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MINI-REVIEW

Second Messengers Derived from Inositol Lipids

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Abstract

Many hormones, growth factors, and neurotransmitters stimulate their target cells by promoting the hydrolysis of plasma-membrane phosphoinositides to form the two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. In such cells, ligand-receptor interaction stimulates specific phospholipases that are activated by guanyl nucleotide regulatory G proteins or tyrosine phosphorylation. In many cells, the initial rise in cytoplasmic calcium due to Ins(1,4,5)P_3-induced mobilization of calcium from agonist-sensitive stores is followed by a sustained phase of cytoplasmic calcium elevation that maintains the target-cell response, and is dependent on influx of extracellular calcium. Numerous inositol phosphates are formed during metabolism of the calcium-mobilizing messenger, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3)], to lower and higher phosphorylated derivatives. The cloning of several phospholipase-C isozymes, as well as the Ins(1,4,5)P_3-5 kinase and the Ins(1,4,5)P_3 receptor, have clarified several aspects of the diversity and complexity of the phosphoinositide-calcium signaling system. In addition to their well-established roles in hormonal activation of cellular responses such as secretion and contraction, phospholipids and their hydrolysis products have been increasingly implicated in the actions of growth factors and oncogenes on cellular growth and proliferation.

Key Words: Phosphoinositides; inositol-phosphates; phospholipase-C; Ca-mobilization; G-proteins; inositol-trisphosphate-receptor; tyrosine kinases.

Introduction

The numerous and diverse target-cell responses to calcium-mobilizing ligands include endocrine and exocrine secretion, control of intermediary metabolism and cell differentiation, and stimulation of neural transmission

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and muscle contraction. These processes are controlled by a ubiquitous signaling mechanism in which receptor-mediated hydrolysis of plasma-membrane phospholipids generates a set of second messengers (inositol phosphates, diacylglycerol, and arachidonic acid metabolites) with specific actions on calcium-dependent cell responses (Berridge and Irvine, 1989; Nishizuka, 1988; Rana and Hokin, 1990). These include the promotion of calcium mobilization from intracellular stores and enhanced calcium influx across the plasma membrane, as well as the regulation of calcium- and phospholipid-dependent protein kinases that control the phosphorylation of key proteins, including receptors, ion channels, and enzymes present in the plasma membrane, cytoplasm, and nucleus.

Role of Phosphoinositide Hydrolysis in Transmembrane Signalling

The recognition of the second messenger roles of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) was preceded by a long period of research on phospholipids and their metabolites. This was initiated by the discovery of the "PI effect" by Hokin and Hokin, who observed that cholinergic stimulation of the exocrine pancreas increased the ^{32}P-labeling of an "RNA" fraction that was subsequently found to contain a contaminating phospholipid (reviewed in Hokin, 1987). These investigators also showed that this rapid, agonist-induced phosphorylation is essentially confined to phosphatidylinositol (PtdIns) and phosphatidic acid (Hokin and Hokin, 1955a,b). The increased ^{32}P-labeling of phosphatidic acid was proposed to result from hydrolysis of PtdIns by phospholipase C, followed by rapid conversion of the resulting diacylglycerol to phosphatidic acid, and ^{32}P-labeling of PtdIns secondary to restoration of the PtdIns pool from phosphatidate (Hokin and Hokin, 1964). These and other important details were clarified by these pioneering studies, but no specific function was assigned to the "PI response" until the late 1960s. At that time, it was realized that agonist-stimulated ^{32}P-labeling of PtdIns is not only associated with enzyme or ion secretion, but also with other cellular responses, most notably cell proliferation (Fisher and Mueller, 1968, 1971; Maino *et al.*, 1975). In this respect the "PI response" began to be considered as a more general response of cells to the interactions of specific ligands with their cell-surface receptors (Durell *et al.*, 1969; Lapetina and Michell, 1973b).

In a review published in 1975—which is a cornerstone in the inositol-lipid field—Robert Michell integrated the existing information into a model in which the primary effect of calcium-dependent hormones on their target cells was the stimulation of PtdIns hydrolysis in the plasma membrane. Michell noted that only ligands which act on cell surface receptors were active in this

regard, and that these did not include hormones which act through cyclic AMP. He therefore proposed that the "PI response" is part of the signal transmission mechanism through which certain receptors trigger various cellular responses that are not mediated by the *c*AMP messenger system (Michell, 1975).

From this point, phosphoinositide research assumed an increasingly important place in studies on the mechanism of action of hormones which act on cell surface receptors to produce changes in cell calcium metabolism, cyclic GMP formation, and eicosanoid production (Berridge, 1981). These studies raised the question of how receptor-stimulated PtdIns hydrolysis leads to enhanced Ca^{2+} entry (which at that time was believed to be the primary mechanism responsible for increases in cytoplasmic Ca^{2+} concentration). Early candidates for a second messenger role were inositol 1 : 2-cyclic phosphate (Lapetina and Michell, 1973a), diacylglycerol (Takai *et al.*, 1979; Kuo *et al.*, 1980), and phosphatidic acid, which was proposed to function as a natural Ca^{2+} ionophore (Putney *et al.*, 1980; Salmon and Honeyman, 1980). Of these molecules, only DG proved to be an important messenger, by acting as the major lipid regulator of the phospholipid-dependent, Ca^{2+}-sensitive enzyme, protein kinase C (Nishizuka, 1988).

An important further step in phosphoinositide research was the realization that the primary target of agonist-stimulated phospholipase-C hydrolysis in the plasma membrane is not PtdIns but its more highly phosphorylated derivatives, PtdIns-4-phosphate and PtdIns 4,5-bisphosphate [PtdIns(4,5)P_2] (Kirk *et al.*, 1981; Creba *et al.*, 1983; Berridge, 1983). These compounds represent only a minor fraction of the total cellular phosphoinositides, but show very active metabolic "turnover" of their phosphomonoester groups. Their rapid breakdown following agonist stimulation resolved the problem posed by the slow onset of PtdIns hydrolysis, a major criticism raised against the messenger role of phospholipid turnover (Cockcroft, 1981; Hawthorne, 1981). In fact, agonist-induced hydrolysis of polyphosphoinositides had been noticed as early as 1964 (Santiago-Calvo *et al.*, 1964), and was confirmed by several subsequent reports (Durrell, 1969; Abdel-Latif *et al.*, 1977); however, it was thought to be initiated by increased cytoplasmic Ca^{2+} and was therefore considered to be a consequence, rather than a cause, of Ca^{2+}-signal generation (Akhtar and Abdel-Latif, 1978). This argument challenged the primary role of phosphoinositide hydrolysis in the development of the Ca^{2+} signal for many years (Cockcroft, 1981; Hawthorne, 1981). Much of the controversy was caused by the fact that the phosphoinositide-hydrolyzing enzyme, phospholipase C, is Ca^{2+}-sensitive and can be inactivated by depletion of intracellular Ca^{2+}. Since many cells become depleted of calcium during incubation in Ca^{2+}-free solutions, especially when EGTA is present, several studies concluded erroneously that calcium influx rather than phosphoinositide hydrolysis was the primary event in agonist action.

