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Stimulation of Ca²⁺-activated human platelet phospholipase A₂ by diacylglycerol

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We examined the effect of diacylglycerol on Ca²⁺-dependent phospholipase A₂ from human platelets. Phospholipase A₂ was solubilized and partially purified to a stable form in the presence of n-octyl β-D-glucopyranoside (octyl glucoside), and its enzymic activity was determined with sonicated 2.5 μM-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (arachidonoyl-PC) as substrate. Phospholipase A₂ activity was increased when diacylglycerol was incorporated into the substrate arachidonoyl-PC. Stimulation was maximal in the presence of ≥ 29 mol % (1 μM) diacylglycerol, and was greater than 4-fold for both 1,2-dioleoylglycerol and 1-stearoyl-2-arachidonoylglycerol. 1-Stearoyl-2-arachidonoylglycerol at concentrations of 2–5 mol % increased phospholipase A₂ activity 1.3–1.8-fold. Exogenously added 1-oleoyl-2-acetylglycerol also enhanced phospholipase A₂ activity, producing a maximal stimulation of 1.6-fold at a concentration of 25 μM. Comparative studies conducted with pancreatic, bee-venom and snake-venom phospholipase A₂ showed that the activity of these extracellular phospholipases towards the arachidonoyl-PC substrate was also increased by diacylglycerol, but stimulation was less than observed for platelet phospholipase A₂. Our results suggest that diacylglycerol, known to be generated in stimulated platelets, may enhance Ca²⁺-activated phospholipase A₂.

INTRODUCTION

Phospholipase A₂ catalysed hydrolysis of membrane phospholipids liberates the precursors of biologically active lipids, such as eicosanoids and platelet-activating factor [1–5]. The mechanisms of activation and regulation of cellular phospholipase A₂ are not completely understood [6–8]. In human platelets, phospholipase A₂ activity is induced by physiological stimuli [9–12] as well as calcium ionophores [12,13]. Halenda et al. [14] demonstrated that phospholipase A₂-mediated liberation of arachidonic acid in response to ionophore A23187 was enhanced by activators of protein kinase C, including phorbol esters and 1-oleoyl-2-acetylglycerol. These results suggest that protein kinase C may be involved in phospholipase A₂ activation. Dawson and co-workers [15,16] have shown that diacylglycerols have an unique stimulatory effect on a number of phospholipases, including a phospholipase of the A₂ type from rat intestinal mucosa. It therefore seemed possible that in ionophore-treated platelets 1-oleoyl-2-acetylglycerol, a synthetic analogue of diacylglycerols, may itself directly promote phospholipase activity, in addition to causing enhanced activity of phospholipase A₂ via protein kinase C activation.

We have reported that Ca²⁺-dependent phospholipase A₂ can be extracted from human platelets in the presence of octyl glucoside and is obtained in stable form upon partial purification by ion-exchange chromatography [17]. In the present study we show that diacylglycerols are able to stimulate this soluble platelet phospholipase A₂ with sonicated arachidonoyl-PC as substrate.

EXPERIMENTAL

Materials

1-Palmitoyl-2-[1-¹⁴C]arachidonoyl-sn-glycero-3-phosphocholine (52–55 mCi/mmol) was obtained from NEN Research Products. rac-1,3-Dioleoylglycerol was purchased from Pharmacia P-L Biochemicals; sn-1-oleoyl-2-acetylglycerol and sn-1,2-dioleoylglycerol were obtained from Avanti Polar Lipids; arachidonic acid was from Nu-Chek. sn-1-Stearoyl-2-arachidonoylglycerol, octyl glucoside, bovine serum albumin (essentially fatty-acid-free) and phospholipases A₂ from pig pancreas (900 units/mg of protein), bee venom (760 units/mg of protein) and Naja naja venom (980 units/mg of protein) were obtained from Sigma.

Platelet phospholipase A₂ preparation

Platelet-rich plasma was prepared from fresh human blood as previously described [18]. Platelets were pelleted at 2500 g for 10 min at 4 °C, washed with 120 mM-NaCl/2 mM-EGTA/30 mM-Tris/HCl, pH 7.5 (buffer A), suspended in this buffer at a concentration of 2 × 10⁹ platelets/ml, frozen in solid-CO₂/acetone and stored at −70 °C. After thawing and addition of phenylmethanesulphonyl fluoride (final concn. 0.5 mM), the platelets (1.6 × 10⁹–2.0 × 10⁹ platelets/ml, equivalent to 4–5 mg of protein/ml) were sonicated at 4 °C with a probe sonicator (model W-225; Heat Systems–Ultrasonics) for 6 × 15 s, with 45 s intervals, by using a standard tapered micro-tip at an output setting of 4.

