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Differential regulation of phospholipase D and phospholipase A_2 by protein kinase C in $P388D_1$ macrophages

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Activation of $P388D_1$ macrophages by phorbol myristate acetate (PMA) resulted in the translocation of the protein kinase C (PKC) isoforms α, δ, and ϵ from the cytosol to membranes. Furthermore, PMA activated phospholipase D (PLD) in these cells, and potentiated the effect of the inflammatory lipid mediator platelet-activating factor (PAF) on PLD activation. PAF also activated phospholipase A_2 (PLA_2) and enhanced arachidonic acid (AA) release in $P388D_1$ macrophages, and bacterial lipopolysaccharide (LPS) increased the responsiveness of these cells to PAF. In contrast with PLD, PLA_2 activation in $P388D_1$ macrophages was found to take place independently of PKC. This was supported by the following evidence: (i) PMA neither induced AA release nor enhanced the PAF response; (ii) inclusion of PMA along with LPS during priming did not have any effect on PAF-stimulated AA release; (iii) down-regulation of PMA-activatable PKC isoforms by chronic treatment with the phorbol ester had no effect on the PAF response; and (iv) the PKC inhibitor staurosporine did not alter the PAF-induced AA release. The present study provides an example of cells in which the direct activation of PKC by phorbol esters does not lead to a primed and/or enhanced AA release. As a unique example in which PKC activation is neither necessary nor sufficient for AA release to occur, this now allows study of the separate and distinct roles for PLD and PLA_2 in signal-transduction processes. This has hitherto been difficult to achieve because of the lack of specific inhibitors of these two phospholipases.

INTRODUCTION

Receptor-mediated hydrolysis of phospholipids is well established as a key event in cellular signalling [1]. Although phospholipase A_2 (PLA_2)-mediated release of arachidonic acid (AA) is a widely occurring phenomenon, the regulatory mechanisms involved are not completely understood. Evidence for a key role for protein kinase C (PKC) in this process has been obtained in a wide variety of cell types with many different agonists. Addition of phorbol esters increases PLA_2 activity and stimulates AA release in many cells, and treatment of cells with PKC inhibitors, down-regulation of the enzyme, or inhibition of PKC expression by antisense RNA technology blocks AA release [2].

Mammalian cells contain structurally diverse PLA_2 forms capable of effecting AA release [3]. Depending on cell type and agonist involved, distinct PLA_2s appear to be involved. In lipopolysaccharide (LPS)-treated $P388D_1$ macrophages, the major component of AA release upon stimulation with platelet-activating factor (PAF) is mediated by secretory PLA_2 ($sPLA_2$) [4,5]; however, a second effector, i.e. the 85 kDa cytosolic PLA_2 ($cPLA_2$), also plays an active role in AA mobilization in these cells [6]. $cPLA_2$ is regulated by phosphorylation, presumably through a kinase cascade that appears to be downstream of PKC activation [7,8].

A large number of agonists that increase PLA_2 activity in mammalian cells also stimulate phospholipase D (PLD) [2]. By hydrolysing phospholipids, PLD generates phosphatidate, which in turn can be dephosphorylated to yield diacylglycerol. The sustained activation of PLD is believed to be the major route for generation of these two lipid messengers in many cell types [2]. In common with its role in AA release, PKC also appears to play a major role in regulating PLD activation in most cell types [2].

Preliminary data from our laboratory failed to detect prostaglandin E_2 production by PKC activators in $P388D_1$ cells [9]. In the present investigation we have studied the role of PKC in regulating phospholipid turnover in activated $P388D_1$ macrophages. Our results show that PKC is responsible for PLD activation, but does not regulate AA mobilization in these cells. The results therefore establish PLD, but not PLA_2, as a downstream effector of PKC in activated $P388D_1$ macrophages and, hence, demonstrate separate pathways for activation of each of the phospholipases.

