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Bombesin stimulation of fibroblast mitogenesis: specific receptors, signal transduction and early events

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Quiescent cultures of Swiss 3T3 cells can be stimulated to recommence deoxyribonucleic acid (DNA) synthesis by polypeptide growth factors, neuropeptides and various pharmacological agents that act via multiple signal transduction pathways. Neuropeptides of the bombesin family provide novel and potent mitogens to elucidate these pathways. The peptides bind to specific receptors that have been characterized by radioligand binding and sensitivity to antagonists and identified as glycoproteins of relative molecular mass (M_r) 75000–85000 by chemical cross-linking. After binding, bombesin elicits a cascade of early molecular events, including stimulation of phosphorylation of the acidic M_r 80000 cellular protein (80000) that is a major substrate of protein kinase C; Ca^{2+} mobilization mediated by Ins(1,4,5)P_3; Na^+ and K^+ fluxes; transmodulation of (EGF) receptor; enhancement of cyclic adenosine monophosphate (cAMP) accumulation and expression of the proto-oncogenes c-*fos* and c-*myc*. Studies using digitonin-permeabilized 3T3 cells show that a G protein plays a role in the transduction of the mitogenic signal triggered by the binding of bombesin to its receptor.

Introduction

The cells of many tissues and organs *in vivo* are maintained in a non-proliferating state (G_0/G_1). However, such cells remain viable and can be induced to resume deoxyribonucleic acid (DNA) synthesis and cell division when exposed to external stimuli such as hormones, antigens or growth factors. In this manner the growth of individual cells is regulated according to the requirements of the whole organism. The elucidation of the molecular mechanisms by which these mitogens regulate growth and differentiation at the cellular level remains one of the fundamental problems in biomedical research and may prove crucial to understanding both the normal proliferative response and the unrestrained growth of cancer cells.

To avoid the complexities of whole-animal experimentation, a number of cultured cell systems have been developed to examine the factors involved in growth control. In this respect the non-tumourigenic murine Swiss 3T3 fibroblast line has proved useful for both identifying the extracellular factors that modulate cell growth and for elucidating the early signals and molecular events that lead to mitogenesis. These cells cease to proliferate when the medium is depleted of its growth-promoting activity and can be stimulated to re-initiate DNA synthesis and cell division either by replenishing the medium with fresh serum, or by the addition of purified growth factors or pharmacological agents in serum-free medium (Rozengurt 1985). Studies done using such quiescent cells and defined combinations of growth factors have revealed the existence of potent and specific synergistically acting signal transduction pathways initiated almost immediately after mitogen addition (Rozengurt 1986).

A new and intriguing development is the discovery that neuropeptides localized in neural and neuroendocrine cells of mammalian tissues can also act as growth factors for cells in culture

(Woll & Rozengurt 1989). Furthermore, indirect evidence is accumulating that the mitogenic effects of neuropeptides may be relevant for a variety of long-term biological processes, including development and oncogenesis (reviewed in Zachary *et al.* (1987)). In this context, the peptides of the bombesin family (table 1) are of particular significance. These peptides are potent mitogens for Swiss 3T3 cells (Rozengurt & Sinnett-Smith 1983) and may act as autocrine growth factors for small-cell lung cancer (reviewed in Woll & Rozengurt (1988*a*)). Here we summarize our recent studies by using bombesin-like peptides for elucidating the signal transduction pathways leading to mitogenesis and compare these pathways with those elicited by other growth factors.

Table 1. Amino acid sequences of peptides of the bombesin family

mammalian																
GRP(1–27) human	Val	Pro	Leu	Pro	Ala	Gly	Gly	Gly	Thr	Val	Leu	Thr	Lys	—		
	—	Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	-NH_2
GRP(1–27) porcine	Ala	Pro	Val	Ser	Val	Gly	Gly	Gly	Thr	val	Leu	Ala	Lys	—		
	—	Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	-NH_2
GRP(14–27) porcine	—	Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	-NH_2
GRP10 (neuromedin C)						Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	-NH_2
neuromedin B						Gly	Asn	Leu	Trp	Ala	Thr	Gly	His	Phe	Met	-NH_2
amphibian																
bombesin	pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met	-NH_2	
bombesin (8–14)								Trp	Ala	Val	Gly	His	Leu	Met	-NH_2	
ranatensin				pGlu	Val	Pro	Gln	Trp	Ala	Val	Gly	His	Phe	Met	-NH_2	
Litorin					pGlu	Gln	Trp	Ala	Val	Gly	His	Phe	Met	-NH_2		

Mitogenic response of Swiss 3T3 cells to bombesin

In serum-free medium, addition of bombesin to Swiss 3T3 cells induces DNA synthesis and cell division in the absence of other growth-promoting agents with a half-maximal effect at 1 nM (Rozengurt & Sinnett-Smith 1983). The ability of bombesin, like platelet-derived growth factor (PDGF), to act as a sole mitogen for Swiss 3T3 cells contrasts with other growth factors that are only active in synergistic combinations (Rozengurt 1986). The stimulation of DNA synthesis by bombesin is markedly potentiated by insulin which increases the maximal response and decreases the bombesin concentration required to produce a half-maximal effect (from 1 nM to 0.3 nM). Mammalian bombesin-like peptides (table 1) including gastrin-releasing peptide (GRP) behave similarly in the stimulation of DNA synthesis (Zachary & Rozengurt 1985*a*).

