Biochem. J. (1990) 269, 281–291 (Printed in Great Britain)

REVIEW ARTICLE

The regulation and cellular functions of phosphatidylcholine hydrolysis

M. Motasim BILLAH and John C. ANTHES
Department of Allergy and Inflammation, Schering–Plough Research, 60 Orange Street, Bloomfield, NJ 07003, U.S.A.

INTRODUCTION

Ca^{2+}-mobilizing agonists, including hormones, neurotransmitters and growth factors, cause profound changes in cellular lipid metabolism. These changes are initiated by receptor-mediated activation of various phospholipases including phospholipase A_2, phospholipase C and phospholipase D. Phospholipase A_2 hydrolyses several phospholipids including phosphatidylinositol, phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) to liberate arachidonic acid and the respective lysophospholipids. Arachidonic acid is converted via the cyclo-oxygenase and lipoxygenase pathways to eicosanoids, including prostaglandins, thromboxanes, prostacyclins, leukotrienes and lipoxins [1]. Certain lysophospholipids such as ether-linked lysophosphatidylcholine are acetylated by a specific acetyltransferase to form platelet-activating factors [2]. Platelet-activating factors and eicosanoids exert profound biological actions on a variety of tissues [1,2]. Thus, phospholipase A_2 activation constitutes an important component in cell–cell communication.

Intracellular signalling by Ca^{2+}-mobilizing agonists is believed to be initiated by receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate by specific phospholipases C [3,4]. This hydrolysis leads to the generation of two signalling substances, inositol 1,4,5-trisphosphate and diacylglycerol (DG). Inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from intracellular stores [3,4]. DG, in concert with Ca^{2+}, activates a phospholipid-dependent protein kinase, protein kinase C (PKC) [5,6]. This ubiquitous protein kinase phosphorylates a multitude of cellular proteins and thereby controls a host of cellular processes.

Although most attention has been focused on phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate as an intracellular signalling system, it is now clear that Ca^{2+}-mobilizing agonists induce rapid hydrolysis of PtdCho by both phospholipase C and phospholipase D, generating DG and phosphatidic acid (PtdOH), respectively. PtdOH, which itself could be a potential intracellular mediator, can be further degraded by a PtdOH phosphohydrolase to DG.

Various aspects of phospholipase A_2 and phosphoinositide-specific phospholipase C have been reviewed extensively [7–9]. In the present article, we will focus on the regulation and function of phosphodiesteratic cleavage of PtdCho during cell activation. This subject has been briefly discussed in several recent articles [10–13].

COMPOSITION OF PtdCho

PtdCho is the principal phospholipid class in mammalian tissues and can account for up to 50% of the total cellular phospholipid content [14]. It consists of 1,2-diacyl-*sn*-glycero-3-phosphocholine, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine and 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine [15]. In most tissues, 1,2-diacyl-*sn*-glycero-3-phosphocholine is the predominant subclass. However, in several cells of myeloid origin, namely neutrophils, eosinophils and macrophages, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine may account for 30–70% of the cellular PtdCho with 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine being a minor component [16,17]. In contrast, in heart and several other electrically active tissues, about 30% of the PtdCho exists as 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine [18].

PtdCho from mammalian tissues normally contains a saturated fatty acid residue at the C-1 position and an unsaturated fatty acyl residue at the C-2 position of the glycerol moiety. Compared to phosphoinositides, which are relatively enriched in stearic acid and arachidonic acid, 1,2-diacyl-*sn*-glycero-3-phosphocholine is relatively deficient in arachidonic acid, and contains mostly oleic acid and linoleic acid at the C-2 position [17,19]. However, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine is relatively enriched in arachidonic acid [17,19]. Thus, phosphodiesteratic cleavage of PtdCho can lead to multiple molecular species of DG and PtdOH, with important ramifications in cell activation.

PHOSPHODIESTERASES THAT HYDROLYSE PtdCho

Phosphodiesteratic cleavage of PtdCho occurs by two distinct types of phospholipases. A phospholipase C activity produces DG and phosphocholine, whereas a phospholipase D activity generates PtdOH and choline. In addition to hydrolysis, phospholipase D catalyses a unique transfer reaction whereby the phosphatidyl moiety of the phospholipid substrate is transferred to primary alcohols to produce phosphatidylalcohols [20,21]. This transphosphatidylation may be considered the general reaction, with hydrolysis representing a specific case in which the acceptor is water.

Mammalian phospholipase C

Cell-free preparations from a variety of rat tissues exhibit phospholipase C activities that degrade PtdCho [22–26]. Phospholipase C activities of lysosomal origin with acid pH optima also hydrolyse phosphatidylethanolamine (PtdEtn), phosphatidylinositol and phosphatidylglycerol [22]. PtdCho-cleaving phospholipases C with alkaline pH optima have been detected in rat brain cytosol [23] and rat liver membranes [24]. Cell-free preparations from endothelial cells also degrade exogenous PtdCho [25,26]. More recently, phospholipases C that utilize PtdCho as substrate have been partially purified from dog heart cytosol [27], bull seminal plasma [28] and promonocytic U937 cells [29]. Utilizing exogenous phospholipids as substrates, these activities exhibit neutral pH optima and do not hydrolyse

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; PKC, protein kinase C; DG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; GTPγS, guanosine 5′-[γ-thio]triphosphate.

phosphatidylinositol. However, myocardial phospholipase C degrades both PtdCho and PtdEtn, with PtdCho being the preferred substrate. These activities appear to be distinct from phospholipases C of lysosomal and bacterial origins by several criteria, including pH profile, substrate specificity, requirements for detergents and divalent cations, subcellular localization and molecular size.

Mammalian phospholipase D

Although phospholipase D activities of plant origin (reviewed in [30]) have been known since 1948 [31], the existence of mammalian phospholipase D acting on PtdCho was first detected in 1975 using a microsomal preparation from rat brain [32]. Subsequent studies have demonstrated PtdCho-preferring phospholipase D in homogenates and membranes from various tissues and cells including lung, liver, adipose tissue, endothelial cells, HL-60 cells and spermatozoa, with lung and brain being the richest sources [33–38]. Phospholipase D activities have been partially purified from rat brain [39] and human eosinophils [40] and found to be associated primarily with particulate fractions [33–35,41,42]. Mammalian phospholipases D from various systems also exhibit a transphosphatidylation activity [37,42–44]. Requirements of Ca^{2+}, fatty acids and detergents for phospholipase D expression vary from cell to cell [34–38,42], suggesting that multiple phospholipase D isoforms may exist. PtdCho is the preferred substrate for phospholipase D from both mammalian and non-mammalian sources [30,34,37], although under certain conditions, phosphatidylinositol and PtdEtn may also be degraded [39,45].

AGONIST-INDUCED PHOSPHODIESTERATIC CLEAVAGE OF CELLULAR PtdCho

Products of the phosphodiesteratic cleavage of PtdCho can be measured by both isotopic labelling and mass measurements. Isotopic labelling involves selective labelling of endogenous PtdCho in the acyl chains, phosphate or the choline moiety. Choline, fatty acids (e.g. myristic acid) and lysophosphatidylcholine have been widely used as radiolabelled precursors. Sensitive methods for the measurement of absolute mass of DG [46], choline [47], phosphocholine [48] and PtdOH [34,49,50] are available.

Formation of choline and phosphocholine

Isotopic labelling studies demonstrate that diverse cell types produce choline metabolites from endogenous PtdCho in response to a wide variety of agents including hormones, neurotransmitters, growth factors and phorbol esters (Table 1). The product profile and kinetics of product appearance vary amongst cell types and even within the same cell depending on the agonists. For instance, in neutrophils (our unpublished observation) and endothelial cells [89,90], choline, and not phosphocholine, is detected after specific stimulation. By contrast, upon stimulation with specific agents, MDCK-D1 cells [91,92] and preadipocytes [86] produce primarily phosphocholine. On the other hand, muscarinic stimulation of astrocytoma cells produces both phosphocholine and choline with choline appearing only at later time points [80]. However, in this system, choline is the only product found following stimulation with PMA. In most systems, receptor agonists rapidly and transiently stimulate the formation of choline metabolites, whereas PMA-induced responses are delayed in onset and prolonged. Effects of stimulation on cellular levels of glycerophosphocholine, a water-soluble product generated by sequential deacylation of PtdCho by phospholipase A_2 and lysophospholipase, have not been examined except for endothelial cells [89,90] and REF52 embryo cells [102]. In these two systems, the contents of glycerophosphocholine do not change appreciably during stimulation. A common theme emerging from these studies is that receptor agonists and tumour-promoting phorbol esters induce phosphodiesteratic cleavage of PtdCho in a wide variety of cells.

Formation of PtdOH and DG

Using isotopic labelling and mass measurements, the extent and duration of PtdOH and DG have been measured in a wide variety of tissues and cells. In response to various Ca^{2+}-mobilizing agonists, many cells produce DG in a biphasic manner (Table 1). The early phase peaks within initial 30 s whereas the delayed, quantitatively larger phase reaches a maximum within 2–15 min of stimulation and is long-lasting.

The initial rise in DG temporally correlates with inositol 1,4,5-trisphosphate generation [66,77,84,85,94,98]. In addition, the fatty acid composition and molecular species distribution of early DG closely resemble those of phosphoinositides [99]. These observations indicate that the early DG is formed by phospholipase C action on phosphatidylinositol 4,5-bisphosphate. Two major lines of evidence suggest that sustained DG formation is derived from sources other than phosphoinositides. First, DG mass that accumulates after stimulation is kinetically distinct from and exceeds the mass of inositol phosphates [66]. Second, phorbol esters [56,63], certain growth factors [98,99]. *ras* oncogene product [105] and certain interleukins [70] induce formation of DG without provoking phosphoinositide hydrolysis (see Table 1 for additional references).

