p38 Mitogen-activated Protein Kinase Phosphorylates Cytosolic Phospholipase A₂ (cPLA₂) in Thrombin-stimulated Platelets

EVIDENCE THAT PROLINE-DIRECTED PHOSPHORYLATION IS NOT REQUIRED FOR MOBILIZATION OF ARACHIDONIC ACID BY cPLA₂

(Received for publication, June 20, 1996, and in revised form, August 13, 1996)

Ruth M. Kramer‡, Edda F. Roberts, Suzane L. Um, Angelika G. Börsch-Haubold§, Steve P. Watson§, Matthew J. Fisher, and Joseph A. Jakubowski

From Lilly Research Laboratories, Indianapolis, Indiana 46285 and §University of Oxford, Oxford OX1 3QT, United Kingdom

The Ca²⁺-sensitive 85-kDa cytosolic phospholipase A₂ (cPLA₂) is responsible for thrombin-stimulated mobilization of arachidonic acid for the synthesis of thromboxane A₂ in human platelets. We have previously shown that thrombin activates p38 kinase, a recently discovered new member of the mitogen-activated protein kinase family (Kramer, R. M., Roberts, E. F., Striffer, B. A., and Johnstone, E. M. (1995) J. Biol. Chem. 270, 27395−27398) and also induces phosphorylation of cPLA₂, thereby increasing its intrinsic catalytic activity. In the present study we have examined the role of p38 kinase in the phosphorylation and activation of cPLA₂ in stimulated platelets. We have observed that activation of p38 kinase accompanies receptor-mediated events in platelets and coincides with cPLA₂ phosphorylation. Furthermore, in the presence of inhibitors of p38 kinase, the proline-directed phosphorylation of cPLA₂ was completely blocked in platelets stimulated with the thrombin receptor agonist peptide SFLLRN and was suppressed during the early (up to 2 min) phase of platelet stimulation caused by thrombin. Unexpectedly, we found that prevention of proline-directed phosphorylation of cPLA₂ in stimulated platelets did not attenuate its ability to release arachidonic acid from platelet phospholipids. We conclude that: 1) cPLA₂ is a physiological target of p38 kinase; 2) p38 kinase is involved in the early phosphorylation of cPLA₂ in stimulated platelets; and 3) proline-directed phosphorylation of cPLA₂ is not required for its receptor-mediated activation.

On activation of platelets with physiological agonists such as thrombin, significant amounts of arachidonic acid are rapidly liberated for transformation to thromboxane A₂ via the cyclooxygenase-thromboxane synthase pathway. There is substantial evidence to indicate that this efficient receptor-mediated mobilization of arachidonic acid is mediated by a phospholipase A₂ pathway (1, 2) and that the involved phospholipase A₂ is the Ca²⁺-sensitive cytosolic phospholipase A₂ (cPLA₂)³ (3, 4).

Many studies with different cellular systems, including platelets, have documented that phosphorylation of cPLA₂ by receptor-mediated events accompanies the stimulated release of arachidonic acid from cellular phospholipids (5). Lin et al. (6) established that this phosphorylation is “activating” (i.e. it increases the catalytic activity of cPLA₂ severalfold) and occurs at Ser⁵⁰⁵ residing within a MAP kinase consensus sequence (Pro-Leu-Ser⁵⁰⁵-Pro). In fact, cPLA₂ was phosphorylated and activated by the MAP kinase ERK2 in vitro and in vivo (i.e. in cultured cells overexpressing cPLA₂ and ERK2; Ref. 6), and accordingly, the ERKs were taken to be responsible for the proline-directed phosphorylation of cPLA₂ observed in various cellular systems. Surprisingly, we have noted that phosphorylation of cPLA₂ occurred in the absence of ERK activation in human platelets stimulated with the thrombin receptor agonist peptide SFLLRN (7). Furthermore, under conditions in which ERK activation was completely suppressed by protein kinase C inhibitors, cPLA₂ phosphorylation induced by thrombin or collagen was unaffected (8). Last, PD 098059, a specific inhibitor of the activation of ERKs, did not block thrombin-induced cPLA₂ phosphorylation (9). Taken together, these findings suggested that kinases other than the ERKs may be involved in receptor-mediated phosphorylation of cPLA₂.

