# Synergistic Release of Arachidonic Acid from Platelets by Activators of Protein Kinase C and $Ca^{2+}$ Ionophores. Evidence for the Role of Protein Phosphorylation in the Activation of Phospholipase $A_2$ and Independence from the $Na^+/H^+$ Exchanger†

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ABSTRACT: The protein kinase C activators phorbol myristate acetate (PMA), mezerein, oleoylacetylglycerol, and (−)-indolactam V, although without direct effect on arachidonic acid release, greatly enhance the release of platelet arachidonic acid caused by the $Ca^{2+}$ ionophores A23187 and ionomycin. In contrast, 4α-phorbol 12,13-didecanoate and (+)-indolactam V, which lack the ability to activate kinase C, do not potentiate arachidonate release. Release of arachidonic acid occurs without activation of phospholipase C and is therefore mediated by phospholipase $A_2$. Synergism between PMA and A23187 is not affected by inactivation of the $Na^+/H^+$ exchanger with dimethylamiloride. The time course and dose–response for the effect of PMA at 23 °C closely correlate with the phosphorylation of a set of relatively "slowly" phosphorylated proteins (P20, P35, P41, P60), but not the rapidly phosphorylated P47 protein. P20 is myosin light chain, and P41 is probably Giα, but the other proteins have not been positively identified. Depletion of metabolic ATP stores by antimycin A plus 2-deoxyglucose abolishes both protein phosphorylation and the potentiation of arachidonate release by PMA, but does not prevent fatty acid release by the ionophores. Similarly, the kinase C inhibitors H-7 and staurosporine produce, respectively, partial and complete inhibition of PMA-potentiated arachidonic acid release and protein phosphorylation, without affecting the direct response to ionophores. These results indicate that protein phosphorylation, mediated by kinase C, promotes the phospholipase $A_2$ dependent release of arachidonic acid in platelets when intracellular $Ca^{2+}$ is elevated by $Ca^{2+}$ ionophores.

Platelet agonists, such as thrombin and collagen, evoke the rapid release of a substantial fraction of the membrane arachidonic acid that is esterified in phospholipids. Two pathways for the release of arachidonic acid have been described. Arachidonic acid is mobilized in part from phosphoinositides by phospholipase C to yield 1-stearoyl-2-arachidonyldiacylglycerol followed by the concerted action of di- and monoacylglycerol lipases to liberate arachidonic acid (Prescott & Majerus, 1983). A much larger amount of the arachidonic acid release by thrombin is catalyzed by a phospholipase $A_2$ and comes from phosphatidylcholine (Smith et al., 1985). Platelet phospholipase $A_2$ is a $Ca^{2+}$-dependent enzyme (Baron & Limbird, 1988), and $Ca^{2+}$ ionophores are effective releasers of arachidonate. Thrombin, in addition to mobilizing $Ca^{2+}$, can also decrease the $Ca^{2+}$ requirement for arachidonic acid release (Halenda & Rehm, 1987), indicating that there are additional agonist-generated signals that affect phospholipase $A_2$. Some factors proposed to modulate phospholipid hydrolysis by phospholipase $A_2$ include the $Na^+/H^+$ exchanger that regulates $pH_i$[1] (Sweatt et al., 1986), GTP-binding proteins (Jelsema & Axelrod, 1987), and lipocortins (calpactins) (Touqui et al., 1986).

Activators of protein kinase C, such as tumor-promoting phorbol diesters and diacylglycerol, greatly enhance the release of arachidonic acid by $Ca^{2+}$ ionophores in platelets (Halenda et al., 1985; Mobley & Tai, 1985). A similar synergism between phorbol esters and $Ca^{2+}$ ionophores also occurs in neutrophils (Volpi et al., 1985; McColl et al., 1986), permeabilized adrenal medullary chromaffin cells (Frye & Holz, 1985), HL-60 cells (Billah & Siegel, 1987), and Madin–Darby canine kidney cells (Parker et al., 1987; Slivka & Insel, 1988; Portilla et al., 1988). However, in some studies there is evidence that kinase C is not involved in the release of arachidonic acid. In these cases the effects of phorbol diesters and diacylglycerol were attributed instead to some type of membrane perturbation affecting the enzyme or its substrates (Hofmann & Majerus, 1982; Dawson et al., 1983; Matsumoto et al., 1988; Tao et al., 1989; Bauldry et al., 1988; Pollock et al., 1986). In this paper we report new evidence indicating that the potentiation of arachidonic acid mobilization by phorbol diesters, and several other kinase C activators, in human platelets is dependent upon protein phosphorylation and is completely eliminated by staurosporine, a potent inhibitor of protein kinase C.

## Experimental Procedures

*Materials.* Human thrombin (≥1000 NIH units/mg) from United States Biochemical Corp. was stored in 50% (v/v) glycerol at −20 °C. H-7 and staurosporine were from Seikagaku America, Inc., and Calbiochem, respectively. All other

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[1] Abbreviations: $[Ca^{2+}]_i$, cytoplasmic free calcium ion concentration; $pH_i$, intracellular pH; $TXB_2$, thromboxane $B_2$; $TXA_2$, thromboxane $A_2$; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; PMA, 4β-phorbol 12-myristate 13-acetate; PDBu, 4β-phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; OAG, 1-oleoyl-2-acetyl-glycerol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

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chemicals were of reagent grade and obtained from various sources. [$^{14}$C]Arachidonic acid (58.4 mCi/mmol) from Amersham Corp. was stored as a toluene solution under nitrogen at −20 °C. Purity was greater than 98% (thin-layer chromatography, solvent system 1). Ionophores A23187 and ionomycin, phorbol myristate acetate, phorbol dibutyrate, 4α-phorbol 12,13-didecanoate (4α-PDD), and mezerein were obtained from Sigma Chemical Co., and the (−)- and (+)-indolactam V stereoisomers were from LC Services, Inc. These reagents were all stored as single-use aliquots in anhydrous dimethyl sulfoxide at −80 °C. The maximum final concentration of dimethyl sulfoxide present in cell incubations did not exceed 0.3% (v/v) and had no effect of its own on arachidonic acid metabolism.