Specific bombesin receptors in intact Swiss 3T3 cells

Binding measurements and chemical cross-linking experiments show that bombesin-like peptides interact with specific, high-affinity receptors located on the cell surface. ^{125}I-labelled GRP binds to intact, quiescent cells in a specific, saturable and reversible manner (Zachary & Rozengurt 1985). Scatchard analysis shows the presence of a single population of high affinity sites of K_d about 1 nM. ^{125}I-labelled GRP binding is inhibited by various bombesin-like peptides in proportion to their ability to stimulate DNA synthesis. Two potent bombesin antagonists [DArg^1, DPhe^5, DTrp^{7,9}, Leu^{11}]substance P (Woll & Rozengurt 1988*b*) and [Leu^{13}-ψ(CH_2NH)Leu^{14}] bombesin (Coy *et al.* 1988) inhibit both GRP biding and bombesin–GRP-stimulated mitogenesis (Woll & Rozengurt 1988*c*). These structure–activity relations strongly

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suggest that bombesin and related peptides interact with receptors that are distinct from those for other mitogens in Swiss 3T3 cells.

To investigate the physical properties of the bombesin–GRP receptor, we used an affinity-labelling method to identify surface components of Swiss 3T3 cells that specifically recognize ^{125}I-labelled GRP. Analysis of extracts of cells that have been preincubated with ^{125}I-labelled GRP and then treated with disuccinimidyl cross-linking agents reveals the presence of a major band with relative molecular mass (M_r) 75000–85000 (Zachary & Rozengurt 1987*a*). Several lines of evidence support the conclusion that this protein is the receptor or a major component of the receptor for peptides of the bombesin family in these cells:

1. The formation of the M_r 75000–85000 affinity-labelled complex is promoted by a variety of disuccinimidyl cross-linking agents, including EGS, DSS, BSOCOES, and the thiol-clearable agent DSP.

2. The inhibition of ^{125}I-labelled GRP affinity-labelling of this band with unlabelled GRP corresponds closely with the ability of GRP to inhibit binding of the labelled ligand in parallel cultures. Indeed, the ability of various nonradioactive peptide agonists and antagonists to inhibit the formation of the M_r 75000–85000 affinity-labelled complex correlates extremely well ($r = 0.994$) with the relative capacity of these peptides to inhibit ^{125}I-labelled GRP specific binding.

3. Timecourses carried out at different temperatures show that the M_r 75000–85000 band is the earliest cross-linked complex detected in cells incubated with ^{125}I-labelled GRP.

4. ^{125}I-labelled GRP affinity-labelling of the M_r 75000–85000 band is specific, as shown by the lack of effect of a panel of other mitogens and neuropeptides on cross-linking.

5. The M_r 75000–85000 protein was not found in other cell lines that do not exhibit receptors for bombesin-like peptides.

6. The dependence of affinity labelling of the M_r 75000–85000 protein on the concentration of ^{125}I-labelled GRP closely paralleled the ability of the unlabelled peptide to stimulate DNA synthesis and a variety of other biological responses in Swiss 3T3 cells (Zachary & Rozengurt 1987*a*; Sinnett-Smith *et al.* 1988; Woll & Rozengurt 1988*a*). All these findings taken together strongly suggest that an M_r 75000–85000 surface protein is the receptor or a major component of the receptor for peptides of the bombesin family in Swiss 3T3 cells.

A solubilized preparation of the radiolabelled M_r 75000–85000 protein binds to wheatgerm lectin-sepharose columns and can be eluted with *N*-acetyl-*D*-glucosamine, suggesting that it is a glycoprotein. In addition, treatment with endo-β-*N*-acetyl glucosaminidase F reduced the relative molecular mass of the affinity-labelled band from 75000–85000 to 43000, showing the presence of *N*-linked oligosaccharide groups (Kris *et al.* 1987; Sinnett-Smith *et al.* 1988). The small size of the core polypeptide distinguishes the bombesin–GRP receptor from receptors for other growth factors (for example, EGF or PDGF), but it is consistent with a receptor recently described for the neuropeptide substance K (Masu *et al.* 1987).

Specific ^{125}I-labelled GRP binding to cell membranes

As all previous studies on the mitogenic bombesin receptor discussed in the preceding section were performed with whole 3T3 cells, it was important to examine the properties of the binding reaction in membrane preparations. Initial experiments revealed that membrane fractions of Swiss 3T3 cells prepared according to various procedures that were used for other growth factor receptors (EGF, PDGF, transforming growth factor β (TGF-β)) failed to exhibit any

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consistent specific binding of ^{125}I-labelled GRP. However, we recently found that addition of Mg^{2+} (5–10 mM) during the homogenization of the cells, as well as during the binding assay results in a striking increase in the specific binding of ^{125}I-labelled GRP to membranes. A variety of mitogens for cultured fibroblasts (for example, PDGF, EGF, fibroblast growth factor (FGF), insulin, vasopressin, bradykinin) and the neuropeptides substance P, substance K and vasoactive intestinal peptide that exhibit slight carboxyl-terminal homology with GRP did not inhibit the binding of ^{25}I-labelled GRP to membrane preparations. In contrast, the binding was reduced considerably by addition of either unlabelled bombesin or GRP at 100 nM. These results show that ^{125}I-labelled GRP binding to membrane preparations of Swiss 3T3 cells is specific.

