J. Cell Sci. Suppl. 3, 229–242 (1985)
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SYNERGISTIC SIGNALS IN MITOGENESIS: ROLE OF ION FLUXES, CYCLIC NUCLEOTIDES AND PROTEIN KINASE C IN SWISS 3T3 CELLS

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SUMMARY

A fundamental feature in the action of most mitogenic agents when added to quiescent cells in serum-free medium is that they exhibit striking synergistic effects when applied in specific combinations. A tenable hypothesis of growth control must provide a cogent explanation for the molecular mechanisms underlying this complex pattern of synergistic effects. To gain an understanding of the mechanisms by which these synergistic effects arise, we studied the initial cellular responses associated with the interaction of mitogenic factors and hormones with the cell, including changes in cation fluxes, cyclic nucleotides and cellular phosphoproteins. In this paper, some of our recent results on the early signals and responses elicited by multiple growth-promoting agents in quiescent cultures of Swiss 3T3 cells will be summarized. On the basis of the emerging information, we propose a framework that integrates early events and synergistic effects in a unified hypothesis of growth control.

INTRODUCTION

Quiescent cultures of the mouse 3T3 cell line, which has provided a useful model system for many studies on growth control, can be stimulated to reinitiate DNA synthesis and cell division by a variety of exogenous agents added to serum-free medium (Rozengurt, 1980, 1983). The mitogenic effectiveness of a variety of growth-promoting agents in Swiss 3T3 cells is summarized in Table 1. The striking feature is that only platelet-derived growth factor (PDGF) and related factors (Dicker, Pohjanpelto, Pettican & Rozengurt, 1981; Lopez-Rivas, Stroobant, Waterfield & Rozengurt, 1984) and the peptides of the bombesin family (Rozengurt & Sinnett-Smith, 1983) can induce DNA synthesis when added in the absence of other growth factors. In contrast, the remaining array of agents only stimulate initiation of DNA synthesis in Swiss 3T3 cells when added in specific combinations (see Rozengurt, 1980, 1983, 1985, for references). The pattern of these striking and specific synergistic interactions among defined growth-promoting molecules has critical mechanistic implications which must be explained by any hypothesis of growth control.

In an effort to gain an understanding of the mechanism by which these synergistic effects arise, our attention has been focused on the initial cellular responses

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Table 1. *Synergistic effects among mitogenic agents added to quiescent cultures of Swiss 3T3 cells maintained in serum-free medium*

	PDGF/ FDGF	Bombesin	Vaso-pressin	Phorbol esters, teleocidin	Diacyl-glycerol (OAG)	Insulin	EGF	Cholera toxin	PGE_1	NECA	cAMP analogues	Anti-micro-tubule agents
PDGF/FDGF	+++	++++	+++	+++	+++	++++	++++	+++	+++	+++	+++	++++
Bombesin	++++	++	++	++	++	++++	+++	++	++	++	++	+++
Vasopressin	+++	++	–	–	–	+++	++	++	++	++	++	–
Phorbol esters teleocidin	+++	++	–	–	–	+++	++	++	++	++	++	–
Diacylglycerol (OAG)	+++	++	–	–	–	+++	++	++	++	++	++	–
Insulin	++++	++++	+++	+++	+++	–	+++	+++	+++	+++	+++	–
EGF	++++	+++	++	++	++	+++	–	+	+	+	+	–
Cholera toxin	+++	++	++	++	++	+++	+	–	–	–	–	–
PGE_1	+++	++	++	++	++	+++	+	–	–	–	–	–
NECA	+++	++	++	++	++	+++	+	–	–	–	–	–
cAMP analogues	+++	++	++	++	+++	+	–	–	–	–	–	
Antimicrotubule	++++	+++	–	–	–	+	–	–	–	–	–	–

The original data can be obtained from references given in the text and in recent reviews (Rozengurt, 1980, 1983, 1984, 1985; Rozengurt *et al.* 1985). ++++, +++, ++, +, –, represent 80–100 %, 60–80 %, 20–40 %, 5–20 % and <2 % of the maximal response ([^{3}H]thymidine incorporation into acid-precipitable material) achieved. In some cases, a factor causes a shift in the dose-response of another factor but does not alter the maximal response; for clarity these situations are not described in the Table. They can be found in the original publications.

associated with the interaction of mitogenic factors and hormones with the cell, in the expectation that the early events will provide useful clues to primary regulatory mechanisms. Some of our recent studies on the early signals and cellular responses elicited by growth factors in quiescent cultures of Swiss 3T3 cells will be briefly described. Subsequently, a framework that integrates these early events and the synergistic effects among mitogens in a unified hypothesis of growth control will be discussed.