*Incorporation of [$^{14}$C]Arachidonic Acid into Platelet Phospholipids.* Human platelet concentrates from the American Red Cross, Farmington, CT, were obtained within 24 h of collection. Contaminating blood cells were removed by centrifugation at 125*g* for 10 min after addition of 1 mM EGTA, and platelets were harvested by centrifugation at 750*g* for 15 min. Platelets (approximately $2.5 \times 10^{10}$ cells) were resuspended in 10 mL of plasma containing 5 μM (2.92 μCi) [1-$^{14}$C]arachidonic acid that had been dissolved in 0.025 mL of 100 mM $Na_2CO_3$, pH 10, before dilution in plasma. The cell suspension was incubated at 37 °C for 60 min and then cooled to room temperature. Unincorporated [$^{14}$C]arachidonic acid, amounting to 10–20% of the total radioactivity, was removed by diluting the platelet suspension to 40 mL with nonradioactive plasma (from the same donor) and centrifuging at 750*g* for 15 min. Radiolabeled platelets were resuspended in washing medium containing 145 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 0.2 mM EGTA, and 10 mM PIPES, pH 6.5. After sedimentation at 750*g* for 10 min, platelets were resuspended to a cell density of $10^9$/mL in incubation medium of 145 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 0.04 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4, and 0.2 mg/mL fatty acid free bovine serum albumin. Intracellular pH was measured with the fluorescent pH indicator BCECF, as described by Zavoico et al. (1986).

*Analysis of [$^{14}$C]Arachidonic Acid and Metabolites.* Agents that affect arachidonic acid mobilization were added to aliquots (0.5 mL) of platelet suspension incubated in siliconized glass tubes at 23 °C. Incubations were terminated by adding 1.88 mL of ice-cold methanol/chloroform (2:1 v/v). Phases were separated by adding 0.6 mL each of chloroform and 0.1% (v/v) formic acid followed by vigorous mixing and centrifugation to extract arachidonic acid and metabolites. The lower chloroform-rich phases were collected and concentrated under nitrogen, and the radioactive arachidonic acid and metabolites were separated by thin-layer chromatography using solvent system 1 [chloroform/methanol/acetic acid/water (90:8:1:0.8 v/v)], in which phospholipids remain at the origin.

*Analysis of [$^{14}$C]Arachidonate- and [$^{32}$P]Phosphate-Labeled Phospholipids.* Phospholipids were analyzed as previously described (Halenda et al., 1985) after extraction of platelets (0.5 mL) with 1.88 mL of ice-cold methanol/chloroform/HCl (100:50:1 v/v). Phases were separated with 0.6 mL each of chloroform and 2 mM KCl. After concentration of the chloroform-rich phases under nitrogen, the phospholipids were chromatographed on Merck silica gel 60 thin-layer plates with solvent system 2 [chloroform/methanol/20% (w/v) aqueous methylamine (60:36:10 v/v)]. Radioactive lipid bands located by autoradiography and identified by comigration with standards were scraped and quantitated by scintillation counting. All extraction solvents contained 0.01 mg/mL of the antioxidant butylated hydroxytoluene. [$^{14}$C]Arachidonic acid release is expressed either as counts per minute (mean percent SD for triplicate determinations, unless otherwise indicated) from a single representative experiment or, when averaged results from more than one experiment are shown, as percent release of [$^{14}$C]arachidonic acid: percent release (cpm) = 100 × cpm (arachidonic acid + $TXB_2$ + HHT + HETE)/cpm (phospholipids + arachidonic acid + $TXB_2$ + HHT + HETE). Measurement of the total amount of [$^{14}$C]arachidonic acid released from phospholipids allows comparison of data from platelets from different donors, which incorporated [$^{14}$C]arachidonic acid to varying extents. [$^{32}$P]Phosphate-labeled phosphoinositides and phosphatidic acid were analyzed as previously described (Zavoico et al., 1985).

*Protein Phosphorylation.* Suspensions of washed platelets ($3 \times 10^9$ to $10^{10}$/mL) were incubated for 45 min at 37 °C with 0.2 mCi/mL of ortho[$^{32}$P]phosphoric acid (carrier-free, New England Nuclear, Boston, MA). Buffer containing 0.2 mM EGTA was added, and the suspension was centrifuged for 10 min at 750*g*. The platelet pellet was resuspended in incubation buffer containing 1 mM $MgCl_2$ and 40 μM $CaCl_2$ to a concentration of $5 \times 10^8$ cells/mL and then diluted to $2.5 \times 10^8$ cells/mL in the experiments. Reactions were stopped with a solution of 2.5% dithiothreitol, 2% sodium dodecyl sulfate, 12.5% glycerol, and a trace of bromophenol blue and then placed in a boiling-water bath for 5 min. The samples were electrophoresed essentially as described previously (Feinstein et al., 1983) on straight 10% or 5–15% gradient polyacrylamide gels containing 0.1% SDS. The gels were stained with Coomassie Brilliant Blue R, destained, and dried, the phosphorylated proteins located by radioautography, and the radioactive gel bands cut out, digested, and counted by liquid scintillation spectrometry.

## Results

*Potentiation of Ionophore-Induced Arachidonic Acid Release by Activators of Protein Kinase C.* $Ca^{2+}$ ionophores release arachidonic acid in platelets treated with aspirin plus ADP scavengers, which prevents activation of phospholipase C via thromboxane and ADP receptors (Rittenhouse & Horne, 1984). Platelets treated in this way did not hydrolyze phosphatidylinositol 4,5-bisphosphate or produce phosphatidic acid from diglyceride in response to A23187, in sharp contrast to the receptor–agonist thrombin (Figure 1A). These data, and those previously presented (Halenda et al., 1985), show that the release of arachidonic acid by ionophores plus PMA is most probably catalyzed solely by phospholipase $A_2$. A23187 stimulated the appearance of free [$^{14}$C]arachidonic acid and its radioactive metabolites, $TXB_2$, HHT, and 12-HETE, in washed platelet suspensions. Figure 1 shows the total amount of [$^{14}$C]arachidonic acid (arachidonic acid plus metabolites) released from phospholipids as a function of ionophore concentration. Release of radioactive fatty acid was just detectable at 0.5 μM A23187 (0.5 nmol of A23187/$10^9$ platelets), and the maximal release (approximately 20%) was attained at about 5 μM ionophore (5 nmol of A23187/$10^9$ platelets). In untreated platelets the free [$^{14}$C]arachidonic acid plus metabolites was only 0.19% (±0.01% SE; eight exp) of the total radioactive arachidonic acid in phospholipids.

