Biochemical Pharmacology, Vol. 55, pp. 1759–1767, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00632-1

Effects of the Mitogen-Activated Protein (MAP) Kinase Kinase Inhibitor 2-(2′-Amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059) on Human Platelet Activation*

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ABSTRACT. The role of mitogen-activated protein (MAP) kinase cascades in platelet function remains to be determined. Several studies have suggested a role in the activation of phospholipase A_2; however, other functions seem likely. The object of the present study was to determine the role of the MAP kinase cascade in platelet function. An inhibitor of the mitogen-activated protein kinase kinase MEK1, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059), was used, at concentrations consistent with those reported to inhibit MEK1, to examine the role that this enzyme plays in platelet function. PD98059 inhibited aggregation in response to low-dose collagen and arachidonic acid, but not that in response to high-dose collagen, thrombin, thrombin receptor-activating peptide (TRAP), 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), or phorbol ester. Thrombin, thrombin receptor-activating peptide, U46619, collagen, and arachidonic acid each caused the release of [^{3}H]serotonin from dense granules, but only that elicited by low-dose collagen and arachidonic acid was inhibited by PD98059. The release of [^{3}H]arachidonic acid in response to thrombin or collagen was unaffected by PD98059 pretreatment. In contrast, collagen- and arachidonic acid-induced thromboxane formation was inhibited by PD98059. These data suggest that MEK1 is not involved in the platelet response to thrombin or U46619. Furthermore, the inhibitory effects of PD98059 on collagen- and arachidonic acid-induced responses suggest that PD98059 may inhibit the conversion of arachidonic acid to thromboxane, in addition to its reported effects on MEK1. BIOCHEM PHARMACOL **55**;11:1759–1767, 1998.

KEY WORDS. platelets; collagen; arachidonic acid; MAP kinase; cyclooxygenase

Platelets play a central role in the haemostatic process, including adhering at the site of injury, recruiting additional platelets, and subsequently incorporating into a haemostatic plug or aggregate [1–3]. To achieve this, platelets undergo a series of biochemical and physical changes that constitute the platelet response. These responses include a change in platelet shape, pseudopod extension, expression of adhesive receptors on the platelet surface, and an alteration in the cytoskeleton [1–3]. These changes are accompanied and enhanced by the release of important mediators that act in a positive feedback, pro-aggregatory manner; ADP, released from dense granules, is a platelet agonist that recruits additional platelets to the aggregate, and adhesive proteins (notably fibrinogen), released from alpha granules, act to stabilize the forming aggregate. In addition TxA_2[‖] is synthesized in, and released from, platelets, and acts as an important platelet agonist and vasoconstrictor [1–4]. The biochemical mechanisms underlying platelet activation have been the focus of considerable interest. Numerous studies have shown that phospholipase C-mediated inositol phospholipid metabolism is the primary biochemical event underlying platelet activation induced by some (e.g. thrombin, Tx mimetics) but not all (e.g. ADP) agonists [5–8]. The consequences of phospholipase C activity are the production of second messengers, inositol trisphosphate and diacylglycerol, which, in turn, elevate $[Ca^{2+}]_i$ and stimulate protein kinase C, respectively [3–7]. These two well-defined pathways mediate the variety of physiological platelet responses.

* Some of these data were presented at the XVIth Congress on Thrombosis and Haemostasis, Florence, June 1997 (McNicol A, Philpott CL, Shibou TS and Israels SJ, *Thromb Haemost* **77:** SC2357, 1997). Similar observations were presented independently at the same meeting (Barnes MJ, Rawlinson L, Saklatvala J and Farndale RW, *Thromb Haemost* **77:** OC2454, 1997).

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Received 19 June 1997; accepted 7 October 1997.

‖ *Abbreviations:* $[Ca^{2+}]_i$, cytosolic calcium concentration; $cPLA_2$, cytosolic phospholipase A_2; MAP, mitogen-activated protein; PMA, phorbol myristate acetate; TRAP, thrombin receptor-activating peptide; and Tx, thromboxane.

Stimulation of platelets by collagen remains, from a mechanistic point of view, an enigma. At higher concentrations, collagen causes tyrosine phosphorylation, and activation of phospholipase Cγ [9, 10]. Activation of platelets with a peptide corresponding to a portion of collagen also stimulates the phospholipase Cγ pathway [11]. In addition, in single adherent cells, collagen causes an indomethacin-independent, but phosphotyrosine-dependent, increase in $[Ca^{2+}]_i$ [12].