IONIC RESPONSES ELICITED BY GROWTH FACTORS IN QUIESCENT CELLS

Monovalent ion fluxes

One of the earliest responses elicited by the addition of serum and growth factors to quiescent cultures of 3T3 fibroblasts is an increase in the activity of the ouabain-sensitive Na^+/K^+ pump (Rozengurt & Heppel, 1975). The activity of the Na^+/K^+ pump in intact fibroblasts is limited and regulated by cytosolic Na^+ (Smith & Rozengurt, 1978; Mendoza, Wigglesworth, Pohjanpelto & Rozengurt, 1980). Since serum and growth factors stimulate the entry of Na^+ into quiescent cells (Rozengurt, 1981*a*), it has been suggested that one of the initial events that occurs in fibroblastic cells that have been stimulated to proliferate is an increase in the rate of Na^+ influx into the cells with a subsequent stimulation of Na^+/K^+ pump activity.

Because the translocation of Na^+ across the plasma membrane of 3T3 cells is mediated, at least in part, by an amiloride-sensitive Na^+/H^+ antiport system, the stimulation of Na^+ entry by mitogenic agents leads to an increase in intracellular pH (Schuldiner & Rozengurt, 1982; Burns & Rozengurt, 1983, 1984; Lopez-Rivas *et al.* 1984). The fact that mitogenic stimulation leads to cytoplasmic alkalinization has been substantiated using different combinations of growth factors, cell types and techniques (see Rozengurt & Mendoza, 1986, for review). The ability of growth factors to induce cytoplasmic alkalinization suggests that the activation of Na^+/H^+ exchange is a primary effect of the mitogens rather than a secondary mechanism for the extrusion of protons resulting from a growth factor-induced acceleration of cellular metabolism. It is plausible that the stimulation of a proliferative response in quiescent cells depends on maintaining intracellular pH (Mendoza & Rozengurt, unpublished) and K^+ concentration (Lopez-Rivas, Adelberg & Rozengurt, 1982; Burns & Rozengurt, 1984) above critical threshold levels. As discussed recently (Rozengurt & Mendoza, 1986), these threshold concentrations may play a permissive and, or, a triggering role in mitogenesis.

Divalent cation fluxes

Addition of serum to quiescent cultures of Swiss 3T3 cells and other fibroblast cell lines induces a marked increase in the rate of Ca^{2+} efflux from radioactively labelled cells (Lopez-Rivas & Rozengurt, 1983). This is one of the earliest events (15 s) that takes place in quiescent fibroblasts after stimulation with serum. Changes in Ca^{2+} distribution are not restricted to serum-stimulated cells since vasopressin stimulates

the efflux of $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$-loaded cells (Lopez-Rivas & Rozengurt, 1984). Likewise, homogeneous preparations of PDGF cause a marked and dose-dependent (half-maximal effect at 6×10^{-10}M) stimulation of $^{45}Ca^{2+}$ efflux from quiescent 3T3 cells (Lopez-Rivas & Rozengurt, unpublished results). Since the stimulation of $^{45}Ca^{2+}$ efflux can be elicited in the absence of extracellular Ca^{2+}, it seems that serum, vasopressin and PDGF release this cation from an intracellular store(s). Indeed, vasopressin caused a rapid 50% decrease in the total Ca^{2+} content of the cells (Lopez-Rivas & Rozengurt, 1984). This release would be likely to increase the concentration of cytosolic Ca^{2+} [Ca_i^{2+}] which, in turn, leads to Ca^{2+} efflux mediated by the plasma membrane Ca^{2+}-ATPase. This interpretation was confirmed by the finding that vasopressin, PDGF and bombesin caused a rapid two- to three-fold increase in [Ca_i^{2+}] measured by changes in the fluorescence of quin-2. The increases in [Ca_i^{2+}] were brief, with [Ca_i^{2+}] falling to or near basal levels in 3–5 min (Fig. 1). This Ca^{2+} mobilization may be mediated by inositol 1,4,5-triphosphate (IP_3), which has been implicated as a second messenger in the action of ligands that induce receptor-mediated inositol lipid turnover and Ca^{2+} release (Berridge & Irvine, 1984). Interestingly, neither insulin nor EGF induces Ca^{2+} mobilization or increases [Ca_i^{2+}] in 3T3 cells (Lopez-Rivas & Rozengurt, 1983; Fig. 1).