There is now considerable evidence that cellular PtdCho is a major source of the sustained DG. Early studies with PMA-stimulated myoblasts have shown that PMA-induced DG has a fatty acid composition similar to that of PtdCho [83]. Subsequent studies have confirmed that, in neutrophils stimulated with fMet-Leu-Phe [51,54,56,57] and in hepatocytes stimulated with vasopressin [34,87], the fatty acid composition and distribution of *sn*-1 bonds of PtdOH and DG formed differ from those of phosphoinositides and phosphatidylethanolamine but closely resemble those of PtdCho. Two recent studies have thoroughly analysed molecular species of DG in antigen-induced mast cells by argentation chromatography [67] and in thrombin-stimulated fibroblasts by capillary gas chromatography of derivatized DG [99]. This latter study has also compared the molecular species distribution of DG to those of various potential precursors and identified PtdCho as the major source of the sustained DG formed after thrombin stimulation. Many cells that produce PtdOH and DG also generate choline and phosphocholine (Table 1). Phosphodiesteratic cleavage of PtdCho has been further confirmed by experiments in which intact cells have been selectively labelled in PtdCho with [^{3}H]lysophosphatidylcholine [38,54,56,61,91] and [^{3}H]myristic acid [80,90,102]. Stimulation of these cells produces [^{3}H]PtdOH and [^{3}H]DG, with [^{3}H]DG having the characteristics of a sustained response.

Thus, in various cell types, phosphodiesteratic cleavage of PtdCho is enhanced in response to diverse stimuli, resulting in the formation of water-soluble and lipid products. Various types of determinations further indicate that, in several cells, PtdCho breakdown is a major route to both PtdOH and sustained DG during stimulation.

AGONIST-INDUCED PHOSPHODIESTERATIC CLEAVAGE OF PtdEtn

PtdEtn, the second most abundant phospholipid in mammalian tissue, is poorly degraded by partially purified phospholipase C and phospholipase D [27,39]. In hepatocyte membranes and in

Table 1. Agonist-induced cleavage of cellular PtdCho via phospholipase C and phospholipase D

Key: ⇒, no change; ⇑, increase; ⇓, decrease; –, not measured; *, increases in unidentified water-soluble choline metabolites. Abbreviations for agonists: fMLP, formyl-methionyl-leucyl-phenylalanine. OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PAF, platelet-activating factor; CCK8, cholecystokinin octapeptide; GnRH, gonadotropin-releasing hormone; DiC_8, 1,2-dioctanoyl-*sn*-glycerol; PGE_2, prostaglandin E_2; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; FSG, fucose sulphate glycoconjugate.

Cell system	Agonist	Product... Phosphocholine	Choline	DG Early (⩽ 1 min)	DG Late (> 1 min)	PtdOH	Phosphatidylethanol	References
Inflammatory/immune								
Neutrophils	fMLP, OAG, PMA, A23187	⇓	–	⇑	⇑	⇑	⇑	[48–58]
HL-60 cells	fMLP, OAG, PMA, A23187	–	–	⇑	⇑	⇑	⇑	[59–64]
Monocytes	PMA	–	–	–	–	–	⇑	[65]
Macrophages	PMA, A23187, PAF	⇒	⇑	⇑	⇑	–	–	[66]
Mast cells	Antigen	–	–	⇑	⇑	⇑	⇑	[67,68]
R6-XE.4 cells	Interleukin-3	*	*	–	–	–	–	[69]
Jurkat/T lymphocytes	Interleukin-1	⇑	–	⇑	⇑	–	–	[70]
Platelets	Thrombin	–	–	–	–	–	⇑	[71]
Endocrine/exocrine								
Pancreatic	A23187, PMA, carbachol, CCK8, bombesin	⇑	⇑	⇑	⇑	⇑	–	[72–75]
Ovarian granulosa cells	GnRH, PMA	–	–	–	–	⇑	⇑	[76]
Adrenal granulosa cells	Angiotensin II	–	–	⇑	⇑	–	–	[77]
Nerve								
NG108-15 neuroblastoma cells	PMA, DiC_8	⇒	⇑	–	–	⇒	⇑	[78,79]
1321N1 astrocytoma cells	PMA, carbachol	⇑	⇑	⇒	⇑	⇑	–	[80]
Rat cortex	Bethanechol	–	⇑	–	–	–	–	[12,81]
Striatal tissue	Current	–	⇑	–	–	–	–	[82]
Muscle								
BC3H-1 myocytes	Insulin, PMA	⇑	⇒	⇑	⇑	⇑	–	[11,83]
A-10 aortic smooth muscle cells	Vasopressin	⇑	⇒	⇒	⇑	–	–	[84]
Vascular smooth muscle cells	Angiotensin II, A23187	–	–	⇑	⇑	–	–	[85]
Chicken heart	Carbachol	–	⇑	–	–	⇑	–	[12,81]
Metabolic								
3T3-L1 preadipocytes	PDGF, PMA, serum	⇑	⇒	⇑	–	–	–	[86]
Hepatocytes	Vasopressin, angiotensin, adrenaline, ATP, PMA, A23187	–	–	⇑	⇑	⇑	⇑	[34,44,87]
Endothelial	Thrombin, bradykinin, ATP, A23187	⇒	⇑	⇑	⇑	⇑	⇑	[88–90]
Epithelial								
MDCK	PMA	⇑	⇑	–	⇑	⇑	–	[91]
MDCK-DI	Adrenaline	⇑	⇒	⇑	–	–	–	[92]
A431	PMA	⇑	⇒	–	⇑	–	–	[93]
Fibroblast								
BALB/c-3T3	PDGF, PGE_2	–	–	⇑	⇑	–	–	[94]
CCL39	Thrombin, FGF, PMA	–	–	⇑	⇑	–	–	[95]
C3H10T 1/2	PMA	⇑	⇑	–	–	–	–	[96]
HEF	PMA	⇑	⇒	–	⇑	–	–	[93]
HeLa	PMA, A23187	⇑	⇑	–	–	–	–	[97]
IIC9	Thrombin, EGF	⇒	⇑	⇑	⇑	–	–	[98,99]
NIH-3T3	*ras* p21	⇑	–	–	⇑	–	–	[105]
REF52	Vasopressin, serum, PMA	⇒	⇑	–	⇑	⇑	⇑	[101–104]
Swiss-3T3	PDGF, bombesin, *ras* p21, PMA	⇑	⇑	⇑	⇑	⇒	–	[100,106]
Other								
Spermatozoa	FSG, gramicidin S	⇒	⇑	⇒	⇑	⇑	⇑	[38]

granulocyte homogenates, GTPγS-stimulated phospholipase D activities exhibit strict specificity for PtdCho [34,37]. However, several recent studies using cells labelled in PtdEtn with [^{3}H]ethanolamine suggest that PtdEtn may also be degraded by agonist-stimulated phosphodiesterases [64,80,107]. Thus, when stimulated with phorbol esters, several cells including HL-60 cells, NIH 3T3 fibroblasts and baby hamster kidney cells produce both choline and ethanolamine, but [^{3}H]ethanolamine is detected only in the presence of a large excess of unlabelled ethanolamine [64]. Similarly, muscarinic stimulation of astrocytoma cells causes generation of choline metabolites as well as ethanolamine metabolites [80]. However, in this system, ethanolamine metabolites are quantitatively minor products. Both phosphocholine and phosphoethanolamine are also detected in *ras* oncogene-transfected fibroblasts [105]. However, in IIC9 fibroblasts stimulated with α-thrombin, ethanolamine metabolites are not found [99]. Interestingly, mesengial cells stimulated with interleukin 1 generate only phosphoethanolamine, and no choline metabolites are detected [107]. Thus, although PtdEtn can be degraded by phosphodiesterases, in most cells this degradation is unlikely to make a major contribution to PtdOH and DG generation during cell stimulation. There is at present no information regarding phospholipase D-catalysed degradation of phosphoinositides in stimulated intact cells.

DISTINCTION BETWEEN AGONIST-STIMULATED PHOSPHOLIPASE C AND PHOSPHOLIPASE D HYDROLYSING CELLULAR PtdCho

The products of phospholipase C (DG and phosphocholine) and phospholipase D (PtdOH and choline) are rapidly interconvertible through highly active, specific, kinases and phosphatases. Indeed, some of these enzymes are also activated during stimulation. For instance, in cultured fibroblasts stimulated with growth factors and *ras* oncogene transfection, choline kinase activity is greatly enhanced [108,109]. Thus, while product identification, quantification and 'finger-printing' establish agonist-stimulated phosphodiesteratic cleavage of cellular PtdCho, such determinations do not identify the actual phosphodiesterase(s) involved.

Kinetic analysis

Kinetic comparisons between various products can provide important clues to the identity of the activated phosphodiesterases. In some cells such as neutrophils (our unpublished observation) and endothelial cells [90], PtdOH is formed with rates comparable with those of choline formation, providing strong evidence for phospholipase D activity. Similarly, concurrent formation of DG and phosphocholine has been observed in 3T3-L1 preadipocytes [86], indicating PtdCho hydrolysis by phospholipase C. However, without a knowledge of the activity status of relevant kinases and phosphatases, conclusions from kinetic studies must be considered tentative, especially in systems where the responses are prolonged.

