British Journal of Pharmacology (1998) 125, 1601–1609
http://www.stockton-press.co.uk/bjp

Distinct PKC isoforms mediate the activation of $cPLA_2$ and adenylyl cyclase by phorbol ester in RAW264.7 macrophages

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1 The modulatory effects of protein kinase C (PKC) on the activation of cytosolic phospholipase A_2 ($cPLA_2$) and adenylyl cyclase (AC) have recently been described. Since the signalling cascades associated with these events play critical roles in various functions of macrophages, we set out to investigate the crosstalk between PKC and the $cPLA_2$ and AC pathways in mouse RAW 264.7 macrophages and to determine the involvement of individual PKC isoforms. The $cPLA_2$ and AC pathways were studied by measuring the potentiation by the phorbol ester PMA of ionomycin-induced arachidonic acid (AA) release and prostagladin E_1 (PGE_1)-stimulated cyclic AMP production, respectively.

2 PMA at 1 μM caused a significant increase in AA release both in the presence (371%) and absence (67%) of ionomycin induction, while exposure of RAW 264.7 cells to PMA increased PGE_1 stimulation of cyclic AMP levels by 208%.

3 Treatment of cells with staurosporine and Ro 31-8220 inhibited the PMA-induced potentiation of both AA release and cyclic AMP accumulation, while Go 6976 (an inhibitor of classical PKC isoforms) and LY 379196 (a specific inhibitor of PKCβ) inhibited the AA response but failed to affect the enhancement of the cyclic AMP response by PMA.

4 Long term pretreatment of cells with PMA abolished the subsequent effect of PMA in potentiating AA release, but only inhibited the cyclic AMP response by 42%.

5 Neither PD 98059, an inhibitor of MEK, nor genistein, an inhibitor of tyrosine kinases, had any effect on the ability of PMA to potentiate AA or cyclic AMP production.

6 The potentiation of AA release, but not of cyclic AMP formation, by PMA was sensitive to inhibition by wortmannin. This effect was unrelated to the inhibition of PKC activation as deduced from the translocation of PKC activity to the cell membrane.

7 Western blot analysis revealed the presence of eight PKC isoforms (α, βI, βII, δ, ε, μ λ and ξ) in RAW 264.7 cells and PMA was shown to induce the translocation of the α, βI, βII, δ, ε and μ isoforms from the cytosol to the cell membrane within 2 min.

8 Pretreatment of cells with PMA for 2–24 h resulted in a time-dependent down-regulation of PKCα, βI, βII, and δ expression, while the levels of the other four PKC isozymes were unchanged after PMA treatment for 24 h. A decrease in the potentiation of AA release by PMA was observed, concomitant with the time-dependent down-regulation of PKC.

9 These results indicate that PKCβ has a crucial role in the mediation of $cPLA_2$ activation by the phorbol ester PMA, whereas PMA utilizes PKC ε and/or μ to up-regulate AC activity.

Keywords: PKC isoform; $cPLA_2$; adenylyl cyclase; RAW 264.7; signalling crosstalk

Introduction

The activation of intracellular signalling pathways by extracellular stimuli usually requires the involvement of one or more integral membrane proteins. For example, multimodal regulation of cytosolic phospholipase A_2 ($cPLA_2$) and cyclic AMP synthesis has recently been described (for reviews see Taussig & Gilman, 1995; Sunahara *et al*., 1996; Dennis, 1997). Modulation of enzymatic activity by phosphorylation is a common mode of both downstream and feedback regulation within signal transduction cascades and protein kinase C (PKC), which belongs to a family of serine/threonine specific kinases, acts as a regulator in this context.

PKC plays a critical role in a plethora of cellular responses. It is activated by lipid second messengers, predominantly diacylglycerol (DAG), in response to various extracellular agonists such as hormones, neurotransmitters, growth factors and cytokines (for reviews see Hug & Sarre, 1993; Dekker & Parker, 1994; Nishizuka, 1995; Jaken, 1996). Tumour-promoting phorbol esters, e.g. phorbol 12-myristae 13-acetate (PMA), can substitute for DAG as activators of PKC, thus enhancing the association of the cytosolic enzyme with the membrane (i.e. translocation). To date, at least 12 PKC isoforms have been characterized at the molecular level. Based on primary structure and *in vitro* activation requirements, the PKC family can be grouped into three major classes: Ca^{2+}-dependent and DAG-activated, conventional PKCs (α, βI, βII and γ); Ca^{2+}-independent, but DAG-activated novel PKCs (δ, ε, η, θ and μ); and Ca^{2+}-independent and DAG-nonactivated atypical PKCs (ζ, λ and ι). Both conventional and novel PKCs are known to translocate to the plasma membrane following phorbol ester stimulation, and some of them appear to be down-regulated in a cell type-specific manner by prolonged stimulation with phorbol ester, as the membrane-associated forms of the enzyme is more susceptible to proteolysis than the cytosolic forms (Young *et al*., 1987). Atypical PKCs are believed not to be responsive to DAG, but are activated by PIP_3, ceramide and arachidonic acid (AA) (Nakanishi *et al*, 1993; Muller *et al*., 1995). At present, tissue specific expression and differential intracellular location suggest that individual PKC isotypes have distinct functions in signal

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transduction and cellular metabolism, although it has been difficult to ascribe specific functions to PKC isoforms with certainty.

