Role of phospholipases in adrenal steroidogenesis

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Abstract

Phospholipases are lipid-metabolizing enzymes that hydrolyze phospholipids. In some cases, their activity results in remodeling of lipids and/or allows the synthesis of other lipids. In other cases, however, and of interest to the topic of adrenal steroidogenesis, phospholipases produce second messengers that modify the function of a cell. In this review, the enzymatic reactions, products, and effectors of three phospholipases, phospholipase C, phospholipase D, and phospholipase A_2, are discussed. Although much data have been obtained concerning the role of phospholipases C and D in regulating adrenal steroid hormone production, there are still many gaps in our knowledge. Furthermore, little is known about the involvement of phospholipase A_2, perhaps, in part, because this enzyme comprises a large family of related enzymes that are differentially regulated and with different functions. This review presents the evidence supporting the role of each of these phospholipases in steroidogenesis in the adrenal cortex.

Key Words
- adrenal cortex
- angiotensin
- intracellular signaling
- phospholipids
- signal transduction

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Introduction

Phospholipids serve a structural function in the cell in that they form the lipid bilayer that maintains cell integrity. However, phospholipids are also involved in some very important signaling processes, as they can serve as precursors for signals created as a result of the action of phospholipases that hydrolyze them to yield various second messengers. There are three main types of these phospholipases, phospholipase C, phospholipase D, and phospholipase A (Fig. 1), and they have each been shown to play a role in regulating steroidogenesis in the adrenal cortex. This review discusses each in turn, as well as the evidence linking them to adrenal steroid hormone production.

Phospholipase C

A hormone binding to its receptor induces a conformational change in the receptor such that an associated GTP-binding protein exchanges a bound GDP for a GTP. The G protein with GTP bound can then activate the enzyme, phospholipase C (PLC), that cleaves the minor membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield diacylglycerol (DAG) and the polar head group, inositol 1,4,5-trisphosphate (IP_3). The liberated IP_3 binds to its receptor, IP_3 receptors, of which all the three are expressed in the adrenal cortex ((Enyedi *et al*. 1994) and reviewed in (Bollag 2014)). Upon binding to these receptors on the intracellular calcium store (the endoplasmic reticulum), IP_3 releases calcium to elevate the intracellular calcium concentration. This increase, in turn, activates calcium/calmodulin-dependent protein kinases that phosphorylate various proteins to trigger a cellular response. DAG, however, remains in the membrane, where it activates proteins such as members of the protein kinase C (PKC) family. One or more PKC isoforms, or other DAG-sensitive enzymes

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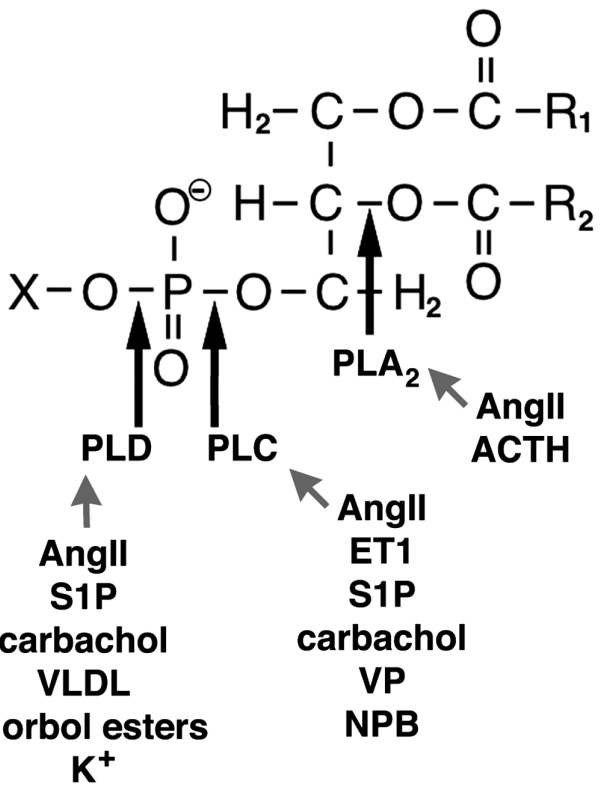

Figure 1
The reactions catalyzed by phospholipases C, D, and A_2. In adrenocortical cells treated with agonists such as angiotensin II (AngII), endothelin-1 (ET1), sphingosine 1-phosphate (S1P), carbachol, vasopressin (VP), and neuropeptide B (NPB), activated phospholipase C (PLC) cleaves on one side of the phosphate linker, as indicated, to produce diacylglycerol and the phosphorylated head group. In contrast, phospholipase D (PLD) hydrolyzes the phospholipid on the other side of the phosphate linker to generate phosphatidic acid and the headgroup; in adrenocortical cells, PLD has been demonstrated to be activated by AngII, S1P, carbachol, very-low-density lipoprotein (VLDL), phorbol esters, and an elevated extracellular potassium concentration (K^+). Finally, as illustrated, phospholipase A_2 (PLA_2) removes the fatty acid from the 2 position of the glycerol backbone of a phospholipid, whereas PLA_1 deacylates the phospholipid at position 1 (not shown). The products resulting from this activity are a lysophospholipid and a free fatty acid. The fatty acids can be further metabolized to bioactive lipids such as eicosanoids.

such as protein kinase D (PKD), can also phosphorylate a set of proteins to sustain the cellular response (Fig. 2).

