phospholipase-D-derived PtdOH in phosphocholine and of DRG kinase on DRG. Thus, phospholipase D to generate phosphatidic acid (PtdOH) and choline formed by phospholipase C degradation of PtdCho, hydrolysis of phosphatidylcholine (PtdCho) by PtdOH phosphohydrolase to guanosine 5’-(y-thio)di-phosphate, respectively; PMA, phorbol 12-myristate 13-acetate.

Abbreviations used: PtdCho, phosphatidylcholine; PtdOH and choline, two immediate products of phospholipase D action on PtdCho, may also be formed by phospholipase C degradation of PtdCho, and subsequent action of a specific phosphatase on phosphocholine and of DRG kinase on DRG. Thus, identification and kinetic analysis of PtdOH and choline may not provide unequivocal proof for phospholipase-D activity. A more direct approach to phospholipase D identification in intact cells is based on the unique ability of phospholipase D to catalyse a transphosphatidylation reaction between PtdCho and primary alcohols to produce phosphatidylalcohols (e.g. phosphatidylethanol in the presence of ethanol) [5, 6]. Since this reaction is not catalysed by phospholipase C, transphosphatidylation has been widely exploited to detect phospholipase D activity in intact cells (Table 1). While measurements of choline, PtdOH and phosphatidylalcohols provide an indication of phospholipase D activity, a definitive proof for phospholipase-D-mediated lipid turnover in intact cells, however, requires specific labelling of cellular PtdCho with 32P under conditions where cellular ATP is not labelled. This is achieved by incubating cells with 32P-labelled 2-lysoPtdCho, which readily enters the cell and becomes rapidly acylated into membrane-associated 32P[P]PtdCho. [32P]PtdOH formation from [32P]PtdCho can occur only by phospholipase D action. This approach has so far been applied successfully to several cell types, including: human neutrophils [7–9], HL-60 granulocytes [15, 16], human eosinophils [17] and monocytic U937 cells [18], with the conclusion that various agonists activate phospholipase D which acts on PtdCho to generate PtdOH and that transphosphatidylation is a valid measure of phospholipase D activity in intact cells. Table 1 lists the cells in which phospholipase D action can be definitively measured.

<table>
<thead>
<tr>
<th>Abbreviations used: PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; DRG, 1,2-diradylglycerol; GTP[S], GDP[S], guanosine 5’-(y-thio)triphosphate; guanosine 5’-(y-thio)diphosphate, respectively; PMA, phorbol 12-myristate 13-acetate.</th>
</tr>
</thead>
</table>

Receptor-coupled phospholipase D: regulation and functional significance
M. Motasim Billah, John C. Anthes and Theodore J. Mullmann
Schering-Plough Research, Department of Allergy, Bloomfield, NJ 07003, U.S.A.

Introduction
Phospholipase-C-catalysed hydrolysis of phosphatidylinositol-4,5-bisphosphate has been studied extensively as an intracellular signalling system for Ca2+-mobilizing agonists [1]. Recent studies clearly indicate that many of these agonists cause rapid hydrolysis of phosphatidylcholine (PtdCho) by phospholipase D to generate phosphatidic acid (PtdOH) and choline [2, 3]. PtdOH can be further degraded by PtdOH phosphohydrolase to 1,2-diradylglycerol (DRG) [2]. DRG activates protein kinase C, a ubiquitous protein that controls a wide array of cellular responses, such as secretion and proliferation [4]. Rapid and transient formation of PtdOH during cell activation [2], coupled with its ability to modify a number of receptor-linked cellular processes [3], underscores the potential importance of phospholipase-D-derived PtdOH in cell signalling.

