Inhibition of mitogen-activated protein kinase kinase does not impair primary activation of human platelets

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Mitogen-activated protein kinases (MAPKs), a family of protein serine/threonine kinases regulating cell growth and differentiation, are activated by a dual-specificity kinase through phosphorylation at threonine and tyrosine. We used a recently described selective inhibitor of the p42/p44^{mapk}-activating enzyme, PD 98059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one], to investigate the role of the p42/p44^{mapk} pathway in human platelets. PD 98059 inhibited p42/p44^{mapk} activation in thrombin-, collagen- and phorbol ester-stimulated platelets, as determined from in-gel renaturation kinase assays, with an IC_{50} of approx. 5 μM (thrombin stimulation). It also prevented activation of MAPK kinase, which was measured in whole-cell lysates with glutathione S-transferase/p42^{mapk} fusion protein (GST–MAPK) as substrate. Inhibition of p42/p44^{mapk} did not affect platelet responses to thrombin or collagen such as aggregation, 5-hydroxytryptamine release and protein kinase C activation. In addition, PD 98059 did not interfere with release of arachidonic acid, a response mediated by cytosolic phospholipase A_2 (cPLA$_2$), or with cPLA$_2$ phosphorylation. This suggests that platelet cPLA$_2$ is not regulated by p42/p44^{mapk} after stimulation with physiological agonists. In contrast, phorbol ester-induced phosphorylation of cPLA$_2$ and potentiation of arachidonic acid release stimulated by Ca^{2+} ionophore A23187 were inhibited by PD 98059, indicating that p42/p44^{mapk} phosphorylates cPLA$_2$ after activation of protein kinase C by the non-physiological tumour promoter.

INTRODUCTION

Activation of the mitogen-activated protein kinase (MAPK) pathway downstream of receptor tyrosine kinases or G-protein-coupled receptors leads to cell differentiation and cell growth. The kinase cascade, consisting of the three components MAPK kinase kinase, MAPK kinase and MAPK, is conserved throughout eukaryotes [1]. The 42 and 44 kDa isoforms of MAPK [also named extracellular-regulated protein kinases (ERKs) 2 and 1 respectively] are phosphorylated at threonine and tyrosine by MAPK/ERK kinase (MEK). On activation, MAPKs translocate to the nucleus and phosphorylate transcription factors. MAPKs can also phosphorylate cytosolic substrates, including microtubule-associated protein, MAPK-activated protein kinase 1α and 1β [2–4] and cytosolic phospholipase A_2 (cPLA$_2$) [5,6].

Although much is known about the regulation and role of MAPK in various cell lines, the function of platelet MAPK is not resolved. As platelets are terminally differentiated cells that lack a nucleus, studies on the MAPK pathway focus on its cytosolic substrates. For example, cPLA$_2$ becomes phosphorylated in stimulated platelets [7–9], and this increases the intrinsic activity of the phospholipase to release arachidonic acid from phospholipids [7]. The ensuing production of thromboxane A_2, a potent pro-aggregatory agent, through the activity of cyclo-oxygenase is a major positive-feedback stimulus during platelet aggregation [10].

From studies *in vitro* and in transfected cells, it was suggested that p42^{mapk} phosphorylates cPLA$_2$ at Ser-505 [5,6], which lies within a consensus sequence for MAPK (Pro-Xaa-Ser/Thr-Pro) [11]. After this observation, many groups reported the concomitant activation of MAPK and phosphorylation of cPLA$_2$ in stimulated cells [12–17]. Several recent reports, however, dissociate cPLA$_2$ phosphorylation from MAPK activation. Stimulation of human neutrophils by tumour necrosis factor α leads to phosphorylation of cPLA$_2$ but not to activation of p42^{mapk} or p44^{mapk} [18]; similarly, low concentrations of lipopolysaccharide do not stimulate MAPK but induce phosphorylation of cPLA$_2$ [19]. In human platelets, cPLA$_2$ is phosphorylated after stimulation with a thrombin receptor agonist peptide that does not induce MAPK phosphorylation [8]. We have previously used the protein kinase C inhibitor Ro 31-8220 to block activation of p42/p44^{mapk} in platelets stimulated by thrombin or collagen and demonstrated that this affected neither phosphorylation of cPLA$_2$ nor release of arachidonic acid [9].

The present study uses a flavone compound, PD 98059, which has been described as a potent and highly selective inhibitor of MEK [20,21], to investigate the role of p42/p44^{mapk} in platelets. PD 98059 blocks MEK activation, probably through an allosteric mechanism without affecting other kinases such as protein kinase C, protein kinase A, Raf or MAPK itself [21]. We found that inhibition of the MAPK cascade by PD 98059 did not affect platelet responses to the physiological stimuli thrombin and collagen. In particular, cPLA$_2$ phosphorylation and activation were not impaired. The results argue against an important physiological role for p42/p44^{mapk} in primary platelet activation.

