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Cyclic Phosphatidic Acid – A Unique Bioactive Phospholipid

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Abstract

Cyclic phosphatidic acid (CPA) is a naturally occurring analog of the growth factor-like phospholipid mediator, lysophosphatidic acid (LPA). The *sn*-2 hydroxy group of CPA forms a 5-membered ring with the *sn*-3 phosphate. CPA affects numerous cellular functions, including antimitogenic regulation of the cell cycle, induction of stress fiber formation, inhibition of tumor cell invasion and metastasis, and regulation of differentiation and survival of neuronal cells. Interestingly, many of these cellular responses caused by CPA oppose those of LPA despite the activation of apparently overlapping receptor populations. Since the early 1990s, studies on CPA actions gradually developed, and we are now beginning to understand the importance of this lipid. In this review, we focus on the current knowledge about CPA, including enzymatic formation of CPA, unique biological activities and biological targets of CPA, and we also explore metabolically stabilized CPA analogs.

Keywords

cyclic phosphatidic acid; transphosphatidylation; cancer cell invasion; tumor metastasis; ATX inhibitor; anti-cancer drugs

Introduction

In 1992, the first cyclic phosphatidic acid (1-acyl-sn-glycerol-2,3-cyclic phosphate; CPA) species was isolated from the myxoamoebae of a true slime mold, *Physarum polycephalum*, and was named *Physarum* lysophosphatidic acid (PHYLPA) [1]. PHYLPA is structurally related to lysophosphatidic acid (LPA), chemically, the smallest and simplest glycerophospholipid. PHYLPA comprises cyclopropane-containing hexadecanoic acid at *sn*-1 position and a cyclic phosphate at *sn*-2 and *sn*-3 position of the glycerol carbons. A derivative of PHYLPA, having cyclopropane-containing octadecanoic acid, was also isolated from the same organism [2]. These substances inhibit eukaryotic DNA polymerase α and the proliferation of human fibroblasts cultured in a chemically defined serum free medium [3]. A variety of PHYLPA analogs with a cyclic phosphate and various fatty acyl chains were synthesized [4], and these compounds are now referred to as CPA. The biological activities of CPA and the structure-activity relationships indicated that the cyclic phosphate was necessary for the anti-proliferative activity [5]. Subsequent to the discovery of PHYLPA, CPA analogs with various fatty acyl chains were detected in human serum and some other organisms [6]. CPA activates $LPA_{1–5}$ G-protein coupled receptors (GPCRs) [7–10]. However, the cellular effects of CPA can be remarkably different from those of LPA. CPA inhibits cell proliferation

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[3,5,11], induces actin stress fiber formation [11], promotes differentiation and survival of cultured embryonic hippocampal neurons [12], inhibits LPA-induced platelet aggregation [13], and inhibits cancer cell invasion and metastasis *in vitro* [14,15] and *in vivo* [15,16]. Recently, metabolically stabilized carba derivatives of CPA (CCPA) were described as a novel inhibitors of metastatic cancer [10,15]. This finding provides the proof of concept for the anti-metastatic application of CCPA and led us to develop a second generation of CCPA analogs ([17], and see Prestwich *et al*. in this issue). CCPA and its analogs could be promising anti-cancer drug candidates but their anti-metastatic mechanism of action has not been fully elucidated. Further studies focusing on the molecular target(s) and the mechanism of inhibition of cancer cell invasion and metastasis by CPA will increase the feasibility of use of CPA derivatives in the treatment of cancer. This review summarizes current knowledge about CPA, including enzymatic formation of CPA, unique biological activities of CPA and also biological targets of CPA.

