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Early Signals in the Mitogenic Response

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Polypeptide growth factors, regulatory peptides, and a variety of pharmacological agents acting alone or synergistically induce mitogenesis in cultured fibroblasts. The early signals in the membrane, cytosol, and nucleus promoted by these extracellular factors, together with their mitogenic effectiveness, are integrated in a unified hypothesis for the regulation of fibroblast growth.

GROWTH FACTORS ARE IMPLICATED IN A WIDE VARIETY OF physiological and pathological processes. These include embryogenesis, growth and development, selective cell survival, hemopoiesis, tissue repair, immune responses, atherosclerosis, and neoplasia (*1*). An important link between growth factors or their receptors and oncogene products has also been established (*2*). Thus, the elucidation of the mechanism of action of growth factors has emerged as one of the fundamental problems in biological sciences and may prove a crucial prerequisite for understanding the cause or causes underlying the unrestrained proliferation of cancer cells.

Much of the information concerning the cellular and molecular responses evoked by growth factors comes from the use of cultured cells, in particular, murine 3T3 cell lines. Cultures of these lines become "quiescent" in the G_1 to G_0 phase of the cell cycle when deprived of exogenous growth factors. The arrest of growth is reversible; addition of fresh serum or defined mitogens leads to synthesis of DNA and cell division (*3*). Defined mitogens include purified polypeptide growth factors, mitogenic hormones, and various pharmacological agents, all of which exhibit potent synergistic interactions when added to cell cultures maintained in medium devoid of serum (*3, 4*). The existence of synergistic effects has important mechanistic implications because these effects follow a specific pattern (summarized in Table 1) that must be accounted for by any hypothesis of growth control.

The first step in the interaction of many polypeptide growth factors with their target cell is binding of the factors to specific, high-affinity receptors, which upon occupancy undergo rapid phosphorylation, redistributions in the plane of the membrane, and endocytosis (*4*). The binding of the growth factors promotes the generation of early signals in the membrane and cytosol. Within minutes, the mitogenic signal is propagated into the nucleus. These early events are followed in parallel sequences by multiple molecular and cellular responses, which eventually converge into a common final path leading to synthesis of DNA and cell division. Since the initiation of DNA synthesis is a late event, occurring 10 to 15 hours after the addition of the mitogens, attention has focused on the initial cellular responses associated with the interaction of mitogenic factors with the cell in the expectation that the early events will provide clues to primary regulatory mechanisms. In this context, the early events evoked by platelet-derived growth factor (PDGF) (*5*) are of considerable significance. In contrast to many other mitogens, PDGF stimulates DNA synthesis and cell division in Swiss 3T3 cells in the absence of any other synergistic factor (Table 1). Furthermore, PDGF is closely related both to growth factors produced by embryonic and cancer cells (*6*) and to the product of the *sis*[28] oncogene (*7*). This article describes the early signals and molecular events initiated by PDGF and other growth factors and proposes a framework that integrates early mitogenic events and synergistic effects in a hypothesis of growth control.

Early Events Elicited by PDGF and Other Growth Factors in Quiescent Cells

Binding measurements (*8*) and chemical cross-linking studies (*9*) show that PDGF interacts with specific, high-affinity receptors

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located on the plasma membrane. The receptor is a transmembrane glycoprotein of relative molecular mass (M_r) of 175,000 with tyrosine-specific protein kinase activity (*10*). Since a number of growth factor receptors and retroviral oncogene products exhibit this activity (*11*), increased tyrosine phosphorylation could be involved in the triggering of mitogenesis. However, its functional significance in the transmission of the mitogenic signal remains unknown. After binding, PDGF stimulates a complex set of early events in the membrane, cytosol, and nucleus, which are described below and depicted schematically in Fig. 1.

