REVIEW

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Calcium-dependent growth regulation of small cell lung cancer cells by neuropeptides

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Abstract

Approximately 15–25% of all primary cancers of the lung are classified histologically as small cell lung carcinoma (SCLC), a subtype characterized by rapid growth and a poor prognosis. Neuropeptide hormones like bombesin/gastrin-releasing peptide, bradykinin or galanin are the principal mitogenic stimuli of this tumour entity. The mitogenic signal is transmitted into the cell via heptahelical neuropeptide hormone receptors, which couple to the heterotrimeric G proteins of the $G_{q/11}$ familiy. Subsequent activation of phospholipase Cβ (PLCβ) entails the activation of protein kinase C and the elevation of the intracellular calcium concentration. There is mounting evidence to support the notion that calcium mobilization is the key event that initiates different mitogen-activated protein kinase cascades. Neuropeptide-dependent proliferation of SCLC cells relies on parallel activation of the $G_{q/11}$/PLCβ/Ras/extracellular signal-regulated kinase and the c-jun N-terminal kinase pathways, while selective engagement of either signalling cascade alone results in growth arrest and differentiation or apoptotic cell death. Basic experimental research has the potential to identify and validate novel therapeutic targets located at critical points of convergence of different mitogenic signal transduction pathways. In the case of SCLC, targeting the distinct components of the Ca^{2+} influx pathway as well as critical Ca^{2+}-dependent cellular effectors may be rewarding in this regard.

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Growth regulation of small cell lung carcinoma

Due to over one million cases of lung cancer diagnosed every year, lung cancer is still the leading cause of cancer death in the world (Parkin *et al.* 2001). Human lung cancers are classified into two major groups, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (N-SCLC), the latter consisting of adenocarcinoma, squamous-cell carcinoma, bronchio-alveolar carcinoma and large-cell carcinoma (Travis 2002). Adenocarcinoma and squamous-cell carcinoma are the most frequently occurring N-SCLC subtypes, and adenocarcinoma is the most common form in women and never-smokers. The predominant risk factor for SCLC is cigarette smoking with smokers facing a 20–30-fold higher incidence of developing SCLC than non-smokers. Within the context of the next 20 years, the incidence of lung cancer will rise with an increasing number of cases unrelated to smoking (MacKinnon *et al.* 1999). At present, there is no solid evidence for a genetic predisposition to SCLC. However, genetic polymorphisms in alleles at loci of carcinogen-activating and -detoxifying enzymes as well as in DNA repair genes have been found, which augment the susceptibility to lung cancer after tobacco consumption (Kiyohara *et al.* 2002, Husgafvel-Pursiainen 2004, Lee *et al.* 2005).

Approximately 20% of all lung neoplasms are histologically classified as SCLC, which is characterized by rapid growth and a high metastatic potential. Cytotoxic chemotherapy for SCLC appears to have reached its maximal efficacy. Despite initial radio- and chemosensitivity, patients diagnosed with SCLC display a 5-year survival rate of $<5\%$ (Clark & Ihde 1998, Laskin *et al.* 2003). Contributing to this devastating outcome is the fact that 70% of the patients are found to have metastases at the time of diagnosis, rendering this disease one of the most difficult to cure.

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As SCLC tumours are seldom resected, fresh tumour tissue is difficult to obtain and continuous cell lines are usually utilized for laboratory studies.

Key events in the pathogenesis of SCLC are mutations in tumour suppressor genes and chromosomal alterations like loss of heterozygosity (LOH) of the short arm of chromosome 3 (3p[14–25]) in 100% of the cases examined (Lindblad-Toh *et al.* 2000). Among the known tumour suppressor genes regularly inactivated in SCLC are *p53* (approximately. 90%) and *Rb* (approximately 90%) (Wistuba *et al.* 2001). Oncogenes such as *myc* and *bcl2* are frequently amplified in SCLC. In addition, epigenetic changes like changes in methylation patterns are frequently observed (Minna *et al.* 2002).

A major advance in our understanding of the ontogeny and molecular pathogenesis of SCLC was achieved by the development of the first mouse model for this sort of lung cancer. Conditional inactivation of both *Trp53* and *Rb1* was necessary and sufficient to induce SCLC-like tumours in mice, while *p53* inactivation alone gave rise to an adenocarcinoma phenotype (Meuwissen *et al.* 2003).

SCLC cells display many neuroendocrine characteristics and express markers of neuroendocrine cells like neural cell adhesion molecule (Ncam1), neuron-specific enzymes (e.g. L-DOPA decarboxylase, neuron-specific enolase), proteins involved in vesicle fusion like synaptophysin as well as a whole set of neuropeptide hormones like bombesin/gastrin-releasing peptide (GRP), galanin, bradykinin or arginine–vasopressin, and their cognate receptors. Furthermore, SCLC cells express a variety of voltage-gated Ca^{2+} channels (VGCCs). Since multiple neuroendocrine markers are expressed by 80–100% of SCLC tumours and cell lines, compared with 0–20% of N-SCLC tumours and cell lines, the hypothesis has been put forward that SCLC arises from neoplastic transformation of neurocrest-derived neuroendocrine cells in the bronchial mucosa (Williams 1997). Pulmonary neuroendocrine cells are a minor cell population of the airway epithelium and may function as oxygen sensors (Ito *et al.* 2000). An alternative hypothesis states that SCLC and N-SCLC are derived from the same pluripotent endodermal cell line that gives rise to all types of bronchial epithelial cells and that the neuroendocrine features of SCLC cells are a consequence of discrete transforming mutations (Williams 1997).

Human ASH1, a basic helix–loop–helix transcription factor that determines the onset and maintenance of neuroendocrine differentiation, is another prominent marker for human SCLC and can be detected in early hyperplastic lesions as well as in neuroendocrine tumours of the murine SCLC model (Meuwissen *et al.* 2003), supporting the hypothesis that SCLC arises from neoplastic transformation of neuroendocrine cells in the pulmonary epithelium. Moreover, human SCLC cells express ASH1 and its expression level correlates with neuroendocrine markers such as GRP, L-DOPA decarboxylase activity and calcitonin (Pedersen *et al.* 2003, Jiang *et al.* 2004). The *Drosophila* orthologues of ASH1 (Achaete and Scute) and the growth factor independence-1 (GFI1) oncoprotein (senseless) have been shown to interact genetically to inhibit Notch signalling. Thus, it did not come as a surprise that GFI1 as well as ASH1 are expressed in neuroendocrine lung cancer cell lines (Kazanjian *et al.* 2004). GFI1 expression was shown to closely correlate with ASH1 and several neuroendocrine markers indicating a functioning, highly conserved proneural developmental pathway that appears to be active in human lung cancer. The additional observation that the hedgehog signalling pathway, usually operative in embryonic lung development to mediate epithelial–mesenchymal interaction, induces human ASH1 in regenerating adult airway epithelium and in SCLC cells, lends further credence to a direct neuroendocrine lineage of SCLC (Watkins *et al.* 2003).

