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# CHARACTERISATION OF CYTOSOLIC PHOSPHOLIPASE $A_2$ AS MEDIATOR OF THE ENHANCED ARACHIDONIC ACID RELEASE FROM DIMETHYL SULPHOXIDE DIFFERENTIATED U937 CELLS

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**Abstract**—Studies were performed to characterise the phospholipase $A_2$ ($PLA_2$) responsible for the greatly increased capacity to release arachidonic acid (AA) of dimethyl sulphoxide (DMSO) differentiated U937 monocytic cells compared to undifferentiated cells (18-fold increase in response to $Ca^{2+}$ ionophore A23187). Cytosolic $PLA_2$ ($cPLA_2$) activity could be measured in homogenates of differentiated cells, and the highly selective $cPLA_2$ inhibitor arachidonic acid trifluoromethyl ketone reduced A23187 induced [$^3$H]AA release from pre-labelled cells by at least 80%, with an $IC_{50}$ (12.7 ± 1.4 μM) not significantly different from that for inhibiting authentic $cPLA_2$ (9.3 ± 2.0 μM). On the other hand, type II $PLA_2$ activity was not detected in cell homogenates, and [$^3$H]AA release was not inhibited by heparin (1 mg/mL), which binds secreted type II $PLA_2$ and reduces its ability to degrade membrane phospholipids. Stimulation of intact cells with A23187 plus phorbol myristate acetate (PMA) under conditions that released [$^3$H]AA did not increase $cPLA_2$ activity of the cell homogenate, and there was little difference between DMSO differentiated and undifferentiated cells in $cPLA_2$ protein content, $cPLA_2$ specific activity of homogenates, or distribution of $cPLA_2$ between membrane and cytosol in the resting cell. Following stimulation with A23187 plus PMA, no increase in [$^{33}$P] labelling of $cPLA_2$ immunoprecipitates was seen in cells pre-labelled with [$^{33}$P] orthophosphate, nor a change in electrophoretic mobility of $cPLA_2$. It was concluded that $cPLA_2$ releases the bulk of AA from stimulated, DMSO differentiated U937 cells. The failure to observe increased $cPLA_2$ specific activity following cellular stimulation could be explained by increased [$^3$H]AA release requiring the activation of only a small proportion of the cell pool of $cPLA_2$ or, alternatively, by increased release reflecting greater $Ca^{2+}$-dependent association of $cPLA_2$ with membrane substrate rather than increased specific activity *per se*. There was no evidence that any such increased membrane association resulted from $cPLA_2$ phosphorylation. The relative inability of undifferentiated cells to release AA was not due to the absence of $cPLA_2$ or an altered distribution between membrane and cytosol, but suggested the presence of a repressor mechanism that prevents elevated $Ca^{2+}$ from functionally activating the enzyme intracellularly.

*Key words:* cytosolic phospholipase $A_2$; arachidonate mobilisation; differentiated U937 cells

Phospholipases $A_2$ ($PLA_2$; E.C. 3.1.1.4) are widely distributed intra- and extra-cellular enzymes that specifically hydrolyse *sn*-2 acyl ester linkages of membrane glycerophospholipids to yield fatty acids and lysophospholipids. The action of $PLA_2$ in releasing AA† from membrane phospholipids is considered to be rate-limiting in the biosynthesis of pro-inflammatory prostaglandins, leukotrienes, and hydroxy fatty acids [1], whereas 1-0-alkyl-2-lysophospholipids are precursors of platelet activating factor [2]. $PLA_2$s are thus key enzymes in initiating and propagating the inflammatory response. We have been interested in the regulation of $PLA_2$ activity in monocytes and macrophages [3], and have used human monocytic leukaemic U937 cells, which resemble immature human peripheral blood monocytes, as an experimental model. When induced to differentiate along the monocyte-macrophage lineage, U937 cells undergo morphological and functional changes, acquiring the ability to respond to chemotactic agents, produce superoxide anion, release lysosomal enzymes, and perform antibody-dependent cytolysis [4]. Undifferentiated U937 cells have very low capacity to release eicosanoids, but culture in the presence of DMSO [5], as used in the present studies, or with phorbol ester [6, 7] results in a many-fold increase in stimulated AA release. It is not clear whether or not these dissimilar reagents cause the up-regulation by identical mechanisms.

In principle, two classes of $PLA_2$ could be responsible for increased AA release, the high molecular weight (85–110 kDa) cytosolic enzyme ($cPLA_2$) or the 14kDa type II enzyme, or conceivably both could be involved, since they have been reported to co-exist in certain inflammatory cells [8, 9]. The intracellular $cPLA_2$ has a number of properties that make it a likely mediator of responses to acute cell stimulation. It is activated by the submicromolar concentrations of $Ca^{2+}$ found in stimulated cells, and preferentially hydrolyses phospholipids with AA in the *sn*-2 position [10–12]; its activity in several cell types has been shown to be regulated by phosphorylation [13], and the phosphorylated enzyme translocates and binds in a $Ca^{2+}$-dependent manner to the cell membrane, where it is juxtaposed to its substrate [11, 14]. Whilst type II $PLA_2$ is up-regulated by inflammatory cytokines and is thought to play an important role in inflammation by being secreted into extracellular fluids (reviewed in refs. [9, 15]), there is now abundant

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† Abbreviations: $cPLA_2$, cytosolic phospholipase $A_2$; AA, arachidonic acid; DMSO, dimethyl sulphoxide; PMA, phorbol myristate acetate; BSA, bovine serum albumin; FMLP, N-formyl methionyl leucyl phenylalanine; PMSF, phenyl methyl sulphonyl fluoride; HBSS, Hanks balanced salt solution; $AACOCF_3$, arachidonic acid trifluoromethyl ketone; $LTD_4$, leukotriene $D_4$; MAP kinase, mitogen activated protein kinase; ECL, enhanced chemiluminescence; PAGE, polyacrylamide gel electrophoresis.

evidence that this enzyme can also generate AA in response to cell stimulation. In a number of cells and cell lines, including macrophage-like P388 D1 cells [16], AA release has been shown to result from type II $PLA_2$ being secreted to the outside of the cell and binding to membrane proteoglycan [15–18], and an extracellular membrane receptor for type II and other secretory $PLA_2$s has been characterised [19]. It is generally considered that 14kDa $PLA_2$s require millimolar concentrations of $Ca^{2+}$ for activation [20, 24], but a recent report demonstrated that under certain conditions type II $PLA_2$ can be activated by low micromolar $Ca^{2+}$ concentrations, and the case for an intracellular mechanism of action was discussed [21].