PMA had two effects on arachidonic acid release (Figure 1B). One effect was to increase sensitivity to the ionophore; e.g., at a minimally effective concentration of ionophore (i.e., 0.5 μM) pretreatment with 100 ng/mL PMA increased total arachidonic acid release by about 8-fold, and maximum release occurred at 1 μM rather than 5 μM A23187. Second, PMA

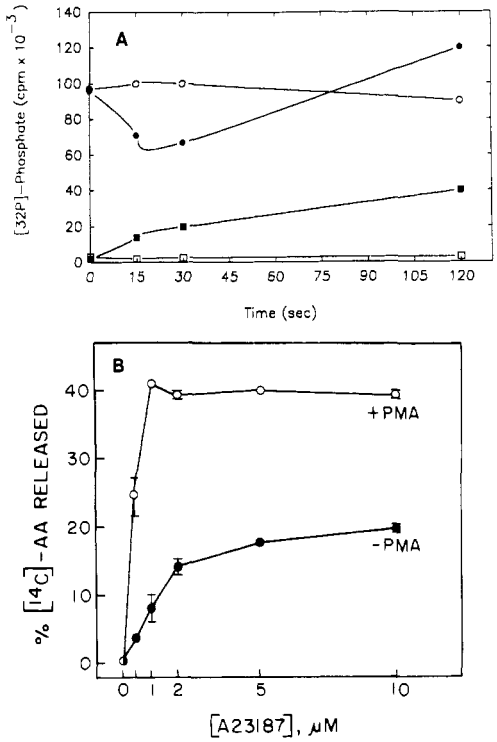


FIGURE 1: (A) Phosphatidylinositol 4,5-bisphosphate and phosphatidic acid in platelets stimulated with A23187 or thrombin. Washed platelets ($10^9$/mL) labeled with [$^{32}$P]phosphate were treated with 1 mM aspirin for 20 min, incubated in the presence of 5 mM creatine phosphate/creatine phosphokinase (40 units/mL), and then stimulated with 1 μM A23187 (○, □) or 1 unit/mL thrombin (●, ■) for the times indicated. [$^{32}$P]Phosphatidylinositol 4,5-bisphosphate (circles) and [$^{32}$P]phosphatidic acid (squares) were analyzed as described under Experimental Procedures. (B) Potentiation of arachidonic acid release by PMA. Washed platelets ($10^9$/mL), prelabeled with [$^{14}$C]arachidonic acid, were stimulated for 5 min with indicated concentrations of A23187. Total [$^{14}$C]arachidonic acid released (free arachidonate plus all radioactive metabolites) was measured as described under Experimental Procedures: (●) A23187; (○) treated with PMA 100 ng/mL (160 nM) for 10 min prior to A23187. Temperature was 21–23 °C.

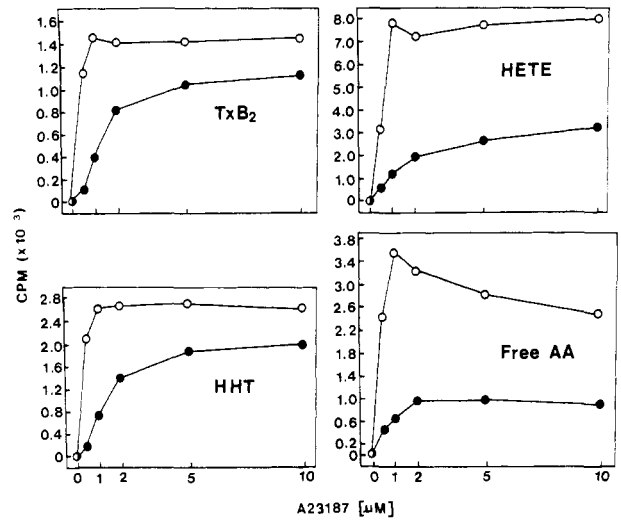


FIGURE 2: Effects of PMA on A23187-induced release of arachidonic acid and formation of its metabolites. Washed platelets as in Figure 1 (but not treated with aspirin) were stimulated with indicated concentrations of A23187 alone (●) or after pretreatment with 160 nM PMA (○). [$^{14}$C]Arachidonic acid and $^{14}$C-labeled metabolites were assayed as described under Experimental Procedures. Note the differences in scale of radioactivity for different metabolites.

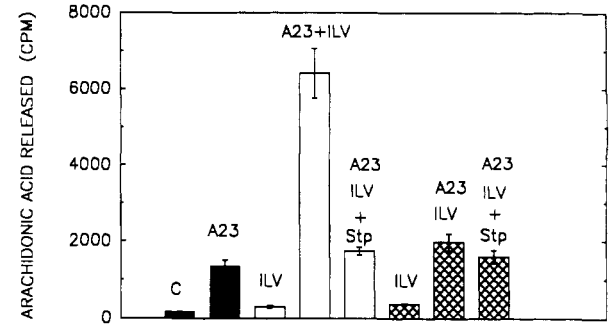


FIGURE 3: Effect of indolactam V stereoisomers on arachidonic acid release. [$^{14}$C]Arachidonic acid labeled platelets were treated as follows: (solid bars) not treated with indolactams, (C) controls, (A23) 1 μM A23187 for 5 min; (open bars) (ILV) 20 μM (−)-indolactam V for 10 min, (A23 + ILV) pretreated with (−)-ILV (20 μM) for 10 min prior to A23187, (A23,ILV + Stp) pretreated with 1 μM staurosporine for 3 min prior to indolactam and then A23187; (hatched bars) same experiment as open bars but using (+)-indolactam V. Total [$^{14}$C]-arachidonic acid (plus metabolites) was measured as in Figure 1B.

increased the *maximum* amount of arachidonic acid that could be released from phospholipids by 2-fold (from 20% to 40%) (Figure 1B). Similar results were obtained with the $Ca^{2+}$ ionophore ionomycin (see below).

