The effect of various phospholipase A<sub>2</sub> 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 PLA<sub>2</sub> (cPLA<sub>2</sub>), since inhibitors of this enzyme markedly inhibited arachidonic acid formation. However, group VI Ca<sup>2+</sup>-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) seems to contribute to the arachidonic acid liberation too, since the relatively specific iPLA<sub>2</sub> 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 31–8220, respectively, and by the p38 MAP kinase inhibitor SB 202190 suggests that the activation of the PLA<sub>2</sub> by TPA is mediated via PKC and p38 MAP kinase.

**Key words**: Phospholipase A<sub>2</sub>, Protein kinase inhibitors, 12-O-tetradecanoylphorbol–13-acetate, Bovine platelets

**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 Ca<sup>2+</sup>-sensitive group IV cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>).<sup>1–4</sup>

The activity of the cPLA<sub>2</sub>, which is present in many mammalian cells, can be regulated by two important mechanisms. The first appears to be a rise in the intracellular Ca<sup>2+</sup> concentration, which causes translocation of the cPLA<sub>2</sub> from the cytosol to the internal membranes where it binds through a Ca<sup>2+</sup>-dependent lipid binding domain. A second mechanism of regulation of cPLA<sub>2</sub> is its phosphorylation by protein kinases.<sup>1,4</sup> So the phorbol ester 12-O-tetradecanoylphorbol–13-acetate (TPA), which is known to exhibit its physiological activities via stimulation of protein kinases,<sup>5</sup> induced an increase in phosphorylation and catalytic activity of cPLA<sub>2</sub> as well as arachidonic acid release in macrophages, neutrophiles, keratinocytes and glomerular mesangial cells.<sup>6–10</sup>

We have previously reported that TPA is able to stimulate the liberation of arachidonic acid also in platelets.<sup>11</sup> In these cells different protein kinases have been found: protein kinase C<sub>C</sub>,<sup>12</sup> p44 MAP kinase (also named ERK1), p42 MAP kinase (also named ERK2)<sup>13–15</sup> and p38 MAP kinase.<sup>16</sup> 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-2H-pyran-2-one), RHC 80267 (1,6-bis(cyclohexyloximinocarbonylaminomethyl)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)-1H-imidazole), GF 109203X (2-[1-(3-dimethylaminoethylamino)-1H-indol-3-yl]-3-(1H-indol-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-Na<sub>2</sub>, dimethyl sulfoxide
(DMSO) (Merck, Darmstadt), ML 3116 (1-(7-carboxy-heptyl)-3-dodecanoyl-1H-indole-2-carboxylic acid) was synthesized by the published procedure;\textsuperscript{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-Na\(_2\) 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.\textsuperscript{18} The platelets were stored at +4°C. All experiments were performed within 8 h after isolation of the platelets.

Measurement of inhibition of cPLA\(_2\)-activity
Inhibition of cPLA\(_2\) was determined by measuring the TPA- or calcium ionophore A23187-induced arachidonic acid release from bovine platelets with HPLC/UV-detection as previously described.\textsuperscript{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 \times 10\(^8\) cells/2 ml). Then the cPLA\(_2\) was activated by TPA (2 \(\mu\)M) or A23187 (20 \(\mu\)M). 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.\textsuperscript{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.\textsuperscript{11} The time course of arachidonic acid 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 ETYA (10 \(\mu\)M) which inhibits the further metabolism of arachidonic acid to thromboxane B\(_2\) and 12-HETE.

Contribution of the cPLA\(_2\) to the arachidonic acid release in TPA-treated platelets
The arachidonic acid release in A23184-treated platelets is catalysed by group IV cytosolic phospholipase A\(_2\) (cPLA\(_2\)).\textsuperscript{20–23} To investigate, whether cPLA\(_2\) 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 PLA\(_2\) inhibitors. Methyl arachidonylfluorophosphonate (MAFP),\textsuperscript{24,25} which is known as dual inhibitor of cPLA\(_2\) and group VI Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)),\textsuperscript{26} blocked the arachidonic release to about 82\% at 10 \(\mu\)M (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 \(\mu\)M. A recent study has demonstrated that both cPLA\(_2\) and iPLA\(_2\) are involved in protein kinase dependent arachidonic acid liberation in macrophage-like P388D\(_1\) cells.\textsuperscript{27} Therefore, we also examined the
**Effect of the selective iPLA$_2$ inhibitor bromoeno(l) lactone (BEL) on TPA-induced arachidonic acid release.** At a concentration of 5 μM BEL, at which a maximal effect on iPLA$_2$-activity has been ascertained, the arachidonic acid liberation was decreased by about 27%. On the other hand, with the cPLA$_2$ inhibitor ML 3116, 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. 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 PLA$_2$s, cPLA$_2$ and iPLA$_2$, 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 A$_2$ 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.

