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Morphine-Potentiated Platelet Aggregation in in vitro and Platelet Plug Formation in in vivo Experiments

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Key Words
Morphine · Thrombosis · α_2-Adrenoceptors · Cyclic AMP · Na^+/H^+ exchanger

Abstract
The detailed mechanisms underlying morphine-signaling pathways in platelets remain obscure. Therefore, we systematically examined the influence of morphine on washed human platelets. In this study, washed human platelet suspensions were used for in vitro studies. Furthermore, platelet thrombus formation induced by irradiation of mesenteric venules with filtered light in mice pretreated with fluorescein sodium was used for an in vivo thrombotic study. Morphine concentration dependently (0.6, 1, and 5 μM) potentiated platelet aggregation and the ATP release reaction stimulated by agonists (i.e., collagen and U46619) in washed human platelets. Yohimbine (0.1 μM), a specific α_2-adrenoceptor antagonist, markedly abolished the potentiation of morphine in platelet aggregation stimulated by agonists. Morphine also potentiated phosphoinositide breakdown and intracellular Ca^{2+} mobilization in human platelets stimulated by collagen (1 μg/ml). Moreover, morphine (0.6–5 μM) markedly inhibited prostaglandin E_1 (10 μM)-induced cyclic AMP formation in human platelets, while yohimbine (0.1 μM) significantly reversed the inhibition of cyclic AMP by morphine (0.6 and 1 μM) in this study. The thrombin-evoked increase in pH_i was markedly potentiated in the presence of morphine (1 and 5 μM). Morphine (2 and 5 mg/g) significantly shortened the time require to induce platelet plug formation in mesenteric venules. We concluded that morphine may exert its potentiation in platelet aggregation by binding to α_2-adrenoceptors in human platelets, with a resulting inhibition of adenylate cyclase, thereby reducing intracellular cyclic AMP formation followed by increased activation of phospholipase C and the Na^+/H^+ exchanger. This leads to increased intracellular Ca^{2+} mobilization, and finally potentiation of platelet aggregation and of the ATP release reaction.

Introduction

Narcotic analgesics, of which morphine is the prototype, produce a large variety of pharmacological responses by interacting with opioid receptors in the nervous system. Morphine is one of the agonist ligands for

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multiple opioid receptors, and therefore the majority of its effects are attributed to specific drug-receptor interactions.

The cardiovascular effects of morphine have been extensively studied [16]. Intravenous administration of morphine in anesthetized rats and dogs produces dose-dependent hypotension. The cardiovascular action of morphine in humans depends on the physiological state of the subject. Intravenous administration of 10–30 mg of morphine to supine, healthy subjects does not consistently change blood pressure [16]. However, a similar dose given to healthy subjects in a head-up position produces a fall in blood pressure and bradycardia, and in some subjects, syncope [16]. Furthermore, in conscious patients undergoing coronary artery catheterization, morphine can decrease the cardiac index, stroke volume, and myocardial contractility, sometimes causing a fall in blood pressure, even when subjects are supine [1]. Taken together, cardiovascular mechanism(s) of morphine may involve interactions of morphine with its tissue receptors or secondary changes in activity of the autonomic nervous system or release of mediators such as histamine or serotonin [16].

Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. Initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Thus, platelet aggregation may play a crucial role in atherothrombotic processes. Indeed, antiplatelet agents (e.g., ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients [12]. On the other hand, studies of the effects of morphine on platelets are relatively rarely compared with studies of its effects on blood vessels. Gryglewski et al. [10] presented data to indicate that morphine antagonizes prostaglandin E_1 (PGE_1)-mediated inhibition of human platelet aggregation; the antagonism between PGE_1 and morphine is similar to that described in rat brain homogenate and has also been demonstrated in human platelets [7]. They also speculated that adenylate cyclase may be the common target for PGE_1 and morphine in platelets as it is in neurons [7]. Furthermore, Ballesta and Orts [2] also demonstrated that morphine and semisynthetic antagonist derivatives inhibit the binding of [^{3}H]-clonidine to human platelet α_2-adrenoceptors, indicating that morphine binds to human platelets through α_2-adrenoceptors. However, the detailed mechanisms underlying the morphine-signaling pathways in platelets still remain obscure. We therefore systematically examined the influence of morphine on washed human platelets, and utilized the findings to characterize the mechanisms involved in this influence. In addition, we previously reported that platelet thrombi were induced by irradiation with filtered light in the microvasculature of mice pretreated with fluorescein sodium [25]. Therefore, we used this model to further evaluate the potentiation of platelet plug formation by morphine in vivo.

Materials and Methods

Materials

Collagen (type I, bovine Achilles tendon), sodium citrate, luciferin-luciferase, indomethacin, fluorescein sodium, nigericin, Dowex-1 (100–200 mesh; X_8, chloride form), myoinositol, PGE_1, nitroglycerin, apyrase, heparin, morphine, and yohimbine were purchased from Sigma (St. Louis, Mo., USA). Fura 2-AM and BCECF-AM were purchased from Molecular Probe (Eugene, Oreg., USA). U46619 was purchased from Biomol Research Lab. (Plymouth Meeting, Pa., USA). Myo-2-[^{3}H]-inositol was purchased from Amersham (UK). A cyclic AMP EIA kit was purchased from Cayman (Ann Arbor, Mich., USA).

