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Induction of cyclo-oxygenase-2 expression by methyl arachidonyl fluorophosphonate in murine J774 macrophages: roles of protein kinase C, ERKs and p38 MAPK

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1 Methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of phospholipase A_2 (PLA_2), has been widely used to assess the roles of PLA_2 in various cell functions. Here, we report on a novel action of this compound at concentrations similar to those used for PLA_2 inhibition.

2 The murine macrophage J774 released a large amount of prostaglandin E_2 (PGE_2) by MAFP (1–30 μM), which was abolished by indomethacin and NS-398 but not by valeryl salicylate, and results from increased cyclo-oxygenase-2 (COX-2) protein levels and gene expression.

3 This PGE_2 release was blocked by inhibitors of tyrosine kinase (genistein), protein kinase C (PKC) (Ro 31-8220, Go 6976 or LY 379196), mitogen-activated protein kinase kinase (MEK) (PD 098059) or p38 mitogen-activated protein kinase (MAPK) (SB 203580).

4 Consistent with these results, MAFP caused membrane translocation of PKCβI and βII isoforms and activated extracellular signal-regulated kinase (ERK) and p38 MAPK.

5 In accordance with these effects of MAFP, PKC activator phorbol 12-myristate 13-acetate (PMA) increased PGE_2 release and caused activation of PKCβ, ERKs and p38 MAPK.

6 This is the first report that the PLA_2 inhibitor, MAFP, can induce COX-2 gene expression and PGE_2 synthesis *via* the PKC-, ERK- and p38 MAPK-dependent pathways. Thus, the use of MAFP as a PLA_2 inhibitor should be treated with caution.

Keywords: MAFP; COX-2 expression; PKC; ERK; p38 MAPK; J774 macrophage

Abbreviations: AA, arachidonic acid; COX, cyclo-oxygenase; $cPLA_2$, cytosolic phospholipase A_2; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; Go 6976, 12-(-2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-indolo(2,3-a)pyrrolo(3,4-c) carbazole; $iPLA_2$, Ca^{2+}-independent PLA_2, MAFP, methyl arachidonyl fluorophosphonate; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulphonamide; PD 098059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PG, prostaglandin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Ro 31-8220; 1-[3-(amidinothio) propyl-1H-indoyl-3-yl]-3-(1-methyl-1H-indoyl-3-yl)-maleimide-methane sulphate; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfonyl-phenyl)-5(4-pyridyl)imidazole; $sPLA_2$, secretory PLA_2

Introduction

Phospholipases (PLA_2s) consist of a group of extracellular and intracellular enzymes that catalyze the hydrolysis of the sn-2 fatty acyl bond of phospholipids to yield AA and lysophospholipids; they play crucial roles in cellular processes involving phospholipid digestion, metabolizm and signal transduction and also produce rate-limiting precursors for the biosynthesis of eicosanoids and platelet-activating factor (Dennis, 1997; Leslie, 1997). The extracellular secretory PLA_2s ($sPLA_2$, group I, II, III, V, IX and X), intracellular cytosolic PLA_2s ($cPLA_2$, group IV) and Ca^{2+}-independent PLA_2 ($iPLA_2$, group VI, VII and VIII) have been purified and cloned (Balsinde & Dennis, 1997; Dennis, 1997; Leslie, 1997). To understand the chemical and functional differences between these enzymes, the discovery of selective inhibitors would be helpful. Recently, methyl arachidonyl fluorophosphonate (MAFP) was identified as a potent inhibitor of $cPLA_2$ and $iPLA_2$ (Ackermann *et al*., 1995; Lio *et al*., 1996) and has been widely used to explore the cellular functions of PLA_2 in a variety of cell types (Teslenko *et al*., 1997; Lin & Chen, 1998a).

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Prostaglandins (PGs), the arachidonic acid (AA) metabolites of the cyclo-oxygenase (COX) pathway, are major mediators in the regulation of inflammation and immune function. COX exists in two major isoforms, the constitutive form, COX-1, and the inducible form, COX-2. COX-1 is constitutively expressed in a wide range of cells and tissues (Funk *et al*., 1991) and may undergo slow changes in levels of expression associated with cellular differentiation (Smith *et al*., 1993), whereas COX-2 is highly expressed in stimulated inflammatory cells (e.g. macrophages) by a variety of pro-inflammatory agents, including cytokines, bacterial endotoxin and diverse mitogens (O'Sullivan *et al*., 1992). The characteristics of their expression suggest that COX-1 may be the isoform important for the production of PGs mediating homeostatic functions, while COX-2 may make a major contribution to increased PG production localized to specific tissues affected by inflammatory pathology. In this context, it is surprising that MAFP at concentrations comparable to those generally used to assess PLA_2 function in cells markedly stimulate COX-2 expression. We therefore undertook a study to investigate the mode of action of this compound on COX-2 expression.

Methods

Activation of J774 cells

The mouse macrophage cell line, J774, obtained from the ATCC, was cultured, as described previously (Lin & Chen, 1997), in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum and antibiotics (100 U ml^{-1} of penicillin, and 100 $\mu g\ ml^{-1}$ of streptomycin). Except for the RNA blot analysis, in which cells were grown in 10 cm Petri dishes, cells in 0.5 ml of DMEM were seeded into 24-well plates; once they had reached confluence, 0.5 ml of fresh culture medium, with or without drugs, was added to each well to activate the cells. After 24 h (unless otherwise indicated), the medium was collected for PGE_2 assay and the cells harvested for immunoblot analysis.