ACTIVATION OF PROTEIN KINASE C IN INTACT CELLS

Ca^{2+}-sensitive, phospholipid-dependent protein kinase (protein kinase C), which is stimulated by unsaturated diacylglycerol and serves as a major receptor for the tumour promoters of the phorbol ester family (see Nishizuka, 1984, for a review), may play an important role in signalling a variety of cellular responses including cell growth. Accordingly, it is of importance to determine which mitogenic agents lead to activation of protein kinase C in intact, quiescent cells. Recently, we reported that a rapid increase in the phosphorylation of a $M_r = 80\,000$ cellular protein (termed 80k) reflects the activation of protein kinase C in intact cells (see Rozengurt, Rodriguez-Pena & Sinnett-Smith, 1985, for a review). For example, addition of biologically active phorbol esters stimulates a rapid (15 s) phosphorylation of this protein (Rozengurt, Rodriguez-Pena & Smith, 1983*c*). Further, phospholipid breakdown induced by exogenous phospholipase C, which generates diacylglycerol, causes a rapid enhancement of 80k phosphorylation (Rozengurt *et al.* 1983*c*). In addition, the synthetic diacylglycerol 1-oleoyl-2-acetyl-glycerol (OAG) stimulates the phosphorylation of the same 80k phosphoprotein in quiescent 3T3 cells (Rozengurt, Rodriguez-Pena, Coombs & Sinnett-Smith, 1984; Rozengurt *et al.* 1985). Finally, prolonged pretreatment of the cells with phorbol ester, which leads to a marked decrease in the number of specific phorobol ester binding sites (Collins & Rozengurt, 1982*a*,*b*, 1984) and to disappearance of protein kinase C activity measured in cell-free preparations (Rodriguez-Pena & Rozengurt, 1984; Rozengurt *et al.* 1985), prevents the increase in 80k phosphorylation elicited by all these diverse agents: namely, phorbol esters, phospholipase C and OAG (Rozengurt *et al.* 1983*c*, 1984; Rodriguez-Pena & Rozengurt, 1985). Although the 80k phosphoprotein has not been