Preparation of Human Platelet Suspensions

Human platelet suspensions were prepared as previously described [26]. In brief, blood was collected from healthy volunteers following informed consent. The subjects had taken no medications during the preceding 2 weeks. Blood was mixed with acid/citrate/glucose (9:1, v/v). After centrifugation at 120 *g* for 10 min at room temperature, the supernatant (platelet-rich plasma; PRP) was supplemented with prostaglandin E_1 (PGE_1) (0.5 μM) and heparin (6.4 IU/ml), then incubated for 10 min at 30°C and centrifuged at 500 *g* for 10 min. The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (3.5 mg/ml) and adjusted to a concentration of 4.5×10^8 platelets/ml. The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Platelet Aggregation

The turbidimetric method was applied to measure platelet aggregation [4], using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (4.5×10^8 platelets/ml, 0.4 ml) were prewarmed to 37°C for 2 min (stirring at 1,200 rpm) in a silicone-treated glass cuvette. Morphine (0.6–5 μM) was added 3 min before the addition of platelet-aggregation inducers. The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light transmission units. When measuring ATP release, 20 µl of the luciferin/luciferase mixture was added 1 min before addition of the agonists, and ATP release was compared with that of the control. For the other experiments, platelets (4.5×10^8/ml, 0.4 ml) were preincubated with morphine (5 μM) for 30 min followed by two washings with Tyrode's solution. The resuspended platelets were incubated with morphine (5 μM) for 3 min followed by the addition of collagen (1 µg/ml) to trigger platelet aggregation for 6 min.

Labeling of Membrane Phospholipids and Measurement of the Production of [^{3}H]-Inositol Phosphates

The method was carried out as previously described [15]. Briefly, citrated human PRP was centrifuged, and the pellets were suspended in Tyrode's solution containing [^{3}H]-inositol (75 µCi/ml). Platelets

were incubated for 2 h followed by centrifugation, and were finally resuspended in Ca^{2+}-free Tyrode's solution (5×10^8/ml). Morphine (1 and 5 μ*M*) was preincubated with 1 ml of loaded platelets at room temperature for 3 min, and collagen (1 μg/ml) was then added to trigger aggregation. The reaction was stopped after 6 min, and the samples were centrifuged at 1,000 *g* for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [^{3}H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of Platelet $[Ca^{2+}]_i$ Mobilization by Fura 2-AM Fluorescence

Citrated whole blood was centrifuged at 120 *g* for 10 min. The supernatant was protected from light and incubated with Fura 2-AM (5 μ*M*) at 37°C for 1 h. Human platelets were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 m*M*. The $[Ca^{2+}]_i$ rise was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Japan) at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. $[Ca^{2+}]_i$ was calculated from the fluorescence, using 224 n*M* as the Ca^{2+}-Fura 2 dissociation constant [11].

Estimation of Platelet Cyclic AMP

The method of Karniguian et al. [18] was followed. Platelet suspensions (4.5×10^8/ml) were preincubated with morphine (0.6, 1, and 5 μ*M*), or morphine (0.6 and 1 μ*M*) combined with yohimbine (0.1 μ*M*) for 3 min followed by the addition of PGE_1 (10 μ*M*) for 6 min at 37°C. Incubation was stopped by the addition of 10 m*M* EDTA and by immediately boiling the mixture for 5 min. The reaction mixtures were cooled to 4°C, and the precipitated protein was collected as sediment after centrifugation in an Eppendorf centrifuge. The supernatant (400 μl) was freeze-dried, and the residue was dissolved in 100 ml of distilled water. To determine the cyclic AMP concentration, 50 μl of the reconstituted supernatant was acetylated and used for EIA as described by the manufacturer (Cayman).

Platelet pH_i Measurement

Platelet pH_i was measured with the fluorescent probe, BCECF-AM, according to a previously described method [28]. Washed platelets were incubated with 5 μ*M* BCECF-AM at 37°C for 30 min in an Hepes-buffered solution (HBS) and then centrifuged at 450 *g* for 8 min. Washed pellets were finally suspended in buffer and adjusted to 4.5×10^8/ml. Leukocyte contamination was less than 0.01%. Aliquots of this platelet suspension (50 μl) were transferred into a cuvette containing 2 ml HBS (pH 7.4, 37°C) in a dual-excitation wavelength spectrofluorometer (CAF 110, Jasco). Fluorescence signals for BCECF-AM were recorded at 430- and 490-nm excitation wavelengths with an emission wavelength of 530 nm (5-nm slit). The background fluorescence of platelets was subtracted from each reading. Calibration was carried out after diluting the BCECF-loaded platelets in a high-K^+ buffer (120 m*M* KCl, 30 m*M* NaCl, 1 m*M* $MgSO_4$, and 5 m*M* glucose) in the presence of nigericin (0.2 mg/ml), as described by Horne et al. [14]. In all experiments, platelets were stimulated by thrombin (0.03 U/ml) to trigger the Na^+/H^+ exchanger.

Fluorescein Sodium-Induced Platelet Thrombi in Mesenteric Microvessels of Mice

As we previously described [25], male ICR strain mice (20–24 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After a tracheotomy was performed, an external jugular vein was cannulated with polyethylene tubing (PE-10) for administration of the dye and drug (by i.v. bolus), while additional tubing was cannulated through the femoral artery to monitor blood pressure. A segment of the small intestine with its mesentery attached was loosely exteriorized through a midline incision in the abdominal wall and was placed onto a transparent culture dish for microscopic observation. Frequent rinsing of the mesentery with warm saline solution maintained at 37 ± 0.5°C was performed to prevent the mesentery from drying out. Microvessels in the mesentery were observed under transillumination from a halogen lamp. Venules with diameters of 30–40 μm were selected for irradiation to produce a microthrombus. In the epi-illumination system, light from a 100-watt mercury lamp was filtered through a filter (B-2A, Nikon, Tokyo, Japan) with a dichromic mirror (DM 510, Nikon). Filtering the light eliminated wavelengths below 520 nm, and this light was used to irradiate a microvessel (the area of irradiation was about 100 μm in diameter on the focal plane) through an objective lens (20×). Doses of fluorescein sodium used were 10 and 20 μg/kg. The injected volume of the test solution or normal saline (control) was smaller than 50 μl. Five minutes after administration of the dye, a timer and irradiation with filtered light were simultaneously started, and platelet aggregation was observed on a monitor. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured. The elapsed time for inducing platelet plug formation was repeatedly measured every 5 min during irradiation of the venules.