At lower concentrations, collagen, in common with other agonists, causes increased $[Ca^{2+}]_i$, the formation of both inositol trisphosphate and diacylglycerol, as well as full platelet aggregation. However, in contrast to the other agonists, these effects of collagen at low concentrations are attenuated by cyclooxygenase inhibitors and, therefore, mediated by released TxA_2 [13–15]. These observations are consistent with the release of arachidonic acid, the precursor of TxA_2, being a crucial first step in the process of collagen-induced platelet aggregation [13]. The mechanism of arachidonic acid release under these circumstances is unclear. The sequential actions of phospholipase C and diglyceride lipase are believed to account for a relatively minor proportion of the released arachidonic acid [16, 17]. The direct action of a phospholipase A_2, in particular the calcium-dependent cytosolic form ($cPLA_2$), on membrane phospholipids has attracted the most attention [18–21]. However, a role for $cPLA_2$ is unlikely, as the arachidonic acid release precedes, and therefore occurs in the absence of, elevated $[Ca^{2+}]_i$ [13], and a $cPLA_2$ inhibitor does not affect collagen-induced release [22]. Several studies have provided evidence that a PLA_2 is activated in platelets by a mechanism (to date undefined) other than increased $[Ca^{2+}]_i$ [13, 23–25]. Of particular interest with respect to collagen were studies using a phosphotyrosine phosphatase inhibitor, orthovanadate, which demonstrated that phosphotyrosine phosphorylation plays a role in arachidonic acid release [26].

Therefore, it is probable that collagen-induced platelet activation, at any concentration, involves tyrosine phosphorylation. However, the pathway involved is ill-defined, although a role for MAP kinases is possible. MAP kinases are a family of phosphotyrosine- and phosphothreonine-activated protein serine threonine kinases [27, 28]. The presence and role of these enzymes in platelets are controversial. Papkoff and colleagues [29] reported the presence of two forms of MAP kinase, ERK1 and ERK2, in platelets, with only the latter being activated in response to thrombin. In contrast, two groups reported that both ERK1 and ERK2 were activated by thrombin [21, 30]. In addition, a third form, $p38^{mapk}$, has been identified [18, 19, 31], and Kramer and colleagues [18, 19] suggested that ERK1, ERK2, and $p38^{mapk}$ were all present and activated in thrombin-stimulated platelets. In addition, Saklatvala and colleagues [31] suggested that $p38^{mapk}$ plays a role in collagen- and Tx mimetic-induced platelet activation, potentially arachidonic acid release or exocytosis.

The upstream regulation of MAP kinase(s) in other cells involves activation by tyrosine- and threonine-phosphorylation in a cascade mechanism [27, 28]. Watson and colleagues [20] have demonstrated that protein kinase C precedes ERK1 and ERK2 activity in collagen- or thrombin-stimulated platelets. Specific MAP kinase kinases have also been identified in several other cell types [27, 28]. This family of kinases, which includes MEK1 and MEK2, phosphorylate and, therefore, activate the MAP kinases. In the present study, the effects of an inhibitor of MEK1, PD98059 [32, 33], have been examined to determine the role that the MAP kinase pathway plays in the platelet-activation process.

MATERIALS AND METHODS

Materials

PD98059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] was obtained from New England Biolabs Inc. and resuspended in DMSO. Collagen and arachidonic acid were obtained from Helena Laboratories. Thrombin, PMA, and bovine serum albumin were obtained from the Sigma Chemical Co. The stable Tx mimetic U46619 [9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$] and TxB_2 were obtained from Cayman Chemicals. TRAP (single letter code SFLLRN) was synthesised with an Applied Biosystems model 431A peptide synthesizer using Fmoc chemistry by Dr. D. Litchfield (University of Western Ontario). The anti-TxB_2 antisera and BW755C [3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride] were gifts from Dr. J. Gerrard (University of Manitoba). [^{3}H]Serotonin and [^{3}H]arachidonic acid were obtained from Amersham. All other chemicals were of the highest grade available.

Preparation of Platelets

Blood was collected into acid citrate dextrose (3.8 mM of citric acid, 7.5 mM of trisodium citrate, 125 mM of dextrose; 1.9 mL of anticoagulant/8.1 mL of blood) by venipuncture of healthy human volunteers who had denied taking medication known to interfere with platelet function within the previous 2 weeks. Platelet-rich plasma was obtained by centrifugation at 800 *g* for 5 min. Plasma-free platelet suspensions were obtained by centrifugation of platelet-rich plasma at 800 *g* for 15 min, and the resultant pellet was resuspended in the appropriate buffer [34].

Platelet Aggregation

Platelets were resuspended in the plasma volume of HEPES-buffered Tyrode's solution (134 mM of NaCl, 12 mM of $NaHCO_3$, 2.9 mM of KCl, 0.34 mM of Na_2HPO_4, 1 mM of $MgCl_2$, 10 mM of HEPES, 5 mM of dextrose, 0.3% BSA; pH 7.4). Aliquots (400 μL; 300–700 × 10^6/mL) containing 1 mM of $CaCl_2$ were dispensed into aggregometer cuvettes. Aggregation in response to agonists, in the presence of the inhibitor or vehicle control, was monitored

photometrically in a Payton dual channel aggregometer at 37° with continuous stirring [34].

