Early Events Elicited by Bombesin and Structurally Related Peptides in Quiescent Swiss 3T3 Cells. I. Activation of Protein Kinase C and Inhibition of Epidermal Growth Factor Binding

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Abstract. Addition of bombesin to quiescent cultures of Swiss 3T3 cells caused a rapid increase in the phosphorylation of an Mr 80,000 cellular protein (designated 80k). The effect was both concentration and time dependent; enhancement in 80k phosphorylation could be detected as early as 10 s after the addition of peptide. Recently, a rapid increase in the phosphorylation of an 80k cellular protein after treatment with phorbol esters or diacylglycerol has been shown to reflect the activation of protein kinase C in intact fibroblasts (Rozengurt, E., A. Rodriguez-Pena, and K. A. Smith, 1983, Proc. Natl. Acad. Sci. USA., 80:7244-7248; Rozengurt, E., A. Rodriguez-Pena, M. Coombs, and J. Sinnett-Smith, 1984, Proc. Natl. Acad. Sci. USA., 81:5748-5752). The 80k phosphoproteins generated in response to bombesin and to phorbol 12,13-dibutyrate were identical as judged by one- and two-dimensional PAGE and by peptide mapping after partial proteolysis with Staphylococcus aureus V8 protease. In addition, prolonged pretreatment of 3T3 cells with phorbol 12,13-dibutyrate, which leads to the disappearance of protein kinase C activity, blocked the ability of bombesin to stimulate 80k. Bombesin also caused a rapid (1 min) inhibition of 125I-labeled epidermal growth factor (125I-EGF) binding to Swiss 3T3 cells. The inhibition was both concentration and temperature dependent and resulted from a marked decrease in the affinity of the EGF receptor for its ligand. Peptides structurally related to bombesin, including gastrin-releasing peptide, also stimulated 80k phosphorylation and inhibited 125I-EGF binding; both effects were selectively blocked by a novel bombesin antagonist. These results strongly suggest that these responses are mediated by specific high-affinity receptors that recognize the peptides of the bombesin family in Swiss 3T3 cells. While an increase in cytosolic Ca²⁺ concentration does not mediate the bombesin inhibition of 125I-EGF binding, the activation of protein kinase C in intact Swiss 3T3 cells by peptides of the bombesin family may lead to rapid inhibition of the binding of 125I-EGF to its cellular receptor.

A large number of new regulatory peptides have been identified in the brain, gastrointestinal tract, and other tissues (13, 23, 45). These peptides, which elicit a multiplicity of biological responses, appear to behave as local hormones or neurotransmitters acting in a paracrine or autocrine fashion on adjacent cells. Bombesin, a tetradecapeptide originally isolated from amphibian skin (1), has potent pharmacological effects in animals (22, 47, 67, 68), and peptides structurally related to bombesin, including gastrin-releasing peptide (GRP) and the neuromedins have been identified in a number of mammalian tissues (21, 36, 38-40). Recently, several reports have also demonstrated the presence of high levels of bombesin-like peptides in human pulmonary (19, 41, 52, 72) and thyroid (35) carcinomas.

Bombesin is a potent mitogen for Swiss 3T3 cells (56). Strikingly, in contrast to other growth-promoting agents, bombesin shares with both platelet-derived growth factor (PDGF) (51) and the PDGF-like fibroblast-derived growth factor (FDGF) (18, 58) the ability to stimulate DNA synthesis and cell division in the absence of other mitogens. Recently, we demonstrated that mammalian analogues of bombesin are also mitogenic for Swiss 3T3 cells. The mitogenic response to these peptides is mediated by specific, high-affinity receptors that are distinguishable from those of other growth factors for these cells, including PDGF (73). Furthermore, both receptor binding and mitogenesis are selectively blocked by a novel bombesin antagonist (27, 73). Thus, this family of peptides...
offers a novel and attractive model for testing the validity of current hypotheses regarding the control of cellular proliferation (53, 55).

In this and the following paper (37), we have examined the effect of bombesin and structurally related peptides on some of the initial cellular responses that are being implicated in the control of a proliferative response (53). These studies on the mechanism of bombesin-induced mitogenesis assume an added significance in view of recent findings implicating bombesin in a self-stimulatory (autocrine) growth circuit that contributes to the unrestrained growth of small cell carcinoma of the lung (14, 65).

Ca$^{2+}$-sensitive, phospholipid-dependent protein kinase (protein kinase C) which is stimulated by diacylglycerol and by tumor promoters of the phorbol ester family (42), may play a fundamental role in the stimulation of fibroblast proliferation (for review see reference 60). It was, therefore, of importance to determine whether bombesin and related peptides activate protein kinase C in intact cells. Recently, we found that a rapid increase in the phosphorylation of an M$_r$ 80,000 cellular protein (designated 80k) reflects the activation of protein kinase C in intact cells (60, 61). The salient features of the evidence supporting this conclusion are as follows. (a) Biologically active phorbol esters or diacylglycerols either exogenously added (59) or endogenously generated (61) stimulate a rapid phosphorylation of this protein. (b) Prolonged pretreatment of 3T3 cells with phorbol ester, which leads to a marked decrease in the number of specific phorbol ester-binding sites (8–10, 54) and to the disappearance of protein kinase C activity in vitro (48, 60) blocks the increase in 80k phosphorylation elicited by phorbol esters or diacylglycerol. (c) The same 80k phosphorylation can be generated in cell-free extracts by activation of protein kinase C (i.e., by addition of phosphatidylserine, Ca$^{2+}$, and phorbol 12,13-dibutyrate [PBD$_2$] in the presence of [$\gamma$-$^32$P]ATP) (50). The nature of 80k remains to be established, but the detection of changes in its phosphorylation provides a novel approach with which to assess the mitogenic agents that activate protein kinase C in intact cells.

