Overexpression of myristoylated alanine-rich C-kinase substrate enhances activation of phospholipase D by protein kinase C in SK-N-MC human neuroblastoma cells

Sherry C. MORASH, Sergio D. ROSÉ, David M. BYERS, Neale D. RIDGWAY and Harold W. COOK¹

Atlantic Research Centre, Departments of Pediatrics and Biochemistry, Dalhousie University, Halifax, Nova Scotia, B3H 4H7, Canada

Signal transduction can involve the activation of protein kinase C (PKC) and the subsequent phosphorylation of protein substrates, including myristoylated alanine-rich C kinase substrate (MARCKS). Previously we showed that stimulation of phosphatidylcholine (PtdCho) synthesis by PMA in SK-N-MC human neuroblastoma cells required overexpression of MARCKS, whereas PKC α alone was insufficient. We have now investigated the role of MARCKS in PMA-stimulated PtdCho hydrolysis by phospholipase D (PLD). Overexpression of MARCKS enhanced PLD activity 1.3–2.5-fold compared with vector controls in unstimulated cells, and 3–4-fold in cells stimulated with 100 nM PMA. PMA-stimulated PLD activity was blocked by the PKC inhibitor bisindolylmaleimide. Activation of PLD by PMA was linear with time to 60 min, whereas stimulation of PtdCho synthesis by PMA in clones overexpressing MARCKS was

observed after a 15 min time lag, suggesting that the hydrolysis of PtdCho by PLD preceded synthesis. The formation of phosphatidylbutanol by PLD was greatest when PtdCho was the predominantly labelled phospholipid, indicating that PtdCho was the preferred, but not the only, phospholipid substrate for PLD. Cells overexpressing MARCKS had 2-fold higher levels of PKC α than in vector control cells analysed by Western blot analysis; levels of PKC β and PLD were similar in all clones. The loss of both MARCKS and PKC α expression at higher subcultures of the clones was paralleled by the loss of stimulation of PLD activity and PtdCho synthesis by PMA. Our results show that MARCKS is an essential link in the PKC-mediated activation of PtdCho-specific PLD in these cells and that the stimulation of PtdCho synthesis by PMA is a secondary response.

INTRODUCTION

The activation of protein kinase C (PKC) by cellular agonists or directly with the phorbol ester PMA results in the stimulated turnover of phosphatidylcholine (PtdCho) in many cell types [1,2]. However, a direct role of phosphorylation by PKC in the PtdCho biosynthetic pathway has not been demonstrated [3–5]. Recently PKC has been shown to mediate the degradation of PtdCho via the activation of phospholipase D (PLD) [6–8].

The hydrolysis of PtdCho by PLD generates choline and phosphatidic acid, which can be metabolized to the signalling molecules diacylglycerol, an endogenous activator of PKC [9], or lysophosphatidic acid [10]. Phosphatidic acid itself can also function as a second messenger [2,11]. Several activators of PLD have been described, including PKC α , PtdInsP₂ and the small GTP-binding proteins, ADP-ribosylation factor (ARF), Rho, Rac and Cdc42 [7,8]. A PLD activated by unsaturated fatty acids but not by PtdInsP₂ or GTP-binding proteins has also been described [12]. Two mammalian genes for PLD have been cloned. PLD1 required PKC α , PtdInsP₂ and ARF for activation and was localized to perinuclear membranes [13,14]. PLD2 localized to the plasma membrane and required only PtdInsP₂ for activation [14]. The activation of PLD by ARF and Rho proteins points to a role for PLD in vesicular trafficking and cytoskeletal organization [7,8,14].

In previous studies with several neural-derived cell lines, we correlated the PKC-dependent stimulation of PtdCho synthesis with the expression of PKC α and a major PKC substrate, myristoylated alanine-rich C-kinase substrate (MARCKS) [15,16]. MARCKS is a heat- and acid-stable protein of 30 kDa

that runs anomalously on SDS/PAGE at approx. 80 kDa. The protein contains a centrally located basic domain in close proximity to the PKC phosphorylation site, termed the effector domain, that binds actin, Ca²⁺/calmodulin and acidic phospholipids [17,18]. Phosphorylation of MARCKS prevents binding to the effector domain and is associated with the translocation of MARCKS to the cytosol [17–19]. MARCKS has been shown to inhibit phospholipase C *in vitro* by sequestering its substrate, PtdInsP₂, in membranes [20]; inhibition was released by the phosphorylation of MARCKS. Although the functions of MARCKS are not known, it has been implicated in actin–membrane interactions [18,21,22], phagocytosis [23] and secretion [24], functions similar to those proposed for PLD [7,8].

We have overexpressed MARCKS or PKC α in SK-N-MC human neuroblastoma cells that are normally unresponsive to PMA [25]. Clones overexpressing MARCKS, but not those overexpressing PKC α only, increased PtdCho synthesis in response to PMA [26]. We now show that the overexpression of MARCKS increases both the basal and the PMA-stimulated PLD activity, suggesting that the presence of MARCKS is essential for the PKC-mediated activation of PtdCho-specific PLD in these cells.

EXPERIMENTAL

Materials

[methyl-³H]Choline chloride (80 Ci/mmol), [9,10-³H]oleic acid (9.2 Ci/mmol), [9,10-³H]myristic acid (50 Ci/mmol) and [1-

Abbreviations used: ARF, ADP-ribosylation factor; bIM, bisindolylmaleimide hydrochloride; DMEM, Dulbecco's modified Eagle's medium; MARCKS, myristoylated alanine-rich C-kinase substrate; PKC, protein kinase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

¹ To whom correspondence should be addressed (e-mail h.cook@dal.ca).