Previously published work implies that $cPLA_2$ is responsible for AA generation in U937 cells, but the information is largely circumstantial. Thus, $cPLA_2$ is known to be present at low levels in undifferentiated cells and in DMSO or phorbol myristate acetate (PMA) differentiated cells [10–12, 22], whereas it has been difficult to detect type II activity in these cells [8, 10, 22], although this enzyme is present in monocytes and macrophages [8, 16, 23]. In one of these studies, PMA-induced differentiation was shown to cause increased membrane association of $cPLA_2$ in the resting cell, and this was hypothesised to be the reason for increased AA release on cell stimulation [22]. $PLA_2$ activity in the cytosol of DMSO differentiated cells was largely abolished by acid treatment, which inactivates $cPLA_2$ but not type II $PLA_2$, and was not inhibited by a transition-state analogue selective for type II $PLA_2$ [8]. However, this does not preclude a functional role for type II $PLA_2$, since it is likely that any type II present would have separated with the membrane fraction on cell disruption [15]. In the present study we have attempted to define a causal relationship between either $cPLA_2$ or type II $PLA_2$ activity and stimulated AA release, and have characterised some properties of the responsible enzyme in differentiated and undifferentiated U937 cells.

## MATERIALS AND METHODS

### *Reagents*

RPMI 1640 cell culture medium, DMSO, fatty-acid free bovine serum albumin (BSA), calcium ionophore A23187, leukotriene $D_4$, N-formyl methionyl leucyl phenylalanine (FMLP), L-3-phosphatidyl choline 1-stearoyl-2-arachidonoyl, 1,2 dioleoyl-*sn*-glycerol, heparin (sodium salt), and general biochemical reagents were purchased from Sigma (Poole, U.K.). PMA and protein A-Pansorbin beads were from Calbiochem (Nottingham, U.K.). Foetal calf serum was supplied by Advanced Protein Products (Brierley Hill, U.K.), and L-glutamine and penicillin/streptomycin by ICN Flow (Thame, U.K.). Hanks balanced salt solution (HBSS) with or without added $Ca^{2+}$ and $Mg^{2+}$ was from Gibco (Paisley, U.K.). [5,6,8,9,11,12,14,15-$^3$H] arachidonic acid (150–230 Ci/m mol), L-3-phosphatidyl choline 1-stearoyl-2-[1-$^{14}$C] arachidonoyl (50–60 mCi/mmol), [9,10-$^3$H] oleic acid (2–10 Ci/mmol), horse radish peroxidase (HRP)-conjugated donkey anti-rabbit antiserum, and ECL detection kit were all from Amersham International (Aylesbury, U.K.). [$^{33}$P] orthophosphoric acid (40–158 Ci/mg) was from DuPont, Stevenage, U.K. Gold (5 nm)-conjugated donkey anti-rabbit antiserum and Immunogold reagents were from Bio Cell (Cardiff, U.K.). Rabbit polyclonal anti-$cPLA_2$ antiserum raised against the peptide sequence 53–72 of human $cPLA_2$ [25] was kindly provided by Dr. Y. Ivashchenko, Rhône-Poulenc Rorer Ltd. Sorbsil C60, 40–60H silica used for separating [$^{14}$C] arachidonic acid in the $cPLA_2$ enzyme assay was purchased from the Crossfield Group (Warrington, U.K.). The $cPLA_2$ inhibitor arachidonic acid trifluoromethyl ketone ($AACOCF_3$) was obtained from Affiniti Research Products (Nottingham, U.K.), and a stock 30 mM solution in ethanol stored under nitrogen. Protein concentration was determined using Coomassie blue reagent purchased from Bio-Rad (Hemel Hempstead, U.K.) using BSA as standard.

### *Cell culture*

Human monocytic leukaemic U937 cells obtained from American Type Culture Collection were grown in RPMI 1640 medium containing 10% foetal calf serum, 2 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10 μM β-mercaptoethanol at 37°C in a humidified 5% $CO_2$ incubator, and passaged when cell density reached approximately $10^6$ cells/mL. DMSO (1.3% v/v) was added to cultures ($2 \times 10^5$ cells/mL) for up to 96 hr to differentiate the cells along the monocyte-macrophage lineage [5]. Viability of undifferentiated or differentiated cells was consistently greater than 95%, as assessed by exclusion of Trypan blue dye.

### *[$^3$H]AA release from pre-labelled cells*

U937 cells were suspended at $2.5–5 \times 10^6$ cells/mL in culture medium containing 0.5 μCi/mL [$^3$H]AA (added in 0.5 μL/mL ethanol), to which DMSO was added back at 1.3% in the case of cells being differentiated. Culture was continued for 18 hr. Cells were centrifuged (180 g, 5 min) and washed by resuspension once in culture medium, then twice in HBSS with added $Ca^{2+}$ and $Mg^{2+}$, supplemented with 0.2% BSA ($Ca^{2+}$, $Mg^{2+}$-HBSS/BSA) before being resuspended at $0.8–1.6 \times 10^7$ cells/mL in the latter salt solution. Aliquots (0.1 mL) were added to Eppendorf tubes containing test reagent or vehicle diluted in $Ca^{2+}$, $Mg^{2+}$-HBSS/BSA (0.1 mL), equilibrated to 37°C with shaking for 10 min, then A23187 and/or PMA or vehicle (<0.25% final DMSO) (5 μL) was added, normally for a further 15 min. Incubations were terminated by adding 0.75 mL ice-cold $Ca^{2+}$ and $Mg^{2+}$-free HBSS containing 2 mM EDTA, and by transferring tubes to an ice bath prior to centrifugation. Radioactivity in cell supernatants was quantified by liquid scintillation counting, and calculated as a percentage of the total radioactivity incorporated by the cells. T.L.C. analysis showed that 80–90% of the released radioactivity co-chromatographed with authentic arachidonic acid. In the text, released radioactive material is referred to as [$^3$H] arachidonic acid.

### *Cell fractionation*

Identical numbers ($2.5–5 \times 10^7$ cells) of undifferentiated or 96 hr differentiated U937 cells were centrifuged and washed with phosphate buffered saline. In most experiments, washed cells were resuspended in $Ca^{2+}$, $Mg^{2+}$-HBSS (1 mL), and stimulated with A23187 (5 μM) and PMA (30 nM) for 2 or 5 min at 37°C to activate $PLA_2$ before being centrifuged again and resuspended in ice-cold homogenising buffer (1 mL). Cells were homogenised at 0–4°C using an all-glass Potter-Elvehjem type homogeniser. The basic homogenising buffer, com-

prising 20 mM HEPES, pH 7.5, 10 μg/mL leupeptin, 1 mM sodium orthovanadate, and 0.4 mM phenyl methyl sulphonyl fluoride (PMSF) (freshly added), was employed when the distribution of $cPLA_2$ between membranes and cytosol was to be measured. For all other studies, $CaCl_2$ (0.1 mM) was added to the basic homogenising buffer. Membranes were pelleted by centrifuging the homogenate at 100,000 g for 60 min at 4°C, then dispersed in homogenising buffer (1 mL).

