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Mechanism Involved in the Antiplatelet Activity of Naloxone in Human Platelets

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In this study, naloxone was tested for its antiplatelet activity in human platelet suspensions. In platelet suspensions (4.5×10^8/ml), naloxone (0.1-0.5 mM) significantly inhibited platelet aggregation and ATP-release stimulated by various agonists (i.e., thrombin, collagen, U46619, and ADP). Furthermore, naloxone (0.5 and 0.8 mM) dose-dependently inhibited the intracellular free Ca^{2+} rise of Fura 2-AM loaded platelets stimulated by collagen. Additionally, naloxone (0.5 and 1.0 mM) did not influence the binding of FITC-triflavin to platelet glycoprotein (GP) IIb/IIIa complex. On the other hand, naloxone (0.5 mM) markedly decreased the fluorescence of platelet membranes tagged with diphenylhexatriene (DPH). In addition, naloxone (0.1-0.5 mM) did not significantly affect cyclic-AMP levels in human washed platelets. It is concluded that the antiplatelet activity of naloxone may possibly be due to the induction of conformational changes in the platelet membrane and the inhibition of the intracellular Ca^{2+} ($[Ca^{2+}]i$) mobilization as well as the release reaction of platelets stimulated by agonists.

Naloxone, an opiate antagonist, can reverse narcotic coma and neuroleptic anesthesia (1). Naloxone has been demonstrated to be beneficial in the treatment not only of ischemic insults to the central nervous system but also of endotoxic shock, anaphylactic shock and adult respiratory distress syndrome (ARDS) (2,3). Pulmonary platelet trapping is thought to play an important role in the evolution of ARDS. Injection of endotoxin is known to decrease the number of circulating platelets and white blood cells, and to increase platelet aggregability (4). The decrease in the number of circulating platelets is accompanied by sequestration of platelets in the lung (5). In addition to platelet plugs in the lung, the release of platelet vasoactive substances from aggregating platelets may contribute to pulmonary vasoconstriction (6). This condition is in turn followed by increased pulmonary artery pressure and decreased cardiac output (5). Naloxone effectively blocks these effects (7), and also is effective in attenuating a decrease in blood pressure and improving survival after hemorrhagic and endotoxin shock (5). Furthermore, animals treated with naloxone show no increase in platelet aggregability, indicating the applicability of naloxone therapy for ARDS (8). Opiate antagonists exert considerable effects on cerebral circulation in both physiological and pathological conditions (9). The explanation for this phenomenon is unclear and various mechanisms including direct cerebral vasculature (10), interactions with neurotransmitters (9), interference with autoregulation and metabolic effects have been suggested (11).

Despite uncertainties about the pathogenic significance of alterations in blood platelet activity in cerebral circulatory disorders, the contribution of platelet aggregates to cerebral blood flow disturbances is experimentally well documented. There is evidence that blood platelets may possess opiate receptors and this fact implies the possibility of direct interaction between naloxone and blood platelets (12). Therefore, the experiments in this study were designed to evaluate the antiplatelet activity and try to elucidate the inhibitory mechanism of naloxone on platelet aggregation.

MATERIALS AND METHODS

Materials. Naloxone, human thrombin, ADP, epinephrine, collagen, DPH, prostaglandin E_1 (PGE_1), apyrase, EGTA and luciferase/luciferin mixture were purchased from Sigma (U.S.A.). Fura 2-AM and FITC were purchased from Molecular Probe Inc. (U.S.A.). *Trimeresurus flavoviridis* venom was purchased from Latoxan (France), and U46619 compound was obtained from Biomol. Res. Lab. (U.S.A.).

Preparation of human platelet suspensions. Human platelet suspensions were isolated from healthy humans as previously described

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(13). The washed platelets were finally suspended in Tyrode's solution and adjusted to about 4.5×10^8/ml.