We have recently shown that in addition to the ERKs, platelets contain p38 kinase (10), a recently discovered MAP kinase typically activated by inflammatory cytokines and environmental stress. On the other hand, we were unable to detect kinases belonging to the Jun nuclear kinase subfamily of the stress-activated MAP kinases (11). Thrombin induces a rapid and robust activation of p38, suggesting that this kinase may play a role in platelet function (10). It was recently reported that p38 kinase provides a signal crucial for platelet aggregation at low agonist concentrations (12). The present study was undertaken to further elucidate the functional role of p38 in cPLA₂ activation. Using inhibitors of p38 kinase we found that cPLA₂ is a physiological target of p38 kinase in platelets stimulated via the thrombin receptor. Our results further indicate that prevention of cPLA₂ phosphorylation by such p38 kinase inhibitors does not attenuate the receptor-mediated liberation of arachidonic acid, suggesting that proline-directed phosphorylation of cPLA₂ is not a prerequisite for its activation in stimulated platelets.

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† To whom correspondence should be addressed: Lilly Research Laboratories, Cardiovascular Research, Indianapolis, IN 46285-0444. Tel.: 317-276-1264; Fax: 317-277-2934; E-mail: KRAMER_RUTH_M@Lilly.Com.

‡ To Lilly Research Laboratories, Cardiovascular Research, Indianapolis, IN 46285-0444. Tel.: 317-276-1264; Fax: 317-277-2934; E-mail: KRAMER_RUTH_M@Lilly.Com.

§ The abbreviation used is: cPLA₂, cytosolic phospholipase A₂; MAP, mitogen-activated protein ERK, extracellular signal-regulated kinase; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; MAPF, methylarachidonyl fluorophosphonate; PAGE, polyacrylamide gel electrophoresis; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SB 202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5(4-pyridyl)-1H-imidazole; U46619, 911-dideoxy-11a, 9a-epoxymethano-prostaglandin F₂α.
**cPLA₂ Phosphorylation by p38 Kinase in Activated Platelets**

**EXPERIMENTAL PROCEDURES**

**Materials**—Thrombin and collagen were obtained from Enzyme Research Laboratories and Hormon-Chemie (Munich, Germany), respectively. Calcium ionophore A23187, phorbol 12,13-dibutyrate, progaglandin E₁ (PGF₂α), and U46619 were from Sigma, 4-(4-fluorophenyl)-2-(4-methylthiophenyl-1)-H-imidazole (SB 203580) and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5(4-pyridyl)-1-H-imidazole (SB 202190) were synthesized at Lilly Research Laboratories following the procedure described by Adams et al. (13). Specifically, SB 203580 was prepared by condensing 4-(methylthio)-benzaldehyde with 4-fluoro-2-hydroxyimino-2-(4-pyridyl)acetophenone, then the presence of unusual acetate, which provided 4-(4-fluorophenyl)-N-1-hydroxy-2-(4-methyl-thiophenyl)-5(4-pyridyl)imidazole. Reduction of this intermediate with triethylphosphite followed by oxidation of the sulfur with K₂S₂O₈ provided SB 203580, which was purified by column chromatography and recrystallization. SB 202190 was prepared in a like manner starting from 4-fluoro-2-hydroxyimino-2-(4-pyridyl)acetophenone and 4-hydroxybenzaldehyde. Methylarachidonoyl fluorophosphonate (MAFP) was from Cayman, and 3-(3-acetamido-1-benzyl-2-ethylindolyl-5-oxyl)-propane phosphonic acid (LY311727) was kindly provided by Sue Dihlau (Lilly Research Laboratories). Platelet Isolation and Incubation—Blood from healthy volunteers that had taken regular aspirin (325 mg) the day before and on the morning of the phlebotomy was drawn into 0.16 volume acid-citrate-dextrose (55 mM triosodium citrate, 64 mM citric acid, 111 mM glucose, and 3 µM prostaglandin E₁). Platelets were isolated as described previously (14) and suspended in 140 mM NaCl, 27 mM KCl, 1 mM MgCl₂, 5.5 mM glucose, 0.2 mM EGTA, and 10 mM Hepes, pH 7.4. After addition of CaCl₂ and the GPIIb-IIIa antagonist cycloS,S-Mpr(Har)GDWPFPen-NH₂ (kindly provided by Dr. Robert Scarborough, COR Therapeutics) to final concentrations of 1 mM, and 50 µM, respectively, platelet suspensions (final concentration, 1 × 10⁹/ml or as indicated) were preincubated with inhibitors or vehicle (≤1% Me₂SO final concentration) at 37 °C as indicated. Platelets were then incubated at 37 °C with α-thrombin (3500 NIH units/ml, SFL-LRN (C-terminal amide) synthesized as detailed before (15), or other agonists as indicated. The reaction was stopped by adding (final concentrations) 1% Triton X-100, 5 mM EGTA, 1 mM dithiothreitol, 0.2 mM Na₂VO₃, 0.1 µM microcystin, 5 mM sodium pyrophosphate, 25 mM sodium fluoride, 100 µM leupeptin, 0.2 mg/ml aprotinin, 1 mM Pefabloc (Centerchem), and 50 mM β-glycerophosphate, pH 7.5, followed by SDS-PAGE sample buffer (7). For cPLA₂ assays in platelet lysates, incubations were terminated, and platelet sonicates were prepared as detailed previously (14).