^{14}C jeicosapentaenoic acid (52.2 mCi/mmol) were obtained from Mandel Scientific. PMA, BSA, DMSO, oleic acid, digitonin, protease inhibitors, trifluoperazine, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide, HCl (W7), brefeldin A, colchicine, cytochalasin D and calmidazolium were from Sigma Chemical Co. Bisindolylmaleimide hydrochloride I (bIM) was purchased from CalBiochem. Triton X-100 (Surfact-Amps) was from Pierce Chemical Co. Fetal bovine serum and culture media were from Flow Laboratories. G418 was from Life Technologies. Equipment and reagents for SDS/PAGE and Western blotting were from Bio-Rad Canada. Enhanced chemiluminescence Western blotting kits were purchased from Amersham Canada.

Cell culture

SK-N-MC human neuroblastoma clones stably overexpressing human PKC α or rat MARCKS or containing vector alone were prepared as described previously [26]. Cells were maintained in 75 cm² flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum and 350 $\mu\text{g}/\text{ml}$ G418. Cells were grown at 37 °C in a humidified atmosphere of air/CO₂ (19:1) and subcultured every 7 days. For most experiments, cells were grown to subconfluence in 35 mm dishes in DMEM with 10% fetal bovine serum; medium was changed to DMEM without serum 18 h before use.

Determination of PLD activity

Cells were prelabelled with [^3H]oleic acid or [^3H]myristic acid for 18 h or with [^{14}C]jeicosapentaenoic acid (0.5 μCi) for 48 h. Radiolabelled medium was removed; cells were then washed and incubated in 1 ml of DMEM for 1 h. PMA (100 nM) suspended in DMSO or DMSO alone (less than 0.2%, v/v) and butan-1-ol (0.6%, v/v) were added and incubation was continued for 1 h. Where indicated, bIM was added 15 min before other additions and was present for the entire incubation period. Cellular lipids were extracted and the distribution of radioactivity was determined by TLC on silica HL 60 plates with the organic phase from ethyl acetate/iso-octane/acetic acid/water (65:10:15:50, by vol.) [27]. Plates were scanned with a Bioscan Imaging Scanner System 200-IBM; lipids and derivatives were identified by comparison with standards and phosphatidylbutanol synthesized with plant PLD [28].

Determination of PtdCho synthesis

Cells were incubated with [*methyl*- ^3H]choline (4 μCi) and β -PMA or DMSO for 1 h. Oleic acid was added to medium suspended in sterile BSA [29]. Cells were harvested, extracted into lipid and aqueous fractions and quantified as described previously [30]. TLC of the lipid fraction indicated that more than 98% of the radioactivity was in PtdCho after 1 h of incubation. Choline-containing, water-soluble metabolites were separated by TLC [30].

Western blotting

Cells were treated with 100 nM PMA or DMSO for 15 min and washed twice with 20 mM Tris/HCl, 0.1 M NaCl (pH 7.5) (Tris-buffered saline). Cytosol and membrane fractions were prepared by sequential extraction in buffers containing digitonin (100 $\mu\text{g}/\text{ml}$) or Triton-X100 (1%) [15,25]. Total protein in each fraction was quantified with a micro-bicinchoninic acid assay (Pierce). Proteins were precipitated with acetone and resuspended by boiling in 1.25% (w/v) SDS/60 mM Tris/HCl/12% (v/v)

glycerol/0.001% Bromophenol Blue/3.5% (v/v) 2-mercaptoethanol for detection of PKC and PLD. Proteins (10–20 μg) were separated by SDS/PAGE and transferred to membranes (0.45 μm pore size) with a Bio-Rad Mini-Protean II system [15]. MARCKS antibody was prepared from a MARCKS-GST fusion construct in pGEX-5X3 vector (Invitrogen). The fusion protein contained the C-terminal half of rat MARCKS extending from residue 138 to 309 including the calmodulin-binding domain [31]. MARCKS was detected with primary sera from rabbits [15]. PKC antibodies were from Santa Cruz; PLD antibody was from Upstate Biotechnology. Secondary antibody was goat anti-rabbit IgG antibody conjugated to horseradish peroxidase. Proteins were detected with an enhanced chemiluminescence kit. Bands were scanned with an Apple One Scanner with Ofoto software (Light Source) and analysed with NIH Image version 1.55 software (Research Services Branch, NIMH).

RESULTS

Effects of MARCKS and PKC α overexpression on PMA-stimulated PLD activity

In previous studies we observed that four separately isolated clones derived from human SK-N-MC cell lines and stably overexpressing rat MARCKS showed a doubling in incorporation of [*methyl*- ^3H]choline into PtdCho in response to treatment with PMA [26]. PtdCho synthesis was not stimulated by PMA in clones overexpressing human PKC α or in vector controls. As activation of PKC by PMA is known to stimulate PLD activity [6–8], we assessed PMA-stimulated PLD activity in the overexpressing clones by measuring the formation of [^3H]phosphatidylbutanol in cells prelabelled with myristic acid, oleic acid or jeicosapentaenoic acid (Table 1). The vector control and PKC α clone ($\alpha 6$) responded to PMA with stimulation of PLD activity; however, both basal (in M12 and M22) and PMA-stimulated

