

Full-length article

Prostaglandin A1 inhibits increases in intracellular calcium concentration, TXA₂ production and platelet activation¹

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Key words

prostaglandin A1; platelet aggregation; calcium; TXA₂

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Abstract

Aim: In our previous studies we found that cyclopentenane prostaglandin A1 (PGA1) had neuroprotective effects in a rodent ischemic model. In the present study we aimed to investigate the inhibitory effect of PGA1 on platelet function.

Method: The rate of aggregation of human platelets was measured by using turbidimetry. The rate of adhesion of platelets to cultured endothelial cells was determined by using [³H]-adenine labeled platelets. 5-Hydroxytryptamine release from platelets was measured with *O*-phthalaldehyde fluorospectrophotometry. The levels of TXB₂, a stable metabolite of TXA₂, were determined by radioimmunoassay. Alterations in platelet morphology were observed using an electron microscope, and the intraplatelet free calcium concentrations were measured with Fluo-3/AM FCM assay. **Results:** PGA1 significantly inhibited thrombin-, collagen- and ADP-induced aggregation and adhesion of platelets. The morphological changes of platelets induced by thrombin were blocked by PGA1. PGA1 inhibited the release of 5-hydroxytryptamine from dense granules and the synthesis of TXA₂. **Conclusion:** PGA1 inhibits the activation of platelets probably through blocking increases in intracellular calcium concentration and TXA₂ synthesis.

Introduction

Prostaglandins are derived from arachidonic acid liberated from the cell membrane. They are produced in a variety of tissues and mediate an array of physiologic and pathologic processes, including regulation of respiratory, vascular, intestinal and inflammatory activities^[1]. Cyclopentenone prostaglandins (cycPG), including the A and J series, are formed from the enzyme-induced dehydration of prostaglandin E and D. They have some unique biological activities that are different from those of classic prostaglandins, such as inducing cell differentiation^[2], anti-tumor^[3–5] and anti-virus activities^[6,7]. Prostaglandin A1 (PGA1) has many pharmacological actions, including inhibiting tumor growth, inflammation and viral replication^[8–11]. We have reported previously that PGA1 inhibits excitotoxin-induced apoptosis of striatal neurons *in vivo* and rotenone-induced apoptosis of cultured SH-SY5Y cells^[12,13]. In a recent study, we found that PGA1 significantly reduced infarction volume in rodent

models of focal cerebral ischemia^[14,15]. PGA1 influences several cellular signaling pathways, including activation of peroxisome proliferator-activated receptor γ (PPAR γ)^[16], inhibition of nuclear factor-kappaB (NF- κ B)^[11,17], and induction of heat shock proteins^[18,19]. The molecular signaling pathways involved in its neuroprotection, however, remain to be determined.

The activation and aggregation of platelets play an important role in the pathological process of cerebral ischemia through interactions with endothelial cells and formation of thrombosis^[20,21]. It has been shown that drugs inhibiting platelet function, including aspirin, could be beneficial in preventing ischemic attack. The effects of some prostaglandins on platelets have been reported^[22,23]. However, information about the effects of PGA1 on platelets is incomplete and inconsistent; therefore, the aim of the present study was to determine if PGA1 had an inhibitory effect on the functions of human platelets. We found that PGA1 inhibited platelet aggregation, release of TXB₂ and platelet adhesion

to endothelial cells. These actions could contribute to its neuroprotective effects in rodent models of stroke.

Materials and methods

Drugs and reagents PGA1, thrombin, Fluo-3 acetoxy-methyl ester (Fluo-3 AM), sulfinpyrazone, and A23187 were purchased from Sigma (St Louis, MO, USA); adenosine 5' diphosphate (ADP), *N*-(2-hydroxyethylpiperazine)-*N'*-(2-ethanesulfonic acid) (HEPES), and ethylene glycol bi- β -aminoethylether)-*N,N,N'*-tetraacetic acid (EGTA) were purchased from Shanghai Sangon Bioengineering and Technology Service (Shanghai, China). Collagen was purchased from the Jiangsu Institute of Hematology (Suzhou, China).