The platelet lysate was then extracted with octyl

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glucoside as previously described [17]. A concentrated solution of octyl glucoside (120 mM in buffer A; 1.25 ml) was added to 5 ml of lysate (4 mg of protein/ml) and incubated on ice for 30 min. The mixture was then centrifuged at 150000 g for 60 min, and the resulting supernatant containing the soluble phospholipase A_2 was stored at 4 °C. For DEAE-cellulose chromatography, this supernatant was diluted 2.4-fold with 30 mM-Tris/HCl, pH 8.0, and applied to a column (1.6 cm × 13 cm) of DEAE-cellulose (Whatman DE-52) pre-equilibrated with 1 mM-EGTA/10 mM-octyl glucoside/30 mM-Tris/HCl, pH 8.0 (buffer B), containing 50 mM-NaCl. The column was washed with 50 ml of this buffer, eluted at a flow rate of 0.5 ml/min with a linear gradient of 50–500 mM-NaCl in 120 ml of buffer B by using an Ultragrad gradient mixer (model 11300; LKB Instruments) and then washed with 50 ml of buffer B containing 500 mM-NaCl. Phospholipase A_2 activity was eluted at 250 mM-NaCl as a single peak. Active fractions were pooled and stored at 4 °C (without apparent loss of activity for at least 2 months).

Assay for platelet phospholipase A_2 activity

[^{14}C]Arachidonoyl-PC (10 nmol) and long-chain diacyl-glycerols (0.4 nmol/nmol of phosphatidylcholine or as indicated) were dried under N_2. The lipids were suspended in 1 ml of 100 mM-Tris/HCl, pH 9 (assay buffer), by sonication at 4 °C with a probe sonicator (see above) for 4 × 15 s with 45 s intervals, with the stepped micro-tip at an output setting of 2.

Platelet phospholipase A_2 (5 μg of protein) was incubated with 0.5 nmol of labelled arachidonoyl-PC, containing diacylglycerols as indicated, in a total volume of 0.2 ml of assay buffer containing 5 mM-$CaCl_2$, 0.5 mg of fatty-acid-free bovine serum albumin/ml and 1 mM-2-mercaptoethanol. Incubations were carried out at 37 °C for various time periods and terminated by adding 2 ml of Dole's reagent (propan-2-ol/heptane/0.5 M-H_2SO_4, 40:10:1, by vol.). After addition of 10 μg of arachidonic acid as carrier, 1.2 ml of heptane and 1 ml of water were added. The mixtures were briefly vortex-mixed and the upper phases transferred to tubes containing 2 ml of heptane and 150 mg of Bio-Sil A (Bio-Rad Laboratories; activated at 120 °C for 2 h before use). The tubes were thoroughly vortex-mixed and centrifuged (400 g-min), and the supernatants pipetted into scintillation vials. After addition of 10 ml of Hydrofluor (National Diagnostics), radioactivity was counted in a Packard Tri-Carb 460C scintillation counter. Released [^{14}C]-arachidonic acid was quantitatively recovered by this procedure; and in control assays (containing no Ca^{2+} or heat-denatured enzyme) less than 0.5% of the total [^{14}C]phosphatidylcholine was detected in the heptane phase after adsorption with silica gel.

Other methods

Protein concentration was measured, with bovine serum albumin as standard, by the method of Lowry et al. [19], modified to include 1 % SDS in the alkaline reagent.