Recently the effects of phorbol esters on the expressed adenylyl cyclase (AC) isoforms have been reported. Among the mammalian ACs known, PKC has been implicated both in the stimulation of AC type I (AC I), AC type II (AC II) and AC type V (AC V) and in the inhibition of AC type VI (AC VI) (Choi *et al*., 1993; Jacobowitz & Iyengar, 1994; Kawabe *et al*., 1994; Lai *et al*., 1997). Although there is no consensus of opinion on the effects of PKC on ACs, there are some reports concerning the regulation and activation of ACs by different PKC isoforms. In NIH-3T3 cells, a facilitatory role for PKCγ and an inhibitory role for PKCα have been suggested in cyclic AMP-generating systems (Gusovsky & Gutkind, 1991). On the other hand, in SK-N-MC cells, PKCα has been implicated in the potentiation of AC activity (Zhou *et al*., 1994). Direct interactions between PKC and AC have recently been explored. The results revealed that AC V is a substrate for PKCα and PKCζ both *in vitro* and intact cells (Kawabe *et al*., 1994; 1996). Moreover, AC II and AC V have been shown to be phosphorylated *in vivo* in response to PMA (Jacobowitz & Iyengar, 1994). Zimmermann & Taussig (1996) reported that PKCα markedly increases the G protein α-subunit-stimulated activity of AC II, but reduces that of AC IV.

With respect to the PKC-dependent activation of cPLA$_2$, although this has been demonstrated in response to different stimuli in a variety of cells (Wijkander & Sundler, 1991; Qiu & Leslie, 1994; Xing & Insel, 1996), the involvement of specific PKC isoform(s) is not yet clear. The roles of PKC isozymes in cPLA$_2$ activation have recently been shown to include the participation of PKCα in phorbol ester-mediated AA release by MDCK cells, as evidenced by antisense technique (Godson *et al*., 1993), and the involvement of PKCβ in the PMA-induced activation of the AA cascade in liver macrophages (Duyster *et al*., 1993).

Macrophages are able to process and present foreign antigens, and are known to play a key role in many respects of acute and chronic inflammation. Macrophages themselves secrete a range of cytokines as well as lipid-derived mediators that may participate in amplification of inflammatory responses at sites of infection (Serhan *et al*., 1996). cPLA$_2$ is pivotal in the biosynthesis of lipid-derived mediators, AA and eicosanoids. Intracellular cyclic AMP concentrations are also known to affect a variety of functions of macrophages, particularly those functions that are involved in inflammatory processes. Phagocytosis, presentation of antigenic particles, and the production of reactive oxygen species. AA metabolites and cytokines are all inhibited by elevation of intracellular cyclic AMP levels (Dent & Rabe, 1996).

Since the mechanisms of AC and cPLA$_2$ regulation by the PKC system in macrophages is still unclear, it is interesting to explore the role of the PKC isozymes in mediating the effects of PMA on cPLA$_2$ and AC activation. Using RAW 264.7 mouse macrophages as a model system, the aim of this study was to investigate the crosstalk between PKC activtion and the cPLA$_2$ and AC systems, and to study whether specific PKC isoforms are involved in these signal transduction pathways. Our findings suggest that in RAW 264.7 macrophages, PKCβ modulates the regulation of cPLA$_2$ activity in response to PMA, and PKC ε and/or μ is responsible for the potentiation of AC activity.

Methods

Cell culture

RAW 264.7 cells, obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.), were grown in 24-well plates (for AA release assays), 35 mm Petri dishes (for cyclic AMP measurement) or 10 cm dishes (for preparation of immunoblots) at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, in a humidified atmosphere of 95% air/5% CO_2.

AA release

AA release was measured as described before (Lin & Lee, 1996). In brief, cells were prelabelled with 0.3 μCi ml^{-1} of [^{3}H]-AA in DMEM for 24 h at 37°C. The cells were washed twice with serum-free DMEM and incubated in medium containing 0.5% fatty acid-free bovine serum albumin (BSA) before stimulation with ionomycin (1 μM) at 37°C for 30 min. At the end of the incubation, the medium was removed and centrifuged at 250 × *g* for 5 min to remove floating cells. The radioactivity in the supernatant measured.

Measurement of intracellular cyclic AMP

Confluent cells were washed with physiological saline solution (in mM): PSS: 118 NaCl; 4.7 KCl; 1.8 $CaCl_2$; 1.2 KH_2PO_4; 11 glucose; 20 HEPES; pH 7.4) and incubated for 20 min at 37°C in the presence of 500 μM 3-isobutyl-methylxanthine (IBMX). The reaction was initiated by the addition of 1 μM prostaglandin E_1 (PGE_1) for 10 min and then terminated by aspirating the reaction mixture and adding 0.1 N HCl. The cells were detached from the plates by scraping and the suspensions were centrifuged. The supernatant was neutralized and assayed for cyclic AMP levels with the [^{3}H]-cyclic AMP assay kit (Amersham International) according to the manufacturer's instructions. The level of cyclic AMP was expressed as pmol mg^{-1} protein.

Immunoblotting analysis

Cells were placed on ice, washed with PBS, resuspended in homogenization buffer (in mM): 20 Tris-HCl, 0.5 EGTA; 2 EDTA; 2 DTT; 0.5 p-methylsulphonyl fluoride; 10 μg ml^{-1} leupeptin; pH 7.5) and sonicated. In some experiments, cell homogenates were fractionated into cytosol and membrane parts by centrifugation at 40,000 × *g* for 1 h. The protein levels in cell homogenates and cell fractions were assayed by the Bradford method. Samples containing equal masses of protein (60 μg) were then separated by 9% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate (SDS–PAGE) and proteins were electroblotted onto nitrocellulose membranes (Amersham ECL grade). Blots were then blocked in TBST (100 μM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) containing 2% nonfat dry milk powder overnight at 4°C, washed four to six times with TBST, and incubated with PKC isoform- or AC II-specific primary antibody, diluted by 1 : 1000 in TBST, for 1 h. After further washing, the blots were incubated for 45 min with horseradish peroxidase conjugate of anti-mouse IgG or anti-rabbit IgG antibody, diluted by 1 : 1000 in TBST, washed again and processed for visualization using the Enhanced ChemiLuminescence (ECL) system (Amersham International) according to the manufac-

turer's recommendations. The blots were exposed to Kodak XAR-5 film to obtain the fluorographic images.

