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Calcium-mobilizing Receptors, Polyphosphoinositides, and the Generation of Second Messengers*

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I. Introduction

CELLULAR functions are regulated by neurotransmitters, hormones, and a wide variety of regulatory and growth-promoting factors. The agonists produce a host of physiological responses in their target tissues as a result of their interactions with specific cell surface receptors. In addition to receptors that regulate cyclic AMP formation, there is another major type of cell surface receptors which mediate their effects via an increase in cytosolic Ca^{2+}. These are referred to as Ca^{2+}-mobilizing receptors, and there is compelling experimental evidence which indicates that these receptors affect intracellular Ca^{2+} levels via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2)† into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DG). Phosphatidylinositol 4-phosphate (PIP) and PIP_2, collectively referred to as polyphosphoinositides (PPI), are minor plasma membrane phospholipids (in eucaryotic cells they constitute about 0.8 to 1.5% of total phospholipids) and have metabolic turnover which is more rapid than any other membrane phospholipid. In the last decade, there has been increasing interest in the PPI as a result of two major findings: (*a*) that their hydrolysis by phospholipase C (also referred to as phosphoinositide phosphodiesterase) is coupled to the activation of Ca^{2+}-mobilizing receptors such as muscarinic cholinergic, α_1-adrenergic, vasopressin, etc., in a wide variety of tissues; and (*b*) that the products of the phospholipase C hydrolysis, namely DG and IP_3, may serve as intracellular second messengers. Further degradation of DG by specific lipases also leads to the release of arachidonic acid (AA), the precursor of prostaglandins (PGs), thromboxanes, and leukotrienes. There is now growing experimental evidence which suggests that IP_3 and DG synergistically mediate signal transduction in receptor systems linked to Ca^{2+} mobilization. IP_3 has been shown to be involved in the release of Ca^{2+} from intracellular stores, such as the endoplasmic reticulum (ER; sarcoplasmic reticulum in muscle, SR), and DG activates the phospholipid-sensitive protein kinase C (C-kinase), which phosphorylates specific proteins. In many cell types the IP_3-Ca^{2+}-calmodulin and DG systems interact synergistically to produce physiological responses such as metabolism, secretion, muscle contraction, and cell growth.

In the present review, attention will be focused on the biochemical and pharmacological characteristics of the agonist-stimulated breakdown of PPI (PPI response), properties of the enzymes involved in phosphoinositide metabolism, some general properties of Ca^{2+}-mobilizing receptors which are coupled to PPI turnover, and finally on the pharmacological and physiological significance of the phosphoinositide-derived second messenger molecules, namely IP_3, DG, and AA in cellular functions. Several reviews have recently appeared on various aspects of this topic (214, 59, 408, 3, 218, 396, 260, 239, 278, 35, 197, 203, 629, 62, 444, 341, 188, 261, 284, 125, 413, 57, 362, 198, 176, 306, 477a, 270, 529a), and papers presented at two international meetings are also an excellent source of detailed studies of various systems during the last decade (622, 78).

II. Historical Aspects

Since the initial findings of Mabel and Lowell Hokin on the effects of neurotransmitters and hormones on

† Abbreviations used are: DG, 1,2-diacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate (formerly diphosphoinositide, DPI); PIP_2, phosphatidylinositol 4,5-bisphosphate (formerly triphosphoinositide, TPI); PPI, polyphosphoinositides; IP, myo-inositol 1-phosphate; IP_2, myo-inositol bisphosphate; IP_3, myo-inositol 1,4,5-trisphosphate; AA, arachidonic acid; PG, prostaglandin; NE, norepinephrine; ACh, acetylcholine; CCh, carbachol; C-kinase, calcium/phospholipid-dependent protein kinase. For a recent review on nomenclature and stereochemistry of inositol phospholipids, see ref. 458a.

phospholipid turnover more than three decades ago in which they reported that phospholipids, later identified as phosphatidylinositol (PI) and phosphatidic acid (PA), may play an important role in receptor-mediated cell responses (280, 281; for a review of the early literature, see ref. 277), studies on neurotransmitter and hormonal control of phosphoinositide metabolism went through three stages of development: (*a*) the studies on the agonist-stimulated turnover of PA and PI; (*b*) the agonist-stimulated breakdown of PPI; and (*c*) the current excitement about the physiological roles of PPI-derived second-messenger molecules. To clarify some of the confusion in this area, especially for the newcomers to the phosphoinositide field, I have divided the historical perspective into those studies which have centered mainly on the PI response and those which have dealt mainly with the PPI response. Only the latter will be emphasized in this review.

A. Agonist-stimulated Turnover of PI

Briefly, the Hokins first reported that acetylcholine (ACh) plus eserine increased the incorporation of $^{32}P_i$ into PA and PI of brain slices and that the stimulatory effect of the neurotransmitter was blocked by the cholinergic muscarinic antagonist, atropine, but not by *d*-tubocurarine, a cholinergic nicotinic antagonist. This phenomenon has been referred to by a variety of terms such as the "phospholipid effect" (279); "ACh lipid effect" (518); "neurotransmitter effect" (16); and "PI effect" (624, 106), and it was demonstrated in a wide variety of tissues including pancreas (280), brain and its subcellular fractions, mainly the synaptosome (518, 644, 1, 517), sympathetic ganglia (106), pineal gland (189), parotid (454), and the smooth muscles of the vas deferens (111) and iris (2). While these studies indicated that PI metabolism is coupled to the activation of muscarinic cholinergic and α_1-adrenergic receptors, the molecular mechanism and physiological significance of this phenomenon remained unclear. In 1974, it became clear to many investigators that agonists act on PI metabolism by stimulating the breakdown, and not synthesis, of PI. To put it in another way, the enhanced $^{32}P_i$ incorporation into PA and PI is a secondary synthetic reaction, which occurs as a result of the agonist-stimulated PI breakdown and increase in DG formation at the plasma membrane. This concept gained support from the studies of Hokin-Neaverson and coworkers (283), working with pancreas, and Michell and coworkers (319), working with rat-parotid fragments, who demonstrated a decrease in mass of PI and an equivalent increase in mass of PA by ACh and other agonists. In the study on the parotids there was a loss of 26% in mass of PI and a 34% loss ^{32}P radioactivity from this phosphoinositide in ^{32}P-labeled tissue by ACh (319). In his 1975 review (408) Michell listed a number of tissues which showed a PI effect in response to various stimuli, and since then more tissues have been added (for review, see refs. 214, 59, 3, and 278) including the following: exocrine pancreas (168, 580, 115); pancreatic islets (124, 527, 65); adipocytes (228, 226a, 416); blood vessels (603, 578, 658); atria (99, 531); trachealis muscle (45), ileal smooth muscle (314); vas deferens (187); heart (481); parotid acinar cells (156, 414, 591); blowfly salivary gland (199, 200, 378); anterior pituitary cells (112, 570, 487, 489); thyroid (232, 594); platelets (54, 91, 388, 287); neutrophils (126, 508, 648); hepatocytes (345, 554, 587, 475, 584); adrenal cells (215, 40); lymphocytes (296, 256); mast cells (133); leukocytes (273); WRK-1 mammary tumor cells (418); and retina (30, 31, 524, 523). As can be seen from these studies, up to the past 3 yr work on the phosphoinositides was aimed mostly at the PI effect, and several functions were proposed for the enhanced PI turnover including possible involvement in: Ca^{2+}-gating (408, 57, 198, 477a, 409), later modified to include Ca^{2+}-mobilization from both extracellular and intracellular stores (413, 410) (for another view, see refs. 125 and 259); membrane fusion (261); AA release (54); and activation of C-kinase (443).

In the past few years the roles of PI in Ca^{2+}-gating and in receptor function have been reevaluated by several investigators, and there is more or less general agreement at the present time that at the plasma membrane it is the PPI, and not PI, which is directly coupled to the activation of Ca^{2+}-mobilizing receptors (for another point of view, see refs. 278, 169, and 16a).

B. Agonist-stimulated Breakdown of PPI

Until the mid 1970s little attention had been paid to the role of PPI in receptor function (408, 218, 396, 260, 278). The experimental evidence reported by a number of investigators on the effects of external stimuli on PPI in various tissues was confusing and in many instances contradictory (5). Thus, Hokin and coworkers (515), working with salt-gland slices, reported an inhibition of ^{32}P incorporation into PPI by ACh. Durell et al. (184), working with crude mitochondrial fractions of guinea pig brain, noted a possible increased production of IP_2, but not in that of IP_3, by ACh. Schacht and Agranoff (517, 519) observed a decreased labeling of PPI with ^{32}P in guinea pig brain cortex subfractions incubated with ACh, but this effect was insensitive to atropine, and it was equally elicited by choline. In his review in 1975 (408), Michell concluded that PIP and PIP_2 may have a function in nervous conduction and in the nicotinic cholinergic receptor.

Efforts in the author's laboratory and in collaboration with John Hawthorne, at the University of Nottingham, England, in 1975–1976 to demonstrate a decrease in radioactivity in PI in rabbit iris smooth muscle prelabeled with $^{32}P_i$ or myo-[^{3}H]inositol by ACh were unsuccessful; instead we repeatedly obtained an increase in the ^{32}P labeling of PA and PI (4, 5). However, when the stimulated iris was extracted with acidified chloroform-methanol, which extracts the PPI, instead of extracting with neutral chloroform-methanol, we observed a signif-

icant loss (up to 30%) of ^{32}P radioactivity from PIP_2 and a corresponding increase in the labeling of PA and PI by ACh (4, 5). Since this PIP_2 response was first characterized in the iris smooth muscle (for review, see refs. 6, 15, 25, and 9), it is appropriate to review here the historical background which led to these findings. (*a*) Time-course studies revealed that the ACh-stimulated breakdown of PIP_2 occurs at short time intervals (4, 5, 25), as compared to the PI effect reported in other tissues. (*b*) Pharmacological studies revealed that enhanced PIP_2 breakdown is coupled to the activation of muscarinic cholinergic (5, 25) and α_1-adrenergic (8, 25) receptors. (*c*) The PPI response was demonstrated in vivo, in response to electrical stimulation of the sympathetic nerve of the eye, thus indicating that this phenomenon can occur under physiological conditions (8). (*d*) Dose-response studies on agonist-stimulated PIP_2 breakdown and agonist-induced muscle contraction in sympathetically denervated (7, 26) and in normal irides (249c, 26, 9) revealed that both the biochemical and physiological responses are governed by the same type of receptor. (*e*) Studies on the mechanism of the PPI response revealed a partial requirement (i.e., potentiation of the PPI response) for Ca^{2+} in this phenomenon (23), and furthermore it showed that phospholipase C is both membranous and cytosolic in this tissue and that it is sensitive to Ca^{2+} (22). (*f*) Further studies on the mechanism of this phenomenon revealed a close correlation between the loss of radioactivity from PIP_2 in iris muscle prelabeled with myo-[^3H] inositol and the release of IP_3, thus demonstrating for the first time that the mechanism underlying the PPI response is a phosphodiesteratic cleavage of PIP_2 into IP_3 and DG, as depicted in fig. 1 (24).

Working with nervous tissue, Soukup et al. (559) demonstrated a PPI response in rat brain in vivo; Griffin and Hawthorne (249a) reported on a Ca^{2+}-activated hydrolysis of PIP and PIP_2 in guinea pig synaptosomes; Gispen and coworkers (317, 318), employing corticotropin (1–24)-tetracosapeptide (ACTH), showed a close relationship between polyphosphoinositide metabolism and protein phosphorylation; and Fisher and Agranoff (213) reported that in brain synaptosomes the turnover of PPI, in addition to that of PA and PI, is linked to the activation of muscarinic receptors, and this was enhanced by the ionophore A23187. More recently Warenycia and Vohra (616) reported that ionophore A23187-induced muscle contraction in vas deferens is blocked by phentolamine, and Liang et al. (372) demonstrated that the Ca^{2+} ionophores X537A and A23187 produced dose-dependent release of catecholamines from chopped rat hypothalamus and brainstem. In the iris muscle the ionophore A23187-stimulated accumulation of inositol phosphates was inhibited by prazosin (25), an α_1-adrenergic blocker, thus suggesting that the ionophore stimulation of PIP_2 breakdown reported on previously (23, 24) was secondary to the release of NE by the ionophore.

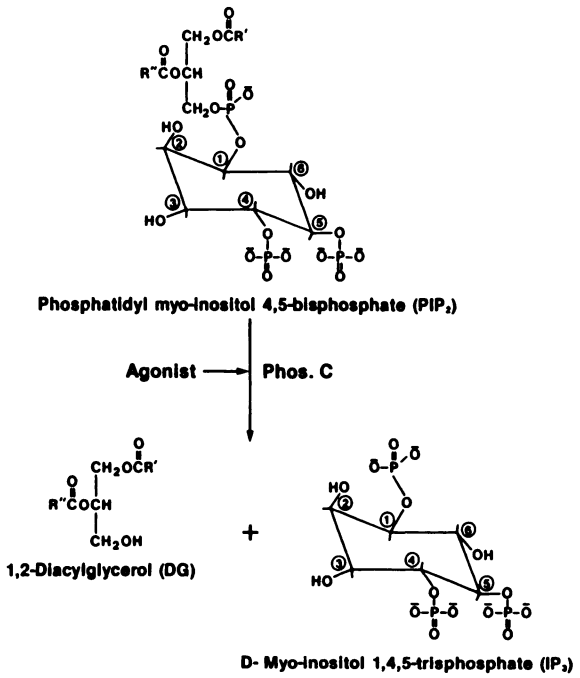


FIG. 1. Scheme showing the mechanism of the polyphosphoinositide (PPI) response as first reported in smooth muscle. For details, see text.

The mechanism of the effects of Ca^{2+} ionophores on phosphoinositide metabolism in various excitable tissues, such as brain, is still unclear.

In late 1981, in a short communication, Kirk et al. (344) reported a PPI response in hepatocytes, a nonexcitable tissue, and in addition these workers measured the vasopressin-stimulated breakdown of PIP_2 at shorter time intervals (30 s). As with the PI effect, a Ca^{2+} requirement for the PPI response in hepatocytes (344, 492) and other tissues has become controversial. It must be emphasized that, in all tissues in which this phenomenon has been investigated, including the early studies on the iris muscle (23), added Ca^{2+} is not essential for demonstration of the PPI response; it simply potentiates it, and only removal of Ca^{2+} with a chelator such as EGTA inhibits this biochemical response. The precise mechanisms underlying EGTA actions on cell metabolism are unclear, and there is general agreement now that, while the PPI response needs some Ca^{2+}, it is not regulated by intracellular Ca^{2+}. The mechanism of Ca^{2+} potentiation of the agonist-stimulated breakdown of PPI is still unresolved. As to early suggestions on possible functions for the PPI response, initially we proposed that, since this phenomenon does occur at the plasma membrane in the iris muscle, it could precede depolarization of the plasma membrane and consequently may be involved in muscle contraction (23, 6, 15); later Michell proposed that it could be involved in Ca^{2+} fluxes (410).

In the past 3 yr the PPI response was confirmed in all the tissues in which the PI effect was previously investigated (see above), and in addition there is now accumulating experimental evidence which indicates that the

primary products of this biochemical response, namely IP_3 and DG, could act as potential second-messenger molecules (59, 444). In this review emphasis will be placed on these new developments.

III. Distribution and Metabolism of Polyphosphoinositides

A. Distribution of Polyphosphoinositides

PPI have been demonstrated in all membranes of animal tissues that have been investigated, and with the exception of brain, they are usually found in trace amounts. In contrast to PI, which can be extracted with neutral chloroform-methanol, PPI are extracted with acidified chloroform-methanol. The acid is employed to separate the highly charged PPI from membrane proteins. Because of their rapid hydrolysis postmortem by phospholipases, estimates of PPI concentrations in various tissues should be taken as minimum figures. Table 1 shows the phosphoinositide levels of rat brain and rabbit iris smooth muscle. Phosphoinositides constitute about 3 and 4.9% of the total phospholipids of brain and iris muscle, respectively, and PPI constitute about 1.3 and 0.8% of the total phospholipids of these tissues, respectively. At the present time, there is a paucity of reports on the phosphoinositide composition of various tissues. This will have to await the development of more sensitive methods for their quantitative determinations.

Although it is generally thought that PPI are localized in the cytoplasmic leaflet of the plasma membrane (59, 408, 3, 278, 176), there is a need for more experimental evidence to support this conclusion. These conclusions were mostly based on the findings that PPI are enriched in the myelin sheath and in erythrocyte membranes and on the fact that PI kinase is a plasma membrane enzyme (see below). Accurate analyses of PPI in plasma membranes are difficult because of rapid hydrolysis during homogenization and subcellular fractionation.

B. Polyphosphoinositide Cycle: Pathways for Synthesis and Degradation and Generation of Second-Messenger Molecules

A summary of the major pathways for biosynthesis and degradation of phosphoinositides and phosphoinositide-derived second-messenger molecules is given in fig. 2. Both PA and myo-inositol are synthesized from D-glucose. Briefly, CDP-diacylglycerol (CDP-DG or CMP-PA), formed from PA and CTP in the presence of CTP-PA cytidyltransferase (step 1), is the key intermediate in phosphoinositide biosynthesis. Biosynthesis of PI from CDP-DG and myo-inositol, via PI synthetase (step 2) occurs at the ER, from which it is transported via a specific protein, called "PI-exchange protein," to the plasma membrane. A characteristic of inositol-containing phospholipids from animal tissues is that they exhibit predominantly 1-stearoyl-2-arachidonyl fatty acid composition on the *sn*-glycerol backbone (384, 128). This is in marked contrast with the composition of PA, which generally possesses low contents of stearate and AA. PI accumulates AA by acyltransferase reactions rather than in its initial synthesis from CDP-DG (384). Thus, Holub and Kuskis (288) have shown that PI acquires its characteristic fatty acid composition by a deacylation-acylation cycle (360). Phosphorylation of PI by ATP in the presence of specific kinases to PIP (step 3) and to PIP_2 (step 5) occurs at the plasma membrane. Phosphorylation of PI by ATP results in formation of the PPI in which the phosphodiester originates from PA and the monoester directly from ATP. Thus PI, PIP, and PIP_2 are interconvertible in the plasma membrane through the action of two kinases and two phosphatases. Phosphodiesteratic breakdown of phosphoinositides into DG and water-soluble inositol phosphates is achieved by phospholipase C (step 7), which is found in the soluble and particulate fractions and is activated by Ca^{2+}. DG is either metabolized via lipases (step 11), to release AA for eicosanoid biosynthesis, or is phosphorylated by ATP in the presence of the plasma membrane enzyme DG kinase (step 12) to regenerate PA, the precursor of CDP-DG and consequently the PIP_2 cycle. The other product of phospholipase C hydrolysis, namely IP_3, is degraded via specific myo-inositol phosphatases (steps 8 to 10) into free myo-inositol and inorganic phosphate.

C. Key Enzymes of the Polyphosphoinositide Cycle: General Properties and Subcellular Distribution

All of the pathways involved in the PIP_2 cycle (fig. 2) have been investigated, and several reviews dealing with

TABLE 1

Recoveries of phosphoinositides from rat forebrain and rabbit iris smooth muscle†*

Tissue	Pretreatment	Total lipid P (μmol/g tissue)	Phosphoinositides (nmol/g tissue)		
			PI	PIP	PIP_2
Brain	Freeze blown	61.5	1110 (1.8)‡	315 (0.51)	763 (1.2)
	Microwave irradiated	62.2	1210 (1.9)	264 (0.42)	619 (1.0)
	Frozen in liquid N_2	61.3	1050 (1.7)	198 (0.32)	469 (0.77)
	Unfixed sample	61.9	1050 (1.7)	224 (0.36)	330 (0.53)
Iris	Frozen in liquid N_2	9.8	406 (4.1)	42.3 (0.43)	36.7 (0.37)

* Data taken from ref. 439.

† Data taken from S. Naderi and A. A. Abdel-Latif (unpublished work).

‡ Numbers in parentheses, percentage of total lipid P.

