Inhibitory Effects of PD98059, SB203580, and SP600125 on α - and δ -granule Release and Intracellular Ca^{2+} Levels in Human Platelets

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Platelets are activated at sites of vascular injury via several molecules, such as adenosine diphosphate, collagen and thrombin. Full platelet aggregation is absolutely essential for normal hemostasis. Moreover, this physiological event can trigger circulatory disorders, such as thrombosis, atherosclerosis, and cardiovascular disease. Therefore, platelet function inhibition is a promising approach in preventing platelet-mediated circulatory disease. Many studies reported the involvement of mitogen-activated protein kinases (MAPKs) signaling pathways in platelet functions. However, these studies were limited. Thus, we examined MAPK signaling pathways in human platelets using specific MAPK inhibitors, such as PD98059, SB203580, and SP600125. We observed that these inhibitors were involved in calcium mobilization and influx in human platelets. They also suppressed thrombin-induced α - and δ -granule release. These results suggest that PD98059, SB203580, and SP600125 exhibit Ca^{2+} antagonistic effects.

Key Words: MAPK signaling pathways, Calcium mobilization, Calcium influx, Granule release

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that are divided into three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38. Studies have detected MAPKs in human platelets and have shown them to be phosphorylated by thrombin for full activation (Kramer et al., 1995; Bugaud et al., 1999; Nadal-Wollbold et al., 2002). Furthermore, the roles of MAPKs have been studied in hemostasis and thrombosis (Adam et al., 2008). In platelets, ERKs have been extensively investigated among MAPKs. ERKs may be activated via growth factors, receptor tyrosine

kinases and G protein-coupled receptors in various cell types (van Blesien et al., 1995). Its signaling process is initiated by the small G protein Ras, which activates the Raf-1/MAPK/ERK (MEK)/ERK phosphorylation pathway (Kyriakis et al., 1992). ERK2 is reportedly activated by the dual-specificity kinase, MEK1/2, and protein kinase C (PKC)-dependent pathway in platelets (Börsch-Haubold et al., 1995; Börsch-Haubold et al., 1996). Regarding the ERK1/2 upstream molecule specific inhibitors of conventional PKCs (Ro-318425 and Ro-318220) reportedly inhibit MEK1/2 and ERK1/2 (Börsch-Haubold et al., 1995; Nadal-Wollbold et al., 2002). Furthermore, H-Ras and Raf-1 in the platelets do not influence ERK1/2 activation (Nadal-Wollbold et al., 2002). A specific PKC δ inhibitor (Rottlerin) also reportedly suppresses

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the thrombin-induced phosphorylation of MEK1/2, ERK1/2, and p38 (Yacoub et al., 2006). Src kinase inhibitor PP2 and phospholipase C inhibitor U73122, have also been shown to inhibit the von Willebrand factor induced ERK2 phosphorylation (Ramakrishnan et al., 2001). Considering these previous reports, it appears that ERK2 may be regulated by PKC α and PKC δ . Furthermore, the study of JNK1 in platelets has identified, a key role for it in platelet function and thrombosis (Adam et al., 2010). JNK1-deficient mice were shown to reduce thromboxane A₂ (TXA₂) production despite normal platelet counts, ultrastructures, and expression of adhesive receptors such as GPIIb α , GPVI, and glycoprotein IIb/IIIa (α IIb/ β 3). Moreover, JNK1^{-/-} showed diminished ERK2 levels after thrombin stimulation, which suggested that JNK1 may be an upstream molecule of ERK2 or at least associated with each other (Adam et al., 2010). Thrombin-activated ERK2 and JNK1 were also reportedly down-regulated by α IIb/ β 3 inhibition, which suggested that the activation probably involved a common pathway (Bugaud et al., 1999).

p38 is activated by thrombin (Kramer et al., 1995) and the phospholipases C β , and PKC δ , are known as upstream mediators (Yacoub et al., 2006). Activated p38 increases the phosphorylation of cytosolic phospholipase A₂, resulting in arachidonic acid release and TXA₂ production; however, SB203580 inhibits TXA₂ production (Kramer et al., 1996). Furthermore, PD98059 and SB203580, specific inhibitors of ERK1/2 and p38, block COX-1 directly and SB203580 further inhibits the conversion of PGH₂ to TXA₂ (Börsch-Haubold et al., 1998), which means that MAPKs may influence endogenous enzyme activity leading to TXA₂ production. Various studies have revealed the MAPK functions in platelets; however, their complete regulatory functions have been elusive. Therefore, we investigated MAPK signaling pathways in human platelet using specific inhibitors.