[^{3}H]Serotonin Release

Platelet-rich plasma was incubated with 1 μCi/mL of [^{3}H]serotonin for 30 min at 37°. The platelets were subsequently washed and resuspended in the plasma volume of HEPES-buffered Tyrode's solution. Aggregation was carried out, as outlined above, and the release was terminated by the addition of an equal volume (400 μL) of 0.1% glutaraldehyde in White's saline. The entire sample was transferred to a microfuge tube and centrifuged to pellet the platelets. An aliquot of the supernatant was removed and subjected to liquid scintillation counting, and the remainder was discarded. The pellet was digested by incubation in 50 μL of formic acid and subjected to liquid scintillation counting. The [^{3}H]serotonin in the supernatant was expressed as a percentage of the total (supernatant plus pellet) [^{3}H]serotonin [34].

[^{3}H]Arachidonic Acid Release

Platelet-rich plasma was centrifuged at 800 *g* for 15 min. The subsequent pellet was resuspended in 2 mL of platelet-depleted plasma containing 100 μM of aspirin and incubated with 1 μCi/mL of [^{3}H]arachidonic acid for 60 min at 37°. The excess radiolabel was removed by the addition of 2 mL of homologous platelet-depleted plasma and 1 mL of acid citrate dextrose, and centrifugation at 800 *g* for 15 min [22]. The platelets were resuspended in the plasma volume of HEPES-buffered Tyrode's solution. Aggregation was carried out, as outlined above, and the release was terminated by the addition of an equal volume (400 μL) of 0.1% glutaraldehyde in White's saline. The entire sample was transferred to a microfuge tube and centrifuged to pellet the platelets. An aliquot of the supernatant was removed and subjected to liquid scintillation counting, and the remainder was discarded. The pellet was digested by incubation in 50 μL of formic acid and subjected to liquid scintillation counting. The [^{3}H]arachidonic acid in the supernatant was expressed as a percentage of the total (supernatant plus pellet) [^{3}H]arachidonic acid. Initial studies determined that under these conditions the results were qualitatively similar to those obtained following [^{3}H]arachidonic acid extraction and separation by thin-layer chromatography [22].

TxB_2 Measurement

TxB_2 release was measured by ELISA as previously reported [22, 34]. Briefly platelets were resuspended in the plasma volume of HEPES-buffered Tyrodes, and aggregation in response to agonists was carried out at 37° as outlined above. Three minutes following agonist addition, release was terminated by the addition of an equal volume (400 μL) of ice-cold acid citrate dextrose. The platelets were removed by centrifugation, and the supernatant was analysed for TxB_2 by the method of Docherty and Gerrard [35].

RESULTS

Previous studies using specific antibodies raised against various members of the MAP kinase cascade have demonstrated the presence of two forms of MAP kinase, ERK1 (p44) and ERK2 (p42), in platelets [20, 29, 30]; a third form, ERK3, could not be identified.¶ Similarly, MEK1 and MEK2, two forms of MAP kinase kinase, have been found to be present.¶

To examine the potential role that one of these enzymes, MEK1, plays in platelet function, the MEK1 inhibitor PD98059 was used at concentrations (1–10 μM) consistent with inhibition of enzyme activity [32, 33].

Pretreatment of platelets for 2 min with PD98059 (1–10 μM) inhibited, in a concentration-dependent manner, aggregation in response to a low (1 μg/mL; Fig. 1a), but not a high (50 μg/mL; Fig. 1b), concentration of collagen. Similarly, PD98059 (1–10 μM) inhibited aggregation in response to arachidonic acid (60 μM; Fig. 2a) but not to the stable Tx mimetic U46619 (1 μM; Fig. 2b). Similar preincubations with PD98059 had no effect on platelet aggregation in response to thrombin (0.1 U/mL; Fig. 3a), TRAP (10 μM; Fig. 3b), or the phorbol ester PMA (30 nM; Fig. 3c). Aggregation in response to lower concentrations (0.025 U/mL) of thrombin showed some donor variability; however, on those occasions where aggregation was observed, it was unaffected by PD98059 pretreatment (data not shown).

Platelet activation is accompanied, and enhanced in a positive feedback manner, by the secretion of dense granule components and the release of arachidonic acid. Arachidonic acid is subsequently converted *in vivo* and *in vitro* to the active platelet agonist TxA_2. Both dense granule release and arachidonic acid liberation can be examined by prelabeling techniques.

Thrombin (0.025 to 1 U/mL) and TRAP caused the release of [^{3}H]serotonin from platelet dense granules, and this was not affected significantly by pretreatment with 1–10 μM of PD98059 (Fig. 4). Both high (50 μg/mL) and low (1 μg/mL) concentrations of collagen caused the release of [^{3}H]serotonin (Fig. 5). The former was unaffected by pretreatment with PD98059; however, the [^{3}H]serotonin release stimulated by 1 μg/mL of collagen was inhibited significantly by PD98059 (1–10 μM; Fig. 5). Similarly, preincubation with the dual cyclooxygenase/lipoxygenase inhibitor BW755C (80 μM) inhibited [^{3}H]serotonin release elicited by 1 μg/mL of collagen (Fig. 5). The stable Tx mimetic U46619 caused the PD98059-insensitive release of [^{3}H]serotonin; however, that induced by arachidonic acid was inhibited by both PD98059 and BW755C (Fig. 6).

