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# Bombesin stimulation of DNA synthesis and cell division in cultures of Swiss 3T3 cells

(growth control/cAMP/insulin/vasopressin/litorin)

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**ABSTRACT** **Bombesin is shown to be a potent mitogen for Swiss 3T3 cells. At nanomolar concentrations the peptide markedly enhances the ability of fresh serum to stimulate DNA synthesis in confluent and quiescent cultures of these cells. In the presence of a low concentration (3.5%) of serum, bombesin stimulates 3T3 cell proliferation. In serum-free medium, bombesin induces DNA synthesis in the absence of any other added growth factor; half-maximal effect is obtained at 1 nM. The mitogenic effect of bombesin is dependent on dose and time, is mimicked by litorin, and is markedly potentiated by insulin, colchicine, platelet-derived growth factor, and fibroblast-derived growth factor. These mitogens increase the maximal response elicited by bombesin and decrease the bombesin concentration required to produce half-maximal effect (from 1 nM to 0.3 nM). In contrast, vasopressin, phorbol esters, or cAMP increasing agents fail to enhance the maximal level of DNA synthesis induced by bombesin. Bombesin and litorin may provide useful model peptides for studies on the mechanism(s) by which extracellular ligands control cell proliferation.**

In recent years, a considerable number of new regulatory peptides have been identified in the brain, gastrointestinal tract, and other tissues (1–3). Bombesin, a tetradecapeptide originally isolated from frog skin (4), and bombesin-like peptides have been detected in mammalian brain (5, 6), gut (5, 7), and lung (8, 9). This peptide has potent pharmacological effects on the central nervous system (10–12) and elicits the release of other peptide hormones including insulin (13, 14), glucagon (13, 14), gastrin (5, 7, 14), cholecystokinin (14), and prolactin and growth hormone (15, 16). Bombesin binds to specific surface receptors in pancreatic acinar cells (17) and stimulates ion fluxes (17, 18) and enzyme secretion (17, 18) in these cells.

Recently, several reports demonstrated the presence of high concentrations of bombesin in human pulmonary tumors (19–21). This observation and the report that repeated administration of bombesin induced pancreatic hyperplasia in the rat (22) raises the possibility that bombesin could participate in the control of cell proliferation, a proposition that hitherto remained unproven. Indeed, it is difficult to obtain unambiguous evidence for a direct growth-promoting activity of bombesin *in vivo* because the administration of this peptide stimulates the release of many other biologically active peptides (see above) which could act as proximal effectors of the action of bombesin.

Cultured cells provide a useful experimental system for elucidating the extracellular factors that control cell proliferation without the many complexities of whole animal experimentation. Many mammalian cells in culture, and Swiss 3T3 cells in particular, cease to proliferate and become arrested in the $G_1/G_0$ phase of the cell cycle when they deplete the nutrient medium of its growth-promoting activity (23). Addition of fresh serum or defined growth-promoting factors to such quiescent cells stimulates reinitiation of DNA synthesis and cell division (23). Ion fluxes (23, 24), cyclic nucleotides (25–27), or cytoskeletal changes (28) may play a role in mediating the action of the extracellular ligands.

In the present paper we report that bombesin is a potent mitogen for Swiss 3T3 cells. At low (namolar) concentrations, bombesin enhances the ability of fresh serum to initiate and support cell proliferation and stimulates initiation of DNA synthesis in Swiss 3T3 cells maintained in serum-free medium. The mitogenic effect is dose- and time-dependent, is specific, and is markedly enhanced by insulin and other growth-promoting agents.

## MATERIALS AND METHODS

**Cell Culture.** Stock cultures of Swiss 3T3 cells (29) were maintained in Dulbecco–Vogt modified Eagle's medium (DME medium) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 $\mu$g/ml) in a humidified atmosphere of 10% $CO_2$/90% air at 37°C. For experimental purposes, $10^5$ cells were subcultured in 33-mm Nunc Petri dishes with 2 ml of DME medium containing 10% fetal bovine serum and used at least 6 days after the last change of medium. These cells were arrested in the $G_1/G_0$ phase of the cell cycle as judged by cytofluorometric analysis and by the fact that only 1% of cells were autoradiographically labeled after a 40-hr continuous exposure to [$^3$H]thymidine.