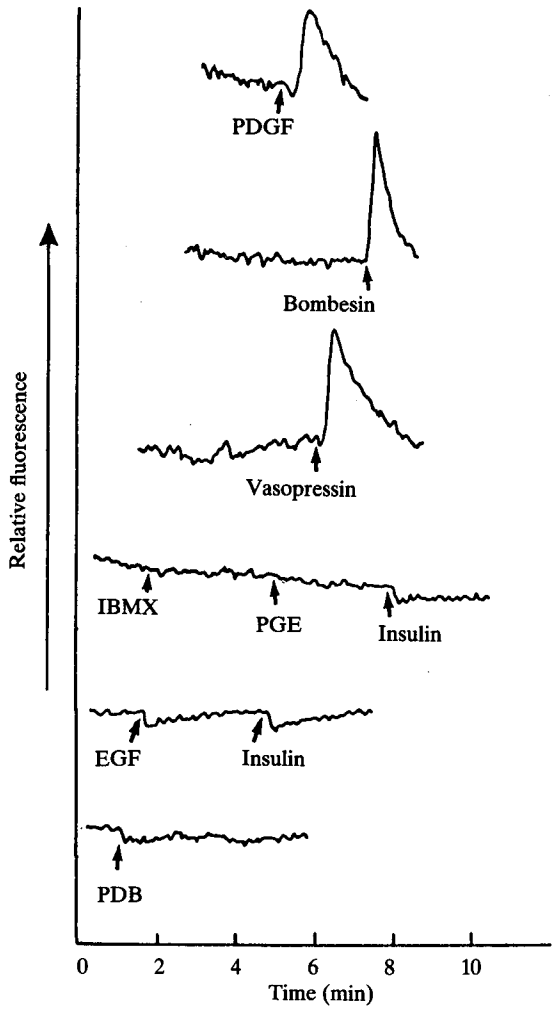

Fig. 1. For these studies, Swiss 3T3 cells were grown on Cytodex 2 beads suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and constantly stirred at 37°C. After 6 days, the medium was changed to DMEM containing 1% FBS. Twenty-four hours later, beads were washed with DMEM and then incubated for 45 min in this medium containing 15 μM-quin-2 acetoxymethylester. They were then washed three times with a solution containing 140 mM-NaCl, 5 mM-KCl, 0·9 mM-$MgCl_2$, 1·8 mM-$CaCl_2$, 25 mM-glucose, amino acids at the same concentration as in DMEM, 20 mM-Hepes and sufficient Tris to bring the pH to 7·2. After washing, beads containing about 2·5 mg of cell protein were suspended and stirred at 37°C in a 10 mm cuvette. Fluorescence was followed in a Perkin-Elmer LS-5 fluorimeter (excitation wavelength, 339 nm; emission wavelength, 492 nm). At the times indicated by the arrows, vasopressin (20 ng ml^{-1}), bombesin (10 ng ml^{-1}), PDGF (1·25 μg ml^{-1}), phorbol dibutyrate (200 ng ml^{-1}), insulin (10 μg ml^{-1}), EGF (15 ng ml^{-1}), PGE_1 (250 ng ml^{-1}) or methylisobutyl-xanthine (IBMX) (50 μM) was added. Control (Ca_i^{2+}) was 158±8 nM ($n = 55$).

identified, the detection of changes in its phosphorylation provides a useful approach to determine which mitogenic agents activate protein kinase C in intact cells.

In the course of studies designed to determine which extracellular agents activate protein kinase C in intact fibroblastic cells we found that addition of PDGF (Rozengurt *et al.* 1983*c*; Rodriguez-Pena & Rozengurt, 1985) or of mitogenic peptides such as vasopressin and bombesin (Zachary & Rozengurt, unpublished data) causes a potent and extremely rapid increase in the phosphorylation of the 80k cellular protein. Hence, protein kinase C might not only mediate the multiple biological actions of phorbol esters but it may play a fundamental role in effecting the proliferative response elicited by serum, and certain growth factors and mitogenic hormones in their target cells. Substantial evidence for this hypothesis has come from recent studies demonstrating that the synthetic diacylglycerol OAG, which stimulates protein kinase C in intact cells, is a potent mitogen for Swiss 3T3 cells acting as a phorbol ester agonist (Rozengurt *et al.* 1984).

Protein kinase C and ion fluxes

In view of the possibility that protein kinase C may play a role in the control of the proliferative response, it was important to define whether activation of protein kinase C elicits monovalent ionic fluxes in quiescent 3T3 cells. Recently, Vara, Schneider & Rozengurt (1985) reported that addition of OAG or PBt_2 to Swiss 3T3 cells rapidly enhances amiloride-sensitive Na^+/H^+ antiport activity leading to stimulation of the Na^+/K^+ pump. Previously, Dicker & Rozengurt (1981*a*) showed that the biologically active phorbol esters PBt_2 and TPA stimulated Na^+ entry and Na^+/K^+ pump in 3T3 cells; the half-maximal concentration of PBt_2 needed to elicit this early effect was virtually identical to the K_d of [^{3}H]PBt_2 for its high-affinity receptor in these cells (Collins & Rozengurt, 1982*a*). In addition, the activators of protein kinase C, e.g. PBt_2 and OAG, increase pH_i (Burns & Rozengurt, 1983; Vara *et al.* 1985). In contrast, phorbol esters fail to stimulate $^{45}Ca^{2+}$ efflux from quiescent 3T3 cells preloaded with this isotope (unpublished results) or to increase (Ca_i^{2+}) as judged by changes in the fluorescence of quin-2 (Fig. 1).