PGE_2 assays

PGE_2 released into the medium was measured using commercial kits, following the manufacturer's instructions.

Immunoblot analysis of COX-2 and protein kinase C (PKC) isoforms

Following drug treatment, the cells were washed twice in ice-cold PBS, solubilized in buffer A (in mM: Tris-HCl 20, EGTA 0.5, EDTA 2, DDT 2, p-methylsulphonyl fluoride 0.5 and leupeptin 10 $\mu g\ ml^{-1}$, pH 7.5), then sonicated. For the PKC translocation experiment, the crude cell lysate was centrifuged at 40,000 x *g* for 40 min to obtain the cytosolic and membrane fractions. Samples of equal amount of protein (50–100 μg) were subjected to 9% SDS–PAGE under reducing condition, and the separated proteins transferred onto a nitrocellulose membrane, which was then incubated in mM: NaCl 150, Tris 20, Tween 0.02%, pH 7.4 containing 5% milk, before being probed with antibodies specific for COX-2, PKCβI or PKCβII. After washing, the blots were probed with horseradish peroxidase-conjugated IgG and immunoreactivity detected by ECL, following the manufacturer's instructions.

RNA blotting

Confluent cells, grown in 10 cm Petri dishes, were treated with MAFP for different periods, then harvested. Equal amounts (about 20 μg) of total RNA, purified using RNAzol reagent, were applied to each lane of 1.2% formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Amersham Pharmacia Biotech). After UV cross-linking and prehybridization for 1 h at 42°C, the membranes were then sequentially probed for 16–24 h with COX-2 cDNA probes labelled with α-^{32}P-dCTP by random priming (approximately 2×10^8 c.p.m. μg^{-1}). Hybridization reactions were performed in 50% formamide, 5 × SSPE, 10 × Denhardt's solution, 0.5% SDS and 0.1 mg ml^{-1} salmon sperm DNA, then the membranes were washed twice in 2 × SSC, 0.1% SDS at room temperature for 15 min, followed by twice in 0.1 × SSC, 0.1% SDS at 65°C for 30 min before being exposed for 1 week to Kodak film using intensifying screens. Densitometrical analyses were performed on a Molecular Dynamics densitometer.

Assay of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)

Following drug treatment, equal amounts of lysate proteins, prepared in immunoprecipitation buffer (Tris 20 mM, pH 7.5, $MgCl_2$ 1 mM, NaCl 125 mM, Triton X-100 1%, p-methylsulphonyl fluoride 1 mM, leupeptin 10 $\mu g\ ml^{-1}$, aprotinin 10 $\mu g\ ml^{-1}$, NaF 50 mM, β-glycerophosphate 25 mM, Na_3VO_4 100 μM) were incubated with anti-p38 MAPK or anti-ERK antibody and protein A-sepharose beads overnight at 4°C, then the beads were washed three times with 1 ml of ice-cold immunoprecipitation buffer and immune-complex kinase assays performed on the antibody immunoprecipitates at 30°C for 30 min in 20 μl of kinase reaction buffer (in mM: HEPES 25, pH 7.4, $MgCl_2$ 20, Na_3VO_4 0.1, dithiothreitol 2) containing myelin basic protein (MBP) 50 $\mu g\ ml^{-1}$, ATP 100 μM and [γ-^{32}P]-ATP 10 μCi). The reaction was terminated with 5 × Laemmli sample buffer, the products separated on 15% SDS–PAGE gels and the phosphorylated MBP was visualized by autoradiography. A PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) was used to quantify band intensity.

Materials

Antibodies for COX-2, PKCβI, βII and p38 MAPK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). COX-2 cDNA, the PGE_2 assay kits, valeryl salicylate and MAFP were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). RNAzol was obtained from Biotecx Laboratories, Inc. (Houston, Texas, U.S.A.). DMEM, foetal bovine serum, penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). [α-^{32}P]-dCTP (3,000 Ci $mmol^{-1}$), [γ-^{32}P]-ATP (5,000 Ci $mmol^{-1}$), horseradish peroxidase-coupled antibody, and the enhanced chemiluminescence detection agent were purchased from Amersham Pharmacia Biotech. Genistein, PD 098059 and NS-398 were from RBI (Natick, MA, U.S.A.). LY 379196 was a generous gift from Eli Lilly (Indianapolis, IN, U.S.A.). SB 203580, Ro 31-8220, Go 6976, H-8 and H-89 were purchased from Calbiochem (La Jolla, CA, U.S.A.). D609 was obtained from Biomol (Plymouth Meeting, PA, U.S.A.). All materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Phorbol 12-myristate 13-acetate (PMA), MBP and other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Anti-ERK antibody and protein A-sepharose beads were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). [^{3}H]-Myoinositol (20 Ci $mmol^{-1}$) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Statistical evaluation

Values are expressed as the mean ± standard error of the mean (s.e.mean) of at least three experiments. Student's *t*-test was used to assess the statistical significance of the differences, a *P* value of less than 0.05 being considered statistically significant.

Results

MAFP induced COX-2 induction and PGE_2 secretion

Murine J774 cells were chosen to investigate the effect of MAFP on PGE_2 formation, as they show a marked induction of inflammatory response genes, such as COX-2 following LPS stimulation. Treatment with MAFP (1–30 μM) for 24 h stimulated PGE_2 release into the medium in a concentration-dependent manner (Figure 1a), this effect being time-dependent, reaching a steady-state at 12 h (Figure 1b). After 24 h stimulation with 30 μM MAFP, the PGE_2 level increased approximately 28 fold (from 0.6 ± 0.1 to 16.8 ± 1.5 ng ml^{-1}, $n = 10$).