Preincubation of platelets with PD98059 (1–10 μM) did

¶ McNicol A and Shibou TS, unpublished observations.

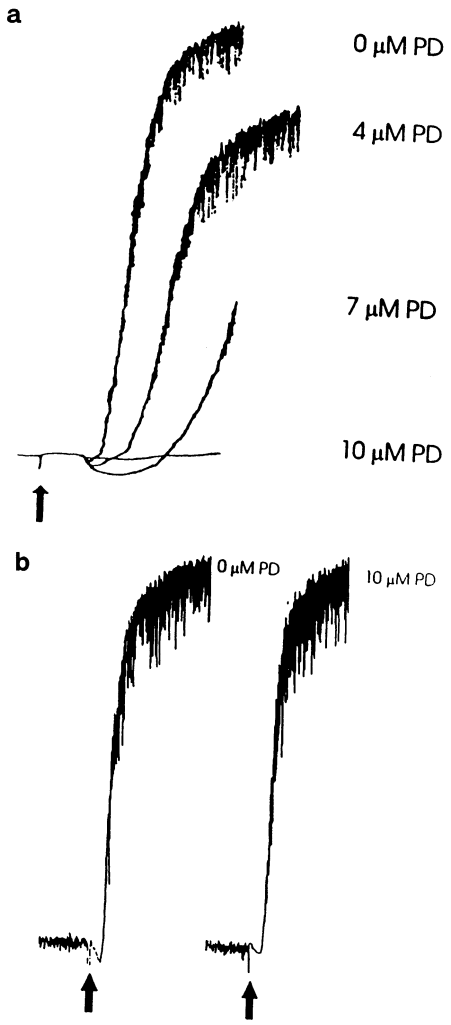

FIG. 1. Effects of PD98059 on collagen-induced platelet aggregation. Washed human platelets were preincubated with 1–10 μM of PD98059, or 0.25% DMSO vehicle control, for 2 min prior to the addition (↑) of (a) 1 μg/mL or (b) 50 μg/mL of collagen. Aggregation was monitored continuously as an increase in light transmission.

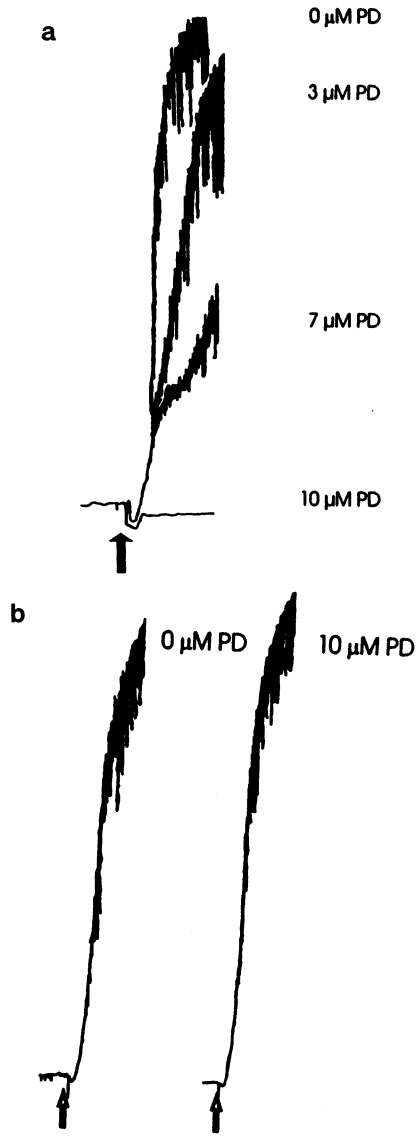

FIG. 2. Effects of PD98059 on arachidonic acid- and U46619-induced platelet aggregation. Washed human platelets were preincubated with 1–10 μM of PD98059, or 0.25% DMSO vehicle control, for 2 min prior to the addition (↑) of (a) 60 μM arachidonic acid or (b) 1 μM of U46619. Aggregation was monitored continuously as an increase in light transmission.

not inhibit collagen- or thrombin-induced [^{3}H]arachidonic acid release (Fig. 7).

Collagen, arachidonic acid, thrombin, and TRAP each stimulated the formation of TxB_2; however, thrombin elicited significantly more TxB_2 than the other three agonists. Pretreatment of the platelets with 10 μM of PD98059 inhibited TxB_2 formation in response to all four agonists (Table 1). Interestingly, 1 μM of PD98059 inhibited collagen- but not arachidonic acid-induced TxB_2; indeed, the release in response to arachidonic acid was apparently potentiated by 1 μM of PD98059. The reason for this is unclear; however, it may reflect differences in the amount of arachidonic acid available for conversion to thromboxane in response to the two agonists. The levels of exogenously added arachidonic acid available for conversion may exceed those produced endogenously by collagen, and thus a higher concentration of PD98059 would be required for inhibition. Indeed, in the case of arachidonic acid, the inhibition of TxB_2 production mimics inhibition of aggregation.

