Multiple Neuropeptides Stimulate Clonal Growth of Small Cell Lung Cancer: Effects of Bradykinin, Vasopressin, Cholecystokinin, Galanin, and Neurotensin

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Abstract

We tested whether Ca2+-mobilizing neuropeptides can function as growth factors for small cell lung carcinoma cells. The neuropeptides bradykinin, neurotensin, cholecystokinin, and vasopressin at nanomolar concentrations stimulated a rapid and transient increase in the intracellular concentration of Ca2+. Crucially, these peptides in the same concentration range also caused a marked increase in colony formation in semisolid medium in responsive small cell lung carcinoma cell lines. At optimal concentrations bradykinin, neurotensin, cholecystokinin, vasopressin, galanin, and gastrin-releasing peptide were equally effective in promoting clonal growth. These findings support the hypothesis that small cell lung carcinoma growth is sustained by an extensive network of autocrine and paracrine interactions involving multiple neuropeptides.

Introduction

Lung cancer is the commonest fatal malignancy in the developed world. SCLC2 constitutes 25% of the total and follows a rapid and 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, 4–9). Among these, only bombesin-like peptides, which include GRP, have been shown to act as autocrine growth factors for certain SCLC cell lines (10). The role of other neuropeptides in the proliferation of SCLC cells remains poorly understood.

Multiple neuropeptides, including bradykinin, vasopressin, cholecystokinin, galanin, neurotensin, and GRP, stimulate a rapid, transient increase in the intracellular concentration of Ca2+ ([Ca2+]i) in SCLC cell lines (11–14). Since a rise in [Ca2+]i is one of the early signals in a complex signaling cascade leading to mitogenesis in fibroblast model systems (15, 16), it has been hypothesized that SCLC growth is regulated by multiple autocrine and/or paracrine circuits involving Ca2+-mobilizing neuropeptides (11, 12). A crucial test of this hypothesis is to determine whether Ca2+-mobilizing neuropeptides increase the ability of SCLC cell lines to form colonies in semisolid medium. We now report that bradykinin, vasopressin, cholecystokinin, neurotensin, galanin, and GRP, at optimal concentrations, are equally effective in stimulating clonal growth of responsive SCLC cell lines. This supports the hypothesis that SCLC growth is regulated by multiple autocrine and paracrine circuits.

Materials and Methods

Cell Culture. SCLC cell lines H345 and H510 were the kind gift of Dr Adi Gazdar (National Cancer Institute, Bethesda, MD). H69 was purchased from the American Type Culture Collection. Stocks were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 57°C for 1 h) in a humidified atmosphere of 10% CO2:90% air at 37°C. They were passaged every 7 days. For experimental purposes, the cells were grown in HITESA (17).

Determination of [Ca2+]i Concentration. Aliquots of 4–5 × 106 SCLC cells cultured in HITESA for 3–5 days were washed and incubated for 2 h at 37°C in 10 ml of fresh HITESA medium. Fura-2-tetra-acetoxy methyl ester (1 μM) in dimethyl sulfoxide was then added and the cells were incubated for a further 5 min. The cell suspension was centrifuged at 2000 rpm for 15 s, and the cells were resuspended in 2 ml of electrolyte solution (140 mM NaCl, 5 mM KCl, 0.9 mM MgCl2, 1.8 mM CaCl2, 25 mM glucose, 16 mM N-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 16 mM Tris, and a mixture of amino acids at pH 7.2), transferred to a quartz cuvette, and stirred continuously at 37°C. Fluorescence was recorded continuously in a Perkin-Elmer LS5 luminescence spectrometer with an excitation wavelength of 336 nm and an emission wavelength of 510 nm. [Ca2+]i was calculated as previously described (12).

Clonogenic Assay. SCLC cells, 3–5 days postpassage, were washed and resuspended in HITESA. Cells were then disaggregated into a single cell suspension by two passages through a 19-gauge needle and then through a 15-μm pore size nylon gauze. Viability was judged by trypan blue exclusion on a standard hemocytometer. Approximately 1 × 104 viable cells/ml, as determined using a Coulter Counter, were suspended in culture medium and 0.3% agarose. One ml of the mixture was plated in 5 replicate 35-mm plastic dishes containing a 2-ml base layer of 0.5% agarose in culture medium that had hardened. Both layers contained neuropeptide at the same concentration. Cultures were incubated at 37°C in a humidified atmosphere at 10% CO2:90% air for 21 days and then stained with the vital stain Nitro blue tetrazolium. Colonies with diameters of >120 μm (16 cells) were counted under a microscope.