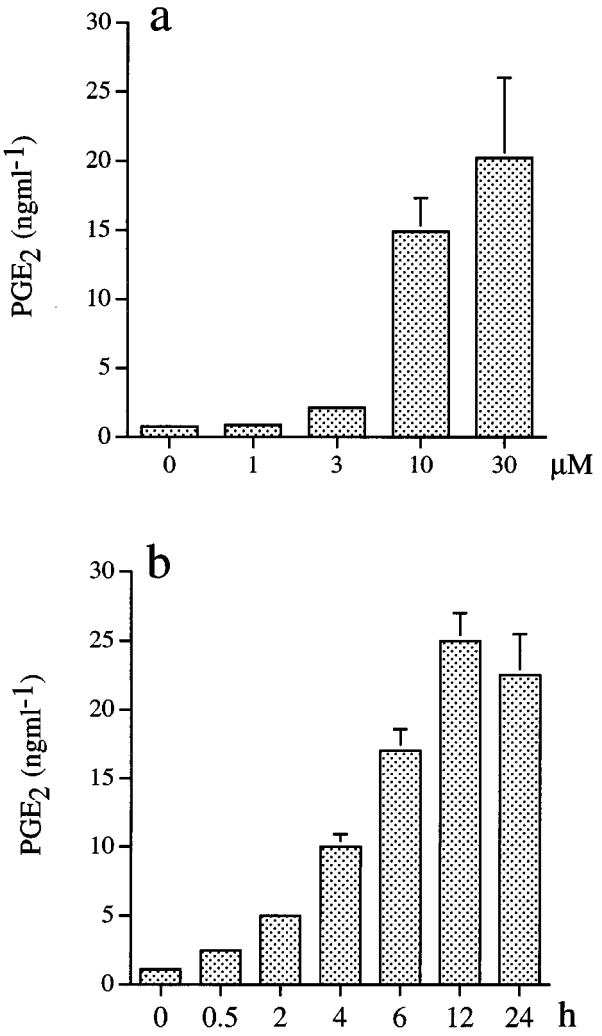

Figure 1 Concentration- and time-dependent effects of MAFP on PGE_2 formation. (a) Cells were treated with vehicle or MAFP (1–30 μM) for 24 h and PGE_2 released into medium was measured. (b) After treating cells with 30 μM MAFP for different intervals, released PGE_2 was measured. The data represents the mean ± s.e.mean from three independent experiments performed in duplicate.

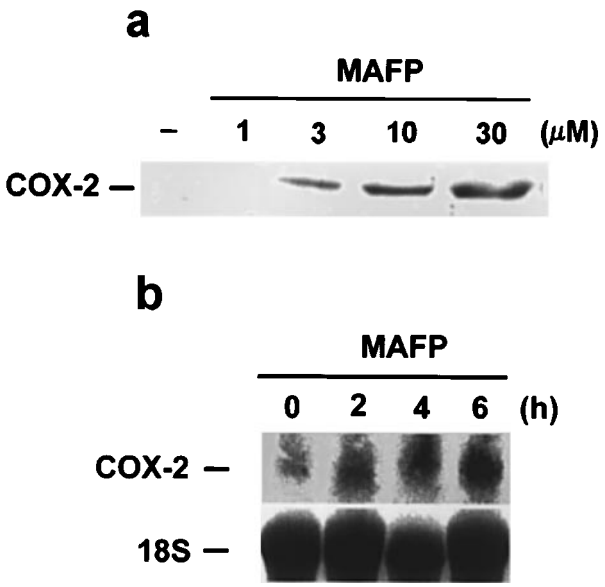

Figure 2 MAFP-induced COX-2 expression in J774 macrophages. (a) Cells were treated with MAFP at different concentrations for 24 h, then COX-2 protein levels were determined. (b) Cells were treated with 30 μM MAFP for different periods, then COX-2 mRNA levels were determined. The results are representative of three experiments.

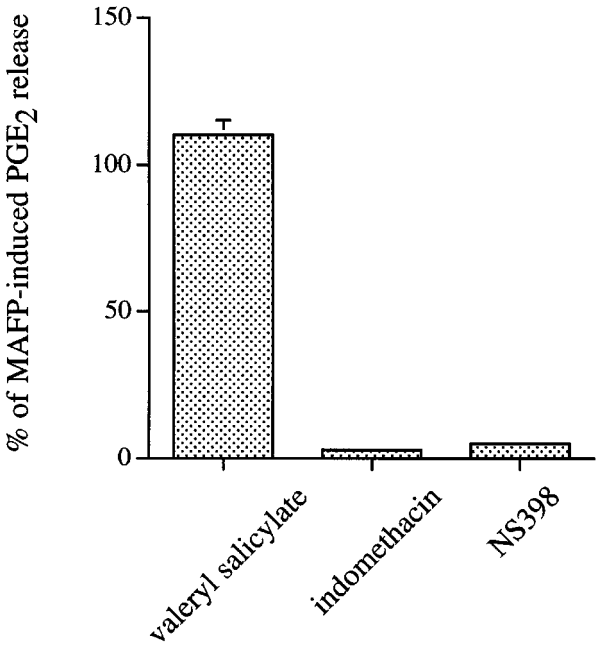

Figure 3 Effects of COX inhibitors on MAFP-induced PGE_2 release. Cells were pretreated with indomethacin (3 μM), valeryl salicylate (10 μg ml^{-1}) or NS-398 (3 ng ml^{-1}) for 20 min, then MAFP (30 μM) was added and incubation continued for 24 h. PGE_2 released into the medium was measured. The data represent the mean ± s.e.mean from 3–4 independent experiments performed in duplicate.