PMA also enhanced the formation of arachidonic acid metabolites (Figure 2). We previously showed that PMA did not affect the metabolism of exogenous arachidonic acid or its esterification into phospholipids (Halenda et al., 1985), so the production of the metabolites solely reflects the much greater availability of substrate (free arachidonic acid). At 0.5 μM A23187 PMA increased free arachidonic acid 5-fold and thromboxane $B_2$ and HHT by more than 10-fold. At low concentrations of A23187 the products of the cyclooxygenase/thromboxane synthetase pathway (thromboxane $B_2$ plus HHT) roughly equaled the amount of 12-HETE formed by the lipoxygenase pathway. At higher concentrations of A23187 free arachidonic acid plus 12-HETE comprised the greatest portion (i.e., 75%) of the total amount of arachidonic acid released after 5 min. In some cultured cells PMA increases the spontaneous appearance of arachidonic acid metabolites in the medium, which is a very slow process compared to the explosive events in platelets. In these cells, PMA stimulates synthesis of prostaglandin G/H synthase (Wu et al., 1988; Goerig et al., 1988) and thromboxane synthase (Goerig et al., 1988), and this effect is blocked by cycloheximide. As expected, in anucleate platelets, cycloheximide (10 μg/mL) did not alter the enhancement of arachidonic acid release by PMA (not shown).

The potentiating effect of PMA was mimicked by the synthetic diacylglycerol OAG and phorbol dibutyrate, but not by the phorbol diesters 4α-PDD or 4α-PMA, which do not stimulate protein kinase C (Halenda et al., 1985; Halenda & Rehm, 1987). These results implicated protein kinase C in the release of arachidonic acid. To further test this hypothesis, we employed other kinase C activators, i.e., mezerein, a non-phorbol ester diterpene lacking a diacylglycerol-like moiety (Miyake et al., 1984), and (−)-indolactam V, which is similar to the active structural element of tumor-promoting teleocidins (Fujiki et al., 1984). Mezerein potentiated arachidonic acid release, and activated protein phosphorylation, to about the same extent as PMA (see Table I and Figure 5C). Figure 3 shows experiments using indolactams. (−)-Indolactam V (20 μM), an activator of kinase C, produced a 4.3-fold increase of arachidonic acid release by 1 μM A23187, similar to the potentiation by PMA at the same concentration of ionophore (Figure 1B). In contrast, the optical stereoisomer (+)-indolactam V, which does not stimulate kinase C, also did not potentiate arachidonic acid release (Figure 3).

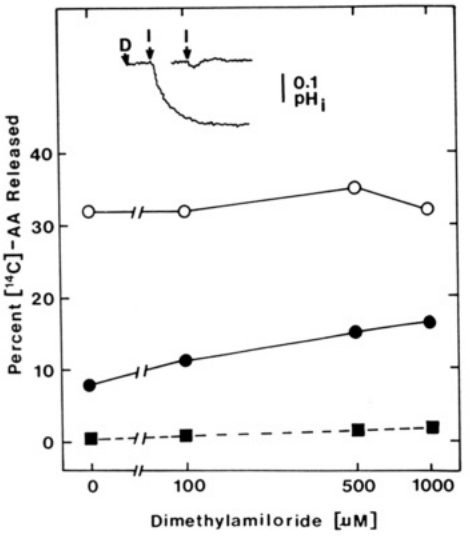


FIGURE 4: Potentiation of arachidonic acid release by PMA in platelets treated with dimethylamiloride. Platelets prelabeled with [$^{14}$C]arachidonic acid were stimulated with 1 μM ionomycin alone (●) or ionomycin after 160 nM PMA (○) in the presence of indicated concentrations of dimethylamiloride. Controls (■) received equivalent amounts of vehicle (DMSO). Percent [$^{14}$C]arachidonic acid release is calculated as described under Experimental Procedures. [Insert (above)] Effect of ionomycin alone (I) or ionomycin added after 100 μM dimethylamiloride (D) on intracellular pH measured with the fluorescent probe BCECF. A decrease in fluorescence indicates a fall of pH$_i$. The initial pH$_i$ was 7.0.

*An Inhibitor of $Na^+/H^+$ Exchange Does Not Affect Enhancement of Arachidonic Acid Release by PMA.* Stimulation of the $Na^+/H^+$ antiporter by PMA might facilitate arachidonic acid release by elevating the pH$_i$ to a more favorable level for phospholipase A$_2$ activity (Banga et al., 1986; Baron & Limbird, 1988). When platelets were pretreated with 0.1–1 mM dimethylamiloride, the $Na^+/H^+$ antiporter was totally inactivated (Zavoico et al., 1986). Under these conditions dimethylamiloride did not hinder potentiation of arachidonic acid release by PMA, despite a fall of more than 0.2 pH$_i$ caused by A23187 or ionomycin (Figure 4). The potentiation of arachidonic acid release by PMA is therefore not dependent on a rise in pH$_i$ or stimulation of the $Na^+/H^+$ antiporter.

*Effects of PMA on Protein Phosphorylation.* We used an incubation temperature of 22–23 °C to slow responses, enabling temporal resolution of events that are nearly concurrent at 37 °C. At 37 °C the full potentiation of arachidonic acid release by 100 ng of PMA/$10^9$ platelets occurs in only 2–3 min (not shown), whereas at 23 °C half-maximal potentiation develops in about 5 min and the full effect is attained in about 15 min (Halenda et al., 1985). PMA, mezerein (Figure 5), (−)-indolactam V, OAG, and thrombin (not shown) stimulated phosphorylation of the 47-kDa protein (P47), 20-kDa myosin light chains (P20), and unidentified polypeptides of 60–65, 41, (P41), and 35–37 kDa (P35). The rate and dose–response relationship for PMA-induced phosphorylation of P47 at 23 °C differed substantially from potentiation of arachidonic acid release (Halenda et al., 1985). Other proteins were phosphorylated more slowly than P47; e.g., P20, P35, and P41 phosphorylation was low when P47 phosphorylation was maximal (Figure 5A,B). Most interesting, both the time course and dose–response for potentiation of arachidonic acid release by PMA at 23 °C (Halenda et al., 1985) corresponded closely to phosphorylation of P41, P35, and P20 induced by PMA (Figure 5).

