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PHOSPHATIDYLINOSITOL TURNOVER IN RECEPTOR MECHANISM AND SIGNAL TRANSDUCTION

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INTRODUCTION

The activation of cellular functions and proliferation are frequently initiated by the interaction of external stimuli with their specific cell-surface receptors. Early events of the intracellular response include a metabolic cascade of membrane phospholipids. Investigations of the target site of biochemical changes in cell membranes have focused particularly on the immediate breakdown of inositol phospholipids and the accumulation of phosphatidic acid.

As early as 1953, Hokin & Hokin (1) observed that one effect of acetylcholine on pancreas slices was the rapid enhancement of phospholipid metabolism, specifically, the incorporation of $^{32}P_i$ into phosphatidylinositol (PtdIns) and phosphatidic acid. Later, the physiological significance of this original observation was found in the cholinergic release of amylase from pancreatic acinar cells (2). A specific increase in the turnover of PtdIns has since been documented repeatedly in a number of tissues in response to stimulation by a wide variety of neurotransmitters, hormones, and many other biologically active substances [for reviews, see (3–6)]. The activation of the receptors involved, for example, muscarinic rather than nicotinic and α- rather than

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β-adrenergic, which constitute a class distinct from those acting through cyclic AMP, results in the increased availability of other second messengers such as Ca^{2+} (7). In no system does cyclic AMP or its derivatives elicit such phospholipid metabolic effects. The precise consequences and physiological significance of these effects are not yet fully understood. However, many functions for the stimulated PtdIns turnover thus far have been proposed: e.g. membrane fusion (8,9), early events in cell proliferation (10, 11), elevation of intracellular Ca^{2+} concentration, arachidonic acid release (12–14), increases in cyclic GMP levels (15–17), and visual signal transduction (18, 19).

In recent years, polyphosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2), which represent less than a few percent of the total inositol phospholipids (20) and are relatively enriched in the nervous system (21), have been studied with renewed interest. Rapid disappearance of the phospholipid has been observed in stimulated cells, and more recently the water soluble product, inositol 1,4,5-trisphosphate, has been proposed by Berridge and his colleagues (22,23) to be an intracellular mediator for the release of Ca^{2+} from internal stores. On the other hand, Nishizuka and colleagues (24,25) have demonstrated that a small amount of another product, diacylglycerol, acts as a novel intracellular mediator and stimulates a unique Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C). Thus, the receptor-mediated turnover of inositol phospholipid appears to represent a multifunctional second messenger–transducing mechanism (Figure 1).

THE ASSOCIATION OF CYTOSOLIC Ca^{2+} ELEVATION

The stimulation of the receptors relating to inositol phospholipid breakdown is usually accompanied by an elevation of intracellular Ca^{2+}. The second-messenger function for Ca^{2+} has been proposed in many stimulated tissues (26–28). The intracellular Ca^{2+} can be raised both by external influx and by release from its internal stores. When blowfly salivary gland is stimulated with 5-hydroxytryptamine in Ca^{2+}-free media, the decline in secretion is not immediate, but there is a short initial period during which a normal secretory response may be observed. Prince & Berridge (29) have suggested that this temporary independence of external Ca^{2+} is probably maintained by the internal mobilization of this divalent cation. In addition, in the liver, the glycogenolytic action of α_1-adrenergic agonists and vasoactive peptide hormones is mediated by an initial increase in the cytosolic Ca^{2+} concentration that is probably released from intracellular compartments (30, 31). Farese et al (2) have reported that in rat pancreatic slices the removal of Ca^{2+} from the media abolishes both the cholinergic release of amylase and the chemically measurable loss of PtdIns. Thus, in some tissues at least, external Ca^{2+} influx appears to have a role in the long-term potential of physiological cell responses.

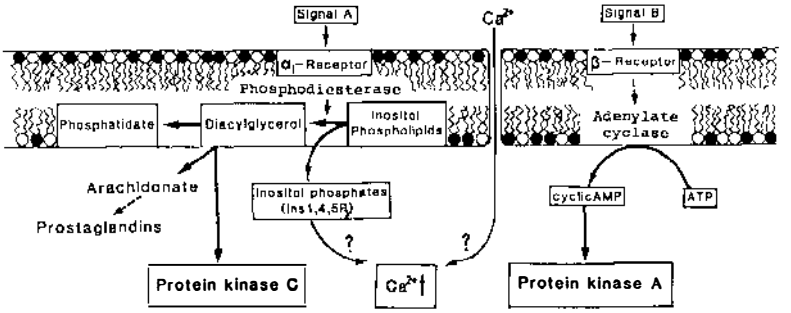

Figure 1 Two major receptor mechanisms in transmembrane signaling. α_1- and β-receptors are used as representatives. Ins1,4,5P_3: inositol 1,4,5-trisphosphate; protein kinase A: cyclic AMP-dependent protein kinase.

In 1975, Michell (4) proposed that breakdown of inositol phospholipids is responsible for opening the Ca^{2+} gate. Some of the implications of this proposal have been reviewed by Cockcroft (32) and Hawthorne (33). Many studies have been made as to whether Ca^{2+}-ionophores provoke the degradation of inositol phospholipids. Creba et al (34) have reported that disappearance of ^{32}P-labeled phosphatidylinositol 4-phosphate (PtdIns4P) and PtdIns4,5P_2 is not observed when cells are incubated with the Ca^{2+} ionophore A23187. In Ca^{2+}-depleted cells, hormone-stimulated polyphosphoinositide disappearance is reduced but not abolished.