Ras genes are the most frequently mutated oncogenes detected in human tumours. Aberrantly activated *Ras* has been implicated in virtually all aspects of a malignant phenotype including proliferation, invasion or metastasis (Campbell & Der 2004). In humans, *K-Ras* mutations are found in nearly all pancreatic adenocarcinomas and are common in lung and colorectal cancer (Bos *et al.* 1987, Almoguera *et al.* 1988, Rodenhuis *et al.* 1988). The relevance of *Ras* in tumour formation has been confirmed in mouse tumour models that mimic somatically obtained oncogenic *Ras* mutations. Oncogenic *K-Ras* predisposes transgenic mice to a variety of tumours, specifically lung tumours of the adenocarcinoma type (Jackson *et al.* 2001, Johnson *et al.* 2001). In contrast to N-SCLC, transforming Ras mutations, mutations in heptahelical receptors or overexpression of receptor tyrosine kinases (RTKs) are usually not encountered in SCLC. Instead, expression of GTPase-deficient Ras mutants induces cell differentiation and growth arrest (Mabry *et al.* 1988, 1989, Rozengurt 1999). Furthermore, overexpression of constitutively active Raf-1 leads to apoptosis in SCLC cells (Ravi *et al.* 1998). However, it should be noted that juxtamembrane gain-of-function mutations have recently been described in the RTK c-MET, which might be associated with a more aggressive phenotype of SCLC (Ma *et al.* 2003).

In seminal experiments using monoclonal antibodies against the GRP receptor or broadspectrum neuropeptide receptor antagonists, the proliferation of SCLC cells was efficiently attenuated *in vitro* as well as in xenografts of human SCLC (Sethi *et al.* 1992, Halmos & Schally 1997, Seckl *et al.* 1997, Maruno *et al.* 1998, MacKinnon *et al.* 1999). These findings support the notion that calcium-mobilizing neuropeptides acting via auto- and paracrine growth loops represent the main driving force for the high proliferative potential and metastasis of this tumour entity (Gudermann *et al.* 2000, Rozengurt 2002).

Neuropeptide-mediated growth of small cell lung cancer cells

Receptors for neuropeptide hormones belong to the large superfamily of heptahelical G protein-coupled receptors (GPCRs) and have been shown to couple to a portfolio of G proteins belonging to the $G_{q/11}$, $G_{i/0}$ and $G_{12/13}$ families (Fig. 1) (Wittau *et al.* 2000, Heasley 2001). Neuropeptide-initiated cascades resulting in mitogenic responses are primarily related to G_q proteins and are known to signal via the phospholipase Cβ (PLCβ) pathway, thereby activating protein kinase C (PKC) as well as increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The critical role of receptor-initiated PLCβ and subsequent ERK activation for the anchorage-independent phenotype and proliferation of SCLC cells has been proven by overexpression of a dominant-negative PLCβ construct and pharmacological MEK inhibition (Beekman *et al.* 1998). Even though PKC plays a pivotal role in neuropeptide-induced signal transduction, events downstream of PKC are still not well understood in SCLC cells. PKC, activated through neuropeptides, has been implicated in cell cycle control via the Ras/Raf/extracellular signal-regulated kinase (ERK) cascade in several cell types (Rozengurt 1998). Another target of PKC in neuropeptide-mediated signal transduction in SCLC cells is represented by protein kinase D (PKD), a serine/threonine kinase distinct from known PKC isoforms. Stimulation of PKC with phorbol ester leads to activation of PKD raising the possibility of a PKC/PKD pathway in SCLC cells (Paolucci & Rozengurt 1999). In Swiss3T3 cells, neuropeptides trigger DNA synthesis and cell proliferation via PKC-induced PKD activation (Sinnett-Smith *et al.* 2004).