**Assays of Growth-Promoting Activity.** All determinations of DNA synthesis were performed in a 1:1 mixture of DME medium and Waymouth medium. The cultures were washed twice with DME medium to remove residual serum immediately prior to assay. For determinations of DNA synthesis, the medium (2 ml) contained either 0.2 $\mu$M (5 $\mu$Ci/ml; 1 Ci = $3.7 \times 10^{10}$ Bq) or 1 $\mu$M (0.5 $\mu$Ci/ml) [$^3$H]thymidine for autoradiography or incorporation into acid-precipitable DNA, respectively (30). Cell number was determined by removing the cells from the dish with a trypsin solution (0.05% trypsin in $Ca^{2+}$- and $Mg^2$-free phosphate-buffered saline with EDTA) and counting a portion of the resulting cell suspension in a Coulter Counter. Each point represents an average of at least two determinations.

**Materials.** Bombesin, [Arg]vasopressin, vasoactive intestinal peptide, substance P, somatostatin, gastrin, and bovine insulin (26 international units/mg) were obtained from Sigma. Litorin and neurotensin were purchased from UCB-Bioproducts, Brussels, Belgium. Fibroblast-derived growth factor (FDGF) was isolated and purified from the medium conditioned by simian virus 40-infected baby hamster kidney cells as described

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Abbreviations: DME medium, Dulbecco–Vogt modified Eagle medium; PDGF, platelet-derived growth factor; FDGF, fibroblast-derived growth factor.

(31, 32). [$^3$H]Thymidine was from the Radiochemical Centre, Amersham, England. The serum used was fetal bovine (Flow Laboratories). All other materials used were of reagent grade.

## RESULTS

**Effect of Bombesin on Serum-Stimulated DNA Synthesis.** To determine whether bombesin can modulate the proliferative response of Swiss 3T3 cells, confluent and quiescent cultures of these cells were transferred to medium containing various concentrations of fresh fetal bovine serum in the absence or presence of bombesin. The cumulative incorporation of [$^3$H]-thymidine into DNA was measured after a 28-hr incubation. Bombesin added at 0.3 or 1.2 nM caused a marked shift of the dose–response relationship for the effect of serum on DNA synthesis in quiescent cultures of Swiss 3T3 cells (Fig. 1). A marked synergistic effect was observed between the peptide and low (1–3%) concentrations of fetal bovine serum that alone are incapable of inducing a substantial stimulation of DNA synthesis.

**Bombesin Stimulates DNA Synthesis in Serum-Free Medium.** Addition of bombesin to confluent and quiescent Swiss 3T3 cells in serum-free medium stimulated DNA synthesis in the absence of any other exogenously added growth factor (Fig. 2). Bombesin elicited a maximal effect at 3 nM; half-maximal effect was obtained at 1 nM. The results were similar whether DNA synthesis was assessed by incorporation of [$^3$H]thymidine into acid-insoluble material or by autoradiography of labeled nuclei. At 3 nM, bombesin stimulated 42% of the cells to enter DNA synthesis after a 40-hr incubation.

The DNA synthesis response elicited by bombesin was markedly potentiated by addition of insulin at 1 $\mu$g/ml (Fig. 2); this hormone increased the maximal response promoted by bombesin and decreased the peptide concentration required to produce half-maximal effect (from 1 nM to 0.3 nM). In the presence of insulin, bombesin at 1.5 nM stimulated 76% of the cells to initiate DNA synthesis after a 40-hr incubation. In separate experiments, we found that a marked potentiation of bombesin stimulation of DNA synthesis was also achieved when insulin was added at 0.1 $\mu$g/ml (results not shown).