Table 1 Phospholipase D activity in SK-N-MC clones

SK-N-MC clones, stably transfected with vector and cDNA for rat MARCKS (M9, M12, M15, M22) or human PKC α ($\alpha 6$), were incubated for 18 h with [^3H]myristic acid, [^3H]oleic acid or [^{14}C]jeicosapentaenoic acid. Cells were washed and incubated for 1 h in fresh medium containing 0.6% (v/v) butan-1-ol and DMSO or 100 nM PMA. Where indicated, 5 μM bIM was added 15 min before butan-1-ol and DMSO or PMA. The formation of [^3H]phosphatidylbutanol was determined as described in the Experimental section. Results are expressed as percentages of total lipid radioactivity in phosphatidylbutanol and are means $\pm$ S.E.M. for the number of independent determinations shown in parentheses. Significant differences between vector and overexpressing clones for DMSO controls and PMA treatment were determined by using analysis of variance for normal data and the Tukey test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. Abbreviation: n.d., not determined.

Clone	DMSO	PMA	PMA + bIM
Prelabelling with 14:0			
Vector	0.69 $\pm$ 0.04 (10)	1.66 $\pm$ 0.07 (10)	0.90 $\pm$ 0.08 (4)
M9	0.73 $\pm$ 0.09 (6)	2.06 $\pm$ 0.21 (6)***	n.d.
M12	0.91 $\pm$ 0.05 (15)*	3.02 $\pm$ 0.08 (15)***	0.98 $\pm$ 0.20 (4)
M15	0.79 $\pm$ 0.07 (6)	3.01 $\pm$ 0.1 (6)***	n.d.
M22	1.03 $\pm$ 0.03 (6)**	3.32 $\pm$ 0.19 (6)***	0.56 $\pm$ 0.05 (6)
$\alpha 6$	0.67 $\pm$ 0.06 (10)	1.19 $\pm$ 0.07 (10)*	0.69 $\pm$ 0.13 (4)
Prelabelling with 18:1,n=9			
Vector	0.23 $\pm$ 0.03 (10)	0.66 $\pm$ 0.07 (10)	0.35 $\pm$ 0.04 (6)
M12	0.59 $\pm$ 0.07 (10)***	2.71 $\pm$ 0.23 (10)***	0.78 $\pm$ 0.04 (6)
$\alpha 6$	0.39 $\pm$ 0.04 (14)	0.78 $\pm$ 0.06 (14)	0.44 $\pm$.13 (4)
Prelabelling with 20:5,n=3			
Vector	0.12 $\pm$ 0.02 (6)	0.58 $\pm$ 0.03 (6)	n.d.
M12	0.13 $\pm$ 0.01 (6)	1.25 $\pm$ 0.12 (6)***	n.d.
$\alpha 6$	0.11 $\pm$ 0.02 (6)	0.50 $\pm$ 0.08 (6)	n.d.

Figure 1 Effects of PMA concentration on PLD activity in cells transfected with vector, MARCKS or PKC α

Cells were labelled for 18 h with [3 H]myristic acid (1 μ Ci per dish), then washed and incubated for 1 h in fresh medium containing 0.6% (v/v) butan-1-ol and various concentrations of PMA. Lipids were extracted and separated by TLC, and radioactivity in phosphatidylbutanol (PtdBuOH) was determined. Results are percentages of total lipid radioactivity in phosphatidylbutanol and are means $\pm$ S.E.M. for three separate experiments. Symbols: $\bullet$, vector control cells; $\blacktriangledown$, M12 cells; $\circ$, $\alpha 6$ cells.

PLD activities were significantly increased in cells overexpressing MARCKS, irrespective of the fatty acid label used. PMA-stimulated PLD activity was inhibited by co-incubation with 5 μ M bLM, a concentration previously shown to inhibit PKC activity completely in glioma cells [28]. Stimulation of PLD activity was dependent on the PMA concentration, with maximal stimulation observed at 100 nM PMA (Figure 1).

Although most studies indicate that PtdCho is the preferred substrate for agonist-stimulated PLD, degradation of phosphatidylethanolamine (PtdEtn) has also been reported [6]. To determine whether MARCKS-dependent PLD activity was specific for PtdCho, we compared the relative distributions of radioactivity in phospholipids and the levels of phosphatidylbutanol formed in cells prelabelled with [3 H]myristic acid or [14 C]eicosapentaenoic acid (Table 1). Maximum activity for all clones was observed with [3 H]myristic acid-labelled phospholipids; under these conditions, more than 75% of total phospholipid radioactivity was in PtdCho and only 15% was in PtdEtn. Lowest levels of PLD activity were observed in cells prelabelled with [14 C]eicosapentaenoic acid, which predominantly labelled PtdEtn (60%); only 20% of radioactivity was incorporated into PtdCho. However, PLD activity was stimulated 10-fold by PMA in cells prelabelled with [14 C]eicosapentaenoic acid, in comparison with a 3.5-fold stimulation in [3 H]myristic acid-labelled cells. Together these results indicate that PtdCho is probably the preferred substrate for PLD but contributions from PLD hydrolysis of PtdEtn cannot be excluded.

