Phospholipase A₂ and Signal Transduction

Joseph V. Bonventre

J.V. Bonventre, Medical Services, Massachusetts General Hospital, Department of Medicine, Harvard Medical School, Boston, MA

ABSTRACT
Phospholipases A₂ (PLA₂) comprise a family of enzymes that hydrolyze the acyl bond at the sn-2 position of phospholipids to generate free fatty acids and lysophospholipids. Different forms of PLA₂ are involved in digestion, inflammation, and intercellular and intracellular signal transduction. The sn-2 position of phospholipids in mammalian cells is enriched in arachidonic acid, the precursor of eicosanoids, which have diverse physiologic and pathophysiologic effects on the kidney and other organs. Thus, the regulation of PLA₂ activity has important implications for kidney function. PLA₂ regulation involves: calcium, pH, protein kinases, GTP-binding proteins, inhibitory and activating proteins, metabolic product inhibition, and transcriptional control. The various roles of arachidonic acid and cyclooxygenase, lipoxygenase, and cytochrome P450 monooxygenase products of arachidonic acid metabolism, as intracellular messengers, in the regulation of membrane channel activities, intracellular enzyme activities, cellular calcium homeostasis, mitogenesis, differentiation, cytokine and early response gene expression are discussed.

Key Words: Eicosanoids, kidney, calcium, phospholipids, proliferation, prostaglandins, lipoxygenases, cytochrome P450, leukotrienes, arachidonic acid

Phospholipases are enzymes that hydrolyze phospholipids. They fall into two broad classes: acyl hydrolases (phospholipase A₁, PLA₁; phospholipase A₂, PLA₂; and phospholipase B, PLB) and phosphodiesterases (phospholipase C, PLC, and phospholipase D, PLD). The nomenclature for the phospholipases is depicted schematically in Figure 1.

Phospholipases A₂ comprise a family of enzymes that act at the sn-2 position of phospholipids to generate a free fatty acid and lysophospholipid. This stereospecificity means that the enzymes act on naturally occurring L-phospholipids but not on D-phospholipids. These enzymes are involved in digestion, membrane remodeling, and membrane repair. In addition, because the sn-2 position of the phospholipids of mammalian cells is enriched with arachidonic acid, the precursor for eicosanoids, PLA₂ activity has important implications for the control of eicosanoid production. Although arachidonic acid can be released from phospholipids by mechanisms not involving PLA₂ (1), it is likely that in many cells PLA₂ is the primary enzyme regulating arachidonic acid release. The free arachidonic acid generated by PLA₂ activity is converted, by cyclooxygenases, lipoxygenases, and monooxygenases, to a large number of products that have many effects on cells, both as intercellular and intracellular messengers. PLA₂ is rate limiting for prostaglandin synthesis (2); however, the synthesis of noncyclooxygenase products requires both the release of arachidonic acid as well as the activation of other enzymes (3). In addition to its role in the physiologic responses of the cell, PLA₂ activity is important for pathophysiologic states in which activation is associated with membrane degradation and changes in plasma and mitochondrial membrane bioenergetics and permeability (4-7). Furthermore, when 1-O-alkyl-2-acylglycerophosphocholine is the substrate for PLA₂, the resultant product becomes the precursor for platelet-activating factor, an important lipid mediator.

By virtue of the involvement of arachidonic acid and its products, as well as lysophospholipids, in a multitude of fundamental cellular regulatory events, it has become increasingly evident that PLA₂ plays a critical role in intracellular signaling. The products of PLA₂ activity have been implicated in membrane channel activity, receptor binding, intracellular enzyme regulation, cell volume regulation, Ca²⁺ homeostasis, gene regulation, and growth. In this brief review, I will provide an overview of this rapidly expanding area of investigation.

CHARACTERISTICS OF PLA₂s

There are secretory and intracellular forms of PLA₂ (Table 1). Most of the investigative efforts to characterize this enzymatic activity have been focused on secretory forms from snake venoms and mammalian pancreas that have molecular masses in the range of
Figure 1. Site of hydrolysis of phospholipids by various phospholipases. PLA₁ (A₁) hydrolyzes the 1-acyl ester; PLA₂ (PLA₂), the 2-acyl ester. Phospholipase B (B) hydrolyzes at both 1- and 2-acyl ester bonds. Phospholipase C (C) cleaves the glycerol-phosphate bond, whereas the base is removed by phospholipase D (D). R₁ and R₂ represent fatty acids.

TABLE 1. Characteristics of PLA₂⁹

<table>
<thead>
<tr>
<th>Type</th>
<th>Size (kd)</th>
<th>Origin</th>
<th>Ca²⁺ Dependency</th>
<th>pH Optimum</th>
<th>Acyl Group Selectivity</th>
<th>Other Distinguishing Characteristics</th>
</tr>
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<tbody>
<tr>
<td>Group I</td>
<td>13–15</td>
<td>Snake venom Pancreas Gastric mucosa Spleen</td>
<td>Yes (mM)</td>
<td>8–9</td>
<td>None</td>
<td>Seven disulfide bonds including Cys¹¹–Cys²⁷</td>
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<td></td>
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<tr>
<td>Group II</td>
<td>13–15</td>
<td>Snake venom Platelets Synovial fluid Mesangial cells Ascitic fluid Gastric mucosa Vascular smooth muscle cells Mitochondria of liver, heart, kidney, brain Membrane-associated form in platelet, spleen, macrophage cell line (P388D₁)</td>
<td>Yes (&gt;1 μM)</td>
<td>8–9</td>
<td>None</td>
<td>Cys⁴⁵–Cys¹³² replaces Cys¹¹–Cys²⁷</td>
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<tr>
<td>Cytosolic</td>
<td>85–110</td>
<td>Kidney U937 cells Spleen Mesangial cells Platelets Macrophage cell lines (RAW 264.7 and J774)</td>
<td>Yes (&gt;100 nM)</td>
<td>8–9</td>
<td>Arachidonic acid</td>
<td>Translocates to membrane in Ca²⁺-dependent manner</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Membrane</td>
<td>40</td>
<td>Heart</td>
<td>No</td>
<td>6.4</td>
<td>None</td>
<td>Plasmalogen selective Lysophospholipase activity (PLB)</td>
</tr>
<tr>
<td>Lysosomal</td>
<td>?</td>
<td>Liver, heart</td>
<td>No</td>
<td>4.5</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

⁹ ? indicates not known.
regions of sequence homology among mammalian, insect, and reptilian secretory forms (25). Recently, high-resolution crystal structures of secretory PLA₂ have been published (26,27). On the basis of structural information, it has been proposed that optimal binding and catalytic activity of secretory PLA₂ at the lipid-water interface are due to facilitated diffusion of the substrate phospholipid from the interfacial binding site rather than to allosteric modifications in the enzyme itself (27).

Although the secretory forms of PLA₂ likely play important roles in digestion and inflammation their localization to granules in the cell (28), inactivity at free calcium concentrations present intracellularly, and nonspecificity for arachidonic acid at the sn-2 position of fatty acids suggest that they do not play an important role in signal transduction. Likewise, the lysosomal form with its acid pH optimum (29) likely has a role limited to intracellular digestion.

Less is known about the intracellular nonsecretory membrane-associated and cytosolic PLA₂s that are involved in membrane remodeling and the biosynthesis of lipid mediators that act as intercellular and intracellular messengers. These PLA₂s have been much more difficult to purify than the secretory forms. Recent success, however, in purification, has led to the realization that there are many different types of intracellular nonsecretory PLA₂, with different sizes, substrate specificities and calcium requirements for activation. There are membrane-associated microsomal and mitochondrial forms (18,28,30–34) of PLA₂ that are 13 to 18.5 kd in molecular mass. Membrane-bound nonmitochondrial forms isolated from the platelet and spleen have apparent molecular masses of 13 to 14 kd, whereas those from sheep erythrocytes and the mouse macrophage-like cell line P388D1, have apparent sizes of 18 to 18.5 kd. These forms belong to the group II class of PLA₂, have alkaline pH optima, and are Ca²⁺ dependent. The microsomal enzymes require millimolar concentrations of Ca²⁺ for full activity (18,35) and therefore are less likely to be responsible for physiologic arachidonic acid release than are the larger molecular mass forms described below.