Recent findings obtained with intact cells (15, 26, 64) and cell-free systems (7, 25) suggest that activation of protein kinase C may play a central role in the modulation of the affinity of epidermal growth factor (EGF) receptors for its ligand by a set of structurally unrelated mitogens (3, 5, 11, 30, 34, 57, 58, 62–64). It has also been reported that bombesin reduces the association of 125I-EGF to 3T3 cells (4) and to pancreatic acinar cells, an effect apparently mediated by an increase in the cytosolic Ca$^{2+}$ concentration (28, 31, 46). In the course of our studies concerning the early events elicited by peptides of the bombesin family in quiescent Swiss 3T3 cells, we examined the effect of these peptides on the binding and internalization of 125I-EGF into Swiss 3T3 cells, and the parts played by protein kinase C and an increase in cytosolic Ca$^{2+}$ in mediating the inhibition of 125I-EGF association caused by bombesin.

On the basis of the results presented here, we conclude that the activation of protein kinase C is one of the earliest events that follows the interaction of peptides of the bombesin family with specific, high-affinity receptors. Our findings further show that activation of protein kinase C leads to the rapid decrease in 125I-EGF binding caused by these peptides.

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**Materials and Methods**

**Cell Culture**

Swiss 3T3 cells (69) were maintained as previously described and subcultured to 33-mm Nun dishes (10$^5$ cells/dish) in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum. After 5–7 d, the cultures were confluent and quiescent as shown by autoradiography (<1% labeled nuclei) after a 40-h exposure to [$^3$H]thymidine (16).

**Labeling of Cells with $^32$P$_i$**

The cultures were washed twice with DME without phosphate and incubated with this medium containing 200 or 400 ng/ml of carrier-free $^32$P$_i$ for both one- and two-dimensional PAGE at 37°C for 4 h to label the endogenous ATP pool. Bombesin, PBD$_2$, or other agents were then added for various times. The reaction was stopped by removing the medium and rapidly washing the cultures twice with ice-cold Tris-saline solution (0.15 M NaCl, 20 mM Tris-HCl, pH 7.5). The cells were immediately extracted with 5% trichloroacetic acid at 4°C for 20 min. The acid-soluble pools were removed; the precipitated protein was washed twice with Tris-saline and dissolved with 100 $\mu$l of a solution containing 3% SDS, 5% glycerol, 10 mM Tris-HCl, pH 7.8, 2% 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride heated at 100°C. After 2 min, the samples were placed in a boiling water bath for 10 min before resolution by gel electrophoresis.

**SDS PAGE**

Slab gel electrophoresis was performed using a 5–15% acrylamide gradient and 0.1% SDS (29). After electrophoresis, the slabs were stained, destained, and dried down onto paper for autoradiography with Fuji x-ray film (Fuji Photo Film Co. Ltd., Japan). Dried gels were exposed to film for 1–3 d.

Two-dimensional PAGE was carried out as described by O‘Farrell (43), using isoelectric focusing in the first dimension and SDS PAGE (10% polyacrylamide) in the second dimension. The samples were prepared for isoelectric focusing containing 1.4% LKB ampholytes, pH 5–7, plus 0.6% LKB ampholytes, pH 3–10, 0.1 M urea, and 2% Nonidet P-40. The approximate pH range of the two-dimensional gels shown throughout the present paper was 4.5 (left) to 6 (right).

 Autoradiograms were scanned using a Joyce-Loebl double-beam microdensitometer and the incorporation of $^32$P$_i$ into a specific protein band was quantitated by measuring the peak area (Hewlett-Packard digitizer) above background in densitometry tracing as previously described (61).

**Peptide Mapping by Limited Proteolysis**

Peptide mapping of the 80k phosphoprotein was carried out according to Cleveland et al. (6). The 80k band visualized by autoradiography was excised from several lanes of an SDS gel. Individual gel bands containing the same amount of counts were rehydrated for 2 h in a solution containing 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA, and placed into separate wells of a discontinuous SDS PAGE (15% acrylamide). The gel bands were overlayed with 100 $\mu$l of the same buffer containing 15% (wt/vol) glycerol, a trace amount of bromophenol blue, and different amounts of Staphylococcus aureus V8 protease as indicated. Electrophoresis was carried out at 60 V.