In the MARCKS-transfected clone, there was a linear, time-dependent increase in PLD activity in response to PMA up to 60 min without an initial lag (Figure 2, top panel) but no increase

Figure 2 Effects of incubation time on PMA-stimulated PLD activity and PtdCho synthesis in cells transfected with MARCKS

Top panel: M12 cells were prelabelled with [3 H]myristic acid (1 μ Ci per dish), then washed and incubated in fresh medium containing 0.6% (v/v) butan-1-ol and DMSO ($\bullet$) or 100 nM PMA ($\circ$) for the indicated times. Results are percentages of total lipid radioactivity in phosphatidylbutanol (PtdBuOH). Lower panel: cells were preincubated with [3 H]choline for 30 min; DMSO ($\bullet$) or 100 nM PMA ($\circ$) was added and the incubation was continued for the indicated times. Lipids were extracted and analysed as described in the Experimental section. Results are means $\pm$ S.E.M. for duplicate dishes from three independent experiments for each panel.

was seen in unstimulated cells at 30 or 60 min. In contrast, there was a distinct lag of approx. 15 min in [3 H]choline incorporation into PtdCho after treatment with PMA (Figure 2, bottom panel). The lag in synthesis of labelled PtdCho was not due to a delay in labelling of intracellular water-soluble precursors because cells were preincubated for 30 min with [3 H]choline before stimulation with PMA. Other experiments (results not shown) showed that preincubation for 15 min with PMA eliminated the lag in PtdCho synthesis. Overexpression of MARCKS or PKC α had no effect on choline uptake from medium or conversion to phosphocholine. However, in the M12 clone after 15 min of treatment with PMA, radioactivity in phosphocholine was less than in cells treated with DMSO alone. These results are consistent with the delayed activation of cytidylyltransferase, the rate-limiting step in PtdCho synthesis, in response to PMA.

Expression of MARCKS, PKC α , PKC β and PLD

The expression of PLD1 in membrane and cytosol fractions prepared from vector controls and cells overexpressing MARCKS or PKC α was monitored with a commercial antibody (Figure 3). The relative intensities of the immunoreactive band at 120 kDa were similar for all three clones, indicating that the overexpression of MARCKS or PKC α had no effect on PLD1 expression. PLD1 was observed only in membrane fractions and its distribution was unaltered by PMA treatment. Although the

Figure 3 PLD1 expression in vector control, M12 and $\alpha 6$ clones

Cells were treated with (+) or without (–) 100 nM PMA (TPA) for 15 min. Soluble and membrane fractions were obtained by sequential extraction with digitonin and Triton X-100. Proteins (20 μ g) were separated by SDS/PAGE, transferred to membranes and probed with antibody to PLD1. The control lane (con) contained 20 μ g of EGF-stimulated A431 cell lysate provided by the commercial supplier as a positive control for the PLD1 antibody. The locations of molecular mass markers (in kDa) are indicated at the right. Results shown are representative of three separate experiments.

120 kDa immunoreactive band was absent from the cytosol fractions, several bands between 56 and 40 kDa were observed. The expressions of MARCKS, PKC α , PKC β and PLD were compared between a vector control and four MARCKS clones (Figure 4). Compared with the M12 clone, the expression of MARCKS in the others ranged from 0.96 for M9 to 1.72 for M15. Although not an absolute proportional correlation, there was a general trend for increased PLD activity (Table 1) as the level of MARCKS overexpression increased. Levels of PKC α were increased 1.7-fold in lysates from the MARCKS clones compared with vector control cells (Figure 4). When the membrane fractions from unstimulated cells were examined (Figure 4b), PKC α levels were increased 11-fold over control cells. In contrast, levels of endogenous PKC β or PLD1 expression did not differ appreciably from those in control cells in any of the MARCKS clones.

Figure 4 Western blot analysis of MARCKS, PKC α , PKC β and PLD1 expression in the vector control and MARCKS-overexpressing clones

Total cell lysates (a) from vector control cells (lane 1) and four MARCKS-expressing clones [M9 (lane 2), M12 (lane 3), M15 (lane 4) and M22 (lane 5)] were obtained by extraction with Triton X-100 as described in the Experimental section. Proteins (10 μ g for PKC α and PKC β , and 20 μ g for PLD1 and MARCKS) were separated by SDS/PAGE, transferred to membranes and probed with the indicated antibody. For MARCKS, cell lysates were heated for 10 min at 80 °C before precipitation with acetone; proteins equivalent to 20 μ g of unheated sample were loaded on gels. Membrane-associated PKC α (b) was obtained by Triton X-100 extraction after the removal of cytosolic proteins by digitonin. Results shown are representative of three separate experiments for each antibody.

Figure 5 Effect of PKC inhibition on translocation of MARCKS and PKC α by PMA

M12 cells were preincubated with or without 5 μ M bIM for 15 min followed by a 15 min incubation with DMSO or 100 nM PMA (TPA). Soluble and membrane fractions were obtained by sequential extraction with digitonin and Triton X-100. Proteins (10 μ g) were separated by SDS/PAGE, transferred to nitrocellulose and probed with antibody against PKC α or MARCKS.

Table 2 Translocation of PKC α and MARCKS

M12 cells were treated as described in the legend to Figure 3. Autoradiographs were scanned and results are expressed relative to DMSO controls. Results are means $\pm$ S.E.M. for three to seven separate experiments. Significant differences between PMA and PMA + bIM were calculated by using analysis of variance for normal data and the Tukey test. * P < 0.005; ** P < 0.01.