Membrane fractions of Swiss 3T3 cells prepared and assayed with solutions containing Mg^{2+} at 5 mM were used to define the kinetic and equilibrium characteristics of ^{125}I-labelled GRP binding. Binding of ^{125}I-labelled GRP to membrane preparations from Swiss 3T3 cells as a function of increasing concentrations of the radiolabelled ligand is shown in figure 1 (left).

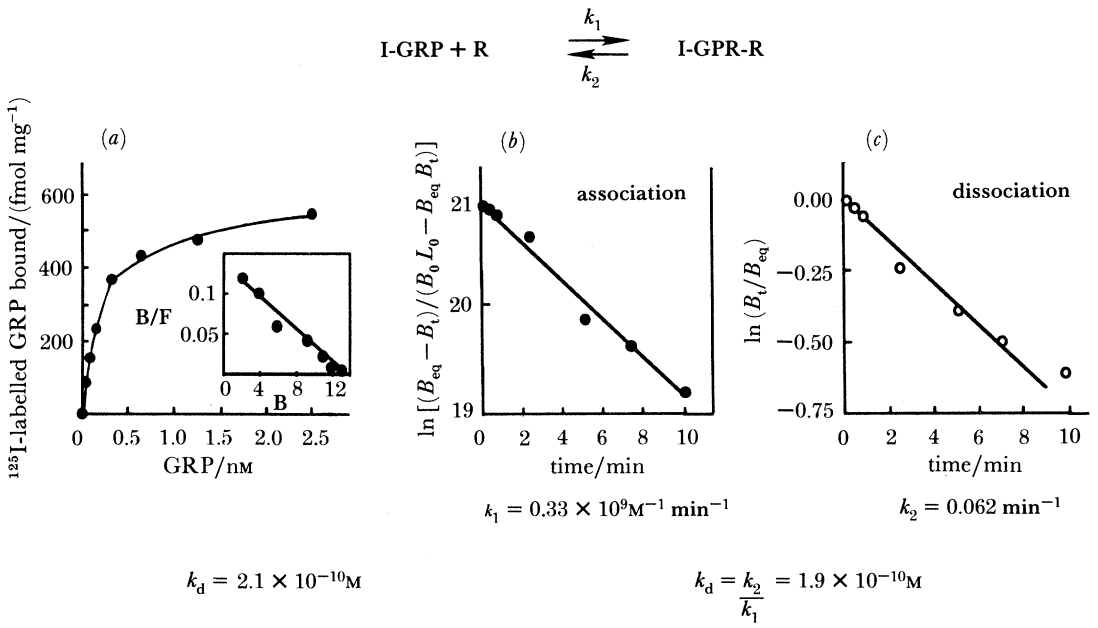

FIGURE 1. (*a*) Analysis of binding as a function of ^{125}I-labelled GRP concentration to Swiss 3T3 membranes. Membranes were incubated in the presence of various concentrations of ^{125}I-labelled GRP at 15 °C. Specific binding was determined after 30 min at 15 °C using 25 μg of membrane protein. Non-specific binding was measured by the addition of at least 1000-fold excess unlabelled bombesin or 1 μM bombesin for concentrations of ^{125}I-labelled GRP below 1 nM. The binding reactions were terminated by rapid filtration on glass fibre filters. The inset shows Scatchard analysis of the data. (*b*) Semi-logarithmic plot of the time course of ^{125}I-labelled GRP association to Swiss 3T3 membranes. ^{125}I-labelled GRP (0.5 nM) was incubated with 25 μg of membrane protein in 100 μl of the binding medium at 15 °C for the indicated times. Time was plotted on the abscissa and $\ln [(B_{eq} - B_t)/(B_0 L_0 - B_{eq} B_t)]$ was plotted on the ordinate. B_0, the initial concentration of free receptors, was estimated from the Scatchard analysis (*a*) to be 0.137 nM. B_{eq}, the equilibrium concentration of occupied receptors, was taken as 0.095 nM, that obtained after 30 min. L_0 was the initial ligand concentration (0.5 nM). The slope of the linear regression line through the initial points (0–8 min) gives the second-order association rate constant k_1 according to the relation $k_1 = \text{slope} \times (B_{eq}/B_{eq}^2 - B_0 L_0)$. Derivation of the equation is presented by Maelicke *et al.* (1977). (*c*) Semi-logarithmic plot of the time course of ^{125}I-labelled GRP dissociation from Swiss 3T3 membranes. Membranes were incubated with ^{125}I-labelled GRP (0.5 nM) in 100 μl of binding medium at 15 °C for 30 min. Excess bombesin (1 μM) was then added to each tube, and ^{125}I-labelled GRP specific binding was determined at the indicated times. Time was plotted on the abscissa and $\ln (B_t/B_{eq})$ was plotted on the ordinate. The slope of the line gave the first-order rate constant k_2.

