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Topical Review

The Role of Phosphoinositides in Signal Transduction

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Introduction

The binding of agonists to protein receptors at the cell surface is well established, but the mechanism by which the "message" generated at the receptor is transferred to the inside of the cell is not clearly understood for all types of stimuli. One class of receptors mediates its response through formation of cAMP, catalyzed by adenylate cyclase (a protein present on the cytoplasmic side of the membrane). The coupling of adenylate cyclase to the receptor is further regulated by GTP-binding proteins [186, 258]. It has been suggested that conversion of phosphatidylethanolamine to phosphatidylcholine plays a role in the coupling of receptors to adenylate cyclase [120], although evidence against this hypothesis has also been presented [216; *see* 202].

Another class of receptors mediates its response through Ca^{2+} mobilization. Evidence is rapidly mounting that a specific class of phospholipids, i.e., the phosphoinositides, plays an important role in signal transduction from the receptors at the plasma membrane.

There have been numerous recent reviews on the various aspects of phosphoinositide metabolism and its role in signal transduction [*see* 1, 3, 23, 25, 71, 81, 82, 88, 89, 118, 119, 125, 148, 197, 207, 211, 220, 237, 239]. The emphasis of this review will be to analyze the recent developments[1] and how they relate to earlier observations.

Early Observations

In animal cells, there are three myoinositol-containing phosphatides: phosphatidylinositol [1-(3-*sn*-phosphatidyl)-D-*myo*-inositol) (PI)], which usually accounts for well over 90% of the total inositol lipid, phosphatidylinositol-4-phosphate or diphosphoinositide (PIP), and phosphatidylinositol-4,5-bisphosphate or triphosphoinositide (PIP_2) [*see* 1, 3, 25, 71, 119, 206].

It was observed more than three decades ago [132] that stimulation of enzyme secretion in pigeon pancreas slices by acetylcholine (ACh) or carbamylcholine [132, 133] or cholecystokinin/pancreozymin [127] was associated with an enhanced incorporation of $^{32}P_i$ into the total phospholipid fraction. Later, the phospholipids showing the enhanced incorporation in pigeon pancreas were identified as PI (15-fold increase) and phosphatidic acid (PA) (threefold increase) [126, 128]. Similar "phospholipid effects" were shown in a variety of other tissues within the next two decades [*see* 124, 135, 206]. An increase in the incorporation of [^{3}H]inositol into PI on stimulation was also observed in pancreas [128], brain [129], and many other tissues [*see* 206].

If avian salt gland slices were incubated with $^{32}P_i$ in the absence and presence of ACh, the incorporation of ^{32}P into PA and PI was increased 15-fold and threefold, respectively, by ACh [130, 135]. Detailed kinetic studies of the turnover of PA and PI under various conditions revealed several interesting features. If ACh was added to salt gland slices preincubated with $^{32}P_i$, a fraction of PA amounting to no more than 20% of the total PA became rapidly labeled and continued to turn over [130, 135]. Under these conditions, in contrast to the addition of ACh and $^{32}P_i$ at zero time, there was only a small increase in radioactivity in PI due to ACh [135], presumably as a result of simultaneous breakdown of prelabeled PI and its increased turnover (*see below*). If the action of ACh was blocked with atropine, the renewing fraction of PA rapidly disappeared and was accompanied by a rapid spurt in radioactivity in PI [135]. The rise in radioactivity in PI was quite close to the loss in radioactivity in PA.

Key Words phosphoinositides · phosphatidylinositol · inositol-1,4,5-trisphosphate · diacylglycerol · arachidonic acid · calcium

[1] This review covers material up to January 30, 1985.

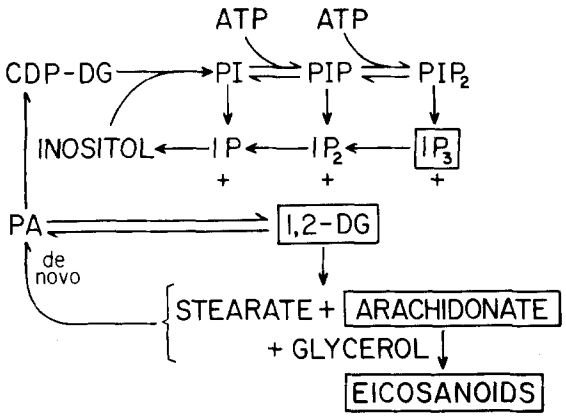

Fig. 1. The phosphoinositide cycle. The bordered substances are second messengers. *CDP-DG*: cytidine diphospho-diglyceride. Other abbreviations are given in the text. (Reproduced by permission of Annual Reviews, Inc.)

A similar rise in radioactivity in PI could be seen with [^{3}H]myoinositol. If the blocking action of atropine was overridden by addition of a concentration of ACh 33 times higher than the initial ACh concentration, the labeled PI which had been formed on adding atropine disappeared and PA gained the label lost from PI. This latter observation was the first demonstration of PI "breakdown" on stimulation with agonist.

If albatross salt gland slices were incubated without and with ACh, the average PI-phosphorus in the stimulated tissue was 20% lower than that in the control, but this difference did not quite reach statistical significance [130]. However, later calculations showed that if the percentage difference between controls and ACh-stimulated values were taken for each animal and these differences averaged, there was a 40% decrease in PI-phosphorus which was statistically significant [*see* 138]. These chemical studies on PI breakdown confirmed the radioactive studies showing PI breakdown.