Treatment	PKC α		MARCKS	
	Cytosol	Membrane	Cytosol	Membrane
DMSO	1.00	1.00	1.00	1.00
PMA	0.78 $\pm$ 0.06	3.19 $\pm$ 0.14	1.57 $\pm$ 0.13	0.77 $\pm$ 0.09
PMA + bIM	0.59 $\pm$ 0.16	3.51 $\pm$ 0.87	0.91 $\pm$ 0.17*	1.01 $\pm$ 0.10**

Translocation of MARCKS and PKC α by PMA

The phosphorylation of MARCKS after stimulation and translocation of PKC to a membrane-bound active form is associated with the translocation of MARCKS from membranes to the cytosol [18,19]. Under basal conditions, 48 % of overexpressed MARCKS protein in the M12 clone was associated with the cytosolic fraction and 52 % with membrane (Figure 5). Treatment with 100 nM PMA for 15 min resulted in a 30–50 % increase in cytoplasmic MARCKS with a concomitant decrease in membrane-associated MARCKS (Table 2), whereas PKC α was translocated from a predominantly cytosolic location to the membrane. Co-incubation with the PKC inhibitor bIM had no effect on translocation of PKC α but bIM inhibited translocation of MARCKS to the cytosol (Table 2) and inhibited PLD activation in the presence of PMA (Table 1). Results suggest that the phosphorylation of MARCKS by PKC is necessary for the activation of PLD and the translocation of MARCKS.

Correlation of loss of MARCKS and PKC α expression with a decline in PMA-stimulated PLD and PtdCho synthesis

As the passage number of MARCKS-expressing clones increased, there was a loss of PMA-stimulated PLD activity and PtdCho synthesis (Table 3). When MARCKS expression in M12 cells was compared at low (subculture 8) and high (subculture 26) passage numbers, levels of rat MARCKS were decreased by more than 60 % in the older cells (Figure 6). Increased expression

Table 3 Correlation of MARCKS expression with PMA-stimulated PLD activity and PtdCho synthesis

Vector control cells, M12 cells at low passage number (fewer than 17 subcultures; < 17) and at high passage number (more than 17 subcultures; > 17) and $\alpha 6$ cells were tested for PMA-stimulated PLD activity and PtdCho synthesis as described in the Experimental section. PLD activity is expressed as the percentage of total lipid radioactivity in phosphatidylbutanol; PtdCho synthesis is expressed as the ratio of lipid radioactivity in PMA-treated cells relative to DMSO. Results are means $\pm$ S.E.M. for the number of independent experiments shown in parentheses. Significant differences were calculated by using analysis of variance and the Tukey test. *Significantly different from M12 (< 17); $P > 0.005$.

Clone	PLD activity	PtdCho synthesis
Vector	1.66 $\pm$ 0.07 (9)	1.07 $\pm$ 0.02 (26)
M12 (< 17)	3.02 $\pm$ 0.08 (15)	1.77 $\pm$ 0.03 (18)
M12 (> 17)	1.20 $\pm$ 0.12 (4)*	1.24 $\pm$ 0.05 (14)*
$\alpha 6$	1.19 $\pm$ 0.07 (10)	1.05 $\pm$ 0.03 (14)

Figure 6 Co-ordinate loss of MARCKS and PKC α expression at high subculture number

Total cell lysates from vector control cells, M12 cells at low passage number (sc8), M12 cells at high passage number (sc26) and $\alpha 6$ cells were prepared as described in the Experimental section. Proteins (10 μ g) from duplicate dishes were probed with antibody against PKC α or rat MARCKS.

of PKC α in M12 cells seen at low passage number was decreased to the levels observed in control cells as the passage number increased.

Possible role of Golgi and cytoskeleton in PLD activation

As both PLD and MARCKS have been proposed to function in cytoskeletal rearrangements [7,8,20,21,23,24], we evaluated whether the observed effects of MARCKS overexpression on PLD activity might be dependent on an intact cytoskeleton. M12 cells were treated with 10 μ M cytochalasin D or colchicine, which disrupt microfilaments and microtubules, respectively. These agents caused appreciable rounding up and contraction of cells, characteristic of cytoskeletal rearrangements [32]; however, they had no effect on the ability of PMA to activate PLD (Table 4) or to stimulate PtdCho synthesis (results not shown). Treatment of M12 cells with 10 μ g/ml brefeldin A, a fungal metabolite that disrupts the Golgi apparatus [33], did not affect basal or PMA-stimulated PLD activity. As MARCKS binds Ca²⁺/calmodulin near the PKC phosphorylation site [17–19], we also tested the effect of several calmodulin antagonists, including calmidazolium, *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide, HCl and trifluoperazine, on PMA-stimulated PLD activity. None of these agents altered the ability of PMA to activate PLD in M12 cells.

DISCUSSION

In this study we have shown that MARCKS enhances the PMA-dependent activation of PLD in SK-N-MC cells. Activation of PLD by PMA and degradation of PtdCho preceded the increased

Table 4 Effect of cytoskeletal and Golgi disruption and calmodulin inhibition on PMA-stimulated PLD activity

PLD activity was determined in M12 cells prelabelled with [³H]myristic acid as described in the Experimental section. Before the assay, cells were treated for 30 min with 10 μ M cytochalasin D or colchicine, for 10 min with brefeldin A (10 μ g/ml) or for 5 min with 10 μ M calmidazolium, 1 μ M trifluoperazine or 1 μ M *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide, HCl (W7). Results are expressed as the percentage of total lipid radioactivity in phosphatidylbutanol and are means $\pm$ S.E.M. for the number of independent determinations shown in parentheses. Significant differences between treatments were calculated by using analysis of variance and the Tukey test. *Significantly different from no addition; $P < 0.05$.