PKC activation

PKC activities in cytosolic and membrane fractions were measured using kits (Amersham International) to determine DAG and phospholipid-dependent phosphotransferase activities, according to the manufacturer's instructions. The kinase activity was expressed as pmol phosphate min^{-1} mg protein^{-1}.

Materials

DMEM, foetal bovine serum, penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). [^{3}H]-AA (100 Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]-cyclic AMP assay system, ECL system, HRP-conjugated goat anti-mouse and sheep anti-rabbit antibodies and the PKC enzyme assay system was purchased from Amersham International (Arlington Heights, IL, U.S.A.). Staurosporine, PMA, ionomycin, wortmannin, PGE_1, IBMX and pertussis toxin (PTX) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PD 98059 and genistein were from RBI (Natick, MA, U.S.A.). Ro 31-8220 and Go 6976 were purchased from Calbiochem (La Jolla, CA, U.S.A.). LY379196 was kindly supplied by Lilly Research Laboratories (Indianapolis, IN, U.S.A.). Monoclonal antibodies specific for PKC α, γ, δ, ε, θ, λ, μ, ι and ζ were purchased from Transduction Laboratories (Lexington, KY, U.S.A.) and polyclonal antibodies specific for PKC βI, βII, η and ACII were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All the materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Statistical analysis

In each experiment all samples were set up in duplicate, and the data shown represent the mean$\pm$s.e.mean from several independent experiments. $P<0.05$ was considered significant by evaluation of the data with Student's *t*-test. The error bar was omitted when it was within the symbol representing the mean value.

Results

PMA-mediated potentiation of AA release and cyclic AMP formation

Figure 1 shows the dose-dependency of PMA-mediated potentiation of stimulus-induced AA release and cyclic AMP formation in RAW 264.7 cells. Treatment of cells with ionomycin (1 μM) results in an increase of [^{3}H]-AA release from 693$\pm$51 to 1335$\pm$120 c.p.m. ($n=6$) within 30 min. In the presence of IBMX (500 μM), PGE_1 (1 μM) caused an increase in cyclic AMP accumulation from 30$\pm$3 to 450$\pm$63 pmol mg protein^{-1} ($n=22$) within 10 min. As shown in Figure 1a and b, in cells pretreated with PMA for 20 min, the AA and cyclic AMP responses induced by ionomycin and PGE_1, respectively, were potentiated in a concentration dependent manner. The cyclic AMP response was more sensitive to PMA than AA release, as 30 nM PMA was sufficient to achieve a significant increase in PGE_1-induced cyclic AMP formation, whereas at least 100 nM PMA was needed to potentiate ionomycin-induced AA release. At a concentration of 1 μM, PMA alone was seen to upregulate AA production by 67$\pm$14% ($n=6$),

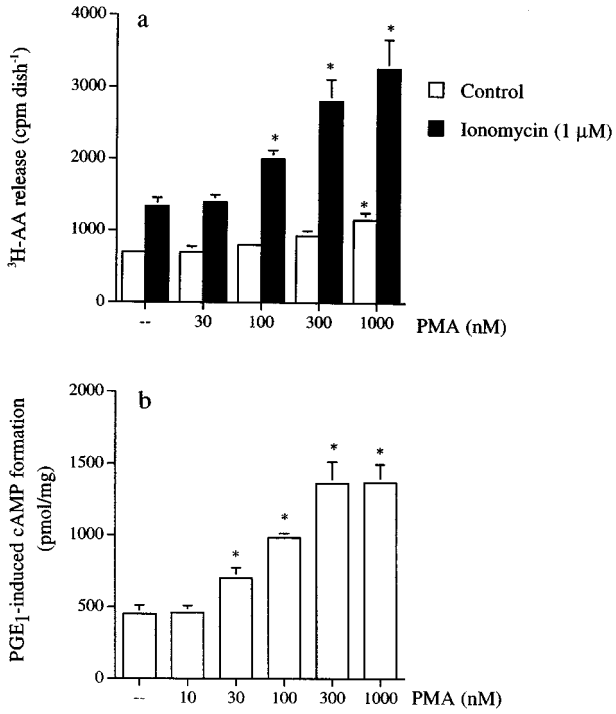

Figure 1 Concentration-dependent potentiation effects of PMA on stimulus-induced AA release and cyclic AMP formation. RAW264.7 cells were pretreated with PMA at the indicated concentrations for 20 min prior to the stimulation with 1 μM ionomycin for AA release (a) or 1 μM PGE_1 for cyclic AMP accumulation (b). Released [^{3}H]-AA and cytosolic cyclic AMP levels were assayed as described in Methods. The data represent the mean$\pm$s.e.mean from at least three independent experiments performed in duplicate. *Indicates AA or cyclic AMP levels significantly different ($P<0.05$) from controls without PMA pretreatment.

while potentiating the ionomycin-induced release of AA from 93$\pm$17% above background (in the absence of PMA) to 371$\pm$98% above background ($n=6$) (Figure 1a). In contrast, PMA at 1 μM had no effect on basal cyclic AMP formation (data not shown), but increased the PGE_1-induced response by 208$\pm$24% ($n=6$) (Figure 1b). Unlike PMA, the inactive phorbol ester, 4αPMA, was not seen to potentiate either of the two signalling cascades at concentrations up to 1 μM (data not shown).

Roles of PKC, extracellular Ca^{2+} and G proteins

As shown in Figure 2, the effects of PMA on ionomycin-induced AA release and PGE_1-induced cyclic AMP accumulation were both inhibited by the pretreatment of cells with 1 μM staurosporine or 1 μM Ro 31-8220 for 20 min. Pretreatment with Go 6976 (1 μM) for 20 min inhibited the PMA-mediated potentiation of AA release by 83$\pm$6%, but had no effect on the cyclic AMP response. To determine any involvement of PKCβ in AA and cyclic AMP production, we used a specific PKCβ inhibitor, LY 379196 (Gillig, personal communication). We found that LY 379196 at concentrations selective for PKCβ inhibition ($\leqslant$100 nM) could effectively inhibit the AA response, while leaving the cyclic AMP response unchanged. The increase in AA release, due to PMA treatment was reduced to 39$\pm$7% and 12$\pm$2% by LY 379196 at 10 nM and 30 nM, respectively. Long-term (24 h) pretreatment with 1 μM PMA almost abolished the AA response and inhibited the potentiation of cyclic AMP production by about 42$\pm$17% ($n=4$), following rechallenge with PMA.