On the basis of these studies, a scheme was proposed, called the "phosphatidylinositol-phosphatidate cycle" [135, 136] (Fig. 1). According to this model, on stimulation with ACh, PI breaks down to diacylglycerol (DG), catalyzed by PI phosphodiesterase [62, 162], and DG is phosphorylated from ATP to form PA [134]. On removal of ACh, PA is converted back to PI by the sequential actions of CTP-DG cytidyl transferase and PI synthetase [4, 227]. The important feature of this cycle is that at least a certain fraction of the DG moiety is conserved, and it is only the head group which undergoes turnover. Support for such a mechanism in salt gland was obtained by the lack of enhancement of labeled glycerol incorporation during stimulated PI turnover [135].

In exocrine pancreas [128], stimulation led to increased incorporation of labeled glycerol into PI and PA, indicating enhanced *de novo* synthesis. A similar increase in labeled glycerol incorporation into PI has also been observed in thyroid [97]. Breakdown of prelabeled PI to its individual building blocks, an essential observation to explain enhanced glycerol incorporation, has recently been reported in exocrine pancreas [67]. Recent observations suggest that enhanced glycerol and phosphate incorporation may involve different molecular species of PI. While agonist-stimulated ^{32}P incorporation in both PI and PA were observed mainly in the tetraene species, the [^{3}H]glycerol incorporation was predominantly elevated in molecular species of PI and PA other than tetraene [294].

The phospholipid effect, as described above, where breakdown of phosphoinositides is followed by an enhanced resynthesis, is probably different from the stimulated net synthesis of phosphoinositides by trophic hormones [*see* 88, 89].

Functional Significance

In 1975, Michell [206] noted a correlation between the Ca^{2+} mobilizing action of certain agonists and the PI effect in various tissues. In addition to this, the independence or only partial dependence of the PI effect on Ca^{2+} in most of the tissues which had been studied at that time, as well as stimulation by Ca^{2+} ionophore of the physiological response but not of PI breakdown, led him to suggest that the PI effect may be antecedent and causally related to Ca^{2+} mobilization [*see* 206]. In the years following 1975, the list of stimuli which mediate their response through Ca^{2+} mobilization and which are also capable of producing the PI effect has been extended to a large number of tissues [*see* 1, 23, 207], thereby indirectly supporting the Ca^{2+}-gating hypothesis. Support for the Ca^{2+}-gating hypothesis, although circumstantial, came from the blowfly salivary gland, where stimulation with 5-hydroxytryptamine (5HT) caused PI loss and the entry of Ca^{2+} into the epithelial cells [83]. When a supramaximal concentration of 5HT was used, this effect was followed by receptor desensitization and abolition of Ca^{2+} uptake. Incubation of washed glands with myoinositol restored both PI sensitivity to 5HT and Ca^{2+} transport [84]. Recently, it has been shown that recovery of Ca^{2+} gating in blowfly salivary gland is dependent on the synthesis of both PI and PIP_2 [267].

In spite of all the circumstantial evidence suggesting a role for PI turnover in Ca^{2+} gating, some of the following points did not neatly fit into this hypothesis: (a) nonparallel dose-response curves

for the PI effect and the physiological response [133, 153]; (b) longer time period for the PI effect to be elicited; (c) Ca^{2+} dependence of the PI effect in certain tissues; (d) the endoplasmic reticulum (ER) as the site of most of the PI effect [250], as shown by [^{3}H] inositol autoradiography and differential centrifugation [99, 137, 138, 250]; (e) unanswered questions concerning the mechanism of Ca^{2+} mobilization.

The first question was explained by a good correlation between receptor occupancy and the PI effect and that maximum physiological responses occur before all receptors are occupied by agonist [210; *see* 206, 211].

Some of the remaining four points could be more satisfactorily answered by a recent modification of the "Ca^{2+}-gating" hypothesis [*see* 25, 71, 207, 211]. According to this, polyphosphoinositide (most probably only PIP_2) rather than PI breakdown is the primary event, and this may play a role in mobilization of cytosolic Ca^{2+} (Ca_c^{2+}) from intracellular stores [291; *see* 25, 125] rather than Ca^{2+} gating at the plasma membrane (*see below*).

Polyphosphoinositides

There have been several reviews in recent years dealing mainly with the various aspects of polyphosphoinositide metabolism and its significance *vis-a-vis* receptor activation [1, 25, 71, 119, 125].

Folch, working with brain lipids, was the first to identify a phosphoinositide fraction, which, on the basis of chemical analysis, appeared to be a "diphosphoinositide" [94, 95]. When more sophisticated chromatographic methodology became available, it was shown that PIP and PIP_2 were present in addition to PI [105]. In the earlier nomenclature, these compounds were referred to as diphosphoinositide and triphosphoinositide. The deacylation products of these two polyphosphoinositides were fully characterized as 1-(α-glycerylphosphoryl)-L-myoinositol 4-phosphate and 1-(α-glycerylphosphoryl)-L-myoinositol 4,5-diphosphate, respectively [37].

The rapid turnover of polyphosphoinositides was first described in brain in 1962 [38] and shortly after that in non-neural tissue [271]. In a brain cytoplasmic particulate fraction, muscarinic receptor stimulation with ACh caused a 30% loss in "spot C" [29] (later identified as PIP) [271]. In avian salt gland slices, ACh depressed the steady-state levels of PIP and PIP_2 [271]. Durell and associates [78, 79] also observed the formation of inositol mono and bisphosphate in synaptosomes in response to ACh, and they suggested that the primary reaction in response to ACh was phosphodiesteratic cleavage of PIP and PIP_2. In the late seventies, muscarinic cholinergic or noradrenergic stimulation of iris smooth muscle was found to cause the breakdown of PIP_2 within 5 min [2], and this was accompanied by an increase in inositol-1-phosphate (IP), inositol-4,5-bisphosphate (IP_2), and inositol-1,4,5-trisphosphate (IP_3) [6]. Because of the Ca^{2+} requirement of this response [2] and due to the ability of a Ca^{2+} ionophore to mimic the above breakdown, it was not considered as a candidate for Ca^{2+} mobilization.