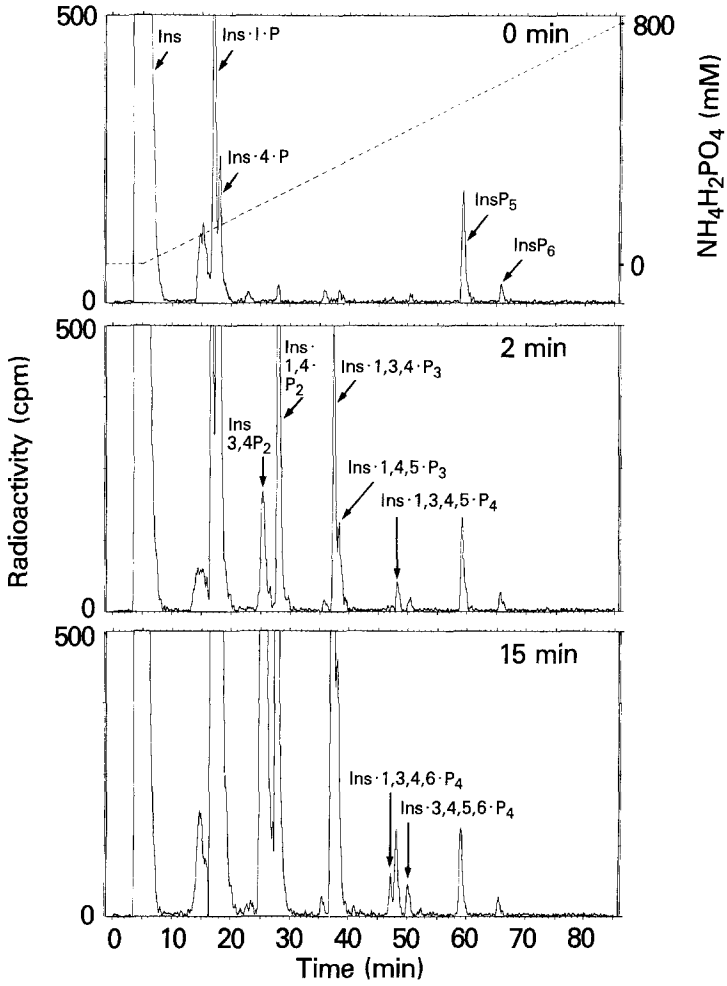

Fig. 1. Production of inositol phosphates in agonist-stimulated bovine adrenal glomerulosa cells. After incubation with [^{3}H]inositol for 36 h, cultured glomerulosa cells were stimulated with angiotensin II (50 nM) for 2 and 10 min, then extracted and fractionated by strong anion exchange HPLC (Balla *et al.*, 1987a).

Although receptor-mediated depletion of the PtdIns[4,5]P_2 pool was generally interpreted as hydrolysis catalyzed by a phospholipase-C, the direct demonstration of agonist-stimulated formation of $InsP_3$ and $InsP_2$ by ion-exchange chromatography helped to clarify the nature of these reactions (Berridge, 1983). Subsequently, measurement of the production of water-soluble inositol phosphates in [^{3}H]inositol-labeled cells by anion-exchange HPLC has become the method of choice to analyze receptor-mediated phosphoinositide hydrolysis (Fig. 1). In addition, the use of Li^+ ions to

amplify the agonist-induced increases in several of the inositol phosphates (Berridge *et al.*, 1982) further facilitated the progress of research on inositol phospholipid turnover. This manoeuver was based on observations that Li^+ ions inhibit the dephosphorylation of inositol monophosphate to inositol, thereby causing accumulation of inositol monophosphate during agonist-stimulated phosphoinositide hydrolysis (Allison *et al.*, 1976; Sherman *et al.*, 1981). This finding raised the possibility that the therapeutic effects of Li^+ in manic-depressive illness could be related to its ability to interfere with the metabolism of inositol phosphates (Berridge *et al.*, 1982).

The primary water-soluble product of PtdIns(4,5)P_2 cleavage, Ins(1,4,5)P_3, was found to be rapidly hydrolyzed by a specific 5-phosphatase to form Ins(1,4)P_2 (Downes *et al.*, 1982). This activity was mainly associated with the plasma membrane (Seyfred *et al.*, 1984; Storey *et al.*, 1984) [except in platelets, where high 5-phosphatase activity is present in the cytosol (Connolly *et al.*, 1985)], suggesting that Ins(1,4,5)P_3 could be a second messenger since its production as well as its elimination is controlled at a site adjacent to the plasma membrane. This proposal was confirmed by the demonstration that Ins(1,4,5)P_3 mobilized Ca^{2+} from an ATP-ase loaded nonmitochondrial Ca^{2+} pool in permeabilized cells (Streb *et al.*, 1983) and subcellular membrane preparations (Prentki *et al.*, 1984). Similar observations in a wide variety of cells and cell-free preparations clearly established the key role of Ins(1,4,5)P_3 in the coupling of receptor-activated phosphoinositide hydrolysis to Ca^{2+} signal generation.

