The kinase inhibitors SB 203580 and PD 98059 have been reported to be specific inhibitors of the 38- and 42/44-kDa mitogen-activated protein kinase (MAPK) pathways, respectively. In this study, the two inhibitors were found to decrease platelet aggregation induced by low concentrations of arachidonic acid, suggesting that they also interfere with the metabolism of arachidonic acid to thromboxane A2. In support of this, SB 203580 and PD 98059 inhibited the conversion of exogenous [3H]arachidonic acid to [3H]thromboxane in intact platelets. Measurement of platelet cyclooxygenase-1 activity following immunoprecipitation revealed that SB 203580 and PD 98059 are direct inhibitors of this enzyme. Both compounds were shown to inhibit purified cyclooxygenase-1 and -2 by a reversible mechanism. In addition, SB 203580 (but not PD 98059) inhibited platelet aggregation induced by prostaglandin H2 and the conversion of prostaglandin H2 to thromboxane A2 in intact platelets. SB 203580 also inhibited this pathway in platelet microsome preparations, suggesting a direct inhibitory effect on thromboxane synthase. These results demonstrate that direct effects of the two kinase inhibitors on active arachidonic acid metabolites have to be excluded before using these compounds for the investigation of MAPKs in signal transduction pathways. This is of particular relevance to studies on the regulation of cytosolic phospholipase A2 as these two MAPKs are capable of phosphorylating cytosolic phospholipase A2, thereby increasing its intrinsic activity.