Platelet preparations Venous blood was obtained from healthy donors with no drug history for at least 14 d, and immediately mixed with a one-ninth volume of citrate acid (3.8%). Platelet enriched plasma (PRP) was obtained by centrifugation at $200\times g$ for 10 min. For experiments with thrombin, platelets were washed with HEPES-buffed solution (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L glucose and 20 mmol/L HEPES), and then resuspended in Tyrode's solution (137 mmol/L NaCl, 2.7 μ mol/L KCl, 0.36 μ mol/L NaH₂PO₄, 12 μ mol/L NaHCO₃, 1 μ mol/L MgCl₂, 5 μ mol/L HEPES, 5 μ mol/L glucose, 2 μ mol/L CaCl₂).

Platelet aggregation assay Aggregation of platelets was evaluated by using an MPG-3E aggregometer (Shanghai Silong Medical Instrument Factory, Shanghai, China) according to the manufacturer's instructions. PRP was first incubated with ethanol (with a final concentration of 0.8%, as a vehicle control), or different concentrations of PGA1 at 37 °C for 5 min. Platelet aggregation was triggered by adding 20 μ L of ADP, collagen and thrombin to 200 μ L of PRP to achieve final concentrations of 40 μ mol/L, 40 μ g/mL and 0.25 U/mL, respectively.

Platelet adhesion assay Platelets were washed and treated as described earlier. Human umbilical endothelial cells were cultured in 96-well plates and grown to cover the entire surface of wells. Endothelial cells were pre-treated with Triton X-100 (0.5%) before use. Thrombin-stimulated platelet suspension was added to each well and incubated for 30 min. The adherent platelets were stained with Rose Bengal (0.25%) at 37 °C and then dissolved in absolute ethanol 30 min later. The number of adherent platelets was determined by measuring absorption using a wavelength of 570 nm.

Assay of 5-hydroxytryptamine release from platelets Levels of 5-hydroxytryptamine (5-HT) released from platelets were determined by using O-phthaldialdehyde (OPT)

fluorospectrophotometry as described elsewhere^[2]. Briefly, platelets were obtained and washed as described earlier, and different concentrations of PGA1 (20–80 μ mol/L) or vehicle (final concentration=0.8% ethanol) were incubated with washed human platelets for 5 min. 5-HT was extracted after stimulation with thrombin (0.25 μ mol/L) for 5 min and reacted with OPT in 10 mol/L HCl for 15 min. Fluorescence was then measured with a 960 CRT fluorospectrophotometer (Shanghai Jinmi Scientific Instrument Company, Shanghai, China) using excitation and emission wavelengths of 365 nm and 480 nm, respectively. The concentration of 5-HT was calculated as follows: $[5\text{-HT}] = (F_{\text{sample}} - F_{\text{blank}}) / (F_{\text{standard}} - F_{\text{blank}}) \times 0.5$ (μ g/mL)

Evaluation of ultrastructure of platelets The effect of PGA1 on ultrastructural changes of platelets induced by thrombin was examined with an electron microscope. Washed platelets were pretreated with PGA1 (20–80 μ mol/L), then thrombin (0.25 U/mL) was added to activate platelets 5 min later. Platelets were then fixed with 4% (w/v) glutaraldehyde after a 5 min incubation with thrombin, stained with osmium tetroxide, dehydrated, and embedded in Araldite. Ultrathin sections were examined with a Philips CM-120 electron microscope.

TXB₂ assay Platelets were obtained as described earlier. Washed platelets were prepared and pre-incubated with PGA1 (20–80 μ mol/L) or vehicle (final concentration=0.8% ethanol) for 5 min. Then thrombin was added and the reaction was terminated (500 μ L of ice-cooled stop solution containing 50 mmol/L EDTA; 2 mmol/L indomethacin, 130 mmol/L NaCl) 5 min later. Samples were then centrifuged at $500\times g$ for 10 min at 4 °C. Supernatants were used for determination of the levels of TXB₂ with radioimmunoassay (TXB₂ Radioimmunoassay kit, Jiangsu Institute of Hematology).