Complexity of Inositol–Phosphate Metabolism

The model of receptor-stimulated hydrolysis of PtdIns(4,5)P_2 producing two intracellular messengers, Ins(1,4,5)P_3 and DAG, was rapidly accepted and has been shown to operate in numerous ligand-regulated cells. However, the phosphoinositide signal transduction system was soon found to be far more complicated than originally realized (Fig. 2). This was first indicated by the observation that most of the $InsP_3$ accumulating in agonist-stimulated cells is not the Ca^{2+}-mobilizing (1,4,5) isomer but an inactive form identified as Ins(1,3,4)P_3 (Irvine *et al.*, 1984a). The origin of this $InsP_3$ isomer was initially unclear, since no evidence was found for an appropriate PtdInsP_2 that could be a precursor of this new molecule. The origin of Ins(1,3,4)P_3 was clarified by the discovery of a new pathway (Irvine *et al.*, 1986) through which Ins(1,4,5)P_3 was phosphorylated to inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], which was then converted to Ins(1,3,4)P_3 by the same 5-phosphatase that dephosphorylates Ins(1,4,5)P_3. The discovery of the conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 also initiated a new concept in which the concerted actions of these two messengers would be responsible for

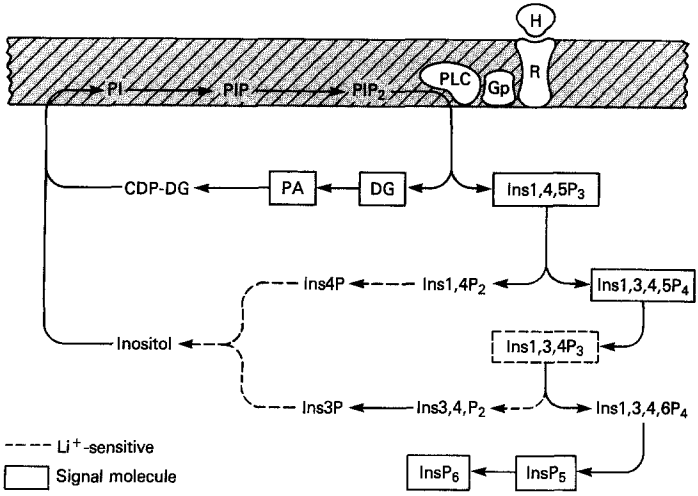

Fig. 2. Outline of phosphoinositide biosynthesis and metabolism in agonist-stimulated target cells. The polyphosphoinositides (PIP and PIP_2) synthesized in the plasma membrane are rapidly degraded by phospholipase C (phosphoinositidase C) during agonist activation of receptors and the associated G protein that mediates signal transduction. The two primary products of phosphoinositide hydrolysis [diacylglycerol (DG) and $Ins(1,4,5)P_3$] are rapidly metabolized after activating protein kinase C and Ca^{2+} mobilization, respectively. DG is converted to phosphatidic acid (PA) and recycled to the lipid biosynthesis pathway; DG also serves as a source of arachidonic acid when hydrolyzed by DG lipase. $Ins(1,4,5)P_2$ binds to specific intracellular receptors to mobilize internal calcium stores and is also rapidly removed by degradative and synthetic pathways, the latter leading to the formation of $Ins(1,3,4,5)P_4$ and subsequently to other metabolites including $Ins(1,3,4)P_3$, $Ins(1,3,4,6)P_4$, and $InsP_5$. The known lithium-sensitive steps in inositol phosphate metabolism are shown by dashed lines, and the proven and putative messenger molecules derived from phospholipid breakdown are shown as boxed intermediates.

the full development of the cytoplasmic Ca^{2+} signal. $Ins(1,3,4,5)P_4$, although ineffective by itself, was shown to act in conjunction with $Ins(1,4,5)_3$ to mimic fertilization in sea urchin eggs (Irvine and Moor, 1986) and to increase a Ca^{2+}-dependent K^+-conductance in lacrimal gland cells (Morris *et al.*, 1987), due to enhanced Ca^{2+} influx. Also, $Ins(1,3,4,5)P_4$ binding sites distinct from those of $Ins(1,4,5)P_3$ were described in HL-60 cells (Bradford and Irvine, 1987) as well as in the adrenal cortex (Enyedi and Williams, 1988), where $Ins(1,3,4,5)P_4$ was found to elicit Ca^{2+} mobilization by a mechanism distinct from the $InsP_3$ receptor (Ely *et al.*, 1990). While these data suggest a role of $Ins(1,3,4,5)P_4$ in the process of Ca^{2+} signal generation, the biological significance of the $InsP_3$–$InsP_4$ pathway is still far from being understood.

The activity of the $InsP_3$-kinase enzyme is modulated by Ca^{2+}-calmodulin (Biden *et al.*, 1987), a property that would accelerate the removal of $Ins(1,4,5)P_3$ by its conversion to $Ins(1,3,4,5)P_4$ during agonist-induced elevations of cytoplasmic Ca^{2+}. The enzyme has recently been purified from bovine (Takazawa *et al.*, 1989) and rat brain (Lee *et al.*, 1990; Johanson *et al.*,

1988), and a *c*DNA clone that encodes Ins(1,4,5)P_3 kinase has been isolated from a rat brain library (Choi *et al.*, 1990). The calculated molecular mass (50 kDa) of the cloned enzymes is in good agreement with that of the purified protein (53 kDa). The $InsP_3$-kinase enzyme can be phosphorylated by both *c*AMP-dependent protein kinase and protein kinase C, the former increasing and the latter decreasing its activity *in vitro* (Sim *et al.*, 1990). Whether such potential regulation by agonist-induced phosphorylation operates *in vivo* is still to be determined.

The use of highly resolving HPLC systems revealed several additional Ins-phosphate isomers in mammalian cells, the most interesting of which were highly phosphorylated inositols corresponding to $InsP_4$, $InsP_5$, and $InsP_6$ (Heslop *et al.*, 1985). Several $InsP_2$ and InsP isomers were identified as intermediates produced by enzymes that remove phosphate groups from specific positions of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and their metabolites (Majerus *et al.*, 1988). In addition, the Ins-monophosphate ultimately produced after the degradation of Ins(1,4,5)P_3 to Ins(1,4)P_2 was found to be Ins(4)P rather than Ins(1)P as formerly assumed. The Ins(4)P metabolite thus reflects the rate of polyphosphoinositide hydrolysis (Morgan *et al.*, 1987; Balla *et al.*, 1986), whereas Ins(1)P is derived primarily from the breakdown of phosphatidylinositol. It was also recognized that Li^+ ions inhibit several of the Ins-phospatases in addition to the Ins-monophosphatase, most notably the Ins-polyphosphate-1-phospatase which converts Ins(1,4)P_2 to Ins(4)P as well as Ins(1,3,4)P_3 to Ins(3,4)P_2 (Majerus *et al.*, 1988). In general, the use of Li^+ ions to enhance the accumulation of Ins-phosphates is not recommended in studies in inositol phosphate metabolism, since it causes major distortions in their relative proportions in agonist-stimulated cells.