The stimulation of DNA synthesis by bombesin and the synergistic interaction between bombesin and insulin were also assessed as a function of time. Bombesin or the combination of this peptide and insulin stimulated [$^3$H]thymidine incorporation after a lag period of about 15 hr (Fig. 3), which is comparable to that produced by addition of 10% fetal bovine serum to parallel cultures. The synergistic effect between bombesin

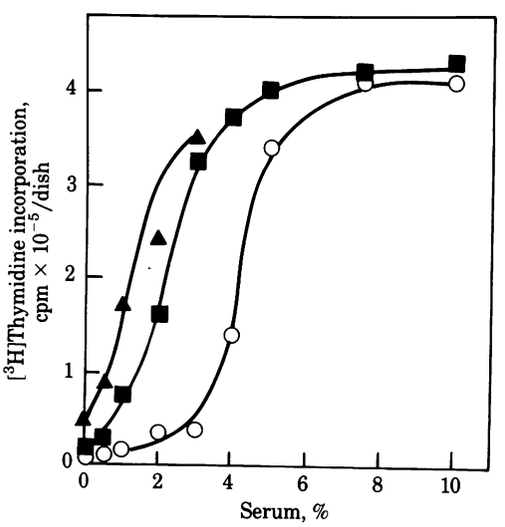


FIG. 1. Dose–response curves for the effect of serum on DNA synthesis by quiescent cultures of Swiss 3T3 cells in the absence (○) or presence of bombesin at 0.3 nM (■) or 1.2 nM (▲). The serum and the peptide were added to the cultures in 2 ml of DME/Waymouth medium containing [$^3$H]thymidine. Incorporation of radioactivity into acid-insoluble material was determined 28 hr later.

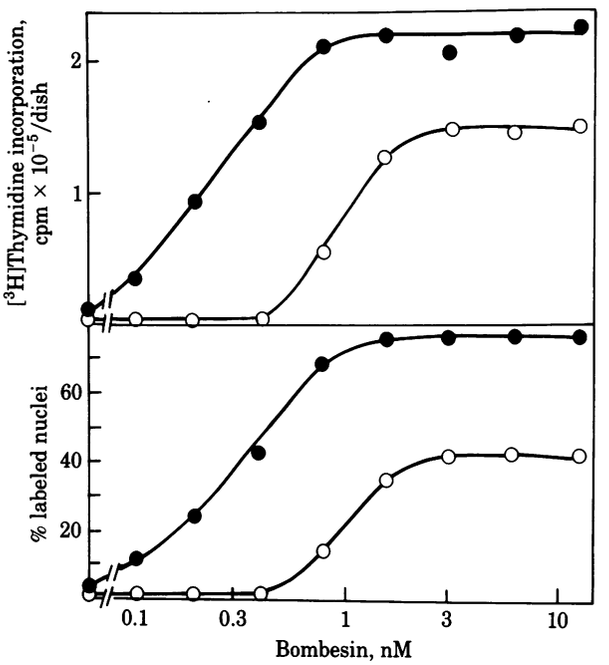


FIG. 2. Dose–response curves for the effect of bombesin on DNA synthesis by quiescent cultures of Swiss 3T3 cells in the absence (○) or in the presence (●) of insulin at 1 $\mu$g/ml. The peptides were added to confluent and quiescent cultures in 2 ml of DME/Waymouth medium containing [$^3$H]thymidine. DNA synthesis was assessed either by incorporation into acid-insoluble material (*Upper*) or by autoradiography (*Lower*) at 40 hr after continuous exposure to radioactive thymidine. In this experiment, addition of 10% fetal bovine serum produced 2.5 × $10^5$ cpm per dish incorporated into acid-insoluble material and 95% labeled nuclei.

