Oxytocin- and vasopressin-induced growth of human small-cell lung cancer is mediated by the mitogen-activated protein kinase pathway

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Abstract

Malignant growth of small-cell lung carcinoma is promoted by various neuroendocrine autocrine/paracrine loops. Therefore, to interfere with this mitogenic process, it is crucial to elucidate the mechanisms involved. It is known that the oxytocin (OT) and vasopressin (VP) genes, normally transcriptionally restricted in their expression, are activated in small-cell lung cancer (SCLC), concomitantly with expression of their receptors (OTR, V1aR, V1bR/V3R and V2R). The aim of the present study was to characterize, in concentrations close to physiological and pharmacological conditions, intracellular signalling events triggered by OT and VP binding to their specific receptors in SCLC cells and to identify factors mediating OT- and VP-induced mitogenic effects on SCLC. Known agonists for OTR ([Thr4,Gly7]OT) and V1aR (F180), in addition to OT and VP, were able to elicit increases in cytosolic Ca2+ levels and this effect could be blocked using an OTR antagonist (OVTA) or a V1aR antagonist (SR49059) respectively. There was no activation of the cAMP pathway detected after VP, dDAVP (a V2R agonist), or OT treatment. Stimulation of SCLC cells with OT and VP led to an increase of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, maximal at 5 min, and the subsequent phosphorylation of its downstream target p90 ribosomal S6 kinase (p90RSK). Pre-incubation with OVTA and SR49059, and with inhibitors of phospholipase C (PLC), protein kinase C (PKC), mitogen-activated protein kinase/ERK kinase (MEK) 1/2 and a Ca2+ chelator significantly reduced OT- and VP-induced ERK1/2 phosphorylations. OVTA, SR49059 as well as MEK1/2 and PKC inhibitors also downregulated OT- and VP-induced p90RSK phosphorylation. In [3H]thymidine-uptake experiments, we subsequently observed that PLC, Ca2+, PKC and ERK1/2 are absolutely required for the OT- and VP-stimulated SCLC cellular growth process. In conclusion, the results presented here indicate that OT- and VP-induced mitogenic effects on SCLC are respectively mediated by OTR and V1aR signalling and that this mitogenic signalling passes through the phosphorylation of ERK1/2 and p90RSK in a PLC-, Ca2+-, PKC- and MEK1/2-dependent pathway.

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Introduction

As a basic component of oncogenesis, neuroendocrine tumour cells usually develop very potent autocrine/paracrine signalling pathways that play a crucial part in the dysregulation of cellular growth (Hanahan & Weinberg 2000, Zochbauer-Muller et al. 2002). Among such autocrine/paracrine loops, the neurohypophyseal peptide system is implicated in the pathogenesis of an increasing number of cancers and particularly in small-cell lung carcinoma (SCLC) (North 2000, Cassoni et al. 2001). Ligands of this system include the neurohypophysial...
hormones oxytocin (OT) and vasopressin (VP). There is only one known receptor for OT, OTR, and three main receptors for VP, V1aR, V1bR/V3R and V2R, by which respective hormones exert their physiological actions (Birnbaumer et al. 1992, Kimura et al. 1992, de Keyzer et al. 1994, Sugimoto et al. 1994, Thibonnier et al. 1994, Jard 1998). Tumour production, secretion of VP and its mitogenic action on SCLC cellular growth are well documented (Woll & Rozengurt 1989a, Sethi & Rozengurt 1991, Friedmann et al. 1994, North 2000, Rozengurt 2002). Moreover, it is now established that these tumours also express and secrete OT (North et al. 1993, Péqueux et al. 2002). In SCLC, OT and VP have been shown to induce a dose-dependent (from 1 nM) and time-persistent increase of tumour cell proliferation. This mitogenic effect of OT was completely abolished by the OTR antagonist (dCH2)5Tyr(Me)2Tyr9-NH2, Orn8, Tyr5NH2-vasotocin (OVTA) which when used alone induces a reduction of SCLC cellular growth (Péqueux et al. 2002). However, the subsequent signalling pathways involved in this OT-related mitogenic effect have not been fully investigated.

The biological effects of neuropeptides result from the activation of complex intracellular networks rather than linear signalling cascades (Heasley 2001, Rozengurt 2002). The OTR, V1aR, V1bR/V3R and V2R all belong to the superfamily of G-protein-coupled receptors (GPCRs), which are structurally characterized by seven transmembrane domains. These GPCRs form constitutive homo-and heterodimers, although no differences in pharmacological and signalling properties have been shown as a result of these interactions (Terrillon et al. 2003, Devost & Zingg 2004). The OTR, as well as the V1aR and V1bR/V3R, mediate phosphatidylinositol hydrolysis and cytosolic Ca2+ increase, whereas V2R is coupled to adenylate cyclase and the second messenger cAMP (Barberis et al. 1998). In certain cell types expressing OTR or V1aR, OT and VP can induce phosphorylation and activation of mitogen-activated protein kinases (MAPKs, Ohmichi et al. 1995, Chiu et al. 2002, Zingg & Laporte 2003). All known neurohypophyseal peptide GPCRs are expressed in SCLC (Friedmann et al. 1994, North et al. 1997, North et al. 1998a, 1998b, Péqueux et al. 2002). Moreover, all these receptors have the ability to bind VP and/or OT with varying affinity. Thus both ligands are capable of initiating signalling cascades mediated by either receptor (Barberis et al. 1999). Altogether, these data suggest that the neurohypophyseal hormone system displays a high degree of complexity in the tumour modulation of SCLC cellular growth. Moreover, the oxytocinergic system (OT and OTR) is implicated in the growth modulation of various neoplastic cells where it may inhibit or stimulate cell proliferation. These discordant effects have the potential to be a consequence of the activation of different signal transduction pathways which result in increased cytosolic Ca2+ or the formation of cAMP (Bussolati & Cassoni 2001, Cassoni et al. 2001, Cassoni et al. 2002). Additionally, the activation of particular signalling intermediates by the OTR depends on its localization inside or outside caveolin-enriched domains resulting in a mitogenic or anti-mitogenic response to OT stimulation, respectively (Guzzi et al. 2002, Rimoldi et al. 2003).