However, to date there has been no conclusive agreement among previous reports, probably because the phosphodiesterase must be activated not only by Ca^{2+} but also by unknown mechanisms such as proteolytic activation (35). Unless the phosphodiesterase is activated, the inositol phospholipids will not be hydrolyzed, even if the intracellular Ca^{2+} is increased by the addition of a Ca^{2+} ionophore. The question of what is responsible for opening channels for external Ca^{2+} still remains to be resolved. Recently, Cachelin et al (36) have proposed that in some tissues cyclic AMP–dependent phosphorylation of Ca^{2+} channels primarily promotes the forward rate constants that lead to the open state of Ca^{2+} channels during depolarization.

INOSITOL PHOSPHOLIPID TURNOVER AND POLYPHOSPHOINOSITIDES

In pancreas, an enhanced turnover of PtdIns in response to acetylcholine is accompanied by the formation of phosphatidic acid, which can be approximately equal to the decrease in the level of PtdIns (37). In stimulated tissues the lipid-soluble products of inositol phospholipid breakdown appear as phosphatidic acid, CDP-diacylglycerol, or diacylglycerol (38). The overall pathway of PtdIns turnover is known as the PtdIns-resynthesis cycle. The first step of this

cycle is initiated by a Ca^{2+}-dependent phospholipase C-type phosphodiesterase(s) that hydrolyzes inositol phospholipids to produce diacylglycerol and inositol phosphates (39–42). The diacylglycerol is then immediately phosphorylated to phosphatidic acid with ATP by the reaction of diacylglycerol kinase (43). The measurement of enhanced $^{32}P_i$ incorporation into PtdIns and phosphatidic acid is therefore a secondary effect of external stimuli on inositol phospholipids breakdown. Results obtained using this method have lead to considerable confusion over the stimulated PtdIns turnover, for example, over the important question of which inositol phospholipids are really hydrolyzed by the phosphodiesterase in response to external stimuli. Earlier reports do not appear to support a primary role for polyphosphoinositides in stimulated phosphatidic acid and PtdIns labeling (44–46), possibly because the changes seen are relatively small and rapid. However, two polyphosphoinositides, PtdIns4P and PtdIns4,5P_2, are potentially more interesting as membrane constituents because of their unique physicochemical properties. Particularly PtdIns4,5P_2 is a multiply-charged anion that has a very high affinity for Ca^{2+} (greater than that of EDTA) and exhibits a rapid turnover in vivo. Its hydrophilic/hydrophobic solubility partition coefficient is markedly altered when Ca^{2+} replaces monovalent phosphate counterions (47–49).

The phosphorylated PtdIns-derivatives PtdIns4P and PtdIns4,5P_2 are synthesized by the stepwise phosphorylation of PtdIns by the enzyme PtdIns kinase and PtdIns4P kinase. PtdIns kinase has been described in a variety of tissues, erythrocytes (50), brain (51–54), liver (55, 56), and kidney (57), where subcellular fractionation studies have suggested that it is localized in the plasma membrane. One area of recent interest has been the relationship between the transforming genes and phosphorylation of PtdIns and diacylglycerol (58, 59). Phosphomonoesterases that degrade PtdIns4,5P_2 to PtdIns4P and thence to PtdIns are also present (Figure 2).

THE DISAPPEARANCE OF INOSITOL PHOSPHOLIPIDS

PtdIns4P and PtdIns4,5P_2, very minor phospholipids in the inner leaflet of cell membranes, have been considered alternative candidates to PtdIns in the early breakdown of inositol phospholipids by receptor-mediated hydrolysis (60). Like PtdIns, these phospholipids may be degraded by phosphodiesterase action to inositol phosphates and diacylglycerol (41, 61).

It has recently been suggested that the breakdown of PtdIns4P and PtdIns4,5P_2 may precede the previously known PtdIns response. Rapid signal-induced breakdown of PtdIns4P and PtdIns4,5P_2 has been reported in a variety of tissues [for a review, see (62)]. For example, results from iris smooth muscle have shown that the activation of muscarinic cholinergic and α_1-adrenergic receptors results in the immediate breakdown of PtdIns4,5P_2 but not of PtdIns

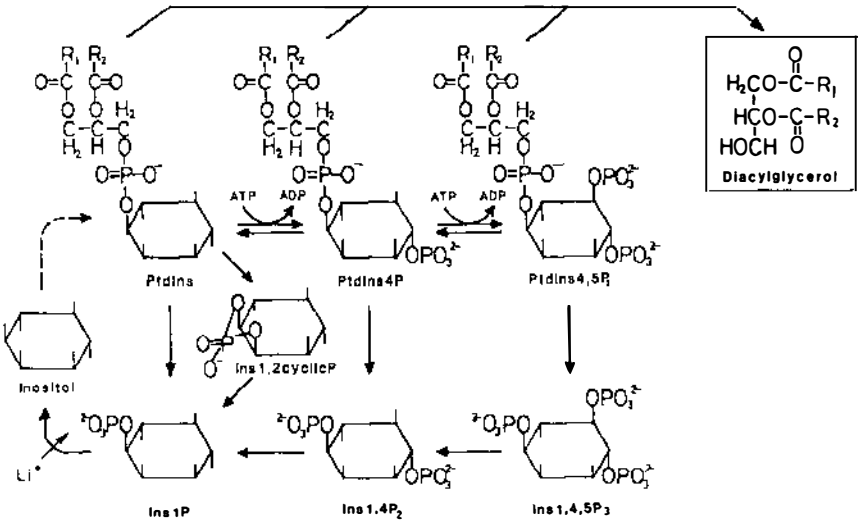

Figure 2 Production of diacylglycerol and inositol phosphates in inositol phospholipid metabolism. PtdIns: phosphatidylinositol; PtdIns4P: phosphatidylinositol 4-phosphate; PtdIns4,5P_2: phosphatidylinositol 4,5-bisphosphate; Ins1P: inositol 1-phosphate; Ins1,2 cyclic P: inositol 1,2-cyclic phosphate; Ins1,4P_2: inositol 1,4-bisphosphate; Ins1,4,5P_3: inositol 1,4,5-trisphosphate.

