Regulation of prostaglandin synthesis by protein kinase C in mouse peritoneal macrophages

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Resident mouse peritoneal macrophages synthesized and released prostaglandins (PGs) when challenged with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or 1,2-dioctanoyl-*sn*-glycerol (DiC₈). Both stimuli were found to activate Ca²⁺/phospholipid-dependent protein kinase C (PKC). 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine ('H-7') and D-sphingosine, known to inhibit PKC by different mechanisms, were able to decrease the PKC activity of macrophages in a dose-dependent manner. Addition of either PKC inhibitor decreased PG synthesis and also the release of arachidonic acid (AA) from phospholipids induced by TPA or DiC₈. Simultaneously TPA or DiC₈ also decreased incorporation of free AA into membrane phospholipids of macrophages. AA incorporation could be restored, however, by pretreatment with the PKC inhibitors. Our results demonstrate an involvement of PKC in the regulation of PG synthesis in mouse peritoneal macrophages and provide further evidence that reacylation of released fatty acids may be an important regulatory step.

INTRODUCTION

Macrophages play a central role in the stimulation and expression of immune responses [1] and possess the ability to secrete a wide array of biologically active compounds [2]. These secretory products include inflammatory mediators such as prostanoids, leukotrienes and hydroxy fatty acids. Several soluble and particulate stimuli can induce the synthesis of these arachidonic acid (AA) metabolites in macrophages [3–5]. It is widely accepted that, in most tissues, the synthesis of eicosanoids is limited by the availability of their common precursor, free arachidonic acid (AA), which has to be liberated from membrane phospholipids [6]. The concentration of free AA is very low both intra- and extra-cellularly, and represents a balance between the liberation of the fatty acid by hydrolysis and its re-esterification into membrane lipids by effective acyl-CoA synthetases (EC 6.2.1.3) and lysophosphatide acyltransferases (EC 2.3.1.23) [6,7]. It has been suggested that, in macrophages and many other cells, the activities of lysophosphatide acyltransferases (LAT) rather than those of phospholipases determine the cellular level of free AA [8–11]. Considerable efforts have been made to identify the pathways of AA mobilization from cellular phospholipids [12,13]. However, the control of AA availability is not completely understood at present.

It has been shown that AA release can be induced by tumour-promoting phorbol diesters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [14]. TPA is also known to directly activate the Ca²⁺/phospholipid-dependent protein kinase C (PKC). Increasing evidence

suggests that PKC has a crucial role in cell regulation and signal transduction for a variety of extracellular messengers such as growth factors, hormones and other agents [15,16]. These agents may induce phosphatidylinositol turnover, including transient production of 1,2-diacyl-*sn*-glycerol [17]. Endogenous diacylglycerol also activates PKC by a mechanism whereby the enzyme undergoes translocation from the cytosol to membrane sites [18].

We [19] and others [20] showed previously that macrophages respond to synthetic diacylglycerols with enhanced prostaglandin (PG) synthesis, suggesting an involvement of PKC in the regulation of eicosanoid synthesis. At high concentrations, diacylglycerols can also directly inhibit the key enzyme for the reacylation of released fatty acids, LAT, which may also lead to induction of increased PG synthesis [21].

In the present paper we demonstrate the effect of 12-*O*-tetradecanoylphorbol 13-acetate or 1,2-dioctanoyl-glycerol (DiC₈) treatment upon AA metabolism and subcellular distribution of PKC in resident macrophages. Our observations using 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) and D-sphingosine [22–24], both PKC inhibitors, demonstrate that PKC may be centrally involved in the regulation of AA availability for PG synthesis.

MATERIALS AND METHODS

Materials

All phospholipids, bovine serum albumin (essentially fatty-acid-free), CHAPS {3-[(3-cholamidopropyl)di-

Abbreviations used: AA, arachidonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate; DiC₈, 1,2-dioctanoyl-glycerol; DTE, 2,3-dihydroxybutane-1,4-dithiol; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; LAT, lysophosphatide acyltransferase [acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine *O*-acyltransferase (EC 2.3.1.23)]; PG(E₂), prostaglandin (E₂); PKC, protein kinase C (Ca²⁺/phospholipid-dependent protein kinase); sphingosine, *trans*-D-erythro-2-amino-4-octadecane-1,3-diol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate (phorbol myristate acetate, 'PMA'); DMEM, Dulbecco's modified Eagle medium; BSA, bovine serum albumin; OAG, 1-oleoyl-2-acetyl-glycerol.

