Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 185-190

SUBSTRATE LEVEL MODULATION OF THE ACTIVITY OF PHOSPHOLIPASE A2 IN VITRO BY 12-O-TETRADECANOYLPHORBOL-13-ACETATE

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Received April 13, 1992

The action of porcine pancreatic phospholipase A2 towards fluorescent phospholipid analogs is either enhanced or suppressed by 4β-12-O-tetradecanoylphorbol-13-acetate (TPA), depending on the chemical structure of the substrate and the concentration of Ca^{2+}. In the presence of nmolar Ca^{2+} concentrations increasing [TPA] enhanced by approx. 5-fold the rate of hydrolysis of the pyrene-labelled acidic alkyl-acyl phospholipid, 1-octacosanyl -2- [6-(pyrene-1-yl)] hexanoyl -*sn*- glycero -3- phosphatidylmethanol. Maximal effect was obtained at high TPA/substrate molar ratios approaching 1:2. In the presence of 4 mM $CaCl_2$ maximal activation was reduced to ≈1.5-fold. With the corresponding phosphatidylcholine derivative as a substrate increasing [TPA] reduced fatty acid release maximally by 90% both at low [Ca^{2+}] as well as in the presence of 4 mM $CaCl_2$. Essentially identical results were obtained using 4α-TPA, a stereoisomer which does not activate protein kinase C.

Phospholipase A2 (E.C. 3.1.1.4., PLA2) is generally taken as a paradigm for Ca^{2+} activated lipolytic enzymes (1, 2). Intracellular PLA2 has been found to be ubiquitously present in cells. Their catalytic activity and the liberation of arachidonic acid is currently considered to represent the rate-limiting step in the formation of eicosanoids (3, 4). PLA2 reaction is also involved in the cellular formation of the platelet activating factor (2).

Unlike most enzymes acting on soluble substrates a distinct feature of lipases is the high degree of dependency of the measured reaction rates on the physicochemical properties of the substrate/water interface. For instance, PLA2 is most active towards phospholipids at the phase transition temperature (5, 6). Similarly, the catalytic rate is greatly enhanced when the critical micellar concentration of the substrate lipid is exceeded. Several mechanisms for the interfacial activation of PLA2 have been proposed (2). We have provided experimental evidence favouring

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Abbreviations: PLA2, phospholipase A2; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; C_{28}-O-PHPM, 1-octacosanyl-2-[6-(pyrene-1-yl)]hexanoyl -*sn*- glycero -3-phosphatidylmethanol; C_{28}-O-PHPC, 1-octacosanyl-2-[6-(pyrene-1-yl)] hexanoyl- *sn*-glycero-3-phosphatidylcholine; PPHPG,1-palmitoyl-2-[6-(pyren-1-yl)] hexanoyl -*sn*- glycero -3- phosphatidyl -*sn*' -*rac* -glycerol; PPHPC, 1- palmitoyl -2- [6-(pyren-1-yl)] hexanoyl -*sn* -glycero -3-phosphatidylcholine; PKC, protein kinase C.

0006-291X/92 $4.00

aggregation-induced changes in the conformation of the substrates (7-9). This view is now gaining more credibility due to confirming data from X-ray diffraction studies on PLA2 crystals and the use of conformationally restricted substrates (10). In a recent paper direct indications for lipid packing allowing the initiation of PLA2-reaction were visualized using fluorescence microscopy of lipid monolayers (11).

Conclusive evidence supports phorbol ester (TPA) tumor promoters to function as diacylglycerol analogs so as to activate protein kinase C (12). It has been suggested that part of the effects of TPA is mediated by its binding to membrane lipid domains (13). Direct membrane perturbation has been proposed to contribute to the enhanced release of arachidonic acid by phorbol esters (14, 15). Results from studies on the effects of phorbols on the activity of PLA2 in cells do not exclude the possibility of a direct, non PKC-mediated effect on PLA2 activity (20-22). Indirect evidence indicating modulation of PLA2 catalyzed hydrolysis of dipalmitoylphosphatidylcholine by TPA has been forwarded (16). Diacylglycerols have been shown to activate PLA2 in vitro (17-19).

To exclude the possibility that the recorded liposome permeability alterations due to TPA and PLA2 (16) would result from nonenzymatic processes similarly to insulin enhancement of vesiculation of liposomes by lysophosphatidylcholine (23), we quantitated the influence of the tumor promoter TPA on porcine pancreatic PLA2 catalyzed hydrolysis of phospholipids in vitro. As substrates we used fluorescent phospholipid analogs developed for the sensitive and specific measurement of PLA2 activity (24-26).