*Determination of $cPLA_2$ activity*

$cPLA_2$ in U937 cell fractions was assayed using sonicated liposomes containing 1-stearoyl-2-[$^{14}$C] arachidonoyl-phosphatidylcholine and *sn*-1,2-dioleoyl glycerol at a molar ratio of 2:1 as described by Kramer *et al.* [12]. The assay mixture contained 2 μM radioactive phosphatidyl choline (50,000 cpm), 1 μM dioleoyl glycerol, 80 mM glycine, pH 8, 150 mM NaCl, 3 mM $Ca^{2+}$, 2 mg/mL BSA, 2 mM dithiothreitol, and 50 μL U937 cell fraction (25–150 μg protein) in a total volume of 0.2 mL. In studies of the $Ca^{2+}$ requirement of U937 $cPLA_2$, $Ca^{2+}$ was replaced by EGTA (2 mM). After 30 min at 37°C, incubations were terminated by acidification, and liberated [$^{14}$C]AA extracted into hexane-isopropanol (1:1). The upper organic phase was applied to silica minicolumns, [$^{14}$C]AA eluted with hexane, and quantified by liquid scintillation counting. The recovery of AA through extraction and chromatography was 85 ± 2% ($n$ = 20). To ensure linear kinetics, measurements were made at <3% substrate conversion. This assay is considered to be selective for $cPLA_2$ in view of the instability of type II $PLA_2$ towards the reducing agent dithiothreitol present in the assay medium [24].

Potency of the $cPLA_2$ inhibitor $AACOCF_3$ was determined in a triton X-100 mixed micelle assay using partially purified $cPLA_2$ obtained by cloning the cDNA for U937 cell $cPLA_2$ in yeast cells (kindly provided by Dr. Y. Ivashchenko, Rhône-Poulenc Rorer Ltd.). The enzyme had a specific activity of 4.6 nmol AA/min per mg protein in the assay described. The assay mixture contained 50 μM 1-stearoyl-2-[$^{14}$C]arachidonoyl-phosphatidylcholine substrate (50,000 cpm), 80 mM glycine, pH 8, 200 μM triton X-100, 50% glycerol, 2.5 mM $Ca^{2+}$, 2 mg/mL BSA, and 2 mM dithiothreitol in a total volume of 0.2 mL. [$^{14}$C]AA released during 20 min at 37°C was quantified as described for the liposomal assay. The assay showed linear kinetics up to 10% substrate conversion.

*Determination of type II $PLA_2$ activity*

This assay employing [$^3$H] oleic acid-labelled *E. coli* cells (strain K12C600) was performed essentially as described previously [20, 26] using 50–100 μg cell homogenate protein. It was validated using type II $PLA_2$ in peritoneal exudate fluid from oyster glycogen primed rats [27], which gave concentration-related increases in [$^3$H] oleate release. The assay is widely used to measure type II $PLA_2$ activity because the enzyme shows a preference for substrate presented in the physical form of *E. coli* membranes [20]; it was confirmed using the yeast cell derived material that $cPLA_2$ is not active.

*Immunoprecipitation and Western blotting*

U937 cell incubates ($5 \times 10^7$ cells) were centrifuged (3000 rpm, 1 min), and the cells lysed in 0.5 mL buffer containing 1% triton X-100, 0.5% nonidet P-40, 10 mM tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 10 μg/mL leupeptin, and 25 mM sodium fluoride by continuous agitation at 4°C for 30 min. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. Lysate (600 μg protein) was immunoprecipitated by incubation with $cPLA_2$ antiserum (1 in 500) for 1 hr at 4°C before adding Pansorbin beads (50 μL suspension) and continuously agitating the suspension for 3 hr at 4°C. Beads were centrifuged at 12,000 g for 5 min at 4°C and washed 3 times by resuspension in lysis buffer. The beads were finally suspended in 30 μL 2× sample buffer (125 mM tris HCl, 4% SDS,. 13 mM dithiothreitol, and 0.02% bromophenol blue, with or without 10% glycerol), boiled for 5 min, centrifuged at 12,000 g for 5 min at 4°C, and the supernatant collected for PAGE. PAGE and Western blotting were also performed directly on aliquots of lysate, which were mixed 4:1 with 5× sample buffer and boiled. PAGE employed 7.5% gels with the Pharmacia Phast System or 6% gels with the Novex system and separated proteins were blotted onto nitrocellulose. Blots were blocked with 5% milk powder in PBS/0.1% Tween 20, then incubated with $cPLA_2$ antiserum (1 in 5000) for 1 hr at room temperature. Either HRP conjugated donkey anti-rabbit antiserum (1 in 5000) or gold-conjugated donkey anti-rabbit antiserum (1 in 500) was used as secondary antibody. Bands were visualised according to the recommendations of the suppliers of ECL or Immunogold reagents, respectively.

*Measurement of [$^{33}$P] orthophosphate incorporated into $cPLA_2$*

Differentiated or undifferentiated U937 cells ($5 \times 10^7$) were centrifuged (180 g, 5 min) and washed 3 times with 20 mL phosphate-free buffer (PFB) (10 mM tris, pH 7.4, 150 mM NaCl, 3.7 mM KCl, 1 mM $CaCl_2$, 0.1% glucose, 1 mg/mL BSA). They were resuspended in 0.5 mL PFB containing 125 μCi [$^{33}$P] orthophosphate, incubated at 37°C for 1 hr, then diluted with 0.5 mL PFB. Samples were treated with A23187 (5 μM) plus PMA (30 nM) or with vehicle for 2 min at 37°C before pelleting the cells in a microfuge (3000 rpm, 1 min). Cells were immediately lysed and the lysate immunoprecipitated. Radioactivity in 5 μL aliquots of 2× sample buffer was determined by liquid scintillation counting.

*Statistical analysis*

Unless stated otherwise, data in figures and in the text are means ± SEM from at least three experiments with samples in triplicate or quadruplicate. The interaction between A23187 and PMA in stimulating [$^3$H]AA release from U937 cells was evaluated by analysis of variance followed by the Student-Newman-Keuls multiple comparison test. $P < 0.05$ was considered significant.

## RESULTS

*Effects of DMSO differentiation on [$^3$H]AA release*

[$^3$H]AA release induced by $Ca^{2+}$ ionophore A23187 was measured in undifferentiated cells and in cells cultured in the presence of 1.3% DMSO for up to 96 hr. Incorporation of radiolabel was similar for undifferentiated and differentiated U937 cells (0.05–0.07 μCi/$10^6$ cells). Unstimulated (basal) release of [$^3$H]AA was less than 1% of incorporated radioactivity for undifferentiated cells and less than 2% for DMSO-treated cells at 24,

48, or 96 hr (Fig. 1). A23187 (5 μM) induced an approximate 10-fold increase in [$^3$H]AA release from cells cultured with DMSO for 48 or 96 hr but at best caused only an approximate doubling of release from undifferentiated cells at any time of measurement. The ratio of A23187-induced [$^3$H]AA release from 96 hr DMSO-differentiated cells compared to undifferentiated was 18 ± 3: 1 ($n$ = 8 expts.), and this time of differentiation was adopted for subsequent studies. The amount of incorporated [$^3$H]AA released from differentiated cells by A23187 (up to approx. 20%) was similar to that in another recent study [28]. The incubation with 5 μM A23187 did not reduce cell viability, which was consistently >95%.

