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Expression of Cytosolic Phospholipase $A_2\alpha$ in Murine C12 Cells, a Variant of L929 Cells, Induces Arachidonic Acid Release in Response to Phorbol Myristate Acetate and Ca^{2+} Ionophores, but Not to Tumor Necrosis Factor-α

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Abstract. Tumor necrosis factor-α (TNFα)-induced cell death is regulated through the release of arachidonic acid (AA) by group IVA cytosolic phospholipase A_2 (cPLA$_2\alpha$) in the murine fibroblast cell line L929. However, the signaling pathway by which TNFα activates cPLA$_2\alpha$ remained to be solved. We examined AA release in L929 cells, in a variant of L929 (C12 cells) lacking cPLA$_2\alpha$, and in C12 cells transfected with cPLA$_2\alpha$ expression vectors. In transient and stable clones of C12 cells expressing cPLA$_2\alpha$, Ca^{2+} ionophore A23187 and phorbol myristate acetate (PMA) stimulated AA release within 90 min, although no response to TNFα was observed within 6 h. These results suggest that C12 cells may lack the components necessary for TNFα-induced AA release, in addition to cPLA$_2\alpha$. PMA is known to stimulate AA release via phosphorylation of Ser^{505} in cPLA$_2\alpha$ by activating extracellular signal-regulated kinases (ERK1/2). However, PMA-induced AA release from C12 cells expressing mutant cPLA$_2\alpha$S505A (mutation of Ser^{505} to Ala), which is not phosphorylated by ERK1/2, was similar to that from L929 cells and C12 cells expressing wild-type cPLA$_2\alpha$. The role of Ser^{505} phosphorylation in AA release induced by PMA is also discussed.

Keywords: cytosolic phospholipase $A_2\alpha$, tumor necrosis factor, Ca^{2+} ionophore, phorbol myristate acetate, L929 and C12 cells

Introduction

Tumor necrosis factor α (TNFα) exerts cytotoxic action against several cells including mouse L929 fibrosarcoma cells and fibroblasts (1 – 4). TNFα-evoked responses such as cell death (1 – 3), the accumulation of reactive oxygen species (4, 5), and gene expression (6, 7) are mediated by the activation of phospholipase A_2 (PLA_2) and/or arachidonic acid (AA). Group IVA cytosolic PLA_2 (cPLA$_2\alpha$) selectively releases AA from the *sn*-2 position of glycerophospholipids (8, 9). The L929 variant C12, which expresses undetectable levels of cPLA$_2\alpha$, is resistant to TNFα-induced AA release and cell death (1, 2, 10). The C12 cell line was found to be analogous to the parental L929 cell line in all parameters of TNFα receptor binding and internalization, and transfection of C12 cells with cPLA$_2\alpha$ cDNA recovered TNFα-induced AA release and cell death (1, 10). Since TNFα was able to activate nuclear factor κB (NFκB) in both L929 cells and C12 cells (3), functions of the TNFα receptor appears to be intact in C12 cells. Clones of C12 cells expressing cPLA$_2\alpha$ appear to be useful for studying TNFα-induced signaling pathways, particularly the mechanism of cPLA$_2\alpha$ activation. In the present study, we tried to express cPLA$_2\alpha$ protein in C12 cells using two vector systems (pcDNA4/HisMax and pEB6 CAG).

Activation of cPLA$_2\alpha$ is regulated by phosphorylation and Ca^{2+}-dependent translocation from the cytosol to membranes (8, 9). The presence of a consensus phosphorylation site for mitogen-activated protein kinase (MAPK) at Ser^{505} of cPLA$_2\alpha$ has led to studies

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demonstrating the role of MAPKs including extracellular signal-regulated kinases (ERK1/2) in the phosphorylation and activation of this enzyme (8, 9). One proposed model for TNFα-induced AA release is that stimulation of the TNFα receptor (type 1, p55) causes the activation of a ceramide-activated protein kinase that phosphorylates Raf-1 kinase, which eventually results in the phosphorylation and activation of ERK1/2 (11). ERK1/2 in turn phosphorylates and activates cPLA$_2\alpha$ (8, 9). However, cPLA$_2$ activation is a necessary component in the pathways leading to TNFα-induced ceramide accumulation in L929 cells (3). Thus, TNFα-induced signaling pathways coupled with activation of cPLA$_2\alpha$ are not fully elucidated. TNFα-induced responses were regulated by a protein kinase C (PKC) pathway (12, 13) and a role for PKC in the regulation of cPLA$_2\alpha$ and AA release has been shown (8, 14, 15). Activation of PKC can trigger a kinase cascade leading to activation of ERK1/2 and the resulting release of AA (8, 15, 16), but the relationship between PKC and the MAPK pathways in AA release is still largely unknown.

In this study, we established two stable C12 cell clones expressing wild-type cPLA$_2\alpha$ and mutant cPLA$_2\alpha$ (Ser^{505} mutation) and examined the release of AA induced by phorbol myristate acetate (PMA), A23187 and TNFα. In stable clones of C12 cells expressing wild-type cPLA$_2\alpha$, PMA and A23187 stimulated AA release but no response to TNFα was observed. Our findings also suggest that the PMA-induced release was not mediated by phosphorylation of Ser^{505} in cPLA$_2\alpha$. We discuss the possibility that C12 cells lack the component(s) essential for TNFα-induced AA release in addition to cPLA$_2\alpha$ and the role of Ser^{505} phosphorylation in AA release induced by PMA.