RESULTS

We have demonstrated previously that treatment of human platelet lysates with 24 mM-octyl glucoside, followed by DEAE-cellulose chromatography of the
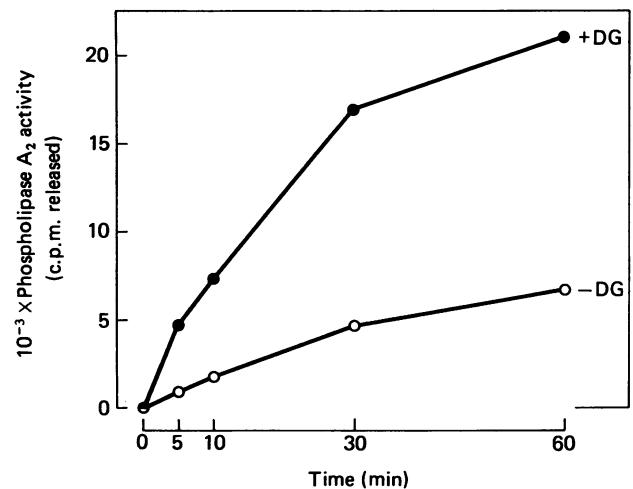

Fig. 1. Effect of 1,2-dioleoylglycerol on platelet phospholipase A_2 activity

Phospholipase A_2 (5 μg of protein) was incubated with 0.5 nmol of [^{14}C]arachidonoyl-PC without (O) or with (●) 0.2 nmol of 1,2-dioleoylglycerol (DG) in a total volume of 0.2 ml of 100 mM-Tris/HCl, pH 9, containing 5 mM-$CaCl_2$, 0.5 mg of albumin/ml and 1 mM-2-mercapto-ethanol. Reactions were stopped and released [^{14}C]-arachidonic acid was extracted as detailed in the Experimental section. Phospholipase A_2 activity is presented as c.p.m. of [^{14}C]arachidonic acid released. Results are shown as means of duplicate determinations and are representative for two separate experiments.

soluble extract, yields a stable preparation of phospholipase A_2 that is devoid of A_1- and lyso-phospholipase activity [17]. With 2.5 μM sonicated [^{14}C]arachidonoyl-PC as substrate, this phospholipase A_2 was shown to be optimally active at pH 9–10 in the presence of 5 mM-$CaCl_2$ and 0.5 mg of albumin/ml, exhibiting a specific activity of approx. 0.5 nmol/min per mg.

In the present study, the effect of diacylglycerol on this platelet phospholipase A_2 was examined by incorporating 1,2-dioleoylglycerol into the arachidonoyl-PC substrate dispersion. We found that addition of 1,2-dioleoyl-glycerol by itself did not induce phospholipase A_2 activity, but greatly enhanced enzymic activity in the presence of Ca^{2+}. The time course of hydrolysis of arachidonoyl-PC in the absence and presence of 1 μM (29 mol %) 1,2-dioleoylglycerol is depicted in Fig. 1. The magnitude of stimulation was most pronounced at short incubation times, and decreased from 4.8-fold after 5 min to 3.1-fold after 60 min of incubation. The dependence of phospholipase A_2 stimulation on the amount of dioleoylglycerol present in the arachidonoyl-PC substrate is shown in Fig. 2. Stimulation was saturable, with a maximal effect occurring at ≥ 29 mol % (1 μM) dioleoylglycerol. Both 1,2- and 1,3-dioleoylglycerol similarly increased enzymic activity, but the extent of activation was always greater with the 1,2- than with the 1,3-isomer.

Halenda et al. [14] reported that externally added 1-oleoyl-2-acetylglycerol increased the release of arachidonic acid from ionophore A23187 treated platelets 2.2-fold. We examined whether this may be due to the fact that 1-oleoyl-2-acetylglycerol directly influences platelet phospholipase A_2 activity. As demonstrated in Fig. 3, 1-
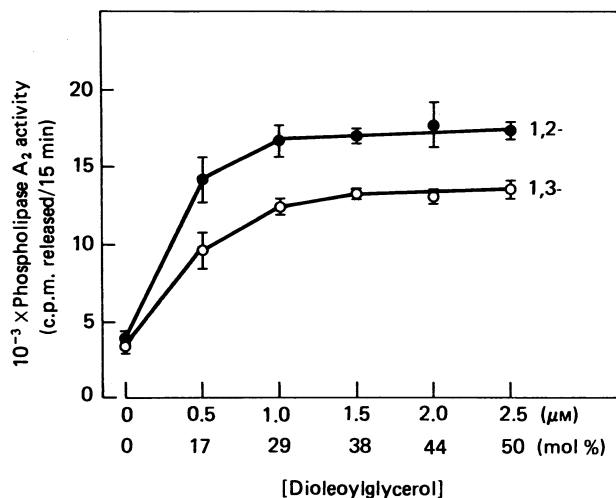
Fig. 2. Concentration-dependence of platelet phospholipase A$_2$ stimulation by dioleoylglycerol