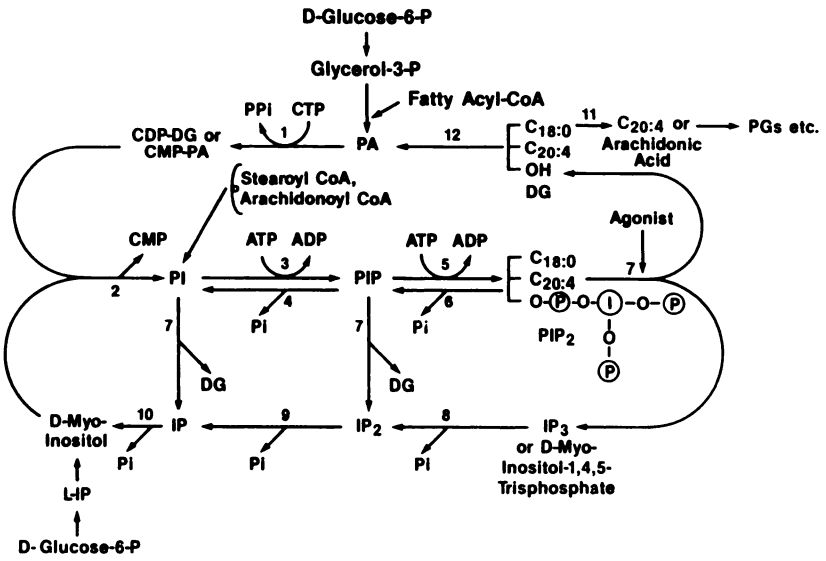


FIG. 2. PIP_2 cycle: pathways for phosphoinositide synthesis and degradation and generation of phosphoinositide-derived messenger molecules. For details, see text.

the enzymes involved with these reactions have appeared in the past few years (214, 3, 260, 278, 176, 306, 78, 540).

1. Phosphoinositide kinases. PI synthesis occurs at the ER (56, 458, 79, 575, 248, 628, 474, 237), and it is transported from there to other nonsynthetic membranes by a PI specific transfer protein (556, 231). The microsomal fraction also contains plasma membrane fragments, and thus one cannot eliminate the possibility that PI could be synthesized at the plasma membrane. Once at the plasma membrane, PI can then be sequentially phosphorylated by specific kinases to generate PIP and PIP_2. Although the PI and PIP kinases have often been considered to be plasma membrane enzymes (59, 408, 176, 264), there is little agreement on this point in the literature. Thus, PI kinase has been found to be enriched not only in plasma membranes, but at least in liver it is largely concentrated in lysosomes and Golgi (138, 145, 315, 135), and in nuclear envelope (550). This enzyme has also been demonstrated in rat brain myelin (162), coated vesicles (109), erythrocyte membranes (282, 228, 480), rat hepatoma cells where it was found to be associated with the insulin receptor (511a), and the protein products of two oncogenes, v-*src* (567) and v-*ros* (384b). Plasma membrane (135, 282, 228, 109, 171, 383, 486, 265, 151), cytosol (171, 323, 596a), and Golgi (145) have all been reported as sites for PIP kinase; however, recently it was found in liver to be localized predominantly at the plasma membrane (135, 383). Recent studies on immunochemical characterization of PIP kinase from rat brain cytosol revealed that the catalytic activity of this enzyme resides in a M_r 45,000 protein (596a). Despite the multiple sites within the cell where PI and PIP kinases have been reported, Seyfred and Wells (534) furnished evidence that, in hepatocytes, the plasma membrane is an exceptionally active site of PPI synthesis. Thus, these investigators observed that the rate of ^{32}P incorporation per mg of protein was 5 to 10 and 25 to 50 times faster into PIP and PIP_2, respectively, in the plasma membrane than for any other subcellular fraction. The activities of PI and PIP kinases are stimulated by Mg^{2+} and inhibited by Ca^{2+}. A recent report has suggested that PIP_2 may decrease PIP kinase activity by product inhibition (598). Thus, by using a PIP kinase preparation from the high speed supernatant of bovine retina, these authors found that exogenously added PIP_2 has an inhibitory effect on PIP kinase.

2. Phospholipase C (PPI phosphodiesterase). Since phospholipase C is involved in the initiating event of the PPI response (fig. 1), several studies have been carried out in recent years on its Ca^{2+} requirement, cellular localization, physical properties, and mechanism of control in both procaryotes and eucaryotes (for review, see refs. 408, 3, 176, 306, 270, and 540). For many years the phospholipase C activities against phosphoinositides were dealt with as soluble enzymes, exhibiting two separate Ca^{2+}-dependent activities which degraded PI and the PPI, respectively. Existing evidence suggests that these enzymes are both cytosolic (306, 308, 34, 11, 401, 382, 22, 311, 631, 227, 495, 577, 190, 536, 546, 434, 41a), as well as membranous (340, 310, 22, 11, 174, 150, 129, 363, 300, 599, 420, 175, 465a), and furthermore that the purified enzyme is capable of hydrolyzing all three phosphoinositides (382, 631, 495, 434, 41a). There is no experimental evidence which indicates that PI, PIP, and PIP_2 are hydrolyzed by different phospholipase C activities. Changes in assay conditions of this enzyme have in general led to conflicting conclusions about its properties and subcellular localization. A convenient and sensitive in vitro assay for phospholipase C was recently reported in which IP_3 released from exogenous PIP_2 was hydrolyzed by alkaline phosphatase (456). The enzyme appears to exist in multiple forms: four forms in bovine

heart (382); two forms in sheep seminal vesicle (631); and two forms in brain and liver (434). Majerus and coworkers (631) have recently isolated two immunologically distinct forms of the enzyme from sheep seminal vesicular gland, both of which show similar sensitivities to Ca^{2+} and hydrolyze perferentially polyphosphoinositides at lower concentrations of Ca^{2+} (<4 μM).

Phospholipase C activity is extremely sensitive to Ca^{2+}, and thus several workers have investigated the cationic requirement of this enzyme. In human erythrocyte membranes the concentration of Ca^{2+} required for half-maximal hydrolysis of PPI ranged between 1 and 3 μM (420, 175). At low ionic strength in the absence of Mg^{2+}, half-maximal activation of the phospholipase was at about 3 μM Ca^{2+} (175). The presence of 1 mM Mg^{2+} shifted the Ca^{2+} activation curve to the right, as did elevation of the ionic strength. When the Ca^{2+}-pump ATPase and the phospholipase were assayed in the same incubations and under conditions of intracellular ionic strength and Mg^{2+} concentration, the ATPase was fully activated, whereas no phospholipase C activity was detected below 100 μM Ca^{2+}. These authors concluded, therefore, that it seems unlikely that the phospholipase C of the erythrocyte membrane ever expresses its activity in a healthy erythrocyte (175). This finding has been used by many workers in support of the idea that the increase in $[Ca^{2+}]_i$ by agonists is not the cause of PIP_2 breakdown. Nishizuka and coworkers (434) have purified two forms (types I and II) of phospholipase C from rat brain and liver cytosol. At pH 7.4 and PI as substrate, type I required lower concentrations of Ca^{2+} (1 μM) than type II (10^{-3} M Ca^{2+}) to hydrolyze the phosphoinositide; however, at pH 5.5 both types required 10^{-3} M Ca^{2+}. Both forms of the enzyme hydrolyzed preferentially the PPI. The phospholipase C of the rat brain soluble fraction is Ca^{2+} dependent, and in Ca^{2+}/EDTA buffers with the pure lipid as substrate, it shows maximal activity at 0.1 μM Ca^{2+} (311). However, if the enzyme is assayed against the same substrate in Ca^{2+}/EGTA buffers with 3 mM Mg^{2+} and 80 mM KCl present, it shows no detectable activity below 100 μM Ca^{2+}, and it is maximal at 1 mM Ca^{2+}. Partially purified phospholipase C from human platelets was reported to have maximal activity in the presence of 10^{-4} M Ca^{2+}, and it displays substrate affinities in the order PI > PIP > PIP_2 and maximum rates in the order PIP > PIP_2 > PI (495). Current studies in our laboratory indicate that addition of 1 μM Ca^{2+} to ^{32}P-labeled membrane fractions derived from bovine iris sphincter smooth muscle, incubated in Ca^{2+}-EGTA Krebs-Ringer bicarbonate buffer, pH 7.4, leads to the specific breakdown of the endogenous PPI and release of inositol polyphosphates (294b). It can be concluded from the above that in all the studies which have been reported, phospholipase C activity was found to require Ca^{2+}, although in many of these investigations levels of Ca^{2+} well above the intracellular cytoplasmic range were employed. Lipid substrate orientation, Mg^{2+}, ionic strength, proteolysis, and pH strongly influence phospholipase C activation by Ca^{2+} in in vitro studies. Utilization of isolated membranes, prelabeled with $^{32}P_i$ or myo-[^{3}H]inositol, in studies on the properties of phospholipase C will shed more light on the mechanism of Ca^{2+} activation of PIP_2 hydrolysis by this enzyme, as well as on the role of this cation in potentiating the PPI response.

3. Myo-inositol polyphosphates phosphomonoesterases. Several studies have been carried out recently on the characterization of the phosphatases which degrade inositol polyphosphates, since if the latter are mediators of physiological functions, then their metabolism could be a signal terminating step. It must also be remembered that these enzymes are involved in regenerating free myo-inositol for phosphoinositide biosynthesis (fig. 2). There are phosphomonoesterases which degrade IP_3 to IP_2, IP_2 to IP, and IP to free myo-inositol and inorganic phosphate in iris muscle microsomal fractions (24), erythrocyte membranes (178, 502), liver plasma membranes (562, 321, 533), homogenates of insect salivary gland (60), and in the soluble fraction of human platelets (140a). IP_3 phosphatase has been shown to specifically remove the 5-phosphate from IP_3 (140a) and from cyclic IP_3 (142) to produce IP_2 and cyclic IP_2, respectively. The PPI phosphatase has also been reported to dephosphorylate IP_4 to inositol 1,3,4-trisphosphate (311a). These enzyme activities are both cytosolic and membranous, they are dependent on Mg^{2+}, and in contrast to IP phosphatase (253), IP_3 phosphatase is much less inhibited by lithium ion. Very recently, Connolly and Majerus (141) reported that IP_3 phosphatase isolated from human platelets is phosphorylated by rat brain C-kinase, resulting in a 4-fold increase in its activity. A similarity was observed between the C-kinase-phosphorylated IP_3 phosphatase observed in vitro and the thrombin-stimulated phosphorylated M_r 40,000 protein known to be phosphorylated by C-kinase in vivo, thus suggesting that these proteins may be the same. These results suggest that platelet Ca^{2+} mobilization may be regulated by C-kinase phosphorylation of IP_3 phosphatase and can explain the observation that phorbol ester treatment of intact human platelets results in decreased production of IP_3 and decreased Ca^{2+} mobilization upon subsequent thrombin addition (see also section V C 1 d).

It can be concluded from the above that the enzymes involved in PPI synthesis, namely the phosphoinositide kinases, and those involved in their degradation, namely phospholipase C and the phosphomonoesterases, are both membranous and cytosolic. This is also true of the subcellular distribution of the inositol polyphosphate phosphomonoesterases. The plasma membrane is capable of both synthesizing and degrading the PPI, and the metabolism of the latter appears to be under stringent and complex control. Phospholipase C, the enzyme in-

volved in the receptor-mediated stimulation of the phosphodiesteratic cleavage of PIP_2, is stimulated by μM concentrations of Ca^{2+}, and this activation is dependent on the ionic and phospholipid environment. Little information is available now on the regulation of the enzymes involved in the PPI cycle. The development of novel pharmacological agents to modulate the activities of key enzymes, such as phospholipase C and PIP kinase, and consequently the level of the PPI at the plasma membrane, will increase our understanding of the role of inositol-containing phospholipids in the mechanism and physiological control of receptor-mediated events in a wide variety of systems.

IV. Calcium-mobilizing Receptors and the Biochemical and Pharmacological Characterization of the Polyphosphoinositide Response

A. Regulation of Calcium Ion in the Cell

The importance of Ca^{2+} for the transmission of information inside living cells has been a subject of many recent reviews (239, 482, 80, 146, 477, 477b, 481b). Calcium is a regulator of many cell functions, and its importance is most appreciated in excitable and secretory cells, where such functions as contraction and secretion are controlled by this cation. In the unstimulated cell, the cytosolic Ca^{2+} concentration is approximately 0.1 μM, which is 10^5-fold lower than the mM extracellular Ca^{2+}, and following a stimulus it rises to 1 to 10 μM (fig. 3). The source of Ca^{2+} could be either extracellular or intracellular. There is accumulating evidence which indicates that the ER is the major source of bound Ca^{2+} released into the cytosol under stimulated conditions. The mobilized Ca^{2+} is then extruded. High concentrations of Ca^{2+} in the cell are toxic (482), and thus cytosolic Ca^{2+} levels are maintained by various Ca^{2+}-regulatory mechanisms in the cell including: (*a*) the Ca^{2+}-dependent plasma membrane ATPase pump; (*b*) the plasma membrane Na^+/Ca^{2+} exchange mechanism; (*c*) the mitochondrial Ca^{2+}-pump; and (*d*) the ATP-dependent Ca^{2+}-pump in the ER.

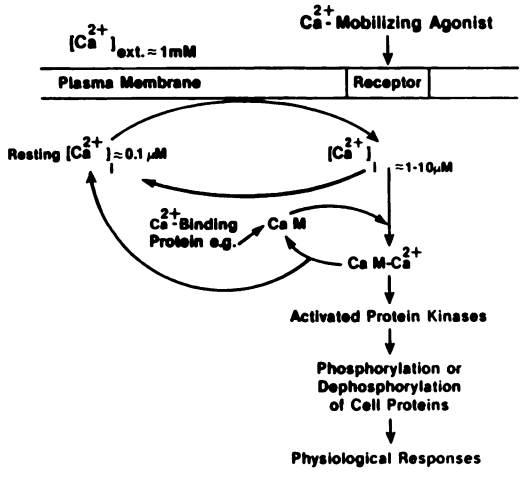


FIG. 3. Diagrammatic representation for Ca^{2+}-dependent regulation of physiological responses in animal cells. In a resting cell, $[Ca^{2+}]_i$ is 0.1 μM, and upon stimulation, it rises to 1 to 10 μM and binds to calmodulin (CaM). The CaM-Ca^{2+} complex then activates protein kinases, which in turn modulate the activities of several enzymes and physiological processes. During relaxation, the $[Ca^{2+}]_i$ returns to resting levels, and this results in the dissociation of the CaM-Ca^{2+} complex and subsequently in deactivation of enzyme activities and cellular processes.

Calcium-mobilizing receptors mediate their effects on cell function by increasing cytosolic Ca^{2+} concentration. Thus, among cell functions which are controlled by these receptors are: smooth muscle contraction by ACh and NE; pancreatic secretion by ACh; platelet aggregation by thrombin; glycogen breakdown in liver by vasopressin; prolactin secretion in pituitary cells by thyrotropin-releasing hormone (TRH); fluid secretion in blowfly salivary gland by serotonin; cell proliferation in fibroblasts by growth factors; and phototransduction by light. How a receptor-mediated change in cytosolic Ca^{2+} occurs has been a subject of great interest to many investigators for decades. There has been accumulating experimental evidence in the past 3 yr which suggests that this could be mediated through PPI breakdown products (see below).

B. Types of Receptors Coupled to the Rapid Breakdown of Polyphosphoinositides

The suggestion that it is the stimulated PPI breakdown, and not that of PI as was thought for many years (see section II above), which is involved in Ca^{2+} mobilization (for review, see refs. 59 and 413), coupled with the demonstration of a PPI response in hepatocytes (344, 492), a nonexcitable tissue in which vasoactive peptide hormones and α_1-adrenergic agonists are known to bring about an increase in the cytosolic free Ca^{2+} concentration through the mobilization of Ca^{2+} from intracellular stores (76), spurred many investigators to study the role of PPI in receptor function in various systems. Thus, in the past 3 yr the stimulated breakdown of PPI in response to Ca^{2+}-mobilizing agonists was reported in a wide variety of tissues (summarized in table 2), including: (*a*) those which are excitable, e.g., smooth muscle, brain, and sympathetic ganglion; (*b*) those which are nonexcitable, e.g., liver hepatocytes, platelets, and neutrophils; and (*c*) those which are either endocrine, e.g., adrenal medulla and pineal, or exocrine, e.g., pancreas, parotids, and blowfly salivary glands. Activation of Ca^{2+}-mobilizing receptors in these systems leads both to the breakdown of PPI and to an increase in $[Ca^{2+}]_i$. However, the recent findings that activation of certain receptors which do not function through Ca^{2+} mobilization can lead to PPI hydrolysis (table 2) are not in accord with this generalization. These receptors are linked to ion channels and, on occupancy by their specific ligands, allow flux of specific ions (cations or anions) through the plasma membrane and thereby trigger electrophysiological responses (73). Examples of this class of receptors are the nicotinic acetylcholine receptor, a cation channel, and

TABLE 2

Examples of receptors coupled to stimulated polyphosphoinositide turnover

Stimulus (receptor)	Target tissue (refs.)
Ca^{2+} mobilizing	
ACh (muscarinic, M1 or M2?)	Smooth muscle (5, 25, 23, 99, 45, 530, 63, 574); brain and other neural tissues (214, 213, 49, 238, 246, 29, 293, 597, 292a, 460, 460a); pancreas (506, 478, 285, 468, 206); parotids (35, 620, 36, 312, 313, 179); adrenal medulla (263, 215, 262, 221a)
NE (α_1-adrenergic)	Smooth muscle (8, 25, 26, 369, 531, 658, 603, 223, 110); brain (560, 316, 337, 460, 460a, 246); hepatocytes (197, 384); brown adipocytes (435); parotids (620)
Histamine (H_1)	Brain (94, 155); chromaffin cells (444a)
Serotonin (5-H_2)	Blowfly salivary gland (378, 510, 58); brain (140, 339); platelets (520)
Peptidergic	
Vasopressin (V_1)	Smooth muscle (429, 180); sympathetic ganglion (84); hepatocytes (344, 492, 384, 117, 582, 535, 379, 583)
Angiotensin II	Smooth muscle (429, 552, 249); hepatocytes (384, 117); kidney (250, 55); anterior pituitary cells (194a)
Bradykinin	Neuroblastoma-glioma hybrid NG 108-15 (649); neuroblastoma hybrid cell line NCB-20 (223a)
Substance P	Smooth muscle (289, 617, 655); parotid (620, 36)
f-Methionyl-leucyl-phenylalanine	Neutrophils (609, 608, 130, 164, 576, 650); leucocytes (172)
Thrombin	Platelets (491, 634, 656, 20, 602, 90, 69, 463, 302, 497, 403, 287)
Thyrotropin-releasing hormone	GH_3 pituitary tumor cells (387, 522, 181, 352, 41, 395, 488, 233, 481a)
Glucose	Pancreatic islets (123, 64, 39, 367, 419, 462)
Platelet-activating factor*	Platelets (543, 402, 386, 541); hepatocytes (210, 542)
Light	Photoreceptors (266, 236, 206a, 71a)
Others	
Glutamate (glutaminergic)	Striatal neurones (548)
ACh (nicotinic)	Cultured myotubes (17)
Electrical (nicotinic)	Skeletal muscle (600, 605)
ATP (P_2-purinergic)	Hepatocytes (116); Ehrlich ascites tumor cells (182a)

* PAF had no effect on PIP_2 breakdown in the iris muscle (653) or in isolated rat hepatocytes (117).

receptors of GABA and glutamate which are chloride channels.

There is mounting experimental evidence which favors a link between the PPI hydrolysis and the agonist-stimulated Ca^{2+} mobilization. A discussion of all the tissues in which the PPI response has been demonstrated is for the most part beyond the scope of this review; instead I shall concentrate my discussion on a few tissues in which this phenomenon has been investigated in more detail, such as hepatocytes as an example of a nonexcitable tissue, and smooth muscle as an example of an excitable tissue. The PPI response has been characterized in these tissues, and the dependency of physiological functions on a rise in $[Ca^{2+}]_i$ is well established for both tissues. Originally, it was reported that in hepatocytes vasopressin produced a 16% decrease in radioactive PIP_2 at 15 s, and that this hormonal effect was transient since by 2 min, no PPI breakdown was evident (492). However, more recently Charest et al. (117) reported that vasopressin (10^{-7} M) increased IP_3 accumulation in isolated rat hepatocytes by 100% at 3 s and 150% at 6 s, and that these increases were maintained for at least 10 min. In excitable tissues, such as the iris smooth muscle (4, 25) and the superior cervical sympathetic ganglia (84), the response to the agonist can be detected in 15 s, and it is sustained for at least 15 min in the iris and 1 h in the ganglia.

It can be concluded from the above that the experimental evidence is overwhelming now in support of the concept that activation of Ca^{2+}-mobilizing receptors in a wide variety of cells leads to PPI breakdown; however, it must also be mentioned that there are probably some receptors which may not function through Ca^{2+} mobilization, but still elicit this response (Table 2). Furthermore, we must keep in mind that, while the basic mechanism of the PPI response is probably the same, its characteristics and functions could vary from tissue to tissue, e.g., excitable and nonexcitable tissues.