Platelet aggregation. The turbidimetric method using a Lumi-Aggregometer (Payton, Canada) was used to measure platelet aggregation as described previously (13). In brief, platelet suspensions (0.4 ml) were prewarmed at 37 °C for 2 min and naloxone was then added 2 min before the addition of agonists. The extent of aggregation was expressed as the percentage of the control (in the absence of naloxone). The degree of aggregation was expressed in light-transmission units. While measuring ATP release, 20 μl of luciferase/luciferin mixture was added 1 min before the addition of agonists and ATP release was compared with that of the control.

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 incubated with Fura 2-AM (5 μM), and protected from light at 37°C for 1 h. Then, the human platelet suspensions was prepared as mentioned. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The $[Ca^{2+}]i$ rise was measured using a Fluorescence Spectrophotometer (CAF110, Jasco, Japan) at excitation and emission wavelengths of 340 and 500 nm, respectively. The $[Ca^{2+}]i$ was calculated from the fluorescence measured using 224 nM as the Ca^{2+}-Fura 2 dissociation constant (14).

Purification and fluorescence-labeling with triflavin. Triflavin, a specific fibrinogen receptor (GP IIb/IIIa complex) antagonist, was prepared as previously described (15). Fluorescence-conjugated triflavin, was also prepared as previously described (16). In brief, 20 μl aliquots of FITC solution was added to the triflavin solution, and this reaction was stopped by adding hydroxylamine (1.5 M). Finally, the concentration of FITC-triflavin was adjusted to 1 mg/ml.

Analysis of platelet surface GP IIb/IIIa complex by flow cytometer. Human platelet suspensions were prepared as stated above. Aliquots of platelet suspensions (4.5×10^8/ml) were preincubation with various concentrations of naloxone (0.5 and 1.0 mM) for 2 min. Then 2 μl of FITC-triflavin (1mg/ml) was added prior to the addition of collagen (5 μg/ml). Following the addition of collagen, the platelet suspension mixture was incubated for 8 min, and finally adjusted to 1 ml/tube with Tyrode's solution and assayed. Fluorescein-labeled platelets were assayed with a Flow Cytometer (Becton Dickinson, FACScan Sys.). Data were collected in a 256-channel resolution and 5,000 platelets were counted per experimental group. All experiments were repeated at least five times to ensure reproducibility.

Measurement of membrane fluidity by fluorescence probe. The fluorescence intensity in human platelets was measured as previously described (17). In brief, platelets (4.5×10^8/ml) were preincubated with naloxone (0.5 mM) for 2 min followed by the addition of DPH (0.5 μM) at 37 °C for another 30 min. The relative fluorescence intensity in platelets was measured in a fluorescence spectrophotometer (Hitachi F4500, Japan) at 37 °C.

Estimation of platelet cyclic-AMP. The method of Karniguian et al. (18) was followed. Platelet suspensions were warmed at 37 °C for 1 min, then PGE_1 (10 μM) or naloxone (0.1, 0.2 and 0.5 mM) were added and incubated for 3 min. The incubation was stopped by adding 10 mM EDTA and immediately boiling for 5 min. After cooling to 4 °C, the precipitated protein was sedimented by centrifugation in an Eppendorf centrifuge. The supernatant (400 μl) was freeze-dried and the residue was dissolved in 100 ml of distilled water. Fifty microliters of supernatant was used to determine the cyclic-AMP content by EIA kits following acetylation of the samples as described by the manufacturer.

Statistical analysis. The experimental results were expressed as the means ± S.E.M. and accompanied by the number of observations. Statistical significance was assessed by the Student's-t-test and a *P* value less than 0.05 was considered significant.

