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Inhibition of the MEK/ERK pathway has no effect on agonist-induced aggregation of human platelets

Archibald McNicol[a,b,*], Elke C.G. Jackson[a]

[a]Department of Oral Biology, University of Manitoba, 780 Bannatyne Avenue, Winnipeg, Man., Canada R3E 0W2
[b]Department of Pharmacology & Therapeutics, University of Manitoba, 753 Mc Dermot Avenue, Winnipeg, Man., Canada R3E 0W2

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Abstract

The activation of human platelets by a variety of agonists is accompanied by the phosphorylation of the extracellular signal-regulated kinase (ERK) isoforms of mitogen-activated protein (MAP) kinases. However, the role(s) of, and the substrate(s) for, these enzymes in platelet function remain unclear. Studies on ERKs in platelets have relied on pharmacological tools, including an inhibitor of ERK activation, U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene]. In the present study, the effects of U0126 and its "inactive" analogue, U0125 [1,4-diamino-2,3-dicyano-1,4-bis(phenylthio)butadiene], on human platelet aggregation and MAP kinase activity were examined. Several agonists with a variety of signaling pathways were studied including thrombin, a thromboxane analogue, arachidonic acid, collagen, calcium ionophores, and the phorbol ester phorbol myristate acetate (PMA). U0126, at concentrations consistent with inhibition of the isolated enzyme, inhibited ERK phosphorylation, and therefore MEK activation, in response to each agonist. Under such conditions, U0126 did not affect the phosphorylation of a second MAP kinase, $p38^{MAPK}$; however, platelet aggregation was also unaffected. Higher concentrations of U0126, and of U0125, inhibited platelet aggregation in response to collagen and PMA with no effect on that induced by the other agonists. These results dissociate ERK activation from platelet aggregation, suggesting an alternative role for ERKs in platelet function. In addition, the effects of higher concentrations of U0126 are likely due to an action on protein kinase C, likely unrelated to ERK inhibition, suggesting that the inhibitor concentration is crucial to the interpretation of such studies.

Keywords: Platelets; MAP kinase; Collagen; Phorbol ester; U0126

1. Introduction

The MAP kinases are a family of threonine/tyrosine-activated serine/threonine kinases that have been implicated in both cellular proliferation and a wide variety of cell functions. They are activated by MAP kinase kinases (e.g. MEK1/2) through common mechanisms involving phosphorylation at two sites with a sequence of T(P)-X-Y(P). In contrast to the MAP kinases, the MAP kinase kinases are serine/threonine phosphorylated, and activated, by several different MAP kinase kinase kinases, including Raf family members, c-Mos, MEK kinases, and multi-lineage protein kinases [1,2]. To date, eleven mammalian MAP kinase and seven MAP kinase kinase genes have been identified [1]. Human platelets contain $p38^{MAPK}$ (α and β2 isoforms and low levels of the γ and δ isoforms) [3], ERK1 ($p44^{MAPK}$), and ERK2 ($p42^{MAPK}$) [4,5] MAP kinases, as well as both the MEK1 and MEK2 MAP kinase kinases [6]. All three MAP kinase enzymes are phosphorylated, and are therefore presumably active, following platelet stimulation.

Thrombin, collagen, TxA_2 mimetics, and calcium ionophores each activate $p38^{MAPK}$ in platelets [7,8]; indeed $p38^{MAPK}$ plays an important role in platelet activation in response to these agonists [8]. However, the specific $p38^{MAPK}$ isoform involved is partially agonist-specific; each isoform is phosphorylated in response to thrombin, whereas only the α and β2 isoforms are phosphorylated in

* Corresponding author. Tel.: +1-204-789-3527; fax: +1-204-789-3913.
E-mail address: mcnicol@ms.umanitoba.ca (A. McNicol).

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; $cPLA_2$, cytosolic phospholipase A_2; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; IP_3, inositol 1,4,5-trisphosphate; MAP, mitogen-activated protein; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol myristate acetate; and Tx, thromboxane.