Materials and Methods

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (214 Ci/mmol, 7.92 TBq /mmol) and 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine (48 mCi/mmol, 1776 MBq/mmol) were purchased from Amersham Bioscience (Buckinghamshire, UK) and Perkin Elmer (Boston, MA, USA), respectively. TNFα was obtained from Pepro Tech EC Ltd. (London, UK). A23187, ionomycin, and PMA were purchased from Sigma (St. Louis, MO, USA). The concentrations of reagents were the same as those in previous reports (14, 17, 18). A23187, ionomycin, and PMA were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide in the medium was under 0.5%. The vehicle containing dimethyl sulfoxide did not stimulate AA release for 6 h.

Cell culture, construction of expression vectors, and isolation of stable clones

L929 cells, C12 cells, and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Thermo Trace Ltd., Noble Park, Australia), 200 U/mL penicillin G sodium, and 200 μg/mL streptomycin sulfate (10, 19). The pcDNA4/HisMax and pEB6 CAG mammalian expression vectors for human cPLA$_2\alpha$ were prepared according to the standard protocol. The vectors for mutants cPLA$_2\alpha$ were prepared using a QuickChange kit (Stratagene, La Jolla, CA, USA) and respective primers. For instance, cPLA$_2\alpha$S505A refers to the mutation of the Ser residue at position 505 to Ala in wild-type cPLA$_2\alpha$. The primers for cPLA$_2\alpha$S505A were CAC ATC ATA TCC ACT GGC TCC CCT GAG AGA GAC TTC AGC and its complement; for cPLA$_2\alpha$S228A, they were CGT TGC TGG TCT TGC GGG CTC CAC CTG G and its complement. The entire coding regions of mutant cPLA$_2\alpha$ vectors were confirmed by DNA sequencing. The vectors for chimeric proteins containing green fluorescent protein (GFP) at the amino terminus of wild-type cPLA$_2\alpha$ and mutant cPLA$_2\alpha$ were prepared as previously reported (17). Transfection was performed with LipofectAMINE (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transfection efficiencies were about 70 – 80% and 5 – 10% in the HEK293T cells and C12 cells transfected with pEGFP-CI-cPLA$_2\alpha$, respectively, and 70 – 80% in the C12 cells transfected with pEB6 CAG-GFP.

C12 cells stably expressing wild-type cPLA$_2\alpha$ and mutant cPLA$_2\alpha$ were generated as previously described (17). Briefly, pcDNA4/HisMax-cPLA$_2\alpha$ and pPUR encoding the puromycin resistance gene (CLONTECH, Palo Alto, CA, USA) were transfected into C12 cells. Clones resistant to puromycin (10 μg/mL, Sigma) were isolated by limiting dilution, tested for cPLA$_2\alpha$ expression, and maintained in the presence of puromycin. The expression of cPLA$_2\alpha$ was confirmed by immunoblotting using anti-cPLA$_2\alpha$ monoclonal antibody (4-4B-3C; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and by measurement of PLA$_2$ activity, as previously reported (19, 20). For immunoblotting, 30 μg of protein per lane was applied to a sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. The levels of β-tubulin in the respective samples from tested cells were almost the same. For the measurement of PLA$_2$ activity, 12.5 μg of protein from cell lysate was used per tube. The values of cPLA$_2$ activity per tube were about 1500 dpm and 500 dpm in HEK293T and C12 cells expressing cPLA$_2\alpha$, respectively, and 200 – 300 dpm in

control HEK293T cells and C12 cells transfected with the control vector.

AA release and PLA_2 activity

The release of AA from intact cells was determined as previously reported (10, 19). Briefly, cells (5×10^4) on 12-well plates were labeled overnight with 0.1 μCi/well of [^{3}H]AA. Cells were washed three times with DMEM supplemented with 10 mM HEPES (pH 7.4) and 0.1% fatty acid-free bovine serum albumin (Sigma, A-7511). Cells were stimulated with 10 μM A23187 for 30 min, 100 nM PMA for 90 min, or 10 nM TNFα for 6 h at 37°C. In some experiments, cells were stimulated for the indicated time with the reagents. Then, the medium was collected and centrifuged at $8{,}000 \times g$ for 5 min. ^{3}H Radioactivity released into the supernatant was expressed as a percentage of the total incorporated radioactivity (20,000 – 40,000 dpm per well). $cPLA_2$ activity was measured using 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine as the substrate as previously described (19).

Confocal microscopy

The translocation of GFP-$cPLA_2\alpha$ protein in cells was determined with a confocal laser scanning microscope (Olympus, Tokyo), as previously described (17).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR analysis was carried out as previously described (20). The primers for mouse TNFα receptors (p55 and p75) were described by Pan et al. (21).