DISCUSSION

The activation of human platelets is a complex process culminating in an alteration in cell shape, expression of adhesive receptors, formation of eicosanoids, granular exocytosis, and aggregation [1–4]. Numerous agonists (e.g. thrombin, TxA_2, ADP, collagen, platelet-activating factor, vasopressin), acting on specific cell surface receptors, elicit platelet activation to varying degrees [3–8]. An intricate array of intracellular pathways have been shown to underlie activation, and indeed the specific pathway(s) involved appears to be dependent on the individual agonist exam-

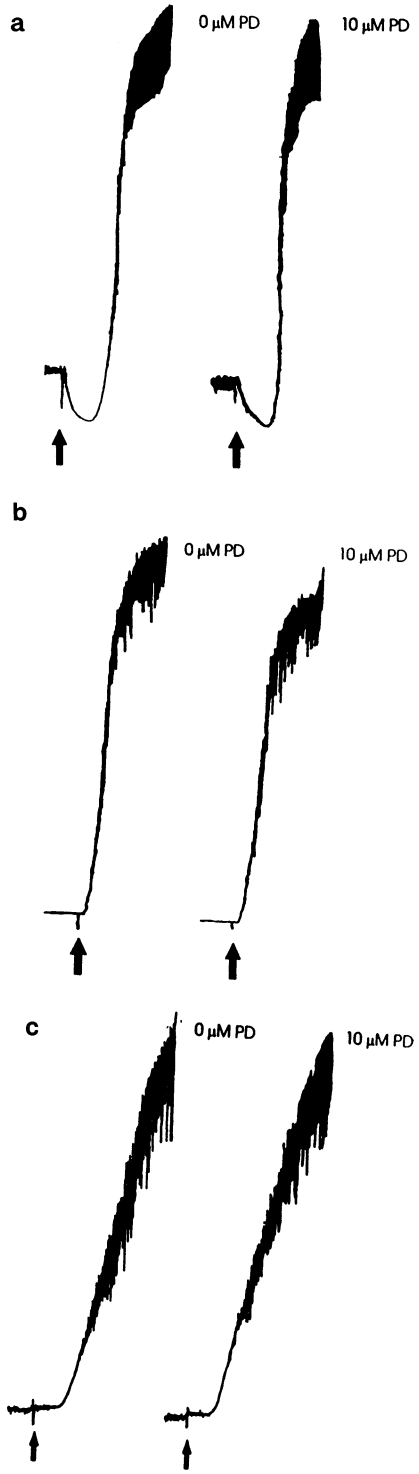

FIG. 3. Effects of PD98059 on agonist-induced platelet aggregation. Washed human platelets were preincubated with 1–10 μM of PD98059, or 0.25% DMSO vehicle control, for 2 min prior to the addition (↑) of (a) 0.1 U/mL of thrombin, (b) 10 μM of TRAP, or (c) 30 nM of PMA. Aggregation was monitored continuously as an increase in light transmission.

ined [3–8]. Receptors for thrombin, TxA_2, platelet-activating factor, and vasopressin are all linked via G-proteins to the activation of phosphoinositide specific phospholipase C [3, 36–40]. In contrast, the post-receptor events associated

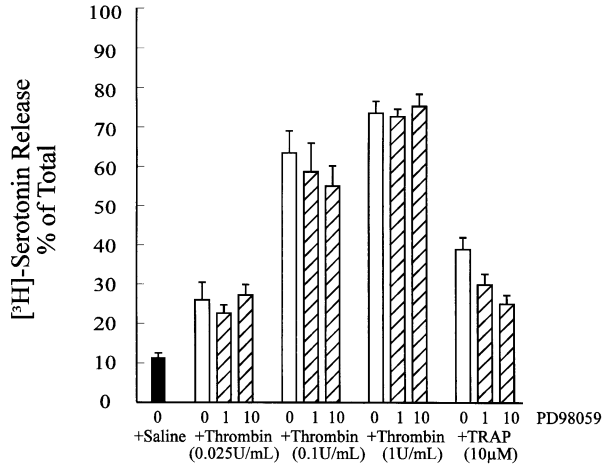

FIG. 4. Effects of PD98059 on thrombin- and TRAP-induced [^{3}H]serotonin release. Human platelets were prelabelled with [^{3}H]serotonin and subsequently washed in a physiological buffer. Aliquots were preincubated with 0.25% DMSO vehicle control (open bars) or 1–10 μM of PD98059 (hatched bars) for 2 min prior to the addition of saline control (closed bars), 0.025 U/mL of thrombin, 0.1 U/mL of thrombin, 1 U/mL of thrombin, or 10 μM of TRAP. Release was terminated, and the [^{3}H]serotonin in the supernatant was determined by liquid scintillation counting and expressed as a percentage of the total [^{3}H]serotonin. Values are means ± SEM; N = 8 for TRAP experiments and N = 10 for thrombin experiments.

with ADP-induced platelet activation continue to be the subject of conjecture. In particular, alterations in $[Ca^{2+}]_i$, Na^+ flux, and an inhibition of adenylate cyclase have all been implicated [8].