or PtdIns4P (63–65). Michell and his colleagues have also observed that Ca^{2+}-mobilizing hormones such as vasopressin, angiotensin II, and epinephrine active at the α_1-receptor provoke rapid degradation of ^{32}P-labeled PtdIns4P and PtdIns4,5P_2 in hepatocytes (34, 66). The maximum disappearance of PtdIns4,5P_2 is detected one minute after the addition of vasopressin. Similar results have been obtained in the early actions of thyrotropin-releasing hormone on hormone-responsive clonal GH_3 pituitary cells (67), carbachol and pancreozymin on pancreatic acinar cells (68), and thrombin on platelets (69). Addition of these stimulators results in the rapid disappearance of labeled polyphosphoinositide(s), whereas levels of PtdIns and other phospholipids remain unchanged. Litosch et al (70) have observed the breakdown of both PtdIns and PtdIns4,5P_2 in response to vasopressin in hepatocytes within 30 seconds. More recently, Orchard et al (71) have described the stimulation by carbachol or pancreozymin of ^{32}P-labeled rat pancreas acinar cells, which causes a decrease of PtdIns4,5P_2 by 30–50% within 10–15 seconds; this decrease is followed by the sequential increase in $^{32}P_i$ incorporation into phosphatidic acid and PtdIns. This signal-induced disappearance of PtdIns4,5P_2 is not dependent on external Ca^{2+}. These authors of this study have suggested that the disappearance may initiate the Ca^{2+}-independent labeling of phosphatidic acid and PtdIns. Ca^{2+} mobilization may follow these

responses and subsequently cause Ca^{2+}-dependent hydrolysis of PtdIns and exocytosis.

These observations raise the possibility that the initial action of various extracellular messengers involves the hydrolysis of polyphosphoinositides. At present, it is still not absolutely clear which inositol phospholipid is specifically cleaved by the phosphodiesterase. Michell (72) has speculated that PtdIns is never hydrolyzed by phospholipase C-type enzyme but disappears due to sequential phosphorylation by the specific kinases. If this is correct, a continuous increase of PtdIns4,5P_2 may be expected during stimulation. The kinase probably has to be activated in order to replenish the polyphosphoinositides, since disappearance of PtdIns continues over rather a long period. Using carbamyl-choline injection as the cholinergic stimulation of polyphosphoinositide metabolism, Soukup et al (73) have demonstrated enhanced incorporation of phosphate as well as inositol into polyphosphoinositides in regions of the rat brain in vivo. In contrast, the stimulation of hepatocytes by vasopressin does not result in an increase of PtdIns4,5P_2 (34). It seems still early to conclude that only PtdIns4,5P_2 is hydrolyzed in response to external stimuli. It is possible that the three inositol phospholipids are hydrolyzed by the phosphodiesterase(s) at different times in different rates, or, as Hokin-Neaverson & Sadeghian (74) have speculated, that polyphosphoinositide and PtdIns responses occur at different sites within the cell.

THE PHOSPHOLIPASE C-TYPE CLEAVAGE

Since the discovery of specific phosphodiesterase in pancreas (39) and liver (75), it has been proposed that the phospholipase C-type cleavage of inositol phospholipids is responsible for the receptor-mediated PtdIns turnover. Until recently this hypothesis has not been fully substantiated, but researchers now agree that the putative initial step of increased PtdIns turnover following receptor stimulation is due to the hydrolysis of inositol phospholipids by specific phosphodiesterase(s). This is supported by recent studies that used direct chemical measurement of the water soluble products, inositol 1-phosphate and inositol cyclic 1,2-phosphate from PtdIns, or inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate from PtdIns4P and PtdIns4,5P_2, respectively. Durell et al (76) have observed that the primary effect of acetylcholine is to stimulate the hydrolysis of the inositol phospholipids. This reaction has been best observed over a short period; a trace of inositol 1,4,5-trisphosphate and the accumulation of inositol 1,4-bisphosphate and inositol 1-phosphate are measured. The enhanced incorporation of $^{32}P_i$ into the phospholipids subsequently observed is thus a secondary synthetic reaction that occurs due to the increased concentration of diacylglycerol bound to active membranes. In addition, when platelets are stimulated to aggregate by throm-

bin (13), diacylglycerol is observed as an early product of inositol phospholipid catabolism. In their serial experiments with insect salivary gland stimulated by 5-hydroxytryptamine, a very short time after stimulation Berridge and his colleagues (77, 78) saw a large and rapid increase in the levels of inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate but no change in the amount of inositol 1-phosphate or inositol present. Similar results have been reported in rat parotid gland after muscarinic stimulation (79) and in GH_3 pituitary cell following stimulation by thyrotropin-releasing hormone (67).

It is known that the inhibitory effect of lithium ion on *myo*-inositol 1-phosphate phosphatase, which is different from PtdIns4,5P_2 phosphomonoesterase, causes an increase in the level of *myo*-inositol 1-phosphate both in vivo (80) and in vitro (81, 82). This may be related to the control of manic-depressive illness. A recent study by Hokin-Neaverson & Sadeghian (74) has shown lithium-induced accumulation of inositol 1-phosphate during cholecystokinin octapeptide and acetylcholine stimulation of mouse pancreas acinar cells. These authors have noted that inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate can not be detected for 15 minutes following stimulation.