To determine whether the PGE_2 release over 24 h resulted from COX-2 activity, protein and mRNA levels of COX-2 and its pharmacological features were analysed. Treatment with MAFP for 24 h resulted in a concentration-dependent induction of COX-2 protein (Figure 2a), while treatment with 30 μM MAFP resulted in a time-dependent induction of COX-2 mRNA (Figure 2b). The effect of MAFP on PGE_2 release was abolished by indomethacin (3 μM), a non-selective COX inhibitor, or NS-398 (3 ng ml^{-1}), a COX-2 selective inhibitor (Futaki *et al*., 1994; Ashraf *et al*., 1996), whereas the COX-1 selective inhibitor, valeryl salicylate (10 μg ml^{-1}) (Bhattacharyya *et al*., 1995) had no effect (Figure 3). Consistent with these findings, MAFP treatment was not accompanied by a change in constitutive COX-1 expression (data not shown). Under the conditions described, none of the drugs had any significant effect on cell viability as assessed by the ability of the cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (a marker of intact mitochondrial metabolic activity) (data not shown).

Protein kinases involved in the MAFP effect

Since both we and others have shown various protein kinases, such as tyrosine kinase lyn, p38 MAPK (our unpublished data), ERKs (Hwang *et al*., 1997) and PKC (Bauer *et al*., 1997), to be involved in LPS-induced COX-2 expression, the events involved in the MAFP response were analysed. As shown in Figure 4a, inhibitor of tyrosine kinase (genistein, 50 μM) (Akiyama *et al*., 1987), MEK (PD 098059, 30 μM) (Dudley *et al*., 1995) or p38 MAPK (SB 203580, 3 μM) (Cuenda *et al*., 1995) markedly inhibited MAFP-induced PGE_2

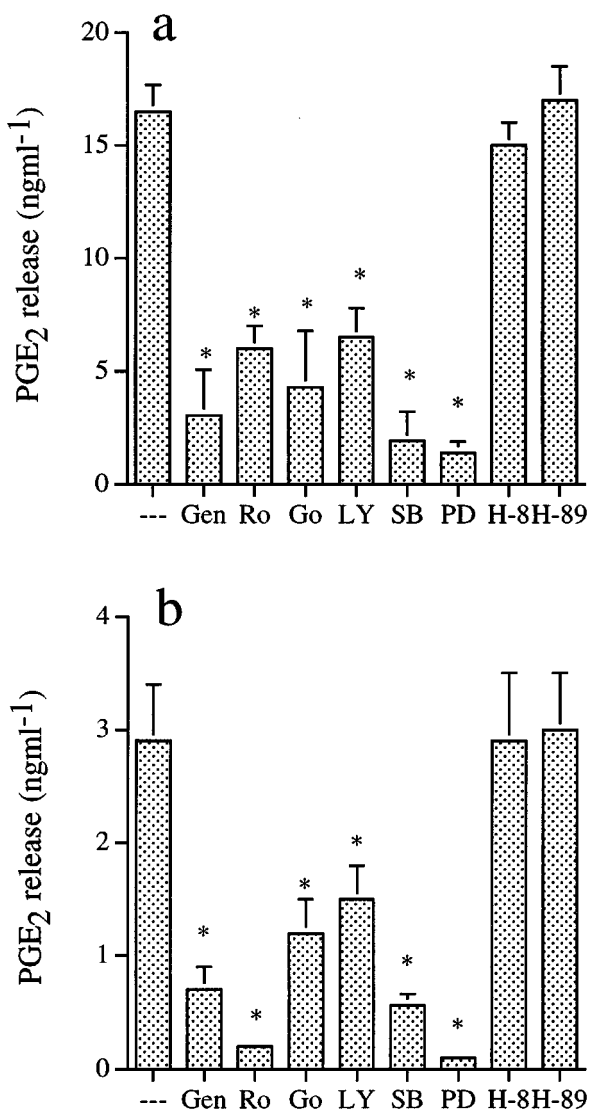

Figure 4 Effects of genistein, SB 203580, PD 098059 and PKC inhibitors on MAFP- and PMA-induced PGE_2 release. Cells were pretreated with 50 μM genistein, 30 μM PD 098059, 3 μM SB 203580, 1 μM Ro 31-8220, 1 μM Go 6976, 100 nM LY 379196, 1 μM H-8 or 1 μM H-89 for 20 min, then 30 μM MAFP (a) or 1 μM PMA (b) was applied and incubation continued for 24 h. PGE_2 released into the medium was measured. The data represents the mean $\pm$ s.e.mean from 3–4 independent experiments performed in duplicate. $^*P<0.05$ as compared to the control PGE_2 release without drug pretreatment.

release by $80\pm12\%$ ($n=4$), $91\pm6\%$ ($n=3$) and $88\pm3\%$ ($n=3$), respectively. In addition, Ro 31-8220 (1 μM; an inhibitor of all PKC isoforms), Go 6976 (1 μM; a selective inhibitor of conventional PKCα, β and γ) (Martinyl-Baron *et al.*, 1993) or LY 379196 (100 nM; a selective inhibitor of PKCβ) (Lin & Chen, 1998b) also resulted in an inhibition of $69\pm6\%$ ($n=3$), $73\pm15\%$ ($n=3$) and $67\pm8\%$ ($n=3$), respectively, while two PKA inhibitors, H-8 and H-89 (1 μM) did not significantly affect the MAFP-induced response. At the concentrations used, none of the protein kinase inhibitors affected the cell viability (data not shown). These results suggest the involvement of PKC, ERKs and p38 MAPK in the signalling cascade involved in the MAFP-induced PGE_2 release.