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methylammonio]propane-1-sulphonate), DiC₈, 1,2-dioleoylglycerol, H-7, histone (type III-S), leupeptin, D-sphingosine, TPA and zinc acetate were obtained from Sigma (Deisenhofen, Germany). Cell-culture medium and its supplements were Gibco (Karlsruhe, Germany) products. AA was from NuChek Prep. (Elysian, MN, U.S.A.), and PGs were from Upjohn (Kalamazoo, MI, U.S.A.). Goat anti-(rabbit γ -globulin) antiserum was obtained from Behring-Calbiochem (Frankfurt, Germany). [γ -³²P]ATP (sp. radioactivity 3000 Ci/mmol) was from New England Nuclear (Dreieich, Germany). [1-¹⁴C]AA (sp. radioactivity 60 mCi/mmol), and [5,6,8,11,12,15(*n*)-³H]PGE₂ (sp. radioactivity 140–170 mCi/mmol) were purchased from Amersham Buchler (Braunschweig, Germany). T.l.c plates type F1500/LS 254 and nitrocellulose membrane filters were from Schleicher and Schüll (Dassel, Germany). ATP, 4-nitrophenyl phosphate and all other Ultrapure chemical products were obtained from Boehringer (Mannheim, Germany) and Merck (Darmstadt, Germany) respectively.

Cell cultures

Resident macrophages were collected by peritoneal lavage from untreated DBA/2 mice as described recently [19]. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with NaHCO₃ (2.0 g/l), glucose (4.5 g/l), penicillin (100 units/ml), streptomycin sulphate (100 μ g/ml), sodium pyruvate (1 mM), L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M) and 1% non-essential amino acids, at a cell density of 5×10^5 /ml in appropriate culture dishes (Nunc G.m.b.h., Wiesbaden, Germany) (see below) at 37 °C in a humidified atmosphere of CO₂/air (1:19). After pre-incubation for 2 h, the non-adherent cells were removed by washing with medium, and the adherent cells were maintained under the conditions specified in different experiments. The cultures were shown by Pappenheim staining and morphological criteria to be predominantly resident macrophages. The viability of these cells, as judged by Trypan Blue exclusion was never below 95%.

Preparation of subcellular fractions

To measure the effect of H-7 or sphingosine on PKC activity, the cytosolic fraction of untreated resting macrophages was used. Purified macrophage cultures in plastic dishes were washed with isolation buffer (consisting of 1 mM-EGTA, 2 mM-EDTA, 5 mM-DTE and 20 mM-Hepes, pH 7.4) and treated as described below. In order to examine the influence of TPA or DiC₈ upon the distribution of PKC activity in resident macrophages, cell cultures in plastic dishes were incubated with the addition of these substances in serum-free DMEM at 37 °C in CO₂/air (1:19) for various times. After incubation the supernatants were collected to assay PG synthesis, and the macrophages were washed with isolation buffer. The cells were then scraped off and disrupted by sonication (three times, 10 s bursts, 50 W) in isolation buffer for determination of PKC activity. The homogenate was sedimented at 100000 *g* for 60 min at 4 °C, the supernatant was carefully decanted and referred to as the 'cytosolic fraction'. The pellet ('crude particulate fraction') was resuspended in isolation buffer containing 5 mM-CHAPS, left for at least 30 min on ice, and was subjected to centrifugation as described above. The supernatant of this centrifugation step was referred to as the 'solubilized particulate fraction'.

Determination of PKC activity

PKC activity was assayed essentially as described by Kikkawa *et al.* [25] by measuring the incorporation of radioactive phosphate from [γ -³²P]ATP into H1 histone in the presence or absence of 1 μ g of 1,2-dioleoyl-*sn*-glycerol and 5 μ g of phosphatidylserine in a final volume of 200 μ l. The reaction medium contained 6 nmol of [γ -³²P]ATP (5×10^4 c.p.m./nmol), 40 μ g of histone, 10 mM-MgCl₂, 1 mM-CaCl₂, 0.4 mM-EGTA and 40 mM-Hepes, pH 7.4, with or without lipids (dispersed by sonication; three 15 s bursts at 50 W). The reaction was started by mixing 100 μ l of reaction medium with 10 μ g of sample protein of the corresponding subcellular fraction in 100 μ l of isolation buffer and was allowed to run for 3 min at 37 °C. During this assay, the reaction was linear and the activity was proportional to the amount of sample protein employed. The incubation was stopped by the addition of 200 μ l of chilled trichloroacetic acid (10%, w/v) containing 1 mM-ATP and 15 mM-K₂HPO₄. The acid-precipitable material was collected on a 1.5 cm² nitrocellulose membrane filter (pore size 0.45 μ m) and washed three times with 2 ml of chilled trichloroacetic acid (5%, w/v) containing 15 mM-K₂HPO₄. The filters were then placed in scintillation vials with 2 ml of scintillation fluid and the radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. PKC activity is expressed as a difference between the values measured in the presence or absence of lipids in units of nmol of phosphate transferred to histone/min per mg of sample protein.