CPA in mammalian body fluids

To answer the question whether CPA is present in mammalian biological fluids, Kobayashi *et al*. extracted lipids from human serum albumin searching for CPA [6]. Preparative TLC and HPLC analysis revealed that CPA was extracted and purified from human serum albumin. Furthermore, ESI-MS/MS analysis determined that the most abundant molecular species was CPA 16:0, whereas, CPA 14:0 and CPA 18:0 were also present as minor species. These authors estimated the concentration of CPA 16:0 in human serum could be about 0.1 μM. This is less than one-tenth of the concentration of LPA, since the concentration of LPA bound to albumin in mammalian serum is 1–5 μM [18]. Nonetheless, the detection of CPA after the harsh conditions of albumin purification underlines the chemical stability of this lipid. Recently, Shan and colleagues developed an LC/ESI/MS/MS method and successfully quantified the level of two CPA species, CPA 16:0 and CPA 18:1 in human serum [19]. The average concentration of CPA 16:0 in the serum of pre-surgery ovarian cancer patients was significantly lower than that of either post-surgery ovarian cancer patients or normal subjects. These data suggest that CPA might exert some anti-cancer activity in human body and also that surgery can affect the serum CPA levels of ovarian cancer patients. Liliom *et al.* [20] also reported the evidence that CPA along with other growth factor-like glycerophosphate mediators are present in the aqueous humor and the lacrimal gland fluid of rabbit eyes. The amount of these mediators increases after injury.

Enzymatic formation of CPA

In 1996, Friedman *et al*. [21] demonstrated that CPA can be formed by intermolecular transphosphatidylation with the hydroxyl on *sn*-2 position of lysophosphatidylcholine (LPC) by the bacterial phospholipase D (PLD) from *Streptomyces chromofuscus*. However, the efficiency of transphospatidylation relative to hydrolysis differs among PLD enzymes and the assay conditions. Murakami-Murofushi *et al*. examined the formation of CPA from LPC by PLD derived from *Actinomadurea* sp. NO 362 and by PLD from *Streptomyces chromofuscus*, and found the *Actinomadurea* enzyme, under certain conditions, preferentially transphosphatidylates rather than hydrolyzes LPA and produces almost exclusively CPA [22]. These results raised the hypothesis that in blood a LPLD/PLD-like enzyme could be responsible for CPA production via transphosphatidylation of abundant LPC.

CPA-producing enzyme in blood

Based on the reaction by *Actinomadurea* PLD and the fact that LPC, a substrate of PLD, is the second most abundant phopholipid in serum [23], Tsuda *et al*. reported significant transphosphatidylation activity capable of CPA generation in fetal bovine serum [24]. Furthermore, they purified the enzyme and the peptide mass matching analysis revealed that

the purified CPA biosynthetic enzyme shared several peptide fragments with autotaxin (ATX), a serum lysoPLD (LPLD) that also produces LPA [25,26]. Immunoblot analysis confirmed that active fraction contains a 100-kDa protein that was immunoreactive with human ATX antibody. Moreover, recombinant ATX expressed in Sf9 cells also generated CPA in addition to LPA, and no significant CPA-producing activity was detected in ATX-depleted serum from bovine and human. However, the conditions used in this study (two-phase buffer) do not mimic the physiological milieu and for this reason, ATX may not be the only and most important source of CPA in blood.

Distinguishing features of LPA and CPA action

Although CPA is structurally close to LPA, CPA shows several distinct biological activities. The major physiological and pathophysiological effects of CPA are shown in Table 1.

i. Anti-mitogenic action

When CPA was added to cultures of human fibroblast cells, TIG-3 or TIG-7, in serum-free medium, proliferation was inhibited [3]. In both cell lines, LPA stimulates cell proliferation. In mouse fibroblast NIH3T3, pertussis toxin (PTX) pretreatment inhibited LPA-induced proliferation, whereas the anti-proliferative effect of CPA was unaffected. Thus, PTX-sensitive Gi protein is unlikely to be involved in the anti-proliferative effect induced by CPA.

ii. cAMP elevation

CPA elevates cyclic AMP (cAMP) level via a non-PTX-sensitive mechanism through the activation of a Ca^{2+}-sensitive adenylyl cyclase [11,22,27]. In contrast, LPA possesses dual action in cAMP mobilization. LPA decreases cAMP level possibly through Gi-coupled $LPA_{1–3}$ receptors [28,29], whereas it increases cAMP level through LPA_4 and LPA_5 receptors [30,31]. These results suggest that CPA could elevate cAMP through the activation of LPA_4 and/or LPA_5. cAMP has been recognized as a modulator of cell proliferation [32,33], and the elevation of cAMP causes a marked growth-inhibiting effect [34,35]. These observations led us to assume that cAMP elevation mediates the anti-proliferative action of CPA. However, another possibility for the cell cycle regulation by CPA has also been proposed [22]. CPA directly inhibits the cell cycle regulatory protein Cdc25, which in turn dephosphorylates the cyclin-dependent kinase 2 (Cdk2) resulting in the arrest of the cell cycle progression and the inhibition of cell proliferation. The contribution of these two mechanisms to the CPA-elicited anti-proliferative effect is not known at the time.