Phospholipase A$_2$ (5 μg of protein) was incubated with sonicated substrate containing 0.5 nmol of [^{14}C]-arachidonoyl-PC and increasing amounts (0–0.5 nmol) of 1,2-dioleoylglycerol (●) or 1,3-dioleoylglycerol (○) in a total volume of 0.2 ml of 100 mM-Tris/HCl, pH 9, containing 5 mM-CaCl$_2$, 0.5 mg of albumin/ml and 1 mM-2-mercaptoethanol. After 15 min of incubation, the reaction was stopped and released [^{14}C]arachidonic acid was extracted by a modification of the Dole procedure as detailed in the Experimental section. Phospholipase A$_2$ activity is presented as c.p.m. of [^{14}C]arachidonic acid released/15 min. Results are shown as means±S.D. of three independent experiments performed in duplicate.
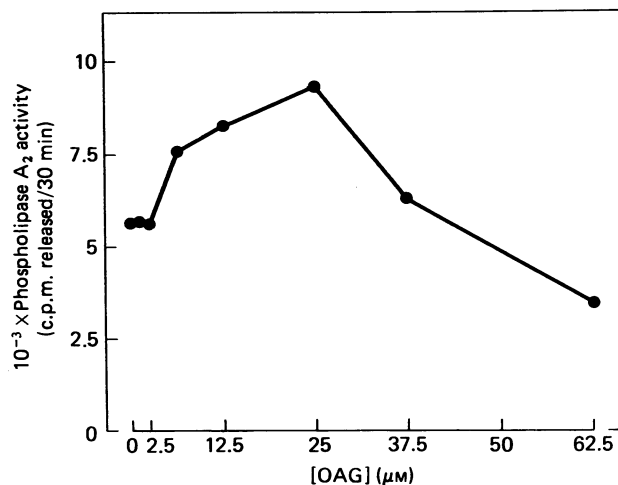

Fig. 3. Effect of 1-oleoyl-2-acetylglycerol on platelet phospholipase A$_2$ activity

Phospholipase A$_2$ (5 μg of protein) was incubated for 30 min with 0.5 nmol of [^{14}C]arachidonoyl-PC in the presence of increasing amounts (0–12.5 nmol) of 1-oleoyl-2-acetylglycerol (OAG) in a total volume of 0.2 ml of 100 mM-Tris/HCl, pH 9, containing 5 mM-CaCl$_2$, 0.5 mg of albumin/ml and 1 mM-2-mercaptoethanol. OAG (150 μg) was dissolved in 9 μl of dimethyl sulphoxide, dispersed into 3 ml of 100 mM-Tris/HCl, pH 9, and then added to incubations as indicated. Dimethyl sulphoxide was adjusted to a concentration of 0.15% in all samples, and had no effect itself on phospholipase A$_2$ activity. Reactions were stopped and liberated [^{14}C]arachidonic acid was extracted as described in the Experimental section. Phospholipase A$_2$ activity is given as c.p.m. of [^{14}C]arachidonic acid released/30 min. Results are shown as means of duplicate determinations and are representative for three separate experiments.

oleoyl-2-acetylglycerol was indeed able to increase platelet phospholipase A$_2$ activity, with maximal stimulation of 1.6-fold occurring at a concentration of 25 μM.

In order to gauge the physiological significance of our observations, we investigated whether the naturally produced diacylglycerol 1-stearoyl-2-arachidonoyl-glycerol also enhances the activity of platelet phospholipase A$_2$. We found that, similar to the results obtained with increasing amounts of 1,2-dioleoylglycerol, maximal enhancement of phospholipase A$_2$ activity by 1-stearoyl-2-arachidonoylglycerol occurred at concentrations ⩾ 29 mol% and was 4.5-fold. The effect of 1-stearoyl-2-arachidonoylglycerol was further explored at concentrations < 10 mol%. As demonstrated in Fig. 4, stimulation of phospholipase A$_2$ activity was increased 1.3–1.8-fold at concentrations of 2–5 mol%.

We then examined whether diacylglycerol also enhances the activity of extracellular phospholipases and studied the effect of 1,2-dioleoylglycerol on pancreatic, bee-venom and snake-venom phospholipase A$_2$. The concentration of the various phospholipases was adjusted so that incubation with sonicated arachidonoyl-PC under standard incubation conditions yielded similar release of arachidonic acid in the absence of diacylglycerol. As shown in Table 1, incorporation of 1,2-dioleoylglycerol (29 mol%, 1 μM) into the substrate arachidonoyl-PC promoted enzymic activity of all phospholipases investigated. However, stimulation was most pronounced for platelet phospholipase A$_2$.