Determination of PGE₂ synthesis

Macrophage cultures were incubated in 96-well microtitre dishes for the appropriate periods under CO₂/air (1:19) at 37 °C in DMEM under serum-free conditions. During this time the cells were exposed to the indicated concentrations of TPA or DiC₈. When the influence of H-7 or sphingosine on PG synthesis was investigated, the macrophages were preincubated with these compounds for 15 min before the actual incubation procedure started. After the times indicated, the supernatants were transferred into 1 ml Eppendorf tubes and centrifuged at 10000 *g* for 1 min to remove all cellular material. The amount of PGs in the culture supernatants was determined by radioimmunoassay with specific antisera using a modified double-antibody method to separate free and specific antibody-bound PG as described in detail by Kaever *et al.* [26]. The detection limit of the immunoassay for PGE₂ was 20 pg/ml of incubation medium.

AA release of labelled macrophages

To assay AA release, macrophages were cultured in 24-well dishes in fresh DMEM supplemented with 1% fetal-calf serum (heat-inactivated) and with the addition of 25 nCi or 50 nCi of [1-¹⁴C]AA. After a labelling period of 18 h, the macrophages were washed three times with DMEM and overlaid with fresh DMEM containing 0.3% bovine serum albumin. After incubation with appropriate stimuli for different times (see individual experiments) at 37 °C in a humidified atmosphere of CO₂/air (1:19), media were removed and collected separately in scintillation vials. Radioactivity of the supernatants was measured by liquid-scintillation counting. When the phospholipids of the remaining macro-

phage monolayers were studied, the extraction procedure was performed as described for AA-incorporation experiments (see below).

AA incorporation into cellular phospholipids of macrophages

Cells were incubated in flat-bottomed tubes with 25 nCi of [^{14}C]AA and unlabelled AA (10^{-5} M) in DMEM supplemented with bovine serum albumin (0.1%) at 37 °C in CO_2 /air (19:1) for 30 min. During this labelling period, 10^{-7} M-TPA or DiC_8 (25 $\mu\text{g}/\text{ml}$) were present and, in the case of H-7, a 15 min preincubation step with this compound preceded the incubation with AA. The reaction was stopped by removing the supernatant. To analyse the individual phospholipids, macrophage monolayers were overlaid with buffer (0.14 M-KCl/20 mM-Hepes, pH 7.0), sonicated for 10 s at 50 W, and the lipids extracted by the method of Bligh & Dyer [27]. Individual phospholipids were separated on silica sheets by t.l.c. with a solvent system of chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as described by Skipski *et al.* [28]. Lipid-containing regions were cut out after comparison with authentic standards, exposed to iodine vapours, and radioactivity was determined by liquid-scintillation counting.

Assay of LAT activity

After serum-free (DMEM) incubation procedures in the presence or absence of 10^{-7} M-TPA and removal of the supernatants to assay PGE_2 synthesis, the macrophages were homogenized by sonication in the assay buffer (0.14 M-KCl/20 mM-Hepes, pH 7.0); where indicated the buffer additionally contained 10 mM-4-nitrophenyl phosphate and 0.5 mM- Zn^{2+} . LAT activity was determined with [^{14}C]lysophosphatidylcholine and arachidonoyl-CoA as substrates [29]. Protein (8–20 μg) was incubated with 25 nmol of lysophosphatidylcholine, 10 nCi of 1-[^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine and 10 nmol of arachidonoyl-CoA in a total volume of 0.5 ml of assay buffer. Before performing the LAT assay, Zn^{2+} -containing tubes were supplemented with 10 mM-EDTA. After incubation for 10 min at 37 °C the enzymic reaction was stopped with methanol. During this assay, the reaction was linear with time and with the amount of sample protein employed. The phospholipids were extracted, separated by t.l.c. and identified by comparison with authentic standards. ^{14}C -containing regions were cut out and radioactivity was determined by liquid-scintillation counting. Enzyme activities of the homogenates were calculated from the conversion of labelled precursor into phosphatidylcholine.

Quantification of protein

Proteins were determined by the method of Bradford, with bovine serum albumin as standard [30].

RESULTS

PKC

When resting macrophages were homogenized in a buffer containing EDTA and EGTA, most of the PKC activity was located in the cytosolic fraction (2.1 ± 0.3 nmol of phosphate $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$), whereas only a small amount of this enzyme activity (about 12%) was detectable in the solubilized particulate fraction. Exposure of macrophages to TPA induced significant

Fig. 1. Cytosolic and membrane-associated PKC activities after exposure of macrophages to TPA and DiC_8 .