There has been relatively slow progress in defining the origins of the highly phosphorylated inositols; a summary of the current views about their formation and actions is shown in Fig. 3. It was clear from the beginning that Ins(1,3,4,5)P_4 is not further phosphorylated to $InsP_5$ and $InsP_6$ (Irvine *et al.*, 1986). However, at least two additional $InsP_4$ isomers have been detected in mammalian cells (Balla *et al.*, 1987b; Stephens *et al.*, 1988c) and in avian erythrocytes (Stephens *et al.*, 1988c), and are increased in agonist-stimulated adrenal cells (Balla *et al.*, 1987a, 1989b). One of these, Ins(1,3,4,6)P_4, was shown to be produced by the phosphorylation of Ins(1,3,4)P_3, by a distinctive 6-kinase (Balla *et al.*, 1987b; Shears *et al.*, 1987), and was later found to be a precursor of $InsP_5$ (Hunyady *et al.*, 1988; Stephens *et al.*, 1988b). The structure of a third $InsP_4$ isomer, produced in chicken erythrocytes (Stephens *et al.*, 1988c) and also in adrenal cells stimulated with angiotensin II (Balla *et al.*, 1989a), was found to be Ins(3,4,5,6)P_4. This $InsP_4$ isomer was also shown to be converted to $InsP_5$ by a specific 1-kinase (Stephens *et al.*, 1988a). One report has suggested that Ins(3,4,5,6)P_4 is also a breakdown product of $InsP_5$ (Menniti *et al.*, 1990), but the complete pathways of its formation are

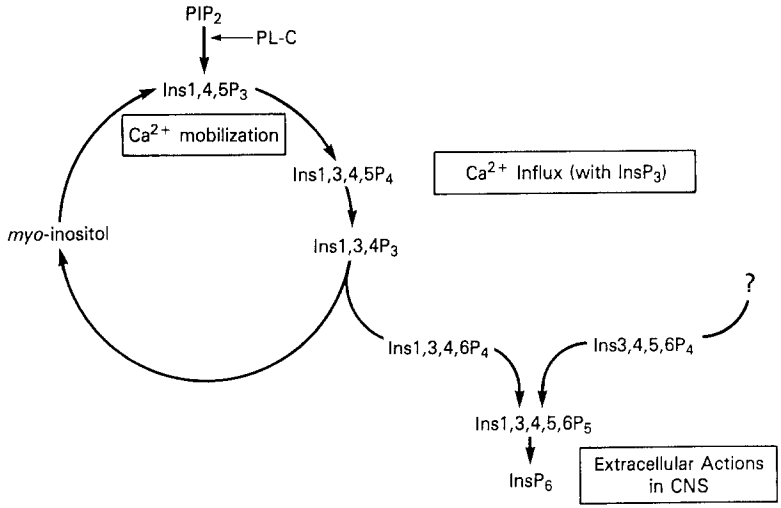

Fig. 3. Origins and functions of higher inositol polyphosphates. The ability of Ins(1,4,5)P_3 to mobilize intracellular calcium is well established, but none of the other polyphosphates has been proven to participate in calcium mobilization. Ins(1,3,4,5)P_4 has been proposed to promote calcium influx at the plasma membrane, acting in conjunction with Ins(1,4,5)P_3. The Ins(1,3,4)P_3 produced by metabolism of Ins(1,3,4,5)P_4 has relatively low calcium-mobilizing activity but may reach sufficiently high levels to have a significant action on release of intracellular calcium. The Ins(1,3,4,6)P_4 produced from Ins(1,3,4)P_3 appears to be a major precursor of $InsP_5$. However, $InsP_5$ is also formed from Ins(3,4,5,6)P_4, the origin of which is not yet known. Both $InsP_5$ and $InsP_6$ have been proposed to act as extracellular messengers to regulate neuronal excitability in the central nervous system (Vallejo *et al.*, 1987).

yet to be clarified. Although the levels of these compounds change only moderately during acute stimulation, they are increased substantially after prolonged exposure to agonists (Balla *et al.*, 1989b) and during maturation of *Xenopus* oocytes (Ji *et al.*, 1989), suggesting that they might be involved in regulation of long-term cellular responses. In this regard, it is interesting that the level of an $InsP_4$ isomer [probably Ins(3,4,5,6)P_4] is greatly increased in fibroblasts transformed with the *v-src* oncogene (Johnson *et al.*, 1989). Some of the more highly phosphorylated inositols ($InsP_5$ and $InsP_6$) have been proposed to serve as extracellular signals to evoke complex neuronal responses in the CNS (Vallejo *et al.*, 1987), but otherwise there is little evidence for specific roles of the higher inositol phosphates in cellular regulation.

Role of G-Proteins in Coupling Calcium-Mobilizing Receptors to Phospholipase C

A role for GTP-binding proteins in the coupling of Ca^{2+}-mobilizing receptors to their second messenger-generating enzymes, similar to those

involved in coupling receptors to the adenylate cyclase system (Freissmuth *et al.*, 1989), was suggested by early studies that demonstrated an inhibitory effect of GTP and/or its nonhydrolyzable analogues on the binding of such agonists to their receptors (Glossmann *et al.*, 1974a,b; GoodHardt *et al.*, 1982). By analogy with the adenylate cyclase messenger system, these findings were consistent with the concept that agonist occupancy of receptors eliciting Ca^{2+}-mediated responses promotes the exchange of GDP for GTP on the α-subunit of a heterotrimeric G-protein, with subsequent dissociation of the activated α-subunit. The free α-subunit regulates the activity of the messenger-generating enzyme, and there is a concomitant decrease in the affinity of the receptors for their ligands (Gilman, 1987). However, it was also necessary to demonstrate that the interaction of such putative G-proteins with GTP analogues can initiate the process(es) by which Ca^{2+}-dependent responses are elicited.