To further confirm the regulatory role of PKC in COX-2 activation, the effect of PMA, a potent and irreversible PKC activator, on PGE_2 synthesis was tested. As shown in Figure 4b, treatment of cells with 1 μM PMA caused an increase in PGE_2 level of 5.9 ± 0.8 fold ($n=5$) after 24 h; this effect was inhibited by the same six protein kinase inhibitors that inhibited the MAFP-induced response.

MAFP activates PKCβ, ERK and p38 MAPK

To directly confirm the crucial roles of the three protein kinases, we determined whether MAFP can indeed activate these protein kinases. The combined results of our previous work showing the presence in J774 macrophages of eight PKC isoforms (βI, βII, δ, ε, η, μ, λ and ζ) (Lin & Chen, 1997), the involvement of PKCβ in COX-2 induction by LPS (Bauer *et al.*, 1997), and the effective inhibition of MAFP-induced response by selective conventional PKC or PKCβ inhibitors (Go 6976 or LY 379196, respectively) (Figure 4a) suggested the involvement of PKCβ in the MAFP-induced PGE_2 release. To confirm this suggestion, PKCβ translocation from the cytosol to the membrane, a well-known index for PKC activation, was studied. Using antibodies specific, respectively, for PKCβI and βII, the results shown in Figure 5 indicated that both PKC isoforms can be activated by treatment with MAFP (30 μM) within 10 min. The PKC activator, PMA (1 μM) was even more effective than MAFP in causing translocation of both PKCβ isoforms from the cytosol to membrane.

Immunocomplex kinase assays were carried out to support the involvement of ERKs and p38 MAPK in MAFP signalling. The results using myelin basic protein as a kinase substrate (Figure 6) indicated that 30 μM MAFP was more effective than 1 μM PMA in stimulating ERKs and p38 MAPK.

Discussion

An increasing number of studies have demonstrated that $cPLA_2$ and/or $sPLA_2$ play a prominent role in chronic inflammation (Glaser, 1995) and have substantiated the proposal that PLA_2s might be a novel pharmacological target for anti-inflammatory therapy. Subsequent to PLA_2 activation, COX catalyzes the first rate-limiting steps in the conversion of AA into PGs and thromboxanes, both of which are biologically active molecules operative in both acute and chronic inflammation. Levels of COX-1 protein appear to be unchanged throughout the inflammation period, whereas COX-2, an immediate-early gene, is undetectable in most normal tissues, but is strongly induced by pro-inflammatory cytokines and mitogens at sites of inflammation, with PGE_2 being the predominant metabolite (Herschmann *et al.*, 1995). In this context, macrophages have been identified as the cells most obviously immunolabelled for COX-2 protein during inflammation. In this study, we found that addition of MAFP to the J774 macrophages caused a rapid and time-dependent induction of COX-2 mRNA over the period of 2–6 h, with COX-2 protein levels similarly increasing 24 h after MAFP addition.

ERK and p38 MAPK, two key enzymes that transmit signals from the cell surface to the nucleus (Nishida & Gotoh, 1993), are known to be activated by a variety of extracellular stimuli, including those mediated by receptor tyrosine kinases and by G protein-coupled receptors (Hawes *et al.*, 1995; Yamauchi *et al.*, 1997). With respect to the PGE_2 release by various cell types, including the monocyte/macrophage lineages, both ERK and p38 MAPK have been shown to play crucial roles in this process by up-regulating COX-2 transcription (Guan *et al.*, 1997; 1998; Ridley *et al.*, 1997). In

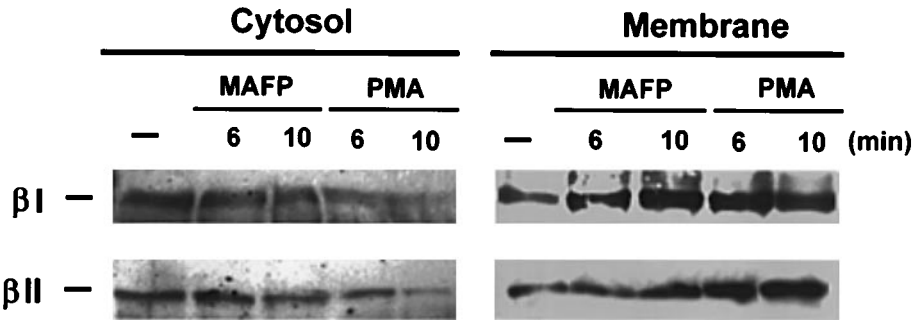

Figure 5 Effects of MAFP on the translocation of PKCβ from the cytosol to the membrane. Homogenate from J774 cells, exposed to 30 μM MAFP or 1 μM PMA for 6 or 10 min, were fractionated into cytosolic and membrane fractions and immunoblotted with antibodies specific for PKC βI and PKC βII, as described in 'Methods'. The results are representative of three experiments.