Prolonged pretreatment with PBt_2 markedly reduces the stimulation of $^{86}Rb^+$ or $^{22}Na^+$ uptake by subsequent addition of either PBt_2 or OAG. This loss of ionic responses to OAG and PBt_2 seen in 3T3 cells with a greatly reduced number of high-affinity phorbol ester receptors and activity of protein kinase C (see above) implicates this phosphotransferase system in the stimulation of monvalent cation fluxes. These findings strongly suggest that activation of protein kinase C leads, either directly or indirectly, to increased activity of the Na^+/H^+ antiport system, which in turn promotes Na^+ influx, increases pH_i and stimulates the Na^+/K^+ pump activity. In this manner, protein kinase C may represent an important molecular link in the sequence of events triggered by the binding of growth-promoting factors to their respective receptors. However, since the activity of the Na^+/H^+ antiport is also enhanced by some mitogens that do not activate protein kinase C in 3T3 cells (e.g. EGF + insulin), it is likely that these ionic fluxes may be regulated by mechanisms not involving protein kinase C (Vara & Rozengurt, 1985).

Protein kinase C and transmodulation of EGF receptor

A set of structurally unrelated ligands including phorbol esters, vasopressin, PDGF and FDGF inhibit the binding of [^{125}I]EGF to specific surface receptors in Swiss 3T3 cells and other cell lines (Rozengurt & Collins, 1983). A feature shared by all the inhibitory ligands is that the modulation of EGF binding is rapid in onset and results from a decrease in the apparent affinity of the EGF receptor population for EGF (Rozengurt, Brown & Pettican, 1981*a*; Rozengurt, Collins, Brown & Pettican, 1982; Collins, Sinett-Smith & Rozengurt, 1983). Since the various ligands that inhibit EGF binding interact with sites that are distinct from EGF receptors, the decrease in the affinity of the EGF receptors occurs through an indirect mechanism termed 'transmodulation' (Rozengurt & Collins, 1983). Although the significance of transmodulation as an early response in mitogenesis remains unclear, it is likely that the decreases in apparent affinity of the EGF receptor induced by various agents could occur by a common mechanism (Rozengurt *et al.* 1982; Collins & Rozengurt, 1983; Rozengurt & Collins, 1983).

Considerable evidence implicates protein kinase C in the transmodulation of the EGF receptor affinity. Recent studies showed that OAG causes a rapid and striking decrease in the apparent affinity of this receptor without changing the total number of sites (Sinnett-Smith & Rozengurt, 1985). Further, the transmodulation induced by OAG or PBt_2 is blocked by greatly reducing protein kinase C by prior treatment of the cells with phorbol esters (Collins & Rozengurt, 1982*a*, 1984; Sinnett-Smith & Rozengurt, 1985). Interestingly, protein kinase C has recently been shown to phosphorylate the EGF receptor of human epidermal carcinoma A431 cells at specific sites (Hunter, Ling & Cooper, 1984). The possibility that the interconversion of EGF receptors from a high-affinity state to a low-affinity one induced in 3T3 cells by phorbol esters, OAG, vasopressin, PDGF and FDGF, results from the covalent modification of the EGF receptor catalysed by protein kinase C, is attractive and warrants further experimental work.

CYCLIC NUCLEOTIDES AND INITIATION OF DNA SYNTHESIS

The role of cyclic nucleotides, cyclic AMP (cAMP) and cyclic GMP, in the control of the proliferative response of quiescent fibroblastic cells has been the subject of a large and controversial amount of literature (Rozengurt, 1981*b*). Recently, we found that increased cellular concentrations of cAMP promoted by cholera toxin (Rozengurt, Legg, Strang & Courtenay-Luck, 1981*b*), adenosine agonists (Rozengurt, 1982*a*), cAMP derivatives (Rozengurt, 1982*b*), prostaglandin E_1 (Rozengurt, Collins & Keehan, 1983*b*) or forskolin act synergistically with other growth-promoting agents to stimulate DNA synthesis in quiescent cultures of 3T3 cells (Table 1; Rozengurt, 1985, for references).

Since a sustained increase in the cellular level of cAMP constitutes a growth-promoting signal for Swiss 3T3 cells, it was important to evaluate whether physiological growth factors added to serum-free medium could alter cAMP metabolism in 3T3 cells. Interestingly, PDGF induced a striking accumulation of cAMP in 3T3

cells incubated in the presence of inhibitors of cyclic nucleotide degradation (Rozengurt *et al.* 1983*a*). In contrast, other growth-promoting factors including EGF, vasopressin, phorbol esters, OAG or insulin failed to increase the level of cAMP (Rozengurt *et al.* 1983*a*; unpublished results). This accumulation of cAMP elicited by PDGF was mediated by increased synthesis of E-type prostaglandins, which in turn stimulated cAMP synthesis through their own receptor (Rozengurt *et al.* 1983*b*). These findings suggest that cAMP may be one of the signals utilized by PDGF to stimulate initiation of cell proliferation in Swiss 3T3 cells.