Calcium-mediated cellular responses, such as secretion of hormones from different kinds of permeabilized cells, were indeed found to be stimulated by GTP or its nonhydrolyzable analogues such as GTPγS (Gomperts, 1983; Haslam and Davidson, 1984a). Direct evidence for G-protein-regulated breakdown of phosphoinositides was subsequently obtained in permeabilized cells and membrane preparations, where agonist-induced formation of Ins(1,4,5)P_3 and/or DAG was found to be dependent on or potentiated by GTP analogues (Haslam and Davidson, 1984b; Hepler and Harden, 1986; Blackmore *et al.*, 1985; Baukal *et al.*, 1988; Enyedi *et al.*, 1986; Litosch and Fain, 1986). It was also shown in several cell types that inhibition of the GTPase activity of the putative G-proteins by the application of NaF (especially in the presence of $AlCl_3$) results in increased breakdown of phosphoinositides, suggesting that the protein–GTP complex is the active form involved in stimulation of second messenger generation (Blackmore *et al.*, 1985; Martin *et al.*, 1986; Litosch, 1987; Strnad *et al.*, 1986).

One of the G-proteins involved in coupling receptors to phospholipase C was identified by the finding that *Bordetella Pertussis* toxin, which specifically ADP-ribosylates and inactivates certain G proteins, inhibits receptor-stimulated phosphoinositide responses (Nakamura and Ui, 1985) and secretion (Nakamura and Ui, 1983) in several cell types. In such cells (mostly originating from bone marrow) the ability of pertussis toxin (PT) to inhibit receptor-mediated activation of phospholipase C indicated that the coupling protein belongs to the G_i family of guanine nucleotide regulatory proteins. The G_i proteins are major substrates of PT-calayzed ADP-ribosylation, and are involved in inhibition of adenylate cyclase (Freissmuth *et al.*, 1989) and coupling to muscarinic receptor-activated ion channels (Brown and Birnbaumer, 1990). It should be emphasized that many agonist-stimulated phosphoinositide responses are not sensitive to PT treatment, and that a unique and yet undiscovered G-protein (termed G_p or G_x) is involved in the coupling of

receptors to phospholipase C in most tissues (Litosch and Fain, 1986). This elusive protein has been variously predicted to be a conventional heterotrimeric G protein with an α-subunit lacking the cysteine residue at which PT ADP-ribosylates G_i proteins, or one of the *ras*-like 21–24-kDa small G proteins that are currently being defined as novel regulators of intracellular and secretory mechanisms (McAtee and Dawson, 1990).

Properties of Phosphoinositide-Specific Phospholipase C

The hydrolysis of plasma membrane phospholipids during receptor activation is performed by phospholipase C (sometimes termed phosphoinositidase C) which exists in membrane-bound and cytosolic forms, and catalyzes the breakdown of phosphoinositides located on the inner aspect of the plasma membrane (Allan and Michell, 1978; Thompson and Dawson, 1964). This reaction leads to the production of 1,2-diacylglycerol (DAG) and several inositol phosphates, including Ins(1)P, Ins(1,4)P_2, and Ins(1,4,5)P_3. The major substrate for phospholipase C during agonist-stimulated phospholipid hydrolysis in PtdIns(4,5)P_2, which is cleaved to form DAG and Ins(1,4,5)P_3 as shown in Fig. 2. Multiple forms of the enzyme have been characterized in various tissues and can hydrolyze all of the three plasma membrane phosphoinositides. Three phospholipase C isoenzymes of MW 85, 145, and 150 kDa have been isolated from bovine brain, and are structurally and immunologically distinct from the 62–68-kDa enzyme present in liver, seminal vesicle, and uterus (Ryu *et al.*, 1987).

The phospholipase C enzymes have recently been designated by Greek letters for their primary structures and numerals to indicate products of alternative splicing or proteolysis (Rhee *et al.*, 1989). By *c*DNA cloning, the major phospholipases were found to be distinct polypeptides of MW 57 (PLC-α), 138 (PLC-β), 148 (PLE-γ), and 86 kDa (PLC-δ) (Suh *et al.*, 1988). The four enzymes show little overall amino acid sequence homology, consistent with their lack of immunological cross-reactivity, but the β, γ, and δ enzymes contain two domains of 150 and 120 amino acids (termed X and Y) with 54 and 42% identity, respectively (Fig. 4). These conserved domains are possibly involved in the catalytic site of the enzyme or its interactions with receptors and/or regulatory proteins controlling enzyme activation. The largest enzyme, phospholipase C-γ, has a relatively long variable region which contains sequences homologous to the noncatalytic domain of the nonreceptor class of tyrosine kinases such as *src*. This enzyme has been recently found to be physically associated with certain growth factor receptors, and to be tyrosine-phosphorylated during stimulation of phospholipid hydrolysis by the cognate ligands, EGF and PDGF.

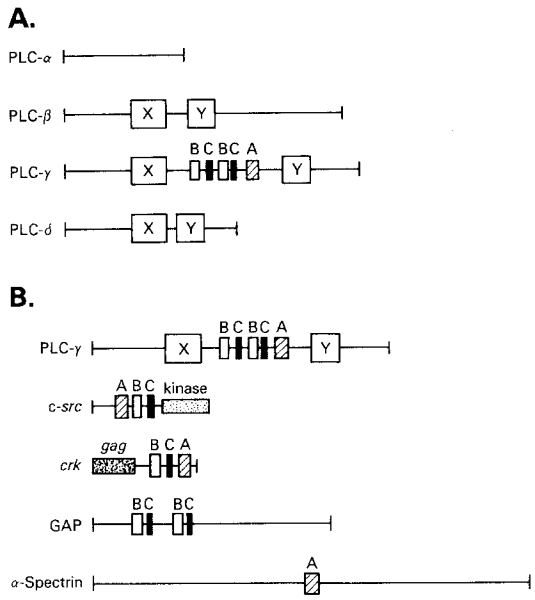

Fig. 4. (A) Linear structures of the major phospholipase C isozymes. The open boxes (X and Y) indicate common sequences of ~120 and ~150 amino acids, respectively, that occur in phospholipases β, γ, and δ. (B) Structural similarities between PLC-γ and *c-src*, *crk*, GAP, and α-spectrin. Boxes A, B, and C indicate similar sequences of ~50, ~40, and ~15 amino acids, respectively (from Rhee *et al.*, 1989).

