Growth of Small Cell Lung Cancer Cells: Stimulation by Multiple Neuropeptides and Inhibition by Broad Spectrum Antagonists *in Vitro* and *in Vivo*¹

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Abstract

Neuropeptides are increasingly implicated in the control of cell proliferation and their mechanisms of action are attracting intense interest. The early complex cascade of events initiated by peptides of the bombesin family including gastrin-releasing peptide is increasingly understood. The cause-effect relationships and temporal organization of these early signals and molecular events provide a paradigm for the study of other growth factors and mitogenic neuropeptides and illustrate the activation and interaction of a variety of signaling pathways. These peptides may also act as autocrine growth factors for certain small cell lung cancer cells. The results discussed here strongly suggest that the autocrine growth loop of bombesin-like peptides may be only a part of an extensive network of autocrine and paracrine interactions involving a variety of Ca²⁺-mobilizing neuropeptides in small cell lung cancer including bradykinin, cholecystokinin, galanin, neuropeitin, and vasopressin. In this context, broad spectrum antagonists that prevent the function of multiple Ca²⁺-mobilizing receptors are of special interest. These antagonists block neuropeptide mediated signals and inhibit small cell lung cancer growth *in vitro* and *in vivo*. Thus, broad spectrum neuropeptide antagonists constitute potential anticancer agents.

Introduction

Lung cancer is the commonest fatal malignancy in the developed world. SCLC³ constitutes 25% of the total and follows an aggressive clinical course, despite initial chemosensitivity (1). Identification of the factors that stimulate the proliferation of SCLC cells will be important in the design of alternative and more effective therapeutic strategies. SCLC is characterized by the presence of intracytoplasmic neurosecretory granules and by its ability to secrete many hormones and neuropeptides (2, 3) including bombesin, neurotensin, cholecystokinin, and vasopressin (2–9). Among these, only bombesin-like peptides, which include GRP, have been shown to act as autocrine growth factors for certain SCLC cell lines (9, 10). In contrast, the role of other neuropeptides in the proliferation of SCLC cells remains poorly understood. Consequently, it is important to understand in detail the receptor and signal transduction pathways that mediate the mitogenic action of bombesin and GRP as well as to elucidate the role played by other neuropeptides in SCLC growth.

Neuropeptides are increasingly recognized to act as cellular growth factors (11) and their mechanisms of action are attracting considerable attention. Many studies to identify the molecular pathways by which neuropeptide mitogens eliciting cellular growth have exploited cultured murine 3T3 cells as a model system (12, 13). These cells cease to proliferate when they deplete the medium of its growth-promoting activity and can be stimulated to reinitiate DNA synthesis and cell division by the addition of various neuropeptide growth factors in serum-free medium (12). In particular, bombesin (14), vasopressin (15), bradykinin (16), vasoactive intestinal peptide (17), endothelin (18), and vasoactive intestinal cryptor (19) can act as growth factors for cultured 3T3 cells. In what follows some fundamental features of the mechanism of action of neuropeptides as growth factors in 3T3 cells will be discussed and subsequently the evidence for multiple neuropeptide growth factor action in SCLC will be considered.

The present article is not intended as an extensive review of the rapidly expanding literature, but rather as a presentation of specific topics and ideas under investigation in our laboratories.

Early Signaling Events

The early cellular and molecular responses elicited by bombesin and structurally related peptides in 3T3 cells (Fig. 1) have been elucidated in detail (20). The cause-effect relationships and temporal organization of these early signals and molecular events provide a paradigm for the study of other growth factors and mitogenic neuropeptides and illustrate the activation and interaction of a variety of signaling pathways (21).

Bombesin/GRP binds to a single class of high affinity receptors in Swiss 3T3 cells (22, 23). The receptors are M, 75,000–85,000 glycoproteins with a M, 43,000 core (24–26). The receptor is coupled to one or more G proteins as judged by the modulation of ligand binding in either membrane preparations or receptor-solubilized preparations and of signal transduction in permeabilized cells (23, 27–29). The bombesin/GRP receptor has recently been cloned and sequenced (30, 31) and shown to be a member of the G protein-coupled receptor family. These receptors have seven predicted transmembrane domains which cluster to form a ligand-binding pocket (32, 33). Other neuropeptide mitogens with receptors of this type include angiotensin, endothelin, serotonin, substance K, and substance P (34, 35).