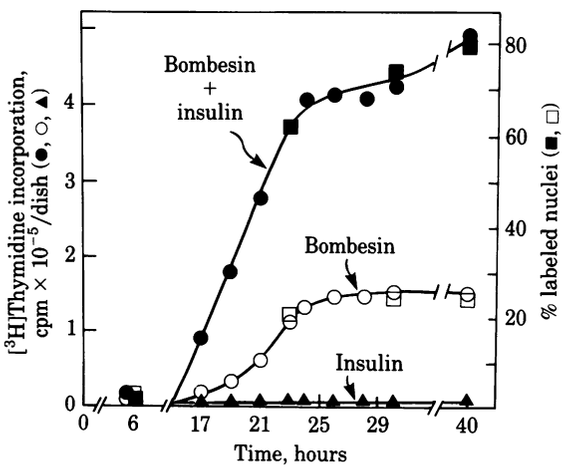


FIG. 3. Stimulation of DNA synthesis by bombesin, insulin, or both as a function of time. The peptides were added at 0 hr. At the times indicated, dishes from each group were washed and either treated with trichloroacetic acid for measuring the incorporation of radioactivity into acid-precipitable DNA or fixed and processed for autoradiography. The cultures were exposed to bombesin at 6 nM (○, □), to insulin at 1 $\mu$g/ml (▲), or to both (●, ■). Addition of medium containing insulin or 10% fetal bovine serum produced 3% and 75% labeled nuclei, respectively, at 40 hr.

and insulin results from a change in both the rate of entry into S and the maximal stimulation achieved. The latter reflects an increase in the fraction of labeled nuclei obtained in the presence of bombesin and insulin as compared with bombesin alone.

**Interaction of Bombesin with Other Growth-Promoting Agents.** FDGF, a potent mitogenic polypeptide produced by a transformed cell line (31, 32), markedly potentiated the stimulation of DNA synthesis by bombesin (Fig. 4 *Left*). FDGF decreased the concentration of bombesin required to produce half-maximal effect (from 0.8 nM to 0.2 nM) and enhanced the maximal effect achieved by saturating concentrations of bombesin. Similar results were obtained when partially purified platelet-derived growth factor (PDGF) was used instead of FDGF (results not shown).

Disruption of the microtubule network by colchicine enhances the stimulation of DNA synthesis induced by certain growth factors in quiescent cells (28). Addition of colchicine at 2.5 μM markedly enhanced the DNA synthetic response of Swiss 3T3 cells to bombesin (Fig. 4 *Right*). The potentiation of bombesin action by colchicine, FDGF, and PDGF was further substantiated when DNA synthesis was assessed by the percentage of labeled nuclei after autoradiography (Table 1).

The neurohypophyseal nonapeptide vasopressin (34, 35) and the potent tumor promoters of the phorbol ester family (36, 37) stimulate initiation of DNA synthesis in Swiss 3T3 cells by a common mechanism (36). Neither vasopressin nor phorbol dibutyrate enhanced the mitogenic effect of a saturating concentration (6 nM) of bombesin (Table 2). The responsiveness of the Swiss 3T3 cells to vasopressin or phorbol ester in these experiments is clearly shown by the striking synergistic increase in the labeling index obtained when these agents were added with insulin instead of with bombesin.

Recently, it was reported that an increase in intracellular cAMP caused by various agents acts synergistically with insulin and other growth factors to stimulate DNA synthesis in Swiss 3T3 cells (25–27). Cholera toxin and isobutylmethylxanthine or 8-bromo cAMP stimulated DNA synthesis when added in the presence of insulin but failed to enhance entry into S phase induced by bombesin (Table 2). Thus, although insulin, colchicine, FDGF, and PDGF potentiated the mitogenic effects of bombesin in Swiss 3T3 cells, neither vasopressin and phorbol esters nor cAMP-increasing agents enhanced the maximal effect of bombesin on DNA synthesis.