Ins(1,4,5)P_3 Releases Ca^{2+} through a Receptor-Mediated Mechanism

The recognition that Ins(1,4,5)P_3 is the major link between receptor activation and intracellular Ca^{2+} stimulated attempts to clarify the underlying mechanism(s) of Ca^{2+} release. The use of Ins(1,4,5)P_3 labeled to high specific activity with ^{32}P facilitated the detection of specific high-affinity binding sites for Ins(1,4,5)P_3 in subcellular membrane fractions and permeabilized cells (Baukal *et al.*, 1985; Spät *et al.*, 1986a,b). These binding sites are distinct from the degradative enzyme, Ins(1,4,5)P_3 5-phosphatase (Guillemette *et al.*, 1987), and in liver are enriched in a plasma-membrane fraction (Guillemette *et al.*, 1988). The relative binding affinities of various inositol phosphates for these sites is in good agreement with their Ca^{2+}-releasing activities (Spät *et al.*, 1986b; Irvine *et al.*, 1984b). However, the affinity of the binding sites for Ins(1,4,5)P_3 (1–30 nM) is significantly higher than the ED_{50} for Ca^{2+} release (50 nM–1 μM) (Guillemette *et al.*, 1987). This discrepancy between binding and activation constants could reflect the need for binding of more than one $InsP_3$ molecule to evoke Ca^{2+} release (Meyer *et al.*, 1988). There is no evidence that guanyl nucleotide regulatory (G) proteins, similar to those

that couple receptors to second messenger generating enzymes, are involved in the Ca^{2+}-mobilizing action of $InsP_3$. However, under special conditions GTP is able to release Ca^{2+} from intracellular pools, and has been proposed to mediate communication and Ca^{2+} transfer between different vesicular compartments within the cell (Mullaney *et al.*, 1987). Although the physiological significance of these observations is not yet known, it is possible that such communication occurs during agonist-stimulated influx of intracellular Ca^{2+} by a recently proposed vesicular uptake mechanism that depends on receptor-mediated endocytosis (Hunyady *et al.*, 1990).

The Inositol(1,4,5)-trisphosphate Receptor

The nature of the Ins(1,4,5)P_3 receptor involved in mobilization of intracellular calcium has been clarified by binding studies with [^{3}H]- or [^{32}P]-labeled Ins(1,4,5)P_3, and by the recent purification and molecular cloning of the brain receptor site. The Ins(1,4,5)P_3 receptor in particulate fractions of several tissues (adrenal, liver, salivary gland, brain, and platelets) is highly specific for Ins(1,4,5)P_3 and related calcium-mobilizing compounds such as Ins(2,4,5)P_3 (Guillemette *et al.*, 1987; Worley *et al.*, 1987; O'Rourke and Feinstein, 1990). The receptors were found to be associated with calcium-mobilizing activity in vesicular structures which in several studies were enriched in the plasma-membrane fraction of cell and tissue homogenates (Guillemette *et al.*, 1988; Dunlop and Larkins, 1988). In some tissues, Ins(1,4,5)P_3 has been proposed to act on a specialized calsequestrin-containing organelle, the calciosome (Volpe *et al.*, 1988), but the more general view is that Ins(1,4,5)P_3 mobilizes calcium from specialized regions of the endoplasmic reticulum (Ross *et al.*, 1989). An action of Ins(1,4,5)P_3 at the plasma membrane level has also been suggested, based on immunolocalization studies (Furuichi *et al.*, 1989) and the ability of Ins(1,4,5)P_3 to stimulate inward calcium currents in lymphocytes (Kuno and Gardner, 1987). However, it is likely that the $InsP_3$ receptor is specifically associated with vesicular Ca^{2+} stores derived from or continguous with the endoplasmic reticulum.

The Ins(1,4,5)P_3 receptor is abundant in the brain, in particular the cerebellum, where it is highly concentrated in the Purkinje cells (Worley *et al.*, 1987). The receptor isolated from the cerebellum is a 260-kDa glycoprotein that is inhibited by heparin and can be phosphorylated by *c*AMP-dependent protein kinase (Supattapone *et al.*, 1987); its binding affinity for Ins(1,4,5)P_3 is increased at alkaline pH and inhibited by Ca^{2+} (Worley *et al.*, 1987). The receptor appears to form tetramers of identical subunits, and has been shown by immunohistochemical studies to be located on particles associated with the endoplasmic reticulum (Ross *et al.*, 1989). The purified

$Ins(1,4,5)P_3$ binding protein from brain membranes has been reconstituted into lipid vesicles and shown to mediate calcium influx stimulated by $Ins(1,4,5)P_3$ and other inositol phosphates with the potencies and specificities which they exhibit in brain microsomes (Ferris *et al.*, 1989). These findings have demonstrated that the brain binding protein is the physiological intracellular receptor for $Ins(1,4,5)P_3$, and, moreover, that a single protein mediates both ligand binding and calcium flux. In this regard the tetrameric $Ins(1,4,5)P_3$ receptor shares features with the nicotinic, GABA, and glycine receptors, in which the associated (dissimilar) subunits contain the ligand binding site and the respective ion channel. However, the $Ins(1,4,5)P_3$ receptor is more analogous to the ryanodine receptor that mediates calcium release from the sarcoplasmic reticulum in skeletal muscle and is a tetramer of similar subunits with four transmembrane regions that form the calcium channel which is gated by the voltage-sensing dihydropyridine receptor present in the T-tubule of the muscle fiber (Lai *et al.*, 1988; Tanabe *et al.*, 1988; Takeshima *et al.*, 1989).

Recently, the $Ins(1,4,5)P_3$ receptor was found to be identical with a previously known 250-kDa membrane glycoprotein (termed P_{400}) that is highly concentrated in Purkinje cells and is deficient in cerebellar ataxic mice with Purkinje cell degeneration (Furuichi *et al.*, 1989). This was achieved by screening mouse cerebellum *c*DNA libraries in λgT11 expression vectors with monoclonal antibodies to purified P_{400}, which led to the cloning of a complete *c*DNA sequence encoding a protein of 2749 amino acids with MW of 313 kDA. The protein sequence contained several potential membrane-spanning regions, two potential cytoplasmic sites for phosphorylation by cyclic AMP-dependent protein kinase, and a short glycosylated C-terminal region that is oriented within the ER lumen. The potential sites for serine phosphorylation are located within the large cytoplasmic domain of the receptor protein. The mRNA for the $Ins(1,4,5)P_3$ receptor is highly abundant in Punkinje cells (and reduced in cerebella of Purkinje cell-deficient mutant mice), and is present at much lower levels in the cerebrum and peripheral tissues. Expression of the cloned *c*DNA in transfected neuronlike (NG108-15 neuroblastoma/glioma hybrid) cells produced immunoreactive P_{400} protein with high affinity for $[^3H]Ins(1,4,5)P_3$ (K_d = 22 nM) and relative binding potencies for inositol phosphates of $Ins(1,4,5)P_3 > Ins(2,4,5)P_3 > Ins(1,3,4,5)P_4 > Ins(1,4)P_2$.