These data emphasize the crucial part played by transduction signalling in mediation of neuropeptide effects. Therefore, in order to clarify the mechanisms leading to SCLC cellular growth dysregulation, we investigated the OT- and VP-induced mitogenic responses and the activation of signal transduction mediators in H345 and H146 SCLC cell lines. Specific agonists and antagonists of the OT and VP GPCRs were employed to further dissect ligand-mediated receptor activation involved in SCLC cellular growth. In order to extrapolate our in vitro findings to relevant physiological and pharmacological conditions, we adhered strictly to the concentrations of OT and VP that would be observed in vivo. The maintenance of this appropriate concentration is crucial for delineating the specificity of ligand binding (i.e. OT and VP) to their appropriate receptors. These results show that the OT- and VP-induced mitogenic effects on SCLC are mediated largely by the OTR and the V1aR, respectively, and that their activation leads to increases in cytosolic Ca2+, but not in production of cAMP. OT and VP stimulation also results in the increased phosphorylation of extracellular signal-regulated kinases (ERKs) 1 (p44MAPK) and 2 (p42MAPK) and of p90 ribosomal S6 kinases (p90RSK). These phosphorylations are mediated by phospholipase C (PLC)-, Ca2+-, protein kinase C (PKC)- and MAPK/ERK kinases (MEK1 and MEK2)-dependent pathways.

Materials and methods
Reagents and cell culture
Dulbecco’s PBS (DPBS), fetal calf serum and RPMI 1640 were purchased from Cambrex (Verviers, Belgium); OT and VP originated from Calbiochem (VWR, Leuven, Belgium). Desmopressin or (deamino-Cys1, D-Arg9)VP (dDAVP; a V2R agonist; Zaoral et al. 1967) and OVTA (an OTR antagonist; Manning et al. 1989) were obtained from Bachem (Bubendorf, Switzerland). F180 (a V1aR agonist; Aurell et al. 1990, Andres et al. 2002) was provided kindly by P. Riviere (Ferring Research, San Diego, CA, USA), [Thr4, Gly7]OT (an OTR agonist; Lowbridge et al. 1977) by M. Manning (Medical College of Ohio, Toledo, OH, USA) and SR49059 (a V1aR agonist; Serradeil-Le Gal et al. 1993) by C. Serradeil-Le Gal (Sanoﬁ Recherche, Montpellier).
France). Indo-1-AM (where AM is acetoxymethyl ester), EGTA-AM and ionomycin (4-bromo A-23187) were purchased from Molecular Probes (Eugene, OR, USA). Insulin–transferin–sodium selenite (ITS) solution (I-1884), BSA (fraction V, A-4503), forskolin, isobutylmethylxanthine (a phosphodiesterase inhibitor) and glucose were obtained from Sigma. Complete™ protease-inhibitor cocktail tablets were from Roche, [3H]thymidine was from Amersham Pharmacia Biotech and scintillation liquid from Perkin Elmer (Courtaboeuf, France).

The origins of the antibodies were as follows: polyclonal anti-β-actin (ab8227) was from Abcam (Cambridge, UK), and polyclonal anti-p42/44MAPK (ERK1/2; #9102), polyclonal anti-phospho-p42/44MAPK (ERK1/2; #9101), polyclonal anti-phospho-p90RSK (#9344) and anti-rabbit IgG horseradish peroxidase-linked (#7074) antibodies were from Cell Signaling Technology (Beverly, MA, USA). U73122 (a PLC inhibitor), GF109203X (a PKC inhibitor) and BAPTA-AM (bis-(o-aminophenoxy)-N,N,N′,N′'-tetra-acetic acid AM; a Ca$^{2+}$ chelator) were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA) and U0126 (a MEK1/2 inhibitor; #9903) was from Cell Signaling Technology.

The classical SCLC cell lines NCI H345 and NCI H146 were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in a humidified atmosphere of 5% CO$_2$ and 95% air at 37°C, according to American Type Culture Collection recommended guidelines.

Flow-cytometric analysis of Ca$^{2+}$ kinetics

H345 cells routinely cultured were washed twice with DPBS. Cells (3 × 10$^6$, cells/ml) were loaded by incubating them for 45 min at 37°C in serum-free medium (RPMI 1640 + ITS 1%) containing 5 μM indo-1-AM. After two washes with DPBS, cells were suspended at 1 × 10$^5$ cells/ml in a Ca$^{2+}$ buffer made of DPBS containing CaCl$_2$ (1 mM), glucose (1 mg/ml) and BSA (0.1%). Loaded cells were kept at room temperature until flow analysis was performed on a Facstar plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Prior to analysis, loaded cells (900 μl) were placed at 37°C for 2–5 min. Fluorescence records of each sample were measured over approximately 4 min, at an excitation wavelength of 356 nm and emission wavelengths of 405 nm (Ca$^{2+}$-bound indo-1-AM) and 485 nm (free indo-1-AM). The various peptide ligands being tested (Table 1) were administered as 100 μl volumes in 1 mM Ca$^{2+}$ buffer to cell samples after 30 s of baseline recording. Proper loading of the cells with indo-1-AM was confirmed (approximately 80–90% of responding cells) by stimulation with ionomycin (4-bromo A-23187; 10 μM), which induces Ca$^{2+}$ influx from the extracellular medium. Incubation of the cells with EGTA-AM (0.01 mg/ml) 5 min prior to flow measurement, as well as administration of Ca$^{2+}$ buffer without ligand, were used as suitable negative controls. Receptor activity was specifically blocked by pre-incubating the cells with antagonists of the OTR or V1aR for 15 min at 37°C prior to measuring their baseline and agonist-stimulated cytosolic Ca$^{2+}$ levels by flow cytometry. The agonists tested were OT (1 nM), VP (1 nM), F180 (V1aR agonist; 20 nM) and [Thr$^4$,Gly$^7$]OT (OTR agonist; 5 nM). The antagonists were SR49059 (V1aR antagonist; 10 nM) and OVTA (OTR antagonist; 0.5 nM). Data were analysed using Flowjo 3.4 software (Tree Star, Ashland, OR, USA) and plotted as the ratio 405 nm/485 nm expressed as the percentage of responding cells over a threshold of 200 (mean ratio 405 nm/485 nm) against time (s).