Unlike PI, which is primarily distributed in intracellular membranes, in particular the ER, polyphosphoinositides are considered to be located in the cytoplasmic side of the plasma membrane. This is based on the localization of Mg^{2+}-dependent PI kinase and PIP kinase in the plasma membrane [71, 98, 114, 131, 209, 243]. Besides the plasma membrane, these kinases have been shown to be present in Golgi membrane fractions of kidney [53], secretory granule membranes [303], and chromaffin granules of adrenal medulla [183]. The localization of polyphosphoinositides at the plasma membrane (specifically at the cytoplasmic side) places them in the right "geographical" location so that they can play a role in signal transduction. The recent revival of interest in polyphosphoinositides began with the finding that Ca^{2+}-mobilizing agonists, such as vasopressin in liver, caused a very rapid breakdown of polyphosphoinositides, particularly PIP_2 [54, 164, 211]. Unlike the effect in iris smooth muscle reported earlier [2], the breakdown of polyphosphoinositides in liver was independent of or only partially dependent on Ca^{2+} [54, 280].

The rapid breakdown of polyphosphoinositides has been confirmed for liver [252, 302] and has also been reported in several other tissues [247] (*see* Tables 1 and 2). In some of the tissues, such as parotid gland [238, 318, 319], platelets [30, 240], superior cervical sympathetic ganglia [36], hepatocytes [54, 87, 165, 280], and human fibroblasts [307], the rapid decrease in PIP_2 was independent of Ca^{2+}. The ionophore-mediated breakdown of polyphosphoinositides in rabbit iris smooth muscle [2] and platelets [255] has now been shown to be a secondary effect due to the release of norepinephrine [7] and cyclooxygenase products [255], respectively. Based on the Ca^{2+} requirements for the phosphoinositide effect in various tissues, it is now generally believed that under physiological conditions of ionic strength and pH the Ca^{2+} concentration required for phosphodiesteric cleavage of at least PIP_2 are at or below resting Ca_c^{2+} (0.1 μM). Therefore, Ca_c^{2+} is not rate limiting for PIP_2 cleavage [149, 170, 197, 211, 245] although it may be rate limiting in some cases of PI and PIP cleavage [125, 170, 197]. It is believed by many that the loss in PI is entirely due to its phosphorylation to polyphosphoinositides rather

Table 1. Tissues where phosphoinositide breakdown, primarily that of polyphosphoinositide, has recently been demonstrated

Tissue	Stimuli	Time	PI	PIP	PIP_2	Reference
Adrenocortical fasciculata cell	Angiotensin II	30 sec	ND[a]	↓[b,c]	↓[c]	[109]
Iris smooth muscle	Carbachol	30 sec	→[d]	→	↓	[7]
Retina	Light	5 & 15 sec	→	→	↓	[102]
NG 108-15 cell	Bradykinin	30 sec	→	→	↓	[325]
Mast cell	Antigen	5 sec	↓	↓	→	[152]
Lacrimal acinar cell	Methacholine	<1 min	→	↓	↓	[103]
Granulosa cell	Gonadotropin-releasing hormone	30 sec	—↑[e]	↓	↓	[59]
Hepatocyte	Vasopressin	<1 min	ND	↓	↓	[54]
	Angiotensin	<1 min	ND	↓	↓	[54]
	α_1 Adrenergic	<1 min	ND	↓	↓	[54]
	ATP	<1 min	ND	↓	↓	[54]
	Vasopressin	30 sec	→	↓	↓	[252]
		15 sec	↓	↓	↓	[302]
Parotid gland	Carbachol	10 min	→	↓	↓	[72]
	Methacholine	1 min	ND	→	↓	[319]
	Norepinephrine	1 min	ND	ND	↓	[319]
	Substance P	1 min	ND	→	↓	[319]
Exocrine pancreas	Carbachol	<1 min	ND	→	↓	[240]
	Caerulein	<1 min	ND	→	↓	[240]
	Carbachol	30 sec	→	→	↓	[225]
	Pancreozymin	30 sec	→	→	↓	[225]
Islet	Glucose	15 min	↑	↓	↓	[77]
GH_3 pituitary cell	Thyrotropin-releasing hormone	10 sec	→	↓	↓	[198]
	Thyrotropin-releasing hormone	10 sec	↓	↓	↓	[193]
	Thyrotropin-releasing hormone	30 sec	↓	↓	↓	[248]
Platelet	Thrombin	10 sec	↓	→	↓	[30]
	Thrombin	5 sec	→	→	↓	[5]
	Thrombin	10 sec	↓	→	↓	[145]
	Thrombin	5–10 sec	ND	→	↓	[39]
	Thrombin	60 sec	→	→	→	[287]
	Thrombin	15 sec	→	↓	↓	[106]
	Thrombin	10–30 sec	↓	↓	↓	[251]
	AGEPC[f]	15 sec	↓	ND	ND	[285]
	AGEPC	10 sec	↓	→	↓	[286]
	Platelet-activating factor	10–60 sec	↑	↑	↓	[203]
	Platelet-activating factor	10 sec	↓	↓	↓	[31]
	ADP	30 sec	ND	↑	↓	[184]
Neutrophil	f-met-leu-phe[g]	30 sec	↓	↓	↓	[326]
	f-met-leu-phe	10–30 sec	→	↓	↓	[310]
	f-met-leu-phe	5 sec	↓	ND	ND	[279]
Leukocyte	leukocidin	30 sec	↓	↓	↓	[122]
	f-met-leu-phe	15 sec	↓	↑	↓	[70]
Insect salivary gland	5-Hydroxytryptamine	6 min	→	↓	↓	[24]

[a] Not determined.
[b] Decrease compared to control.
[c] In PIP and PIP_2, decrease was usually transient.
[d] No change.
[e] Increase after a lag period.
[f] 1-0-alkyl-2-acetyl-*sn*-glycerol-3-phosphorylcholine.
[g] Formyl-methionyl-leucyl-phenylalanine.

than direct action of phospholipase C on PI [5, 24, 25, 211]. This is based on the following observations: (a) faster breakdown of polyphosphoinositides as compared to PI; (b) earlier appearance of IP_3 and IP_2 as compared to IP; and (c) an energy requirement for PI disappearance.