Specific binding of ^{125}I-labelled GRP measured under equilibrium conditions at 15 °C was saturable, whereas non-specific binding increased linearly with increasing ligand concentration (not shown). Scatchard analysis (figure 1, inset) of these equilibrium binding data indicates the presence of a homogeneous population of high-affinity binding sites of $K_d = 2.1 \times 10^{-10}$ and a value for the maximal binding capacity (B max) of 550 fmol mg^{-1} of cell membrane protein.

The rate of ^{125}I-labelled GRP binding to membrane fractions of Swiss 3T3 cells was measured at 15 °C by using ^{125}I-labelled GRP at 0.5 nM (figure 1). Under these conditions, specific binding of ^{125}I-labelled GRP reached 50 % of its equilibrium value within 2.5 min and maximum binding was achieved after 15 min of incubation. The kinetics of ^{125}I-labelled GRP association to membrane fraction was analysed as a bimolecular reaction (see Maelike *et al.* (1977) for the derivation of the equations used in figure 1). The second-order rate constant (k_1) obtained from the slope of the line shown in figure 1*b* was 0.33×10^9 M^{-1} min^{-1}.

The binding of ^{125}I-labelled GRP to membrane preparations was reversible. Addition of a 2000-fold excess of unlabelled bombesin to the membrane fractions incubated with ^{125}I-labelled GRP for 30 min, promoted first-order dissociation of the labelled ligand–receptor complex (figure 1*c*). Half-maximal loss of ^{125}I-labelled GRP binding occurred after 7.5 min and the value of k_2, the rate constant of dissociation was 0.062 min^{-1}. With the values of the rate constant derived from figure 1, the equilibrium dissociation constant ($K_d = k_2/k_1$) can be calculated as 1.9×10^{-10} M. Hence, the kinetically derived equilibrium constant was in excellent agreement with the K_d obtained from equilibrium binding measurements. These results are consistent with the existence of a single population of bombesin–GRP binding sites in membranes of Swiss 3T3 cells.

The availability of membrane preparations that retain specific bombesin receptors will be useful in the characterization of their molecular and regulatory properties. Moreover, such membrane preparations provide an important step for attempting the solubilization and purification of this mitogenic neuropeptide receptor.

Early signals elicited by bombesin

The binding of growth factors to their receptors promotes the generation of early signals in the membrane, cytosol and nucleus that lead to cell proliferation (Rozengurt 1986). Because the initiation of DNA synthesis occurs 10–15 h after the addition of the mitogens, it is expected that knowledge of the early events will provide clues to primary regulatory mechanisms. A summary of the early cellular and molecular responses elicited by bombesin and structurally related peptides is compiled in table 2.

Protein kinase C and initiation of DNA synthesis

Protein kinase C (PKC), which is activated by diacylglycerols and phorbol esters (reviewed by Nishizuka (1984)) and comprises multiple subspecies (Nishizuka 1988) has been implicated in the signal transduction of many short-term cellular responses, including secretion and contraction (Kikkawa & Nishizuka 1986). Of particular interest here is the evidence implicating PKC in mediating long-term responses. Phorbol esters stimulate DNA synthesis and cell division in synergy with insulin and other growth-promoting factors (Dicker & Rozengurt 1978; 1980). The mitogenic effect is mediated by high-affinity binding sites (Collins

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Table 2. Events in the action of bombesin in Swiss 3T3 cells

event	references
binding to specific receptors	Zachary & Rozengurt (1985*a*)
cross-linking to M_r 75000–85000 glycoprotein	Zachary & Rozengurt (1987*a*); Kris *et al.* (1987); Sinnett-Smith *et al.* (1988)
ligand internalization and degradation	Zachary & Rozengurt (1987*b*); Brown *et al.* (1988)
activation of PKC (intact cells)	Zachary *et al.* (1986); Isacke *et al.* (1986); Rodriguez-Pena *et al.* (1986)
activation of PKC (permeabilized cells)	Erusalimsky *et al.* (1988)
elevation of DAG levels	Muir & Murray (1987); Takuwa *et al.* (1987)
Ins(1,4,5)P_3 production	Heslop *et al.* (1986); Takuwa *et al.* (1987); Lopez-Rivas *et al.* (1987)
Ca^{2+} mobilization	Mendoza *et al.* (1986); Takuwa *et al.* (1987); Lopez-Rivas *et al.* (1987)
Ins(1,4,5)P_3 and Ca^{2+} (kinetics)	Nånberg & Rozengurt (1988)
Na^+ influx and Na^+/K^+ pump	Mendoza *et al.* (1986)
transmodulation of EGF receptor	Zachary & Rozengurt (1985*b*); Zachary *et al.* (1986)
arachidonic acid release and prostaglandin synthesis	Millar & Rozengurt (1988)
enhancement of cAMP accumulation	Millar & Rozengurt (1988)
increase in c-*fos* and c-*myc* mRNA levels	Rozengurt & Sinnett-Smith (1987, 1988); Mehmet *et al.* (1989)
elevation of c-*fos* protein	Mehmet *et al.* (1989)
stimulation of DNA synthesis	Rozengurt & Sinnett-Smith (1983)

& Rozengurt 1982) that were identified as PKC (Nishizuka 1984). Furthermore, addition of the synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG) mimics the action of phorbol esters in stimulating reinitiation of DNA synthesis and cell division (Rozengurt *et al.* 1984).