DISCUSSION

Several mechanisms have been implicated in the regulation of cellular phospholipase A$_2$, including cytosolic concentrations of free Ca^{2+} [6], phospholipase-inhibitory proteins [20,21] and perturbations of the structural arrangement of cell-membrane phospholipids [6,22]. In platelets, increased cytosolic Ca^{2+} plays an important role in the activation of phospholipase A$_2$ [13,23,24]. Indirect evidence for a possible involvement of protein kinase C [14,25] and phospholipase-inhibitory proteins [26] in the regulation of platelet phospholipase A$_2$ has been presented. Here, we demonstrate that diacylglycerols known to be generated in stimulated platelets promote platelet phospholipase A$_2$ activity. Taken together, these findings suggest that platelet phospholipase A$_2$ activity may be governed not solely by Ca^{2+} availability but by other factors as well.

Diacylglycerol is generated in response to extracellular stimuli as a result of phospholipase C-mediated phosphoinositide hydrolysis, and is thought to function as a second messenger, initiating a cascade of biochemical reactions [27,28]. Physico-chemical studies have indicated that diacylglycerol also affects the structure and stability of phospholipid bilayer membranes [29,30]. Thus increasing amounts of diacylglycerol in phosphatidylcholine
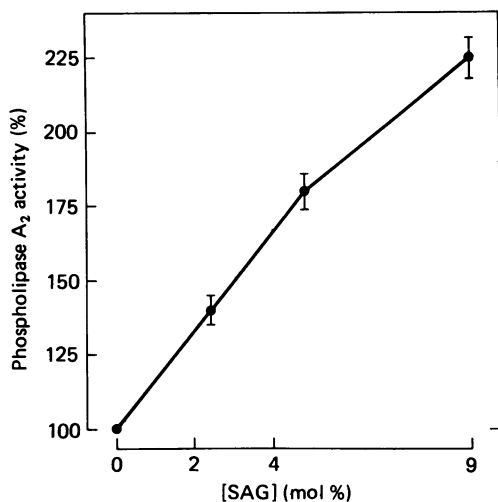
Fig. 4. Enhancement of platelet phospholipase A_2 activity by 1-stearoyl-2-arachidonoylglycerol

Phospholipase A_2 (5 μg of protein) was incubated for 30 min with 0.5 nmol of [^{14}C]arachidonoyl-PC containing 0, 12.5, 25 and 50 pmol (0, 2.4, 4.8 and 9 mol%) of 1-stearoyl-2-arachidonoylglycerol (SAG) in a total volume of 0.2 ml of 100 mM-Tris/HCl, pH 9, containing 5 mM-$CaCl_2$, 0.5 mg of albumin/ml and 1 mM-2-mercapto-ethanol. In the control (100%) incubation without diacylglycerol, hydrolysis of [^{14}C]arachidonoyl-PC was 4342 ± 44 c.p.m./30 min. Results are shown as means$\pm$s.D. for three separate experiments performed in duplicate.

Table 1. Effect of dioleoylglycerol on various phospholipases

Phospholipase A_2 from pig pancreas (10 ng), bee venom (0.01 ng), *Naja naja* venom (0.01 ng) and human platelets (5 μg) were assayed in reaction mixtures of 5 mM-$CaCl_2$ and 0.5 mg of albumin/ml in a total volume of 0.2 ml of 100 mM-Tris/HCl, pH 9, containing 0.5 nmol of [^{14}C]-arachidonoyl-PC without or with 0.2 nmol of 1,2-dioleoylglycerol (DG). After 15 min of incubation, the reaction was stopped and released [^{14}C]arachidonic acid determined as detailed in the Experimental section. The values shown are means$\pm$s.D. for three separate experiments performed in duplicate.

| | Phospholipase A_2 activity | | |
| | [^{14}C]Arachidonic acid released (c.p.m./15 min) | | |
Enzyme source	$-DG$	$+DG$	Increase (fold)
Pig pancreas	3897 ± 374	8842 ± 472	2.3
Bee venom	3300 ± 826	10163 ± 659	3.1
Snake venom	2698 ± 553	4755 ± 1175	1.8
Human platelet	3665 ± 163	15165 ± 1513	4.1

bilayers induced spreading apart of the polar groups and, above 30 mol% (at 37 °C), formation of non-lamellar phases. Such structural changes may enhance the access of proteins to the membrane hydrocarbon core and facilitate the interaction of enzymes with their phospholipid substrates.