EXPERIMENTAL

Materials

$P388D_1$ cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). LPS Re595 was kindly given by Dr. R. J. Ulevitch (Scripps Clinic and Research Foundation, La Jolla, CA, U.S.A.). Iscove's modified Dulbecco's medium (IMDM; endotoxin 0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD, U.S.A.). Fetal bovine serum was from Hyclone Labs. (Logan, UT, U.S.A.). Non-essential amino acids were from Irvine Scientific (Santa Ana, CA, U.S.A.).

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (sp. radioactivity 100 Ci/mmol) and [9,10-^{3}H]palmitic acid (sp. radioactivity 54 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). PAF and staurosporine were from Sigma (St. Louis, MO, U.S.A.). Ionophore A23187, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (αPDD) and antibodies

Abbreviations used: AA, arachidonic acid; LPS, lipopolysaccharide; PA, phosphatidic acid; PAF, platelet-activating factor; αPDD, 4α-phorbol 12,13-didecanoate; PtdEtOH, phosphatidylethanol; PKC, protein kinase C; PLA_2, phospholipase A_2; $cPLA_2$, 85 kDa cytosolic phospholipase A_2; $sPLA_2$, 14 kDa secretory phospholipase A_2; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; IMDM, Iscove's modified Dulbecco's medium.

‡ J. B. and M. A. B. contributed equally to this work; the order of authorship is arbitrary.

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against the γ, δ and ζ isoforms of PKC were from Calbiochem (San Diego, CA, U.S.A.). Antibodies against the α, and ϵ isoforms of PKC were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-PKCβ antibody was from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Cell culture

P388D$_1$ cells were maintained at 37 °C in a humidified air/CO_2 (9:1) atmosphere in IMDM supplemented with 10 % fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and non-essential amino acids. Adherent cells were selected by passage of only adherent cells. Cells were plated at 10^6/well in six- or twelve-well plates, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free IMDM. Radiolabelling of the cells was achieved by including [^{3}H]AA (0.5 μCi/ml) or [^{3}H]palmitic acid (3 μCi/ml) in the medium during the overnight adherence period.

Stimulation of P388D$_1$ cells

The standard regimen for activating cells with LPS and PAF has been described previously [4–6]. Briefly, the cells were placed in serum-free medium for 30–60 min before the addition of LPS (200 ng/ml), PMA or αPDD (1–100 nM), forskolin (100 μM) or combinations of LPS plus one of the listed compounds. After incubation for the time indicated, cells were washed and stimulated with 100 nM PAF in serum-free medium containing 1 mg/ml BSA.

Measurement of [^{3}H]AA release

The cells, prelabelled with [^{3}H]AA, were incubated with the different stimulants as described in the preceding paragraph. After the indicated times, supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid-scintillation counting.

PLD activation

For these experiments, [^{3}H]palmitate-labelled cells were used, and the incubations were carried out in the presence of 1 % ethanol. After stimulation, total lipids were extracted and phosphatidylethanol (PtdEtOH), a specific marker for PLD activity, was resolved from cellular lipids by TLC on silica-gel G plates (Whatman), using the upper phase of a system consisting of ethyl acetate/iso-octane/acetic acid/water (13:2:3:10, by vol.) [10].

Western-blot analyses

Control and PMA-stimulated cells were washed twice with serum-free medium and homogenized by 25 strokes in a Dounce homogenizer in a buffer consisting of 20 mM Tris/HCl, 2 mM EDTA, 10 mM EGTA, 1 mM PMSF, 20 μM leupeptin, 20 μM aprotinin and 0.1 % 2-mercaptoethanol, pH 7.5. The homogenates were centrifuged at 500 ***g*** for 5 min at 4 °C to separate nuclei. The resulting supernatant was centrifuged at 35000 ***g*** for 35 min at 4 °C to separate soluble and membrane fractions. After protein quantification, samples from cell homogenates (50 μg) and their equivalent cytosolic and membrane fractions, were separated by SDS/10 %-PAGE and transferred to Immobilon-P membrane (Millipore). Non-specific binding was blocked by incubating the membranes with 5 % non-fat milk in PBS for 18 h. Membranes were then incubated with anti-PKC antisera for 30 min, and then treated with horseradish peroxidase-conjugated Protein A (Amersham). Bands were detected by enhanced chemiluminiscence (ECL; Amersham International).