Resident peritoneal macrophages were incubated with 10^{-7} M-TPA (■) or DiC_8 (25 $\mu\text{g}/\text{ml}$) (▲) for various times as indicated. Washed cells were homogenized and sub-cellular fractions were prepared. Ca^{2+} /phospholipid-dependent kinase activities of crude cytosolic fractions (a) and solubilized particulate fractions (b) were measured and expressed as a percentage of control enzyme activity (●). Data shown are the mean percentage activities calculated from triplicate determinations from a single representative experiment of five similar ones.

changes in the cytosolic enzyme activity. PKC activity completely disappeared within 10 min (Fig. 1a), whereas the kinase activity associated with cellular membranes was significantly and simultaneously increased (Fig. 1b). After TPA treatment the membrane-bound PKC activity rapidly shifted to a Ca^{2+} /phospholipid-independent protein kinase activity (H.-J. Pfannkuche, V. Kaefer & K. Resch, unpublished work). The addition of DiC_8 to macrophages resulted in an incomplete and transient enzyme translocation (Figs. 1a and 1b). With time the lowered cytosolic enzyme activity (after 5 min incubation, 39% of initial level) rose, reaching 64% of the initial level after 40 min incubation. Alteration of the particulate PKC activity varied inversely with that of the cytosolic enzyme (Fig. 1). In contrast with the effect of TPA exposure, no changes of Ca^{2+} /phospholipid-independent kinase activity were observed during DiC_8 stimulation (results not shown).

Both the PKC inhibitors, H-7 and sphingosine, decreased the original cytosolic PKC activity (see above) in a concentration-dependent manner. Half-maximal inhibition under these conditions was observed at 5.5 μM -sphingosine and 15 μM -H-7 respectively.

Fig. 2. Effect of the PKC inhibitors H-7 and sphingosine on induction of PG synthesis in TPA- or DiC₈-treated macrophages

Resting macrophages were exposed to 10⁻⁷ M-TPA (Δ) in the presence or absence of different concentrations of H-7 (a) or sphingosine (b). Likewise, macrophages were incubated with DiC₈ (12.5 μ g/l; 25 μ g/l) ($\square$) plus additions of various concentrations of H-7 (c) or sphingosine (d). TPA and DiC₈ were omitted from control ($\circ$) cultures. After an incubation of 30 min the content of PGE₂ in the supernatant was quantified. The results presented are means $\pm$ range from duplicate determinations in individual and typical experiments.

Effect of H-7 and of sphingosine on the release of PGE₂ induced by TPA or DiC₈ respectively

The addition of TPA or the cell-permeable diacylglycerol DiC₈ to cultured macrophages caused an immediate and dose-dependent stimulation of PGE₂ release into the supernatants. The absolute amounts of PGE₂ released varied from experiment to experiment, even under identical stimulation conditions. However, the stimulation index (% increase above control values) for each substance was always comparable (results not shown).

H.p.l.c. analysis proved PGE₂ to be the main cyclooxygenase product synthesized by [³H]AA-labelled resident DBA/2 macrophages during exposure to TPA or DiC₈. Prostacyclin (measured as the hydrolysis product 6-oxo-PGF_{1 α}) was synthesized up to two-thirds of the

PGE₂ level, whereas only trace amounts of free AA were liberated into the supernatants under serum-free conditions (results not shown).

The release of PGE₂ stimulated by TPA or DiC₈ was decreased in a concentration-dependent fashion by both the PKC inhibitors (H-7 and sphingosine) (Figs. 2a–2d). The concentrations for half-maximal inhibition (5 μ M for sphingosine and 15–20 μ M for H-7) were in the same range as that seen when investigating their inhibitory action on PKC.

Influence of PKC inhibitors on AA release

In the presence of 0.3% defatted bovine serum albumin ('BSA trap') we measured a 2–3-fold increase of released radioactivity after exposure of the cells to TPA (Figs. 3, 4). The employment of BSA principally allows one to

Fig. 3. Effect of H-7 on the TPA-induced release of AA by macrophages

Resting macrophages labelled with 25 nCi of [^{14}C]AA were incubated for 90 min in the presence (Δ) or absence ($\circ$) of 10^{-7} M-TPA plus various concentrations of H-7, as indicated. The media were removed for subsequent determination of released radioactivity by liquid-scintillation spectrometry. The results presented are means $\pm$ range from duplicate determinations in a single experiment out of a series of three similar ones.

measure the amounts of AA cleaved from phospholipids, since trapping decreases cellular AA utilization [8]. For that reason unmetabolized AA predominantly accumulated in the supernatants under these experimental conditions.