Identification of phospholipase D
PtdOH and choline, two immediate products of phospholipase D action on PtdCho, may also be formed by phospholipase C degradation of PtdCho, and subsequent action of a specific phosphatase on phosphocholine and of DRG kinase on DRG. Thus, identification and kinetic analysis of PtdOH and choline may not provide unequivocal proof for phospholipase D activity. A more direct approach to phospholipase D identification in intact cells is based on the unique ability of phospholipase D to catalyse a transphosphatidylation reaction between PtdCho and primary alcohols to produce phosphatidylalcohols (e.g. phosphatidylethanol in the presence of ethanol) [5, 6]. Since this reaction is not catalysed by phospholipase C, transphosphatidylation has been widely exploited to detect phospholipase D activity in intact cells (Table 1). While measurements of choline, PtdOH and phosphatidylalcohols provide an indication of phospholipase D activity, a definitive proof for phospholipase-D-mediated lipid turnover in intact cells, however, requires specific labelling of cellular PtdCho with 32P under conditions where cellular ATP is not labelled. This is achieved by incubating cells with 32P-labelled 2-lysoPtdCho, which readily enters the cell and becomes rapidly acylated into membrane-associated 32P[P]PtdCho. [32P]PtdOH formation from [32P]PtdCho can occur only by phospholipase D action. This approach has so far been applied successfully to several cell types, including: human neutrophils [7–9], HL-60 granulocytes [15, 16], human eosinophils [17] and monocytic U937 cells [18], with the conclusion that various agonists activate phospholipase D which acts on PtdCho to generate PtdOH and that transphosphatidylation is a valid measure of phospholipase D activity in intact cells. Table 1 lists the cells in which phospholipase D action can be definitively measured by specific agonists has been demonstrated by measuring one or more of phospholipase-D-derived products.

Regulation of phospholipase D
Analysis of available data indicates that physiologically relevant activation of phospholipase D occurs

Received 17 December 1990

Volume 19
Table I

Agonist-induced cleavage of cellular PtdCho by phospholipase D

<table>
<thead>
<tr>
<th>Cell system</th>
<th>Agonist</th>
<th>Choline</th>
<th>PtdOH</th>
<th>Phosphatidyl-ethanol</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory/immune</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>fMLP, C5a, PMA, A23187, sphingosine</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>[7–14]</td>
</tr>
<tr>
<td>HL-60 cells</td>
<td>fMLP, OAG, PMA, A23187</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>[15, 16]</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>C5a, PMA, A23187</td>
<td>—</td>
<td>†</td>
<td>†</td>
<td>[17]</td>
</tr>
<tr>
<td>U937 cells</td>
<td>C5a, fMLP, PMA</td>
<td>—</td>
<td>†</td>
<td>†</td>
<td>[18]</td>
</tr>
<tr>
<td>Monocytes</td>
<td>PMA</td>
<td>—</td>
<td>—</td>
<td>†</td>
<td>[19]</td>
</tr>
<tr>
<td>Macrophages</td>
<td>PMA, A23187</td>
<td>†</td>
<td>—</td>
<td>—</td>
<td>[20]</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Antigen</td>
<td>—</td>
<td>—</td>
<td>†</td>
<td>[21]</td>
</tr>
<tr>
<td>Platelets</td>
<td>Thrombin, collagen</td>
<td>—</td>
<td>—</td>
<td>†</td>
<td>[22, 23]</td>
</tr>
<tr>
<td>Endocrine/exocrine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreatic</td>
<td>A23187, PMA, carbachol, CCK8</td>
<td>†</td>
<td>†</td>
<td>—</td>
<td>[24–26]</td>
</tr>
<tr>
<td>Ovarian granulosa cells</td>
<td>GnRH, PMA</td>
<td>—</td>
<td>†</td>
<td>†</td>
<td>[27]</td>
</tr>
<tr>
<td>Adrenal granulosa cells</td>
<td>Angiotensin II</td>
<td>—</td>
<td>†</td>
<td>†</td>
<td>[28]</td>
</tr>
<tr>
<td>Nerve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NG108-15 neuroblastoma cells</td>
<td>PMA, DiC&lt;sub&gt;6&lt;/sub&gt;</td>
<td>†</td>
<td>—</td>
<td>†</td>
<td>[29–31]</td>
</tr>
<tr>
<td>1321N1 astrocytoma cells</td>
<td>PMA, carbachol</td>
<td>†</td>
<td>†</td>
<td>—</td>
<td>[32]</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic smooth muscle</td>
<td>PMA</td>
<td>—</td>
<td>†</td>
<td>†</td>
<td>[33]</td>
</tr>
<tr>
<td>Chicken heart</td>
<td>Carbachol</td>
<td>†</td>
<td>†</td>
<td>—</td>
<td>[34]</td>
</tr>
<tr>
<td>Metabolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Vasopressin, angiotensin, adrenaline, ATP, PMA, A23187</td>
<td>—</td>
<td>†</td>
<td>†</td>
<td>[35, 36]</td>
</tr>
<tr>
<td>Endothelial</td>
<td>Thrombin, bradykinin, ATP, A23187, PMA</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>[37–39]</td>
</tr>
<tr>
<td>Epithelial</td>
<td>MCDK</td>
<td>PMA</td>
<td>—</td>
<td>†</td>
<td>[33, 40]</td>
</tr>
</tbody>
</table>

