

# Stimulation of Calcium Mobilization but not Proliferation by Bombesin and Tachykinin Neuropeptides in Human Small Cell Lung Cancer Cells<sup>1</sup>

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## ABSTRACT

The tachykinin family of neuropeptides, including substance P and neurokinins A and B, induce a transient increase in intracellular free calcium concentration in human small cell lung carcinoma (SCLC) cells, as measured with a calcium indicator fura-2. The effects are dose dependent and even greater than that of bombesin at equimolar concentrations in these cells. The tachykinins, like bombesin, induce calcium mobilization mainly from intracellular store(s). None of the peptides, however, shows a stimulatory effect on DNA synthesis. In addition, exogenously applied bombesin does not stimulate DNA synthesis at any concentration tested. We also examined the effects of a recently reported bombesin antagonist [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]substance P in SCLC cells, and compared them to those in Swiss 3T3 fibroblasts in which the mitogenic effect of bombesin is well characterized. The antagonist at 10<sup>-5</sup> M completely abolishes the Ca<sup>2+</sup>-mobilizing effect of 10<sup>-7</sup> M bombesin in SCLC cells, and that of 10<sup>-9</sup> M but not 10<sup>-7</sup> M bombesin in Swiss 3T3 cells. The antagonist at this concentration effectively inhibits the mitogenic action of bombesin (10<sup>-9</sup> M) in Swiss 3T3 cells; however, much higher doses (~10<sup>-4</sup> M) are needed to inhibit DNA synthesis in SCLC cells. Moreover, the antagonist inhibits DNA synthesis in bombesin/gastrin-releasing peptide-nonproducing cells with a similar dose dependency as in producing cells. These results indicate that bombesin/gastrin-releasing peptide and other calcium mobilizing peptides do not always act as a growth factor in SCLC cells, and that the bombesin antagonist could inhibit growth of SCLC cells through a mechanism other than bombesin antagonism.

## INTRODUCTION

Human SCLC<sup>3</sup> is a virulent type of cancer that originates from neuroendocrine cells of the lung and retains some of their features. For example, SCLC cells produce and secrete a variety of hormones including adrenocorticotropin, calcitonin, parathyroid hormone, glucagon, vasopressin, somatostatin, neurotensin, and GRP (1-4). Especially, GRP and its related bombesin-like peptides have attracted much interest, since these peptides have recently been reported to act as an autocrine growth factor in SCLC cells (5-7). GRP and its amphibian counterpart bombesin have C-terminal 7-residue sequences in common and both bind to a single class of high affinity receptors on Swiss 3T3 fibroblasts, a well-characterized model system for studying mitogenesis, and thereby induce a marked stimulation in DNA synthesis (8-10). In these cells bombesin/GRP stimulates phospholipase C with the production of two second messengers, inositol-1,4,5-trisphosphate and 1,2-diacylglycerol, resulting in intracellular Ca<sup>2+</sup> mobilization and activation of

protein kinase C (11, 12). These signal generations are thought to contribute to mitogenic action of bombesin/GRP in Swiss 3T3 cells. It has recently been reported that bombesin and its congeners also induce phospholipase C activation and Ca<sup>2+</sup> mobilization in SCLC cells (13, 14).

Substance P, its amphibian counterpart physalaemin, and neurokinins A and B are members of tachykinin family neuropeptides and are known to act in a similar way as bombesin/GRP, *i.e.*, they induce phospholipase C activation and subsequent Ca<sup>2+</sup> mobilization in a number of tissues (15, 16). Moreover, the tachykinin peptides have been shown to be present in the normal lung tissues (17, 18) as well as a tumor extract from SCLC (19). In the present study we examined whether these neuropeptides cause Ca<sup>2+</sup> mobilization and cell growth in SCLC cells. The results show that two out of four SCLC cell lines tested respond to the tachykinin neuropeptides with Ca<sup>2+</sup> mobilization, however, the peptides do not stimulate DNA synthesis in these cells. Similarly, bombesin induces Ca<sup>2+</sup> mobilization in three SCLC cell lines without stimulating DNA synthesis.