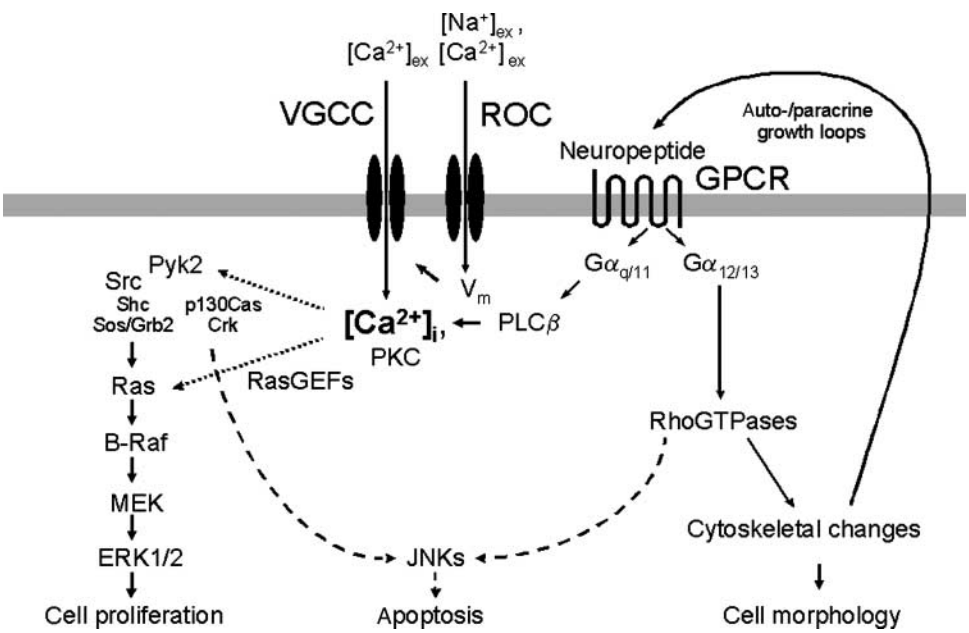


Figure 1 Model of Ca^{2+}-dependent signalling in SCLC cells. Neuropeptide receptors are stimulated by neuropeptide hormones acting in an auto- and paracrine fashion. While $G_{12/13}$ proteins mediate activation of RhoGTPases which impinge on the dynamics of the actin cytoskeleton and contribute to the recruitment of the JNK cascade, activated $G_{q/11}$ proteins engage PLCβ and finally lead to activation of PKC and elevation of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ is also raised via influx through VGCCs. In order to recruit these channels, the membrane potential (V_m) has to be depolarized by the gating of receptor-operated non-selective cation channels (ROC). Increased $[Ca^{2+}]_i$ activates the tyrosine kinase Pyk2 resulting in engagement of the Ras/ERK/MAPK cascade following Src phosphorylation and recruitment of adaptor proteins like Shc and Grb2. Pyk2 activation may also signal to JNKs via p130Cas/Crk. At present it is not known how a common Ca^{2+} signal impinges on either of these two pathways. Furthermore, Ca^{2+} can activate the Ras/ERK cascade via RasGEFs, RasGRF and RasGRP.

In neuroendocrine PC12 cells, Ca^{2+} transients resulting from bradykinin stimulation are sufficient to trigger ERK activation (Zwick *et al.* 1997). It is conceptually important in this context that a neuropeptide- and PLCβ-induced increase of $[Ca^{2+}]_i$ is necessary and sufficient to mediate ERK activation in SCLC cells (Wittau *et al.* 2000). This concept is emphasized by results obtained from experiments using antagonists of neuropeptide receptors such as [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance-P (SP-D) and [Arg^6, D-Trp^{7,9}, NmePhe^8]-substance-P (6–11) (SP-G; Table 1). Substance P displays low sequence homology with GRP and is not produced by SCLC cells. However, the first molecule identified with GRP-antagonistic properties and to be able to inhibit SCLC proliferation was [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P (Orosz *et al.* 1995). Since substance P-analogues not only inhibit GRP-stimulated cell proliferation but also mitogenesis induced by other neuropeptides, they are now referred as broadspectrum neuropeptide receptor antagonists (Sethi *et al.* 1992, Nyeki *et al.* 1998). Analogues of substance P inhibit increases of $[Ca^{2+}]_i$, ERK activation and DNA synthesis after neuropeptide stimulation (Table 1). Most notably, abrogation of the Ca^{2+} signal leads to growth arrest *in vitro* and *in vivo* and to apoptotic cell death of SCLC cells (MacKinnon *et al.* 1999, 2001). Therefore, there is reason to conclude that Ca^{2+} mobilization, besides contributing to PKC activation, may regulate other targets to stimulate ERK activity resulting in mitogenic responses in SCLC cells. So far, targets of increased cellular Ca^{2+} leading to ERK activation have not been identified in SCLC cells. They are likely to include Ca^{2+}/calmodulin-dependent protein kinases, Ras guanine nucleotide exchange factors (RasGEFs) or yet to be defined new targets of Ca^{2+} signalling.

There is mounting evidence that discordant signalling of neuropeptide hormone receptors inhibits proliferation of SCLC cells and even leads to apoptotic cell death. Discordant signalling-induced apoptotic cell death of SCLC cells coincides with activation of another mitogen-activated protein kinase (MAPK) cascade, the c-jun N-terminal kinase (JNK) cascade. Overexpression of GTPase-deficient $G\alpha_{16}$ revealed that constitutive activation of the G_q signalling

Table 1 Substance P analogues as inhibitors of SCLC cell proliferation

Name	Sequence	Biological characteristic
Bombesin	Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	Cell proliferation
Gastrin-releasing peptide	H-Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	Cell proliferation
Substance P	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	n.s. by SCLC cells
SP-D	[D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P	Blockage of bombesin binding, inhibition of bombesin-induced Ca^{2+} mobilization, inhibition of DNA synthesis, inhibition of mitogenesis
	[D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P	Blockage of bombesin binding, inhibition of bombesin-induced Ca^{2+} mobilization, inhibition of DNA synthesis, inhibition of mitogenesis
	[D-Arg^1, D-Trp^{5,7,9}, Leu^{11}]-substance P	Inhibition of neuropeptide-induced Ca^{2+} mobilization, inhibition of neuropeptide-induced MAPK activation, inhibition of cell proliferation
SP-G	[Arg^6, D-Trp ^{7,9}, NmePhe^8]-substance-P (6–11)	Inhibition of bombesin-induced Ca^{2+} mobilization, inhibition of cell proliferation
	pHOPA-D-Trp-Phe-D-Trp-Leu-Leu-NH_2	Blockage of bombesin binding, inhibition of cell proliferation
	pHOPA-D-Trp-Phe-D-Trp-Leu-$\Psi(CH_2NH)$-Leu-NH_2	Loss of affinity for bombesin receptor, inhibition of cell proliferation
	D-MePhe-D-Trp-Phe-D-Trp-Leu-$\Psi(CH_2NH)$-Leu-NH_2	Loss of affinity for bombesin receptor, inhibition of cell proliferation
	D-MePhe-DTrp-Phe-D-Trp-Leu-MPA	Loss of affinity for bombesin receptor, inhibition of cell proliferation
	D-Tyr-D-Trp-Phe-D-Trp-Leu-$\Psi(CH_2NH)$-Leu-NH_2	Blockage of bombesin binding
	D-Tyr(Et)-D-Trp-Phe-D-Trp-Leu-$\Psi(CH_2NH)$-Leu-NH_2	Loss of affinity for bombesin receptor, inhibition of cell proliferation

n.s., not synthezised; pHOPA, 4-hydroxyphenyl-acetyl; MPA, 2-amino-3-methylpentane; SP, substance P.

pathway in SCLC perturbs receptor-regulated mitogenic signalling and proliferation by inhibiting neuropeptide-stimulated Ca^{2+}-mobilization and by enhancing basal JNK activity (Heasley *et al.* 1996). A possible explanation for the fact that constitutively active G_q proteins lead to growth arrest of SCLC cells relates to the previously made observation that chronic activation of PLCβ entails desensitization and downregulation of IP_3 receptors in the endoplasmic reticulum and induces polyphosphatidylinositol phosphatase activity (Wojcikiewicz *et al.* 1994, Sipma *et al.* 1998). Furthermore, overexpression of a dominant-negative PLCβ construct leads to inhibition of clonal growth whereas at the same time, stimulation of JNK was not disturbed in SCLC cells (Beekman *et al.* 1998). This finding indicates that other G protein signalling pathways, probably via $G_{12/13}$ proteins, mediate neuropeptide-induced JNK activation.

