}	V_{1i3}
[^{3}H]-AVP binding			
K_d (nmol·L^{-1})	5.7 ± 2.5	2.6 ± 0.7	1.1 ± 0.6
B_{max} (fmoles mg^{-1})	151 ± 45	103 ± 68	226 ± 140
SP-G K_i (μmol·L^{-1})	4.2 ± 1.3	3.9 ± 1.1	1.5 ± 0.6
PLC activation			
SP-G IC_{50} (μmol·L^{-1})	7.1 ± 0.4	>100	1.0 ± 0.1

CHO-K1 cells were transiently transfected with V_{1A} receptor chimeras as described in the *Methods*. The binding parameters K_d and B_{max} were calculated from competition binding isotherms with unlabelled AVP. The inhibitory constant K_i was calculated from the IC_{50} using the equation of Cheng and Prusoff (1973). [^{3}H]-Inositol phosphate formation was measured as described in the *Methods*. Cells were stimulated with 10 nmol·L^{-1} AVP for 30 min at 37°C in the presence of various concentrations of SP-G. The results represent the mean ± SEM of three independent experiments.

Figure 5B shows that the V_{1i3} chimera potently stimulated ERK in response to AVP, with higher potency than observed

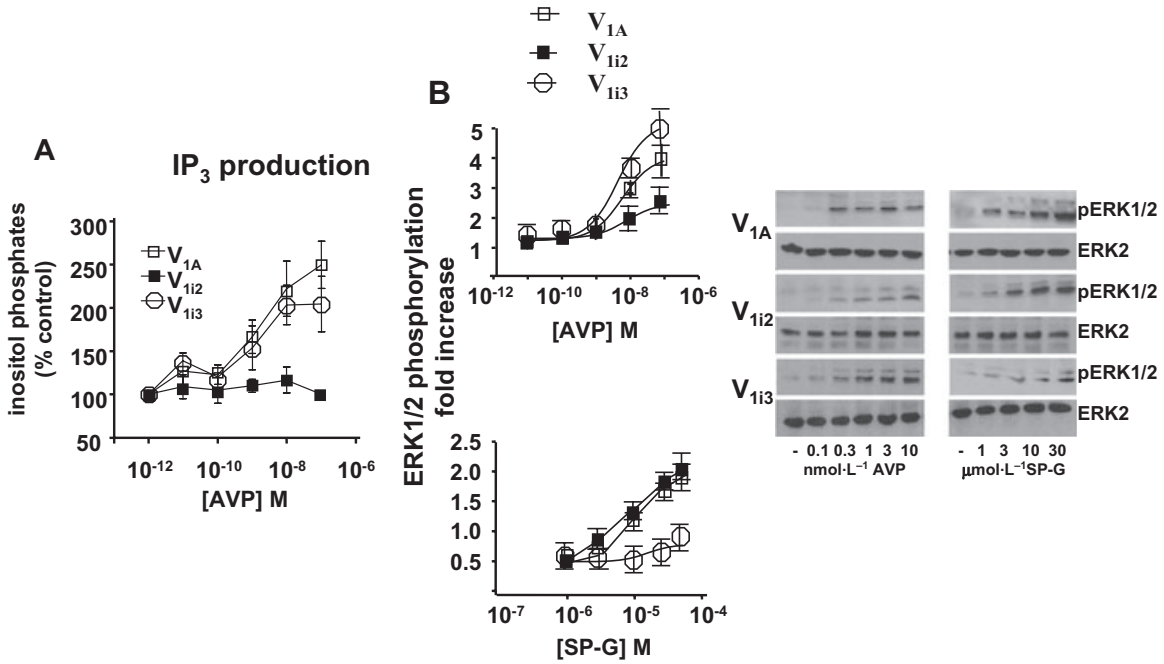

Figure 5 A. PLC activation. [^{3}H]-inositol phosphate formation was measured in cells expressing V_{1A} receptor chimeras and stimulated with the indicated concentrations of AVP for 30 min at 37°C. The results are expressed as % control and represent the mean ± SEM of three independent experiments. B. ERK phosphorylation. Confluent cultures of V_{1A} receptor chimera expressing CHO-K1 cells were rested overnight and stimulated for 5 min with SP-G or AVP, as indicated. Aliquots of cell lysate were resolved by SDS-PAGE and Western blots probed with monoclonal anti-pERK1/2 antibody or anti-ERK2 antibody. Phosphorylation of ERK1/2 from three experiments was quantified by Image J.

in the wild-type receptor. However, SP-G was less able to activate ERK in these cells producing significant stimulation only at 30 µmol·L^{-1}. Conversely, the V_{1i2} chimera which has the second intracellular loop replaced with that of the V_2 receptor, was less able than the wild-type receptor to activate ERK in response to AVP but produced a robust stimulation of ERK in response to SP-G which was similar to its effects in the wild-type cells. It is of note however that the V_{1i2} chimera was able to activate ERK in response to AVP even though it was unable to activate PLC at these concentrations. These data suggest that SP-G induces a conformational change in the V_{1A} receptor at a region within the third intracellular loop which promotes coupling to G_i and subsequent activation of ERK.