Ion fluxes. One of the earliest responses elicited by addition of PDGF and other growth factors to quiescent cells is an increase in the fluxes of Na^+, K^+, and H^+ across the plasma membrane (Fig. 1A). The mitogens stimulate Na^+ entry into Swiss 3T3 cells (*12*) by an amiloride-sensitive Na^+/H^+ antiport. This increases intracellular Na^+ and causes cytoplasmic alkalinization (*13*). Since the activity of the Na^+/K^+ pump is both limited and regulated by intracellular Na^+ (*12*), there is a secondary stimulation of Na^+/K^+ pump activity (*12, 14*), which increases intracellular K^+ and restores the electrochemical gradient for Na^+. The ability of PDGF and other 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. Rapid increases in monovalent ion fluxes are observed with different combinations of growth factors, cell types, and techniques (*15*). Since the stimulation of a proliferative response in quiescent cells depends on maintaining intracellular *p*H and K^+ concentrations above critical threshold levels (*16*), the ionic events shown in Fig. 1A may play a permissive or triggering role, or both, in mitogenesis.

In addition to rapid changes in monovalent ion fluxes, PDGF and other mitogens markedly stimulate Ca^{2+} efflux from radioactively labeled Swiss 3T3 cells (*17*). This efflux is one of the earliest events (15 seconds) to take place in quiescent fibroblasts after mitogenic stimulation. Since the stimulation of $^{45}Ca^{2+}$ efflux can be elicited in the absence of extracellular Ca^{2+}, these growth factors must release

Table 1. Mitogenic effectiveness of a variety of polypeptide growth factors (PDGF, FDGF, EGF, and insulin), regulatory peptides (vasopressin and bombesin), and various pharmacological agents (the tumor promoters phorbol esters and teleocidin, cAMP-increasing agents, and antimicrotubule drugs). All the assays were performed under comparable experimental conditions in quiescent cultures of Swiss 3T3 cells. The maximal level of DNA synthesis (++++) was equivalent to that produced by a saturating concentration of fresh serum (10% v/v). PDGF, FDGF, and bombesin can induce DNA synthesis in the absence of other agents (individual effects are between diagonal lines). All other agents stimulate DNA synthesis when added in specific combinations. Many combinations that give a synergistic effect on DNA synthesis act from the time that both agents are added. Abbreviations: PDGF, platelet-derived growth factor; FDGF, fibroblast-derived growth factor; EGF, epidermal growth factor; cAMP, cyclic adenosine monophosphate; diacylglycerol OAG, diacylglycerol 1-oleoyl 2-acetyl glycerol; PGE_1, prostaglandin E_1; NECA, 5′-*N*-ethyl-carboxamide adenosine.

Agent	PDGF/ FDGF	Bombesin	Vaso-pressin	Phorbol esters Teleocidin	Diacyl-glycerol OAG	Insulin	EGF	Cholera toxin	PGE_1	NECA	cAMP analogs	Antimicro-tubule agents
PDGF/FDGF	+++	++++	+++	+++	+++	++++	++++	+++	+++	+++	+++	++++
Bombesin	++++	++	++	++	++	++++	+++	++	++	++	++	+++
Vasopressin	+++	++	—	—	—	+++	++	++	++	++	++	—
Phorbol esters Teleocidin	+++	++	—	—	—	+++	++	++	++	++	++	—
Diacylglycerol OAG	+++	++	—	—	—	+++	++	++	++	++	++	—
Insulin	++++	++++	+++	+++	+++	—	+++	+++	+++	+++	+++	+
EGF	++++	+++	++	++	++	+++	—	+	+	+	+	—
Cholera toxin	+++	++	++	++	++	+++	+	—	—	—	—	—
PGE_1	+++	++	++	++	++	+++	+	—	—	—	—	—
NECA	+++	++	++	++	++	+++	+	—	—	—	—	—
cAMP analogs	+++	++	++	++	++	+++	+	—	—	—	—	—
Antimicrotu-bule agents	++++	+++	—	—	—	+	—	—	—	—	—	—