So far, our knowledge of neuropeptide-mediated $G_{12/13}$ signalling in SCLC cells is rather limited. Neuropeptide-mediated GTP-loading of $G_{12/13}$ proteins entails the activation of the Rho family of small GTPases which impinge on the dynamics of the actin cytoskeleton and contribute to the recruitment of the JNK cascade (Beekman *et al.* 1998, Wittau *et al.* 2000). Activation of $G_{12/13}$ proteins has been shown to be important for cell morphology of SCLC cells but does not have any impact on cell proliferation (Tokman *et al.* 1997, Varker *et al.* 2003). Numerous studies indicate that Rho family members play a significant role in metastasis by controlling the organization of the cytoskeleton (Jaffe & Hall 2002). Compared with N-SCLC cells, which differ in their metastatic potential and cell morphologies from SCLC cells, the expression level of RhoA is higher in SCLC cells (Varker *et al.* 2003). Inactivation of RhoA by ADP-ribosylation after overexpression of the C3 exoenzyme of *C. botulinum* leads to cadherin-mediated compaction and aggregation of SCLC cells as well as adhesion and spreading on collagen IV. Furthermore, inactivation of Rho proteins by expression of C3 exoenzyme does not alter cell proliferation of SCLC cells, whereas proliferation of N-SCLC cells was decreased (Tokman *et al.* 1997, Varker *et al.* 2003). SCLC cells exist in a rich extracellular matrix (ECM) environment with β1 integrin, representing the predominant integrin mediating adhesion to the ECM. The surrounding ECM appears to be responsible for the high resistance of SCLC cells to chemotherapeutic agents since co-incubation with function-blocking anti-β1 integrin antibodies rendered SCLC cells sensitive to chemotherapy-induced apoptosis. β1 integrin stimulates tyrosine kinase activity leading to diminished caspase activation to protect SCLC cells from apoptosis after cytostatic treatment (Sethi *et al.* 1999). Recently, it has been shown that CXCR4 activation by CXCL12 mediates β1 integrin-dependent adhesion to ECM components and chemoresistance through a pathway involving tyrosine phosphorylation of components of focal adhesion complexes as well as RhoGTPases in SCLC cells (Hartmann *et al.* 2005). It has been proposed that β1 integrin-induced activation of RhoGTPases in epithelial cells regulates their polarity and motility involving downregulation of α-catenin function, and that overexpression of dominant-negative Rho constructs reverses cell scattering and partially restores cadherin-based adhesion (Gimond *et al.* 1999). Therefore, the interplay between RhoGTPases and β1 intregin-mediated signal transduction is worth considering in SCLC cells.

The connection between signalling pathways emanating from $G_{12/13}$ proteins leading to JNK activation and apoptotic cell death remains to be delineated. Originally, substance P analogues were thought to behave as broadspectrum neuropeptide antagonists. However, recent studies suggest that they also display agonistic properties, a phenomenon known as biased agonism, allowing the bombesin receptor to couple to G_i and G_{12} proteins leading to ERK and JNK activation, while the G_q-mediated increase of $[Ca^{2+}]_i$ is inhibited at the same time (MacKinnon *et al.* 2001). These findings suggest a G_{12}-mediated activation of the JNK cascade after neuropeptide stimulation and are in agreement with the finding that overexpression of dominant-negative G_{12} results in diminished JNK activation in SCLC cells (MacKinnon *et al.* 2001).

The neuropeptide-controlled growth of SCLC cells becomes even more complicated by the ability of neuropeptide receptors to couple to G_i proteins (Wittau *et al.* 2000). In general, activation through G_i proteins regulates cellular effects through both α and βγ subunits. Intracellular effectors of $G_{\beta\gamma}$-dimers represent adenylyl cyclase isoforms, G protein-coupled inwardly rectifying potassium channels, PLC isoforms and phosphatidylinositol 3-kinase γ (PI3Kγ) (Marinissen & Gutkind 2001, Schwindinger & Robishaw 2001). Signalling molecules implicated in linking mitogenic $G_{\beta\gamma}$ signal transduction to the ERK cascade are scaffolding proteins for small GTPases proteins galanin, bradykinin or such as β-arrestin or KSR-1, Src tyrosine kinases, protein tyrosine phosphatase PTP1C and RasGRF1, a Ras guanine nucleotide exchange factor also known as Cdc25Mm (Gudermann 2001, Lefkowitz & Shenoy 2005). For the Cos-7 cell model it has been postulated that the tyrosine kinase Src via adaptor protein β-arrestin is responsible for GPCR-mediated

Shc phosphorylation, rendering the adaptor protein a point of entry into the Ras/ERK pathway (Luttrell *et al.* 1996). However, Ras activation and subsequent engagement of the ERK/MAPK can be mediated by the $G\alpha_i$ subunit independently of $\beta\gamma$ subunits and Ras activation (Hedin *et al.* 1999). The contribution of G_i coupling to neuropeptide-mediated signalling events has not been fully delineated (Wittau *et al.* 2000). In SCLC cells, neuropeptide-mediated ERK activation proceeds via a pertussis toxin-insensitive pathway. On the contrary, treatment of SCLC cells with substance P analogues results in pertussis toxin-sensitive stimulation of ERK activity (MacKinnon *et al.* 2001). It has been hypothesized that binding of the natural agonist bombesin to its cognate GPCR does not lead to productive engagement of all possible G protein/receptor interactions, because the GRP receptor may desensitize too rapidly upon exposure to bombesin. Therefore, the active conformation to stimulate G_i proteins does not have a long enough lifespan, although binding of biased agonists may principally result in an interaction of the receptor with G_i proteins (Tsuda *et al.* 1997, MacKinnon *et al.* 2001).