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/p44 MAP kinase inhibitor PD 98059 exhibited only a weak inhibition of the arachidonic acid release (about 26% at 33 μM).

**Table 1. Effect of the PLA$_2$ 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 (%) |
|----------------|---------------------------------------------|
|                | stimulation with TPA | stimulation with A23187 |
| 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$^a$ |
| RHC 80267 (100 μM) | NS$^b$ | NS$^b$ |

**Protein kinase inhibitors**

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.

**Discussion**

The experiments with the PLA$_2$ inhibitors propose that cPLA$_2$ and to a lesser extent iPLA$_2$ 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 PLA$_2$s 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 PLA$_2$s.

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

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft (DFG) for supporting this study and Mrs Monika Klimt for technical assistance.

References

1. Clark JD, Schievella AR, Nalefski EA, Lin LL. Cytosolic phospholipase A2. J Lipid Mediators Cell Signalling 1995; 12: 83–117.
2. Bonventre JV, Huang Z, Tcheri MR, O’Leary E, Li E, Moskowitz MA, Sapatrini A. Reduced fertility and postischemic brain injury in mice deficient in cytosolic phospholipase A2. Nature 1997; 390: 622–625.
3. Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro K, Komagata Y, Miki K, Ikuta K, Ouchi Y, Miyazaki J, Shimizu T. Role of cytosolic phospholipase A2 in allergic response and parturition. Nature 1997; 390: 618–622.
4. Gijon MA, Leslie CC. Regulation of arachidonic acid release and cytosolic phospholipase A2 activity. J Leukocyte Biol 1999; 65: 380–386.
5. Castagnola M, Takiy K, Sano K, Kikkaeva U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 1982; 257: 7847–7851.
6. Qu ZH, Leslie CC. Protein kinase C-dependent and independent pathways of mitogen-activated protein kinase activation in macrophages that stimulate arachidonic acid release and cytosolic phospholipase A2 activity, in human neutrophils by opsonized zymosan. Biochem J 1997; 326: 867–876.
7. Kast R, Fürstenberger G, Marks E. Phorbol ester- and bradykinin-induced arachidonic acid release from keratinocytes is catalyzed by a cytosolic phospholipase A2 (cPLA2). J Invest Dermatol 1993; 101: 567–572.
8. Oldoye ME, Evans CB. 12-O-tetradecanoylphorbol-13-acetate and the induction of prostaglandin E2 generation by human keratinocytes: an evaluation. Carcinogenesis 1994; 15: 141–143.
9. Maxwell A, Goldberg HP, Tay AHN, Li ZQ, Arbus G, Skrebeck KL. Epidermal growth factor and phorbol myristate acetate increase expression of the mRNA for cytosolic phospholipase A2 in glomerular mesangial cells. Biochem J 1993; 295: 763–766.
10. Lehr M. In vitro assay for the evaluation of inhibitors of 85kDa cytosolic phospholipase A2 and its mode of activation in human neutrophils by opsonized zymosan. Biochem Pharmacology 1994; 47: 769–776.
11. Kramer RM, Roberts EE, Striiler BA, Johnstone EM. Thrombin induces activation of p38 MAP kinase in human platelets. J Biol Chem 1995; 270: 27985–27990.
12. Lehr M. Synthesis, biological evaluation, and structure-activity relationships of Sacyldindo-2-carboxylic acids as inhibitors of the cytosolic phospholipase A2. J Med Chem 1997; 40: 2798–2803.
13. Lehr M. In vitro assay for the evaluation of phospholipase A2 inhibitors using bovine platelets and HPLC with UV-detection. Pharym Pharmacol Lett 1992; 2: 176–179.