EXPERIMENTAL

Reagents

PD 98059 was provided by Dr. D. Dudley and Dr. A. Saltiel (Ann Arbor, MI, U.S.A.). *Escherichia coli* transfected with pGEX2T-ERK2 was a gift of Dr. C. J. Marshall (London, U.K.) from which glutathione S-transferase/p42^{mapk} fusion protein (GST–MAPK) was expressed and purified as described [22]. [1,2-

Abbreviations used: cPLA$_2$, cytosolic phospholipase A_2; ERK, extracellular-regulated protein kinase; GST–MAPK, glutathione S-transferase/p42^{mapk} fusion protein; 5-HT, 5-hydroxytryptamine; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK/ERK kinase; PDBu, phorbol 12,13-dibutyrate.

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^{3}H]5-Hydroxytryptamine (5-HT; specific radioactivity 25 Ci/mmol) was supplied by Amersham Corp. [^{32}P]P_i (specific radioactivity 8500–9120 Ci/mmol), [γ-^{32}P]ATP (specific radioactivity 3000 Ci/mmol), and [5,6,8,11,12,14,15-^{3}H]arachidonic acid (specific radioactivity 180–240 Ci/mmol) were obtained from Du Pont–NEN. Prostacyclin and BW4AC were donated by Wellcome Laboratories (Beckenham, Kent, U.K.). Bovine thrombin and phorbol 12,13-dibutyrate (PDBu) were obtained from Sigma. All other reagents were of analytical grade.

Preparation of platelets

Blood was drawn by venepuncture from healthy volunteers and washed platelets were prepared as described previously [9]. They were resuspended in Tyrode's buffer (20 mM Hepes, 135 mM NaCl, 3 mM KCl, 0.35 mM Na_2HPO_4, 12 mM $NaHCO_3$ and 1 mM $MgCl_2$, pH 7.3) containing the cyclo-oxygenase blocker indomethacin (10 μM) at 4×10^8 platelets/ml for functional studies, and at 2×10^9 platelets/ml for immunoprecipitations and kinase assays. Before stimulation, platelets were preincubated with DMSO or PD 98059 for 10 min at 37 °C. For kinase assays and phosphorylation studies, EGTA (1 mM) was present to prevent platelet aggregation. When stimulated by collagen (Nycomed Arzneimittel, Munich, Germany), and for kinase studies, platelets were stirred in a Born lumi-aggregometer at 1200 rev./min.

MAPK immunoprecipitation and in-gel renaturation kinase assay

p42/p44^{mapk} were immunoprecipitated with 1 μg of polyclonal anti-p42/p44^{mapk} antibody (Santa Cruz, Devizes, Wilts., U.K.) and Protein A–Sepharose CL-4B [9]. Immunoprecipitates were resolved on SDS/PAGE [10 % (w/v) gel] with myelin basic protein (MBP; 0.5 mg/ml) copolymerized in the polyacrylamide. After renaturation, gels were incubated at 37 °C with kinase assay buffer containing 20 μCi/ml of [γ-^{32}P]ATP, and washed in 5 % (w/v) trichloroacetic acid/1 % $Na_4P_2O_7$ [9]. Autoradiographs were exposed for up to 2 days at −70 °C with intensifying screens. The regions corresponding to MAPK were cut from the gels and scintillation-counted for radioactivity.

Total tissue MAPK kinase activity

Platelets were stimulated, lysed on ice in 1 % Triton X-100 (containing 20 mM Tris, pH 8, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 100 μM Na_3VO_4, 10 mM EGTA, 2 mM PMSF, 100 nM okadaic acid, 20 μg/ml leupeptin and 20 μg/ml aprotinin), and insoluble material was removed by microcentrifugation at 13000 ***g*** for 15 min at 4 °C. Aliquots (10 μl) were incubated with 30 μl of GST–MAPK buffer (30 mM Tris, pH 7.5, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol, 0.03 % Brij-35, 10 mM $MgCl_2$, 0.2 mM ATP, 100 μg/ml GST–MAPK) at 30 °C for 30 min on a shaking platform. The reaction was terminated by the addition of 40 μl of ice-cold buffer A (50 mM Tris, pH 7.5, 0.1 mM EGTA, 0.4 mM Na_3VO_4, 0.1 % 2-mercaptoethanol) containing 20 mM EDTA and 1 mg/ml BSA. To capture GST–MAPK fusion protein, samples were mixed with 20 μl of glutathione–agarose beads [1:1 (v/v) in buffer A] at 4 °C for 15 min. Background kinase activity due to non-specific binding of kinases to glutathione–agarose was measured by performing the assay in the absence of GST–MAPK. Beads were washed and incubated with 40 μl of kinase buffer (50 mM Tris, pH 7.5, 0.1 mM EGTA, 12.5 mM $MgCl_2$, 125 μM ATP, 400 μg/ml MBP and 56 μCi/ml [γ-^{32}P]ATP) at 30 °C for 10 min. The reaction was stopped by spotting 30 μl of supernatant on to P81 phosphocellulose papers and washing these in 75 mM phosphoric acid. Radioactivity of dried filter papers was measured by scintillation counting.