In the concentration range of 12.5–100 μM , H-7 decreased macrophage AA release induced by TPA (Fig. 3). At 100 μM -H-7, the highest concentration tested, an almost complete inhibition of the TPA-induced AA release was apparent. The release of AA by resting macrophages stimulated with TPA was also susceptible to inhibition with sphingosine. Again, the inhibition was dose-dependent, and 10 μM -sphingosine decreased the AA release to control levels. Similarly, addition of the synthetic diacylglycerol DiC_8 to macrophages induced stimulation of AA release to an even greater extent than observed after TPA treatment (Fig. 4). Increasing concentrations of sphingosine caused a dose-dependent decrease in the release induced by DiC_8 ; however, the release was not reduced to basal levels.

Influence of PKC activators and inhibitors on the incorporation of AA into cellular phospholipids

We next examined whether TPA would also influence the incorporation of free AA into the phospholipids of resident macrophages. The cellular distribution of the incorporated label among cellular constituents proved that the major phospholipid labelled with [^{14}C]AA in resident macrophages was phosphatidylcholine [31]. The

Fig. 4. Effect of sphingosine on the TPA- or DiC_8 -induced release of AA by macrophages

Cultures of resident macrophages labelled overnight with 50 nCi of [^{14}C]AA were washed free of non-incorporated radioactively labelled AA. Various amounts of sphingosine were added in the culture medium in the absence ($\circ$) or presence of 10^{-7} M-TPA (Δ) or DiC_8 (25 $\mu\text{g}/\text{ml}$) ($\square$) and the cells incubated for at least 60 min. Subsequently media were collected to determine the corresponding radioactivity released. Results presented are means from duplicate determinations in a single experiment. This experiment was repeated three times with similar results.

addition of 10^{-7} M-TPA significantly decreased the rate of incorporation of 10^{-5} M-AA into phospholipids. Table 1 demonstrates the incorporation into the main component phosphatidylcholine. This agrees with our earlier finding of reacylation inhibition [19] and caused us to investigate whether the potent PKC activator DiC_8 could suppress the incorporation rate. DiC_8 (25 $\mu\text{g}/\text{ml}$) potently inhibited the incorporation of AA into the phosphatidylcholine component of resident macrophages (Table 1). In addition, H-7 was able to restore, in a dose-dependent manner, the suppressed incorporation rates, and even control cells showed increased reacylation activities above basal levels. The suppression of AA incorporation induced by DiC_8 was only partially compensated for by the addition of H-7 (Table 1).

LAT measurements

In contrast with DiC_8 and 1-oleoyl-2-acetylgllycerol (OAG), TPA did not inhibit macrophage LAT activity by means of direct interactions (results not shown). When resident macrophages were stimulated with optimal concentrations of TPA for the times indicated, no alteration of the cellular LAT activity was detectable in either of the corresponding cell homogenates thereafter (results not shown).

Table 1. Incorporation of AA into phosphatidylcholine by resting macrophages treated with PKC activators and inhibitors

Purified macrophages preincubated in the presence or absence of H-7 (25 μM or 50 μM) were exposed to 10^{-7} M-TPA or DiC_8 (25 $\mu\text{g/ml}$). [^{14}C]AA (25 nCi; 10^{-5} M) in BSA (final concn. 0.1%, w/v) was then added to the culture medium. After 30 min incubation the radioactivity content of the individual phospholipid components was determined by liquid scintillation counting. The values in parentheses are percentages of radioactivity associated with phosphatidylcholine and were calculated by comparison with values for untreated cells (designated as 100%).

Treatment	[H-7] (μM)...	Incorporation of AA into phosphatidylcholine (c.p.m.)		
		0	25	50
Control		987 (100.0)	1082 (109.6)	1429 (144.7)
TPA (10^{-7} M)		670 (67.8)	1029 (104.3)	1223 (123.9)
DiC_8 (25 $\mu\text{g/ml}$)		522 (52.9)	707 (71.6)	928 (94.0)

Table 2. PGE_2 concentration in supernatants and LAT activity in homogenates of TPA-treated resident macrophages

Macrophages were incubated with or without 10^{-7} M-TPA for the times indicated. Subsequently supernatants were collected to determine PGE_2 synthesis, and cells were homogenized in the presence of 10 mM-4-nitrophenyl phosphate and 0.5 mM-zinc acetate before LAT measurements (see the Materials and methods section). Values are means $\pm$ S.E.M. ($n = 4$); similar results were obtained in two separate experiments.

