Different roles of protein kinase C-β and -δ in arachidonic acid cascade, superoxide formation and phosphoinositide hydrolysis

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In contrast with protein kinase C (PKC)-β, PKC-δ is exclusively detectable in the membrane fraction of liver macrophages. After long-term treatment with phorbol 12-myristate 13-acetate (PMA) PKC-β is depleted faster (within 3 h) than PKC-δ (> 7 h). Simultaneously, pretreatment with PMA for 3 h inhibits the PMA- and zymosan-induced generation of superoxide and the PMA-induced formation of prostaglandin (PG) E_2, whereas a preincubation of more than 7 h is required to affect the zymosan-induced release of PGE_2 and inositol phosphates. These results support an involvement of PKC-β in the PMA-induced activation of the arachidonic acid cascade and in superoxide formation and imply an involvement of PKC-δ in zymosan-induced phosphoinositide hydrolysis and PGE_2 formation. Two phorbol ester derivates, sapintoxin A (SAPA) and 12-deoxyphorbol 13-phenylacetate 20-acetate (DOPPA), which have been previously reported to activate preferentially PLC-β but not PKC-δ *in vitro* [Ryves, Evans, Olivier, Parker and Evans (1992) FEBS Lett. **288**, 5–9], induce the formation of PGE_2 and superoxide, down-regulate PKC-δ and potentiate inositol phosphate formation in parallel. SAPA, but not DOPPA, down-regulates PKC-β and inhibits the PMA-induced formation of eicosanoids and superoxide.

INTRODUCTION

Kupffer cells, the resident liver macrophages, respond to many stimuli with the secretion of a wide array of biological active agents, including inflammatory mediators such as prostanoids and superoxide [1]. It has been shown that, e.g., phorbol 12-myristate 13-acetate (PMA), zymosan, arachidonic acid (AA) and Ca^{2+} ionophore (A23187) lead to the formation of prostanoids in cultured rat liver macrophages, whereas superoxide formation is observed after addition of PMA and zymosan only [2,3]. The formation of prostanoids is controlled by a phospholipase (PL) A_2 which liberates AA from phospholipids [4]. Superoxide is generated by a membrane-bound NADPH oxidase [5]. We recently showed that the formation of prostanoids and superoxide is regulated by protein kinase C (PKC) [6]. Both the PMA- and zymosan-induced formation of prostanoids and superoxide could be inhibited by several PKC inhibitors or by PMA-mediated PKC down-regulation [6,7]. In contrast, prostanoid formation from exogenously added AA and by A23187 were not affected. Additionally, we showed that PMA and zymosan, but not AA or A23187, lead to a translocation of PKC from the cytosol to membranes [7,8].

PKC is generally accepted to play a crucial role in cellular signal transduction. One of the major cellular transduction systems involves the breakdown of phosphatidylinositol bisphosphate by a PLC to Ins(1,4,5)P_3 and diacylglycerol (DAG). Whereas InsP_3 induces the release of Ca^{2+} from intracellular stores, DAG activates the Ca^{2+}- and phospholipid-dependent PKC [9]. Recently we showed that zymosan activates a PLC in liver macrophages, leading to the release of inositol phosphates and DAG, and that PLC-induced formation of inositol phosphates can be enhanced by PMA pretreatment of the cells [10,11].

Molecular cloning and biochemical analysis have revealed that PKC exists as a family of closely related enzymes. Besides the conventional Ca^{2+}-dependent PKC isoforms α, β and γ, there exist the more recently identified novel PKC-δ, -ε, -ζ, -η (L) and -θ, which are Ca^{2+}-independent [12–15].

We recently showed by Western- and Northern-blot analysis that liver macrophages contain PKC-β, but not PKC -α, -γ or -ε, and that PKC-β may take part in the prostaglandin (PG) and superoxide formation following PMA stimulation [8]. The aim of this study was to clarify the presence of other PKC isoforms in liver macrophages and their involvement in the signal-transduction pathway leading to the formation of prostanoids, superoxide and inositol phosphates.

