Receptor Regulation of Intracellular Phospholipases

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Regulation of the hydrolysis of phosphatidylcholine in Swiss 3T3 cells
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The binding of a range of hormones, growth factors and neurotransmitters to their specific cell surface receptors stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate \(\text{PtdIns}(4,5)P_2\) by activating a specific phospholipase C via a G-protein [1, 2]. This hydrolytic event generates the two second messenger molecules: inositol 1,4,5-trisphosphate \([\text{Ins}(1,4,5)P_3]\) and \(\alpha,\beta\)-diacylglycerol (DAG). However, it is now clear that PtdIns(4,5)P_2 is not the only phospholipid hydrolysed in response to cellular stimulation and a number of studies have shown that phosphatidylinositol (PtdCho) can be rapidly broken down in response to a range of agonists.

PtdCho is the major phospholipid class found in mammalian cells and it has been demonstrated to constitute up to 50% of the cellular phospholipid. It is found in all cellular membranes and is made up of a number of different molecular species, e.g. 1,2-diacyl-, 1-O-alkyl-2-acyl- and 1-alkyl-1'-enyl-2-acyl-\(\gamma\)-glycerophosphocholine; also, further diversity exists in that the acyl chain structure of PtdCho can vary considerably (see [3] for review). Therefore, phosphodiesteric cleavage of PtdCho could generate multiple species of DAG, phosphaticid acid or fatty acids, which potentially could have important ramifications for cell metabolic responses.

The importance of PtdCho hydrolysis in second messenger generation has been demonstrated by the finding that, in many cells, agonist-stimulated PtdIns(4,5)P_2 hydrolysis is a rapidly desensitized event. In Swiss 3T3 cells, bombesin stimulated the rapid, yet transient, generation of \([\text{Ins}(1,4,5)P_3]\) while the generation of DAG was both biphasic and sustained [4]. Detailed analysis of PtdIns(4,5)P_2 hydrolysis has demonstrated that the transient \([\text{Ins}(1,4,5)P_3]\) response is due to the desensitization of bombesin-stimulated phospholipase C activation [4, 5]; thus demonstrating that the second, sustained phase of DAG must be derived from an alternative phospholipid. The alternative phospholipid in Swiss 3T3 cells appeared to be PtdCho [6]. This phospholipid is hydrolysed in a variety of cell types upon agonist stimulation, although it has been suggested that, at least in NIH 3T3 cells, phosphatidylethanolamine is also hydrolysed (see [3]).

To analyse agonist-stimulated PtdCho hydrolysis, a rapid and simple ion-exchange method for analysing the water-soluble choline metabolites was developed [6]. Use of this method demonstrated that there was an increase in the generation of choline (Cho) and choline phosphate (ChoP) after bombesin stimulation of Swiss 3T3 cells, but that there was no change in the levels of glycerophosphocholine. Analysis of the time course of the generation of choline metabolites demonstrated that the primary detectable product was Cho with ChoP being generated more slowly [6]. Examination of the dose dependency of bombesin-stimulated Cho generation yielded an \(\text{EC}_{50}\) value of approx. 3 nM, which was similar to that for bombesin-stimulated Ins(1,4,5)P_3 generation and for both phases of DAG generation [4].

These experiments suggested that PtdCho was being hydrolysed by the action of a phospholipase D rather than a phospholipase C, as is the

Abbreviations used: Ptd, phosphatidyl; InsP_2, InsP_3, inositol bis-, tri-phosphate with locants designated where appropriate; DAG, \(\alpha,\beta\)-diacylglycerol; Cho, choline; ChoP, choline phosphate; But, butanol; PMA, phorbol 12-myristate 13-acetate.

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case for PtdIns(4,5)P₂. Phospholipase D catalyses a transphosphatidylation reaction and in addition to using water as a nucleophilic acceptor can utilize a short-chain aliphatic alcohol such as butanol. The phosphatidylalcohol product of this reaction is not a substrate for phosphatidate phosphohydrolase and thus accumulates in the cell providing a marker of phospholipase D activity. This reaction is now frequently used as a determinant of phospholipase D activity. However, it is important that parallel experiments demonstrating the generation of the water-soluble products in the absence of the alcohol are performed, to rule out the potential contribution of a base exchange activity.