**125I-EGF Binding**

125I-EGF was prepared by the soluble lactoperoxidase procedure as previously described (37, 38). Cultures in 33-mm dishes were washed twice with DME at 37°C and incubated in 1 ml of binding medium (1:1 mixture of DME and Waymouth's medium containing 0.1% crystalline bovine serum albumin [BSA]) and 50 mM N,N bis [2-hydroxyethyl]-2-aminoethane sulphonic acid, pH 7.0, at 37°C, or phosphate-buffered saline [PBS] containing 0.1% crystalline BSA and 25 mM Hepes, pH 7.0, at 4°C. After the specified incubation period, the monolayer was washed rapidly three times with cold (4°C) PBS (pH 7.2) containing 0.1% BSA, extracted with 0.1 N NaOH containing 2% Na$_2$CO$_3$ and 1% SDS, and cell-associated radioactivity was determined in a gamma counter. Nonspecific binding, determined as cell-associated radioactivity in the presence of a 250-fold excess of unlabeled EGF (or 250 ng/ml of EGF at 37°C and in 1 ml of binding medium (1:1 mixture of DME and Waymouth's) containing 0.1% crystalline bovine serum albumin [BSA]), and 50 mM N,N bis [2-hydroxyethyl]-2-aminoethane sulphonic acid, pH 7.0, at 37°C, or phosphate-buffered saline [PBS] containing 0.1% crystalline BSA and 25 mM Hepes, pH 7.0, at 4°C. After the specified incubation period, the monolayer was washed rapidly three times with cold (4°C) PBS (pH 7.2) containing 0.1% BSA, extracted with 0.1 N NaOH containing 2% Na$_2$CO$_3$ and 1% SDS, and cell-associated radioactivity was determined in a gamma counter. Nonspecific binding, determined as cell-associated radioactivity in the presence of a 250-fold excess of unlabeled EGF (or 250 ng/ml of EGF at concentrations of 125I-EGF below 1 ng/ml) varied linearly with 125I-EGF concentrations and was <5% of the total binding at 50 ng/ml of 125I-EGF and ~2% at 0.5 ng/ml in cultures whether or not pretreated with bombesin. All values shown are the mean of duplicate determinations. Cell numbers of parallel cultures were measured by trypsinization and a Coulter counter.

**Materials**

In all experiments bombesin, PBD$_2$, and BSA (essentially fatty acid and globulin free) were from Sigma Chemical Co. (St Louis, MO). Neuraminid B and the
Results

Bombesin Stimulates Phosphorylation of 80k

The addition of bombesin to quiescent cultures of Swiss 3T3 cells prelabeled with $^{32}$P, markedly increased the phosphorylation of 80k (Fig. 1A). The time course for the stimulation of 80k phosphorylation induced by bombesin is shown in Fig. 1B. After addition of the peptide, an enhancement in 80k phosphorylation was detected as early as 10 s; $^{32}$P incorporation increased rapidly up to 1 min, and thereafter increased more slowly reaching a maximum after 10 min. A similar time course is obtained in response to addition of either PBt2 or serum (49, 61) while, in contrast, PDGF stimulates 80k phosphorylation after a lag of 1–2 min (49). The effect of bombesin on 80k phosphorylation is also concentration dependent (Fig. 1C); the maximal effect was obtained at 1.5 nM bombesin.

Analysis by two-dimensional PAGE of extracts of quiescent 3T3 cells labeled with $^{32}$P showed that the 80k phosphorylation generated in response to bombesin migrates as an acidic protein with an isoelectric point (pl) of ~5 (Fig. 2). A marked enhancement of 80k phosphorylation could be observed 1 min after the addition of bombesin; no further increase in phosphorylation of 80k was observed after 10 min (Fig. 2). In other experiments, a 1.5-fold increase in 80k phosphorylation was detected by two-dimensional PAGE analysis as early as 10 s after addition of bombesin. Quantitation of the increase in 80k phosphorylation relative to another prominent spot of ~55 kD (labeled A in Fig. 2), whose phosphorylation appears to increase more slowly after addition of bombesin, indicated relative increases in the phosphorylation of 80k of 1.9 and 1.6 after 1 and 10 min, respectively. However, when the enhancement of 80k phosphorylation was compared with another less prominent spot of ~52 kD (labeled B), relative increases of 3.7 and 2.8 in 80k phosphorylation were obtained after 1 and 10 min, respectively. Most of the radioactivity corresponding to 80k was eliminated by alkaline treatment (12), suggesting that the phosphorylation of 80k in response to bombesin occurs at serine rather than tyrosine residues (results not shown). This is in agreement with results obtained for the 80k phosphorylation generated in response to the addition of PBt2 or serum (49, 61).

Relationship between the 80k Phosphoproteins Generated in Response to Bombesin and PBt2

The fact that the 80k phosphoproteins generated in response to the addition of either serum, PBt2, or bombesin co-migrated in one-dimensional PAGE (Fig. 1A) suggested that bombesin stimulated the phosphorylation of an identical 80k protein to serum and PBt2. This possibility was substantiated by phosphopeptide mapping using Staphylococcus aureus V8 protease. Peptide mapping of the 80k phosphoprotein bands generated by bombesin and PBt2 excised from one-dimensional PAGE showed the presence of similar phosphopeptide fragments (results not shown). These results suggested that the same 80k protein is generated in response to bombesin or PBt2. To test this conclusion further, extracts of cultures exposed to bombesin or PBt2 for 5 min were subjected to two-dimensional electrophoresis. As shown in Fig. 3, the 80k phosphoproteins produced after treatment with either bombesin or PBt2 co-migrate with identical pl's.