Binding of bombesin/GRP to its receptor initiates a cascade of intracellular signals (summarized in Fig. 1) culminating in DNA synthesis 10–15 h later (13, 21). One of the earliest events to occur after the binding of bombesin to its specific receptor is a rapid mobilization of Ca²⁺ from internal stores, which leads to a transient increase in the intracellular concentration of Ca²⁺ ([Ca²⁺]i) and subsequently to Ca²⁺ efflux and decreased Ca²⁺ content of the cells (36, 37). The mobilization of Ca²⁺ by bombesin is mediated by [Ins(1,4,5)P3], which, as a second messenger, binds to an intracellular receptor and induces the release of Ca²⁺ from internal stores. Bombesin causes a rapid increase in Ins(1,4,5)P3, which coincides with the increase in cytosolic Ca²⁺ (38). Ins(1,4,5)P3 is formed as a result of PLC-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate in the plasma membrane, a process that also generates 1,2-diacylglycerol. Diacylglycerol can also be generated from other sources, such as phosphatidyleholine hydrolysis (39), and acts as a second messenger in the activation of PKC by bombesin.

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³ The abbreviations used are: SCLC, small cell lung cancer; GRP, gastrin-releasing peptide; [Ins(1,4,5)P3], inositol 1,4,5-trisphosphate; PLC, phospholipase C; [Ca²⁺], intracellular [Ca²⁺]; G, guanine nucleotide binding; PKC, protein kinase C; fur-2/AME, fur-2-tetraacetoxy methyl ester; HITESA, RPMI 1640 with 10 nm hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin, 10 nm estradiol, 30 nm selenium, and 0.25% bovine serum albumin.

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In accordance with this, bombesin strikingly increases the phosphorylation of the acidic M, 80,000 protein (28, 40, 41), a major substrate of PKC which has been recently purified from Swiss 3T3 cells (42) and molecularly cloned (43). Bombesin/GRP also stimulates a rapid exchange of Na+, H+, and K+ ions across the cell membrane, leading to cytoplasmic alkalinization and increased intracellular [K+], (36) and induces a striking PKC-dependent transmodulation of the epidermal growth factor receptor (40).

Recently, bombesin, vasopressin, and endothelin have been shown to induce a rapid and potent stimulation of tyrosine phosphorylation of several substrates in quiescent 3T3 cells (44). This response is not mediated by either PKC activation or Ca2+ mobilization (44). The mechanism by which neuropeptide receptors elicit this novel pathway as well as the precise role of tyrosine phosphorylation in neuropeptide-mediated signal transduction are intriguing issues that warrant further experimental work.

In addition, bombesin, but not vasopressin, induces a marked and sustained release of arachidonic acid and its cyclooxygenase metabolite prostaglandin E2 into the medium (45). Thus, bombesin receptors may be coupled both to PLC activation through a putative G protein (Gp) and to arachidonic acid release possibly via phospholipase A2, although other possibilities remain open. Considerable evidence indicates that the liberation of arachidonic acid is an early signal that contributes to bombesin-mediated mitogenesis (45, 46).

In common with many other growth factors, bombesin/GRP stimulates transient expression of the nuclear oncogenes c-fos and c-myc (47). It is likely that the induction of c-fos by bombesin is mediated by the coordinated effects of PKC activation, Ca2+ mobilization, and an additional pathway dependent on arachidonic acid release (47-49). Furthermore, additional pathways of control of c-fos expression that are completely independent of activation of PKC have also been shown (47).

Indeed, bombesin can initiate DNA synthesis via PKC-dependent and -independent pathways (47). This complex network of signals (Fig 1; Ref. 21) involves a degree of redundancy and ensures the amplification of the stimulus. In addition to bombesin, several other regulatory peptides have been characterized as mitogens for Swiss 3T3 cells including vasopressin, bradykinin, and endothelin-related peptides, and their signaling pathways have also been defined in detail. These neuropeptide receptors are also linked to phosphoinositide breakdown and Ca2+ mobilization but the intensity, duration (e.g., PKC activation), and even the occurrence of early signals (e.g., arachidonic acid release) differ substantially (21, 45, 50).

Ca2+ Mobilization in SCLC Cell Lines

Studies with SCLC have demonstrated a similar set of early events to those previously elucidated in murine 3T3 cells. Specifically, GRP stimulates mobilization of intracellular Ca2+ and inositol phosphate turnover in SCLC cells (10, 51, 52). In a subsequent study, multiple neuropeptides were screened for their ability to induce a rapid increase in [Ca2+]i in different SCLC cell lines (53). This assay should be regarded as an indicator of a productive ligand-receptor interaction. Ca2+ mobilization is, as discussed in the preceding section and shown in Fig. 1, one of the components of a complex array of signaling events rather than the signal that promotes cell growth. Woll and Rozengurt (53) demonstrated that bradykinin, cholecystokinin, galanin, neurotensin, and vasopressin induce a rapid and transient increase in [Ca2+]i in SCLC cell lines (Table 1). The expression of these receptors is heterogeneous among these lines. These neuropeptides increased [Ca2+]i in a dose-dependent fashion in the nanomolar range; typical dose-response relationships are depicted in Fig. 2. The Ca2+-mobilizing effects are mediated by distinct receptors as shown by the use of specific antagonists and by the induction of homologous desensitization (53, 54). Studies carried out in other laboratories are in agreement with these findings (55-57).