The phosphoinositide system is an important signaling pathway mediating the response of cells to many different growth factors, hormones, and neurotransmitters. This includes the response of adrenocortical cells to hormones such as angiotensin II (AngII), one of the primary physiological regulators of aldosterone production in the zona glomerulosa of the adrenal gland (reviewed in Barrett *et al*. 1989, Spat & Hunyady 2004, Hattangady *et al*. 2012, Bollag 2014). AngII also stimulates cortisol production in fasciculata cells (Mlinar *et al*. 1995, Rabano *et al*. 2004), although the primary regulator of cortisol secretion, adrenocorticotrophic hormone (ACTH), works instead through the adenylate cyclase-cAMP-dependent protein kinase pathway (reviewed in (Berthon *et al*. 2015)). Many years ago, it was shown that treatment of adrenal zona glomerulosa cells with AngII and elevated potassium levels resulted in an increase in the incorporation of radiolabeled phosphate into the phosphoinositides (Farese *et al*. 1980), suggesting that these agents might be stimulating phosphoinositide turnover. Subsequent studies examining early time points after AngII treatment showed that this hormone triggered a rapid decrease in the levels of radiolabeled PIP_2 and phosphatidylinositol 4-phosphate and an increase in the release of radiolabeled inositol phosphates, inositol bisphosphate and IP_3 (Farese *et al*. 1984, Kojima *et al*. 1984). At the same time, Kojima *et al*. (1984) showed an increase in the levels of radiolabeled DAG, indicating that, indeed, AngII induces the activation of a phosphoinositide-specific phospholipase C. Many ensuing studies confirmed this idea and refined our understanding of the involvement of this pathway in steroidogenesis.

The adrenal cortex expresses two receptors for AngII, the AngII receptor, type 1 (AT1R) and the AngII receptor, type 2 (AT2R); the AT1R is the receptor involved in AngII's stimulation of aldosterone production (reviewed in Barrett *et al*. 1989, Spat & Hunyady 2004, Hattangady *et al*. 2012, Bollag 2014). The binding of AngII to AT1R is known in other systems to activate the heterotrimeric GTP-binding protein $G_{q/11}$, which couples AngII binding to the activation of PLCβ. Although it is clear that AngII's regulation of aldosterone production in adrenal glomerulosa cells involves phosphoinositide hydrolysis, the G protein and PLC isoform mediating this response have not yet been demonstrated conclusively; nevertheless, it is assumed based on other systems (e.g. vascular smooth muscle cells (Wynne *et al*. 2009)) that AngII works through $G_{q/11}$ and PLCβ (reviewed in Spat & Hunyady 2004, Hunyady & Catt 2006). The involvement of $G_{q/11}$ is supported by a study showing a translocation of this G protein to the plasma membrane of AngII-stimulated rat glomerulosa cells (Cote *et al*. 1997). In any case, phosphoinositide hydrolysis is required for AngII-elicited aldosterone production in glomerulosa cells, as indicated by the ability of U73122, a chemical inhibitor of PLC, to inhibit AngII effects in these cells (Czirjak *et al*. 2001), including aldosterone production (Tsai *et al*., manuscript in preparation). U73122 also inhibits the parathyroid hormone (PTH) and PTH-related peptide-induced increase in radiolabeled IP_3 in, and aldosterone and cortisol secretion from, adrenocortical

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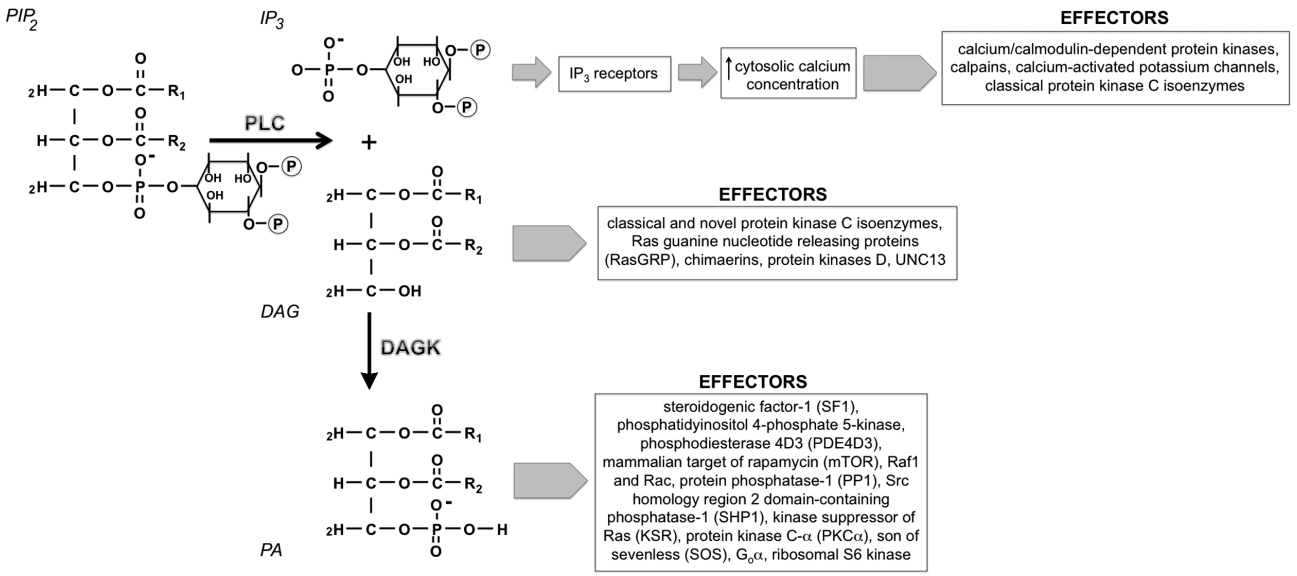