Cyclic AMP and other early responses

Increased cellular level of cAMP stimulates the Na^+/K^+ pump mediated uptake of $^{86}Rb^+$ into Swiss 3T3 (Paris & Rozengurt, 1982). In contrast to the stimulation of the Na/K^+ pump within 1–2 min after the addition of Na^+ flux modulators (serum, PDGF, vasopressin, phorbol esters), the stimulation of ouabain-sensitive $^{86}Rb^+$ uptake by cAMP-elevating agents reached a maximal effect after hours of incubation. Further, increased cAMP failed to augment Na^+ influx into 3T3 cells whereas, under identical conditions, serum markedly increased Na^+ entry into 3T3 cells (Paris & Rozengurt, 1982; Rozengurt & Courtenay-Luck, 1982). These findings suggest that the time-dependent stimulation of Na^+/K^+ pump activity caused by increased cAMP levels contrasts mechanistically with the rapid control of pump activity by other growth factors, which is primarily mediated by increased Na^+ entry into the cells.

In other studies, we found that a rapid increase in cAMP does not activate protein kinase C, stimulate Ca^{2+} mobilization (Fig. 1) or induce transmodulation of EGF receptor affinity (unpublished results). We suggest that cAMP, presumably acting through cAMP-dependent protein kinase, activates a pathway leading to mitogenesis that is clearly separate from that utilized by growth factors and mitogenic factors that enhance Na^+ fluxes, transmodulate EGF receptor and activate protein kinase C. The effect of cAMP on pH_i requires further experimental work. A summary of the early responses elicited by each individual growth-promoting factor is presented in Table 2.

SYNERGISTIC EFFECTS AND EARLY SIGNALS: A UNIFIED FRAMEWORK

As mentioned previously a salient feature in the action of most mitogenic agents when added to quiescent cells in serum-free medium is that they exhibit striking synergistic effects when applied in specific combinations. Indeed, a tenable hypothesis of growth control must provide a cogent explanation for the mechanism(s) underlying this complex pattern of synergistic effects. Inspection of Tables 1 and 2 shows that a group of mitogenic agents, such as the tumour promoters phorbol esters and teleocidin, the synthetic diacylglycerol OAG and the neurohypophyseal hormone vasopressin and its related peptides, elicit a common set of early events: namely, they activate protein kinase C, enhance monovalent ion fluxes and transmodulate the EGF receptor but do not alter the basal level of cAMP. Addition of any

Table 2. *Early response elicited by individual growth promoting factors in Swiss 3T3 cells*

	Na^+ influx/ increase in pH_i	Stimulation Na^+/K^+ pump	Ca^{2+} mobilization	EGF receptor transmodulation	80k phosphorylation	Arachidonic acid release	Elevation of cAMP
PDGF/FDGF	+/+	+	+	+	+	+	+
Bombesin	+/+	+	+	+	+	+	+
Vasopressin	+/+	+	+	+	+	−	−
Phorbol esters	+/+	+	−	+	+	−	−
Diacylglycerol	+/+	+	−	+	+	−	−
Insulin	+/+	+	−	−	−	−	−
EGF	+/+	+	−	−	−	−	−
Cholera toxin	−/ND	+	−	−	−	−	+
PGE_1	−/ND	+	−	−	−	−	+
NECA	−/ND	+	−	−	−	−	+
cAMP analogues	−/ND	+	−	−	−	−	+
Colchicine	−/−	−	−	−	−	−	−

The responses to each factor are shown. The magnitude of these responses vary but are not indicated. The original data can be obtained from references in the respective sections in the text and from recent reviews (Rozengurt, 1985; Rozengurt & Mendoza, 1986; Rozengurt *et al.* 1985). ND, not determined.