14. Lehr M, Griesbach K. Cell-lytic and cPLA2-inhibitory properties in bovine platelets of the commercially available cPLA2 inhibitor, arachidonyl trifluoromethyl ketone, methyl arachidonyfluorophosphonate and palmitoyl trifluoromethyl ketone. Pharm Pharmacol Commun 1999; 5: 389–393.
15. Rienhouse SE. Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclooxygenase products and ADP. Biochem J 1984; 222: 103–110.
16. Mounier C, Faili A, Vargaftig BB, Bon C, Hartmi M. Secretory phospholipase A2 is not required for arachidonic acid liberation during platelet activation. Eur J Biochem 1993; 216: 169–175.
17. Faili A, Emadi S, Vargaftig B, Hartmi M. Disassociation between the phospholipases C and A2 activities in stimulated platelets and their involvement in the arachidonic acid liberation. Br J Haematol 1994; 88: 149–155.
18. Riedenau D, Gauj J, Weech PK, Laliberte E, Yergey J, Li C, Desmarais S, Perrier H, Liu S, Nicolli-Grotheel D, Street I. Arachidonyl trifluoromethyl ketone, a potent inhibitor of 85kDa phospholipase A2, blocks production of arachidonate and 12-hydroxyeicosatetraenoic acid by calcium ionophore-stimulated platelets. J Biochem 1994; 269: 15619–15624.
19. Huang Z, Liu S, Street I, Laliberte E, Abdullah K, Desmarais S, Wang Z, Kennedy B, Payette P, Riedenau D, Wcrech P, Gresser M, Methyl arachidonyl fluorophosphonate, a potent irreversible cPLA2 inhibitor, blocks the mobilization of arachidonic acid in human platelets and neutrophils. Mediators Inflamm 1994; 3: 307–308.
20. Lio YC, Reynolds SJ, Balsinde J, Dennis EA. Irreversible inhibition of Ca2+-independent phospholipase A2 by methyl arachidonyl fluorophospho- nate. Biochim Biophys Acta 1996; 1302: 55–60.
21. Ackermann BJ, Dennis EA. Mammalian calcium-independent phospholipase A2. Biochim Biophys Acta 1995; 128: 125–136.
22. Satoh A, Shingo M, Keisuke K, Misako H, Takashi S. Involvement of group VI Ca2+-independent phospholipase A2 in protein kinase C-dependent arachidonic acid liberation in zymosan-stimulated macrophage-like P388D cells. J Biochem 1997; 32: 15619–15624.
23. Tries S, Neher K, Laef S, Abraham WM, Lehr M. Effects of ML 3116, an inhibitor of cPLA2, on carrageenan-induced paw edema, phospholipase-in- duced mouse ear edema and in sheep model of asthma. Mediators Inflamm 1999; 8: S123–S124.
24. Ambs P, Bucacin M, Schwene H, Fitzke E, Dieter P. Regulation of cytosolic phospholipase A2 in arachidonic acid release of rat-liver macrophages. Adv Exp Med Biol 1997; 407: 479–483.
25. Borsch-Haubold AG, Kramer RM, Watson SF. Inhibition of mitogen-activated protein kinase protein kinase does not impair primary activation of human platelets. Biochem J 1996; 318: 207–212.
26. Kramer RM, Roberts EE, Un SL, Borsch-Haubold AG, Watson SF, Fisher MJ, Jakubowski JA. P38 Mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. J Biol Chem 1996; 271: 27723–27727.
27. Touille D, Pianetti F, Coste H, Bellevergue P, Grand-Perret T, Ajkane M, Bauer V, Boisman P, Bourger E, Lorillie E, Dhameil L, Charon D, Kirilovsky J. The bisindolylmimide GD 102025X is a potent and selective inhibitor of protein kinase C. J Biol Chem 1991; 266: 15771–15781.
28. Beltman J, McCormick E, Cook SJ. The selective protein kinase C inhibitor, Bo–31–8220, inhibits mitogen-activated protein kinase phosphatase–1 (MKP–1) expression, induces c-Jun expression, and activates Jun N-terminal kinase. J Biol Chem 1996; 271: 27018–27024.

Received 18 February 2000; accepted 1 March 2000.