Discussion

The key findings of this study are: (i) SP-G selectively activates G_i and inhibits cell growth of SCLC cells and CHO-K1 cells stably expressing V_{1A} receptors; (ii) SP-G favours coupling to G_i and blocks AVP activation of G_q in V_{1A} CHO-K1 cells thus supporting a biased agonist mechanism; (iii) PTX attenuates SP-G-induced growth inhibition in SCLC cells and V_{1A} CHO-K1 cells suggesting that activation of G_i is an important aspect of its antiproliferative activity; and (iv) Mutant receptors containing the second intracellular loop of the V_2 receptor were unable to respond to AVP in terms of PLC activation but were able to activate ERK in response to SP-G suggesting that V_{1A} receptor coupling to G_i involves a region of the receptor outwith the second intracellular loop. Given that a single receptor can couple to more than one G-protein, traditional receptor theory as proposed by Furchgott (1966) would predict that an agonist at the receptor would have similar efficacies for different signalling pathways. However, there are a number of reports that show differential effector activation by agonists that cannot be explained by this mechanism (Berg *et al.*, 1998). For example, α_{2A} receptor agonists show different efficacies for G_i-mediated adenylyl cyclase inhibition and G_s-mediated adenylyl cyclase stimulation in transfected CHO cells (Brink *et al.*, 2000), and the neurotensin receptor-1 agonists EISA-1 and neuromedin B show reverse potency orders for G_q- and G_s-mediated responses (Skrzydelski *et al.*, 2003). This type of activity can be explained by the hypothesis of agonist-dependent trafficking of receptor stimulus (ADTRS) originally described by Kenakin (1995). This hypothesis predicts that when a receptor couples to more than one stimulus, the relative efficacies of a series of agonists may differ depending on their abilities to stabilise different receptor/G-protein activation states. It is therefore theoretically possible to design 'biased' agonists which activate a selective subset of responses triggered by the receptor. As yet however there has been little description of such pharmacological agents providing any therapeutic benefit in disease.

In CHO cells expressing V_{1A} receptors, SP-G showed the highest antagonist potency in inhibiting calcium elevation ($pA_2 = 0.53$ µmol·L^{-1}), a G_q-coupled response, but its potency for ERK activation and all other functional responses demonstrated IC_{50}/EC_{50} values in the low micromolar range. This suggests that stimulation of G_i correlates better with functional effects on growth and this is corroborated by the finding that PTX blocks these responses. Our results illustrate

the characteristics of ADTRS, namely that two agents can show reverse efficacies for two pathways and that one (SP-G) directs signalling via a G-protein usually considered to be less efficiently coupled to this receptor. The higher potency of SP-G to antagonise calcium elevation may be reflected by a higher affinity of SP-G for the receptor conformation coupled to G_q. This is adequately explained by the ADTRS hypothesis which states that agonists can differ in their rank order of potency/efficacy between responses mediated by the same receptor. The affinity for SP-G for agonist binding (K_i = 3.1 μmol·L^{-1}) is intermediate between its affinity for inhibition of intracellular calcium and ERK activation. SP-G must therefore interact differently with the receptor to induce a different agonist conformation and would suggest that SP-G must act at a site distinct from the AVP binding site on the V_{1A} receptor. This is corroborated by the finding that SP-G-induced ERK activation was dependent on receptor expression, but was non-competitive in nature as shown by its ability to decrease saturable [^{3}H]-AVP binding and accelerate [^{3}H]-AVP dissociation.