Treatment	Radioactivity in phosphatidylbutanol (% of total)	
	DMSO	PMA
No addition	0.64 $\pm$ 0.03 (6)	2.74 $\pm$ 0.10 (6)
Cytochalasin D	0.73 $\pm$ 0.08 (4)	3.43 $\pm$ 0.17 (4)
Colchicine	0.60 $\pm$ 0.01 (3)	3.80 $\pm$ 0.15 (3)*
Brefeldin A	0.80 $\pm$ 0.08 (6)	3.20 $\pm$ 0.16 (6)
Calmidazolium	0.92 (2)	2.44 (2)
Trifluoperazine	0.65 $\pm$ 0.08 (4)	2.91 $\pm$ 0.32 (4)
W7	0.62 $\pm$ 0.04 (4)	2.69 $\pm$ 0.18 (4)

incorporation of [*methyl*-³H]choline in response to PMA, suggesting that stimulation of PtdCho synthesis by PMA is a secondary response to replace PtdCho, possibly to re-establish local membrane integrity. Functional association of MARCKS and PKC α is indicated by the enhanced levels of PKC α in MARCKS-overexpressing clones and the co-ordinate loss of both MARCKS and PKC α in cells at increased passage number.

Many cellular agonists, including PMA, elicit cellular responses by activation of PtdCho-specific PLD [2,6–8]. All SK-N-MC clonal cell lines reacted to PMA with some increase in PLD activity but the response was greatly enhanced in clones overexpressing MARCKS, irrespective of the fatty acid label used. Highest phosphatidylbutanol formation was observed with myristic acid, which was distributed predominantly in PtdCho; lowest levels of phosphatidylbutanol were observed in cells prelabelled with eicosapentaenoic acid, esterified predominantly in PtdEtn. Basal PLD activity was also higher in MARCKS-expressing cells when PtdCho was the major labelled phospholipid. When cells were labelled with eicosapentaenoic acid, basal activity was low in all clones; however, stimulation by PMA was highest under this labelling condition. Differences in basal and activated PLD activities observed with the various fatty acids used might reflect the presence of several isoforms of PLD in these cells that differ in substrate specificity. Overexpression of PLD1 in COS-7 cells [14] exhibited a low basal activity that was stimulated 10-fold by PMA. In contrast, PLD2 overexpression yielded a high basal activity that was stimulated only 2-fold by PMA. All clonal lines in the present study expressed PLD1; however, the presence of other isoforms including PLD2 is currently unknown.

PKC α has been implicated as a major mediator of phorbol ester-stimulated PLD hydrolysis of phospholipids [34–36], however, the mechanism of activation is not clear. In Swiss-3T3 cells, the overexpression of PKC α increased both basal and PMA-stimulated PLD activities, prompting the conclusion that PKC α might have a role in regulating PLD expression [37]. The possibility that the stimulation of PLD1 expression by MARCKS was responsible for enhanced PLD activity in M12 cells seems unlikely, as overexpression of MARCKS or PKC α had no effect on PLD1 levels; however, effects on other PLD isoforms cannot

be excluded. Other workers have proposed that activation of PLD by PKC α occurs independently of phosphorylation but requires association of the regulatory domain of PKC [34]. Inhibition of PKC activity by BIM had no effect on the PMA-mediated translocation of PKC α to membranes, whereas both translocation of MARCKS to the cytosol and PLD activity were inhibited. These results suggest that activation of PLD by PKC is dependent on the phosphorylation of some component, probably MARCKS. In neutrophils, the activation of PLD by conventional isoforms of PKC was dependent on the phosphorylation of a target protein located in the plasma membrane [38].

The overexpression of PKC β in rat fibroblast cells enhanced PMA-stimulated PLD activity [39] and suggested a role for this PKC isoform in PLD regulation. Despite high levels of endogenous PKC β in the parental SK-N-MC cell line and of PKC α in $\alpha 6$ cells, a major increase in PLD activity was observed only in MARCKS-transfected clones, suggesting that MARCKS is a limiting factor in PLD activation by PMA in these cells. Although a role for PKC β cannot be excluded, several observations suggest that PKC α is the isoform responsible for the activation of PLD in these cells. Levels of endogenous PKC α were consistently increased in clones overexpressing MARCKS, whereas no change was observed for PKC β . The loss of PMA-stimulated PLD activity as the number of cell passages increase, concomitant with the loss of both overexpression of MARCKS and enhanced expression of PKC α , suggests that the coexistence, and perhaps the co-ordinated expression, of both MARCKS and PKC α are required for PMA-mediated stimulation of PLD and PtdCho turnover.

To assess whether the effects of MARCKS overexpression on PLD activation and PtdCho synthesis were specific to stimulation by PMA, two other potentially stimulatory agents were examined. The vasoactive peptide endothelin 1 has been shown to stimulate PLD activity in SK-N-MC cells [40]. Endothelin 1 (200 nM)-stimulated PLD activity was 1.8-fold higher in M12 cells than in vector controls (S. C. Morash and H. W. Cook, unpublished work). Thus the presence of MARCKS also enhanced the PLD activity stimulated by an agent that increases endogenous diacylglycerol production and PKC activation. Fatty acids can stimulate PtdCho synthesis [25,30] and have been shown to activate at least one form of PLD [12]. The stimulation of PtdCho synthesis by fatty acids is the result of rapid translocation of cytidyltransferase and is independent of PKC activity [30]. In the present study the stimulation of PtdCho synthesis by oleic acid occurred without a delay and was independent of both the activation of PKC and the overexpression of MARCKS. In addition, fatty acids did not alter PLD activity in these cells (S. C. Morash and H. W. Cook, unpublished work). Thus MARCKS was specific for the PMA-activated synthesis of PtdCho mediated by PLD hydrolysis.