Transphosphatidylation

Since transphosphatidylation is catalysed by phospholipase D and not phospholipase C, this property provides the basis for a novel methodology to distinguish phospholipase D from phospholipase C. Although the acceptor of choice is ethanol which readily crosses the membrane barrier, various other primary alcohols such as methanol, butanol and glycerol may also be used. In several cells, phosphatidylethanol is formed when cells are stimulated with specific agents in the presence of ethanol (0.1–1%) (Table 1) and wherever measured, phosphatidylethanol is formed with rates similar to those for choline and PtdOH [54,61–63,90]. Because of metabolic stability, phosphatidylethanol formation can be conveniently used to detect phospholipase D activity in systems where PtdOH does not accumulate due to rapid dephosphorylation. Unfortunately, in cells such as MDCK cells and preadipocytes where choline accumulation has not been observed [86,92], no information regarding phosphatidylethanol formation is available. The possibility that enhanced base exchange [110] between PtdCho and ethanol may contribute to phosphatidylethanol formation cannot be excluded.

^{32}P labelling

Cellular ATP is readily labelled by incubating cells with [^{32}P]orthophosphate. Subsequent labelling of cellular phospholipids occurs through the reaction cascades involving [^{32}P]ATP and specific kinases. When human neutrophils labelled with [^{32}P]ATP are stimulated with fMet-Leu-Phe, [^{32}P]PtdOH is rapidly formed [49]. The specific activity of this [^{32}P]PtdOH is much lower than that of the [^{32}P]ATP. This observation is the first indication that in stimulated neutrophils, PtdOH is formed through pathways other than that involving phospholipase C and DG kinase and that a phospholipase D may be activated to act directly on phospholipids to produce PtdOH [49]. More recently, this ^{32}P-labelling technique has been used to study phospholipase D activation in vasopressin-stimulated hepatocytes [34]. However, the data obtained by this approach are open to alternative interpretations and provide no information about the source(s) of the PtdOH that is formed.

To identify unequivocally phosphodiesterases and their substrates in intact cells, where various competing reactions operate optimally, a novel approach has recently been developed to label endogenous phospholipids selectively with ^{32}P. A crucial consideration in this approach is that the labelling must be done in the absence of cellular [^{32}P]ATP. In such a system, [^{32}P]PtdOH or [^{32}P]phosphocholine must be formed from [^{32}P]phospholipids exclusively by phospholipase D or phospholipase C, respectively, and not by kinases. This labelling technique has been employed successfully to study PtdCho metabolism in neutrophils [54,62,63]. Selective labelling of cellular PtdCho is achieved by incubation of neutrophils with lyso[^{32}P]phosphatidylcholine which readily enters the cell and becomes acylated into membrane-associated [^{32}P]PtdCho. Stimulation of these ^{32}P-labelled neutrophils with various agents, including fMet-Leu-Phe, phorbol esters, ionophore A23187 or 1-oleoyl-2-acetylglycerol induces rapid generation of [^{32}P]PtdOH [54,62,63], unequivocally demonstrating PtdCho hydrolysis by phospholipase D. This definitive approach has not yet been extended to other cells or to other phospholipids.

RELATIVE CONTRIBUTION OF AGONIST-INDUCED PHOSPHOLIPASE D AND PHOSOPHOLIPASE C TO PtdOH AND DG GENERATION

Recently, a simple approach has been developed to determine the relative contribution of the phospholipase D and phospholipase C pathways [54,62,63] to PtdOH formed by stimulated cells. This approach involves double-labelling of cellular PtdCho with ^{32}P and ^{3}H, with the ^{3}H being in the acyl chain. Following stimulation, these labelled cells would produce [^{32}P]PtdOH exclusively by phospholipase D. However, [^{3}H]PtdOH could be formed by both phospholipase D and the phospholipase C/DG kinase pathway. Therefore, by comparing the ^{3}H/^{32}P ratios of PtdOH and PtdCho, it is possible to determine contribution of the DG kinase pathway to PtdOH accumulation. For instance, in neutrophils, the ^{3}H/^{32}P ratio of

PtdOH formed early during stimulation is identical to that of PtdCho, demonstrating that [^{3}H]PtdOH is derived exclusively by phospholipase D action on PtdCho [54]. At later times, ^{3}H/^{32}P ratios of PtdOH are higher than that of PtdCho, demonstrating a conversion of [^{3}H]DG to [^{3}H]PtdOH. The conclusion that the early formation of PtdOH occurs exclusively by phospholipase D action on PtdCho is supported by the fact that PtdOH resembles PtdCho with respect to fatty acyl composition and *sn*-1 bond distribution [51,54,56]. Determinations of ^{32}P specific activities of PtdOH and PtdCho further indicate that phospholipids other than PtdCho or pathways other than phospholipase D do not contribute significantly to PtdOH accumulation in stimulated neutrophils [54]. It remains to be determined if similar conclusions are valid for other cells including macrophages [66], hepatocytes [34], endothelial cells [90] and spermatozoa [38], where PtdCho is likely to be degraded by phospholipase D.

While it is generally believed that DG formed during stimulation is derived primarily from phospholipase C-catalysed degradation of phospholipid precursors, PtdOH formed by phospholipase D can also be degraded by PtdOH phosphohydrolase to produce DG [111]. In cell-free preparations from rat brain [42] and endothelial cells [35], PtdOH formed by phospholipase D action on PtdCho is rapidly dephosphorylated by PtdOH phosphohydrolase to produce DG. Conclusive evidence for the operation of the phospholipase D/phosphohydrolase pathway in stimulated cells comes from recent experiments using cytochalasin B-primed neutrophils in which endogenous PtdCho has been double-labelled with ^{3}H (acyl chain) and ^{32}P [54]. Upon stimulation of these cells, both [^{32}P]phosphate and [^{3}H]DG, but not [^{32}P]phosphocholine, are formed in parallel with a concomitant decline in PtdOH accumulation. Furthermore, the PtdOH phosphohydrolase inhibitor, propranolol, inhibits formation of both [^{3}H]DG and [^{32}P]phosphate while increasing PtdOH accumulation without affecting the formation of choline and phosphatidylethanol [54]. Propranolol also inhibits DG mass accumulation by 70–90%, confirming the radiolabelling studies. Since propranolol does not inhibit phosphoinositide-specific phospholipase C (our unpublished observation) and also since phosphocholine mass is not increased during neutrophil stimulation [48], it is concluded that sequential action of phospholipase D and PtdOH phosphohydrolase on PtdCho is the major route to DG formation by cytochalasin B-primed neutrophils. In REF52 rat embryonic cells stimulated with PMA [102], DG formation is inhibited by propranolol whereas in gonadotropin-releasing-hormone-stimulated granulosa cells propranolol increases PtdOH accumulation [76]. These observations are consistent with the phospholipase D/phosphohydrolase pathway being involved in DG generation by these cells. This novel pathway is likely to contribute significantly to DG formation in various other cells where phospholipase D is activated to produce PtdOH (see Table 1 for references).

In certain cultured cells, including MDCK-D1 cells [92], A10 smooth muscle cells [84] and 3T3-L1 preadipocytes [86], PtdCho hydrolysis by phospholipase C may be a major route to DG formation, although involvement of phospholipase D/phosphohydrolase cannot be ruled out. Astrocytoma cells degrade PtdCho to produce both choline and phosphocholine. Experiments in which these cells have been double-labelled with [^{14}C]arachidonic acid and [^{3}H]myristic acid suggest that PtdCho hydrolysis accounts for about 60% of the total DG formed during muscarinic stimulation [80]. However, the relative contribution of phospholipase C and phospholipase D to this DG formation is not known. A comprehensive approach involving double-labelling procedures, mass measurements and pharmacological manipulations is needed to establish the existence and relative importance of various pathways to PtdOH and DG generation during stimulation.

REGULATION OF AGONIST-INDUCED PHOSPHOLIPASE D AND PHOSPHOLIPASE C HYDROLYSING PtdCho

With regard to cellular regulation, phospholipase D has been studied more extensively than PtdCho-specific phospholipase C. The picture emerging from these studies indicates that receptor-linked activation of phospholipase D, and probably also phospholipase C, may occur via several distinct mechanisms and may involve multiple factors including Ca^{2+}, DG, PKC and G proteins. Postulated interactions between these factors are illustrated in Fig. 1.

Role of phosphoinositide breakdown

Most of the agonists that cause PtdCho hydrolysis also induce phosphatidylinositol 4,5-bisphosphate breakdown and subsequent mobilization of intracellular Ca^{2+}. Furthermore, both PMA and 1-acyl-2-acetyl-*sn*-glycerol act synergistically with the Ca^{2+} ionophore A23187 to activate phospholipase D in neutrophils [63]. These observations suggest that initial increases in Ca^{2+} and DG due to phospholipase C activation may be necessary for subsequent PtdCho hydrolysis. However, in MDCK-D1 cells stimulated with adrenaline, phosphocholine formation precedes phosphatidylinositol 4,5-bisphosphate hydrolysis and is not inhibited by neomycin pretreatment [92]. Furthermore, certain agonists, including interleukin-1, interleukin-3 and epidermal growth factor, which are known not to stimulate the hydrolysis of inositol lipids, still cause formation of choline metabolites in cultured cells [69,70,98]. In addition, in vascular smooth muscle cells stimulated with angiotensin [85] and in IIC9 fibroblasts stimulated with α-thrombin [98], phosphoinositide breakdown can be completely abolished without attenuating the sustained DG responses. Conversely, in neutrophils stimulated with fMet-Leu-Phe in the absence of cytochalasin B, phosphatidylinositol 4,5-bisphosphate is maximally hydrolysed [58] with minimal stimulation of phospholipase D [54]. Thus, phosphoinositide hydrolysis *per se* may be neither essential nor sufficient for, but may still have modulatory effects on, receptor-mediated PtdCho breakdown.