MATERIALS AND METHODS

Chemicals

Zymosan was purchased from Sigma (München, Germany), Medium RPMI 1640 and newborn-calf serum were obtained from Biochrom (Berlin, Germany); newborn-calf serum was heat-inactivated at 56 °C for 30 min. PMA was obtained from Pharmacia (Freiburg, Germany). Sapintoxin A (SAPA) was a gift from Dr. F. J. Evans (London, U.K.) [16]; 12-deoxyphorbol 13-phenylacetate 20-acetate (DOPPA) was purchased from Scientific Marketing Associates, Barnet, Herts., U.K. The monoclonal antibody MC2, raised against the β-isoform of PKC, was kindly provided by Professor H. Hidaka (Nagoya, Japan) [17]. The polyclonal antibodies against the δ-isoform and the δ-specific peptide were purchased from Gibco (Eggenstein, Germany). Purified recombinant human PKC-α and -β were generously given by B. Fiebich and G. Kochs (Freiburg, Germany) [18].

Cell culture

Livers of male Wistar rats were removed aseptically under

Abbreviations used: PK, protein kinase; PMA, phorbol 12-myristate 13-acetate; PG, prostaglandin; AA, arachidonic acid; PL, phospholipase; DAG, diacylglycerol; SAPA, sapintoxin A; DOPPA, 12-deoxyphorbol 13-phenylacetate 20-acetate.

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Nembutal anaesthesia, and the Kupffer cells were isolated by a centrifugal elutriation procedure [19]. The cells were maintained in primary culture with RPMI medium containing 30% (v/v) newborn-calf serum. All experiments were performed with Kupffer cells kept in primary culture for 72 h.

Preparation of subcellular fractions

Macrophage cultures were incubated in Hanks' solution with or without the various stimuli for the indicated periods of time. Then the cells were scraped off and disrupted in isolation buffer (40 mM Hepes, 4 mM EDTA, 2 mM EGTA, 10 mM dithiothreitol, 100 μg/ml phenylmethanesulphonyl fluoride) by sonication. The homogenate was sedimented at 100000 *g* for 60 min at 4 °C, and the corresponding membrane fraction was resuspended in isolation buffer.

Determination of prostanoid and superoxide release

PGE_2 present in the medium was determined by enzyme-linked immunosorbent assay [7]. Superoxide generation was measured as reduction of cytochrome *c* inhibitable by superoxide dismutase [2].

Analysis of inositol phosphates

Kupffer cells (48 h in primary culture on 24-well plates) were incubated for 24 h in RPMI medium without unlabelled inositol and containing 10% newborn-calf serum and 2 μCi of *myo*-[^{3}H]inositol. Then the media were removed, and the cells were washed thoroughly and incubated without or with various stimuli in 0.5 ml of Hanks' solution containing 10 mM LiCl. After 30 min the incubation was stopped by adding 250 μl of 2 M KOH containing 36 mM $Na_2B_4O_7$, 7.6 mM EDTA and 15.2 mM NaOH. After neutralization with 250 μl of 3–4% HCl, the cell lysates were frozen at −20 °C. Inositol phosphates formed were determined as described previously [10].

Western-blot analysis

Samples (10 μg) of total protein were separated on 7.5–10%-polyacrylamide gels [20] and transferred to Hybond ECL nitrocellulose (Amersham). The blot was probed with monoclonal antibodies raised against the β-isoenzyme and with polyclonal antibodies raised against the δ-isoenzyme of PKC. Detection was performed with the Amersham ECL Western detection system.

RESULTS

In addition to PKC-β [8], liver macrophages also express PKC-δ (Figure 1). In unstimulated liver macrophages PKC-δ is exclusively detectable in the membrane fraction. Stimulation with PMA or zymosan does not alter this distribution (Figure 1). This is in contrast with PKC-β, which has been shown recently to be almost equally distributed between the cytosolic and membrane fraction of unstimulated cells and to translocate to membranes upon addition of zymosan or PMA [8]. In order to distinguish between specific and non-specific bands (Figures 1 and 5), competition experiments with a δ-specific peptide (Gibco) were performed. Preincubation of the antibodies with this peptide led to a specific disappearance of the upper bands of 76–78 kDa (Figures 1 and 5), indicating that these bands represent the δ isoenzyme of PKC (results not shown). The bands of low molecular mass (< 70 kDa) remained unchanged (results not shown). As shown in Figure 5, PKC-δ appears as doublet proteins upon gel electrophoresis and Western-blot analysis. These doublets have been reported recently to represent most probably an unphosphorylated and a phosphorylated form of the enzyme [21].