Figure 2
The phosphoinositide-specific phospholipase C pathway. The action of phosphoinositide-specific PLC on phosphatidylinositol 4,5-bisphosphate (PIP_2) releases inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors to release calcium from the endoplasmic reticulum and increase intracellular calcium levels. The elevated cytosolic calcium concentration activates calcium/calmodulin-dependent protein kinases, calpains (calcium-sensitive proteases), calcium-activated potassium channels, and classical protein kinase C isoenzymes. At the same time, DAG in the membrane activates various effectors such as classical and novel protein kinase C isoenzymes, protein kinase D isoenzymes, guanine nucleotide exchange factors for Ras called Ras guanine nucleotide release proteins (RasGRPs), chimaerins (Rho GTPase-activating proteins), and UNC-13 proteins involved in vesicle exocytosis. Effectors for PA include the nuclear hormone receptor steroidogenic factor-1 (SF1), the PIP_2-synthesizing enzyme phosphatidyinositol 4-phosphate 5-kinase, the cAMP-degrading enzyme phosphodiesterase 4D3 (PDE4D3), mammalian target of rapamycin (mTOR), the small GTP-binding proteins c-Raf and Rac, protein phosphatase-1 (PP1), Src homology region 2 domain-containing phosphatase-1 (SHP1), kinase suppressor of Ras (KSR), protein kinase C-α (PKC-α), son of sevenless (SOS), $G_o\alpha$, and ribosomal S6 kinase (reviewed in Selvy et al. 2011).

cells (Mazzocchi *et al*. 2001), as well as cortisol production in response to AngII and endothelin (Rebuffat *et al*. 2001).

PLC activity should generate two second messengers: IP_3 and DAG. Hunyady *et al*. (1990) demonstrated an AngII-induced increase in absolute DAG levels in cultured bovine adrenal glomerulosa cells, as did Bollag *et al*. (1991); the changes in DAG content in response to AngII exhibit a biphasic time course, with an initial rapid increase followed by a slight decrease and then a sustained plateau (Hunyady *et al*. 1990). Radiolabeled IP_3 levels show a similar time course, and additional data indicate an extracellular calcium-dependent sustained hydrolysis of phosphoinositides (Hunyady *et al*. 1990). This idea is supported by the observed AngII-induced stable rise in radiolabeled inositol phosphate, inositol bisphosphate, and IP_3 levels and the reduction of these sustained elevations in the absence of medium calcium or upon inhibition of calcium influx with various calcium channel blockers (Balla *et al*. 1994). Other studies have also shown an ability of AngII to increase inositol phosphates, using either [^{3}H]inositol prelabeled cells (e.g. Kojima *et al*. 1984, Enyedi *et al*. 1985) or a microspectrophotometric assay to monitor the absolute mass of unlabeled inositol phosphates in adrenocortical cells (Underwood *et al*. 1988).

Many hormones in addition to AngII, working through G protein-coupled receptors other than AT1R, have been demonstrated to activate PLC to induce steroidogenesis. For example, carbachol (a stable acetylcholine receptor agonist), endothelin-1, sphingosine 1-phosphate, and vasopressin have all been reported to activate phosphoinositide hydrolysis to stimulate adrenocortical cell steroid hormone production (Kojima *et al*. 1986, Woodcock *et al*. 1986, Woodcock *et al*. 1990, Bollag *et al*. 1992, Andreis *et al*. 2001, Delarue *et al*. 2004, Brizuela *et al*. 2006, Lucki *et al*. 2012). Neuropeptide B has also been shown to increase IP_3 levels in adrenal fasciculata/reticularis cells, and the PLC inhibitor U73122 reduced neuropeptide-elicited cortisol production (Mazzocchi *et al*. 2005). These data suggest the importance of heterotrimeric G protein-activated PLC and phosphoinositide turnover to adrenocortical steroid hormone production.

In other systems (e.g. vascular smooth muscle cells), it is known that AngII binding to the AT1R stimulates a tyrosine kinase activity that results in the activation of PLCγ to provide an additional mechanism for increasing

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phosphoinositide hydrolysis ((Marrero *et al*. 1994, Marrero *et al*. 1996) and reviewed in (Wynne *et al*. 2009)). An involvement of tyrosine kinases, such as Src and Janus kinase 2, in AngII-induced glomerulosa cell responses has also been reported (Kapas *et al*. 1995, Sirianni *et al*. 2001, Li *et al*. 2003), although to date, no definitive evidence has been presented linking these kinases and AngII activation of PLCγ in glomerulosa cells. Nevertheless, many AngII-elicited changes in cell function appear to be mediated by tyrosine kinases; these include aldosterone production, capacitative calcium influx, steroidogenic acute regulatory (StAR) protein expression, protein kinase D activation, and proliferation (Kapas *et al*. 1995, Aptel *et al*. 1999, Sirianni *et al*. 2001, Olala *et al*. 2014*b*). Another mechanism by which tyrosine kinases and/or PLCγ may be activated in AngII-stimulated cells is via transactivation of the epidermal growth factor receptor (reviewed in Shah & Catt 2004, Wolf 2005), although, to date, in glomerulosa cells such an effect has only been reported for lysophosphatidic acid and endothelin-1 (Shah *et al*. 2005).