Since ligands that bind to G-protein linked receptors mobilise AA in a variety of cells [29], the effect of protein kinase C stimulation was investigated in 96 hr DMSO differentiated cells. PMA (30 nM) alone did not increase [$^3$H]AA release, but it synergised with threshold concentrations (1 and 3 μM) of A23187 (Fig. 2), suggesting that the A23187 dose-response curve was shifted to the left without the maximal response being affected. Somewhat surprisingly, neither FMLP (0.01–1 μM) nor $LTD_4$ (0.01–0.3 μM) induced [$^3$H]AA release, although DMSO differentiated U937 cells have been reported to express receptors for these ligands, stimulation of which elevated intracellular $Ca^{2+}$ [30, 31].

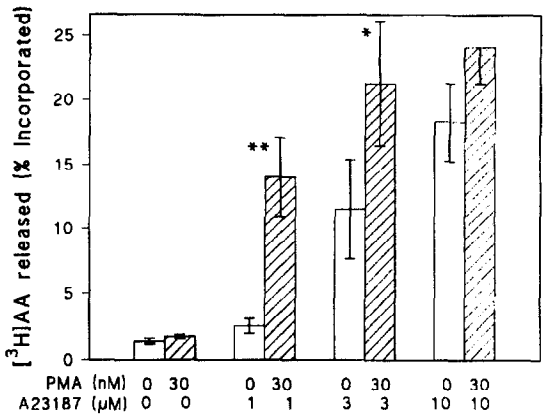


Fig. 2. Synergy between PMA and A23187 in stimulating [$^3$H]AA release from DMSO-differentiated U937 cells. U937 cells (2 × $10^5$/mL) were differentiated with 1.3% DMSO for 96 hr, with [$^3$H]AA being added for the final 18 hr. Washed cells were stimulated for 15 min at 37°C with combinations of PMA (30 nM) and A23187 (1–10 μM) added simultaneously. [$^3$H]AA release was quantified as described in Fig. 1. The significance of the interaction between PMA and A23187 was determined by analysis of variance followed by the Student-Newman-Keuls multiple comparison test. **$P$ < 0.001, *$P$ < 0.01 for PMA + A23187 compared to A23187 alone. Mean ± SEM, n = 3 expts. in quadruplicate.

*Identification of $PLA_2$ responsible for [$^3$H]AA release*

Three approaches were followed to characterise the responsible $PLA_2$. Firstly, $cPLA_2$ and type II activities were measured directly in homogenates of DMSO-differentiated U937 cells using assays selective for the two enzymes. Unlabelled cells were stimulated for 5 min with A23187 (5 μM) and PMA (30 nM) prior to homogenisation, since this provides a strong stimulus for $PLA_2$ activation in the intact cell (Fig. 2). In the $cPLA_2$ assay, the homogenate of differentiated cells consistently increased [$^{14}$C]AA release from 1-stearoyl-2-[$^{14}$C] arachidonoyl phosphatidylcholine above basal release in the presence of homogenising buffer in a totally $Ca^{2+}$-dependent manner (6.0 ± 1.3 fold elevation; $n$ = 3 expts) (Table 1). On the other hand, in the *E. coli* assay, U937 cell homogenate failed to release [$^3$H] oleate (data not shown), suggesting that differentiated U937 cells contain little, if any, type II enzyme.

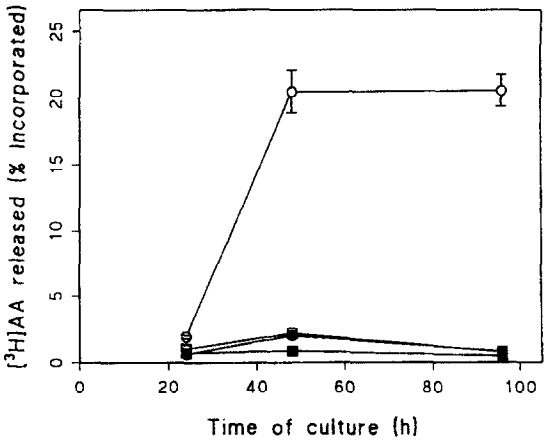


Fig. 1. Effect of DMSO differentiation on stimulated [$^3$H]AA release from prelabelled U937 cells. U937 cells (2 × $10^5$/mL) were cultured in the presence or absence of 1.3% DMSO and allowed to incorporate [$^3$H]AA for the final 18 hr. Cultures were terminated after 24, 48, or 96 hr, and the washed cells stimulated for 15 min at 37°C with A23187 (5 μM) or vehicle. [$^3$H]AA in the supernatant was quantified by liquid scintillation counting and expressed as % of incorporated radioactivity. (■) undifferentiated cells + vehicle; (□) undifferentiated cells + A23187; (●) DMSO differentiated cells + vehicle; (○) DMSO differentiated cells + A23187. Mean ± SD from 1 expt. with quadruplicate samples representative of 3.

Secondly, the effect of arachidonic acid trifluoromethyl ketone ($AACOCF_3$), which was reported to show at least 1000-fold selectivity for inhibiting $cPLA_2$ over type II $PLA_2$ [32], was evaluated on A23187 (5 μM) induced [$^3$H]AA release from pre-labelled DMSO-dif-

Table 1. $Ca^{2+}$-dependent $cPLA_2$ activity is present in homogenates of DMSO-differentiated U937 cells

| Sample | $cPLA_2$ activity (pmol AA/min per mg protein) |
|---|---|
| Homogenising buffer + $Ca^{2+}$ | 0.56 ± 0.25 |
| Homogenate + $Ca^{2+}$ | 2.96 ± 0.95 |
| Homogenate + EGTA | 0.54 ± 0.18 |

U937 cells differentiated for 96 hr with 1.3% DMSO (2.5 × $10^7$ cells total) were washed, then stimulated for 5 min at 37°C with A23187 (5 μM) and PMA (30 nM), before being homogenised at 0–4°C in buffer (1 mL) containing $CaCl_2$ (0.1 mM) and protease and phosphatase inhibitors. $cPLA_2$ activity of aliquots (50 μL) of homogenates was determined as described in Materials and Methods. $Ca^{2+}$-dependence was assessed by replacing $Ca^{2+}$ in the assay buffer with EGTA (2 mM). Data for buffer alone were "corrected" for the protein concentration of the homogenate in each expt. to enable results to be compared as specific activities. Mean ± SEM, $n$ = 3 expts. in triplicate.

ferentiated U937 cells. $AACOCF_3$ dose-dependently inhibited [$^3$H]AA release, achieving 80% inhibition at 30 μM, which was the highest concentration that could be tested without exceeding the ethanol (vehicle) concentration tolerated by the cells (Fig. 3). The concentration-response curve parallelled that for inhibiting enzyme activity of authentic $cPLA_2$ in a mixed micelle assay, and the $IC_{50}$ values for inhibiting the two activities were not significantly different (12.7 ± 1.4 μM for [$^3$H]AA release, 9.3 ± 2.0 μM for $cPLA_2$; $n$ = 3 expts). These data are consistent with $cPLA_2$ being the major source of AA generated on cell stimulation.