In order to clarify the involvement of mitogen activated protein kinase (MAPK) in these PMA-induced responses, we tested the effects of PD 98059, a selective inhibitor of MAPK-activating enzyme (MEK) (Dudley *et al.*, 1995). We found that PD 98059 had no effect on the potentiation of AA or cyclic AMP production by PMA (Figure 2). Pretreatment of cells with 100 μM genistein, an inhibitor of tyrosine kinases, also left both effects of PMA unchanged (Figure 2).

Interestingly, we found that in the absence of PMA, staurosporine increased cyclic AMP production in response to PGE_1 by $91 \pm 20\%$ ($n=5$). Moreover, under these conditions, PMA could not further increase cyclic AMP formation (Figure 2b). Long-term pretreatment with 1 μM PMA, on the other hand, caused inhibition of the ionomycin- and PGE_1-induced responses by $29 \pm 10\%$ and $15 \pm 7\%$ ($n=4$), respectively.

The effects of PMA pretreatment on AA and cyclic AMP production are shown in Figure 3. In cells pretreated with PMA for 2 or 4 h ionomycin-induced AA production was significantly increased in comparison to cells that had not been pretreated. In cells that had received no PMA pretreatment or that had been pretreated for 2 h, rechallenge with PMA (1 μM for 20 min) resulted in a 90–110% increase over control ionomycin-induced AA release. Longer preincubation times with PMA did not increase ionomycin-induced or basal AA release and, with all the preincubation times tested, subsequent rechallenge with PMA did not significantly increase ionomycin-induced AA release (Figure 3a). In the case of cyclic AMP formation, the results shown in Figure 3b reveal that the most marked effect (150% increase over control) on PGE_1-elicited cyclic AMP formation was achieved following a 20 min incubation with PMA without any pretreatment. In the absence of PMA rechallenge, preincubation with PMA for 2 h led to an increase of 50% in the PGE_1-induced cyclic AMP levels, but longer preincubation periods had no effect on cyclic AMP production. However, rechallenge with PMA caused a significant increase in PGE_1-induced cyclic AMP production following all the periods of PMA preincubation tested.

The effects of extracellular Ca^{2+} removal and PTX pretreatment on AA release and cyclic AMP formation are shown in Figure 4. Removal of extracellular Ca^{2+} abolished

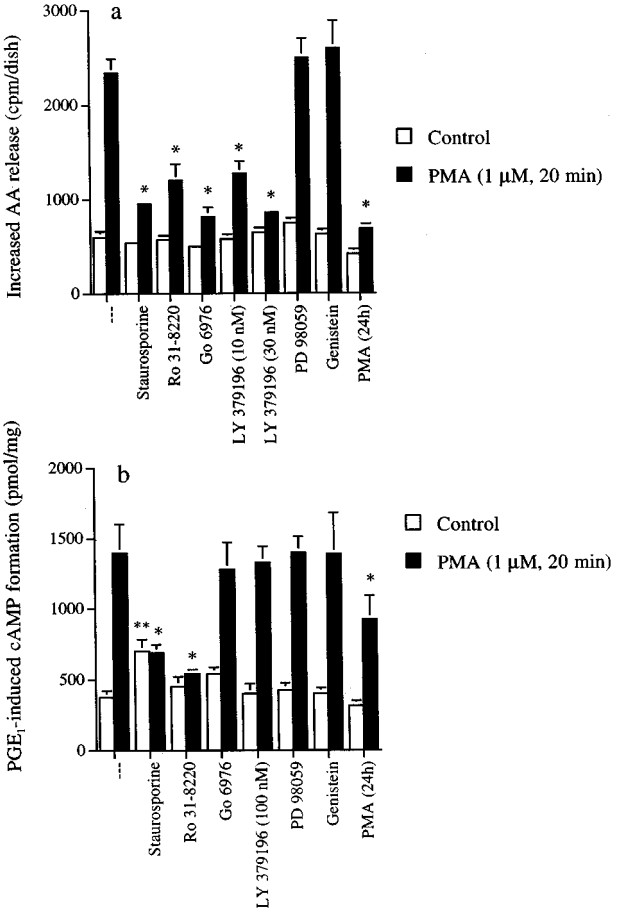

Figure 2 Effects of kinase inhibitors and chronic PMA pretreatment on PMA-induced potentiation of AA and cyclic AMP production. Cells were pretreated with staurosporine (1 μM), Ro 31-8220 (1 μM), Go 6976 (1 μM), LY 379196 (10, 30 or 100 nM), PD 98059 (100 μM), genistein (100 μM) for 20 min or PMA (1 μM) for 24 h prior to the addition of vehicle (control) or 1 μM PMA for 20 min to induce the potentiation of ionomycin (1 μM)-triggered AA release (a) or PGE_1 (1 μM)-triggered cyclic AMP accumulation (b). The data represent the mean $\pm$ s.e.mean from three independent experiments. *Indicates AA or cyclic AMP production signifciantly different ($P<0.05$) to the PMA- induced responses without drug pretreatment. **Indicates cyclic AMP production significantly different ($P<0.05$) to the control (PGE_1-induced) response without PMA treatment.