The heart contains a plasmalogen-selective 40-kd PLA₂, which, in contrast to most forms of PLA₂, is not dependent on calcium for maximal activity and has a pH optimum at 6.4 (36). Calcium-independent forms of the enzyme are also present in cytosolic fractions in a number of other organs, particularly brain, lung, liver, and spleen (37). A 97-kd calcium-independent enzyme with both PLA₂ and lysophospholipase activity has been purified from the intestine (38), although this enzyme is better termed a "phospholipase B" because of its ability to remove fatty acids both at the sn-1 and sn-2 positions of phospholipids. In the kidney (39), platelets (40), human monocytes, and rat macrophage cell line RAW 264.7 (41), there is a 85- to 110-kd PLA₂ that is calcium dependent and specific for arachidonic acid at the sn-2 position.

**KIDNEY FORMS OF PLA₂**

We have characterized PLA₂ activities in cytosolic, mitochondrial, and microsomal fractions of rat kidneys (33). At least two forms of PLA₂ activity were present in the cytosolic fraction. A high-molecular-weight form (Figure 2), active against phosphatidylcholine (PC) and phosphatidylethanolamine (PE), migrates on Superose 12 gel filtration columns (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) with an apparent molecular mass of approximately 60 kd and upon purification has a molecular mass of ap-

Figure 2. PLA₂ activity in rat kidney cytosolic extracts fractionated by fast protein liquid chromatography (FPLC) Superose 12 gel filtration chromatography. The extract was applied to a 24-ml column. One-milliliter fractions were collected and assayed for PLA₂ activity. In panel A, 1-acyl-2-(1-¹⁴C)arachidonoyl-PC was used as substrate for PLA₂ activity. In panel A, 1-acyl-2-(1-¹⁴C)PC was used as substrate for PLA₂ activity. In panel B, 1-acyl-2-(1-¹⁴C)PC was used as substrate for PLA₂ activity.
proximately 110 kd (39). This discrepancy between its pattern of migration on gel filtration and its true molecular mass may relate to hydrophobic interactions of the protein with the gel filtration column matrix. A smaller form with a molecular mass of approximately 14 kd by gel filtration chromatography is active against PE but not PC. A large-molecular-weight form is hormonally regulated in rat mesangial cells (42,43) and is likely to be the same form or closely related to the PLA2 purified from the rat kidney. As such, it represents the first hormonally regulated form of PLA2 to be purified (39,44). A large-molecular-weight form of PLA2, with characteristics similar to those of the kidney form, has been recently cloned by two laboratories from human U937 cells (41,45). The cDNA sequence encodes a protein with a predicted molecular mass of 85.2 kd, with no sequence homology to the secreted, smaller forms of PLA2.

PLA2 activities in mitochondrial and microsomal fractions from the rat kidney migrate on gel filtration chromatography as single peaks with a predicted molecular mass of approximately 14 kd (Figures 3 and 4). Mitochondrial and microsomal PLA2 were active against both PC and PE. Thus, the rat kidney has multiple forms of PLA2 activity, likely representing distinct enzymes. The characteristics of PLA2 activity in the different compartments of the kidney were very similar to the enzymatic activities we found in the fractionated gerbil brain (34).

A 14-kd group II PLA2 is produced and secreted by renal mesangial cells (46–48). The production and secretion of this enzyme are enhanced by cytokines, and this secreted form of PLA2 may play an important role in glomerular inflammatory processes.

REGULATION OF PLA2

In order to understand the role played by PLA2 in signal transduction, it is necessary to understand how the enzymatic activity is regulated. The regulation of PLA2 activity in the cell is complex and poorly understood. Known and proposed regulatory factors are depicted in Figure 5. I will briefly summarize

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Figure 3. PLA2 activity in rat kidney mitochondrial extracts fractionated by FPLC Superose 12 gel filtration chromatography. Fractionation conditions were exactly the same as those used for the cytosolic extracts (Figure 2). PLA2 activity was measured with PC (A) or PE (B) as substrates. Molecular mass markers were identical to those described in the legend to Figure 2. From reference 33 with permission.

Figure 4. PLA2 activity in rat kidney microsomal extracts fractionated by FPLC Superose 12 gel filtration chromatography. Fractionation conditions and molecular mass standards were as described in legend to Figure 2. Either PC (A) or PE (B) was used as a substrate for PLA2 activity in each fraction. From reference 33 with permission.
what is known about each of these mechanisms of regulation.

Calcium

When we first began to study the regulation of kidney cell PLA2 activity, it was known that secretory forms of PLA2 were activated by Ca2+. However, the Ca2+ concentrations reported to be necessary to activate the enzymes (49) were well above those achieved intracellularly, even under conditions of hormonal stimulation (50), thus raising doubts as to the physiologic relevance of this Ca2+ dependence.

Using the mesangial cell as a model, we examined the Ca2+ dependency of PLA2 activation by measuring both arachidonic acid release and prostaglandin E2 production as functions of cytosolic free calcium concentration ([Ca2+]c). Cells were rendered permeable with digitonin, and [Ca2+]c was fixed at varying levels. In this system, we demonstrated that PLA2 activity was enhanced when [Ca2+]c was increased over the physiologic range (100 to 1000 nM). These results demonstrated that [Ca2+]c was an important physiologic regulator of the PLA2 activity in the mesangial cell.

When we examined the [Ca2+]c dependence of rat kidney cytosolic PLA2 activity, we found that enzymatic activity was enhanced when [Ca2+]c was increased over the physiologic range (100 to 1000 nM) (Figure 6). The partially purified 110-kd cytosolic PLA2 also demonstrated similar [Ca2+]c dependency (39). Thus, the cytosolic large-molecular-weight PLA2 demonstrates a dependency on [Ca2+]c that is compatible with physiologic regulation by [Ca2+]c in the intact cell. Mitochondrial PLA2 required higher [Ca2+]c for activation (>10^{-6} M) but responded to changes in [Ca2+]c over the range of variation of the mitochondrial matrix [Ca2+]m. It has been estimated that basal mitochondrial matrix [Ca2+]m is 0.5 to 1.5 μM (51) and that mitochondrial matrix free [Ca2+]m is highly correlated with extramitochondrial [Ca2+]m (52), so that it is reasonable to assume that mitochondrial PLA2 is regulated, at least in part, by changes in matrix levels of [Ca2+]m.

Calcium is also involved in the regulation of the cytosolic large-molecular-weight form of PLA2 by mediating the translocation of the enzyme from the cytosol to the membrane, thus promoting the apposition of the enzyme to its substrate in the membrane. In mesangial cell homogenates, an increase in [Ca2+]c results in a greater percentage of total PLA2 activity associated with the membrane fraction (42).

This observation of the translocation of activity from soluble to membrane fraction was later confirmed in the macrophage cell line RAW 264.7 (53). Using antibodies directed against the large form of PLA2 isolated from human U937 cells, Clark et al. (41) showed that this translocation of activity is due to translocation of the 110-kd form of the enzyme.
authors have also shown that an amino-terminal 138-amino-acid fragment of the large form of PLA₂ translocates to natural membrane vesicles in a Ca²⁺-dependent fashion. Furthermore, this part of the protein contains a 45-amino-acid region that is homologous to a number of enzymes including: protein kinase C isoforms which translocate to the membrane in a Ca²⁺-dependent fashion; the synaptic vesicle protein p65 (54); the GTPase-activating protein (GAP) (55); and phospholipase C (56). It was suggested that this 45-amino-acid region of these proteins determines Ca²⁺-dependent phospholipid binding.

Not all forms of PLA₂ rely on Ca²⁺ for optimal activity. The cardiac lysosomal and cytosolic 40-kd forms are Ca²⁺ independent (29,36).