Flow-cytometric analysis of Ca$^{2+}$ kinetics

Prior to cAMP analysis, H345 and H146 cells were cultured overnight in serum-free medium (RPMI 1640 + BSA 0.1% + ITS 1%) at a density of 1 × 10$^6$ cells/ml under normal conditions. The cells were then treated for periods from 0 to 60 min at 37°C with OT, VP or dDAVP (V2R agonist) in the presence of isobutylmethylxanthine (50 μM). Treatment with the adenylyl cyclase activator forskolin was

<table>
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<th>Ligands</th>
<th>V1aR</th>
<th>V1bR/V3R</th>
<th>V2R</th>
<th>OTR</th>
</tr>
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<tr>
<td>VP</td>
<td>1.0</td>
<td>0.7</td>
<td>3.3</td>
<td>1.6</td>
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<tr>
<td>F180</td>
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<td>2100</td>
<td>&gt;10000</td>
<td>520</td>
</tr>
<tr>
<td>(1-deamino, B-d-arginine)VP</td>
<td>21</td>
<td>22</td>
<td>2.7</td>
<td>203</td>
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<td>SR49059</td>
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<td>119</td>
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<td>1540</td>
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<tr>
<td>[Thr$^4$,Gly$^7$]OT</td>
<td>78.8</td>
<td>–</td>
<td>–</td>
<td>6.6</td>
</tr>
<tr>
<td>OVTA</td>
<td>3.9</td>
<td>10229</td>
<td>929</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Dissociation constant (K$_d$) values are taken from Barberis et al. (1999) and Andres et al. (2002).
performed as a positive control. OT, VP and dDAVP were tested at concentrations of 1 nM, 10 nM, 100 nM and 1 μM and forskolin was used at 1 and 10 μM. After the appropriate time, the reactions were stopped by centrifugation (250 g, 4 °C, 5 min). Supernatants were discarded and the pellets were washed with DPBS (2 ml, 4 °C). After centrifugation, the cell pellets were lysed in 1 ml ethanol (95%)/HCl (0.01 M) at 4 °C, and then vortexed three times for 20 s. Samples were centrifuged (13 600 g, 4 °C, 15 min) and the supernatants (900 μl) were collected in 1.5 ml tubes, lyophilized and stored at −20 °C until assayed. The cAMP content was determined using a RIA kit (RPA 509; Biotrak, Amersham Biosciences).

Preparation of cell lysates and Western blot kinase phosphorylation analysis

H345 and H146 cells in stock culture were washed in DPBS and seeded into 12-well plates in serum-free medium (RPMI 1640 + BSA 1%) at a density of 3 × 10⁶ cells/well, and cultured overnight. Cells were pre-incubated in RPMI 1640 alone (control) or containing U73122 (PLC inhibitor; 2.5 μM), BAPTA-AM (Ca²⁺ chelator; 20 μM), GF109203X (PKC inhibitor; 20 μM) or U0126 (MEK1/2 inhibitor; 10 μM) for 45 min, or they were pre-incubated in RPMI 1640 containing OTVA (OTR antagonist; 0.5 nM) or SR49059 (V1aR antagonist; 10 nM) for 15 min. Then, cells were stimulated with OT or VP at a final concentration of 1 nM for the indicated period of time (basal control is unstimulated). Reactions were stopped by addition of ice-cold DPBS. Ice-cold DPBS and lysing buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM dithiothreitol, 1 mM Na₂VO₄, 25 mM β-glycerophosphate, 1 mM NaF and 1 tablet/50 ml Complete™) were used to wash the cells twice and extract their protein. After centrifugation (18 000 g, 15 min, 4 °C) supernatants were isolated and aliquots were assayed for protein content using the Bradford protein assay (BioRad). Cell lysates were conserved at −20 °C until Western blot procedure.

Cellular proteins (20 μg) were separated by 10% SDS-PAGE and Western transferred on to polyvinylidene difluoride (PVDF; Immobilon™-P; Millipore, Bedford, MA, USA) membranes. After activation in methanol (100%), blots were incubated for 1 h at room temperature, in TBS-T (20 mM Tris/HCl, 140 mM NaCl and 0.2% Tween 20, pH 7.6) containing 5% powdered skimmed milk (Gloria®; Nestlé, Switzerland; TBS-T/milk). After washing, membranes were incubated overnight at 4°C with the appropriate primary polyclonal antibody (see legends of Figs 3 and 4 for specific antibodies) at a concentration recommended by the manufacturer, in TBS-T containing 5% BSA. After washing, the membranes were incubated for 1 h at room temperature with the horseradish peroxidedase-linked anti-rabbit IgG diluted in TBS-T/milk, washed again, and the immunocomplexes visualized by chemiluminescence reaction (ECL™ Western blotting Detection Reagents; Amersham Biosciences). For signal quantification, membranes were densitometrically scanned (Gs-700; BioRad) and analyzed with Quantity One 4.2.1 software (BioRad). Quantification of phosphorylated protein forms was carried out by expressing their intensity relatively to the intensity generated by β-actin or ERK1/2 immunoblotting and as a ratio of basal activation.

Cell proliferation

Cellular growth and toxicity were evaluated for the same experiment by respective measurement of [³H]thymidine incorporation into DNA and of lactate dehydrogenase (LDH) release in culture supernatants using a standard chemical procedure (Roche). H345 and H146 cells in stock culture were washed in DPBS and replated in T culture flasks (25 cm²; VWR, Belgium) in RPMI 1640 added with 5% charcoal-treated fetal calf serum (charcoal treatment discards small molecules and proteins such as OT and VP) at an initial density of 1 × 10⁶ cells/ml and cultured overnight. Then, RPMI 1640 (basal control), OT or VP were added in culture medium at final concentration of 1 nM. After 72 h of culture with hormone or solvent, U73122, BAPTA-AM, GF109203X, U0126 or DMSO (1/2000) diluted in RPMI 1640 (control) were added to culture medium at final concentrations ranging from 2.5 to 10 μM. After 30 min and 1, 2, 4, 24, 48 and 72 h of culture with these inhibitors or solvent, 1 ml cell suspension was taken and distributed in triplicate on 96-well plates (200 μl/well) for [³H]thymidine incorporation. The remaining cell-suspension fraction was centrifuged (250 g, 20 °C, 10 min), and 200 μl supernatant was mixed with Complete™ protease-inhibitor cocktail and 50% glycerol (v/v) and stored at -20°C till LDH assay.