SCLC cells exhibit high basal constitutive PI3K activity resulting in high basal protein kinase B (PKB)/Akt and ribosomal p70 S6 kinase (p70S6K) activities (Moore *et al.* 1998). Tyrosine phosphorylation of the regulatory p85 subunit of PI3K was reported to regulate PI3K activity by several stimuli such as bradykinin or tyrosine kinases, e.g. platelet-derived growth factor receptor (PDGFR) and Src kinases (Kavanaugh *et al.* 1994, Thakker *et al.* 1999, Xie *et al.* 2000). In monocytic cells and blood-derived macrophages, the p85 subunit forms a complex with activated Pyk2, a non-receptor tyrosine kinase, which contains a putative binding site for the p85 subunit of PI3K (Lev *et al.* 1995, Hatch *et al.* 1998, Chen *et al.* 2004). Furthermore, Pyk2 was shown to mediate bradykinin-induced NF-κB activation via PI3K in HeLa cells (Xie *et al.* 2000). PI3K signalling is essentially involved in the survival, proliferation and chemoresistance of SCLC cells. It has been shown that neuropeptides like galanin or bombesin strongly activate PI3K and the downstream located PKB/p70S6K pathway, and that this pathway is important to maintain the anchorage-independent phenotype of SCLC cells (Moore *et al.* 1998). Blocking PI3K activity by pharmacological approaches markedly inhibited SCLC proliferation and rendered SCLC cells sensitive to chemotherapy-induced apoptosis (Krystal *et al.* 2002, Chen *et al.* 2004). Survivin, an inhibitor of apoptosis, is regulated by Akt in SCLC cells. DNA damage after treatment with cisplatin activates the PI3K/Akt/survivin pathway resulting in protection from apoptosis by this anti-cancer agent (Belyanskaya *et al.* 2005). As mentioned before, the surrounding ECM is an important factor for the increased risk of SCLC cells to develop resistance to chemotherapeutic agents. Recently, it has been shown that adhesion to laminin results in activation of the PI3K/Akt/mTOR and MEK/ERK pathways leading to increased cellular survival. Inhibitors of the PI3K/Akt pathway but not the MEK/ERK pathway abrogated this survival benefit. Furthermore, inhibition of the PI3K/Akt/mTOR pathway increased the responsiveness of SCLC cells to treatment with standard therapeutics etoposid and cisplatin and to imatinib, a small molecule inhibitor of c-kit, PDGFR and bcr-Abl (Tsurutani *et al.* 2005).

Ca^{2+}-permeable ion channels and cell proliferation

Rises in $[Ca^{2+}]_i$ are realized by releasing Ca^{2+} from internal stores or by influx of extracellular Ca^{2+} through Ca^{2+} channels (Berridge *et al.* 2000). Intracellular Ca^{2+}-binding proteins, such as calmodulin, act as calcium sensors decoding the information according to distinct increases in $[Ca^{2+}]_i$ (Agell *et al.* 2002). It has become clear that Ca^{2+} signalling is involved in a variety of cellular processes such as cell fertilization, development, proliferation and apoptosis. These diverse cellular processes are controlled by the enormous range of timing as well as spatial and temporal signals that Ca^{2+} can create within the cell. Alteration of the spatial and temporal balances in $[Ca^{2+}]_i$ by extracellular stimuli or intracellular Ca^{2+} effectors can result in cell death (Berridge *et al.* 2000). As mentioned above, PLCβ-initiated rises of $[Ca^{2+}]_i$ are crucial for mitogenic responses in SCLC cells (Wittau *et al.* 2000). Due to their neuroendocrine origin, SCLC cells express dihydropyridine-sensitive L-type as well as dihydropyridine-insensitive P/Q- and N-type VGCCs.

Approximately, 2–3% of all SCLC patients present with Lambert-Eaton myasthenic syndrome (LEMS) caused by autoantibodies against P/Q-type VGCCs leading to generalized deficit in neurotransmitter release (Elrington *et al.* 1991, Sutton & Winer 2002). Autoantibodies that interfere with neurotransmitter release by binding to presynaptic VGCCs have been found in patients with SCLC and autoantibodies from patients affected by LEMS can precipitate these channels. The same antibodies also block influx through VGCCs in SCLC and thereby may inhibit tumour growth. In fact, search of SCLC in patients having

LEMS might lead to early detection of limited-stage SCLC and SCLC patients presenting with LEMS had a significant longer median survival time from the diagnosis of SCLC when compared with SCLC-only patients (Maddison *et al.* 1999, Wang *et al.* 2006).

Apart from VGCCs, SCLC cells also express ligand-gated cation channels (nicotinic acetylcholine receptors) and receptor-operated as well as Ca^{2+}-activated cation channels. In contrast, to the situation in neurons, VGCCs in SCLC cells are not activated by action potentials, but by depolarizing cation currents carried by receptor-operated ion channels (ROCs; Figs 1 and 2). ROCs are gated in response to agonist binding to a membrane receptor distinct from the channel protein itself. Cation influx carried by these type of channels is required to depolarize the cell, thereby recruiting VGCCs which mediate Ca^{2+} influx, neuropeptide secretion and ensuing proliferation of SCLC cells (Gudermann *et al.* 2004, Plummer *et al.* 2005). Consequently, blockade of non-selective cation channels by various imidazole compounds like carboxyamidotriazole inhibits the growth of SCLC cells (Moody *et al.* 2003). Members of the transient receptor potential (TRP) family of cation channels are probable molecular substrates for receptor-operated cation entry and have been implicated in the control of normal and abnormal cell proliferation (Clapham 2003, Montell 2005). TRP proteins constitute a family of nearly 30 members that can be divided into seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMPC) (Pedersen *et al.* 2005). In prostate cancer epithelial cells, diacylglycerol (DAG)-gated Ca^{2+}-permeable cation channels resembling the members of the TRPC3/6/7 family were recently identified (Hofmann *et al.* 1999, Sydorenko *et al.* 2003). In accord with these findings, the proliferation of these cancer cells could be inhibited by blocking α_1-adrenoceptor-activated DAG-sensitive cation channels (TRPC3/6/7 subfamily) (Thebault *et al.* 2003). TRPV6 was characterized as a novel marker for prostate cancer staging and progression (Wissenbach *et al.* 2001, Bodding *et al.* 2003, Fixemer *et al.* 2003). The founding member of the TRPM family, TRPM1 (melastatin) is a putative cation channel, whose expression inversely correlates with melanoma tumour progression and metastasis (Duncan *et al.* 1998). The latest TRPM family member, TRPM8, was initially detected as a tumour marker for prostate cancer with strong predictive power for prostate cancer relapse (Tsavaler *et al.* 2001, Zhang & Barritt 2006). TRPM2 was characterized as an apoptosis-mediating Ca^{2+}-permeable channel activated by oxidative stress, NAD^+ or ADP ribose, while the constitutively active