**pH**

With the exception of the lysosomal form of PLA₂ (57) and the 40-kd calcium-independent cardiac form (36), which have acid pH optima at 4.5 and 6.4, respectively, all of the other forms characterized have neutral or alkaline pH optima (22). Kidney and brain cytosolic, mitochondrial, and microsomal PLA₂ activities are optimal at pH 8.5 to 9.5 (33,34). Although optimal activities of the kidney forms occur at an alkaline pH, there is regulated activity at a physiological pH (42,43,58). Given the pH dependency, it can be expected that intracellular cytosolic alkalinization would enhance PLA₂ activity. This pH dependency may partially explain the findings in platelets, where it has been reported that the stimulation of Na⁺/H⁺ exchange and the resultant cytosolic alkalinization play an important role in the epinephrine-induced stimulation of PLA₂ activity (59,60).

The alkaline optimum of the mitochondrial form of PLA₂ indicates that the enzyme is likely to be near its optimal activity because the mitochondrial matrix pH is significantly higher than that of the cytosol (61). The alkaline pH optimum also likely has important implications for ischemic injury. We have found that acidosis protects kidney tubules and hepatocytes against anoxic damage (61,62). It is possible that this is due to the inhibition of PLA₂. Therefore, the acidosis that occurs in ischemic tissue may be protective and the increase in pH observed with reoxygenation may potentiate and increase in PLA₂ activity, which may partially explain reperfusion injury.

**Protein Kinases**

Agonists such as vasopressin (58) and endothelin enhance PLA₂ activity in mesangial cells. These agonists not only increase [Ca²⁺], but also activate protein kinase C (50,63,64). We evaluated whether protein kinase C might be involved in the activation of PLA₂ by examining whether the phorbol ester phorbol myristate acetate (PMA) modulates the [Ca²⁺]-dependent activation of PLA₂ in the intact mesangial cell (58). PGE₃ production was determined in permeabilized cells where cytosolic Ca²⁺ was fixed at basal cellular levels of [Ca²⁺] (≤200 nM) or at levels of [Ca²⁺] (0.3 to 2.2 μM) that are measured in the presence of agonists such as vasopressin and endothelin.

PMA had no effect on prostaglandin E₂ (PGE₂) production when [Ca²⁺] was fixed at low basal levels. By contrast, at the higher (hormone-stimulated) levels of [Ca²⁺], there was a twofold increase in PGE₂ production when PMA was present. Thus, protein kinase C enhances the Ca²⁺ sensitivity of PLA₂. In further support of this conclusion, we found that protein kinase C activation also potentiated Ca²⁺ ionophore (A23187)-stimulated PLA₂ activity. In the intact mesangial cell, PMA alone had no effect upon arachidonic acid release (58). A23187 increased the release of arachidonic acid minimally. However, when both A23187 and PMA were added, together, there was a markedly synergistic response that equaled or exceeded that seen with vasopressin. PMA alone had no effect upon [Ca²⁺].

To further demonstrate that protein kinase C activation was insufficient to activate PLA₂ in the intact cell in the absence of an increase in [Ca²⁺], we permeabilized cells in the presence of vasopressin and GTPγS, where we could measure an increase in diacylglycerol levels due to the activation of phospholipase C (58). Although protein kinase C activity was likely to be increased, there was no increase in free arachidonic acid release when [Ca²⁺] was maintained at low basal cytosolic levels. Thus, in the mesangial cell, an increase in [Ca²⁺] was necessary in order for protein kinase C to enhance PLA₂ activity.

In order to better understand the effects of protein kinase C activation on PLA₂ activity, we directly examined PLA₂ enzymatic activity in cytosolic fractions of mesangial cells previously stimulated with phorbol esters. After exposure to PMA, cytosolic fractions were prepared by 100,000 × g centrifugation of the cell homogenate. PLA₂ activity was measured with 1-acetyl, 2-[³H]arachidonyl PC or PE as substrates in the presence of a free [Ca²⁺] of 1 mM. Cytosolic fractions from cells that had been incubated with PMA for 10 min had an increased PLA₂ activity (42). After DEAE anion exchange and gel filtration chromatography, the peak of activity was also higher in extracts from PMA-treated cells, suggesting that the PLA₂ protein itself was modified. Prolonged exposure to PMA to down-regulate protein kinase C resulted in the loss of PMA-induced activation as well as of vasopressin-induced activation (43). We concluded from these studies that PLA₂ may be covalently modulated by PMA, likely by serving as a substrate for phosphorylation by protein kinase C.

There is additional evidence for the regulation of
PLA₂ by kinases. In a recent study, Plomelli and Greengard (65) found kinase-regulated bidirectional control of PLA₂ activity in brain synaptosomes. Membrane depolarization in the presence of Ca²⁺ resulted in the inhibition of PLA₂ activity. In lysed synaptosomes, the direct addition of Ca²⁺/calmodulin-dependent protein kinase II inhibited PLA₂ activity. By contrast, cAMP-dependent protein kinase and casein kinase II increased PLA₂ activity. Phorbol esters had no effect upon synaptosomal PLA₂ activity. It is thus possible that in tissues containing high levels of Ca²⁺/calmodulin-dependent protein kinase II, this enzyme may serve to limit the PLA₂ activation normally expected because of increased [Ca²⁺]. If phosphorylation is a major mechanism of the activation of the enzyme, then it is possible that agents that inhibit cellular PLA₂ activity, such as glucocorticoids, may do so by activating protein phosphatases (66).

Phospholipase C-Independent Activation of PLA₂

The increase in [Ca²⁺] and protein kinase C activities associated with many agonists, such as vasopressin, angiotensin, and endothelin, can potentially explain the enhanced PLA₂ activities seen with these agonists. We have found, however, that platelet-derived growth factor (PDGF) increases arachidonic acid release before activating phospholipase C in mesangial cells (67). PDGF increases [Ca²⁺] in mesangial cells. In contrast to vasopressin, endothelin, and platelet-activating factor, however, where [Ca²⁺] increases to peak levels within 10 s of the addition of the agonist, [Ca²⁺] peaks at approximately 60 s after PDGF exposure. At 15 s of stimulation, there was no measurable increase in diacylglycerol or inositol triphosphate, indicating that the delayed increase in [Ca²⁺] was associated with a delayed phospholipase C activation. Although there was no evidence for phospholipase C activity at 15 s, there was a significant increase in free arachidonic acid. These data indicate that the release of arachidonic acid was independent of the activation of phospholipase C.

In order to prove that the phospholipase C-independent release of arachidonic acid was due to PLA₂ activation in these studies, we conducted a subsequent study in mesangial cells with epidermal growth factor (EGF), which did not activate phospholipase C. EGF produced a stable enhancement of cytosolic PLA₂ activity (43). Because phospholipase C was not activated by EGF, other mechanisms—besides increases in [Ca²⁺]—and protein kinase C activation—were responsible for the activation of PLA₂ associated with EGF. Clark and Dunlop (68) subsequently found that EGF-stimulated PLA₂ activation could also be found in Chinese hamster ovarian (CHO) cells transfected with the normal human EGF receptor, where phospholipase C is not activated. Goldberg et al. (69) reported that the EGF-induced activation of PLA₂ was dependent upon the intact tyrosine kinase activity of the EGF receptor. Clark and Dunlop found that CHO cells did not respond to EGF with the activation of PLA₂ if they were transfected with a mutant receptor that showed EGF-stimulated kinase activity but that lacked the major autophosphorylation sites and was unable to undergo EGF-induced receptor internalization.