It should be noted, however, that phosphoinositide-specific PLC is not the only enzyme that can contribute to DAG production in many systems, including adrenocortical cells. For example, there may be a phosphatidylcholine (PC)-specific PLC that hydrolyzes this phospholipid to generate DAG, although, to date, no such enzyme has been isolated or cloned. Why is the phospholipid source of the DAG potentially important? In fact, DAG is not a single molecule but a set of molecules containing various fatty acids in the R_1 and R_2 position (Fig. 1). Because different phospholipids are enriched in different fatty acids, PLC activity on various phospholipids yields different DAG species. Different DAG species, in turn, may be differentially metabolized (see below) or they may differentially activate various enzymes or PKC isoforms. As an example, the PKC family actually consists of three types of acidic phospholipid (i.e. phosphatidylserine)-dependent isoforms: the classical type, the activity of which is also regulated by calcium and DAG (PKC-α, -β, and -γ); the novel, calcium-insensitive isoenzymes PKC-δ, -ε, -η, and -θ), which are nonetheless activated by DAG; and the atypical isoforms (PKC-ζ and -ι (-λ)), which are insensitive to both calcium and DAG. Although not much is known concerning the ability of different DAG species to activate PKC in an intact cell (e.g. Deacon *et al*. 2002), there is strong evidence that different DAGs are differentially metabolized. For instance, a DAG kinase has been identified that preferentially phosphorylates arachidonic acid-containing DAG to yield phosphatidic acid (Lemaitre & Glomset 1992, MacDonald *et al*. 1988*a*,*b*). The phosphatidylinositol phospholipids are enriched in arachidonic acid (Kennerly 1987, Augert *et al*. 1989), suggesting that the DAG from phosphoinositide turnover may be more readily metabolized than that resulting from phosphatidylcholine hydrolysis. In addition, a DAG lipase that releases arachidonic acid from DAG has also been identified (Natarajan *et al*. 1988*b*, 1990); this enzyme would also allow more rapid metabolism of arachidonic acid-containing DAG, while also releasing arachidonic acid that can serve as a precursor of other lipid signals (see below). In fact, arachidonate-DAG is more quickly metabolized than myristate-DAG in adrenal glomerulosa cells (Bollag *et al*. 1991). Thus, the type of DAG produced and the phospholipid from which it is generated (and therefore the phospholipase that creates it) may be important in determining the duration of the signal as well as which effectors are activated.

Phospholipase D

Although DAG is generated directly by PLC, DAG may also be produced indirectly by the action of another phospholipase, phospholipase D (PLD). PLD cleaves phospholipids on the other side of the phosphate group from PLC (that is, closer to the head group), as shown in Fig. 1. Thus, the action of PLD on a phospholipid produces phosphatidic acid (PA), essentially phosphorylated DAG, and the unphosphorylated polar head group, generally choline, as the substrate for the PLD isoforms characterized to date is phosphatidylcholine. The PA, in turn, can be converted to DAG by the activity of the enzyme phosphatidate phosphohydrolase, which removes the phosphate group. The phosphatidate phosphohydrolases are actually now often referred to as lipid phosphate phosphatases (LPP), as many of the members of this family can be active on phosphorylated lipids other than PA, such as sphingosine 1-phosphate or ceramide phosphate. DAG, generated indirectly by PLD or directly by PLC, can be converted to PA by the action of DAG kinase, which phosphorylates DAG. DAG can also be metabolized by DAG lipase, which removes a fatty acid to yield monoacylglycerol. DAG lipase will be discussed again shortly. The bottom line is that PLD activation followed by LPP activity is a pathway by which DAG can also be formed, in addition to its direct production by PLC-mediated cleavage of phospholipids (Fig. 2).

Several hormones in various cell types are able to activate PLD, and it is thought that at least part of the DAG produced during long hormonal exposures

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is formed by the combined action of PLD and LPP. PLD activity can be measured by determining the formation of the products, i.e., PA and the polar head group choline; however, because DAG formed by PLC can be converted to PA by DAG kinase (and the phosphorylated and nonphosphorylated choline head group is also similarly interconvertible), this method is not reliable. A more reliable method relies on the fact that PLD can catalyze not only the hydrolysis (using water) of a phospholipid but also the "alcoholysis" (by primary alcohols such as ethanol and 1-butanol) of the phospholipid. Prelabeling of cells with radioactive fatty acid followed by stimulation with the hormone of interest in the presence of a primary alcohol such as ethanol will result in increased levels of both radiolabeled PA and radiolabeled phosphatidylalcohol (e.g., phosphatidylethanol or PEt), if the hormone of interest is capable of activating PLD (Fig. 3).

Using these methods, it has been shown that numerous receptor ligands (hormones, neurotransmitters, growth factors, etc.) activate PLD. Indeed, AngII increases radiolabeled phosphatidic acid and phosphatidylethanol levels in prelabeled glomerulosa cells and decreases radiolabeled phosphatidylcholine levels (Bollag *et al*. 1990, 1991). The effect of AngII on PLD activity can be inhibited by the AT1R inhibitor losartan and is dose dependent and sustained (Jung *et al*. 1998). Nevertheless, until recently, it has been difficult to prove a role for PLD in sustained DAG production and cellular responses, due to the lack of specific (or selective) inhibitors of PLD (which have now been developed; see below). Two agents have been used to attempt to indirectly demonstrate a role for PLD in sustained DAG formation. One such compound is propranolol. In addition to being a β-blocker, propranolol also inhibits LPP. Therefore, a reduction in hormone-stimulated DAG levels and/or a cellular response upon treatment with this drug is taken as evidence that combined PLD and LPP activity contributes to DAG production, as was observed with the aldosterone secretagogue sphingosine 1-phosphate (Brizuela *et al*. 2006), as well as with cortisol secretion in response to AngII (Rabano *et al*. 2004). Similarly, PLD activity in the presence of ethanol results in a reduced generation of PA, as some of the phospholipid undergoes ethanolysis to yield PEt, which is not readily metabolized to DAG, rather than hydrolysis to give PA. It should be noted that although originally it was thought that PEt was not metabolized by cells, in fact, some cells are capable of metabolizing PEt, albeit at a slower rate than PA. Thus, an ability of ethanol to decrease the DAG formed in response to a hormone indicates that at least a portion of the hormone-elevated DAG originates from PLD/LPP activity. Other primary alcohols can also be utilized; 1-butanol has the advantage of having a related compound, tert-butanol, that can be used to test the specificity of any butanol effect on a cellular response. As tert-butanol is not a primary alcohol, it is not thought to affect PLD signaling, except non-specifically. Using 1-butanol (versus tert-butanol), Bollag and colleagues showed that PLD activity is required for AngII-induced aldosterone production in NCI H295R human adrenocortical carcinoma cells (Zheng & Bollag 2003) and bovine adrenal glomerulosa cells (Bollag *et al*. 2002); PLD is also involved in AngII-induced cortisol secretion (Rabano *et al*. 2004) and sphingosine 1-phosphate-elicited steroidogenesis (Rabano *et al*. 2003, Brizuela *et al*. 2006).