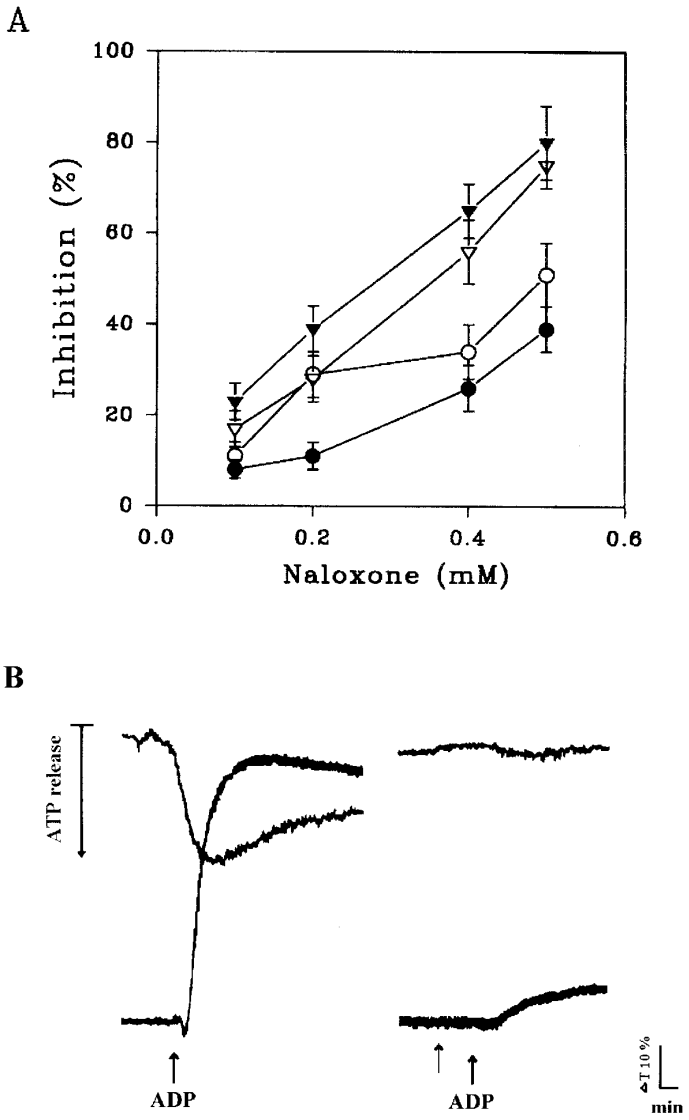

FIG. 1. Effect of various concentrations of naloxone on human platelet suspensions. (A) Dose-inhibition curve of naloxone on thrombin (0.1U/ml, ○)-, collagen (5 μg/ml, ●)-, U46619 (1μM, ▽)-, and ADP (10 μM, ▼)-induced aggregation of platelet suspensions. (B) Typical patterns of the antiplatelet effect of naloxone on ADP (10 μM)-induced aggregation and ATP release of platelet suspensions. Data are presented as percentage of the control (means ± S.E.M., n=4).

RESULTS

Effect of naloxone on platelet aggregation of human platelet suspensions. Naloxone dose-dependently inhibited platelet aggregation stimulated by thrombin (0.1 U/ml), collagen (5 μg/ml) and U46619 (1 μM) in human platelet suspensions (Fig. 1A). It similarly inhibited fibrinogen (200 μg/ml)-induced aggregation of ADP (10 μM)-stimulated platelets (Fig. 1A). Furthermore, naloxone also inhibited the ATP-release reaction when stimulated by these agonists (i.e., ADP) (Fig. 1B). The IC_{50} values of naloxone for platelet aggregation