this cation from an intracellular store (or stores), thereby leading to a rapid decrease in the total Ca^{2+} content of the cells. The finding that PDGF causes a rapid two- to threefold transient increase in cytosolic Ca^{2+} as measured by the fluorescent Ca^{2+}-binding dye quin-2 (*18*) suggests that release from intracellular stores promotes Ca^{2+} efflux by activation of the plasma membrane Ca^{2+}-dependent adenosinetriphosphatase (Ca^{2+}-ATPase) (Fig. 1B). The mobilization of Ca^{2+} may be mediated by inositol 1,4,5-trisphosphate (IP_3), which has been proposed as a second messenger in the action of many ligands that induce receptor-mediated inositol lipid turnover and Ca^{2+} efflux (*19*). IP_3 is formed as a result of increased hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane, a process that also generates 1,2-diacylglycerol (DAG). In addition, other phosphoinositides are degraded rapidly by phospholipase C to form DAG and inositol phosphates (*20*). As described below, DAG may play a role in the signaling cascade initiated by PDGF and other mitogenic peptides.

Activation of protein kinase C in intact cells. DAG is a potent activator of the purified Ca^{2+}-sensitive, phospholipid-dependent protein kinase (protein kinase C). This enzyme has received considerable attention because it is a major receptor for the tumor promoters of the phorbol ester family (*21*). Protein kinase C is also stimulated by the synthetic diacylglycerol 1-oleoyl 2-acetyl glycerol (OAG), which intercalates into the plasma membrane of intact cells more readily than long-chain diacylglycerols. Since phorbol esters (*22*) and OAG (*23*) can act as mitogens for quiescent cells, protein kinase C may play a role in the production of a mitogenic response. Accordingly, it was of importance to test directly whether PDGF and other mitogenic agents lead to activation of this enzyme in intact, quiescent cells. Recently, a rapid increase in the phosphorylation of an acidic cellular protein with an M_r of 80,000 (termed 80K) was shown to reflect the activation of protein kinase C in intact fibroblastic cells (*24*). For example, the phosphorylation of the same 80K protein is stimulated in cells by addition of (i) biologically active phorbol esters; (ii) the synthetic diacylglycerol OAG; and (iii) exogenous phospholipase C, which causes phospholipid breakdown and generates diacylglycerol (Fig. 1C). Furthermore, the same 80K protein is phosphorylated in cell-free systems either by activation of endogenous protein kinase C or by addition of the purified enzyme (*25*). Although the nature and role of the 80K phosphoprotein remain to be identified, its phosphorylation provides a specific marker for assessing which mitogenic agents activate protein kinase C in intact cells.

Another approach to testing the role of protein kinase C 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 (*26*) and to the disappearance of measurable protein kinase C activity in cell-free preparations (*27, 28*). It also prevents the increase in 80K phosphorylation elicited by subsequent addition of phorbol esters, phospholipase C, or OAG (*23–25*). In parallel with this loss of protein kinase C activity, the cells become desensitized to the biological effects elicited by phorbol esters or OAG (*23–26, 28*).

Using the approaches outlined above, researchers concluded that PDGF markedly activates protein kinase C in intact, quiescent Swiss 3T3 cells (*24*). Further, desensitization of the protein kinase C pathway reduces the stimulation of DNA synthesis by low concentrations of PDGF in these cells (*29*). Hence, protein kinase C appears not only to mediate the multiple biological actions of phorbol esters, but its activation constitutes one of the multiple signaling pathways utilized by PDGF in responsive cells.

Protein kinase C and ion fluxes. Activation of protein kinase C may represent an important molecular link in the sequence of events induced by the binding of growth-promoting factors to their respective receptors (*28*). In accord with this, activation of this enzyme leads, either directly or indirectly, to increased activity of the Na^+/H^+ antiport system, which in turn increases intracellular *p*H, promotes Na^+ influx, and stimulates the Na^+/K^+ pump activity (*30–32*). Desensitization of the protein kinase C pathway prevents the stimulation of ion fluxes by subsequent addition of either phorbol esters or OAG (*31, 32*). Since the activity of the Na^+/H^+ antiport is also enhanced in 3T3 cells by mitogens that do not activate protein kinase C (*32*) and because some stimulation of ion fluxes by PDGF persists even after removal of protein kinase C, it is likely that these ionic fluxes are also regulated by other mechanisms.