In the third approach, we examined the effect of exogenous heparin on A23187-induced [$^3$H]AA release from DMSO-differentiated U937 cells. Heparin has been shown to compete with membrane proteoglycan for binding secreted type II $PLA_2$, thereby preventing membrane de-esterification [16–18]. The cells were incubated with heparin for 10 min at 37°C prior to stimulation with A23187 (5 μM). In three separate experiments, heparin at concentrations up to 1.0 mg/mL affected release by less than ±10%, although this concentration markedly inhibited eicosonoid generation in other cell types in which type II $PLA_2$ is the responsible enzyme [16, 17].

*Effect of cellular stimulation on $cPLA_2$ activity against exogenous substrate*

It has been reported that the specific activity of $cPLA_2$ is increased by phosphorylation resulting from cellular stimulation [e.g. 13]. We determined the effects of stimulating differentiated U937 cells for 2 min with A23187 (5 μM) and PMA (30 nM) on $cPLA_2$ activity of the homogenate against exogenous substrate. After stimulation, the cells were rapidly microfuged (3000 rpm, 1 min), then immediately homogenised in buffer with protease, and phosphatase inhibitors added to minimise the possibility of changing the state of the enzyme on cell disruption. In 3 out of 4 experiments, there was no difference in $cPLA_2$ activity between stimulated and non-stimulated cells, and the overall ratio (stimulated/non-stimulated) was 1.4 ± 0.4: 1 ($n$ = 4 expts) (Table 2).

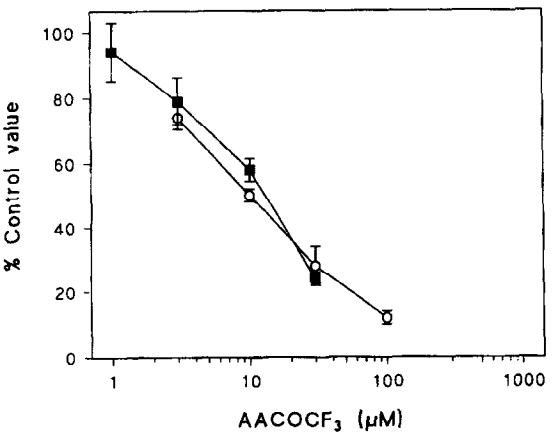


Fig. 3. Comparison of the effects of $AACOCF_3$ on authentic $cPLA_2$ enzyme activity and stimulated [$^3$H]AA release from DMSO-differentiated U937 cells. Effects on enzyme activity were studied in a triton X-100 mixed micelle assay system as described in Materials and Methods. $AACOCF_3$ was equilibrated with substrate, and reaction initiated by adding partially purified $cPLA_2$. Uninhibited activity was 4–5 nmol/min per mg protein. Effects on [$^3$H]AA release were measured in U937 cells ($2 \times 10^5$/mL) differentiated for 96 hr with 1.3% DMSO and labelled with [$^3$H]AA during the final 18 hr of culture. $AACOCF_3$ was incubated with washed cells for 10 min before stimulation for 15 min at 37°C with A23187 (5 μM). [$^3$H]AA released was quantified as described in Fig. 1. Uninhibited release was 13–18% of incorporated radioactivity. (■), U937 cells, (○), $cPLA_2$. Mean ± SEM, $n$ = 3 expts. in triplicate ($cPLA_2$ activity) or quadruplicate ([$^3$H]AA release).

*Comparison of $cPLA_2$ protein concentration and enzyme activity in differentiated and undifferentiated U937 cells*

The extent to which the 18-fold increase in stimulated [$^3$H]AA release from DMSO-differentiated U937 cells reflected an increased cellular content of $cPLA_2$ was investigated. For measurement of $cPLA_2$ mass, identical numbers of undifferentiated or differentiated cells, with or without stimulation by A23187 (5 μM) plus PMA (30 nM), were lysed and identical aliquots of lysate subjected to electrophoresis and Western blotting. Development of the blots with a specific $cPLA_2$ antiserum revealed similar levels of $cPLA_2$ protein in the two cell populations as judged by densitometry (Fig. 4A). $cPLA_2$ activity against exogenous substrate was determined for homogenates stimulated for 5 min with A23187 (5 μM) and PMA (30 nM). Somewhat surprisingly, the activity of DMSO differentiated cells was only 1.6 ± 0.2-fold greater ($n$ = 6 expts) than that of undifferentiated cells (2.27 ± 0.34 compared to 1.53 ± 0.30 pmol AA/min per mg protein). The possibility that $cPLA_2$ may be only transiently activated on cell stimulation was considered. Time-course studies demonstrated that after adding A23187 (5 μM) plus PMA (30 nM) to pre-labelled differentiated U937 cells, the level of [$^3$H]AA in the supernatant was essentially maximal by 5 min and plateaued between 5 and 15 min (Fig. 5), which could imply that the enzyme was down-regulated by the 5 min time point at which specific activity was determined. However, $cPLA_2$ activity in homogenates of cells stimulated for only 2 min, when AA release was still increasing (2.80 ± 0.32 pmol AA/min per mg protein) (Table 2), was not significantly different from that measured at 5 min. $cPLA_2$ activity of cell homogenates was thus similar for non-differentiated and DMSO-differentiated cells.

Increased AA generation in PMA-differentiated U937 cells was ascribed to increased membrane association of $cPLA_2$ in the resting cell [22]. The membrane/cytosol distribution of $cPLA_2$ in unlabelled DMSO differentiated and undifferentiated cells was measured after homogenisation in buffer without added $Ca^{2+}$, a procedure similar to that used by the previous workers [22]. The cells were not stimulated before homogenisation. In contrast to the findings in PMA-differentiated cells, the fraction of membrane-bound $cPLA_2$ activity in DMSO-differentiated cells (calculated from total $cPLA_2$ activities in membrane and cytosol) was actually lower in 2 out of 3 experiments, and overall there was no significant difference between mean values (18.7 ± 2.8% for undifferentiated, 13.4 ± 4.0% for differentiated) (Table 3A). In all three experiments, $cPLA_2$ activity in cytosol was higher in DMSO-differentiated cells (Table 3B), whereas in PMA-differentiated cells cytosolic activity was reported to decline [22]. Furthermore, the ratio of $cPLA_2$ activities in cytosol for DMSO-differentiated compared to undifferentiated cells (2.0 ± 0.2: 1, $n$ = 3