Data presentation

Data concerning immunoblotting and confocal microscopy are from a typical experiment involving two or three independent experiments. For the measurement of AA release and PLA_2 activity, values are the mean ± S.D. for three determinations in a typical experiment, and data are representative of three independent experiments. A statistical analysis for AA release was not performed, since the values were changeable depending on the individual experiment. The variation between each experiment was large and less than 50%.

Results

TNFα-, Ca^{2+} ionophore-, and PMA-induced AA release from L929 and C12 cells

C12 cells expressed undetectable levels of $cPLA_2\alpha$ unlike the control L929 cells (Fig. 1, Panel A), as previously reported (2, 10). Addition of 10 nM TNFα markedly caused AA release for 6 h from L929 cells

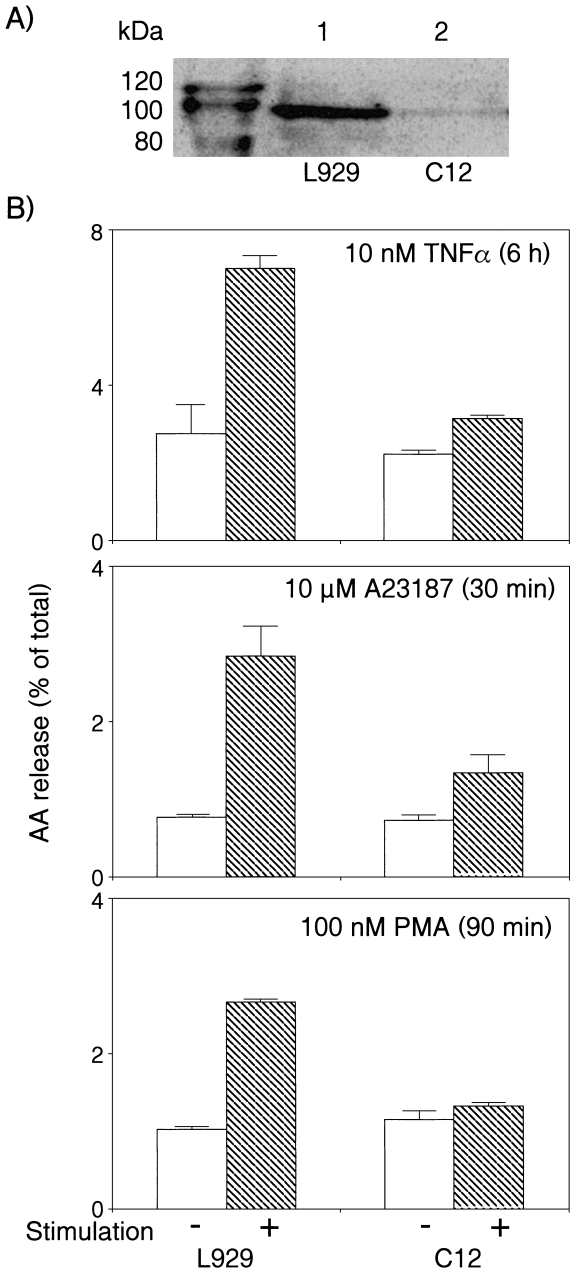

Fig. 1. AA release induced by TNFα, A23187, and PMA in L929 and C12 cells. Soluble fractions from both L929 and C12 cells were analyzed by Western blotting using anti-$cPLA_2\alpha$ antibody in Panel A. In Panel B, labeled L929 and C12 cells on dishes were incubated with 10 nM TNFα for 6 h, 10 μM A23187 for 30 min, or 100 nM PMA for 90 min. ^{3}H Contents in supernatants were calculated as percentages of the total incorporation of [^{3}H]AA. Data are the mean ± S.D. of three determinations in a typical experiment involving three representative experiments.

(Panel B). No response to TNFα in L929 cells was detected in the period for 30 min – 4 h after the addition under our conditions, as previously reported (3, 10). Thus, we measured AA release for 6 h induced by 10 nM TNFα in subsequent experiments. Addition of 30 and

100 nM TNFα caused significant AA release for 6 h from C12 cells in some experiments, although the response in C12 cells was much weaker than that in L929 cells. Addition of 10 μM A23187 caused AA release for 30 min in L929 cells. The AA release induced by A23187 from C12 cells was much weaker than that in L929 cells. Addition of 100 nM PMA, a direct activator of PKC, alone caused AA release for 90 min in L929 cells, but not in C12 cells. The effect of PMA on AA release for 30 min in L929 cells was marginal, but 100 nM PMA enhanced the A23187 (10 μM)-induced AA release for 30 min by about twofold; AA release was 1.9 (% of total) with vehicle, 1.6% with PMA alone, 3.7% with A23187 alone, and 6.7% with PMA plus A23187 in a typical experiment.