Statistical Analysis

The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed using analysis of variance (ANOVA). If this analysis indicated significant differences in the group means, then each group was compared by the Newman-Keuls method. A p value <0.05 was considered statistically significant.

Results

Effect of Morphine on Platelet Aggregation in Washed Human Platelets

Morphine concentration dependently (0.6–5 μ*M*) potentiated platelet aggregation stimulated by collagen (1 μg/ml) and U46619 (0.5 μ*M*), a prostaglandin endoperoxide analogue compound, in washed human platelet suspensions (fig. 1a, 2) and PRP (data not shown). It also similarly potentiated thrombin (0.03 U/ml)- and ADP (2 μ*M*)-induced platelet aggregation in the presence of fibrinogen (200 μg/ml) (data not shown). Furthermore, morphine also potentiated the ATP release reaction when stimulated by agonists (i.e., U46619) (fig. 1a). At the same concentrations, morphine showed a lower activity of potentiation for U46619-induced platelet aggregation (fig. 2). Platelets were preincubated with morphine (5 μ*M*) for 30 min followed by two washings with Tyrode's solution. We found that morphine (5 μ*M*) also

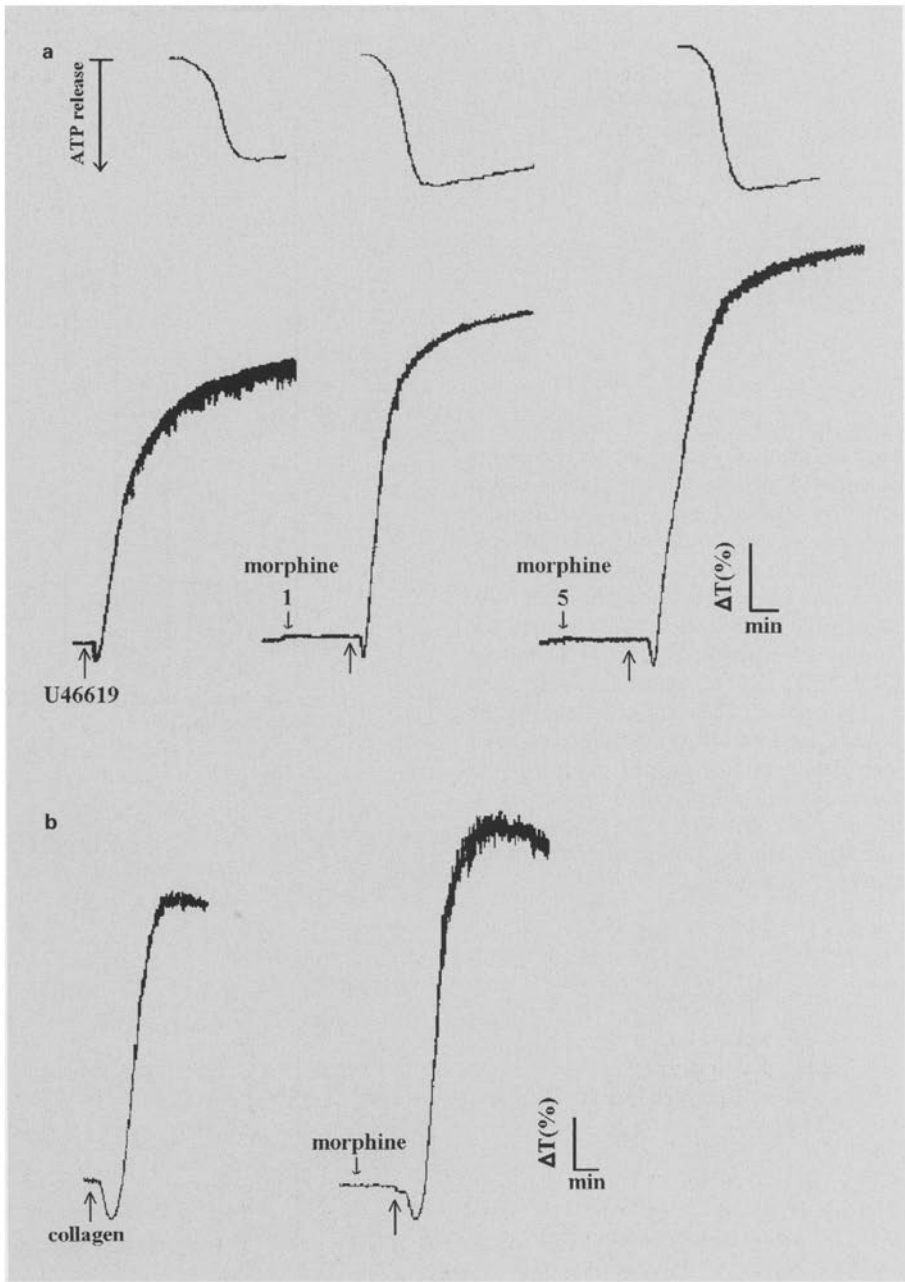

Fig. 1. Typical patterns of the potentiation by morphine of U46619 (0.5 μ*M*)-induced platelet aggregation and the ATP release reaction (**a**), and the tolerant effect of morphine on collagen (1 μg/ml)-induced platelet aggregation in washed human platelets (**b**). **a** Platelets (4.5×10^8/ml) were preincubated with morphine (1 and 5 μ*M*) at 37°C for 3 min, then U46619 (0.5 μ*M*) was added to trigger aggregation (lower tracings) and ATP release (upper tracings). A luciferin-luciferase mixture (20 μl) was added 1 min before the measurement of ATP release. **b** Platelets (4.5×10^8/ml) were preincubated with morphine (5 μ*M*) for 30 min followed by two washings with Tyrode's solution. The resuspended platelets were incubated with morphine (5 μ*M*) for 3 min followed by the addition of collagen (1 μg/ml) to trigger platelet aggregation. The profiles are representative examples of seven similar experiments.