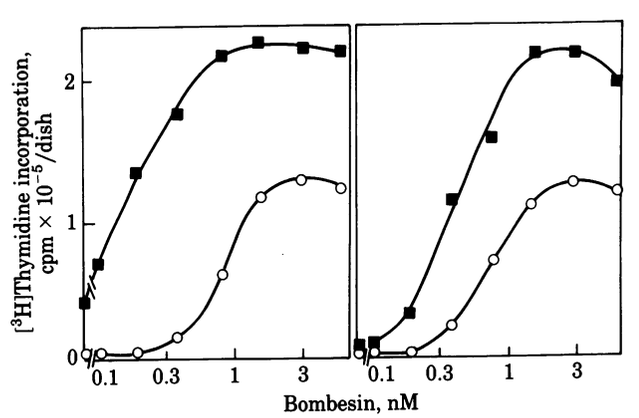


FIG. 4. Effect of FDGF (*Left*) or colchicine (*Right*) on bombesin stimulation of DNA synthesis in Swiss 3T3 cells. Confluent and quiescent cultures of these cells were exposed to 2 ml of DME/Waymouth medium containing [$^3$H]thymidine and various concentrations of bombesin either in the absence (○) or presence (■) of FDGF at 5 μg/ml (*Left*) or colchicine at 2.5 μM (*Right*). Incorporation of radioactivity into acid-insoluble material was assessed at 40 hr of continuous exposure to [$^3$H]thymidine.

Table 1. Effect of colchicine, PDGF, and FDGF on mitogenic activity of bombesin in Swiss 3T3 cells

| Addition* | % labeled nuclei |
|---|---|
| None | 1.5 |
| Colchicine | 1.8 |
| FDGF | 2.4 |
| PDGF | 15 |
| Bombesin | 32 |
| Bombesin + colchicine | 63 |
| Bombesin + FDGF | 69 |
| Bombesin + PDGF | 65 |
| 10% fetal bovine serum | 82 |

*The concentrations of colchicine, FDGF, PDGF, and bombesin were 2.5 μM, 4 μg/ml, 0.3 μg/ml, and 6 nM, respectively. PDGF was partially purified (700-fold) by Sulfadex gel chromatography and heating as described by Deuel *et al.* (33). DNA synthesis was assessed by autoradiography after 40 hr of continuous exposure to [$^3$H]thymidine.

**Specificity of Bombesin Stimulation of DNA Synthesis.** Several other regulatory peptides were tested both alone and with insulin (1 μg/ml) for their ability to stimulate DNA synthesis in quiescent Swiss 3T3 cells. Litorin, which has a COOH-terminal octapeptide in common with bombesin and closely mimics bombesin in its pharmacological effects (10, 17, 18, 38), is a potent mitogen for Swiss 3T3 cells; the maximal effect was identical to that elicited by bombesin and the half-maximal effect was obtained at 2 nM (results not shown). In contrast, substance P ($10–10^4$ ng/ml) or vasoactive intestinal peptide ($10–10^3$ ng/ml), which have slight structural similarity to bombesin but do not inhibit the binding of $^{125}$I-labeled [Tyr$^4$]bombesin to dispersed pancreatic acini (17) or brain membranes (39), failed to stimulate DNA synthesis. Likewise, somatostatin, cholecystokinin, gastrin, and neurotensin were inactive when tested in a wide range of concentrations ($10–10^3$ ng/ml).

Several cell lines, including BALB/c 3T3, BHK, and rat-1, which were rendered quiescent by growth to confluency (BALB/

Table 2. Effect of vasopressin, phorbol dibutyrate, 8-bromo cAMP, or cholera toxin on bombesin stimulation of DNA synthesis in Swiss 3T3 cells

| Addition* | Bombesin | Insulin | % labeled nuclei |
|---|---|---|---|
| None | − | − | 1 |
| | − | + | 5 |
| | + | − | 32 |
| | + | + | 73 |
| Vasopressin | − | − | 1.4 |
| | + | − | 30 |
| | − | + | 50 |
| PDBu | − | − | 1 |
| | + | − | 28 |
| | − | + | 46 |
| 8BrcAMP | − | − | 1 |
| | + | − | 30 |
| | − | + | 36 |
| Cholera toxin/IBMX | − | − | 3 |
| | + | − | 31 |
| | − | + | 58 |