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response to collagen [3]. Activation of $p38^{MAPK}$, primarily the α isoform, subsequently phosphorylates $cPLA_2$, on Ser^{505}, which is a prerequisite for $cPLA_2$ activity, and arachidonic acid release, in response to high concentrations of collagen but not thrombin [9,10]. Interestingly, platelets also contain the $p38^{MAPK}$ substrates Mnk1 and PRAK1, both of which are phosphorylated in response to thrombin, and it has been proposed that Mnk1, or a closely related kinase, is responsible for the thrombin-induced phosphorylation of a second site on $cPLA_2$ (Ser^{727}) [11]. These observations are consistent with a proposed role for the MAP kinase/$cPLA_2$ pathway in arachidonic acid release from cells, such as fibroblasts [12], endothelial cells [13,14], and neutrophils [15]. In contrast, release in response to low concentrations of collagen, where arachidonic acid liberation is critical, does not involve $p38^{MAPK}$ [16]. Furthermore, $p38^{MAPK}$ plays a role in the procoagulant membrane blebbing, but not in phosphatidylserine exposure, in collagen-stimulated platelets [17].

Less is known about the role(s) of the ERKs in platelets. Both ERK1 and ERK2 are active distal to PKC in thrombin-stimulated platelets; however, neither is responsible for the phosphorylation of $cPLA_2$ [7,18]. Thrombin-induced ERK1 phosphorylation and activity are enhanced when fibrinogen binding to the integrin α_{IIb}/β_3 is blocked by either the peptide RGDS or an antibody against α_{IIb}/β_3 [19]. Consistent with this, ERK1 and ERK2, and their active (phosphorylated) forms, associate with the contractile cytoskeleton in thrombin-stimulated platelets. Cytoskeletal MAP kinase activity in platelets is enhanced by the RGDS peptide or the lack of integrin α_{IIb}/β_3 [6]. Recently, a pathway involving the ERKs has been implicated in store-mediated calcium entry in platelets independent of both PKC and the actin cytoskeleton [20]. However, the specific substrates for ERK1 and ERK2 in activated platelets are unknown.

One approach to elucidating the function(s) played by MAP kinases in platelets has been the use of inhibitors [21,22]. The pyridinyl imidazole 4-4(fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580) [23], which inhibits $p38^{MAPK}$, has been used extensively in platelets to clarify the role that $p38^{MAPK}$ plays in aggregation [8], $cPLA_2$ phosphorylation (and arachidonic acid release) [3], and procoagulant membrane blebbing [17]. In contrast, there are no inhibitors available for either ERK isoform, although inhibitors of the MEK upstream mediators are available, notably 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059) [24]. However, both PD98059 and SB203580 are limited as each inhibits cyclooxygenase in addition to their effects on the MAP kinase pathways [25,26]. More recently, a second MEK inhibitor, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126) [27,28], has been used in a variety of cells, including platelets [20]. Studies using U0126 suggest that the ERK pathway regulates the process of store-mediated calcium entry [20], the main mechanism for calcium influx in platelets [29]. In the present study, we have further examined the effects of U0126 on platelet function.

2. Materials and methods

2.1. Materials

U0126 and U0125 [1,4-diamino-2,3-dicyano-1,4-bis-(phenylthio)butadiene] were obtained from CalBiochem. Collagen was obtained from Helena Laboratories. Thrombin, PMA, BSA, and DMSO were obtained from the Sigma Chemical Co. The stable Tx mimetic U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$) was obtained from Caymen Chemicals. Antibodies to ERK and phospho-ERK, as well as anti-mouse IgG-HRP and anti-goat IgG-HRP were obtained from Santa Cruz Biotechnology, and antibodies to $p38^{MAPK}$ and phospho-$p38^{MAPK}$ were from New England Biolabs. ECL reagents, Hyperfilm, and [^{32}P]-*ortho*-phosphate were obtained from Amersham. Carnation milk was obtained from Carnation Inc., and all electrophoresis and immunoblotting supplies were from Bio-Rad. All other chemicals were of the highest grade available.

2.2. Preparation of platelets

Blood was collected, by venipuncture of human volunteers who had denied taking medication known to interfere with platelet function within the previous 2 weeks, into acid citrate dextrose anti-coagulant (3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 1.8 mL anti-coagulant/8.1 mL whole blood) [30]. Platelet-rich plasma was obtained by centrifugation at 800 *g* for 5 min at 20°. Plasma-free platelet suspensions were obtained by centrifugation of platelet-rich plasma at 800 *g* for 15 min at 20°, and the resultant pellet was resuspended in the appropriate buffer [25,30–32]. This study was approved by the Human Research Ethics Board of the University of Manitoba, and informed consent was obtained from all the volunteers.