Since specific inhibitors of the mitogen-activated protein kinase (MAPK) and stress-activated protein kinase (SAPK) cascades were first described, they have been widely exploited to inhibit the MAPK pathway. However, we have obtained evidence that SB 203580 inhibits thromboxane synthase. These compounds were shown to have inhibitory effects on thromboxane synthase. These results demonstrate that direct effects of the two kinase inhibitors on active arachidonic acid metabolites have to be excluded before using these compounds for the investigation of MAPKs in signal transduction pathways. This is of particular relevance to studies on the regulation of cytosolic phospholipase A2 as these two MAPKs are capable of phosphorylating cytosolic phospholipase A2, thereby increasing its intrinsic activity.
were supplied by Amersham Pharmacia Biotech. γ-[32P]ATP (specific activity of 3000 Ci/mmol) was obtained from NEN Life Science Products. Arachidonic acid (Sigma) was dissolved in ethanol and stored at −20 °C. PGG2 (Calbiochem), dissolved in hexane/isopropl alcohol (9:1), was stored at −70 °C; before experimentation, the solvent was evaporated under a stream of N2 and PGG2 was dissolved in H2O (1 µg/ml) (14). Furegrelate (Sigma) was dissolved in Tyrode’s buffer. Prostacyclin was kindly donated by Wellcome Laboratories (Beckenham, Kent, UK). Bovine thrombin and protein A-Sepharose CL-4B were obtained from Sigma. Collagen was purchased from Nycomed Arzneimittel (Munich, Germany). All other reagents were of analytical grade. Preparation of Washed Platelets—Platelet-rich plasma (PRP) from whole blood was used for this study. Washed Platelets—Platelets were prepared as described (17). The suspension was sonicated on ice for a short period of time and centrifuged at 1500 x g for 10 min in the presence of 0.1 µg/ml polymyxin. The platelet pellet was resuspended in Tyrode’s buffer. Stimulation of platelets was stopped with an ice-cold 3 M glucose, 5 mM dithiothreitol, and 1 mM benzamidine HCl. Protein concentrations were determined by the Bradford reaction (Bio-Rad) and p38 was measured by the Bradford reaction (Bio-Rad) (19). Proteins were separated on 10% SDS-polyacrylamide gels that contained 0.5 mg/ml myelin basic protein co-polymerized with the acrylamide. Kinases were denatured in 6 M guanidine HCl and renatured for 16 h at 4 °C as described previously (16). Gels were incubated in kinase buffer (50 mM Tris, pH 8.0, 5 mM MgCl2, 1 mM EGTA, 5 mM dithiothreitol, 0.1% bovine serum albumin, and 0.1% Na3VO4). The amount of protein released was determined by radioimmunoassay or EIA as described (15). Activation of p42 and p38 was measured after immunoprecipitation from platelet lysates using 5 µl of p42 polyconal antibody (18) or 5 µl of anti-p38 polyclonal antibody (19). Proteins were separated on 10% SDS-polyacrylamide gels that contained 0.5 mg/ml myelin basic protein co-polymerized with the acrylamide. Kinases were denatured in 6 M guanidine HCl and renatured for 16 h at 4 °C as described previously (16). Gels were incubated in kinase buffer (50 mM Tris, pH 8.0, 5 mM MgCl2, 1 mM EGTA, 5 mM dithiothreitol, 50 µM AT&F, and 10 mM glucose, 5 mM dithiothreitol, 1 mM benzamidine HCl, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM EGTA, and microsomal fractions were prepared as described (17). The suspension was sonicated on ice four times for 5 s and then centrifuged at 15000 x g. This process was repeated on the pellet, and both sonicates were centrifuged at 13000 x g for 30 min at 4 °C to remove insoluble material. The supernatant was centrifuged at 150000 x g for 60 min at 4 °C, and the pellet, representing the microsomal fraction, was suspended in 20 mM Tris, pH 8.0, containing 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM benzamidine HCl. Protein concentrations were determined by the Bradford reaction (Bio-Rad) using bovine serum albumin as a standard. Microsomes were stored at −70 °C. Thromboxane synthase activity was verified by the capacity of the platelet microsomes to convert PGG2 into TXB2. The amount of TXB2 produced by EIA was compared as described (18). In some experiments, microsomes were preincubated for 15 min at 37 °C with MeSO (0.4%), SB 203580 (20 µM), or PD 98059 (20 µM). Cyclooxygenase and Thromboxane Synthase Activities in Intact Platelets—Platelets were preincubated with MeSO (0.4%), SB 203580 (20 µM), or PD 98059 (20 µM) for 10 min and then incubated for 5 min with 1 µM arachidonic acid containing 1 µCi of [3H]arachidonic acid at 37 °C. The reaction was stopped by acidification (addition of 1 N HCl to reach pH 4), and lipids were extracted by incubation overnight at 4 °C with 3 volumes of ethyl acetate. Samples were concentrated by evaporation and applied to silica thin-layer chromatography plates (Merck). Lipids were separated by ascending thin-layer chromatography in ethyl acetate/isooctane/acidic acid (25:70:10, v/v/v) as described (18). 1-cm fractions of the silica plate were scraped off, and the radioactive products were quantitated by liquid scintillation spectrometry in a Beckman scintillation counter. Radioactive lipids were identified compared with the migration of standards. The 12-oxoprostaglandin E2 (12-oxo-PGJ2) product was immunoprecipitated using polyclonal anti-cyclooxygenase-1 antibody and 25 µl of protein A-Sepharose CL-4B slurry overnight at 4 °C. Immunoprecipitates were recovered by microcentrifugation and washed twice in the ice-cold lysis buffer. Immunoprecipitates were resuspended in 20 µl of 50 mM Tris, pH 8, containing 0.1% bovine serum albumin and 1% phenol at 37 °C and were incubated with buffer, MeSO (2%; control), SB 203580 (20 µM), or PD 98059 (20 µM) for 15 min followed by addition of 50 mM arachidonic acid containing 1 µCi of [3H]arachidonic acid at 37 °C. After 10 min, the reaction was terminated by the addition of 3 volumes of ice-cold Tris-buffered saline and centrifugation for 1 min at 5000 x g. Lipids were extracted from the supernatant in 3 volumes of ethyl acetate and separated by thin-layer chromatography as described above. Cyclooxygenase-1 bound to protein A-Sepharose was solubilized in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Cyclooxygenase-1 immunoprecipitation was verified by immunoblotting. For measuring transformation of arachidonic acid using purified cyclooxygenase, cyclooxygenase-1 and -2 were diluted to give PGE2 production within the linear range of the EIA kit. Cyclooxygenase was incubated with buffer, MeSO, SB 203580, PD 98059, or indomethacin for 15 min, and the reaction was initiated by the addition of arachidonic acid (25 µM) as described above. Formation of PGE2 was measured using EIA according to the instructions of the manufacturer. RESULTS Inhibitory Effects of SB 203580 and PD 98059 on Platelet Responses—Platelet aggregation induced by collagen is dependent on the formation of thromboxane A2 from arachidonic acid by the action of cyclooxygenase and thromboxane synthase. We have reported earlier that SB 203580 and PD 98059 inhibit platelet aggregation induced by low concentrations of collagen. PD 98059 is more powerful than SB 203580 in inhibiting this response (11). Its inhibitory action was only overcome by collagen at 20 µg/ml (Fig. 1A), whereas the effect of SB 203580 was overcome at 5 µg/ml collagen (11). In contrast to collagen, aggregation stimulated by thrombin (0.1 unit/ml) was not dependent on the release of TXA2 as demonstrated by preincubation with the cyclooxygenase inhibitor indomethacin (Fig. 1B). The presence of neither PD 98059 (20 µM) nor SB 203580 (20 µM) altered the aggregation trace to thrombin (Fig. 1B). These concentrations of PD 98059 and SB 203580 are sufficient to inhibit thrombin-induced activation of p42 and p38, respectively, (10, 20). The inclusion of MeSO did not have any significant effect on platelet aggregation as tested with thrombin (0.1 units/ml) and collagen (10 µg/ml) (data not shown). SB 203580 and PD 98059 partially inhibited aggregation stimulated by the α2 agonist I-cyanoephore A23187 (2 µM) (data not shown). A platelet stimulus that induced profound liberation of thromboxane acid from phospholipids (21). In contrast, aggregation induced by A23187 in the presence of the cyclooxygenase blocker indomethacin was not altered in the presence of PD 98059 or SB 203580 (data not shown). Thus, SB 203580 and PD 98059 impaired platelet aggregation only under conditions where formation of TXA2 contributed to the response. Thromboxane formation stimulated by collagen (2 and 5
Inhibition of Cyclooxygenase by SB 203580 and PD 98059