[^{3}H]5-HT and [^{3}H]arachidonic acid release

Platelets were incubated with either 10 μCi of [^{3}H]5-HT for 1 h or with 5 μCi of [^{3}H]arachidonic acid for 2 h at 30 °C, washed, and resuspended in Tyrode's buffer containing 10 μM indomethacin. For [^{3}H]arachidonic acid release, 3 μM BW4AC was added to the final suspension to block lipoxygenase [9]. Stimulation of platelets was stopped with an equal volume of 6 % (v/v) glutaraldehyde, and the radioactivity of the supernatant was determined by liquid-scintillation spectrometry in a Beckman scintillation counter to a 5 % level of significance. Experiments were performed in quadruplicate. Results are expressed as means ± S.E.M. Statistical significance was assessed with Student's *t*-test.

Phosphorylation of cPLA$_2$

cPLA$_2$ was immunoprecipitated from [^{32}P]P_i-radiolabelled platelets [9] and resolved on SDS/PAGE (10 % gel). Autoradiographs were taken at −70 °C with intensifying screens. Electrophoretic mobility, to distinguish phosphorylated from non-phosphorylated cPLA$_2$, was analysed from whole-cell lysates on SDS/PAGE (8 % gel) Maxigels.

Immunoblotting

Membranes were incubated with rabbit cPLA$_2$ antiserum (1:10000) [7] in Tris-buffered saline/Tween-20 (20 mM Tris, 137 mM NaCl, 0.1 % Tween-20, pH 7.6) containing 1 % (v/v) BSA for 2 h or with polyclonal anti-p44^{mapk} antibody (2 μg/ml) for 1 h. Blots were washed five times in Tris-buffered saline/Tween-20 and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit Ig (1:20000). For anti-phosphotyrosine immunoblots, monoclonal antibody 4G10 (1:1000; TCS Biologicals Ltd., Botolph Claydon, Bucks., U.K.) and horseradish peroxidase-conjugated sheep anti-mouse Ig (1:10000) were used. After further washing, blots were developed with the Amersham enhanced chemiluminescence system.

RESULTS

PD 98059 inhibits activation of platelet p42/p44^{mapk}

To determine the inhibiting potential of PD 98059 on p42/p44^{mapk} activation in platelets, we used an in-gel renaturation kinase assay with MBP as substrate. Preincubation of platelets with PD 98059 at 10 μM decreased thrombin-stimulated activation of p42/p44^{mapk} by more than 80 %, and completely blocked the response downstream of collagen or PDBu (Figure 1A). The IC_{50} of approx. 5 μM, as determined in thrombin-activated platelets (Figure 1A), is similar to the published values of 2 and 10 μM [20,21]. Using a portion of the MAPK immunoprecipitate, we investigated the effect of PD 98059 on p42/p44^{mapk} tyrosine phosphorylation, a necessary condition for MAPK activation. PD 98059 prevented tyrosine phosphorylation of p42^{mapk} in response to thrombin, PDBu (Figure 1B, upper panels) and collagen (results not shown) at concentrations similar to the results obtained from the kinase assays. Tyrosine phosphorylation of p44^{mapk} was inhibited correspondingly (visible at longer exposures; results not shown). Immunoblotting with an anti-p42/p44^{mapk} antibody demonstrated that similar amounts of p42/p44^{mapk} shown in Figures 1A and 1B were immunoprecipitated under all conditions (Figure 1B, lower panels).