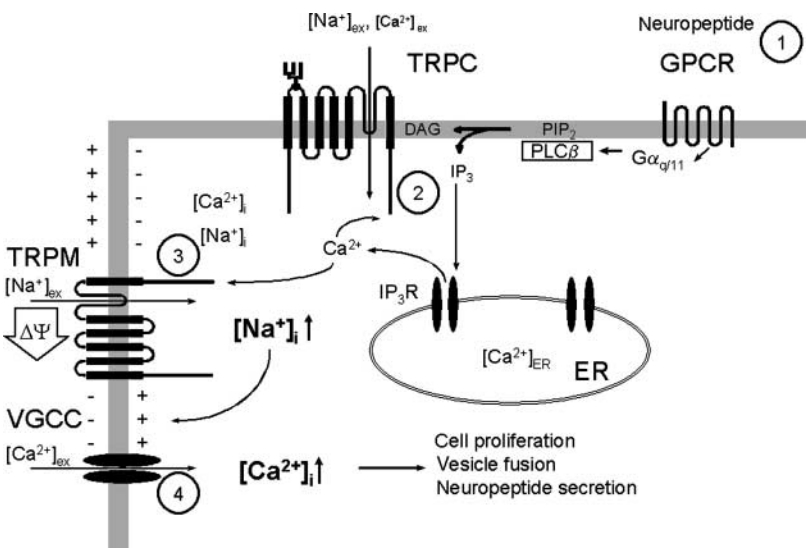


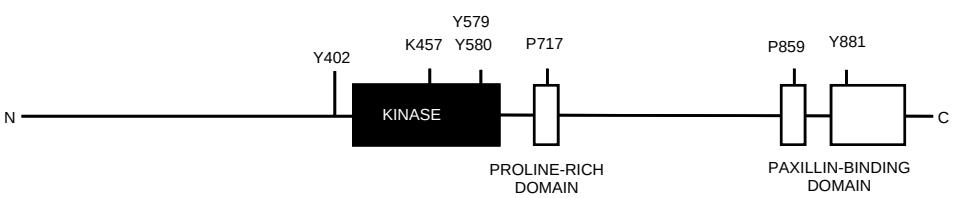
Figure 2 Model of Ca^{2+} entry in SCLC cells. Apart from voltage-gated Ca^{2+}-channels (VGCCs), SCLC cells also express receptor-operated (ROC) cation channels. Receptor-operated cation channels are gated in response to agonists binding to a cell membrane receptor distinct from the channel protein itself. Members of the transient receptor potential (TRP) family of cation channels, which are gated in response to PLC activation, are probable candidates for receptor-operated cation entry. Binding of neuropeptides to their cognate GPCR (1) leads to IP_3-mediated release of Ca^{2+} from intracellular stores (e.g. the endoplasmic reticulum (ER)) and DAG-induced cation entry through TRPC channels (2). Ca^{2+} released from internal stores might further activate TRPM channels (3) raising the intracellular cation concentration. Cation influx is required to depolarize the cell thereby leading to VGCC-mediated Ca^{2+}-influx (4). Increases of $[Ca^{2+}]_i$ are necessary for cell proliferation, vesicle fusion and neuropeptide secretion of SCLC cells.

TRPM7 is regulated by cytosolic MgATP levels and turned out to be indispensable for cell viability (Runnels *et al.* 2001, Hara *et al.* 2002, Clapham 2003). Finally, TRPM5 is mapped to a chromosomal region (11p[15.5]) associated with LOH in a variety of childhood and adult tumours, and with the cancer-predisposing Beckwith–Wiedemann syndrome (Prawitt *et al.* 2000). TRPM5 has been functionally characterized as a Ca^{2+}-activated monovalent selective cation channel and appears to be expressed in sensory cells of the respiratory epithelium (Hofmann *et al.* 2003).

Ca^{2+}-dependent Ras signalling

Considering that a neuropeptide- and PLCβ-induced rise of $[Ca^{2+}]_i$ is necessary and sufficient to mediate neuropeptide-dependent ERK activation in SCLC cells (Wittau *et al.* 2000), it is worth considering the possible interplay between Ca^{2+} and Ras signalling in SCLC cells. Ras and Ca^{2+} signalling are frequently discussed as separate entities, although Ca^{2+} has been known to influence cell proliferation and differentiation for a long time. Nearly 10 years ago, Ca^{2+} influx through VGCCs or release from internal stores was shown to activate Ras in the neuroendocrine PC12 cell model (Rosen *et al.* 1994).

One Ca^{2+}-dependent signalling pathway to Ras activation involves the cytosolic tyrosine kinase Pyk2, which can be independently activated by elevations of $[Ca^{2+}]_i$ and PKC (Fig. 3.) (Lev *et al.* 1995, Avraham *et al.* 2000). Pyk2 is closely related to focal adhesion kinase (FAK) and highly expressed in the central nervous system, cells derived from hematopoietic lineages, renal and liver epithelial as well as vascular smooth muscle cells (VSMCs). In PC12 cells, Pyk2 appears to link the activation of various GPCRs to the ERK/MAPK cascade. Mechanistically, Ca^{2+}-induced activation of Pyk2 is not understood at present. The Ca^{2+} effect may be achieved indirectly through Pyk2 phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II as has been shown in VSMCs (Ginnan & Singer 2002). Yet, the source of Ca^{2+} leading to Pyk2 activation is unknown. Observations in VSMCs underscore a crucial role of voltage-independent, receptor-operated Ca^{2+}-permeable channels for agonist-induced Pyk2 tyrosine phosphorylation (Kawanabe *et al.* 2003). In contrast to FAK, Pyk2 resides in the cytosol and is recruited to focal adhesions in the plasma membrane in response to increases in $[Ca^{2+}]_i$ (Schlaepfer *et al.* 1999). In fibroblasts, a perinuclear localization of Pyk2 has been noted and experimental evidence in favour of Pyk2 shuttling between the cytoplasm and the nucleus has been presented (Aoto *et al.* 2002). Once activated, the kinase activity of Pyk2 chiefly results in autophosphorylation of Y402 and formation of a Pyk2/Src complex at this site (Dikic *et al.* 1996). Activated Src kinases then transphosphorylate Y579 and Y580 in the kinase domain activation loop and Y881, a direct binding site of Grb2/Sos. Src bound to Pyk2 may further directly phosphorylate adjacent