For PKC down-regulation experiments, the cells were treated with 80 nM PMA for 18 h, after which homogenates were prepared. After protein quantification, samples (50 μg) from control and PMA-treated cells were separated by SDS/PAGE and analysed as described above.

Data presentation

Assays were carried out in triplicate. Each set of experiments was repeated three different times with similar results. The data presented are from representative experiments and are given as means ± S.E.M. Data are expressed as the percentage of radioactivity in either [^{3}H]AA or [^{3}H]PtdEtOH compared with total cellular radioactivity.

RESULTS

Translocation of PKC isoforms in response to PMA

Increasing evidence suggests that there is not only a characteristic cell-type specific pattern of PKC isoenzyme expression, but also a cell-type-specific role of individual PKC isoenzymes in triggering certain functional cell responses. Figure 1 demonstrates that, among six isoforms examined, i.e. α, β, γ, δ, ϵ and ζ, the only PKC isoform that P388D$_1$ macrophages do not express is γ. This result is consistent with other observations indicating that the expression of PKC γ is restricted to neural tissues [2]. Stimulation of the cells with 80 nM PMA for 30 min resulted in translocation of the PKC isoforms α, δ and ϵ from the cytosol to membrane fractions, suggesting activation of these isoforms (Figure 1). In contrast, we could not detect translocation of the β and ζ isoforms (Figure 1), indicating either these two isoforms are not activated by PMA in P388D$_1$ cells or the level of translocation is too low to be detectable under the present conditions.

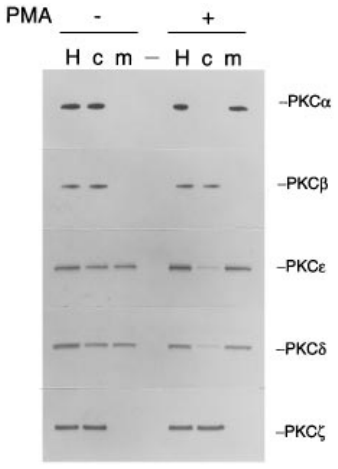

Figure 1 Distribution of PKC isoforms in resting and activated P388D$_1$ cells

Total protein from cell homogenates (H) (50 μg of protein) as well as the cytosolic (c) and membrane (m) fractions arising from 50 μg of protein, was electrophoresed and immunodetected by Western blotting as described in the Experimental section. Samples from control or PMA-activated cells (80 nM, 30 min) were probed in a Western blot with isoenzyme-specific antibodies to PKC (indicated along the right margin).

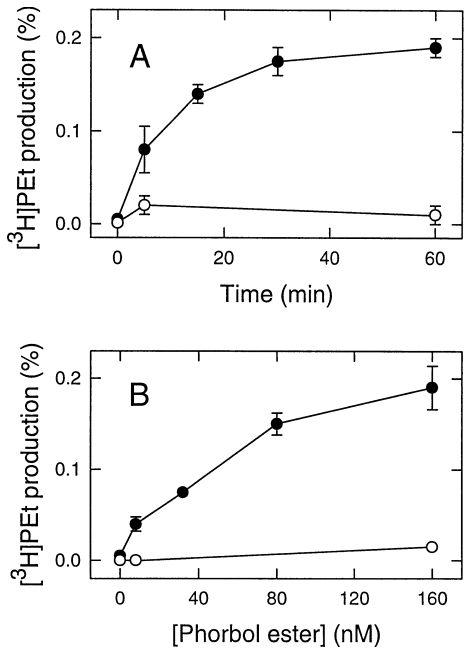

Figure 2 PMA activates PLD in P388D$_1$ macrophages

(**A**) Cells, prelabelled with [^{3}H]palmitic acid, were exposed to 80 nM PMA (●) or 80 nM αPDD (○) as a negative control, for the time indicated in the presence of 1% ethanol. (**B**) The cells were exposed to the indicated concentrations of PMA (●) or αPDD (○) for 30 min in the presence of 1% ethanol. PtdEtOH (PEt) was quantified as described in the Experimental section.