These reports support the disappearance of inositol phospholipids by the phospholipase C-type cleavage as a very early event in the receptor-mediated cell responses. However, results to date do not allow us to conclude that only PtdIns4,5P_2 is hydrolyzed, with PtdIns and PtdIns4P in turn disappearing by the sequential action of PtdIns kinase and PtdIns4P kinase to PtdIns4,5P_2. Lithium ion appears to be a specific inhibitor of inositol 1-phosphate phosphatase, but not of inositol 1,4,5-trisphosphate or inositol 1,4-bisphosphate phosphatases. However, lack of inhibition of the latter phosphatases by lithium ion needs to be more clearly demonstrated. To test the hypothesis that PtdIns4,5P_2 but not PtdIns is hydrolyzed by the phosphodiesterase, a specific inhibitor for inositol 1,4,5-trisphosphate phosphatase must be developed. If, as a result, inositol 1,4,5-trisphosphate accumulation occurs in stimulated cells, and if it is correct that PtdIns disappears by the sequential action of kinases to PtdIns4,5P_2, then a continuous elevation of intracellular Ca^{2+} should be seen, as discussed below.

On the other hand, PtdIns is decreased over rather a long time, and it may be required for the long-term potentiation of the physiological cell responses. Hokin-Neaverson & Sadeghian (74) have speculated that stimulated PtdIns4,5P_2 breakdown and stimulated PtdIns breakdown may be separate processes. In mouse pancreas these authors have observed that the breakdown of PtdIns in response to 10 μM acetylcholine continues in a linear fashion for much longer than it does in response to lower concentrations of acetylcholine. This may suggest an alternative hypothesis: that enhanced breakdown of PtdIns4,5P_2 and PtdIns are two separate processes that may serve different, as yet unknown, functions. The enhanced breakdown of PtdIns4,5P_2 seems to be

an event that is initiated and terminated rapidly, whereas the enhanced breakdown of PtdIns appears to persist for a much longer period of time. When three inositol phospholipids are attacked by the phosphodiesterase in stimulated cells, obviously the ratio of the products, diacylglycerol/inositol 1,4,5-trisphosphate, is largely increased. Because the content of PtdIns4,5P_2 in plasma membrane is so small compared to the other inositol phospholipids, this different amount of production of the second messengers may contribute to the uneven activation of protein kinase C and Ca^{2+} release from an internal store (see below). It would be interesting to know how the cells rearrange such uneven activations into the appropriate intracellular responses.

Ca^{2+}-DEPENDENT INOSITOL PHOSPHOLIPID PHOSPHODIESTERASES

It has now been clearly demonstrated that a Ca^{2+}-dependent phosphodiesterase that hydrolyzes inositol phospholipids plays a central role in receptor-mediated PtdIns turnover.

This phosphodiesterase was first found in sheep pancreas (39) and has since been demonstrated in various tissues, such as rat liver (75), guinea-pig intestinal mucosa (83), rat brain (84), pig lymphocytes (85, 86), and blowfly salivary gland (87, 88). Dawson and colleagues (42, 89) have identified the cleavage products of PtdIns phosphodiesterase as diacylglycerol, D-inositol 1-phosphate, and D-inositol 1,2-cyclic phosphate. The latter can be further hydrolyzed to D-inositol 1-phosphate by a specific phosphatase. As yet no specific physiological function for these water-soluble products has been demonstrated. PtdIns4,5P_2 phosphodiesterase could be involved in the breakdown of PtdIns4,5P_2, since it has been shown to be activated by Ca^{2+} and Mg^{2+} in brain tissue (40, 41) and human erythrocytes (90, 91). However, it has not been clearly resolved whether PtdIns phosphodiesterase and PtdIns4P and PtdIns4,5P_2 phosphodiesterases exist as different enzymes. To date, in vitro studies indicate that the phosphodiesterase(s) seems to attack all three inositol phospholipids; there is not one specific substrate. Rittenhouse (92) has reported that partially purified phospholipase C from human platelets is maximally active in the presence of 0.1 mM Ca^{2+} and displays substrate affinities in the order PtdIns > PtdIns4P > PtdIns4,5P_2 and maximum rates in the order PtdIns4P > PtdIns4,5P_2 > PtdIns. Irvine et al (93) have reported that an enzymological study of the phosphodiesterase using PtdIns4,5P_2 as a substrate has shown very similar profiles of PtdIns phosphodiesterase activity in terms of the Ca^{2+} requirement and the pH response curve. At present, it is not known how the phosphodiesterase is controlled so that a specific substrate, for instance only PtdIns4,5P_2 among all the inositol phospholipids, is hydrolyzed when the cells are stimulated.