PLD was cloned in the mid-1990s and two isoforms of PLD, PLD1 and PLD2 have been sequenced, extensively characterized and shown to possess phospholipase activity ((Hammond *et al*. 1995, Colley *et al*. 1997) and reviewed in (Frohman & Morris 1996, Frohman 2015)). Both of these isoenzymes utilize phosphatidylcholine as a substrate and require PIP_2 as a cofactor. The activity of PLD1 is regulated by ADP-ribosylating factor (ARF) and RhoA, as well as PKC-α (e.g., Du *et al*. 2000). PLD2, however, exhibits constitutive activity, at least when expressed in Sf9 insect cells and assayed *in vitro*. However, in intact cells, PLD_2 may be regulated by both PKC and ARF (e.g. Lopez *et al*. 1998), and apparently also by cytoskeletal elements, as well as the supply of PIP2 (reviewed in Selvy *et al*. 2011). Both PLDs are likely to play a role in vesicle trafficking within the cell, as well as in cellular responses to hormones and other receptor ligands (reviewed in Frohman & Morris 1996, Peng and Frohman 2012, Frohman 2015). Four other PLD isoforms, PLD3-6, have also been identified in the genome based on homology, but these have been much less studied, and in many cases whether or not the gene products actually exhibit PLD activity is not known. Two of these, PLD3 and PLD4, have been shown to be associated with human disease (e.g., Terao *et al*. 2013, Cruchaga *et al*. 2014), and PLD6 is a mitochondrial PLD thought to hydrolyze cardiolipin, the structure of which is essentially di-phosphatidylglycerol, to produce PA and regulate mitochondrial biology and spermatogenesis (Choi *et al*. 2006, Gao & Frohman 2012 and reviewed in Peng & Frohman 2012, Frohman 2015). Nevertheless, these other homologues have not been thoroughly investigated and their exact functions are unclear.

The next question is: how is PLD activated by hormones binding to their receptors? In some systems, PLD activation is direct through a receptor/G-protein-mediated

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mechanism. In other systems, the pathway is indirect involving prior activation of PKC and in still others, PLD activation appears to occur through other mechanisms (reviewed in Selvy *et al*. 2011). Indeed, in glomerulosa cells stimulated with AngII, PKC is involved in PLD activation, as evidenced by the inability of maximal doses of AngII and the PKC-activating phorbol ester, phorbol 12-myristate 13-acetate, to additively increase PLD activity (Bollag *et al*. 2002). In addition, although PLD does not itself bind calcium, its activation in many cases appears to depend on extracellular calcium (Selvy *et al*. 2011), including in AngII-stimulated glomerulosa and fasciculata cells (Rabano *et al*. 2004, Qin *et al*. 2009). Also, as PLD appears to often preferentially hydrolyze phosphatidylcholine, this enzyme may provide a mechanism for the phosphatidycholine hydrolysis that is observed in some systems, including adrenal glomerulosa cells exposed to AngII (Bollag *et al*. 1991). Indeed, AngII induces an increase in the levels of myristate-containing DAG in glomerulosa cells (Bollag *et al*. 1991); phosphatidycholine is enriched in myristate (Bollag *et al*. 1991), suggesting that PLD activity underlies, at least in part, AngII-elicited DAG generation (and again bringing up the issue of DAG species).

It should be noted that the PLD-generated lipid important in regulating aldosterone production is not entirely clear. Thus, PLD can clearly contribute to AngII-induced DAG generation (Bollag *et al*. 1990, Bollag *et al*. 2002, Qin *et al*. 2010); DAG, in turn, can activate PKC isoforms, as discussed above. There are also PKC-related protein kinases, such as the protein kinase D family, which can be activated by DAG (see below), as well as DAG-activated Ras guanine exchange factors, Ras guanine nucleotide-releasing protein 1-3 (RasGRP1-3). However, PA is also itself a second messenger, with targets including mTOR (mammalian target of rapamycin, an important enzyme for regulating cell growth), phosphodiesterase, phosphoinositide synthetic enzymes, and proteins involved in actin polymerization and vesicle trafficking (Fig. 2) (reviewed in Selvy *et al*. 2011, Peng and Frohman 2012, Bollag 2014). Also of particular interest for the adrenal cortex, PA can also serve as a ligand for steroidogenic factor-1 (Li *et al*. 2007), a nuclear hormone receptor important in steroidogenesis (Ye *et al*. 2009). In this case, however, the PA appears to be formed by the action of the DAG kinase, DAGK-θ, on DAG ((Li *et al*. 2007, Cai *et al*. 2014) and reviewed in (Lucki & Sewer 2012)). Also of interest in the context of the adrenal cortex, protein phosphatase-1 (PP1), which has been shown to be inhibited by PA (Kishikawa *et al*. 1999), is expressed in the adrenal cortex, and non-selective inhibitors of protein phosphatase activity, okadaic acid and calyculin A, reduce ACTH-stimulated aldosterone and corticosterone secretion from rat zona glomerulosa and zonae fasciculata/reticularis cells, respectively (Sayed *et al*. 1997). Finally, it should be noted that in primary aldosteronism (aldosterone-producing adenomas and idiopathic hyperaldosteronism), enhanced phosphorylation of mTOR and ribosomal S6 kinase has been detected relative to normal adrenal glands, suggesting that these enzymes are excessively activated in this adrenal disorder (Su *et al*. 2013). Therefore, whether the important steroidogenic signal produced by PLD is DAG or PA, or both, is unclear.