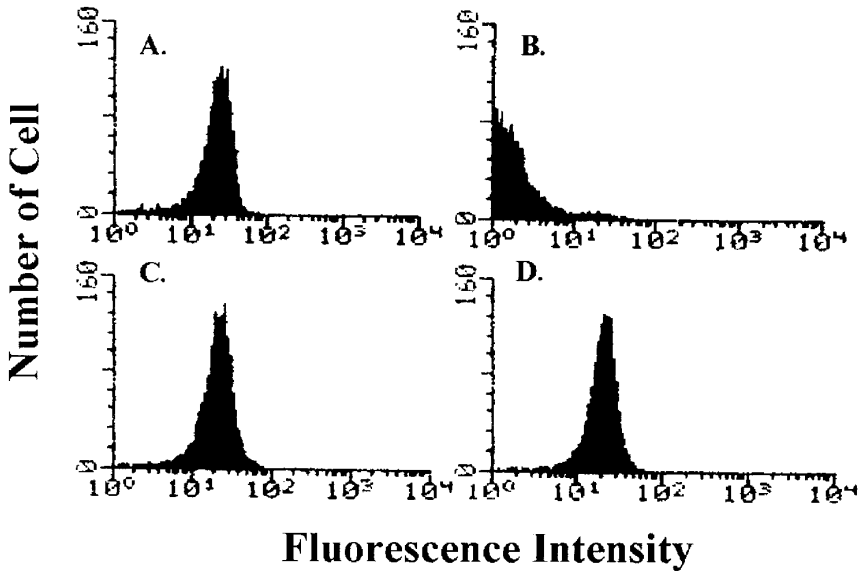

FIG. 2. Flow cytometric analysis of FITC-triflavin binding to human platelets in the absence or presence of various concentrations of naloxone (0.5 and 1.0 mM). (A) The solid lines represent the fluorescence profiles of FITC-triflavin (2 μg/ml) in the absence of naloxone; (B) in the presence of EDTA (10 mM) as the negative control; or (C) in the presence of naloxone (0.5 mM) and (D) (1.0 mM). The profiles are representative examples of five similar experiments.

induced by ADP, U46619, collagen and thrombin were estimated to be about 0.28, 0.34, 0.67 and 0.52 (mM), respectively. At the same concentration (0.5 mM), naloxone showed a lesser degree of antiplatelet activity induced by collagen and thrombin (Fig. 1A).

Effect of naloxone on FITC-triflavin binding to collagen-activated platelets. Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide, purified from *Trimeresurus flavoviridis* snake venom (13). It inhibits I^{125}-labeled fibrinogen binding to activated-platelets, suggesting that triflavin inhibits platelet aggregation through directly interfering with the fibrinogen binding to fibrinogen receptors associated with the GP IIb/IIIa complex (15). Nowadays, it is well established that the binding of fibrinogen to the GP IIb/IIIa complex appears to be the final common pathway for platelet aggregation. Therefore, we would like to further evaluate whether or not naloxone directly binds to platelet GP IIb/IIIa complex, leading to blockade of platelet aggregation induced by agonists.

As shown in Fig. 2A, FITC-triflavin (2 μg/ml) directly bound to collagen (5 μg/ml)-activated platelets (17.9 $\pm$ 1.3, n=4). The intensity of the fluorescence was markedly reduced in the presence of 10 mM EDTA (negative control, 1.52 $\pm$ 0.2, n=4) (Fig. 2B). At a concentration of 0.5 mM, naloxone did not significantly inhibit the FITC-triflavin binding to GP IIb/IIIa in platelet suspensions (16.6 $\pm$ 1.5, n=4), even at a higher concentration (1.0 mM) (17.2 $\pm$ 0.7, n=4) (Fig. 2C, D), indicating that naloxone's effect was not through binding to the GP IIb/IIIa complex, leading to the inhibition of platelet aggregation.

Effect of naloxone on $[Ca^{2+}]i$ mobilization. Free cytoplasmic Ca^{2+} concentrations of human platelets were measured by the Fura 2-AM loading method. As shown in Fig. 3, collagen (5 μg/ml) evoked a rise of $[Ca^{2+}]i$ from 32.6 $\pm$ 1.7 to 205.8 $\pm$ 10.2 (nM) in the presence of external Ca^{2+} (1mM). The collagen evoked rise of $[Ca^{2+}]i$ was markedly inhibited in the presence of naloxone (0.5 mM, 87.5 $\pm$ 6.8 nM, n=4; 0.8 mM, 48.5 $\pm$ 5.2 nM, n=4). Therefore, naloxone exerted a significant effect on $[Ca^{2+}]i$ mobilization of human platelets stimulated by collagen.

Effect of naloxone on platelet membrane fluidity. Platelet membrane fluidity was measured in DPH-labeled human platelets. It was found that naloxone is capable of direct interaction with platelet membranes as revealed by the fluorescence probe technique (Fig. 4). Addition of naloxone (0.5 mM) to the platelet suspensions tagged with DPH resulted in a considerable decrease in the relative fluorescence intensity (Fig. 4).