*Protein Kinase C Inhibitors Prevent Potentiation of Arachidonic Acid Release by PMA.* The kinase C inhibitor H-7

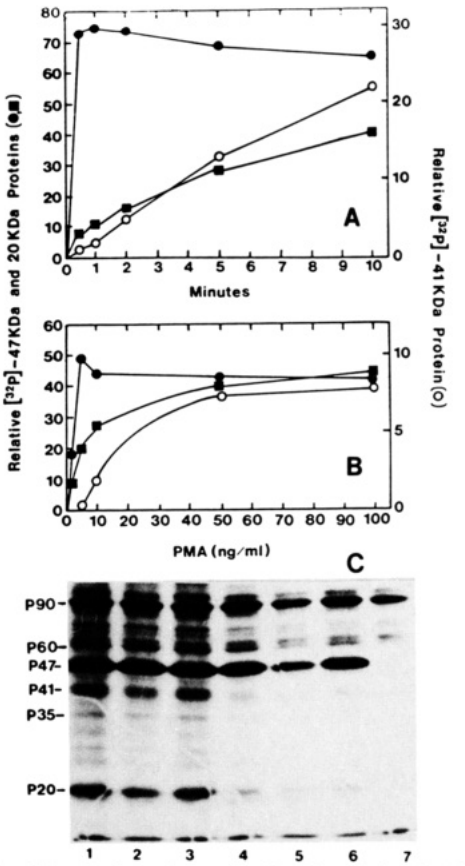


FIGURE 5: Stimulation of protein phosphorylation by PMA and mezerein. (A) Time course of phosphorylation of P47 (●), P41 (○), and P20 (■) by 160 nM (100 ng/mL) PMA. (B) Dose–response for protein phosphorylation induced by PMA (10-min incubation). (C) Phosphorylation of platelet proteins by PMA and mezerein. Washed platelets prelabeled with [$^{32}$P]phosphate were treated as follows and then analyzed after SDS–PAGE by radioautography: (lane 1) 160 nM (100 ng/mL) PMA, 10 min; (lane 2) 100 nM mezerein, 10 min; (lane 3) 1 μM mezerein, 10 min; (lane 4) 160 nM PMA, 1 min; (lane 5) 100 nM mezerein, 1 min; (lane 6) 1 μM mezerein, 1 min; (lane 7) no PMA or mezerein. Temperature was 23 °C.

Table I

| | % inhibition of arachidonic acid release[a] | | |
|---|---|---|---|
| H-7 (μM) | PMA (100 nM) | mezerein (100 nM) | OAG (40 μg/mL) |
| 50 | 6.7 ± 3.9 (4) | 8.4 (2) | 30.9 (2) |
| 100 | 14.3 ± 6.5 (6) | 36.7 ± 8.2 (3) | 54.8 ± 8.6 (6) |
| 150 | 23.5 (2) | | |
| 200 | 33.0 ± 8.6 (6) | 71 (1) | 55.4 ± 8.1 (3) |

[a] Washed platelets were incubated in the presence of H-7 for 5 min and then exposed to kinase C activator for an additional 5 min before 0.5 μM A23187 was added. After 5 min, reactions were terminated and arachidonic acid plus metabolites assayed. A23187 (0.5 μM, 5 min) released 6.1% (±0.7, $n$ = 14) of platelet radioactive arachidonate. The kinase C activators increased total arachidonic acid release as follows: PMA 276% (±32%, $n$ = 12), mezerein 181% (±18%, $n$ = 4), and OAG 193% (±23%, $n$ = 7). The data are expressed as percent inhibition of the potentiated arachidonic acid release (±SE, number of experiments). H-7 did not inhibit A23187 alone.

(50–200 μM) only partially prevented the potentiation of arachidonic acid release by PMA, OAG, and mezerein (Table I). However, staurosporine, a more potent and effective inhibitor of platelet kinase C activity (Watson et al., 1988), completely abolished the potentiation by kinase C activators, including PMA (Figure 6) and (−)-indolactam V (Figure 3), with little or no inhibition of the response to A23187 alone (Figures 3 and 6). Staurosporine inhibited PMA-induced phosphorylation of P60–65, P47, P41, P35, and P20 to a

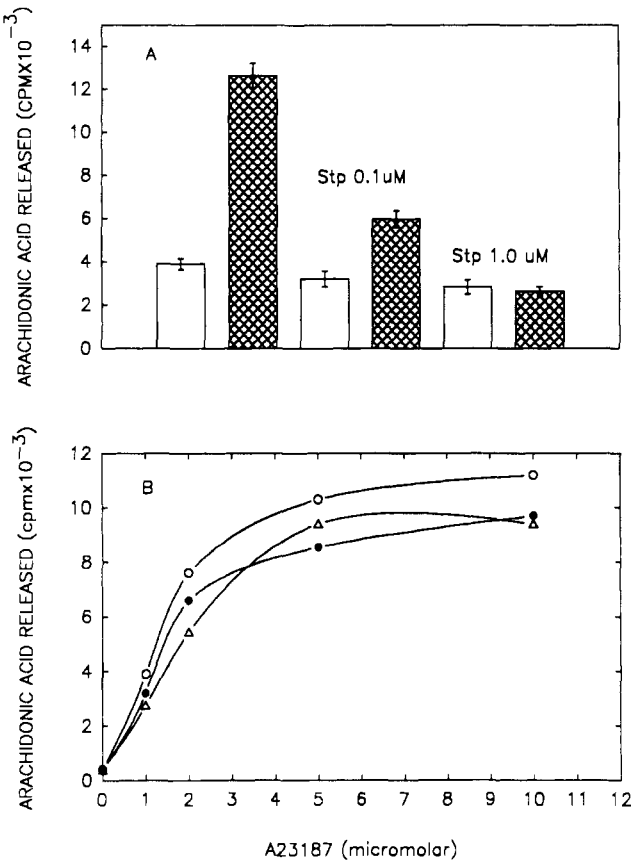


FIGURE 6: Effects of staurosporine on PMA-potentiated release of [$^{14}$C]arachidonic acid. (A) (open bars) DMSO for 10 min followed by 1 $\mu$M A23187 for 5 min; (hatched bars) 160 nM PMA (in DMSO) for 10 min prior to addition of A23187. Staurosporine (Stp) was added at indicated concentrations for 3 min prior to DMSO ± PMA. Total arachidonic acid (plus metabolites) released was measured. (B) Time course of total [$^{14}$C]arachidonic acid release by A23187 alone (○) or after pretreatment with 0.1 $\mu$M (●) or 1.0 $\mu$M (△) staurosporine.