## MATERIALS AND METHODS

**Cell Lines.** Swiss-mouse 3T3 fibroblasts were generously provided by Dr. E. Rozengurt (Imperial Cancer Research Fund, London, England), and were maintained as described previously (20). The establishment and characteristics of the SCLC cell lines used in this study have been described previously (21). Morphologically, TKB-15 and -16 are the classical, and TKB-2 and -17/1 the variant type. Radioimmunoassay of cellular content of bombesin/GRP-related peptides (22) show <10, <10, 39, and 2400 pg/mg protein in TKB-2, -15, -16, and -17/1 cells, respectively. The cells were maintained either in HITES medium (23) or in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 4.5 g/liter of D-glucose in 95% air/5% CO<sub>2</sub> atmosphere.

**Peptides.** Bombesin was purchased from Peninsula Laboratories (Belmont, CA). Substance P, neurokinin A, neurokinin B, physalaemin, and [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]substance P were synthesized by a solid-phase method (24, 25).

**Measurement of Intracellular Free Ca<sup>2+</sup> Concentration ([Ca<sup>2+</sup>]<sub>i</sub>) with Fura-2.** The detailed method for determination of [Ca<sup>2+</sup>]<sub>i</sub> is described elsewhere (26). Approximately 10<sup>6</sup> cells were used in each set of experiments. The free Ca<sup>2+</sup> concentration was calculated from the measurements of the ratio of fluorescence intensities as described by Grynkiewicz *et al.* (27).

**Measurement of DNA Synthesis.** Incorporation of [<sup>3</sup>H]thymidine into DNA was measured as previously described (12, 20, 26). Swiss 3T3 cells were made confluent and quiescent prior to experiments as described. SCLC cells were plated at 1 × 10<sup>4</sup> cells/well (24-well plates, Corning) in growth medium, and on the next day the medium was replaced with serum-free medium containing 0.2% bovine serum albumin, in which SCLC cells tested in the present study still continued to proliferate for at least 48 h, although much slower than in growth medium. The cells were incubated in the presence or absence of test substances for 24 h, and [<sup>3</sup>H]thymidine (1 μCi/ml) was added for the final 4 h of incubations.

## RESULTS

**Effects of Neuropeptides on Intracellular Free Ca<sup>2+</sup> Concentration ([Ca<sup>2+</sup>]<sub>i</sub>) in SCLC Cells.** Shown in Fig. 1, A and B, are

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<sup>3</sup> The abbreviations used are: SCLC, small cell lung carcinoma; GRP, gastrin-releasing peptide.