Normally the enzyme extracted from various tissues is virtually inactive under physiological Ca^{2+} concentrations (10^{-7}–10^{-6} M) and at neutral pH. When the cytosolic supernatant is assayed at a concentration of 1 μM free Ca^{2+}, a low level of activity with an optimum of pH 6.0 is detected; complete activation is achieved by increasing Ca^{2+} concentration to 1 mM (94, 95). Furthermore, if choline-containing phospholipids are present in a lipid bilayer (96, 97) or monolayer (94), the PtdIns phosphodiesterase cannot attack its substrate even at high Ca^{2+} concentrations (10^{-3} M). These observations seem to suggest that the enzymatic hydrolysis of inositol phospholipids requires activation factors that remove or overcome the various inhibitory effects on enzyme activity and alter its requirement for Ca^{2+} concentrations. Researchers have made one suggestion based on the observation that PtdIns breakdown under physiological Ca^{2+} concentrations (10^{-7}–10^{-6} M at pH 7.25) is stimulated by trypsin (98). Hirasawa (35) has proposed that the modification of the enzyme molecule, for example, limited proteolysis, may be essential for the long-term cell responses. A similar characteristic of Ca^{2+} and phospholipid-dependent protein kinase (protein kinase C) is shown after cleavage of the molecule by the proteases. The kinase becomes fully active without Ca^{2+}, phospholipids, and diacylglycerol. A recent report from Tapley & Murray (99) has shown that incubation of intact human platelets with exogenous phospholipase C from *Clostridium perfringens* induces such a proteolytic cleavage of protein kinase C. Interestingly, the possible involvement of proteolytic enzymes in the receptor-mediated triggering mechanism has often been proposed, since protease inhibitors frequently block receptor-linked physiological cell responses such as IgE-mediated histamine release from rat mast cells (100), acetylcholine-stimulated catecholamine release from bovine adrenal medullary cells (101), and antibody secretion from B lymphocytes (102). At present, however, the protease involvement has not been unequivocally clarified in terms of the receptor mechanisms.

On the other hand, Irvine et al (93) have proposed that the initial activation of the phosphodiesterase that acts against PtdIns4,5P_2 can be achieved by a change of the substrate microenvironment, such as the presence of phosphatidylethanolamine near the inositol phospholipid.

HETEROGENEITY AND THE Ca^{2+}-SENSITIVITY OF PHOSPHODIESTERASE

Although some inconsistencies appear in the enzymological characterization of the phosphodiesterase(s), at least two distinct pH optima have been obtained for PtdIns phosphodiesterase from lymphocytes (86) and rat brain (95, 103) if the enzyme is assayed at 0.4 mM and 1 mM Ca^{2+} respectively. However, only one peak is observed when the Ca^{2+} concentration is buffered to 1 μM. These

observations can be resolved on the basis of the enzyme molecules' Ca^{2+} sensitivity at different pH. Kemp et al (75) have also reported some evidence for two pH optima in the enzyme from liver, a major one at 5.7 and a minor one at 6.9. Dawson et al (104) have recently extended these observations to a sheep pancreas supernatant fraction, where strong PtdIns phosphodiesterase activity is seen up to pH 8.5. Similar results have been obtained in rat liver and kidney (105). The activity at an alkaline pH is relatively unstable if the enzyme is submitted to ammonium sulphate fractionation and dialysis, as has been the case in the enzyme preparations of rat brain (84), guinea-pig intestinal mucosa (83), ox brain (61), rabbit smooth muscle (106), and rat liver (107). However, the activity can be maintained by the addition of an SH-residue protector such as dithiothreitol into the enzyme preparation.

In addition, the apparent heterogeneity of the phosphodiesterase has been examined by a standard isoelectrofocusing technique and by a new chromatofocusing technique, both of which have been proven to be valuable aids in probing the heterogeneity of the enzyme. Highly reproducible patterns of enzyme elution have been obtained from the brain (108), liver, and kidney (105). Hirasawa et al (108) have suggested that the brain cytosolic supernatant has at least four different forms of the phosphodiesterase according to their isoelectric points. Takenawa & Nagai (107) presumably have isolated one of these fractions, which is activated under 2 mM Ca^{2+} at neutral pH, from rat liver supernatant. Similarly, two distinct PtdIns phosphodiesterases have been purified from sheep seminal vesicular glands by Hofmann & Majerus (109). Nevertheless, the exact nature of this heterogeneity is not yet unequivocally understood.

It has been reported that the Ca^{2+}-dependent soluble PtdIns phosphodiesterase obtained from pig lymphocytes is fully active in the presence of 1 μM Ca^{2+} at pH 7.0, and such a characteristic is similar to those of certain peaks obtained by chromatofocusing of the trypsinized or the long-term preincubated cytosolic supernatant from rat brain (98). The enzyme forms in lymphocytes, separated by using the chromatofocusing technique, are two different Ca^{2+}-sensitive forms that appear at isoelectric points almost identical to those of the Ca^{2+}-sensitive forms produced in trypsin-treated and preincubated brain supernatants (K. Hirasawa, unpublished data). It is possible that there are active and inactive forms of the phosphodiesterases in physiological Ca^{2+} concentrations and that these can be obtained without artificial treatments such as trypsinization. These observations suggest that one early step in the signal-induced hydrolysis of inositol phospholipids may be an activation of the phosphodiesterase(s). The possibility that there are receptor-sensitive enzymes responsible for activating the phosphodiesterase needs serious consideration in the light of these results.

It is also worth noting that the receptor-mediated breakdown of inositol phospholipids appears to be a complicated process, since recent evidence

suggests that GTP-binding protein(s) may be involved in this stimulus-response coupling (110, 111).

INOSITOL 1,4,5-TRISPHOSPHATE AND Ca^{2+} MOBILIZATION

The stimulation of the α_1-adrenergic receptor increases the intracellular concentration of Ca^{2+} to between 10^{-7} and 10^{-6} M. This increase can be the result of both external Ca^{2+} influx and release from internal stores. The release of Ca^{2+} from internal stores appears to be important for very early cell responses in the liver (27), pancreas (112), cockroach salivary gland (113), and blowfly salivary gland (114). On the other hand, the external Ca^{2+} influx appears to be responsible for long-term cell responses. The most recent suggestion, based on results with permeabilized leaky pancreatic acinar cells (22) and saponin-treated hepatocytes (23, 115, 116), is that the addition of inositol 1,4,5-trisphosphate into media similar to cytosol induces the release of Ca^{2+}, which is previously taken up by nonmitochondrial stores in an ATP-dependent manner. Streb et al (22) have suggested that this Ca^{2+}, released from an internal store by inositol 1,4,5-trisphosphate, is the same as the Ca^{2+} released by acetylcholine. This finding has given biochemical significance to PtdIns4,5P_2 hydrolysis by the phosphodiesterase.