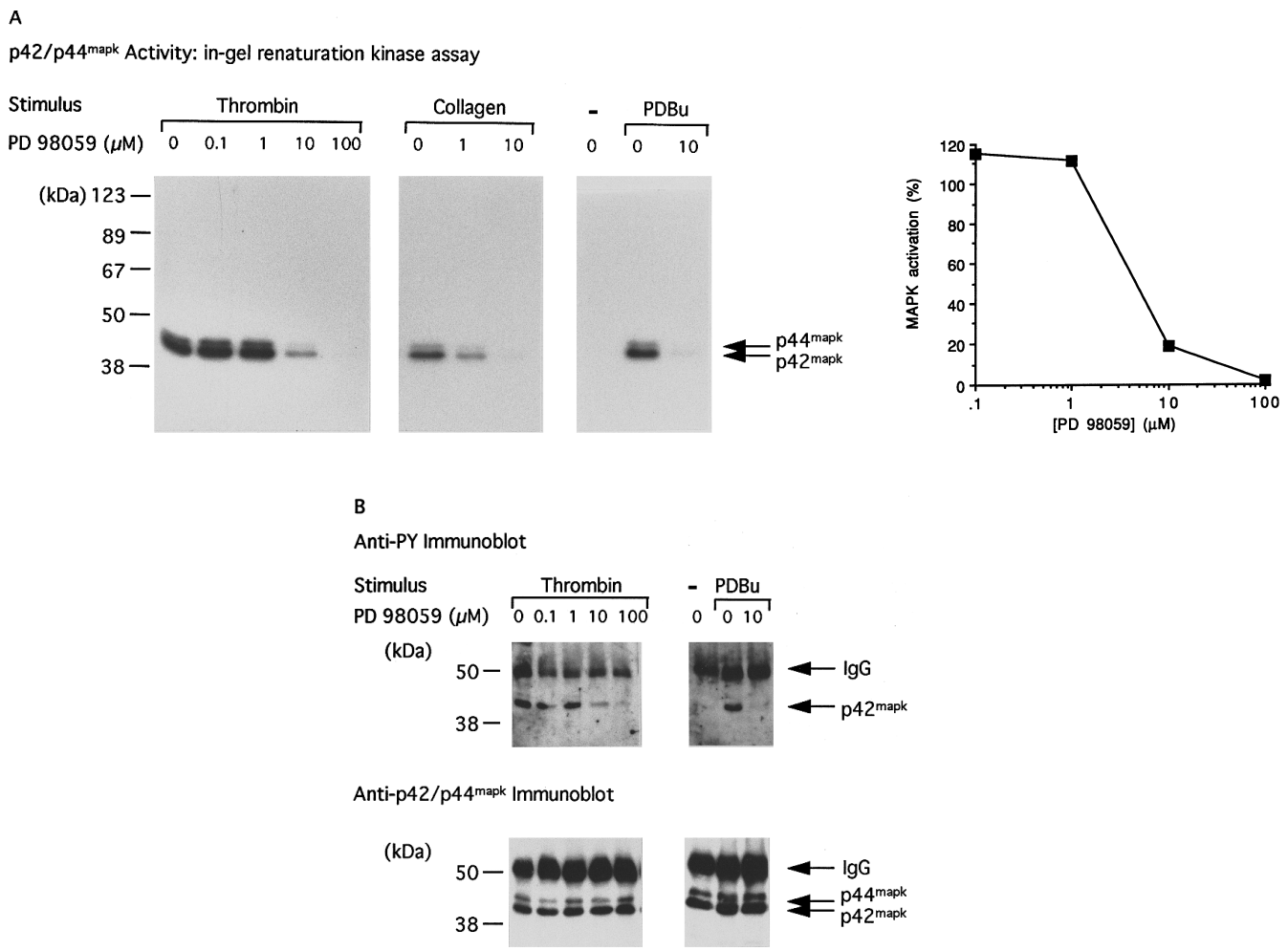

Figure 1 Inhibition of p42/p44^{mapk} activation by PD 98059

Indomethacin-pretreated platelets were incubated with DMSO or PD 98059 for 10 min and then stimulated with 1 unit/ml thrombin (■), 100 μg/ml collagen or 1 μM PDBu for 2 min. (**A**) Immunoprecipitates of p42/p44^{mapk} were resolved on MBP-containing SDS/PAGE (10% gel); gels were renatured and then incubated with 50 μM ATP and 20 μCi/ml of [γ-^{32}P]ATP in kinase buffer for 1 h at 37 °C. After autoradiographs had been taken from dried gels (left three panels), the MAPK bands were excised and scintillation-counted for radioactivity (right panel). Results are expressed as percentages of thrombin activation (100% being 4400 c.p.m.). (**B**) Anti-phosphotyrosine immunoblot of immunoprecipitated p42/p44^{mapk} (upper panels). The same membranes were immunoblotted for p42/p44^{mapk} (lower panel).