2.3. Platelet aggregation

Platelets were resuspended in the plasma volume of HEPES-buffered Tyrode's solution (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM HEPES, 5 mM dextrose; pH 7.4). Aliquots (0.4 mL; 300×10^6–700×10^6/mL) of the platelet suspension, containing 1 mM $CaCl_2$, were dispensed into aggregometer cuvettes. Aggregation in response to various agonists, in the presence of inhibitor or vehicle control (0.25% DMSO), was monitored photometrically in a Payton dual channel aggregometer at 37° with continuous stirring [25,30–32].

2.4. Immunoblotting

Platelets were prepared in the plasma volume of HEPES-buffered Tyrode's solution and aggregation was carried out, as outlined above. Aggregation was terminated by the addition of an equal volume of reducing buffer [62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% (w/v) bromophenol blue], and samples were denatured by boiling for 10 min. Proteins were separated by electrophoresis on a 10% polyacrylamide/SDS vertical slab gel and transferred to a nitrocellulose membrane at 100 V for 1 hr at 4. The

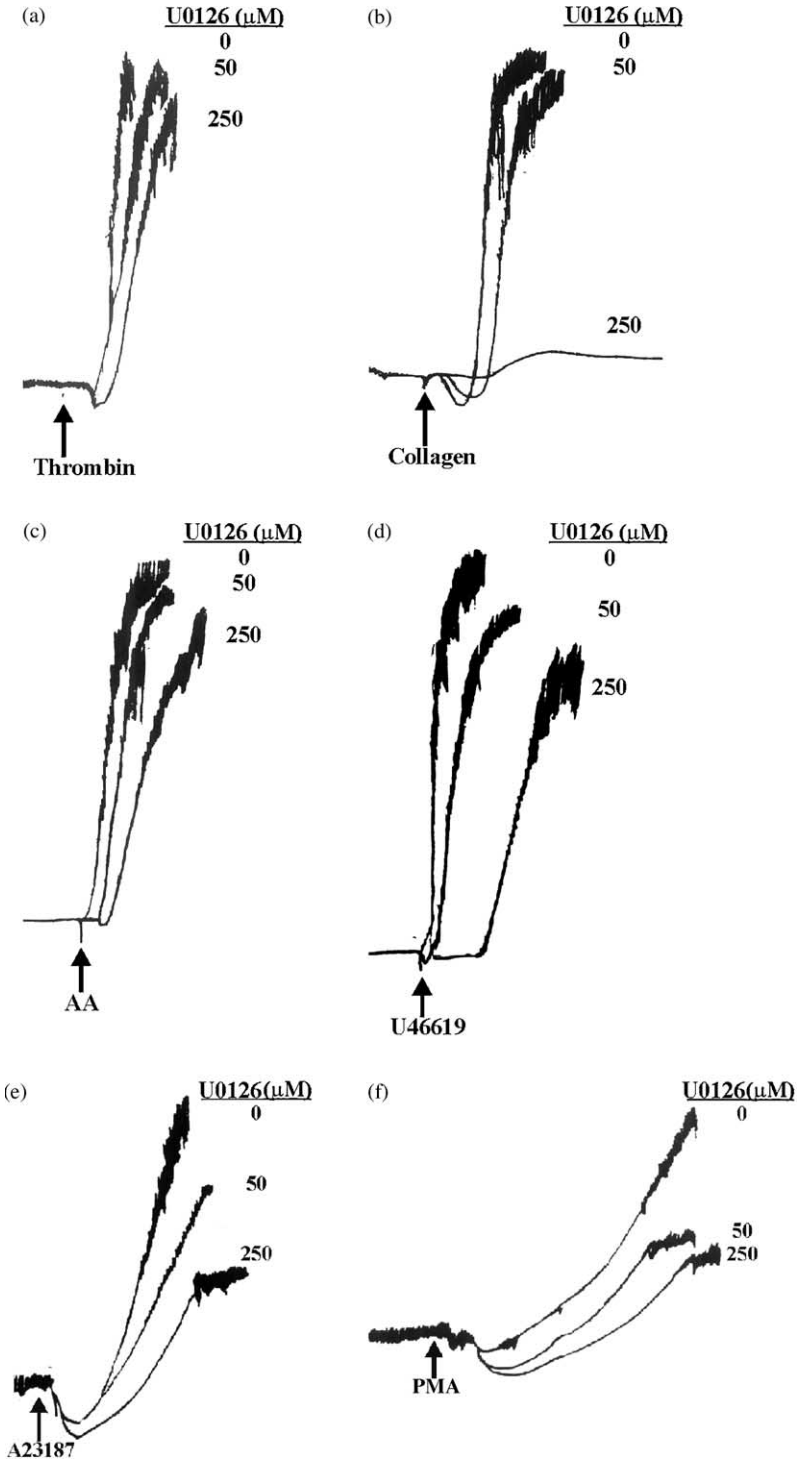

Fig. 1. Effects of U0126 on agonist-induced platelet aggregation. Washed human platelets were incubated for 4 min with U0126 at the concentrations (μM) indicated, or with 0.25% DMSO (control; 0 μM), prior to the addition (arrow) of (a) 0.1 U/mL of thrombin, (b) 1 μg/mL of collagen, (c) 60 μM arachidonic acid, (d) 1 μM U46619, (e) 1 μM A23187, or (f) 15 nM PMA. Aggregation was monitored continuously as an increase in light transmission for 3 min. Each tracing is representative of three experiments.

membrane was then incubated for 2 hr at room temperature in a blocking solution of 5% non-fat powdered Carnation milk in TBS (20 mM Tris base, 130 mM NaCl; pH 7.4) containing 0.1% Tween-20 (TBS-T). Following extensive washing with TBS-T, the membrane was incubated with one of the following antibodies: (i) anti-phospho-specific ERK1/2 diluted 1/1000 in 5% BSA in TBS-T, incubated for 16 hr at 4°; (ii) anti-ERK1/2 diluted 1/1000 in 5% BSA in TBS-T incubated for 16 hr at 4°; (iii) anti-phospho-specific $p38^{MAPK}$ diluted 1/1000 in 5% BSA in TBS-T, incubated for 16 hr at 4°; or (iv) anti-$p38^{MAPK}$ diluted 1/1000 in 5% BSA in TBS-T, incubated for 16 hr at 4°.

In all cases, the membrane was then washed with TBS-T followed by incubation with HRP-conjugated goat anti-mouse IgG diluted in 5% non-fat powdered Carnation milk in TBS-T for 1 hr at room temperature. The membrane was subjected to a final cycle of washes with TBS-T before visualization using the ECL western blotting detection system. Densitometry was carried out using Quantiscan. When the ERK1/2 and phospho-ERK1/2 antibodies, or the $p38^{MAPK}$ and phospho-specific $p38^{MAPK}$ antibodies, were used, the immunoblots were subjected to both antibodies and the phosphorylation data corrected for loading using the non-phosphorylation-specific antibody [25,31,32].

