

THE effect of various phospholipase A₂ and protein kinase inhibitors on the arachidonic acid liberation in bovine platelets induced by the protein kinase activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was studied. TPA stimulates arachidonic acid release mainly by activating group IV cytosolic PLA₂ (cPLA₂), since inhibitors of this enzyme markedly inhibited arachidonic acid formation. However, group VI Ca²⁺-independent PLA₂ (iPLA₂) seems to contribute to the arachidonic acid liberation too, since the relatively specific iPLA₂ inhibitor bromoenol lactone (BEL) decreased arachidonic acid generation in part. The pronounced inhibition of the TPA-induced arachidonic acid release by the protein kinase C (PKC) inhibitors GF 109203X and Ro 31-82220, respectively, and by the p38 MAP kinase inhibitor SB 202190 suggests that the activation of the PLA₂s by TPA is mediated via PKC and p38 MAP kinase.

Key words: Phospholipase A₂, Protein kinase inhibitors, 12-*O*-tetradecanoylphorbol-13-acetate, Bovine platelets

Involvement of different protein kinases and phospholipases A₂ in phorbol ester (TPA)-induced arachidonic acid liberation in bovine platelets

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Introduction

The stimulation of cells by diverse agonists leads to the liberation of arachidonic acid from membrane phospholipids. Arachidonic acid can be metabolized through the cyclooxygenase or lipoxygenase pathways to form the eicosanoids, including prostaglandins and leukotrienes. These are important mediators of acute inflammatory processes. There is substantial evidence that this arachidonic acid release is mediated by the Ca²⁺-sensitive group IV cytosolic phospholipase A₂ (cPLA₂).^{1–4}

The activity of the cPLA₂, which is present in many mammalian cells, can be regulated by two important mechanisms. The first appears to be a rise in the intracellular Ca²⁺ concentration, which causes translocation of the cPLA₂ from the cytosol to the internal membranes where it binds through a Ca²⁺-dependent lipid binding domain. A second mechanism of regulation of cPLA₂ is its phosphorylation by protein kinases.^{1,4} So the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is known to exhibit its physiological activities via stimulation of protein kinases,⁵ induced an increase in phosphorylation and catalytic activity of cPLA₂ as well as arachidonic acid release in macrophages, neutrophils, keratinocytes and glomerular mesangial cells.^{6–10}

We have previously reported that TPA is able to stimulate the liberation of arachidonic acid also in

platelets.¹¹ In these cells different protein kinases have been found: protein kinase C,¹² p44 MAP kinase (also named ERK1), p42 MAP kinase (also named ERK2)^{13–15} and p38 MAP kinase.¹⁶ The aim of our present work was to investigate, which of them are involved in the TPA-induced arachidonic acid release in bovine platelets by using specific inhibitors of the protein kinases.

Materials and Methods

Reagents

MAFP (methyl arachidonylfluorophosphonate), BEL ((E)-6-(bromomethylidene)-3-(1-naphthyl)-3,4,5,6-tetrahydro-2*H*-pyran-2-one), RHC 80267 (1,6-bis(cyclohexyloximinocarbonylamino)hexane), PD 98059 (2'-amino-3'-methoxyflavone), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Biomol, Hamburg); SB 202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole), GF 109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide), Ro 31-8220 (2-[1-[3-(Amidinothio)propyl]-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indole-3-yl)maleimide methane sulphonate) (Calbiochem, Bad Soden); 5,8,11,14-eicosatetraynoic acid (ETYA), staurosporine, calcium ionophore A23187, arachidonic acid, phosphate buffered saline tablets (Sigma, Deisenhofen); EDTA-Na₂, dimethyl sulphoxide

(DMSO) (Merck, Darmstadt), ML 3116 (1-(7-carboxyheptyl)-3-dodecanoyl-1*H*-indole-2-carboxylic acid) was synthesized by the published procedure;¹⁷ bovine blood (local slaughterhouse).

Cells

Immediately after the death of the animal, bovine blood (1 litre) was collected in a polypropylene vessel containing a solution of 0.077 M EDTA-Na₂ in 0.2% (w/v) saline (0.1 litre per 1 litre blood). After dilution of the blood with 0.5 litre of 0.9% (w/v) saline the platelets were isolated by centrifugation as previously described.¹⁸ The platelets were stored at +4°C. All experiments were performed within 8 h after isolation of the platelets.