Determination of cytosolic free calcium The intracellular [Ca²⁺] was determined using Fluo-3 AM, essentially as described elsewhere^[1]. Briefly, PRP was incubated with 8 μ mol/L Fluo-3 AM for 30 min at 37 °C; the dyed platelets were spun down and gently re-suspended at a concentration of approximately 1×10^8 cells/mL in the HEPES-buffered solution containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L glucose and 20 mmol/L HEPES, supplemented with 100 mmol/L sulfinpyrazone to prevent the cellular efflux of Fluo-3 acid. The external [Ca²⁺] was adjusted to 1 mmol/L and the fluorescence was measured with flow cytometer using excitation and emission wavelengths of 488 nm and 526 nm, respectively. Calibration of the ratio of fluorescence signals into pseudo fluorescence was performed using 1 μ mol/L A23187 to obtain the maximal ratio, followed by 5 mmol/L EGTA to obtain the minimal ratio. Platelets were incubated with PGA1 (20–80 μ mol/L) at 37 °C for 5 min in

HEPES-buffered solutions before the addition of thrombin. The full response was obtained in solutions containing 1 mmol/L CaCl₂. Calcium concentration was estimated as follows: $F_p = (F - F_{min}) / (F_{max} - F_{min})$. To clarify whether the rise in intracellular calcium is derived from intracellular reservoirs or the entry of extracellular Ca²⁺, platelets were stimulated in a Ca²⁺-free solution, and calcium concentration was estimated as described earlier.

Statistical analysis Statistical analyses of the differences between vehicle control and PGA1-treated samples were carried out with an unpaired, two-tailed Student's *t*-test, and *P*<0.05 was considered significant.

Results

PGA1 inhibited platelet activation Adding ADP (40 μmol/L), collagen (40 mg/mL) or thrombin (0.25 U/mL) to human platelets triggered robust aggregation. Pretreatment with PGA1 (20–80 μmol/L) dose-dependently inhibited the platelet aggregation induced by collagen, ADP and thrombin. The maximal inhibition of PGA1 (20, 40, and 80 μmol/L) on platelet aggregation induced by collagen was 46.63%, 48.70%, and 64.11% (*P*<0.05); that by ADP was 33.13%, 42.45%, and 49.43% (*P*<0.05); and that by thrombin was 41.63%, 90.29%, and 96.39% (*P*<0.05), respectively (Figure 1). The IC₅₀ values of PGA1 on platelet aggregation induced by collagen, ADP and thrombin were 31.02, 80.56, and 20.67 μmol/L, respectively. Thus, PGA1 had greater effects on the thrombin-

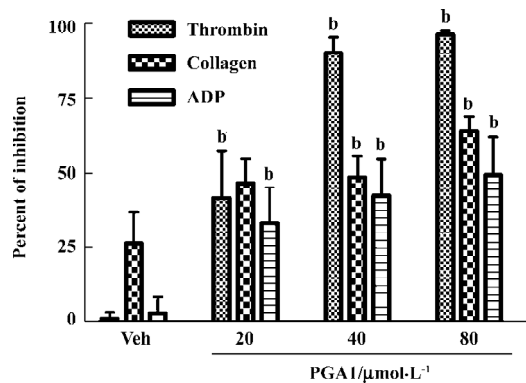


Figure 1. Effects of PGA on aggregation of human platelets induced by thrombin, collagen and ADP. Human platelets were washed and re-suspended in HEPES-Tyrodé's solution. Platelets were pre-incubated with ethanol (vehicle control, 0.8% final concentration) or different concentrations of PGA1 (20, 40, 80 μmol/L) at 37 °C for 5 min. Platelet aggregation was triggered by adding 20 μL of ADP, collagen and thrombin to 200 μL of platelet suspension to final concentrations of 40 μmol/L, 40 μg/mL and 0.25 U/mL, respectively. Platelet aggregation was recorded for 5 min using a MPG-3E aggregometer. *n*=6. Data are mean±SD. *P*<0.05 vs vehicle control.

induced aggregation of platelets. Thrombin-induced adhesion of platelets to endothelial cells was inhibited by pretreatment with PGA1 5 min before thrombin. The inhibitory rates of PGA1 at concentrations of 20 μmol/L, 40 μmol/L, and 80 μmol/L on platelet adhesion were 38.7%, 34.1%, and 40.8%, respectively (*P*<0.05 vs vehicle; Figure 2). PGA1 significantly decreased the release of 5-HT from the dense granules of platelets induced by thrombin (*P*<0.05; Figure 3). The lactate dehydrogenase (LDH) assay showed that there was no difference in LDH leakage among groups with and without PGA1 treatment using the same samples for 5-HT assay, excluding the possibility that the increase in 5-HT in supernatant was caused by damage of platelets during preparation (data not shown).