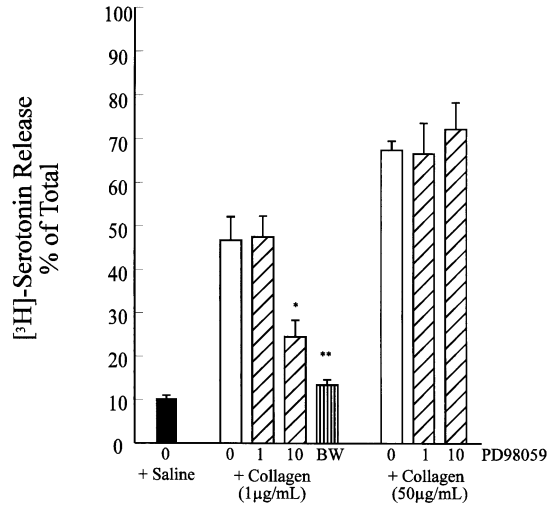

FIG. 5. Effects of PD98059 on collagen-induced [^{3}H]serotonin release. Human platelets were prelabelled with [^{3}H]serotonin and subsequently washed in a physiological buffer. Aliquots were preincubated with 0.25% DMSO vehicle control (open bars), 1–10 μM of PD98059 (hatched bars), or 80 μM of BW755C (striped bars) for 2 min prior to the addition of the saline control (closed bars), 1 μg/mL of collagen, or 50 μg/mL of collagen. Release was terminated, and the [^{3}H]serotonin in the supernatant was determined by liquid scintillation counting and expressed as a percentage of the total [^{3}H]serotonin. Values are means ± SEM, N = 8. *0.05 > P > 0.01 and **P < 0.01.

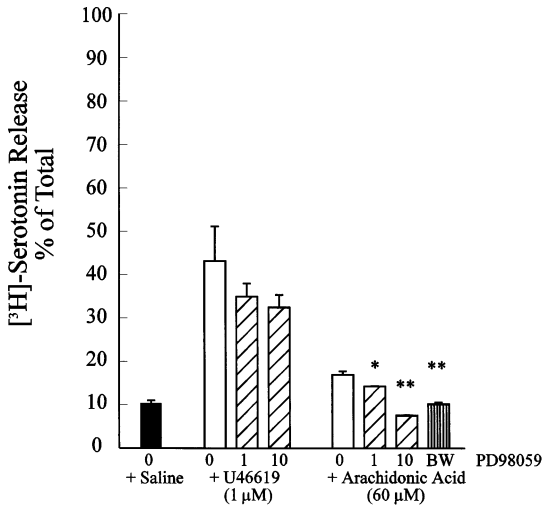

FIG. 6. Effects of PD98059 on U46619- and -arachidonic acid-induced [^{3}H]serotonin release. Human platelets were prelabelled with [^{3}H]serotonin and subsequently washed in a physiological buffer. Aliquots were preincubated with 0.25% DMSO vehicle control (open bars), 1–10 μM of PD98059 (hatched bars), or 80 μM of BW755C (striped bars) for 2 min prior to the addition of the saline control (closed bars), 1 μM of U46619, or 60 μM of arachidonic acid. Release was terminated, and the [^{3}H]serotonin in the supernatant was determined by liquid scintillation counting and expressed as a percentage of the total [^{3}H]serotonin. Values are means ± SEM; N = 10 for U46619 experiments and N = 6 for arachidonic acid experiments. *0.05 > P > 0.01 and **P < 0.01.

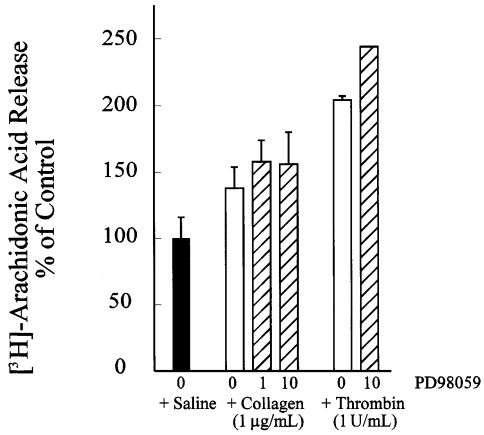

FIG. 7. Effects of PD98059 on [^{3}H]arachidonic acid release. Human platelets were prelabelled with [^{3}H]arachidonic acid and subsequently washed in a physiological buffer. Aliquots were preincubated with 0.25% DMSO vehicle control (open bars) or 1–10 μM of PD98059 (hatched bars) for 2 min prior to the addition of the saline control (closed bars), 1 U/mL of thrombin, or 1 μg/mL of collagen. Release was terminated, and the [^{3}H]arachidonic acid in the supernatant was determined by liquid scintillation counting and expressed as a percentage of the DMSO/saline control. Values are means ± SEM, N = 8.