C. Biochemical and Pharmacological Characterization of the Polyphosphoinositide Response

1. Biochemical. Methods of assay, the use and actions of Li^+ in this assay, and the time-course studies which resulted in the conclusion that Ca^{2+}-mobilizing receptors metabolize PPI instead of PI will be discussed here.

a. METHODS OF ASSAY. In the early studies on the PI effect most workers measured phospholipid changes by separating the individual lipids by means of thin-layer chromatography (TLC) and determining the amount of

^{32}P incorporated into each of the phospholipids. Since both synthesis and degradation of PI occur simultaneously in the cell, these studies proved to be of limited value in showing how receptor activation affects PI metabolism. In a few studies changes in PI concentrations in response to agonists were measured chemically, and a few investigators have performed "chase" experiments to follow loss of radioactivity from PI in ^{32}P-labeled tissues.

An agonist-stimulated PIP_2 breakdown (fig. 1; section II B) can be assayed either by measuring the disappearance of the PPI and appearance of DG, measured as PA, by radiochromatography [TLC and measurement by radiometric methods (317, 27)] or by monitoring the accumulation of IP_3, the water-soluble product, by ion-exchange chromatography and measurement by radiometric methods (58, 25, 312, 26, 384). The separation of inositol phosphates by ion-exchange chromatography was originally developed by Ellis et al. (191a) and then used by Berridge et al. (61) in its present form. This method is now the analytical basis of a large body of work from several laboratories. Myo-inositol phosphates can also be separated by high-voltage paper electrophoresis (20). PPI are found in minute amounts in cell membranes (table 1), and thus changes in their metabolism by pharmacological agents are usually estimated by radiochemical techniques employing either $^{32}P_i$ or myo-[^{3}H]inositol. Recently, Dangelmaier et al. (151a) determined the basal and thrombin-stimulated levels of IP_3 in ^{32}P-labeled human platelets, using enzyme treatment and electrophoresis to isolate the inositol polyphosphates. They found that the concentrations of IP_3 in resting and stimulated platelets to be 1 to 4 and 10 to 30 pmol/10^8 cells, respectively. In such studies, isotopic equilibrium between PI, PIP, and PIP_2 needs to be established. In the iris muscle we find 90 min to be required for isotopic equilibration when myo-[^{3}H]inositol is used as precursor. The following methodology for assaying the PPI response is routinely employed in our laboratory (26, 25) as well as other laboratories.

i. Incubation of tissue. Briefly, to label the phosphoinositides and the tissue inositol phosphates, the tissues are incubated singly (of the pair, one is used as experimental) for 75 min at 37°C in 1 ml of a modified Krebs-Ringer bicarbonate buffer, pH 7.4, that contains 6 to 8 μCi of myo-[^{3}H]inositol. At this time the tissues are transferred to 1 ml of fresh buffer containing the isotope and incubated for 15 min, after which agonist and/or other agents are added, and incubation is continued for specified intervals. Addition of 10 mM Li^+, which acts by inhibiting IP phosphatase (253), 10 min prior to adding the pharmacological agents, increased the production of IP when rat cerebral cortex slices, rat parotid fragments, or blowfly salivary glands were incubated with the appropriate agonists (61). The majority of experiments carried out on the release of inositol phosphates by various agonists are now done in the presence of Li^+. In general, antagonists are added 5 min prior to the addition of agonists. All incubations are gassed with O_2:CO_2 (97:3) periodically before and during the experiment. Incubations are terminated with 1 ml of 10% (w/v) ice-cold trichloroacetic acid (TCA) or by freezing the tissue on solid CO_2.

In experiments where ^{32}P is used as a precursor for phospholipids, the experimental procedure is as described above except that 25 to 30 μCi of $^{32}P_i$ per ml are added instead of myo-[^{3}H]inositol. The PPI are initially labeled in the monoester phosphate groups which rapidly (within 30 min) reach isotopic equilibrium with the labeled ATP pool.

ii. Extraction and analysis of myo-[^{3}H]inositol phosphates by anion-exchange chromatography. To extract the myo-[^{3}H]inositol phosphates, the tissues are homogenized in 5% TCA, and the homogenate is centrifuged at 3000 × *g* for 15 min. The pellet is analyzed for phospholipids (see below). The supernatant is extracted 5 times with 4 ml of anhydrous diethylether, neutralized with 0.1 M NaOH, and then applied to a Bio-Rad AG 1×8, formate form, column (0.7 × 8 cm). The resin is washed with water containing 5 mM myo-inositol until no free myo-[^{3}H]inositol is detected in the eluate. The column is then sequentially washed with 0.2 M, 0.5 M, and 1.0 M ammonium formate in 0.1 M formic acid to elute IP, IP_2, and IP_3, respectively. The total radioactivity in each fraction is determined by scintillation counting, and corrections for quenching of ^{3}H label are made by a quench curve based on the external standard ratio.

iii. Extraction and analysis of phospholipids. Briefly, the TCA-insoluble pellet obtained from the tissue homogenate is extracted once with chloroform:methanol:concentrated HCl (200:200:1, v/v) and once with chloroform:methanol:concentrated HCl (400:200:1.5, v/v). The extracts are combined and evaporated under N_2, and the residue is dissolved in 2 ml of chloroform. After washing the extract with methanolic 0.1 M HCl, the lipids are concentrated in a small volume (60 μl) of chloroform. Carrier PIP and PIP_2 are added to this extract, and the phospholipids are separated by one-dimensional TLC on silica gel high-performance thin-layer plates (e.g., see refs. 317 and 27). The phospholipids are visualized by exposure to I_2 vapor, and spots corresponding to PI, PIP, and PIP_2 are scraped into vials, and their radioactive contents are determined by liquid scintillation counting. The ^{32}P-labeled phospholipids are separated by two-dimensional TLC (5), which also separates the PA, and by one-dimensional TLC, which separates the PPI (317, 27).

In general, the results are reported in dpm of inositol phosphates released, or PIP_2 degraded and PA synthesized per a specified amount of tissue in the presence of a pharmacological agent. Although the increased labeling of PI occurs after the initial breakdown of PPI, the appearance of ^{32}P label in PA is kinetically indistinguish-

able from the phosphoinositide breakdown (214, 25, 512, 131). Assaying for the PPI response by measurement of the release of inositol phosphates, rather than by changes in the label associated with the various phospholipids, will circumvent the problems associated with either the labeling or prelabeling approach. As originally described (61) the ion-exchange method cannot separate the isomers of inositol polyphosphates; however, high-pressure liquid chromatography (HPLC) methods have been worked out that do separate three of the five inositol monophosphates (70, 545a) and two of the inositol trisphosphates (307, 312). Tetra-, penta-, and hexaphosphates have been separated as groups of isomers by HPLC (50). High-voltage paper electrophoresis has been employed to separate ^{32}P-labeled inositol polyphosphates from stimulated platelets (20); however, when the tissue is prelabeled with ^{32}P several nucleotides and sugar phosphates are also labeled, and this is bound to interfere with the separation of inositol polyphosphates as has recently been reported (47, 151a). Nonradiometric measurements of IP in brain (539) and IP_3 in platelets (497) by gas chromatography (GC) have been reported; however, gas chromatography/mass spectroscopy (GC/MS) has not yet been used to isolate the isomers of inositol polyphosphates. Thus, there is a need to develop more sensitive methods (both nonradiometric and radiometric) for the analyses of all of the inositol phosphates that are produced as a result of activation of Ca^{2+}-mobilizing receptors in a wide variety of tissues (Table 2). HPLC and mass spectrometry may be useful as a complement to the above methods (633).

b. ACTIONS OF LITHIUM. In some tissues such as the nervous system, until recently it has been difficult to characterize the PPI response and measure inositol phosphates release. In 1980, Sherman et al. (253, 539) demonstrated that Li^+ is a potent and selective inhibitor of IP phosphatase, the enzyme that hydrolyzes IP, formed during activation of Ca^{2+}-mobilizing receptors, back to free inositol (fig. 2). Berridge et al. (61) exploited the capacity of Li^+ to block the breakdown of IP and directly analyzed agonist-stimulated phospholipase C-specific inositol phospholipid hydrolysis in blowfly salivary gland and rat brain.

In general, inclusion of Li^+ in incubations of certain tissues such as brain (173, 49) and hepatocytes (117, 582) with a variety of receptor agonists leads to a large agonist-dependent accumulation of IP with smaller increases in the IP_2 and IP_3 fractions. Burgess et al. (105) showed that Li^+ increases the levels of the inactive isomer 1,3,4-IP_3 and not the active one IP_3, thus accounting for the fact that Li^+ can augment IP_3 levels but does not enhance the physiological response. In preliminary studies in our laboratory on the effects of 10 mM Li^+ on CCh-stimulated IP_3 release in the iris sphincter, we find a large agonist-dependent accumulation of IP with smaller increases in IP_2 and IP_3 fractions in the presence of the cation at short time intervals (30 s); however, we find that Li^+ alone leads to large increases in accumulation of all the inositol phosphates at longer time intervals (R. A. Akhtar and A. A. Abdel-Latif, unpublished observations). Recently, Batty and Nahorski (49), working with rat cerebral cortex, found that exposure of Li^+ in the presence of CCh produced a concentration- and time-dependent inhibition of IP_3 accumulation that was not related to receptor desensitization. These data suggest sites additional to IP that are affected by Li^+. Very recently, the effects of Li^+ and CCh on phosphoinositide metabolism were investigated in rat parotid glands (178a). Lithium alone had little effect upon $^{32}P_i$ incorporation, but in combination with CCh it greatly reduced the labeling of PI in response to the agonist, increased that of PA, and had no effect on PIP_2 labeling. These lithium effects were reversed if 10 to 30 mM inositol was included in the incubation medium. These findings suggest that an active inositol phosphatase pathway is essential to maintain intracellular inositol levels, but that PIP_2 synthesis is not markedly reduced by a fall in intracellular inositol.

Thus, while inclusion of Li^+ in the incubation mixture has formed a sensitive assay for receptor-stimulated inositol phospholipid breakdown, the precise effects of this cation on the metabolism of both phosphoinositides and their water-soluble products remain unclear. Furthermore, although administration of Li^+ into rats has been shown to increase the accumulation of IP in brain (539), there is no evidence to indicate that a Li^+-induced reduction in intracellular inositol supply in the brain does affect phosphoinositide metabolism in vivo. These observations are in conflict with the hypothesis that depletion of cellular inositol in the presence of Li^+ would be greatest in cells that are heavily stimulated and that the phosphoinositides, being dependent on inositol for their synthesis, could become substrate limited with consequential diminishment of receptor response (61).

c. TIME-COURSE OF INOSITOL PHOSPHATES RELEASE. In the original studies on the PPI response in smooth muscle, it was assumed that the agonist-stimulated breakdown of PIP_2 occurs at the plasma membrane and that it precedes membrane depolarization and is involved in muscle contraction (15). This was based in part on the finding that PPI and their enzymes are localized at the plasma membrane and that the PI response occurs at the ER (278, 285), and thus the PPI response is a separate phenomenon from the PI effect. However, in the above studies no attempt was made to identify which of the three inositol phospholipids (PI, PIP, and PIP_2) is the primary substrate for Ca^{2+}-mobilizing receptors. Time-course studies on agonist-stimulated breakdown of phosphoinositides in a wide variety of tissues (table 2) revealed that the initial action of Ca^{2+}-mobilizing agonists is to stimulate the hydrolysis of PIP_2 into DG and IP_3 (for reviews, see refs. 59, 218, 278, 35, 197, 203, 62,

and 413). Thus, when tissues are prelabeled with myo-[^{3}H]inositol, the label is incorporated into the three phosphoinositides. Upon stimulation with Ca^{2+}-mobilizing agonists for various time intervals (5 s to 15 min), there is an initial release of IP_3 within 5 to 15 s, but not IP, measured by anion-exchange chromatography, and a parallel loss of ^{3}H label from PIP_2, but not from PI, measured by TLC. In general, time-course experiments with myo-[^{3}H]inositol showed that agonists increase the accumulation of IP_3 by 10 to 20% within 15 s; in contrast, a significant increase in IP release is not observed until about 2 min. Thus, kinetic studies revealed that, at short time intervals [5 s in insect salivary glands (58)], there is an increased formation of IP_3 and IP_2, but increased formation of IP occurs at longer time intervals. Similar results have been obtained in several tissues (see table 2 for references) including: hepatocytes in response to vasopressin; parotid acinar cells in response to methacholine; platelets in response to thrombin; pituitary cells in response to TRH; smooth muscle in response to CCh; and pancreatic acinar cells in response to CCh. These observations suggested to many investigators that the primary lipid substrate used by these receptors is PIP_2 rather than PI. The rapid formation of IP_2 may result from action of a phosphomonoesterase on IP_3. There is general agreement now that, in all systems which have been investigated (table 2), PI breakdown is secondary to an initial receptor-linked breakdown of PIP_2 at the plasma membrane; however, there is a debate as to whether the stimulated loss of PI is exclusively via phosphorylation in the plasma membrane to regenerate PPI (278, 35, 179, 58, 149) or partially by direct action of phospholipase C on PI in the ER (278, 285).

2. *Pharmacological.* While a number of agonists have been reported to elicit the PPI response in a variety of tissues (Table 2), most of the information we have on their pharmacological properties relates to muscarinic cholinergic, α_1-adrenergic, and peptidergic systems. In general, the pharmacological studies centered on (*a*) defining the type and nature of the receptors through which agonists stimulate PPI breakdown, and (*b*) establishing, through dose-response studies, relationships between receptor occupancy, generation of IP_3, release of Ca^{2+}, and physiological responses such as enzyme activation, fluid and enzyme secretions, and muscle contraction.

a. RECEPTOR CHARACTERIZATION. Until recently, the most widely used method for identification and characterization of receptors in a variety of tissues is largely based on radioligand binding studies in membrane fragments (646). Characterization of receptors can also be accomplished by investigating the effects of agonists and antagonists on PPI breakdown, which can be monitored by measuring the release of inositol phosphates. This biochemical method is a powerful technique for identification and characterization of receptors, and it gives us more information about the intracellular events mediated by these receptors than the radioligand method.

For most neurotransmitters, different receptor types and subtypes can be identified pharmacologically. Since fairly selective antagonists and agonists are available for the classification of adrenergic and histaminergic receptor subtypes, it was shown that α_1- and H_1-receptors, respectively, mediate the enhancement of PPI turnover (see table 2 for references). In two other receptor systems, serotonin (5-HT) and ACh, contradictory results have been reported. The 5-HT_1-selective agonists 8-hydroxy-2(di-*n*-propylamino-tetralin) (339) appeared not to be effective in enhancing PPI turnover in cerebral cortex slices, whereas the 5-HT_2-selective antagonist ketanserin potently inhibited the biochemical response to serotonin (339, 140). However, no close correlation of the potency of various antagonists to inhibit this phosphoinositide response and high affinity [^{3}H]ketanserine binding could be found (339). At present, experimental evidence suggests that 5-HT_2 receptors could mediate the PPI response to serotonin. The PPI response to ACh is mediated through the muscarinic and not the nicotinic receptor. Thus, muscarinic antagonists such as atropine and quinuclidinylbenzilate (QNB), at low concentrations (10^{-6} to 10^{-8} M), block the ACh- or CCh-stimulated breakdown of PPI in a variety of tissues (see table 2 for references), while nicotinic antagonists such as *d*-tubocurarine are ineffective. From this we can conclude that the muscarinic, but not the nicotinic, cholinergic receptor is coupled to the enhanced PPI turnover. This conclusion seems to contradict the finding of a possible PPI response in skeletal muscle, a tissue enriched with nicotinic receptors, in response to electrical stimulation (refs. 600 and 605; table 2), and thus there is a need to determine whether nicotinic receptors in certain tissues mediate this biochemical response. The muscarinic receptor subtype involved in phosphoinositide hydrolysis has not yet been identified (238, 597). Thus, studies with pirenzepine and McN-A343 in chick heart and astrocytomal cells revealed that the putative M_1 muscarinic receptor regulates cyclic AMP, but does not regulate phosphoinositide hydrolysis (97). In another study on membrane fragments from rat brain, it was found that potencies of muscarinic (m)AChR-antagonists to inhibit the phosphoinositide response to CCh corresponded best with ligand affinities for the M_1AChR, whereas potencies of mAChR-agonists to evoke the biochemical response corresponded best with ligand affinities for the M_2AChR (597). These findings suggest that further study is required to elucidate unequivocal determination of which mAChR-subtype is preferentially coupled to the PPI cycle, and in which manner.

α_1- but not α_2-adrenergic receptors have been implicated in the stimulated PPI breakdown (Table 2). This conclusion is based on the finding that the α_1-adrenergic receptor-specific antagonist prazosin, at a concentration

of 10^{-7} M, completely blocks the response to NE, whereas similar concentrations of yohimbine, an α_2-adrenergic antagonist, and propranolol, a β-adrenergic receptor antagonist, did not have an effect on the neurotransmitter-stimulated IP_3 release (for references, see table 2). Pretreatment of hepatocytes with a specific vasopressin antagonist immediately decreased the vasopressin-induced IP_3 release towards their control values (582).

As more specific receptor antagonists become available, the same approach can be employed to characterize all of those receptors which are linked to the metabolism of PPI (table 2).

b. DOSE-RESPONSE RELATIONSHIPS. Until recently, there has been very little work done on dose-response relationships for agonist-induced PPI breakdown (7, 249c). However, introduction of a more direct assay for the PPI response, in which the agonist-induced IP_3 release is measured, has facilitated such studies, and in the past 3 yr a number of reports have appeared on this topic. Dose-response relationships have been reported for CCh- and NE-induced IP_3 release in smooth muscle (9, 26, 25, 530, 63, 223, 110), in astrocytes (460), and in parotid acinar cells (36), and for serotonin-induced IP_3 release in hepatocytes (384, 117, 582, 149). A particular objective from most of these studies has been to throw more light on the mechanism and physiological significance of the PPI response, rather than to assess the efficacy of pharmacological agents.

Concentration-response curves for CCh-induced IP_3 production and of contractility in smooth muscle revealed a close correlation between the two responses (9, 26, 25, 530, 63, 223, 110). The 50% effective concentration (ED_{50}) of CCh-stimulated IP_3 accumulation is similar to the IC_{50} for muscarinic-receptor occupation as assessed by [^{3}H]QNB binding. Thus, the EC_{50}s for CCh-induced IP_3 accumulation and for muscle contraction in the iris sphincter muscle were found to be 2.5×10^{-6} M and 6×10^{-7} M, respectively (9), and the 50% inhibitory concentration (IC_{50}) for muscarinic receptor occupation in this tissue was found to be 5.3×10^{-6} M (571). Similar data were reported for the guinea pig ileum smooth muscle (530). More recently, Fox et al. (223) examined the effect of α_1-adrenoceptor stimulation on inositol phosphates and on muscle contraction in rat vas deferens and caudal artery in the presence of 10 mM Li^+. They reported that NE increased inositol phosphate accumulation 7-fold in rings from vas deferens and 3-fold in rings from caudal artery. Epinephrine, phenylephrine, and methoxamine were as effective as NE, suggesting that these drugs are full agonists in causing this response. Prazosin, phentolamine, and yohimbine completely blocked the stimulation by NE in both tissues with potencies typical of blockade of α_1-adrenoceptors. Despite a substantial receptor reserve for α_1-adrenoceptor-mediated contractile responses, clonidine, *p*-amino-clonidine, phenylpropanolamine, and ephedrine can only cause a partial contractile response in rat vas deferens. However, all of these partial agonists were either as effective or more effective in increasing inositol phosphate accumulation in rat vas deferens as they were in activating a contractile response. They concluded that α_1-adrenoceptors increase phosphoinositide turnover in rat vas deferens and caudal artery, and that there may be a receptor reserve for α_1-adrenoceptor-mediated increases in inositol phosphate accumulation in these smooth muscles. The latter conclusion is interesting, since no receptor reserve for receptor-mediated increases in inositol phosphates accumulation has been reported yet in other tissues (see below). Thus, in parotid acinar cells the dose-response curve for methacholine activation of IP_3 formation more closely approximates the curve for receptor occupancy than for Ca^{2+}-activated K^+ release (36). Lynch et al. (384), working with liver hepatocytes, investigated the relationship between receptor binding capacity for NE, angiotensin II, and vasopressin and release of IP_3, Ca^{2+} mobilization, and glycogen phosphorylase activation. They found that all three agonists produced the same maximum changes in phosphorylase and cytosolic Ca^{2+}, but the maximum capacity of each agonist to generate IP_3 varied greatly and was correlated with the maximum receptor binding capacity. Similar observations were reported by other investigators working with hepatocytes (582, 149).