Role of Ca^{2+} influx

Although PtdCho hydrolysis can occur in the absence of inositol 1,4,5-trisphosphate-induced mobilization of Ca^{2+}, there is some evidence that receptor-mediated Ca^{2+} flux through the plasma membrane may be involved in phospholipase D activation. In neutrophils, depletion of extracellular Ca^{2+} by EGTA prevents phospholipase D activation by receptor agonists and by A23187 [49,54,62,63]. Maximal activation of phospholipase D in neutrophils requires prior treatment with cytochalasin B [49,54,62], although such treatment affects neither phosphoinositide breakdown nor the extent of the initial Ca^{2+} rise [58]. However, in the presence of cytochalasin B, fMet-Leu-Phe causes a sustained Ca^{2+} increase as opposed to a transient initial response in the absence of cytochalasin B [58], indicating increased Ca^{2+} flux across the plasma membrane. In spermatozoa, fucose sulphate glycoconjugate-stimulated Ca^{2+} influx and phospholipase D activation are both inhibited by the Ca^{2+} channel blocker verapamil [38]. Thus, receptor-mediated Ca^{2+} metabolism at the level of plasma membrane appears to be necessary for enhanced PtdCho hydrolysis.

Effects of Ca^{2+} on phospholipase D and phospholipase C have been investigated in cell-free preparations from several tissues. Partially purified preparations of phospholipase D from rat

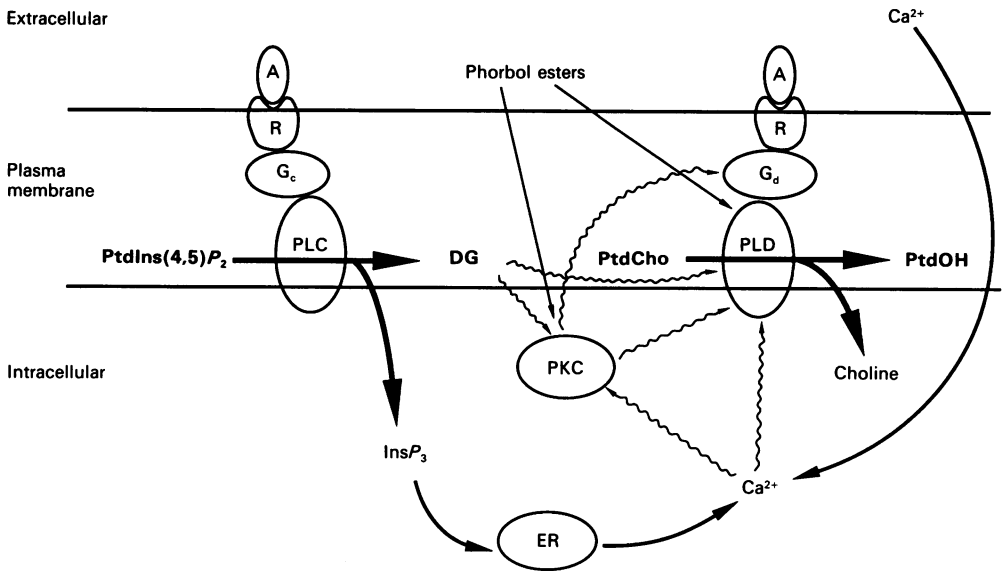

Fig. 1. Hypothetical scheme showing phospholipase D activation by receptor agonists and phorbol esters

Interaction of an agonist (A) with a specific receptor (R) activates phospholipase C (PLC) via a specific G protein (G_c) leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to produce DG and inositol 1,4,5-trisphosphate (InsP_3). InsP_3 mobilizes Ca^{2+} from the endoplasmic reticulum (ER). Ca^{2+} is also mobilized from the external medium through specific Ca^{2+}-channels, raising the level of cytosolic free Ca^{2+}. Ca^{2+} can interact with phospholipase D (PLD) directly or indirectly through regulatory proteins such as calmodulin. DG can directly interact with phospholipase D or act synergistically with Ca^{2+} to activate PKC. PKC can phosphorylate either phospholipase D or regulatory proteins such as a specific G protein (G_d) which links phospholipase D to receptor. These interactions facilitate efficient coupling between agonist-bound receptors and phospholipase D through G_d. Phorbol esters can activate phospholipase D both directly and indirectly through PKC.

brain [39] and phospholipase C from heart [27] and promonocytic U937 cells [29] are enhanced by added Ca^{2+}. Ca^{2+} is required for GTPγS-stimulated phospholipase D activity in granulocyte homogenates [37] and for ATP-induced phosphocholine formation in hepatocyte membranes [24]. By contrast, in several preparations, including hepatocyte membranes [34], homogenates from endothelial cells [35] and spermatozoal extracts [38], phospholipase D activities do not require Ca^{2+} for expression. While it is uncertain as to whether the activities expressed in cell-free preparations truly represent receptor-linked enzymes, such studies raise two important issues. First, the Ca^{2+} requirement for receptor-mediated PtdCho hydrolysis may vary among cell types and second, Ca^{2+} may affect phosphodiesterases indirectly through some unknown mechanism that becomes non-functional following cell rupture.

Role of PKC

Phorbol esters appear to be universally effective in inducing PtdCho hydrolysis by both phospholipase C and phospholipase D (Table 1). Synthetic DGs also activate PtdCho hydrolysis in granulocytes and fibroblasts [63,106]. Rank order of potency of these agents for PtdCho hydrolysis closely correlates with PKC activation [5]. In addition, PKC down-regulation by prolonged PMA treatment or PKC inhibition by inhibitors such as H7 and K252a blocks PMA-induced activation of phospholipase D or phospholipase C, partially in some cells [63,86] and completely in others [79,80,106]. Thus, PMA-induced PtdCho hydrolysis may occur by PKC-dependent as well as PKC-independent mechanisms (Fig. 1). There is no evidence that PKC is the sole target for PMA and that PMA down-regulates only PKC. It is possible that PMA directly interacts with phospholipase D and PtdCho-specific phospholipase C in much the same way as it does with PKC. Consistent with this view is the observation that addition of PMA to the membrane preparations from HL-60 cells stimulates phospholipase D activity [64].

In astrocytoma cells stimulated with carbachol, prolonged PMA pretreatment inhibits formation of choline and not of phosphocholine [80]. This result indicates that muscarinic stimulation of phospholipase D, and not phospholipase C, occurs via a PKC-dependent mechanism. However, in other cells, receptor-mediated PtdCho hydrolysis by phospholipase D [63,76] or phospholipase C [86,92] is not inhibited by protein kinase inhibitors. Apparently, receptor-mediated PtdCho hydrolysis occurs largely through mechanisms that do not involve enhanced protein phosphorylation.

Role of G proteins

GTP-binding regulatory proteins (G proteins) transduce the external signal from surface receptors to appropriate effector systems such as adenylate cyclase and phosphoinositide-specific phospholipase C [112]. Non-hydrolysable GTP analogues (e.g. GTPγS) and pertussis toxin have been used extensively to study the role of G proteins in the activation of phospholipases. Pertussis toxin catalyses an ADP-ribosylation of G proteins to block phospholipase activation by Ca^{2+}-mobilizing agonists in certain cells (e.g. neutrophils) but not in others (e.g. hepatocytes) [10,112].

Evidence is emerging from experiments using GTPγS and pertussis toxin that G proteins control PtdCho hydrolysis by both phospholipase C and phospholipase D. GTPγS stimulates phospholipase D activity in rat hepatocyte membranes [34], in granulocyte homogenates [37] and in saponin-permeabilized endothelial cells [90]. A PtdCho-specific phospholipase C may also be activated by GTPγS in hepatocyte membranes [24,34]. These effects are observed with micromolar concentrations of GTPγS and are inhibited by GDPβS, consistent with G protein

involvement. The p21 oncogene protein resembles G proteins in that it binds GTP and, in fibroblasts, expression of this protein by *ras* oncogene transfection is associated with generation of phosphocholine from PtdCho [100,105]. In granulocytes, pertussis toxin inhibits activation of phospholipase D by fMet-Leu-Phe but not by PMA [56,62]. However, in rat hepatocytes [34] and in A10 aortic smooth muscle [84], vasopressin-induced PtdCho hydrolysis is not inhibited by pertussis toxin. It is apparent that PtdCho-specific phosphodiesterases are regulated by distinct G proteins (Fig. 1).

In granulocyte homogenates, neither Ca^{2+} nor GTPγS is sufficient for phospholipase D activation [37]. A combined presence of both Ca^{2+} and GTPγS is essential. However, in hepatocyte membranes, GTPγS alone is sufficient for phospholipase D activation and Ca^{2+} has no effect on phospholipase D in the absence or presence of GTPγS [34]. Since Ca^{2+} by itself activates phosphoinositide-specific phospholipase C [112], it may be concluded that phospholipase D activation by GTPγS is not merely a consequence of phosphoinositide breakdown and that, in these systems, G protein stimulation is essential for phospholipase D activation. In hepatocyte membranes, PMA augments GTPγS-stimulation of phospholipase D, suggesting that PMA [24,34] and, by inference, DG, may modulate interactions between phospholipase D and G proteins (Fig. 1).