*The concentrations of bombesin, insulin, vasopressin, phorbol 12,13-dibutyrate (PDBu), 8-bromo cAMP (8BrcAMP), cholera toxin, and isobutylmethylxanthine (IBMX) were 6 nM, 1 μg/ml, 20 ng/ml, 100 ng/ml, 2 mM, 100 ng/ml, and 50 μM. DNA synthesis was assessed by autoradiography after 40 hr of continuous exposure to [$^3$H]thymidine.

c 3T3) or by incubation in 0.5% serum for 5 days (BHK and rat-1) failed to respond to bombesin. Conversely, two clonal lines derived from Swiss 3T3 cells responded to bombesin with a dose–response curve similar to that obtained in the cultures used in the present studies (results not shown). Thus, the growth-promoting effects of bombesin appear to be relatively specific for Swiss 3T3 cells.

**Bombesin Stimulation of Swiss 3T3 Proliferation.** The mitogenic activity of bombesin in cultures of Swiss 3T3 cells also could be demonstrated when cell number (rather than [$^3$H]thymidine incorporation into acid-precipitable material) was monitored over a period of several days. In the presence of 3.5% serum, bombesin at 6.7 nM induced a 2.5-fold increase in cell number (Fig. 5). Addition of bombesin with insulin (1 $\mu$g/ml) resulted in a 5-fold increase in cell number. When these peptides were added 3 days after the start of the experiment, they stimulated reinitiation of cell proliferation (Fig. 5*A*, broken line).

Bombesin stimulated cell proliferation in a concentration-dependent manner; the maximal effect was achieved at 3 nM (Fig. 5*B*). The range of bombesin concentration that stimulated increases in cell number was somewhat lower than that required to stimulate DNA synthesis in serum-free medium. This difference can be accounted for by the synergism that exists between the peptide and growth factors contributed by the 3.5% serum present in the assays of cell growth (see Fig. 1). Addition of bombesin at 6 nM to the medium in which confluent and quiescent 3T3 cells were grown (depleted medium) resulted in loss of density-dependent inhibition of growth; the mean (± SEM) saturation density (cells per cm$^2$) of Swiss 3T3 cells increased from 48.85 ± 1.9 × $10^3$ to 79.67 ± 0.5 × $10^3$ after a 72-hr incubation in the presence of bombesin at 6 nM.