**Labeling of Platelets with [³²P]Orthophosphoric Acid and Immuno-precipitation of [³²P]-labeled cPLA₂—To measure thrombin-induced incorporation of phosphate into cPLA₂, washed platelets were prelabeled with 0.5 mM [³²P]orthophosphate for 2 h and stimulated with thrombin, and cPLA₂ was immunoprecipitated with anti-cPLA₂ antisera as detailed previously (8). Immunoprecipitates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes, and cPLA₂ was visualized by autoradiography.

**Labeling of Platelet Phospholipids with [³H]Arachidonic Acid and Agonist-induced Release of [³H]Arachidonic Acid—Platelet-rich plasma (40 ml) was prepared as above and incubated for 1 h at 37 °C with 20 µCi of [5,6,8,9,11,12,14,15-³H]arachidonic acid (–60 Ci/mmol; DuPont NEN) added in 2 ml of 5 mM Tris/HCl, pH 8, bound to fatty acid-free bovine serum albumin (100 µg/ml). Free [³H]arachidonic acid was removed by washing platelets as detailed above. The incorporation of [³H]arachidonic acid into platelet phospholipids was ~35%, and the content of esterified [³H]arachidonic acid was approximately 2 × 10⁶ dpm/10⁹ platelets. [³H]Arachidonic acid-labeled platelets (0.5 × 10⁹/ml) were preincubated with inhibitors and subsequently stimulated with thrombin or SFL-LRN as described above. Reactions were stopped by adding 2 ml of Dole’s reagent (2-propanol/heptane/0.5M sulfuric acid, 40:10:1, containing 10 µg/ml stearic acid), and the released [³H]arachidonic acid and 12-lipoxygenase metabolites were extracted and quantified as described (7).

**Assay for Platelet p38 Kinase—p38 kinase was partially purified from thrombin-stimulated platelets by MonoQ chromatography as described previously (7, 10). Kinase assays were performed as described (7) using the peptide substrate (KRELVEPSGPSEAPNQALLR) (Macromolecular Resources, Colorado State University) and the Spinzyme system (Pierce).

**SDS-PAGE and Immunoblotting—Solubilized samples were subjected to electrophoresis according to Laemmli (16) using the NOVEX system with 10% Tris/glycine gels at 35 mA for 3.5 h (for detection of cPLA₂ gel shift) or at 30 mA for 2 h (for MAP kinase detection) and

**RESULTS**

Receptor-mediated Activation of p38 MAP Kinase Correlates with the Phosphorylation of cPLA₂—We previously determined the kinase activity of the different platelet MAP kinases resolved by MonoQ chromatography and observed that thrombin-induced stimulation of p38 kinase is more rapid and more potent than that of ERK1 and -2 (10). Due to the small shift in electrophoretic mobility and high reactivity with antiphosphotyrosine antibodies, activated p38 can be readily detected in platelet extracts despite its molecular mass being almost the same as that of ERK2 (41.3 versus 41.4 kDa, respectively). Thus, as shown in Fig. 1, p38 kinase activated by thrombin displays only a small decrease in electrophoretic mobility compared with activated ERK2 (lanes 4–6 versus lanes 10–12). However, activated p38 can be readily visualized by antiphosphotyrosine antibodies (lanes 2 and 3), whereas activated ERKs due to comigration with the very abundant actin at 45 kDa react only poorly (lane 3 versus lane 12).