of these agents individually to quiescent 3T3 cells fails to induce a significant mitogenic response. Further, combinations of this class of agents, e.g. vasopressin and phorbol esters (Dicker & Rozengurt, 1980; Collins & Rozengurt, 1984), teleocidin and phorbol esters (Collins & Rozengurt, 1982*b*), teleocidin and vasopressin (Collins & Rozengurt, 1982*b*), diacylglycerol and vasopressin (Rozengurt *et al.* 1984) or even teleocidin, phorbol esters and vasopressin (Collins & Rozengurt, 1982*b*) and diacylglycerol, phorbol esters and vasopressin (Rozengurt *et al.* 1984) also failed to induce DNA synthesis. Agents that increase intracellular cyclic AMP, such as PGE_1 (Rozengurt *et al.* 1983*b*), the adenosine analogue 5′-*N*-ethylcarboxamide adenosine (Rozengurt, 1982*a*), cholera toxin (Rozengurt *et al.* 1981*b*) and cyclic AMP derivatives (Rozengurt, 1982*b*), produce a totally different pattern of early events. These agents do not activate protein kinase C, stimulate Na^+ flux or transmodulate EGF receptors. Furthermore, they fail to stimulate DNA synthesis when added singly or in combination. We suggest that agents sharing a common signalling system fail to act synergistically to stimulate initiation of DNA synthesis.

The critical point is that the agents mentioned above become potent mitogens when added to quiescent 3T3 cells in combinations that elicit the generation of both types of signals and thereby activate cAMP-dependent protein kinase and protein kinase C simultaneously. For example, combinations such as phorbol esters and cholera toxin (Rozengurt *et al.* 1981*b*), teleocidin and cholera toxin (Collins & Rozengurt, 1982*b*), vasopressin and butcAMP (Rozengurt, 1982*b*), vasopressin and cholera toxin (Collins & Rozengurt, 1983), diacylglycerol and PGE_1 (unpublished result) are mitogenic for Swiss 3T3 cells. Insulin, which can synergize with both types of extracellular factors at supramaximal concentrations, does not act in an identical way to either group of agents. In fact, this hormone does not activate protein kinase C (Rozengurt *et al.* 1983*c*), induce Ca^{2+} mobilization (Lopez-Rivas & Rozengurt, 1983; Fig. 1, this paper) or increase the level of cAMP in intact 3T3 cells (Rozengurt *et al.* 1981*b*). Any of these synergistic effects can be further enhanced by disruption of the microtubule network, which acts at a later point in G_1 (Friedkin & Rozengurt, 1981; Wang & Rozengurt, 1983). As proposed recently (Rozengurt, 1985), synergistic effects between extracellular factors appear to result from the generation of separate intracellular signals, which act in concert to elicit the complete array of metabolic processes required for a proliferative response. In this manner, complex synergistic effects and early cellular responses can be readily predicted.

In contrast to many mitogenic ligands, PDGF and FDGF stimulate DNA synthesis in the absence of any synergistic factor (Table 1). This has been verified with homogeneous preparations of PDGF (Lopez-Rivas *et al.* 1984) and poses important questions concerning the model of synergistic signals. Recent findings indicate that PDGF elicits the generation of the early signals that synergistically lead to initiation of DNA synthesis. As indicated in Table 2, PDGF activates protein kinase C, as judged by the increase in the phosphorylation of the 80k phosphoprotein in intact 3T3 cells (Rozengurt *et al.* 1983*a*; Rodriguez-Pena & Rozengurt, 1985). The activation of this phosphotransferase system may contribute to the marked transmodulation of EGF receptor affinity (Collins & Rozengurt, 1983) and to the

stimulation of Na^+ influx, enhancement of pH_i and increase in Na^+/K^+ pump activity caused by PDGF (Lopez-Rivas *et al.* 1984). PDGF also elicits mobilization of Ca^{2+} from intracellular stores (Fig. 1), an effect that could be mediated by IP_3, a water-soluble product that is released by inositol lipid breakdown catalysed by phospholipase C activation (Berridge & Irvine, 1984). An additional striking response elicited by PDGF is a large release of arachidonic acid, which is converted into stable derivatives such as E-type prostaglandins that, in turn, bind to their own receptor to stimulate cAMP synthesis (Rozengurt *et al.* 1983*a,b*). In this manner, PDGF stimulates all the early responses discussed in the preceding sections, which is in line with its potency as a complete mitogen (see Table 1).