Sequence motifs of SH2 and SH3 binding sites, kinase domain and paxillin-binding domain

Pyk2 aa residue	Sequence motif	Function
Y402	YAEI	Src family tyrosine kinases SH2 binding
Y457	AVKTCK	kinase region
Y579, Y580	EDYYKAS	regulatory/kinase activation loop
P717	PPPKPSRPK	p130Cas SH3 binding site/ASAP1
P859	PPQKPPRLG	p130Cas/Graf/PSGAP SH3 binding sites/ASAP1
P881	YLNV	Grb2 SH2 binding site
paxillin-binding domain	875-894, 985-999	paxillin, Hic-5, leupaxin

Figure 3 Protein domains and docking sites of Pyk2. The schematic drawing shows the central kinase domain, phosphorylation sites and proline-rich regions as well as the paxillin-binding domain of Pyk2. The table below lists sequence motifs of SH2 and SH3 binding sites, kinase domain and paxillin-binding site. aa, amino acid.

cellular proteins such as Shc and the constitutively bound adaptor protein p130Cas. Ras activation and subsequent engagement of the ERK cascade can be initiated via Grb2/Sos binding to Y881 or indirectly via Shc–Grb2/Sos interaction at Y402 (Schlaepfer *et al.* 1999). Semaphorin 4D/plexin-B1 stimulated endothelial cell migration requires the sequential activation of PI3K and Pyk2/Src complex formation resulting in Akt activation (Basile *et al.* 2005). Pyk2-K457A has been described as kinase-dead Pyk2 and shown to be impaired in activating Src and associating with the Grb2/Sos complex (Lev *et al.* 1995).

One of the main functions (possibly tyrosine kinase-independent) of Pyk2 is to serve as a precisely localized adaptor platform for the assembly of signalling complexes. Potential SH2 and SH3 as well as proline-rich regions mediate these interactions. Hence, a p130Cas/Crk protein complex formed at the first of two proline-rich domains specifically links Pyk2 with the activation of JNK (Blaukat *et al.* 1999). So far, how the cell manages to transmit a Pyk2-dependent signal either through the ERK or the JNK cascade by means of a common initial Ca^{2+} stimulus is utterly unknown. The situation is complicated even further because Pyk2 may establish a feedback loop to cation channels at the plasma membrane by regulating the activity of voltage-gated K^+ channels through direct phosphorylation (Lev *et al.* 1995). Pyk2 signalling has been revealed as part of the Rho pathway and RhoGTPase-activating proteins (RhoGAPs) Graf and PSGAP have been suggested as mediators (Wu *et al.* 2002). Graf mediates the cross-talk between FAK and Rho family members, and it has been shown that Pyk2 also interacts with Graf. However, a function for Graf in Pyk2 signalling has not been described so far (Hildebrand *et al.* 1996, Xiong *et al.* 1998). It was suggested that PSGAP may be an essential protein for regulation of cytoskeletal organization by Pyk2 via RhoGTPases (Ren *et al.* 2001). Furthermore, Pyk2 binds to an ArfGTPase-activating protein ASAP1, thereby regulating Arf1 activity by phosphorylation building another bridge to reorganization of the actin cytoskeleton (Kruljac-Letunic *et al.* 2003). Pyk2 contains a consensus paxillin binding sequence within its C-terminus and it has been shown that Pyk2 can associate with paxillin or paxillin-related proteins Hic-5 or leupaxin (Schlaepfer *et al.* 1999). Translocation of Pyk2 to focal adhesions was attributed to enhance tyrosine phosphorylation of Pyk2, and its association with the focal adhesion proteins paxillin and p130Cas (Litvak *et al.* 2000). In rat aortic smooth muscle cells angiotensin II, stimulation leads to complex formation of Pyk2 with MAPK/ERK kinase kinase (MEKK4) resulting in tyrosine phosphorylation of MEKK4. Complex formation is sensitive to calcium and facilitated by annexin II (Derbyshire *et al.* 2005). Last but not the least, Pyk2 forms a complex with Nir proteins related to *Drosophila* retinal degeneration protein (Lev *et al.* 1999). A negative regulator of Pyk2 FIP200 binds to its kinase domain and leads to apoptosis of Rat-1 cells (Ueda *et al.* 2000). Interestingly, overexpression of Pyk2, but not FAK, in fibroblast cell lines or cardiomyocytes leads to apoptosis, while PC12 cells that express significant levels of endogenous Pyk2 are not affected (Xiong & Parsons 1997, Melendez *et al.* 2004). However, the cellular mechanism underlying Pyk2-initiated apoptotic cell death as well as the reasons for the discrepant behaviour of different cell lines are still unknown.

A more obvious connection between Ca^{2+} and Ras signalling is provided by two families of Ca^{2+}-dependent RasGEFs, Ras guanine nucleotide exchange factors (RasGRFs) and Ras guanine nucleotide exchange proteins (RasGRPs) which can be differentiated by their cellular control mechanisms (Table 2) (Cullen & Lockyer 2002). Members of the RasGRF family are highly expressed in neurons. RasGRF proteins are IQ motif-containing GEFs with their activity being enhanced following binding of Ca^{2+}-bound calmodulin. RasGRF1 is activated indirectly through calmodulin binding to its IQ motif, whereas Ca^{2+} might exert an additional stimulatory effect on a closely related exchange factor RasGRF2. A dominant-negative form of RasGRF1, GRFβ, is expressed in pancreatic β cells and seems to antagonize Ca^{2+}-dependent signalling of RasGRF1. RasGRP/CalDAG-GEF proteins contain a calcium binding EF hand and a DAG binding site, and are activated following direct binding of Ca^{2+} and DAG. CalDAG-GEF I prefers Rap1a to N-Ras and a splice variant of the same exchange factor, RasGRP2, is inhibited rather than stimulated by Ca^{2+} when functioning as a RasGEF. The production of DAG is the key factor of the spatio-temporal regulation of RasGRP3 and RaGRP4, whereas elevation of $[Ca^{2+}]_i$ does not affect the exchange activities of these GEFs (Farnsworth *et al.* 1995, Fam *et al.* 1997, Ebinu *et al.* 1998, Cullen & Lockyer 2002). The net effect of Ca^{2+} on Ras signalling is not only determined by GEF activity, but also by Ca^{2+}-dependent deactivation through GTPase-activating proteins (GAPs) (Cook & Lockyer 2006). RasGAPs share four conserved structural modules with the tandem C2 domain being responsible for Ca^{2+} binding. p120GAP is widely expressed and was the first RasGAP identified (Bernards 2003). The data concerning Ca^{2+}-dependent activation of p120GAP are controversially discussed. It has been