Prolonged exposure of cells to phorbol esters leads to both
a progressive decrease in the number of phorbol ester receptors in intact cells (8-10, 54) and the disappearance of protein kinase C activity in cell-free preparations (48), and it blocks the stimulation of 80k phosphorylation in response to PBA (61) or the synthetic diacylglycerol 1-oleoyl-2-acetylgllycerol (OAG) (59). If the enhancement in 80k phosphorylation caused by bombesin reflects the activation of protein kinase C, prolonged pretreatment with PBA should also block the stimulation of 80k phosphorylation produced in response to bombesin. To test this possibility, cultures of Swiss 3T3 cells were treated with PBA at a saturating concentration (800 nM). After 40 h, control and pretreated cultures were washed extensively to remove residual PBA (17) and then labeled with ³²P. Prolonged pretreatment with PBA completely blocked the stimulation of 80k phosphorylation caused by a subsequent challenge with bombesin as shown by both one-dimensional (Table I) and two-dimensional (Fig. 4) PAGE. In Table I, the effect of pretreatment on the enhancement of 80k phosphorylation by bombesin is shown for comparison.

The effect of prolonged exposure of 3T3 cells to PBA on the stimulation of 80k phosphorylation by bombesin could also be due to a low rate of turnover of the 80k phosphoprotein generated by PBA during the early stages of the pretreatment. To test this possibility, cultures of 3T3 cells were pretreated

**Figure 2.** Resolution of the 80k phosphoprotein by two-dimensional PAGE of extracts from bombesin-stimulated 3T3 cells. Quiescent cultures of Swiss 3T3 cells were labeled with ³²P for 4 h. Bombesin was then added at a concentration of 6.2 nM and the incubations terminated after the indicated times. In the first dimension, phosphoproteins were separated by isoelectrofocusing from the right (basic) to the left (acidic). The arrows indicate the position of 80k. The letters A and B indicate the positions of two reference proteins whose phosphorylation state was compared with that of 80k (see text for details). On the left, molecular weight standards (X 10⁻³).

**Figure 3.** Resolution of the 80k phosphoprotein by two-dimensional PAGE of extracts from control and either PBA or bombesin-stimulated 3T3 cells. Quiescent cultures of Swiss 3T3 cells were labeled with ³²P for 4 h. They were then treated for 5 min with 200 nM PBA, 6.2 nM bombesin, or with an equivalent volume of solvent (control). Quantitation of the 80k phosphoprotein generated by bombesin relative to spots A and B showed increases of 1.6 and 4.4, respectively. The corresponding values for PBA were 2.5 and 2.7, respectively. Other experimental details were as described in the legend to Fig. 2. The arrow indicates the position of 80k. On the left, molecular weight standards (X 10⁻³).
with PB2 (800 nM) for only 1 h before labeling with $^{32}$P. Under these conditions, there is neither a reduction in the number of phorbol ester–binding sites (10), nor a decrease in the activity of protein kinase C in cell-free extracts (48). Pretreatment with PB2 for 1 h failed to block the increase in 80k phosphorylation induced by a subsequent challenge with either bombesin or PB2.

**Bombesin Inhibition of $^{125}$I-EGF Binding: Dependence on Time, Temperature, and Ligand Concentration**

Treatment with bombesin at 6.2 nM caused a marked reduction in the binding of $^{125}$I-EGF to Swiss 3T3 cells (Fig. 5A). As judged by the selective removal of cell surface–bound $^{125}$I-EGF by treatment with acetic acid (24), 88% of the total $^{125}$I-EGF bound was on the cell surface after 1 min whereas only 37% of the total radioactivity associated with the cell remained surface bound after 12 min of incubation (Fig. 5B). The salient feature of the results shown in Fig. 5B is that bombesin decreased the surface-bound $^{125}$I-EGF as early as 1 min of incubation. This strongly suggests that bombesin inhibits the binding of $^{125}$I-EGF to surface receptors rather than a subsequent step in the interaction of EGF with 3T3 cells.

Since the EGF–receptor complexes are internalized and degraded at 37°C but not at 4°C (44), we examined the effect of temperature on the ability of bombesin to inhibit the binding of $^{125}$I-EGF to 3T3 cells. At 37°C, bombesin inhibited $^{125}$I-EGF binding in a concentration–dependent fashion; half-maximal inhibition was elicited at a concentration of 0.15 nM and maximal inhibition (85%) was achieved at 6.2 nM (Fig. 6). The inhibition of $^{125}$I-EGF binding was strikingly reduced when the temperature of the incubation was decreased from 37°C to 4°C (Fig. 6); at 4°C, bombesin failed to inhibit $^{125}$I-EGF binding to 3T3 cells. However, when the cultures were preincubated with bombesin at 37°C for 1 h (in the absence of $^{125}$I-EGF) and $^{125}$I-EGF binding was subsequently measured at 4°C, a concentration–dependent inhibition of binding was observed (inset, Fig. 6). This indicates that the decrease in $^{125}$I-EGF binding induced by bombesin can be elicited in the absence of EGF and that once established at 37°C the decrease is not readily reversed at 4°C. In this manner, the effect of bombesin on the interaction of $^{125}$I-EGF with cellular receptors can be ascertained in the absence of ligand internalization and degradation. Using this approach, we determined the effect of bombesin on $^{125}$I-EGF binding as a function of ligand concentration. The inhibition by bombesin was competitive, with no change in maximal binding but with a considerable increase in the concentration of $^{125}$I-EGF required for half-maximal binding (Fig. 7). Scatchard analysis of these data (shown in the inset) revealed that $^{125}$I-EGF binds to a population of receptors of mixed affinity with apparent dissociation constants ($K_a$) of 0.1 and 1.7 nM. Treatment with bombesin did not affect the total number of EGF receptors present on the cells (85,000 sites/cell) but markedly decreased the affinity of the receptor population to an almost uniform $K_a$ value of 3 nM.