MATERIALS AND METHODS

Materials

Thrombin was obtained from Chrono-Log Corporation (Havertown, PA, USA). Fura 2-acetoxymethyl (Fura 2-AM) and BAPTA-AM were obtained from Invitrogen Molecular

Probes (Eugene, OR, USA). Anti-phosphor-IP3-receptor type I (Ser¹⁷⁵⁶), anti-phosphor-p38, anti-phosphor-ERK (1/2), anti-phosphor-JNK (1/2), anti-p38, anti-ERK (1/2), anti-JNK (1/2), anti-rabbit IgG-horseradish peroxidase conjugate, and lysis buffer were obtained from Cell Signaling (Beverly, MA, USA). PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), SP6000125 (JNK inhibitor) and anti- β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Thapsigargin was purchased from Cayman Chemical. Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence solution (ECL) were purchased from General Electric Healthcare (Buckinghamshire, UK).

Preparation of washed human platelets

Human platelet-rich plasma (PRP) was obtained from the Korean Red Cross Blood Center (Changwon, Korea), and centrifuged for 10 min at 1,300 g. The platelets were then washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 5.5 mM glucose, and 1 mM Na₂EDTA, pH 6.5), and resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, 0.25% gelatin, pH 6.9). The platelets concentrations were adjusted to a final concentration of 5×10^8 /mL. All aforementioned procedures for platelet activity were performed at 25°C. Experimental approval (PIRB12-072) was obtained from the Public Institutional Review Board at the National Institute for Bioethics Policy (Seoul, Korea).

Determination of platelet aggregation

Platelets (10^8 /mL) were preincubated with or without substances in the 2 mM CaCl₂ for 3 min at 37°C followed by thrombin stimulation (0.05 U/mL). The aggregation was performed for 5 min using an aggregometer (Chrono-Log Corporation). Platelet aggregation rate was determined as an increase in light transmission. MAPK inhibitors were dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 0.1%.

Western blots for analyzing of ERK1/2-, p38-, and JNK1/2-phosphorylation

Platelet aggregation was stopped by adding 1 $\times$ lysis

buffer. The platelet lysates were then measured using a BCA protein assay kit (Pierce Biotechnology, IL, USA). Proteins (15 µg) were analyzed via SDS-PAGE (6%, 1.5 mm), and PVDF membranes were used for protein transfer. The dilutions for the primary and secondary antibodies were 1:1,000 and 1:10,000, respectively. The membranes were visualized using the ECL solution.

Determination of Ca^{2+} mobilization and influx

PRP was incubated with 5 µM of Fura 2-AM at 37°C for 60 min. The washed platelets ($10^8/\text{mL}$), loaded with Fura 2-AM, were prepared using the procedure described above preincubated with MAPK inhibitors for 3 min at 37°C in the presence of 100 µM of EGTA, and stimulated with thrombin (0.05 U/mL) for Ca^{2+} mobilization and thapsigargin (1 µM) for Ca^{2+} influx. After thapsigargin stimulation, 2 mM of calcium was added at 3 min. Fura 2-AM fluorescence was measured using a spectrofluorometer (SFM-25; BioTeek Instruments, Italy) at an excitation wavelength that changed every 0.5 s from 340 to 380 nm; and an emission wavelength set at 510 nm. The $[\text{Ca}^{2+}]_i$ values were calculated using the Grynkiewicz method (Grynkiewicz et al., 1985).

Determination of p-selectin release

Washed human platelets ($10^8/\text{mL}$) were preincubated with or without substances in the presence of 2 mM CaCl_2 for 3 min at 37°C followed by thrombin stimulation (0.05 U/mL). The platelets were reconstituted in 250 µL of ice-cold phosphate-buffered saline (PBS; pH 7.4) and incubated with 10 µL of Alexa Fluor 488 anti-human CD62P in PBS (pH 7.4) containing 0.09% sodium azide and 0.2% bovine serum albumin (BSA) for 60 min at 4°C in a dark room. The platelets were then washed thrice with ice-cold PBS and resuspended in 0.5% paraformaldehyde in PBS. The platelet-bound Alexa Fluor 488 anti-human CD62P was determined using flow cytometry (BD Biosciences, San Diego, CA, USA) and the data was analyzed using the BD cellQuest™ software.