Materials and Methods

Reagents - The pyrene-labeled phospholipids 1-octacosanyl-2-[6-(pyrene-1-yl)]hexanoyl-*sn* glycero-3-phosphatidylmethanol (C28-O-PHPM), 1-palmitoyl -2- [6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidyl-*sn'*-*rac*-glycerol (PPHPG), and the corresponding phosphatidylcholine derivatives (C28-O-PHPC and PPHPC) were made by total organic synthesis and were purchased from KSV Chemical Co. The acidic lipids were Ca^{2+}-free sodium salts. No impurities were detected upon thin layer chromatography on silicic acid coated plates (Merck) using chloroform/methanol/water/ammonia (90:5.5:5.5:5.5, v/v) as the solvent system and examination of the plates for pyrene fluorescence or after staining with iodine vapor. Porcine pancreatic phospholipase A2 was from Boehringer Mannheim. Other reagent grade materials as well as 4β-12-O-tetradecanoylphorbol-13-acetate (TPA) were from Sigma. The 4α stereoisomer of TPA was from LC Services (Woburn, MA, USA).

End-point assay for phospholipase A2 - The assay medium consisted of 31.5 mM substrate in 20 mM Tris-HCl, pH 7.6, in a total volume 200 μL. TPA was added dissolved in ethanol. In the concentration used (9 vol. %) this solvent did not affect the enzyme activity. When indicated $CaCl_2$ was thereafter added to yield the indicated final concentrations. The method described previously (25) was used except that 0.1 mg/ml of oleic acid was included as a carrier in the organic phase consisting of chloroform : methanol: heptane (1.25 : 1.41 : 1.00, by vol.). Each data point represents the mean from a duplicate determination. Standard deviation was < 5 %. In the presence of 1 mM $CaCl_2$ the specific activities with C28-O-PHPM and C28-O-PHPC were 2.4 nmol/min/mg and 25 pmol/min/mg, respectively. In the experiments performed in the absence of exogenous Ca^{2+} the residual concentration of this metal cation due to its presence in the buffering materials and our purified water is approximately 50 nM (24). Complete loss of activity was seen due to the inclusion of excess (0.1 mM) EDTA.

Kinetic assay for phospholipase A2 - Assays were performed in 2.0 mL of 20 mM Tris-HCl, pH 7.6, using thermostated and magnetically stirred cuvettes at 37 °C and were calibrated by the addition of free 6-(pyren-1-yl)hexanoic acid in the absence of enzyme (24, 26, 27). Substrate concentration was 3.1 mM. The progress of enzymatic hydrolysis was monitored with a strip-chart recorder as a function of time by measuring the intensity of pyrene monomer fluorescence emission at 400 nm while illuminating at 344 nm. In the presence of 1 mM $CaCl_2$ the specific activities with

PPHPG and PPHPC were 7.5 nmol/min/mg and 50 pmol/min/mg, respectively. Determined from uncorrected fluorescence emission spectra the addition of phorbol ester in a 1:1 molar ratio to phospholipid liposomes (both PPHPG and PPHPC) increased the ratio of pyrene excimer to monomer emission ≈ 3.5-fold. These fluorescence changes were rapid and took place within < 3 min.

Results

At low Ca^{2+} concentrations TPA enhances in vitro the hydrolysis of C28-O-PHPM by PLA2 by approx. 5-fold, Fig.1. Maximal effect is observed at TPA/lipid molar ratios > 1:2. When this experiment was repeated in the presence of 4 mM $CaCl_2$ the activation was about 1.5-fold, Fig. 1. The rates of PLA2-reaction were then determined as a function of the concentration of exogenous $CaCl_2$, both in the absence of TPA and when the tumour promoter was included in a concentration corresponding to a 1:1 TPA/lipid molar ratio, Fig. 2. It is evident that the reaction rates depend both on $[Ca^{2+}]$ as well as on the content of TPA. Under otherwise identical conditions in the absence of added Ca^{2+} increasing [1,2-dioctanoylglycerol] activated PLA2-catalyzed hydrolysis of C28-O-PHPM two-fold. However, compared to TPA much higher diacylglycerol/phospholipid molar ratios (2:1) were required. As a further distinction inhibition by diacylglycerol was observed when the assay contained 4 mM $CaCl_2$ (data not shown).