Protein kinase C and transmodulation of epidermal growth factor (EGF) receptor. Addition of PDGF inhibits the binding of ^{125}I-labeled EGF to specific surface receptors in Swiss 3T3 cells. The modulation of EGF binding is rapid in onset and results from a

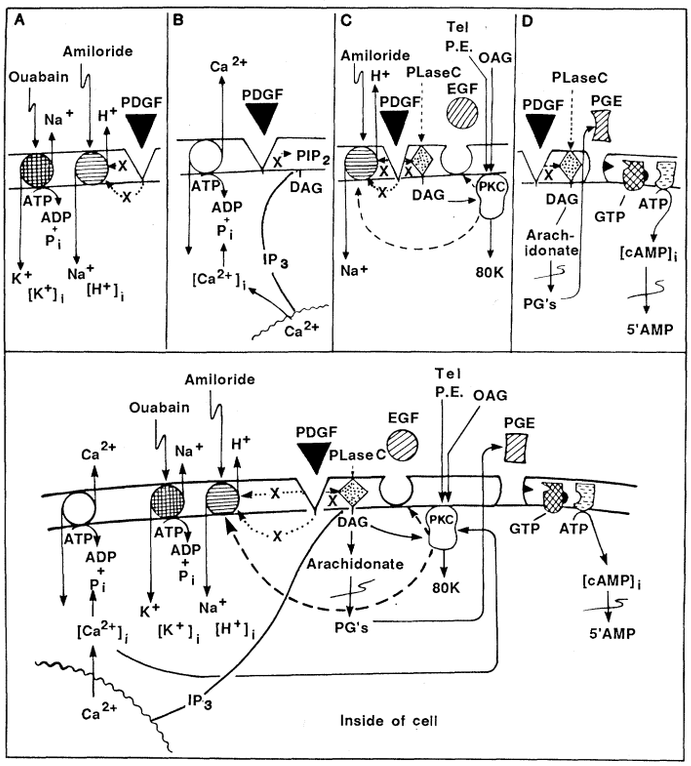

Fig. 1. Binding of platelet-derived growth factor (PDGF) to its receptor rapidly induces a cascade of early events in the membrane and cytosol. Ion concentrations are indicated by the brackets. X implies that the mechanism is unknown. Dotted line, a hypothetical mechanistic connection; broken line, a regulatory relation; s-shaped line crossing an arrow, an inhibitor added at this point will block all of the subsequent reactions. (A) Stimulation of Na^+, K^+, and H^+ fluxes across the plasma membrane. (B) Mobilization of Ca^{2+} from an intracellular store or stores promoted by inositol 1,4,5-trisphosphate (IP_3). In the membrane, phosphatidylinositol 4,5-bisphosphate is PIP_2. (C) Increased phosphorylation of an 80K cellular protein (M_r = 80,000) marks the activation of protein kinase C (PKC) either by PDGF through endogenous diacylglycerol (DAG) or by exogenous phorbol esters (P.E.), teleocidin (Tel) and synthetic diacylglycerol (OAG). PKC is implicated in the transmodulation of epidermal growth factor (EGF) receptors and in the enhancement of activity of the Na^+/H^+ antiport. Exogenous phospholipase C (PLase C). (D) Arachidonate, released from DAG and other sources, is converted into metabolically stable E-type prostaglandins (PG's, PGE), which stimulate adenylate cyclase activity acting through their own receptor. The whole array of these early events is shown in the lower panel. Other abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate (brackets for intracellular concentration); 5′AMP, adenosine 5′-monophosphate; GTP, guanosine triphosphate.

decrease in the apparent affinity of the EGF receptor population for EGF (*33*). Since PDGF does not bind to EGF receptors (*8, 33*), the decrease in the affinity of the EGF receptors occurs through an indirect mechanism termed "transmodulation" (*34*).