Expression of cPLA$_2\alpha$ in HEK293T and C12 cells using a transient expression system with pcDNA4/HisMax

First, we examined the effect of the vector pcDNA4/HisMax on the expression of cPLA$_2\alpha$ in HEK293T cells (Fig. 2, Panels A – C). cPLA$_2\alpha$ was expressed in native HEK293T cells, but at quite low levels. Transfection of HEK293T cells with the cPLA$_2\alpha$ expression vectors (wild-type, S228A, and S505A) caused a marked expression of cPLA$_2\alpha$ protein (Fig. 2, Panel A). The PLA$_2$ activity in the soluble fractions from HEK293T cells expressing wild-type cPLA$_2\alpha$ or cPLA$_2\alpha$S505A was much stronger than that in the control cells (Panel B). The PLA$_2$ activity in HEK293T cells expressing cPLA$_2\alpha$S228A was quite low, as previously reported (8). Next we investigated the effect of a Ca^{2+} ionophore (ionomycin) on the localization of GFP-cPLA$_2\alpha$ (Panel C). Stimulation of the cells with

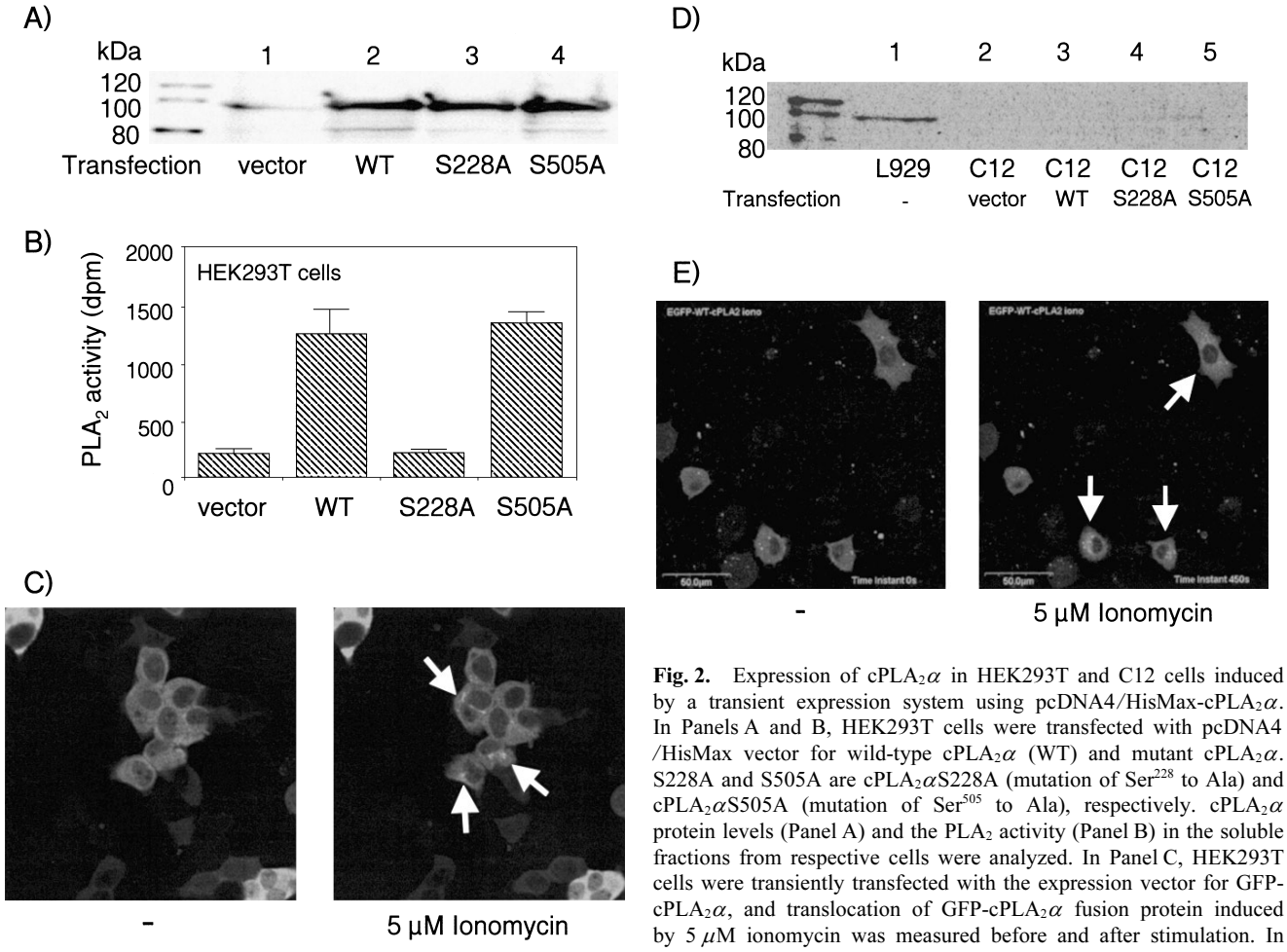

Fig. 2. Expression of cPLA$_2\alpha$ in HEK293T and C12 cells induced by a transient expression system using pcDNA4/HisMax-cPLA$_2\alpha$. In Panels A and B, HEK293T cells were transfected with pcDNA4 /HisMax vector for wild-type cPLA$_2\alpha$ (WT) and mutant cPLA$_2\alpha$. S228A and S505A are cPLA$_2\alpha$S228A (mutation of Ser^{228} to Ala) and cPLA$_2\alpha$S505A (mutation of Ser^{505} to Ala), respectively. cPLA$_2\alpha$ protein levels (Panel A) and the PLA$_2$ activity (Panel B) in the soluble fractions from respective cells were analyzed. In Panel C, HEK293T cells were transiently transfected with the expression vector for GFP-cPLA$_2\alpha$, and translocation of GFP-cPLA$_2\alpha$ fusion protein induced by 5 μM ionomycin was measured before and after stimulation. In Panel D, C12 cells were transfected with the pcDNA4/HisMax expression vector for wild-type and mutant cPLA$_2\alpha$. cPLA$_2\alpha$ protein levels in the soluble fractions from respective cells were analyzed. In Panel E, C12 cells were transiently transfected with the expression vector for GFP-cPLA$_2\alpha$, and the translocation of GFP-cPLA$_2\alpha$ induced by 5 μM ionomycin was measured before and after stimulation.