Effect of naloxone on cyclic-AMP level in human platelets. The level of cyclic-AMP in unstimulated platelets was low (0.27 $\pm$ 0.11 pmol/10^8 platelets). PGE_1 (10 μM) increased the cyclic-AMP level to 5.23 $\pm$ 1.05 pmol/10^8 platelets (n=5). Naloxone (0.1, 0.2 and 0.5 mM) resulted in no significant increase in platelet cyclic-AMP levels (0.49 $\pm$ 0.09, 0.47 $\pm$ 0.13, and 0.59 $\pm$ 0.18 pmol/10^8 platelets, n=5, respectively).

DISCUSSION

It has been suggested that naloxone can be used to inhibit cardiac arrhythmias resulting from coronary ar-

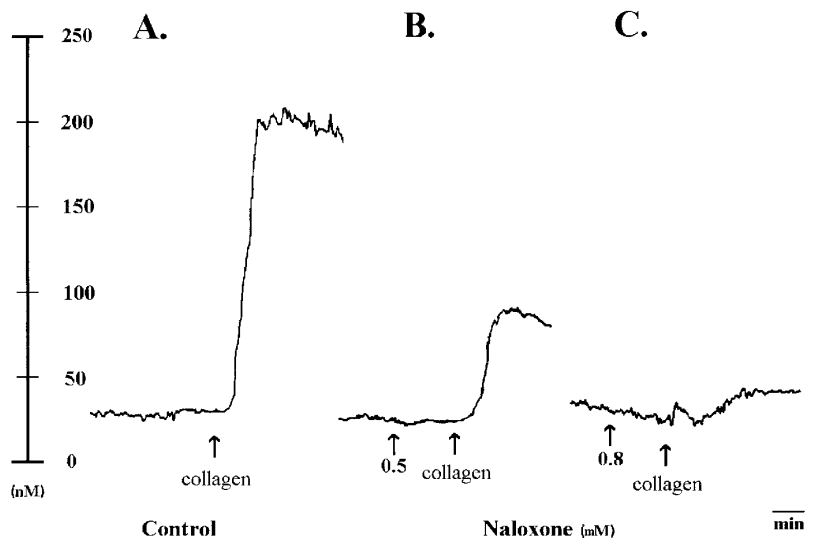

FIG. 3. Effect of naloxone on collagen-induced intracellular Ca^{2+} mobilization of Fura 2-AM loaded human platelets. Platelet suspensions were incubated with Fura 2-AM (5 μM) at 37 °C for 30 min, followed by the addition of collagen (5 μg/ml) in the absence or presence of naloxone (0.5 and 0.8 mM), which was added 2 min prior to the addition of collagen.

tery occlusion in rats and dogs (19). Furthermore, an early manifestation of endotoxin, hemorrhagic and traumatic shock results in an abrupt decrease of circulating platelets accompanied by increased platelet aggregability (4). This condition is subsequent to increase pulmonary vasocontraction and decreased cardiac output (5). Naloxone could effectively inhibit these effects (7).

As stated above, naloxone's multiple effects indicates either a broad spectrum of pharmacological activities of the drug or the existence of a basic pathophysiological mechanism common to all these disorders. There is reasonable evidence that blood platelets may be involved to some extent in the pathogenesis of all these pathological states, thus offering the possibility that the observed beneficial effect of naloxone administration is at least partly mediated by its effect on the activity of blood platelets. However, only a little information relating to the direct effects of naloxone on platelet aggregation has been reported.

The principal objective of this study was to ascertain the mechanisms involved in the inhibition of platelet aggregation by naloxone. In this study, naloxone significantly inhibited the platelet aggregation induced by agonists in human platelet suspensions (Fig. 1A). Concurrently, it also showed that ATP-release induced by ADP apparently were inhibited by naloxone (Fig. 1B). Further-