Considerable evidence implicates protein kinase C in the transmodulation of the EGF receptor affinity. Phorbol esters (*35*) or OAG (*36*) cause a rapid and striking decrease in the apparent affinity of this receptor without changing the total number of sites. The transmodulation induced by these agents is prevented by prolonged treatment of the cells with phorbol esters, which desensitizes the protein kinase C pathway (*26, 36*). Further, protein kinase C phosphorylates the EGF receptor of human epidermal carcinoma A431 cells at a specific threonine residue, located nine amino acids from the transmembrane domain of this receptor (*37*). PDGF stimulates the phosphorylation of the EGF receptor in human fibroblasts at an identical site (*38*). Thus, the transmodulation of EGF receptors may result from the covalent modification of the EGF receptor catalyzed by protein kinase C, but other mechanisms are not excluded (*29*).

Cyclic nucleotides and the initiation of DNA synthesis. The role of cyclic nucleotides in the control of the proliferative response of quiescent fibroblastic cells has been the subject of a large and controversial literature (*39*). It is now recognized that a sustained increase in the cellular level of cyclic adenosine monophosphate (cAMP) constitutes a growth-promoting signal for Swiss 3T3 cells (*40*). PDGF induces a striking accumulation of cAMP in quiescent 3T3 cells incubated in the presence of inhibitors of cyclic nucleotide degradation (*41*). In contrast, other growth-promoting factors (including insulin, EGF, vasopressin, phorbol esters, or OAG) did not increase the level of cAMP. The alteration of cAMP metabolism by PDGF occurs in an indirect fashion (*42*). This mitogen elicits a striking release of arachidonate, the source of which remains unclear, but it is at least partly derived from DAG (*20, 42*). Arachidonate is converted into many biologically active metabolites including stable E-type prostaglandins. The accumulation of cAMP elicited by PDGF is mediated by increased synthesis of E-type prostaglandins, which in turn leave the cell and stimulate cAMP synthesis through their own receptor (Fig. 1D). Therefore, cAMP may be one of the multiple signals utilized by PDGF to stimulate reinitiation of DNA synthesis in Swiss 3T3 cells (*39, 41*). The cascade of early events in the membrane and cytosol evoked by the binding of PDGF to its receptor in a quiescent 3T3 cell is summarized in Fig. 1 (lower panel). Fibroblast-derived growth factor (FDGF), a polypeptide secreted by an SV40-transformed fibroblast cell line, which is structurally and immunologically related to PDGF, elicits an identical set of early events (*29, 43*).