5 μM ionomycin triggered the translocation of GFP-cPLA$_2\alpha$ to the perinuclear region within 1 to 2 min, as previously reported (9). Similarly, ionomycin triggered the translocation of GFP-cPLA$_2\alpha$S505A (data not shown), as previously reported (18). Stimulation with 100 nM PMA did not cause translocation 2 and 10 min after the addition (data not shown). Although cPLA$_2\alpha$ was expressed in the transfected HEK293T cells and functional (enzyme activity and translocation), the addition of a Ca^{2+} ionophore (10 μM A23187 and 5 μM ionomycin) had a marginal or no effect on AA release from the cells (data not shown).

Transfection of C12 cells with these vectors caused little expression of wild-type cPLA$_2\alpha$ and mutant-cPLA$_2\alpha$ proteins (Fig. 2, Panel D). The application of 5 μM ionomycin triggered the translocation of GFP-cPLA$_2\alpha$ to the perinuclear region within 1 to 2 min in the GFP-positive cells (Panel E). The application of 10 μM A23187 showed similar results (data not shown). Treatment with 10 nM TNFα for 6 h, 10 μM A23187 for 30 min in the presence and absence of 100 nM PMA, and 100 nM PMA for 90 min did not stimulate AA release from these vector-transfected C12 cells (data not shown).

Expression of cPLA$_2\alpha$ in C12 cells using pEB6 CAG

Next, we tried to express wild-type cPLA$_2\alpha$ in C12 cells using the expression vector pEB6 CAG, which is useful for a relatively stable expression of proteins (22). In the conditions tested, transfection of C12 cells with pEB6 CAG-cPLA$_2\alpha$ induced marked expression of cPLA$_2\alpha$ protein (Fig. 3, Panel A). The release of AA induced by 10 μM A23187 was greater in C12 cells expressing cPLA$_2\alpha$ than in the native C12 cells or the control cells transfected with pEB6 CAG-GFP (Panel B). However, no TNFα-induced release was detected in C12 cells expressing cPLA$_2\alpha$.

AA release from stable clones of C12 cells expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A

We established two clones of C12 cells expressing wild-type cPLA$_2\alpha$ and two clones of C12 cells expressing cPLA$_2\alpha$S505A (Fig. 4). Although the expression levels of cPLA$_2\alpha$ were dependent on the respective clones, the protein levels of wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A were much higher in these stable clones than in the control C12 cells (Panel A). The PLA$_2$ activity in the soluble fraction from C12 cell clones expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A was about twofold that in the control C12 cells and almost the same as that in L929 cells (data not shown). The reason for the inconsistency between cPLA$_2\alpha$ protein levels and their enzyme activities is not clear at present.