Nevertheless, what has become clear is the importance of PLD signal generation to AngII-induced aldosterone production, and PLD appears to be both necessary and sufficient to trigger steroidogenesis. Thus, Bollag *et al*. (1990) demonstrated that exposure of bovine adrenal glomerulosa cells to bacterial PLD triggered aldosterone secretion. The secretory response to exogenous PLD alone is small (but significant); however, the voltage-dependent calcium channel agonist, BAYK 8644, enhances the effect of the enzyme. This result is consistent with the idea that AngII induces both calcium influx and DAG signals to maintain aldosterone production (Kojima *et al*. 1984). This result also indicates that PLD is sufficient to cause aldosterone production. PLD is also necessary for agonist-induced aldosterone production: thus, as mentioned earlier, inhibition of PLD signal generation with 1-butanol reduces the secretory response to AngII and sphingosine 1-phosphate (Bollag *et al*. 2002, Zheng & Bollag 2003, Brizuela *et al*. 2006). Similarly, the PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (Su *et al*. 2009) also decreases AngII-elicited aldosterone secretion in both bovine glomerulosa cells and NCI H295R human adrenocortical carcinoma cells (Olala *et al*. 2013). Recent studies in which PLD isoforms were overexpressed using adenovirus also demonstrated that PLD2, but not PLD1, can enhance AngII-stimulated DAG levels and aldosterone production in bovine adrenal glomerulosa cells (Qin *et al*. 2010), again indicating the importance of PLD to AngII's steroidogenic ability. The role of PLD in mediating the aldosterone response to other agonists, such as sphingosine 1-phosphate and very-low-density lipoprotein (VLDL), has also been shown (Brizuela *et al*. 2006, Tsai *et al*. 2014). In the case of VLDL as an agonist of aldosterone production in HAC15 human adrenocortical carcinoma cells, both PLD1 and PLD2 appear to underlie secretion (Tsai *et al*. 2014). Finally, PLD activation has been reported in response to an elevated extracellular potassium concentration as well (Betancourt-Calle *et al*. 2001*a*).

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The exact mechanism by which PLD-generated lipid signals in response to AngII result in steroidogenesis is not clear; however, one key player is clearly protein kinase D (PKD), a DAG-activated protein kinase with homology to both PKC and calcium/calmodulin-dependent protein kinases. AngII-induced PKD activation is mediated, in part, by PLD activity, as well as by PKC- and tyrosine kinase-mediated transphosphorylation (Romero *et al.* 2006, Olala *et al.* 2014*b*). PKD, in turn, phosphorylates members of the activating transcription factor/cAMP response element binding family of transcription factors to increase the transcription of steroidogenic acute regulatory protein (Olala *et al.* 2014*a*), the protein mediating the initial rate-limiting step in steroid hormone biosynthesis. Overexpression of a constitutively active mutant PKD has also been shown to increase the expression of CYP11B2 (Romero *et al.* 2006), the gene encoding the late rate-limiting enzyme in aldosterone production, aldosterone synthase, and enhance aldosterone production in NCI H295R human adrenocortical carcinoma cells (Romero *et al.* 2006). This active PKD mutant also increases cortisol-synthesizing 11β-hydroxylase expression and cortisol secretion (Romero *et al.* 2006).

Finally, the potential role of PLD in priming of adrenal glomerulosa cells should be mentioned. Priming refers to the ability of AngII to sensitize glomerulosa cells, following its removal (or blocking of its action with a receptor antagonist), to subsequent agonist exposures to result in enhanced aldosterone secretion. Thus, if glomerulosa cells are pretreated with AngII, a second exposure to AngII within a specific time frame of its washout induces a greater increase in the aldosterone secretory rate than in cells with no prior pretreatment (Barrett *et al.* 1986, Bollag *et al.* 2007). Similarly, agents that increase calcium influx, such as the voltage-dependent calcium channel agonist BAYK 8644 or a small elevation in the extracellular potassium concentration, induce a greater change in aldosterone production in AngII-pretreated cells than when used in "naïve" cells (that have not been previously exposed to AngII (Barrett *et al.* 1986, Bollag *et al.* 1991, Betancourt-Calle *et al.* 2001*b*). Priming does not seem to be the result of either an enhanced second cytosolic calcium increase (the second response is in fact smaller (Kojima *et al.* 1987)) or residual AngII, as the AngII concentration used (10 nM) is a maximal dose in terms of aldosterone secretion and signaling processes (Bollag *et al.* 1991), and neither PLD activation (Bollag *et al.* 2007), [^{3}H]arachidonate-labeled DAG levels nor calcium influx (Barrett *et al.* 1986) is sustained after AngII washout (Kojima *et al.* 1984). As maintained AngII-elicited aldosterone secretion appears to require both a calcium influx and a lipid (i.e. DAG) signal (Kojima *et al.* 1984, Rasmussen *et al.* 1995), the ability of agents that increase calcium influx to increase aldosterone secretion with priming suggested the possibility that AngII generated an elevation in DAG levels that persisted after removal of the hormone. Indeed, Bollag *et al.* (1991) demonstrated a persistent increase in DAG content upon AngII treatment and removal. Further, this DAG is marked by the fatty acid myristate; the fact that phosphatidylcholine is enriched in myristate and that AngII induces phosphatidylcholine hydrolysis suggested that the persistent DAG arose from phosphatidylcholine (Bollag *et al.* 1991). Subsequent studies showed that PLD, which hydrolyzes phosphatidylcholine to (indirectly) produce DAG, likely is the source of this persistent DAG based on several lines of evidence: (1) AngII induces a sustained PLD activation and priming (Bollag *et al.* 1991, Jung *et al.* 1998, Betancourt-Calle *et al.* 2001*b*, Bollag *et al.* 2007), whereas carbachol, which also promotes phosphoinositide hydrolysis (Kojima *et al.* 1986), generates only a transient stimulation of PLD activity and no priming (Bollag *et al.* 1992, Jung *et al.* 1998); (2) the phorbol ester, phorbol 12,13-dibutyrate (PDBu), and exogenous PLD also elicit sustained PLD activation as well as priming (Bollag *et al.* 2007, Qin *et al.* 2010); and (3) overexpression of a specific PLD isoform, PLD2 enhances AngII-induced aldosterone secretion in adrenocortical cells (Qin *et al.* 2010).