Berridge (117) has proposed that the role of very rapid signal-dependent PtdIns4,5P_2 hydrolysis by the phosphodiesterase may be in the release of intracellular Ca^{2+}, which acts as a mediator for subsequent cellular responses. This reaction would not be limited by the intracellular level of ATP, whereas enhanced PtdIns turnover has an energy-requiring step that can be identified as the sequential phosphorylation of PtdIns into PtdIns4,5P_2. Thus, the synthesis of PtdIns4,5P_2 must be inhibited by depression of the internal ATP level. Presumably, the release of Ca^{2+} from the internal store is a part of the initial trigger system. Although the depletion of Ca^{2+} from the bathing media causes the termination of long-term cell responses, the initial trigger response or short-time cell responses could be completed.

The other water-soluble products from the hydrolysis of inositol phospholipids, inositol 1-phosphate, inositol 1,2-cyclic phosphate, and inositol 1,4-bisphosphate, do not appear to show any effect on the elevation of Ca^{2+} release (22). PtdIns4,5P_2, however, is able to chelate Ca^{2+} even more strongly than EDTA. The phospholipid can hold more than one Ca^{2+} in the hydrophilic head group of the inositol ring, the 1, 4, and 5 positions of which are occupied by phosphates. It seems likely that the PtdIns 4, 5P_2 molecule could exist in vivo as a Ptd4,5P_2-Ca^{2+} complex. ^{31}P NMR studies have shown that Ca^{2+} interacts simultaneously with the 4 and 5 positions of phosphate in the Ptd4,5P_2 molecule (118). If both PtdIns4,5P_2-Ca^{2+} and PtdIns4,5P_2 molecules are hydro-

lyzed by a specific phosphodiesterase, the negative charge of the inositol 1,4,5-trisphosphate would be increased to some degree, since the 1 position of the inositol ring would become free. It is possible that inositol 1,4,5-trisphosphate can attract Ca^{2+} from the internal Ca^{2+} store. The less Ca^{2+} bound to the inositol 1,4,5-trisphosphate molecule, the stronger the attraction of Ca^{2+}. The preparation of the inositol 1,4,5-trisphosphate employed for the experiments is therefore very important in identifying which form of inositol 1,4,5-trisphosphate, Ca^{2+}-bound or free form, actually works on the release of Ca^{2+}.

The role of the inositol 1,4,5-trisphosphate in this effect has been discussed as that of an internal stimulus acting on the intracellular organelle. Prentki et al (119) have reported that this highly negatively charged compound causes a rapid release of Ca^{2+} from isolated microsomal fractions of rat insulinoma. At present, it is not clear whether the effect is a direct stimulation of the organelle surface or whether there are several steps involved in the release of Ca^{2+} from its internal store.

DIACYLGLYCEROL AND THE ACTIVATION OF PROTEIN KINASE C

The rapid appearance of diacylglycerol-containing arachidonic acid has been observed in plasma membranes as a result of the concomitant phospholipase C–type cleavage of inositol phospholipids in response to external signals (13, 120–23). It is important to note that normally diacylglycerol is almost absent from membranes; it only appears transiently due both to its rapid phosphorylation to phosphatidic acid by diacylglycerol kinase and/or to its further degradation to monoacylglycerol and arachidonic acid by diacylglycerol lipase for the synthesis of prostaglandin and thromboxane. The diacylglycerol produced in this way initiates the activation of a unique protein kinase C, so that the information from extracellular signals can be directly translated across the membrane to protein phosphorylation (124). This finding has given new biochemical significance to the enhanced turnover of inositol phospholipids.

In the case of human platelets, the two endogenous proteins with approximate molecular weights of 40,000 daltons (40K) and 20,000 daltons (20K) are rapidly and heavily phosphorylated by natural stimuli such as thrombin (121, 123, 125), collagen (123), or platelet-activating factor (122). Protein kinase C is responsible for phosphorylation of the 40K protein (123). Recently, Imaoka et al (126) have confirmed this reaction using a highly purified 40K protein, which they found to have a 47K component with some microheterogeneity shown with isoelectrofocusing. The function of the 40K protein remains unknown. On the other hand, the 20K protein, a myosin light chain, is phosphorylated by a calmodulin-dependent protein kinase, which has an absolute requirement for Ca^{2+} mobilization.

The ability of various synthetic diacylglycerols to be intercalated into the

membrane and to directly activate protein kinase C without inositol phospholipid breakdown has been tested in intact cell systems. 1-Oleoyl-2-acetylglycerol is most effective in activating the enzyme in platelets (15, 127, 128). In intact platelets, the enzyme activity may be monitored by measuring the phosphorylation of the 40K protein. The synthetic diacylglycerol is then rapidly converted in situ to 1-oleoyl-2-acetyl-3-phosphorylglycerol by the action of diacylglycerol kinase.