**FIG. 1**

A. Collagen
- 21 µg/ml
- 5 µg/ml
- PD 98059
- MeSO

B. Thrombin
- 0.1 unit/ml
- PD 98059
- MeSO
- SB 203580

C. Arachidonic acid
- 0.2 µM
- 0.1 µM
- 0.05 µM
- 0.02 µM
- 0.005 µM
- SB 203580
- 20 µM
- 5 µM
- MeSO

D. 
- PGH₂
- PD 98059
- SB 203580

1 min
and measured the formation of 3H-labeled metabolites. To determine the transformation of arachidonic acid to TxB2 independently of the release of endogenous thromboxane. For this reason, we incubated platelets with [3H]arachidonic acid (1 µM) and measured the formation of 3H-labeled metabolites. 29.0 ±

µg/ml, as measured by EIA, was partially inhibited by SB 203580 and completely blocked by PD 98059 (Fig. 2), which is in agreement with the effects of the compounds on aggregation.

In addition, release of 5-hydroxytryptamine stimulated by collagen in the absence of indomethacin was reduced by both compounds, but could be overcome at higher concentrations of collagen (Fig. 3).

Platelet Activation by Arachidonic Acid—To investigate the effect of the compounds on the metabolism of arachidonic acid, we incubated platelets with low concentrations of arachidonic acid, which induce aggregation as a consequence of conversion to TxAr. SB 203580 and PD 98059 inhibited platelet aggregation induced by 0.2 µM arachidonic acid with approximate IC50 values of 3 and 0.8 µM, respectively (Fig. 1C). Aggregation stimulated by 1 µM arachidonic acid was reduced by 50% in the presence of SB 203580 (20 µM) and was blocked by PD 98059 (20 µM) (Fig. 1C). Moreover, both compounds inhibited formation of TxB2 from arachidonic acid (1 µM) as measured by EIA (Table I). These observations demonstrate that both inhibitors interfere with the metabolism of arachidonic acid to TxA2.

Arachidonic acid (0.2 and 1 µM) did not stimulate platelet p42mapk and caused weak activation of p38mapk relative to activation by thrombin (Fig. 4). However, activation of MAPK-activated protein kinase-2, the in vivo substrate of p38mapk, was not significantly enhanced above basal levels by arachidonic acid (data not shown). The TxA2 receptor agonist U46619 has been reported to cause weak phosphorylation of p42mapk and p38mapk (20). Thus, arachidonic acid and its metabolites seem to be only weak stimulators of MAPKs.