iii. Platelet inhibition

The structure-activity analysis of the effect of various LPA analogs on platelet aggregation has been reported [13]. Unlike LPA, CPA with an acyl chain inhibited LPA-induced platelet aggregation, whereas CPA with an alkyl chain was a weak agonist inducing only reversible platelet aggregation. Apparently, cyclization of the phosphate group greatly decreases the aggregating activity of CPA. However, since the alkyl analog of LPA has been found to act on platelets at around 20-fold lower concentrations [36,37], the same difference was observed between the acyl and alkyl CPA analogs. Furthermore, acyl- and alkyl-CPA had only a marginal effect on platelet cAMP levels. This indicates that CPA does not inhibit platelet aggregation by stimulating adenylate cyclase.

iv. Neurotrophic effect

Since the brain tissue has been found to be the richest source of CPA (0.14 μmol per g wet weight of cerebrum) [12], and LPA has pronounced effect on neuronal cell morphology [38–40], the effects of CPA have been tested in primary neurons established from embryonic rat

hippocampi [12]. Nanomolar concentrations of CPA promote neurite outgrowth and enhances the survival of the neuronal cells, suggesting that CPA mimics the effects of the neurotrophin NGF in cultured embryonic hippocampal neurons.

v. Inhibition of tumor cell invasion

Tumor cell invasion across the basement membrane is an important step in the cancer metastasis [41]. An *in vitro* culture system has been developed by Akedo and colleagues [42], in which the tumor cells are seeded on a mesothelial cell monolayer and a peritoneal dissemination is monitored by counting the number of invasion foci underneath the monolayer. This *in vitro* system is considered to be a good model for the first step of experimental cancerous peritonitis. Using this system, MM1 rat ascites hepatoma cells, OC-10 human lung cancer cells, PSN-1 human pancreatic adenocarcinoma cells and B16 mouse melanoma cells have been tested for the effect of CPA on transcellular migration [14]. Although LPA was a potent inducer of transmonolayer migration, CPA significantly inhibited transcellular migration. Among some naturally occurring CPA species, CPA 16:0 was the most potent inhibitor and 25 μM CPA 16:0 inhibited the LPA-induced invasion in all of these cell lines by 70–95% [14].

CPA is a stable LPA analog and it has been shown that in tissue culture medium over 75% remains intact up to 24 hrs after incubation [9]. Furthermore, the chemical breakdown of CPA did yield LPA [9]. Nonetheless, CPA could give rise to the formation of LPA by ring opening. To prevent the opening of the cyclic phosphate ring by phospholipases and phosphodiesterase, metabolically stabilized derivatives of CPA have been synthesized [15]. These derivatives are called carba-CPA (CCPA) because the phosphate oxygen is replaced with a methylene (CH2, carba) group at either the *sn*-2 or *sn*-3 position. 2CCPA 16:0 and 3CCPA 16:0 at 25 μM significantly inhibited serum- and LPA-induced transcellular migration of MM1 cells [15]. Furthermore, CCPA derivatives with unsaturated fatty acids, CCPA 18:1 or CCPA 16:1, inhibit tumor cell migration more strongly than CCPA 16:0. LPA elicits the transient activation of RhoA, which is an essential event in transcellular tumor cell migration [27]. CPA 16:0 and 2CCPA 16:1 inhibited LPA-induced RhoA activation, suggesting that the anti-metastatic effect of CCPA is achieved by the inhibition of RhoA activation.

vi. Anti-metastatic action

A few recent studies have reported the anti-metastatic effect of CPA and CCPA [43] using the B16 melanoma model in C57BL/6 mice [14,15] and the azoxymethane-induced intestinal tumor model in Wistar rats [16]. In the B16 mouse melanoma metastasis model, a single injection of CPA 16:0 concomitantly with the tumor cells into the tail vein of C57BL/6 mice suppressed pulmonary metastasis by approximately 50% at 0.2 mg/kg and 90% at 0.4 mg/kg three weeks after the injection [14]. A single injection of CCPA 16:1 and CCPA 18:1 also significantly suppressed pulmonary metastasis of B16 melanoma [15].