To probe the role of p38 kinase in cPLA₂ activation, we first examined the stimulation of p38 kinase by platelet receptor agonists and reagents that are known to induce cPLA₂ phosphorylation. For comparison, we also monitored activation of the ERK kinases. Collagen-induced platelet activation is dependent on the integrin α₂β₁ complex (19), and we determined its effect on activation of p38 in aspirinized platelets using a concentration of collagen that caused phosphorylation of cPLA₂ and promoted significant mobilization of arachidonic acid (results not shown). As shown in Fig. 2, collagen induced a robust activation of p38 without stimulating the ERKs. In contrast,
The thromboxane A2-mimetic U46619 activating the platelet thromboxane A2 receptor (20) caused a weak activation of both p38 kinase and the ERKs. As further demonstrated in Fig. 2, the Ca\(^{2+}\) ionophore A23187 solely stimulated p38 kinase, and conversely, the protein kinase C activator phorbol-dibutyrate activated only the ERK kinases. Collectively, these results show that p38 kinase is activated by all receptor agonists tested and some, but not all, agents that selectively target intracellular signaling events (i.e. cytosolic free [Ca\(^{2+}\)] or protein kinase C). To examine the temporal relationship between receptor-mediated activation of p38 kinase and cPLA2 phosphorylation, we stimulated platelets with thrombin and the thrombin receptor agonist peptide SFLLRN for 0–5 min. As shown in Fig. 3, in platelets exposed to thrombin, activation of p38 MAP kinase preceded the time-dependent decrease in the electrophoretic mobility of cPLA2 characteristic of its phospho-p38 MAP kinase preceded the time-dependent decrease in the thrombin-induced shift in electrophoretic mobility of cPLA2. As shown in Fig. 4, both SB 202190 and SB 203580 suppressed the thrombin-induced shift in electrophoretic mobility of cPLA2, indicative of the proline-directed phosphorylation, in a dose-dependent fashion. We then studied the time dependence of the inhibition of cPLA2 phosphorylation by 10 \(\mu\)M SB 202190 in platelets that were stimulated for 0–5 min with a high dose of either thrombin or SFLLRN. As demonstrated in Fig. 5, in SFLLRN-stimulated platelets, cPLA2 phosphorylation was completely suppressed in the presence of the p38 inhibitor. Likewise, thrombin-mediated phosphorylation of cPLA2 was inhibited after 1 min of stimulation and attenuated after 2 min compared with control incubations with vehicle. After 5 min of stimulation with thrombin, however, cPLA2 became phosphorylated in platelets despite the presence of the p38 inhibitor. These results provide the first direct evidence that p38 kinase is responsible for cPLA2 phosphorylation in SFLLRN-stimulated platelets and is involved in the early phosphorylation of cPLA2 in thrombin-stimulated platelets. To further analyze the two temporal phases of cPLA2 phosphorylation, we examined the effect of p38 inhibition on thrombin-
Thus, as demonstrated in Fig. 7, after preincubation of $^{32}$P-labeled platelets at 1 x $10^8$/ml were preincubated for 5 min in the presence of 10 $\mu$M SB 202190 or vehicle (1% Me$_2$SO) and then stimulated with thrombin (10 units/ml) or SFLLRN (100 $\mu$M) for 0–5 min. After addition of Triton X-100 stopping mixture and SDS sample buffer, solubilized extracts were subjected to SDS-PAGE and immunoblotting, probing with anti-cPLA$_2$ IgG. The data shown are representative of two independent experiments yielding similar results.