Because the kinase inhibitors could interfere with the release of endogenous arachidonic acid through an effect on the activity of cytosolic phospholipase A2, it was important to determine the transformation of arachidonic acid to TxB2 independent of the release of endogenous thromboxane. For this reason, we incubated platelets with [3H]arachidonic acid (1 µM) and measured the formation of 3H-labeled metabolites. 29.0 ±

4.6% of [3H]arachidonic acid (mean ± S.E., n = 4 (n = number of independent experiments)) added to platelets was metabolized to [3H]TxB2 in 10 min with little formation of the endoperoxide [3H]PGH2 (1.8 ± 0.3%) or its stable product, [3H]PGE2 (2.8 ± 0.7%) (Fig. 5). Untransformed [3H]arachidonic acid and the 12-lipoxygenase product 12-[3H]hydroxyeicosatetraenoic
Acid constituted 42.9 ± 3.7% of total radioactivity. When platelets were incubated in the presence of the cyclooxygenase blocker indomethacin, formation of [3H]TxB2, [3H]PGH2, and [3H]PGE2 was completely inhibited (Fig. 5A). This was in contrast to incubation with the thromboxane synthase inhibitor furegrelate (10 μM) (19, 22), which completely blocked the formation of [3H]TxB2, but increased the formation of its precursor, [3H]PGH2, to 13.5 ± 1.3% (n = 4) and of [3H]PGE2 to 14.4 ± 3.0% (n = 4) (Fig. 5A). In the presence of SB 203580 or SB 203580 plus furegrelate, the metabolism of [3H]arachidonic acid was substantially inhibited with [3H]TxB2, [3H]PGH2, and [3H]PGE2, accounting for 2–5% of total radioactivity (Fig. 5B). Similarly, in the presence of furegrelate, PD 98059 fully inhibited the formation of all three products (data not shown). These results confirm that SB 203580 and PD 98059 inhibit platelet cyclooxygenase.

To test the possibility that both compounds cause direct inhibition of cyclooxygenase, we immunoprecipitated cyclooxygenase-1 from unstimulated platelets and measured its activity in vitro. Under resting conditions, neither p42mapk nor p38mapk is activated (11, 16). Cyclooxygenase-1 converted [3H]arachidonic acid to [3H]PGH2, which was measured as the chemically stable [3H]PGE2 (Fig. 6). There was no significant formation of [3H]PGE2 in the presence of either SB 203580 (20 μM) or PD 98059 (20 μM) (Fig. 6). The presence of Me2SO had no significant effect on enzyme activity (data not shown).

Human platelets contain the constitutively expressed cyclooxygenase-1, but not the inducible cyclooxygenase-2 (23). To further characterize the inhibition of cyclooxygenase by the two kinase inhibitors, we measured the effect of increasing inhibitor concentrations on purified cyclooxygenase-1 and -2. SB 203580 and PD 98059 inhibited cyclooxygenase-1 with approximate IC50 values of 2 and 1 μM, respectively (Fig. 7A). Inhibition was completely reversible for both compounds, as tested by preincubation of cyclooxygenase with 3 μM inhibitor before a 10-fold dilution and incubation with substrate (data not shown). Cyclooxygenase-2 was also inhibited by both compounds in a reversible manner. IC50 values were ~2 and 4 μM for SB 203580 and PD 98059, respectively (Fig. 7B). Inclusion

**Fig. 4. Activation of p42mapk and p38mapk.** Platelets were incubated with buffer (basal), arachidonic acid (aa; 0.2 and 1 μM), or thrombin (1 unit/ml) for 2 min at 37 °C under stirred conditions. p42mapk and p38mapk were immunoprecipitated from platelet lysates and run on SDS-polyacrylamide gels containing myelin basic protein (MBP). Gels were denatured, renatured, and incubated with kinase buffer. After extensive washing, gels were dried. Phosphorylated proteins were visualized by autoradiography, cut from the gels, and Cerenkov-counted for incorporation of 32P into myelin basic protein.