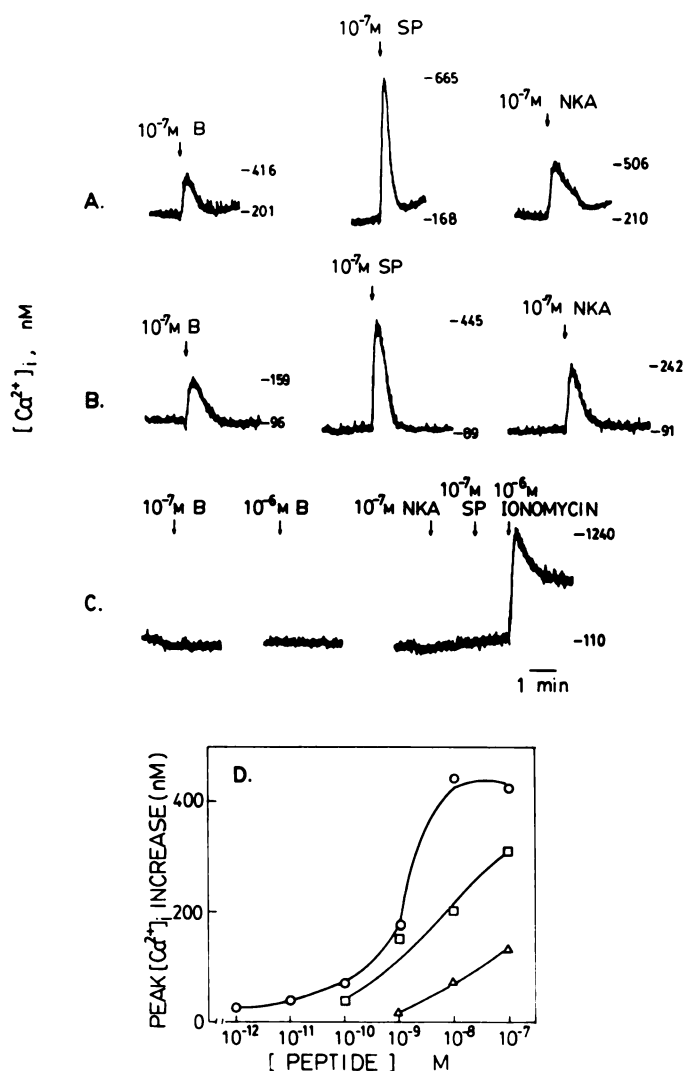


Fig. 1. Effects of various neuropeptides and a calcium ionophore ionomycin on the intracellular free  $\text{Ca}^{2+}$  concentration  $[\text{Ca}^{2+}]_i$  in SCLC cells TKB-2 (A), TKB-17/1 (B), and TKB-15 (C).  $[\text{Ca}^{2+}]_i$  was measured by use of a  $\text{Ca}^{2+}$  indicator fura-2 as described in "Materials and Methods." B, bombesin; SP, substance P; NKA, neurokinin A. D, dose-dependent relationships between the neuropeptide concentration and the peak  $[\text{Ca}^{2+}]_i$  increase in TKB-2 cells.  $\circ$ , substance P;  $\square$ , neurokinin A; and  $\Delta$ , bombesin. The results represent the mean values of four independent experiments.

typical tracings representing the effects of various neuropeptides on  $[\text{Ca}^{2+}]_i$  in SCLC cell lines TKB-2 and TKB-17/1, respectively. In both cell lines bombesin, substance P, and neurokinin A all induce a transient rise in  $[\text{Ca}^{2+}]_i$  at  $10^{-7}$  M. Neurokinin B and physalaemin, an amphibian counterpart of substance P, also induce a similar  $\text{Ca}^{2+}$  transient in these cells (see Fig. 2). In TKB-16, by contrast, only bombesin induces a comparable change in  $[\text{Ca}^{2+}]_i$  (see Fig. 5B). In TKB-15, none of the peptides tested induces a detectable increase in  $[\text{Ca}^{2+}]_i$  (Fig. 1C). In responsive cells, the effects of the peptides on  $[\text{Ca}^{2+}]_i$  are dose dependent. Fig. 1D depicts dose-dependent increases in  $[\text{Ca}^{2+}]_i$  in TKB-2 cells, in which the  $\text{Ca}^{2+}$  mobilizing effects of substance P and neurokinin A are larger than that of bombesin. All these peptides induce substantial increases in  $[\text{Ca}^{2+}]_i$  even in the presence of 1 mM EGTA without added  $\text{Ca}^{2+}$  (Fig. 2), indicating that the peptides mobilize  $\text{Ca}^{2+}$  at least from an intracellular store(s).

**Effects of Neuropeptides on DNA Synthesis in SCLC Cells.** In order to know whether these  $\text{Ca}^{2+}$ -mobilizing peptides have any effect on DNA synthesis in SCLC cells, we measured  $[\text{H}]$ -

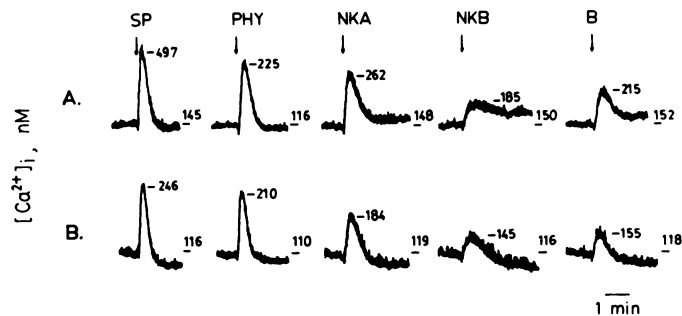


Fig. 2. Effects of various neuropeptides on  $[\text{Ca}^{2+}]_i$  in TKB-2 cells in the presence of 1.25 mM  $\text{Ca}^{2+}$  (A) and in the absence of  $\text{Ca}^{2+}$  but in the presence of 1 mM EGTA (B). SP, substance P; PHY, physalaemin; NKA, neurokinin A; NKB, neurokinin B; B, bombesin. The concentration of each neuropeptide is  $10^{-7}$  M.