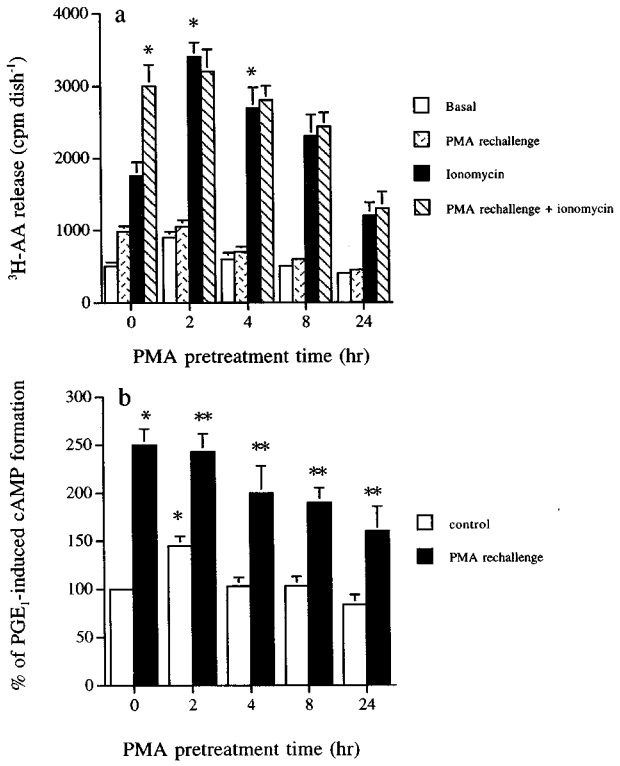

Figure 3 Time-dependent effects of PMA on AA release and cyclic AMP potentiation. Cells were pretreated with 1 μM PMA for 2, 4, 8, or 24 h prior to the addition of stimuli. In (a), cells were changed to serum-free fresh medium containing 0.5% BSA, rechallenged with 1 μM PMA for 20 min, then stimulated with 1 μM ionomycin for 30 min as indicated. [^{3}H]-AA released into the medium was measured. In (b), cells were washed with IBMX-containing PSS, rechallenged with 1 μM PMA for 20 min, then stimulated with 1 μM PGE_1 for 10 min as indicated. Intracellular cyclic AMP level was measured and expressed as the percentage of control PGE_1 response without PMA treatment. The data represent the mean $\pm$ s.e.mean from two independent experiments which were performed in duplicate. *Indicates a significant increase ($P<0.05$) in response to PMA pretreatment as compared to the control response of ionomycin or PGE_1. **Indicates a significant increase in response ($P<0.05$) following rechallenge with PMA.

the AA responses caused by ionomycin both in the absence and presence of PMA (Figure 4a). Overnight pretreatment with PTX inhibited the induction of AA in response to ionomycin by 36±4%. However, PMA treatment still increased ionomycin-induced AA release by 140% following PTX pretreatment. Neither Ca^{2+} removal nor PTX treatment had any effect on PGE_1-induced cyclic AMP formation in the presence or absence of PMA (Figure 4b).

Effects of wortmannin

As shown in Figure 5, wortmannin pretreatment inhibited PMA-induced potentiation of AA induction in a dose-dependent manner with an IC_{50} of 0.2 μM, but had no significant effect on PMA-mediated potentiation of cyclic AMP induction at concentrations up to 1 μM. In the absence of PMA, wortmannin (1 μM) did not alter the effects of ionomycin and PGE_1 on AA and cyclic AMP induction, respectively (data not shown).

In order to determine whether wortmannin had any effect on PMA-induced PKC activation, PKC activities in the cytosolic and membrane fractions of RAW 264.7 cells, before and after PMA treatment, were determined. Figure 6 shows that treatment with 1 μM PMA for 10 min decreased cytosolic PKC activity by 35±5% ($n=3$) with a concomitant increase in the membrane PKC activity by 56±7% ($n=3$). Pretreatment with 1 μM wortmannin for 20 min had no significant effect on PMA-induced PKC translocation.

Presence of AC II in RAW 264.7 macrophages

A Western blot of RAW 264.7 cel lysate was incubated with a polyclonal antibody specific for AC II. A lysate of J774 macrophages, which have previously been shown to express AC II (Lin & Chen, 1997), was used as a positive control. The results shown in Figure 7 demonstrate that RAW 264.7 cells also express AC II (~100 kDa).

Involvement of PKC isoforms in cPLA$_2$ and AC signalling pathways

Specific antibodies were used in Western blot analysis to show that RAW 264.7 cells express eight PKC isozymes, α, βI, βII, δ, ε, μ, λ and ζ (Figure 8). The other isozymes (γ, θ, η and ι) were not detected in this cell line (data not shown). When cells were treated with 1 μM PMA for 2–24 h, the immunoreactivities of

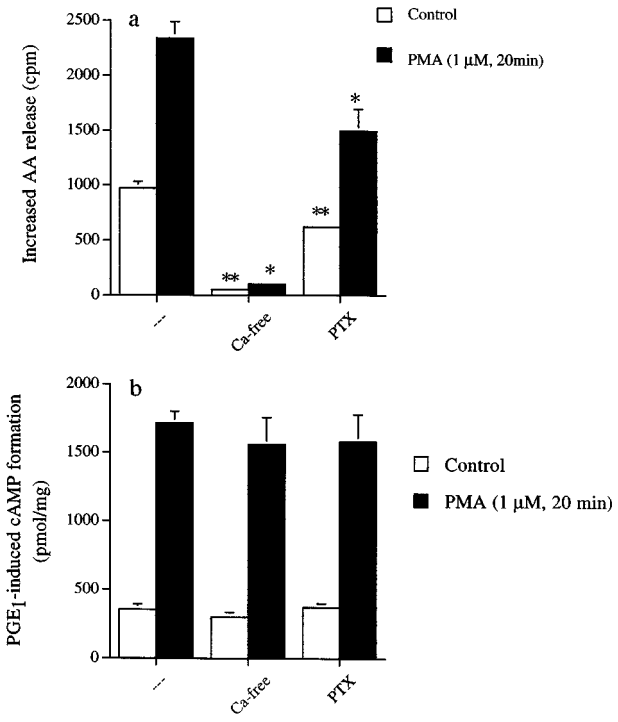

Figure 4 Effects of extracellular Ca^{2+} removal and pertussis toxin (PTX) on PMA-induced AA and cyclic AMP production. Cells were pretreated with Ca^{2+}-free PSS (containing 0.5 mM EGTA) four 20 min or with PTX (100 ng ml^{-1}) overnight prior to the addition of vehicle (control) or 1 μM PMA to potentiate 1 μM ionomycin-induced AA release (a) and 1 μM PGE_1-induced cyclic AMP accumulation (b). The data represent the mean±s.e.mean from four independent experiments. *Indicates a significant decrease ($P<0.05$) in PMA-enhanced response in cells subject to PTX treatment or Ca^{2+} removal. **Indicates $P<0.05$ for comparison of the ionomycin-induced response of cells in normal PSS and those subject to PTX treatment or Ca^{2+} removal.