induced a potentiation curve of platelet aggregation similar to that induced by collagen (1 μg/ml) (fig. 1b). These results indicate that morphine can concentration dependently potentiate agonist-induced platelet aggregation; however, it did not significantly induce a tolerant reaction in platelets.

On the other hand, Ballesta and Orts [2] reported that morphine binds to α_2-adrenoceptors in human platelets. Therefore, we decided to evaluate whether or not morphine-potentiated agonist-induced platelet aggregation is mediated by α_2-adrenoceptors. As shown in figure 2, yohimbine (0.1 μ*M*), a specific α_2-adrenoceptor antagonist [17], markedly abolished the potentiation induced by morphine (1 and 5 μ*M*); however, by itself, it did not affect the aggregation stimulated by agonists (data not shown). This result indicates that the potentiation of morphine is due, at least partly, to the mediation of α_2-adrenoceptors in human platelets.

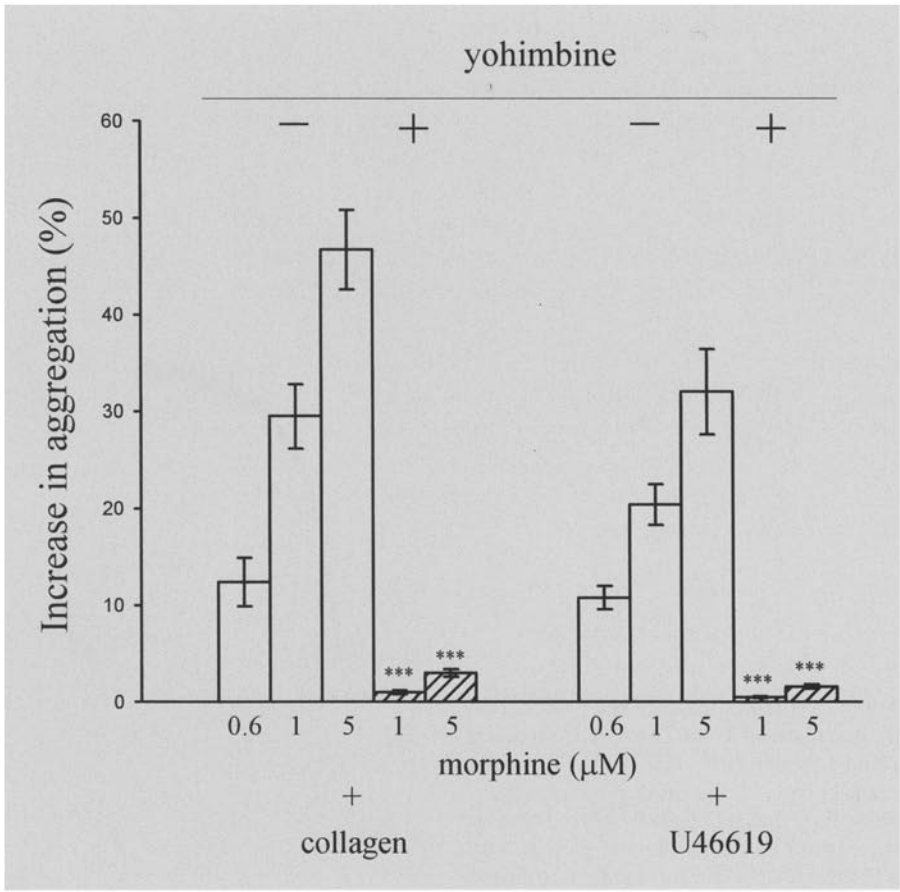

Fig. 2. Effect of yohimbine on morphine-potentiated agonist-induced platelet aggregation in washed human platelets. Platelet suspensions (4.5 × 10^8/ml) were preincubated with or without yohimbine (0.1 μ*M*) for 3 min, followed by the addition of morphine (0.6, 1, and 5 μ*M*) with stirring for 3 min, after which collagen (1 μg/ml) or U46619 (0.5 μ*M*) was added to trigger platelet aggregation. Data are expressed as the percent increase in aggregation compared with the individual agonist group (in the absence of morphine) and are presented as the means ± SEM (n = 7). *** $p < 0.001$ compared with the individual group (in the absence of yohimbine).