Determination of ATP and serotonin release

Washed platelets ($10^8/\text{mL}$) were stimulated with thrombin (0.05 U/mL) with or without MAPK inhibitors in the pre-

sence of 2 mM CaCl_2 . The reaction was terminated and centrifuged at $200 \times g$ for 10 min at 4°C, and the supernatant was used for ATP and serotonin release assay. ATP and serotonin release were measured using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Statistical analyses

The experimental results are indicated as mean $\pm$ standard deviation accompanied by the number of observations. The data was determined via analysis of variance (ANOVA). Significant differences among the group means were compared using the Newman-Keuls method. Statistical analysis was performed using SPSS 21.0.0.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of MAPK inhibitors on thrombin-induced human platelet aggregation

Based on a previous report, 0.05 U/mL of thrombin was used as it maximally aggregated human platelets (Shin et al., 2015). The thrombin-induced aggregation rate was $92.5 \pm 1.2\%$, however, PD98059, SB203580 and SP600125 at 20 µM could not inhibit thrombin-induced platelet aggregation (Fig. 1A). Thus, we used 40 µM of the MAPK inhibitors on thrombin-induced platelet aggregation. As shown in Fig. 1A, PD98059, SB203580 and SP600125 at 40 µM suppressed human platelet aggregation. Therefore, we used these concentrations to evaluate anti-platelet effects.

Effects of MAPK inhibitors on ERK1/2-, p38, and JNK1/2-phosphorylation

MAPK phosphorylation is known to be positively regulated in human platelet aggregation. Thus, we examined if PD98059, SB203580, and SP600125 inhibited the MAPK phosphorylation. Compared to unstimulated platelets thrombin (0.05 U/mL) elevated ERK2-, p38-, and JNK1-phosphorylation (Fig. 1B, 1C, and 1D). However, 40 µM of PD98059, SB203580, and SP600125 reduced ERK2-, p38-, and JNK1-phosphorylation. Furthermore, 0.1% DMSO did not influence phosphorylation (Fig. 1B, 1C, and 1D).

Fig. 1. Effects of MAPK inhibitors on thrombin-induced human platelet aggregation and MAPK phosphorylation. (A) Effects of MAPK inhibitors on thrombin-induced human platelet aggregation. (B) Effects of PD98059 on ERK-phosphorylation. (C) Effects of SB-203580 on p38-phosphorylation. (D) Effects of SP600125 on JNK-phosphorylation. Platelet aggregation and Western blots were carried out as described in the "Materials and Methods" section. The data is expressed as mean $\pm$ standard deviation ($n = 4$). * $P < 0.05$ versus thrombin-stimulated human platelets.

Effects of MAPK inhibitors on $[Ca^{2+}]_i$ mobilization

We investigated the effect of PD98059, SB203580 and SP600125 on Ca^{2+} antagonistic activity. As shown in Fig. 2A, thrombin-increased Ca^{2+} mobilization from 100.5 ± 1.3 nM to 835.9 ± 39.7 nM in the presence of 2 mM $CaCl_2$. However, we eliminated the indirect effects of Ca^{2+} influx to accurately measure $[Ca^{2+}]_i$ mobilization by using EGTA (100 μ M), which was recorded as 370.5 ± 1.1 nM (Fig. 2B). Thrombin-induced $[Ca^{2+}]_i$ mobilization suppressed PD-98059, SB203580, and SP600125 (Fig. 2C, 2D, and 2E). The percentage inhibition by 40 μ M of MAPK inhibitors were 33.6%, 36.2%, and 57.4%, respectively (Table 1).

Effects of MAPK inhibitors on IP_3RI -phosphorylation

IP_3 receptor type I (IP_3RI) phosphorylation leads to the inhibition of $[Ca^{2+}]_i$ mobilization (Quinton and Dean, 1992). Therefore, phosphorylation of IP_3RI is used to evaluate Ca^{2+} -antagonistic effects. As shown in Fig. 3, all MAPK inhibitors strongly increased IP_3RI phosphorylation, which proved the involvement of MAPK signaling pathways in $[Ca^{2+}]_i$ mobilization.