Time (min)		[PGE_2] ($\text{pg}\cdot\text{ml}^{-1}$)	LAT activity ($\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$)
30	Control	111 $\pm$ 15	20.4 $\pm$ 1.2
	TPA (10^{-7} M)	1428 $\pm$ 140	20.3 $\pm$ 2.0
60	Control	300 $\pm$ 44	18.7 $\pm$ 1.7
	TPA (10^{-7} M)	3091 $\pm$ 449	20.9 $\pm$ 1.1

Since LAT enzyme modification via direct phosphorylation by PKC seemed conceivable enzyme measurements were performed in the presence of 4-nitrophenyl phosphate and Zn^{2+} to prevent dephosphorylation steps *in vitro* via cellular phosphatases. Even in the presence of the phosphatase inhibitors, there was no lowering of enzyme activities measured in homogenates of TPA-treated macrophages (Table 2). All experiments performed showed that TPA stimulation did not induce inhibition of the macrophage LAT activity still measurable after cell homogenization.

DISCUSSION

General interest in PKC arose when it was shown to be the phorbol ester receptor (reviewed in [15]). Active phorbol esters intercalate into cellular membranes and mediate a high-affinity interaction between PKC and the membranes, thus mimicking the effect of diacylglycerol, the endogenous activator (see [18] for a review). The redistribution of the enzyme is complete, and PKC activation is virtually irreversible, since the association persists for an unphysiologically long period. Phorbol ester metabolism proceeds very slowly, whereas PKC degradation into regulatory and catalytically active

domains may happen immediately. Physiological membrane association and activation of PKC in response to several naturally occurring agonists which promote inositol phospholipid hydrolysis is known to be transient and of lower magnitude than those induced by phorbol esters. Endogenous and synthetic diacylglycerols are metabolized rapidly by diacylglycerol kinase or lipase [32]. Thus employment of synthetic diacylglycerols more closely mimics the physiological activation of PKC.

Resident mouse peritoneal macrophages exhibit a high PKC activity in their cytosol, whereas the activity in their particulate fraction is of a lower order of magnitude. In this cellular system the phorbol ester TPA induced a complete depletion of cytosolic PKC activity, whereas the synthetic diacylglycerol, DiC_8 , caused partial and reversible translocation (Fig. 1a). Simultaneously we found an increase of kinase activity in the solubilized particulate fractions of TPA- or DiC_8 -treated cells (Fig. 1b). As expected, PKC association with membrane sites induced by DiC_8 stimulation directly corresponded to the cytosolic enzyme alterations, whereas a rapid modification of TPA-induced membrane-bound PKC activity was observed. Since membrane-bound Ca^{2+} /phospholipid-independent kinase activity increased simultaneously with the advancing loss of membrane-bound PKC activity, our findings would give support to a model of highly membrane-integrated TPA-kinase complexes, active without the addition of lipids, as discussed previously for granulocytes [33].

Hidaka *et al.* [22] described the synthesis and properties of a class of protein kinase inhibitors based on isoquinoline sulphonamides. These agents possess potent inhibitory activity against cyclic nucleotide (cyclic AMP and cyclic GMP)-dependent protein kinase as well as PKC by competitively interacting with the ATP-binding site of the enzymes. The most potent of the inhibitors tested, H-7, has been used here. More recently, Hannun *et al.* [23,34] have reported the ability of sphingosine and lysosphingolipids to inhibit reversibly the activity, and phorbol-ester-binding capacity of PKC. Bazzi & Nelsestuen [24] examined the mechanism of sphingosine inhibition of Ca^{2+} /phospholipid-dependent PKC *in vitro*. Their results suggest that sphingosine exerts its function by decreasing the availability of phosphatidylserine (as the essential phospholipid component) by charge neutralization. Indeed, both PKC inhibitors dose-dependently decreased the PKC activity of resident macrophages.

These results enabled us to employ both inhibitors in investigating their effects on intact macrophages treated with PKC activators.

We [19,21] and others [20,35,36] have previously shown that both phorbol esters and more physiological PKC activators (synthetic diacylglycerols) were capable of stimulating PG synthesis. This finding was related to an involvement of PKC in regulating eicosanoid synthesis. PKC inhibitors were utilized in several cell systems to clarify whether PKC would contribute to the corresponding cellular function examined. As these inhibitors are not totally specific for PKC, they were used only in combination with the direct PKC activators TPA or DiC_8 . Our results indicate that H-7 and sphingosine are very effective in inhibiting PG synthesis induced by TPA or DiC_8 in resident macrophages (Fig. 2).