Our data suggest different pathways for ERK activation recruited by AVP and SPG. SP-G-induced ERK activation was abolished by PTX whereas AVP-stimulated ERK activation was largely unaffected. Moreover, the SP-G-induced ERK activation was blocked by the EGFR tyrosine kinase inhibitor AG1478. These data support a multi-track signalling complex leading from the neuropeptide receptor to ERK activation, where one track (stabilised by AVP) is mediated by G_q and most likely involves PKC, whereas another (stabilised by SP-G) feeds into a receptor transactivation pathway (Wetzker and Bohmer, 2003). Although originally described for the EGF receptor activated by thrombin and LPA (Daub *et al.*, 1996), this mechanism occurs in many cell types and via many different GPCRs and RTKs. GPCRs can also induce persistent ERK activation via a G-protein independent recruitment of β-arrestins (see Reiter and Lefkowitz, 2006). Activation of ERK by β-arrestin is slower in onset, persistent and sequestered in the cytosol, whereas G-protein-mediated activation of ERK is normally transient and translocates to the nucleus. The finding that SP-G-induced ERK activation was inhibited by PTX suggests the involvement of G_i and argues against a β-arrestin-mediated pathway.

Persistent ERK activation has been suggested to be consistent with G_i-dependent growth inhibition by the oxytocin 'biased agonist' atosiban (Rimoldi *et al.*, 2003; Reversi *et al.*, 2005). Selective activation of G_i has been suggested to inhibit proliferation and induce apoptosis in other endocrine tumours. Specific activation of G_i by GnRH antagonists may induce apoptosis in type 1 GnRH receptor expressing tumour cells (Maudsley *et al.*, 2004). Although activation of ERK is normally associated with cell survival, prolonged ERK activation has been shown to induce apoptosis by RRR-α-tocopheryl succinate (You *et al.*, 2001; Yu *et al.*, 2001) and phenethyl isothiocyanate (Xiao and Singh, 2002), via mechanisms involving p53. In addition, prolonged ERK activation has been shown to have a primary role in the regulation of neuronal cell apoptosis (Cheung and Slack, 2004; Subramaniam *et al.*, 2004), and in particular, sustained ERK is reported to be involved in G1-specific cell cycle arrest of human breast cancer cells (Alblas *et al.*, 1998), NIH 3T3 murine fibroblasts (Sewing *et al.*, 1997) and human myeloblastic leukaemia cells (Yen *et al.*, 1998).

In endocrine cancers such as SCLC, mitogenic neuropeptide receptors such as the V_{1A} receptor and the GRP receptor activate downstream signals which control proliferation and differentiation but also activate death signals leading to JNK activation and apoptosis. An agent that blocks the proliferative effects of Ca^{2+} mobilising mitogenic neuropeptides, while stimulating receptor-dependent apoptosis, would be highly advantageous in the management of tumour growth. This present study suggests that SP-G acts as a biased agonist promoting an agonist state of the V_{1A} receptor which couples to G_i leading to prolonged activation of ERK but which blocks AVP-induced activation of PLC and subsequent elevation of intracellular Ca^{2+}. Selective activation of G_i coupled with an inhibition of elevation of intracellular Ca^{2+} may represent a common sequence of events leading to growth arrest and apoptosis in endocrine tumours (Figure 6). As our previous work has shown that SP-G causes G_i-dependent ERK activation in cells expressing GRP receptors, we hypothesise that SP-G may interact with a common binding pocket within these neuropeptide receptors to induce biased agonism.

Many GPCRs interact with their various G-proteins via their intracellular domains. Previous studies have shown that the second intracellular loop of the V_{1A} receptor is critical to interact with G_q and subsequently activate PLC (Liu and Wess, 1996; Erlenbach and Wess, 1998). Moreover, active peptide mimetics of the second intracellular loop specifically block AVP probably by disrupting intermolecular interactions between the i2 region of endogenous V_{1A} receptors and another core region of the receptor (Demene *et al.*, 2003). The present study shows that replacement of the second intracellular loop with that of the V_2 receptor (V_{1i2}) resulted in an abolition of PLC activation without affecting AVP-induced binding of G_q, suggesting that this region is involved in receptor coupling to G_q. However, the V_{1i2} chimera was able to partially activate ERK in response to AVP suggesting that AVP can activate ERK by additional mechanisms. The V_{1i2} mutation did not markedly affect the stimulation of ERK by SP-G, although the V_{1i3} chimera was less able to activate ERK in response to SP-G, than the wild-type receptor. Given that substitution of the second intracellular region of the V_{1A} receptor did not affect SP-G-induced ERK activation coupled with the finding that SP-G increases ERK via G_i suggests that the i2 region may not be involved in V_{1A} receptor coupling to G_i although it is crucial for receptor binding to G_q. This lends further credence to the existence of agonist selective states, as different agonists would be expected to alter receptor conformation to expose these different G-protein interacting sequences leading to selective activation of downstream signalling events.