Another approach to testing the role of PKC in the production of biological responses is to exploit the selective removal of this enzyme caused by a prolonged pretreatment of the cells with phorbol ester. Chronic exposure to phorbol esters leads to a marked decrease in the number of specific phorbol ester-binding sites (Collins & Rozengurt 1982, 1984) and to the disappearance of measurable PKC activity in cell-free preparations (Rodriguez-Pena & Rozengurt 1984; Ballester & Rosen 1985; Stabel *et al.* 1987). In parallel with this downregulation of PKC activity, the cells become desensitized to the mitogenic effects elicited by phorbol esters (Collins & Rozengurt 1982, 1984) or OAG (Rozengurt *et al.* 1984). Hence, activation of PKC is a potential pathway leading to mitogenesis.

Activation of protein kinase C in intact fibroblasts

As activation of PKC may play a role in eliciting mitogenesis, it was of importance to test directly whether growth factors, including bombesin, lead to activation of this enzyme in intact, quiescent cells. A rapid increase in the phosphorylation of an acidic cellular protein with an M_r of 80000 (termed 80 kDa) has provided a specific signal for activation of PKC in intact fibroblastic cells (Rozengurt *et al.* 1983*a*; 1984; Rodriguez-Pena & Rozengurt, 1985, 1986*b*). For example, the phosphorylation of the same 80 kDa protein is stimulated in cells by addition of: (i) biologically active phorbol esters; (ii) the synthetic diacylglycerols; and (iii) exogenous

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phospholipase C, which causes phospholipid breakdown and generates diacylglycerol. Down regulation of PKC activity prevents the increase in 80 kDa phosphorylation by subsequent addition of phorbol esters, phospholipase C, or OAG (reviewed in Rozengurt (1986)). Furthermore, the same 80 kDa protein is phosphorylated in cell-free systems either by activation of endogenous PKC or by addition of the purified enzyme (Rodriguez-Pena & Rozengurt 1986*a*; Blackshear *et al.* 1986). Recently, a phosphoprotein closely related to 80 kDa has been purified 3800-fold from rat brain (Morris & Rozengurt 1988). The preparation appears homogeneous by one and two-dimensional polyacrylamide gel electrophoresis (PAGE), is an effective substrate of PKC and contains an unusually high proportion of acidic amino acids (glu, asp) and of alanine. Although the nature and role of the 80 kDa phosphoprotein remain to be elucidated, its phosphorylation provides a specific marker for assessing which mitogenic agents activate PKC in intact cells.

Addition of bombesin causes a potent and rapid increase in 80 kDa phosphorylation (Zachary *et al.* 1986). An enhancement in phosphorylation can be detected as early as 15 seconds after the addition of bombesin, and maximal phosphorylation is obtained in less than 1 min. The increase in 80 kDa phosphorylation produced by bombesin is concentration-dependent, induced by bombesin agonists and blocked by bombesin antagonists. Removal of bombesin results in rapid dephosphorylation of 80 kDa (Rodriguez-Pena *et al.* 1986).

Downregulation of PKC prevents both the increase in 80 kDa phosphorylation (Zachary *et al.* 1986) and the stimulation of DNA synthesis induced by bombesin in the absence of insulin (Rozengurt & Sinnett-Smith 1987). These findings show that PKC may play a central role in the stimulation of bombesin-induced fibroblast proliferation and suggest that 80 kDa phosphorylation could represent a cytoplasmic bridge for the transduction of the mitogenic signal from the plasma membrane to the nucleus.

Transmodulation of EGF binding

^{125}I-labelled EGF binding to specific surface receptors in Swiss 3T3 cells is markedly inhibited by bombesin and other growth factors (reviewed by Zachary & Rozengurt 1985*b*). The effect is rapid in onset and results from a decrease in the apparent affinity of the EGF receptor population for EGF. Considerable evidence implicates PKC in the regulation of EGF receptor affinity by bombesin and other transmodulating agents (Zachary & Rozengurt 1985*b*). In particular, the inhibition of EGF binding induced by either PBt_2 or bombesin is prevented by downregulation of PKC (Zachary *et al.* 1986). The EGF receptor is phosphorylated by PKC at specific sites both *in vitro* and *in vivo* (Lin *et al.* 1986; Schlessinger 1986). Thus transmodulation of the EGF receptor may result from the covalent modification of the EGF receptor catalysed by PKC, though other mechanisms are not excluded.