Swiss 3T3 cells were labelled to isotopic equilibrium with [³H]palmitic acid and stimulated with bombesin in the presence of 30 mM-butanol. This generated a phospholipid with identical chromatographic properties to a standard [³C]phosphatidylbutanol (PtdBut) in a dose- and time-dependent manner. Its formation was dependent upon the concentration of butanol used and a similar product was formed when unlabelled cells were stimulated with agonist in the presence of [³H]butanol. Using a maximal dose of bombesin (617 nM), PtdBut was detectable after 15 s of stimulation and was thus produced later than Ins(1,4,5)P₃ which peaked after 5 s. However, the EC₅₀ value for bombesin-stimulated PtdBut formation was again similar to that found for Ins(1,4,5)P₃ and DAG generation.

The identical EC₅₀ values for bombesin-stimulated Ins(1,4,5)P₃ generation, and the two phases of DAG formation and of PtdCho hydrolysis, suggest that each event is controlled by the activation of a single receptor. However, the kinetic differences between the hydrolysis of the two phospholipids could imply that PtdCho hydrolysis is causally dependent upon prior PtdIns(4,5)P₂ breakdown. Support for this proposal came from experiments with phorbol 12-myristate 13-acetate (PMA) and additional agonists. Vasopressin and prostaglandin F₂α, two mitogens which have been reported to stimulate the hydrolysis of PtdIns(4,5)P₂, also stimulate the generation of Cho and the activation of phospholipase D with EC₅₀ values virtually identical to their effects upon inositol phosphate generation [7]. Pretreatment of Swiss 3T3 cells for 15 min with PMA caused a dose-dependent inhibition of bombesin-stimulated inositol phosphate generation with an IC₅₀ of 5.58 ± 3.84 nM. However, PMA addition stimulated the dose-dependent generation of Cho and, in the presence of 30 mM-butanol, the formation of PtdBut, with EC₅₀ values of 4.95 ± 3.63 nM and 2.80 ± 1.02 nM, respectively. These EC₅₀ and IC₅₀ values for PMA compare favourably with the EC₅₀ value for activation of protein kinase C by the phorbol ester [8]. Therefore, it is possible that the initial generation of DAG in response to bombesin stimulation activates protein kinase C to both inhibit phospholipase-C-catalysed PtdIns(4,5)P₂ hydrolysis and stimulate phospholipase-D-catalysed PtdCho breakdown.

To test this hypothesis, two approaches were adopted to inhibit the activation of protein kinase C: First, C-kinase activity was downregulated by treatment of Swiss 3T3 cells with 400 nM-PMA for 4 h. Under these conditions, the ability, both of agonists such as bombesin, vasopressin and prostaglandin F₂α, and of PMA, to stimulate the activation of phospholipase D was abolished [6, 7]. Secondly, cells were stimulated in the presence of Ro-31-8220, a C-kinase inhibiting staurosporine analogue [9]. The inhibitor completely abolished PMA-stimulated phospholipase D at concentrations effective in inhibiting protein kinase C activity. However, the inhibitor was only able to inhibit bombesin-stimulated phospholipase D activity by some 40% (Fig. 1). These results indicate that while activation of phospholipase D can be achieved by an increase in protein kinase C activity, it is not the only mechanism involved in agonist-stimulated PtdCho-phospholipase D activity.

![Fig. 1](image_url)