In permeabilized endothelial cells, ATP stimulates phospholipase D by stimulating P_2-purinergic receptors and the effects of ATP and GTPγS are synergistic at low concentrations [90]. Similarly, ATP and ADP, but not vasopressin, enhance GTPγS-stimulated PtdCho hydrolysis in rat hepatocyte membranes [24]. Thus, although GTPγS-stimulated G proteins may couple functional receptors to phospholipase D, unequivocal proof for G protein regulation of receptor-mediated PtdCho hydrolysis must await appropriate reconstitution studies.

REGULATION OF CELLULAR LEVELS OF PtdOH AND DG DURING STIMULATION

PtdOH and DG are readily interconvertible through PtdOH phosphohydrolase and DG kinase, and may be further metabolized through the phosphoinositide cycle and the CDP-choline pathway (Fig. 2). DG may also be deacylated by lipases [113] or reacylated by acyltransferases [114]. In cells where PtdCho is degraded to produce DG, there is little information to indicate that deacylation and reacylation constitute major routes of DG metabolism. There is now increasing evidence that PtdOH phosphohydrolase and DG kinase, as well as cytidylyltransferase, the rate-limiting step in the CDP-choline pathway [115], are under important regulatory controls. Translocation to the cellular membranes has emerged as an important mode of activation for these predominantly cytosolic enzymes [111,115–117]. Thus, these enzymes are ideally suited to serve as regulators of the cellular levels of PtdOH and DG during stimulation.

The early generation of DG from phosphoinositides is usually transient. By contrast, DG derived from PtdCho is almost invariably sustained, although PtdCho hydrolysis is rapid and transient [54,56,62,66,84,86,90,92,101]. In many cells, receptor activation leads to increased degradation and subsequent synthesis of both phosphoinositides and PtdCho [13,115,118]. However, PMA stimulates only PtdCho synthesis without altering degradation or synthesis of phosphoinositide [106], although PMA-stimulated cells hydrolyse PtdCho to produce both PtdOH and DG in large amounts (Table 1). Apparently, DG and PtdOH derived from PtdCho do not enter the phosphoinositide cycle; instead they are metabolized through the CDP-choline pathway.

It is likely that phosphoinositides and PtdCho are hydrolysed at the plasma membrane [7,55] and DGs formed from either source can then be transported to the endoplasmic reticulum [119], the major site for the resynthesis of both PtdCho and

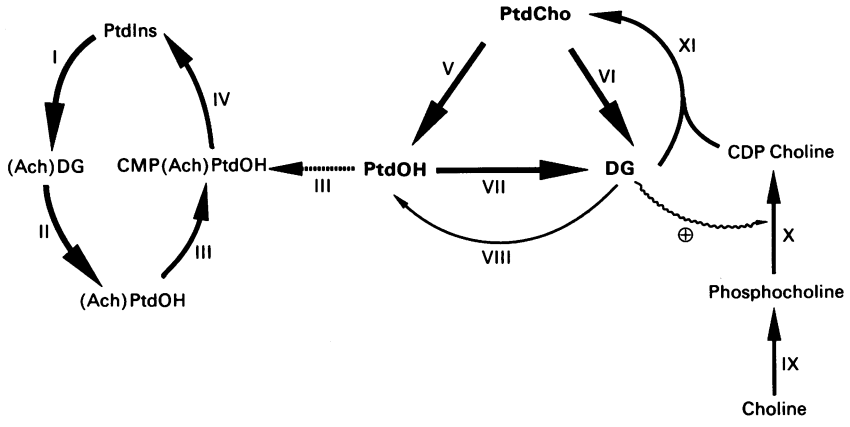

Fig. 2. Proposed regulation of cellular levels of PtdOH and DG during cell activation

Phosphoinositides (PtdIns) are degraded by a phospholipase C (I) to produce arachidonoyl-DG [(Ach)DG]. This is preferentially phosphorylated by a membrane-bound DG kinase (II) to produce arachidonoyl-PtdOH [(Ach)PtdOH] which is converted by a cytidylyltransferase (III) to CMP-arachidonoyl-PtdOH [CMP-(Ach)PtdOH]. A phosphatidate transferase (IV) then catalyses the condensation of CMP-(Ach)PtdOH with inositol to produce PtdIns. This increased turnover of PtdIns results in a rapid removal of DG derived from PtdIns. Arachidonate-poor PtdCho is hydrolysed by phospholipase D (V) or phospholipase C (VI) to produce arachidonate-poor PtdOH or DG, respectively. This PtdOH is not converted to PtdIns; instead it is dephosphorylated by a PtdOH phosphohydrolase (VII) to DG. PtdCho-derived DG can be phosphorylated, albeit poorly, by a cytosolic DG kinase (VIII). During stimulation, translocation of this DG kinase to the membrane further limits its ability to phosphorylate arachidonate-poor DG, resulting in a sustained accumulation of DG. Subsequently, DG is converted back to PtdCho through the CDP-choline pathway involving choline kinase (IX), cytidylyltransferase (X) and phosphocholinetransferase (XI). In this pathway, the cytidylyltransferase is the rate-limiting step. DG activates cytidylyltransferase to facilitate its own conversion to PtdCho with an eventual termination of the signal. According to this proposal, the fatty acid composition of PtdOH and DG determines the flux of these molecules through the PtdIns cycle and the CDP-choline pathway.

phosphoinositides. Thus, PtdCho and phosphoinositide cycles may not be topographically separated. However, one feature that distinguishes DGs derived from phosphoinositides and PtdCho is their acyl composition. DG derived from phosphoinositides is rich in arachidonic acid, whereas DG species coming from PtdCho contain little arachidonic acid [51,83,99,113]. This feature may provide a basis for differential regulation of DGs derived from different sources.

Recent studies have demonstrated that the membrane-bound form of DG kinase prefers arachidonyl-DG as substrate, whereas the cytosolic form shows no apparent specificity for acyl composition [120,121]. It may, therefore, be hypothesized that during activation the translocation of the enzyme to the cellular membrane increases membrane-bound activity and thereby facilitates the conversion of arachidonyl-DG to arachidonyl-PtdOH and subsequent conversion to phosphatidylinositol (Fig. 2), so that arachidonyl-DG accumulates only transiently. By contrast, because of reduced levels of cytosolic DG kinase activity, DG derived from PtdCho would be poorly phosphorylated to PtdOH, resulting in a sustained DG response.

Although the DG signal from phosphoinositide breakdown is rapidly attenuated by DG kinase [118], there is currently no information as to how the sustained signal terminates. However, a plausible hypothesis can be based on the fact that DG and PMA activate cellular cytidylyltransferase [13,115,123–125]. By virtue of its ability to positively affect cytidylyltransferase, sustained DG may enhance its own conversion to PtdCho (Fig. 2) with an eventual termination of the signal.

FUNCTIONAL SIGNIFICANCE OF AGONIST-INDUCED PtdCho HYDROLYSIS

The fact that PtdCho hydrolysis is a wide-spread receptor-linked process (Table 1) suggests its critical importance in signal transduction. Mass measurements in chemotactic peptide-stimulated neutrophils demonstrate that 3–5% of the total cellular PtdCho is phosphodiesteratically cleaved [54]. Isotopic labelling studies confirm a similar conclusion in other cells (Table 1). Since PtdCho accounts for about 50% of the total cellular phospholipid content, PtdCho hydrolysis ensures large quantities of PtdOH and DG without drastic alterations in membrane integrity. Fig. 3 summarizes the possible ways in which PtdCho breakdown by phospholipase C and phospholipase D may be involved in cellular functions.

Role of DG

It is widely accepted that the primary role of DG is to activate PKC which phosphorylates a range of cellular proteins [5,6]. However, there exists evidence to indicate that DG may also directly affect several other enzymes including DG kinase [117], cytidylyltransferase [115] and phospholipase D [63,64]. Numerous studies since its discovery in 1977 have established PKC as a central component for the activation of a large array of biological processes, including metabolism, secretion, contraction, proliferation and differentiation [5]. PKC requires Ca^{2+} and phosphatidylserine for activity. DG and phorbol esters bind to PKC and thereby increase the affinity of the enzyme for Ca^{2+} so as to permit its full activation at the ambient concentrations of intracellular Ca^{2+}. In intact cells, PKC is predominantly cytosolic and undergoes stimulus-induced translocation to the membrane which provides an appropriate lipid environment for activation.