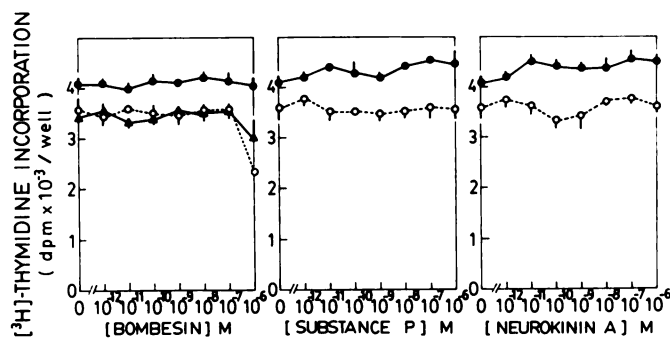


Fig. 3. Effects of various concentrations of neuropeptides on  $[\text{H}]$ thymidine incorporation into DNA in TKB-2 ( $\bullet$ ), TKB-16 ( $\blacktriangle$ ), and TKB-17/1 ( $\circ$ ). The results represent the mean  $\pm$  SD of three determinations. Where bars are absent, SD values are smaller than half the sizes of the symbols.

thymidine incorporation into DNA in the presence of various concentrations of the peptides (Fig. 3). By contrast to their effects on  $[\text{Ca}^{2+}]_i$ , none of the peptides shows a significant increase in  $[\text{H}]$ thymidine incorporation at any concentration from  $10^{-12}$  to  $10^{-6}$  M in TKB-2, -16, or -17/1 cells. A high concentration of bombesin ( $10^{-6}$  M) rather inhibits DNA synthesis in TKB-16 and TKB-17/1 ( $P < 0.01$  and  $P < 0.001$ , respectively). In another set of experiments total incubation time was extended to 48 h, but similar results were obtained (data not shown).

**Effects of a Bombesin Antagonist [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]Substance P on  $[\text{Ca}^{2+}]_i$  and DNA Synthesis in SCLC Cells and Swiss 3T3 Cells.** We further tried to evaluate the role of bombesin in growth of SCLC cells by employing a recently reported potent bombesin antagonist [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]substance P (28). First we studied the effect of the antagonist in Swiss 3T3 fibroblasts, in which the mitogenic effect of bombesin is well characterized (8). As shown in Fig. 4A, the antagonist at  $10^{-5}$  M completely abolishes the  $\text{Ca}^{2+}$ -mobilizing effect of  $10^{-9}$  M bombesin, but the subsequent addition of  $10^{-7}$  M bombesin overcomes the antagonizing effect. Concomitantly,  $10^{-5}$  M of the antagonist inhibits the mitogenic effect of  $10^{-9}$  M bombesin, but  $10^{-7}$  M bombesin overcomes the inhibitory effect of the antagonist (Fig. 4B). These results confirm the findings reported by Woll and Rozengurt (28), and verify the usefulness of the antagonist. As shown in Fig. 5, in bombesin responsive SCLC cells, TKB-2 and -16, the antagonist at  $10^{-5}$  M completely abolishes the  $\text{Ca}^{2+}$ -mobilizing effect of  $10^{-7}$  M bombesin, indicating that the antagonist is also a fully effective bombesin-antagonist in these SCLC cells. However, the antagonist at this concentration ( $10^{-5}$  M) is without a significant effect on DNA synthesis in TKB-2 and TKB-17/1, and is only partially (25%) inhibitory in TKB-16 and TKB-15. At higher concentrations of