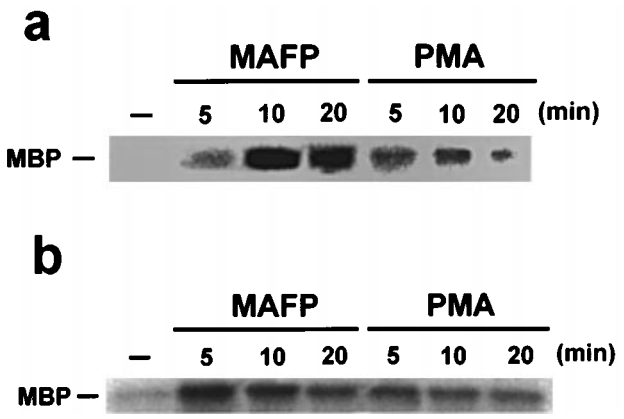

Figure 6 Effects of MAFP or PMA on ERKs and p38 MAPK activity. Cells in 60 mm plates were stimulated with 30 μM MAFP or 1 μM PMA for 5–20 min, then the immunocomplex kinase assay for ERKs (a) and p38 MAPK (b) were performed as described in 'Methods'.

addition, PKC activation and/or increased $[Ca^{2+}]_i$ have been demonstrated to be involved in COX-2 induction (Bauer *et al*., 1997; Ledwith *et al*., 1997; Mestre *et al*., 1997). Because of the important roles of ERKs, p38 MAPK and PKC in COX-2 induction, we performed several experiments to investigate their involvement in the MAFP response. In addition, we compared the effects of MAFP with those induced by the PKC activator, PMA.

Firstly, we confirmed the activating effect of PMA on PGE_2 release in J774 macrophages with an approximately 5 fold increase in the presence of PMA. Although the extent of activation by PMA was less than with MAFP, similar degree of PGE_2 stimulation by PMA (about a 3–4 fold increase) have been seen in human oral epithelial cells (Mestre *et al*., 1997) and mouse liver cells (Ledwith *et al*., 1997). Secondly, the effects of inhibitors of PKC (Ro 31-8220, Go 6976 and LY379196), MEK (PD 098059) or p38 MAPK (SB203580) on MAFP- or PMA-induced PGE_2 stimulation suggest the involvement of these kinases in PGE_2 synthesis. It needs to point out a recent study of Borsch-Haubold *et al*. (1998) who demonstrated a direct inhibitory effect of SB 203580 and PD 098059 on COX activity. Thirdly, both MAFP and PMA can activate PKCβI and βII by translocating them from the cytosol to the membrane, with PMA being more effective than MAFP. Regarding the PKC isoforms involved in COX-2 induction, our previous work have shown the presence of eight PKC isoforms (βI, βII, δ, ε, η, μ, λ and ζ) in J774 macrophages (Lin & Chen, 1997). The reasons why we chose to specifically investigate PKCβ activation in this study was the previous observation that LPS-induced COX-2 transcription is inhibited by Go 6976 (an inhibitor of classical PKCα, β and γ) in microglia (Bauer *et al*., 1997) and the present findings that Go 6976 and LY 379196 (a selective PKCβ inhibitor) effectively inhibit PGE_2 production induced by either MAFP and PMA. Fourthly, both ERKs and p38 MAPK were activated following stimulation of J774 macrophages by MAFP and PMA; in this case, MAFP being more effective than PMA. Fifthly, although we cannot, as yet, provide direct evidence for the relative importance of these three protein kinases in COX-2 induction, we suggest that ERKs and p38 MAPK are more involved than PKCβ. Our results showed that the potent PKC activator, PMA, induced less PGE_2 release and weaker activation of ERKs and p38 MAPK, but more PKCβ activation, than did MAFP. The possibility that signalling pathways other than the PKC-dependent pathway contribute to the MAFP-induced ERKs and p38 MAPK activation requires further investigation.

Since increased $[Ca^{2+}]_i$ are known to induce COX-2 expression (Ledwith *et al*., 1997), we measured change in the $[Ca^{2+}]_i$ after MAFP treatment in order to see if intracellular Ca^{2+} levels are relevant to the MAFP effect. We found that MAFP, at a concentration of 30 μM, did not affect the $[Ca^{2+}]_i$ (data not shown). The cyclic AMP signalling pathway was reported to be a functionally important mechanism in regulating COX-2 expression. In line with findings that the COX-2 promoter contains a cyclic AMP response element (CRE) binding motif (Appleby *et al*., 1994), membrane-permeable analogues of cyclic AMP, forskolin and some cyclic AMP elevating agents (e.g. A2a agonist and inhibitor of cyclic nucleotide phosphodiesterase) have been shown to be inducers of COX-2 expression in microglial (Fiebich *et al*., 1996), and mesangial cells (Nusing *et al*., 1996). To explore the possible cyclic AMP-dependent mechanisms associated with MAFP action, we directly measured intracellular cyclic AMP levels following MAFP treatment and also tested the effects of two PKA inhibitors, H-8 and H-89. We found that MAFP (30 μM) had no significant effect on intracellular cyclic AMP levels (data not shown) and that its effect on PGE_2 stimulation was unaffected by PKA inhibitors.

Since PKCβ is activated by endogenous diacylglycerol (DAG), we wished to verify whether the MAFP-induced PKC was due to DAG formation sebsequent to phosphatidylinositide and/or phosphatidylcholine breakdown. We therefore determined the effect of MAFP on phosphatidylinositide turnover by quantifying the accumulation of inositol monophosphate and found that MAFP, at concentrations as high as 50 μM, only slightly increases the inositol phosphate accumulation ($30 \pm 5\%$; $n = 3$) (data not shown), indicating that MAFP is not a significant stimulator of phosphoinositide-specific

phospholipase C. Moreover, since phosphatidylcholine-specific phospholipase C and phospholipase D-derived phosphatidic acid are other sources of DAG, we addressed these possibilities using pharmacological approaches and found that neither D609 (30 μM, a phosphatidylcholine-specific phospholipase C inhibitor) nor wortmannin (100 nM, a phospholipase D inhibitor) had effects on MAFP-induced stimulation of PGE_2 production (data not shown).