G Proteins

It is likely that there is direct coupling between guanine nucleotide-binding (G) proteins and PLA₂ activation. In addition, there is indirect coupling due to the involvement of G proteins of the G₁ (70,71) and G₂ (72) families in receptor-phospholipase C activation (Figure 5). Evidence for direct coupling derives from experiments in which the addition of GTP analogs to permeabilized cells increases PLA₂ activity independently of phospholipase C activation (73). Jelsma reported that transducin, a major G protein of the rod outer segments of bovine retina, is involved in the activation of PLA₂ in response to light (74). In addition, another G protein has been implicated in the inhibition of light-induced PLA₂ activation, suggesting dual control of PLA₂ by G proteins. In collaboration with Drs. Block, Neer, and Seidman, we have found that transfection of the α₁ subunit cDNA into a murine adrenal cell line results in a marked increase in the expression of this protein and is associated with an increase in the GTP-γ-S-stimulated release of arachidonic acid (75).

Recently reported evidence indicates that, at least in CHO cells, regulation by G₁ of PLA₂ also occurs distal to receptor-G protein coupling, phospholipase C activation, and the increase in [Ca²⁺] (76). In those studies, a chimeric cDNA was constructed in which the last 38 amino acids of α₁ were replaced with the last 36 amino acids from α₂. When this chimera, α₁(38), was transfected into CHO cells and expressed, the receptor-stimulated PLA₂ activity was inhibited. In addition, the iomycin-stimulated [³H]arachidonic acid release was also inhibited. The expression of this chimera did not alter the increase in [Ca²⁺] observed with thrombin. Although the expression of this chimera did result in the inhibition of phospholipase C, the expression of α₂(38), a construct in which both ends are α with the middle α₂, had no effect on thrombin-stimulated phospholipase C but had an effect equivalent to α₁(38) to inhibit PLA₂ activity. Although most of the data linking heterotrimeric G proteins to PLA₂ activation involve possible regulation by the α subunits, there are some data suggesting that the βγ subunit may be involved in PLA₂ regulation in retinal rods (77) and cardiac cells (78).
Inhibitory and Activating Proteins

A good deal of attention has been devoted to the possibility that inhibitory or activating proteins may play an important role in the regulation of PLA2 activity. It had been proposed that a family of proteins, referred to as lipocortins, might mediate the anti-inflammatory effects of glucocorticoids (79,80). Pepinsky and colleagues purified and cloned two proteins with PLA2 inhibitory activity that they called lipocortin I and lipocortin II (81,82). These proteins were found to be substrates of pp60<sup>src</sup> and the EGF receptor kinase (82). A theory evolved whereby phosphorylation of a PLA2 inhibitory lipocortin would result in decreased inhibition of PLA2 (83). We found that mesangial cells have large amounts of both lipocortins I and II and could easily detect the corresponding mRNA (84). It was soon recognized, however, that lipocortins inhibited PLA2 activity by competing with the enzyme for interaction with the phospholipid substrate, rather than directly inhibiting the enzyme itself (85). The lipocortins are thus phospholipid-binding proteins and not enzyme inhibitors. Much of the initial enthusiasm regarding the role of these proteins in the regulation of PLA2 activity has rapidly waned. It remains possible, however, that as-yet-uncharacterized PLA2 inhibitory proteins are functionally important in the regulation of intracellular PLA2 activity (86).

There is evidence that PLA2-activating proteins (PLAP) exist. It has been reported that leukotriene D<sub>4</sub> (LTD<sub>4</sub>)-induced prostanoid production in BC3H<sub>1</sub> smooth muscle cells and an endothelial cell line involved PLA2 activation that was dependent upon protein and RNA synthesis (87). Clark <i>et al.</i> (88) used antibodies prepared against mellitin to isolate a protein that stimulated murine smooth muscle cell sonicate PLA2 activity, directed against PC, but that had no effect upon phospholipase C or snake venom or pancreatic PLA2 activities. In a subsequent study, that group found that tumor necrosis factor induced the synthesis of this PLAP in endothelial cells (89). This protein has recently been cloned (90). In both smooth muscle and endothelial cells, there were rapid increases in PLAP mRNA levels by 1 min and in cellular PLAP content by 2 min of stimulation with LTD<sub>4</sub>. Levels returned to baseline by 8 min. When cells treated with antisense oligonucleotides were stimulated with LTD<sub>4</sub>, the increase in PLAP was inhibited, as was the normally seen increase in [<sup>3</sup>H] arachidonic acid release and eicosanoid synthesis. When the clone was expressed in cells under the control of a metallothionein promoter, copper increased the endogenous PLA2 activity, as was determined by arachidonic acid release. Although an increase in PLAP may explain PLA2 activation in some cells with some agonists, it is not universally involved in PLA2 regulation (90).

Metabolic Product Inhibition

Unesterified cis-unsaturated, but not trans-unsaturated, fatty acids inhibit PLA2 and may function as endogenous inhibitors of PLA2 activity (91,92). When cis-fatty acids are oxidized, their inhibitory action is lost. This may help to explain the increased PLA2 activity with ischemia and reperfusion when cis-unsaturated fatty acids are likely to be oxidized by reactive oxygen species (5), although other factors are also likely to be involved (33). The polymerization of the inactive oxidative products of cis-fatty acids, however, yields a potent inhibitor, both in vitro and in situ, of PLA2 activity (92). The activity of PLA2 may also be regulated by some of the metabolic products of arachidonic acid. The lipoxygenase products of arachidonic acid, 5-hydroxy-6,8,11,15-eicosatetraenoic acid (5-HETE), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE), inhibit platelet PLA2 (93).

Transcriptional Regulation

Although there is no information about the possible regulation of intracellular forms of PLA2 by alterations in the transcription of their genes, there is evidence for the transcriptional regulation of the group II secretory form of PLA2 (94). Forskolin and tumor necrosis factor (TNF) increase mRNA levels of group II PLA2 in cultured smooth muscle cells (95). Glucocorticoids inhibit the increase in group II PLA2 mRNA induced by forskolin, suggesting that the anti-inflammatory action of glucocorticoids may be due, in part, to decreased transcription or altered mRNA stability of PLA2. In addition, glucocorticoids inhibit the TNF-enhanced PLA2 secretion from these cells without affecting TNF-induced increases in PLA2 mRNA levels, indicating that steroids also act at a posttranscriptional level to decrease PLA2 secretion. In the mesangial cell, Sedor, Pfleischchter, and their colleagues have shown that interleukins 1α and 1β (IL-1α and IL-1β), TNF-α, forskolin, and lipopolysaccharide, but not serum, EGF, PDGF, or IL-2, enhance the secretion of a group II PLA2 (46–48,96,97). Nakazato <i>et al.</i> (46) found a sixfold increase in the mRNA levels of group II PLA2 after 24 h of treatment of mesangial cells with IL-1α. PDGF-BB inhibits the IL-1β- and forskolin-induced increases in group II PLA2 mRNA. This inhibition is blocked by genistein, a tyrosine kinase inhibitor, leading the authors to conclude that tyrosine phosphorylation is involved in the PDGF-induced effect on PLA2 gene expression (47).

ARACHIDONIC ACID AND OTHER FATTY ACIDS AS MESSENGERS

Arachidonic acid itself, one of the two initial products of PLA2 activity, may be involved in various
aspects of cell signaling. Arachidonic acid alters ligand-receptor interactions. It inhibits the binding of progestin, androgen, and glucocorticoids to their respective receptors in the brain, uterus, and prostate (98) and potentiates the binding of estrogens (98) and hemicholinum-3 (99) to brain receptors.

Arachidonic acid and other fatty acids that are not substrates for cyclooxygenase or lipoxygenase enzymes modulate the activity of a number of different membrane channels (100). They activate K⁺ channels in smooth muscle cells (101) and the ATP-insensitive K⁺ channel in rat atrial (102) and ventricular (103) cells, as well as the muscarinic-gated K⁺ channel in atrial cells (78). By contrast, fatty acids inhibit an ATP-dependent K⁺ channel in rat ventricular cells (103). Two groups have found that arachidonic acid, as well as other cis-unsaturated fatty acids, acts directly on isolated membrane patches to block Cl⁻ channels in airway epithelial cells (104,105), channels that are implicated in the pathophysiology of cystic fibrosis (106). Arachidonic acid has also been reported to close gap junction channels in rat lacrimal glands as the result of a direct effect of the fatty acid (107). Fatty acids may affect channels by a more complex mechanism than that of direct interaction. For example, cis-unsaturated fatty acids attenuate Na⁺ and Ca²⁺ currents in neuroblastoma cells by a mechanism proposed to involve the activation of protein kinase C with subsequent channel phosphorylation (108).