TPA or DiC_8 increased the release of AA from prelabelled cellular phospholipids (Fig. 3 and 4). In resting macrophages preincubated with H-7 or sphingosine and subsequently stimulated with TPA or DiC_8 , we found a retention of labelled AA in phospholipids (results not shown) as well as a diminished release of AA into the supernatant (Fig. 3 and 4). These findings with respect to AA release further demonstrate that stimulation and inhibition of PG synthesis can take place not only at the level of the cyclooxygenase/lipoxygenase enzyme system, but also occur at the level of the mechanism responsible for AA-precursor availability. The control of free AA availability depends on the activities of all enzyme systems involved in the deacylation-reacylation cycle (first described by Lands & Crawford [7]). AA is liberated in most tissues by the action of phospholipase A_2 and is rapidly reincorporated into phospholipids by LAT. Though some divergent results exist, there is good evidence that, in macrophages, liberation of AA by phospholipase A_2 represents the main pathway [37]. Many possibilities exist regarding the regulation of phospholipase A_2 activity. (1) Most phospholipases A_2 were shown to be Ca^{2+} -dependent, although it is uncertain whether these enzymes are regulated by Ca^{2+} [38]. (2) The localized accumulation of diacylglycerol in a cell membrane might play a role in activating intracellular phospholipases [39,40]. (3) Phospholipase A_2 -inhibitory proteins are believed to be involved in the regulation of enzyme activity [41,42]. These 'lipocortins' inhibit phospholipase A_2 activity, probably by blocking interaction of enzyme and substrate. Finally (4), phospholipases A_2 have been shown to be coupled to receptors by guanine nucleotide-binding regulatory proteins ('G-proteins') which present further pathways for controlling enzyme activity in those cells [43].

Since previous studies [6,8–11] have suggested that the amount of free AA is also substantially controlled by LATs, we examined the role of AA-reincorporating LAT in regulating the availability of this fatty acid. Preliminary results suggested an involvement of LAT [19]. In the present study, experiments were performed which demonstrated that the incorporation capacity of cells was markedly decreased by exposure to TPA or DiC_8 (Table 1). Therefore it appears likely that, in TPA- or DiC_8 -treated macrophages, an accumulation of AA available for PG synthesis occurs. The inhibitory effect of these PKC activators in this experimental system can be substantially attributed to the action of PKC in modulating LAT activity, since H-7 was able to com-

pletely restore reacylating capacity (Table 1). The finding that control cells also increased their AA-incorporating activities by addition of H-7 suggests a basal regulation by PKC in resting cells.

Recently, we were able to show that PKC activators like OAG or DiC_8 were able to inhibit the membrane-bound LAT by a direct interaction independent of PKC [21]. In macrophages a direct inhibition of LAT would also increase the pool of free AA available for PG synthesis. To reveal PKC-mediated changes in AA metabolism it was necessary to employ H-7 or sphingosine. The PKC inhibitors, even at high concentrations, were not able to completely antagonize DiC_8 -induced PG synthesis and AA release (Fig. 2 and 4). This may be attributed to the additional direct impairment of LAT activity, at least at high concentrations of DiC_8 [21].

Since TPA lacks the capacity to directly impair LAT activity, the phorbol diester was suitable for investigating mechanisms modulating LAT activity. Enzyme measurements with homogenates of TPA-treated macrophages never showed LAT activities significantly below control levels. Even the employment of phosphatase inhibitors did not unveil LAT activity impairments in consequence of direct PKC activation (Table 2). These results do not support the idea of enzyme modulation by direct phosphorylation via PKC, but lead to a conception of regulatory proteins linked to LAT enzymes. As LAT is an integral constituent of plasma membranes, direct enzyme phosphorylation may be more unlikely than modulation by regulatory proteins, which are good candidates for phosphorylation by PKC [44,45]. Cell homogenization would uncouple LAT from the corresponding regulatory protein, resulting in an inability to measure modified enzyme activities.

The present results strongly suggest that PKC regulates PG synthesis in mouse resident peritoneal macrophages. Our data further suggest that the activity of LAT may be crucially involved in the regulation of the availability of AA for further metabolism. A tentative model proposes that PKC modulates the AA-reacylating activity in macrophages via phosphorylation of a regulatory protein linked to LAT. Both LAT and phospholipase A_2 may therefore represent linking mechanisms between PKC activation and PG synthesis.