Enzymological studies have shown that the activity of protein kinase C is induced by the addition of membrane fractions containing phospholipids, especially phosphatidylserine, and Ca^{2+}. A small amount of diacylglycerol dramatically increases the apparent affinity of protein kinase C for Ca^{2+} (129). Apparently, one of the fatty acid chains of the diacylglycerol must be unsaturated for activation of the enzyme both in vivo and in vitro (130, 131). In mammalian tissues, inositol phospholipids are usually a rich source of arachidonic acid at the 2 position (132, 133). Ohki et al (134) have suggested that the effect of diacylglycerol on protein kinase activation may be the enhancement of Ca^{2+}-induced phase separation in the phosphatidylserine-containing membranes. Acidic phospholipids are indispensable for full protein kinase C activity, with phosphatidylserine the most effective. This phospholipid possesses a strong affinity for Ca^{2+}. Unimolecular films of the lipid are able to complex with Ca^{2+} through coordination-chelation binding (135). It is well known that the stimulatory and inhibitory effects of solvents and surface active lipids on many lipolytic enzymes may often be dependent on the physicochemical status of the lipid substrate (136). A common feature of phospholipases is that their activities are markedly affected by the ζ potential, which is influenced by the electrostatic charge of membrane components or by the binding of metal ions to the membrane substrate.

Protein kinase C and Ca^{2+}-dependent inositol phospholipid phosphodiesterase have very similar characteristics in terms of the sensitivity of their activities to other phospholipids (96, 137); choline-containing phospholipids are inhibitory and acidic phospholipids are stimulatory. It is probable that the Ca^{2+} dependency of both enzymes is associated with these effects. Therefore, it is attractive to suggest that these two receptor-mediated enzymes are both partly controlled by modification of the microenvironment of the inner leaflet of cell membranes. Several other aspects of protein kinase C system have been reviewed elsewhere (25, 138).

PROTEIN KINASE C AS A MOBILE RECEPTOR FOR A TUMOR PROMOTER

Recent studies from this laboratory have provided evidence about the transmembrane signaling of phorbol esters. Protein kinase C is a prime target for the action of phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA),

which is known as a potent tumor promoter (139). TPA, which possesses a diacylglycerol-like structure in its molecule, is able to substitute for diacylglycerol at extremely low concentrations and directly activates the enzyme both in vivo and in vitro (139, 140). As observed in the diacylglycerol requirement for protein kinase C activation, TPA also dramatically increases the affinity of this enzyme for Ca^{2+} to the 10^{-7} M range (140), resulting in its full activation without detectable cellular mobilization of Ca^{2+} (140, 141).

Kinetic analysis (142) indicates that the binding of ^{3}H-labeled phorbol-12, 13-dibutyrate to purified protein kinase C also has an absolute requirement for Ca^{2+} and phospholipid. The phorbol ester does not appear to bind directly to the enzyme; all four components combine together to form a strong quaternary complex, contributing to the activation of the protein kinase. This idea has been supported by recent reports that Ca^{2+} and phospholipids are needed for the binding of the tumor promoter to a specific direct binding site of the membrane. Delclos et al (143) have reported that the phorbol ester interacts primarily with phospholipid but not with protein in membranes. The tumor promoter presumably activates the enzyme by modifying its phospholipid microenvironment.

It is possible that, in intact cells where phospholipid and Ca^{2+} are sufficiently available, each molecule of the tumor-promoting phorbol ester intercalated into the plasma membrane phospholipid bilayer intracts with one molecule of protein kinase C. The movement of the enzyme toward the complex of Ca^{2+}, phospholipid, and tumor promoter in the plasma membrane is just like a mobile receptor (144). The protein kinase is usually recovered in the cytosolic soluble fraction before the tumor promoters are added, but after its addition protein kinase C is tightly associated with the membrane fraction. Supporting evidence for this hypothesis comes from Niedel et al (145) and Leach et al (146), who have partially co-purified a TPA binding protein with protein kinase C from brain tissues.

In physiological processes, it is possible that one molecule of diacylglycerol produced from inositol phospholipid hydrolysis in the stimulated plasma membrane can activate one molecule of protein kinase C. If so, it may be that, even though PtdIns4,5P_2 is a very minor membrane component, its breakdown provides sufficient diacylglycerol to activate all the protein kinase C within the cell.

SYNERGISM OF Ca^{2+} AND DIACYLGLYCEROL

Stimulation of the receptors relating to the turnover of inositol phospholipids often causes an increase of Ca^{2+} mobilization as well as a transient accumulation of diacylglycerol. The activation of protein kinase C by diacylglycerol appears to be a prerequisite but not a sufficient requirement for physiological responses of target cells, because the cellular responses to synthetic diacyl-

glycerol or to tumor promoter per se are always incomplete. Under appropriate conditions it is possible to demonstrate that in an intact cell system the independent induction of both protein kinase C activation and Ca^{2+} mobilization can be obtained by the exogenous addition of synthetic diacylglycerol or TPA and a Ca^{2+} ionophore respectively (15, 127, 128, 140). Attempts have thus been made to obtain full physiological cellular responses by replacing the natural stimuli with synthetic diacylglycerols or TPA and Ca^{2+} ionophores. When the platelets are incubated with thrombin, collagen, and platelet-activating factor, especially two endogenous proteins with approximate molecular weights of 40K and 20K are rapidly and heavily phosphorylated concomitantly, in association with the release of various constituents of platelet granules such as serotonin (121–123). If only the synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol, is added to the media, the 40K protein is phosphorylated, indicating activation of protein kinase C. On the other hand, if a Ca^{2+} ionophore is added separately the 20K protein (myosin light chain) is phosphorylated, indicating that calmodulin-dependent phosphorylation of this protein absolutely requires the mobilization of Ca^{2+}. In neither case does serotonin secretion from the platelets occur (127, 128, 140). However, when these cells are incubated with diacylglycerol or TPA in the presence of a low concentration of A23187, the release reactions are dramatically enhanced. The Ca^{2+} ionophore alone at the same concentration has little effect. Again, it is plausible that protein phosphorylation by protein kinase C and mobilization of Ca^{2+}, both of which are evoked by a single extracellular messenger, are equally indispensable and synergistically effective for causing full physiological responses (Figure 3). Tumor-promoting phorbol esters at low concentration, by inducing the activation of protein kinase C alone, may leave the cell ready to function once Ca^{2+} becomes available.