Effects of MAPK inhibitors on $[Ca^{2+}]_i$ influx

Thapsigargin is an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase, which is used to trigger Ca^{2+} influx

Fig. 2. Effects of MAPK inhibitors on $[\text{Ca}^{2+}]_i$ mobilization. (A) Thrombin-induced $[\text{Ca}^{2+}]_i$ mobilization. (B) Thrombin-induced $[\text{Ca}^{2+}]_i$ mobilization in the presence of EGTA (100 μM). (C) Effects of PD98059 on $[\text{Ca}^{2+}]_i$ mobilization in the presence of EGTA (100 μM). (D) Effects of SB203580 on $[\text{Ca}^{2+}]_i$ mobilization in the presence of EGTA (100 μM). (E) Effects of SP600125 on $[\text{Ca}^{2+}]_i$ mobilization in the presence of EGTA (100 μM). $[\text{Ca}^{2+}]_i$ mobilization was determined as described in the "Materials and Methods" section. The data is expressed as mean $\pm$ standard deviation ($n = 3$). $*P < 0.05$ versus thrombin-stimulated human platelets.

from extracellular spaces. after adding 2 mM CaCl_2 , 1 μM of thapsigargin triggers Ca^{2+} influx from 101.5 ± 1.2 nM (the basal level) to 860.5 ± 21.2 nM (Fig. 4A). However, 40 μM of PD98059, SB203580, and SP600125 inhibits Ca^{2+} influx (Fig. 4B, 4C, and 4D). The percentage inhibition by 40 μM of MAPK inhibitors were observed to be 67.0%,

69.5%, and 78.9%, respectively (Table 2).

Effects of MAPK inhibitors on p-selectin expression

In order to verify the inhibitory mechanism of PD98059, SB203580 and SP600125 on α -granule release, we investigated p-selectin expression. PD98059, SB203580, and SP-

600125 (40 μ M each) strongly inhibited p-selectin expression (Fig. 5A and 5B), which reflected their inhibitory effect on

[Ca²⁺]_i levels. The intracellular Ca²⁺ chelator, BAPTA-AM also strongly suppressed thrombin-induced p-selectin expression (Fig. 5A and 5B).

Table 1. Effects of MAPK inhibitors on thrombin-induced [Ca²⁺]_i mobilization

	[Ca ²⁺] _i mobilization (nM)	Δ (%)
Thrombin (0.05 U/mL)	370.5 $\pm$ 1.1	
+ PD98059 (20 μ M)	259.1 $\pm$ 2.3*	30.1
+ PD98059 (40 μ M)	246.1 $\pm$ 1.1*	33.6
+ SB203580 (20 μ M)	274.5 $\pm$ 2.4*	25.9
+ SB203580 (40 μ M)	236.5 $\pm$ 4.3*	36.2
+ SP600125 (20 μ M)	228.5 $\pm$ 3.3*	38.3
+ SP600125 (40 μ M)	157.8 $\pm$ 0.7*	57.4

Data from Fig. 2. Δ (%) = [(thrombin + substances) - thrombin] / thrombin $\times$ 100.
Data is expressed as mean $\pm$ standard deviation (n = 4).
*P < 0.05 versus thrombin-stimulated human platelets.

Effects of MAPK inhibitors on ATP and serotonin release

Since the MAPK inhibitors decreased the thrombin-

Fig. 3 Effects of MAPK inhibitors on IP₃RI-phosphorylation. Western blots were analyzed as described in the "Materials and Methods" section. The data is expressed as mean $\pm$ standard deviation (n = 3).

Fig. 4. Effects of MAPK inhibitors on Ca²⁺ influx. (A) Thapsigargin-induced Ca²⁺ influx in the presence of EGTA (100 μ M). (B) Effects of PD98059 on Ca²⁺ influx in the presence of EGTA (100 μ M). (C) Effects of SB203580 on Ca²⁺ influx in the presence of EGTA (100 μ M). (D) Effects of SP600125 on Ca²⁺ influx in the presence of EGTA (100 μ M). Ca²⁺ influx was determined as described in the "Materials and Methods" section.

elevated $[Ca^{2+}]_i$ levels, we investigated the involvement of the MAPK signaling pathways in the inhibition of ATP and serotonin release from δ -granules. Thrombin (0.05 U/mL)

strongly increased ATP (Fig. 6A) and serotonin release (Fig. 6B); however, all the MAPK inhibitors suppressed ATP and serotonin release. Moreover, BAPTA-AM decreased ATP and serotonin release, indicating the involvement of intracellular Ca^{2+} levels in granule release (Fig. 6A and 6B).