Phospholipase A_2

Another phospholipase family involved in phospholipid metabolism and cell signaling is the phospholipases A, such as phospholipase A_2 (PLA_2). PLA_2 removes the fatty acid from the 2 position of the glycerol backbone of a phospholipid as illustrated in Fig. 1 (whereas PLA_1 deacylates the phospholipid at position 1, thus removing R1). The result is a lysophospholipid and a free fatty acid. Lysophospholipids, formed as a result of the activity of PLA_2 activity, as well as that of DAG lipase (see below), may serve as second messengers: for instance, lysophosphatidylcholine has been shown to stimulate cAMP phosphodiesterase activity (Pichard & Cheung 1977) and the activities of some PKC isoenzymes (e.g. Murohara *et al.* 1996, Scott *et al.* 2007). Lysophosphatidic acid has also been implicated in cytoskeletal rearrangements and activation of the Ras/Raf/MEK/MAP kinase pathway (reviewed in Xiang *et al.* 2013). Finally, alkyl-lysophosphatidylcholine produced by PLA_2 from alkyl-phosphatidylcholine can be acetylated to yield

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platelet activating factor, a first messenger that binds to receptors on many cell types.

However, the other product of PLA_2 activity, free fatty acids, also appear to serve important messenger functions. One such fatty acid is arachidonic acid, which may itself be a second messenger. For instance, it and other unsaturated fatty acids have been found to enhance PKC activation by DAG (Nakamura & Nishizuka 1994). In addition, arachidonic acid has been shown to stimulate sphingomyelinase activity (Jayadev *et al*. 1994). Arachidonic acid also binds Ras-GAP and inhibits its activity (Tsai *et al*. 1989, 1991). Moreover, arachidonic acid can also modulate the activity of the TREK-1 potassium channel in bovine adrenal glomerulosa cells (Enyeart *et al*. 2004). Finally, arachidonic acid also serves as a precursor for several paracrine signaling molecules, as described below.

PLA_2 constitutes a large family of various enzyme groups, including secretory, cytosolic (calcium-dependent), and calcium-independent PLA_2 enzymes (reviewed in Murakami & Kudo 2002, Burke and Dennis 2009). In many systems, PLA_2 (the cytosolic type) is activated secondarily to activation of phosphoinositide turnover and increased cytosolic calcium levels and/or phosphorylation (Murakami & Kudo 2002, Burke & Dennis 2009). The activation of this form of PLA_2, cytosolic calcium-dependent PLA_2 ($cPLA_2$), is accompanied by a calcium-dependent translocation to the membrane where it can interact with its substrate(s). A group of non-arachidonic acid-selective, calcium-insensitive PLA_2 enzymes has also been identified (Murakami & Kudo 2002). Note that arachidonic acid, in addition to other free fatty acids, may be released from DAG by the action of DAG lipase as well as from phospholipids via PLA_2 activity, as will be discussed below.

Arachidonic acid release by PLA_2 is also important because this free fatty acid serves as a precursor for the synthesis of prostaglandins, thromboxanes, hydroxyeicosatetraenoic acids (HETEs), and leukotrienes. Collectively, these molecules are known as eicosanoids and each has been implicated in paracrine signaling in one system or another. Prostaglandins and thromboxanes are synthesized from arachidonic acid via the action of the enzyme