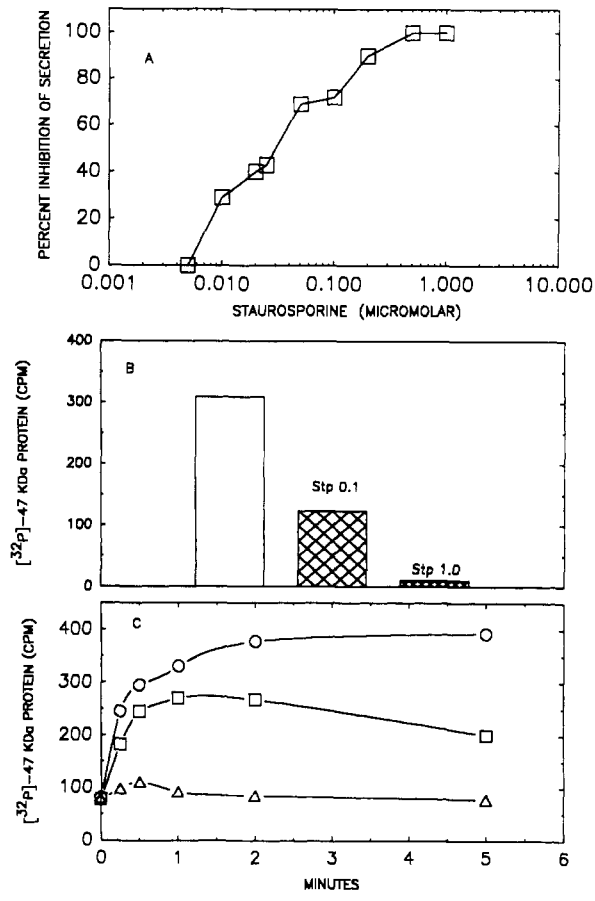


FIGURE 7: Inhibition of PMA-induced ATP secretion and protein phosphorylation by staurosporine. (A) Platelets ($10^9$/mL) were stimulated by 160 nM PMA, and ATP secretion was measured with luciferin/luciferase in a lumiaggregometer. Controls, treated with DMSO alone, secreted approximately 20 nmol of ATP/$10^9$ platelets. Staurosporine (in DMSO), at concentrations indicated, was added 3 min prior to PMA. Temperature was 22 °C. (B) Phosphorylation of P47; (open bar) 160 nM PMA for 10 min; (hatched bars) pretreatment with staurosporine (Stp) at indicated concentrations (0.1 and 1.0 $\mu$M), added 3 min before PMA). (C) Time course of phosphorylation of P47 by 160 nM PMA (○), PMA + 0.1 $\mu$M Stp (□), and PMA + 1.0 $\mu$M Stp (△). Note that after the initial burst of phosphorylation, dephosphorylation ensues in the presence of staurosporine. P47 was measured because of greater $^{32}$P incorporation. Results were similar for other substrate proteins as well.

comparable degree (not shown). The dose–response for inhibition of PMA-induced secretion of dense granule ATP by staurosporine (Figure 7A) was similar to that for inhibition of protein phosphorylation (Figure 7B) and arachidonic acid release (Figure 6A).

*Metabolic Inhibitors Prevent PMA-Potentiated Arachidonic Acid Release and Protein Phosphorylation.* Blockade of oxidative phosphorylation and glycolysis by incubation of platelets with 4 $\mu$g/mL antimycin A (or carbonyl cyanide *m*-fluorophenylhydrazone) plus 32 mM 2-deoxyglucose decreased the metabolic pool of ATP by 75% in 30 min (Holmsen et al., 1982). This treatment of our platelets was without effect on arachidonic acid release caused by A23187 (or ionomycin) alone (Figure 8), but it nearly abolished protein phosphorylation by PMA and totally blocked PMA's enhancement of arachidonic acid release by 0.5–1.0 $\mu$M A23187 (or ionomycin, not shown). Metabolic inhibitors were earlier shown to prevent arachidonic acid release by thrombin (but not A23187) (Rittenhouse-Simmons & Deykin, 1977). The depletion of ATP (and GTP) would be expected to impair the $Ca^{2+}$-mobilizing effect of thrombin (but not A23187) by reducing the amount of available phosphatidylinositol 4,5-bisphosphate and its GTP-dependent hydrolysis necessary for inositol 1,4,5-trisphosphate production.

## Discussion

The tumor promoters PMA and phorbol dibutyrate and the diacylglycerol OAG strongly potentiated arachidonic acid release by $Ca^{2+}$ ionophores without any direct effect by themselves (Halenda et al., 1985; Mobley & Tai, 1985; Toqui et al., 1986; Halenda & Rehm, 1987; this study). Since 4$\alpha$-phorbol diesters were ineffective, it appeared that kinase C was involved in the potentiation of arachidonic acid release by ionophores. To test this hypothesis more thoroughly, we used two other kinase C activators, which differ significantly from the phorbol diesters in chemical structure. Mezerein and (−)-indolactam V were also found to facilitate release of arachidonic acid in intact platelets. In contrast, (+)-indolactam V has very low affinity for kinase C (Fujiki et al., 1984) and did not potentiate arachidonic acid release by A23187. The inactivity of the latter compound is especially significant because it is the optical stereoisomer of the strong potentiator (−)-indolactam V.

To further understand the mechanism of action of these agents, the effects of PMA were more thoroughly investigated. PMA has four characteristic effects on arachidonic acid release by ionophores: (1) enhancement of release requires that ionophores elevate $[Ca^{2+}]_i$ to some threshold level, which by itself is sufficient to initiate only a small but detectable release of fatty acid; (2) at concentrations of ionophores that produce minimal effects, PMA can increase arachidonic acid release

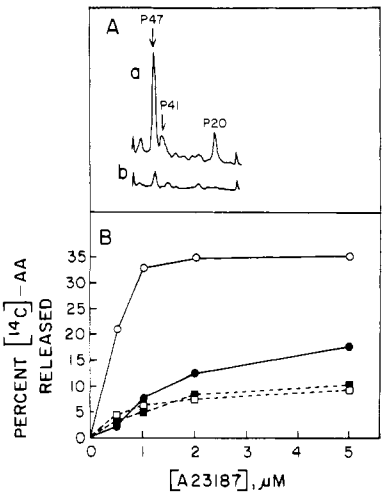


FIGURE 8: Inhibition of protein phosphorylation and PMA-potentiated arachidonic acid release by metabolic inhibitors. Washed platelets prelabeled with [$^{32}$P]phosphate and [$^{14}$C]arachidonate were incubated 30 min at 37 °C with deoxyglucose (DOG) plus antimycin A (antiA) to deplete the metabolic pool of ATP. (A) Densitometric scans of radioautograph of $^{32}$P-labeled proteins from platelets incubated 10 min with 160 nM PMA under normal conditions (a) or after 30 min of incubation with metabolic inhibitors (b). (B) [$^{14}$C]Arachidonic acid release: Normal platelets plus 1 μM A23187 (□), A23187 after 160 nM PMA (○); metabolically inhibited platelets plus 1 μM A23187 (■) and A23187 after PMA (●). Same protocol as Figure 1B.

as much as 10-fold, with a corresponding increase in the production of thromboxane; (3) at saturating concentrations of ionophore, PMA can further increase the maximum amount of arachidonic acid release by 2-fold; and (4) PMA does not affect the metabolism of free arachidonic acid.