LDH baseline and positive control were respectively LDH measures in culture medium or when 1 ml cell suspension was lysed with RPMI 1640 with 2% Triton X-100. On 96-well plates, [³H]thymidine (25 μl, 11.5 μCi/ml) was added and plates were incubated for 4 h in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cellular DNA was harvested from the cells using a Tittertek cell harvester (Flow Laboratories, McLean, VA, USA). One-minute sample counts were obtained in a Beckman liquid scintillation counter (Beckman, Fullerton, CA, USA).

Statistical analyses

Variations in [³H]thymidine uptake were analyzed statistically with GraphPad Prism™ software (GraphPad.

**Results**

**OT- and VP-induced Ca\(^{2+}\) kinetics**

In order to assess whether OT, in addition to VP, could initiate in SCLC, under ligand concentrations within the range *in vivo*, the inositol-mediated pathway usually associated with OTR, V1aR and V1bR/V3R, changes in H345 cytosolic Ca\(^{2+}\) level were measured by flow cytometry. There was a 40% increase in the cytosolic Ca\(^{2+}\) levels observed in response to OT or VP at 1 nM (Fig. 1). Specific activation of the OTR or the V1aR located in the H345 cell membrane was tested using receptor agonists at selective concentrations (see Table 1). Stimulation with \([\text{Thr}^4, \text{Gly}^7]\)OT (OTR agonist) at 5 nM or with F180 (V1aR agonist) at 20 nM resulted in a clear increase (35%) in cytosolic Ca\(^{2+}\) (Fig. 1). Depending on their concentrations, OT and VP can bind each of the neurohypophysial peptide GPCRs (see Table 1). To identify which receptor(s) mediate the OT- or VP-induced increase in cytosolic Ca\(^{2+}\), cells were exposed to OTR antagonist (OVTA) or V1aR antagonist (SR49059) at concentrations (0.5 and 10 nM respectively) that selectively block those receptors (see Table 1). Cell pre-incubation with antagonists was followed by stimulation.

![Figure 1](image)

**Figure 1** Effect of OT and VP on Ca\(^{2+}\) kinetics with or without antagonist pre-incubation. For antagonist pre-incubation, indo-1-AM-loaded H345 cells were exposed to the V1aR antagonists SR49059 (10 nM), or the OTR antagonist OVTA (0.5 nM) for 15 min at 37°C. Then, changes in cytosolic free calcium were measured using flow-cytometric analysis to determine the 405/485 nM emissions ratio over a 200-s period. After approximately 30 s of baseline, samples were injected with OT (1 nM), OTR agonist \([\text{Thr}^4, \text{Gly}^7]\)OT (5 nM), VP (1 nM) or V1aR agonist F180 (20 nM). Data are expressed as the percentage of responding cells over a threshold of 200 (mean ratio 405 nM/485 nM) against time (s). Kinetics shown are representative of two independent experiments.
with OT, VP, [Thr4, Gly7]OT or F180 at pharmacological concentrations. Pre-incubation with SR49059 almost completely blocked the increases of cytosolic Ca\(^{2+}\) induced by VP at 1 nM or F180 at 20 nM, while OT at 1 nM and [Thr4, Gly7]OT at 5 nM were still able to elicit increases in the cytosolic Ca\(^{2+}\) levels. Pre-incubation with OVTA was able to block the OT-induced but not the VP-induced increase in cytosolic Ca\(^{2+}\) (Fig. 1).

**cAMP kinetics**

To discern whether adenylate cyclase-mediated signal transduction, generally associated with the V2R response, could be initiated in SCLC cells after stimulation with OT or VP, measurements of intracellular cAMP kinetics were performed on H345 and H146 cells. When the cells were incubated with VP, OT or the V2R agonist dDAVP, each at 1 nM, there was no increase observed in the intracellular cAMP levels, even after 60 min exposure (Fig. 2A). Various incubation times (15–60 min) and ligand concentrations (1 nM to 1 \(\mu\)M) were tested; however, there were no significant changes in either SCLC cell line in the intracellular levels of cAMP detected. The results obtained from H345 and H146 cells after 30 min incubation with various ligand concentrations are shown in Fig. 2B.
OT- and VP-induced ERK1/2 phosphorylations and pathway

MAPKs, and particularly ERK1/2, two highly conserved serine/threonine kinases activated via protein phosphorylation cascades, have been shown to be associated in proliferation processes of various cell types (Johnson & Lapadat 2002). Moreover, phosphorylation of ERK1/2 is implicated in the growth of cells transfected with the OTR (Rimoldi et al. 2003) or the V1aR (Thibonnier et al. 2000). Therefore, SCLC H345 and H146 cells were treated with either OT or VP at 1 nM and cell lysates were analysed for ERK1/2 phosphorylation. After 5 min treatment, OT and VP induced comparable increases of ERK1/2 phosphorylation in both cell lines. There was a 3-fold (n = 16) and a 2.5-fold (n = 22) average OT- or VP-induced increase in ERK1/2 phosphorylation measured in H345 and H146 cells respectively, compared to the baseline levels of phospho-ERK1/2, and these increases started to decline after 15 min (Fig. 3A). Indeed, it must be noted that an ERK1/2 basal level of activation was usually observed in both SCLC cell lines (Fig. 3). Knowing that these cells secrete endogenous OT and VP (Pe低于ex et al. 2002), the effect of antagonists of these neuropeptides was evaluated.