The mechanism of collagen-induced platelet activation, arguably the most physiologically relevant agonist, is unclear. Collagen stimulates the tyrosine phosphorylation, and activation, of phospholipase Cγ2 [9, 10], which contrasts to the G-protein-linked phospholipase Cβ activated by platelet agonists such as thrombin and TxA_2 [3]. However, these effects of collagen are observed at concentrations (50–100 μg/mL) that are substantially higher than those required to cause full platelet aggregation. Full aggregation and the accompanying increase in $[Ca^{2+}]_i$, formation of phosphatidic acid, and generation of inositol triphosphate can be stimulated with collagen concentrations as low as 1–2 μg/mL. These effects of collagen are abolished by pretreatment either *in vivo* or *in vitro* with cyclooxygenase inhibitors and are, therefore, TxA_2 mediated [13–15, 22], which correlates with a prolonged template bleeding time [41]. The early events in the platelet response to low collagen concentrations, including the release of the TxA_2 precursor arachidonic acid, are therefore of interest.

Roles have been proposed for $cPLA_2$ and diglyceride lipase in collagen-induced arachidonic acid release. However, studies suggest that the diglyceride lipase pathway plays only a minor role in collagen-induced arachidonic acid release [17, 42].

Some interest has centered on the MAP kinase pathway as a mediator of platelet activation, although to date the role of these pathways is unclear [19–21, 29–31, 43, 44]. In other cell types, MAP kinase cascades have been shown to regulate a diverse range of responses, including cell maturation and differentiation [27, 28].

Of particular interest is the evidence that members of the MAP kinase cascade are upstream mediators of arachidonic acid release, primarily, but not exclusively, by the phosphorylation of $cPLA_2$ [45]. This MAP kinase/$cPLA_2$ pathway has been implicated in arachidonic acid release from numerous cells including fibroblasts [46], endothelial cells [47, 48], and neutrophils [49], as well as human [30, 31] and rabbit [50] platelets. Such a pathway is an attractive explanation of the observed phosphotyrosine phosphorylation associated with arachidonic acid release in activated

TABLE 1. Effects of PD98059 on agonist-induced thromboxane production

Additions	Thromboxane*
DMSO + saline	4 ± 2
DMSO + 1 μg/mL collagen	255 ± 112
1 μM PD98059 + 1 μg/mL collagen	15 ± 10†
10 μM PD98059 + 1 μg/mL collagen	18 ± 7†
DMSO + 60 μM arachidonic acid	152 ± 78
1 μM PD98059 + 60 μM arachidonic acid	267 ± 5
10 μM PD98059 + 60 μM arachidonic acid	28 ± 16‡
DMSO + 1 U/mL thrombin	2025 ± 346
10 μM PD98059 + 1 U/mL thrombin	52 ± 10§
DMSO + 10 μM TRAP	272 ± 62
10 μM PD98059 + 10 μM TRAP	11 ± 2‖

*Data are expressed as pg of thromboxane B_2 released per 10^7 platelets.
Values are means ± SEM of triplicate experiments.
†$P < 0.05$, compared with DMSO + collagen.
‡$P < 0.05$, compared with DMSO + arachidonic acid.
§$P < 0.05$, compared with DMSO + thrombin.
‖$P < 0.05$, compared with DMSO + TRAP.

platelets [26]. Kramer and colleagues [19, 21] demonstrated that ERK1, ERK2, and p38 are all activated in response to thrombin and that this, in turn, phosphorylates and activates $cPLA_2$. However, studies by Börsch-Haubold *et al.* [20] apparently contradicted these observations by reporting that thrombin- or collagen-induced phosphorylation of $cPLA_2$ occurs independently of MAP kinase (or of protein kinase C). In addition, TRAP-induced phosphorylation and activation of $cPLA_2$ occur distal to the action of only p38 [19, 21]. Therefore, the role(s) that various members of the MAP kinase cascades play in platelet activation remains unclear.

In the present study, the MEK1 inhibitor PD98059 attenuated low-dose (1 μg/mL), but not high-dose (50 μg/mL), collagen-induced aggregation and dense granule release, indicative of a role for the MEK1 MAP kinase pathway in collagen-induced platelet activation. At this lower concentration, collagen-induced aggregation and dense granule release are both exquisitely sensitive to cyclooxygenase inhibition and, therefore, presumably TxA_2 mediated. Aggregation in response to the stable Tx mimetic U46619, however, was unaffected by PD98059. Therefore, the inhibitory effects of PD98059 on collagen are entirely consistent with a role for the MAP kinase cascade in the TxA_2 synthetic pathway rather than an action distal to the Tx receptor. These results confirm earlier studies by Börsch-Haubold and colleagues [43], who initially reported no inhibitory action of PD98059 on collagen-induced aggregation. In that study, only supramaximal concentrations (100 μg/mL) of collagen were used where no TxA_2 dependence is observed, and, therefore, any role of the MEK1 MAP kinase pathway in the early responses of collagen (i.e. arachidonic acid release, TxA_2 formation) would not be observed [43]. However, in a later study, Börsch-Haubold and colleagues [44] reported that PD98059 inhibited aggregation in response to 2 μg/mL of collagen and urged caution over interpretation of results using PD98059.