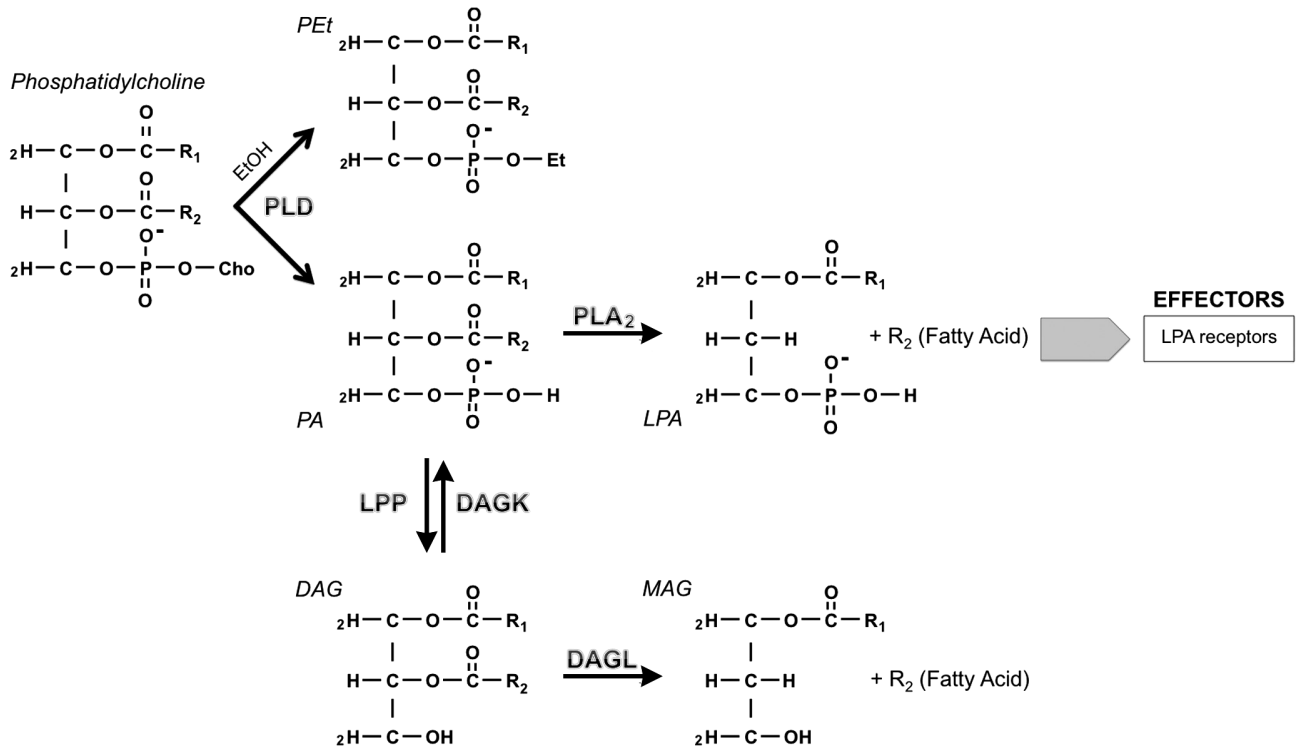

Figure 3
Phospholipase D-catalyzed reactions, lipid signals, and effectors. Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine in the presence of water to produce phosphatidic acid (PA) or a transphosphatidylation of phosphatidylcholine in the presence of a primary alcohol such as ethanol to generate the phosphatidylalcohol (e.g. phosphatidylethanol, PEt) and choline (Cho). PEt is not readily metabolized; however, PA can be dephosphorylated by lipid phosphate phosphatases (LPPs) to yield DAG (with effectors as indicated in Fig. 2) or deacylated to release the free fatty acid and generate lysophosphatidic acid (LPA), which can activate G protein-coupled LPA receptors. DAG can also be phosphorylated by DAG kinases (DAGK) to give PA (with effectors also as indicated in Fig. 2) or deacylated to monoacylglycerol (MAG) with the release of a fatty acid by DAG lipase (DAGL).

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cyclooxygenase. It is this enzyme that is inhibited by the non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, indomethacin, acetoaminophen, and ibuprofen. Presumably by preventing production of prostaglandins and thromboxanes, these drugs interfere with inflammatory processes to reduce pain and fever. The steroidal anti-inflammatory drugs appear to exert their effects at the level of PLA_2 to prevent the release of arachidonic acid in the first place. Both prostaglandins and thromboxanes have very short half-lives in the body.

The HETEs are produced from arachidonic acid by lipoxygenases. There are, in fact, at least three lipoxygenases that add a hydroperoxy (-OOH) group to the 5, 12, or 15 position of arachidonic acid, to yield 5-, 12-, or 15-hydroperoxyeicosatetraenoic acid (HPETE), unstable molecules that are rapidly converted to 5-, 12-, or 15-HETE. 5-HPETE can also be converted to leukotrienes. Eicosanoids are very important in inflammatory processes as well as proliferation and other cell responses (reviewed in Schneider & Pozzi 2011, Lone & Tasken 2013). In each case, these molecules appear to act locally (i.e., within a tissue) by binding to receptors on the plasma membrane; in other words, they act as ligands for G protein-coupled receptors. HETEs have also been found to be involved in the secretion of aldosterone. Thus, Nadler and colleagues have shown that 12-HETE mediates AngII-induced aldosterone production by increasing cytosolic calcium levels and p38 MAP kinase and PKC activities (Nadler *et al*. 1987, Natarajan *et al*. 1988*a*, 1994, 2002, Gu *et al*. 2003, Stern *et al*. 1993). However, in this case, the arachidonic acid does not appear to be released by a PLA_2 but by a DAG lipase (Natarajan *et al*. 1988*b*, 1990). Thus, PLA_2 does not appear to play a role in 12-HETE's production and mediation of AngII-induced aldosterone production, and the involvement of PLA_2 activity in steroidogenesis is not entirely clear.

PLA_2 activity can result in arachidonic acid liberation, and a limited number of investigations have found that AngII can elicit release of this fatty acid from glomerulosa cells (Kojima *et al*. 1985), suggesting that AngII may activate PLA_2, although others have argued against a role for this enzyme (Hunyady *et al*. 1985). Likewise, a few studies have demonstrated that inhibitors of PLA_2 activity, such as quinacrine and p-bromophenacyl bromide, reduce AngII-stimulated aldosterone production in glomerulosa cells (Kojima *et al*. 1985), and exogenous PLA_2 can induce transient steroidogenesis (Kojima *et al*. 1985). Similarly, PLA_2 inhibitors also decrease cortisol secretion from fasciculata/reticularis cells in response to ACTH (Omura *et al*. 2007). In contrast, Andreis *et al*. (1999) have reported that the PLA_2 inhibitor and trifluoromethyl ketone analog of arachidonic acid, AACOCF3, dose-dependently increases the secretion of basal aldosterone and corticosterone from rat glomerulosa and fasciculata/reticularis cells, respectively, as well as that of aldosterone and cortisol from human adrenocortical cells. AACOCF3 also enhances cortisol production induced by submaximal doses of ACTH (Andreis *et al*. 1999). These results, along with the fact that other steroid intermediates are also increased by this PLA_2 inhibitor, suggest that PLA_2, or its products (e.g. arachidonic acid and/or eicosanoids), may inhibit early steps in steroidogenesis (Andreis *et al*. 1999). Obviously, additional studies are needed to determine the role of PLA_2, and/or its downstream metabolites, in the adrenal production of steroid hormones.

Conclusion

In conclusion, the cumulative evidence indicates that two phospholipases, phospholipase C and phospholipase D, are critically important to the adrenal steroidogenic response to AngII, as well as other aldosterone agonists and some cortisol secretagogues. These phospholipases function by generating various second messengers, including those that increase cytosolic calcium levels (IP_3) and activate lipid-sensitive enzymes (DAG and PA and possibly lysophosphatidic acid). Although all steps of the pathway from agonist binding to its receptor to the secretion of the adrenal steroids are still incompletely understood, the importance of the phospholipases and their products to steroid hormone production indicate that further study is necessary for a comprehensive understanding of steroidogenesis.

Declaration of interest
There is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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