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Advances in Brief

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 Ca^{2+}-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 Ca^{2+}. 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. SCLC[2] 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 Ca^{2+} ($[Ca^{2+}]_i$) in SCLC cell lines (11–14). Since a rise in $[Ca^{2+}]_i$ 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 Ca^{2+}-mobilizing neuropeptides (11, 12). A crucial test of this hypothesis is to determine whether Ca^{2+}-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% CO_2:90% air at 37°C. They were passaged every 7 days. For experimental purposes, the cells were grown in HITESA (17).

Determination of $[Ca^{2+}]_i$ Concentration. Aliquots of 4–5 × 10^6 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 $MgCl_2$, 1.8 mM CaCl, 25 mM glucose, 16 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 16 mM Tris, and a mixture of amino acids at pH 7.2), transferred to a quartz cuvete, 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. $[Ca^{2+}]_i$ 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 passes 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 10^4 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% CO_2:90% 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-tetraacetoxy 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 Ca^{2+}-sensitive indicator fura-2 increased $[Ca^{2+}]_i$ without any measurable delay (Fig. 1). Peak $[Ca^{2+}]_i$ was reached 20–30 s after addition of the peptide. Bradykinin, at 100 nM, increased $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ in a

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[2] The abbreviations used are: SCLC, small cell lung carcinoma; GRP, gastrin-releasing peptide; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; 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.

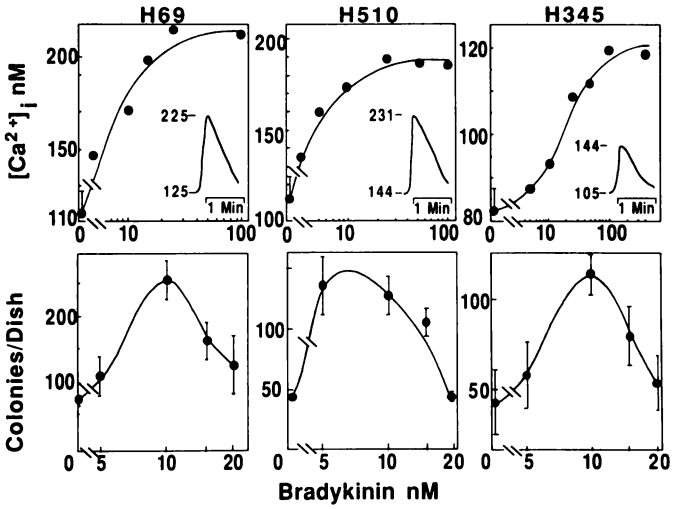

Fig. 1. Dose-dependent effects of bradykinin on $[Ca^{2+}]_i$ (*top*) and on colony formation (*bottom*) in SCLC cells. SCLC cell lines H69 (*left*), H510 (*middle*), and H345 (*right*) were cultured in HITESA for 3–5 days. $[Ca^{2+}]_i$ and colony formation were determined at the concentrations of peptide indicated as described in "Materials and Methods." *Inset*, the fluoresence tracing obtained when 100 nM bradykinin was added to cells loaded with fura-2. Typical $[Ca^{2+}]_i$ dose-response curves are shown for each cell line. The basal $[Ca^{2+}]_i$ is the mean value for that experiment ± SEM. The increases in $[Ca^{2+}]_i$ induced by 100 nM bradykinin were repeated in several independent experiments and the data are given in the text. Each *point* in the colony formation assay represents the mean ± SEM (*bars*) of 3–4 independent experiments (each with 5 replicates).

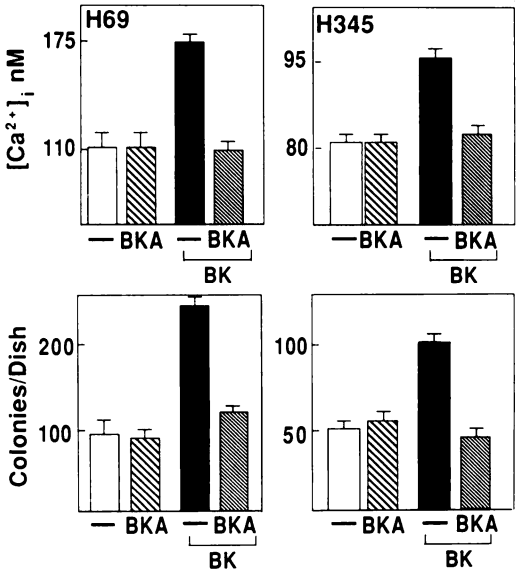

Fig. 2. Effect of the bradykinin antagonist [D-Arg^0,Hyp^3,Thy^{5,8},D-Phe^7]bradykinin on bradykinin induced $[Ca^{2+}]_i$ mobilization and colony formation in SCLC cells H69 (*left*) and H345 (*right*). Bradykinin (*BK*) and [D-Arg^0,Hyp^3,Thy^{5,8}] bradykinin (*BKA*) were added at 10 nM and 10 μM, respectively. *Top*: $[Ca^{2+}]_i$ was determined as described in "Materials and Methods." □, Basal $[Ca^{2+}]_i$. Each column represents the mean ± SEM of 3–6 experiments. *Bottom*: 10^4 cells in 0.3% agarose were layered onto 0.5% agarose containing bradykinin either in the absence (—) or presence (*BKA*) of the bradykinin antagonist. After 21 days colonies >16 cells were counted under a microscope. □, spontaneous colony formation. Each *bar* represents the mean ± SD (*bars*) of 5 replicates.

dose-dependent fashion in the nanomolar range; typical dose-response relationships are depicted in Fig. 1.