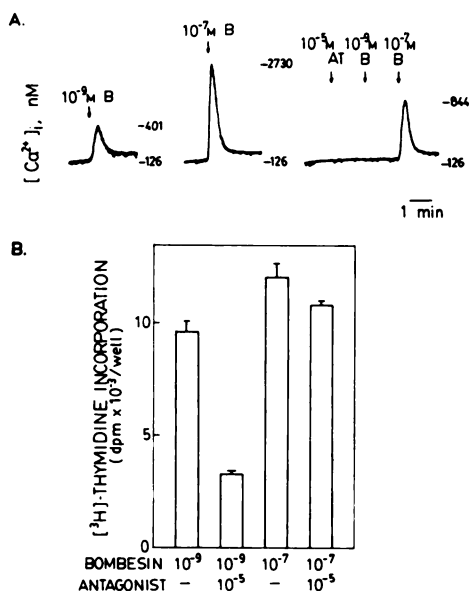


Fig. 4. *A*, effects of bombesin (*B*) and the bombesin antagonist [D-Arg<sup>1</sup>, D-Phe<sup>3</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]substance P (*AT*), on  $[Ca^{2+}]_i$  in Swiss 3T3 fibroblasts. *B*, effects of bombesin and the antagonist on  $[^3H]$ thymidine incorporation into DNA in Swiss 3T3 fibroblasts. The concentrations of the peptides are given in M at the bottom. The results are the mean  $\pm$  SD of three determinations.

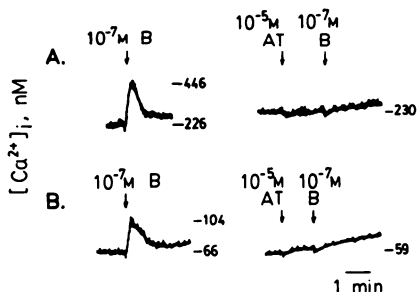


Fig. 5. Effects of bombesin (*B*) and the bombesin antagonist (*AT*) on  $[Ca^{2+}]_i$  in TKB-2 (*A*) and TKB-16 (*B*).

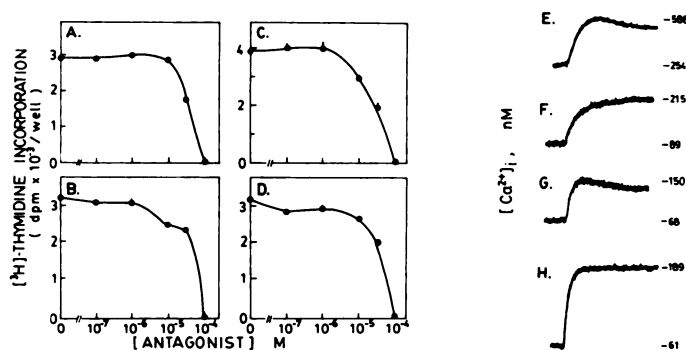


Fig. 6. *A-D*, effects of various concentrations of the bombesin antagonist on  $[^3H]$ thymidine incorporation into DNA in TKB-2 (*A*), TKB-15 (*B*), TKB-16 (*C*), and TKB-17/1 (*D*). The results are the mean  $\pm$  SD of three determinations. *E-H*, effects of the bombesin antagonist ( $10^{-5}$  M) on  $[Ca^{2+}]_i$  in TKB-2 (*E*), TKB-15 (*F*), TKB-16 (*G*), and TKB-17/1 (*H*).

the antagonist, it potently inhibits DNA synthesis in all four SCLC cells (Fig. 6*A-D*). It is to be noted that TKB-2 and TKB-15 do not produce measurable amounts of bombesin/GRP-related peptides (see "Materials and Methods"). Moreover, TKB-15 fails to respond with an increase in  $[Ca^{2+}]_i$  to bombesin for up to  $10^{-6}$  M (Fig. 1*C*). Yet the dose-response curves for the inhibitory effects of the antagonist on  $[^3H]$ -thymidine incorporation into DNA are quite similar among the