PD 98059 inhibits MAPK kinase in platelets

Because PD 98059 interfered with tyrosine phosphorylation of MAPK, and because the in-gel kinase assay measured MAPK activity under conditions where PD 98059 was already washed away, the effect of the compound must occur upstream of MAPK rather than at MAPK itself. We therefore measured total MAPK kinase activity from platelet lysates by using a GST–MAPK fusion protein and a kinase assay *in vitro* with MBP as substrate. Incubation of platelets with thrombin for 2 min stimulated MAPK kinase activity by 8.8 ± 1.9-fold over basal activity (after subtraction of background radioactivity; $n = 6$). Stimulation in the presence of PD 98059 caused inhibition of MAPK kinase in a concentration-dependent manner (Figure 2), similar to the effect on MAPK. When PD 98059 was included during incubation of lysates from thrombin-activated platelets with GST–MAPK, it did not reduce MAPK kinase activity (results not shown). These results are in agreement with former reports [20,21,23] and show that PD 98059 inhibits activation of MAPK kinase rather than MAPK itself.

PD 98059 does not affect platelet functional responses

We were interested to see whether the inhibition of MEK/MAPK would interfere with primary platelet stimulation. Aggregation and shape change (the initial increase in optical density after stimulation) induced by thrombin (0.05–1 unit/ml) and PDBu-stimulated aggregation were not changed in the presence of PD 98059 (0.1–100 μM; example traces are shown in Figure 3A). Similarly, PD 98059 did not alter collagen-induced increase in light transmission, which is believed to result from a combination of adhesion and aggregation events (Figure 3A) and Ca^{2+}-ionophore-induced shape change/aggregation (results not shown). Release of [^{3}H]5-HT or [^{3}H]arachidonic acid stimulated by thrombin or collagen for up to 2 min was not affected significantly in the presence of the MEK inhibitor ($P > 0.05$; Figures 3B and 3C). There was a small but significant decrease in [^{3}H]arachidonic acid release in platelets activated by thrombin for 5 min ($P < 0.05$). PD 98059 did not alter [^{3}H]5-HT or [^{3}H]arachidonic acid release induced by sub-maximal thrombin concentrations (results not shown). Consistent with these results,

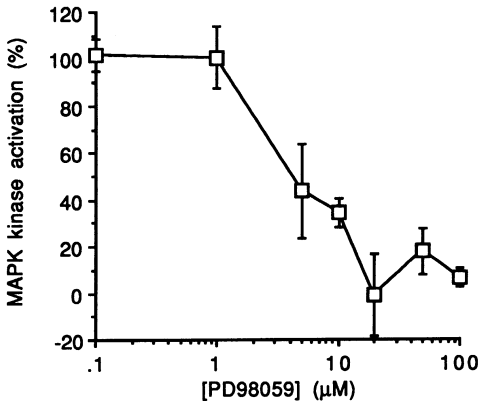

Figure 2 Inhibition of thrombin-induced MAPK kinase activation by PD 98059

Indomethacin-treated platelets were incubated with DMSO or PD 98059 for 10 min and then with 1 unit/ml thrombin for 2 min. Aliquots of platelet lysates were incubated with 100 μg/ml GST–MAPK. Activation of GST–MAPK was determined in a kinase reaction as described in the Experimental section. The radioactivity of controls where the platelet lysate was replaced by H_2O was subtracted. Typical values for basal (unstimulated platelets, set as 0%) and thrombin-stimulated platelets (set as 100%) were 500 and 2500 c.p.m. respectively. Results are given as means $\pm$ S.E.M. ($n = 3$–6).

PD 98059 did not inhibit the increase in intracellular Ca^{2+} concentration stimulated by thrombin or collagen, as measured in fura 2-labelled platelets (results not shown).

Because PD 98059 is a kinase inhibitor, we checked whether it would inhibit protein kinase C, an important kinase mediating platelet responses [10,24]. The compound had no significant effect on thrombin- or collagen-stimulated ^{32}P phosphorylation of pleckstrin, the protein kinase C substrate [25]. Some kinase inhibitors can, paradoxically, induce activation of kinases or protein phosphorylation through inhibition of negative regulatory mechanisms [26]. The presence of PD 98059 did not change the pattern or intensities of total tissue ^{32}P phosphorylation or of tyrosine phosphorylation, which was determined by immunoblots using the monoclonal anti-phosphotyrosine antibody 4G10 (results not shown). This indicates that the compound does not unspecifically affect kinase activity, which is in agreement with the lack of inhibition of PD 98059 on protein serine/threonine kinase activities *in vitro* [23].