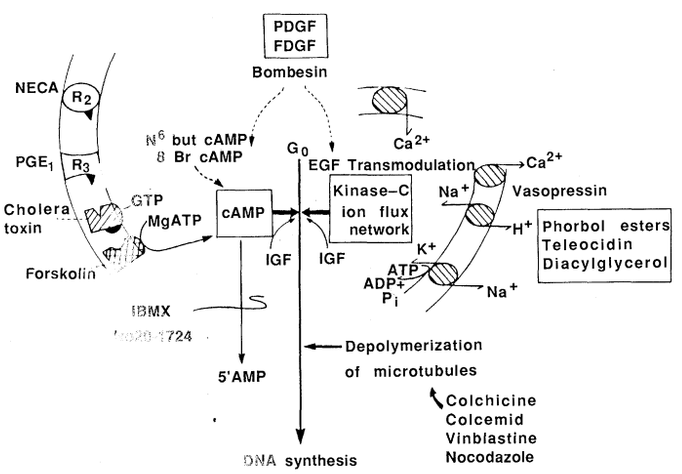

Fig. 2. Early responses and synergistic effects elicited by growth factors, mitogenic hormones, and pharmacological agents in quiescent Swiss 3T3 cells in serum-free medium. A set of extracellular agents, shown on the left, share the ability to increase cellular cAMP; addition of any of these agents to quiescent 3T3 cells in serum-free medium is not sufficient to stimulate exit from G_0 and entry into DNA synthesis. A different set of extracellular agents, shown on the right (vasopressin, phorbol esters, teleocidin, or diacylglycerol), activate protein kinase C, enhance ion fluxes, and transmodulate EGF receptor but do not alter cAMP levels. These agents, either singly or in combination, are not mitogenic. However, a combination comprising any of the agents that increase cAMP with any of the agents shown on the right is effective in stimulating initiation of DNA synthesis in 3T3 cells. Insulin-like growth factors (IGF and insulin at high concentrations) act synergistically with both types of signals. PDGF, FDGF, and bombesin stimulate all these early responses and act as mitogens in the absence of any other synergistic partner. Disruption of the microtubule network acts at a later point and enhances the mitogenic response to any combination that initiates DNA replication. PDGF and FDGF may bind to a common receptor. Protein kinase C is the main cellular target of phorbol esters, teleocidin, and diacylglycerol. Colchicine, colcemid, vinblastine, and nocodazole bind to tubulin. Detailed references for the mitogenic activity of the growth factors, hormones, neuropeptides, tumor promoters, and pharmacological agents shown can be found in the text. Other abbreviations: NECA, 5′-*N*-ethylcarboxamide adenosine; N^6 but cAMP, N^6-butyryl cAMP; 8 Br cAMP, 8-bromo cAMP; IBMX, isobutyl methylxanthene; R020-1724, 4-(3-butoxy-4-methoxybenzyl) 2-imidazolidine.

Early nuclear events. In addition to the events in the membrane and cytosol described above, PDGF and other growth factors rapidly and transiently induce the expression of the cellular oncogenes c-*fos* and c-*myc* in quiescent fibroblasts (*44*). The enhanced expression of c-*fos* messenger RNA (mRNA) occurs within minutes of PDGF addition followed by increased expression of c-*myc*. The induction of c-*fos* mRNA is one of the earliest nuclear events that follow the addition of PDGF. Since these cellular oncogenes encode nuclear proteins (*45*) it is plausible that their transient expression may play a role in the transduction of the mitogenic signal in the nucleus.

The early signals in the membrane and cytosol illustrated in Fig. 1 may participate in the regulation of the expression of c-*fos* and c-*myc*. The fact that phorbol esters increase the expression of these genes (*44*) and that desensitization of the protein kinase C pathway induced by prolonged exposure to phorbol esters reduces the induction of c-*myc* by PDGF (*29, 46*) implicates the activation of this phosphotransferase in the pathway or pathways leading to increased expression of c-*myc*.

Studies on the synergistic effects among PDGF, EGF, and insulin-like growth factors (IGF's) in BALB c/3T3 cells (clone A_{31}) suggested that PDGF renders the cells competent to replicate their DNA in response to EGF and IGF. PDGF-induced competence persists after removal of the growth factor and is apparently promoted by the increased expression of a family of genes ("competence genes") that also includes the protooncogenes c-*fos* and c-*myc* (*47*). This hypothesis, like the one developed below, assumes that various growth factors modulate cell proliferation through different mechanisms. However, both the pathways by which an external mitogenic signal is transduced into the cell to induce competence and the biochemical basis of this state remain to be defined.

Testing the Role of the Early Signals in Mitogenesis

If the early events induced by PDGF and illustrated in Fig. 1 play a role in the transmission of the mitogenic signal, two predictions can be made.

Prediction 1. If a ligand, acting via a distinct receptor, elicits the same set of early events as PDGF, it should act as a growth factor for Swiss 3T3 cells in medium devoid of serum or other mitogens, that

is, it should behave as a PDGF agonist. Recently, my colleagues and I have demonstrated that the amphibian tetradecapeptide bombesin and mammalian peptides structurally related to bombesin, including gastrin-releasing peptide (GRP) and the neuromedins (*48*), are potent mitogens for Swiss 3T3 cells (*49, 50*). The mitogenic response to this family of peptides (which exhibit a highly conserved carboxyl-terminal heptapeptide) depends on their binding to specific, high-affinity receptors that are distinguishable from those of other growth factors for these cells, including PDGF (*50*). Both ligand binding and mitogenesis are selectively blocked by a bombesin antagonist (*50*). Since bombesin and related peptides stimulate DNA synthesis and cell division in the absence of other mitogens (*49*), this family of peptides offers a novel and attractive tool for testing the validity of prediction 1. 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 (*51*).