Arachidonic acid can also affect intracellular enzyme systems involved in signal transduction. Arachidonic acid increases the activity of guanylate cyclase by direct interaction with the enzyme (109). Arachidonic acid and some of its metabolites may couple some receptors to soluble guanylate cyclase activation (110). Arachidonic acid stimulates prolactin release from GH3 cells, (111) and human placental lactogen release (112) and stimulates phospholipase C in these cells by mechanisms unaffected by inhibitors of cyclooxygenase or lipoxygenase metabolism. Arachidonic acid inhibits the Ca²⁺/calmodulin-dependent protein kinase II purified from brain and also inhibits kinase activity in intact brain synaptosomes (113). Because this enzyme is believed to be important in the regulation of neurotransmitter release, arachidonic acid may modulate synaptic transmission.

Arachidonic acid inhibits the GTPase-activating protein, GAP (114). Arachidonic acid was more potent than any other fatty acid tested. Thus, arachidonic acid would be predicted to decrease the GAP-stimulated rate at which the cellular H-Ras protein converts bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP). Because this conversion of GTP to GDP is believed to be the mechanism by which the Ras protein is inactivated, arachidonic acid will enhance Ras activity. An additional effect of arachidonic acid on the hydrolysis of GTP is predicted by the finding that arachidonic acid stimulates the activity of a GTPase inhibitory protein (115). Thus, by inhibiting a GTPase-activating protein and stimulating a GTPase inhibitory protein, GTPase activity will be reduced and cellular Ras activity will increase. Thus, arachidonic acid may represent a critical link between ligand-receptor interaction and cellular Ras activity, which is believed to be important for mitogenesis.

Arachidonic acid activates protein kinase C (116,117). Recently, the specificity of this effect of arachidonic acid for the various isoforms of protein kinase C (118) has been examined. Arachidonic acid alone activates the γ isoform of protein kinase C but not the α or β forms (119). Arachidonic acid potentiates the effects of diacylglycerol on all subspecies of protein kinase C. This synergistic effect of arachidonic acid can be mimicked by other cis-unsaturated naturally occurring fatty acids, such as oleic, linoleic, linolenic, and docosahexaenoic acids. These fatty acids, when present with diacylglycerol, dramatically increase the apparent affinity of protein kinase C for Ca²⁺. We have reported that arachidonic acid can release Ca²⁺ from intracellular stores of mesangial cells, although this release is not as great as that seen with some lipoxygenase and P450 mixed-function oxidase products (120).

It is probable that at least some of these effects of fatty acids are mediated through interactions with cellular fatty acid-binding proteins. Although fatty acid-binding proteins have been identified in a number of tissues (121–123), little is known regarding their possible involvement in signaling pathways.

ARACHIDONIC ACID METABOLITES AND SIGNALING

Arachidonic acid metabolites can be both intercellular as well as intracellular messengers. Metabolites derived from arachidonic acid can be released from cells and function as first messengers or autacoids (124). By acting via specific receptors, these metabolites can then trigger a cascade of events involving intracellular second and third messengers. An appreciation of the importance of arachidonic acid metabolites, and hence PLA₂, in cell signaling has increased with the realization that these metabolites can also serve important regulatory roles as intracellular messengers.

Metabolism of Arachidonic Acid

Arachidonic acid can be metabolized via the PGH synthase (cyclooxygenase/peroxidase), lipoxygenase, or P-450 mixed-function oxidase systems (Figure 7).
The oxidized metabolites are collectively referred to as eicosanoids.

The PGH synthase enzyme converts arachidonic acid to PGH₂, which is then converted by specific isomerases or synthases to prostaglandins and thromboxanes. PGH synthase has both a cyclooxygenase component, which catalyzes the insertion of two molecules of oxygen into arachidonic acid to form PGG₂, and a hydroperoxidase, which catalyzes two-electron reduction of the 15-hydroperoxy group of PGG₂ to yield PGH₃. Nonsteroidal anti-inflammatory agents inhibit the cyclooxygenase component of PGH₂ synthase. PGH synthase has been cloned from sheep vesicular gland by DeWitt and Smith (125) and Yokoyama et al. (126). The molecular weight of the unglycosylated enzyme, lacking the 24-amino-acid signal sequence, is 65,621. The enzyme is a glycoprotein. The site of acetylation by aspirin is at serine⁵³⁹, close to the carboxyl terminus. Acetylation likely results in interference with arachidonic acid binding (127).

The lipoxigenases introduce oxygen at positions 5, 12, and 15 of eicosanoid acids. The immediate products are hydroperoxy fatty acids. When the substrate is arachidonic acid, the initial products are hydroperoxyeicosatetraenoic acids (HPETEs). These products can then be hydroxylated, converting the hydroperoxy group to an alcohol, resulting in the formation of hydroxyeicosatetraenoic acids (HETEs). Alternatively, HPETEs can be acted upon by lipoxigenases at another site, resulting in the formation of two hydroperoxy groups, which upon hydroxylation, form diHETEs. HPETEs can also be dehydrated to form epoxy fatty acids. Epoxy fatty acids can also be hydrolyzed to form diHETEs. Epoxy eicosatetraenoic acids and their products (other than diHETEs) that contain a conjugated triene unit are called leukotrienes. Alternatively, the eicosanoid ring can open and react with glutathione to form peptide derivatives in which glutathione is attached to the fatty acid via a sulfoether bond, forming peptidoleukotrienes.

The lipoxins are another class of metabolites of arachidonic acid that are generated by lipoxigenases. These compounds are trihydroxylated derivatives of arachidonic acid that are likely generated as a product of the interaction of both 5- and 15-lipoxigenases with arachidonic acid.

Cytochrome P-450 monoxygenases represent a family of enzymes (128). Mixed-function oxidase activity involves the hemoprotein cytochrome P-450, together with a flavoprotein reductase (NADPH cytochrome P-450 reductase) and phosphatidylcholine (128). Various epoxyeicosatrienoic acids (EETs) are generated by the action of these enzymes on arachidonic acid. EETs can then be metabolized to dihydroxyeicosatrienoic acid (DHETs) by epoxide hydroxylases.

**Figure 7. Metabolic pathways for arachidonic acid (AA) with some of the products of the pathways listed. FAs, fatty acids. Other abbreviations are defined in the Text.**

**Production of Arachidonic Acid Metabolites in the Kidney and Their Role as Intercellular Messengers**

Many of the products of arachidonic acid have important roles as messengers communicating information between cells. The cyclooxygenase system, consisting of prostaglandin endoperoxide synthase and endoperoxide peroxidase, is responsible for the conversion of arachidonic acid to prostaglandins. In the kidney, there is significant production of prostaglandins in the glomerulus, outer medulla, and inner medulla (129–133). PGE₃ is the predominant form of prostaglandin produced. Cortical proximal tubule and thick ascending limb production of prostaglandins is very low (129–131). Cortical and medullary collecting ducts, as well as thin descending limbs, are the major tubular sites of prostaglandin synthesis (132). Medullary interstitial cells contain large amounts of prostaglandin endoperoxide synthase and produce large amounts of PGE₃ (134).

Intrarenal eicosanoids can have multiple effects upon kidney function (135). Many of the cyclooxygenase products are vasoactive. Platelet-derived thromboxanes and endothelium-derived prostacyclin as well as medullary PGE₃ production likely play important roles in the regulation of intrarenal vascular tone and the distribution of RBF. Prostaglandins are potent stimuli of renin production (136–139). In addition, prostaglandins inhibit sodium reabsorption from the thick ascending limb (140–142) and collecting duct (143–145). Prostaglandins of the E class inhibit arginine vasopressin-induced water flux across the collecting tubule (146).