Our previous results have demonstrated that SCLC tumours which have become resistant to chemotherapy express higher levels of neuropeptide receptors and become more sensitive to substance P analogues (Waters *et al.*, 2003). Through direct activation of anti-proliferative effects, SP-G and its analogues would be more valuable anti-tumour agents than silent antagonists as they would not be dependent on high levels of circulating AVP and may be more beneficial in cancers which have become resistant to conventional chemotherapy.

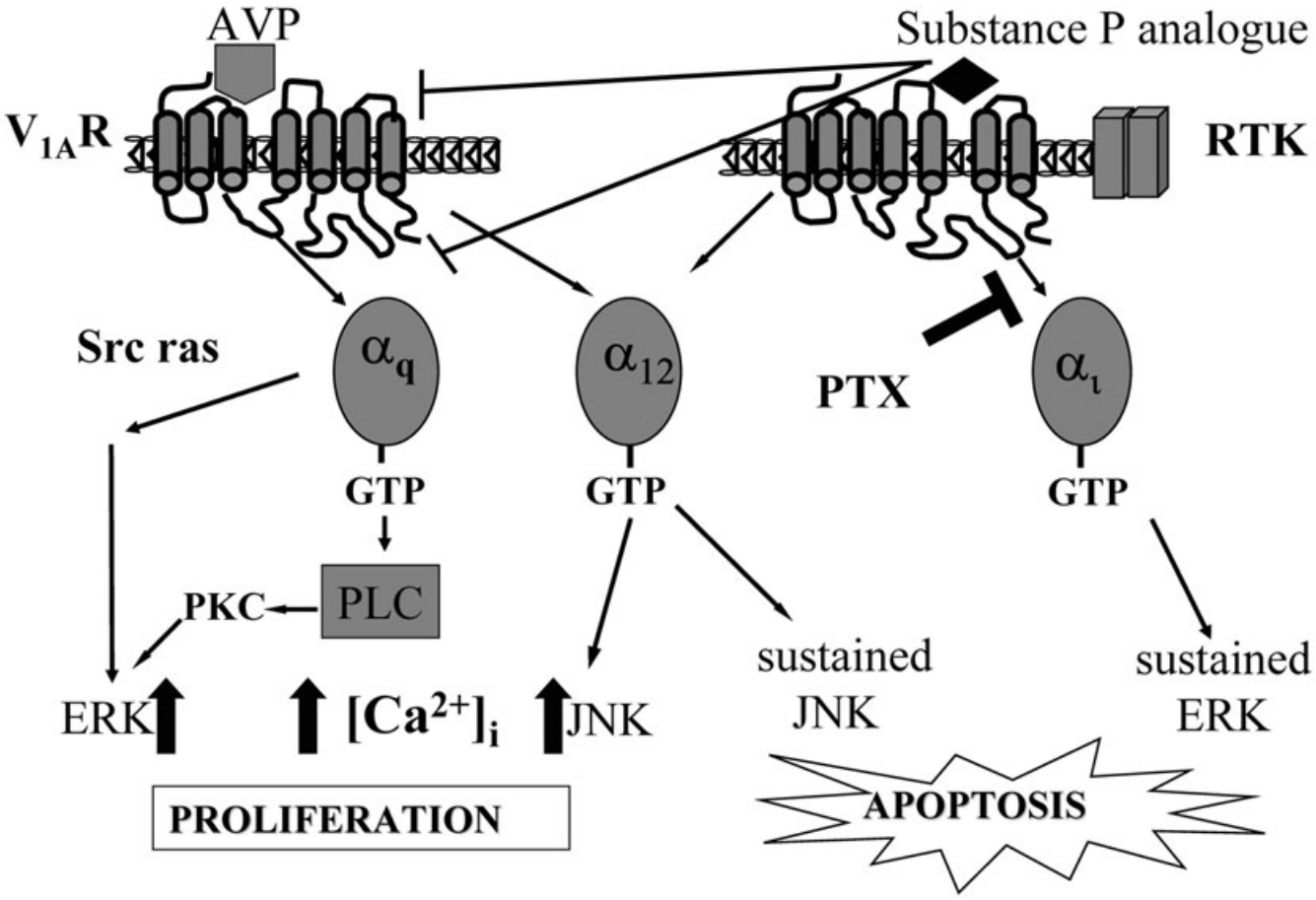

Figure 6 Schematic representation showing activation of V_{1A}-mediated signalling pathways by AVP and substance P analogues. Substance P analogues such as SP-G inhibit calcium mobilisation but induce sustained ERK and JNK activation leading to apoptosis.

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Conflict of interests

The authors state no conflict of interest.

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