Although MAFP was shown to be an irreversible inhibitor of $cPLA_2$, (Huang *et al*., 1996), it was recently shown to have non-selective effects other than those shown in the present study. For example, it induces irreversible inhibition of $iPLA_2$ with an IC_{50} of 0.5 μM (Lio *et al*., 1996), inhibits the enzymic hydrolysis of the endogenous cannabinoid receptor agonist, arachidonoyl ethanolamide (anandamide) ($IC_{50} = 1-3$ nM) (Deutsch *et al*., 1997), and is an irreversible cannabinoid receptor antagonist (Fernando & Pertwee, 1997). Thus, extreme caution is required when working with this product if any valid conclusions are to be drawn on the physiological and pathological roles of PLA_2.

In summary, the principle findings of this study were that, in murine J774 macrophages, the PLA_2 inhibitor, MAFP, can cause COX-2 gene expression and protein induction *via* activation of PKCβ, ERKs and p38 MAPK.

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References

ACKERMANN, E.J., CONDE-FRIEBOES, K. & DENNIS, E.A. (1995). Inhibition of macrophage Ca^{2+}-independent phospholipase A_2 by bromoenol lactone and trifluoromethyl ketones. *J. Biol. Chem.,* **270,** 445–450.

AKIYAMA, T., ISHIDA, J., NAKAGAWA, S., OGAWARA, H., WATANABE, S.I., ITOH, N., SHIBUYA, M. & FUKAMI, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.,* **262,** 5592–5595.

APPLEBY, S.B., RISTIMAKI, A., NEILSON, K., NARKO, K. & HLA, T. (1994). Structure of the human cyclooxygenase-2 gene. *Biochem. J.,* **302,** 723–727.

ASHRAF, M., MURAKAMI, M. & KUDO, I. (1996). Cross-linking of the high-affinity IgE receptor induces the expression of cyclooxygenase 2 and attendant prostaglandin generation requiring interleukin 10 and interleukin 1β in mouse cultured mast cells. *Biochem. J.,* **320,** 965–973.

BALSINDE, J. & DENNIS, E.A. (1997). Function and inhibition of intracellular calcium-independent phospholipase A_2. *J. Biol. Chem.,* **272,** 16069–16072.

BAUER, M.K.A., LIEB, K., SCHULZE-OSTHOFF, K., BERGER, M., GEBICKIE-HAERTER, P.J., BAUER, J. & FIEBICH, B.L. (1997). Expression and regulation of cyclooxygenase-2 in rat microglia. *Eu. J. Biochem.,* **243,** 726–731.

BHATTACHARYYA, D.K., LECOMTE, M., DUNN, J., MORGANS, D.J. & SMITH, W.L. (1995). Selective inhibition of prostaglandin endoperoxide synthase-1 (cyclooxygenase-1) by valerylsalicyclic acid. *Arch. Biochem. Biophys.,* **317,** 19–24.

BORSCH-HAUBOLD, A.G., PASQUET, S. & WATSON, S.P. (1998). Direct inhibition of cyclooxygenase-1 and -2 by the kinase inhibitors SB 203580 and PD 98059. *J. Biol. Chem.,* **273,** 28766–28772.

CUENDA, A., ROUSE, J., DOZA, Y., MEIER, R., COHEN, P., GALLAGHER, T., YOUNG, P. & LEE, T. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.,* **364,** 229–233.

DENNIS, E.A. (1997). The growing phospholipase A_2 superfamily of signal transduction enzymes. *Trends Biochem. Sci.,* **22,** 1–2.

DEUTSCH, D.G., OMEIR, R., ARREAZA, A., SALEHANI, D., PRESTWICH, G.D., HUANG, Z. & HOWLETT, A. (1997). Methyl arachidonyl fluorophosphonate: a potent irreversible inhibitor of anandamide amidase. *Biochem. Pharmacol.,* **53,** 255–260.

DUDLEY, D.T., PANG, L., DECKER, S.J., BRIDGES, A.J. & SALTIEL, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7686–7689.

FERNANDO, S.R. & PERTWEE, R.G. (1997). Evidence that methyl arachidonyl fluorophosphonate is an irreversible cannabinoid receptor antagonist. *Br. J. Pharmacol.,* **121,** 1716–1720.

FIEBICH, B.L., BIBER, K., LEIB, K., VAN CALKER, D., BERGER, M., BAUER, J. & GEBICKE-HAERTER, P.J. (1996). Cyclooxygenase 2 expression in rat microglia is induced by adenosine A_{2a}-receptors. *Glia,* **18,** 152–160.

FUNK, C.D., FUNK, L.B., KENNEDY, M.E., PONG, A.S. & FITZGERALD, G.A. (1991). Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J.,* **5,** 2304–2312.

FUTAKI, N., TAKAHASHI, S., YOKOYAMA, M., ARAI, S., HIGUCHI, S. & OTOMO, S. (1994). NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins.,* **47,** 55–59.

GLASER, K.B. (1995). Regulation of phospholipase A_2 enzymes: selective inhibitors and their pharmacological potential. *Adv. Pharmacol.,* **32,** 31–66.