Effect of PD 98059 on $cPLA_2$ phosphorylation

In transfected Chinese hamster ovary cells, MAPK phosphorylates, and thereby regulates, $cPLA_2$ [5,6]. Autoradiographs of immunoprecipitated $cPLA_2$ from [^{32}P]P_i-labelled platelets show that thrombin- and collagen-induced phosphorylation was not altered significantly in the presence of PD 98059 (Figure 4). To investigate the time course of $cPLA_2$ phosphorylation more closely, we performed electrophoretic mobility shift assays [27] whereby the phosphorylated form of $cPLA_2$, which moves more slowly on SDS/PAGE, is detected as a second band in immunoblots. Thrombin-induced $cPLA_2$ phosphorylation was detectable after 2 min of stimulation (approximately half of the protein converted to the phosphorylated form) and $cPLA_2$ was completely transformed to the more slowly migrating band after 10 min of stimulation; PD 98059 did not change this pattern (results not shown). Similar

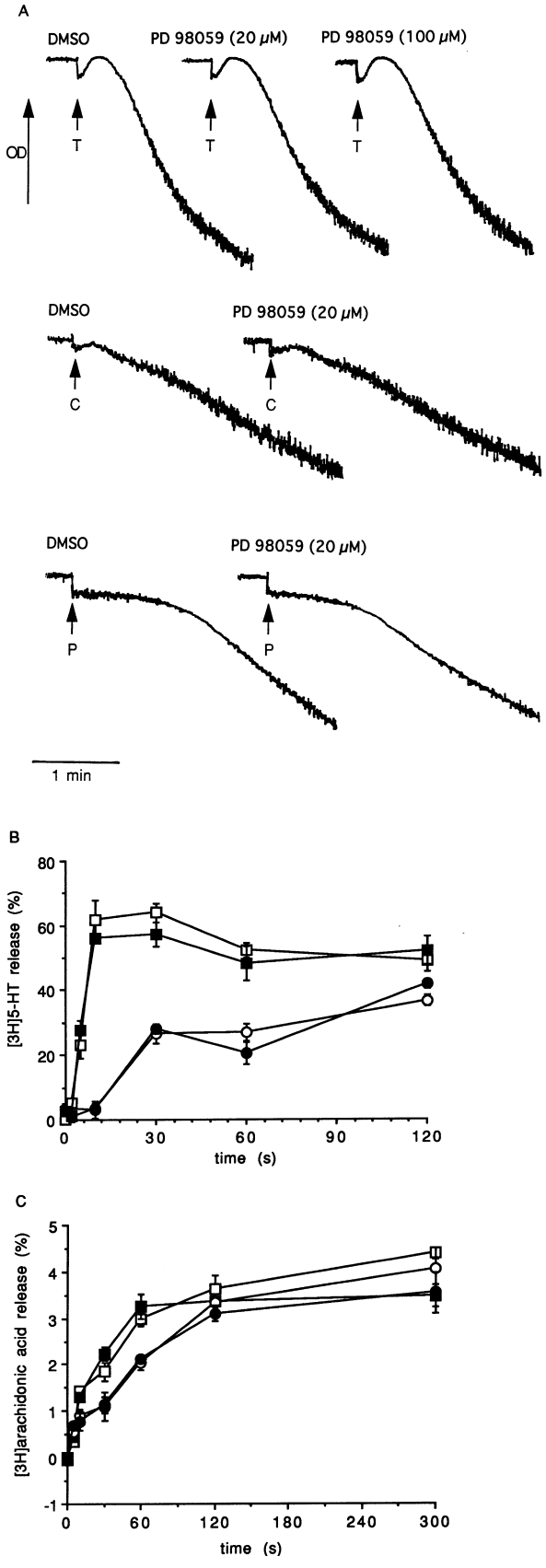

Figure 3 Effect of PD 98059 on platelet responses

Platelets were incubated with 10 μM indomethacin. (**A**) Aggregation was measured in a Born lumi-aggregometer as decrease in attenuance (OD) under stirred conditions. Platelets were

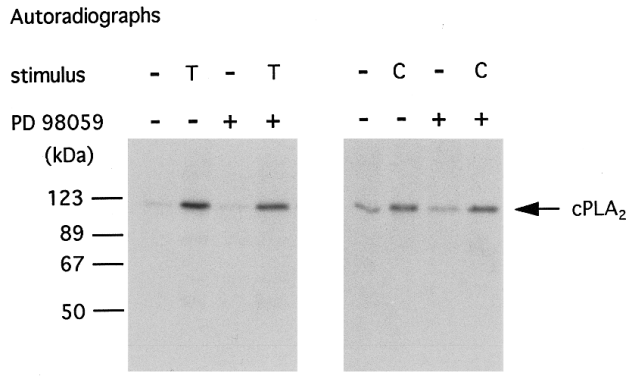

Figure 4 Phosphorylation of cPLA$_2$

Indomethacin-treated [^{32}P]P_i-labelled platelets were incubated with 1% DMSO or 20 μM PD 98059 for 10 min and then stimulated with 1 unit/ml thrombin (T) for 2 min or with 100 μg/ml collagen (C) for 5 min. cPLA$_2$ was immunoprecipitated, resolved on SDS/PAGE (10% gel) and autoradiographed. Similar amounts of cPLA$_2$ were immunoprecipitated in all samples (results not shown).