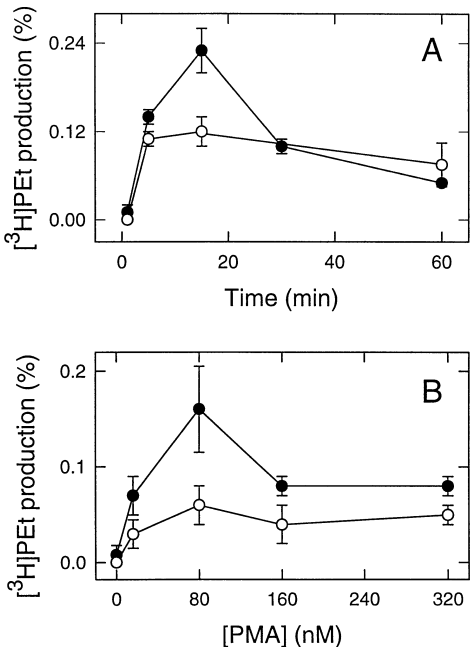

Figure 3 PMA pretreatment potentiates PAF-induced PLD activation in P388D$_1$ macrophages

(**A**) Cells, prelabelled with [^{3}H]palmitic acid, were incubated with 80 nM PMA for the indicated times. Subsequently, the cells were washed and treated with 100 nM PAF (●) or buffer (○) in the presence of 1% ethanol for 30 min. (**B**) The cells were preincubated with the indicated PMA doses for 15 min, washed and re-stimulated with 100 nM PAF in the presence of 1% ethanol for 30 min. PtdEtOH (PEt) was quantified as described in the Experimental section.

PLD activation depends on PKC

PLD has been widely reported to be activated by PMA and, in many situations, activated PLD is regarded as a hallmark of PKC activation [2,11]. In the presence of ethanol, PLD catalyses a transphosphatidylation reaction, giving rise to PtdEtOH. Since there is no known cellular mechanism other than PLD for producing PtdEtOH, quantification of this product is a specific and sensitive assay for PLD activity. As shown in Figure 2, PMA induced a time- and dose-dependent accumulation of PtdEtOH in P388D$_1$ cells, indicating PLD activation. The effect of PMA on PLD activity was not mimicked by the inactive phorbol diester αPDD (Figure 2). This demonstrates that the PMA effect on PLD is stereospecific and therefore, enzyme-mediated. In turn, these data further emphasize the existence of 'activatable' PKC in P388D$_1$ macrophages.

Interestingly, when the cells were first treated with PMA, washed and re-stimulated with PAF, an increased accumulation of PtdEtOH was detected (Figure 3). Thus, in P388D$_1$ cells, PMA is able to activate PLD (Figure 2) and to potentiate the response to a second stimulus, namely PAF (Figure 3). The amplified response to PAF induced by PMA (Figure 3) was hardly observed if the cells were treated with more than 80 nM phorbol ester or for more than 15 min, suggesting that PAF-receptor-induced stimulatory signals are desensitized by PKC activation. This phenomenon has been observed previously for other PAF-stimulated effectors (for a review, see [12]).

PLA$_2$ activation does not depend on PKC

As had been previously done for PLD, the role of PKC in the stimulation of AA release in P388D$_1$ macrophages was initially investigated by assessing the acute effects of PMA. When [^{3}H]AA-prelabelled P388D$_1$ macrophages were incubated with various concentrations of PMA (or αPDD as a negative control) (1–100 nM) for different periods of time (up to 1 h), no release of radiolabel could be detected (Table 1 and results not shown). When the cells were first treated with PMA for different times, and subsequently stimulated with either ionophore A23187 or PAF, PMA was unable to prime the response to the second stimulus (Figure 4). Moreover, when PMA was added together with LPS during priming, again no effect of PMA was observed in the amount of AA released after PAF stimulation (Table 1).