An accumulation of IP_3 in amounts far exceeding the total amount of PIP_2 initially present and the energy requirement for PI disappearance clearly suggests that part of the PI is lost as a result of phosphorylation, but this does not completely rule out a direct breakdown of a portion of PI. Fain [82]

believes that PI as well as polyphosphoinositides located in the plasma membrane are capable of direct breakdown in response to receptor activation. This hypothesis is supported by the observation that some loss (10% of total) of PI could be observed in salivary gland homogenates on addition of 5-HT [86]. Two groups have reported a small stimulation in PI breakdown by vasopressin [87, 312] or epinephrine [112] in plasma membrane of liver without an added energy source, although deoxycholate was required for one study [312] and cytosol for the other [112]. The requirement of cytosol is understandable as it acts as a source of phospholipase C.

More recently, cholinergic- and α-adrenergic-stimulated breakdown of [^{3}H]inositol-labeled phosphoinositides from isolated parotid gland membranes has been reported [217]. Unlike epinephrine-mediated breakdown of PI in liver membranes [112], cytosol was not required for receptor-stimulated phosphoinositide breakdown in parotid gland [217], although incubation of membranes with cytosol or medium of low ionic strength by itself caused loss of phosphoinositides. Vasopressin (200 nM) produced a 20% decrease in PIP_2 in the isolated plasma membrane of hepatocytes. The above effect was observed even in the presence of only 200 nM free Ca^{2+} [280].

Kinetic flux studies indicate that during thrombin stimulation a large portion of PI in platelets undergoes direct degradation [323]. Calculations showed that during the first 30 sec of thrombin stimulation there is a decrease in PI mass of 7 nmol/10^9 platelets, while the flux of PI to PIP during the same period is <1 nmol/10^9 platelets. Further evidence for direct breakdown of PI has recently been obtained in studies where the energy requirements for PI breakdown were studied in pancreatic minilobules. Incubation of pancreatic minilobules with 10^{-4} M dinitrophenol produced a 50% drop in ATP level and a 40% drop in agonist-stimulated IP_2 accumulation but no effect on PI breakdown (J.F. Dixon & L.E. Hokin, *unpublished observations*). The breakdown of 70% of the total cellular PI during stimulation of pancreatic minilobules certainly indicates that PI located at sites other than plasma membrane must be degraded. Another line of evidence suggesting direct phosphodiesteratic cleavage of PI is the formation of cyclic inositol-1,2-monophosphate on stimulation of pancreatic minilobules with caerulein (J.F. Dixon & L.E. Hokin, *unpublished observations*). This is an intermediate in phospholipase C action on PI. It is possible that while the primary receptor-activated breakdown of PI in plasma membrane is through its conversion to polyphosphoinositides, a later direct breakdown of PI could occur at intracellular sites. That would be consistent with an earlier finding that

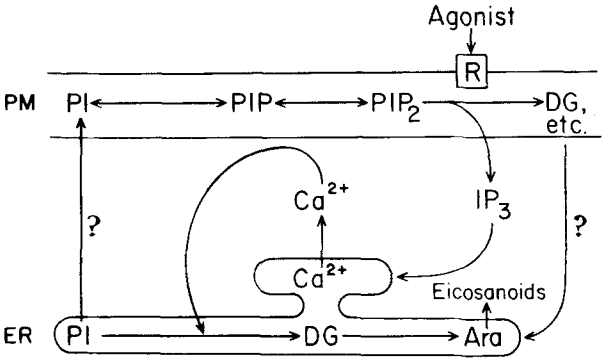

Fig. 2. Possible interactions between the plasma membrane and the endoplasmic reticulum. *Abbreviations*: *PM*, plasma membrane; *R*, receptor; *Ara*, arachidonate; *ER*, endoplasmic reticulum. Other abbreviations are given in the text. (Reproduced by permission of Annual Reviews, Inc.)

the majority of the labeled [^{3}H]inositol, [^{32}P]P_i, or [^{14}C]glycerol incorporated in pancreas during receptor activation was found in endoplasmic reticulum [99, 123, 137, 250]. Similar radioautographic results have recently been obtained with photoreceptor cells of the retina in response to light [13, 275]. If PI "breakdown" is to occur only at the plasma membrane, PI and one or more of the phosphoinositide-cycle intermediates must be shuttled back and forth between the plasma membrane and the ER (Fig. 2).

Lithium and Inositol Phosphates

The antimanic cation, lithium, inhibits IP phosphatase, resulting in an increased concentration of IP and a decreased concentration of inositol in the brain [10, 11]. Lithium amplified the agonist-stimulated accumulation of IP (five- to 20-fold) in blowfly salivary gland, rat brain slices, and rat parotid gland [27]. Following a 10-min lag period, there was a linear increase in the accumulation of IP [27]. In later studies, increased levels of IP_3 and IP_2 were also observed in blowfly salivary gland [24], brain slices, and parotid gland [26]. Since the elevation in the levels of IP_3 and IP_2 during receptor activation occurred at an earlier time period, it was consistent with the current belief that polyphosphoinositide breakdown is the primary event of receptor stimulation. Over the past two years, an increased level of inositol phosphates during the phosphoinositide effect has been observed in several tissues (*see* Table 2).