After binding to specific, high-affinity receptors, bombesin and structurally related peptides elicit a complex set of early events in quiescent Swiss 3T3 cells (*52*). These ligands rapidly stimulate (i) the phosphorylation of the 80K cellular protein, which reflects the activation of protein kinase C in intact 3T3 cells, (ii) the mobilization of Ca^{2+} from an intracellular store, which leads to a transient increase in the concentration of cytosolic Ca^{2+} and Ca^{2+} efflux, (iii) the enhancement of activity of the Na^+/H^+ antiport, which increases intracellular *p*H, and, in turn, increases Na^+ influx and Na^+/K^+ pump activity, and (iv) the transmodulation of EGF-receptor affinity (*52*). The activation of protein kinase C mediates, at least in part, the changes in monovalent ionic fluxes and the transmodulation of EGF-receptor affinity. Bombesin also increases cAMP synthesis in an indirect fashion and induces the expression of the nuclear oncogenes c-*fos* and c-*myc* (*29*). The fact that bombesin promotes the generation of signals virtually identical to those evoked by PDGF (Fig. 1) is entirely consistent with prediction 1. The stringency of this conclusion is emphasized by two lines of evidence. (i) In contrast to many other factors that are mitogenic only when added in synergistic combinations, bombesin and PDGF stimulate DNA synthesis in Swiss 3T3 cells in the absence of other mitogenic factors (Table 1). (ii) Agents that induce mitogenesis only when added in specific combinations (Table 1) stimulate some but not all of the early events evoked by PDGF and bombesin (see below). These considerations lead to another important prediction concerning the relation between early events and synergistic mitogenic effects.

Prediction 2. Agents that elicit part of the early responses stimulated by PDGF or bombesin should become mitogenic when added in combinations that reconstitute the early events shown in Fig. 1. This prediction is of particular significance, because it provides a conceptual basis for interpreting many complex synergistic effects (summarized in Fig. 2). The availability of a panel of defined mitogens that are biologically active in a nutrient medium devoid of serum provides a tool for elucidating the nature of the regulatory signals and molecular events implicated in these synergistic effects. For example, a group of agents such as the tumor-promoting phorbol esters and teleocidin (*22, 26*), the synthetic diacylglycerol OAG (*23*), and the neurohypophyseal hormone vasopressin and its related peptides (*53*) 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 of these agents either individually or in combination to quiescent 3T3 cells fails to induce a mitogenic response (Table 1). Agents that increase intracellular cAMP such as prostaglandin E_1 (PGE_1), the adenosine analog 5′-*N*-ethylcarboxamide adenosine (NECA), cholera toxin, and cAMP derivatives (*40*) produce a totally different pattern of early events. These agents do not activate protein kinase C, induce Ca^{2+} mobilization, or transmodulate EGF receptors. Furthermore, they do not stimulate DNA synthesis when added singly or in combination. We suggest that agents sharing a common signaling system, such as those illustrated on the right or left of Fig. 2, cannot act synergistically to stimulate initiation of DNA synthesis.