2.5. Pleckstrin phosphorylation

Plasma-free platelet suspensions were prepared in a phosphate-free HEPES–Tyrode's buffer, as outlined above, and incubated with 1 mCi of [^{32}P]-*ortho*-phosphate for 60 min at 37°. Platelets were isolated by centrifugation and resuspended in HEPES–Tyrode's buffer, as outlined above. Platelets were incubated at 37°, in the presence or absence of U0126 or the DMSO control, prior to the addition of the agonist. At the appropriate time the reactions were terminated by the addition of a denaturing solution, the samples were separated by SDS–PAGE, the gels were dried and subjected to autoradiography, and pleckstrin was excised and quantified as previously reported [32]. Pleckstrin is a 40- to 47-kDa protein that is the major PKC substrate in platelets and, as such, its phosphorylation is regarded as an index of PKC activity [33–35].

2.6. Statistical analysis

Data are presented as means ± SEM. Raw data were analyzed by ANOVA, and significance was determined at $P < 0.05$.

3. Results

Previous studies have shown that U0126 inhibits thapsigargin-induced elevation in cytosolic free calcium levels ($[Ca^{2+}]_i$) [20], consistent with a role for the ERK pathway in calcium regulation. As $[Ca^{2+}]_i$ is a mediator of platelet aggregation, the effects of U0126 on agonist-induced aggregation were examined. Thrombin (0.1 U/mL), collagen (1 μg/mL), arachidonic acid (60 μM), the Tx analogue U46619 (1 μM), the calcium ionophore A23187 (1 μM), and the phorbol ester PMA (15 nM) were used at concentrations that produced full platelet aggregation. Platelets were pretreated for 4 min with U0126 (100 nM–250 μM) prior to the addition of the respective agonist. U0126 at 50–250 μM inhibited aggregation, to varying degrees, in response to each agonist (Fig. 1a-f). The aggregation responses to thrombin and arachidonic acid were delayed, to U46619, A23187, and PMA markedly reduced, and to collagen abolished by 250 μM U0126. However, there was no detectable effect of U0126 on aggregation in response to any agonist at U0126 concentrations of less than 50 μM. A similar inhibitory profile was observed when platelets were pretreated with U0125, the inactive analogue of U0126 [27,28], prior to the addition of collagen, PMA (Fig. 2), thrombin, arachidonic acid, U46619, or A23187 (data not shown).