Interestingly, in the present study, aggregation and serotonin release caused by thrombin, TRAP, or the Tx mimetic U46619 were not affected by PD98059. These observations suggest that the MEK1 pathway may play a role that is unique to collagen responses and not shared with thrombin and Tx. Furthermore thrombin- and TRAP-induced TxB_2 was inhibited by PD98059. Previous studies have shown thrombin to be relatively insensitive to cyclooxygenase inhibitors [7, 51–53], although arachidonic acid is clearly liberated from these platelets ([7, 17, 22]; present study). In addition, arachidonic acid is released from TRAP-stimulated platelets; however, at concentrations above 6 μM, aggregation is not thromboxane mediated [54]. Similarly, inhibition of cyclooxygenase has little effect on platelet aggregation in response to Tx mimetics [55]. Taken together, these data are consistent with released TxA_2 playing a relatively minor role in platelet responses to thrombin, TRAP, or Tx mimetics.

PD98059 had no effect on phorbol ester-induced platelet aggregation, although a previous study reported that PD98059 attenuates phorbol ester-induced Na^+/H^+ exchange [56]. Indeed, a role for the MEK MAP kinase kinases as a point of intracellular convergence from either tyrosine kinase or protein kinase C leading to stimulation of the Na^+/H^+ exchanger was presented [56]. The role of Na^+/H^+ exchange in platelet activation depends to a large degree on the individual agonist and, indeed, on the concentration used [57]. Therefore, these data suggest that inhibition of PMA-induced Na^+/H^+ exchange by PD98059 [56] does not affect PMA-induced aggregation (present study). Alternatively, the 500 nM of PMA used in the previous study causes effects that are not observed at the lower concentration (30 nM) of PMA used in the present study, and which are inhibitable by PD98059. In addition, the data are consistent with released TxA_2 playing a relatively minor role in PMA-induced platelet aggregation.

To further evaluate the role of the MAP kinase pathway in collagen-induced arachidonic acid release, the effects of PD98059 on collagen-induced [^{3}H]arachidonic acid release from [^{3}H]arachidonic acid-prelabelled platelets were examined. PD98059 failed to inhibit this release. This would suggest that the MEK1 MAP kinase pathway is not involved in the collagen-induced liberation of arachidonic acid. This conclusion supports the studies of Börsch-Haubold *et al.* [20], which indicated that collagen-induced arachidonic acid release, by the phosphorylation and activation of $cPLA_2$, occurs independently of MAP kinases. In addition, it supports studies by the same authors which suggested that inhibition of MEK1 did not affect primary platelet activation in response to collagen [43]. However, as discussed earlier, both of these studies used substantially higher concentrations of collagen than that used in the present study.

PD98059 inhibited the formation of TxB_2 in response to collagen, arachidonic acid, thrombin, and TRAP and also the aggregation observed in response to exogenously added arachidonic acid. Arachidonic acid-induced aggregation is absolutely dependent on its conversion to the active TxA_2 by the combined activities of cyclooxygenase and Tx synthase. Therefore, these data, combined with the absence of any inhibition of U46619-induced aggregation or agonist-induced [^{3}H]arachidonic acid release, can only be explained by an action of PD98059 to inhibit the conversion of arachidonic acid to TxA_2. Such inhibition would also account for PD98059 inhibiting collagen-induced aggregation, secretion, and Tx formation in the absence of an action on [^{3}H]arachidonic acid release.

The mechanism of inhibition has not been addressed. A role for a MAP kinase in the regulation of cyclooxygenase and/or Tx synthase would explain the observed effects of PD98059. However, this is unlikely, as, under conditions where the platelets are otherwise inactive (i.e. no MAP kinase activity), the conversion of exogenous arachidonic acid to Tx is inhibited by PD98059. A more plausible explanation is that PD98059, in addition to its reported

action on MEK1, inhibits either cyclooxygenase or Tx synthase.

In conclusion, the present study supports the observation that components of the MAP kinase cascades are present in human platelets. Studies using PD98059, a reported inhibitor of MEK1, suggest that the MEK1 pathway may not be involved in the release of arachidonic acid in collagen-activated platelets. However, PD98059 inhibits the conversion of arachidonic acid to TxA_2, potentially demonstrating an additional platelet-inhibiting action of this compound.

This study was funded by a grant from the Heart and Stroke Foundation of Canada.

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