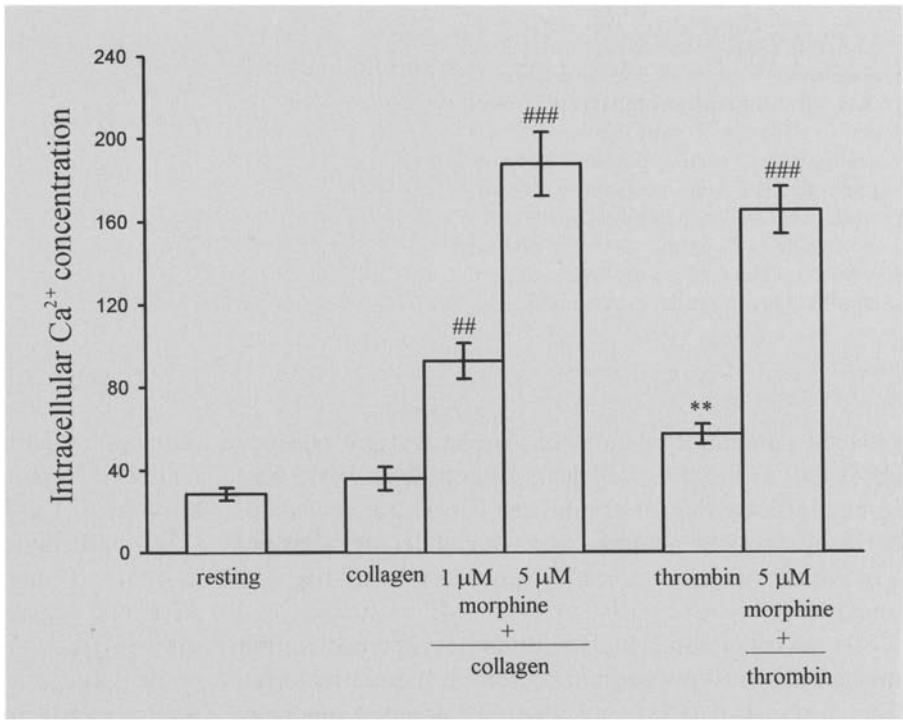

Fig. 3. Effect of morphine on agonist-induced intracellular Ca^{2+} mobilization in Fura 2-AM-loaded human platelets. Platelets were preincubated with Fura 2-AM (5 μ*M*) for 30 min, followed by the addition of collagen (1 μg/ml) or thrombin (0.03 U/ml) in the absence or presence of morphine (1 and 5 μ*M*), which was added 3 min prior to the addition of agonists. Data are presented as the means ± SEM (n = 4). ** $p < 0.01$ compared with the resting group; ## $p < 0.01$ and ### $p < 0.001$ as compared with the collagen group.

Effect of Morphine on $[Ca^{2+}]_i$ Mobilization in Human Platelets

Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. Collagen did not significantly evoke an increase in $[Ca^{2+}]_i$ in human platelets (35.8 ± 5.7 n*M*) at a lower concentration (1 μg/ml). However, morphine (1 and 5 μ*M*) markedly potentiated collagen (1 μg/ml), evoking an increase in $[Ca^{2+}]_i$ in human platelets (1 μ*M*, 92.5 ± 8.7 n*M*; 5 μ*M*, 187.4 ± 15.3 n*M*) (fig. 3). On the other hand, morphine also markedly potentiated thrombin (0.03 U/ml)-induced $[Ca^{2+}]_i$ mobilization in human platelets (fig. 3). In contrast, morphine (10 μ*M*) alone did not evoke a significant increase in $[Ca^{2+}]_i$ (data not shown). This result suggests that morphine potentiates $[Ca^{2+}]_i$ mobilization in human platelets stimulated by agonists (i.e., collagen).

Effect of Morphine on Phosphoinositide Breakdown in Human Platelet Suspensions

Phosphoinositide breakdown occurs in platelets activated by many different agonists [5]. In this study, we found that collagen (1 μg/ml) induced the rapid formation of radioactive IP, IP_2, and IP_3 in human platelets loaded with [^{3}H]-inositol. We only measured [^{3}H]-IP formation as an index of total inositol phosphate formation. As shown in figure 4, the addition of collagen (1 μg/ml) resulted in a rise in IP formation of about 1.4-fold compared to that in resting platelets. In the presence of morphine (5 μ*M*), the radioactivity of IP formation in collagen-stimulated human platelets was increased. These results indicate that morphine increases phosphoinositide breakdown in human platelets stimulated by collagen.

Effect of Morphine on Cyclic AMP Levels in Human Platelets

As shown in table 1, the level of cyclic AMP in unstimulated platelets was low (41.7 ± 6.9 pmol/ml). Addition of PGE_1 (10 μ*M*) increased the cyclic AMP level to 217.5 ± 19.5 pmol/ml. When platelet suspensions were preincubated with various concentrations of morphine (0.6, 1, and 5 μ*M*) for 3 min followed by the addition of PGE_1 (10 μ*M*), we found that morphine markedly inhibited PGE_1-induced cyclic AMP formation in human platelets (table 1). Yohimbine (0.1 μ*M*) significantly reversed the inhibition of cyclic AMP formation by morphine (0.6 and 1 μ*M*) in the presence of PGE_1 (10 μ*M*). We also performed similar studies measuring GMP responses, and found that morphine (5 μ*M*) did not significantly inhibit nitroglycerin (10 μ*M*)-induced cyclic GMP formation in human platelets (data not shown). These results indicate