The effects of U0126 on the phosphorylation (activation) of ERK were examined by immunoblotting. Thrombin, collagen, arachidonic acid, U46619, and PMA each

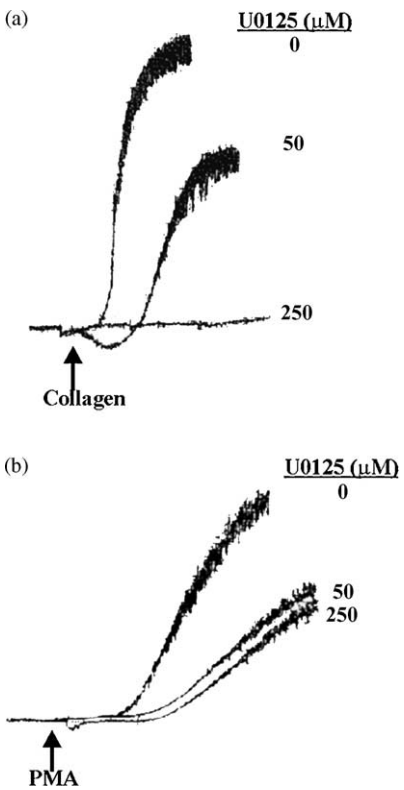

Fig. 2. Effects of U0125 on agonist-induced platelet aggregation. Washed human platelets were incubated for 4 min with U0125 at the concentrations (μM) indicated, or with 0.25% DMSO (control; 0 μM), prior to the addition (arrow) of (a) 1 μg/mL of collagen or (b) 15 nM PMA. Aggregation was monitored continuously as an increase in light transmission for 3 min. Each tracing is representative of three experiments.

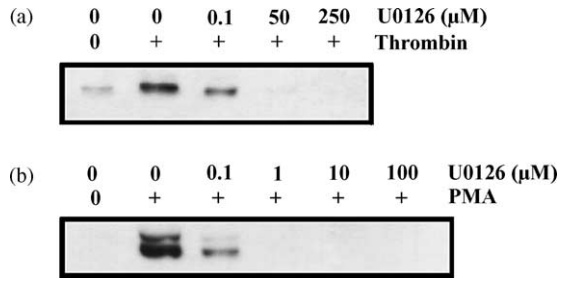

Fig. 3. Effects of U0126 on thrombin- and PMA-induced ERK phosphorylation. Washed human platelets were incubated for 4 min with U0126 at the concentrations indicated, or with 0.25% DMSO (control), prior to the addition of DMSO (0.25%) or either (a) 0.1 U/mL of thrombin (immunoblot is representative of five similar experiments) or (b) 15 nM PMA (immunoblot is representative of six similar experiments) for 3 min. Aggregation was monitored as in Fig. 1. Proteins were extracted, separated, transferred to nitrocellulose, and immunoblotted using an anti-phospho-specific ERK antibody and visualized by ECL. Blots were re-probed with anti-ERK antibody and visualized by ECL to confirm equal loading (not shown).

caused the phosphorylation of the ERKs, as has been reported in numerous studies [4–7,18–22]. Pretreatment with U0126 (100 nM–250 μM) blocked the phosphorylation of the ERKs in response to thrombin (Fig. 3a), PMA (Fig. 3b), collagen (Fig. 4), U46619 (Fig. 5), and arachidonic acid (data not shown), with total inhibition observed in each case at 1 μM U0126. These data are consistent with the reported IC_{50} values for U0126 of 72 nM for MEK1 and 58 nM for MEK2 [27]. Pretreatment of platelets with 1 μM U0125 had a modest effect on ERK phosphorylation, compared to U0126 (Figs. 3–5), in response to thrombin, collagen, A23187, PMA (Fig. 6), U46619 or arachidonic