Measurement of inhibition of cPLA₂-activity

Inhibition of cPLA₂ was determined by measuring the TPA- or calcium ionophore A23187-induced arachidonic acid release from bovine platelets with HPLC/UV-detection as previously described.^{11,18} Briefly, to a solution of ETYA in DMSO, which inhibits formation of arachidonic acid metabolites in platelets, was added the DMSO solution of a test compound or DMSO alone (in case of the control tests and the kinetic experiments) followed by the platelet suspension and a solution of calcium chloride at 37°C (final platelet concentration: about 8×10^8 cells/2 ml). Then the cPLA₂ was activated by TPA (2 μM) or A23187 (20 μM). The solution of TPA was freshly prepared each time. When using TPA as stimulant the incubation time was variable (kinetic experiments) or 60 min (experiments with enzyme inhibitors). In the experiments with A23187 the incubation time was 1 min. After terminating the enzyme reaction the produced arachidonic acid was cleaned up by solid-phase extraction and quantified with HPLC/UV-detection at 200 nm.

Solubility of the test compounds

All compounds were soluble under the test conditions.

Cell lytic potency of the test compounds

The cell lytic potency of the test compounds was measured by turbidimetry according to a procedure previously described.¹⁹ The compounds did not show cell lytic properties at the concentrations applied.

Results

Recently, we have reported that TPA stimulates the liberation of arachidonic acid in washed bovine platelets.¹¹ The time course of arachidonic acid

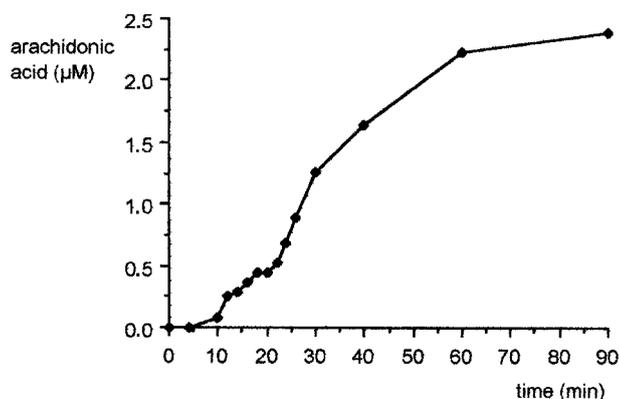


FIG. 1. Kinetics of the release of arachidonic acid by bovine platelets (8×10^8 cells/2 ml) after stimulation with TPA (2 μM) in the presence of the dual cyclooxygenase-1/12-lipoxygenase-inhibitor ETYA (10 μM), which inhibits the further metabolism of arachidonic acid to thromboxane B₂ and 12-HETE.

release was sigmoid reaching a plateau after about 15 min. To avoid metabolism of arachidonic acid via the cyclooxygenase-1 and the 12-lipoxygenase pathways, the dual cyclooxygenase-1/12-lipoxygenase-inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA) was added to the platelets in these experiments. We now have found that a second even more pronounced arachidonic acid liberation occurs if the incubation was extended (Fig. 1). However, this second step of arachidonic acid release only could be measured when fresh (1–2 days old) platelets were used, while the first step still appeared 3–4 days after the isolation of the platelets from bovine blood.

Contribution of the cPLA₂ to the arachidonic acid release in TPA-treated platelets

The arachidonic acid release in A23184-treated platelets is catalysed by group IV cytosolic phospholipase A₂ (cPLA₂).^{20–23} To investigate, whether cPLA₂ is also predominantly responsible for the arachidonic acid liberation induced by TPA, we monitored the inhibition of the whole arachidonic acid production after TPA stimulation (incubation time 60 min) by several PLA₂ inhibitors. Methyl arachidonylfluorophosphate (MAFP),^{24,25} which is known as dual inhibitor of cPLA₂ and group VI Ca²⁺-independent phospholipase A₂ (iPLA₂),²⁶ blocked the arachidonic release to about 82% at 10 μM (Table 1). Experiments with higher MAFP concentrations have not been performed, since we had found that MAFP shows cytotoxic properties at concentrations not far above 10 μM. So a significant loss of lactate dehydrogenase from the cells could be determined at 33 μM.¹⁹

A recent study has demonstrated that both cPLA₂ and iPLA₂ are involved in protein kinase dependent arachidonic acid liberation in macrophage-like P388D₁ cells.²⁷ Therefore, we also examined the

effect of the selective iPLA₂ inhibitor bromoenol lactone (BEL) on TPA-induced arachidonic acid release. At a concentration of 5 µM BEL, at which a maximal effect on iPLA₂-activity has been ascertained,²⁷ the arachidonic acid liberation was decreased by about 27%. On the other hand, with the cPLA₂ inhibitor ML 3116,²⁸ which is able to block the arachidonic acid release in A23187-stimulated platelets nearly completely, the TPA-induced arachidonic acid liberation could only be inhibited to about 70%. The increase of ML 3116 concentration from 10 µM to 33 µM did not cause a further decrease of arachidonic acid release.