Lipoxigenase products can be generated in the kidney by trapped macrophages, monocytes, neutrophils, and platelets. Leukotrienes can activate mes-
Angial cells and reduce GFR. They are potent vasoconstrictors, particularly of the efferent arteriole (147). They reduce the glomerular capillary ultrafiltration coefficient, possibly because of their effect to contract mesangial cells (148), and have been implicated in the pathogenesis of glomerulonephritis (149). There is no evidence for 5-lipoxygenase activity and subsequent leukotriene production by renal cells. Leukotrienes have been found in glomeruli, but it has not been excluded that they derive from trapped blood cells (150). In contrast to the absence of evidence for 5-lipoxygenase activity, there are data indicating that 12- and 15-lipoxygenase activities are present in renal cells. 12-HETE and 15-HETE are generated by kidney homogenates (151,152). 12-HETE is produced by homogenized glomeruli and glomerular epithelial cells in culture (152). 12- and 15-HETE have been reported to be produced by glomerular mesangial cells in culture (153). 12-HETE and 15-HETE and their precursors 12- and 15-HPETE (12- and 15-hydroperoxyeicosatetraenoic acids) inhibit renin secretion from rat cortical slices (154).

Cytochrome P-450 monooxygenase metabolism of arachidonic acid is present in a number of organs including the kidney (151,155). Two P-450 products of arachidonic acid have been reported to be produced by thick ascending limb cells (156). One of these products inhibited the (Na⁺,K⁺) ATPase and the other relaxed vascular smooth muscle (157). Epoxygenase products have been reported to inhibit vasopressin action in the toad bladder (158). We have reported the presence in mesangial cells of an arachidonic acid product that migrates with P-450 products on reverse-phase HPLC. This metabolite is modulated by EGF and vasopressin (159). Furthermore, gas chromatography–mass spectrometry revealed the presence of a compound with characteristics identical to those of EET. We concluded that mesangial cells produce products of the cytochrome P-450 monooxygenase system.

We have recently reviewed the many effects of these metabolites of arachidonic acid on the kidney as intercellular messengers (1). Although these metabolites act as “first messengers” and their response in cells is mediated through various intracellular “second messengers,” we will focus the remainder of this review on the intracellular effects of these metabolites as “second” and “third” messengers, because this is most reflective of the theme of the effects of PLA₂ on signal transduction.

Metabolites of Arachidonic Acid as Intracellular Messengers

In many cases, by using pharmacologic blockers of pathways of arachidonic acid metabolism, it is possible to implicate products of a specific pathway (i.e., cyclooxygenase, lipoxygenase, or P-450 monooxygenase) as intracellular messengers. Further support for a particular pathway, as well as insight into which specific metabolite is important in that pathway, is provided by adding specific metabolites directly to the system being studied. It is not always possible, however, to implicate a specific metabolite as a mediator of a biologic effect for a number of reasons including: (1) the inhibitors are not entirely specific; (2) the inhibitors may not block the production of all products of a particular metabolic pathway; (3) the effects of inhibitors may be partially masked by the enhanced production of products of other metabolic pathways because the blocked metabolic pathway results in more available arachidonic acid for use by the unblocked pathways; (4) the distinction between the inhibition of the lipoxygenase pathways and the cytochrome P-450 monooxygenase pathways with available inhibitors is not ideal. This is particularly important with the widespread use of nordihydroguaiaretic (NDGA) as a “lipoxygenase inhibitor,” although it also inhibits the monooxygenase system (160); (5) the products added to a biologic system, especially if intact cells are involved, may be rapidly metabolized before arrival at the site of action or may trigger responses through membrane receptors that do not relate directly to the potential intracellular action of the added metabolite; and (6) the knowledge of the presence of particular metabolites in the cell type being studied is frequently incomplete, so that, even if an effect is observed, it is not clear that it is physiologically relevant.

In some cases, arachidonic acid metabolites have been implicated in the transduction of cellular events by the inhibition of the event by PLA₂ inhibitors, even though, in some cases, the inhibition cannot be reproduced by metabolic pathway inhibitors (161, 162). It must be considered, however, that other fatty acid products of PLA₂ action may be mediating these effects. In addition, lysophospholipids, other products of PLA₂ action, may be the important intracellular mediators in some of these responses that are blocked by PLA₂ inhibitors.

Cyclooxygenase Metabolites as Intracellular Messengers

Products of the cyclooxygenase system can act at plasma membrane receptors to trigger intracellular signaling events. For example, thromboxane activates phospholipase C in platelets (163) and renal mesangial cells (164). Recently, the platelet thromboxane receptor has been cloned and found to be a member of the family of serpentine receptors with seven transmembrane domains (165). In most cases when PGE₂, the dominant renal prostaglandin, has
been implicated in the modulation of cell function, cytokine production, cytokine gene expression, and mitogenesis; its effect is generally attributed to the stimulation of adenyl cyclase (161, 166–168), with cAMP serving as the effector (166, 169, 170). There is some evidence, however, that in some cells, prostaglandins may act independently of adenyl cyclase. Cyclooxygenase products of arachidonic acid have been proposed to be inhibitory for atrial muscarinic K⁺ channels (171). Although prostaglandins can stimulate and indomethacin can inhibit mitogenesis in a number of vertebrate and invertebrate systems (172–174), the mechanisms involved are not defined. Because cyclooxygenase activity is present in the nuclear membrane (175), prostaglandins produced at this site may have ready access to the nucleus, where they might modulate transcription. In glomerular mesangial cells, we were unable to find any effect of indomethacin on vasopressin, EGF, or phorbol ester-induced increases in ³H]thymidine incorporation. Likewise, we found no effects of PGE₂ on the immediate-early response gene Egr-1 and found no modulatory effect of PGE₂ on the vasopressin-induced stimulation of Egr-1 (159).

Lipoxygenase Metabolites as Intracellular Messengers

Membrane Channels. In contrast to the sparse amount of data directly linking cyclooxygenase products to intracellular signaling events, there are large amounts of data implicating lipoxygenase products in intracellular signal transduction. Evidence that arachidonic acid metabolites serve as second messengers for membrane channel regulation was reported in Aplysia sensory neurons (176). Arachidonic acid, via lipoxygenase metabolism, and 12-HPETE mimicked the effect of FMRFamide to increase the open time of S channels and thereby increase net outward K⁺ current. Neither 5-HETE nor other products of lipoxygenase activity were able to mimic the action of FMRFamide. The effects of FMRFamide were unaffected by indomethacin but inhibited by NDGA, which can inhibit lipoxygenases and cytochrome P-450 monoxygenases (160). 12-HPETE also mimics the effects of histamine on L14 cells in Aplysia, an action believed to be due to effects on more than one type of ion channel (177). Subsequent studies indicate that it may not be 12-HPETE, but rather a metabolite other than 12-HETE, that is directly active to modulate channel activity. In inside-out cell-free patches of Aplysia sensory neurons, 12-HPETE, when applied to the cytosolic side, had no effect upon the K⁺ channel current (178). In the presence of hematin, however, 12-HPETE increased K⁺ channel activity. The addition of hematin to the HPETE in vitro results in the generation of metabolites such as 8- and 10-hydroxy-11,12-epoxyeicosatrienoic acids (179). In tissues containing the epoxide hydratase enzyme, these unstable epoxides would likely be converted to trihydroxy-eicosatrienoic acids, which may be the active metabolites.