The observation that galanin, a 29-amino acid neuropeptide, causes Ca2+ mobilization in SCLC is of special interest. In pancreatic cells galanin activates an ATP-sensitive K+ channel, hyperpolarizes the plasma membrane, and inhibits the activity of voltage-dependent Ca2+ channels (58). In this manner it reduces Ca2+ influx and blocks the activity of various agents...
that increase the intracellular concentration of Ca\(^{2+}\). Surprisingly, in SCLC cell lines galanin caused a rapid and transient increase in [Ca\(^{2+}\)], (53). Recent studies showed that galanin induced rapid mobilization of Ca\(^{2+}\) from internal stores and stimulated early production of inositol phosphates, particularly Ins(1,4,5)P\(_3\), (54). In contrast to the pancreatic cells addition of galanin to SCLC cell lines did not alter membrane potential. Thus, these studies suggest that SCLC express a novel type of galanin receptors that are coupled to Ca\(^{2+}\) mobilization.

Collectively, the studies discussed in this section indicate that SCLC exhibit receptors for multiple neuropeptides and that the expression of these receptors is heterogeneous among SCLC cell lines.

Multiple Neuropeptides Stimulate Clonal Growth in SCLC Cells

In view of the findings discussed in the preceding section, it has been hypothesized that SCLC growth is regulated by multiple autocrine and/or paracrine circuits involving Ca\(^{2+}\)-mobilizing neuropeptides (53, 54, 59, 60). A crucial test of this hypothesis is to determine whether Ca\(^{2+}\)-mobilizing neuropeptides can act as growth factors for SCLC cell lines. Consequently, we determined the effect of multiple Ca\(^{2+}\)-mobilizing neuropeptides to promote clonal growth in semisolid medium in different SCLC cell lines (Fig. 3) (60). The results shown in Fig. 3 demonstrate that, at optimal concentrations, bradykinin, neurotensin, vasopressin, cholecystokinin, galanin, and GRP induce comparable increases of SCLC clonal growth in responsive cell lines. Thus, multiple Ca\(^{2+}\)-mobilizing neuropeptides, via distinct receptors, can act directly as growth factors for SCLC.

It is known that GRP, vasopressin, cholecystokinin, and neurotensin are secreted by some SCLC tumours (2–10). Other peptides may be released by a variety of normal cells in the lung or, like bradykinin, produced extracellularly as a result of the proteolytic cleavage of plasma precursors in the damaged tissue surrounding tumors (61). Collectively, these findings support the hypothesis that SCLC growth is sustained by an extensive network of autocrine and paracrine interactions involving multiple neuropeptides. Approaches designed to block SCLC growth must take into account this mitogenic complexity.

Blocking the Action of Multiple Neuropeptides: Broad Spectrum Antagonists

As understanding of the effects of growth factors in cancer increases, it has become possible to plan rational therapeutic interventions. If an autocrine growth loop is considered, in which cells synthesize, secrete, bind, and respond to the same growth factor, it is evident that interruption of this cycle at any point will block mitogenesis. Paracrine growth could be blocked in the same way. As discussed in the preceding sections, SCLC constitutes a special case in which unrestrained proliferation appears driven, at least in part, by multiple autocrine and paracrine circuits involving Ca\(^{2+}\)-mobilizing neuropeptides.

Secreted factors can be cleared by antibodies, such as the bombesin monoclonal antibody 2A11 used to retard the growth of SCLC xenografts in nude mice (9). We have directed our effort to develop peptide antagonists which are not antigenic and should have higher tissue penetration than antibody proteins. We have characterized neuropeptide antagonists in the model Swiss 3T3 fibroblast system and then tested their effects on SCLC in vitro and in vivo.

The first antagonist to be studied was an analogue of substance P, [DArg\(^{1}\),DPro\(^{2}\),DTrp\(^{9}\),Leu\(^{11}\)]substance P [antagonist A (Table 2)]. Substance P is structurally unrelated to the bombesin-like peptides, but antagonist A, which is a substance P antagonist, was found to block the secretory effects of bombesin.

Table 2 Bombesin/GRP and broad spectrum antagonists

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Fig. 2. Effect of bradykinin, neurotensin, cholecystokinin, galanin vasopressin and GRP on [Ca\(^{2+}\)] in SCLC cells. SCLC cell lines H69, H510, and H345 were cultured in HITESA for 3–5 days. Aliquots of 4–5 x 10\(^{9}\) cells were washed and incubated in 10 ml fresh HITESA medium for 2 h at 37°C. Then, 1 μM fura-2 AME was added for 5 min. The cells were washed and resuspended in 2 ml of electrolyte solution (53, 54). This cell suspension was placed in a quartz cuvet and stirred continuously. Fluorescence was monitored in a Perkin-Elmer LS5 luminescence spectrophotometer and basal and peak [Ca\(^{2+}\)] values were determined as described (54). Each curve represents a typical dose-response relationship for the neuropeptide in the SCLC cell line indicated. The basal [Ca\(^{2+}\)], represents the mean value for that experiment ± SEM (bars).