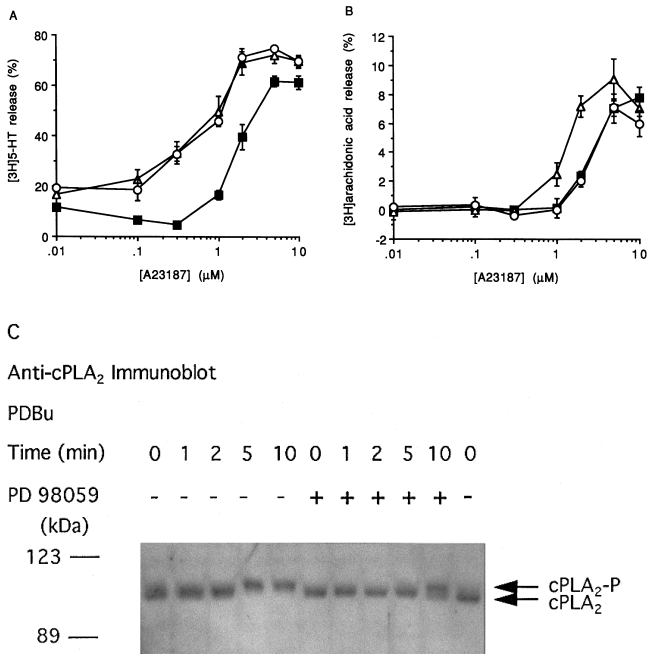

Figure 5 Stimulation of platelets with the combination A23187 and PDBu

(**A**) Platelets were labelled with [^{3}H]5-HT, treated with indomethacin and incubated with 1% DMSO (■, △) or 20 μM PD 98059 (○) for 10 min. Buffer (■) or 1 μM PDBu (△, ○) was added, and the samples were stimulated with A23187 for 2 min. (**B**) Platelets were labelled with [^{3}H]arachidonic acid, treated with indomethacin/BW4AC and stimulated as described in **A**. In **A** and **B**, radioactivity released into the buffer was measured by scintillation counting. Results are presented as percentages of total ^{3}H radioactivity incorporated (see Figure 3). Results are shown as means ± S.E.M. from quadruplicate determinations and are representative of at least two other similar experiments. **C**, Platelets were incubated with 1% DMSO or 20 μM PD 98059 and stimulated with 1 μM PDBu. The shift in electrophoretic mobility of cPLA$_2$ was monitored from whole-cell lysates.

results were obtained from collagen-treated platelets where the phosphorylated form of cPLA$_2$ can be detected at 5 and 10 min (results not shown). cPLA$_2$ phosphorylation was therefore not altered significantly under conditions when p42/p44^{mapk} activation was inhibited.

Stimulation of platelets by Ca^{2+} ionophore and phorbol ester

Phorbol ester-induced activation of protein kinase C has a synergistic effect on Ca^{2+} ionophore-activated release of 5-HT and arachidonic acid [24,28]. We were interested to test the role of p42/p44^{mapk} in these responses. Preincubation of platelets with PD 98059 did not interfere with the synergistic release of [^{3}H]5-HT stimulated by A23187 in the presence of PDBu (Figure 5A) but blocked the effect of PDBu on A23187-induced [^{3}H]arachidonic acid release (Figure 5B; stimulation time 2 min). PDBu-induced phosphorylation of cPLA$_2$ can be detected after 5 min of stimulation (Figure 5C). In agreement with the inhibition of [^{3}H]arachidonic acid release by PD 98059, PDBu-induced cPLA$_2$ phosphorylation was fully blocked in the presence of the compound after 5 min of stimulation (Figure 5C). However, partial phosphorylation of cPLA$_2$ was detected after 10 min of stimulation in the presence of PD 98059, which might be due to activation of a kinase that is not blocked by PD 98059.

preincubated with DMSO or PD 98059 for 10 min and stimulated with 0.1 unit/ml thrombin (T), 100 μg/ml collagen (C), or 1 μM PDBu (P). The aggregation traces are representative of three independent experiments. The presence of DMSO did not effect the responses (results not shown). (**B**) [^{3}H]5-HT-labelled platelets and (**C**) [^{3}H]arachidonic acid-labelled platelets (treated with BW4AC) were incubated with DMSO (1%; □, ○) or PD 98059 (■, 20 μM; ●, 10 μM) for 10 min, and stimulated with 1 unit/ml thrombin (□, ■) or 100 μg/ml collagen (○, ●). Reactions were stopped with 6% (v/v) glutaraldehyde solution, and release of radioactivity into the buffer was measured by scintillation counting. The results are presented as the percentage of the total ^{3}H radioactivity incorporated into the platelet after subtraction of the radioactivity released from unstimulated platelets (basal). Typical values for basal and stimulated release of ^{3}H radioactivity were 2500 d.p.m. (basal) and 8500 d.p.m. (thrombin, 2 min) for [^{3}H]5-HT release, and 2000 d.p.m. (basal) and 6000 d.p.m. (thrombin, 2 min) for [^{3}H]arachidonic acid release. Results shown are means ± S.E.M. from quadruplicate determinations; they are representative of at least two other similar experiments.