Recently it was suggested, that in rat liver macrophages TPA leads to an arachidonic acid release via an activation of PLC and DAG lipase.²⁹ If at all, in platelets this pathway does not play a greater role, since the arachidonic acid formation was not affected by the DAG lipase inhibitor RHC 80267 (concentration: 100 µM).

In conclusion, we propose that two different PLA₂s, cPLA₂ and iPLA₂, are mainly responsible for the liberation of arachidonic acid from platelet phospholipids after stimulation with TPA.

Effect of protein kinase inhibitors on the TPA-induced liberation of arachidonic acid in platelets

To investigate the role of the different protein kinases present in platelets on the TPA-induced phospholipase A₂ activation, we measured the inhibition of the arachidonic acid formation by the potent broad spectrum protein kinase inhibitor staurosporine, the

PKC inhibitors GF 109203X and Ro 31-8220, the p44/p42 MAP kinase inhibitor PD 98059 and the p38 MAP kinase inhibitor SB 202190. The inhibitors were applied at concentrations, at which a full inhibition of the appropriate protein kinase had been achieved in intact cells according to data in the literature.^{6,7,30-33}

Staurosporine strongly inhibited arachidonic acid formation (Table 1). Similar results were obtained with the PKC inhibitors GF 109203X and Ro 31-8220, and with the p38 MAP kinase inhibitor SB 202190. However, their inhibition values were a little bit lower than that of staurosporine. In contrast, the p42/44 MAP kinase inhibitor PD 98059 exhibited only a weak inhibition of the arachidonic acid release (about 26% at 33 µM).

Effect of the protein kinase inhibitors on the A23187-induced liberation of arachidonic acid in platelets

Staurosporine, GF 109203X, Ro 31-8220, SB 202190 and PD 98059 did not inhibit the arachidonic acid release in A23187-stimulated bovine platelets at the concentrations applied in the experiments with TPA, thus protein kinases do not seem to be involved in the A23187-induced arachidonic acid liberation. The ineffectiveness of the DAG lipase inhibitor RHC 80267 (concentration 100 µM) in these experiments confirm the results of previous studies, which demonstrated that the PLC/DAG lipase pathway is not involved in the arachidonic acid liberation occurring after the stimulation of platelets with A23187.^{20,22}

Discussion

The experiments with the PLA₂ inhibitors propose that cPLA₂ and to a lesser extent iPLA₂ are responsible for the TPA-induced arachidonic acid release in bovine platelets.

As far as the applied protein kinase inhibitors have the specificity ascribed in literature, the pronounced inhibition of TPA-induced arachidonic acid release by the PKC inhibitors GF 109203X and Ro 31-8220 and by the p38 MAP kinase inhibitor SB 202190 suggests that the activation of the PLA₂s in platelets by TPA is mediated via an activation of PKC and the p38 MAP kinase. Although the effect of PD 98059 on TPA-induced arachidonic acid release was significantly less pronounced than that of the other protein kinase inhibitors investigated, p42/p44 MAP kinase may also be involved in the activation of the PLA₂s.

In conclusion, the results indicate that the TPA-induced arachidonic acid liberation is mediated via other mechanisms than the arachidonic acid release observed after stimulation with A23187. Further investigations will be necessary to elucidate the

Table 1. Effect of the PLA₂ inhibitors MAFP, ML 3116 and BEL, the DAG lipase inhibitor RHC 80267 and different protein kinase inhibitors on the TPA- and A23187-induced release of arachidonic acid in bovine platelets

| Compound | Inhibition of arachidonic acid release (%) ^a | |
|----------------------------------|---|---------------------------------|
| | stimulation with TPA (60 min) | stimulation with A23187 (1 min) |
| MAFP (10 µM) | 82 ± 5 | 61 ± 5 |
| ML 3116 (10 µM) | 66 ± 3 | 82 ± 2 |
| (33 µM) | 70 ± 2 | 94 ± 3 |
| BEL (5 µM) | 27 ± 13 | NS ^b |
| RHC 80267 (100 µM) | NS ^b | NS ^b |
| <i>Protein kinase inhibitors</i> | | |
| Staurosporine (1 µM) | 92 ± 6 | NS ^b |
| GF 109203X (10 µM) | 89 ± 1 | NS ^b |
| Ro 31-8220 (5 µM) | 78 ± 2 | NS ^b |
| SB 202190 (33 µM) | 73 ± 16 | NS ^b |
| PD 98059 (33 µM) | 26 ± 3 | NS ^b |

^a Means ± SD, *n* = 3; in the case of SB 202190 and PD 98059, *n* = 4; in the case of BEL, *n* = 6.

^b NS, not significant.

sequence of the events occurring after TPA-stimulation and the reasons for the biphasic arachidonic acid liberation observed.

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