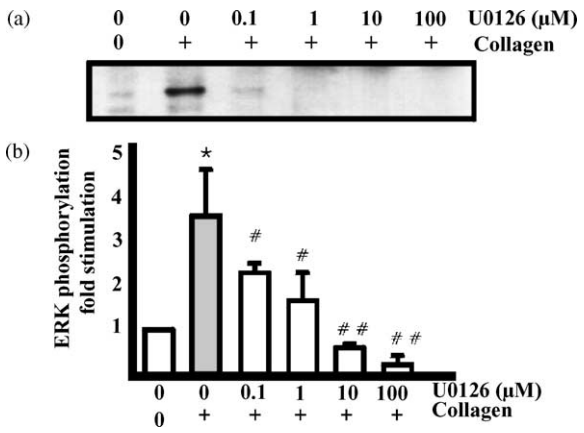

Fig. 4. Effects of U0126 on collagen-induced ERK phosphorylation. Washed human platelets were incubated for 4 min with U0126 at the concentrations indicated, or with 0.25% DMSO (control), prior to the addition of 0.25% DMSO or 1 μg/mL of collagen for 3 min. Aggregation was monitored as in Fig. 1. Proteins were extracted, separated, transferred to nitrocellulose, and immunoblotted using an anti-phospho-specific ERK antibody and visualized by ECL. The immunoblot (a) is representative of three experiments. The blots were re-probed with anti-ERK antibody and visualized by ECL to confirm equal loading (not shown), and data were expressed (b) as fold stimulation of the unstimulated control (mean ± SEM; N = 3). Key: (∗) $P < 0.05$ vs DMSO/DMSO; (#) $P < 0.05$ vs DMSO/collagen; and (##) $P < 0.01$ vs DMSO/collagen.

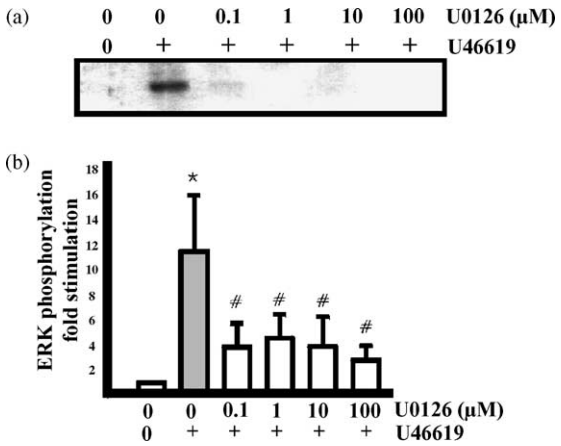

Fig. 5. Effects of U0126 on U46619-induced ERK phosphorylation. Washed human platelets were incubated for 4 min with U0126 at the concentrations indicated, or with 0.25% DMSO (control), prior to the addition of 0.25% DMSO or 1 μM U46619 for 3 min. Aggregation was monitored as in Fig. 1. Proteins were extracted, separated, transferred to nitrocellulose, and immunoblotted using an anti-phospho-specific ERK antibody, and visualized by ECL. The immunoblot (a) is representative of three experiments. Blots were re-probed with anti-ERK antibody and visualized by ECL to confirm equal loading (not shown), and data were expressed (b) as fold stimulation of the unstimulated control (mean ± SEM; N = 3). Key: (∗) $P < 0.05$ vs DMSO/DMSO; and (#) $P < 0.05$ vs DMSO/U46619.

acid (data not shown), although complete inhibition was observed at 100 μM.

Each agonist also caused the phosphorylation of $p38^{MAPK}$, as has been reported previously [3,7,8]. Pretreatment with U0126 (100 nM–250 μM) had no effects on $p38^{MAPK}$ phosphorylation in response to thrombin (Fig. 7a), collagen (Fig. 7b), U46619 (Fig. 7c), arachidonic acid, A23187, or thapsigargin (data not shown). In contrast, PMA-induced $p38^{MAPK}$ phosphorylation was abolished by U0126 (Fig. 7d). PMA induced a 2.25 ± 0.06-fold phosphorylation of pleckstrin, the signature PKC substrate in platelets, which was not inhibited significantly by

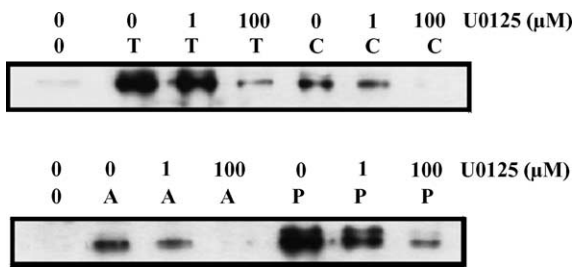

Fig. 6. Effects of U0125 on thrombin-, collagen-, A23187-, and PMA-induced ERK phosphorylation. Washed human platelets were incubated for 4 min with U0125 at the concentrations indicated, or with 0.25% DMSO (control), prior to the addition of 0.25% DMSO or either 0.1 U/mL of thrombin (T), 1 μg/mL of collagen (C), 1 μM A23187 (A), or 15 nM PMA (P) for 3 min. Aggregation was monitored as in Fig. 1. Proteins were extracted, separated, transferred to nitrocellulose, and immunoblotted using an anti-phospho-specific ERK antibody and visualized by ECL. Blots were re-probed with anti-ERK antibody and visualized by ECL to confirm equal loading (not shown). The immunoblot is representative of five similar experiments.