DISCUSSION

The MEK inhibitor PD 98059 has recently been used to confirm the role of p42/p44^{mapk} in cell differentiation and growth [20,21] and in insulin and insulin-like growth factor signalling [29,30]. Alessi et al. [23] propose in a detailed study on the effects of PD 98059 *in vitro* and *in vivo* that PD 98059 acts by binding to the inactivated form of MAPK kinase and thereby prevents its phosphorylation through the activity of c-Raf. Thus the degree of inhibition of MAPK and downstream kinases by PD 98059 *in vivo* depends on the strength of Raf stimulation brought about by the agonist used. The increased potency of PD 98059 on MAPK inhibition in platelets compared with Swiss 3T3 cells [23] might be due to a smaller activation of the MAPK pathway in indomethacin-treated platelets by the stimuli employed.

The subtypes of MAPK kinases that are present in platelets are not known. PD 98059 seems to be more selective in blocking activation of the MAPK kinase 1 isoform (IC_{50} 2–7 μM) than MAPK kinase 2 (IC_{50} 50 μM) [23], and because activation of GST–MAPK by MAPK kinase in whole-cell lysates was completely inhibited in the presence of 20 μM PD 98059, MAPK kinase 1 might be the predominant isoform present in platelets. By immunoblotting with a polyclonal antibody (Santa Cruz), we detected p74$^{Raf\text{-}1}$ in platelet lysates (results not shown), which might act as an MAPK kinase kinase [3]. Whether Raf is activated in human platelets has still to be determined.

In the present study we verified the inhibitory action of PD 98059 towards MAPK kinase, towards p42/p44^{mapk} tyrosine phosphorylation and activation, and its specificity. Platelet responses, such as increase in Ca^{2+} concentration, phosphorylation of pleckstrin, and release of 5-HT and arachidonic acid, were not altered significantly by the inhibitor. The results obtained with PD 98059 on $cPLA_2$ phosphorylation are in agreement with our recent report [9], where we show that inhibition of the p42/p44^{mapk} cascade does not interfere with thrombin- and collagen-stimulated $cPLA_2$ activation or phosphorylation. Interestingly, PDBu-induced potentiation of A23187-stimulated arachidonic acid release and PDBu-induced $cPLA_2$ phosphorylation were blocked by PD 98059. This suggests that p42/p44^{mapk} are able to phosphorylate $cPLA_2$ in platelets and thereby enhance phospholipase activity, but only after activation by phorbol esters. Thus the physiological activation of phospholipase C, which leads to an increase in intracellular Ca^{2+} concentration and activation of protein kinase C, stimulates a different pathway mediating $cPLA_2$ phosphorylation compared with combination of Ca^{2+} ionophore and phorbol ester. One possible explanation of this paradoxical observation lies in the complexity of signalling networks. Stimulation at the receptor level is expected not only to have effects on activatory pathways, but also to start inhibitory mechanisms, for example an increase in phosphatase activity, that are responsible for the termination of stimulation and the return of cell activity to basal. It might be that stimulation at the level of protein kinase C by PDBu fails to activate such negative regulatory mechanisms, or that an unphysiologically robust activation of protein kinase C gives rise to effects that are not observed otherwise. Alternatively, PDBu might activate protein kinase C subtypes in different time courses or compartments of the cell, or with different intensities compared with thrombin and collagen. Additionally, the use of PD 98059 has demonstrated that potentiation of 5-HT release by PDBu in Ca^{2+} ionophore-treated platelets is not related to activation of p42/p44^{mapk}.

Previous studies in platelets [8,9] and the present report suggest that a proline-directed kinase other than p42/p44^{mapk} phosphorylates $cPLA_2$. Candidate kinases would be members of the stress-activated protein kinase family [31,32], which are not inhibited by PD 98059 [23,30]. Recently Kramer et al. [33] measured the activity of a proline-directed kinase in fractions obtained from platelet lysates by partial purification, which contained the p38 isoform of stress-activated protein kinase. To investigate further the regulation of $cPLA_2$ in platelets it will be interesting to see whether immunoprecipitates of this stress-activated protein kinase are stimulated in platelets by thrombin and collagen, and whether specific inhibition of this kinase would affect $cPLA_2$ phosphorylation and activation.

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