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MULTIPLE NEUROPEPTIDES MOBILISE CALCIUM IN SMALL CELL LUNG CANCER: EFFECTS OF VASOPRESSIN, BRADYKININ, CHOLECYSTOKININ, GALANIN AND NEUROTENSIN

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SUMMARY: The neuropeptides vasopressin, bradykinin, cholecystokinin, galanin, neurotensin and gastrin-releasing peptide stimulate rapid, transient increases in cytosolic Ca^{2+} in small cell lung cancer cell lines at nanomolar concentrations. Responsiveness to individual peptides is heterogeneous among the diverse cell lines, but the ability to respond to regulatory peptides is a general phenomenon. Peptide responses demonstrate homologous desensitisation and are blocked by ligand-specific antagonists, indicating that they are mediated by distinct receptors. Many neuropeptides are also secreted by small cell lung cancer. Here we suggest that multiple autocrine and paracrine interactions regulate its growth.

Lung cancer remains the commonest fatal malignancy in the developed world. Small cell lung cancer (SCLC) constitutes 25% of the total, and follows a rapid and aggressive clinical course, despite initial chemosensitivity (1). Increased understanding of SCLC growth regulation may identify novel targets for treatment. SCLC is characterised by the presence of intracytoplasmic neurosecretory granules and by its ability to secrete a variety of ectopic hormones and neuropeptides (2,3). Among these, only the bombesin-like peptides, which include gastrin-releasing peptide (GRP), have been shown to induce rapid increases in cytoplasmic Ca^{2+} (6,7) and to act as autocrine growth factors for SCLC (8).

Abbreviations: ACTH, adrenocorticotrophin; CCK, cholecystokinin; FMLP, N-formyl-met-leu-phe; FSH, follicle stimulating hormone; fura-2/AM, fura-2 tetraacetoxymethyl ester; GIP, gastric inhibitory peptide; GHRH, growth hormone releasing hormone; GRP, gastrin-releasing peptide; Hyp, L-4-hydroxyproline; PBt_2, phorbol dibutyrate; Pmp, 1-(β-mercapto-β,β-cyclopentamethylene-propionic acid; SCLC, small cell lung cancer; Thi, β-(2-thienyl)-L-alanine; TRH, thyrotropin releasing hormone.

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Evidence is rapidly accumulating that neuropeptides acting through distinct receptors and signal transduction pathways (9) can control proliferation in a variety of cell types (10). In view of this, we decided to investigate the hypothesis that SCLC exhibits multiple neuropeptide receptors. We have screened neuropeptides and hormones secreted by SCLC for the ability to rapidly mobilise intracellular Ca^{2+} in five SCLC cell lines. We now report that vasopressin, bradykinin, CCK, galanin and neurotensin induce marked increases in cytoplasmic Ca^{2+} through distinct receptors, raising the possibility that SCLC growth is regulated by multiple autocrine or paracrine circuits.

MATERIALS AND METHODS

SCLC cell lines H69 and H128 were obtained from the American Type Culture Collection, Rockville, Maryland, USA. H209, H345 and H510A were a gift from Dr Adi Gazdar, National Cancer Institute, Bethesda, Maryland, USA. Stocks were grown in RPMI 1640 medium with 10% heat-inactivated, fetal bovine serum, and experimental flasks in RPMI 1640 medium with 10 nM hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin, 10 nM estradiol, 30 nM selenium and 0.25% bovine serum albumin (modified from 12).

Intracellular $[Ca^{2+}]$ was measured with the fluorescent Ca^{2+} indicator fura-2/AM (modified from 11). Aliquots of 2-5 x 10^6 cells were equilibrated for 3 h in 10 ml fresh medium, then 1 µM fura-2/AM was added for 5 min. The cell suspension was centrifuged at 2000 rpm for 10 s, and the cells resuspended in 2 ml electrolyte solution (13). Fluorescence was recorded continuously in a Perkins-Elmer LSS luminescence fluorimeter with an excitation wavelength of 335 nm and an emission wavelength of 510 nm.

Materials: Acetyl choline, ACTH, angiotensin I, II and III, bradykinin, [Thi^{5,8},DPhe^{7}]bradykinin, CCK-8, chorionic gonadotrophin, dynorphin, α-endorphin, epinephine, FMLP, FSH, galanin, GHRH, GIP, glucagon, GRP, histamine, 5-hydroxy tryptamine, leu-enkephalin, neuropeptide Y, neurotensin, parathyroid hormone, substance K, substance P, thrombin, TRH and vasopressin were from Sigma. Atrial peptide, [DesArg^{9},Leu^{8}]bradykinin, calcitonin, endothelin and [Pmp^{1},OMeTyr^{2},Arg^{8}]vasopressin were from Peninsula. [DArg^{0},Hyp^{3}, Thi^{5,8},DPhe^{7}]bradykinin and [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin were from Bachem. Fura-2/AM was from Calbiochem.