Table 2. Effects of MAPK inhibitors on thapsigargin-induced Ca^{2+} influx

	Ca^{2+} influx (nM)	Δ (%)
Thapsigargin (1 μ M)	860.5 $\pm$ 21.2	
+ PD98059 (20 μ M)	344.2 $\pm$ 5.6*	60.0
+ PD98059 (40 μ M)	284.0 $\pm$ 8.0*	67.0
+ SB203580 (20 μ M)	352.1 $\pm$ 5.6*	59.1
+ SB203580 (40 μ M)	262.4 $\pm$ 3.6*	69.5
+ SP600125 (20 μ M)	254.5 $\pm$ 5.2*	70.4
+ SP600125 (40 μ M)	181.3 $\pm$ 2.2*	78.9

Data from Fig. 4. Δ (%) = [(thapsigargin + substances) - thapsigargin] / thrombin $\times$ 100.

Data is expressed as mean $\pm$ standard deviation (n = 4).

* P < 0.05 versus thapsigargin-stimulated human platelets.

DISCUSSION

Complete platelet aggregation is essential for hemostatic plug formation. This physiological event is achieved by free cytosolic Ca^{2+} . Thus, inhibiting intracellular Ca^{2+} is useful in preventing circulatory disorders. Elevation of $[Ca^{2+}]_i$ by agonists is dependent on its mobilization from the endoplasmic reticulum and influx from extracellular spaces. Thrombin, a platelet agonist, is known to stimulate platelet aggregation by binding to the Gq-coupled proteinase-activated receptor, which activates phospholipase C_β (PLC β). The activated PLC β hydrolyzes phosphatidylinositol 4, 5-bisphosphate

Fig. 5. Effects of MAPK inhibitors on p-selectin expression. (A) Flow cytometry histograms representing p-selectin expression. a, Intact platelets (base); b, Thrombin (0.05 U/mL); c, Thrombin (0.05 U/mL) + DMSO (0.1%); d, Thrombin (0.05 U/mL) + PD-98059 (40 μ M); e, Thrombin (0.05 U/mL) + SB203580 (40 μ M); f, Thrombin (0.05 U/mL) + SP600125 (40 μ M); g, Thrombin (0.05 U/mL) + BAPTA-AM (10 μ M). (B) Effects of MAPK inhibitors on thrombin-induced p-selectin expression (%). Determination of p-selectin expression was carried out as described in the "Materials and Methods" section. The data is expressed as mean $\pm$ standard deviation (n = 3). * P < 0.05 versus thrombin-stimulated human platelets.

Fig. 6. Effects of MAPK inhibitors on serotonin-, and ATP-release. (A) Effects of MAPK inhibitors on serotonin release in thrombin-activated platelets. (B) Effects of MAPK inhibitors on ATP release in thrombin-activated platelets. Determination of ATP and serotonin release was carried out as described in the "Materials and Methods" section. The data is expressed as mean $\pm$ standard deviation ($n = 3$). * $P < 0.05$ versus thrombin-stimulated human platelets.

(PIP₂) to inositol 1, 4, 5-trisphosphate (IP₃) and diacylglycerol (Berridge and Irvine, 1984; Cattaneo, 2005; Guidetti et al., 2008; Jennings, 2009). Moreover, IP₃ mobilizes free cytosolic Ca²⁺ from dense tubular systems by binding to IP₃ receptor type I (IP₃RI). The increased Ca²⁺ activates Ca²⁺/calmodulin-dependent kinase and the DG-dependent kinase. Myosin light chain kinase (MLCK) is the most representative Ca²⁺/calmodulin-dependent enzyme and phosphorylates myosin light chain (20 kDa) in platelets. The phosphorylation of myosin light chain is associated with the activation of platelets and subsequent release of platelet granules. Therefore, the increase of calcium plays an important role in inducing the granule secretion (i.e., dense body and α -granule) and platelet aggregation (Nishikawa et al., 1980; Kaibuchi et al., 1982).