In another cancer metastasis animal model, the administration of the gastrointerstinal peptide bombesin significantly increased the metastasis to the peritoneum from intestinal adenocarcinomas induced by azoxymethane in rats [44]. When 3 or 6 mg/kg of CPA was delivered subcutaneously every other day for 30 weeks [16], CPA blocked bombesin-induced metastasis by up to 95% in that model. CPA, at either dose, significantly also decreased the incidence of lymphatic vessel invasion of adenocarcinomas. However, CPA had little or no effect on the body weight, location, histologic type, and depth of involvement or infiltrating growth pattern of the tumors. Although these milestone studies established the profound inhibitory effect of CCPA on metastasis, they fell short of identifying the molecular target(s) of CCPA. (see section *ii. Lysophospholipase D, autotaxin*)

vii. Respiration and cardiovascular functions

The effects of CPA and CCPA on cancer cell invasion and metastasis hint at the possible usage of these compounds for cancer treatment. For clinical use, it is important to identify the effects of systemically administered compound *in vivo* on autonomic functions. A study on the effect of CCPA on respiratory and cardiovascular functions in anesthetized rats has recently been reported [45]. Intravenous administration of 3CCPA 18:1 (0.13 or 0.39 mg/kg) increased in tidal volume and respiratory frequency, resulting in an increase in total ventilation. Heart rate was slightly decreased at the 0.39 mg/kg dose, while systemic blood pressure was not affected. Although LPA has a similar structure to CPA, LPA has a potent hypertensive action on systemic blood pressure [46,47]. CCPA at these doses caused neither vasopressor nor tachycardiac effects, suggesting that systemic administration of CPA will not have major adverse effects.

Biological targets of CPA

i. Receptors

Seven LPA-specific cell surface receptors, $LPA_{1–7}$, have been identified to date. The $LPA_{1–3}$ receptors belong to the endothelial differentiation gene (EDG) family of G-protein-coupled receptors (GPCRs) and they are well-characterized. More details of the properties of LPA receptors and signaling can be found in previous reviews [29,48] and also reviews in this issue. More recently $LPA_{4–7}$ have been identified as LPA receptors [30,49–51], which are more closely related to the purino-receptor cluster of GPCRs. Although, CPA is known to activate $LPA_{1–5}$ receptors [7–10], CPA activates $LPA_{1–4}$ receptors with a significantly higher EC_{50} concentration than LPA [10]; however, it has as high an efficacy as LPA for LPA_5 receptor (Y Fujiwara, in preparation). Interestingly, LPA_4 and LPA_5 have been shown to elevate cAMP by LPA [30,31]. These results lead us to hypothesize that an elevation of cAMP by CPA reported previously could be mediated by the activation of LPA_4 or/and LPA_5. However, the possibility of the existence of as-yet-unknown CPA selective receptor(s) cannot be ruled out. Identification and characterization of CPA receptors will be important to shed more light on the understanding of CPA actions and signaling.