Fig. 3. Effect of bradykinin (BK), neuropeptide (NT), cholecystokinin (CCK), galanin (Gal), vasopressin (VP), and GRP on colony formation in SCLC cells. SCLC cell line H69, H510, and H345 were used as indicated. Cells 3–5 days postpassage were washed and resuspended in serum-free medium. Cells were then disaggregated into an essentially single cell suspension, judged by microscopy, bypassing the cells through a 19-gauge needle and then through a 0.4-μm nylon gauze. Viability was judged by trypan blue exclusion on a standard hemocytometer. Cell number was determined using a Coulter Counter and approximately 10\(^{4}\) viable cells/ml were suspended in culture medium and 0.3% agarose. One ml of the mixture was plated in 5 replicates in 35-mm plastic dishes containing a base layer of 0.5% agarose in culture medium that had hardened. Both layers of the mixture was plated in 5 replicates in 35-mm plastic dishes containing a base layer of 0.5% agarose in culture medium that had hardened. Both layers of the mixture was plated in 5 replicates in 35-mm plastic dishes containing a base layer of 0.5% agarose in culture medium that had hardened. Both layers...
growth factors, such as epidermal and platelet-derived growth
47). It did not affect mitogenesis stimulated by polypeptide
 radically. The broad spectrum antagonists inhibit the basal and stimulated clonal growth of SCLC cell lines in liquid and semisolid media (54, 59, 64). Antagonist D and G were equipotent, with half-maximal effect at about 20 μM, whereas antagonist A was 5-fold less potent.

The broad spectrum antagonists (D and G) caused a dramatic decrease of the cloning efficiency of these cells in the absence of any exogenously added peptide (i.e., basal colony formation). Broad spectrum antagonists also decrease clonal growth in the presence of neuropeptide stimulation (54). For example, antagonist G profoundly inhibited the clonal growth of SCLC H69 or H345 cells in the absence as well as in the presence of either galanin or vasopressin (Fig. 4). The striking finding that antagonists D and G inhibit the basal and stimulated clonal growth of so many cell lines (54, 59), regardless of positivity for bombesin receptors, suggests that broad spectrum antagonists could be more useful anticancer drugs than ligand-specific growth factor antagonists.

As a first step to test this possibility, we examined the effect of antagonist G on the growth of a H69 SCLC xenograft. Fragments of the H69 xenograft were implanted s.c. in the flanks of nude mice and allowed to grow to a measurable size (30 mm³). Then, a group of animals were treated with antagonist G given peritumorally once a day for 1 week. Fig. 5 shows that the antagonist profoundly inhibited the growth of the tumor, as compared with the control group. The inhibitory effect was clearly maintained beyond the duration of administration. These results demonstrate that antagonist G can inhibit SCLC growth in vivo as well as in vitro.

Fig. 5. Effect of antagonist G on H69 SCLC xenograft growth in nude mice. Fragments of the NCI-H69 xenograft (previously established from the cell line) were implanted s.c. into female nude mice. After 6 weeks, when tumors had reached a mean volume of 30 mm³, groups of 7 mice were given injections of either PBS containing 0.9 mg of antagonist G (G) or phosphate-buffered saline alone (C) s.c. adjacent to the tumor once a day for 7 days. Tumor volume was determined by means of vernier calipers and estimated according to the formula $0.5 \times length \times width^2$. For each individual tumor, the change in volume was compared to the value at the start of the treatment. Points, mean of 7 values is indicated. *P < 0.05 (t test).
Conclusions

Neuropeptides are increasingly implicated in the control of cell proliferation and their mechanisms of action are attracting intense interest. The peptides of the bombesin family including GRP bind to specific surface receptors and initiate a complex cascade of signaling events (Fig. 1) that culminates in the growth of SCLC cells. The results discussed here strongly suggest that the autocrine growth loop of bombesin-like peptides may be at an early stage in SCLC as tumor promoters in initiated cells and later as growth factors in the unrestrained growth of the fully developed SCLC tumor. A detailed understanding of the receptors and signal transduction pathways that mediate the mitogenic action of neuropeptides may identify novel targets for therapeutic intervention. In this context, broad spectrum antagonists that prevent the function of multiple Ca2+-mobilizing receptors are of special interest. These antagonists block neuropeptide-mediated signals in the 3T3 and SCLC cells and inhibit SCLC growth in vitro and in vivo. Thus, broad spectrum neuropeptide antagonists constitute potential anticancer agents.

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