Table 2. Effect of stimulation with (A23187 + PMA) on $cPLA_2$ activity in homogenates of differentiated U937 cells

| Experiment | $cPLA_2$ activity in homogenate (pmol AA/min per mg protein) | | |
|---|---|---|---|
| | Non-stimulated | (A23187 + PMA) | (A23187 + PMA)/Non-stimulated |
| 1 | 2.4 | 2.5 | 1.0 |
| 2 | 3.9 | 3.7 | 1.0 |
| 3 | 2.1 | 2.3 | 1.1 |
| 4 | 1.1 | 2.6 | 2.4 |

U937 cells differentiated for 96 hr with 1.3% DMSO ($2.0–2.5 \times 10^7$ cells per treatment) were washed, then incubated for 2 min at 37°C with A23187 (5 μM) plus PMA (30 nM) or with vehicle in $Ca^{2+}/Mg^{2+}$-HBSS buffer (1 mL). Cells were rapidly centrifuged (1 min), then immediately homogenised at 0–4°C in buffer (1 mL) containing 0.1 mM $CaCl_2$ and protease and phosphatase inhibitors. $cPLA_2$ activity of aliquots (50 μL) of the homogenates was determined as described in Materials and Methods. Mean values from triplicate determinations in each experiment.

expts) was similar to the ratio of activities in the cell homogenate (1.6 ± 0.2: 1), again consistent with there being no marked change in intracellular distribution of $cPLA_2$ during differentiation.

### *Is $cPLA_2$ activated without phosphorylation in DMSO-differentiated cells?*

The question of whether a difference in extent of phosphorylation of $cPLA_2$ on cellular activation accounts for the difference in AA release between non-differentiated and differentiated cells was addressed in two ways: by direct measurement of [$^{33}$P] orthophosphate incorporated into $cPLA_2$ immunoprecipitates, and by looking for an electrophoretic mobility shift due to the slower-moving phosphorylated $cPLA_2$ [25, 33]. Control studies in which U937 cell lysates were immunoprecipitated and analysed by Western blotting confirmed the ability of the antiserum to immunoprecipitate $cPLA_2$ (Fig. 4B). Cells ($5 \times 10^7$) incubated for 60 min in medium containing [$^{33}$P] orthophosphate as the only phosphate source had low basal incorporation of radiolabel into $cPLA_2$, which was 2–3 fold higher in DMSO-differentiated cells (non-differentiated 369 ± 12 cpm/5 μL, differentiated 1111 ± 141 cpm/5 μL; mean ± range, $n$ = 2 expts). In both cell types no increase in [$^{33}$P] labelling of $cPLA_2$ immunoprecipitates resulted from stimulation for 2 min with A23187 (5 μM) plus PMA (30 nM) before preparing cell lysates (non-differentiated 337 ± 14 cpm/ 5 μl, differentiated 970 ± 92 cpm/5 μl; mean ± range, $n$ = 2 expts). This was unlikely to be an artefact due to a poor physiological state of the cells, since they retained good viability in the two experiments (undifferentiated 95, 90%; differentiated 93, 85%). Furthermore, the antibody has been shown to recognise phosphorylated $cPLA_2$ [25]. In additional studies with unlabelled non-differentiated or differentiated cells, identical numbers ($5 \times 10^7$) were stimulated for 2 min with A23187 (5 μM) plus PMA (30 nM), or treated with vehicle before being lysed, and aliquots of lysate subjected to Western blotting. Electrophoresis was allowed to continue until the 97 kDa marker neared the top of the gel in order to increase the resolution of non-phosphorylated and phosphorylated $cPLA_2$ [25, 33]. All samples showed a similar pattern of one major band of molecular size expected for $cPLA_2$ and a minor band with slightly greater migration (Fig. 4A). There was no evidence of a slower-moving component resulting from A23187 plus PMA stimulation of DMSO differentiated cells.

## DISCUSSION

### *$cPLA_2$ mediation of increased AA generation in DMSO-differentiated U937 cells*

It is unwise to assign functions in U937 cells to different $PLA_2$ enzymes based on activity measurements alone, since these cells have been reported to contain endogenous materials that interfere with the determination of both type II $PLA_2$ [34] and $cPLA_2$ [10, 11]. The fact that we could measure only $cPLA_2$ activity in homogenates thus does not necessarily preclude the involvement of type II $PLA_2$ in AA release. On the other hand, the observation that the $cPLA_2$ inhibitor $AACOCF_3$ inhibited A23187-induced [$^3$H]AA release from DMSO-differentiated cells by at least 80% is more persuasive evidence that $cPLA_2$ is responsible for the great majority of AA generation. This compound inhibits $cPLA_2$ by forming a stable hemiketal with a serine or threoinine group involved in the catalytic mechanism [32, 35]. Its reported selectivity of more than 1000 fold for $cPLA_2$ over type II $PLA_2$ [32] probably reflects the fact that such groups are not required for catalysis by type II $PLA_2$ [36]. The similarity of $AACOCF_3$ concentration-response curves for inhibiting $cPLA_2$ activity and stimulated [$^3$H]AA release from U937 cells, and similar $IC_{50}$ values (9.3 ± 2.0 and 12.7 ± 1.4 μM, respectively) strongly suggest a causal relationship between these two activities of $AACOCF_3$. Whilst this work was in progress, another group reported that the rank order of potency of analogues of $AACOCF_3$ in inhibiting AA release from U937 cells correlated with that for inhibiting $cPLA_2$ [37], $AACOCF_3$ itself having an $IC_{50}$ against release (8 μM) similar to our own value. Our results corroborate those findings and support the validity of using $AACOCF_3$ as a pharmacological tool for investigating the role of $cPLA_2$.

Evidence that type II $PLA_2$ acts by being secreted, and binding to the external surface of the cell, has arisen from the observations that exogenously added type II $PLA_2$ can amplify eicosanoid release [18, 38], and that agonist-induced release is inhibited by antibodies to type II $PLA_2$ [18, 38], by exogenous addition of competing glycoproteins such as heparin [16–18] or by enzymatic removal of membrane proteoglycan with heparitinases [17]. The fact that heparin, at a concentration that inhibited type II $PLA_2$ mediated eicosanoid generation in other cell types [16, 17], had no effect on A23187-induced [$^3$H]AA release from DMSO-differentiated U937