PMA treatment of liver macrophages for 3 h completely down-regulates the β-isoform (Figure 2). In contrast, PKC-δ down-regulation is achieved only after 7 h of PMA treatment (Figure 2); down-regulation of PKC-δ is completed after 16 h. The time course of PKC-β down-regulation correlates with the inhibition of superoxide generation and PMA-induced PGE_2 formation (Figure 3a). At 3 h after PMA treatment, superoxide formation and PMA-induced PGE_2 formation are completely blocked. The zymosan-induced formation of PGE_2, however, is not inhibited by 3 h pretreatment of the cells with the phorbol

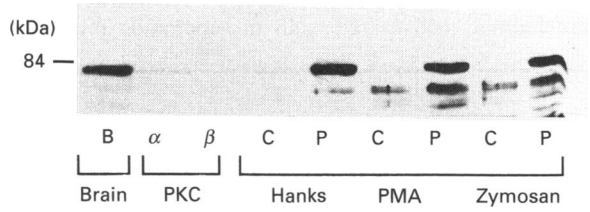

Figure 1 Western-blot analysis of PKC-δ expression

Cells (72 h in primary culture) were incubated in Hanks' solution without or with zymosan (15 min, 0.5 mg/ml) or PMA (10 min, 1 μM); cytosolic (C) and particulate (P) fractions were prepared as described in the Materials and methods section. Cell lysates, samples of purified PKC-α and -β and a rat brain homogenate (B) were electrophoresed, transferred on to nitrocellulose and probed with a polyclonal antibody against PKC-δ. The antibody recognizes PKC-δ in brain and does not cross-react with purified human PKC-α and -β (three left lanes).

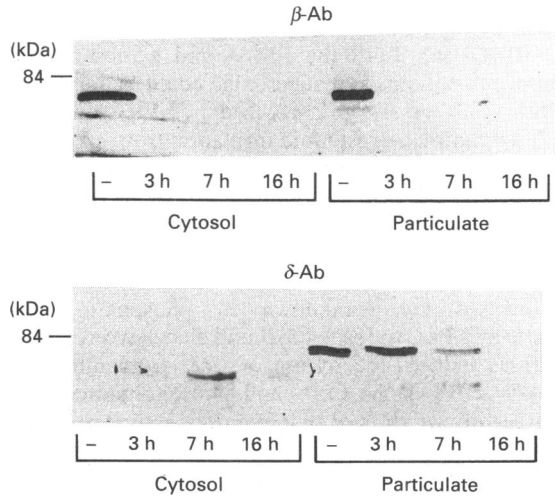

Figure 2 Time-dependence of down-regulation of PKC-β and PKC-δ by PMA

Cells (48 h in primary culture) were incubated in RPMI medium containing 30% newborn-calf serum without (–) or with PMA (100 nM) for the indicated periods of time. Then the cells were washed thoroughly; cytosolic and particulate fractions were prepared, electrophoresed, transferred on to nitrocellulose and probed with monoclonal antibodies (Ab) raised against PKC-β and polyclonal antibodies raised against PKC-δ as described in the Materials and methods section.

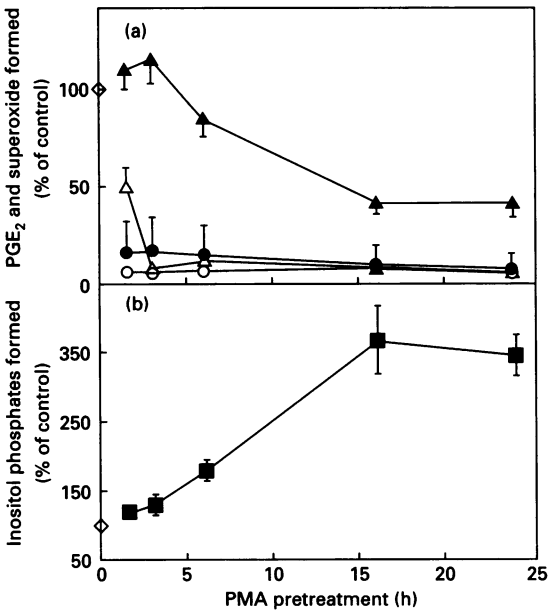

Figure 3 Effect of PMA pretreatment on the formation of PGE_2, superoxide and inositol phosphates

Cells were incubated in RPMI medium containing 30% newborn-calf serum for the indicated periods of time without (◇) or with PMA (100 nM). Then the cells were washed thoroughly and incubated in Hanks' solution without or with PMA (1 μM, white symbols) or with zymosan (0.5 mg/ml, black symbols). The release of PGE_2 (▲, △), superoxide (●, ○) and inositol phosphates [Ins4P+Ins(1,4)P_2+Ins(1,4,5)P_3; ■] was determined as described in the Materials and methods section. A value of 100% (◇; non-pretreated cells) corresponds to a release of 65±12 (PMA) and 58±8 (zymosan) pmol of $PGE_2/10^6$ cells, 28±5 (PMA) and 32±6 (zymosan) nmol of superoxide/10^6 cells and 13758±3089 c.p.m. of inositol phosphates/10^6 cells. Results are means±S.D. of three to five independent experiments.