**Figure 3** Effect of OT and VP on ERK1/2 phosphorylation with or without pre-incubation with specific signal transduction inhibitors. Proteins were extracted and separated by 10% SDS-PAGE and Western blot analysis was performed using a polyclonal anti-phospho-ERK1/2 antibody. Cells were incubated (+) or not (−) with OT (1 nM) or VP (1 nM) for 3, 5 and 15 min. A polyclonal anti-ERK1/2 antibody was used to normalize loading (A). Cells were pre-incubated (+) with OTR antagonist OVTA (0.5 nM) (B), V1aR antagonist SR49059 (10 nM) (C), or with solvent (−) for 15 min. Cells were pre-incubated (+) with PLC inhibitor U73122 (2.5 μM) (D), Ca²⁺ chelator BAPTA-AM (20 μM) (E), PKC inhibitor GF109203X (20 μM) (F), MEK1/2 inhibitor U0126 (10 μM) (G), or with solvent (−) for 45 min. Then, after these pre-incubations, cells were stimulated (+) or not (−) with OT (1 nM) or VP (1 nM) for 5 min. A polyclonal anti-β-actin antibody was used to normalize loading. Western blots shown are representative of at least three independent experiments, each performed on H345 and H146 cells.
on the ERK1/2 basal level of phosphorylation. In both SCLC cell lines studied, the OTR antagonist OVTA and the V1aR antagonist SR49059, used at selective concentrations of 0.5 and 10 nM respectively, reduced the basal level of phosphorylated ERK1/2 (Table 2, Fig. 3B and C). These antagonists were also used to check the specificity of ERK1/2 phosphorylation increases induced by exogenous OT and VP. In H345 and H146 cells, treatment with the OTR antagonist OVTA prevented OT-induced ERK1/2 phosphorylation, while it presented a minor effect on the VP-induced ERK1/2 phosphorylation (Table 3, Fig. 3B). Similarly, the effect of the V1aR antagonist SR49059 prevented VP-induced ERK1/2 phosphorylation, while its effect on the OT-induced ERK1/2 phosphorylation remained weaker (Table 3, Fig. 3C).

In order to identify the upstream mediators involved in the observed ERK1/2 phosphorylations, specific pharmacological inhibitors of signal transduction were examined for their effect on OT- and VP-induced ERK1/2 phosphorylation (Fig. 3, Table 3). OT and VP increase cytosolic Ca$^{2+}$, and this pathway is activated when OTR or V1aR are coupled to Gαq/11; thus contributions of PLC and Ca$^{2+}$ to the mediation of ERK1/2 phosphorylation were examined. Pre-incubation of H345 and H146 cells with the PLC inhibitor U73122 (2.5 μM; Fig. 3D) and the Ca$^{2+}$ chelator BAPTA-AM (20 μM; Fig. 3E) reduced OT- and VP-induced ERK1/2 phosphorylation in a similar manner in both cell lines (Table 3). OT- and VP-induced ERK 1/2 phosphorylation was downregulated by the PKC inhibitor GF109203X (20 μM) similarly in both SCLC cells (Table 3, Fig. 3F). Moreover, these phosphorylations were almost completely blocked by the MEK1/2 inhibitor U0126 (10 μM), confirming their specificity (Table 3, Fig. 3G).

### OT- and VP-induced p90RSK phosphorylation

A downstream target of ERK1/2 implicated in cell proliferation is the serine/threonine kinase p90RSK (Frodin & Gammeltoft 1999). Incubation of SCLC H345 and H146 cells with OT or VP (1 nM) induced an increase of p90RSK phosphorylation (Fig. 4). Compared to the basal level of p90RSK phosphorylation observed in these cells, a 1.7-fold (n = 15) and a 2.5-fold (n = 13) average p90RSK phosphorylation increase was observed after 5 min treatment with OT and VP respectively in H345 cells. Similarly in H146 cells, a 2-fold (n = 6) and a 3-fold (n = 6) average p90RSK phosphorylation increase was observed after 5 min treatment with OT and VP respectively. Next, OVTA and SR49059 were used to examine

### Table 2 Quantified inhibition of basal ERK1/2 and p90RSK phosphorylation after cell treatment with OT or VP antagonists. Means ± S.D. are shown.

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<thead>
<tr>
<th>Antagonist</th>
<th>H345 cells</th>
<th>H146 cells</th>
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<td>SR49059 (V1aR antagonist)</td>
<td>41 ± 4 (n=4)</td>
<td>54 ± 8 (n=3)</td>
<td>38 ± 7 (n=7)</td>
<td>30 ± 10 (n=3)</td>
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### Table 3 Quantified inhibition of OT- and VP-induced ERK1/2 and p90RSK phosphorylation after cell treatment with pharmacological inhibitors and antagonists. Means ± S.D. are shown.

<table>
<thead>
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<th>Inhibitors</th>
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<td><strong>% ERK1/2 phosphorylation inhibition</strong></td>
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<td>OVTA (OTR antagonist)</td>
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<td>30 ± 5 (n=3)</td>
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<td>SR49059 (V1aR antagonist)</td>
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<td>U73122 (PLC inhibitor)</td>
<td>72 ± 10 (n=3)</td>
<td>76 ± 9 (n=3)</td>
<td>85 ± 9 (n=3)</td>
<td>86 ± 11 (n=3)</td>
</tr>
<tr>
<td>BAPTA-AM (Ca$^{2+}$ chelator)</td>
<td>89 ± 6 (n=3)</td>
<td>90 ± 5 (n=5)</td>
<td>88 ± 10 (n=3)</td>
<td>91 ± 11 (n=5)</td>
</tr>
<tr>
<td>GF109203X (PKC inhibitor)</td>
<td>63 ± 9 (n=3)</td>
<td>77 ± 8 (n=6)</td>
<td>72 ± 8 (n=3)</td>
<td>74 ± 6 (n=6)</td>
</tr>
<tr>
<td>U0126 (MEK1/2 inhibitor)</td>
<td>91 ± 7 (n=3)</td>
<td>94 ± 4 (n=4)</td>
<td>95 ± 3 (n=3)</td>
<td>97 ± 5 (n=4)</td>
</tr>
<tr>
<td><strong>% p90RSK phosphorylation inhibition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVTA (OTR antagonist)</td>
<td>44 ± 12 (n=10)</td>
<td>58 ± 6 (n=3)</td>
<td>25 ± 7 (n=3)</td>
<td>26 ± 9 (n=3)</td>
</tr>
<tr>
<td>SR49059 (V1aR antagonist)</td>
<td>23 ± 12 (n=10)</td>
<td>12 ± 5 (n=3)</td>
<td>56 ± 13 (n=4)</td>
<td>49 ± 10 (n=3)</td>
</tr>
<tr>
<td>GF109203X (PKC inhibitor)</td>
<td>62 ± 9 (n=3)</td>
<td>57 ± 7 (n=3)</td>
<td>85 ± 7 (n=5)</td>
<td>78 ± 10 (n=3)</td>
</tr>
<tr>
<td>U0126 (MEK1/2 inhibitor)</td>
<td>64 ± 12 (n=4)</td>
<td>60 ± 8 (n=3)</td>
<td>80 ± 14 (n=4)</td>
<td>75 ± 7 (n=3)</td>
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to a lesser extent the effect of the exogenous VP on p90RSK phosphorylation (Table 3, Fig. 4A). Similarly, treatment with the V1aR antagonist SR49059 resulted in a decrease in VP-induced p90RSK phosphorylation, while its inhibition of OT-induced p90RSK phosphorylation was weaker (Table 3, Fig. 4B). Moreover, the p90RSK level of phosphorylation, resulting from the action of OT and SR49059 on OT- and VP-induced p90RSK phosphorylation respectively, was under the basal level of p90RSK phosphorylation observed in these SCLC cells (Fig. 4A and B).