Ionic fluxes

Ca^{2+} *mobilization*

One of the earliest events to occur after the binding of bombesin to its specific receptor is a rapid mobilization of Ca^{2+} from intracellular stores, which leads to a transient increase in the concentration of cytosolic Ca^{2+} (Mendoza *et al.* 1986). This Ca^{2+} flux can be distinguished from that of PDGF on the basis of kinetics and sensitivity to phorbol ester inhibition (Lopez-Rivas *et al.* 1987; Nånberg & Rozengurt 1988). The mobilization of Ca^{2+} by bombesin may be

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mediated by inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), which acts as a second messenger in the action of many ligands that stimulate inositol lipid turnover and Ca^{2+} efflux (Berridge 1987). Bombesin causes a rapid increase in Ins(1,4,5)P_3 (Heslop *et al.* 1986; Lopez-Rivas *et al.* 1987; Takuwa 1987 that coincided with the increase in cytosolic Ca^{2+} (Nånberg & Rozengurt, 1988). Ins(1,4,5)P_3 is formed as a result of phospholipase C catalysed hydrolysis of phosphatidyl inositol 4,5-bisphosphate in the plasma membrane, a process that also generates 1,2-diacylglycerol (DAG). DAG can also be generated from other sources such as phosphatidylcholine hydrolysis (Muir & Murray 1987) and acts as second messenger in the activation of PKC by bombesin (Morris & Rozengurt 1988*a*).

Monovalent ion fluxes

The stimulation of the monovalent K^+, H^+ and Na^+ ion fluxes is a general early response seen in most types of quiescent cells stimulated to proliferate by multiple combinations of growth promoting factors (reviewed in Rozengurt & Mendoza (1986)). This ubiquity suggests a possible role for enhanced ion fluxes in the mitogenic response. Addition of bombesin to quiescent 3T3 cells also causes a rapid increase in the activity of the ouabain-sensitive Na^+/K^+ pump (Mendoza *et al.* 1986). The activity of the Na^+/K^+ pump in intact fibroblasts is limited and regulated by the supply of Na^+ (Rozengurt 1985). Growth-promoting agents, including bombesin, stimulate the pump by increasing Na^+ entry into the cells. This translocation of Na^+ across the plasma membrane is mediated in part by an amiloride-sensitive Na^+/H^+ antiport system that is driven by the Na^+ electrochemical gradient. Studies to determine the mechanisms by which bombesin stimulates these monovalent ion fluxes have shown the existence of PKC dependent and independent pathways of activation (Vara & Rozengurt 1985; Vara *et al.* 1985; Mendoza *et al.* 1986).

Cyclic AMP

The role of cyclic nucleotides in the control of the proliferative response in quiescent fibroblasts has been the subject of a large and controversial literature (Rozengurt 1985). The following lines of evidence indicate that a sustained increase in the cellular level of cyclic adenosine monophosphate (cAMP) constitutes a growth-promoting signal for Swiss 3T3 cells:

1. A variety of agents that promote cAMP accumulation in Swiss 3T3 cells, including prostaglandin E (PGE_1), the adenosine agonist 5′-*N*-ethylcarboxamide (NECA), cholera toxin, and permeable cAMP analogues stimulate DNA synthesis by acting synergistically with insulin, phorbol esters, and other mitogens (Rozengurt *et al.* 1981; Rozengurt 1982).

2. The neuropeptide vasoactive intestinal peptide (VIP) both promotes rapid accumulation of cAMP and stimulates re-initiation of DNA synthesis in 3T3 cells when added in the presence of insulin and modulators of cAMP metabolism, such as forskolin or inhibitors of cAMP phosphodiesterase (Zurier *et al.* 1988).

3. PDGF, one of the most potent mitogens in Swiss 3T3 fibroblasts (Ross *et al.* 1986), induces a striking increase in intracellular cAMP mediated, at least in part, by increased synthesis of E type prostaglandins that in turn leave the cell and stimulate cAMP synthesis through their own receptors (Rozengurt *et al.* 1983*b*).

Recent work to elucidate the molecular mechanisms underlying the cAMP-mediated mitogenic response have shown that an elevation in cAMP rapidly increases the phos-

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phorylation of a $M_r = 58000$ cellular protein identified as vimentin, the main protein subunit of the intermediate filaments of mesenchymal cells, including Swiss 3T3 cells (Escribano & Rozengurt 1988). A direct role of cAMP-dependent protein kinase in this phosphorylation has been substantiated by the finding that identically migrating phosphorylated tryptic fragments of vimentin are generated both *in vivo* in response to an increase in CAMP and *in vitro* by addition of the catalytic subunit of cAMP-dependent protein kinase (Rozengurt & Ober 1989). In contrast, the phosphorylation of 80 kDa is not changed by any of the agents used to increase cAMP. Thus the stimulation of the PKC and cAMP signalling pathways can be monitored by distinct phosphorylation events.

Cross-talk between protein kinase C and cAMP

While, as shown above, cAMP and PKC represent separate signal transduction pathways, recent results show the existence of interactions between these major transmembrane signalling systems. Specifically, activation of PKC by either phorbol esters or diacylglycerols considerably enhances the accumulation of cAMP in response to forskolin or cholera toxin, whereas downregulation of PKC blocks this enhancing effect (Rozengurt *et al.* 1987).

A further example of cAMP–PKC signal pathway interactions has come from studies with the neuropeptides of the bombesin family. Bombesin cause a marked enhancement of cAMP accumulation (in the presence of forskolin); this increase is partially diminished both by downregulation of PKC and by the cyclooxygenase inhibitor indomethacin (Millar & Rozengurt 1988). The inhibitory effects are additive in nature, suggesting the existence of two mechanisms by which bombesin can enhance cAMP-accumulation. These findings suggest that cAMP could contribute to the signalling of growth factors that primarily act through the PKC pathway.