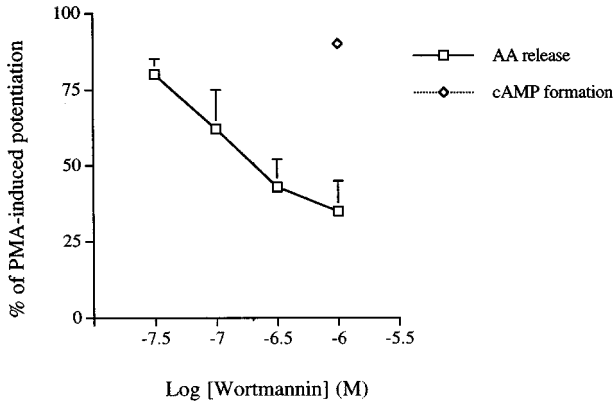

Figure 5 Effects of wortmannin on PMA-induced potentiation of the effects of ionomycin and PGE_1 on AA and cyclic AMP production. Cells were pretreated with wortmannin at the indicated concentrations for 20 min prior to the addition of 1 μM PMA to potentiate ionomycin (1 μM)- and PGE_1 (1 μM)-induced AA release and cyclic AMP accumulation, respectively. The data represent the mean±s.e.mean from 3–5 independent experiments.

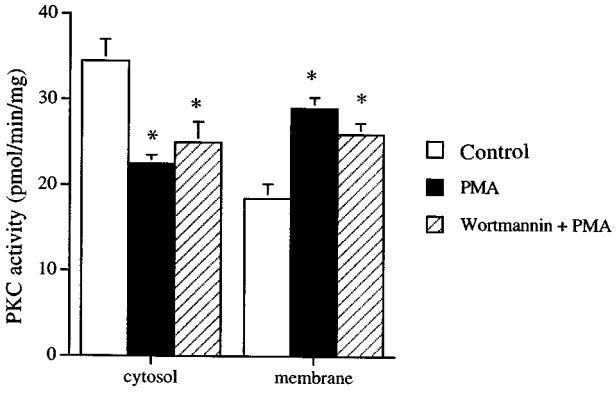

Figure 6 Effects of wortmannin on PMA-stimulated PKC translocation. Cells were treated with vehicle, 1 μM PMA or pretreated with 1 μM wortmannin for 20 min before the challenge with 1 μM PMA for 10 min. PKC activities in the cytosolic and membrane fractions were then assayed as described in Methods. The data represent the mean±s.e.mean from four independent experiments. *Indicates $P<0.05$ for PKC activities in PMA-treated samples compared to controls.

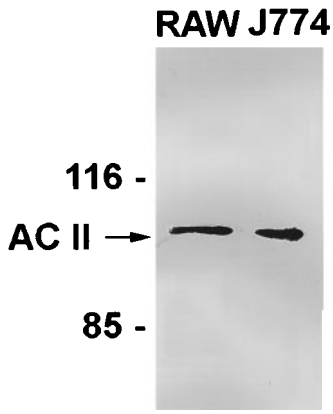

Figure 7 Immunodetection of AC II in RAW 264.7 cells. Homogenates from RAW 264.7 and J774 cells were fractionated by SDS–PAGE and immunoblots were incubated with a polyclonal antibody against AC II, as described in Methods.

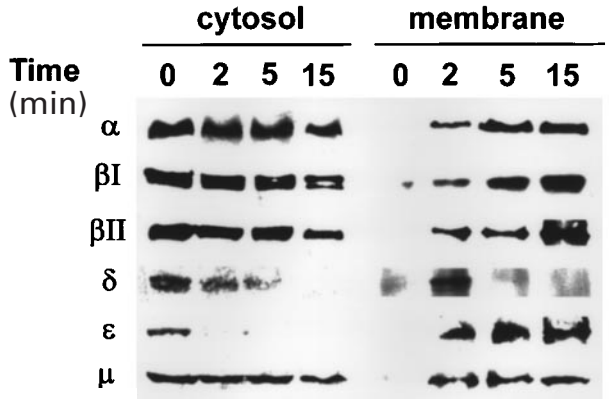

Figure 9 PMA-induced PKC translocation in RAW 264.7 cells. Cytosolic and membrane fractions were prepared from homogenates of RAW 264.7 cells, that had been exposed to 1 μM PMA for different times (2, 5 and 15 min). Immunoblots of the fractions were incubated with antibodies against each of the PKC isoforms, as described in Methods. The results are representative of three experiments.