Table 2 Ca^{2+}-regulated Ras exchange factors and GTPase-activating proteins

Name	Modulator	Effectors/effects	Tissue expression
RasGRF1	Ca^{2+}/CaM	H-Ras, R-Ras, Rac1 ERK/MAPK activation	Neurons, pancreatic β cells
RasGRF2	Ca^{2+}/CaM	H-Ras, Rac1 ERK/MAPK activation	Ubiquitous
Mouse GRFβ		Dominant-negative regulator of RasGRF1 in βcells	Pancreatic β cells
RasGRP/CalDAG-GEF II	Ca^{2+}↑, DAG↑↑	H-Ras, R-Ras	Forebrain
CalDAG-GEF I	Ca^{2+}↑, DAG ↑	Rap1a	Forebrain
RasGRP2 (splice variant of CalDAG-GEF I)	Ca^{2+}↑, DAG ↑	N-Ras, K-Ras	Brain
RasGRP3/CalDAG-GEF III	DAG ↑	H-Ras, R-Ras, TC21, M-Ras, Rap1a, Rap2a ERK/MAPK activation	Glial cells of the brain, kidney
RasGRP4	DAG ↑	H-Ras	Blood leukocytes, myeloid cell lines
p120GAP	Ca^{2+}↑	H-Ras, R-Ras, Rab5	Ubiquitous
CAPRI	Ca^{2+}↑	H-Ras	n.d.
RASAL	Ca^{2+}↑	n.d.	n.d.
SynGAP	CaMK II ↑	n.d.	Neurons

n.d., not determined; CaM, calmodulin; CaMK II, Ca^{2+}calmodulin – dependent kinase II.

shown that recruitment of p120GAP to the plasma membrane is Ca^{2+}-dependent and proceeds via binding of Ca^{2+} to the C2 domain. However, the p120GAP C2 domain does not contain any aspartate residues responsible for interaction with Ca^{2+} in other Ca^{2+}-binding C2 domains. It was proposed that p120GAP is indirectly regulated by Ca^{2+} via association with the Ca^{2+} sensor annexin VI (Cullen & Lockyer 2002). Another indirectly Ca^{2+}-modulated RasGAP is p135 synGAP, a neuron-specific GAP which is regulated by CaMK II (Oh *et al.* 2004). SynGAP associates with PSD-95 and SAP102 and is involved in the regulation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptor-mediated activation of Ras signalling at excitatory synapses (Kim *et al.* 1998). The two known directly Ca^{2+}-triggered RasGAPs are calcium-promoted Ras inactivator (CAPRI) and a related protein, Ras GTPase-activating-like (RASAL). Upon agonist-stimulated rises of $[Ca^{2+}]_i$, CAPRI and RASAL translocate to the plasma membrane where their GAP activity is initiated (Lockyer *et al.* 2001, Walker *et al.* 2003). Thus, in a given cell Ca^{2+} can directly shape the temporal Ras signalling pattern through the relative abundance of Ca^{2+}-activated RasGEFs and RasGAPs. However, different Ras proteins and Ca^{2+}-dependent RasGEFs and GAPs are not equally distributed between the plasma membrane and endomembranes like the Golgi apparatus and the endoplasmic reticulum (Hancock 2003). For instance, Ras signalling at the plasma membrane can be suppressed by CAPRI, while a preferential translocation of RasGRP1 to the Golgi sustains Ras activation at this location (Bivona *et al.* 2003). Furthermore, due to differential Ca^{2+} sensitivities, Ras signalling may occur mainly from the Golgi in cells displaying prolonged increases in $[Ca^{2+}]_i$, whereas transient Ca^{2+} rises predispose Ras signalling at the plasma membrane. To summarize, Ca^{2+} can determine the spatio-temporal pattern of Ras signalling in a given cell, thereby directing a cell's fate towards proliferation or differentiation.

While the impact of Ca^{2+} on cell proliferation and Ras signalling has been fairly established in various cell models, the biological relevance of a given signalling pathway for the growth regulation of SCLC cells still needs to be demonstrated definitively, thus opening rewarding avenues for future research.

Molecularly targeted therapeutic approaches for SCLC

Despite the improvement of 'conventional' cytostatic regimens, SCLC still is a fatal diagnosis and cure remains elusive for most patients. Therefore, novel molecular targets for signal transduction-based pharmacological interventions would be beneficial. Molecularly targeted therapies include the application of receptor and non-receptor tyrosine kinase inhibitors, immunological approaches as well as the use of inhibitors of autocrine and paracrine growth factor loops (Pisick *et al.* 2004, Christodoulou & Skarlos 2005). Clinical trials testing imatinib for the treatment of SCLC patients yielded disappointing results, necessitating a re-evaluation of c-kit expression and its biological role in SCLC (Soria *et al.* 2003, Dy *et al.*

2005). Similar disillusioning results were obtained with matrix metalloproteinase inhibitors (Bonomi 2002). Targeting of GPCRs expressed on SCLC cells by monoclonal antibodies or broadspectrum neuropeptide receptor antagonists attenuates the proliferation of SCLC cells *in vitro* and the growth of tumour xenotransplants in nude mice (Rozengurt 1999). In the meantime, one of these substance P derivatives, SP-G, has completed clinical phase I testing with acceptable toxicity and new dimeric antagonists are currently being developed (Chan *et al.* 2002). Most likely, simultaneous disruption of the multiple variably expressed neuropeptide growth loops will be required to efficiently inhibit SCLC cell proliferation. Basic experimental research has the potential to identify and validate novel therapeutic targets located at critical points of convergence of different mitogenic signal transduction pathways. In the case of SCLC, distinct components of the Ca^{2+} influx pathway such as cation-permeable ion channels as well as critical Ca^{2+}-dependent cellular effectors like Ca^{2+}-activated protein kinases may be promising therapeutic targets.

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