We also previously reported that PMA did not affect the incorporation of arachidonic acid into platelet phospholipids (Halenda et al., 1985). However, Fuse et al. (1989) recently reported that PMA caused inactivation of two enzymes that are responsible for uptake of arachidonate and its esterification into platelet phospholipids (i.e., arachidonyl-coenzyme A synthase and arachidonyl-CoA lysophosphatide acyltransferase). Inactivation of these enzymes would prevent the reesterification of released arachidonate and might account for an *apparent* potentiated release of arachidonate by the combined action of PMA and ionophores. We have reinvestigated this problem and find no interference by PMA with arachidonate uptake into platelets or its incorporation into phospholipids; indeed, arachidonate incorporation into PI and PA was increased.[2] Furthermore, 100 μM 8,11,14-eicosatrienoic acid totally blocked uptake of arachidonic acid (5 μM) into platelets, but unlike PMA had no effect on the release of arachidonic acid by A23187. From these data we conclude that interference with the reincorporation of arachidonic acid released by ionophore is not a valid explanation for the potentiation of arachidonate release by PMA.

In $Ca^{2+}$-depleted "ionophore-permeabilized" platelets arachidonic acid is released as a function of $Ca^{2+}$ in the medium over the range 0.1–10 μM, similar to the concentration attained in the cytoplasm of stimulated platelets (Rehm & Halenda, 1987). Under these conditions PMA or thrombin cause a leftward shift (potentiation) of the $Ca^{2+}$ dose–response curve and an increase of the maximum amount of arachidonic acid released. This effect is similar to the leftward shift of the ionophore dose–response curves by PMA by intact platelets and shows that PMA, although lacking the ability to directly stimulate phospholipase $A_2$, potentiates the ability of elevated calcium to cause phospholipid hydrolysis.

On the basis of these findings the initial focus of our investigations was on two known mechanisms of action of phorbol diesters, i.e., increase of intracellular pH ($pH_i$) mediated by the amiloride-sensitive $Na^+/H^+$ exchanger and phosphorylation of proteins mediated by protein kinase C. Intracellular pH can modulate the release of arachidonate by weak platelet agonists (e.g., epinephrine, ADP) and low concentrations of thrombin (Sweatt et al., 1986). Because arachidonic acid release was inhibited by amiloride derivatives or by removal of extracellular $Na^+$, Sweatt et al. concluded that the $Na^+/H^+$ exchanger was important for arachidonic acid release by $Ca^{2+}$-mobilizing agonists. The $Na^+/H^+$ exchanger is responsible for a sustained alkalinization of the cytosol after stimulation by thrombin (Zavoico et al., 1986), which would favor hydrolysis of phospholipids because of the alkaline pH optimum of phospholipase $A_2$ (Baron & Limbird, 1988). Amiloride derivatives or lack of extracellular $Na^+$ converts the normal increase of $pH_i$ (approximately 0.15 unit) caused by thrombin to a sustained fall of 0.35 $pH_i$ below the normal resting level of $pH_i$ 7.0 (Zavoico et al., 1986). This fall of $pH_i$ could inhibit responses to weak agonists, and similarly stimulation of platelet $Na^+/H^+$ exchanger by PMA (Siffert & Scheid, 1986; Siffert & Akkerman, 1988) might account for its potentiation of arachidonic acid release. However, PMA's potentiation of arachidonic acid release by ionophores is not inhibited by the potent $Na^+/H^+$ exchange inhibitor dimethylamiloride, despite the substantial fall of $pH_i$ caused by ionophore under these conditions. This demonstrates conclusively that PMA/ionophore synergism is not mediated by the $Na^+/H^+$ antiporter. Indeed, treatment with PMA appears to make phospholipase $A_2$ behave as if it were at high pH and may ultimately affect enzyme conformation and activity in a similar way. It is important to point out that our results do not dispute the findings of Sweatt et al. (1986) regarding weak agonists, and in some cases inhibition of the $Na^+/H^+$ antiporter may also attenuate the mobilization of $Ca^{2+}$ (Sweatt et al., 1986; Zavoico et al., 1986; Siffert & Akkerman, 1988).

Potentiation of arachidonic acid release by PMA could also be mediated by protein phosphorylation, to produce an activator, or to inhibit an antagonist, of phospholipase $A_2$. Several studies in other types of cells (e.g., neutrophils) did not support the involvement of kinase C, because the kinase C inhibitor H-7 failed to block the response to PMA (Billah & Siegel, 1987; Matsumoto et al., 1988; Tao et al., 1989). It is also reported that the alkylacyl analogue of OAG, 1-*O*-(9-octadecenyl)-2-acetylglycerol, stimulates arachidonic acid release in neutrophils, although it lacks the ability to activate kinase C (Bauldry et al., 1988). These studies suggest that diacylglycerols and phorbol diesters have nonspecific effects on membrane properties or directly enhance phospholipase activity (Dawson et al., 1983). It should be borne in mind, however, that agonist-induced arachidonic acid mobilization and metabolism in neutrophils differ quite substantially from that in platelets and may therefore be subject to different types of control. In platelets, we believe a strong case can be made for involvement of kinase C (or some other kinase) in the response to PMA because the effect is (a) produced by kinase C activators of several different chemical classes, but not by closely related compounds or stereoisomers that do not stimulate kinase C, (b) correlated temporally, and with respect to dose, with PMA-induced phosphorylation of certain proteins, (c) abolished by depletion of ATP, and (d) partially or com-

[2] Halenda, Banga, Lau, and Feinstein, unpublished results.

pletely inhibited by H-7 and staurosporine, respectively. The efficacy of these inhibitors against arachidonic acid release is well correlated with their potency to block protein phosphorylation. H-7, which is a weak and only partial inhibitor of protein kinase C dependent protein phosphorylation in platelets (Watson et al., 1988), only partially inhibited the potentiation of arachidonic acid release by OAG, mezerein, and PMA without affecting arachidonic acid release by A23187 alone. In contrast, staurosporine, a much more potent inhibitor of kinase C in platelets (Watson et al., 1988), was able to totally abolish potentiation of arachidonic acid release by PMA and (-)-indolactam V. Over a concentration range of 0.02–1.0 $\mu$M, staurosporine produced equivalent inhibition of PMA-induced secretion, protein phosphorylation, and arachidonic acid release.