Crucially, 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 reconstitute the complex pattern of early events elicited by either PDGF or bombesin. For example, combinations such as phorbol esters and cholera toxin, teleocidin and cholera toxin, vasopressin and butyryl cAMP, vasopressin and cholera toxin, diacylglycerol and PGE_1 are mitogenic for Swiss 3T3 cells. Insulin, which can synergize with both groups of extracellular factors at supramaximal concentrations (that is, in lieu of insulin-like growth factor) does not act identically to either group of agents. In fact, this hormone does not activate protein kinase C, induce Ca^{2+} mobilization, or increase the level of cAMP in intact 3T3 cells. Similar conclusions can be drawn when EGF is added instead of insulin (Table 1). Disruption of the microtubule network acts at a later point (Fig. 2) and enhances the mitogenic response to any combination that stimulates DNA synthesis (*54*). As would be expected, these drugs do not elicit any of the events shown in Fig. 1. Thus, synergistic effects between extracellular factors appear to result from the generation of complementary intracellular signals that 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.

Regulatory Signals and Obligatory Events

In line with the hypothesis of growth control discussed above, the key events elicited by growth factors in quiescent cells can be broadly divided into two major categories: regulatory signals and obligatory events (*55*). The former class represent intracellular processes that mediate the action of specific growth-promoting agents; although they are crucial in eliciting biological responses by a given factor, they can be bypassed by another group of factors. In contrast, obligatory events are envisaged as molecular steps that must take place for the stimulation of DNA synthesis and cell division to occur regardless of the regulatory signals utilized to activate the cells. In order to distinguish between regulatory signals and obligatory events one approach is to ascertain the effect of a wide panel of defined mitogenic agents. While regulatory signals are elicited by certain mitogens but not others, obligatory events should be stimulated by all mitogenic combinations. In this context, Ca^{2+} mobilization, activation of protein kinase C, and elevation of the intracellular level of cAMP must be regarded as regulatory signals, as can be inferred from Fig. 2. Whether the increased expression of the oncogenes c-*fos* and c-*myc* represents a regulatory nuclear signal in the action of specific mitogens or an obligatory event in the sequence of molecular steps leading to DNA synthesis remains to be determined. Within the framework of these considerations, it is plausible that alternate regulatory signals may elicit a common set of obligatory events leading to DNA synthesis. This concept may prove useful in the analysis of cause-effect relationships between various events that precede the initiation of DNA synthesis. In addition, drugs designated to block regulatory signals rather than obligatory events should be more selective in inhibiting cell proliferation.

Concluding Remarks

The identification of the extracellular and intracellular signals that stimulate cultured cells to grow and divide is providing remarkable insights into the multiple pathways that control cell growth. Nevertheless, many molecular and cellular aspects of the flow of information leading to a mitogenic response remain unclear. The molecular mechanisms of signal transduction between the extracellular and intracellular domains of receptor molecules, the molecular steps by which receptor occupancy is coupled to the generation of the early regulatory signals, and the pathways by which regulatory signals modulate other processes in the cell including the expression of genes associated with mitogenesis remain important areas for future research. In this regard, the role of tyrosine phosphorylation (*2, 10, 11*) and of guanosine 5′-triphosphate (GTP)–binding proteins (*56*) in the transmission of the mitogenic signal is of considerable importance. The analysis of many cellular and molecular responses in terms of regulatory signals and obligatory events will require critical evaluation. Finally, the effectiveness of the extracellular and intracellular signals discussed above may depend on cell type and state of differentiation. In spite of many obvious gaps, the model of multiple control of initiation of DNA synthesis by alternate regulatory signals developed here provides a framework for understanding the complex organization of the mechanisms by which diverse extracellular factors regulate cell proliferation.

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55. Many cellular and molecular responses (of which a detailed description is outside the scope of this article) are also associated with the reinitiation of cell proliferation. These include increases in nutrient uptake, energy metabolism, and overall rate of protein synthesis; induction of gene expression, enzymes, receptors and transport systems; morphological and cytoskeletal changes, and augmented cell locomotion. These events can be classified as obligatory and nonobligatory according to whether they are necessary or not for the stimulation of DNA synthesis and cell division. Many of the events discussed in this article fall into a different class, which does not fit neatly in either of the groups mentioned above. They are "obligatory" in the action of a given mitogen but not even stimulated by other mitogens. The activation of protein kinase C constitutes a prime example. Here we propose the term "regulatory signals" to designate this type of early event.
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