It can be concluded from the above observations that the dose-response curves for IP_3 accumulation are more closely associated with the curves for receptor occupancy, but are shifted to the right relative to the curves obtained for physiological responses. These conclusions are in accord with the hypothesis that the receptor-mediated breakdown of PPI at the plasma membrane is an early event in the coupling of receptor occupation to Ca^{2+} mobilization, and that the physiological response may occur when only a small percentage of receptors is occupied (for reviews, see refs. 59, 408, 35, 197, 62, 413, 57, and 343). The latter has been attributed to the presence of spare receptors. In pharmacological terms, there is a large "receptor reserve" for the physiological response (336), but the depletion of PIP_2 in the plasma membrane by the agonist is rather closely coupled to receptor occupation (i.e., it displays little or no "receptor reserve"). A very small and submaximal elevation of IP_3 is sufficient to maximally elevate Ca^{2+} and brings about a physiological response. While the time-course and dose-response studies in hepatocytes suggest a close relationship between activation of Ca^{2+}-mobilizing receptors, PPI breakdown and the mobilization of Ca^{2+}, and cellular responses (384), there is a need to extend these observations to other tissues. There is a lack of information on correlative studies showing close relationships between the biochemical, pharmacological, and physiological responses under the same experimental conditions.

V. Significance and Possible Biological Functions of the Polyphosphoinositide Response

A. Overview—A Scheme

The experimental evidence, derived from the studies of several investigators, indicates that the phosphodiesteratic breakdown of PPI is coupled, in an as yet unknown manner, to the activation of Ca^{2+}-mobilizing receptors and that an important function of this response is to raise $[Ca^{2+}]_i$, which is an essential link in the mechanism of stimulus-response coupling. Based on our observations in the iris smooth muscle (15, 9, 26, 25, 653) and those reported by several other investigators, I propose the following scheme (fig. 4) showing PIP_2 breakdown mediating the step between activation of Ca^{2+}-mobilizing receptors and cellular responses and emphasizing the role of both extracellular and intracellular Ca^{2+} in mediating the biochemical and physiological responses. Briefly, the binding of a Ca^{2+}-mobilizing agonist to its receptor at the plasma membrane initiates the phosphodiesteratic hydrolysis of PIP_2 to yield IP_3 and DG, the two putative intracellular second messengers, and subsequent resynthesis of the phosphoinositides and the increase of $[Ca^{2+}]_i$. IP_3, the water-soluble product of PIP_2 hydrolysis, diffuses from the plasma membrane to the ER, where it may bind to specific intracellular receptors which control Ca^{2+} release and thus mobilize Ca^{2+} from this subcellular organelle (62). The release of Ca^{2+} from the ER into the cytoplasm results in the initial rise in $[Ca^{2+}]_i$ which in smooth muscle couples the agonist-receptor interaction to the phasic (fast) component of the contraction response. Extrusion of Ca^{2+} from the ER by IP_3 could signal the influx of extracellular Ca^{2+}. The influx of extracellular Ca^{2+} is not required for the initial-phase elevation of $[Ca^{2+}]_i$; however, it is required for maintenance of the elevated $[Ca^{2+}]_i$, needed for a maximal and sustained response and to reload the agonist-sensitive stores (83, 588, 71, 48, 132, 133). The elevation of $[Ca^{2+}]_i$ may induce muscle contraction either by binding to calmodulin which can then activate myosin light chain kinase to phosphorylate the M_r 20,000 light chains of myosin, or by interacting synergistically with DG and phosphatidylserine to activate C-kinase, which phosphorylates the M_r 20,000 light chains of myosin. The ATPase activity of phosphorylated myosin can then be activated by actin. DG can also be metabolized by lipases to liberate AA for eicosanoid biosynthesis, or it can be phosphorylated at the plasma membrane, via DG kinase, to PA. The fate of PA has not yet been settled. There is no experimental evidence for the presence of an exchange protein to transport PA from the plasma membrane to ER for incorporation into PI. PA, produced from the DG liberated during PIP_2 breakdown, has been proposed to act as an endogenous Ca^{2+} ionophore for the transport of Ca^{2+} (477a, 512, 479, 532), but this has recently been disputed on the basis that PA failed to facilitate Ca^{2+} fluxes across liposomal membranes (286). Some have suggested that the time course of the appearance of PA is delayed after agonist stimulation so that it is unlikely for it to be responsible for the initial elevation in intracellular Ca^{2+} concentration (131, 208). A phospholipase A_2 action on PA has also been reported in platelets (361), but it was shown later that half of the AA was released on stimulation of platelets with thrombin before any increase in PA (437). An action of phospholipase A_2 on PI to liberate AA for PG synthesis has been reported in transformed mouse BALB/3T3 cells (290), platelets (68), pancreas (168), and iris smooth muscle (653, 652). The affinity of phospholipase A_2 for Ca^{2+} is much lower than that of phospholipase C, and thus the rise in $[Ca^{2+}]_i$ which results from the activation of Ca^{2+}-mobilizing

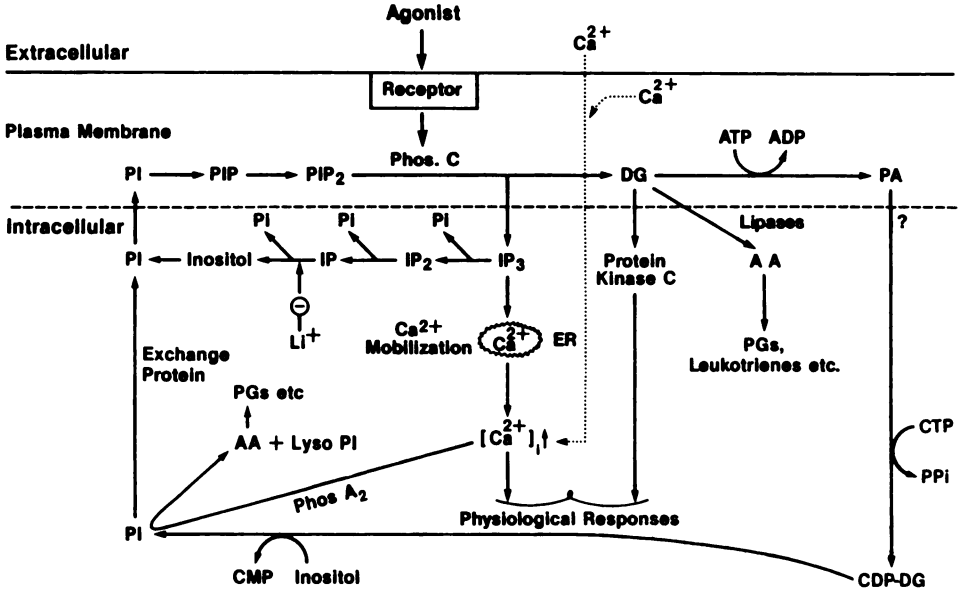


FIG. 4. Scheme showing PIP_2 breakdown mediating the step between activation of Ca^{2+}-mobilizing receptors in smooth muscle and other tissues and cellular responses. See text for details.

receptors can activate phospholipase A_2 to release AA from PI and other phospholipids.

B. Enhanced Polyphosphoinositide Turnover and plasma Membrane Functions

1. Does agonist-stimulated breakdown of polyphosphoinositides regulate a Ca^{2+} gate at the plasma membrane? Although there is little, if any, experimental evidence at the present time to support the hypothesis that enhanced PI and/or PPI turnover is (are) involved in Ca^{2+} gating at the plasma membrane, there is a need to discuss these concepts here for the following reasons. (*a*) The suggestion that accelerated phosphoinositide turnover could be involved in Ca^{2+} mobilization had a considerable impact on this field. (*b*) There is evidence now which indicates that there are probably some receptors which metabolize phosphoinositides, but normally do not function through Ca^{2+} mobilization (e.g., see table 2), and (*c*) the mechanism for the partial requirement (i.e., potentiation of the phosphoinositide response by the cation) in this phenomenon for Ca^{2+} in certain systems is still unclear.

In 1975, Michell (408) after surveying the literature made the interesting observation that PI metabolism is triggered only by those receptors that control a rise in $[Ca^{2+}]_i$ which then acts as the second messenger for stimulating the functional response of the cell. He suggested that PI breakdown precedes the entry of Ca^{2+} into cells and could therefore be a universal biochemical event intrinsic to the Ca^{2+}-gating mechanism (408, 412). In general, the following arguments were put forward by some investigators to support this hypothesis: (*a*) the PI response [later PIP_2 (410)] should be observed with Ca^{2+}-mobilizing receptors only; (*b*) the receptor-mediated breakdown of phosphoinositides should be independent of both the extracellular Ca^{2+} and of the increase in $[Ca^{2+}]_i$ due to receptor activation; and (*c*) the phosphoinositide response should be directly controlled by receptor occupancy.

The first argument has received much support; however, there are examples of receptors which elicit the PPI response, but do not function through Ca^{2+} mobilization (see table 2). These include: (*a*) glutamatergic and nicotinic stimulation of PPI hydrolysis in striatal neurones (548), cultured myotubes (17), and skeletal muscle (600, 605), respectively, and (*b*) the adrenal medulla where the secretory response is elicited via nicotinic cholinergic receptors and requires an influx of extracellular Ca^{2+} (525). However, the inositol lipid metabolism is enhanced by the activation of the muscarinic cholinergic receptor whose function is to inhibit the response of the nicotinic receptor (263, 215, 262). This is an example of a muscarinic receptor which stimulates inositol lipid metabolism, and instead of mobilizing Ca^{2+}, it inhibits the secretory response (262, 127). More recently, studies on the effects of ACh and specific muscarinic ligands on cytosolic free Ca^{2+} in isolated bovine adrenal chromaffin cells, using Quin-2, showed that a small component of the ACh-evoked rise in cytosolic Ca^{2+} is independent of extracellular Ca^{2+} and that this component is mediated by muscarinic receptors on the surface of bovine chromaffin cells (331, 415, 120). However, despite the existence of an intracellular Ca^{2+} store mobilized by muscarinic receptor activation in bovine chromaffin cells, the muscarinic stimulation did not effect secretion itself or secretion induced by either nicotine or high K^+ (120). Very recently, Stoehr et al. (561b) investigated the ability of IP_3 to induce Ca^{2+} release from intracellular sites within suspended bovine adrenal chromaffin cells permeabilized with digitonin. They found that IP_3 specifically triggered an immediate and dose-dependent release of Ca^{2+} from intracellular stores and suggested that, during brief exposure of chromaffin cells to ACh in the functioning bovine adrenal medulla, both the muscarinic receptor-induced release of IP_3 and the nicotinic receptor-induced Ca^{2+} influx could contribute to the rise in cytosolic Ca^{2+} and the triggering of exocytosis. (*c*) In some cells, such as heart, there is evidence that stimulation of muscarinic receptors causes an inhibition of adenylate cyclase that is not Ca^{2+} mediated by an increase in phosphoinositide breakdown, although distinct muscarinic subtypes have been postulated to exist on these cells (97, 98). In platelets, collagen can stimulate phosphoinositide hydrolysis without increasing the intracellular concentration of free Ca^{2+} (493). (*d*) In synaptosomes and other CNS tissues, there is no evidence to show that the PPI response is involved in Ca^{2+} mobilization.

The second argument for the Ca^{2+}-gating hypothesis was that the receptor-mediated breakdown of phosphoinositides should be independent of both the extracellular Ca^{2+} and of the increase in $[Ca^{2+}]_i$ due to receptor activation. Thus, it was argued that removal of Ca^{2+} from the external medium should abolish the functional but not the PI response, and introduction of Ca^{2+} with an ionophore should provoke the former but not the latter. This criterion has been controversial (125, 259, 410, 127), and Cockcroft (127) has recently listed the tissues which show and those which do not show a Ca^{2+} requirement for the stimulated inositol lipid breakdown. Examples of systems in which partial requirement for external Ca^{2+} has been reported include neutrophils (608, 132), pancreatic islets (367, 399, 39), pancreas (276, 205), hepatocytes (344, 492, 149, 117, 583), iris smooth muscle (23, 25), vas deferens (187), synaptosomes (249a, 213, 212, 249b), and cerebral cortical slices (338). Examples of tissues which do not show Ca^{2+} requirement include parotid (620), GH_3 pituitary tumor cells (488), adrenal glomerulosa (204), hepatocytes (379), insect salivary gland (199), blood platelets (67), pancreas (478), and vas deferens (187). These findings were interpreted by some (59) as indicating that agonist-stimulated PIP_2 breakdown can occur in many cell types at resting levels of Ca^{2+}. In support of this conclusion are the findings: (*a*) that when the activity of rat brain phospholipase C was

studied using ionic conditions that match the intracellular environment, there was little change in activity when Ca^{2+} was varied over its physiological range of 10^{-7} to 10^{-5} M (311); and (*b*) that the human erythrocyte phospholipase C requires minimal concentrations of Ca^{2+} for activity (175). With the exception of the studies on neutrophils (132) and pancreatic islets (39), where the stimulation of phosphoinositide metabolism does seem to depend upon an increase in $[Ca^{2+}]_i$, there is no conclusive experimental evidence to demonstrate that inositol lipid breakdown is a consequence of a receptor-mediated rise in the cytosol Ca^{2+} concentration. On the contrary, there is mounting experimental evidence which indicates that the agonist-stimulated breakdown of PPI is not dependent on a rise in the cytosol Ca^{2+} concentration (117, 233, 582). Very recently, Martin et al. (395a), working on the effects of TRH on PPI breakdown in permeabilized GH_3 cells, suggested that TRH activates phospholipase C by increasing its sensitivity to Ca^{2+}.

Conclusions on whether the phosphoinositide response does or does not require Ca^{2+} were mostly based on the following experimental approaches: (*a*) omission of Ca^{2+} from the medium. This method simply reduces, but does not block, the PPI effect (23, 25, 117). (*b*) The use of Ca^{2+} ionophores to introduce Ca^{2+} into the cell. (*c*) Depolarization of the plasma membrane with high K^+ concentrations. (*d*) Chelation of Ca^{2+} with EGTA. Each of these methods has its drawbacks. The effects of ionophore A23187 on PPI breakdown in the iris muscle (25) and probably in synaptosomes are secondary to the release of neurotransmitters by the ionophore. Thus, like the stimulatory effect of NE, the ionophore A23187-stimulated accumulation of IP_3 in the iris muscle was inhibited by prazosin, an α_1-adrenergic blocker (25). In accord with this conclusion were the findings that ionophore A23187-induced muscle contraction in vas deferens is blocked by phentolamine (616), and that the Ca^{2+} ionophores X537A and A23187 produced dose-dependent release of catecholamines from chopped rat hypothalamus and brain stem (372). Ionophore A23187 has also been shown to cause release of NE from adrenergic nerve terminals in smooth muscle (136) and from synaptosomes (87). The Ca^{2+} ionophore has also been reported to increase AA release in smooth muscle (652), platelets (68, 252), and macrophages (193). Potassium depolarization, which causes influx of extracellular Ca^{2+} in smooth muscle and other tissues, did not change the level of IP_3 in GH_3 pituitary tumor cells (488) and in iris smooth muscle (25); however, in other tissues such as sympathetic ganglion, it provoked the PPI response (84). The precise mechanism for the action of EGTA in Ca^{2+} metabolism is still unclear; it is possible that the treatment of the tissue with EGTA depletes intracellular Ca^{2+} to the extent that the activity of the Ca^{2+}-requiring phospholipase C is inhibited. This suggests that the Ca^{2+} dependency of the effects of Ca^{2+}-mobilizing agonists on phospholipase C could be explained if the enzyme is dependent on Ca^{2+} for its activity. There is disagreement, however, as to whether this enzyme is Ca^{2+} regulated or merely Ca^{2+} dependent (413, 381, 549). Recently, Kendall and Nahorski (338) concluded from studies on the Ca^{2+} requirement for agonist-dependent breakdown of PI and PIP_2 in rat cerebral cortex that different receptors mediating PI/PIP_2 breakdown in this tissue have quantitatively different Ca^{2+} requirements.

Taken together, we can conclude from the studies on the role of Ca^{2+} in this phenomenon that, although phospholipase C requires a certain minimal level of Ca^{2+} which could be necessary for the PPI response, there is little evidence to indicate that this response is regulated by intracellular Ca^{2+}. However, the mechanism for a partial requirement for some Ca^{2+}, needed in certain tissues to elicit a maximal PPI response, remains unclear.

The third argument for the Ca^{2+}-gating hypothesis was that the agonist concentration requirement for enhanced phosphoinositide breakdown should closely match that for receptor occupancy. As was discussed above (secion IV C 2 b), concentration-response curves for agonist-induced IP_3 production and of biological response and receptor occupancy, as assessed by ligand binding studies, revealed a close correlation between the three responses in some tissues, but this was not observed invariably (132, 621). Thus, in neutrophils inositol lipid breakdown does not follow the receptor occupancy curve as predicted but instead lies close to the functional response of the cell (132). This appears to be also true of the vas deferens (223). There is a need for more rigorous experiments showing in the same tissue and under the same experimental conditions dose-response curves for receptor occupation, PPI hydrolysis, and physiological responses.

The most direct support for the Ca^{2+}-gating hypothesis came from the work of Berridge and Fain (199, 200, 510) working with the blowfly salivary gland where stimulation with serotonin caused PI loss and entry of Ca^{2+} into the epithelial cells. Supramaximal stimulation with serotonin caused a loss of the small pool of responsive PI and a fall in Ca^{2+} transport (desensitization). Incubation of washed glands with myo-inositol restored both PI sensitivity to serotonin and Ca^{2+} transport. In light of the developing concept that it is PIP_2, and not PI, which is the substrate for Ca^{2+}-mobilizing receptors, these findings need to be reevaluated.

Although there is little experimental support for the hypothesis that phosphoinositide turnover is involved in Ca^{2+}-gating, both the hypothesis and the arguments developed for its demonstration have contributed appreciably to the current concept that enhanced PPI hydrolysis is involved in the mobilization of Ca^{2+} from intracellular stores. Recent findings suggest that the phosphoinositide system may subserve receptors which may not function through Ca^{2+} mobilization (table 2). This ought to be

established in other tissues. There is a lack of information on how phospholipase C is controlled. It is extremely sensitive to Ca^{2+}, and the relationship of this cation to the activation of the enzyme by the stimulus could be crucial to our understanding of the mechanism of the PPI response. Better controlled dose-response studies on the agonist-induced IP_3 release and of physiological response and receptor occupancy in the same tissue and under the same experimental conditions will increase our understanding of the physiological and pharmacological significance of this phenomenon.

2. Does agonist-stimulated breakdown of polyphosphoinositides regulate the plasma membrane Ca^{2+}-pump? Another possible function for the stimulated PIP_2 hydrolysis is to regulate the plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase and consequently the level of $[Ca^{2+}]_i$. In support of this concept are the following observations. (*a*) Inositol phospholipid interconversions can lead to changes in Ca^{2+} binding by membranes, as has been shown in erythrocytes (101), in which the amount of Ca^{2+} bound to erythrocyte membranes increased in parallel with the incorporation of ^{32}P into the PPI. Membranes with elevated levels of PPI also have much higher Ca^{2+}-ATPase activity than membranes with normal levels. (*b*) PIP_2 stimulated Ca^{2+}-ATPase activity in erythrocyte membranes (122, 424), in platelet membranes (424), in parotid ER (581), and in brain (122). Thus, Penniston and coworkers (122) reported a strong stimulation of the erythrocyte plasma membrane Ca^{2+}-pump by PA and PIP_2, but relatively small effects of DG and PI. Furthermore, they showed that PIP_2, at low concentrations, has a stimulatory effect on the similar Ca^{2+}-ATPase from rat brain synaptic plasma membranes. (*c*) Ca^{2+}-mobilizing agonists, such as vasopressin, angiotensin II, epinephrine, and phenylephrine, inhibited $(Ca^{2+} + Mg^{2+})$-ATPase activity in liver plasma membranes (374, 476), a protocol that would have led to the breakdown of the PPI. Thus, the activity of $(Ca^{2+} + Mg^{2+})$-ATPase can be regulated by the level of the PPI. According to this, activation of Ca^{2+}-mobilizing receptors leads to depletion of PIP_2 at the plasma membrane, and this could inhibit the Ca^{2+}-pump mechanism and subsequently increases the $[Ca^{2+}]_i$.