In cells such as neutrophils, phosphoinositide hydrolysis by phospholipase C is accompanied by increases in DG levels [53,58] and transient activation of PKC [126]. However, under conditions where neutrophils are stimulated to produce DG from PtdCho [54,56], a prolonged translocation of PKC is

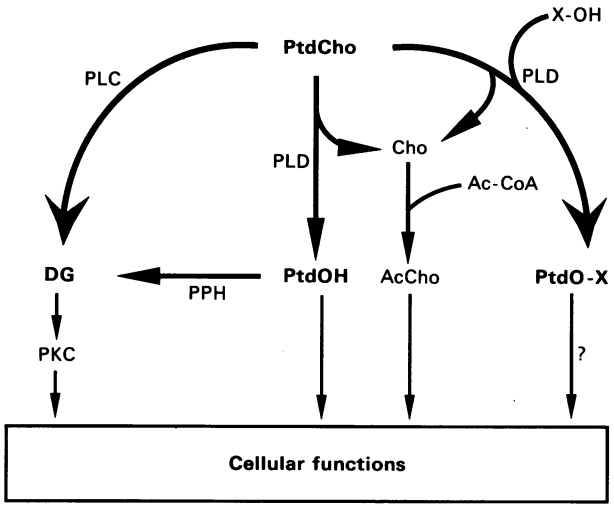

Fig. 3. Functional significance of PtdCho hydrolysis

PtdCho breakdown by either phospholipase C (PLC) or sequential actions of phospholipase D (PLD) and PtdOH phosphohydrolase (PPH) leads to a sustained DG response. This DG causes prolonged activation of PKC to maintain a sustained physiological response even after the Ca^{2+} signal subsides. PtdOH either alone or in combination with other second messengers (e.g. DG, Ca^{2+}) might subserve mediator functions that are yet to be identified. Transphosphatidylation between PtdCho and putative intracellular acceptor compounds (X-OH) such as nucleosides may produce 'phosphatidylated' compounds (PtdO-X) such as phosphatidyl-nucleosides with potential mediator functions. An acetyltransferase transfers the acetyl group from acetyl-CoA to choline (Cho) to form acetylcholine (AcCho).

observed [126,127]. Furthermore, in MDCK-D1 cells stimulated with adrenaline, PKC translocation correlates with PtdCho hydrolysis [92]. In most cells, the physiological response persists long after the initial Ca^{2+} signal has subsided to basal levels [128]. It has been suggested that PKC activation may be responsible for maintaining a response initiated by inositol 1,4,5-trisphosphate-induced mobilization of intracellular Ca^{2+} [128]. Postulated prolongation of PKC activation could be readily achieved by sustained DG generation from PtdCho hydrolysis. Sustained DG generation may also be of critical importance for long-term responses such as proliferation. Consistent with this suggestion is the observation that various growth factors, and oncogenic transformation, cause PtdCho hydrolysis in cultured cells (Table 1).

Phosphoinositide-derived DG consists primarily of a 1-stearoyl-2-arachidonyl species, whereas DG from PtdCho degradation is composed of multiple molecular species with regard to both acyl composition and *sn*-1 bond distribution [51,54,56, 83,87,99]. DG subspecies formed during cell stimulation are determined by cell type and by agonists within the same cell. Thus, neutrophils produce both 1,2-diacyl-*sn*-glycerol and 1-*O*-alkyl-2-acyl-*sn*-glycerol in roughly equal amounts when stimulated with fMet-Leu-Phe or PMA, but predominantly 1,2-diacyl-*sn*-glycerol after stimulation with opsonized zymosan particles [57].

DG species containing various fatty acids with different chain lengths are capable of activating PKC. It has been suggested that DG subspecies with unsaturated fatty acids are most active [5]. Several studies have indicated that 1-*O*-alkyl-2-acyl-*sn*-glycerol does not activate, but in fact inhibits, PKC [129,130]. However, a recent study using purified PKC from rat brain concludes that 1-*O*-alkyl-2-acyl-*sn*-glycerol does activate PKC but only at relatively high Ca^{2+} concentrations compared with 1,2-diacyl-*sn*-

glycerol [131]. The differential responsiveness of PKC to DG molecular species underscores the possible significance of the existence in tissues of multiple PKC isoforms. These isoforms, of which there are at least seven, vary considerably with respect to both tissue distribution and sensitivity to various activating factors, including DG and PMA [6]. It is possible that, *in vivo*, different DG subspecies affect different PKC isoforms differently and thereby produce distinct PKC activation patterns with respect to extent, duration, substrate recognition and subcellular translocation.

Role of PtdOH

Although PtdOH is degraded to provide DG, it is unlikely that this is the only function of phospholipase D activation. Phospholipase D-derived PtdOH has several attributes suggestive of its potential role as a second messenger. Upon receptor activation, PtdOH is formed rapidly and in large quantities [34,38,49,54], and appears to be localized at the plasma membrane [55]. An earlier conclusion that PtdOH is a potent Ca^{2+} ionophore in artificial phospholipid membranes [132] has not been confirmed by subsequent studies [133]. However, exogenous PtdOH causes Ca^{2+} influx across the plasma membrane in intact cells [134]. A possible explanation for this Ca^{2+} influx may be based on a recent finding that PtdOH binds to specific membrane sites (receptors?) on cultured fibroblasts [135]. Indeed, when added to fibroblasts, PtdOH causes rapid DNA synthesis and cell proliferation [136,137]. These observations raise the intriguing possibility that PtdOH might act as an autocoid to modulate cellular functions.

Several studies have raised the possibility that PtdOH may be involved in stimulus–secretion coupling. In platelets stimulated with various agonists, PtdOH formation through the phospholipase C/DG kinase pathway exhibits a close kinetic correlation with serotonin secretion from dense granules [138,139]. In chemotactic peptide-stimulated neutrophils, PtdOH generation through phospholipase D coincides with the release of azurophilic granules with respect to time-course, Ca^{2+} requirement and augmentation by cytochalasin B [49]. Several other secretory cells including mononuclear cells, neuronal cells and ovarian granulosa cells, contain an activatable phospholipase D [65,66,76,79,80]. In granulosa cells, gonadotropin-releasing hormone-stimulated aldosterone secretion is enhanced by propranolol with concomitant enhancement of PtdOH accumulation, and addition of phospholipase D from *Streptomyces chromofuscus* to intact cells induces both aldosterone secretion and PtdOH production [76]. The observed fusogenic ability of PtdOH is consistent with its potential involvement in secretory responses [140].

Role of transphosphatidylation

This reaction exhibits several important features that are consistent with its potential involvement in signal transduction (Fig. 3). First, transphosphatidylation is rapidly enhanced by various receptor agonists and phorbol esters (Table 1). Second, the reaction appears to be widespread in mammalian tissues (Table 1). Third, on a molar basis, primary alcohols are much better acceptors for the phosphatidyl group than is water. Although no systematic search for putative cellular acceptors during cell activation has yet been made, phospholipase D activities of non-mammalian origin have been shown to catalyse the transfer of the phosphatidyl moiety of PtdCho to nucleosides and phosphatidylinositol to produce phosphatidylnucleosides and bis-phosphatidylinositol, respectively [141,142]. Many other cellular components including proteins may be similarly 'phosphatidylated'. In addition to providing a potentially large array of modified molecules, transphosphatidylation might constitute a novel means of rapidly 'anchoring' specific cytosolic components to the membrane bilayer.

A recent study has shown that peripheral lymphocytes from alcoholic subjects exhibit a significantly greater potential to synthesize phosphatidylethanol than do lymphocytes from the control population [143]. Various organs, including brain, liver and kidney, from ethanol-intoxicated rats produce phosphatidylethanol in large quantities [144]. An additional relevant finding is that phosphatidylethanol can substitute for phosphatidyserine in activating a brain-specific PKCγ [145]. These observations indicate that phospholipase D-catalysed transphosphatidylation may play a role in the development of alcohol dependency and alcohol-related pathology.

Role of choline

There is no evidence to indicate a signalling role for choline or phosphocholine. However, a coupling between PtdCho hydrolysis and acetylcholine synthesis in the brain has been proposed [12,81,82]. During muscarinic or electrical stimulation of rat brains, choline is mobilized in a sustained manner and acetylcholine is synthesized at the expense of cellular PtdCho [81,82]. In addition, using synaptosomal preparations, it has been demonstrated that choline released from endogenous PtdCho by endogenous phospholipase D is subsequently acetylated into acetylcholine [41]. Brain is a very rich source of phospholipase D [33] and in cultured neuronal cells, phospholipase D acting on PtdCho is stimulated by muscarinic stimulation [79,80]. Thus, phospholipase D activation serves to provide not only PtdOH and DG to mediate sustained release of acetylcholine but also choline for acetylcholine resynthesis to replenish the depleted acetylcholine pool. Apparently, the released acetylcholine is capable of establishing a positive feedback regulation of acetylcholine synthesis. Such a regulation may be critical under conditions where a normal supply of choline from plasma is severely restricted.

CONCLUSION

Phosphodiesteratic cleavage of PtdCho, the principal cellular phospholipid, is emerging as a wide-spread transduction pathway. An intriguing feature of PtdCho hydrolysis is that it is universally activated by phorbol esters and related compounds. Kinetic analyses of products of PtdCho breakdown suggest activation of either phospholipase C, phospholipase D or both, depending on cell types and stimulants. However, except for neutrophils, a clear distinction between phospholipase C and phospholipase D in stimulated cells is still lacking. Such distinctions are crucial, in view of rapid interconversion between hydrolytic products through highly active kinases and phosphatases. Available evidence suggest that phospholipase D is regulated by multiple factors including G proteins, Ca^{2+}, DG and PKC. Little information is available on the regulation of PtdCho-specific phospholipase C. There is convincing evidence that PtdCho-derived DG may cause sustained activation of PKC to initiate and maintain long-term responses. No such second-messenger role for PtdOH has yet been described, but it is unlikely that the only function of PtdOH is to provide DG. The physiological significance of phospholipase D-catalysed transphosphatidylation involving PtdCho is also not known. It seems likely that both sustenance and termination of the DG signal involves several regulatory enzymes including PtdOH phosphohydrolase, DG kinase and cytidylyltransferase. These enzymes are subject to regulation by DG, and are, therefore, ideally suited for feedback regulation. Future studies will focus on rigorous identification of PtdCho-specific phosphodiesterases in stimulated cells, determination of relative contributions of

various pathways to PtdOH and DG formation, purification and molecular cloning of various key enzymes regulating PtdCho metabolism and elucidation of complex interactions between various transduction pathways.