In contrast to the results with the acidic alkyl-acyl lipid C28-O-PHPM, both in the absence of added Ca^{2+} as well as in the presence of 4 mM $CaCl_2$ TPA inhibited in a concentration dependent manner the hydrolysis by PLA2 of the alkyl-acyl phosphatidylcholine C28-O-PHPC , Fig. 3. This inhibition was maximal (> 90%) at TPA/lipid molar ratios exceeding 1:1. The activity of PLA2 towards this lipid was enhanced approx. 2- and 4-fold by $CaCl_2$ concentrations exceeding 1 mM when measured in the absence and presence of TPA (in a 1:1 phorbol:phospholipid molar ratio), respectively, Fig. 4. Importantly, essentially identical results

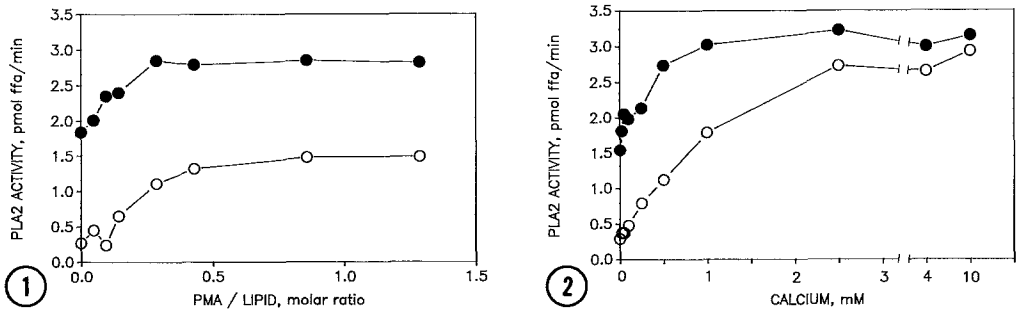

Fig. 1: Enhancement of PLA2 catalyzed hydrolysis of C28-O-PHPM by increasing concentrations of TPA. The medium consisted of 31.5 mM substrate in 20 mM Tris-HCl, pH 7.6, in a total volume 200 µL, either in the absence of exogenous Ca^{2+} (O–O) or in the presence of 4 mM $CaCl_2$ (●–●). Degree of hydrolysis was quantitated by the end-point assay. Each data point represents mean from a duplicate determination. Reactions were started by the addition of 0.7 ng of porcine pancreatic PLA2.

Fig. 2: Enhancement of PLA2 activity towards C28-O-PHPM by $CaCl_2$ in the absence (O–O) and in the presence of 31.5 mM TPA (●–●). Amount of enzyme was ≈ 0.7 ng. Other details are as in the legend for Fig.1.

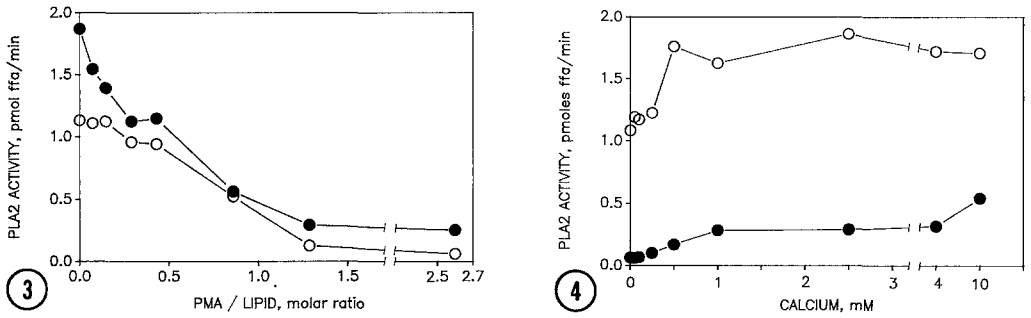

Fig. 3: Inhibitory effect of increasing concentrations of TPA on PLA2-activity towards C28-O-PHPC. The medium consisted of 20 mM Tris-HCl, pH 7.6, 31.5 μM substrate either in the absence of added Ca^{2+} (O–O) or containing 4 mM $CaCl_2$ (●–●). The amount of enzyme was ≈ 70 ng. Other details are as described in the legend for Fig. 1.

Fig. 4: Enhancement of PLA2 activity towards C28-O-PHPC by $CaCl_2$ in the absence (O–O) and presence of 31.5 mM TPA (●–●). The reactions were started by the addition of ≈ 70 ng of enzyme.

were obtained both with C28-O-PHPM and C28-O-PHPC when the above experiments were repeated substituting 4α-TPA for 4β-TPA (data not shown). The former stereoisomer does not activate protein kinase C

In order to exclude the possibility that the above results would just reflect the aberrant structural features of these alkyl-acyl phospholipid analogs, we also studied the effects of TPA using two fluorescent diacyl phospholipids, 1-palmitoyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidyl-*sn*'-*rac*-glycerol (PPHPG) and the corresponding phosphatidylcholine, PPHPC. Similarly to C28-O-PHPM in the absence of added Ca^{2+} the addition of TPA resulted in a ≈ 4-fold enhancement of the hydrolysis of PPHPG. In the presence of 4 mM $CaCl_2$ the activation by TPA was ≈ 3-fold, Fig. 5. Interestingly, results obtained with PPHPC as a substrate differed from those using the alkyl-acyl phosphatidylcholine C28-O-PHPC, Fig. 5. With PPHPC in the absence of added calcium a 1.5 to 1.8-fold activation by TPA was evident. Presence of 4 mM $CaCl_2$ abolished the activation. Using a 1:1 molar ratio of diacylglycerol to phospholipid without exogenous Ca^{2+}