![Diagram](Image)

**Fig. 5.** Time course of inhibition of cPLA$_2$ phosphorylation by p38 inhibitors. Platelets at 1 x $10^8$/ml were preincubated for 5 min in the presence of 10 $\mu$M SB 202190 or vehicle (1% Me$_2$SO) and then stimulated with thrombin (10 units/ml) or SFLLRN (100 $\mu$M) for 0–5 min. After addition of Triton X-100 stopping mixture and SDS sample buffer, solubilized extracts were subjected to SDS-PAGE and immunoblotting, probing with anti-cPLA$_2$ IgG. The data shown are representative of two independent experiments yielding similar results.

mediated cPLA$_2$ phosphorylation after preincubation of platelets with 30 $\mu$M SB 203580 followed by stimulation for 2 or 5 min with increasing concentrations of thrombin. As shown in Fig. 6, after 2 min of thrombin treatment, as expected, the phosphorylation of cPLA$_2$ was completely suppressed in the presence of SB 203580. In contrast, following the 5-min stimulation, cPLA$_2$ phosphorylation was prevented by the p38 inhibitor at low doses of thrombin but only partially suppressed at higher doses. Altogether these results are consistent with the notion that proline-directed phosphorylation of cPLA$_2$ is initially mediated by p38 kinase and may subsequently occur via p38 kinase-independent mechanisms. However, we noted that under conditions in which the gel shift is completely prevented by p38 inhibitors (i.e., stimulation for 2 min), the enhancement of cPLA$_2$ activity in lysates from platelets stimulated with thrombin or SFLLRN is suppressed by only 50% (Table I). Likewise, thrombin-induced phosphorylation of cPLA$_2$ monitored by $^{32}$P incorporation is only partially blocked. Thus, as demonstrated in Fig. 7, after preincubation of $^{32}$P-labeled platelets with SB 203580 and exposure to thrombin for 2 min, the $^{32}$P labeling of cPLA$_2$ was reduced to 50% of the control. Collectively, these results suggest that a kinase that phosphorylates cPLA$_2$ at a site distinct from Ser$^{505}$ and therefore does not induce a gel shift of cPLA$_2$ also participates in the early receptor-mediated phosphorylation and activation of cPLA$_2$.

**Fig. 6.** Effect of p38 inhibition on cPLA$_2$ phosphorylation induced by increasing concentrations of thrombin. Platelets at 1 x $10^8$/ml were preincubated for 5 min in the presence of 30 $\mu$M SB 203580 or vehicle (1% Me$_2$SO) and then stimulated with thrombin (THR, 0–5 units/ml) for 2 or 5 min as detailed under “Experimental Procedures.” After addition of Triton X-100 stopping mixture and SDS sample buffer, solubilized extracts were subjected to SDS-PAGE and immunoblotting, probing with anti-cPLA$_2$ IgG. The data shown are representative of two independent experiments yielding similar results.

Effect of p38 Inhibitor on Arachidonic Acid Mobilization and Thromboxane $A_2$ Generation in Stimulated Platelets—As shown above, preincubation with p38 inhibitors resulted in complete inhibition of proline-directed phosphorylation of cPLA$_2$ in platelets exposed to thrombin up to 2 min or SFLLRN up to 5 min. We therefore questioned whether suppression of this cPLA$_2$ phosphorylation may affect its ability to hydrolyze platelet phospholipids in response to these agonists. Aspirinized platelets were prelabeled with [H]$^3$H]arachidonic acid, incubated with and without p38 inhibitor, and then exposed to either SFLLRN or thrombin. The released arachidonic acid was then measured as described under “Experimental Procedures.” Unexpectedly, as shown with a dose dependence study for thrombin (Fig. 8A) and a time course experiment for thrombin (Fig. 8B) and SFLLRN (Fig. 8C), the stimulus-induced arachidonic acid mobilization was not affected at any dose or time in the presence of p38 inhibitors. Importantly, as depicted in Fig. 9, the thrombin- and SFLLRN-mediated liberation of arachidonic acid was effectively inhibited by MAFP, a recently described irreversible inhibitor of cPLA$_2$ (22), but not LY331727, a potent inhibitor of secretory group II phospholipase A$_2$ (23). Likewise, bromoelolactone, a known inhibitor of Ca$^{2+}$-independent PLA$_2$ activities (24), did not significantly affect stimulus-induced mobilization of arachidonic acid (data not shown). Taken together, these data demonstrate that effective inhibition of early agonist-induced, proline-directed phosphorylation of cPLA$_2$ does not attenuate its ability to release arachidonic acid from platelet phospholipids, suggesting that this phosphorylation is not required for activation of cPLA$_2$.