There is a need to test these findings and concepts more rigorously in other tissues and to show whether the Ca^{2+}-pump can be regulated by the synergistic interactions of DG and IP_3 on C-kinase.

3. Is there a role for GTP-binding proteins in the receptor-stimulated polyphosphoinositide breakdown? Although there is general agreement that Ca^{2+}-mobilizing agonists exert their physiological effects on cells by activating phospholipase C that specifically hydrolyzes PIP_2 at the plasma membrane to produce DG and IP_3, the mechanism involved in coupling the receptors to this enzyme is still unclear. In the early studies on the PPI response, we (for summary, see ref. 15) and others (for reviews, see refs. 214 and 260) have speculated that Ca^{2+}, which is released at the plasma membrane following receptor activation, could be involved in coupling the receptor to the activation of phospholipase C. Fisher et al. (217, 211), working with brain synaptosomes, suggested that the enhancement of phosphoinositide turnover in brain is caused by agonist-mediated conformational changes in the muscarinic receptor and that the ability of an agonist to induce this conversion may be predicted by its differential binding to the high and low affinity forms of the receptor. In more recent studies Evans et al. (195), working with $1321N_1$ human astrocytoma cells, found that CCh and methacholine were "full" agonists as regards phosphoinositide breakdown and Ca^{2+} mobilization, whereas bethanechol, arecoline, and oxotremorine were "partial" agonists for these two responses. Pilocarpine was the least efficacious of the six drugs tested. They speculated that the difference between full and partial agonists would be that a high-affinity GTP-sensitive state is formed in the presence of full agonists, but not with partial agonists.

There is convincing experimental evidence at present which suggests a role of GTP-binding proteins in the PPI response, in a manner having similarity with the control of adenylate cyclase. According to this hypothesis, Ca^{2+}-mobilizing receptors stimulation is initiated by an agonist-receptor interaction which results in the promotion of GTP binding to the nucleotide binding protein; the complex formed then activates the phospholipase C to cleave PIP_2 into DG and IP_3 (given in fig. 5). In support of this scheme are the following findings.

a. HORMONAL AND NEUROTRANSMITTER EFFECTS ON PHOSPHOINOSITIDE METABOLISM IN MEMBRANE FRACTIONS. Vasopressin and α_1-adrenergic agonists have been shown to cause loss of PI when added to rat liver plasma membranes (254, 373), cholinergic and α_1-adrenergic agonists stimulated phosphoinositide breakdown in isolated rat parotid membranes (430), and in pancreatic

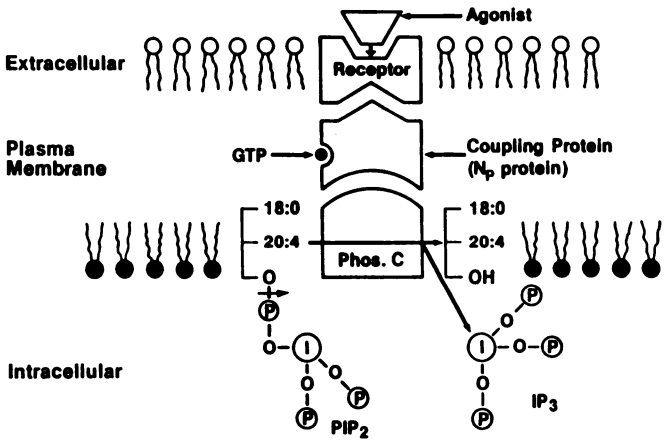


FIG. 5. Schematic representation of a possible role for GTP-binding proteins in coupling Ca^{2+}-mobilizing receptors to the activation of phospholipase C to cleave PIP_2 into DG and IP_3. The coupling protein is termed Np, with the "p" referring to phospholipase C. See text for details.

islet cell membranes (183), serotonin induced breakdown of PI in cell-free preparations of the blowfly salivary glands (202), and an increased phosphorylation by ACTH in rat brain (317, 318) and rabbit iris smooth muscle (27) membranes has been reported. Seyfred and Wells (535) reported that vasopressin may cause a preferential loss of PIP_2 when added to ^{32}P-labeled rat hepatocyte plasma membranes. Very recently, TRH was reported to activate IP_3 formation in crude membrane preparations isolated from rat pituitary cells (562a). The above is in contrast to previous studies where all demonstrations of receptor-mediated alterations in phosphoinositide metabolism and levels have required intact cellular structure (for review, see refs. 408, 3, 278, and 277).

b. THE COUPLING FACTOR LINKING RECEPTOR AND PHOSPHOLIPASE C IS PROBABLY A GTP-BINDING PROTEIN. There is good experimental evidence for the involvement of GTP-binding proteins in the coupling of various Ca^{2+}-mobilizing receptors to PIP_2 hydrolysis and Ca^{2+} mobilization. Thus, in permeabilized cells, nonhydrolyzable analogues of GTP introduced and then trapped in the cytosol are able to substitute for external ligands in stimulating histamine release, a well-defined Ca^{2+}-dependent process (244). These GTP analogues also stimulate serotonin release and DG formation in permeabilized platelets (257), and they stimulate PIP_2 hydrolysis in: human neutrophil membranes (134); membranes of hepatocytes (612, 592, 75, 592a, 462a); cell-free systems from blowfly salivary gland membranes (381, 377, 376); smooth muscle membranes (516); human polymorphonuclear leukocyte membranes (549, 548a); WRK1 cell membranes (249d); permeabilized GH_3 cells (395a); permeabilized rat parotid gland (578b); exocrine pancreas (407c); membrane fractions isolated from rat pituitary cells (562a); and cerebral cortical membrane fractions (245). In some of these studies additional evidence was obtained from a hormonal stimulation of phospholipase C which is potentiated by GTP analogues (381, 377, 75, 516, 549, 395a, 562a, 578b). Thus, addition of membrane fractions from smooth muscle of the porcine coronary artery (516) and from blowfly salivary gland (377) to exogenous PIP_2 resulted in a rapid, time-dependent breakdown of the substrate and liberation of IP_3, and this was enhanced significantly upon the addition of ACh and 5-methyltryptamine, respectively. Vasopressin (1 μM) stimulated the liberation of IP_3 from plasma membranes prepared from myo-[^{3}H]inositol-prelabeled WRK1 cells (249d). GTP did not increase IP_3 release; however, the nucleotide was necessary for hormonal stimulation of phospholipase C. In contrast, nonhydrolyzable analogues of GTP alone stimulated the phospholipase C activity. Straub and Gershengorn (562a), working with membrane fractions from rat pituitary cells, demonstrated that TRH and GTP act synergistically to stimulate the accumulation of IP_3 in these preparations, and that ATP was necessary for this effect. The TRH-stimulated IP_3 release in permeable GH_3 cells was also stimulated by GTP analogues (395a).

In spite of the fact that crude membrane preparations, which consisted of nuclear, mitochondrial, microsomal, and plasma membranes, were employed in most of these studies, the above findings suggest that a GTP-binding regulatory protein is probably involved in coupling Ca^{2+}-mobilizing receptors to phospholipase C that hydrolyzes PIP_2.

c. PERTUSSIS TOXIN AND THE STIMULATED HYDROLYSIS OF POLYPHOSPHOINOSITIDES. Several groups have now provided additional support for an involvement of GTP-binding proteins in the PPI response by making use of pertussis toxin (derived from the whooping cough bacterium *Bordetella*). It is a specific modifier of the GTP-binding regulatory protein, and it inhibits the ability of some Ca^{2+}-mobilizing agonists to promote PIP_2 hydrolysis. In the adenylate cyclase system it causes an NAD-dependent ADP-ribosylation of the M_r 41,000 α-subunit of the GTP regulatory protein (Ni) and inhibits its action (294). Two GTP-binding proteins, Ni and Ns, function in the adenylate cyclase system. Ni mediates the effects of inhibitory agonists such as the α_2-adrenergic amines on attenuation of adenylate cyclase, while Ns mediates the effects of stimulatory agonists such as the β-adrenergic amines on activation of adenylate cyclase activity. Pretreatment of neutrophils (449, 448, 417, 607), human polymorphonuclear leukocyte membranes (549), human leukemic (HL-60) cells (88), rat renal mesangial cells (463a), and mast cells (433) with this toxin inhibited the agonist-stimulated PIP_2 hydrolysis. In mast cells, prior treatment with the toxin results in the ADP-ribosylation of a M_r 41,000 protein in the cell membrane and inhibition of the Ca^{2+} influx, AA release, PIP_2 turnover, and histamine release induced by compound 48/80 (433). In neutrophils, the peptide N-formyl-Met-Leu-Phe stimulates chemotaxis and aggregation, PIP_2 breakdown, AA release, and a rise in $[Ca^{2+}]_i$. All of these actions of the peptide are inhibited by prior pretreatment of the cells with pertussis toxin (449, 448, 607, 88). These results suggest that Ni could be involved in mediating the transduction of signals causing degranulation in these cells. It is not yet known whether this mechanism is applicable to all systems. Thus, in the 1321 N1 astrocytoma cells, ADP-ribosylation of Ni does not abolish the Ca^{2+}-linked responses to muscarinic agonists (196). Moreover, pertussis toxin treatment does not abolish the phosphoinositide response to angiotensin or vasopressin in hepatocytes (75, 467), to muscarinic stimulation in cultured chick heart cells or in 1321 N1 cells (397), to α_1-adrenergic stimulation in brown adipocytes (521a), and to TRH stimulation in permeable GH_3 cells (395a). The reason for these differences is not yet clear, but it is possible that different Ca^{2+}-mobilizing receptors may activate the phospholipase C system by interaction

with an as-yet unidentified GTP regulatory protein (i.e., Np; fig. 5). In rat hepatocytes, the GTP-binding protein does not appear to be Ni (384a). This is also true of the exocrine pancreas (407c). In human platelets, PAF and U44069, which stimulate phosphoinositide hydrolysis in these cells, exert actions through a GTP-regulatory protein which is distinct from Ns and Ni (294a). Different receptors are likely to interact with separate but maybe related families of N-proteins. Other evidence for the possible involvement of GTP in the PPI response comes from experiments showing that GTP and its analogues inhibit agonist binding to Ca^{2+}-mobilizing receptors (148a, 247).

It can be concluded from the above studies that there is now good experimental evidence which indicates that hormonal and neurotransmitter stimulation of PIP_2 hydrolysis could be demonstrated in membrane fractions, and that the coupling of the receptors for these agonists to the hydrolysis of the PPI by phospholipase C in certain cells is probably through an as-yet unidentified GTP-binding regulatory protein. The role and the nature of the putative GTP binding proteins involved in these mechanisms remain to be established. By analogy with hormonally regulated adenylate cyclase systems, these proteins may exhibit a GTPase activity and may be responsible for the reversal of phospholipase C activation. In certain systems, such as liver plasma membranes (592) and permeable GH_3 cells (395a), GTP and its analogues could act by decreasing the Ca^{2+} requirement of the phospholipase C as well as increasing its activity at maximal Ca^{2+} concentrations (592). There is a need to extend these observations to other cell systems and to identify the GTP-binding protein(s) involved in the PPI response. An important objective in the future will be to characterize regulatory proteins involved in the receptor-stimulated hydrolysis of PPI.

C. Enhanced Polyphosphoinositide Turnover-Generation of Second Messengers

1. Inositol-1,4,5-trisphosphate (IP_3)—a putative Ca^{2+} mobilizer. This section will be divided into (*a*) IP_3 and the release of Ca^{2+} from intracellular stores in various cell types, (*b*) possible mechanisms for IP_3-induced Ca^{2+} release from the ER, and (*c*) the significance and metabolism of other inositol polyphosphates.

a. IP_3 AND THE RELEASE OF Ca^{2+} FROM INTRACELLULAR STORES IN VARIOUS CELL TYPES. Evidence for the hypothesis that IP_3 may function as a second messenger to mobilize Ca^{2+} from the ER has been gathered through studies of a wide variety of cells permeabilized by Ca^{2+}-free solutions, by high voltage discharge, and by saponin or digitonin treatment and was reported first for rat pancreatic acinar cells (565) and later for several other systems (summarized in table 3). Thus, it was observed in permeabilized acinar cells, which accumulate Ca^{2+} within the ER through an ATP-dependent mechanism, that addition of micromolar amounts of IP_3, as low as 0.2 μM, caused a rapid release of Ca^{2+}, and this was followed by slow re-uptake of the cation (565). This effect had a K_m for IP_3 of 1.1 μM and released the cation from the same store that is released by CCh. The IP_3-induced Ca^{2+} release is specific, since there was no release with IP_2 and IP. In later studies these investigators showed that secretagogue-induced Ca^{2+} release is accompanied by a parallel production of IP_3 and that blockade of IP_3 production by neomycin, which chelates PIP_2 and thus inhibits phospholipase C action, inhibited the release of Ca^{2+} by the agonist (564).

Studies with saponin-permeabilized hepatocytes (320, 102) showed that IP_3 caused a release of Ca^{2+} only from a nonmitochondrial intracellular Ca^{2+} pool with half-maximal and maximal effects at 0.1 and 0.5 μM, respectively. The time required to cause Ca^{2+} mobilization after vasopressin addition to intact hepatocytes and that taken by IP_3 to cause Ca^{2+} release from permeabilized cells are compatible with the postulated role for IP_3 in the action of vasopressin (629). The rate of IP_3 production in intact cells appears to be such that it could reach concentrations of 20 to 30 μM within 1 min (102, 35, 105). These calculations are a rough estimate of IP_3 that may be physiologically active, since the exact quantity of active IP_3 isomer, that is, inositol-1,4,5-trisphosphate, present in the cell or in the IP_3 preparation is unknown. Thus, it is not yet possible to make quantitative comparisons of IP_3 action in intact and permeabilized cells.

Particularly interesting systems, which may also involve IP_3 as an intracellular mediator, are muscle contraction and visual transduction (table 3). Thus, in vascular smooth muscle, IP_3 (0.5 to 30 μM) caused Ca^{2+} release and tension development in rabbit main pulmonary artery smooth muscle permeabilized with saponin or digitonin (558). Sustained contractions were induced by IP_3. The amount of Ca^{2+} released by IP_3, measured with a Ca^{2+}-selective electrode, was also sufficient to stimulate contraction in intact smooth muscle. These investigators presented experimental evidence which supports the hypothesis that, in smooth muscle, IP_3 is the messenger or one of the messengers involved in transmitter-induced (pharmacomechanical) Ca^{2+} release from the SR. Another interesting finding is that IP_3 can be microinjected into photoreceptor cells of the horseshoe crab (*Limulus polyphemus*) to produce a transient rise in intracellular Ca^{2+} concentration (95) and a voltage response that mimics that produced by light (207, 96). The sensitivity of the photoreceptor to a light flash is decreased after IP_3 injection and, conversely, light decreases the sensitivity of the photoreceptor to IP_3. Illumination causes a fall in PIP_2 and increased the IP_3 content of the *Limulus* eye (96). It is not known whether IP_3 exerts its effect on the photoreceptor by releasing Ca^{2+} from intracellular stores or through other mechanisms. The authors concluded that IP_3 may be an intra-

TABLE 3

Examples of systems in which an IP_3-induced Ca^{2+} release has been either demonstrated or implicated

System	IP_3-induced Ca^{2+} release	References
Permeabilized pancreatic cells	Yes	421, 471, 564, 638, 65a, 565, 322
Pancreatic microsomes	Yes	470, 563
Permeabilized hepatocytes	Yes	320, 102, 103, 561
Liver microsomes	Yes	157a, 426
Permeabilized human and rabbit neutrophils	Yes	472, 561
Human platelet membranes	Yes	38, 455, 19
Adipocyte ER	Yes	160b
Permeabilized GH_3 pituitary tumor cells	Yes	234, 66
Permeabilized peritoneal macrophages	Yes	272
Smooth muscle		
Permeabilized cells		
Vascular	Yes	553, 558, 645, 566
Mesenteric artery	Yes	256a
Microsomes (uterine)	Yes	113
Skeletal muscle		
Permeabilized fibers (frog semitendinosus)	Yes	600
Microsomes (lobster abdominal muscle)	No	521
Skinned fibers	Yes	444b, 580a
Cardiac muscle		
Permeabilized cells (canine)	Yes	272
Permeabilized cells (rat)	No	425
Microsomes (rat)	No	425
Skinned fibers	Yes	444b
Photoreceptors	Yes	207, 96, 95, 613

cellular messenger in the cascade of reactions mediating visual excitation in *Limulus* photoreceptors (206a).

The site at which IP_3 acts has been shown to be a vesicular ATP-dependent nonmitochondrial Ca^{2+} pool, probably the ER. This conclusion is based on the following findings: this pool is ATP dependent; it is vesicular, since Ca^{2+} ionophores discharge it and prevent a subsequent response to IP_3; and it is nonmitochondrial, since normal responses to IP_3 are observed at free Ca^{2+} concentration below the threshold for mitochondrial uptake or in the presence of mitochondrial inhibitors (103, 579). Further support for the conclusion that ER is the site of the intracellular IP_3-sensitive Ca^{2+} pool comes from the findings that IP_3 can release Ca^{2+} when added directly to microsomes obtained from a wide variety of tissues (see table 3), but was without effect on Ca^{2+} release from mitochondrial fractions (563, 470, 157a).

It can be concluded from the above that these data are consistent with the hypothesis that IP_3 is released from the plasma membrane by Ca^{2+}-mobilizing agonists and can act as a signal for Ca^{2+} release from intracellular stores. The recent finding by Spat et al. (561a) that [^{32}P]IP_3 preferentially binds to a crude microsomal fraction, relative to cytosolic and mitochondrial fractions, lends strong support to this conclusion.

b. POSSIBLE MECHANISMS FOR IP_3-INDUCED Ca^{2+} RELEASE FROM THE ENDOPLASMIC RETICULUM. The mechanism of IP_3-induced Ca^{2+} release from the ER is not known. The results of structure-activity studies on the ability of inositol phosphates to release Ca^{2+} are consistent with the idea of a "receptor" for IP_3, and evidence associated with the ER has just been reported (561). Binding sites for IP_3 have been identified in microsomal fractions from bovine adrenal cortex, employing [^{32}P]IP_3 from human erythrocytes radiolabeled with [^{32}P]ATP (52). IP_3 was bound to the microsomes with high affinity (K_d = 5nM) and low capacity (186 fmol/mg of protein). Half-maximal binding was reached in 1 min at 4°C, and dissociation was even more rapid with $t½$ of about 10 s. [^{32}P]IP_2 was ineffective. In another study they demonstrated that [^{32}P]IP_3 binds to a specific saturable site in permeabilized guinea pig hepatocytes and rabbit neutrophils, and they concluded that the properties of this binding site suggest that it is the physiological receptor

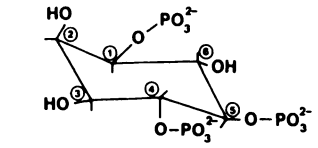
for IP_3 (561). Hirata et al. (271) synthesized an arylazide derivative of IP_3 for photoaffinity labeling [IP_3 coupled to *p*-azidobenzoic acid (IP_3-pAB) using N,N′-carbonyldiimidazole] and showed that IP_3-pAB, but not an arylazide derivative of IP_2, causes the irreversible inhibition of IP_3-induced release of Ca^{2+} in saponin-permeabilized photoirradiated macrophages. This inhibition was reversed by a 10-fold excess of IP_3. Working with cultured vascular smooth muscle cells, Smith et al. (553) reported that IP_3 activates a Ca^{2+} channel, rather than a carrier, by ligand binding mechanism, that is independent of the metabolism of ATP or IP_3. IP_3 added to a liver rough ER vesicle preparation activates a Ca^{2+} channel that is distinct from the electrogenic ATP-dependent Ca^{2+}-pump and allows Ca^{2+} efflux into the cytosol in exchange for K^+ uptake (426). Very recently, Gill and coworkers (240, 591a), working with a detergent-permeabilized neuroblastoma cell line, reported that approximately half of the Ca^{2+} accumulated by ER can be released by exposure to micromolar concentrations of GTP, and that the GTP-induced and IP_3-induced Ca^{2+} release can occur through two distinct and separate systems. The finding of Dawson et al. (157) that, in rat liver microsomal fractions, IP_3-induced Ca^{2+} release is strongly stimulated by GTP is in accord with these observations.

Although these studies are still preliminary, they do support the hypothesis that the IP_3 effects on Ca^{2+} release are specific, and it is not unreasonable to speculate that IP_3 could bind to a specific receptor at the surface of the ER, opening an ion-conducting channel for Ca^{2+} transport in a manner similar to that reported for the nicotinic cholinergic receptor (46, 511, 191).