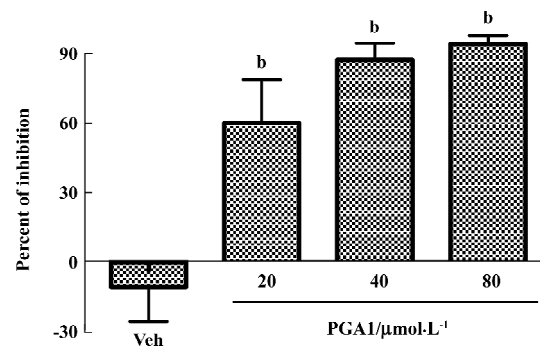


Figure 2. Effects of PGA1 on platelet adhesion. Human umbilical endothelial cells were cultured in 96-well plates. Human platelets were washed and pre-incubated with ethanol (vehicle control, 0.8% final concentration). Then platelets were stimulated with thrombin (0.25 U/mL) and co-cultured with endothelial cells. Adhesion of platelets to endothelial cells was quantified as described in the Materials and Methods. *n*=4. Data are mean±SD. *P*<0.05 vs vehicle control.

PGA1 inhibited the ultrastructural changes of platelets Stimulated platelets transform from a discoid form to a spiny spherical shape with numerous pseudopodia (shape change); simultaneously, the fibrinogen receptors that mediate aggregation are exposed. We investigated the effects of PGA1 on platelet morphology after thrombin stimulation with an electron microscope. We found that thrombin elicited robust changes in platelet morphology, including formation of numerous pseudopodia and loss of dense granules. Pretreatment with PGA1 almost completely blocked the thrombin-induced shape changes (formation of pseudopodia) and aggregation of human platelets (Figure 4).

PGA1 inhibited TXA₂ synthesis and calcium influx in platelets TXA₂ is a potent activator of platelets. Addition of thrombin to platelets stimulated synthesis of TXB₂, a stable

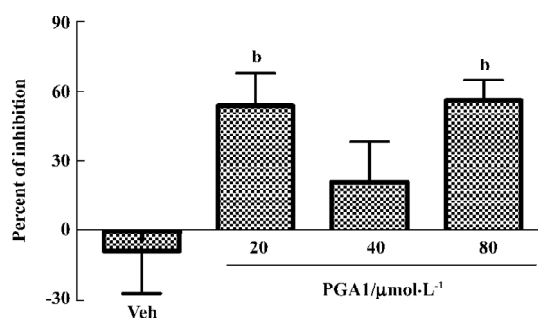


Figure 3. Effects of PGA1 on thrombin-induced release of 5-HT from platelets. Human platelets were washed and pre-incubated with PGA1 (20–80 μmol/L) or vehicle (final concentration=0.8% ethanol) for 5 min. 5-HT was extracted after stimulation with thrombin (0.25 μmol/L) for 5 min and reacted with OPT. Fluorescence was then measured using a 960 CRT fluorospectrophotometer. $n=6$. Data are mean±SD. $P<0.05$ vs vehicle control.

metabolite of TXA₂. PGA1 dose-dependently inhibited the production of TXB₂ ($P<0.05$). The IC₅₀ of PGA1 on TXB₂ production was 34.1 μmol/L ($P<0.05$; Figure 5). Calcium is a key player in activation of platelets. PGA1 (20–80 μmol/L) downregulated the increase in calcium concentration inside the platelets induced by thrombin ($P<0.05$; Figure 6A). When calcium was absent in the extracellular solution, we failed to observe an inhibition of calcium increase by PGA1 (Figure 6B), suggesting that PGA1 inhibited calcium influx from the extracellular space.

Discussion

Most actions of the cyclopentenone prostaglandins, including PGA2, PGA1, and PGJ2, are not mediated by binding to G-protein-coupled prostanoid receptors, but result from their direct interaction with other cellular target proteins^[8].