Tumor and transformed cells, including SCLC, are able to form colonies in agarose medium. Indeed, there is a positive correlation between cloning efficiency of the cells and the histological involvement and invasiveness of the tumor in specimens taken from SCLC (18). Consequently, we determined the effect of bradykinin on the ability of H69, H510, and H345 cells to form colonies in semisolid medium. Fig. 1 (*bottom*) shows that bradykinin markedly increased colony growth of these SCLC cell lines in a steeply dose-dependent manner. Optimal colony stimulation was achieved at 10 nM bradykinin in H69 and H345 cell lines and at 5–10 nM bradykinin in H510 cells. At higher concentrations the stimulatory effect decreased, presumably due to homologous desensitization in this long-term assay (Fig. 1). Time-dependent mitogenic desensitization has been reported in other cellular systems (19, 20).

The role of bradykinin receptors in mediating Ca^{2+} mobilization and cell growth was tested using [D-Arg^0,Hyp^3,Thy^{5,8},D-Phe^7]bradykinin, a specific competitive antagonist of the β_2 receptor (21). The antagonist, at 10 μM, completely blocked the increase in both $[Ca^{2+}]_i$ and colony formation induced by 10 nM bradykinin in either H69 cells or H345 cells (Fig. 2). [D-Arg^0,Hyp^3,Thy^{5,8},D-Phe^7]bradykinin at 10 μM had no effect on the basal $[Ca^{2+}]_i$ 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^{2+} mobilization and colony growth. The peptides neurotensin, cholecystokinin, and vasopressin are secreted by SCLC (5–9, 22), and increase $[Ca^{2+}]_i$ in responsive SCLC cell lines through distinct receptors (11, 14). Fig. 3 shows that these peptides increased $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ and did not stimulate colony formation in these cell lines (Table 1).

The ability of multiple Ca^{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^{2+} mobilization, and colony formation in H69 and H510 cells (12), and GRP were also included in parallel experiments

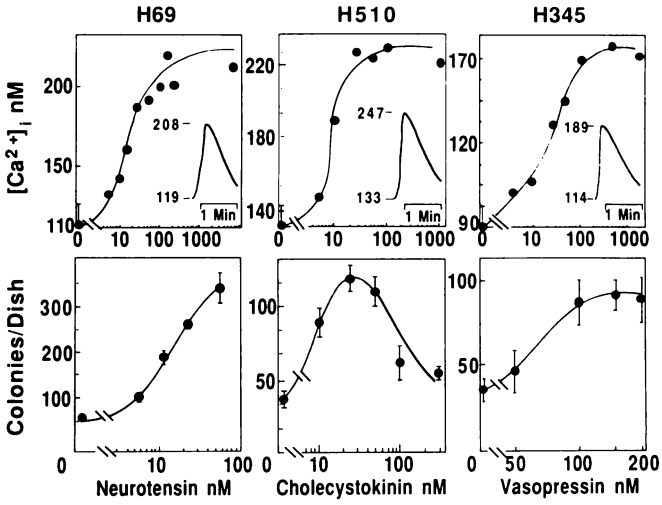

Fig. 3. Effect of neurotensin, cholecystokinin, and vasopressin on $[Ca^{2+}]_i$ and colony formation in H69, H510, and H345 SCLC cell lines. $[Ca^{2+}]_i$ and colony formation were determined as described in "Materials and Methods" *Inset*, fluorescence tracing obtained when 100 nM concentrations of the peptide indicated were added to cells loaded with fura-2. Typical $[Ca^{2+}]_i$ dose-response relationships are shown. The basal $[Ca^{2+}]_i$ is the mean value for that experiment ± SEM (*bars*). Each *point* in the colony assay represents the mean ± SEM of 2–3 independent experiments (each with 5 replicates).

Table 1 *Multiple Ca^{2+}-mobilizing neuropeptides stimulate clonal growth of SCLC cell lines*

$[Ca^{2+}]_i$ was measured with the fluorescent indicator fura-2 as described in "Materials and Methods." The positivity of the $[Ca^{2+}]_i$ response reflects a productive ligand-receptor complex. Colony formation was determined using the clonogenic assay as described in "Materials and Methods." Spontaneous colony formation, *i.e.*, in the absence of any exogenously added peptide (—), 98 ± 4, 57± 4, and 56 ± 6 in H69, H510, and H345, respectively, is normalized to 100%. The percentage of colony formation is expressed as the mean ± SEM. The number of 35 mm dishes counted are indicated in parentheses at the concentration or range of concentrations indicated. In H69 cells, GRP, vasopressin, and cholecystokinin had little or no effect on both $[Ca^{2+}]_i$ and colony formation. In H345 cells, galanin neither increased $[Ca^{2+}]_i$ nor stimulated clonal growth.

Cell line	Peptide	[nM]	$[Ca^{2+}]_i$	% of colony formation
H69	—			100 (80)
	Bradykinin	10	+	360 ± 19 (24)
	Galanin	50	+	252 ± 8 (34)
	Neurotensin	50	+	455 ± 37 (9)
H510	—			100 (70)
	Bradykinin	5–10	+	359 ± 23 (19)
	Galanin	50	+	368 ± 21 (23)
	Vasopressin	100	+	344 ± 12 (18)
	Cholecystokinin	25	+	291 ± 8 (9)
H345	—			100 (50)
	Bradykinin	10	+	321 ± 37 (20)
	Vasopressin	150	+	257 ± 19 (9)
	GRP	5–10	+	232 ± 7 (10)

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 extracellulary 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.

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