RESULTS AND DISCUSSION

It has long been known that SCLC secrete a variety of biologically active peptides. Despite this, the effects of these peptides on the tumours have not previously been investigated in detail. In the

Table 1

Peptides tested for Ca^{2+}-mobilising ability in SCLC cell lines

	NCI H-510A	NCI H-345	NCI H-209	NCI H-128	NCI H-69
Acetylcholine	-	+	++	-	-
ACTH	-	+	-	-	-
Angiotensin I	-	-	-	-	-
Angiotensin II	-	n	n	n	-
Angiotensin III	-	-	-	-	-
Atrial peptide	-	-	-	-	-
Bradykinin	++	++	++	-	++
Calcitonin	-	-	-	-	-
CCK-8	++	+	++	-	+
Chorionic gonadotrophin	-	-	-	-	-
Dynorphin	-	-	-	-	-
α-endorphin	-	-	n	-	-
Endothelin	-	-	-	-	-
Epinephrine	-	-	-	-	-
FMLP	-	-	-	-	-
FSH	-	-	-	-	-
Galanin	++	+	-	-	++
GHRH	-	-	-	-	-
GIP	n	-	-	n	-
Glucagon	-	-	-	-	-
GRP	+	++	-	-	+
Histamine	-	-	-	-	-
5-hydroxy tryptamine	-	-	-	-	-
Leu-enkephalin	-	-	-	-	-
Neuropeptide-Y	-	-	-	-	-
Neurotensin	-	++	++	n	++
Parathyroid hormone	-	-	-	n	-
Substance K	-	-	-	-	-
Substance P	-	-	-	-	-
Thrombin	++	-	-	++	-
TRH	-	-	-	-	-
Vasopressin	++	++	++	++	+

Intracellular Ca^{2+} was measured with the indicator fura-2/AM as described in Materials and Methods. The peptides were used at 1 μM except for the following: acetylcholine 2.5 μM, ACTH 2 u/ml, FSH 0.5 u/ml, GIP 2 μM, glucagon 10 μg/ml, chorionic gonadotrophin 10 u/ml, parathyroid hormone 100 nM, thrombin 1 u/ml. Responses have been scored: -, no response; +, small response; ++, consistently large response; n, not done. Examples are shown in the Figures.

present study, we have examined the effect of 32 neuropeptides and hormones on Ca^{2+}-sensitive fluorescence in fura-2/AM loaded SCLC cells (Table 1). Because SCLC are heterogeneous, 5 cell lines of diverse phenotypes were studied (14).

Vasopressin caused a marked increase in the cytosolic Ca^{2+} in H345 cells (Fig. 1), but repeated additions resulted in no further responses, indicating that homologous desensitisation occurs. The cells remained sensitive to GRP, which acts through a different

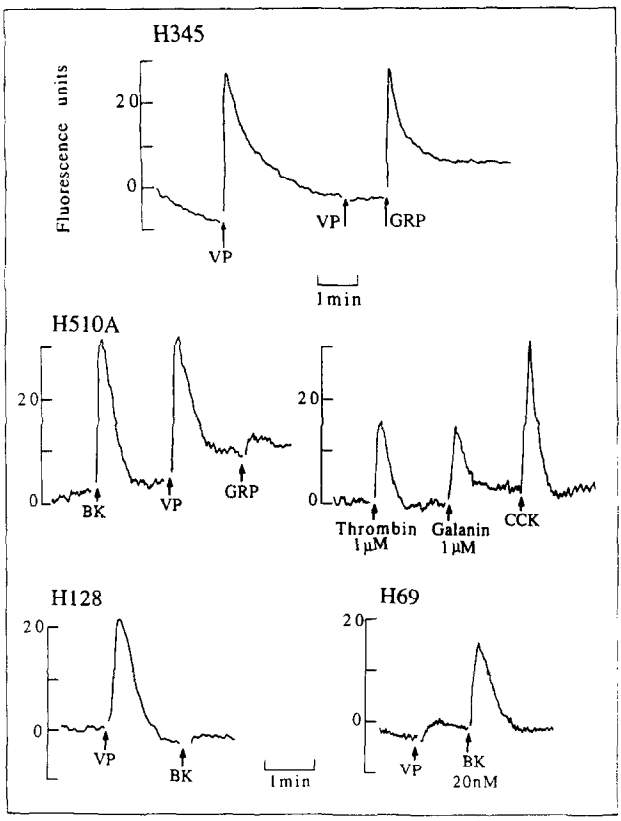

Fig. 1: Multiple peptides cause rapid and transient increases in intracellular Ca^{2+} in SCLC cell lines. Cytosolic Ca^{2+} was measured in cells loaded with fura-2/AM (see Materials and Methods) and suspended in electrolyte solution containing: 140 mM NaCl, 5 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 16 mM HEPES, 6 mM Tris and a mixture of amino acids (13) at pH 7.2. VP, vasopressin; BK, bradykinin. Peptides were used at 100 nM, except where otherwise indicated.