Long-term incubation (hours to days) of the cells with PMA triggers a proteolytic degradation of PKC, which is associated with the loss of PKC-mediated cell responses [2]. Data in Figure

Table 1 Effect of PKC activation on [^{3}H]AA release in P388D$_1$ macrophages

The cells were treated without (left) or with 200 ng/ml LPS (right) for 1 h. The incubation medium contained either 80 nM PMA, 80 nM αPDD or neither, as indicated. Afterwards the cells were washed and incubated with buffer (basal conditions) or 100 nM PAF (black bars) for 30 min. Extracellular [^{3}H]AA release was quantified as described in the Experimental section.

	[^{3}H]AA release (% with respect to cellular radioactivity)			
	Without LPS		With LPS	
	Basal	PAF	Basal	PAF
PMA	0.8 ± 0.1	1.7 ± 0.1	0.7 ± 0.1	3.4 ± 0.1
αPDD	0.9 ± 0.1	1.5 ± 0.1	0.7 ± 0.1	3.1 ± 0.2
Neither	0.6 ± 0.1	1.5 ± 0.2	0.7 ± 0.1	3.3 ± 0.2

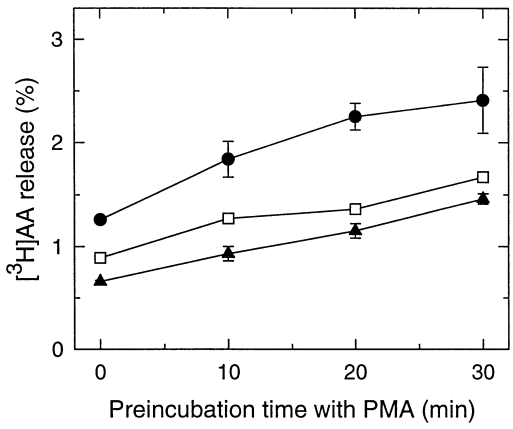

Figure 4 PMA pretreatment does not potentiate AA release in P388D$_1$ cells

Cells, prelabelled with [^{3}H]AA, were incubated with 80 nM PMA for the indicated times. Subsequently, the cells were washed and treated with 100 nM PAF (□), 10 μM ionophore A23187 (●) or buffer (▲) for 30 min.

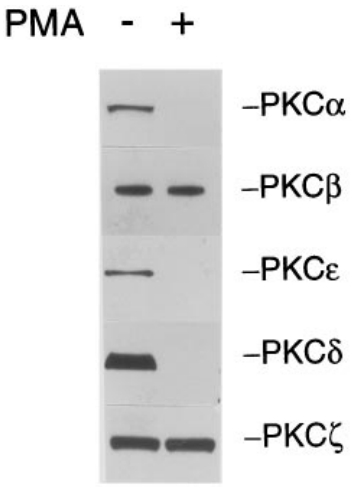

Figure 5 Down-regulation of PKC isoforms in P388D1 macrophages

Cells were incubated with 80 nM PMA for 18 h. After that time, homogenates were prepared, and 50 μg of protein was separated by SDS/PAGE. The expression of the different PKC isoforms was analysed by Western Blot.

Table 2 Effect of PKC down-regulation on [^{3}H]AA release in P388D$_1$ macrophages

The cells were incubated with 80 nM PMA, 80 nM αPDD or neither for 20 h. Afterwards, the cells were washed and incubated without (left) or with 200 ng/ml LPS (right) for 1 h. Afterwards, the cells were washed and incubated without (basal) or with 100 nM PAF for 30 min. Extracellular [^{3}H]AA release was quantified as described in the Experimental section.