The involvement of the two synergistic pathways for release reactions may explain, at least in part, the signal-selectivity that is often observed during release reactions. For instance, in platelets, serotonin and adenine nucleotides are released from dense bodies in response to a variety of signals such as thrombin, collagen, ADP, epinephrine, and platelet-activating factor (PAF), while the release of lysosomal enzymes are observed only at higher concentrations of thrombin and collagen. By using permeabilized platelets, Knight et al (147) have elegantly shown that such signal selectivity of secretion is not related to Ca^{2+} concentrations because there is no difference in their sensitivity to Ca^{2+}. It is possible that the two signal pathways may exert differential control over release reactions from different granules within a single activated platelet.

This synergism of protein kinase C activation and Ca^{2+} mobilization has been recently extended to many other systems, such as the release of lysosomal enzymes from neutrophils (148), catecholamine release from bovine adrenal

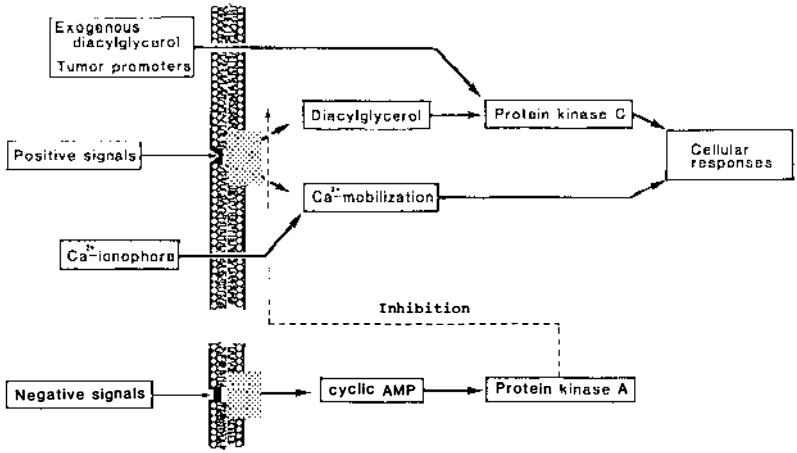

Figure 3 Synergistic role of protein kinase C activation and Ca^{2+}-mobilization for eliciting full cellular responses. Protein kinase A: cyclic AMP-dependent protein kinase.

medullary cells (149), aldosterone secretion from porcine adrenal glomerulosa cells (150), amylase secretion from pancreatic acinar cells (151), acetylcholine release from ileal nerve endings (152), histamine release from mast cells (153), insulin secretion from rat pancreatic islets (154), glycogenolysis in rat hepatocytes (155, 156), and concanavalin A–induced activation of bovine lymphocytes (157, 158). However, for the proliferation of macrophage-depleted peripheral lymphocytes, a very low concentration of phytohaemagglutinin is required in addition to both internal Ca^{2+} elevation and protein kinase C activation (158). This suggests that an additional receptor may also be involved in producing the cell-proliferation responses. These observations have confirmed that protein kinase C and Ca^{2+} mobilization act synergistically to elicit the physiological responses.

CONCLUSION

Enhanced PtdIns turnover is an early event in the cell receptor mechanism and is initiated by the hydrolysis of inositol phospholipids by the phospholipase C-type phosphodiesterase. This has been demonstrated by the large accumulation of a water-soluble product, inositol 1-phosphate, when lithium ion is used as an inhibitor of inositol 1-phosphate phosphatase. Recently, it has been suggested that two phosphorylated derivatives of PtdIns, PtdIns4P and PtdIns4,5P_2, rather than PtdIns are responsible for the receptor-mediated inositol phospholipid breakdown; PtdIns may disappear due to sequential phosphorylation by PtdIns kinase. However, this hypothesis has not yet been fully supported by the experimental evidence. The development of an inhibitor

of inositol 1,4,5-trisphosphate phosphatase is urgently required to substantiate it.

Since an elevation of internal Ca^{2+} is observed together with enhanced PtdIns turnover, interest in Ca^{2+}-mobilization has recently focused on the role of inositol 1,4,5-trisphosphate as an internal second messenger for the release of Ca^{2+} from nonmitochondrial internal stores, such as the endoplasmic reticulum. If this hypothesis is correct, then one cycle of inositol phospholipid turnover may generate two intracellular mediators, diacylglycerol and inositol 1,4,5-trisphosphate, for subsequent signal transduction at the expense of four molecules of ATP. The evidence presented thus far appears to be plausible, but this attractive hypothesis is yet to be substantiated. On the other hand, the control of external Ca^{2+} influx essential for the long-term potentiation of physiological responses is still not understood.

The role of diacylglycerol in the receptor mechanism has been well established as that of a second messenger for the activation of novel protein kinase C, which can also act as the mobile receptor for tumor-promoting phorbol esters. Moreover, a synergism of Ca^{2+} and diacylglycerol has been demonstrated in various tissues as causing full physiological responses such as secretion, exocytosis, and glycogenolysis, as well as cell proliferation. At present, it is not known how the Ca^{2+}-dependent inositol phospholipid phosphodiesterase is activated once the receptor is stimulated. The mechanism may involve modification of the enzyme molecule itself or it may involve a change in the membrane physicochemical properties surrounding the substrate. In addition, some still-unknown event involving a GTP-requiring process probably occurs before inositol phospholipid hydrolysis.

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