After preincubation for 5 min at 37°C with 30 $\mu$M SB 203580 or vehicle (1% Me$_2$SO), platelets (0.5 x 10$^9$/ml) were incubated for 2 min in the presence of buffer, thrombin (2.5 units/ml), or SFLLRN (25 $\mu$M) and lysed by sonication. Lysates (20 $\mu$g of protein) were assayed for cPLA$_2$ activity as detailed under “Experimental Procedures.” Values in parentheses indicate the fold increase in enzymatic activity of cPLA$_2$ in lysates from stimulated compared with nonstimulated platelets. The gel shift of cPLA$_2$ was completely prevented by SB 203580, as verified by SDS-PAGE and immunoblotting analyses. The data are representative of three different experiments; values shown for cPLA$_2$ activity are means ± SD, assaying three separate platelet incubations in duplicate.

**Table I**

| Treatment | SB 203580 | SB 203580 |
|-----------|-----------|-----------|
| No agonist | 1383 ± 35 | 1368 ± 109 |
| Thrombin   | 2658 ± 164 (1.78) | 1884 ± 179 (1.38) |
| SFLLRN     | 2086 ± 162 (1.51) | 1722 ± 34 (1.26) |

**Fig. 7.** Inhibition of thrombin-induced $^{32}$P labeling of cPLA$_2$ by p38 inhibitors. Platelets metabolically labeled with $^{32}$P[orthophosphoric acid (at 1 x 10$^8$/ml) were preincubated in the presence of 10 $\mu$M SB 203580 or vehicle (1% Me$_2$SO) for 20 min and then stimulated with thrombin (1 unit/ml) for 2 min. The reaction was stopped, and cPLA$_2$ was immunoprecipitated and subjected to SDS-PAGE and autoradiography as described under “Experimental Procedures.” Phosphorylation quantified by densitometric analysis was in the presence of thrombin (+THR; - SB 203580, 100%; + SB 203580, 52%) and in the absence of thrombin (-THR; - SB 203580, 0%; + SB 203580, below basal levels). An equal amount of cPLA$_2$ was immunoprecipitated in each sample, as verified by immunoblotting analysis (not shown).
paired thromboxane A₂ formation. In fact, we observed that nonaspirinized platelets preincubated for 5 min with 10 μM SB 203580 generated significantly less thromboxane A₂ in response to SFLLRN than control platelets (Fig. 10A). Since our studies described above clearly showed that prevention of p38 kinase-mediated cPLA₂ phosphorylation did not affect its ability to release arachidonic acid from platelet phospholipids, we reasoned that SB 203580 could affect the metabolism of newly released arachidonic acid rather than its liberation. To bypass cPLA₂, we therefore used arachidonic acid to stimulate platelets. As depicted in Fig. 10B, on preincubation of platelets with 10 μM SB 203580, the amount of thromboxane A₂ generated in response to arachidonic acid was indeed markedly decreased compared with control platelets. Hence, in SFLLRN-stimulated platelets (Fig. 10A) the p38 inhibitor SB 203580 attenuated thromboxane A₂ formation downstream of cPLA₂ via inhibition of cyclooxygenase (or thromboxane synthase). Our findings thus differ from those of Saklatvala et al. (12), who stated that SB 203580 does not act as a cyclooxygenase inhibitor.

**DISCUSSION**

We believe that p38 kinase is involved in the receptor-mediated phosphorylation of cPLA₂ in stimulated platelets for the following reasons. First, the early (within 2 min) onset of proline-directed cPLA₂ phosphorylation correlates with the rapid activation of p38 kinase by thrombin, consistent with cPLA₂ being a target of p38 kinase. Second, p38 inhibitors effectively suppress the thrombin-mediated phosphorylation of cPLA₂ during this early phase. Third, in SFLLRN-stimulated platelets, in which p38 is the only activated MAP kinase, proline-directed phosphorylation of cPLA₂ is prevented by p38 inhibitors at all times. Last, we have previously demonstrated that cPLA₂, but not the S505A mutant cPLA₂, is a substrate for p38 kinase in vitro, suggesting that p38 mediates the "activating" phosphorylation at Ser^{505} of cPLA₂ (7).