	[^{3}H]AA release (%)			
	Without LPS		With LPS	
Basal	PAF	Basal	PAF	
PMA	1.1 ± 0.1	1.7 ± 0.1	1.0 ± 0.2	3.1 ± 0.1
αPDD	0.9 ± 0.1	1.5 ± 0.1	1.0 ± 0.2	3.0 ± 0.1
Neither	0.9 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	3.0 ± 0.3

5 demonstrate that complete down-regulation of the PMA-activated PKC isoforms in P388D$_1$ cells, i.e. α, δ, and ϵ (see Figure 1), was achieved in P388D$_1$ macrophages after treatment with 80 nM PMA for 18 h. Lack of PKCβ down-regulation under these conditions is consistent with the finding that this isoform appears not to be activated by PMA in P388D$_1$ cells, as shown in Figure 1. Table 2 shows that PAF-stimulated AA release in P388D$_1$ macrophages was unaffected by down-regulation of PMA-activated PKC isoforms.

We next examined the effect of staurosporine (10–500 nM), a well-established PKC inhibitor, on AA release from P388D$_1$ cells. Staurosporine has previously been shown to inhibit PKC-mediated release of AA in murine peritoneal macrophages [13]. This compound blocks the active site of the kinase and thus inhibits all isoforms. In the range of concentrations tested, staurosporine did not have any effect on PAF-induced AA release. That staurosporine blocks PKC activity in our cellular system was confirmed by the finding that PMA-induced PLD activation in P388D$_1$ cells was almost completely inhibited by this drug at 500 nM (results not shown).

Role of protein kinase A (PKA) in AA release

Recently the idea has evolved that cAMP-dependent PKA may act in an analogous manner to PKC in the regulation of certain biochemical responses. Particularly relevant to this study is the modulatory effect that these two kinases appear to exert on the turnover of phospholipids. PKA has been shown to phosphorylate PLCγ [14] and diacylglycerol kinase [15] on the same residues as does PKC, thereby inducing a similar change in the activity of these enzymes. Thus, in P388D$_1$ cells, PKA might replace PKC in regulating AA release. Since PKA is activated by intracellular elevations of cAMP, the following pharmacological approaches were conducted to study this hypothesized role for PKA. First, the cells were treated with forskolin (100 μM) or prostaglandin E$_2$ (100 nM) for 30 min and then stimulated with PAF. We have previously demonstrated that both forskolin and prostaglandin E$_2$ activate PKA in P388D$_1$ cells by inducing large increases in cAMP [16]. However, neither compound had any effect on PAF-stimulated AA release. Secondly, we studied the effect of the non-hydrolysable cAMP analogue dibutyryl-cAMP (1 mM, preincubation of cells for 30 min prior to stimulation with PAF). Again no effect was seen on PAF-induced AA release. Combining dibutyryl-cAMP with prostaglandin E$_2$ was also ineffective in enhancing PAF-stimulated AA release. Adding any of the above-mentioned compounds together with LPS during priming produced no effect on the amount of AA released following PAF stimulation. These data do not support a role for cAMP, and hence for PKA, in AA release in LPS-treated P388D$_1$ macrophages.

DISCUSSION

Stimulation of murine P388D$_1$ macrophages with nanomolar amounts of the receptor agonist PAF results in a very modest mobilization of free AA. However, preincubation of the cells with LPS prior to stimulation with PAF increases the release of AA by these cells by about 2–3-fold ([9]; Tables 1 and 2). PAF-induced AA mobilization in LPS-primed P388D$_1$ macrophages can be viewed as a two-step process, involving the participation of two different PLA$_2$s, namely sPLA$_2$ and cPLA$_2$. The two enzymes, working in concert, account for the AA mobilized during PAF activation [5,6]. Therefore, in these cells, quantification of AA release relates directly to PLA$_2$ activation.