Diacylglycerol activates protein kinase C [31] and promotes the activity of a number of phospholipases when incorporated into their phosphatidylcholine or phosphatidylinositol substrate, including phospholipase C from sheep seminal vesicles [32] and human platelets [15], phospholipase A_2 from rat intestinal mucosa and phospholipase A_1 from rat liver [15]. Our data extend these findings, demonstrating that diacylglycerol is also able to enhance human platelet phospholipase A_2 activity. Diacylglycerols did not, however, promote platelet phospholipase A_2 activity in the absence of Ca^{2+}. This is consistent with the observation that their addition to intact platelets does not cause release of arachidonic acid [33]. Exogenously added 1-oleoyl-2-acetylglycerol caused an increase in enzymic activity. Long-chain diacylglycerols, directly incorporated into the substrate arachidonoyl-PC, markedly enhanced platelet phospholipase A_2 activity. 1,2-Dioleoylglycerol and 1-stearoyl-2-arachidonoylglycerol, naturally produced diacylglycerols, were more effective than 1,3-dioleoylglycerol in promoting enzymic activity. Stimulation of platelet phospholipase A_2 increased as the dioleoylglycerol content of the phosphatidylcholine substrate was raised, and was maximal near 30 mol%. This optimal concentration coincides with concentrations of diacylglycerol that were reported to produce maximal spreading apart of polar groups of phosphatidylcholine and induce structural transitions in model membranes [30]. Higher molar ratios of diacylglycerol (up to 50 mol%) did not result in a decline in platelet phospholipase A_2 activity. Hofmann & Majerus [32] made similar findings for diacylglycerol-stimulated hydrolysis of phosphatidylinositol by phospholipase C purified from sheep seminal vesicles. Dawson *et al.*, [15,16], on the other hand, reported that stimulation of both phospholipase C from human platelets and phospholipase A_2 from rat intestinal mucosa decreased at diacylglycerol concentrations above 30 mol%.

Extracellular phospholipases A_2 from various sources were less stimulated by diacylglycerol than were platelet phospholipase A_2 and other intracellular phospholipases [15,16,32], suggesting that the stimulatory effect of diacylglycerol may be most pronounced on intracellular phospholipases.

As to the biological significance of our observations, the question arises whether sufficient diacylglycerol is generated in activated platelets to have an effect on phospholipase A_2. We have observed a significant increase in phospholipase A_2 activity when the phosphatidylcholine substrate contained $\geqslant 2$ mol% 1-stearoyl-2-arachidonoylglycerol. Upon stimulation with physiological agonists, an estimated 4–11 nmol of diacylglycerol may be formed per 10^9 platelets [4]. Given the fact that such platelets contain approx. 400 nmol of phospholipids and that phosphatidylinositol is distributed asymmetrically [34], the global amounts of diacylglycerol generated on the cytoplasmic membrane surface would be 2–6 mol%. As a result of heterogeneity of lateral organization of phospholipids [35], and depending on rates of production, diffusion and further enzymic conversion, the local concentration of diacylglycerol is likely to be even higher. Hence it seems possible that in agonist-stimulated platelets sufficient 1,2-diacylglycerol may be generated to accomplish local perturbation of platelet membrane phospholipids, thereby facilitating their hydrolysis by phospholipase A_2.

Interestingly, evidence was presented recently that diacylglycerols mediate protein-kinase-C-independent priming of human neutrophils and cause enhanced activation of phospholipase A_2 in response to stimulation with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine [36,37]. Moreover, it was reported that in HL60 granulocytes augmentation of Ca^{2+}-activated phospholipase A_2 by the peptide was mediated by endogenously generated diacylglycerol in a mechanism independent of protein kinase C [38].

In summary, we conclude that in stimulated platelets Ca^{2+}-activated phospholipase A_2 may be affected by newly generated diacylglycerols not only biochemically, via protein-kinase-C-mediated events, but also directly, through structural changes imposed on cellular membrane phospholipids.

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