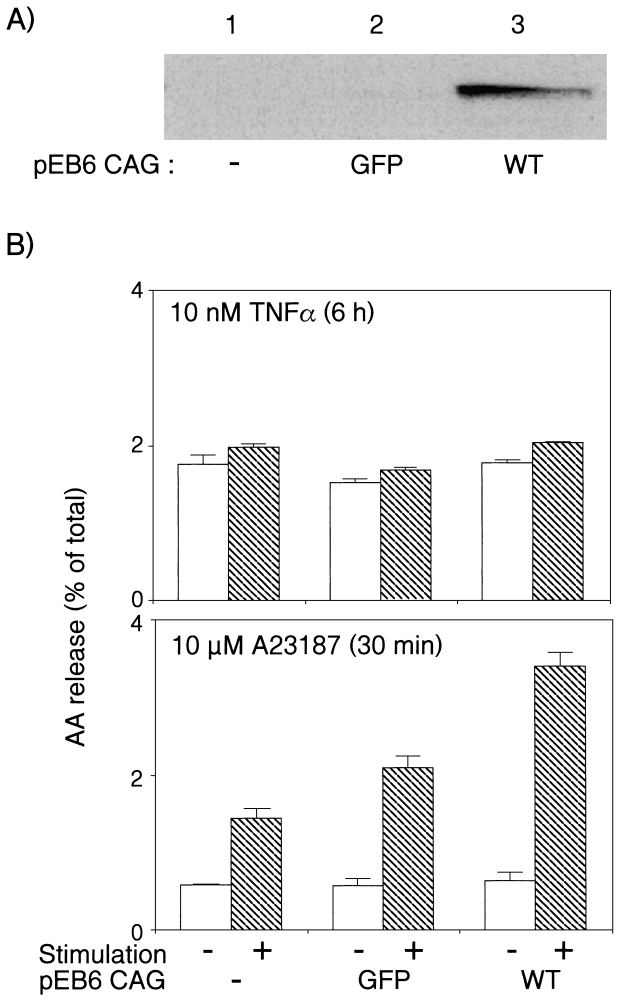

Fig. 3. AA release in C12 cells transfected with pEB6 CAG-cPLA$_2\alpha$. C12 cells were transfected with the pEB6 CAG expression vector for GFP and wild-type cPLA$_2\alpha$ (WT). cPLA$_2\alpha$ levels in soluble fractions from the respective C12 cells are shown in Panel A. In Panel B, respective C12 cells were labeled with [^{3}H]AA and then incubated with 10 nM TNFα or 10 μM A23187 for 6 h and 30 min, respectively. Data are the mean ± S.D. of three determinations in a typical experiment involving three representative experiments.

In a typical experiment (Panel B), the responses to A23187 were greater in C12 cells expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A than in the control C12 cells. Treatment with 100 nM PMA for 90 min caused AA release from C12 cells expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A, but not from control C12 cells. However, treatment with TNFα at 10 nM (Panel B) and 30 nM (data not shown) did not stimulate AA release for 6 h in the C12 cells expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A, as in the control C12 cells. In preliminary experiments, similar amounts of TNFα receptors (p55 and p75) transcripts were detected in L929 cells, C12 cells, and C12 cells expressing

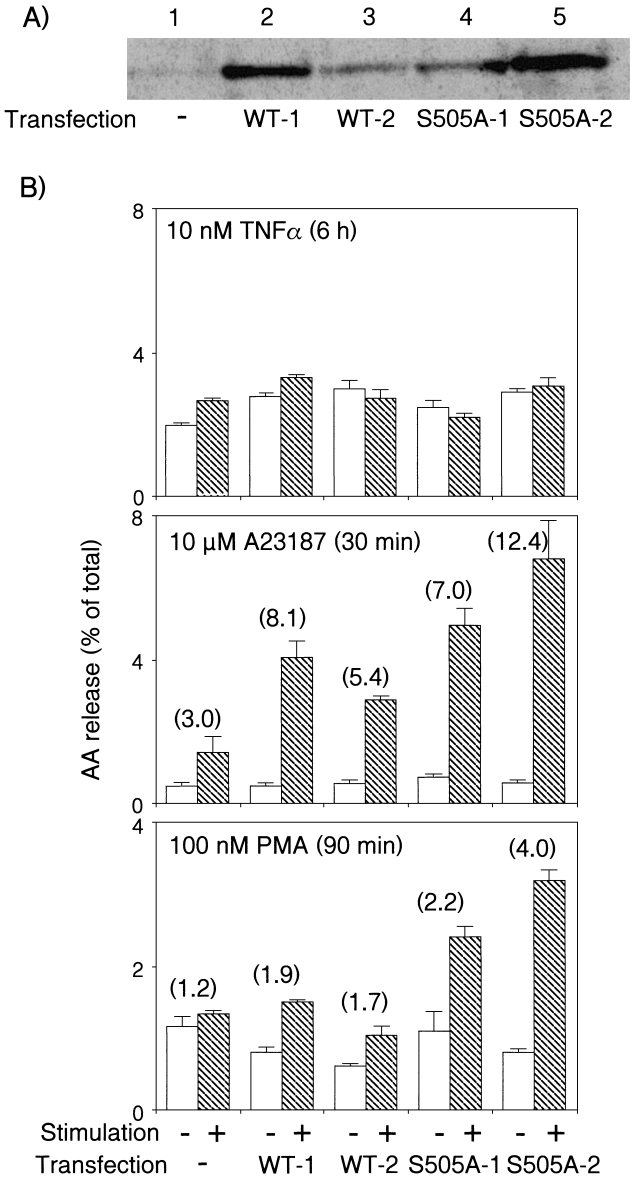

Fig. 4. AA release from stable clones of C12 cells expressing wild-type and mutant cPLA$_2\alpha$. WT-1 and WT-2 show two independent C12 cell clones expressing wild-type cPLA$_2\alpha$, and S505A-1 and S505A-2 show two independent C12 cell clones expressing cPLA$_2\alpha$S505A. The cPLA$_2\alpha$ levels in soluble fractions from the respective C12 cells are shown in Panel A. In Panel B, labeled C12 cells were incubated with 10 nM TNFα for 6 h, 10 μM A23187 for 30 min, or 100 nM PMA for 90 min. Data are the mean ± S.D. of three determinations in a typical experiment. The fold-increase in AA release induced by stimulants compared with that from the respective control cells is shown in parentheses.