Regulation of bombesin-stimulated phospholipase D activity

[³H]Palmitate-labelled Swiss 3T3 cell were stimulated with 100 nM-bombesin (Bb) or 5 µM-A23187, in the presence or absence of 10 µM-Ro-31-8220 (Ro) for 5 min in a buffer containing 30 mM-butanol, and the generation of PtdBut was determined as described in Cook & Wakelam [7]. Cells stimulated in normal buffer; □, cells stimulated in buffer containing EGTA to reduce [Ca²⁺] to 150 nM.
In addition to activating protein kinase C, stimulated PtdIns(4,5)P₂ breakdown results in an increase in intracellular free [Ca²⁺]. Therefore, the potential role for changes in [Ca²⁺] in the regulation of phospholipase D was examined. Experiments were performed where the extracellular [Ca²⁺] was reduced, by the use of a calcium-EGTA buffer, to equal the resting intracellular concentration, i.e. \( \sim 150 \text{ nm} \). Stimulation of cells with bombesin in the presence of the low extracellular [Ca²⁺], resulted in an approximately 50% inhibition of PtdBut formation (Fig. 1). The generation of both of these PtdCho metabolites is similar to that observed for the activation of phospholipase D activity via the release of intracellular Ca²⁺, it is possible that there is a component of bombesin-stimulated activity that remains inexplicable by changes in protein kinase C or [Ca²⁺]. Thus, in this experiment with bombesin in the presence of the protein kinase C inhibitor in a low Ca²⁺ medium, approximately 30-40% of the normal increase in PtdBut formation was still observed (Fig. 1). While these experiments are unable to rule out the possibility that the agonist stimulates phospholipase D activity via the release of intracellular Ca²⁺, it is possible that there is a component of bombesin-stimulated activity that remains inexplicable by changes in protein kinase C or [Ca²⁺]. Thus, a direct receptor activation of phospholipase D, mediated perhaps by a G-protein, may occur, as proposed by Bocckino et al. [10] for vasopressin-stimulated PtdCho hydrolysis in hepatocytes.

In addition to being hydrolysed by a phospholipase-D-catalysed pathway, PtdCho is acted upon by a phospholipase A₂ in bombesin-stimulated Swiss 3T3 cells. This response is clearly observed as an increase in the generation of free \([¹H]arachidonate in prelabelled cells and in the transient generation of lysophosphatidylcholine. The generation of both of these PtdCho metabolites is observed within 2 s of agonist stimulation and reaches a maximum within 10 s. Bombesin-stimulated arachidonate generation was dose dependent with an EC₅₀ value of 2.7 ± 0.6 nm, which is similar to that observed for the activation of inositol lipid hydrolysis and for PtdCho—phospholipase D activity. Experiments, similar to those described above for investigating the regulation of phospholipase D activity demonstrated that bombesin-stimulated phospholipase A₂ activity is completely independent both of protein kinase C activity and the entry of Ca²⁺. This would suggest that the enzyme is directly activated by occupation of the bombesin receptor, perhaps via the interaction of a G-protein as has been suggested [11].

Surprisingly, we have been unable to detect any significant increase in either lipoygenase or cyclo-oxygenase products in bombesin-stimulated cells. It would, therefore, appear that arachidonic acid itself may have a function in the cells. Indeed, arachidonate has been demonstrated to act both as an activator of protein kinase C in \emph{vitro} [12] and as an inducer of Ca²⁺ release from intracellular stores [13].

Phosphatidylic acid, the lipid product of phospholipase D action, can be converted to DAG by the action of phosphatidate phosphohydrolase; however, it may be that phosphatidate itself has important functions within the cell. Both phosphatidic acid and arachidonic acid can inhibit the activity of the p21⁰-regulating protein GAP [14] and together with DAG stimulate the activity of a p21⁰ GTPase-inhibiting protein [15] in \emph{vitro}. Phosphatidic acid can also function as a mitogenic agonist in its own right [16].

The bombesin receptor has recently been purified from Swiss 3T3 cells and subtypes do not appear to exist [17]. Therefore, it would seem likely that the three pathways, i.e. PtdIns(4,5)P₂ breakdown catalysed by phospholipase C and PtdCho breakdown catalysed by both phospholipases D and A₂, are stimulated in response to the activation of a single population of bombesin receptors. However, if each of these responses is so regulated then the concept of receptor--G-protein--effector fidelity must be questioned.

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Receptor-coupled phospholipase D: regulation and functional significance
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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 phosphatidylinositol (PtdIns) 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 transphosphatidylolation 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, transphosphatidylolation 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]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 transphosphatidylolation is a valid measure of phospholipase D activity in intact cells. Table 1 lists the cells in which phospholipase D activation 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...