GUAN, Z., BAIER, L.D. & MORRISON, A.R. (1997). p38 Mitogen-activated protein kinase down-regulates nitric oxide and up-regulates prostaglandin E_2 biosynthesis stimulated by interleukin-1β. *J. Biol. Chem.,* **272,** 8083–8089.

GUAN, Z., BUCKMAN, S.Y., MILLER, B.W., SPRINGER, L.D. & MORRISON, A.R. (1998). Interleukin-1β-induced cyclooxygenase-2 expression requires activation of both c-Jun NH_2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. *J. Biol. Chem.,* **273,** 28670–28676.

HAWES, B.E., VAN BIESEN, T., KOCH, W.J., LUTTRELL, L.M. & LEFKOWITZ, R.J. (1995). Distinct pathway of Gi- and Gq-mediated mitogen-activated protein kinase activation. *J. Biol. Chem.,* **270,** 17148–17153.

HERSCHMANN, H.R., GILBERT, R.S., XIE, W., LUNER, S. & REDDY, S. (1995). The regulation and role of TIS 10 prostaglandin synthase-2. *Adv. Prost. Thromb. Leuk. Res.,* **23,** 23–28.

HUANG, Z., PAYETTE, P., ABDULLAH, K., CROMLISH, W.A. & KENNEDY, B.P. (1996). Functional identification of the active-site nucleophile of the human 85-kDa cytosolic phospholipase A_2. *Biochemistry,* **35,** 3712–3721.

HWANG, D., JANG, B.C., YU, G. & BOUDREAU, M. (1997). Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-kappa B signaling pathways in macrophages. *Biochem. Pharmacol.,* **54,** 87–96.

LEDWITH, B.J., PAULEY, C.J., WAGNER, L.K., ROKOS, C.L., ALBERTS, D.W. & MANAM, S. (1997). Induction of cyclooxygenase-2 expression by peroxisome proliferators and non-tetradecanoylphorbol 12,13-myristate-type tumor promoters in immortalized mouse liver cells. *J. Biol. Chem.,* **272,** 3707–3714.

LESLIE, C.C. (1997). Properties and regulation of cytosolic phospholipase A_2. *J. Biol. Chem.,* **272,** 16709–16712.

LIN, W.W. & CHEN, B.C. (1997). Involvement of protein kinase C in the UTP-mediated potentiation of cyclic AMP accumulation in mouse J774 macrophages. *Br. J. Pharmacol.,* **121,** 1749–1757.

LIN, W.W. & CHEN, B.C. (1998a). Pharmacological comparison of UTP- and thapsigargin-induced arachidonic acid release in mouse RAW 264.7 macrophages. *Br. J. Pharmacol.,* **123,** 1173–1181.

LIN, W.W. & CHEN, B.C. (1998b). Distinct PKC isoforms mediate the activation of $cPLA_2$ and adenylyl cyclase by phorbol ester in RAW 264.7 macrophages. *Br. J. Pharmacol.,* **125,** 1601–1609.

LIO, Y.C., REYNOLDS, L.J., BALSINDE, J. & DENNIS, E.A. (1996). Irreversible inhibition of Ca^{2+}-independent phospholipase A_2 by methyl arachidonyl fluorophosphonate. *Biochim. Biophys. Acta,* **1302,** 55–60.

MARTINYL-BARON, G., KAZANIETZ, M.G., MISCHAK, H., BLUMBERG, P.M., KOCHS, G., HUG, H., MARME, D. & SCHACHTELE, C. (1993). Selective inhibition of protein kinase C isozymes by the indolo-carbazole Go 6976. *J. Biol. Chem.,* **268,** 9194–9197.

MESTRE, J.R., SUBBARAMAIAH, K., SACKS, P.G., SCHANTZ, S.P., TANABE, T., INOUE, H. & DANNENBERG, A.J. (1997). Retinoids suppress phorbol ester-mediated induction of cyclooxygenase-2. *Cancer Res.,* **57,** 1081–1085.

NISHIDA, E. & GOTOH, Y. (1993). The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.,* **18,** 128–131.

NUSING, R.M., KLEIN, T., PFEILSCHIFTER, J. & ULLRICH, V. (1996). Effect of cyclic AMP and prostaglandin D_2 on the induction of nitric oxide- and prostanoid-forming pathways in cultured rat mesangial cells. *Biochem. J.,* **313,** 617–623.

O'SULLIVAN, M.G., CHILTON, F.H., HUGGINS, E.M. & MCCALL, C.E. (1992). Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase. *J. Biol. Chem.,* **267,** 14547–14550.

RIDLEY, S.H., SARSFIELD, S.J., LEE, J.C., BIGG, H.F., CAWSTON, T.E., TAYLOR, D.J., DEWITT, D.L. & SAKLATVALA, J. (1997). Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J. Immunol.,* **158,** 3165–3173.

SMITH, C.J., MORROW, J.D., ROBERTS, L.J. & MARNETT, L.J. (1993). Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1). *Biochem. Biophys. Res. Commun.,* **192,** 787–793.

TESLENKO, V., ROGERS, M. & LEFKOWITH, J.B. (1997). Macrophage arachidonate release via both the cytosolic Ca^{2+}-dependent and -independent phospholipases is necessary for cell spreading. *Biochem. Biophys. Acta,* **1344,** 189–199.

YAMAUCHI, J., NAGAO, M., KAZIRO, Y. & ITOH, H. (1997). Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. *J. Biol. Chem.,* **272,** 27771–27777.

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