PKC inhibitor GF109203X (20 μM; Fig. 4C) and MEK1/2 inhibitor U0126 (10 μM; Fig. 4D) downregulated OT- and VP-induced p90RSK phosphorylation in both SCLC cell lines (Table 3). GF109203X and U0126 resulted in an acute decrease in VP-induced p90RSK phosphorylation compared to the OT-induced p90RSK phosphorylation.

**Inhibition of OT- and VP-induced mitogenic effect by acting on the OT- and VP-signalling pathway**

In order to define the signalling pathways contributing to the OT- and VP-induced mitogenic action on SCLC cellular growth, inhibitors described above were employed to determine their effects on H345 and H146 [3H]thymidine incorporation experiments as a measure of proliferation (Fig. 5). We have demonstrated previously that OT and VP induce a significant increase in H345 cell proliferation (Péqueux et al. 2002). Both SCLC cell lines were therefore stimulated for 72 h with OT (1 nM) or VP (1 nM) prior to inhibitor addition. Compared to unstimulated cells, OT and VP treatments led to increases in H345 cellular growth of 20 and 35% respectively (P < 0.001), a range similar to that already described (Péqueux et al. 2002). These neuropeptides induced an increase in H146 cell proliferation as well. Indeed, H146 cellular growth was 15 and 20% higher after OT and VP stimulation respectively (P < 0.001 compared with unstimulated cells; Fig. 5A). After 30 min inhibitor treatment, the OT- and VP-stimulated proliferation of H345 and H146 cells was significantly reduced by PLC inhibitor U73122 (2.5 μM), Ca²⁺ chelator BAPTA-AM (10 μM), PKC inhibitor GF109203X (10 μM) and MEK1/2 inhibitor U0126 (10 μM) (Fig. 5A). This effect persisted through 4 h of exposure to the inhibitors (time, 76 h; Fig. 5C). Over 24 h of PLC inhibitor U73122 treatment (time, 96 h) cells returned to their basal growth rate and then H345 cells remained quiescent through the 72 h incubation period while H146 cells continued to grow. The growth rates of cells incubated with Ca²⁺ chelator BAPTA-AM were 5–20% and 25–35% of basal levels for H345 and H146 cells respectively. After 24 h of

**Figure 4** Effect of OT and VP on p90RSK phosphorylation with or without pre-incubation with specific signal transduction inhibitors. Cells were pre-incubated (+) with OT, VP or OTR antagonist OVTA (0.5 nM) (A), V1aR antagonist SR49059 (10 nM) (B) or with solvent (−) for 15 min. Cells were pre-incubated (+) with PKC inhibitor GF109203X (20 μM) (C), MEK1/2 inhibitor U0126 (10 μM) (D) or with solvent (−) for 45 min. Then, after these pre-incubations, cells were stimulated (+) or not (−) with OT (1 nM) or VP (1 nM) for 5 min. After extraction, proteins were separated by 10% SDS-PAGE and Western blot analysis was performed with a polyclonal anti-phospho-p90RSK antibody. A polyclonal anti-β-actin antibody was used to normalize loading. Western blots shown are representative of at least three independent experiments, each performed on H345 and H146 cells.

The contribution of endogenous OT and VP to the basal level of p90RSK phosphorylation observed in H345 and H146 cells. In both SCLC cell lines, OVTA and SR49059, used at selective concentrations of 0.5 and 10 nM respectively, partially inhibited the p90RSK basal level of phosphorylation in comparable proportions to what was observed for the ERK1/2 basal level of phosphorylation (Table 2, Fig. 4A and B). Incubation of H345 and H146 with the OTR antagonist OVTA reduced the stimulatory effect of exogenous OT on p90RSK phosphorylation, and
Figure 5 Effect of signalling pathway inhibitors on OT- and VP-stimulated SCLC H345 and H146 cellular growth. (A) Cellular growth of H345 and H146 cells was estimated by [3H]thymidine incorporation when cells were stimulated with OT or VP (1 nM) for 72 h, prior to addition of U73122 (2.5 μM), BAPTA-AM (10 μM), GF109203X (10 μM), U0126 (10 μM) or solvent (−) for 30 min. For each sample, the variation in [3H]thymidine incorporation was evaluated with respect to control unstimulated cultured H345 or H146 cells in at least three independent experiments each performed in quadruplicate. Data are expressed as percentage variations in [3H]thymidine ([3H]dThd) uptake (means ± S.D.; *P < 0.05, **P < 0.001) against solvent under OT- or VP-stimulated conditions. (B) LDH measurements in supernatants of H345 and H146 cells during OT- and VP-stimulated proliferation experiments. LDH was measured in culture medium as negative control (−; left-hand bar) and in supernatants of cells stimulated with OT or VP (1 nM) for 72 h, then added to U73122, BAPTA-AM, GF109203X or U0126 against solvent under OT- or VP-stimulated conditions. Positive control (+) is LDH level obtained in supernatants when all H345 or H146 cells are lysed. Data are expressed as LDH (ng/ml), means ± S.D., n = 3. (C) Proliferation of OT-stimulated H345 and H146 cells in the presence of U73122 (2.5 μM, ▲), BAPTA-AM (10 μM, ◇), GF109203X (10 μM, ★), U0126 (10 μM, ▲) or solvent (■) was estimated by [3H]thymidine incorporation in at least three independent experiments each performed in quadruplicate. H345 and H146 cells were stimulated with OT (1 nM) for 72 h prior to inhibitor addition (time, 72 h). Data are expressed as [%] variations in [3H]thymidine uptake, (means ± S.D.) against time (h), normalized for each sample to 100% at time 72 h; *P < 0.05, **P < 0.001; U73122, BAPTA-AM, GF109203X or U0126 against solvent under OT-stimulated conditions.
incubation in the Ca^{2+} chelator (time, 96 h), both cell lines reinitiated proliferation. PKC inhibitor GF109203X reduced the H345 and H146 proliferation rate to about 15 and 40% of the basal rate respectively, and both cell lines remained under 40% of the basal level throughout the exposure. In both cell lines the MEK1/2 inhibitor significantly prevented cellular proliferation and thus induced quiescence. This indicates that ERK1/2 is required in the H345 and H146 cell proliferation process. During OT- and VP-stimulated proliferation assays, LDH measurements in supernatants of H345 and H146 cells remained at negative control level (9.5 ± 0.8 ng/ml, n = 3) from 30 min to 72 h of exposure with various inhibitors (Fig. 5B and results not shown). LDH values obtained from H345 and H146 cell lysates were 56.1 ± 1.4 ng/ml (n = 3) and 65.2 ± 1.5 ng/ml (n = 3) respectively. Altogether, these results demonstrate that signalling inhibitors, used in proliferation assays, do not induce cell lysis and therefore growth inhibitions are not a consequence of toxicity. In addition, the absence of toxicity was confirmed by re-establishment of cell growth after treatment (Fig. 5C). Proliferation curves obtained for H345 and H146 cells under VP-stimulated conditions (results not shown) were comparable to those obtained under OT-stimulated conditions (Fig. 5C).