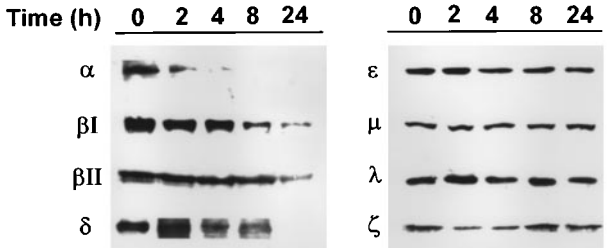

Figure 8 Time-dependent down-regulation of PKC isoforms by PMA. Cells were pretreated with 1 μM PMA for different times (2, 4, 8 and 24 h). Cell homogenates were separated by SDS–PAGE and immunoblots were incubated with antibodies specific for each of the PKC isoforms, as described in Methods.

the α, βI, βII and δ isozymes decayed in a time-dependent manner (Figure 8). After 2 h treatment, the amounts of PKC α and δ decreased by $65\pm12\%$ ($n=3$) and $74\pm10\%$ ($n=3$), respectively. As compared to the rapid down-regulation of PKC α and δ, the decay of βI and βII occurred slowly, with a $45\pm9\%$ ($n=3$) decrease in the amount of PKC βI after 4 h PMA treatment and a $43\pm10\%$ ($n=3$) decrease in the level of PKC βII after 8 h treatment. The levels of the ε, μ, λ and ζ isozymes were unaffected by PMA treatment for up to 24 h.

Further exploration of the PMA-mediated translocation of PKC to the cell membrane revealed that PKC α, βI, βII, δ, ε and μ were translocated from the cytosol to the cell membrane after treatment with PMA for only 2 min (Figure 9). In contrast, PKC λ and ζ remained in the cytosol after 30 min of PMA treatment (data not shown).

Discussion

In this study we have demonstrated that PKC-dependent potentiation of cPLA$_2$ and AC activities by a phorbol ester, PMA, in macrophage RAW 264.7 cells by measurement of AA and cyclic AMP production, respectively. In line with the results of studies using other cell lines (Wijkander & Sundler, 1991; Qiu & Leslie, 1994; Xing & Insel, 1996), we have shown that PKC activation can amplify cPLA$_2$ activity in RAW 264.7 cells. Several groups have demonstrated that phorbol ester treatment of cells expressing AC II exogenously, results in an increase in AC activity (Jacobowitz *et al.*, 1993; Lustig *et al.*, 1993). These findings, together with the observation that AC II is phosphorylated following PMA treatment (Jacobowitz & Iyengar, 1994), suggest that AC II may be phosphorylated and stimulated in response to PKC activation. Recently, Zimmermann & Taussig (1996) demonstrated *in vitro* that PKCα increased not only the basal activity of AC II but also the responsiveness of AC II to G protein α and $\beta\gamma$ subunits, particularly Gsα. Likewise, Tsu & Wong (1996) showed that receptors coupled to Gq proteins can stimulate AC II in a synergistic manner and that this stimulation is mediated by PKC. Our detection of AC II in RAW 264.7 cells (Figure 7) is in full agreement with the occurrence of crosstalk between AC-mediated signalling pathway and PMA-mediated PKC activation. This effect of PMA is consistant with our previous findings observed in mouse J774 macrophages where UTP can potentiate AC II activation *via* a PKC-dependent pathway (Lin & Chen, 1997).

In an attempt to assess the importance of PKC as a signalling mechanism in the activation of cPLA$_2$ and AC, we have utilized pharmacological approaches to inhibit and down-regulate PKC. Firstly, the inhibitory effects of staurosporine (a non-selective inhibitor of Ser/Thr protein kinases) and Ro 31-8220 (an inhibitor of PKC), but not genistein (an inhibitor of tyrosine kinases) and PD 98059 (an inhibitor of MEK) (Figure 2), on PMA-mediated potentiation of AA and cyclic AMP production indicate the specific involvement of PKC. Secondly, Go 6976, a selective inhibitor of the conventional PKC family (i.e. α, β, and γ) (Martiny-Baron *et al.*, 1993), substantially inhibited the ability of PMA to potentiate induction of AA, but had no effect on cyclic AMP production. These results suggest that classical PKC isoforms mediate AA release, while novel PKC isoforms mediate cyclic AMP production. Thirdly, additional support for this suggestion comes from the results of experiments with LY 379196, which is a selective inhibitor of PKC βI and βII with half-maximal inhibitory concentrations of 50 nM and 30 nM, respectively. At a concentration of 600 nM, the compound will show non-specific kinase inhibition. The IC_{50} values of LY 379196 against other PKCs are as follows (in μM): 0.3 for PKCη; 0.6 for PKCα and γ; 0.7 for PKCδ; 5 for PKCε; 48 for PKCζ (Gillig, personal communication). In this study LY 379196 at 10 or 30 nM effectively attenuated the upregulation of AA release in response to PMA, but at 100 nM had no effect on the PMA-mediated potentiation of cyclic AMP production.

Thus, we confirmed that PKC β (βI and/or βII) is involved in AA release. Fourthy, PMA can indeed activate and cause translocation to the cell membrane of the classical (α, βI, and βII) and novel (δ, ε and μ), but not atypical (λ and ζ) PKCs, in RAW 264.7 cells (Figure 9). Moreover, PKC α, βI, βII and δ were down-regulated following treatment of RAW 264.7 cells with PMA for 24 h. The observed lack of activation of PKC λ and ζ by PMA is in line with the absence of binding sites for DAG on atypical PKC family members. Fifthly, the potentiation by PMA of AA release, but not cyclic AMP production, was substantially inhibited after 24 h pretreatment with phorbol ester (Figures 2 and 3). After 24 h PMA preincubation, rechallenge with PMA can still elicit cyclic AMP potentiation, although less effectively. Sixthly, the time-dependent down-regulation of PKCβ by PMA within 2–24 h (Figure 8) is paralleled by a loss of ability to potentiate AA release (Figure 3a). Therefore, it is conceivable that PKC isoforms that are not down-regulated by PMA, but are PMA-responsive (i.e. PKC ε and μ) may play crucial roles in the potentiation of AC activity. These results all indicate that different PKC isoforms play specific modulatory roles, and suggest that PKC β is involved in cPLA$_2$ activation, while PKC ε and/or μ participate in AC induction.