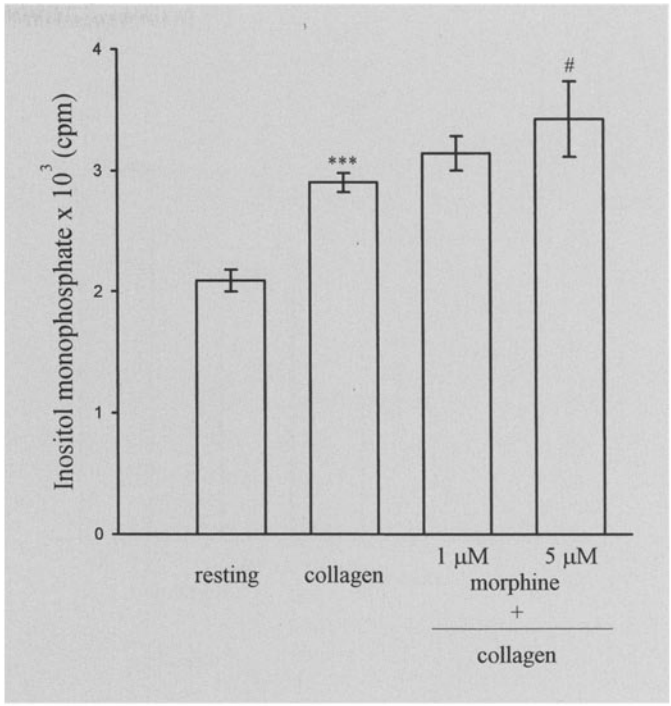

Fig. 4. Effect of morphine on collagen-induced inositol monophosphate formation in human platelet suspensions. Platelets were labeled with [^{3}H]-inositol and stimulated with collagen (1 μg/ml) in the presence (1 and 5 μ*M*) or absence of morphine. Data are presented as the means ± SEM (n = 4). *** $p < 0.001$ compared with the resting group; # $p < 0.05$ compared with the collagen group.

Table 1. Effect of yohimbine on morphine inhibition of PGE_1-induced cyclic AMP formation in washed human platelets

Treatment	Cyclic AMP, pmol/ml
Resting	41.7±6.9
PGE_1 (10 μ*M*)	217.5±19.5[a]
PGE_1 (10 μ*M*)	
+ morphine (0.6 μ*M*)	98.4±0.3[b]
+ morphine (1.0 μ*M*)	61.2±5.3[b]
+ morphine (5.0 μ*M*)	62.7±4.5[b]
+ yohimbine + morphine (0.6 μ*M*)	187.4±14.6
+ yohimbine + morphine (1.0 μ*M*)	174.8±18.2

Platelet suspensions (4.5 × 10^8/ml) were preincubated with morphine (0.6, 1, and 5 μ*M*, or morphine (0.6 and 1 μ*M*) combined with yohimbine (0.1 μ*M* for 3 min followed by the addition of PGE_1 (10 μ*M*) for 6 min at 37 °C. Addition of PGE_1 in platelet suspensions represents the positive control. The data are presented as the means ± SEM (n = 7).

[a] $p < 0.001$ compared with the resting group.

[b] $p < 0.001$ compared with the PGE_1 group.

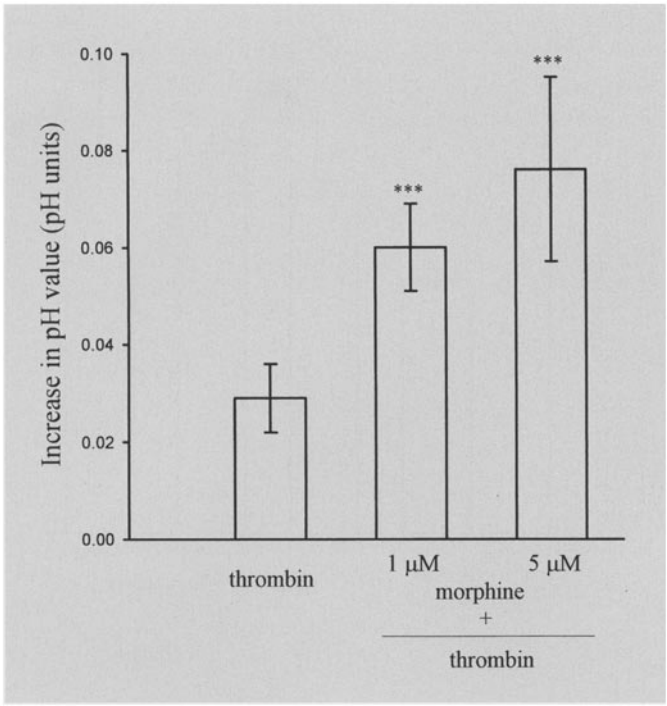

Fig. 5. Effect of thrombin-triggered intracellular pH increase on BCECF-AM-loaded platelets. Platelet suspensions (4.5×10^8/ml) were preincubated with BCECF-AM (5 μ*M*) at 37°C for 30 min, followed by the addition of thrombin (0.03 U/ml) to trigger intracellular alkalinization in the presence or absence of morphine (1 and 5 μ*M*). Profiles are representative examples of five similar experiments. Data are presented as the means ± SEM (n = 5). *** $p < 0.001$ compared with the thrombin-treated group without morphine.

that morphine inhibition of cyclic AMP formation may be mediated, at least partly, through α_2-adrenoceptors.