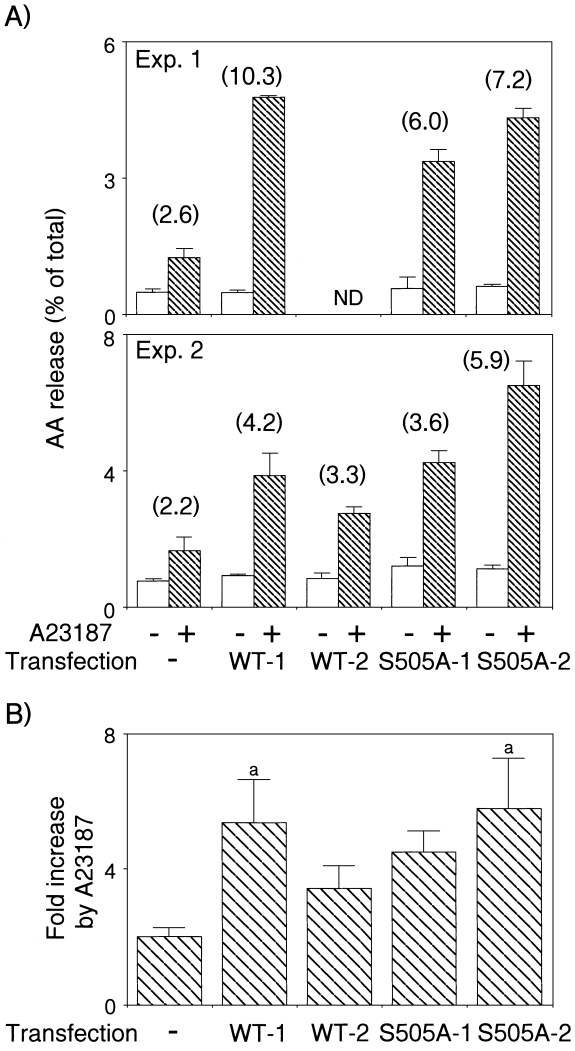

Fig. 5. A23187-induced AA release from stable clones of C12 cells expressing wild-type and mutant cPLA$_2\alpha$. Labeled C12 cells were incubated with and without 10 μM A23187 for 30 min. Panel A shows the data from two independent experiments, and the data are the mean ± S.D. of three determinations in the respective experiments. ND, not determined. Panel B shows the results of the quantitative analyses of fold-stimulation induced by A23187 in the respective stable clones. Values are means ± S.E.M. for four to six independent experiments. [a]$P<0.05$, significantly different from the value in control cells.

wild-type cPLA$_2\alpha$ by RT-PCR analysis (data not shown). Figure 5 shows the responses to A23187 in other typical experiments (Panel A) and quantitative analyses of the A23187 responses in the respective cells (Panel B). The fold-increase induced by A23187 was significantly larger in the stable clones expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A than in the control cells. As in L929 cells, 100 nM PMA enhanced 10 μM A23187-induced AA release for 30 min by about twofold in stable clones of C12 cells expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A (data not shown).

Discussion

Induced expression of cPLA$_2\alpha$ and its function in C12 cells

To investigate the regulatory mechanism of cPLA$_2\alpha$

activation in cells, we tried to express wild-type and mutant cPLA$_2\alpha$ in a C12 cell clone, a L929 variant lacking cPLA$_2\alpha$. On transient transfection using the pcDNA4/HisMax vector, cPLA$_2\alpha$ was expressed effectively in HEK293T cells but barely in C12 cells (Fig. 2). In the C12 cells treated with the pcDNA4 /HisMax-cPLA$_2\alpha$ vector, no response was detected, probably because the percentage of transfected cells was quite low. However, the expressed cPLA$_2\alpha$ was translocated on stimulation with Ca^{2+} in the vector-transfected C12 cells (Fig. 2, Panel E). On transfection using the pEB6 CAG vector, cPLA$_2\alpha$ protein was effectively expressed and Ca^{2+}, but not TNFα, stimulated AA release in the transfected C12 cells (Fig. 3). Further analysis of the release of AA in C12 cells transfected with pEB6 CAG-cPLA$_2\alpha$ was difficult since the response to Ca^{2+} was limited. Then, we established several stable clones of C12 cells expressing wild-type cPLA$_2\alpha$ and mutant cPLA$_2\alpha$, and we found that they are useful for studying AA release responses.

In the present study, the Ca^{2+} ionophore-induced AA release was observed in stable C12 cell clones expressing wild-type cPLA$_2\alpha$ (and cPLA$_2\alpha$S505A). PLA$_2\alpha$ activity increased in stable C12 cell clones expressing wild-type cPLA$_2\alpha$ (and cPLA$_2\alpha$S505A). In addition, PMA-induced AA release was observed in stable C12 cell clones expressing cPLA$_2\alpha$, like in L929 cells. These findings suggest that the cPLA$_2\alpha$ expressed in C12 cells is functionally active. Hayakawa et al. (10) reported that the release of AA induced by 300 ng/mL (about 18 nM) of TNFα for 2 – 4 h was observed in stable clones of C12 cells transfected with wild-type cPLA$_2\alpha$ cDNA. In our established C12 cell clones expressing wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$S505A, however, 10 and 30 nM TNFα did not stimulate AA release. Possible reasons for this discrepancy are discussed below.