Table 1 Effect of PMA and zymosan on the formation of inositol phosphates in non-pretreated and in 3 h- and 24 h-PMA-pretreated cells

Cells were incubated in RPMI medium containing 30% newborn-calf serum for the indicated periods of time without or with PMA (100 nM). Then the cells were washed thoroughly and incubated in Hanks' solution without PMA or zymosin (Control), with PMA (1 μM) with zymosan (ZY; 0.5 mg/ml) or with PMA+zymosan. The release of inositol phosphates [Ins4P+Ins(1,4)P_2+Ins(1,4,5)P_3] was determined as described in the Materials and methods section. Results are means±S.D. of three to five independent experiments. P values were calculated by Student's t test: $^*P < 0.002$.

	10^{-3} × Inositol phosphates (c.p.m./30 min per 10^6 cells)			
Pretreatment	Control	PMA	ZY	PMA+ZY
0 h	3.0±0.5	3.6±1.0	16.2±2.1	11.8±1.9*
3 h	2.8±0.4	3.3±0.8	23.8±3.4	18.5±2.3*
24 h	3.2±0.2	3.0±0.6	72.4±9.6	75.2±8.2

ester; an inhibition (50–60%) is obtained only after 16–24 h (Figure 3a). In contrast with PGE_2 and superoxide formation, pretreatment of the cells with PMA enhances the zymosan-induced inositol phosphate formation (Figure 3b). This enhancement is detectable 7 h after the addition of PMA and reaches a plateau after 16 h. Thus, this time course correlates with down-regulation of PKC-δ, but not of PKC-β.

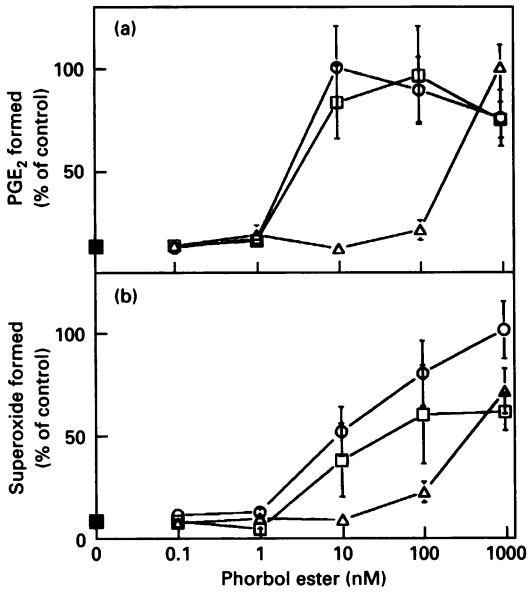

Figure 4 Effect of PMA, DOPPA and SAPA on the formation of PGE_2 and superoxide

Cells (78 h in primary culture) were incubated for 60 min in Hanks' solution without (■) or with different concentrations of PMA (○), DOPPA (△) or SAPA (□). The release of PGE_2 and superoxide was determined as described in the Materials and methods section. A value of 100% (PMA, 1 μM) corresponds to a release of 63±8 pmol of $PGE_2/10^6$ cells and 32±11 nmol of superoxide/10^6 cells. Results are means±S.D. of four to five independent experiments.

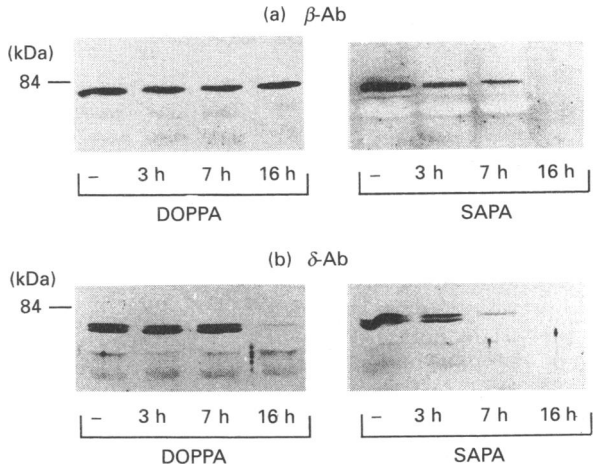

Figure 5 Time-dependence of down-regulation of PKC-β and PKC-δ by SAPA and DOPPA

Cells (48 h in primary culture) were incubated in RPMI medium containing 30% newborn-calf serum without (–) or with SAPA (100 nM) or DOPPA (100 nM) for the indicated periods of time. Then the cells were washed thoroughly; homogenates were prepared, electrophoresed, transferred on to nitrocellulose and probed with a monoclonal antibody (Ab) raised against PKC-β (**a**) and polyclonal antibodies against PKC-δ (**b**) as described in the Materials and methods section.