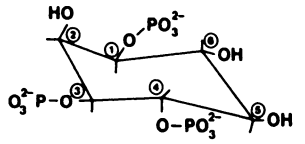
c. OTHER REPORTED FUNCTIONS OF IP_3. Studies on other possible cellular functions of IP_3 include: reports of stimulatory effects on protein phosphorylation (365, 625), but this could not be confirmed in another laboratory (242), and more recently it was withdrawn by one of these laboratories (626); activation of pyruvate dehydrogenase in isolated fat cells (350); its microinjection into immature oocytes, or egg cells, of the frog *Xenopus* can mimic the muscarinic depolarizing chloride current (453); and that microinjection triggers activation of sea urchin eggs (623, 547a), frog (*Xenopus laevis*) eggs (107, 429a), and amphibian and starfish oocytes (465), all presumably, through release of Ca^{2+} from the ER by the inositol polyphosphate.

It can be concluded from the above studies that the experimental evidence from the studies on permeabilized cells supports the hypothesis that IP_3 could function as the link between activation of Ca^{2+}-mobilizing receptors and the internal stores of Ca^{2+}. However, with the exception of few studies, among them the leaky pancreatic acinar cells (565, 564) where evidence was presented that the muscarinic agonist CCh acts on the same pool of Ca^{2+} that is sensitive to the direct effect of IP_3, there has been little work done to determine if the cell-surface receptors are still functional in these permeabilized cells. An advantage of the saponin-treated cell preparations is that it offers a system in which the internal function of the cell is apparently intact, yet the environment surrounding the organelles can be precisely controlled (104). The detergent interacts with cholesterol in the cholesterol-rich plasma membrane to form pores which make the membrane freely permeable to small molecules and ions. The ER, which contains negligible amounts of cholesterol, remains functionally intact. Although saponin treatment preferentially alters cell membrane permeability, it also affects ER, and dependent on time of exposure, temperature, and concentration, changes in a variety of enzyme activities occur (143). Thus, there is a need to develop more physiological methods for introducing IP_3 into cells; this will then allow correlative studies on the effects of agonists and IP_3 on Ca^{2+} release from the ER and on Ca^{2+}-dependent physiological responses in various cell types.

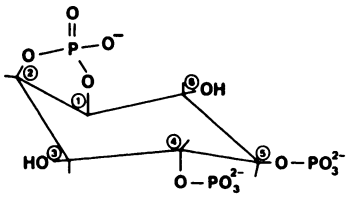
d. SIGNIFICANCE AND METABOLISM OF OTHER INOSITOL POLYPHOSPHATES. While the above studies have established that IP_3, but not IP_2 or IP, is probably the messenger which links receptors to Ca^{2+} mobilization, recent reports indicate that activation of Ca^{2+}-mobilizing receptors also leads to the formation of inositol polyphosphates other than IP_3. Thus, a compound identified as D- or L-myo-inositol 1,3,4-trisphosphate (see fig. 6 for structures) has been demonstrated in parotid gland frag-



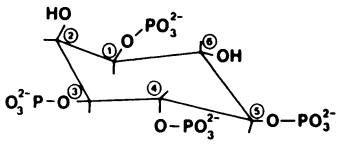
Myo-Inositol-1,4,5-Trisphosphate (IP_3)



Myo-Inositol-1,3,4-Trisphosphate



Myo-Inositol-1:2-Cyclic,4,5-Trisphosphate (cyclic IP_3)



Myo-Inositol-1,3,4,5-Tetrakisphosphate (IP_4)

FIG. 6. Structures of myo-inositol polyphosphates which have been reported to have or may have physiological significance.

ments after stimulation with CCh (312). The appearance of this compound lags behind that of IP_3, but with increasing time, a progressively greater proportion of total inositol trisphosphate is inositol 1,3,4-trisphosphate which is much less readily degraded than IP_3 (307). In guinea-pig hepatocytes, where inositol trisphosphate increases for at least 30 min after hormone application, inositol 1,3,4-trisphosphate made up about 90% of the total inositol trisphosphate by 5 to 10 min (105). Recently, a more polar inositol polyphosphate, inositol 1,3,4,5-tetrakisphosphate (IP_4), and its probable hydrolysis product, inositol 1,3,4-trisphosphate, have been reported to accumulate in CCh-stimulated brain slices (50). Thus, CCh stimulation of muscarinic receptors in rat cortical slices prelabeled with myo-[^{3}H]inositol caused rapid formation of IP_4, and it was suggested by these authors that IP_4 is likely to be a second messenger, and that it is the precursor of inositol 1,3,4-trisphosphate and possibly of IP_3. IP_4 could arise from a tetraphosphoinositide; however, while the existence of such a lipid was previously reported in brain (514), it was later withdrawn by these authors (515). Recently, Irvine et al. (311a) and Hansen et al. (253b) presented evidence for a novel ATP-dependent kinase that converts IP_3 to IP_4. While such a pathway may be involved in the metabolism of IP_3, a possible role for IP_4 in Ca^{2+} signalling has not yet been demonstrated. IP_4 can then be metabolized by a specific phosphatase to give the myo-inositol 1,3,4-trisphosphate.

Majerus and coworkers (632) demonstrated that the water-soluble product of PIP_2 cleavage by purified ram seminal vesicles, phospholipase C, also contains, in addition to IP_3, cyclic IP_3 (fig. 6). The inositol cyclic phosphates were detected by performing the enzyme hydrolysis of PIP_2 in the presence of $H_2{}^{18}O$; the resultant phosphomonoesters will contain ^{18}O. In later studies these investigators examined the physiological effects of the inositol cyclic phosphate in two systems: saponin-permeabilized platelets loaded with $^{45}Ca^{2+}$ and intact *Limulus* photoreceptors (633). Both cyclic IP_3 and IP_3 released $^{45}Ca^{2+}$ from permeabilized platelets in a concentration-dependent manner. Injection of cyclic IP_3 into *Limulus* ventral photoreceptor cells induces both a change in membrane conductance and a transient increase in intracellular calcium ion concentration similar to those induced by light. When both cyclic IP_3 and IP_3 were injected into the same photoreceptor cell, they observed that the cyclic compound was about 5 times more potent than the noncyclic compound in stimulating a conductance change. They speculated that cyclic IP_3 may function as a second messenger in stimulated cells. More recently, these authors reported on a phosphatase which inactivates cyclic IP_3 (ref. 142; section III C 3). Cyclic IP_3 is an extremely labile compound and can be rapidly metabolized to IP_3 by phosphomonoesterases. However, while the above evidence indicates that cyclic IP_3 could be involved in Ca^{2+} mobilization, there is a need to demonstrate that it can be formed in response to receptor activation under physiological conditions. The number of inositol phosphates has grown rapidly in the past few months, which could become confusing at times (411). However, in spite of the confusion one finds in the literature, our current knowledge of the metabolism of inositol polyphosphates can be summarized as shown in fig. 7.

From the above it can be concluded that there is a need to determine which of the inositol polyphosphates, IP_3, cyclic IP_3, IP_4, is the physiological second messenger. So far, the experimental evidence points to IP_3 and to a lesser extent to its cyclic form, namely cyclic IP_3. However, the presence of the latter needs to be demonstrated in stimulated cells.

2. 1,2-Diacylglycerol and activation of C-kinase—a link between polyphosphoinositide metabolism and protein phosphorylation. Another primary product of the stimulated PPI hydrolysis is DG (figs. 1 and 4), which has been proposed to function as a second messenger (444, 341, 270). The neutral lipid, which is formed transiently at the plasma membrane, stimulates C-kinase activity, which is dependent on Ca^{2+} and acidic phospholipids, particularly phosphatidylserine, to catalyze the phosphorylation of serine and threonine residues of various cellular proteins (for review, see refs. 444, 134, 270, 32, 33, 358, 393, 432, and 389). Kinetically, a minute amount of DG enormously increases the affinity of C-kinase for Ca^{2+} to the 10^{-7} M range and renders this enzyme fully active without a net increase in the intracellular Ca^{2+} concentration (324). The physiological substrates for this enzyme have not yet been defined in most tissues.

a. GENERAL PROPERTIES OF C-KINASE. The Ca^{2+}/phospholipid-dependent protein kinase was first purified by Nishizuka and coworkers from brain as a cyclic nucleotide-independent protein kinase which could be activated by a Ca^{2+}-dependent protease also present in brain (572, 304). Without proteolysis, activation of the enzyme required phospholipids. Later the same group demonstrated a protein kinase which required Ca^{2+}, phospha-

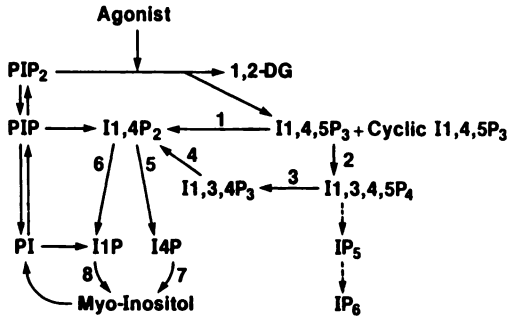


FIG. 7. Scheme showing suggested pathways for the metabolism of myo-inositol polyphosphates. Abbreviations: *I*, myo-inositol. The enzyme activities referred to in the figure are indicated as follows: *1*, IP_3-5′-phosphomonoesterase; *2*, IP_3-3-kinase; *3*, IP_4-5′-phosphomonoesterase; *4*, I-1,3,4-trisphosphatase; *5* and *6*, I-1,4-bisphosphatase; *7*, I-4-phosphatase; *8*, I-1-phosphatase.

tidylserine, and DG for maximum activity which they named C-kinase (144, 347). The enzyme is both cytosolic and membranous, has a wide tissue distribution, and its widespread occurrence is further demonstrated by the presence of its activity in tissues from various species and phyla of the animal kingdom (357). The enzyme from brain is a monomer of M_r 80,000 to 90,000 protein, and it contains a hydrophobic domain, which interacts with Ca^{2+} and phospholipid, and a hydrophilic domain, which is catalytically active (342). Very recently, a rapid purification procedure for its preparation from this tissue, employing HPLC (Pharmacia HPLC system), was described (340a). The affinity for Ca^{2+} was tremendously increased by DG, so that in the presence of the neutral lipid the enzyme is activated at physiological Ca^{2+} concentrations (10^{-7} M). This finding suggested that a control mechanism which increases DG levels at the plasma membrane (namely, phosphoinositide turnover) could activate C-kinase without prior mobilization of Ca^{2+} (393, 347, 484). In unstimulated cells, the enzyme is localized in the cytosol and presumably inactive; however, when the cell is activated, DG is produced from PIP_2, and the enzyme binds to phosphatidylserine at the plasma membrane (642, 182, 274, 404). This shifts the Ca^{2+} dependence of the enzyme from high concentrations (10^{-5} to 10^{-4} M) to low concentrations (μM), thereby activating the enzyme. It is thought that changes in C-kinase distribution by agonists probably serve as a regulatory mechanism to alter the endogeneous protein substrates readily available for phosphorylation by the enzyme. C-Kinase can be activated by synthetic diacylglycerols and also by tumor-promoting phorbol diesters, which possess a DG-like structure in their molecules (see below).

While the cellular substrates of C-kinase have not yet been defined, the enzyme does have a broad substrate specificity, which differs from those of both cyclic nucleotide-dependent and Ca^{2+}/calmodulin-dependent protein kinases. Thus, the enzyme phosphorylates histone H_1 (342), troponin T (333), smooth muscle myosin light chain (194, 440, 299), myosin light chain kinase (299, 441, 593, 442), α_1-adrenergic receptor (545), myelin basic protein (590, 346), erythrocyte band 4.1 (375, 485), ribosomal protein S (370), glucose transporter (637), glycogen synthetase (21), tyrosine hydroxylase (610), IP_3 phosphatase (141), brain B-50 protein (28), and synaptosomal M_r 87,000 protein (630), among others.

b. DIACYLGLYCEROLS AND ACTIVATION OF C-KINASE. Diacylglycerol is formed transiently in the stimulated cell, and under resting conditions, its concentration in the plasma membrane is too low to allow for its detectability. The lack of DG accumulation in the plasma membrane is due both to its rapid phosphorylation to PA by DG kinase and to its further degradation by lipases (see figs. 2 and 4). In spite of this rapid metabolism, changes in DG levels have been reported on stimulation of phosphoinositide breakdown in platelets (498, 334, 473), pancreas (42), mast cells (298), pituitary cells (395, 387), liver (583, 81, 295), and smooth muscle (652). In these studies increase in DG formation was demonstrated either by an increase in its mass or an increase in its radioactivity after prelabeling of phosphoinositides with specific radioactive precursors, usually AA. More recently, Bocckino et al. (81) measured DG in neutral extracts of stimulated hepatocytes by means of HPLC followed by refractive index detection. In both pancreas (42) and hepatocytes (81), the hormonally derived DG was partially derived from sources other than phosphoinositides.

In the original in vitro studies of Nishizuka et al. on C-kinase, they suggested that at least one unsaturated fatty acid was required within the DG for activity (347). Thus, 1,2-DGs containing two long-chain saturated fatty acids were far less active than diolein, the DG commonly used as exogenous activator. *sn*-1-Oleoyl-2-acetylglycerol (OAG), which can penetrate the plasma membrane and directly interact with C-kinase, proved to be an effective activator in vitro (325). In 1984, however, Bell and coworkers (618) and Cabot and Jaken (108) showed that some saturated DGs, such as 1,2-didecanoylglycerol and 1-palmitoyl-2-butyrylglycerol, were also capable of stimulating protein phosphorylation when used exogenously. Since then, several papers coauthored by these authors (139, 364) have appeared on the biological activity for a number of saturated symmetrical 1,2-DGs containing low-molecular-weight fatty acid moieties (C_3–C_{10}) in lengths. 1,2-DiC:8 *sn*-1,2-dioctanoylglycerol) was found to be the most effective of the set of even carbon saturated 1,2-DGs for C-kinase activation and stimulation of pituitary luteinizing hormone release (139). In a study on the nature of the kinase activation by physically defined phospholipid vesicles and DG, it was found that the specificity of the enzyme activation is directed at the glycerol backbone but not at the fatty acid side chain (85). Thus, it was shown that only 1,2-*sn*-diolein, but not its 2,3-*sn*-enantiomer or its 1,3-isomer, was capable of activating the kinase. It appears then that, although an unsaturated fatty acid is not required in DG for C-kinase activation, the acyl ester must satisfy certain balanced physical properties for water/lipid solubility or orientation so that it can partition favorably into a bilayer for an effective access to the kinase (108, 364). In more recent studies on C-kinase activation in mixed micelles, with emphasis on the mechanistic implications of phosphatidylserine, DG, and Ca^{2+}, it was reported that four or more molecules of the phospholipid are required to activate monomeric C-kinase (253a). 1,2-Dioleolglycerol was found to modulate the amount of Ca^{2+} required for maximal activity of the enzyme.

Diacylglycerols and their analogues provide useful tools to investigate the function of DGs as bioregulators in intact cells. Thus, OAG has been reported to have the

following effects: stimulation of serotonin secretion and aggregation in platelets (325); degranulation in neutrophils (446); DNA synthesis and cell division in mouse 3T3 cells (504); and exocytosis of neurotransmitters in the PC12 neurosecretory cell line (469). Among other effects, it was found that OAG increased $^{32}P_i$ incorporation into PIP_2 in intact human platelets, suggesting a role in resynthesis of PPI (159). OAG possibly modulates epidermal growth factor in cells (405) and increases superoxide generation in neutrophils (461). Evidence that the DG formed by PIP_2 breakdown activates C-kinase in intact cells comes from the studies of Nishizuka and coworkers (444, 334). They have demonstrated a correlation between the increase in DG induced by thrombin in platelets and the selective phosphorylation of a M_r 40, 000 protein, which is a substrate for C-kinase. However, in brain synaptosomes, neither ACh nor NE addition results in an increase in protein phosphorylation (356). Similarly, in dissociated avian salt gland cells, muscarinic receptor activation results in a marked increase in PA labeling but has no detectable effect on protein phosphorylation (216).

Since DG formed by phosphoinositide breakdown is usually stearate at C_1 and since arachidonate is at C_2, there is a need to show an activation of C-kinase by 1-stearoyl-2-arachidonyl-*sn*-glycerol. In spite of this, the experimental evidence discussed above strongly suggests that DG could serve as a link between receptor-mediated PIP_2 hydrolysis and the C-kinase system.

c. PHORBOL ESTERS AND ACTIVATION OF C-KINASE. Another exciting discovery in this field has been the demonstration that phorbol esters, such as phorbol-12-myristate-13-acetate (PMA) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA), which are potent cell activators and cocarcinogens, are able to substitute for DG at extremely low concentrations (1000-fold lower than DG) and directly activate C-kinase both in vivo and in vitro (114, 647, 438). This circumvents the physiological pathway of DG production via receptor activation of phosphoinositide hydrolysis. The phorbol ester functions as a nonmetabolizable analogue of DG, by the phosphorylation of specific cellular proteins in the same manner as DG, the endogenous activator. It now appears that the physiological activity of the lipophilic phorbol esters is in part related to their ability to partition into membranes and mimic the action of DGs (108). The tumor promoter could activate the enzyme by modifying its phospholipid microenvironment or through proteolytic modification. Thus, treatment of neutrophils with PMA results in the binding of the native kinase to the membranes, followed by its limited proteolysis (407a). The resulting Ca^{2+}/phospholipid-independent form of the kinase is then released from the membrane, is recovered as an irreversible activated soluble kinase, and is possibly involved in phosphorylation of cytosolic or nuclear proteins. C-Kinase has also been shown to be the intracellular receptor for the tumor promoters (114, 438). Diacylglycerol competitively inhibits phorbol ester binding to a brain cytosolic phorbol ester aporeceptor (537, 466). TPA causes a rapid decrease in cytosolic C-kinase and a corresponding increase in the amount bound to the plasma membrane; this transfer would bring the enzyme in contact with the membrane phosphatidylserine and thus activate it. Phorbol ester activation of C-kinase has been employed by several investigators as a tool to investigate the DG-activated C-kinase pathway in intact cells. An example is the stimulation of superoxide generation in human neutrophils by phorbol esters and OAG and the inhibition of this stimulation by retinal, a known inhibitor of C-kinase (225). As with the DG requirement for C-kinase activation, TPA also dramatically increases the affinity of this enzyme for Ca^{2+} to the 10^{-7} M range (647), resulting in its full activation without detectable cellular mobilization of Ca^{2+}. Exogenous OAG and TPA evoke similar secretion and aggregation in human platelets without elevating $[Ca^{2+}]_i$ above the basal level of 0.1 μM (493). The pattern of secretion resembles that produced by collagen and thrombin when $[Ca^{2+}]_i$ remains at basal levels.

In general, phorbol esters have been reported to reduce the formation of inositol phosphates under stimulatory conditions (452, 497, 619, 359, 601, 385, 450, 368, 89, 417a), to increase the synthesis of PPI under stimulatory conditions (86, 619, 159, 251), and to increase AA release under stimulatory conditions (see table 4), among others. The effects of phorbol esters on phosphoinositide metabolism and Ca^{2+} mobilization in various secretory cells can be summarized as follows: (*a*) they increase the incorporation of $^{32}P_i$ into PIP and PIP_2; and (*b*) pretreatment of the tissue with these agents inhibits both the agonist-induced degradation of phosphoinositides leading to impaired formation of inositol phosphates and the agonist-induced increase in cytosolic Ca^{2+} concentrations. Support for these conclusions comes from the recent studies on the effects of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), a potent C-kinase inhibitor, on phosphoinositide turnover and $[Ca^{2+}]_i$ mobilization in PMA-pretreated human platelets exposed to thrombin (586). These studies demonstrated that H-7 reversed the PMA-induced inhibition of phosphoinositide metabolism and Ca^{2+} mobilization in thrombin-activated platelets, thereby providing evidence that the inhibitory effects of PMA are mediated by C-kinase activation. Very recently, phorbol esters have been reported to stimulate amylase secretion from rat parotid cells (578a), potentiate synaptic transmission in the rat hippocampus (390), inhibit enzymatic hydrolysis of diacylglycerols in vitro (114a), stimulate phosphate accumulation synergistically with A23187 in cultured renal tubular cells (342a), promote depletion of cytosolic C-kinase in rat adipocytes (243), and increase ADP-ribosylation catalyzed by pertussis toxin, and inhibit the

GTPase stimulated by formyl-Met-Leu-Phe in rabbit neutrophils (400).