Recently, we found that the regulatory tetradecapeptide bombesin is a potent mitogen for Swiss 3T3 cells (Rozengurt & Sinnett-Smith, 1983). At low (nanomolar) concentrations, bombesin and its analogues stimulate the initiation of DNA synthesis in a significant fraction (25–40 %) of the 3T3 cell population. This effect is elicited in serum-free medium and in the absence of any added factors. Thus, like PDGF, bombesin provides an interesting model peptide to test further the interpretation of the data summarized in Tables 1 and 2. Our current results show that bombesin binds to a specific receptor and stimulates ion fluxes, activates protein kinase C, transmodulates EGF receptor and alters cAMP metabolism (Zachary & Rozengurt, 1985; Rozengurt & Sinnett-Smith; Mendoza, Schneider & Rozengurt, unpublished data). The possibility that neuropeptides may play a physiological role in the control of target cell growth (e.g. in a trophic manner) deserves further consideration.

The integration of early signals and multiple synergistic effects in a unified framework, as proposed above, has important implications. So, this hypothesis leads to the conclusion that the signals that elicit cell growth are the same as those used by a variety of hormones and neurotransmitters to evoke short-term cellular responses. It is reasonable to question whether the proposition that cells use the same molecular signals for ostensibly different purposes is tenable, in particular since the decision to grow and divide is of paramount importance in the life cycle of a cell. A key feature in the action of most mitogenic signals is that they have to occupy their receptors for

Table 3. *Ca^{2+} mobilization, activation of protein kinase C and, or, elevation of intracellular cAMP are not required for the mitogenic responses elicited by insulin in combination with PBt_2, PGE_1 or EGF*

Mitogenic combination	Monovalent ion fluxes	Ca^{2+} mobilization	Activation of protein kinase C	Elevation of cAMP	DNA synthesis
Insulin + PBt_2	+	−	+	−	+++
Insulin + PGE_1	+	−	−	+	+++
Insulin + EGF	+	−	−	−	+++

In contrast, we have not observed stimulation of DNA synthesis by any combination of growth-promoting agents without an increase in monovalent ion flux.

hours before they stimulate increased DNA synthesis in the cell population (Rozengurt, Legg & Pettican, 1979; Dicker & Rozengurt, 1980; 1981*b*; Rozengurt, 1982*a*). For example, a large (100-fold) but transient (15 min) increase in the cellular levels of cAMP is not sufficient to act as a mitogenic signal for quiescent 3T3 cells; cAMP concentration must remain elevated for several hours before it acts as a mitogenic stimulus (Rozengurt, 1982*a*,*b*). Likewise, a transient exposure to PBt_2 or to vasopressin is not sufficient to stimulate mitogenesis (Dicker & Rozengurt, 1981*b*). When time is taken into consideration, it can be envisaged that the same molecular signals that elicit short-term responses can recruit more functional units if they continue to operate. In this manner, the long-recognized link between cellular function and growth becomes a logical consequence of the use of common signals.

It should be pointed out that there are many aspects of the signalling of fibroblast cell proliferation that remain poorly understood. It is not known whether the tyrosine-directed protein kinase activity associated with the receptors for EGF, PDGF, IGF and insulin plays a role in the elicitation of the early responses and signals that contribute to cell proliferation. In addition, there is only fragmentary knowledge of the mechanism by which such mitogenic signals are translated into the cell nucleus to initiate the program of cell growth. For example, Ca^{2+} mobilization, activation of protein kinase C and elevation of cAMP are not required for eliciting mitogenesis in the presence of insulin (Table 3). Nevertheless, the multiple control of the initiation of DNA synthesis by identifiable intracellular signals (i.e. ion fluxes, diacylglycerol, cAMP and cytoskeletal organization) provides a flexible model for understanding the organization and strategy of the mechanisms whereby extracellular agents may regulate animal cell proliferation.

The authors greatly appreciate the expert technical aasistance of Mr James Sinnett-Smith in performing the measurements of cytosolic Ca^{2+} concentration. The manuscript was written during the tenure of an American Cancer Society Eleanor Roosevelt International Cancer Fellowship awarded by the International Union Against Cancer to S.A.M.

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