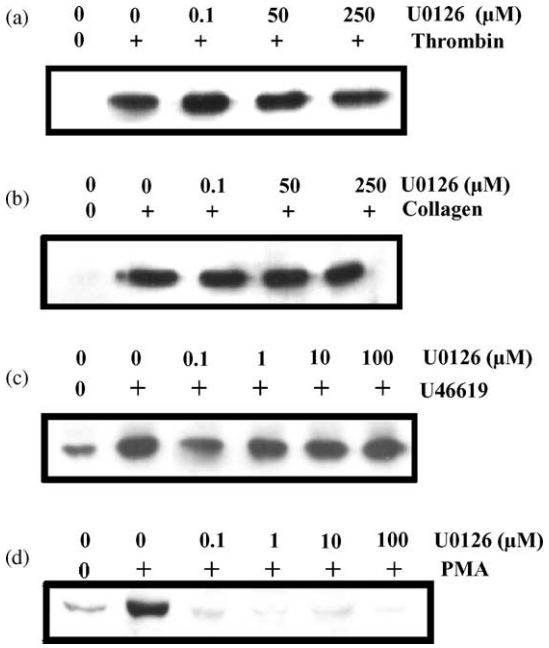

Fig. 7. Effects of U0126 on thrombin-, collagen-, U46619-, and PMA-induced p38^{MAPK} phosphorylation. Washed human platelets were incubated for 4 min with U0126 at the concentrations indicated, or with 0.25% DMSO (control), prior to the addition of DMSO (0.25%) or either (a) 0.1 U/mL of thrombin (immunoblot is representative of two similar experiments), (b) 1 μg/mL of collagen (immunoblot is representative of two similar experiments), (c) 1 μM U46619 (immunoblot is representative of two similar experiments), or (d) 15 nM PMA (immunoblot is representative of three similar experiments) for 3 min. Aggregation was monitored as in Fig. 1. Proteins were extracted, separated, transferred to nitrocellulose, and immunoblotted using an anti-phospho-specific p38^{MAPK} antibody and visualized by ECL. Blots were re-probed with anti-p38^{MAPK} antibody and visualized by ECL to confirm equal loading (not shown).

pretreatment with 0.1 μM (1.92 ± 0.41-fold), 1.0 μM (2.95 ± 1.05-fold), 50 μM (3.02 ± 1.18-fold), or 250 μM (1.63 ± 0.24-fold) U0126.

4. Discussion

Human platelets contain p38^{MAPK} (α, β2, γ, and δ isoforms), ERK1 (p44^{MAPK}), and ERK2 (p42^{MAPK}) members of the MAP kinase family [3]. Although p38^{MAPK} has been implicated in both cPLA$_2$-mediated arachidonic acid release [3] and procoagulant membrane blebbing [17], the role(s) of the ERKs is less well defined. Both ERK1 and ERK2 translocate to the cytoskeleton of thrombin-activated platelets, and both translocation and enzyme activity are enhanced by the blockade of integrin α_{IIb}/β_3 [6,19,36]. Recent studies have shown that prolonged preincubation with the MEK inhibitor U0126 blocks the entry of calcium into thrombin- and thapsigargin-stimulated platelets [20]. This is consistent with a role for the ERK pathway in store-mediated calcium entry and provides the first potential role for this pathway in platelets.

In the present study, U0126, at concentrations consistent with those that inhibit the isolated MEK enzymes [27], abolished ERK phosphorylation in response to a variety of agonists. In contrast, at these concentrations U0125 had a minimal effect. Similarly, with the exception of PMA, U0126 had no effect on agonist-induced p38^{MAPK} phosphorylation. Taken together, these data support the notion that U0126, but not U0125, inhibits MEK but has no comparable direct inhibitory effect on the p38^{MAPK} pathway.