Kinetics of Formation of Inositol Phosphates

The relative duration and the extent of the increase in levels of IP_3 and IP_2 differ among various tissues

Table 2. Tissues where inositol phosphate accumulation has been reported

Tissue	Stimuli	Li	Inositol Phosphates			Reference
			IP	IP_2	IP_3	
		(mM)				
Synaptosomes	Acetylcholine	—	↑	↑	→	[78]
Brain	Carbachol	—	↑ ↑[a,b]	↑	↑	[26]
Parotid	Carbachol Phenylephrine Substance P	—	↑	↑	↑	[26]
Insect salivary gland	5-Hydroxytryptamine	—	↑	↑	→[c]	[26]
Parotid acinar cell	Methacholine	10	↑ ↑	↑	↑	[15]
Insect salivary gland	5-Hydroxytryptamine	—	—↑[d]	↑	↑	[24]
Swiss 3T3 cell	Platelet-derived growth factor	—	↑ ↑	↑	↑	[28]
Exocrine pancreas	Carbachol Caerulein	—	↑	↑	↑	[266]
Hepatocyte	Vasopressin	—	—↑	ND[e]	ND	[301]
Hepatocyte	Vasopressin	+	↑	↑	↑	[301]
Astrocytoma cell	Muscarinic agonist	10	—↑	ND	ND	[199]
Brain	Histamine Carbachol Phenylephrine 5-Hydroxytryptamine	10	—↑	ND	ND	[27]
Insect salivary gland	5-Hydroxytryptamine	5	↑	ND	ND	[27]
Parotid gland	Carbachol	10	↑	ND	ND	[27]
	Phenylephrine		↑	ND	ND	
	Substance P		↑	ND	ND	
	Isoprevaline		↑	ND	ND	
Superior cervical sympathetic ganglia	Vasopressin Cholinergic	10	↑ ↑	↑	↑	[36]
Dispersed mouse pancreas	Acetylcholine Choleocystokinin	58	↑	→	→	[139]
Ileum smooth muscle	Carbachol	10	↑	↑ ↑	↑	[276, 277]
Ileum smooth muscle	Substance P	+	↑ ↑	↑	↑	[316]
Iris smooth muscle	Carbachol	—	↑ ↑	↑	↑	[7]
Hippocampus slices	Carbachol	10	↑	ND	ND	[175]
GH_3 pituitary cell	Ca-ionophore	—	↑	↑	→	[170]
GH_3 pituitary tumor cell	Thyrotropin	+	↑	↑	↑	[74]
BC3H-1 muscle cell	Phenylephrine	+	—↑	—↑	—↑	[12]
Platelet	Thrombin	10	—↑	↑	↑	[317]
Platelet	Thrombin	10	—↑	↑	↑	[308]
Pituitary cell	Thyrotropin-releasing hormone	—	↑	↑	↑	[248]
	Thyrotropin-releasing hormone	+	↑	↑	↑	[274]

[a] Larger increase compared to other inositol phosphates.
[b] Increase compared to control.
[c] No change.
[d] Increase after a lag period.
[e] Not determined.

(Table 2). In salivary gland [24], a transient increase (lasting less than 60 sec) in the level of IP_3 was observed. On the other hand, there are other examples (Table 2) where IP_3 remained high or continued to increase over a 30-min period.

Some of the differences in the level of inositol phosphates among tissues may reflect the relative activities of the various inositol phosphate phosphatases, and this may have some physiological significance. In insect salivary gland, IP_3 phosphomonoesterase was suggested to be more active than IP_2 phosphomonoesterase, accounting for the accumu-

Table 3. Inositol-1,4,5-trisphosphate-stimulated calcium release

Permeabilized cell	Method of measurement	$[IC]_{50}$ of IP_3 (μM)	Reference
Neutrophil	Ca^{2+} selective electrode	0.5	[234]
Insulin-secreting tumor cell	Fluorescent Ca^{2+} indicator	0.025–0.1	[155]
Mammotropic pituitary cell	Fluorescent Ca^{2+} indicator	1	[100]
Porcine coronary artery	Labeled Ca^{2+}	<1	[292]
Swiss mouse 3T3 cell	Labeled Ca^{2+}	0.3	[147]
Hepatocyte	Fluorescent Ca^{2+} indicator	1	[301]
Liver microsomes	Calcium electrode	1	[61]
Pancreatic acinar cell	Calcium-sensitive microelectrode	1[a]	[291]
Insulin-secreting cell	Labeled Ca^{2+}		[29]
Hepatocyte	Labeled Ca^{2+}	<1	[43]
Hepatocyte	Fluorescent Ca^{2+} indicator	0.5	[154]
Leucocyte	Labeled Ca^{2+}	5[a]	[44]
Peritoneal macrophage	Labeled Ca^{2+}	1	[121]
Limulus photoreceptor	Fluorescent Ca^{2+} indicator	—	[41]

[a] Ca^{2+} release was studied at only one IP_3 concentration.

lation of IP_2 during 5HT stimulation [26]. An alternate explanation for IP_3 accumulation could be isomerization. In rat parotid gland, only a small fraction of the IP_3 which accumulated during carbachol stimulation was the 1,4,5-isomer [150]. The remainder appeared to be the 1,3,4-isomer, which was not susceptible to IP_3 phosphomonoesterase from red blood cells. The functional significance of this 1,3,4-isomer, how it is derived from IP_3, and how it is degraded is unknown at present [150].