The $Ins(1,4,5)P_3$ receptor bears a striking resemblance to the ryanodine receptor (Takeshima *et al.*, 1989), which mediates calcium release from the sarcoplasmic reticulum in skeletal muscle. The ryanodine receptor also has a large cytoplasmic N-terminal domain and several C-terminal membrane-spanning regions, two of which show homology with the $Ins(1,4,5)P_3$ receptor. Both receptors are localized in the endoplasmic reticulum or its derivatives,

and form tetramers which have a similar square shape on electron microscopy. This partial homology with the ryanodine receptor was also noted in studies in which a previously described Purkinje cell *c*DNA, identified in a normal mouse cerebellar library by its failure to hybridize to mRNA sequences from cerebella of Purkinje cell-degeneration mice, was found to encode the Ins(1,4,5)P_3 receptor (Mignery *et al.*, 1989). The 260-kDa protein product was highly enriched in the cerebellum but was also present in all peripheral tissues tested (especially liver and testis), and was found to have at least four putative transmembrane regions. The Ins(1,4,5)P_3 receptor shows marked amino acid sequence similarity with the ryanodine receptor over transmembrane regions believed to be part of the ryanodine-sensitive calcium channel, suggesting that the channel is also intrinsic to the Ins(1,4,5)P_3 receptor protein. The otherwise dissimilar regions of the two receptors sharing a similar calcium channel are presumably responsible for the recognition of their different activating ligands.

The rat brain (Ins(1,4,5)P_3 receptor has also been cloned recently, and shows an extremely high degree of conservation when compared with the mouse cerebellar receptor (Mignery *et al.*, 1990). The rat receptor is a 260-kDa protein containing more than 2700 amino acids and forms tetramers similar to the native receptor. The carboxyl-terminal 600 amino acids contain eight putative transmembrane regions related to the intrinsic calcium channel of the receptor, and the carboxyl-terminal tail is located in the cytoplasm. The last four transmembrane regions are flanked by net negative charges and show significant homology with the ryanodine receptor. Since the $InsP_3$ receptor has four independent binding sites that are cooperative for calcium channel opening, it is possible that each 260-kDa subunit binds $InsP_3$ at an amino-terminal cytoplasmic site that regulates a Ca^{2+} channel formed by the last four of the eight carboxyl-terminal transmembrane domains (Mignery *et al.*, 1990).

Growth Factors and Phosphoinositide Hydrolysis

In addition to mediating many of the rapid responses of cells to hormonal stimulation, the products of phosphoinositide hydrolysis also participate in the regulation of intracellular events involved in cell growth and proliferation. Several growth factors, including platelet-derived growth factor (PDGF), EGF, and bombesin, are known to activate phospholipase C and to cause changes in intracellular calcium and pH that depend on the signals ($InsP_3$ and diacylglycerol) produced by hydrolysis of PtdIns(4,5)P_2 (Berridge, 1987). In some tissues, these effects appear to be mediated by a pertussis-sensitive G protein, but this is not a common feature of growth factor-induced inositol

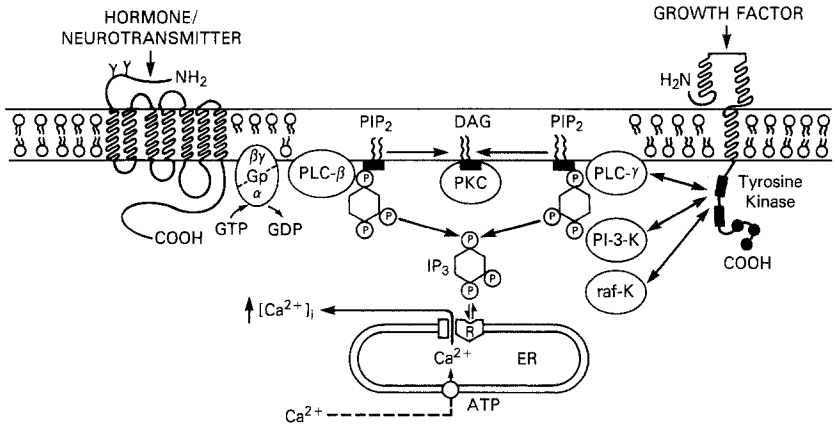

Fig. 5. Comparison of the mechanisms by which receptors for hormones and neurotransmitters (G protein-coupled receptors) and growth factor (tyrosine kinase) receptors activate phospholipase C and stimulate the phosphoinositide–calcium signal transduction pathways (modified from Boyer *et al.*, 1989). Ligands that bind to receptors of the seven membrane-spanning or rhodopsin type promote coupling through a G protein to phospholipase C, probably the β isoform of the enzyme. In contrast, growth factors that bind to tyrosine kinase-containing receptors (EGF, PDGF, FGF, and CSF-1) promote the association of the activated intracellular receptor domain with several cytoplasmic proteins, including phospholipase C-γ as well as PI-3-kinase, the GTP-activating protein of *ras* (GAP) and *raf*-1 protein kinase. These dissimilar types of stimuli both cause hydrolysis of PIP_2 and generation of $Ins(1,4,5)P_3$ and DAG, with mobilization of intracellular calcium and influx of extracellular calcium.

lipid hydrolysis (Taylor *et al.*, 1988). It is now recognized that activation of phospholipase C by hormones and tyrosine kinase growth factors occurs by two quite different pathways, and probably involves two distinct enzymes, the β and γ isoforms (Fig. 5).

An important step in elucidating the mechanism of growth factor-induced $PtdInsP_2$ hydrolysis was the finding that EGF-stimulated phospholipase C activity could be precipitated from A431 cell extracts by anti-phosphotyrosine (Wahl *et al.*, 1988). This finding showed that tyrosine phosphorylation of the enzyme, or of a tightly associated protein, is rquired for EGF-stimulated $PtdInsP_2$ turnover. Subsequently, both EGF and PDGF have been shown to induce tyrosine phosphorylation of phospholipase C, specificaly at two identical tyrosine residues in the γ form of the enzyme. Antibodies to phospholipase C-γ precipitate about 1% of the EGF receptors in A431 cells, and antibodies to EGF were found to co-immunoprecipitate phospholipase C-γ (Margolis *et al.*, 1989). In addition, an inhibitor of tyrosine phosphorylation (tyrphostin) blocked EGF-induced phosphorylation and activation of phospholipase C-γ, and prevented its association with the EGF receptor, as well as the EGF-stimulated Ca^{2+} response. Inhibition of

tyrosine phosphorylation had no effect on the Ca^{2+} release induced by bradykinin and bombesin, which act through G proteins to activate phospholipase C, probably the β form of the enzyme (Margolis *et al.*, 1989).