ii. Lysophospholipase D, autotaxin

It has been more than two decades since LPLD activity that produced LPA from LPC was detected in rat plasma [23]. However, in contrast to the numerous studies on LPA signaling mediated by the LPA receptors, the enzymes regulating LPA production and degradation had not been characterized until recently. In 2002, plasma LPLD was purified and found to be identical to ATX [25,47]. ATX was known to be a tumor motility-stimulating protein and ATX enhances the invasion and metastatic dissemination of tumors [52,53]. Affymetrix gene chip assays of 12,000 genes revealed that ATX was one of the 40 most unregulated genes associated with highly metastatic cancers [54]. More interestingly, van Meeteren et al. recently showed that ATX is under feedback inhibition by its hydrolysis products LPA and sphingosine-1-phosphate [55]. Thus, LPA generated by ATX can inhibit its own production and this inhibition is overcome by the constitutive secretion of ATX in cancer cells. This finding gave us the incentive to screen various LPA analogs for the ability to inhibit ATX. Analysis of ATX activity by measuring ATX-mediated hydrolysis of fluorescent substrates revealed that 2- and 3-CCPA analogs strongly inhibited ATX activity and LPA production [10]. 2CCPA 16:1 was the most effective inhibitor with an IC_{50} of 140 nM, which is more than 100-fold lower than LPA in this assay system. LPA receptor activation studies showed that the 3CCPA analogs neither activate nor inhibit $LPA_{1–4}$ receptors, and 2CCPA 16:1 and 2CCPA 18:1 were partial agonists of these receptors. The activation profiles of these analogs are important because LPA receptors have been reported to be associated with tumor cell invasion [56]. Cell invasion assay using matrigel-coated modified Boyden chamber showed that co-treatment of CCPA analogs with ATX and LPC resulted in a significant decrease of tumor cell invasion [10]. Furthermore,

exogenously added LPA can overcome this inhibitory effect of CCPA on ATX-induced cell invasion, suggesting that CCPA elicits its inhibitory effect on cancer cell invasion by targeting ATX and decreasing the production of LPA without either activating or inhibiting LPA receptors.

It is important to point out that addition of LPC 18:1, the substrate of ATX, does not induce transcellular migration in MM1 cells [43], suggesting that the LPLD activity of ATX generated by these cells does not appear to produce sufficient LPA 18:1 to elicit cell migration. Therefore, the mechanism of inhibition described in this cell line does not solely depend on ATX inhibition and point out a multiplicity of targets.

B16F10 mouse melanoma cells are widely used as a model to assess the therapeutic effect of anti-metastatic compounds *in vivo*. In this model, intraperitoneal injections 3CCPA 16:1 and 3CCPA 18:1 blocked B16F10 tumor cell-derived lung metastasis by 35 and 57%, respectively, with only two 0.25 mg/kg doses administrated at 15 min and 48 hrs after inoculation. These data suggest that CCPA inhibited the formation of metastatic foci. Taken together these results validate ATX as a target of anti-metastatic treatment, and provide a proof of concept for the anti-metastatic effect of the CCPA analogs.

Summary and Future Direction

Over the last 15 years, our understanding of CPA actions has progressed gradually. CPA has emerged as a potential anti-metastatic drug candidate but the mechanisms responsible for this effect remain unresolved. Clinically, the promising drug candidates would be the compounds that inhibit both ATX and LPA receptor subtypes that promote invasion of cancer cells. Molecular pharmacological studies are underway by several groups, aiming to identify novel non-lipid ligand scaffolds using in silico screening and to synthesize antagonists that selectively block lysophospholipid targets ([17,57] and see Prestwich *et al*. in this issue). Further studies on molecular target(s) as well as the mechanism of these compounds will increase the feasibility of the compounds in the treatment of cancer metastasis.