We thank Lisa Ramirez for typing the manuscript and Drs. M. I. Siegel, R. W. Egan, W. Kreutner and R. W. Bryant for helpful comments.

REFERENCES

1. Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R. & Lefkowith, J. B. (1986) Annu. Rev. Biochem. **55**, 69–102
2. Hanahan, D. J. (1986) Annu. Rev. Biochem. **55**, 483–509
3. Berridge, M. J. (1984) Biochem. J. **220**, 345–360
4. Berridge, M. J. & Irvine, R. F. (1989) Nature (London) **341**, 197–205
5. Nishizuka, Y. (1986) Science **233**, 305–312
6. Nishizuka, Y. (1989) Nature (London) **334**, 661–665
7. Abdel-Latif, A. (1986) Pharmacol. Rev. **38**, 227–272
8. Billah, M. M. (1987) Annu. Rep. Med. Chem. **22**, 223–233
9. Rhee, S. G., Suh, P.-G., Ryu, S.-H. & Lee, S. Y. (1989) Science **244**, 546–550
10. Exton, J. (1988) FASEB J. **2**, 2670–2676
11. Farese, R. V. (1988) Am. J. Med. Sci. **296**, 223–230
12. Loffelholz, K. (1989) Biochem. Pharmacol. **38**, 1543–1549
13. Pelech, S. L. & Vance, D. E. (1989) Trends Biochem. Sci. **14**, 28–30
14. White, D. A. (1973) in Form and Function of Phospholipids (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), pp. 444–458, Elsevier, Amsterdam
15. Horroks, L. A. & Sharma, M. (1982) In Phospholipids (Hawthorne, J. N. & Ansell, G. B., eds.), pp. 51–93, Elsevier, Amsterdam
16. Mueller, H. W., O'Flaherty, J. T. & Wykle, R. L. (1982) Lipids **17**, 72–77
17. Ojima-Uchiyama, A., Masuzawa, Y., Suqiura, T., Waku, K., Saito, H., Yui, Y. & Tomioka, H. (1988) Lipids **23**, 815–817
18. Arthur, G., Mock, T., Zaborniak, C. & Choy, P. C. (1985) Lipids **20**, 693–698
19. Mueller, H. W., O'Flaherty, J. T., Green, D. G., Samuel, M. P. & Wykle, R. L. (1984) J. Lipid Res. **25**, 383–388
20. Dawson, R. M. C. (1967) Biochem. J. **102**, 205–210
21. Yang, S., Freer, S. & Benson, A. (1967) J. Biol. Chem. **242**, 477–484
22. Matsuzawa, K. & Hostetler, K. Y. (1980) J. Biol. Chem. **255**, 646–652
23. Edgar, A. D. & Freysz, L. (1982) Biochim. Biophys. Acta **711**, 244–228
24. Irving, H. R. & Exton, J. H. (1987) J. Biol. Chem. **262**, 3440–3443
25. Clark, M. A., Littlejohn, D., Conway, T. M., Mong, S., Steiner, S. & Crooke, S. T. (1986) J. Biol. Chem. **261**, 10713–10718
26. Martin, T. W., Wysolmerski, R. B. & Lagunoff, D. (1987) Biochim. Biophys. Acta **917**, 296–307
27. Wolf, R. A. & Gross, R. W. (1985) J. Biol. Chem. **260**, 7295–7303
28. Sheikhnejad, R. G. & Srivastava, P. N. (1986) J. Biol. Chem. **261**, 7544–7549
29. Clark, M. A., Shorr, R. G. L. & Bomalski, J. S. (1986) Biochem. Biophys. Res. Commun. **140**, 114–119
30. Heller, M. (1978) Adv. Lipid Res. **16**, 267–326
31. Hanahan, D. J. & Chaikoff, I. L. (1948) J. Biol. Chem. **172**, 191–196
32. Saito, M., Bourque, E. & Kanfer, J. N. (1975) Arch. Biochem. Biophys. **164**, 420–428
33. Chalifour, R. J. & Kanfer, J. N. (1980) Biochem. Biophys. Res. Commun. **96**, 742–747
34. Bocckino, S. P., Blackmore, P. F., Wilson, P. B. & Exton, J. H. (1987) J. Biol. Chem. **262**, 15309–15315
35. Martin, T. W. (1988) Biochim. Biophys. Acta **962**, 282–296
36. Tettenborn, C. S. & Mueller, G. C. (1988) Biochem. Biophys. Res. Commun. **155**, 249–255
37. Anthes, J. C., Eckel, S., Siegel, M. I., Egan, R. W. & Billah, M. M. (1989) Biochem. Biophys. Res. Commun. **163**, 657–664
38. Domino, S. E., Bocckino, S. B. & Garbers, D. L. (1989) J. Biol. Chem. **264**, 9412–9419
39. Taki, T. & Kanfer, J. N. (1979) J. Biol. Chem. **254**, 9701–9705
40. Kater, L. A., Goetzl, E. J. & Austen, K. F. (1976) J. Clin. Invest. **57**, 1173–1180
41. Hattori, H. & Kanfer, J. N. (1985) J. Neurochem. **45**, 1578–1584
42. Chalifour, R. & Kanfer, J. N. (1982) J. Neurochem. **39**, 299–305
43. Kobayashi, M. & Kanfer, J. N. (1987) J. Neurochem. **48**, 1597–1603
44. Bocckino, S. P., Wilson, P. B. & Exton, J. H. (1987) FEBS Lett. **225**, 201–204
45. Balsinde, J., Diez, E. & Mallinedo, F. (1988) Biochem. Biophys. Res. Commun. **154**, 502–508
46. Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. & Bell, R. M. (1986) J. Biol. Chem. **261**, 8597–8600
47. Muma, N. A. & Rowell, P. P. (1985) J. Neurosci. Methods **12**, 249–257
48. Truett, A. P., III, Synderman, R. & Murray, J. J. (1989) Biochem. J. **260**, 909–913
49. Cockcroft, S. (1984) Biochim. Biophys. Acta **795**, 37–46
50. Agwu, D. E., McPhail, L. C., Wykle, R. L. & McCall, C. E. (1989) Biochem. Biophys. Res. Commun. **159**, 79–86
51. Cockcroft, S. & Allan, D. (1984) Biochem. J. **222**, 557–559
52. Rider, L. G. & Neidel, J. E. (1987) J. Biol. Chem. **262**, 5603–5608
53. Reibman, J., Korchak, H. M., Vosshall, L. B., Haines, K. A., Rich, A. M. & Weissmann, G. (1988) J. Biol. Chem. **263**, 6322–6328
54. Billah, M. M., Eckel, S., Mullmann, T., Egan, R. W. & Siegel, M. I. (1989) J. Biol. Chem. **264**, 17069–17077
55. Gelas, P., Ribbes, G., Record, M., Terce, F. & Chap, H. (1989) FEBS Lett. **251**, 213–218
56. Agwu, D. E., McPhail, L. C., Chabot, M. C., Daniel, L. W., Wykle, R. L. & McCall, C. E. (1989) J. Biol. Chem. **264**, 1405–1413
57. Tyagi, S. R., Burnham, D. N. & Lambeth, J. D. (1989) J. Biol. Chem. **264**, 12977–12982
58. Truett, A. P., III, Verghese, M. W., Dillon, S. B. & Snyderman, R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1549–1553
59. Preiss, J. E., Bell, R. M. & Niedel, J. E. (1987) J. Immunol. **138**, 1542–1545
60. Tettenborn, C. S. & Mueller, G. C. (1987) Biochim. Biophys. Acta **931**, 242–250
61. Pai, J.-K., Siegel, M. I., Egan, R. W. & Billah, M. M. (1988) Biochem. Biophys. Res. Commun. **150**, 355–364
62. Pai, J.-K., Siegel, M. I., Egan, R. W. & Billah, M. M. (1988) J. Biol. Chem. **263**, 12472–12477
63. Billah, M. M., Pai, J.-K., Mullmann, T. J., Egan, R. W. & Siegel, M. I. (1989) J. Biol. Chem. **264**, 9069–9076
64. Kiss, Z. & Anderson, W. B. (1989) J. Biol. Chem. **264**, 1483–1487
65. Kinsky, S. C., Loader, J. E. & Benedict, S. H. (1989) Biochem. Biophys. Res. Commun. **162**, 788–793
66. Uhing, R. J., Prpic, V., Hollenbach, P. W. & Adams, D. O. (1989) J. Biol. Chem. **264**, 9224–9230
67. Kennerly, D. A. (1987) J. Biol. Chem. **262**, 16305–16313
68. Gruchalla, R. S., Dinh, T. T. & Kennerly, D. A. (1989) FASEB J. **3**, A788
69. Duronio, V., Nip, L. & Pelech, S. L. (1989) Biochem. Biophys. Res. Commun. **164**, 804–808
70. Rosoff, P. M., Savage, N. & Dinarello, C. A. (1988) Cell **54**, 73–81
71. Rubin, R. (1988) Biochem. Biophys. Res. Commun. **156**, 1090–1096
72. Peter-Riesch, B., Fathi, M., Schlegel, W. & Wollheim, C. B. (1988) J. Clin. Invest. **81**, 1154–1161
73. Matozaki, T. & Williams, J. A. (1989) J. Biol. Chem. **264**, 14729–14734
74. Wolf, B. A., Easom, R. A., Hughes, J. H., McDaniel, M. L. & Turk, J. (1989) Biochemistry **28**, 4291–4301
75. Dunlop, M. & Metz, S. A. (1989) Biochem. Biophys. Res. Commun. **163**, 922–928
76. Liscovitch, M. & Amsterdam, A. (1989) J. Biol. Chem. **264**, 11762–11767
77. Kojima, I., Kojima, K., Kreutter, D. & Rasmussen, H. (1984) J. Biol. Chem. **259**, 14448–14457
78. Liscovitch, M., Blusztajn, J. K., Freese, A. & Wurtman, R. J. (1987) Biochem. J. **241**, 81–86
79. Liscovitch, M. (1989) J. Biol. Chem. **264**, 1450–1456
80. Martinson, E. A., Goldstein, D. & Brown, J. H. (1989) J. Biol. Chem. **264**, 14748–14754
81. Corradetti, R., Lindmar, R. & Loffelholz, K. (1983) J. Pharmacol. Exp. Ther. **226**, 826–832
82. Blusztajn, J. K., Liscovitch, M., Mauron, C., Richardson, U. I. & Wurtmann, R. J. (1987) J. Neural Transm. **24**, 247–259
83. Grove, R. I. & Schimmel, S. D. (1982) Biochim. Biophys. Acta **711**, 272–280
84. Grillone, L. R., Clark, M. A., Godfrey, R. W., Stassen, F. & Crooke, S. T. (1988) J. Biol. Chem. **263**, 2658–2663