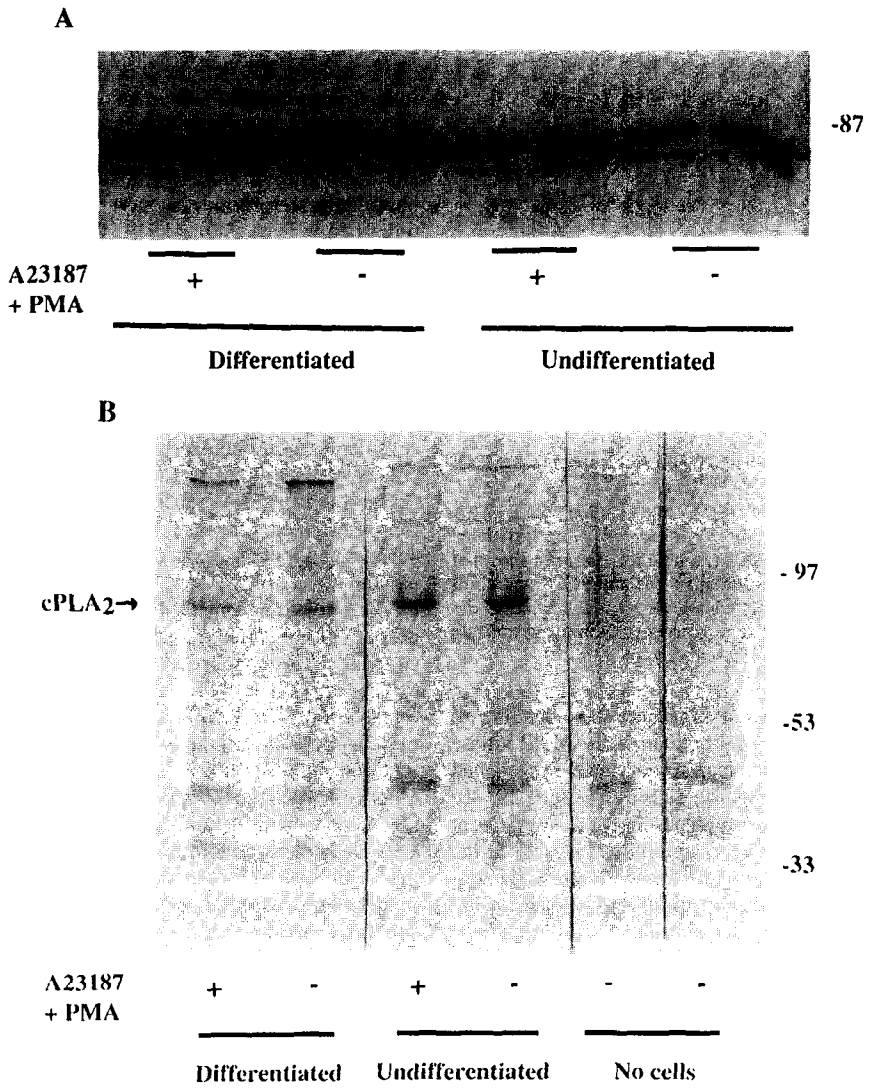


Fig. 4. Quantification of $cPLA_2$ in U937 cell lysates by Western blotting and immunoprecipitation. Identical numbers ($5 \times 10^7$ cells) of 96 hr DMSO-differentiated or undifferentiated cells were incubated for 2 min at 37°C with A23187 (5 μM) plus PMA (30 nM) or vehicle before being lysed. (A) Identical aliquots of lysate were subjected to PAGE on a 6% gel. $cPLA_2$ on Western blots was visualized with specific $cPLA_2$ antiserum and ECL detection. For the blot shown, electrophoresis was continued until the 97 kDa marker neared the top of the gel. (B) Identical aliquots of lysates of stimulated differentiated or undifferentiated cells were immunoprecipitated with $cPLA_2$ antiserum, and immunoprecipitated proteins separated by PAGE on a 7.5% gel. $cPLA_2$ on Western blots was visualised with $cPLA_2$ antiserum and Immunogold detection. Single representative experiments shown.

cells is supportive evidence of the lack of involvement of this isoform. The current studies thus extended previous observations on eicosanoid generation in DMSO-differentiated U937 cells by showing by two criteria that type II $PLA_2$ is not involved, but that $cPLA_2$ is causally linked to AA release. This conclusion is further strengthened by the recent observation that A23187 stimulated the release of [$^3$H]AA, but not that of [$^3$H] oleate, from pre-labelled, DMSO-differentiated U937 cells [28], consistent with the known *in vitro* selectivity of $cPLA_2$ [10–12].

*Regulation of $cPLA_2$ activity in U937 cells*

Whilst $cPLA_2$ was shown to be responsible for AA generation in DMSO-differentiated cells, the lack of response to A23187 stimulation of undifferentiated cells was clearly not due to a lack of $cPLA_2$ protein or a lack of functional catalytic activity in broken cell preparations. The fact that other workers have purified $cPLA_2$ from undifferentiated U937 cells also testifies to the presence of significant amounts of the enzyme in these cells [10, 12]. The proposal of Rehfeldt and co-workers [22] that increased AA release from PMA-differentiated U937 cells resulted from increased membrane association of $cPLA_2$ in the resting cell (3-fold elevation) was not tenable for DMSO differentiated cells. It is difficult to interpret unambiguously the results from enzyme distribution studies because the association of $cPLA_2$ with membrane through its $Ca^{2+}$-lipid binding domain is relatively weak [11, 14], and the proportion bound may change if the $Ca^{2+}$ concentration changes on cell disruption. Nevertheless, using a homogenizing buffer without added $Ca^{2+}$, similar to the procedure of Rehfeldt and co-workers, we saw neither an increase in the membrane/cytosol ratio in differentiated cells nor a decrease in cytosolic $cPLA_2$ activity.

It has been shown in a number of cell types that cel-

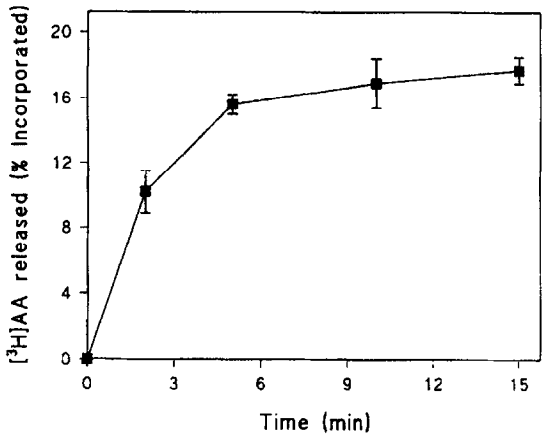


Fig. 5. Time course of stimulated [$^3$H]AA release from DMSO-differentiated U937 cells. U937 cells ($2 \times 10^5$/mL) were differentiated for 96 hr with 1.3% DMSO, and labelled with [$^3$H]AA during the final 18 hr of culture. Washed cells were stimulated with A23187 (5 μM) and PMA (30 nM) for the times shown at 37°C before stopping the reaction by chelating $Ca^{2+}$ and chilling the samples on ice. [$^3$H]AA release was quantified as described in Fig. 1. Mean ± SD, 1 experiment with quadruplicate samples representative of 3.