Mechanism of Lithium Action

If lithium inhibits only IP phosphatase [10, 283] and has no effect on the dephosphorylation of IP_3 and IP_2, then only IP would be expected to accumulate in the presence of lithium during receptor activation [27, 36, 139, 199]. On the other hand, there are at least some tissues, such as parotid cells [15], guinea pig ileum [276, 277], and pancreatic acini [265], where 10 mM lithium (concentrations similar to that employed by Berridge et al. [27]) resulted in the enhanced accumulation of polyphosphates. The effects of varying lithium concentrations on the accumulation of inositol phosphates in hepatocytes indicated that lithium inhibited the three enzymes, IP phosphatase and IP_2 and IP_3 phosphomonoesterase, with a half-maximal concentration of 0.5, 1, and 5 mM, respectively [301].

Based primarily on the time course of the formation of inositol phosphates, it is generally believed that in most cases IP_2 is derived from IP_3, rather than by the breakdown of PIP. But in macrophages, it was observed that ionophore increased the IP_2 level without a prior elevation of IP_3 [80]. In GH_3 cells, this was considered to indicate a direct breakdown of PIP but not PIP_2 by elevated Ca_c^{2+} [170].

Inositol Trisphosphate in Calcium Mobilization

Cytosolic Ca^{2+} may be mobilized from either intracellular stores or from the extracellular compartment or both. It appears that IP_3 is an important link between receptor-activated phosphoinositide breakdown and Ca^{2+} mobilization from intracellular stores. It was first demonstrated in 1983 that the addition of IP_3 (1,4,5-isomer) to "permeabilized" pancreatic acinar cells resulted in the release of Ca^{2+} from these cells [291]. Within the past two years or so, a similar result has been demonstrated in several permeabilized preparations (Table 3) as well as from the microsomal fraction of exocrine pancreas [290], rat insulinoma cells [233], and liver [61]. The release of Ca^{2+} is rapid and occurs at less than micromolar concentrations of IP_3 (Table 3). Specificity of the 1,4,5-isomer of IP_3 for Ca^{2+} release has been demonstrated in pancreas [291] and Swiss mouse 3T3 cells [147]. IP and IP_2, which are the two other products seen during phosphoinositide breakdown, were unable to release Ca^{2+}. The release of Ca^{2+} by IP_3 is transient due to rapid hydrolysis of IP_3, and this is accompanied by Ca^{2+} reuptake [121, 291]. Based on the amount of PIP_2

breakdown, it has been calculated that the IP_3 concentration will reach 15 μM within 2 min, but this concentration may not be achieved *in vivo* due to the presence of inositol trisphosphatase which is estimated to have a $t_{1/2}$ of 4 sec in hepatocytes [289]. Recently, intracellular injection of IP_3 has been shown to mimic the muscarinic-stimulated depolarizing chloride current in *Xenopus* oocyte [226], stimulation of visual photoreceptor of *Limulus* [42, 90], and an injection of IP_3 also produces a reversible hyperpolarization of salamander rod membrane [313].

There is no direct evidence that phosphoinositide metabolism plays any role in the opening of Ca^{2+} gates at the plasma membrane to permit entry of extracellular Ca^{2+}. The formation of PA, which is a weak ionophore as compared to A23187, has been postulated to function in Ca^{2+} gating at the plasma membrane [113, 177, 224, 237, 242, 268, 319]. Results with PA as an ionophore in liposomes have been conflicting [49, 140, 278].

More recently, IP_3 has been shown to stimulate phosphorylation of a 62,000-dalton protein in monkey fibroblast and bovine brain cell lysates [322]. In permeabilized fat cells, IP_3-activated pyruvate dehydrogenase is attributed to its capacity to mobilize Ca^{2+} [168]. It is possible, though purely speculative at this stage, that phosphorylation by IP_3 or the DG-activated protein kinase C (PKC) system (discussed later) may play a role in mobilization of extracellular Ca^{2+}.

Phosphoinositides may also indirectly affect Ca^{2+} levels by regulating the calcium pump. Support for this hypothesis comes from studies where incorporation of a small amount of PA, PIP, or PIP_2 into liposomes containing reconstituted erythrocyte or brain calcium pump enhanced its activity, while other lipids such as DG and PI had little effect [51].

Diacylglycerol

Diacylglycerol is the other immediate product of the phosphodiesteratic cleavage of phosphoinositides. A rapid and usually transient accumulation of DG associated with stimulated phosphoinositide breakdown has been demonstrated in platelets [21, 63, 116, 146, 148, 204, 236, 254], pancreas [18, 67], mast cells [144], 3T3 cells [108], pituitary cells [74, 198, 249], GH_3 cells [75], and liver [142, 302]. Based on the fatty acid composition and mass of this newly generated DG, it has been suggested to be derived from phosphoinositides in platelets [21, 204], aortic epithelial cells [108], and pituicytes [249] and from PA in neutrophils [52]. Some studies [18, 142] have suggested that only a part of this newly generated DG is due to phosphoinositide breakdown, and the rest is considered to arise from other lipids.

Interest in DG has been quite recent. The starting point was the recognition of a phospholipid-dependent, DG-stimulated, and Ca^{2+}-activated protein kinase, termed protein kinase C (PKC), in various mammalian tissues [174, 212]. It was originally thought that PKC requires at least one unsaturated fatty acid in DG [166], but more recently diacylglycerol containing saturated fatty acids has been shown to activate PKC [60, 179]. Activation by DG occurs by a shift in affinity of the enzyme for Ca^{2+} from the mM to the μM range [166, 297]. Though phosphatidylserine was essential for the PKC activity, the enzyme could be further modulated by other phospholipids [156]. Phosphatidylethanolamine in a concentration of 20 μg/ml activated PKC by 50%, PI and PA (two intermediates in phosphoinositide turnover) had no effect and phosphatidylcholine and sphingomyelin acted as inhibitors [156].