85. Griendling, K. K., Rittenhouse, S. E., Brock, T. A., Ekstein, L. S., Gimbrone, M. A. & Alexander, R. W. (1986) J. Biol. Chem. **261**, 5901–5906
86. Besterman, J. M., Duronio, V. & Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6785–6789
87. Bocckino, S. B., Blackmore, P. F. & Exton, J. H. (1985) J. Biol. Chem. **260**, 14201–14207
88. Ragab-Thomas, J. M.-F., Hullin, F., Chap, H. & Douste-Blazy, L. (1987) Biochim. Biophys. Acta **917**, 388–397
89. Martin, T. W. & Michaelis, K. C. (1988) Biochem. Biophys. Res. Commun. **157**, 1271–1279
90. Martin, T. W. & Michaelis, K. (1989) J. Biol. Chem. **264**, 8847–8856
91. Daniel, L. W., Waite, M. & Wykle, R. L. (1986) J. Biol. Chem. **261**, 9128–9132
92. Slivka, S. R., Meier, K. E. & Insel, P. A. (1988) J. Biol. Chem. **263**, 12242–12246
93. Mueller-Decker, K. (1989) Biochem. Biophys. Res. Commun. **162**, 199–205
94. Fukami, K. & Takenawa, T. (1989) J. Biol. Chem. **264**, 14985–14989
95. Magnaldo, I., L'Allemain, G., Chambard, J. C., Moenner, M., Barritault, D. & Pouyssegur, J. (1986) J. Biol. Chem. **261**, 16916–16922
96. Mufson, R. A., Okin, E. & Weinstein, I. B. (1981) Carcinogenesis **2**, 1095–1102
97. Guy, G. R. & Murray, A. W. (1982) Cancer Res. **42**, 1980–1985
98. Wright, T. M., Rangan, L. A., Shin, H. S. & Raben, D. M. (1988) J. Biol. Chem. **263**, 9374–9380
99. Pessin, M. S. & Raben, D. M. (1989) J. Biol. Chem. **264**, 8729–8738
100. Price, B. D., Morris, J. D., Marshall, C. J. & Hall, A. (1989) J. Biol. Chem. **264**, 16638–16643
101. Cabot, M. C., Welsh, C. J., Zhang, Z., Cao, H., Chabbott, H. & Lebowitz, M. (1988) Biochim. Biophys. Acta **959**, 46–57
102. Cabot, M. C., Welsh, C. J., Cao, H. & Chabbott, H. (1988) FEBS Lett. **233**, 153–157
103. Cabot, M. C., Welsh, C. J., Zhang, Z. & Cao, H. (1989) FEBS Lett. **245**, 85–90
104. Welsh, C. J., Cao, H.-T., Chabbott, H. & Cabot, M. C. (1988) Biochem. Biophys. Res. Commun. **152**, 565–572
105. Lacal, J. C., Moscat, J. & Aaronson, S. A. (1987) Nature (London) **330**, 269–272
106. Muir, J. G. & Murray, A. W. (1987) J. Cell Physiol. **130**, 382–391
107. Kester, M., Simonson, M. S., Mene, P. & Sedor, J. R. (1989) J. Clin. Invest. **83**, 718–723
108. Warden, C. H. & Friedkin, M. (1985) J. Biol. Chem. **260**, 6006–6011
109. Macara, I. G. (1989) Mol. Cell. Biol. **9**, 325–328
110. Kanfer, J. N. (1980) Can. J. Biochem. **58**, 1370–1379
111. Brindley, D. N. (1984) Progr. Lipid Res. **23**, 115–133
112. Cockroft, S. (1987) Trends Biochem. Sci. **12**, 75–78
113. Prescott, S. M. & Majerus, P. W. (1983) J. Biol. Chem. **258**, 764–769
114. Haagsman, H. P., de Haas, C. G. M., Geelen, M. J. H. & van Golde, L. M. G. (1982) J. Biol. Chem. **257**, 10593–10598
115. Pelech, S. L. & Vance, D. E. (1984) Biochim. Biophys. Acta **779**, 217–251
116. Besterman, J. M., Pollenz, R. S., Booker, E. L., Jr. & Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9378–9382
117. Maroney, A. C. & Macara, I. G. (1989) J. Cell. Biochem. **40**, 165–172
118. Michell, R. H. (1975) Biochim. Biophys. Acta **415**, 81–147
119. Pagano, R. E. & Longmuir, K. J. (1985) J. Biol. Chem. **260**, 1909–1916
120. MacDonald, M. L., Mack, K. F., Richardson, C. N. & Glomset, J. A. (1988) J. Biol. Chem. **263**, 1575–1583
121. MacDonald, M. L., Mack, K. F., Williams, B. W., King, W. C. & Glomset, J. A. (1988) J. Biol. Chem. **263**, 1584–1592
122. Bishop, W. R., Ganong, B. R. & Bell, R. M. (1986) J. Biol. Chem. **261**, 6993–7000
123. Liscovitch, M., Fleese, A., Blusztajn, J. K. & Wurtman, R. J. (1986) J. Neurochem. **47**, 1936–1941
124. Kolesnick, R. N. (1987) J. Biol. Chem. **262**, 14525–14530
125. Kolesnick, R. N. & Paley, A. E. (1987) J. Biol. Chem. **262**, 9204–9210
126. Horn, W. & Karnovsky, M. L. (1986) Biochem. Biophys. Res. Commun. **139**, 1169–1175
127. Nishihira, J., McPhail, L. C. & O'Flaherty, J. T. (1986) Biochem. Biophys. Res. Commun. **158**, 587–594
128. Rasmussen, H. & Barret, P. Q. (1984) Physiol. Rev. **64**, 938–984
129. Daniel, L. W., Small, G. W., Schmitt, J. D., Ishaq, K. & Piantadosi, C. (1988) Biochem. Biophys. Res. Commun. **151**, 291–297
130. Ganong, B. R., Loomis, C. R., Hannun, Y. A. & Bell, R. M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1184–1188
131. Ford, D. A., Miyake, R., Glaser, P. & Gross, R. W. (1989) J. Biol. Chem. **264**, 13818–13824
132. Serhan, C., Anderson, P., Goodman, E., Dunham, P. & Weissmann, G. (1981) J. Biol. Chem. **256**, 2736–2741
133. Holmes, R. P. & Yoss, N. L. (1983) Nature (London) **305**, 637–638
134. Putney, J. W., Jr., Weiss, S. J., Van De Walle, C. M. & Haddas, R. A. (1980) Nature (London) **284**, 345–347
135. Murayama, T. & Ui, M. (1987) J. Biol. Chem. **262**, 5522–5529
136. Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J. & deLaat, S. W. (1986) Nature (London) **323**, 171–173
137. Yu, C.-L., Tsai, M.-H. & Stacey, D. W. (1988) Cell **52**, 63–71
138. Lapetina, E. G. & Cuatrecasas, P. (1979) Biochim. Biophys. Acta **573**, 394–402
139. Holmsen, H., Dangelmaier, C. A. & Rongved, S. (1984) Biochem. J. **222**, 157–167
140. Simmonds, A. C. & Halsey, M. J. (1985) Biochim. Biophys. Acta **813**, 331–337
141. Shuto, S., Ueda, S., Itoh, H., Endo, E., Fukukawa, K., Imamura, S., Tsujino, M., Matsuda, A. & Ueda, T. (1986) Nucleic Acids Res. **17**, 73–76
142. Clarke, N. G., Irvine, R. F. & Dawson, R. M. C. (1981) Biochem. J. **195**, 521–523
143. Mueller, G. C., Fleming, M. F., LeMahieu, M. A., Lybrand, G. S. & Barry, K. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9778–9782
144. Alling, C., Gustavasson, L. & Ånggard, E. (1983) FEBS Lett. **152**, 24–28
145. Asaoka, Y., Kikkawa, U., Sekiguchi, K., Shearman, M. S., Kosaka, Y., Nakano, Y., Satoh, T. & Nishizuka, Y. (1988) FEBS Lett. **231**, 221–224