Effect of Morphine on Thrombin-Evoked pH_i Changes in Platelets

Thrombin can trigger more pronounced pH_i changes in human platelets. Therefore, we used thrombin instead of collagen as the agonist to explore the potentiation of morphine on pH_i changes in platelets. Figure 5 shows pH_i changes triggered by thrombin (0.03 U/ml) in BCECF-AM-loaded platelets. The resting platelet pH_i value was about 7.11 ± 0.02 (n = 5) in washed human platelets. Addition of thrombin (0.03 U/ml) resulted in an increase in BCECF fluorescence equivalent to an increase in pH_i values of about 0.029 ± 0.007 (fig. 5). This thrombin-evoked increase in pH_i values was markedly potentiated in the presence of morphine (1 μ*M*, 0.060 ± 0.009; 5 μ*M*, 0.076 ± 0.019) (fig. 5).

Effect of Morphine on Thrombus Formation in Microvessels of Mice Pretreated with Fluorescein Sodium

The latency period for inducing platelet plug formation was shortened as the administered dose of fluorescein sodium increased. When 10 and 20 μg/kg of fluorescein sodium were given, the occlusion time required was 217 ± 19 and 145 ± 15 s, respectively (fig. 6). Morphine potentiated platelet aggregation induced by agonists; therefore, we further examined its effect on the formation of platelet-rich thrombi in this model. When morphine was administered at 2 and 5 mg/g in mice pretreated with 10 μg/kg of fluorescein sodium, the occlusion time was concentration dependently shortened. In contrast, morphine (2 mg/g) did not significantly shorten the occlusion time of fluorescein sodium (20 μg/kg)-induced platelet plug formation until it was administered at 5 mg/g (fig. 6). Morphine also exhibited potentiation in arterioles (data not shown). However, arterioles sometimes showed slight vasoconstriction when fluorescein sodium was irradiated [26], thus venules were chosen for induction of platelet plug formation in this study.

On the other hand, aspirin exhibited antithrombotic activity in this experiment. When 150 μg/g of aspirin was administered, the occlusion time was not significantly prolonged. But with 250 μg/g of aspirin, the occlusion time to the formation of a fluorescein sodium (10 μg/kg)-induced platelet plug was prolonged (fig. 6). However, aspirin (250 μg/g) did not significantly prolong the occlusion time when 20 μg/kg of dye was used for pretreatment (fig. 6).

Discussion

The principal objective of this study was to describe the detailed mechanisms of the potentiation of agonist-induced human platelet aggregation by morphine. The most important finding in this study was the demonstration that morphine exerts a potentiation role on agonist-induced platelet aggregation that has not been described previously. This potentiation was directly proportional to the amount of morphine used. Matos et al. [21] reported that the minimal extracellular morphine concentration in the spinal cord required to produce a significant increase in the nociceptive threshold is approximately 100 pg/25 μl, which corresponds to a tissue concentration of about 10 mg/g of morphine. In this study, morphine was employed at concentrations which potentiated platelet aggregation induced by agonists at concentrations of

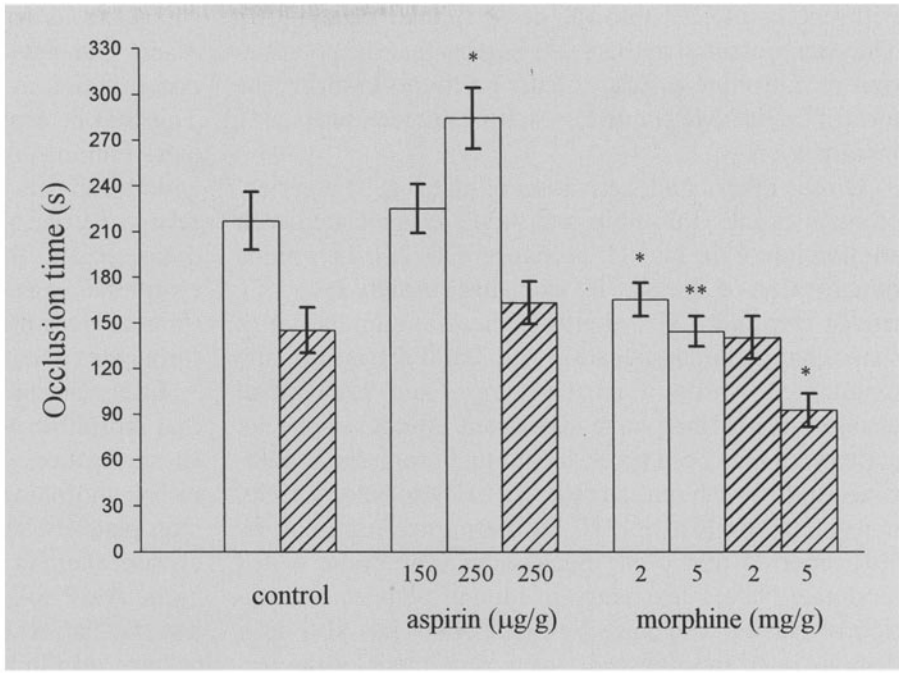

Fig. 6. Effect of morphine (2 and 5 mg/g) and aspirin (150 and 250 μg/g) on occlusion time for inducing thrombus formation upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (10 μg/kg, open bars; 20 μg/kg, hatched bars). Data are presented as the means ± SEM of occlusion time in seconds of platelet plug formation (n = 5). * $p < 0.05$ and ** $p < 0.01$ compared with the individual control group.

about 0.6–5 μ*M*. These results indicate that the concentrations of morphine employed to potentiate platelet aggregation in vitro are reasonable compared to the blood concentrations obtained during morphine-induced analgesia in in vivo studies. In this study, both platelet aggregation and the ATP release reaction induced by agonists (i.e., U46619) appeared to be potentiated in the presence of morphine. Therefore, we can infer that morphine may increase $[Ca^{2+}]_i$ release from intracellular Ca^{2+} storage sites (i.e., dense tubular systems or dense bodies), which is in accord with the concept that $[Ca^{2+}]_i$ release is responsible for the ATP release reaction [6].