The above findings indicate that phorbol esters can mimic the action of endogenously generated DG, and that adds additional support to the concept that the DG released by PIP_2 hydrolysis can activate C-kinase in the intact cell. While the above studies suggest that the inhibitory effects of phorbol esters are mediated by activation of C-kinase, the precise mechanism by which the activation of this enzyme exerts a negative feedback has not yet been elucidated. The recent finding by Connolly and Majerus (141) that C-kinase phosphorylates IP_3 5′-phosphatase in human platelets and increases its activity can explain the reported observations by many that phorbol ester treatment of intact tissues under stimulated conditions results in decreased production of IP_3 (see above). Thus, it must be emphasized that, although phorbol esters may be useful pharmacological tools for determining the role of C-kinase in biological responses, the multiplicity of their effects on cellular metabolism should be taken into consideration.

The steady-state level of DG for activation of C-kinase is determined by its production from phosphoinositides and its phosphorylation to PA and/or deacylation to AA, stearic acid, and glycerol. Either phosphorylation or deacylation could terminate the second messenger function of DG.

3. Release of arachidonic acid (AA)—precursor of the eicosanoids. Increased release of AA and subsequent synthesis of cyclooxygenase and lipooxygenase products have been shown to be related to the agonist-stimulated breakdown of phosphoinositides in a wide variety of tissues. In general, most agents that enhance phosphoinositide turnover also increase AA and consequently eicosanoid (prostaglandins, thromboxane, leukotrienes, etc.) synthesis. This conclusion is supported by the findings that activation of the same receptors which control PPI metabolism (table 2) also results in the liberation of AA and eicosanoid biosynthesis (table 4). Thus, various cell types have been shown to release AA from their phospholipids in response to specific Ca^{2+}-mobilizing agonists such as CCh, NE, serotonin, thrombin, angiotensin II, bradykinin, etc. (table 4). The stimulatory effects of these agonists on AA release, as with the PPI response, can be inhibited specifically by their respective antagonists. Because of these findings the mechanism of coupling between the activation of Ca^{2+}-mobilizing receptors and liberation of AA from membrane phospholipids has been the subject of considerable study in the past few years.

a. METHODS OF ASSAY FOR AA RELEASE. In general, two methods have been employed to assay for the release of AA from tissue phospholipids and subsequent conversion into eicosanoids. (*a*) Assay of PG release by radioimmunoassay (RIA). While the RIA method is indicative of eicosanoid synthesis, it does not necessarily indicate AA release, and furthermore, it does not tell us about the type of phospholipid involved. (*b*) Another method to study the modulation of AA release from tissue lipids is based on the use of intact cells and tissues previously incubated with [^{14}C]AA. This leads to a specific labeling of various lipid fractions and allows the measurement of release of [^{14}C]AA metabolites in the medium (PGs, etc.) as well as the decrease of radioactivity in those lipid fractions which have been preferentially hydrolyzed in the presence of the pharmacological agent. In our work on the iris smooth muscle, we find both methods to yield the same type of data (652, 653).

b. METABOLIC PATHWAYS INVOLVED IN THE AGONIST-STIMULATED ARACHIDONATE RELEASE AND THE EFFECTS OF AGONISTS. In mammalian tissues, AA is found mainly esterified to phospholipids, almost exclusively in the 2-acyl position, from which it can be liberated by the action of specific phospholipases. The esterification of AA in the *sn*-2-position can then occur through different enzyme activities such as specific long-chain acyl-CoA synthetases and CoA-dependent or CoA-independent transacylases (305, 568, 309, 596, 589, 505, 354, 328, 355, 137, 490, 366). Since eicosanoids are not stored in mammalian tissues and since the concentration of free AA in cells under basal conditions is almost nil, it is generally believed that the rate-limiting step for their synthesis is the release of AA from membrane phospholipids through the activation of phospholipases (604, 391, 258, 305).

In phosphoinositides, the fatty acids at C_1 are usually stearate and that at C_2 is AA. Although these phospholipids are considered to be exceptionally enriched in AA on a mole-fraction basis, the largest amounts of cellular AA are in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and their respective plasmalogens. Two pathways have been proposed for the release of AA from phosphoinositides (fig. 8). (*a*) The first is indirect release by phospholipase C, which produces DG, followed by the actions of DG- and monoacylglycerol lipases on DG to release AA (54, 451, 119, 473). This reaction occurs in platelets stimulated to aggregate by thrombin, collagen, and the Ca^{2+} ionophore A23187 and also in neutrophils. (*b*) The second proposed pathway is a direct release by phospholipase A_2. Although there is little information about the properties of the phospholipase A_2 against phosphoinositides, the stimulated formation of lyso-PI has been demonstrated in a wide variety of systems including platelets (68), transformed BALB/3T3 cells (290), neutrophils (508, 615), aortic endothelial cells (291), and iris smooth muscle (653, 652). Lapetina and coworkers (361) have suggested that the PA derived from the agonist-stimulated breakdown of phosphoinositides (fig. 4) could be deacylated by a PA-specific phospholipase A_2 to liberate AA. Polyphosphoinositides are also enriched with AA; however, there is lack of information on their role in AA release and the extent to which they may be deacylated (12). In a more recent study (650),

TABLE 4

Examples of tissues in which a stimulus-induced arachidonic acid liberation and/or prostaglandin release has been reported

Stimulus	Target tissue (refs.)
Thrombin	Platelets (54, 91, 388, 287, 501, 406, 639, 68, 551, 547, 494, 119, 473, 137a, 388a)
Muscarinic cholinergic	Pancreas (43, 51, 394); vascular smooth muscle (82); iris smooth muscle (652, 12); C62B glioma cells (160a)
α_1-Adrenergic	Spleen (241, 269, 100); kidney (436, 371, 267, 431, 144); brain (392, 72, 640); brain microvessels (72); iris smooth muscle (652, 12, 651)
Serotonin	Vascular smooth muscle cells (148); blowfly salivary gland (380)
Peptidergic	
Vasopressin	Renal mesangial cells (464); renal medullary interstitial cells (37)
Angiotensin II	Kidney (529); mesenteric artery (163)
Bradykinin	Kidney (529); aortic endothelial cells (291); mouse fibrosarcoma cells (53); HSDM1C$_1$ cells (526); mouse BALB/3T3 cells (290)
Substance P	Iris smooth muscle (655)
f-Methionyl-leucyl-phenylalanine (FMLP)	Neutrophils (508, 615, 650)
Caerulein	Pancreas (168)
Adrenocorticotropin (ACTH)	Adrenocortical cells (528)
Platelet-activating factor (PAF)	Platelets (538); iris smooth muscle (653); renal epithelial cells (333a)
Phorbol esters (TPA)	Madin-darby canine kidney cells (153, 152); neutrophils (507, 614, 606); human rheumatoid synovial cells (186); platelets (252); murine epidermal cell line (614)
Electroconvulsive shock	Brain (52a)
Ionophore A23187	Platelets (252, 68, 496, 348, 494, 303, 209, 137a); neutrophils (152, 507, 606); adrenocortical cells (528, 220); renal-medullary interstitial cells (37); murine epidermal cell line (226); macrophages (192, 193); cultured endothelial cells (585); mast cells (301); splenic capsular strips (222); brain synaptosomes (87); iris smooth muscle (652, 651); guinea pig *Taenia coli* (166); vascular endothelial cells (93, 627)

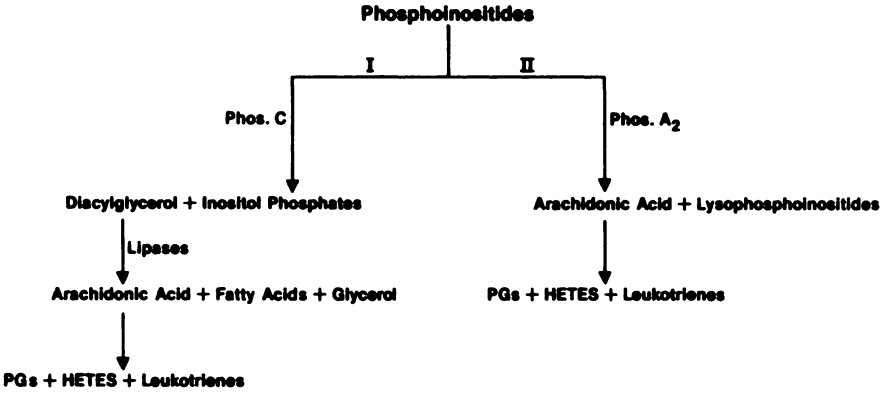


FIG. 8. Scheme showing the two pathways through which AA can be released from phosphoinositides: in *I*, phosphoinositides can be hydrolyzed by phospholipase C, an enzyme which hydrolyzes only phosphoinositides, followed by hydrolysis of DG via lipases to liberate AA; in *II*, AA can be released from phosphoinositides via phospholipase A_2. Abbreviation: *HETES*, 5- and 12-hydroxy-6,8,11,14-eicosatetraenoic acid.

when [^{3}H]AA-labeled neutrophils were stimulated with formyl-Met-Leu-Phe, the radioactivities in PIP and PIP_2 significantly decreased in parallel with initial efflux of Ca^{2+}, thus suggesting that these phospholipids can serve as a source of AA.

Experimental support for the phospholipase C-DG lipase pathway has been well documented in platelets in response to thrombin. Majerus and coworkers (54) reasoned that, since both phospholipase C and DG lipase are present in platelets, they could also act in concert to produce the AA following treatment with thrombin. Rittenhouse-Simmons (498) has detected DG in thrombin-treated platelets, and Bell et al. (54) have demonstrated that platelets possess considerable DG lipase activity. The latter authors have suggested that PI→DG→AA is the major pathway for AA release from platelet membrane phospholipids. Subsequent studies by these authors (473) and others (451, 119) revealed that DG was deacylated first at *sn*-1 as evidenced by the accumulation of 2-arachidonyl monoglyceride but not of 1-stearoyl monoglyceride. Subsequent release of AA from monoglyceride required the action of a monoglyceride lipase. In a more recent study on the pathways involved in AA release in human platelets, Mahadevappa and Holub (388a) concluded that the DG lipase pathway represents only a minor (≈15%) source of the free AA that is released upon thrombin stimulation in these cells.

It can be concluded from the above findings that the DG formed transiently as a result of agonist-stimulated breakdown of phosphoinositides can be degraded, at least in platelets, through a sequential action of a DG lipase and a monoglyceride lipase to liberate AA for eicosanoid synthesis (fig. 8). However, it must be emphasized that this pathway has not yet been rigorously investigated in other cell types, and even in platelets the amount of its previously reported contribution to AA release has now been challenged (388a).

Support for the involvement of the phospholipase A_2 pathway in the agonist-stimulated AA release comes from the fact that lyso-PC (406), lyso-PI (68), and lyso-PA (361) have been detected in thrombin-treated platelets, as well as in other systems (see table 4 for references), thus indicating that phospholipase A_2 is also functional in the stimulated cell. Kinetic studies in the iris smooth muscle on the effects of NE and ACh on PG release, measured by RIA, and the type of phospholipase activated by NE in which PI was doubly prelabeled with myo-[^{3}H]inositol and [^{14}C]AA, quantitated by radiometric and chromatographic methods, revealed that both AA release and PG production by the neurotransmitters are time and dose dependent (652, 12). The NE- and ACh-induced AA liberation and PG release are mediated by α_1-adrenergic and muscarinic receptors, respectively. The neurotransmitters increased the formation of lyso-PI from PI, presumably via phospholipase A_2, in a time-dependent manner (652). Further support for the involvement of phospholipase A_2 in AA release from phosphoinositides in stimulated cells comes from studies on the effects of the platelet-activating factor (PAF) on AA liberation and PG release in the iris muscle (653, 12). In platelets, PAF was reported to increase phosphoinositide turnover, however, and in contrast, this bioactive lipid had no effect on phosphoinositide turnover in the iris muscle, implying that it does not behave as a Ca^{2+}-mobilizing agonist in this tissue. Instead, it activated phospholipase A_2 to release AA and increase the formation of lyso-PI (653). Briefly, (*a*) both the removal of AA and the formation of lyso-PI, by PAF (10^{-9} M), occurred concomitantly in a time-dependent manner; (*b*) Ca^{2+} is required for the PAF-induced AA release: and (*c*) PAF (10^{-12} to 10^{-5} M) had no effect on myo-[^{14}C]inositol phosphate release or ^{32}P labeling of PA and PI. All of these findings implicate the phospholipase A_2 pathway in the PAF actions. Ca^{2+}-channel antagonists, such as nifedipine, verapamil, and diltiazem, inhibited the PAF-induced AA liberation in a dose-dependent manner, probably by interfering with PAF binding to its receptor. More recently, PAF was found to have no effect on inositol phosphate release in hepatocytes (117). Recently, Hwang et al. (297), working with rabbit platelet membranes, reported that the PAF receptor may be coupled with the adenylate cyclase system via an inhibitory GTP regulatory protein.

Further support for the involvement of phospholipase A_2 in AA release from phosphoinositides comes from the Ca^{2+} and Ca^{2+} ionophore studies (for references, see table 4). This is in contrast to the PPI response which does not require extracellular Ca^{2+}. In contrast to phospholipase C (see section III C 2) which is activated by μM concentration of Ca^{2+}, phospholipase A_2 requires millimolar concentrations of Ca^{2+} (596, 330, 611). Although calmodulin has been implicated in the stimulation of phospholipase A_2 (121, 641, 423, 422), more recently Ca^{2+} activation of this enzyme was found to be independent of calmodulin in pancreas (635), platelets (636, 616a), and liver mitochondria (165).

It can be concluded from the above studies that both the phospholipase C and phospholipase A_2 pathways (fig. 8) are probably involved in the liberation of AA from phosphoinositides in cells stimulated with Ca^{2+}-mobilizing agonists. However, neither the relationship between the two pathways, nor the relative amounts each contributes to AA liberation are clear. The Ca^{2+} recruited by IP_3 as a result of activation of Ca^{2+}-mobilizing receptors could liberate AA from phosphoinositides via phospholipase A_2 activation (fig. 4).

c. EFFECTS OF PHOSPHOLIPASE INHIBITORS. Although several inhibitors have been employed in investigations on the role of phospholipases responsible for AA liberation from phospholipids, most of these compounds were found to have other effects on metabolism of the cell beside inhibiting AA liberation. Thus, the cationic am-

phiphilic drug mepacrine, which was originally employed by Flower and Blackwell (219, 77) in their studies on the importance of phospholipase A_2 in AA release and PG synthesis in slices of guinea pig spleen, has more recently been shown to interfere with the metabolism of phospholipids and AA in various tissues including: platelets (275); erythrocytes (167); and iris smooth muscle (13, 14). Similarly, indomethacin, a potent inhibitor of the cyclooxygenase pathway, can inhibit phospholipase A_2 in the test tube depending on Ca^{2+} concentrations (224). In another study, inhibition by this drug of DG lipase has been correlated with the accumulation of DG in the platelet (499), but in the same cell, it also inhibits the release of AA from phosphatidylcholine (500). Another lipase inhibitor, which was employed recently by several investigators to evaluate the relative contribution of the DG lipase pathway to the release of AA, is RHC 80267 {1,6-di[0-(carbamoyl)cyclohexanone]hexane}, an inhibitor of DG lipase activity in microsomes from canine platelets (569). However, various investigators working on the effects of this compound on phospholipases and lipases found it to inhibit DG lipase (118), DG- and monoglyceride lipases (92), and the activities of DG lipase, phospholipase A_2, phospholipase C, and cyclooxygenase (447) in stimulated platelets. Furthermore, it inhibited the activities of DG lipase and PG synthesis in the iris smooth muscle (654). In the majority of these studies, it was concluded that RHC 80267, because of its lack of specificity at concentrations needed to inhibit DG lipase, is an unsuitable inhibitor for studying the release of AA in intact tissues. Very recently, R59 022, a DG kinase inhibitor, was found to inhibit DG kinase in human red blood cells (160). Furthermore, when the platelets inositol lipid turnover was accelerated by thrombin, further addition of R59 022 (6-[2-(4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo[3,2-α]pyrimidin-5-one) resulted in a marked elevation of DG levels, a decreased formation of PA, and an increased C-kinase activity as compared with the controls. It remains to be established whether these effects can be demonstrated in other tissues, and whether this compound will have an effect on AA liberation from phosphoinositides in stimulated cells.

The above findings indicate that the use of phospholipase inhibitors in studies designed to establish the pathway(s) responsible for AA liberation in eicosanoid synthesis should be viewed with caution.

In summary, while the role of phospholipases in the release of AA from phosphoinositides and other phospholipids has been investigated extensively in the past decade, their regulation and precise relationships to Ca^{2+}-mobilizing receptors and the "arachidonate cascade" remain unclear. It is possible that the increase in $[Ca^{2+}]_i$ which results from the activation of these receptors could activate phospholipase A_2 to release AA from phosphoinositides and other phospholipids depending on the type of cell. This could explain the relationship between enhanced phosphoinositide turnover and the release of AA and eicosanoid biosynthesis. It is not yet clear how much each of the DG lipase pathway, which is under the control of Ca^{2+}-mobilizing receptors, and of the phospholipase A_2 pathway contributes to the AA liberated, nor is it clear which phospholipid fraction contributes most of the AA liberated. Development of more specific phospholipase inhibitors will help to resolve these issues. More recently, Rittenhouse (496) has presented evidence that phospholipase A_2 is the first phospholipase activated when platelets are stimulated by Ca^{2+} ionophore A23187 and that PGE_2 produced from the released arachidonate is responsible for the activation of phospholipase C. These observations need to be confirmed in other tissues. Among the functions of a controlled AA release from membrane phosphoinositides and other phospholipids are the regulation of the eicosanoid level in the cell, which in turn plays an essential role in the control of numerous physiological processes (640, 168, 391, 513, 74), and the activation of certain key enzyes, such as guanylate cyclase (459, 235) and C-kinase (407, 427, 253b, 253c). Furthermore, AA and its metabolites have been reported to be involved in Ca^{2+} mobilization in both cellular and subcellular fractions (209, 353, 326). More recently, data were presented which suggest that Ca^{2+}-mobilizing receptors mediate cyclic GMP formation by involvement of arachidonate and lipooxygenase without a rise in intracellular Ca^{2+} (555). Thus, it is not unreasonable to conclude from the above that the enhanced phosphoinositide turnover serves as a signal-generating system producing the two intracellular putative second messengers, IP_3 and DG, and liberating AA for eicosanoid biosynthesis.

D. Synergistic Interactions between 1,2-Diacylglycerol and Inositol 1,4,5-Trisphosphate, the Two Arms of the Polyphosphoinositide Cycle

Several investigators have pointed out that the IP_3 and second messenger systems could interact either cooperatively or synergistically to affect the metabolism and physiological actions of these messenger molecules (59, 444, 484, 270). A synergistic interaction between Ca^{2+} and C-kinase has been reported to underlie the agonist-induced changes in various cell activities. It has been suggested that an important consequence of having this bifurcating signal pathway is that it provides the versatility necessary to introduce subtle variations in the control mechanisms (59). Thus, for example, one pathway phosphorylates proteins by activation of calmodulin by the elevated $[Ca^{2+}]_i$ brought about by the IP_3-induced Ca^{2+} release mechanism, and other pathway phosphorylates proteins by activation of C-kinase (fig. 9). Release of AA could also add more versatility to this signalling system. The following description of platelet aggregation and smooth muscle contraction will serve to illustrate

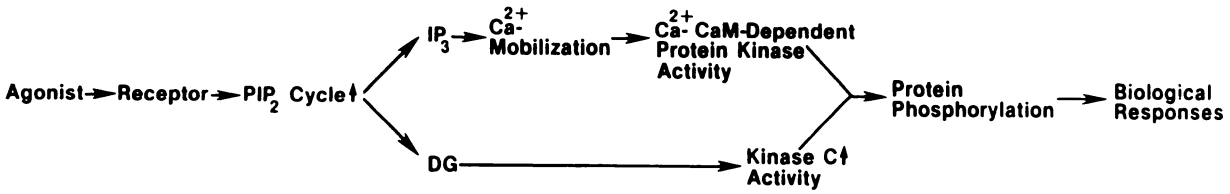


FIG. 9. Scheme showing the synergistic interaction which could occur between the pathway that phosphorylates proteins by activation of calmodulin (*CaM*) by the elevated $[Ca^{2+}]_i$ brought about by the IP_3-induced Ca^{2+} release mechanism, and the pathway that phosphorylates proteins by activation of C-kinase.

the synergistic interactions between the Ca^{2+}- and DG-signalling pathways.