The addition of PMA alone does not elicit formation of inositol phosphates in these cells [10]; however, added together with zymosan, PMA exerts an inhibitory effect on the zymosan-induced formation of inositol phosphates (Table 1). This inhibitory effect is achieved in non-pretreated cells and in cells

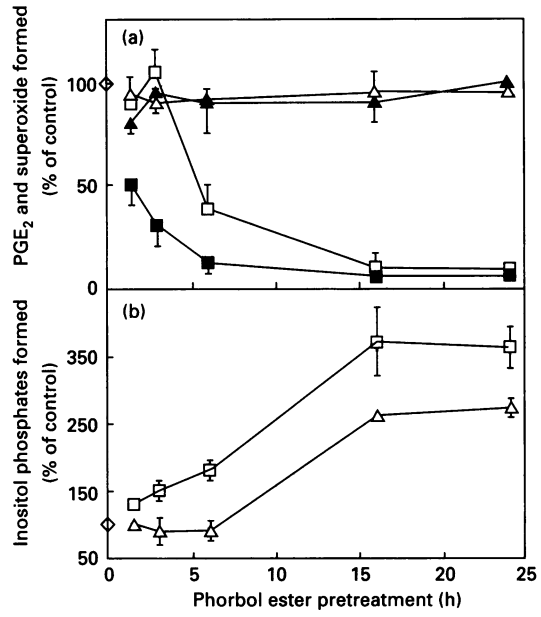

Figure 6 Effect of DOPPA and SAPA pretreatment on the formation of PGE_2, superoxide and inositol phosphates

Cells were incubated in RPMI medium containing 30% newborn-calf serum for the indicated periods of time without (◇) or with DOPPA (100 nM; ▲, △) or SAPA (100 nM; ■, □). Then the cells were washed thoroughly and incubated in Hanks' solution without or with PMA (1 μM) for stimulation of PGE_2 (**a**, black symbols) and superoxide (**a**, white symbols) generation for 60 min or with zymosan (0.5 mg/ml) for triggering inositol phosphate (**b**) formation for 30 min. The release of PGE_2, superoxide and inositol phosphates [Ins4*P*+Ins(1,4)P_2 +Ins(1,4,5)P_3] was determined as described in the Materials and methods section. A value of 100% (◇; non-pretreated cells) corresponds to a release of 58±14 pmol of $PGE_2/10^6$ cells, 26±3 nmol of superoxide/10^6 cells and 16789±4234 c.p.m. of inositol phosphates/10^6 cells. Results are means±S.D. of three to five independent experiments.

pretreated with PMA for 3 h, but not in cells which have been pretreated with phorbol ester for 24 h (Table 1).

To elucidate further the role of PKC-β and PKC-δ in PGE_2, superoxide and inositol phosphate formation, we used two other phorbol ester derivatives, SAPA and DOPPA. Both have been described previously to activate preferentially PKC-β, but not PKC-δ, *in vitro* [16]. Both SAPA and DOPPA are able to induce superoxide and PGE_2 formation (Figure 4), but possess no potency to induce a release of inositol phosphates (results not shown) in liver macrophages. Although SAPA has a similar potency to PMA, DOPPA is 2–3 orders of magnitude less potent than SAPA or PMA (Figure 4). Nevertheless, these results are in line with the assumption that superoxide and PGE_2 formation can both be triggered by PKC-β. Furthermore, both phorbol esters differ in their capacity to down-regulate PKC-β and -δ (Figure 5). Although PKC-β is not affected by DOPPA treatment up to 16 h, a decrease in PKC-β by SAPA is measured already after 3 h; down-regulation of PKC-β by SAPA is complete after 16 h (Figure 5a). SAPA possesses a similar potency to down-regulate PKC-δ (Figure 5b). In contrast, PKC-δ is not affected by DOPPA up to 7 h; however, after treatment of the cells with DOPPA for 16 h, PKC-δ is also completely down-regulated (Figure 5b). The two phorbol esters differ also in their capacity to suppress the PMA-induced formation of PGE_2 and superoxide (Figure 6a). Although pretreatment of the cells with SAPA for 16 h completely blocks formation of superoxide and PGE_2, DOPPA pretreatment for the same period of time does not influence the generation of both mediators. Furthermore, both phorbol esters affect the zymosan-induced formation of inositol phosphates (Figure 6b). At 16 h after addition of DOPPA and SAPA, the zymosan-induced formation of inositol phosphates is enhanced 2–3-fold.