**Table 1. Pretreatment with PB2 Blocks the Stimulation of 80k Phosphorylation by Bombesin and PB2**

<table>
<thead>
<tr>
<th>Additions</th>
<th>80k phosphorylation (% of control)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>PB2</td>
<td>335</td>
</tr>
<tr>
<td>Bombesin</td>
<td>295</td>
</tr>
</tbody>
</table>

Quiescent cultures of Swiss 3T3 cells were incubated either in the absence (control) or presence (pretreated) of 800 nM PB2 or 6.2 nM bombesin, or an equivalent volume of solvent. The reaction was terminated after 5 min. Samples were analyzed for $^{32}$P incorporation into 80k as described in Materials and Methods. Values represent the mean of results obtained from two separate experiments. Other experimental details were performed as described in Materials and Methods.

**Isoelectric Focusing**

![Isoelectric Focusing](image)

**Figure 4.** (Left) Resolution of the 80k phosphoprotein by two-dimensional PAGE of extracts from control and PB2-pretreated 3T3 cells. Subsequent addition of PB2 or bombesin to stimulate 80k phosphorylation. Quiescent cultures of Swiss 3T3 cells were incubated either in the absence (left and center) or presence (right) of 800 nM PB2 for 40 h. The cultures were washed, labeled with $^{32}$P for 4 h and exposed to either 200 nM PB2 or bombesin, or an equivalent volume of solvent. The reaction was terminated after 5 min. Control and pretreated cultures were allowed to migrate to an equivalent volume of solvent showed no differences in the level of 80k phosphorylation from the control panel. Quantitation of the 80k phosphoprotein generated by bombesin relative to spots A and B showed that PB2 pretreatment reduced bombesin stimulation of 80k phosphorylation to the control value. Other experimental details were as described in the legend to Fig. 2. In both control and pretreated cultures, bombesin markedly stimulated the phosphorylation of a spot migrating with a molecular mass of ~93 kD. However, this spot was not observed in the other experiments shown in this paper. The arrow indicates the position of 80k.
The Effect of Bombesin on 80k Phosphorylation and EGF Receptor Affinity is Mediated by the Bombesin Receptor

To determine whether the stimulation of 80k phosphorylation and the transmodulation of the EGF receptor by peptides of the bombesin family were mediated by specific high-affinity receptors for these peptides, we tested the ability of a range of bombesin agonists to induce these responses. GRP is a 27-amino acid peptide identified in mammalian gut (36, 70), brain (40, 70), and lung (21, 71). Neuromedin B is one of another class of bombesin-like peptides isolated from brain and spinal cord (38, 39). As shown in Table II, these peptides exhibit striking sequence homology with the highly conserved COOH-terminal heptapeptide of bombesin (the 8–14 fragment of bombesin). GRP, neuromedin-B, and the 8–14 fragment of bombesin, designated bombesin(8–14), all markedly increased 80k phosphorylation (Fig. 8) and inhibited 125I-EGF binding (see Fig. 10, upper panel) in a concentration-dependent fashion. In contrast, the amino-terminal fragment of GRP, designated GRP(1–16), did not elicit these responses even at concentrations as high as 5 μg/ml (see Fig. 10 upper panel; and results not shown).

The substance P (SP) analogue (d-Arg1, d-Pro2, d-Trp7,9, Leu11) SP (33), recently described as a novel bombesin antagonist (27), markedly inhibits both 125I-Tyr1 GRP binding, and bombesin- and GRP-induced mitogenesis in a concentration-dependent manner (73). The antagonist at 100 μM completely abolished the stimulation of 80k phosphorylation promoted by bombesin and GRP (Fig. 9). Furthermore, (d-Arg1,
Figure 7. Effect of bombesin on the binding of $^{125}$I-EGF to Swiss 3T3 cells as a function of $^{125}$I-EGF concentration. Confluent, quiescent cultures of Swiss 3T3 cells were transferred to 1 ml of DME with no addition (squares) or with 6.2 nM (circles) bombesin at 37°C for 1 h. Cultures were washed five times with DME containing 0.1% BSA at 37°C. All cultures were then chilled in 1 ml of binding medium on ice for 5 min, and incubated for 2 h at 4°C in 1 ml of binding medium containing $^{125}$I-EGF at the concentrations shown. Detailed analysis of the time course of the binding of various levels of $^{125}$I-EGF to Swiss 3T3 cells demonstrated that even at the lowest levels of $^{125}$I-EGF used, equilibrium binding was obtained after this time. Cell-associated radioactivity was then determined as described in Materials and Methods. The values shown represent specific binding, measured for each condition as that displaced by the presence of 250-fold excess (or 250 ng/ml at $^{125}$I-EGF levels below 1 ng/ml) unlabelled EGF. The inset shows Scatchard analysis of the data (B/F vs. B); B, bound $^{125}$I-EGF, is expressed as pmol/10$^6$ cells, whereas F, the concentration of $^{125}$I-EGF in the binding solution, is in pmol/ml.