Lipoxygenase products have also been implicated in long-term potentiation in the hippocampus (180), a model for the cellular mechanisms involved in learning and memory (181). Schweitzer et al. reported that lipoxygenase products may mediate the somatostatin-induced augmentation of the time- and voltage-dependent K⁺ current that persists at slightly depolarized membrane potentials (M-current, iM) in hippocampal pyramidal neurons (182). The PLA₂ inhibitors quinacrine and 4-bromophenacylbromide, as well as NDGA, inhibited the somatostatin-induced augmentation of iM, whereas indomethacin had no effect. LTC₄ overcame the inhibition of NDGA, and 5-lipoxygenase inhibitors also blocked the somatostatin effect, leading those authors to suggest that a 5-lipoxygenase product was the responsible second messenger for the somatostatin effect. There were no data presented, however, to confirm that the cells have 5-lipoxygenase activity. Thus, lipoxygenase products may act as first messengers, autacoids, and second messengers in the regulation of presynaptic function and postsynaptic response.

There is evidence that lipoxygenase products alter K⁺ channel activity in the heart. In inside-out patches of neonatal rat cardiac atrial membranes, Kim et al. (78) found that the direct addition of the βγ subunit of GTP-binding proteins to the patch resulted in the activation of a K⁺ channel that is linked to muscarinic receptors. Antibodies raised to the pancreatic group I 14-kd PLA₂ (183) block the effect of the βγ subunit. Arachidonic acid also activated the channel, an effect inhibited by NDGA but not by indomethacin. 5-HPETE and LTb₄ activated the channel in a manner not inhibitable by NDGA. Because 12-HETE also sometimes activated the channel, the authors concluded that perhaps several lipoxygenase products regulate channel activity. The data could not distinguish, however, between a direct effect of lipoxygenase products on the channel or an indirect effect, perhaps mediated via a G protein α subunit. Arachidonic acid, acting via (a) NDGA-inhibitable metabolite(s), activates the acetylcholine-stimulated atrial muscarinic K⁺ channel by altering the function of the GTP-binding protein, which couples the acetylcholine receptor to the ion channel (171, 184). Similar modulatory effects were found in inside-out patches of A6 renal epithelial cells when Na⁺ channel activity was evaluated by Cantillo et al. (185), who reported that Gα₂,3, GTPγS, or mellitin, an activator of PLA₂, increased amiloride-sensitive Na⁺ channel activity in a manner inhibitable by quinacrine or NDGA. 5-HPETE or LTD₄ overcame the effect of NDGA.
Chloride channel activity may also be modulated by lipoxygenase metabolites. In the bovine chromaffin cell, GTPγS stimulates chloride channels by a mechanism inhibited by a PLA₂ inhibitor or by NDGA (186). It was concluded that chloride channel activation involves the G protein-induced activation of PLA₂ and the subsequent production of a lipoxygenase product.

**Intracellular Enzymes.** Lipoxygenase products can modulate the activity of intracellular enzymes. Graff et al. (187) found that HPETE, but not HETE, activated soluble spleen guanylate cyclase activity. Hansson et al. (117) reported that oxygenated products of arachidonic acid can directly stimulate protein kinase C in vitro. The most potent product was lipoxin A. Other arachidonic acid products, including 15-HETE, 5(S), 15(S)-DHETE, 8(S), 15(S)-DHETE, and LTC₄, but not 15-HPETE, also had stimulatory effects, albeit less pronounced than those of lipoxin A. 12(S)-HPETE inhibits Ca²⁺/calmodulin-dependent protein kinase from rat brain cortex with an IC₅₀ of 0.7 μM (113).

**Intracellular Calcium Homeostasis.** In rat mesangial cells, we have found that 15-HPETE released Ca²⁺ from intracellular storage sites (120). Although PGE₂ and 15-HETE also had an effect to increase Ca²⁺ release, this effect was much less than that of 15-HPETE (Figure 8). This represents a possible mechanism for positive feedback in a signaling pathway. An agonist that initially increased PLA₂ activity, arachidonic acid production, and lipoxygenase product production will potentially have effects sustained by the further increase in cytosolic free [Ca²⁺] due to lipoxygenase product production. The increased cytosolic free [Ca²⁺] would further trigger the release of Ca²⁺ from pools sensitive to raised levels of cytosolic free [Ca²⁺], which would then, in turn, potentiate increased PLA₂ activity. We are presently attempting to distinguish whether noncyclooxygenase products of arachidonic acid exert their effects on the inositol trisphosphate-sensitive or insensitive Ca²⁺ storage sites (188).

Hepoxilin A₃, a metabolite of 12-HPETE, mobilizes Ca²⁺ from intracellular stores of neutrophils (189). It is possible, however, that this effect recapitulates a potential autacoid effect of this 12-lipoxygenase product.

![Figure 8. Effects of various arachidonic acid products on release of Ca²⁺ from intracellular nonmitochondrial stores of mesangial cells. Cells were permeabilized with digitonin and exposed to either 14, 15-EET (A), 15-HPETE (B), 15-HETE (C), or PGE₂ (D). The calcium concentration of the solution was monitored with fura-2 free acid. An increase in the bath calcium concentration reflects release from intracellular nonmitochondrial stores. Modified from reference 120 with permission.](image-url)
Figure 9. ([3H]thymidine incorporation in mesangial cells stimulated with vasopressin (AVP; 100 nM), (100 ng/mL), or phorbol myristate acetate (PMA; 10 nM) and the effects of NDGA (10 μM), SKF-525A (25 μM) ketoconazole (KETO) (20 μM), or indomethacin (INDO) on thymidine incorporation. Before stimulation, cells had been serum deprived for 72 h. Thymidine uptake was measured 18 to 24 h after the stimulus. Data not shown. Data from 12 experiments are presented. Δ, P < 0.0005; +, P <0.01 when compared to controls; *, P <0.01 when compared with mitogen alone. SKF-525A, ketoconazole, and to a lesser extent NDGA also inhibit serum-induced cell growth as determined by counting cells. From reference 159 with permission.

Figure 10. Effects of NDGA or ketoconazole (KETO) on vasopressin (AVP)-induced enhancement of Egr-1 mRNA levels in mesangial cells. Total RNA (10 μg) was loaded on each lane of these Northern blots. Blots were hybridized with either Egr-1 or P31 cDNA. The latter probe is a "housekeeper" ribosomal protein (210) and confirms that equal amounts of RNA were loaded on each lane. Note that pretreatment with either NDGA (10 μM) or ketoconazole (20 μM) inhibited vasopressin (100 nM)-enhanced mRNA levels. Modified from reference 159 with permission.

Mitogenesis, Differentiation, and Early Response Genes. We have reported that NDGA, but not indomethacin, significantly inhibited serum-stimulated cell proliferation, as determined by [3H]thymidine incorporation and cell counts, in glomerular mesangial cells (159) (Figure 9). We also found that NDGA also inhibited the vasopressin, EGF-, and phorbol ester-induced increase in mRNA levels of the immediate-early genes, Egr-1 and c-fos (Figure 10). NDGA had no effects upon cellular ATP levels. We suggested, however, that a P-450 rather than a lipoxygenase product was responsible for these effects because: (1) NDGA inhibits the cytochrome P-450 as well as the lipoxygenase metabolism of arachidonic acid (160); (2) many of the effects of NDGA could also be mimicked with SKF-525A and ketoconazole, inhibitors of the cytochrome P-450 system; (3) a 5-lipoxygenase inhibitor, caffeic acid (190), had no effect upon cell growth; and (4) as described above, we found evidence for regulated P-450 metabolism in mesangial cells. Because NDGA also decreases the production of lipoxygenase products, however, and because caffeic acid at the doses we used would likely only affect 5-lipoxygenase activity, it is possible that, in addition to cytochrome P-450 products, 12- or 15-lipoxygenase products might also be involved in the regulation of growth and immediate-early gene product, operating through a plasma membrane receptor-mediator mechanism, because it is inhibitable by the pertussis toxin pretreatment of the cells.
expression in the mesangial cell. Setty et al. (191) found that NDGA, but not indomethacin, inhibited the proliferation of endothelial cells in culture.