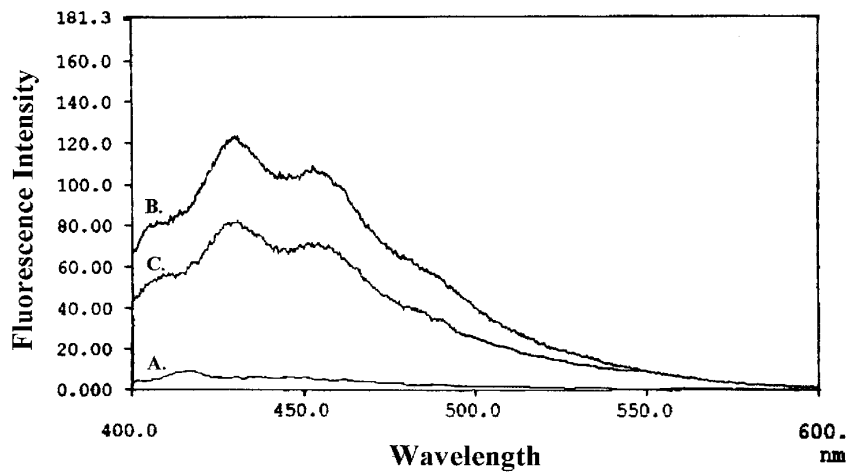

FIG. 4. Fluorescence emission spectra of platelet membranes in the (A) absence or (B) presence of DPH (0.5 μM). Curve C is the emission spectrum of membranes labeled with DPH in the presence of naloxone (0.5 mM). The profiles are representative examples of four similar experiments.

more, we also found that naloxone did not significantly affect the level of cyclic-GMP in human platelets (data not shown). In this study, naloxone significantly inhibited the increase in the $[Ca^{2+}]$i level of Fura 2-loaded platelets stimulated by collagen. Therefore, it appears that naloxone should affect the Ca^{2+} influx and Ca^{2+} release from some intracellular Ca^{2+}-storage sites. This result is consistent with the concept that $[Ca^{2+}]$i release is responsible for the release reaction (20). Mehrishi et al. (21) reported that $[^3H]$-naloxone bound rapidly to human platelets and these authors also suggested that at least some naloxone binding sites on platelets are specific opioid receptor sites of the mu (μ) type. After pretreatment of PL017 (1 mM) (22), a specific mu (μ) receptor agonist 2 min before the addition of naloxone, we found that PL017 did not significantly affect the antiplatelet effect of naloxone (data not shown), indicating that other mechanisms are involved rather than a specific blockade of opioid receptors that are present in platelet membranes. In addition, the importance of cyclic-AMP in modulating platelet reactivity is well established (18). From this observation we suggest that the antiplatelet activity of naloxone is not due to increasing the level of cyclic-AMP.

Triflavin acts by binding to the GP IIb/IIIa complex on platelet membranes, resulting in interfering with the interaction of fibrinogen with its specific receptor (15). In this study, naloxone had no significant effect on FITC-triflavin binding to the GP IIb/IIIa complex, indicating that the antiplatelet activity of naloxone is not directly due to interference with the binding of fibrinogen to GP IIb/IIIa complex on the platelet membrane surface. Furthermore, the platelet membrane has a dominant role in regulating and performing platelet functions, particularly platelet aggregation. Conformational changes in plasma membrane and/or changes in membrane fluidity are the generally accepted mechanism for the antiplatelet effects of numerous drugs, such as chlorpromazine, local anesthetics, and β-blockers. Therefore, we wondered whether naloxone might also inhibit platelet aggregation through influencing of membrane fluidity and to further verify this hypothesis a fluorescent probe DPH was employed. In this study, naloxone (0.5 mM) apparently decreased the relative fluorescence intensity of platelet membranes tagged with DPH. Therefore, based on the above observations, we suggest that the change in platelet membrane fluidity is the primary mechanism, followed by the inhibition of the $[Ca^{2+}]$i mobilization and then inhibition of the release reaction stimulated by agonists.

In conclusion, we report here that naloxone has an evident effect on antiplatelet aggregation. Our work suggests that naloxone inhibits platelet aggregation-induced by agonists (i.e., thrombin, collagen, ADP and U46619) in human platelet suspensions, probably by influencing the plasma membrane fluidity, and the inhibition of $[Ca^{2+}]$i mobilization as well as the release reaction. This effect indicates that naloxone may be used as an effective tool in treating pathological disorders such as ARDS, endotoxic and anaphylactic shock associated with platelet hyperaggregability.

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