**Fig. 5. Conversion of [3H]arachidonic acid to metabolites.** Washed platelets were incubated with Me2SO (●), furegrelate (10 μM; □), indomethacin (10 μM; ▲), SB 203580 (20 μM; ○) or SB 203580 and furegrelate (▲) for 10 min at 37 °C and stimulated with arachidonic acid (1 μM) containing [3H]arachidonic acid (1 μCi). The reaction was stopped after 10 min by acidification, and lipids were extracted by ethyl acetate and separated on silica thin-layer chromatography plates. Fractions of the silica plate were scraped off, and radioactivity was measured by liquid scintillation spectrometry. The positions of TxB2 and PGH2 were determined in comparison with standards ([3H]TxB2 and unlabeled PGH2, visualized by iodine vapor); PGE2 was identified by its RF value. The background represented 1–2% of total radioactivity. Data shown are the means ± S.D. from triplicate determinations and are representative of four independent experiments. 12-HETE, 12-[3H]hydroxyeicosatetraenoic acid.
of the solvent Me₂SO did not reduce enzyme activity. The degree of inhibition of cyclooxygenase-1 and -2 by SB 203580 and PD 98059 (20 μM) was similar to that by a maximally effective concentration of the cyclooxygenase inhibitor indomethacin (data not shown). Inhibition by SB 203580 could be overcome by increasing arachidonic acid concentrations; in the presence of SB 203580 (3 μM), the \( K_m \) for arachidonic acid was increased without a change in the \( V_{max} \), suggesting a competitive mechanism of inhibition (data not shown). In contrast, PD 98059 (3 μM) decreased the \( V_{max} \) even at the highest concentration of arachidonic acid used (50 μM) (data not shown).

Platelet Activation by PGH₂—To distinguish between the effects of the kinase inhibitors on cyclooxygenase and thromboxane synthase, we stimulated platelets with the cyclooxygenase product PGH₂. SB 203580 inhibited PGH₂-induced platelet aggregation, whereas PD 98059 did not alter the response (Fig. 1D). In addition, the formation of TxB₂ from 1 μg/ml PGH₂ was inhibited by SB 203580, but not by PD 98059, as determined by EIA (Table I).

It was important to determine the activity of thromboxane synthase independent of the stimulation of receptor-activated pathways and of activation of cytosolic phospholipase A₂. We therefore measured the transformation of PGH₂ to TxB₂ on platelet microsomes, a membrane fraction that contains thromboxane synthase (17). Microsomes were obtained from unstimulated platelets, which means that p42/p44 MAPK and p38 MAPK were not activated. PGH₂ added to microsomes was transformed to TxB₂ as determined by radioimmunoassay (Table I). The presence of PD 98059 in this assay did not significantly alter the response, whereas the addition of SB 203580 blocked TxB₂ formation (Table I). These results demonstrate that SB 20350 (but not PD 98059) acts as a direct inhibitor of thromboxane synthase.

DISCUSSION

Several studies have confirmed the selectivity of the kinase inhibitors SB 203580 and PD 98059 against a variety of kinases. SB 203580 was tested against 16 protein kinases and two phosphatases and was found to be selective against p38 and p38β (SAPK2a and SAPK2b), but to have no inhibitory effect on SAPK3, SAPK4, p42/p44 MAPK, c-Jun N-terminal kinase (SAPK1), or any of the upstream kinases (3, 6). Similarly, the selectivity of PD 98059 has been described in several studies (7–9). We (Refs. 11 and 20 and this study) and others (24) have observed that platelet responses dependent on the formation of TxA₂ are inhibited by these compounds. However, since
SB 203580 has been developed from drugs that are inhibitors of cylooxygenase and lipooxygenase, it is uncertain whether the inhibitory actions of SB 203580 on platelet responses can be interpreted as effects of p38mapk or whether they are due to inhibition of arachidonic acid metabolism. Furthermore, we have also observed inhibition of platelet activation by PD 98059 under conditions that have little effect on p42

For these reasons, we set out to investigate the effects of these compounds on the arachidonic acid cascade.

We obtained evidence for direct inhibition of platelet cylooxygenase by SB 203580 from a variety of approaches: first, inhibition of platelet aggregation induced by stimuli that are dependent on TxA2 to elicit aggregation; second, inhibition of arachidonic acid metabolism. Furthermore, we have also observed inhibition of platelet activation by PD 98059 under conditions that have little effect on p42mapk activation. For these reasons, we set out to investigate the effects of these compounds on the arachidonic acid cascade.

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