IP₃RI function is inhibited by its phosphorylation and IP₃RI phosphorylation involves in the inhibition of [Ca²⁺]_i mobilization (Quinton and Dean, 1992). Therefore, phosphorylating IP₃RI is very useful for evaluating the Ca²⁺-antagonistic effect of substances or compounds. As shown in Fig. 2, all MAPK inhibitors inhibited the thrombin-induced [Ca²⁺]_i mobilization and also showed strong IP₃RI

(Ser¹⁷⁵⁶) phosphorylation (Fig. 3).

Stromal interaction molecule 1 (STIM1) has been identified as a key element of the store-operated Ca²⁺ entry mechanism. STIM1 is a transmembrane protein with an N-terminal EF hand domain (Varga-Szabo et al., 2009). Upon Ca²⁺ release in the endoplasmic reticulum, STIM1 recognizes the depletion of Ca²⁺ and facilitates Ca²⁺ influx. Recently, STIM1 was reported to have phosphorylation sites on the serine/threonine and tyrosine residues (Lopez et al., 2012; Lang et al., 2013), and thapsigargin was reported to trigger STIM1 tyrosine phosphorylation in human platelets (Lopez et al., 2012). Moreover, both ERK1/2 and the Src family of tyrosine kinases were reported to be involved in STIM1 phosphorylation (Elvers et al., 2012; Lopez et al., 2012). In HEK293 cells, ERK1/2 mediates STIM1 phosphorylation at Ser575, Ser608 and Ser621 during Ca²⁺ store depletion, and that Ca²⁺ entry and store refilling restore phosphorylation to basal levels (Pozo-Guisado et al., 2013). As shown in Fig. 4B, PD98059 (an ERK inhibitor) suppressed Ca²⁺ influx, thereby associating the ERK signaling pathway with Ca²⁺ influx. SB203580 and SP600125 also suppressed the thapsigargin-induced [Ca²⁺]_i influx (Fig. 4C and 4D). These

results suggested that the JNK and p38 signaling pathways were also involved in Ca^{2+} influx. To date, the relationship between stim1 and MAPKs has been reported in various cells and ERK is most closely related. However, in our experiments, it was suggested that p38 and JNK also down-regulated thapsigargin-induced calcium influx in human platelets, which means that p38 and JNK may interact with STIM1. Thus, the specific interaction between JNK, p38, and STIM1 in human platelets will be tested in the future.

MAPK inhibitors showed a potent calcium inhibitory effect (20, 40 μM) but did not suppress at low concentrations in human platelet aggregation reactions (Fig. 1A). Because the platelet aggregation reaction is the final reaction, a variety of signal transduction processes trigger the final platelet aggregation reaction. MAPK inhibitors could affect the detailed signaling of platelets, but the inhibitory effects may be limited. Therefore, MAPK inhibitors (20 μM) could not have a strong effect on the final platelet aggregation inhibition.

The markers of α - and δ -granule release used in this study are p-selectin, ATP, and serotonin. p-selectin is located in the inner membrane of α -granule, and ATP, serotonin are located in the δ -granule. Therefore, we examined the MAPK signaling pathways that were associated with α - and δ -granule release. Thrombin-elevated p-selectin expression (Fig. 5A, 5B) and ATP and serotonin release (Fig. 6A, 6B). However, these reactions were inhibited by MAPK inhibitors. Because $[\text{Ca}^{2+}]_i$ is the most important regulator of α - and δ -granule release, p-selectin expression, ATP and serotonin release are also dependent on $[\text{Ca}^{2+}]_i$ levels. This result reflects that BAPTA-AM (10 μM) treated platelets significantly downregulated the p-selectin expression (Fig. 5A-g, 5B), ATP, and serotonin release (Fig. 6A, 6B). Therefore, these inhibitory effects on α - and δ -granule release by MAPK inhibitors are mediated by downregulation of $[\text{Ca}^{2+}]_i$ mobilization and $[\text{Ca}^{2+}]_i$ influx.

In conclusion, the precise mechanism of the MAPK signaling pathway is still not fully understood. However, our results suggest that the MAPK signaling pathway plays a role in α - and δ -granule release by down-regulating Ca^{2+} mobilization and influx. Our results also suggest that the MAPK signaling pathway is involved in the regulation of calcium and granule release.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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