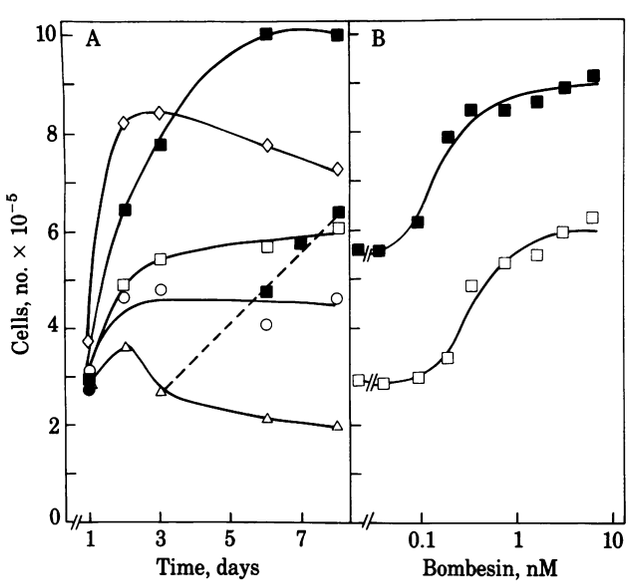


FIG. 5. (*A*) Effect of bombesin, insulin, or both on the proliferation of Swiss 3T3 cells. The cells were seeded at 2 × $10^5$ per 5-cm plastic dish containing 5 ml of DME/Waymouth medium supplemented with 3.5% fetal bovine serum. After 24 hr, the medium was replaced by fresh DME/Waymouth medium containing 3.5% fetal bovine serum (△). Some cultures received bombesin (□) at 6.7 nM, insulin (○) at 1 $\mu$g/ml, or both hormones (■, solid line). Growth of cultures in medium with 10% serum is also shown for comparison (◇). Some cultures plated in DME/Waymouth medium containing 3.5% fetal bovine serum received bombesin and insulin (at the concentrations stated above) 3 days after the start of the experiment (■, broken line). Cells were counted by removing them from the dish with a trypsin solution and counting a portion of the resulting suspension in a Coulter Counter. Each point represents the mean of two determinations. (*B*) Dose–response curves for the effect of bombesin on growth of Swiss 3T3 cells. The cells were seeded as above. After 24 hr, the medium was changed and the cultures received different concentrations of bombesin in the absence (□) or presence (■) of insulin at 1 $\mu$g/ml. Cells were counted at 4 days after the start of the experiment.

## DISCUSSION

The present results demonstrate that the synthetic tetradecapeptide bombesin is a potent mitogen for quiescent cultures of Swiss 3T3 cells. The peptide can induce initiation of DNA synthesis and cell division at concentrations (0.1–1 nM) that are close to the level of bombesin-like immunoreactivity detected in rat plasma [0.5 nM (40)]. The mitogenic effectiveness of bombesin is markedly enhanced by insulin, colchicine, FDGF, and PDGF, suggesting that bombesin does not act identically to any of these factors. The mitogenic effects of bombesin can be mimicked by litorin, which has a COOH-terminal octapeptide in common with bombesin (10, 17, 38), but not by a number of other regulatory peptides including high concentrations of substance P. In view of the potency and specificity of the long-term effects of bombesin on Swiss 3T3 cells, it is reasonable to suggest that homogeneous cultures of this cell line may provide a useful system for *in vitro* study of the mechanism of action and functional significance of the peptides of the bombesin family.

Bombesin stimulates reinitiation of DNA synthesis in serum-free medium in the absence of any other added growth factor. This contrasts with many other ligands including insulin (34–37), vasopressin (34, 35), phorbol esters (36, 37), cAMP-increasing agents (25–27), colchicine (28), and EGF (36) which stimulate DNA synthesis in Swiss 3T3 cells only when added in the presence of other synergistic growth factors. Interestingly, vasopressin and phorbol esters which modulate diverse cellular functions by a common mechanism (34–37) or the cAMP-increasing agents cholera toxin and 8-bromo cAMP (25, 27) fail to enhance the maximal level of DNA synthesis induced by bombesin in 3T3 cells. These findings suggest that the pathways activated by bombesin partly converge with those utilized by two different classes of mitogenic agents. The fact that cAMP is synergistic with vasopressin (25, 27) or phorbol esters (25, 27, 41) in stimulating DNA synthesis in Swiss 3T3 cells raises the intriguing possibility that bombesin or litorin interacts with these cells to elicit the generation of two complementary mitogenic signals which lead synergistically to cellular DNA synthesis.

In recent years, there has been a considerable interest in regulatory peptides that act locally in a paracrine (1) or autocrine (42) fashion. An array of recently discovered peptides in the brain and in the gut, including bombesin and bombesin-like peptides, appear to exert modulatory influences on adjacent cells as local hormones or neurotransmitters (1, 2). Paracrine or autocrine modes of action of biologically active peptides are becoming a matter of intense interest in another recent and rapidly evolving field—namely, that of growth factors released by tumor cells. Production of growth factors by tumor cells may play a role in sustaining a self-stimulatory (autocrine) circuit which may contribute to the unregulated growth of malignant cells (31, 32, 42–47). Recently, the level of bombesin has been found greatly increased in samples of small cell carcinoma of the lung (19), in cell lines established from this type of tumor (20), and in extracts of tumors grown in the *nude* mouse (21). In view of the findings reported here on the potent mitogenic effects of bombesin, it is plausible that enhanced production of bombesin by small carcinoma cells may play a role in the pathogenesis of this important cancer as part of an autocrine circuit which contributes to the rapid growth of the tumor.

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