lular stimulation induces a kinase cascade leading to $cPLA_2$ phosphorylation and activation by MAP kinase [13, 39]. In most reports, the phosphorylated enzyme had a 3–4 fold increase in specific activity [13, 33, 39, 40] and, more importantly, was able to bind to membranes at lower (intracellular) $Ca^{2+}$ concentrations [41, 42]. In the present studies, the synergy of PMA with threshold concentrations of A23187 in inducing [$^3$H]AA release from DMSO-differentiated cells is consistent with these cells being able to initiate a kinase cascade. However, there was no evidence that $cPLA_2$ is phosphorylated following stimulation of either differentiated or undifferentiated cells, as deduced from the lack of [$^{33}$P] orthophosphate incorporation and the lack of an observed electrophoretic mobility shift due to the slower-moving phosphorylated enzyme [25, 33]. It is possible that both these techniques lack the sensitivity to detect a small increment in phosphorylation, the former because of low specific radioactivity of the intracellular [$^{33}$P] ATP pool, and the latter because of methodological difficulties in resolving adequately phosphorylated and non-phosphorylated $cPLA_2$ by electrophoresis [33]. On the other hand, the implication that increased AA release from differentiated U937 cells occurs in the absence of $cPLA_2$ phosphorylation is not inconsistent with the known properties of the enzyme, in that the non-phosphorylated form can bind to cell membranes, and exert catalytic activity, at the $Ca^{2+}$ concentrations typically found in an activated cell [43]. Furthermore, A23187 is likely to induce a larger, and more sustained, increase in intracellular $Ca^{2+}$ than is achieved following receptor stimulation, and this could explain why FMLP and $LTD_4$ failed to stimulate [$^3$H]AA release from DMSO differentiated cells [44], despite the reported presence of functional receptors coupled to phospholipase C and elevated intracellular $Ca^{2+}$ [30, 31]. The ability of A23187 to achieve maximal release of [$^3$H]AA without requiring PKC stimulation may support the argument that phosphorylation is not required, but it is not conclusive, since in at least one other cell type A23187 stimulation was shown to induce $cPLA_2$ phosphorylation, presumably by activating a $Ca^{2+}$-dependent protein kinase [40]. The failure of cellular stimulation to increase $cPLA_2$ activity in homogenates of differentiated cells could be considered supportive evidence that the enzyme is not extensively phosphorylated in view of the reports noted above

Table 3. Effect of DMSO differentiation on distribution of $cPLA_2$ activity between cytosol and membrane fractions

(A)

| | % membrane – associated $cPLA_2$ activity | | |
|---|---|---|---|
| Experiment | Undifferentiated | Differentiated | Differentiated/undifferentiated |
| 1 | 23.4 | 14.5 | 0.62 |
| 2 | 19.1 | 19.7 | 1.0 |
| 3 | 13.7 | 6.1 | 0.45 |

(B)

| | $cPLA_2$ activity in cytosol (pmol AA/min per mg protein) | | |
|---|---|---|---|
| Experiment | Undifferentiated | Differentiated | Differentiated/undifferentiated |
| 1 | 5.5 | 12.5 | 2.3 |
| 2 | 5.9 | 12.5 | 2.1 |
| 3 | 7.2 | 11.7 | 1.6 |

Undifferentiated or 96 hr DMSO-differentiated U937 cells ($2.5 \times 10^7$ cells per treatment) were washed, then homogenised at 0–4°C in buffer (1 mL) containing protease and phosphatase inhibitors but without added $Ca^{2+}$. The homogenates were centrifuged at 100,000 g for 60 min, and the membrane pellet resuspended in homogenising buffer (1 mL). $cPLA_2$ activity of aliquots (50 μL) of membrane and cytosol fractions was determined as described in Materials and Methods. (A) % membrane-associated $cPLA_2$ activity calculated from total $cPLA_2$ activity of membrane and cytosol fractions. (B) actual $cPLA_2$ specific activity in cytosols from the same three experiments. Mean values from triplicate determinations in each experiment.

demonstrating an increase in specific activity on phosphorylation. Stimulated [$^3$H]AA release in the absence of a change in $cPLA_2$ specific activity could thus be explained by release requiring the phosphorylation of only a small proportion of the total pool of $cPLA_2$ (undetectable in the present studies) or by increased membrane association of the non-phosphorylated enzyme in response to elevated intracellular $Ca^{2+}$.

The aforegoing discussion does not adequately explain why DMSO-differentiated cells generated more AA than undifferentiated cells. Increased release was not merely a consequence of increased incorporation of radiolabel into membrane phospholipids, since uptake did not change during differentiation. Furthermore, there was shown to be no difference in mass of esterified AA between undifferentiated and PMA-differentiated U937 cells [7], which show a similar increase in AA-generating capacity to DMSO-differentiated cells. A decrease in the activity of lysophosphatide acyltransferase, which re-incorporates free AA into membrane phospholipids, could lead to increased AA release in the absence of elevated $cPLA_2$ activity. As far as we are aware, the activity of this enzyme in DMSO-differentiated U937 cells has not been reported and is worthy of future study, although no difference was found between undifferentiated and phorbol ester differentiated U937 cells [7]. A redistribution of AA to membrane phospholipids that are more preferred substrates for $cPLA_2$ could theoretically improve the efficiency of catalysis, but this seems unlikely since in undifferentiated U937 cells AA is primarily incorporated into phosphatidyl ethanolamine [45], which is a good substrate for $cPLA_2$ [42]. The most likely explanation for the low activity of undifferentiated cells is either that they lack an essential factor required to couple elevated intracellular $Ca^{2+}$ to functional $cPLA_2$ activity or that they contain an endogenous inhibitor or repressor of $cPLA_2$ activity. It would be hypothesized that the repressor function is lost on differentiation or when activity is measured in broken cell preparations in assays employing exogenous substrate and high (mM) $Ca^{2+}$ concentrations. With regard to the first possibility, it is of interest that a similar suggestion has been made for HL60 granulocytes, where differentiation with dibutyryl cyclic AMP also led to a marked increase in stimulated eicosanoid generation attributable to $cPLA_2$, whereas $cPLA_2$ activity in homogenates was not increased [46]. With regard to the second possibility, there are numerous reports of endogenous cellular inhibitors of $PLA_2$, including inhibitors of $cPLA_2$ in U937 cell homogenates [10, 11] that remain to be characterised.

In summary, $cPLA_2$ was shown to mediate increased AA release induced by A23187 in DMSO-differentiated U937 cells. Stimulated release did not reflect increased $cPLA_2$ specific activity *per se*, but probably resulted from increased $Ca^{2+}$-dependent membrane association of the enzyme. The enhanced ability to generate AA accompanying differentiation was not caused by a large increase in cellular content of $cPLA_2$ or a change in membrane/cytosol distribution of the enzyme in the resting cell, but appeared due to the removal of a repressor that prevents functional activation of $cPLA_2$ in undifferentiated cells. Further work is required to characterise the nature of this postulated repressor and to determine unequivocally whether or not partial phosphorylation of the $cPLA_2$ pool is required for increased AA release from DMSO-differentiated cells.

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