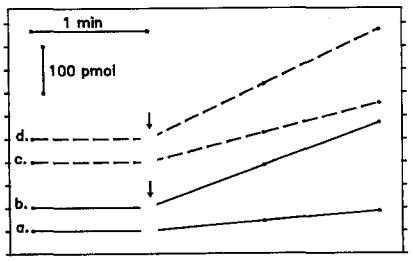

Fig. 5: Time courses of hydrolysis of PPHPG by pancreatic phospholipase A2 (*trace a*) and the effect of 4.1 μM TPA (*trace b*). Additions of PLA2 (30 ng) are indicated with arrows. Traces marked with broken lines depict the time courses of hydrolysis of PPHPC by PLA2 (*trace c*) and the effect of TPA (*trace d*). Addition of PLA2 (1 μg) is marked with an arrow.

both 1-stearoyl-2-arachidonoyl-glycerol and dioctanoylglycerol produced both qualitatively and quantitatively similar enhancement of hydrolysis of PPHPC as did TPA (data not shown).

Discussion

The enhanced release of arachidonic acid from membrane phospholipids observed in cells due to phorbol esters has been assigned to protein kinase C mediated activation of PLA2 and/or inhibition of arachidonoyl-coenzyme A synthase and lysophosphatide acyltransferase (20, 28-30). Phorbol esters have also been suggested to alter the calcium requirement of PLA2 (31). Attempts to demonstrate direct activation of PLA2 by phorbol esters have not been successful (30). Yet, TPA has been shown to modulate PLA2-induced permeability changes of dipalmitoylphosphatidylcholine liposomes (16). The present results clearly reveal that the action of PLA2 can be effectively modulated by TPA in vitro.

Neglecting any possible interference due to possible headgroup specific lipid-TPA interactions, comparison of the results with C28-O-PHPM and PPHPG suggests that the lack of the carbonyl moiety in the former alkyl-acyl compound does not lead to qualitative differences in the effects of TPA on their hydrolysis by PLA2. This is contrasted by C28-O-PHPC and PPHPC. Hydrolysis of the former lipid is inhibited by TPA whereas the latter diacyl phospholipid provides a better substrate when TPA is included. Accordingly, the susceptibilities of different phospholipid species to PLA2 action respond very differently to the inclusion of TPA. The reciprocal effects of TPA on the hydrolysis of C28-O-PHPM and -PC by PLA2 argue against direct effects of the phorbol ester on the enzyme.

The present data together with our previous results on the rather similar effects by polyamines (24), adriamycin (32), which bind to the membrane surface, and the lipid derivative platelet activating factor (27) do suggest that in spite of their structural diversity the effects of TPA and the above compounds on PLA2 all reflect a change in some common physical property of the substrate, such as surface potential (32-34). The results by Smaby and Brockman emphasize the differencies in the electrical properties of membranes formed by acidic, zwitterionic, neutral, diacyl, as well as alkyl-acyl lipids (34). We have shown that the action of PLA2 can be triggered by electric fields imposed across a substrate monolayer (35).

It was shown in a recent study that inspite of the lack of PKC activation by the 4α stereoisomer of TPA this compound in high concentrations enhances arachidonic acid release in epidermal cells (36). This finding would be compatible with our interpretation of the activation of PLA2 being due to a direct membrane effect by both 4β-TPA and 4α-TPA. The magnitude of this change in the membrane, as sensed by PLA2, does not depend on the stereochemistry of the tumor promoter.

When considering the possible implications of the present data it is important to emphasize that it is the actual phospholipid/TPA molar ratio which is important. In cells the situation may well be much more complex as an enrichment of TPA in specific membrane domains (37) can take place. Nevertheless, because of the use of phospholipid analogs as well as the use of the porcine pancreatic phospholipase A2 as a model enzyme any direct significance of the present results regarding the action of TPA remains uncertain and warrants further studies.

Acknowledgments: Financial support was from the Finnish State Medical Research Council (P.K.J.K.), Magnus Ehnrooth Foundation, and from the Research and Science Foundation of Farmos (P.M.). We wish to thank Dr. Tom Thurén for critical reading of the manuscript and Mrs. Birgitta Rantala for skillful technical assistance.

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