DISCUSSION

Here we have shown that, in addition to PKC-β, liver macrophages also express PKC-δ. Recently we showed that Kuppfer cells do not express PKC-α, -γ or -ϵ [8]. In contrast with PKC-β, which is almost equally distributed between the cytosolic and membrane fraction of unstimulated cells and which translocates to membranes upon addition of PMA or zymosan [8], PKC-δ is exclusively detectable in the membrane fraction. The cellular localization of PKC-δ seems to depend on the cell type; whereas in rat brain cells and rat renal mesangial cells PKC-δ is detected predominantly in a membrane-associated form [21,22], in human platelets and GH_4C_1 cells this isoenzyme of PKC is mainly found in the cytosol [23,24].

Long-term exposure of eukaryotic cells to phorbol ester is known to result in down-regulation of PKC and disappearance of PKC-mediated cellular functions [6,25]. A differential down-regulation of PKC isoforms has been reported previously for various cell types [21,26–28]. Here we show that in liver macrophages PKC-β and PKC-δ are differentially down-regulated by PMA and two other phorbol ester derivatives (DOPPA and SAPA), which have been reported to activate PKC-β, but not PKC-δ, *in vitro* [16]. The kinetics of the down-regulation of PKC-β and PKC-δ and the effects of the different phorbol esters on the formation of PGE_2, superoxide and inositol phosphates are almost identical and are in accordance with the assumption that PKC-β mediates the generation of superoxide and PMA-induced formation of PGE_2, whereas PKC-δ may be involved in the zymosan-induced regulation of PGE_2 formation and phosphoinositide hydrolysis. (*a*) Down-regulation of PKC-β and inhibition of superoxide generation and PMA-induced PGE_2 formation are obtained after treatment of the cells with PMA for 3 h (Figure 2) or with SAPA after 16 h (Figure 5); DOPPA does not affect PKC-β (Figure 5), nor does it influence the PMA-induced formation of PGE_2 or superoxide (Figure 5). (*b*) Formation of inositol phosphates is augmented by all three phorbol esters only after pretreatment of the cells for 16 h (Figures 3 and 6); the same duration of exposure of the cells to the phorbol ester is needed for down-regulation of PKC-δ (Figures 2 and 5) and inhibition of the zymosan-induced formation of PGE_2 (Figure 3a). (*c*) The inhibitory effect of PMA on the zymosan-induced release of inositol phosphates (see Table 1) is only achieved in cells which express PKC-δ; the existence of PKC-β, however, seems not to be essential for this action of PMA. Therefore, this inhibitory effect on phosphoinositide hydrolysis is probably due to activation of PKC-δ by PMA. The fact that the basal release of inositol phosphates is almost the same in non-pretreated and in 3 h- and 24 h-PMA-pretreated cells (Table 1) suggests that pretreatment with phorbol ester does not affect the expression or activity of PLC and is in line with the assumption that PKC-δ negatively regulates PLC-catalysed phosphoinositide hydrolysis.

In summary, we have presented evidence for a role of PKC-δ in negative feedback inhibition of phosphoinositide hydrolysis and in zymosan-induced formation of PGE_2, and of PKC-β in PMA-induced PGE_2 formation and in superoxide generation. A negative feedback inhibition of PKC isoenzymes on inositol phosphate hydrolysis has been proposed recently also for rat basophilic leukaemia cells and rat mesangial cells [22,27,29]. In

our and most of these studies prolonged treatment of cells with phorbol ester has been used to discriminate between PKC-dependent and -independent processes and to identify a specific PKC isoenzyme to be involved in the cellular reaction. These data, however, must be interpreted with caution, since treatment of cells with phorbol ester for prolonged periods of time may result in an altered pattern of gene expression and other individual non-identified PKC isoenzymes (λ, η, ξ, θ) may not be susceptible to down-regulation [22,30,31]. The use of other methods, including over-expression studies, antisense RNA techniques and the development of selective PKC-isoenzyme-specific inhibitors, is necessary to identify the selective action of different PKC isoenzymes unequivocally.

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