Table II. Amino Acid Sequence of Peptides of the Bombesin Family

<table>
<thead>
<tr>
<th>Peptide Family</th>
<th>Amino Acid Sequence</th>
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<tbody>
<tr>
<td>GRP (1-27)</td>
<td>Ala Pro Val Ser Val Gly Gly Thr Val Leu Ala Lys Met Tyr Pro Arg Gly Asn His Trp Ala Val Gly His Leu Met</td>
</tr>
<tr>
<td>Bombesin</td>
<td>Glu Gin Arg Leu Gly Asn Gin Trp Ala Val Gly His Leu Met</td>
</tr>
<tr>
<td>GRP (14-27)</td>
<td>Met Tyr Pro Arg Gly Asn His Trp Ala Val Gly His Leu Met</td>
</tr>
<tr>
<td>Litorin</td>
<td>Glu Gin Trp Ala Val Gly His Leu Met</td>
</tr>
<tr>
<td>Neuromedin C</td>
<td>Gln Asn His Trp Ala Val Gly His Leu Met</td>
</tr>
<tr>
<td>Neuromedin B</td>
<td>Gly Asn Leu Trp Ala Thr Gly His Phe Met</td>
</tr>
<tr>
<td>Bombesin (8-14)</td>
<td>Trp Ala Val Gly His Leu Met</td>
</tr>
<tr>
<td>GRP (1-16)</td>
<td>Ala Pro Val Ser Val Gly Gly Thr Val Leu Ala Lys Met Tyr Pro</td>
</tr>
</tbody>
</table>

Figure 8. Dose-response for the stimulation of 80k phosphorylation in quiescent Swiss 3T3 cells by the bombesin-related peptides GRP, neuropeptide B (NMB), and bombesin(8-14)[B (8-14)]. Cultures were washed and labeled with $^{32}$P$i$ for 4 h. After this time, either peptide was added at the concentrations indicated, or cultures were challenged with an equivalent volume of solvent (C). Incubations were terminated after 5 min. The arrow indicates the position of 80k. On the far left of each series of gels, molecular weight standards ($\times 10^3$).
d-Pro\(^2\), d-Trp\(^{7,9}\), Leu\(^{11}\)} SP had no effect on \(^{125}\)I-EGF binding in the absence of bombesin, but caused an eightfold increase in the concentration of bombesin required to induce half-maximal inhibition of \(^{125}\)I-EGF binding (Fig. 9) or the inhibition of \(^{125}\)I-EGF binding by either PDGF or PBt\(_2\) (Fig. 10, lower panel). The effect of the antagonist on both the stimulation of 80k phosphorylation and the inhibition of \(^{125}\)I-EGF binding by bombesin was selective; at 100 \(\mu\)M, the antagonist had no effect upon either the increase in 80k phosphorylation caused by PBt\(_2\) (Fig. 9) or the inhibition of \(^{125}\)I-EGF binding by either PDGF or PBt\(_2\) (Fig. 10, lower panel).

**Role of Increased Cytosolic Ca\(^{2+}\) and Protein Kinase C in Mediating Transmodulation of the EGF Receptor**

It has been suggested that a variety of secretagogues, including bombesin, inhibit \(^{125}\)I-EGF association to pancreatic acinar cells by increasing cytosolic Ca\(^{2+}\) in the cells (28, 31, 46). The following paper (37) reports that bombesin and bombesin-related peptides induce Ca\(^{2+}\) mobilization from intracellular stores and increase cytosolic Ca\(^{2+}\) concentration in 3T3 cells (37). To evaluate the role of Ca\(^{2+}\) in mediating the inhibition of \(^{125}\)I-EGF binding by these peptides, we tested the effect of the Ca\(^{2+}\) ionophore A23187 on \(^{125}\)I-EGF binding to cultures of quiescent Swiss 3T3 cells. At 37\(^\circ\)C, the drug caused inhibition of cell-associated \(^{125}\)I-EGF in a concentration-dependent manner; however, even at 1 \(\mu\)M, A23187 produced an inhibition of only 34\% (Fig. 11). The maximal effect of bombesin at 37\(^\circ\)C was 85\% and it is shown for comparison. In addition, treatment of the cells at 37\(^\circ\)C with A23187 followed by binding at 4\(^\circ\)C markedly decreased the ability of the drug to inhibit \(^{125}\)I-EGF binding (Fig. 11). In contrast to bombesin, which affected surface-bound \(^{125}\)I-EGF as early as 1 min (Fig. 5B), A23187 reduced internalized \(^{125}\)I-EGF only after 15–30 min of incubation (Table III). The striking differences between bombesin and A23187 in their ability to decrease \(^{125}\)I-EGF binding to 3T3 cells indicates that the inhibition of \(^{125}\)I-EGF binding to 3T3 cells by peptides of the bombesin family is not mediated by an increase in the intracellular concentration of Ca\(^{2+}\) in these cells.