TNF, which stimulates arachidonic acid release, can be both mitogenic and cytotoxic for cells (192). In the study where these dual actions were demonstrated (192), both the mitogenic and the cytotoxic effects were blocked by PLA2 inhibition but the authors did not identify what product(s) of PLA2 were involved. TNF, as well as arachidonic acid itself, induces the expression of c-fos in the adipogenic cell line TA1 by a process blocked by NDGA (193). Indomethacin had no effect on the TNF induction of c-fos. TNF induces the production of lipoxygenase products in TA1 cells. 5-, 12-, and 15-HPETE, but not the HETE or the leukotrienes, when added directly to the cells, resulted in increases in c-fos mRNA levels.

Beckman et al. (194) reported that erythropoietin activates PLA2 with the subsequent production of 12-HETE and LTB4 in erythroid progenitor cells. The addition of 12-HPETE or LTB4 to the cells mimicked the effects of erythropoietin to significantly increase the number of colonies derived from colony forming units-erythroid.

**Cytokine Gene Expression.** In human HL-60 promyelocytic leukemia cells, phorbol esters increase TNF gene expression through the formation of noncyclooxygenase products of arachidonic acid (195). Phorbol ester-stimulated TNF gene expression was inhibited by quinacrine and 4-bromophenacyl bromide as well as ketoconazole, the latter at doses (100 \( \mu \)M) that inhibit leukotriene production (196) as well as the cytochrome P-450 system (197). Because LTB4 also increased TNF gene expression, the authors concluded that the 5-lipoxygenase pathway was involved in TNF gene regulation. In a subsequent study, this group (170) also found that lipopolysaccharide also increased TNF mRNA levels in HL-60 cells via a process that was blocked by NDGA and ketoconazole.

The enhanced expression of the colony-stimulating factor gene, induced by TNF, was inhibited by PLA2 inhibitors but not affected by caffeic acid, NDGA, or indomethacin. It was nevertheless suggested that a noncyclooxygenase metabolite, perhaps a lipoxin, which is known to directly activate protein kinase C \textit{in vitro} (117), might be the intracellular mediator enhancing colony-stimulating factor gene expression.

**Epoxygenase Products as Intracellular Messengers**

**Membrane Channels.** As described above, a metabolite of 12-HPETE is believed to mediate the FMRFamide-induced increased opening of the S-K\(^+\) channel in Aplysia neurons (178). SKF-525A, an inhibitor of cytochrome P-450, inhibits the response to FMRFamide, arachidonic acid, and 12-HPETE. Therefore, the authors concluded that the cytochrome P-450 system, which is known to convert HPETE to epoxyhydroxy and trihydroxy fatty acids (197), plays an important role in S-K\(^+\) channel regulation.

**Intracellular Calcium Homeostasis.** We have reported that SKF-525A and NDGA inhibit the vasopressin-induced increase in \([Ca^{2+}]\), in glomerular mesangial cells without altering the vasopressin-induced increase in inositol phosphates (120, 198). 14,15-EET (100 nM) overcame this inhibition without increasing PLC activity. 14,15-EET increased \([Ca^{2+}]\), when added to intact single mesangial cells and also induced Ca\(^{2+}\) release from the intracellular stores of permeabilized mesangial cells. These effects on vasopressin-induced \([Ca^{2+}]\) transients may explain the inhibitory effects that cytochrome P-450 metabolites have been known to have on vasopressin action in the collecting duct (199).

Madhun et al. (200) have reported that an epoxygenase metabolite of arachidonic acid mediates the angiotensin II-induced increase in \([Ca^{2+}]\) in rabbit proximal tubule epithelial cells. Ketoconozole inhibited the increase in \([Ca^{2+}]\). Of the epoxygenase products tested, 5,6-EET had the largest effect on \([Ca^{2+}]\). In contrast to our results in mesangial cells where 14,15-EET increased \([Ca^{2+}]\), even in the presence of very low levels of extracellular \([Ca^{2+}]\), the effect of 5,6-EET on \([Ca^{2+}]\) in proximal tubule cells was lost when extracellular \([Ca^{2+}]\) was reduced. These data, together with the inhibition of the effect of 5,6-EET by nifedipine and verapamil, led Madhun et al. to conclude that 5,6-EET enhanced Ca\(^{2+}\) influx through voltage-sensitive channels.

**Intracellular Enzymes.** Another intracellular effect of epoxygenase products may be as second messengers regulating Na\(^+\).K\(^+\)-ATPase activity. A P-450 product that inhibits microsomal Na\(^+\).K\(^+\)-ATPase activity has been identified in cells derived primarily from the rabbit kidney medullary thick ascending limb (157).

**Mitogenesis and Gene Expression.** As described above, because many of the inhibitory effects of NDGA on immediate-early gene expression in mesangial cells could be mimicked by SKF-525A and ketoconazole, we suggested that epoxygenase products of arachidonic acid may be important intracellular messengers for these genes and hence represent important modulators of growth (159). Hannigan and Williams (201) found that a PLA2 inhibitor, bromophenacyl bromide, as well as a nonspecific inhibitor of arachidonic acid oxygenation, 5,8,11,14-eicosatetraynoic acid, blocked the action of interferon-\(\alpha\), a growth inhibitor, to induce the binding of nuclear factors to the interferon-stimulated response ele-
ment (ISRE) in fibroblasts. In addition, bromophenacyl bromide inhibited interferon-induced ISRE-dependent transcription in transient transfection systems. By contrast, indomethacin and NDGA together enhanced the effect of interferon on nuclear factor binding to the ISRE and enhanced ISRE-dependent gene expression. It was concluded that the effects of indomethacin and NDGA were primarily on the cyclooxygenase and lipoxgenase systems and that these inhibitors may have forced the available arachidonic acid into the epoxygenase system, a product of which acted as an intracellular messenger regulating the association of nuclear factors necessary to bind to the ISRE.

Clearly, at present, the importance of epoxygenase products may have been underestimated because of the difficulty involved with working with these unstable compounds and the possibility that many of the effects attributed to lipoxygenase products may, in fact, be due to epoxygenase products.

LYSOPHOSPHOLIPIDS

When reviewing the role of PLA$_2$ in signal transduction, it is important to consider the importance of lysophospholipids, which along with free fatty acids, are the products of PLA$_2$ action on membrane phospholipids. Lysophospholipids have been found to have a number of biologic effects on cells. Lysophosphatidylcholine (lyso-PC) stimulates guanylate cyclase activity (202). Lyso-PC also stimulates basal adenyl cyclase activity (203) but inhibits sodium fluoride-stimulated adenyl cyclase activity (202). Lyso-PC inhibits Na$^+$,K$^+$-ATPase activity in sarcolemma membranes from dog and rabbit (204). It is interesting to note that lyso-PC was isolated from heart tissue by Hajdu et al. In 1957 (205), was found to have digitalis-like ionotropic activity in the frog heart, and was thought to possibly mediate the effects of digitalis and toxic tissue damage. Lysophospholipids increase membrane fluidity (206) and can be cytotoxic (207). They can alter the activity of transport proteins, as is described for the red blood cell glucose transporter (208).

Lysophosphatidic acid does not induce the membrane "leakiness" induced by lyso-PC. It acts on an extracellular site of fibroblasts to increase GTP-dependent phospholipase C activity with resultant phosphoinositide breakdown and increase in [Ca$^{2+}$] (208,209). Lysophosphatidic acid is also mitogenic for these cells.

CONCLUSIONS

I have attempted to provide an overview of the various ways in which PLA$_2$, arachidonic acid, its metabolic products, and lysophospholipids are involved in signal transduction. The information is very incomplete at the present time. A better understanding of: (1) the various forms of PLA$_2$; (2) the modes of regulation of PLA$_2$ activity; and (3) the factors governing the metabolic conversion of arachidonic acid to its many products; together with the introduction of more specific inhibitors of PLA$_2$ and the metabolic pathways of arachidonic acid metabolism, will result in greater insight into the various roles played by PLA$_2$ in signal transduction.

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"The conclusion to which one is forced is that there is commonly a degree of inevitability about scientific discovery: within a shortish period, if one person does not put forward a hypothesis or discover a fact, another will. No scientist can afford to be arrogant about the degree of originality he achieves."