receptor. Thus, multiple responses could be demonstrated by sequential additions of different peptides. Using this approach we found that bradykinin, neurotensin, CCK and galanin also induced Ca^{2+}mobilisation in H345 cells and there were minor effects with acetylcholine and ACTH, whereas 23 other ligands were ineffective (Table 1).

Multiple neuropeptides also elicited Ca^{2+} responses in other SCLC cell lines (Fig. 1 and Table 1). Although responses to both bradykinin and vasopressin were found in H345, H209 and H510A, they were dissociated in H128 (vasopressin only) and H69 (bradykinin predominant). CCK, galanin, GRP and neurotensin caused rapid and transient increases in intracytoplasmic Ca^{2+} in several of the cell

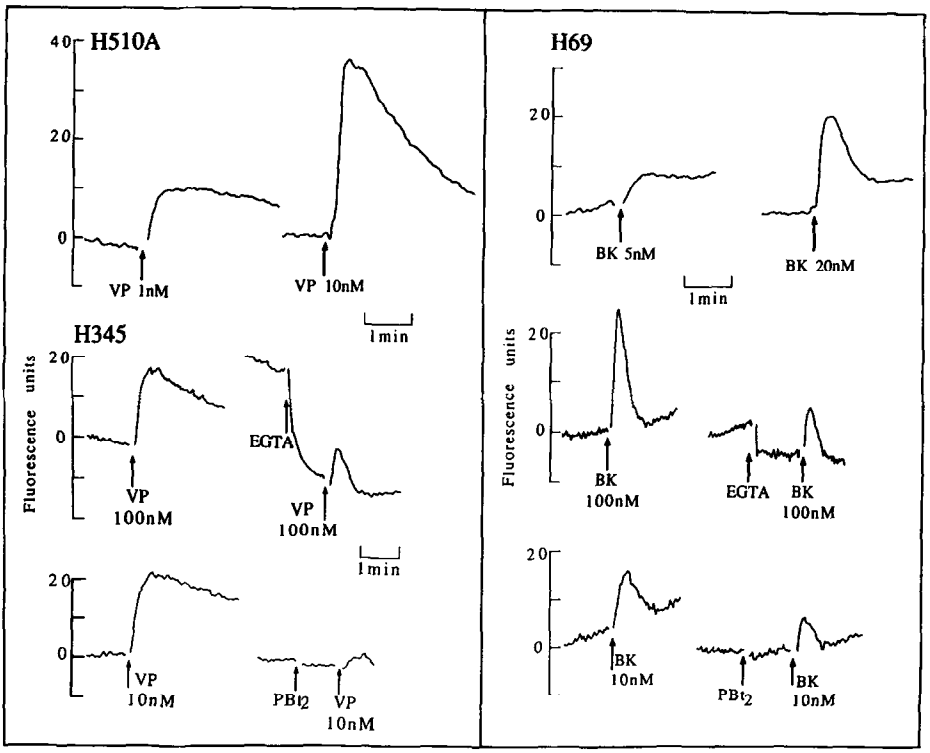

Fig. 2: Effects of vasopressin (VP, left) and bradykinin (BK, right) on intracellular Ca^{2+} in SCLC. Upper: dose-dependence of responses. Middle: effects of treatment with the Ca^{2+} chelator EGTA 1.8 mM. Lower: effects of protein kinase C stimulation with PBt_2 200 nM.

lines, but vasopressin was active in all of them and bradykinin in 4 of 5 cell lines studied (Fig. 1). The effects of vasopressin and bradykinin were therefore characterised in more detail.

Vasopressin and bradykinin increased cytoplasmic Ca^{2+} in a dose-dependent fashion in the nanomolar range (Fig. 2). Both peptides exhibited rapid homologous desensitisation. They appear to elevate intracellular Ca^{2+} by two distinct mechanisms: the initial phase results from Ca^{2+} mobilisation from intracellular stores, since it still occurred after chelation of extracellular Ca^{2+} with 1.8 mM EGTA (Fig. 2). The sustained phase of Ca^{2+} elevation is probably due to Ca^{2+} influx, as it was abolished by treatment with EGTA. As in other systems, including Swiss 3T3 cells (15) and the response of SCLC to GRP (5), stimulation of protein kinase C with 200 nM PBt_2 attenuated the responses to both vasopressin and bradykinin (Fig. 2).