This is the first description of a role for MARCKS (or any direct PKC-specific protein substrate) in the activation of PLD. Several important aspects of interactions between PKC, MARCKS, PLD and PtdCho turnover have been described, although precise mechanisms involved in the regulation of phospholipid hydrolysis by MARCKS remain unclear. Both MARCKS and PLD are membrane-associated proteins with purported roles in membrane trafficking and in the regulation of the actin cytoskeleton [7,8,18–21]. However, intact cytoskeletal elements did not seem to be necessary for the activation of PLD by PMA, as indicated by the limited effects of disruption of F-actin by cytochalasin D or the depolymerization of microtubules by colchicine. These agents also had no effect on PMA-stimulated PtdCho synthesis (S. C. Morash and H. W. Cook, unpublished work). In C6 cells, inhibition of actin polymerization and treat-

ment with calmodulin antagonists had no effect on MARCKS phosphorylation or translocation to cytosol by PMA [32]. PLD has been localized to perinuclear regions including the Golgi [14], where it might function in the ARF-mediated formation of coated vesicles [41]. Disruption of the Golgi by brefeldin A prevented the binding of ARF to membranes and the subsequent activation of PLD [42]. The lack of effect of brefeldin A in these cells suggests that MARCKS-dependent PLD activity is not Golgi-associated. This result is consistent with the observation that MARCKS localizes to the plasma membrane rather than the Golgi [22]. The regulation of PLD activity in HL-60 cells has recently been shown to be independent of brefeldin A-sensitive ARF [43]. PLD2 has been localized to the plasma membrane in REF-52 cells [14]. Serum stimulation resulted in the redistribution of PLD2 to filopodium-like projections similar to those observed for MARCKS during membrane ruffling and cell spreading [22], suggesting that this or another as yet unidentified PLD might be the MARCKS-dependent isoform.

MARCKS co-localizes with PKC α at focal adhesion sites [23] and PKC α is an activator of PLD in several cell systems [34–36]. It is possible that MARCKS might have a role in localizing PLD and PKC α at appropriate membrane sites for subsequent interactions and enzymic activation. The association of MARCKS and PKC α might explain the higher levels of membrane-associated PKC α and the higher basal levels of PLD activity in the MARCKS-overexpressing cells. MARCKS can facilitate the formation of lateral domains in membranes enriched in the acidic phospholipids phosphatidylserine and PtdInsP $_2$ [44]. In studies with phospholipid vesicles, MARCKS inhibited PLC by sequestering PtdInsP $_2$; the phosphorylation of MARCKS by PKC released PtdInsP $_2$, making it accessible as a substrate for PLC [20]. Possibly MARCKS colocalizes PKC α and PLD and sequesters PtdInsP $_2$, a cofactor for PLD activity, so that on phosphorylation of MARCKS by PKC, PtdInsP $_2$ would be released to participate in the activation of PLD. Alternatively, MARCKS might bind to an endogenous inhibitor of PLD in a phosphorylation-dependent manner. In the unstimulated cell the inhibitor would be bound to PLD; agonist stimulation and phosphorylation of MARCKS would increase the affinity of the inhibitory protein for MARCKS, thus releasing PLD for activation. In cells, including the SK-N-MC cell line, that express no or low levels of MARCKS, PLD would be constitutively repressed. The overexpression of MARCKS in these cells would then permit the agonist-dependent derepression of PLD. Several inhibitors that seem to associate directly with PLD have been described, including a small (18 kDa), heat-stable, PLD2-specific inhibitor from bovine brain [14] and the synapse-specific clathrin assembly protein 3 [45].

It is apparent from our studies that MARCKS is required for the activation of at least one isoform of cellular PLD, although the mechanism and physiological significance have not yet been elucidated. Changes in cellular morphology resulting from activation of PKC are most probably mediated by MARCKS. Whether the known effects of MARCKS on actin binding and cytoskeletal rearrangements are direct or mediated entirely or in part by PLD remains to be determined.

The skilled technical assistance of Anne Murphy in performing experiments and Robert Zwicker and Gladys Keddy in cell culture is gratefully acknowledged. This study was supported by a Program Grant (PG-11476) and Scholarship (N.D.R.) from the Medical Research Council of Canada and the Lockwood Foundation (S.C.M.).

REFERENCES

- 1 Pelech, S. L. and Vance, D. E. (1989) Trends Biochem. Sci. **14**, 28–30
- 2 Exton, J. H. (1994) Biochim. Biophys. Acta **1212**, 26–42