PDGF causes phosphoinositide hydrolysis and calcium mobilization in 3T3 cells, with kinetics that differ from the responses induced by bombesin or vasopressin. The somewhat slower and more prolonged time course of the PDGF-induced changes suggests that the growth factor acts through a different transduction pathway (Nanberg and Rozengurt, 1988). In 3T3 cells, the stimulation of phosphoinositide breakdown by PDGF is accompanied by phosphorylation of serine and tyrosine residues in phospholipase C-γ. Furthermore, the PDGF receptor co-immunoprecipitates with phospholipase C-γ, suggesting that the receptor can directly phosphorylate the enzyme (Meisenhelder *et al.*, 1989). The FGF receptor and the HER2/*neu* protein also phosphorylate and associate with phospholipase C-γ. However, not all growth factors act in this manner, since insulin and CSF-I do not promote the tyrosine phosphorylation of phospholipase C-γ (Margolis *et al.*, 1989). An association between activation of phospholipase C and tyrosine phosphorylation has also been observed for the T-cell antigen receptor, and the tyrosine kinase inhibitor, genistein, prevents phospholipase C activation and T-cell proliferation (Mustelin *et al.*, 1990). Although tyrosine phosphorylation is an early and probably essential requirement for T-cell activation, it is not yet clear whether the enzyme itself, or an associated regulatory protein, is the site at which such phosphorylation occurs.

In addition to stimulating phosphoinositide hydrolysis, several growth factors and oncogene products with tyrosine kinase activity are associated with a novel PtdIns kinase that catalyzes the synthesis of small amounts of unusual phosphoinositides in the plasma membrane. This recently identified enzyme (termed Type I PtdIns kinase) produces a phospholipid that is phosphorylated in the 3-position of the inositol ring to produce phosphatidylinositol-3-phosphate [PtdIns(3)P], instead of the phosphatidylinositol-4-phosphate [PtdIns(4)P] previously thought to be the only form of $PtdInsP_2$ (Whitman *et al.*, 1988). This new enzyme was identified because of its tight association with several tyrosine kinases, including the middle $T/pp60^{c\text{-}src}$ complex from 3T3 cells transformed with polyoma virus. The middle T antigen of this DNA tumor virus associates with and activates tyrosine kinases of the *src* family, which in turn phosphorylate an 85-kDa protein corresponding to PtdIns 3-kinase. The PtdIns 3-kinase activity in 3T3 cells also associates with the ligand-activated PDGF receptor, and is immunoprecipitated from extracts of PDGF-stimulated cells by antiphosphotyrosine (Whitman and Cantley, 1988). The enzyme has been shown to be regulated by receptor activation in several cell types, including fibroblasts, neutrophils, vascular smooth muscle, astrocytes, and platelets, where it is activated by

thrombin treatment to produce PtdIns(3)P and PtdIns(3,4)P_2 (Nolan and Lapetina, 1990). Cells stimulated with PDGF, EGF, CSF-I, and insulin also show increased levels of PtdIns(3,4)P_3, and sometimes PtdIns(3,4,5)P_3. (Pignataro and Ascoli, 1990; Varticovski *et al.*, 1989; Ruderman *et al.*, 1990). It is not yet clear whether these new inositol lipids are hydrolyzed to produce inositol phosphates, or themselves serve as novel second messengers or as cofactors for other membrane-bound enzymes. However, the association of the PtdIns 3-kinase with several receptor-associated tyrosine kinases clearly reflects its potential role in the actions of certain growth factors and oncogenes.

The mechanism of action of insulin also involves several facets of phospholipid production and metabolism, but these do not include the established form of agonist-induced phophoinositide hydrolysis. Recent work has shown that inositol glycans are released from membrane glycophospholipids during insulin action and mimic several of the metabolic effects of insulin when added to intact or broken cells (Low and Saltiel, 1988). Such cleavage of plasma-membrane glycolipids has been attributed to a specific form of phospholipase C, which catalyzes the production of inositol glycans and diacylglycerol during insulin action, and may be activated by a G protein that is in turn phosphorylated by the tyrosine-specific kinase of the insulin receptor (Walass and Walass, 1988). Furthermore, DAG levels are increased in insulin-stimulated cells and appear to regulate, via activation of protein kinase C, the expression of several insulin-sensitive genes (Standaert and Pollet, 1988). Such insulin-induced increases in DAG production may arise from both increased *de novo* synthesis of membrane glycerolipids and the cleavage by phospholipase C of the phosphatidylinositol glycan described above. Finally, the insulin receptor is associated with phosphatidylinositol kinase activity, in addition to tyrosine kinase, and could thereby also regulate the biosynthesis of phospholipids involved in the transduction of mitogenic signals.

Early events in the actions of many growth factors and mitogens include the activation of Ca^{2+} fluxes and of an amiloride-sensitive Na^+/H^+ exchange mechanism, with a consequent rise in $[Ca^2]_i$ and transient alkalinization of the cytosol (Macara, 1986). Since activation of protein kinase C by phorbol esters stimulates the plasma-membrane Na^+/H^+ exchanger, it has been suggested that the Ca^{2+} mobilization and phospholipid hydrolysis caused by growth factors leads to increased DAG production and activation of protein kinase C, which in turn stimulates the Na^+/H^+ exchanger (Boron, 1984). However, other studies have indicated that the actions of growth factors on Na^+/H^+ exchange and Ca^{2+} influx can be independent of phosphoinositide turnover (Macara, 1986), and that the regulatory mechanism may differ with cell type. Protein kinase C is also involved in modulating the binding affinity

and tyrosine kinase activity of the EGF receptor through phosphorylation of sites in the cytoplasmic domain of the receptor (Hunter, 1984). In addition to such homologous modulation by EGF itself, the EGF receptor is also subject to transmodulation by PDGF, which stimulates phospholipid hydrolysis and diacylglycerol production on binding on its own receptor and thus leads to kinase C activation and phosphorylation of coexistent EGF receptors, with a decrease in their binding affinity for EGF (Newmark, 1985).

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