†, Increase; ‡, no change; —, not measured; fMLP, formyl-methionyl-leucyl-phenylalanine; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PAF, platelet-activating factor; CCK8, cholecystokinin octapeptide; GnRH, gonadotropin-releasing hormone; DiC<sub>6</sub>, 1,2-dioctanoyl-sn-glycerol; EGF, epidermal growth factor; FSG, fucose sulphate glycoconjugate.
### Table 1 - continued

<table>
<thead>
<tr>
<th>Cell system</th>
<th>Agonist</th>
<th>Choline</th>
<th>PtdOH</th>
<th>Phosphatidyl-ethanol</th>
<th>Refs.</th>
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<tbody>
<tr>
<td>Fibroblast</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3H10T 1/2</td>
<td>PMA</td>
<td>↑</td>
<td>—</td>
<td>—</td>
<td>[41]</td>
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<tr>
<td>HeLa</td>
<td>PMA, A23187</td>
<td>↑</td>
<td>—</td>
<td>↑</td>
<td>[42, 43]</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>EGF</td>
<td>—</td>
<td>↑</td>
<td>↑</td>
<td>[44]</td>
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<tr>
<td>REF52</td>
<td>Vasopressin, serum, PMA</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>[45–48]</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>FSG, gramicidin S</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>[49]</td>
</tr>
<tr>
<td>Erythroleukaemia cells</td>
<td>Thrombin, PAF, ADP, A23187</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>[50]</td>
</tr>
</tbody>
</table>

**Fig. 1**

Proposed mechanisms of phospholipase D activation and its coupling to cellular functions

![Diagram](https://via.placeholder.com/150)

by two distinct mechanisms (Fig. 1). One mechanism is activated by receptor-linked agonists and involves GTP-binding regulatory proteins (G-proteins). In cell-free preparations from several cells, including: hepatocytes [35], HL-60 granulocytes [51, 52], endothelial cells [38] and cerebral cortex [53, 54], the stable GTP analogue, guanosine 5′-(γ-thio)triphosphate (GTP[S]) causes a dose-dependent stimulation of phospholipase D activities. This stimulation requires Mg²⁺ and is inhibited by guanosine 5′-(γ-thio)diphosphate (GDP[S]). More significantly, at low concentrations, GTP[S] acts synergistically with certain receptor agonists, such as ATP in hepatocyte membranes [35] and in permeabilized endothelial cells [38], and carbachol in cerebral cortex membranes [54]. While these observations implicate G-proteins in receptor-linked activation of phospholipase D in intact cells, final proof must await appropriate reconstitution experiments.

The other mechanism with physiological relevance is expressed by nanomolar concentrations of phorbol esters (see Table 1 for specific references) and probably involves protein kinase C. Phorbol ester stimulation of phospholipase D is a universal phenomenon (see Table 1). Tumour-promoting phorbol esters activate phospholipase D with potencies and a structure–activity relationship that are indistinguishable from those for protein kinase C (see Table 1 for specific references). In addition, protein kinase C inhibitors (e.g. staurosporine) inhibit phorbol 12-myristate 13-acetate (PMA)-induced phospholipase D activity in several cells, including: neutrophils [12, 14, 16], granulosa cells [27], neuroblastoma cells [30], endothelial cells [39] and REF52 fibroblasts [47]. In some instances, prolonged treatment of cells to downregulate protein kinase C diminishes phospholipase D activity [27, 35, 47]. These observations suggest the importance of protein-kinase-C-catalysed protein phosphorylation in phospholipase D activation.