Agonist-induced aggregation is the final platelet response that follows, and is dependent upon, many biochemical events [37,38]. In the present study, we used agonists that stimulate particular signaling pathways. U0126, at concentrations (≤1 μM) that blocked ERK activity, had no effect on aggregation in response to any agonist used. These data strongly suggest that the ERKs play, at most, a minimal role in agonist-induced platelet activation. These studies apparently contradict those of Rosado and Sage [20], who demonstrated a dramatic effect of U0126 on calcium flux, and suggested that the ERKs function in the regulation of store-mediated calcium flux. It is well accepted that one of the intracellular mediators of platelet activation is an elevation in $[Ca^{2+}]_i$ [37,38]. By extension, therefore, regulation of calcium flux by the ERKs places them in an important position in the platelet activation cascade. However, it should be noted that Rosado and Sage [20] did not measure a platelet activation end point in their study and, in addition, used a preincubation period of 30 min with U0126 [20], whereas in the present study a 4-min preincubation was used. Finally, Rosado and Sage [20] used a U0126 concentration of 10 μM, whereas in the present study complete inhibition of ERK phosphorylation was observed at 0.1–1 μM. A possible explanation for the difference in interpretation of the role of the ERKs in platelet function may involve inhibitory effects of higher concentrations of U0126 unrelated to its action on MEK. Indeed, in the present study, short-term preincubation with higher concentrations of both U0126 and U0125 produced inhibitory effects, such as collagen-, PMA-, and A23187-induced aggregation, with similar profiles, suggesting that the effects were unrelated to an inhibitory action on MEK.

The agonists used in the present study act by a variety of mechanisms. Thrombin and U46619 each act via PLCβ-mediated pathways [37–40] leading to both the diglyceride-mediated activation of PKC and the IP$_3$-induced elevation in $[Ca^{2+}]_i$ [37–39]. Neither thrombin- nor U46619-induced aggregation was affected by U0126, suggesting that it does not affect, either directly or indirectly, the stimulation of PLC. In addition, the lack of inhibitory effects rules out any action of U0126 at a common end point necessary for aggregation, such as the expression of the integrin α_{IIb}/β_3 or its association with the adhesive protein fibrinogen.

Platelet aggregation in response to thrombin or U46619 occurs independently of arachidonic acid release and subsequent Tx formation [40,41]. In contrast, both collagen-, at the concentrations used in the present study [42–44], and

arachidonic acid-induced aggregation are Tx-mediated. Previous studies have shown that a second MEK inhibitor, PD98059, blocked aggregation in response to these agonists; however, this was due to the direct inhibition of cyclooxygenase/Tx synthetase rather than to an effect mediated by the ERKs [25]. Arachidonic acid-induced aggregation was unaffected by U0126, suggesting that the inhibition of cyclooxygenase/Tx synthetase was not the mechanism involved. Interestingly, however, collagen-induced aggregation was abolished by the higher doses of both U0126 and U0125, consistent with an action, unrelated to inhibition of MEK, prior to arachidonic acid release.

Collagen releases arachidonic acid via a mechanism independent of $cPLA_2$, possibly involving a combination of diacylglyceride lipase and monoglyceride lipase [45,46] distal to PLCγ2 [16]. It, therefore, is plausible that the higher concentrations of both U0126 and U0125 block any of these enzymes leading to an attenuation of arachidonic acid release and subsequent Tx production. However, PMA-induced aggregation, which involves neither PLCγ2 activity nor significant arachidonic acid release [47,48], was also inhibited by the higher concentrations of U0126 and U0125. PMA stimulates platelets by a direct activation of PKC [37] and, significantly, a central role for PKC in the initial stages of the collagen response has been suggested [49]. Taken together, these observations are consistent with an inhibitory effect on the PKC pathway, although the lack of an effect on PMA-induced pleckstrin phosphorylation suggests that PKC *per se* is not blocked. Consistent with such an inhibition of the PKC pathway, $p38^{MAPK}$ phosphorylation in response to PMA, a PKC-mediated event, was also inhibited by U0126, although phosphorylation by other agonists, where multiple intracellular pathways are invoked, remained unaffected.

In conclusion, the MEK inhibitor U0126 blocked agonist-induced ERK phosphorylation at concentrations consistent with those reported to inhibit the isolated enzyme. Inhibition of ERK phosphorylation was not accompanied by an effect on platelet aggregation, although at higher U0126 concentrations aggregation was blocked, possibly due to an effect distal to PKC. These results dissociate ERK activation from platelet aggregation, suggesting an alternative role for ERKs in platelet function, and also reiterating that inhibitor concentration is crucial in such studies.

Acknowledgments

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