As shown in Fig. 4 and Table I, prolonged pretreatment of quiescent Swiss 3T3 cells with PBt\(_2\), which reduces the num-
rinlation of 80k by phorbol esters (61) or by OAG (59) has the phosphorylation of 80k. The effect is concentration dependent and can be detected as early as 10 s after the addition of bombesin. As shown in Fig. 12, treatment of bombesin to intact, quiescent Swiss 3T3 cells rapidly enhances the phosphorylation of 80k phosphoester-binding sites (8-10, 54) and the activity of protein kinase C measured in cell-free preparations (48, 60), prevents the activation of protein kinase C by bombesin. If the inhibition of 125I-EGF binding by bombesin is mediated by activation of protein kinase C, pretreatment with PBt2 should reduce the inhibition of binding caused by a subsequent exposure to bombesin. As shown in Fig. 12, treatment of 3T3 cells with PBt2 for 40 h followed by extensive washing to remove residual PBt2 (17) prevented the inhibition of 125I-EGF binding by a subsequent addition of either bombesin or PBt2 to these pretreated cells.

Discussion

The findings presented here demonstrate that addition of bombesin to intact, quiescent Swiss 3T3 cells rapidly enhances the phosphorylation of 80k. The effect is concentration dependent and can be detected as early as 10 s after the addition of bombesin. Recently, a rapid stimulation in the phosphorylation of 80k by phorbol esters (61) or by OAG (59) has been shown to reflect the activation of protein kinase C in intact cells (60). The possibility that the same 80k phosphoprotein is generated in response to both bombesin and PBt2 is supported by analysis in one- and two-dimensional PAGE.

Table III. Effect of the Ca2+ Ionophore A23187 on Surface-bound and Internalized 125I-EGF

<table>
<thead>
<tr>
<th>Addition</th>
<th>Time</th>
<th>Surface-bound</th>
<th>Internalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>5</td>
<td>48 ± 1.0</td>
<td>48 ± 0.5</td>
</tr>
<tr>
<td>A23187</td>
<td>5</td>
<td>42.5 ± 1.8</td>
<td>41 ± 1.0</td>
</tr>
<tr>
<td>—</td>
<td>15</td>
<td>48 ± 0.9</td>
<td>90 ± 2.0</td>
</tr>
<tr>
<td>A23187</td>
<td>15</td>
<td>48 ± 2.0</td>
<td>70 ± 2.0</td>
</tr>
<tr>
<td>—</td>
<td>30</td>
<td>49 ± 0.5</td>
<td>180 ± 1.2</td>
</tr>
<tr>
<td>A23187</td>
<td>30</td>
<td>50 ± 2.0</td>
<td>112 ± 5.0</td>
</tr>
</tbody>
</table>

Quiescent cultures of Swiss 3T3 cells were incubated at 37°C in 1 ml of binding medium containing 1 ng/ml (125,000 cpm/ng) 125I-EGF either in the absence or in the presence of 1 μM A23187. At the indicated times the surface-bound 125I-EGF was determined by extracting with 0.5 ml of a solution containing 0.2 M acetic acid/0.5 M NaCl at 4°C for 6 min. The internalized radioactivity was measured as described in Materials and Methods. The figures represent mean ± SEM; n = 4.
and by peptide mapping after limited proteolysis with V8 protease. The conclusion that the enhancement in 80k phosphorylation by peptides of the bombesin family also reflects the activation of protein kinase C is further substantiated by experiments in which 3T3 cells are exposed to PBt2 for 40 h before the assay. This prolonged pretreatment leads to a striking reduction in the number of specific [3H]-PBt2 binding sites in intact cells (8-10, 54) in the protein kinase C activity measured in partially purified cell-free extracts (48, 60), and in the early and late responses of the cells to a subsequent challenge with phorbol esters (8-10, 54, 59-61). The results presented here demonstrate that prolonged pretreatment of 3T3 cells with PBt2 blocks the stimulation of 80k phosphorylation elicited by bombesin. Taken together, these findings indicate that the peptides of the bombesin family rapidly activate protein kinase C activity in intact Swiss 3T3 cells. Since the activation of protein kinase C acts as a mitogenic signal for Swiss 3T3 cells (60), the stimulation of this phosphotransferase system may play a fundamental role in effecting the proliferative response elicited by bombesin and structurally related peptides.

Our results also show that bombesin causes a rapid and striking inhibition of 125I-EGF binding to cultures of Swiss 3T3 cells. The data strongly suggest that the inhibition of 125I-EGF binding by bombesin occurs at the level of the interaction of 125I-EGF with its receptor at the cell surface, rather than at some subsequent step such as ligand internalization and degradation. A decrease in the specific binding of a ligand can be due to a decrease in the apparent number of available receptors (e.g., after down regulation) or to a change in the apparent affinity of the receptor population or to a combination of these mechanisms. Bombesin treatment did not decrease the apparent maximum number of EGF receptors but caused a considerable decrease in the apparent affinity of the receptors from a mixed population with KD values of 0.1 and 1.7 nM to receptors with a KD of ~3 nM. Thus, bombesin inhibition of EGF binding in Swiss 3T3 cells has many properties similar to that induced by other ligands that elicit receptor transmodulation (54), including phorbol esters (5, 30, 63), synthetic diacylglycerol OAG (64), vasopressin (57), PDGF (3, 11), and FDGF (58). All these tumor- and growth-promoting agents decrease the apparent affinity of the EGF receptor population in a temperature-sensitive manner (transmodulation) without any decrease in the EGF receptor number due to down regulation.