Protein kinase C is mainly a cytosolic enzyme, but during DG or phorbol ester activation (*see below*) it is translocated to the membrane fraction [173; *see* 245, 300]. It is not clear how this occurs. Translocation of PKC to the membrane may be the result of the membrane-perturbing characteristic of DG, as has been demonstrated in artificial bilayers [58, 223]. Very recently, the physiological significance of the PKC pathway has been supported by the stereospecific requirement of the *sn* form of DG for PKC activation [45, 244].

Interest in the DG-activated PKC pathway has been further stimulated by the observation that tumor-promoting agents, such as phorbol esters, cause direct activation of PKC [46, 219, 269]. In many different tissues, similar distribution patterns for the enzyme and phorbol ester binding site have been observed [13, 163, 284]. Protein kinase C activity and the phorbol ester binding sites have been copurified [181, 219] and DG competes for the phorbol ester binding site [282], further supporting the earlier conclusion that PKC is a receptor for phorbol esters.

Protein Kinase C-Mediated Phosphorylation

Activation of platelets with thrombin, collagen, or platelet-activating factor causes phosphorylation of both a 40K and a 20K protein [116, 117, 143, 160, 180, 190, 270], while the addition of 1-oleyl-2-acetyl glycerol (a synthetic DG) causes a rapid phosphorylation of only the 40K protein [270]. This suggests that the 40K protein is a substrate for PKC. Even though phosphorylation of the 40K protein temporally correlates with serotonin release, its

functional significance is still not known. In HL60 cells, phorbol myristate mediated phosphorylation of 17K and 27K proteins, and this was suggested to play a role in the conversion of HL60 cells to monocytes [91]. In cell-free systems, PKC phosphorylates a large number of proteins in many different tissues [*see* 220]. Recently, PKC has been shown to phosphorylate proteins varying in molecular weight from 11–95K in the retinal photoreceptor outer segment [158] and probably may also be responsible for the enhanced phosphorylation of PI to polyphosphoinositides observed in platelets with phorbol myristate acetate [47, 110].

In cultured hepatoma cells, PKC activation by phorbol myristate acetate results in phosphorylation of the insulin receptor and inhibition of insulin-stimulated glycogen synthase and tyrosine aminotransferase activity [298]. A purified preparation of PKC has been shown to phosphorylate and activate a partially purified preparation of tyrosine hydroxylase [8]. The serine phosphorylated by PKC during activation was the same as that phosphorylated by cAMP [8].

Significance of Multiple Messengers

There are some advantages to a pathway which can generate multiple second messengers. Such a branched pathway would have multiple control points, providing the cell with a capability for fine tuning. Stimuli which cause the breakdown of polyphosphoinositides (Table 1) would result in the formation of IP_3 and DG. Under normal circumstances, IP_3 would mobilize Ca^{2+} from intracellular stores, and DG would activate PKC, resulting in the generation of maximum physiological response. A synergistic interaction between the Ca^{2+}-mobilizing pathway and the PKC pathway by the use of Ca^{2+} ionophore and synthetic DG or phorbol esters was initially demonstrated in platelets [157, 180, 253] and has now been extended to lymphocytes [200], hepatocytes [256], adrenal glomerulosa cells [169], exocrine pancreas [231], mast cells [159], adrenocortical cells [56], pancreatic islets [328], neutrophils [57, 257, 281], GH_4C_1 cells [64], ileum [299], and parotid gland [241]. Synergism between the two pathways was also observed during internalization of epidermal growth factor receptor [188], contraction of rabbit vascular smooth muscle [246], and activation of glycogen phosphorylase in hepatocytes [85]. In neutrophils, DG was found to enhance the β-glucosaminidase and lysozyme-releasing activity of leukotriene β_4, platelet-activating factor, formylated oligopeptide, and C5a by 10- to 30-fold [221]. In contrast to the above-mentioned synergistic effects, a negative coupling between the two branches has been reported in rat hippocampal slices [175].

Further regulation and interaction of these two pathways may be modulated by the kinetics of IP_3 and DG metabolism. Depending upon the tissue, IP_3 accumulation is transient or prolonged (*see* Table 2).

Stimuli or agents which cause the breakdown of PI or PIP rather than PIP_2 [80, 152] will be able to bypass the effect of IP_3 while retaining the DG effect. Though IP and IP_2 are ineffective in releasing Ca^{2+} from ER [291], they may yet have some functional significance. The relative rate and extent of conversion of DG to PA and arachidonate and its metabolites may provide further regulatory control.

Arachidonate and Its Metabolites

Arachidonate metabolites, i.e., prostaglandins, leukotrienes, and thromboxanes, which are collectively referred to as eicosanoids, are potent regulators of various physiological responses. The levels of these compounds are regulated by the availability of free arachidonate, which must be liberated mainly from esterified lipids [22, 69, 176, 309, 311]. It is generally believed that PI in all mammalian tissues is rich in arachidonate [197, 206]. This has been demonstrated in pancreas [18, 161], platelets [194], brain [295], iris [327], and a few other tissues. The polyphosphoinositides have also been shown to contain a large proportion of arachidonate [16, 204]. Besides PI, phosphatidylcholine and phosphatidylethanolamine are also esterified with arachidonate to varying degrees. Irvine [146] has recently discussed in detail the various mechanisms by which arachidonate levels are modulated in mammalian cells.

There are several tissues where a release of free arachidonate as well as a phosphoinositide response have been demonstrated [67, 178, 187, 197, 237, 254, 263]. It is now well established that arachidonate is released from phospholipids with certain stimuli, but it is still not known for most tissues what proportion of the total arachidonate released is derived from phosphoinositides and what is its mechanism of release.