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Abbreviation used

CPA
: cyclic phosphatidic acid

GPCR
: G-protein coupled receptors

LPA
: lysophosphatidic acid

CCPA
: carba-CPA

PLD

phospholipase D

LPC

lysophosphatidyl choline

ATX

autotaxin

PTX

pertussis toxin

cAMP

cyclic AMP

EDG

endothelial differentiation gene

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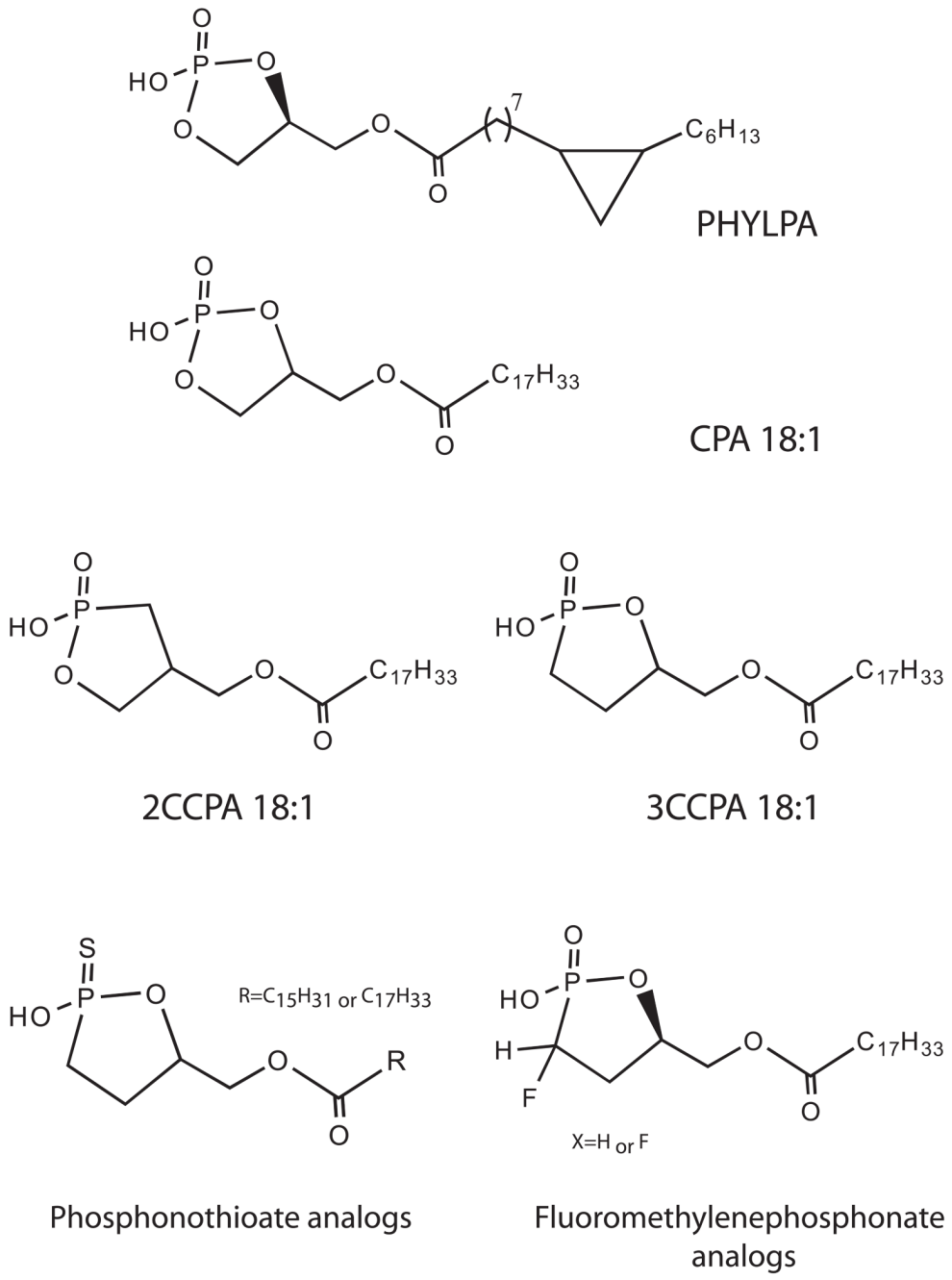

Figure 1. Structures of PHYLPA, CPA, CCPA and CCPA analogs

For details of phosphonothioate and fluoromethylene phosphonate analogs, see [58] and Prestwich *et al*. in this issue