four SCLC cells. These findings strongly suggest that the inhibitory effect on DNA synthesis of high concentrations of the bombesin antagonist may be mediated through a mechanism other than bombesin receptor antagonism. In fact, the antagonist at  $10^{-4}$  M by itself induces an unexplained sustained increase in  $[Ca^{2+}]_i$  in all four SCLC cell lines including TKB-2 and TKB-15 (Fig. 6*E-H*), indicating that the antagonist has as yet an unidentified effect on SCLC cells at this concentration. Moreover, careful analysis of the dose-response curve for the inhibitory effect of the antagonist on DNA synthesis in Swiss 3T3 cells has revealed a paradoxical stimulatory effect of the antagonist: as shown in Fig. 7*A*,  $10^{-5}$  to  $3 \times 10^{-5}$  M of the antagonist by itself moderately stimulates  $[^3H]$ thymidine incorporation into DNA in Swiss 3T3 cells. Accordingly, the apparent dose-response curve for the inhibitory effect of the antagonist on bombesin ( $10^{-9}$  M)-induced  $[^3H]$ thymidine incorporation shows a small hump at  $3 \times 10^{-5}$  M of the antagonist, which is a sum of the paradoxical stimulatory effect of the antagonist by itself and the inhibitory effect on bombesin action. Interestingly, the stimulatory effect of the antagonist on DNA synthesis is enhanced in the presence of other growth factors such as insulin-like growth factor I and endothelin, which we have recently reported to be mitogenic for this cell type (26) (Fig. 7*B*).

## DISCUSSION

The present study demonstrates for the first time that tachykinin neuropeptides, including substance P, neurokinins, and physalaemin, induce a transient increase in  $[Ca^{2+}]_i$  in a subset of SCLC cells (Fig. 1). The responding cells include both bombesin/GRP-producing (TKB-17/1) and -nonproducing (TKB-2) cells. The effects of substance P and neurokinin A in these cells are even greater than that of bombesin at equimolar concentrations (Figs. 1 and 2). All these peptides induce  $Ca^{2+}$

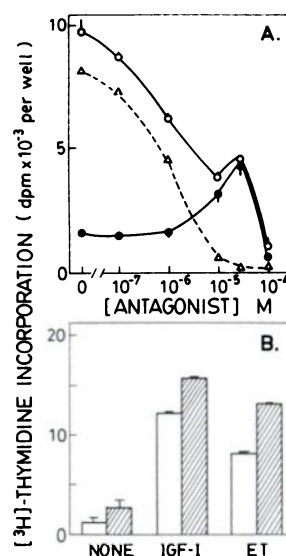


Fig. 7. *A*, effects of various concentrations of the bombesin antagonist on  $[^3H]$ thymidine incorporation into DNA in Swiss 3T3 fibroblasts, in the presence ( $\circ$ ) or absence ( $\bullet$ ) of  $10^{-9}$  M bombesin. The data are the mean  $\pm$  SD of three determinations. The difference between the two curves, plotted with a dotted line, represents the true dose-response curve as a bombesin antagonist: note that  $10^{-3}$  M antagonist almost completely abolishes the mitogenic effect of  $10^{-9}$  M bombesin. *B*, effects of insulin-like growth factor-I (*IGF-I*) and endothelin (*ET*) on  $[^3H]$ -thymidine incorporation into DNA in Swiss 3T3 fibroblasts in the presence ( $\blacksquare$ ) or absence ( $\square$ ) of the bombesin antagonist. The concentrations used are: *IGF-I*, 30 ng/ml; *ET*,  $10^{-9}$  M; the bombesin antagonist,  $3 \times 10^{-5}$  M. The results are the mean  $\pm$  SE of three determinations.

mobilization mainly from an intracellular store(s) (Fig. 2), suggesting that the  $\text{Ca}^{2+}$  mobilization by these peptides is most likely mediated by a second messenger inositol-1,4,5-trisphosphate as demonstrated in other types of cells (12, 15, 16).