Stimulation of platelets by agonists (i.e., collagen) resulted in phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5-trisphosphate and diacylglycerol [20]. There is strong evidence that inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from intracellular stores [3]. Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction. In this study, phosphoinositide breakdown of collagen-activated platelets was potentiated by morphine, suggesting that potentiation of platelet aggregation by morphine is related to the increase in phosphoinositide breakdown.

Gryglewski et al. [10] showed that morphine inhibits the anti-aggregating effect of PGE_1 on ADP- and adrenaline-induced platelet aggregation, and suggested that the inhibition by morphine is mediated through platelet adenylate cyclase activity. Local interactions between α_2-adrenoceptors and an opioid agonist or antagonist have been reported [9, 24]. Human platelets have been shown to have α_2-adrenoceptors in two affinity states: a high-affinity state (α_{2H}), and a low-affinity state (α_{2L}) [2]. [^{3}H]-yohimbine binds both the high- and low-affinity states [13]. Ballesta and Orts [2] demonstrated that morphine interacts with human platelet α_2-adrenoceptors that are in the high-affinity state. In this study, we found that morphine-potentiated agonist-induced platelet aggregation could be abolished by pretreatment with yohimbine (fig. 2b). We also found that yohimbine (0.1 μ*M*) significantly reversed the inhibition of cyclic AMP formation by morphine (0.6 and 1 μ*M*) in the presence of PGE_1 (10 μ*M*) (table 1). These results indicate that the effects of morphine may be due to binding with α_2-adrenoceptors in human platelets, resulting in potentiation of platelet aggregation. In many systems, α_2-adrenoceptors are coupled to the inhibition of adenylate cyclase through an inhibitory G protein termed G_i [29]. Therefore, reductions in intracellular concentrations of cyclic AMP thereby increase the activation of platelets. This inverse relationship of cyclic AMP in modulating platelet reactivity is well established [18]. In addition to inhibiting most platelet responses, elevated levels of cyclic AMP decrease $[Ca^{2+}]_i$

with uptake of Ca^{2+} into the dense tubular system [30]. The data presented in this study suggest that the potentiation of morphine is due, at least partly, to lowering the level of cyclic AMP (table 1), resulting in increased $[Ca^{2+}]_i$ mobilization.

On the other hand, activation of platelets by a variety of agonists (i.e., thrombin and ADP) is associated with stimulation of the Na^+/H^+ exchanger [19, 27]. This mode of activation of the Na^+/H^+ exchanger usually induces a rise in cytosolic Ca^{2+}, granule secretion, stimulation of shape change, and aggregation [19]. Basal pH_i is normally maintained within a narrow range, and even small changes in pH_i may have significant effects on platelet activity. In many cell types, including fibroblasts, hepatocytes, and smooth muscle cells, Na^+/H^+ exchange activity is regulated by $[Ca^{2+}]_i$ [22]. Furthermore, Kimura et al. [19] reported that cyclic nucleotides (i.e., cyclic AMP) modulate Na^+/H^+ exchange in human platelets. Inhibition of Na^+/H^+ exchange by cyclic AMP has also been demonstrated in other cells, such as epithelia of the urinary system and in osteoblast-like cells [8, 23]. Thus, an agent (i.e., parathyroid hormone or dopamine) known to stimulate adenylate cyclase in these cells can also inhibit the Na^+/H^+ exchanger [8, 23]. Relationships among phosphoinositide breakdown, $[Ca^{2+}]_i$, cyclic nucleotides, and the Na^+/H^+ exchanger may play an important role in mediating the antiplatelet activity of morphine.

Platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, potentiation of platelet aggregation by drugs may represent an increased possibility for these diseases. In this study, we further evaluated the potentiation of thrombus formation by morphine in vivo, and found that morphine significantly increased platelet plug formation. It shortened the occlusion time to thrombus formation induced by irradiation of fluorescein sodium in venules or arterioles. Since the light beam covered the entire microscopic field, a simultaneous observation of arterioles and venules was made. Our data reveal that platelet aggregation usually occurred first in the venules rather than in the arterioles. This may be explained by a higher flow velocity in arterioles, resulting in delayed adhesion of platelets to arteriolar endothelial cells. In this system, the occlusion time was related to the blood flow rate, size of the microvessel diameter, and the dose of fluorescein dye. In this study, morphine caused significant shortening of occlusion times in mice pretreated with fluorescein sodium mainly through its potentiation of platelet aggregation.

In conclusion, the observations in this study suggest that morphine potentiates agonist-induced human platelet aggregation. This may involve the following mechanisms: morphine activation of α_2-adrenoceptors in human platelets, with the resulting inhibition of adenylate cyclase, thereby reducing intracellular concentrations of cyclic AMP followed by activation of phospholipase C and the Na^+/H^+ exchanger. This leads to increased intracellular alkalinization and intracellular Ca^{2+} mobilization, and ultimately potentiation of platelet aggregation. Platelet aggregation plays a role in a variety of thromboembolic disorders, including myocardial infarction, atherosclerosis, and stroke. Results of this study imply that the abuse of morphine may increase the risk of thromboembolic-related disorders, and patients undergoing analgesic regimens with morphine may experience increased morbidity due to arterial thrombotic complications. However, the physiological relevance of a direct pro-aggregatory effect of morphine is unclear and remains to be further studied.

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