One of the most intriguing areas of PKC–cAMP 'cross-talk' involves the molecular basis for these pathway interactions. Further studies using phorbol esters, cAMP-increasing agents and bombesin have shown that this 'cross-talk' is abolished by treatment with pertussis toxin in a time and dose-dependent fashion (Millar & Rozengurt, 1988). As pertussis toxin does not itself promote cAMP accumulation in Swiss 3T3 (Rozengurt *et al.* 1987), it is unlikely to act by removing a tonic inhibitory influence on the adenylate cyclase via G_i. An attractive possibility is that a novel pertussis toxin substrate mediates the 'cross talk' between the PKC and the cAMP pathways.

Induction of the proto-oncogenes c-*fos* and c-*myc*

In addition to the events in the membrane and cytosol described above, serum and other growth factors rapidly and transiently induce the expression of the cellular oncogenes c-*fos* and c-*myc* in quiescent fibroblasts (reviewed in Rozengurt & Sinnett-Smith (1988)). As these cellular oncogenes encode nuclear proteins it is plausible that their transient expression may play a role in the transduction of the mitogenic signal in the nucleus. The recent demonstration that the product of the phorbol ester-inducible proto-oncogene c-*jun*, identified as the *trans*-acting factor, AP-1 (Bohmann *et al.* 1987; Angel *et al.* 1988; Bos *et al.* 1988) forms a tight complex with FOS protein (Rauscher *et al.* 1988) is consistent with a role for c-*fos* in the regulation of gene transcription (Schonthal *et al.* 1988).

Bombesin rapidly and transiently induces the expression of the cellular oncogenes c-*fos* and

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c-*myc* (Letterio *et al.* 1986; Rozengurt & Sinnett-Smith 1987). Enhanced expression of c-*fos* occurs within minutes of bombesin addition and is followed by increased expression of c-*myc*. The timecourse and magnitude of these effects are similar to those induced by a saturating concentration of PDGF (Rozengurt & Sinnett-Smith 1987).

There has been considerable interest in elucidating the signal transduction pathways involved in c-*fos* induction. There is increasing evidence implicating PKC activation in the sequence of events linking receptor occupancy and proto-oncogene induction (reviewed in Rozengurt & Sinnett-Smith (1988)). Accordingly, bombesin-induced oncogene expression is markedly reduced by downregulation of PKC. However, direct activation of PKC is not sufficient to evoke a maximal increase in c-*fos* and c-*myc* mRNA levels. It is likely that the induction of these cellular oncogenes by bombesin is mediated by the coordinated effects of Ca^{2+} mobilization and activation of PKC (Rozengurt & Sinnett-Smith 1987). Furthermore, additional pathways of control of c-*fos* expression that are completely independent of activation of protein kinase C have also been shown (Rozengurt & Sinnett-Smith 1988).

PKC activation, c-*fos induction and stimulation of* DNA *synthesis*

Bombesin stimulates DNA synthesis in the absence of any other growth factor (Rozengurt & Sinnett-Smith 1983). Insulin markedly potentiates the mitogenic activity of bombesin without activating PKC (Rozengurt & Sinnett-Smith 1983, 1988) or enhancing the large induction of c-*fos* mRNA expression (Rozengurt & Sinnett-Smith 1987, 1988). Indeed, this hormone removes the requirement for PKC in DNA synthesis mediated by bombesin (Rozengurt & Sinnett-Smith 1987, 1988). Hence, bombesin can initiate DNA synthesis through PKC-dependent and PKC-independent pathways, thereby providing an ideal system to gain further insight into the cause–effect relations between PKC, c-*fos* expression and the reinitiation of the cell cycle.

Mehmet *et al.* (1989) found a direct correlation between c-*fos* induction and PKC-dependent mitogenesis in bombesin-treated cells. On the other hand, these investigators have also shown that Swiss 3T3 cells can be stimulated to divide by bombesin and insulin through PKC-independent pathways with 20–30-fold lower levels of c-*fos* mRNA and protein expression. These findings are consistent with other observations (Rozengurt & Sinnett-Smith 1987, 1988; Mehmet *et al.* 1988). In particular, quiescent Swiss 3T3 cells can be stimulated through a cAMP-dependent pathway (by using a combination of forskolin and insulin) with only a marginal increase in c-*fos* mRNA and protein expression (Mehmet *et al.* 1988). An increase in intracellular cAMP can stimulate mitogenesis in these cells in the absence of PKC (Rozengurt *et al.* 1981, 1983*a*). We conclude that a high level of FOS protein is not an obligatory requirement for mitogenesis in Swiss 3T3 cells, but may be important in the transduction of specific intracellular signals, such as PKC. These findings support the hypothesis that multiple signal transduction pathways (Rozengurt 1986; Rozengurt *et al.* 1988) are involved in the initiation of a proliferative response.