There is ample evidence that PKC activation constitutes a critical step in the cascade of events leading to AA release in response to many receptor agonists. Phorbol esters such as PMA, which directly activate PKC, have been repeatedly observed to prime the cells for an enhanced AA release in response to a second stimulus, whether soluble or receptor-mediated [17–23]. PKC is known to be a target for LPS in macrophages [24,25]. We therefore surmised that preincubating the cells with PMA prior to exposure to PAF would mimic the effect of LPS. To our surprise, we found that PMA was totally unable either to activate AA release or to substitute for LPS in priming the P388D$_1$ cells. Under identical experimental conditions, PMA induced translocation from cytosol to membranes of three PKC isoenzymes present in these cells, namely α, δ and ϵ, activated PLD in a stereospecific manner, and potentiated PLD activation in response to PAF. The effects of PMA on PLD activity are similar to those previously observed in U937 cells [26] and Chinese-hamster ovary cells expressing the PAF receptor [27].

On the basis of the data reported herein, the P388D$_1$ cell line constitutes, to our knowledge, the only cell system reported so far in which direct PKC activation by phorbol esters does not lead to a primed and/or enhanced AA release response. Thus, while previous studies have suggested the existence of receptor-modulated pathways for AA release that may not be regulated by PKC [28,29], the present results establish that PKC activation is neither necessary nor sufficient for AA release to occur. This notion is particularly important in view of the recent findings that one major route for activating AA release in various cell types involves the phosphorylation of cPLA$_2$ via a kinase cascade initiated by PKC [7,8]. It is noteworthy that even though cPLA$_2$ generally needs an increase in the intracellular calcium levels for full translocation to the membranes, PMA [8], tumour necrosis factor-α [30] and transforming growth factor-α [31], three stimuli that do not raise intracellular calcium, have been demonstrated to phosphorylate and activate cPLA$_2$ and hence to promote AA release. PMA is unable to do so in P388D$_1$ cells; it seems that a key component of PKC signalling, placed upstream of PLA$_2$, is either non-functional or missing in P388D$_1$ macrophages. Therefore, the P388D$_1$ cell system constitutes a useful model to study signal-transduction processes leading to PLA$_2$ activation that appear not to involve PKC activity. It is also noteworthy that P388D$_1$ cells are unique among macrophages in that they release AA only in response to PAF, being unresponsive to many other inflammatory stimuli to which other macrophage cells are highly responsive [32]. For example, P388D$_1$ cells do not respond to zymosan particles by releasing AA [32]; however, peritoneal macrophages [8,13,18], alveolar macrophages [33,34], Kupffer cells [35], and RAW 261.7 macrophage-like cells [36] do release high amounts of AA in response to zymosan. Since zymosan-stimulated release of AA is highly dependent on PKC [8,13,18], it is tempting to suggest that the unresponsiveness of P388D$_1$ cells in regard to AA release is due to the lack of a PKC-mediated mechanism for activation of PLA$_2$ in these cells, similar to that operating in mouse peritoneal macrophages [8].

The one other important finding of the present study is the demonstration that at least two separate routes leading to phospholipid turnover exist in activated P388D$_1$ macrophages. The first one is independent of PKC and leads to PLA$_2$ activation; the second, mediated by PLD, depends on PKC and leads to accumulation of PA and possibly, diacylglycerol [2,37]. The existence of two different routes for phospholipid breakdown within a single cell type raises the intriguing question of whether these two routes are independent of each other. PLD activity has been shown to be activated *in vitro* by *cis*-unsaturated fatty acids, which has led to speculation about the possible existence of cross-talk between the activation mechanisms of PLA$_2$ and PLD *in vivo* [2]. Previous studies conducted in Madin–Darby canine kidney cells have suggested the existence of pathways for PLD activation that do not involve PLA$_2$ activation [10]. The finding that PMA triggers PtdEtOH formation in P388D$_1$ macrophages in the absence of AA mobilization conclusively demonstrates that notion. To the best of our knowledge, this report is the first to show activation of PLD in the absence of AA mobilization.

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