It is well known that bombesin/GRP stimulates growth of diverse types of cells, including rat gastrin-secreting cells, normal human bronchial epithelial cells, and Swiss-mouse 3T3 fibroblasts (8, 29, 30). In addition, the peptide is proposed to act as an autocrine growth factor on SCLC cells (5–7). In Swiss 3T3 fibroblasts, previous studies by us and other investigators have clearly shown that bombesin-induced phospholipase C activation triggers the following reaction cascade leading to DNA synthesis (11, 12). Based on these observations, it would be quite natural to expect that tachykinin neuropeptides, by acting in a similar way as bombesin/GRP, stimulate growth of SCLC cells. Unexpectedly, however, neither substance P, neurokinin A, nor bombesin stimulates DNA synthesis in TKB-2, -16, or -17/1 (Fig. 3). The inability to detect any stimulatory effect of the peptides is not due to inappropriate assay conditions adopted, since we could reproducibly detect stimulatory effects of other mitogens including serum growth factors and a tumor promoter (see below). These findings indicate that  $\text{Ca}^{2+}$  mobilizing agonists do not always act as a mitogen on SCLC cells. In our previous study on Swiss 3T3 fibroblasts we showed that mitogenic potency of  $\text{Ca}^{2+}$  mobilizing agonists correlates to their effects on sustained production of 1,2-diacylglycerol, a product of the phospholipase C action, rather than their effects on the initial transient  $\text{Ca}^{2+}$  mobilization (26). It is possible that in these cells production of 1,2-diacylglycerol is for some reason inadequate to effectively activate protein kinase C, since a potent protein kinase C activator phorbol-12,13-dibutyrate (100 nM) does stimulate DNA synthesis in these cells.<sup>4</sup> Alternatively, the  $\text{Ca}^{2+}$  mobilizing agonists may indeed fully activate protein kinase C, which, however, is insufficient as a signal to induce DNA synthesis in SCLC cells. Under such an occasion the observed mitogenic effect of the phorbol ester could be mediated by a mechanism other than protein kinase C activation (31, 32).

Another interesting finding in the present study is the fact that a bombesin antagonist [D-Arg<sup>1</sup>, D-Phe<sup>5</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]-substance P, which potently inhibits mitogenic effects of bombesin in Swiss 3T3 cells (Fig. 4B) (28), does not inhibit DNA synthesis of bombesin/GRP-producing SCLC cells at a concentration ( $10^{-5}$  M) that effectively antagonizes bombesin ( $10^{-7}$  M)-induced  $\text{Ca}^{2+}$  mobilization in these cells (Figs. 5 and 6). At higher concentrations ( $\sim 10^{-4}$  M) the antagonist inhibits DNA synthesis in bombesin/GRP-nonproducing (TKB-2) and -nonproducing/nonresponding (TKB-15) SCLC cells as well, with a similar dose dependency as in producing (TKB-16 and 17/1) cell lines (Fig. 6). These observations strongly suggest that the antagonist inhibits growth of SCLC cells through a mechanism other than bombesin antagonism, and further support the view that bombesin/GRP, either produced by SCLC cells or exogenously applied, does not act as a mitogen on SCLC cells tested in the present study. Although the mechanism of inhibition of DNA synthesis in SCLC cells by high concentrations of the antagonist is not known at present, it may be related to the sustained elevation in  $[\text{Ca}^{2+}]_i$ , which was partially abolished by extracellular  $\text{Ca}^{2+}$  removal (data not shown). Recently, Layton *et al.* have reported a detailed study on growth of bombesin/GRP-producing SCLC cell lines by using two bombesin antagonists, spantide and [D-Arg<sup>1</sup>, D-Pro<sup>2</sup>, D-Trp<sup>7,9</sup>, Leu<sup>11</sup>]-substance

P. They concluded that growth of SCLC cells is not dependent on bombesin under all culture conditions tested *in vitro*, and that the bombesin antagonists inhibit the growth through a mechanism which is not mediated by the bombesin receptor (33). Their findings are in accordance with the present results.

In summary, the present study demonstrates that tachykinin neuropeptides and bombesin induce  $\text{Ca}^{2+}$  mobilization in a subset of SCLC cells, but not stimulation of DNA synthesis. Although many SCLC cells both produce and respond to bombesin/GRP or its related peptides, it seems that these peptides do not always act as an autocrine growth factor for SCLC. It is possible, however, that these peptides may indeed act as a growth factor *in vivo* especially during the early critical period of tumor evolution to sustain clonal expansion of preneoplastic cells, which through the course of "multistep" progression, may eventually transform to fully malignant cancer cells (34). Further studies are needed to elucidate the role of the peptides in the pathogenesis of SCLC.

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