Discussion

Neurohypophysial peptides are involved in potent autocrine/paracrine signalling loops which are essential for cellular growth processes developed by SCLC tumours during oncogenesis (North et al., 1998, Pequeux et al., 2002, Rozengurt, 2002). The fact that all neurohypophysial peptide GPCRs are expressed by SCLC cells suggest that OT- and VP-initiated signalling is part of a complex array of interactions culminating in the development of these tumours (North et al., 1998b, Pequeux et al., 2002). Therefore, understanding the mechanistic action of such hormones and their respective receptors in SCLC cellular growth is essential for the development of new and specific therapeutics.

To define the signalling pathways leading to OT- and VP-induced mitogenic effects on SCLC, the two major signal transduction mechanisms proposed to be associated with the neurohypophysial peptide GPCRs (Barberis et al., 1998) were examined: (1) the inositol-mediated pathway (which is generally associated with OTR, V1aR or V1bR/V3R activation) leading to increases in cytosolic Ca^{2+} and (2) the adenylate cyclase-mediated pathway (which is principally associated with V2R activation) leading to increases in cAMP, and which has also been shown to be activated by OT (Cassoni et al., 1997, 1998). By stimulating H345 SCLC cells with the OTR agonist [Thr^3, Gly^7]OT and with the V1aR agonist F180 at selective concentrations of 5 and 20 nM respectively, we have demonstrated that the cell surface-expressed OTR and V1aR are functional as they can mediate increases in cytosolic Ca^{2+}. OT-induced increases in cytosolic Ca^{2+} levels were found to be comparable to those observed after VP stimulation. Since these increases could be blocked using antagonists to OTR or V1aR, it appears that those receptors are responsible for mediating the majority of the OT- or VP-induced increases in cytosolic Ca^{2+} levels. Additionally, we have shown that neither OT, VP nor dDAVP (V2R agonist) was able to alter the cAMP levels in the two SCLC cell lines H345 and H146, under the conditions used in the following study.

Although a previous study showed that elevations in cytosolic Ca^{2+} can be induced by OT (Hong & Moody, 1991), they were obtained using ligand concentrations in the range of 0.1–10 μM. The use of such high concentrations raised the probability that this Ca^{2+} response to OT was mediated by VP receptors. In the experiments presented here we performed Ca^{2+} kinetics using 1 nM OT, which corresponds to the order of magnitude of the Kd value of OT towards its receptor. We also demonstrated that the cytosolic Ca^{2+} elevation induced by this level of OT was mediated principally through the OTR and not the V1aR. First, OT was able to elicit an increase in Ca^{2+} in the presence of the V1aR antagonist SR49059 (10 nM) and secondly this increase could be abolished by the OTR antagonist OVTA (0.5 nM) thus indicating specific activation of OTR by OT. VP has already been demonstrated to elevate cytosolic Ca^{2+} in various SCLC cell lines and particularly in H345 cells (Woll & Rozengurt, 1998, Hong & Moody, 1991, Bunn et al., 1994, North et al., 1997), but we further examined the effects of VP at the physiological range of 1 nM and established that the Ca^{2+} signal induced at this concentration is largely mediated by the V1aR. Indeed, the V1aR antagonist SR49059 (10 nM) almost completely inhibits the Ca^{2+} signal, whereas OVTA (OTR antagonist) has no measurable effect. For the experimental conditions used here, we were not able to demonstrate any detectable increase in cAMP, in two different SCLC cell lines, in response to VP, dDAVP (V2R agonist) or OT over a wide range of concentrations (1 nM–1 μM). These data suggest that the action of VP at V2R in H345 and H146 cells does not normally produce an activation of the adenylate cyclase cascade. This may be a result of the expression of an abnormally truncated V2R form by these cells, which may block V2R function (North et al., 1998a, b). The lack of an OT-dependent cAMP pathway, the presence of an OT-activated Ca^{2+} response, and a clear mitogenic effect of OT observed on H345 and H146 SCLC cells (Pequeux et al., 2002 and data shown here) is in agreement with the conclusions of...
Bussolati, Cassoni and colleagues (Bussolati & Cassoni 2001, Cassoni et al. 2001). Indeed, the latter report associated the cAMP pathway with an anti-proliferative effect, and the Ca$^{2+}$-cascade with proliferative effects.