1. Platelet aggregation. Nishizuka and coworkers have proposed that two events—an increase in DG (which activates C-kinase) and an increase in cytosolic Ca^{2+}—may be involved in stimulus-secretion coupling in platelets and other cell types (444, 270, 158). Thus, when the platelets are incubated with thrombin or collagen, two endogenous proteins with molecular weights of 40,000 and 20,000 are rapidly and heavily phosphorylated concomitantly, in association with the release of various constitutents of platelets, such as serotonin. However, if only the synthetic DG, OAG, is added to the medium, only the M_r 40,000 protein is phosphorylated, implying activation of C-kinase. On the other hand, if Ca^{2+} ionophore 23187 is added separately, the M_r 20, 000 protein (myosin light chain, MLC) is phosphorylated, indicating that calmodulin (CaM)-dependent phosphorylation of this protein requires the mobilization of Ca^{2+}. In neither case does serotonin secretion from platelets occur. However, when these cells are incubated with DG or TPA in the presence of ionophore A23187, serotonin release was dramatically enhanced. These findings provided the first indication of a synergistic interaction between these two signal pathways. It remains to be established how the phosphorylation of these two proteins is involved in causing the full physiological responses.

The synergism of C-kinase activation and Ca^{2+} mobilization has later been demonstrated in systems by elevating $[Ca^{2+}]_i$ with ionophore A23187 and by stimulating C-kinase with phorbol ester or OAG. In most of these studies, neither agent alone produces maximal biological response seen on stimulation with the physiological agonist, but when the two were added together, a maximal response was achieved. Synergism between phorbol esters and ionophore A23187 has been recently extended to many other cell systems, such as the release of lysosomal enzymes from neutrophils (327), catecholamine release from bovine adrenal medullary cells (349), aldosterone secretion from porcine adrenal glomerulosa cells (351), amylase secretion from pancreatic acinar cells (161), ACh release from ileal nerve endings (573), histamine release from mast cells (332), insulin secretion from rat pancreatic islets (657), glycogenolysis in rat hepatocytes (201, 230), concanavalin A-induced activation of bovine lymphocytes (398), superoxide production by human neutrophils (461), and AA release and production of PA in rabbit neutrophils (606). All of these observations indicate that C-kinase and Ca^{2+} mobilization can act synergistically to elicit physiological responses.

2. Smooth muscle contraction. Another example where synergism between the two arms of the PPI cycle could play an important role is in smooth muscle contraction. According to this, the agonist-induced hydrolysis of PIP_2 at the plasma membrane could bring about the following events (fig. 10): (*a*) release of IP_3, followed by: Ca^{2+} mobilization from the SR, increases in cytosolic free Ca^{2+}, activation of MLC kinase by Ca^{2+}-CaM to phosphorylate the MLC proteins and consequently leading to muscle contraction; and (*b*) formation of DG, followed by: activation of C-kinase, phosphorylation of MLC kinase (299, 441, 593, 442), MLC protein (194, 440, 299), or other proteins such as the actin-binding proteins, vinculin and filamin (335), and consequently muscle contraction. The contraction of smooth muscle in response to various agonists is usually composed of two phases: a fast "phasic" component followed by a slower more sustained "tonic" component. This biphasic response has been attributed to a dual source of Ca^{2+} in smooth muscle (595, 557, 83, 503, 268, 445). According to this hypothesis, the phasic component would result from Ca^{2+} release from intracellular stores, and the tonic component would be a consequence of Ca^{2+} influx across the cell plasma membrane. It is possible that IP_3-induced Ca^{2+} release from the SR is associated with the phasic response, while DG activation of C-kinase is involved with the tonic response. The above conclusions are based on the following findings in smooth muscle reported by several investigators. (*a*) Ca^{2+}-mobilizing agonists provoke the phosphodiesteratic breakdown of PPI in a wide variety of smooth muscles (for references, see table 2), and IP_3 has been demonstrated to mobilize Ca^{2+} from the SR in a number of smooth muscle cell types (for references, see table 3). (*b*) There is general agreement that activation of myosin filaments by actin in smooth muscle requires phosphorylation of MLC proteins by MLC kinase that is activated upon the binding of Ca^{2+} to CaM (for review, see refs. 18, 255, and 329). However, while myosin phosphorylation certainly plays a prominent role, it may not be the only Ca^{2+}-dependent regulatory mechanism operating during the sustained phase of smooth muscle contraction (329, 185, 428). (*c*) A close

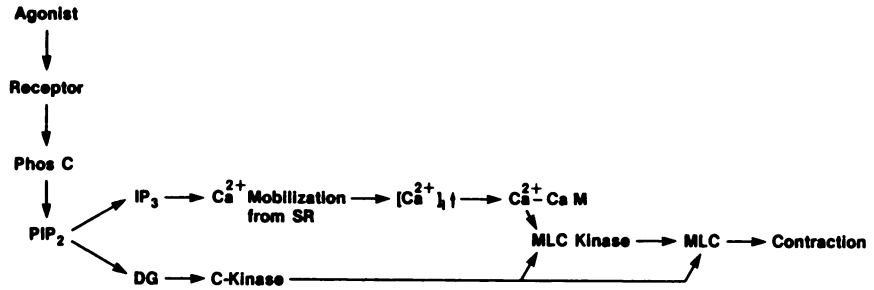


FIG. 10. Scheme showing a possible synergism between the two arms of the polyphosphoinositide cycle in eliciting the tonic phase of smooth muscle contraction. *CaM*, calmodulin; *MLC*, myosin light chain protein. See text for detail.

relationship between CCh-induced IP_3 accumulation, MLC phosphorylation, and muscle contraction in the iris sphincter muscle has recently been reported (9, 10, 294b). In another study, Doctrow and Lowenstein (170), working with a subcellular fraction prepared from calf aorta smooth muscle which contains contractile proteins and membranes, showed that adenosine and certain of its analogues, which decrease contraction in this muscle, decrease also the phosphorylation of PI and MLC, implying a possible relationship between PPI metabolism and MLC phosphorylation. Honeyman and coworkers (110) correlated NE-induced phosphoinositide hydrolysis with the agonist-induced Ca^{2+} flux and contraction in rabbit aorta. They demonstrated a close correlation between agonist-stimulated PIP_2 hydrolysis and stimulated Ca^{2+} release, and furthermore, they showed that the time course of these events parallels the time course of the rapid phase of tension development in the contractile response of this muscle. (*d*) Smooth muscle is enriched with C-kinase (357), and the phosphorylation of MLC kinase (299, 441, 593, 442) and of MLC protein (194, 440, 299) by this enzyme has been demonstrated. (*e*) Finally, synergism between Ca^{2+} and activators of C-kinase, such as phorbol esters, in tracheal smooth muscle contraction (457, 407b) and in other smooth muscles (483, 221, 44, 154, 294b, 407b) has recently been reported. Thus, perfusion of rabbit ear arteries with solutions containing the phorbol ester, TPA (10 to 333 nM), led to slowly developing, sustained, Ca^{2+}-dependent contractions. In these studies on the rabbit ear arteries, the muscle was exposed to TPA for 30 to 70 min. In more recent studies, Park and Rasmussen (457) investigated the effects of the ionophores A23187 and ionomycin, Ca^{2+} channel agonist (BAY K8644), and C-kinase activators, PMA and mezerein, on bovine tracheal smooth muscle contraction. They reported that A23187 (5 μM) and ionomycin (0.5 μM) produced a prompt but transient contraction. C-kinase activators either produced no effect, e.g., PMA at 200 nM, or produced a rise in tension that was slow in onset but then gradually increased, e.g., mezerein at 400 nM. In contrast, ionophores and C-kinase activators, in combination, acted synergistically to produce a prompt and sustained contractile response that is reminiscent of that observed in response to CCh. These observations are in accord with the hypothesis that activation of C-kinase by DG, the other arm of the PPI cycle (fig. 10), is probably involved in the tonic phase of smooth muscle contraction. The above findings were criticized on the basis of multiplicity of the effects that phorbol esters have on drug-induced effects (557). Thus, it was argued, for example, that the adenylate cyclase activity of duck erythrocytes is uncoupled from the β-receptors by phorbol diester-induced activation of C-kinase that phosphorylates the β-adrenergic receptor (544).

Phorbol esters have been shown to inhibit α-adrenergic-mediated contraction of rat aorta (154) and to cause a marked decrease of NE stimulation of inositol phospholipid metabolism and a 3-fold decrease in agonist affinity for ^{125}I-HFAT (2-[β-(4-hydroxyphenyl)-ethylaminomethyl]tetralone) binding to α_1-adrenoceptors in the intact smooth muscle cells (147). Phorbol esters have also been reported to have multiplicity of effects on PPI metabolism in stimulated cells (see section V C 2 c).

It can be concluded from the above studies that there is reasonable experimental evidence which suggests that a synergistic interaction of C-kinase activators and Ca^{2+} on C-kinase could be involved in the tonic phase of smooth muscle contraction. Stimulated PIP_2 hydrolysis may elevate $[Ca^{2+}]_i$ by rapidly causing its release, from the SR, which in turn provides an effective environment for DG activation of C-kinase. However, there is a need to conduct more correlative studies on the effects of Ca^{2+}-mobilizing agonists and C-kinase activators on protein phosphorylation, PPI hydrolysis, and contraction before we can say with certainty that IP_3 and DG, the two arms of the PPI cycle, interact synergistically to elicit the contractile response in smooth muscle.

E. Receptor Supersensitivity and Subsensitivity

After chronic interruption of neurotransmission by surgical or chemical denervation procedures, a variety of peripheral organs and tissues exhibit an exaggerated response to submaximal concentrations of the normal neurotransmitter substance. In smooth muscle, the postjunctional denervation supersensitivity is manifest as leftward shifts of the dose-response curves of a number of unrelated excitatory drugs and ions (for review, see ref. 218a). Unlike in skeletal muscle, the denervation

supersensitivity that occurs in smooth muscle is not due to an increase in the number of receptors controlling muscle contraction. Previously we suggested that a post-receptor mechanism response for the supersensitivity of surgically denervated iris dilator muscle is an enhanced receptor-stimulated breakdown of PIP_2 into DG and IP_3 (7, 26). Janowsky et al. (314a) reported that sympathetic denervation increases α_1-adrenoceptor-mediated IP accumulation in rat hippocampus. More recently, we suggested that, in the iris smooth muscle, adrenergic desensitization is probably associated with attenuation of α_1-adrenergic receptor stimulation of PIP_2 turnover (654a). Thus, desensitization of the rabbit eye with epinephrine resulted in attenuation of the agonist-stimulated breakdown of PIP_2 in the iris, accompanied by decreases in IP_3 accumulation, AA liberation, and PG release and muscle contraction. In contrast, sensitization of the iris, through surgical sympathetic denervation, resulted in potentiation of these biochemical and pharmacological responses (summarized in fig. 11). Phosphoinositide hydrolysis does not desensitize in 1321 N1 astrocytoma cells (397a); in contrast, Lurie et al. (383a) reported that incubation of rabbit aorta ring segments with epinephrine (10^{-6} M) for 7 h resulted in a 10-fold loss in sensitivity of the tissue to α_1-adrenergic receptor-mediated contraction with no change in maximal force of contraction and no changes in receptor number or affinity; however, desensitization was associated with a blunting of α_1-receptor stimulation of PI turnover. More recently, Lefkowitz and coworkers (367a) reported a NE-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to stimulation of PI metabolism in the DDT_1 MF-2 hamster vas deferens smooth muscle cell line.

An increase in α_1-receptor IP_3 accumulation by NE in the sensitized smooth muscle could result in an increase in intracellular Ca^{2+} concentration and subsequently in contraction, while a decrease in α_1-receptor IP_3 accumulation in the adrenergically desensitized muscle may result in a decrease in intracellular Ca^{2+} concentration and subsequently in contraction (fig. 11). Possible mechanisms underlying the phenomenon of adrenergic sub- and supersensitivity in smooth muscle include changes in receptor number, changes in coupling through GTP-binding proteins, or changes in PIP_2 synthesis.

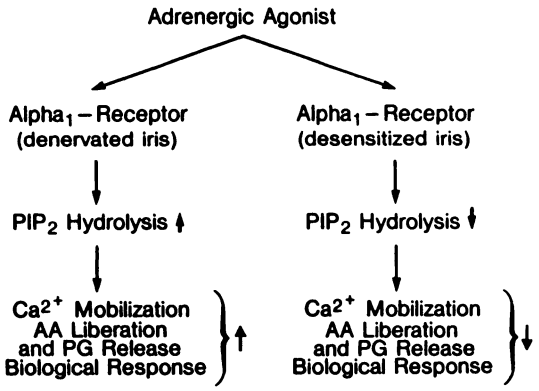


FIG. 11. Scheme showing the possible involvement of phosphoinositide hydrolysis in the mechanism of subsensitivity and supersensitivity in the iris smooth muscle.

VI. Summary and Conclusions

The objective of this review has been to summarize the advances made in the past decade in our understanding of the physiological roles of PPI and its derived second messenger molecules, namely IP_3, DG, and AA, in mediating the actions of neurotransmitters, hormones, and several other pharmacological agents which function through mobilization of Ca^{2+} in a wide variety of tissues. The background for this topic, including historical developments, distribution and metabolism of PPI, emphasizing the key enzymes involved in generating the second messenger molecules, and the biochemical and pharmacological characterization of the PPI response, was reviewed. A scheme showing PIP_2 breakdown mediating the step between activation of Ca^{2+}-mobilizing receptors and cellular responses was proposed (fig. 4). The first step in agonist-induced stimulation of phosphoinositide turnover is the phosphodiesteratic cleavage of PIP_2, via phospholipase C, to yield IP_3 and DG. At later time intervals, there is a loss of PI. The loss of PI, which was the object of intense study for decades, appears now to be due (*a*) to its conversion, through phosphoinositide kinases, to PIP_2 rather than to a direct phospholipase C-mediated cleavage, although there is disagreement on this point, and (*b*) to its deacylation through phospholipase A_2 to liberate AA for eicosanoid biosynthesis. The key enzymes involved in the PPI cycle, namely, the phosphoinositide kinases, phospholipase C, and the myo-inositol polyphosphate phosphatases, have recently been shown to reside also at the plasma membrane, in addition to being cytosolic as well as associated with other subcellular organelles. The rapid loss of PIP_2 at the plasma membrane by agonist stimulation enhances the metabolic conversion of PI and PIP to PIP_2. The intracellular concentrations of IP_3 and DG are determined by the rate of their production by phospholipase C and the rate of their degradation by IP_3 phosphatase and DG lipases (alternatively by DG kinase), respectively. There is little information available on the absolute concentrations of phosphoinositides and the inositol polyphosphates in resting and stimulated cells. There is a lack of information on how the activities of these enzymes are regulated, and thus the development of pharmacological inhibitors for the key enzymes involved in phosphoinositide metabolism is urgently needed. There is good evidence for the involvement of GTP-binding proteins in the coupling of various Ca^{2+}-mobilizing receptors to phospholipase C and Ca^{2+} mobilization, and thus an important objective in the future will be to characterize regulatory proteins involved in the receptor-stimulated hydrolysis of PPI.

Receptor-stimulated hydrolysis of PIP_2 does not appear to be involved in Ca^{2+}-gating, and the partial re-

quirement (i.e., potentiation of the PPI response by the cation) for Ca^{2+} in eliciting this phenomenon in some tissues occurs probably at the phospholipase C level. In certain systems, such as liver plasma membranes and permeable GH_3 cells, GTP and its analogues appear to act by decreasing the Ca^{2+} requirement of the phospholipase C as well as increasing its activity at maximal Ca^{2+} concentrations. Although induced conformational changes and/or increase in membrane fluidity which follows PIP_2 breakdown could bring about permeability changes, a role for the stimulated PIP_2 breakdown at the plasma membrane has not yet been identified. Instead, in several systems it has been established that the appearance of IP_3, following receptor activation, precedes the elevation of $[Ca^{2+}]_i$. There is now compelling evidence which indicates that IP_3 can release Ca^{2+} from the ER, at least in permeabilized cells. Very recently, receptors for IP_3 have been reported in microsomes and in permeabilized hepatocytes and neutrophils. In smooth muscle the release of Ca^{2+} from the SR by IP_3 results in the initial elevation of $[Ca^{2+}]_i$ which couples receptor activation to the phasic component of contraction. This elevated level of $[Ca^{2+}]_i$ is maintained by the agonist-induced increase of influx of extracellular Ca^{2+}, which sustains the tonic component of contraction. The mechanism of control of extracellular Ca^{2+} influx is still unclear. There is a need to clarify the roles, if any, of cyclic IP_3 and IP_4 in Ca^{2+} signalling, and to show whether or not the receptors reported for IP_3 on the ER surface are also receptors for all of these myo-inositol polyphosphates. The source of myo-inositol 1,3,4-triphosphate appears now to originate from myo-inositol 1,3,4,5-tetrakisphosphate (IP_4), a compound formed from IP_3 via IP_3-3-kinase. The physiological significance of inositol 1,3,4-P_3 and inositol 1,3,4,5-P_4 has not yet been determined. There is a need to develop methods for introducing IP_3 and other inositol polyphosphates into intact cells and tissues under physiological conditions; this will then allow correlative studies on the effects of agonists and of IP_3 on Ca^{2+} release from the ER and on Ca^{2+}-dependent biological responses in various cell types.

Concomitant with the effects of IP_3, phosphorylation of proteins involved in biological responses may be stimulated by DG, via activation of C-kinase. C-kinase can also act as the mobile receptor for tumor-promoting phorbol esters. In many cell types, a synergism of Ca^{2+} and DG has been demonstrated in bringing about full physiological responses such as secretion and exocytosis. A synergism of Ca^{2+} and DG has not yet been unequivocally demonstrated in smooth muscle as causing contraction. Although the DG formed by phosphoinositide breakdown is usually stearate at C_1 and AA is at C_2, there is evidence which now indicates that an unsaturated fatty acid is not required in DG for C-kinase activation. Little work has been done on demonstrating whether C-kinase is activated during stimulation of Ca^{2+}-mobilizing receptors in intact tissues, and furthermore there is a need to identify the protein substrates of C-kinase which may be responsible for the enhanced biological responses. In spite of this, there is mounting evidence which indicates that DG acts as a putative second messenger linking receptor activation by Ca^{2+}-mobilizing agonists to activation of C-kinase. Both Ca^{2+} mobilization and DG-induced C-kinase activation are consequences of the action of the Ca^{2+}-mobilizing agonist, and both are coupled to the receptor-stimulated hydrolysis of PIP_2 (fig. 4).

The second proposed function for DG is that it can be degraded sequentially via lipases to release AA for eicosanoid biosynthesis. While the DG lipase/monoglyceride lipase pathway has been amply demonstrated in platelets, both this pathway (via phospholipase C) and phospholipase A_2 are involved in the liberation of AA from phosphoinositides in cells stimulated with Ca^{2+}-mobilizing agonists. However, neither the relationship between the two pathways, nor the relative amounts each contributes to AA liberation are well understood at the present time. Since phospholipase A_2 requires much higher concentrations of Ca^{2+} for activation than phospholipase C, the Ca^{2+} recruited by IP_3 as a result of activation of Ca^{2+}-mobilizing receptors may activate this enzyme to liberate AA from phosphoinositides and other phospholipids. This will then allow phospholipase A_2 to express its role as an amplifying system through the formation of the eicosanoids. Development of more specific phospholipase inhibitors will help to resolve some of these issues.

The use of Li^+ to inhibit the degradation of IP, by IP phosphatase, that accumulates following activation of Ca^{2+}-mobilizing receptors has increased the sensitivity of the assay for the PPI response. However, Li^+ has also been reported to exert some inhibitory effects on the phosphatases which degrade IP_2 and IP_3, and thus there is a need to investigate these effects in various tissues. Furthermore, the effects of Li^+ and Li^+ plus agonists on the inositol-containing phospholipids, both in vivo and in vitro, remain uninvestigated. Development of better methodology, such as the use of high-pressure liquid chromatography, for the determination of phosphoinositides, IP_3, the various isomers of myo-inositol polyphosphates, and DG will help advance our understanding of the roles of PPI and their derived messenger molecules in signal transduction at the plasma membrane and in the physiological functions of Ca^{2+}-mobilizing receptors. The use of the PPI response as a tool to characterize receptors and their subtypes and to investigate the dose-response relationships for pharmacological agents in both excitable and nonexcitable tissues will increase in the coming years as we develop more sensitive methods for its assay, develop better pharmacological tools for probing the various reactions involved in the PPI cycle, develop more specific receptor antagonists, and gain

more understanding of its metabolic and pharmacological control.

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