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REFERENCES

1. Gernsma, D., Leser, H.-G., Seitz, M., Debatin, M., Bärlin, E., Deimann, W. & Kramer, W. (1982) *Agents Actions Suppl.* **11**, 93–114
2. Nathan, C. F. (1987) *J. Clin. Invest.* **79**, 319–326
3. Scott, W. A., Zrike, J. M., Hamill, A. L., Kempe, J. & Cohn, Z. A. (1980) *J. Exp. Med.* **152**, 324–335
4. Humes, J. L., Sadowski, S., Galavage, M., Goldenberg, M., Subers, E., Bonney, R. J. & Kuehl, F. A., Jr. (1982) *J. Biol. Chem.* **257**, 1591–1594
5. Brune, K., Aehringhaus, U. & Peskar, B. A. (1984) *Agents Actions* **14**, 729–734
6. Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
7. Lands, W. E. M. & Crawford, C. G. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., ed.), vol. 2, p. 3, Plenum Press, New York

8. Kröner, E. E., Peskar, B. A., Fischer, H. & Ferber, E. (1981) *J. Biol. Chem.* **256**, 3690–3697
9. Schmidt, B., Flesch, I., Ecker, B., Hovestadt, I. & Ferber, E. (1984) *Agents Actions* **15**, 21–27
10. Körner, C.-F., Hausmann, G., Gemsa, D. & Resch, K. (1984) *Agents Actions* **15**, 28–30
11. Goppelt-Strübe, M., Körner, C.-F., Hausman, G., Gemsa, D. & Resch, K. (1986) *Prostaglandins* **32**, 373–385
12. Emilsson, A. & Sundler, R. (1985) *Biochim. Biophys. Acta* **816**, 265–274
13. Lokesh, B. R. & Kinsella, J. E. (1985) *Biochim. Biophys. Acta* **845**, 101–108
14. Humes, J. L., Davies, P., Bonney, R. J. & Kuehl, R. A., Jr. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1318 (abstr.)
15. Ashendel, C. L. (1985) *Biochim. Biophys. Acta* **822**, 219–242
16. Nishizuka, Y. (1986) *Science* **233**, 305–312
17. Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
18. Niedel, J. E. & Blackshear, P. J. (1986) in *Phosphoinositides and Receptor Mechanisms* (Putney, J. W., Jr., ed.), vol. 7, pp. 47–88, Alan R. Liss, New York
19. Pfannkuche, H.-J., Kaever, V. & Resch, K. (1986) *Biochem. Biophys. Res. Commun.* **139**, 604–611
20. Emilsson, A., Wijkander, J. & Sundler, R. (1986) *Biochem. J.* **239**, 685–690
21. Goppelt-Strübe, M., Pfannkuche, H.-J., Gemsa, D. & Resch, K. (1987) *Biochem. J.* **247**, 773–777
22. Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) *Biochemistry* **23**, 5036–5041
23. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 12604–12609
24. Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochem. Biophys. Res. Commun.* **146**, 203–207
25. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 13341–13348
26. Kaever, V., Goppelt-Strübe, M. & Resch, K. (1988) *Prostaglandins* **35**, 885–902
27. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
28. Skipski, V. P., Peterson, R. F. & Barclay, M. (1964) *Biochem. J.* **90**, 374–378
29. Resch, K., Schneider, S. & Szamel, M. (1983) *Biochim. Biophys. Acta* **733**, 142–153
30. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
31. Bonney, R. J., Wightman, P. D., Davies, P., Sadowski, S. J., Kuehl, F. A., Jr. & Humes, J. L. (1978) *Biochem. J.* **176**, 433–442
32. May, W. S., Lapetina, E. G. & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1281
33. Wolfson, M., McPhail, L. C., Nasrallah, V. N. & Snyderman, R. (1985) *J. Immunol.* **135**, 2057–2062
34. Hannun, Y. A. & Bell, R. M. (1987) *Science* **235**, 670–674
35. Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. (1985) *J. Biol. Chem.* **260**, 12484–12491
36. Pfeilschifter, J., Kurtz, A. & Bauer, Ch. (1986) *Biochem. J.* **234**, 125–130
37. Emilsson, A. & Sundler, R. (1986) *Biochim. Biophys. Acta* **876**, 533–542
38. Chang, J., Musser, J. H. & McGregor, H. (1987) *Biochem. Pharmacol.* **36**, 2429–2436
39. Dawson, R. M. C., Hemington, N. L. & Irvine, R. F. (1983) *Biochem. Biophys. Res. Commun.* **117**, 196–201
40. Kramer, R. M., Checiani, G. C. & Deykin, D. (1987) *Biochem. J.* **248**, 779–783
41. Davidson, F. F., Dennis, E. A., Powell, M. & Glenney, J., Jr. (1987) *J. Biol. Chem.* **262**, 1698–1705
42. Haigler, H. T., Schlaepfer, D. D. & Burgess, W. H. (1987) *J. Biol. Chem.* **262**, 6921–6930
43. Burgoyne, R. D., Cheek, T. R. & O'Sullivan, A. J. (1987) *Trends Biochem. Sci.* **12**, 332–333
44. Smith, C. D., Uhing, R. J. & Snyderman, R. (1987) *J. Biol. Chem.* **262**, 6121–6127
45. Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) *Eur. J. Biochem.* **151**, 431–437

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