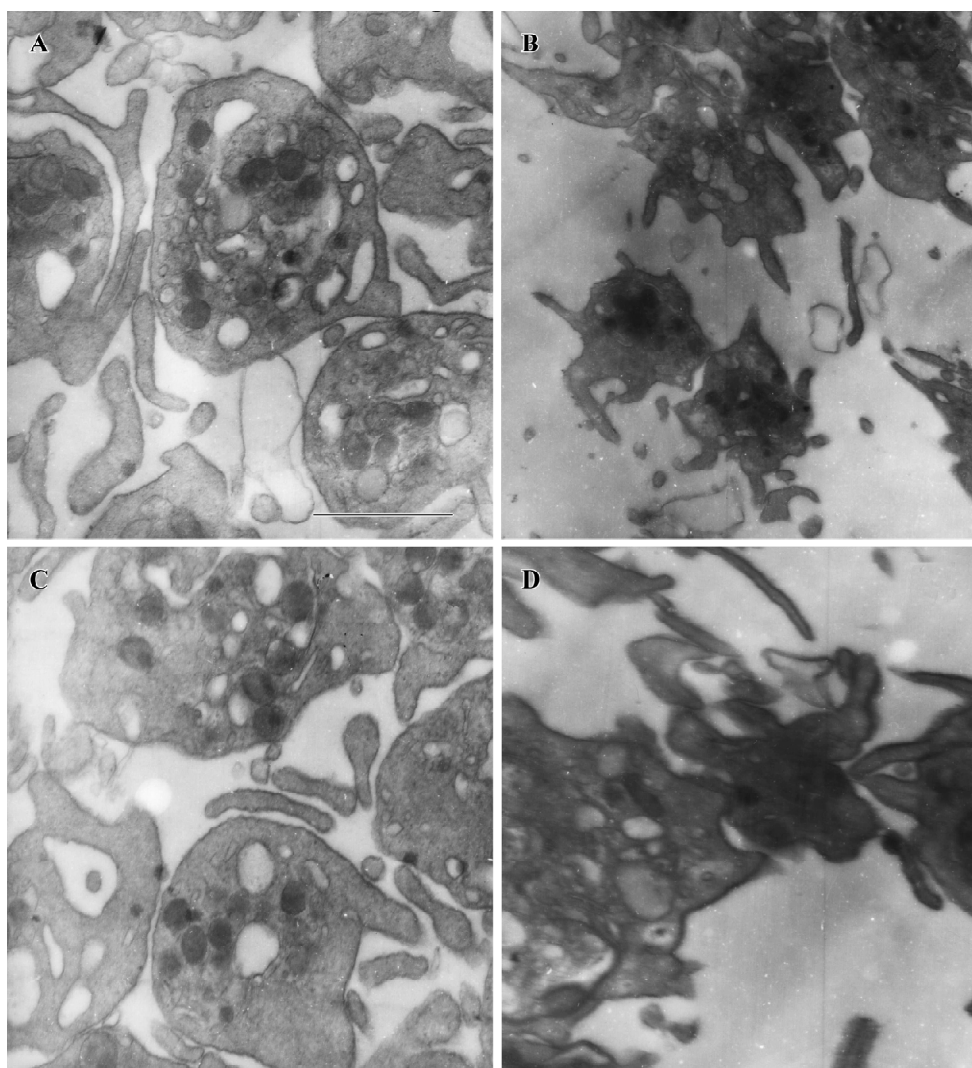


Figure 4. Effects of PGA1 on thrombin-induced ultrastructural changes. Human platelets were washed and pre-incubated with PGA1 (20–80 μmol/L) or vehicle (0.8% ethanol). Activation of platelets was induced by incubation with thrombin (0.25 U/mL) for 5 min. Platelets were then fixed for electron microscopic examination. (A) Normal control; (B) platelets activated by thrombin (0.25 U/mL); (C) PGA1 80 μmol/L+ thrombin (0.25 U/mL); (D) vehicle+ thrombin (0.25 U/mL). After activation with thrombin (0.25 U/mL), there was a reduction in the density of granules inside the platelets due to release reaction to thrombin stimulation and robust formation of pseudopodia. These morphological changes were inhibited by pretreatment with PGA1. Scale bar=1 μm.

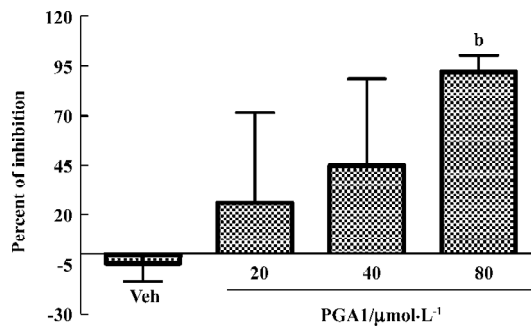


Figure 5. Effects of PGA1 on production of TXB₂ in platelets. Human platelets were washed and pre-incubated with PGA1 (20–80 μmol/L) or vehicle (0.8% ethanol). The production of TXB₂ was determined by radioimmunoassay after stimulation with thrombin (0.25 U/mL) for 5 min. *n*=6. Bars represent data are mean±SD. *P*<0.05 vs vehicle control.