Mechanisms of Arachidonate Liberation

In platelets [196, 197, 254] and pancreatic minilobules [67], there is agonist-evoked liberation of free arachidonate, and at least in pancreas this proceeds via DG-lipase action on DG liberated from the phosphoinositides. In erythrocytes [208] and platelets [205], DG lipase is shown to be present in

the plasma membrane [204]. In pancreatic minilobules, stimulation of phosphoinositide breakdown with the secretogogue, caerulein, is associated with an increase in steady-state level of 1-stearoyl, 2-arachidonoyl-*sn*-glycerol and a release of substantial amounts [up to 50% of the total PI breakdown (*see below*)] of stearate, arachidonate, and glycerol [67]. Unlike the situation in platelets, there is no appreciable accumulation of MG. In the pancreas model system, the DG-lipase inhibitor, RHC 80267 [296], reduces the secretogogue-stimulated liberation of free stearate, arachidonate, and glycerol, and elevates the steady-state level of 1-stearoyl, 2-arachidonoyl-*sn*-glycerol [67]. These observations in the pancreas provide strong evidence that stimulation of phosphoinositide breakdown generates arachidonate via the sequential action of phospholipase C and DG lipase. A similar mechanism of arachidonate release has also been suggested in the thyroid lobes [185] as thyrotropin-stimulated prostaglandin release was inhibited by RHC 80267. RHC 80267 (10 μM), which inhibited DG lipase in broken platelets, had no effect on intact platelets [48, 222], and at the very high concentrations required to inhibit DG lipase, RHC 80267 produced nonspecific effects [222].

In platelets, the presence of phospholipase A_2 specific for PA has been described [33], and it has been implicated in the release of arachidonate from PA produced during PI turnover [177]. But it has been shown more recently that half of the arachidonate was released on stimulation of platelets with thrombin before any rise in PA [218]. The fatty acid composition of resynthesized PI in platelets following thrombin stimulation was different from the fatty acid composition of PI in the unstimulated state [235]. The resynthesized PI was rich in oleate and linoleate, while PI in the basal state was rich in arachidonate. This further supports the notion that arachidonate is released during thrombin-stimulated phosphoinositide turnover in platelets. In GH_3 cells, the formation of prostaglandins in response to bradykinin is attributed to the release of arachidonate from PI by a phospholipase A_2 action [141].

Another pathway which is independent of phosphoinositide turnover and has been implicated in arachidonate release in platelets is a phospholipase A_2-mediated breakdown of PC, PE, and PI [32, 34, 40, 195, 254, 264, 306]. The affinity of this enzyme for Ca^{2+} is much lower (higher K_D) than that of phospholipase C [32], suggesting that phospholipase A_2 action on phospholipids is likely to be stimulated by agonist-evoked rises in Ca_c^{2+} (from ~0.1 to ~1.0 μM). Thus arachidonate release may respond to the transient rises in Ca^{2+} evoked by IP_3 [*see* 245].

Role of Released Arachidonate

As noted earlier, free arachidonate is the limiting factor in the synthesis of eicosanoids, which mediate or modulate numerous physiological functions [*see* 111, 213, 324], including many which involve phosphoinositide metabolism [197, 237, 264]. Not surprisingly, increased arachidonate release is reflected in increased prostaglandin synthesis in several tissues which show a phosphoinositide effect [19, 20, 67, 96, 185, 304]. However, in the exocrine pancreas the elevation in PI-derived arachidonate on stimulation of enzyme secretion with several agonists is several orders of magnitude higher than the stimulated formation of PGE_2 and $PGF_{2\alpha}$ [19, 20, 67]. A similar relationship, although less striking, was noted on stimulation of perfused heart or kidney with bradykinin [151]. The difference between the amount of arachidonate released and prostaglandin formed could possibly be due to increased arachidonate in compartments which are inaccessible to prostaglandin-synthesizing enzymes. Such a possibility is supported by the following observations. First, in human platelets, arachidonate, released from phospholipids is preferentially utilized for cyclooxygenase rather than lipooxygenase formation [272]. Second, in neutrophils, while zymosen and ionophore both liberate arachidonate, only the arachidonate released by ionophore was converted to hydroxyicosatetraenoic acid [314].

The fact that only a small portion of liberated arachidonate is metabolized to eicosanoids in some tissues raises the interesting possibility that arachidonate itself may mediate or modulate some effects. Arachidonate as well as a few other fatty acids is capable of activating guanylate cyclase [101, 104, 228], which suggests that cGMP levels may be regulated by arachidonate levels. An elevation in cGMP but not cAMP levels has been observed in several tissues which show enhanced phosphoinositide turnover [*see* 206].

Arachidonate-generated cGMP may act as a negative feedback inhibitor in some instances. For example, choleocystokinin/pancreozymin binding to pancreatic acini which respond to this hormone with increased phosphoinositide turnover and arachidonate release [67] is inhibited by cGMP [229]. It has generally been thought that the guanylate cyclase system requires Ca^{2+} since Ca^{2+} deprivation prevents rises in cGMP [263] and rises in cGMP can be elicited by the Ca^{2+} ionophore A23187 [263]. It is a common view that the Ca^{2+} dependence for cGMP formation may be due to a Ca^{2+} dependency for the release of arachidonate. This would be particularly true if the arachidonate re-

glycerol appear to be necessary for maximal responses. The elevated Ca_c^{2+} may also activate reactions not dependent on calmodulin. The third physiologically active breakdown product of phosphoinositides in many but perhaps not all cells is arachidonate, which is the obligatory substrate for synthesis of eicosanoids which mediate or modulate a wide variety of physiological (autocrine) functions. There is some evidence that arachidonate itself may directly activate certain processes, particularly formation of cGMP and efflux of Ca^{2+} from the cell.

There is a considerable body of evidence, although primarily of a circumstantial nature, that phosphoinositide turnover is involved in proliferation. Studies are beginning to emerge suggesting that phosphoinositides may be involved in oncogene action. This suggested function is intriguing but unproven.

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