Signal transduction

Molecular aspects

A central problem in understanding the molecular basis of the potent mitogenic response initiated by bombesin-like peptides is to elucidate how the occupied receptor communicates

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with effector molecules in the cell. Transmembrane signalling mechanisms involving either a tyrosine kinase or a guanine nucleotide-binding regulatory protein (*G* protein) have been proposed to couple growth factor receptors to intracellular effectors (see, for example, Rozengurt 1986). However, it has not been shown that any of these mechanisms is physiologically relevant to the molecular events induced by bombesin. Bombesin stimulation of tyrosine phosphorylation has been reported (Cirillo *et al.* 1986; Gaudino *et al.* 1988) and the possibility that bombesin receptor signalling is associated with this kinase activity was raised. However, bombesin associated tyrosine kinase activity was not detected by another laboratory (Isacke *et al.* 1986) and the relative molecular mass of the tyrosine phosphorylated band does not coincide with that of the putative receptor identified by affinity cross-linking (Zachary & Rozengurt 1987*a*; Kris *et al.* 1987; Sinnett-Smith *et al.* 1988).

It was suggested that a pertussis toxin-sensitive G protein could couple the bombesin receptor to the enzymes that hydrolyse polyphosphoinositides (Letterio *et al.* 1986). However, the demonstration that pertussis toxin did not inhibit bombesin stimulation of inositol phosphate formation, Ca^{2+} mobilization or activation of protein kinase C in Swiss 3T3 cells (Zachary *et al.* 1987*a*), did not support this possibility. In addition, a recent report (Fisher & Shonbrum 1988) showed that GTP analogues cause only a small reduction of bombesin binding to 3T3 cell membranes, in contrast to a profound inhibition of bombesin binding to membranes from GH_4C_1 pituitary cells in which this neuropeptide promotes short-term secretion rather than cell proliferation. Thus further experimental evidence was needed to assess whether a G protein is involved in the coupling of the bombesin receptor to the generation of any intracellular signal related to mitogenesis.

Evidence for the role of G proteins in signal transduction pathways can be obtained by assessing the effects of guanine nucleotide analogues on receptor mediated responses in permeabilized cells. Recent work from this laboratory has characterized the phosphorylation of 80 kDa in digitonin-permeabilized Swiss 3T3 cells and employed this technique to study the mechanism of bombesin-induced activation of protein kinase C (Erusalimsky *et al.* 1988). A salient feature of the results is that the GDP analogue GDP-β-S inhibited the stimulation of 80 kDa phosphorylation by bombesin in a selective manner. GDP-β-S is known to prevent the activation of G proteins by inhibiting the binding of GTP. The fact that GTP can reverse the inhibitory effect of GDP-β-S is consistent with this notion. The findings show that guanine nucleotides modulate the transduction of the signal from the bombesin receptor and suggest that a G protein couples the bombesin receptor to the generation of an intracellular signal, which in turn activates protein kinase C in Swiss 3T3 cells. Whereas the G protein is not sensitive to pertussis toxin, its nature as well as that of its effector (phospholipase C or other phospholipases) remains to be elucidated. Further experimental work, by using membrane or receptor preparations, is necessary to elucidate whether the putative G protein(s) is physically associated to the receptor and to clarify the role of tyrosine phosphorylation in the transduction of the bombesin signal.

Regulatory aspects

Exposure of cells to many peptide hormones or neurotransmitters decrease the subsequent response of target cells to further challenge with the same ligand (homologous desensitization) or with a structurally unrelated ligand that elicits responses through a separate receptor (heterologous desensitization). Desensitization has been well-documented for hormones that elicit short-term metabolic responses such as those mediated through adenylate cyclase-coupled

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receptors. However, little is known about desensitization of long-term responses such as cellular growth and differentiation. As Swiss 3T3 cells have been extensively used to analyse the mechanisms of mitogenic stimulation by several mitogenic neuropeptides (Woll & Rozengurt 1989), these cells provide an ideal model system for investigating the role of cellular desensitization in the control of cell proliferation. Collins & Rozengurt (1983) described the homologous desensitization to mitogenic stimulation by the neuropeptide vasopressin in Swiss 3T3 cells. Recently, long-term heterologous desensitization of cellular mitogenic responsiveness has been characterized. Millar & Rozengurt (1989) found that prolonged pretreatment with vasopressin can induce a selective heterologous desensitization of the mitogenic activity of bombesin and structurally related peptides, including gastrin-releasing peptide (GRP), in Swiss 3T3 cells. Desensitization requires prolonged incubation (half-maximal desensitization occurring after approximately 20 h of pretreatment) and continuous protein synthesis. Bombesin responsiveness is restored by incubation in the absence of vasopressin. Pretreatment does not alter the number, affinity or internalization capacity of the bombesin receptors. However, the induction of the proto-oncogene c-*fos* by bombesin is profoundly inhibited following vasopressin pretreatment. The findings suggest that the block to bombesin-stimulated mitogenesis occurs at a post-receptor locus and may involve an uncoupling of ligand bound bombesin receptor from the generation of its early signals.

Neuropeptides have been implicated in the unrestrained growth of certain tumours and consequently the mechanisms by which these peptides stimulate cell growth are attracting considerable attention. For example, bombesin and its structurally related counterparts have been implicated as autocrine growth factors in the growth of small cell lung cancer (Woll & Rozengurt 1988*a*). Disruption of the inhibitory mechanism(s) the cell employs to regulate mitogenic stimulation by neuropeptides (desensitization) could result in the development of unrestrained cell growth.

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