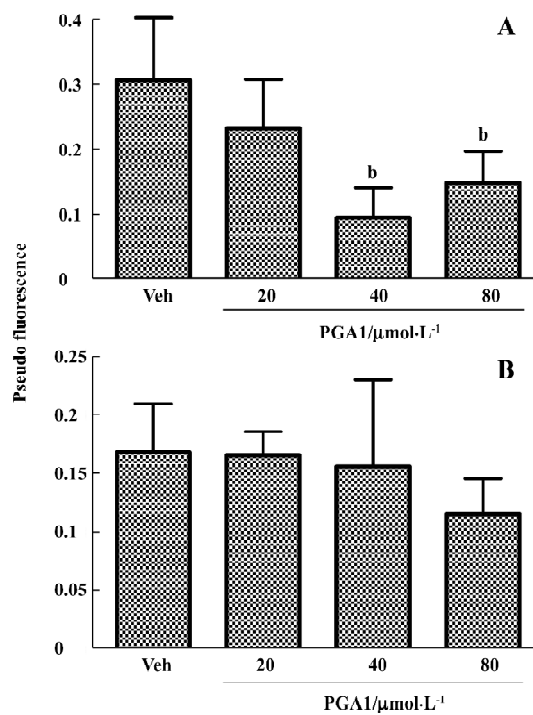


Figure 6. Effects of PGA1 on free calcium concentration in platelets. Human platelets were washed and pre-incubated with PGA1 (20–80 μmol/L) or vehicle (0.8% ethanol). Thrombin (0.25 U/mL) was added to the platelet suspension. The intraplatelet free calcium concentrations were measured with Fluo-3 AM FCM assay. (A) Effects of PGA1 on intracellular calcium concentration after thrombin treatment. (B) Effects of PGA1 on intracellular calcium concentration after thrombin treatment in calcium free buffer. *n*=6. Data are mean±SD. *P*<0.05 vs vehicle treatment.

The pharmacological actions of PGA1 with respect to its anti-inflammatory, anti-neoplastic, and anti-viral activities

have been well documented. Recently, our *in vivo* and *in vitro* studies suggested that PGA1 has neuroprotective actions^[12,14,15], but the mechanisms underlying its neuroprotective effects have not been fully understood.

Platelet aggregation, adhesion to endothelial cells and release of TXA₂ play important roles in the formation of thrombosis. TXA₂ causes constriction of blood vessels, which could further reduce blood flow in the brain. Inhibitors of platelet function are commonly used in therapeutic approaches in the treatment of brain ischemia^[24,25]. Previous studies have found inconsistent results with respect to the effect of PGA1 on platelet aggregation. PGA1 has been reported to have no effect on aggregation of rabbit platelets induced by ADP, or formation of thrombus in rats^[26,27], or weakly inhibited platelet aggregation at high concentrations^[28]. Since platelets from different species commonly respond differently to platelet inhibitors, we thought that human platelets might be sensitive to PGA1. The present studies thus examined the effects of PGA1 on the functions of human platelets. The results showed that PGA1 significantly inhibited thrombin-induced human platelet aggregation, the release of 5-HT, and production of TXA₂. Similar inhibitory results were obtained with respect to platelet aggregation induced by collagen and ADP with less potency. PGA1 inhibited the thrombin-induced increase in cytosolic [Ca²⁺]. These actions could be part of mechanisms by which PGA1 inhibits platelet activation. It has been well documented that in almost every step of platelet activation, calcium is required. We found that the inhibitory effect of PGA1 on rises in calcium concentration was not seen in the absence of extracellular calcium, suggesting that PGA1 may inhibit calcium influx. However, additional studies are required to confirm whether PGA1 blocks calcium entry into platelets. In our study of the effect of PGA1 on thrombin-induced 5-HT release and increases in intraplatelet calcium concentration, the medium dose of PGA1 (40 μmol/L) was less effective. The reason for this is unknown at present. Also, the effects of PGA1 on calcium entry into platelets need to be further investigated under other experimental conditions.

In addition to the above-discussed pharmacological actions, PGA1 inhibits IκB-α degradation and NF-κB activation, induces expression of heat shock proteins and activates PPAR-γ. The significance of these pharmacological actions of PGA1 with respect to the ability of PGA1 to inhibit platelets and neuronal injury in ischemia need to be further investigated.

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