To further identify the impact of OT and VP on the SCLC mitogenic response, the induced phosphorylation of ERK1/2 and p90RSK was assessed on two different SCLC cell lines. Our results indicate that stimulation of SCLC with exogenous OT or VP (1 nM) mediates an increase in phosphorylation of ERK1/2 and of its downstream target p90RSK, peaking 5 min after exposure. Since studies of Ca$^{2+}$ kinetics indicated that at the hormone concentration used there was specific activation of OTR and V1aR, it appears that increases in ERK1/2 and p90RSK phosphorylation are also regulated by these receptors. Moreover, the OTR antagonist OVTA and the V1aR antagonist SR49059 reduced the basal level of MAPK activation in SCLC. Additionally, ERK1/2 and p90RSK phosphorylation induced by exogenous OT or VP was inhibited by OVTA or SR49059 respectively, indicating that the actions of these hormones are mediated primarily through their corresponding receptors. It must be noted that SCLC secretes various neuropeptides, which were shown to activate MAPK phosphorylation as well (Rozengurt 2002). The impact of specific inhibitors of signal transduction intermediates, tested with OT- and VP-initiated increases of ERK1/2 phosphorylation, suggests the involvement of PLC-, Ca$^{2+}$- and PKC-dependent pathways. Similarly, OT- and VP-induced p90RSK phosphorylation was PKC-dependent. Moreover, the use of these inhibitors in [3H]thymidine-uptake assays indicates that PLC, Ca$^{2+}$-, PKC and ERK1/2 are essential in the OT- and VP-stimulated H345 and H146 SCLC cellular growth process. Indeed, arrest of cellular growth was not due to toxicity, but rather to blocking of mitogenic transduction cascades.

The VP-induced phospho-ERK1/2 activation we observed in H345 SCLC cells corroborates findings of Seufferlein & Rozengurt (1996). However, the VP dose they used was 100 nM. Thus, the 1 nM concentration used in this study was chosen to dissect specific contributions from the downregulation of PLC, Ca$^{2+}$- and PKC-dependent pathways. Similarly, OT- and VP-induced p90RSK phosphorylation was PKC-dependent. Moreover, the use of these inhibitors in [3H]thymidine-uptake assays indicates that PLC, Ca$^{2+}$-, PKC and ERK1/2 are essential in the OT- and VP-stimulated H345 and H146 SCLC cellular growth process. Indeed, arrest of cellular growth was not due to toxicity, but rather to blocking of mitogenic transduction cascades.

The VP-induced phospho-ERK1/2 activation we observed in H345 SCLC cells corroborates findings of Seufferlein & Rozengurt (1996). However, the VP dose they used was 100 nM. Thus, the 1 nM concentration used in this study was chosen to dissect specific contributions from each neurohypophyseal peptide and to further analyze the upstream pathway leading to the phosphorylation of ERK1/2, as well as its downstream target p90RSK. We demonstrated that in H345 and H146 SCLC cells OT- and VP-induced phospho-ERK1/2 activation was transient and PLC-, Ca$^{2+}$- and PKC-dependent. These results are in agreement with the VP-mediated mitogenic signalling studied in rat intestinal epithelial cells (Chiu et al. 2002) and in V1aR-transfected cells (Thibonier et al. 2000), where this ERK1/2-associated pathway could be activated simultaneously with Ca$^{2+}$/calmodulin kinase II and phosphoinositide 3-kinase cascades. The transient OT-induced ERK1/2 phosphorylation detected in SCLC H345 and H146 cells is in agreement with data obtained on human uterine myometrial cells and on OTR-transfected cells (Ohmichi et al. 1995, Strakova et al. 1998). In addition, Rimoldi et al. (2003) showed that an OT-induced mitogenic effect, related to OTR located in caveolin-enriched microdomains, was indeed mediated through transient activation, whereas an OT-induced anti-proliferative effect was associated with sustained ERK1/2 phosphorylation. The PLC inhibitor U73122 was shown to prevent the growth of OTR-transfected HEK293 cells; however, it did not inhibit the growth of HEK-293 cells that expressed an OTR-caveolin 2 construct, which had the effect of localizing the OTR to caveolin-enriched microdomains (Rimoldi et al. 2003). In H345 and H146 SCLC cells, we observed that PLC inhibitor U73122 abolished phospho-ERK1/2 activation and cellular growth, which indicates that H345 and H146 cellular growth is dependent on PLC-mediated signalling and that OTR may not be associated with caveolin-enriched surface invaginations of these cells. combine this with the facts that OT induced cytosolic Ca$^{2+}$ increase and that Ca$^{2+}$ is absolutely required for OT-stimulated SCLC proliferation, the PLC-dependent OT-induced cellular growth pathway demonstrated in this study supports the coupling of OTR to Gqz/11, when Gzq mediates the PLC-dependent inhibitory response of OT in OTR-transfected HEK-293 cells (Rimoldi et al. 2003). The downstream ERK1/2 target p90RSK is involved in the regulation of gene expression and protein synthesis, as well as in the cell-cycle progression through regulation of various components of the cell-cycle machinery (Frodin & Gammeltoft 1999, Willard & Crouch 2001). We observed that inhibitors of PKC and MEK1/2 abolished the OT- and VP-induced p90RSK phosphorylation and inhibited H345 and H146 SCLC cellular growth. Similarly, the inhibition of p90RSK was shown to abolish growth stimulation of multicellular prostate tumour spheroids (Sauer et al. 2001). Inhibition of PKC also exhibits anti-tumour activity in various cancer types, in studies in vitro and in clinical trials (Mackay & Twelves 2003). Thus our observations are in support of factors involved in cellular growth and differentiation transduction signalling representing potential targets for anticancer therapy.

In conclusion, we demonstrate that, under pharmacological and physiological levels, OT- and VP-induced mitogenic effects on SCLC pass through the specific and respective binding of OTR and V1aR and culminate in the activation of ERK1/2 and p90RSK phosphorylation through PLC-, Ca$^{2+}$-, PKC- and MEK1/2-dependent.
pathways (Fig. 6). Moreover, we demonstrate that growth of OT- and VP-stimulated SCLC could be downregulated by signalling inhibitors and was not a consequence of toxicity. Thus the results identify pathways activated by OT- and VP-mitogenic action on SCLC and show that blocking of their receptor signalling represents viable pharmacological targets for the treatment of neuroendocrine tumours expressing autocrine/paracrine neurohypophysial system loops, such as SCLC.

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