In most of the cells listed in Table 1, phospholipase D is activated by both phorbol esters and receptor agonists, suggesting that the two activation pathways coexist in a cell. However, protein kinase inhibitors and protein kinase C downregulation do not attenuate phospholipase D activity in receptor-stimulated cells [12, 14, 27]. These results suggest that the protein phosphorylation which invariably occurs during receptor activation is irrelevant to phospholipase D activation by receptor agonists, and that the phorbol-ester-sensitive pathway is not stimulated during receptor activation. It may well be
that protein phosphorylation alone is not sufficient for phospholipase D activation via the phorbol-ester-sensitive pathway. A direct interaction of phorbol esters with phospholipase D, either alone or in conjunction with G-proteins, may also be necessary; because in certain cell-free systems, PMA can directly activate phospholipase D and this activation is enhanced by GTP[S] [35, 39, 55]. Alternatively, the phorbol-ester-sensitive pathway may involve protein kinase C isoenzymes that might be specifically activated by phorbol esters and not by receptor agonists. The possibility that receptor agonists and phorbol ester activate distinct phospholipase D isoforms has yet to be investigated.

In certain tissues, phospholipase D requires Ca^{2+} for activity [51]. When added in the presence of Ca^{2+}, the Ca^{2+} ionophore, A23187, activates phospholipase D in many cells (Table 1) and extracellular Ca^{2+} is essential for receptor-mediated activation of phospholipase D in various cells, including: neutrophils [7, 8, 15] and U937 cells [18]. In HL-60 granulocytes, A23187 acts synergistically with PMA to activate phospholipase D [16]. Thus, mobilization of Ca^{2+} may exert important regulatory controls on both phospholipase-D-activation pathways. Phospholipase D activation by sphingoid bases [14] and membrane-permeant synthetic 1,2-diacylglycerols [16, 30] might be a consequence of membrane perturbation, favouring substrate availability to the enzyme. Such membrane perturbations may in turn facilitate phospholipase D activation by G-proteins and protein kinases. Indeed, in neutrophils, sphingosine acts synergistically with fMet-Leu-Phe and PMA to promote phospholipase D activation [14].

**Significance of phospholipase D**

Phospholipase D activation is a widespread phenomenon (Table 1), suggesting its critical involvement in signal transduction (Fig. 1). PtdOH accumulates rapidly upon stimulation, presumably in the plasma membrane (see Table 1 for specific references). This PtdOH accumulation may cause a transient increase of negative charge density at the cytoplasmic face of the membrane, thereby facilitating translocation to the membrane of specific cytosolic proteins with a net positive charge.

Several recent studies indicate that phospholipase-D-derived PtdOH may be involved in receptor-linked exocytosis. In chemotactic-peptide-stimulated neutrophils, PtdOH accumulation correlates with the release of azurophilic granules [10]. Ethanol reduces both PtdOH accumulation and exocytosis in stimulated neutrophils ([7] and our unpublished work) and mast cells [20]. In addition, exogenously added phospholipase D from *Streptomyces chromofuscus* causes aldosterone secretion in granulosa cells from ovary and adrenal gland [27, 28], and insulin secretion in pancreatic islets [56], presumably as a result of PtdOH formation. Additional experiments are clearly needed to confirm the possible involvement of PtdOH in stimulus-secretion coupling.

PtdOH formed by phospholipase D may be dephosphorylated by PtdOH phosphohydrolase to DRG [57]. Definitive proof that the phospholipase D/phosphohydrolase pathway functions in intact cells has come from experiments using human neutrophils [7-9] and monocytic U937 cells [18]. In neutrophils stimulated, with fMet-Leu-Phe, C5a or PMA, the majority of the DRG formed is derived through phospholipase D action on PtdCho [7-9]. Several other cells, including: mast cells [21], endothelial cells [37, 38], REF52 fibroblasts [46] and MDCK cells [33], appear to utilize this pathway for DRG generation during stimulation. The cytosolic form of phosphohydrolase can be readily translocated to the membrane by unsaturated fatty acids [57]. Whether the substrate PtdOH, that accumulates in the plasma membrane as a consequence of phospholipase D activity, could be a trigger for phosphohydrolase translocation remains to be determined. It has recently been demonstrated in neutrophils that sphingosine, a well-established inhibitor of protein kinase C, inhibits phosphohydrolase in a protein-kinase-C-independent mechanism, thereby preventing DRG formation [14]. Since sphingosine is a product of sphingomyelin metabolism, these findings suggest an important role for sphingomyelin metabolism in the regulation of DRG formation by the phospholipase D/phosphohydrolase pathway.