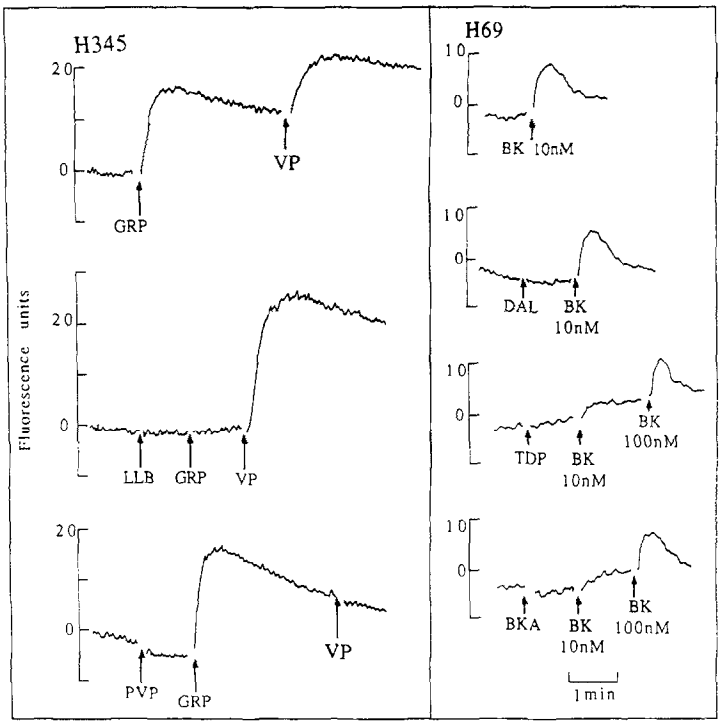

Fig. 3: Effects of ligand-specific antagonists on Ca^{2+} mobilisation in SCLC$_1$ (Left) [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin (LLB) 1 μM and [Pmp^1,OMeTyr^2,Arg^8]vasopressin (PVP) 100 nM with GRP 2 nM and vasopressin (VP) 5 nM. (Right) [desArg^9,Leu^8]bradykinin (DAL) 10 μM, [Thi^{5,8},DPhe^7]bradykinin (TDP) 10 μM and [DArg^0,Hyp^3,Thi^{5,8},DPhe^7] bradykinin (BKA) 7.7 μM with bradykinin (BK) at the concentrations shown.

We used ligand-specific antagonists to identify the receptors mediating these effects (16). Vasopressin acts through a V_1 receptor, as shown by blockade with the selective antagonist [Pmp^1,OMeTyr^2,Arg^8] vasopressin (Fig. 3, left). Blockade of the bombesin receptor with [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin did not preclude a response to vasopressin, and vice versa (Fig. 3, left). Bradykinin stimulated Ca^{2+} mobilisation through a classical B_2 receptor, as shown by competitive blockade with B_2-specific antagonists [Thi^{5,8},DPhe^7]bradykinin and [DArg^0,Hyp^3,Thi^{5,8},DPhe^7]bradykinin, but the absence of effect with the B_1 antagonist [desArg^9,Leu^8]bradykinin (Fig. 3B, right) and either [Pmp^1,OMeTyr^2,Arg^8]vasopressin or [Leu^{13}-ψ(CH$_2$NH)Leu^{14}]bombesin. Furthermore, the B_2 antagonists did not preclude a response to GRP. This evidence suggests that SCLC cell lines exhibit multiple distinct neuropeptide receptors that can stimulate Ca^{2+} mobilisation.

The observation that several neuropeptides can stimulate Ca^{2+} mobilisation in SCLC has implications for the growth of these cells. Although the precise role of Ca^{2+} in the control of cell proliferation remains undefined, studies in Swiss 3T3 cells have demonstrated that this ionic response is part of a mitogenic signalling cascade (9) and this system has provided a model for the response of SCLC to GRP (17). Bradykinin and galanin have not been sought in SCLC, but CCK, neurotensin and vasopressin are known to be secreted by some SCLC tumours (2,5,18-22). Other neuropeptides may be secreted by a variety of normal cells in the lung, but have effects on adjacent tumour cells. Binding sites for CCK have been described in SCLC (23) but binding of other peptides has not been demonstrated.

Despite the phenotypic heterogeneity of SCLC, manifest in different growth patterns (14), oncogene expression (24,25) and secretion of neuropeptides and expression of their receptors (22), we have observed a general phenomenon, that all the cell lines studied respond to some regulatory neuropeptides. We suggest that the autocrine growth loop of bombesin-like peptides (8) is only a part of an extensive network of autocrine and paracrine interactions involving a variety of neuropeptides in SCLC. Future approaches to the diagnosis and treatment of SCLC must accommodate this mitogenic complexity.

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