PKC isoforms have been shown to differ in their sensitivities to stimulation by activators, and to proteolytic degradation (Ha & Exton, 1993). The translocation of PKC isoforms to the membrane is known to facilitate proteolysis of the cell membrane associated form of the enzyme. Certain isoforms of PKC have also been shown to be differentially resistant to down-regulation by PMAs. In our analysis of RAW 264.7 cells we found that PMA stimulation caused a rapid translocation of PKC βI and βII from the cytosol to the membrane fraction, which corresponded to the rapid potentiation of AA release. Furthermore, prolonged PMA treatment resulted in the down-regulation of PKCβI and βII, which was consistent with loss of potentiation of AA induction under these conditions. With regard to PKC ε and μ, both isoforms can be rapidly activated by PMA, but are resistant to proteolytic degradation. These phenomena are in accordance with the features that characterize potentiation of cyclic AMP production by PMA. The resistance to down-regulation of PKCε by long term phorbol ester treatment shown here is consistent to the results observed in other cell types (Berg *et al*., 1994). In contrast, PKC λ and ζ were neither translocated to the cell surface nor down-regulated by long term treatment with 1 μM PMA. With respect to PKC α and δ, although they can be both activated and preferentially down-regulated by PMA, their involvement in the two signalling cascades elicited by PMA can be ruled out. This is based on our observations of efficient inhibition of AA release by the PKC β inhibitor LY 379196 and participation of PKC isoforms that are not down-regulated by PMA in the cyclic AMP response.

Consistent to our previous observation that endogenous PKC activity can facilitate Ca^{2+} signal-evoked cPLA$_2$ activation (Lin, 1997; Lin & Chen, 1998), we found a slight attenuation of AA induction by ionomycin after 24 h PMA treatment. This attenuation might be due to the down-regulation of PKC and the decrease in endogenous PKC activity. The possibility that PMA might affect cPLA$_2$ expression was ruled out on the basis that the immuno-reactivity of cPLA$_2$ from PMA-treated cells, as recognized by a specific antibody (a gift from Genetics Institute, Massachusetts, MA, U.S.A.), was found to be the same as in untreated cells (data not shown).

The results of our exploration of the mechanisms responsible for cPLA$_2$ activation, imply the involvement of pertussis toxin- and wortmannin-sensitive pathways downstream of Ca^{2+}- and PKC-mediated cPLA$_2$ activation, respectively. With respect to the former pathway, we have suggested the possible involvement of a pertussis toxin-sensitive Go/Gi protein in cPLA$_2$ activation in response to $[Ca^{2+}]_i$-upregulating agents, such as thapsigargin (Lin & Chen, 1998), UTP (Lin and Lee, 1996) and ionomycin (present data). Additionally, it has been proposed that the $\beta\gamma$ subunits released from Gi/Go proteins are able to stimulate PLA$_2$ activity (Jelsema & Axelrod, 1987). Despite the partial inhibition by pertussis toxin of the ionomycin-induced AA response, PKC-elicited potentiation of the AA response still occurs to similar extents in the presence or absence of PTX treatment. Thus, the possibility that a G protein is linking PKC activation to cPLA$_2$ activation can be ruled out. With respect to the latter pathway, wortmannin, a microbial product, was found to be a potent inhibitor of PI 3-kinase (Ui *et al*., 1995) and thus has been extensively used to explore the crucial roles of PI 3-kinase in cellular functions. However, extensive studies have also revealed the nonspecific inhibition of myosin light chain kinase (Nakanishi *et al*., 1992), phospholipase D (Thompson *et al*., 1991) and PI 4-kinase (Nakanishi *et al*., 1995) by high concentrations of wortmannin. Here we have demonstrated a concentration-dependent inhibition by wortmannin of PMA-, but not ionomycin-, elicited AA production. Furthermore, the inability of wortmannin to inhibit PMA-induced PKC activity, consistent with previous findings (Nakanishi *et al*., 1992; Ferby *et al*., 1994), and its inability to affect cPLA$_2$ activity triggered by Ca^{2+} in permeabilized RAW 264.7 cells (Lin & Chen, 1998) imply that the target molecule for wortmannin may lie downstream of PKC and upstream of cPLA$_2$ activation. Further investigations are needed to clarify these points.

A unique property of staurosporine, not observed for other PKC inhibitors was revealed in this study. Our results demonstrate that staurosporine markedly potentiates the induction of cyclic AMP by PGE$_1$. In contrast, Ro 31-8220 failed to enhance the PGE$_1$-induced response. The mechanism by which staurosporine potentiates cyclic AMP activity is at present not known. It is possible that staurosporine is having an additional effect that is unrecognized and that somehow leads to the potentiation of the effect of PGE$_1$ on cyclic AMP production. Other effects unique to staurosporine have been previously described. For example, staurosporine potentiated the effect of IL-1β on CoxII mRNA expression while another PKC inhibitor, calphostin C, totally inhibited IL-1-mediated Cox II mRNA expression in renal mesangial cells (Rzymkiewicz *et al*., 1996). In bovine chromaffin cells, staurosporine activates a 60 kDa kinase by a mechanism that does not require PKC isoforms (Pavlovic-Surjancev *et al*., 1993). In subcultured fibroblast-like rheumatoid synovial cells, staurosporine can amplify PGE$_2$ production by IL-1α while inhibiting the effect produced by PMA (Taylor *et al*., 1990). In the context of phospholipase D (PLD) stimulation in rat hepatocytes (Gustavsson *et al*., 1994), rabbit peritoneal macrophages (Kanaho *et al*., 1992) and MDCK-D1 cells (Balboa *et al*., 1994), staurosporine has been described as an activator of PLD, while other PKC inhibitors inhibited it.

In summary, our findings indicate that PKC β plays a major role in phorbol ester-induced cPLA$_2$ activation, while PKC μ and/or ε appear to potentiate AC II activity in RAW 264.7 macrophages.

This research was supported by a research grant from the National Science Council of Taiwan (NSC 88-2314-B002-108).

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(Received Septebmer 15, 1997
Revised September 2, 1998
Accepted September 9, 1998)