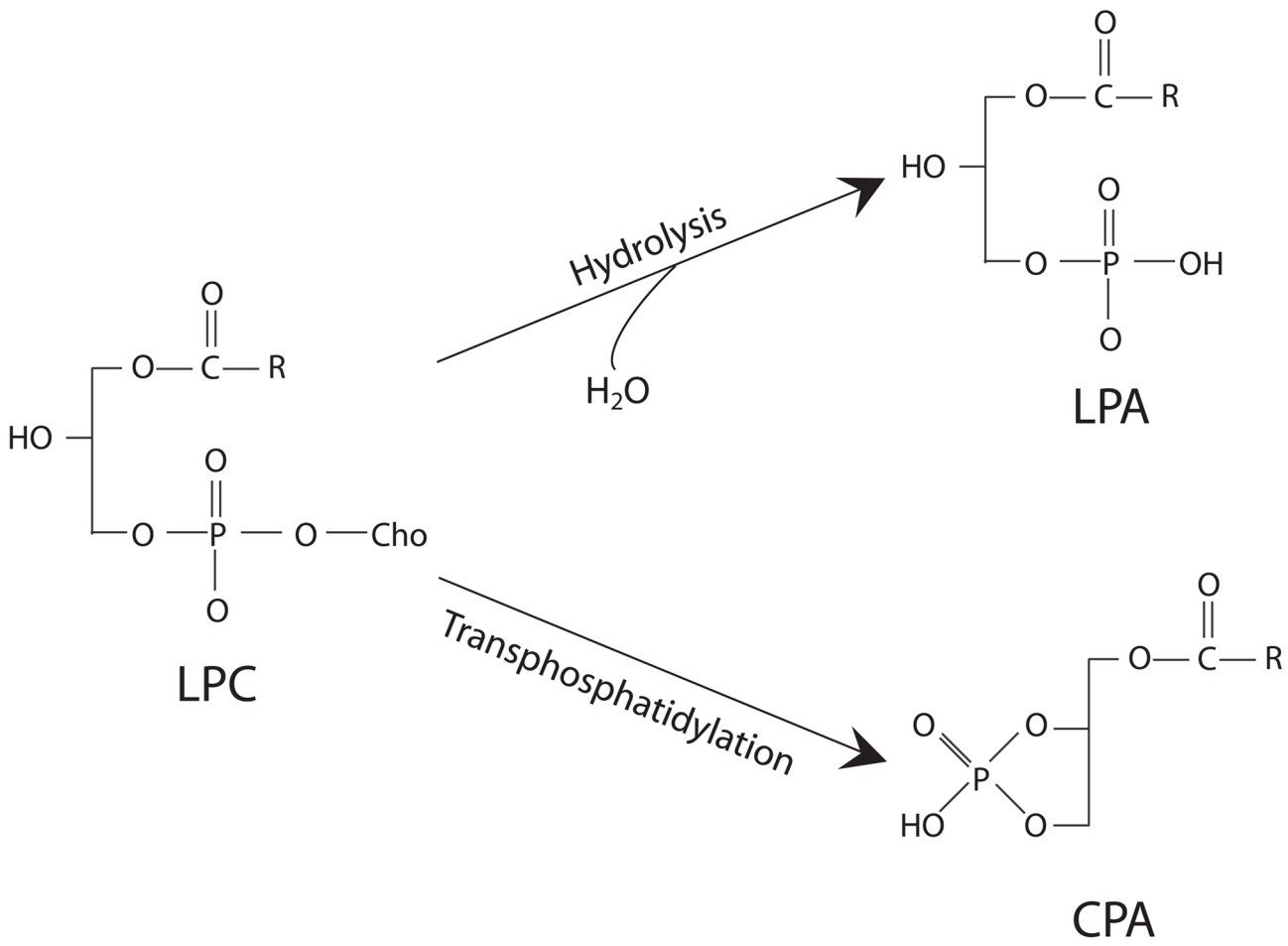

Figure 2.
Enzymatic synthesis of CPA from LPC by PLD

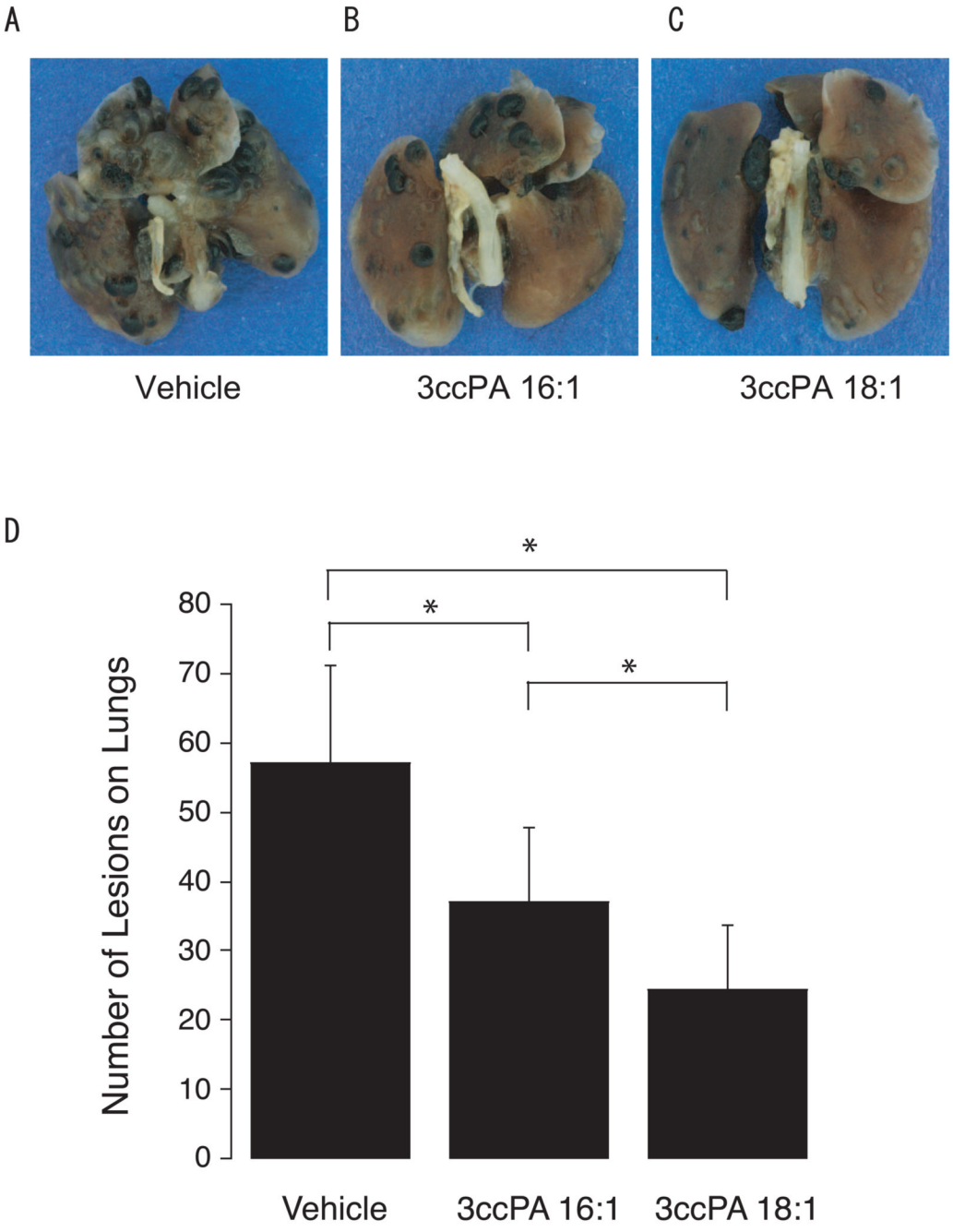

Figure 3.
Inhibition of metastasis *in vivo*: B16F10 melanoma cells were injected in the tail vein of C57BL/6 mice, followed by i.p. injections of vehicle (PBS) (A), 3CCPA 16:1 (B) or 3CCPA 18:1 (C). Animals were sacrificed 3 weeks later and lung nodules were counted (D). (* $p < 0.01$ ANOVA). For details see [10].

Table 1

Major biological effects of CPA

Effects	References
Anti-mitogenic regulation of cell cycle	[3,5,20]
Actin polymerization and stress fiber formation	[20]
Inhibition of platelet aggregation	[13]
Activation of Cl^- current	[59]
Enhancement of neurite outgrowth and survival	[12]
Mobilization of intracellular calcium	[3,9,10]
Inhibition of tumor cell invasion and metastasis	[10,14–16,22,27,60]