- 3 Cook, H. W. and Vance, D. E. (1985) *Can. J. Biochem. Cell Biol.* **63**, 145–151
- 4 Utal, A. K., Jamil, H. and Vance, D. E. (1991) *J. Biol. Chem.* **266**, 24084–24091
- 5 Wang, Y. and Kent, C. (1995) *J. Biol. Chem.* **270**, 17843–17849
- 6 Kiss, Z. (1996) *Chem. Phys. Lipids* **80**, 81–102
- 7 Exton, J. H. (1997) *J. Biol. Chem.* **272**, 15579–15582
- 8 Cockcroft, S. (1997) *Prog. Lipid Res.* **35**, 345–370
- 9 Nishizuka, Y. (1992) *Science* **258**, 607–614
- 10 Moolenaar, W. H. (1995) *J. Biol. Chem.* **270**, 12949–12952
- 11 Ohguchi, K., Kasai, T. and Nozawa, Y. (1997) *Biochim. Biophys. Acta* **1346**, 301–304
- 12 Okamura, S. and Yamashita, S. (1994) *J. Biol. Chem.* **269**, 31207–31213
- 13 Hammond, S. M., Altschuller, Y. M., Sung, T. C., Rudge, S. K., Rose, K., Engebrecht, J., Morris, A. J. and Frohman, M. A. (1995) *J. Biol. Chem.* **270**, 29640–29643
- 14 Colley, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altschuller, Y., Bar-Sagi, D., Morris, A. J. and Frohman, M. A. (1997) *Curr. Biol.* **7**, 191–201
- 15 Rosé, S. D., Cook, H. W., Palmer, F. B. S. C., Ridgway, N. D. and Byers, D. M. (1994) *J. Neurochem.* **63**, 2314–2323
- 16 Byers, D. M., Palmer, F. B. S. C., Spence, M. W. and Cook, H. W. (1993) *J. Neurochem.* **60**, 1414–1421
- 17 Blackshear, P. J. (1993) *J. Biol. Chem.* **268**, 1501–1504
- 18 Aderem, A. (1995) *Biochem. Soc. Trans.* **23**, 587–591
- 19 McLaughlin, S. and Aderem, A. (1995) *Trends Biochem. Sci.* **20**, 272–276
- 20 Glaser, M., Wanaski, S., Buser, C. A., Boguslavsky, V., Rashidzade, W., Morris, A., Rebecchi, M., Scarlata, S. F., Runnels, L. W., Prestwich, G. D. et al. (1996) *J. Biol. Chem.* **271**, 26187–26193
- 21 Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C. and Aderem, A. (1992) *Nature (London)* **356**, 618–622
- 22 Myat, M. M., Anderson, S., Allen, L. H. and Aderem, A. (1997) *Curr. Biol.* **7**, 611–614
- 23 Allen, L. A. H. and Aderem, A. (1995) *J. Exp. Med.* **182**, 829–840
- 24 Goodall, A. R., Turner, N. A., Walker, J. H., Ball, S. G. and Vaughan, P. F. T. (1997) *J. Neurochem.* **68**, 392–401
- 25 Sproull, S. A., Morash, S. C., Byers, D. M., Palmer, F. B. S. and Cook, H. W. (1995) *Neurochem. Res.* **20**, 1397–1407
- 26 Rosé, S. D., Morash, S. C., Ridgway, N. D., Byers, D. M. and Cook, H. W. (1996) *J. Neurochem.* **66**, 1766–1769
- 27 Lavie, Y. and Liscovitch, M. (1990) *J. Biol. Chem.* **265**, 3868–3872
- 28 Van Iderstine, S. C., Byers, D. M., Ridgway, N. D. and Cook, H. W. (1996) *J. Lipid Mediat. Cell Signal.* **15**, 175–192
- 29 Cook, H. W. and Spence, M. W. (1987) *Biochim. Biophys. Acta* **918**, 217–229
- 30 Cook, H. W., Byers, D. M., Palmer, F. B. S. C. and Spence, M. W. (1989) *J. Biol. Chem.* **264**, 2746–2752
- 31 Erusalimsky, J. D., Brooks, S. F., Herget, T., Morris, C. and Rozengurt, E. (1991) *J. Biol. Chem.* **266**, 7073–7080
- 32 Douglas, D. N., Fink, H.-S., Rosé, S. D., Ridgway, N. D., Cook, H. W. and Byers, D. M. (1997) *Biochim. Biophys. Acta* **1356**, 121–130
- 33 Donaldson, J. G. and Klausner, R. D. (1992) *Nature (London)* **360**, 350–352
- 34 Conricode, K. M., Brewer, K. A. and Exton, J. H. (1992) *J. Biol. Chem.* **267**, 7199–7202
- 35 Singer, W. D., Brown, H. A., Jiang, X. J. and Sternweis, P. C. (1996) *J. Biol. Chem.* **271**, 4504–4510
- 36 Balboa, M. A., Firestein, B. L., Godson, C., Bell, K. S. and Insel, P. A. (1994) *J. Biol. Chem.* **269**, 10511–10516
- 37 Eldar, H., Ben-Av, P., Schmidt, U.-S., Livneh, E. and Liscovitch, M. (1993) *J. Biol. Chem.* **268**, 12560–12564
- 38 Lopez, I., Burns, D. J. and Lambeth, J. D. (1995) *J. Biol. Chem.* **270**, 19465–19472
- 39 Pai, J.-K., Pachter, J. A., Weinstein, I. B. and Bishop, W. R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 598–602
- 40 Challiss, R. A. J., Wilkes, L. C., Patel, V., Pyrkiss, J. R. and Boarder, M. R. (1993) *FEBS Lett.* **327**, 157–160
- 41 Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C. and Roth, M. G. (1996) *J. Cell Biol.* **134**, 295–306
- 42 Ktistakis, N. T., Brown, H. A., Sternweis, P. C. and Roth, M. G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4952–4956
- 43 Guillemain, I. and Exton, J. H. (1997) *Eur. J. Biochem.* **249**, 812–819
- 44 Yang, L. and Glaser, M. (1996) *Biochemistry* **35**, 13966–13974
- 45 Lee, C., Kang, H.-S., Chung, J.-K., Sekiya, F., Kim, J.-R., Han, J.-S., Kim, S. R., Bae, Y. S., Morris, A. J. and Rhee, S. G. (1997) *J. Biol. Chem.* **272**, 15986–15992