Lack of TNFα-induced AA release from C12 cells expressing wild-type cPLA$_2\alpha$

Inhibitors of transcription and translation acted synergistically with TNFα to cause the activation of cPLA$_2$ and/or release of AA in cells (23, 24). The absence of TNFα-induced AA release in C12 cells expressing cPLA$_2\alpha$ may be due to protein inhibitors that directly regulate cPLA$_2\alpha$ activity. PLA$_2$ activity in stable C12 cell clones expressing cPLA$_2\alpha$ was twofold higher than that in control C12 cells, but the activity was much lower than in HEK293T cells expressing cPLA$_2\alpha$. However, the Ca^{2+} and PMA-evoked responses (cPLA$_2\alpha$ translocation and AA release) were observed in C12 cells with the expression of cPLA$_2\alpha$. In a previous report by Hayakawa et al. (10), the sensitivity of TNFα-induced cell death in C12 cell clones expressing cPLA$_2$, in which the PLA$_2$ activity was over 60% of that in the control L929 cells, was tenfold lower compared with that in L929 cells. Thus, molecules and/or processes that regulate the TNFα signaling pathway upstream from cPLA$_2\alpha$ may be modified in C12 cells, in addition to a lack of cPLA$_2\alpha$.

TNFα elicits various biological effects as the result of complex signaling events that are initiated through polymerization of two distinct transmembrane receptors: TNFα receptor type 1 (p55) and type 2 (p75). Although L929 cells express both receptors (25, 26), cell death and/or apoptosis and NFκB activation induced by TNFα in L929 cells are mediated by p55 (25, 27). Human TNFα is shown to bind to mouse p55 but not to mouse p75, although human TNFα binds to both human receptors (28, 29). In the present study, we used human TNFα and the cells derived from mouse. Thus, AA release induced by TNFα from L929 cells is mediated by p55. Since C12 cells are analogous to L929 cells in TNFα receptor binding (1) and TNFα-induced activation of NFκB (3) and p55 transcripts were detected by RT-PCR analysis in native C12 cells and a stable clone of C12 cells expressing cPLA$_2\alpha$, functions of TNFα receptor type 1 (p55) appeared to be intact in C12 cells.

It is reported that C12 cells expressed latent NFκB protein at twice the level of L929 cells (30), and the constitutive activity of superoxide dismutase in the TNFα-resistant subclone L929.12 was significantly greater than that in L929 cells (31). The TNFα-induced death of L929 cells is dependent on the production of reactive oxygen species (32, 33). Molecules in the TNFα signaling pathway such as TNFα receptor-associated factor 2 may be changed or the mechanisms keeping levels of reactive oxygen species low may exist in C12 cells. In addition, TNFα triggers the activation of phospholipase D 3 – 6 h after stimulation and concomitant phosphatidic acid production followed by a sustained diacylglycerol accumulation in human myeloblastic cells (34). Diacylglycerol acts through a variety of kinases including PKC and MAPK, and these kinases are able to phosphorylate and activate cPLA$_2\alpha$ (8, 9). The changes in levels of reactive oxygen species and diacylglycerol production induced by TNFα in both L929 and C12 cells should be determined. There is a need to identify the components necessary for TNFα-induced AA release in C12 cells, in addition to cPLA$_2\alpha$.

No involvement of phosphorylation of cPLA$_2\alpha$ at Ser^{505} in PMA-induced AA release in intact cells

Activation of cPLA$_2\alpha$ is regulated by phosphorylation, in addition to Ca^{2+}. Since Ser^{505} and Ser^{727} are

conserved among distinct species, these two amino acids appeared to regulate the activity in the presence of Ca^{2+} (8, 9). Ser^{505} is in the PXSP motif that represents a consensus MAPK phosphorylation site and phosphorylation of Ser^{505} in cPLA$_2\alpha$ increases the enzymatic activity probably by slowing the dissociation of membrane-bound cPLA$_2\alpha$ (18). Since the activation of PKC can trigger a kinase cascade leading to the activation of MAPK in cells, cPLA$_2\alpha$ activation and/or AA release by PMA appeared to be mediated at least partially through MAPKs including ERK1/2 (8, 15, 16). AA releases induced by A23187 with and without PMA from Chinese hamster ovary (CHO) cells expressing cPLA$_2\alpha$S505A were significantly lower compared with those from CHO cells expressing wild-type cPLA$_2\alpha$ (14). In the present study, however, PMA stimulated AA release for 90 min and enhanced A23187-induced AA release for 30 min from C12 cells expressing cPLA$_2\alpha$S505A, as it did from L929 cells and C12 cells expressing wild-type cPLA$_2\alpha$. Thus, phosphorylation sites other than Ser^{505} in cPLA$_2\alpha$ are involved in the PMA-induced AA release from cells. Xu et al. (15) proposed that PKC activation stimulates two events in murine astrocytes; phosphorylation of cPLA$_2\alpha$ at a site(s) different from Ser^{505} through an ERK1/2-independent pathway and phosphorylation of cPLA$_2\alpha$ at Ser^{505} through an ERK1/2-dependent pathway. As previously proposed (35), the role of phosphorylation at Ser sites in the activation of cPLA$_2\alpha$ by protein kinases including PKC should be re-examined.

In conclusion, care should be taken when using a C12 cell clone as a cPLA$_2\alpha$-deficient clone of L929 cells since C12 cells may lack components necessary for TNFα-induced AA release in addition to lacking cPLA$_2\alpha$. PMA causes activation of cPLA$_2\alpha$ in a Ser^{505} phosphorylation-independent manner in C12 cells expressing cPLA$_2\alpha$.

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