Materials. Bradykinin, vasopressin, neurotensin, cholecystokinin, GRP, and galanin were purchased from Sigma Chemical Co., St. Louis, MO; fura-2-tetra-acetoxy methyl ester from Calbiochem Corp., La Jolla, CA; and agarose from SeaKem, Rockland, ME. All the other reagents were of the highest grade commercially available.

Results and Discussion

Addition of bradykinin to H69, H510, or H345 cells loaded with the Ca2+-sensitive indicator fura-2 increased [Ca2+]i, without any measurable delay (Fig. 1). Peak [Ca2+]i was reached 20–30 s after addition of the peptide. Bradykinin, at 100 nM, increased [Ca2+]i from 100 ± 8 (n = 6) to 192 ± 9 (n = 6) nm in H69 cells, from 134 ± 17 (n = 5) to 206 ± 17 (n = 5) nm in H510 cells, and from 89 ± 7 (n = 4) to 126 ± 9 (n = 4) nm in H345 cells. In each cell line bradykinin increased [Ca2+]i in a...
The basal [Ca\textsuperscript{2+}] is the mean value for that experiment ± SEM. The increases in [Ca\textsuperscript{2+}], induced by 100 nM bradykinin were formed at the concentrations of peptide indicated as described in "Materials and Methods." Inset, the fluorescence tracing obtained when 100 nM bradykinin was added to cells loaded with fura-2.

Figure 2. Effect of the bradykinin antagonist [D-Arg\textsuperscript{10},Hyp\textsuperscript{3},Thy\textsuperscript{5-8},D-Phe\textsuperscript{7}]bradykinin on [Ca\textsuperscript{2+}] mobilization and colony formation in SCLC cells. Bradykinin (BK) and [D-Arg\textsuperscript{10},Hyp\textsuperscript{3},Thy\textsuperscript{5-8},D-Phe\textsuperscript{7}]bradykinin at 10 \textmu M had no effect on the basal [Ca\textsuperscript{2+}], or on spontaneous colony formation in the absence of bradykinin (Fig. 2).

The results obtained with bradykinin prompted us to test other neuropeptides for their effects on Ca\textsuperscript{2+} mobilization and colony growth. The peptides neurotensin, cholecystokinin, and vasopressin are secreted by SCLC (5-9, 22), and increase [Ca\textsuperscript{2+}] in H69, H510, and H345 cells, respectively, in a dose-dependent fashion. Crucially, neurotensin, cholecystokinin, and vasopressin at nanomolar concentrations stimulated clonal growth in semisolid medium (Fig. 3). Cholecystokinin, vasopressin, and GRP in H69 cells or galanin in H345 cells caused little or no rise in [Ca\textsuperscript{2+}], and did not stimulate colony formation in these cell lines (Table 1).

The ability of multiple Ca\textsuperscript{2+}-mobilizing neuropeptides to promote clonal growth in semisolid medium in different SCLC cell lines is shown in Table 1. The neuropeptide galanin, recently shown to stimulate inositol phosphate accumulation, Ca\textsuperscript{2+} mobilization, and colony formation in H69 and H510 cells (12), and GRP were also included in parallel experiments dose-dependent fashion in the nanomolar range; typical dose-response relationships are depicted in Fig. 1.
for comparison. The results demonstrated that, at optimal concentrations, bradykinin, vasopressin, cholecystokinin, galanin, neurotensin, and GRP induce comparable increases of SCLC clonal growth in responsive cell lines (Table 1). 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 tumors (5-9, 22). 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 (21). Collectively, these findings support the hypothesis that SCLC growth is sustained by an extensive network of autocrine and paracrine interactions involving multiple neuropeptides. Broad spectrum neuropeptide antagonists (23) provide a strategy to block SCLC growth which takes into account this mitogenic complexity.

References

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