From the selective action of the metabolic inhibitors and staurosporine we deduce that a protein kinase may mediate the action of PMA, but not the direct effect of $Ca^{2+}$ on phospholipase $A_2$. Although staurosporine is reportedly most selective against the serine/threonine kinase C, we cannot discount the possibility that it may affect other kinases, such as platelet tyrosine kinase (Golden & Brugge, 1989). The situation is further complicated by the fact that platelets have two classes of phorbol diester binding sites (Cech et al., 1987) and at least two kinase C isozymes, one of which is quite insensitive to $Ca^{2+}$ (Tsukuda et al., 1988).

Phosphorylation of the major platelet phosphoprotein P47 does not correlate temporally or in dose–response with PMA-induced arachidonic acid release [see also Halenda et al. (1985)]. However, reducing the temperature to 21–23 °C causes both the time course of potentiation of arachidonic acid release by PMA and the protein phosphorylation to be greatly slowed, permitting greater resolution of these events. A group of proteins, which include P41, P35, P20, and possibly P60, are slowly phosphorylated compared to P47. Their phosphorylation closely corresponded both in time course and in dose–response to the potentiation of arachidonic acid release by PMA.

P20 is the myosin light chain that is phosphorylated by kinase C (Nishikawa et al., 1984) and $Ca^{2+}$/calmodulin-dependent protein kinase. The 35–37-kDa polypeptide is similar in molecular weight and p*I* (pH $\geq$7.5, not shown) to platelet lipocortin, a protein that inhibits phospholipid hydrolysis by pancreatic phospholipase $A_2$ in vitro (Toqui et al., 1986). Lipocortins (calpactins) can be inactivated by phosphorylation by kinase C (Schlaepfer & Haigler, 1988; Varticovski et al., 1988) or tyrosine kinases (Varticovski et al., 1988; Toqui et al., 1986). There are conflicting data about the biochemical mechanism of action of lipocortins (Davidson et al., 1987; Haigler et al., 1987; Huang et al., 1987; Nevalainen & Evilampi, 1984; Parente & Flower, 1985), and their physiological significance as regulators of arachidonic acid release remains to be established.

P41 is phosphorylated in intact PMA-treated platelets and on 2-dimensional gels (SDS–PAGE/isoelectric focusing) migrates identically with the ADP-ribosylated pertussis toxin substrate 41 kDa $G_i\alpha$ (Halenda et al., 1986). The GTP-binding protein $G_i\alpha$ is phosphorylated by kinase C in vitro (Katada et al., 1985), but its role in platelet arachidonic acid release is not known. GTP-binding proteins have been indirectly implicated in platelet arachidonic acid release (Nakashima et al., 1987a; Fuse & Tai, 1987). In permeabilized platelets GTP$\gamma$S only slightly increased arachidonate release, but it potentiated the much larger release by thrombin (Nakashima et al., 1987b), suggesting an interaction between a GTP-binding protein and some other signal(s) generated by thrombin. A more definitive link between G-proteins and arachidonic acid release has been provided by experiments utilizing purified G-protein components. The $\beta\gamma$ subunits of the GTP-binding protein transducin are reported to stimulate phospholipase $A_2$ activity (Jelsema & Axelrod, 1987; Kim et al., 1989). It has been suggested that $\beta\gamma$ may stimulate phospholipase $A_2$ activity by counteracting calpactin (Jelsema, 1989). Various $G\alpha$ subunits may also directly or indirectly affect phospholipase $A_2$ (Jelsema, 1989).

Although our evidence supports the hypothesis that kinase C can potentiate arachidonic acid release by $Ca^{2+}$ ionophores in platelets, the enzyme's role in responses mediated by receptor agonists has not been established. In one study the kinase C antagonist H-7 failed to prevent arachidonic acid release by thrombin in intact platelets (Fuse & Tai, 1988), but inhibition was observed by other investigators when the intracellular free $Ca^{2+}$ was fixed (Halenda & Rehm, 1987). In intact platelets we find that the more potent kinase inhibitor staurosporine slightly, but consistently, enhanced arachidonic acid release by thrombin.[3] Staurosporine most likely prevents the negative feedback that is normally exerted on thrombin-induced phosphoinositide hydrolysis and $Ca^{2+}$ mobilization (Rittenhouse & Sasson, 1985; Zavoico et al., 1985; Yoshida & Nachmias, 1987). This negative feedback, which results from activation of kinase C caused by the stimulus-induced formation of diacylglycerol, may serve to limit arachidonic acid release. The existence of this kinase C mediated negative feedback pathway complicates the attempt to demonstrate a direct stimulatory role of the enzyme in receptor-mediated arachidonic acid release. Also, we must consider the possibility that thrombin operates to release arachidonic acid through a different pathway than PMA plus $Ca^{2+}$ ionophores, perhaps involving a protein kinase that is unaffected by staurosporine or GTP-binding protein components (Jelsema, 1989).

In conclusion, the magnitude of the synergism between ionophores and kinase C activators demonstrates that although kinase C is not necessary for arachidonic acid release mediated by elevated $Ca^{2+}$, the enzyme is a potentially important regulator of phospholipase $A_2$, either directly or indirectly. Even if the responses to PMA/$Ca^{2+}$ ionophore and thrombin proceed by somewhat different pathways, it is possible that they ultimately converge at a common site, or interacting sites of action, such as some inhibitor, or activator (Park, 1989), of phospholipase $A_2$.

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