Previous studies have convincingly documented that the majority of the released arachidonic acid in stimulated platelets is provided by the action of a phospholipase A₂ and not via a phospholipase C-diacylglycerol hydrolase pathway (1, 2). Platelets, in addition to cPLA₂, also possess secretory group II phospholipase A₂ that is rapidly secreted on platelet activation (25).
effectively suppress thrombin receptor-mediated liberation of arachidonic acid from platelet phospholipids (3, 4). To promote activation of p38 kinase (10, 12). The functional consequences of p38 activation have not been clear. The p38 inhibitor SB 203580 was used to demonstrate that MAPKAP kinase 2 is a physiological target of p38 kinase in cells exposed to interleukin 1, cellular stresses, and bacterial endotoxin (36). Furthermore, activation of p38 kinase by its activators MKK3 and MKK6 in cultured cells confirmed that the transcription factors ATF2 and Elk-1 are substrates of p38 kinase (37). We observed previously that p38 kinase partially purified from thrombin-stimulated platelets readily phosphorylates cPLA₂ in vitro (7). In this present study we demonstrate that receptor-mediated phosphorylation of cPLA₂ is prevented by inhibitors of p38 kinase and thereby establish that cPLA₂ is also a target for p38 kinase in stimulated platelets. It was previously reported that bacterially expressed p38 kinase does not act on cPLA₂ (35). However, it should be noted that the enzymatic activity of bacterially expressed MAP kinases is orders of magnitude smaller than that of cellular MAP kinases physiologically activated by phosphorylation on both Thr\(^{180}\) and Tyr\(^{182}\) (38). Furthermore, the substrate specificity of recombinant p38 kinase activated due to some autophosphorylation may differ from that of physiologically activated platelet p38 kinase. Lin et al. (6) observed that in cultured cells overexpressing cPLA₂ the stimulus-induced release of arachidonic acid was markedly enhanced compared with control cells. In sharp contrast, when SS05A mutant cPLA₂ was similarly overexpressed, no such increase was observed. This finding suggested that phosphorylation at Ser\(^{505}\) is required for receptor-mediated activation of cPLA₂ to release arachidonic acid from cellular phospholipids. Many subsequent reports have proposed that such phosphorylation of cPLA₂ is a critical step in the sequence of events leading to the mobilization of arachidonic acid in stimulated cells, but direct evidence to support this hypothesis has not been provided (5). Surprisingly, we observed that prevention of the proline-directed phosphorylation of cPLA₂ in stimulated platelets only partially suppressed the enhancement of the enzymatic activity of cPLA₂ and did not affect its ability to rapidly mobilize arachidonic acid in stimulated platelets. Our findings thus indicate that, at least in platelets, proline-directed phosphorylation is not a prerequisite for receptor-mediated activation of cPLA₂.

We conclude that the early thrombin-induced, proline-directed phosphorylation of cPLA₂ that contributes to its increased catalytic activity is mediated by p38 MAP kinase. However, in platelets this phosphorylation of cPLA₂ is not a critical factor for the rapid mobilization of arachidonic acid by cPLA₂, suggesting that other as yet unidentified kinases may be involved. In fact, recently, novel phosphorylation sites of cPLA₂ have been identified (39) that may be targeted by such kinases, and it will be of great interest to study their involvement in the regulation of cPLA₂.

Acknowledgments—We thank John Sharp, Neal Roehm, and Faming Zhang for their support and helpful discussions and Larry Froelich for his contributions to this work.

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FIG. 10. Effect of p38 inhibitor on SFLRN and arachidonic acid induced thromboxane A₂ production in human platelets. Gel-filtered platelets at 0.5–3 × 10⁹/ml were preincubated for 5 min in the presence of 10 μM SB 203580 (p38 inhibitor) or 0.1% Me₂SO (Control) and then stimulated with SFLRN (A) or arachidonic acid (B) for 2 min as indicated. The reaction was stopped by adding indomethacin to a final concentration of 10 μM, platelets were pelleted, and supernatants assayed for thromboxane B₂ (the stable metabolite of thromboxane A₂) as described under “Experimental Procedures.” The data represent four different experiments, and values shown are means ± S.E. (bars).
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