Peptides of the bombesin family bind to specific, high-affinity sites in Swiss 3T3 cells that mediate their mitogenic effects in these cells (73). Our results demonstrate a close correspondence between the ability of peptides of the bombesin family to stimulate 80k phosphorylation and both their relative mitogenic potency and ability to inhibit GRF binding (73). Further, the novel bombesin antagonist, (D-Arg1, D-Pro2, D-Trp7,9, Leu10) SP (27) abolished the increase in 80k phosphorylation induced by bombesin and GRF at a concentration (100 μM) similar to that required to block both mitogenesis and receptor binding (73). Likewise, the relative abilities of bombesin-related peptides to inhibit 125I-EGF binding closely correspond to their capacity to displace [125I]-Tyr19 GRF from its high-affinity binding site (73). Further, (D-Arg1, D-Pro2, D-Trp7,9, Leu10) SP (27, 73) competitively inhibited the effect of bombesin on 125I-EGF binding. These structure-activity relationships strongly suggest that both the activation of protein kinase C and the modulation of 125I-EGF binding to its receptor by peptides of the bombesin family are mediated by the specific high-affinity receptors which have been characterized in Swiss 3T3 cells (73).

The nature of the intracellular signals generated by bombesin and other mitogenic ligands which lead to the activation of protein kinase C remains unclear, though it has been suggested that biologically active peptides or hormones that activate protein kinase C do so through phospholipid breakdown leading to diacylglycerol formation and to an increase in cytosolic Ca2+ (2, 42). Indeed, in the following paper (37) we show that peptides of the bombesin family stimulate a rapid (15 s) mobilization of Ca2+ from intracellular stores (37). Since it has been proposed that an increase in the cellular concentration of Ca2+ decreases the association of 125I-EGF to pancreatic acinar cells (28, 31, 46) and to the human epidermoid carcinoma cell line KB (20), it was important to evaluate the role of this putative second messenger in the action of bombesin on the binding of 125I-EGF to 3T3 cells. We found that the Ca2+ ionophore A23187 does not mimic the effect of bombesin in terms of either maximal inhibition of binding or dependence on temperature. Further, when A23187 was added at 50 nM, a concentration that caused an increase in cytosolic Ca2+ comparable to that induced by a saturating dose of bombesin (37), the ionophore had no effect on cell-associated 125I-EGF. Finally, while bombesin strikingly decreased surface-bound 125I-EGF as early as 1 min, the ionophore only reduced the amount of internalized 125I-EGF at later times of incubation. We conclude that an increase in cytosolic concentration of Ca2+ does not mediate the inhibitory effect of bombesin on 125I-EGF binding in Swiss 3T3 cells.

A salient feature of the results presented here is that prolonged incubation with PBt2, which prevents the activation of protein kinase C by bombesin, also abolished the inhibition of 125I-EGF binding by a subsequent addition of either PBt2 or bombesin. Scatchard analysis of 125I-EGF binding at 4°C after the prolonged PBt2 treatment demonstrated that this procedure did not affect the apparent number or affinity of the EGF receptor. In addition, EGF was mitogenic in PBt2-treated cells (10). These important controls indicate that the EGF receptor is present and functional in PBt2-desensitized cells. The loss of response to bombesin in cells with greatly reduced numbers of phorbol ester receptors and protein kinase C activity strongly implicates this phosphotransferase system in mediating transmodulation of the EGF receptor by bombesin in intact 3T3 cells. This enzyme phosphorylates the EGF receptor at a specific site (7, 15, 25, 26) located in the intracellular portion of the receptor, nine residues from the proposed hydrophobic transmembrane domain (25). Although the precise effect of this phosphorylation on the affinity of the EGF receptor remains unclear, it is plausible that protein kinase C may play a direct role in mediating transmodulation of EGF receptors in intact cells.

The results presented in this and the following paper (37) demonstrate that bombesin and structurally related peptides elicit a complex set of early events in quiescent Swiss 3T3 cells (summarized in Fig. 13). These peptides, which exhibit a highly conserved carboxyl-terminal heptapeptide, bind to specific, high-affinity receptors (73). After binding, these ligands rapidly stimulate (a) the phosphorylation of 80k, which reflects the activation of protein kinase C in intact 3T3 cells;
Figure 13. Early events elicited by bombesin and structurally related peptides in quiescent Swiss 3T3 cells. The cause-effect relationships indicated by the arrows are discussed in detail here and in the following paper (37).

(b) mobilization of Ca++, from an intracellular store which leads to a transient increase in the concentration of cytosolic Ca++ and Ca++ efflux (37); and (c) the activity of Na+/K+ pump activity and increases intracellular pH (37). The activation of protein kinase C mediates, at least in part, the changes in monovalent cation transport (37). As shown in this paper, activation of this phosphotransferase system is also implicated in inducing transmodulation of EGF receptor affinity. These early responses are strikingly similar to those elicited by PDGF and FDGF, a PDGF-like growth factor (3, 11, 32, 49, 58, 61, 66).

In contrast to many other factors which are mitogenic only when added in synergistic combinations (53, 55), bombesin, PDGF, and FDGF stimulate DNA synthesis in Swiss 3T3 cells in the absence of other mitogenic factors (32, 56, 66). It has been hypothesized that initiation of a proliferative response from the sympathetic interaction of multiple complementary signals (discussed in 53, 55); the fact that bombesin, like PDGF and FDGF, triggers a wide variety of signaling processes (Fig. 13) is entirely consistent with this hypothesis. Further studies comparing the early biological responses elicited by these mitogens may provide important clues regarding the nature and role of the signals and early events that precede fibroblastic cell proliferation.

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References