Many cells produce DRG in a biphasic manner [2] and the phospholipase D/phosphohydrolase pathway is responsible for a late, sustained phase [7, 8], whereas degradation of phosphoinositides by phospholipase C gives rise to an early phase. The late phase of DRG may lead to a sustained activation of protein kinase C, prolonging responses initiated by the early signalling events, such as phosphatidylinositol-4,5-bisphosphate hydrolysis. One such functional response that appears to be dependent on this signalling sequence is generation of superoxide anion by chemotactic-peptide-stimulated neutrophils. In this system, DRG formation temporally correlates with the response [13] and occurs initially by phosphoinositide-
specific phospholipase C activation and subsequently by the phospholipase D/phospholipase pathway [7, 13].

Conclusion

In a wide variety of cells, phospholipase D is activated by diverse agents to produce PtdOH and subsequently DRG. PtdOH and DRG possess properties that are consistent with their involvement in cell signalling. Agonist control of phospholipase D appears to occur by at least two distinct mechanisms characterized by the involvement of either G-proteins or protein kinases. Interplay between these mechanisms and their modulation by Ca²⁺ may lead to various complex activation patterns, depending on the cell types and the stimuli used.

We thank Lisa Ramirez for typing the manuscript.

Intracellular Phospholipases


Received 17 December 1990

Regulation of phospholipase A₂ by receptors in MDCK-D1 cells
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Defining the mechanism(s) for signal transduction by cell surface receptors has been an intensely studied problem in biochemistry over the last 20 years. Nature appears to have evolved a relatively limited number of pathways (at least as identified thus far) to accomplish the goal of transmission of extracellular information to produce changes in intracellular metabolism and functional activities. Within the past decade much attention has been focused on one of these mechanisms: activation of phospholipase C (PLC) involved in hydrolysis of membrane phosphoinositides to water-soluble inositol phosphates and sn-diacylglycerol (DAG) [1–3]. The discovery that both of those types of products could function as second messengers, i.e. inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and perhaps other inositol polyphosphates, leading to increases in cytosolic calcium, and DAG contributing to the activation of a calcium/phospholipid-dependent protein kinase, made it attractive to imagine that phosphoinositide (or polyphosphoinoside)-specific PLC would be a key effector molecule in signal transduction.

This notion was furthered by the proposal that DAG could serve a precursor function whereby its hydrolysis by one or more glyceride lipases would generate arachidonic acid from the sn-2 position of DAG, and this arachidonic acid would in turn be a precursor for formation of eicosanoids through the action of cyclo-oxygenases and lipoxygenases [1, 2, 4]. Since the latter products play a prominent autocrine/paracrine role, phosphoinositide-specific PLC then assumed an even more central role in cell and tissue regulation. Although many cell types contain phospholipases that hydrolyse phosphoinositides, it has become increasingly clear that a variety of cells use alternative means for DAG and arachidonic acid formation. The cells that we shall describe herein, and the approach that we have taken, provide, we believe, a useful paradigm that leads to a quite different formulation for signal transduction and phospholipid metabolism.

Madin–Darby canine kidney (MDCK) cells are a widely studied renal epithelial cell line [5, 6]. These cells are well differentiated and express many of the properties of renal tubular cells, in particular of the distal tubule-collecting duct [6]. Several types of hormones and extracellular ‘first messengers’, including: the peptide bradykinin, the catecholamine adrenaline and the nucleotide ATP, have been shown to hyperpolarize these cells by activation of outward-rectifying potassium channels [6, 7]. MDCK cells are quite heterogeneous and, thus, we believe that it is important to study clonal isolates of the parental cell line. Most of our efforts have involved a clonally derived isolate that we have termed MDCK-D1 [8, 9].

A prominent response previously observed in parental MDCK cells was the generation of prostaglandins, in particular prostaglandin E₂ (PGE₂), in

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Abbreviations used: PLC, phospholipase C; DAG, sn-diacylglycerol; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; PGE₂, prostaglandin E₂; PLA₂, phospholipase A₂; PMA, phorbol 12-myristate 13-acetate.