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AGGREGATION OF BLOOD PLATELETS BY BIOGENIC AMINES AND ITS INHIBITION BY ANTIADRENERGIC AND ANTISEROTONINERGIC AGENTS

WOLFGANG BARTHEL and FRITZ MARKWARDT
Institute of Pharmacology and Toxicology, Medical Academy Erfurt, GDR

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Abstract—Rabbit blood platelets were aggregated in Tyrode's solution by epinephrine and norepinephrine. Serotonin was found to be ineffective, but it potentiated the aggregation induced by epinephrine and norepinephrine. Several inhibitors of epinephrine-induced aggregation were studied. α-Receptor blocking agents proved to be the most potent inhibitors. Dihydroergotamine especially exerted a strong inhibitory effect at about 10^{-11} M. This specific inhibitory effect on the epinephrine-induced aggregation was competitive, while the antiserotoninergic agent BOL 148 inhibited this aggregation in a non-competitive unspecific manner.

THE BIOGENIC amines epinephrine, norepinephrine and serotonin aggregate human blood platelets in platelet-rich plasma,[1–4] but isoprenaline is ineffective.[5]

The aggregation of blood platelets by epinephrine especially is important in the development of thrombosis. In certain pathological states and in those where there is a high incidence of thrombosis, epinephrine has been detected in the circulating blood in concentrations which cause aggregation of blood platelets *in vitro*.[6–9] The finding that epinephrine potentiates the aggregation of blood platelets induced by adenosine diphosphate (ADP), collagen and thrombin[5,10,11] also accents the importance of epinephrine in initiating thrombosis.

The aggregation of human blood platelets by epinephrine seems to be mediated by stimulation of α-receptors. As epinephrine-induced platelet aggregation is most effectively inhibited by the classical α-receptor blocking agents.[2,5,11,12]

Blood platelets in platelet-rich plasma of other mammalian species, such as rat, guinea-pig and rabbit, are not or only slightly aggregated by epinephrine and serotonin.[13–17] Epinephrine, however, potentiates the weak serotonin-induced aggregation of rabbit blood platelets in platelet-rich plasma.[16,17]

In this study we investigated the aggregation of washed rabbit blood platelets induced by epinephrine, norepinephrine, serotonin and isoprenaline in Tyrode's solution and determined the effectiveness of several specific inhibitors of this type of aggregation, such as antiadrenergic and antiserotoninergic drugs. In order to demonstrate the specificity of these inhibitors on epinephrine and serotonin induced aggregation of blood platelets some experiments were also performed on the aggregation of blood platelets induced by "tendon extract".

Washed rabbit blood platelets suspended in Tyrode's solution proved to be a highly sensitive model for detecting adrenergic effects and for evaluating adequate inhibitors, e.g. α-receptor blockers.

MATERIALS AND METHODS

The drugs used in the experiments were: epinephrine (Adrenalin 1 : 1000-Ampullen, VEB Arzneimittel Naumburg, GDR); norepinephrine (Noradrenalin-Ampullen, VEB Arzneimittel Naumburg, GDR); isopropylnorepinephrine (Novodrin®, VEB Berlin-Chemie, GDR); serotonin (5-Hydroxy-tryptamin-creatininsulfat, Fluka AG. Buchs SG, Switzerland); BOL 148 (Bromlysergsäurediäthylamid, Forschungsabteilung Sandoz AG Basel, Switzerland); dibenamine hydrochloride (Dibenamine®, Smith, Kline and French Laboratories Philadelphia, U.S.A.); dihydroergotamine (Dihytamin®, VEB Arzneimittelwerk Dresden, GDR); papaverine hydrochloride (Papaverin®, Isis-Chemie KG Zwickau, GDR); phentolamine (Regitin®, CIBA AG, Wehr/Baden, GFR); propranolol (Obsidan®, Isis-Chemie KG Zwickau, GDR); and yohimbine (Yohimbin AWD®, VEB Arzneimittelwerk Dresden, GDR).

Rabbits weighing between 2·5 and 3·0 kg were anesthetized with hexobarbital (50 mg/kg) and a polyethylene cannula was inserted into the carotid artery. Blood was collected in centrifuge tubes containing 0·1 volume of a 1% EDTA solution in physiological saline. Blood platelets were isolated in the usual manner by differential centrifugation.[18] They were washed twice in calcium-free Tyrode's solution, counted under a phase contrast microscope, and resuspended after centrifugation in full Tyrode's solution. The final number of blood platelets in the experimental samples was $0{\cdot}5 \times 10^9$/ml. The pH of Tyrode's solution was adjusted to 7·4.

The collagen-containing "tendon extract" was prepared from rabbit tendons according to Hovig.[20] Six g of tendons from rabbit hind legs were homogenized in 50 ml physiological saline and then treated as described in a previous publication.[21]

Aggregation of blood platelets was measured using a technique based on the turbidimetric method of Born[19] involving continuous recording of the optical density of a stirred suspension of blood platelets. Platelet aggregation is accompanied by an increase in light transmission of the platelet suspension.

Experiments were performed at room temperature (22–24°) and siliconized glass cuvettes were used. Three minutes after the beginning of stirring serotonin or "tendon extract" was added to the samples. Epinephrine was added 1 min after serotonin addition, inhibitors at the beginning of the corresponding experiment. The change in the optical density was recorded every 30 sec in a modified "Magnephot II, Type 2213" (Orion EMG, Hungary).

Platelet aggregation and its inhibition by the drugs was quantified by the change in the optical density of the suspensions occurring after 10 min. The recorder scale of the aggregometer was calibrated with Tyrode's solution (100 per cent light transmission = 0 per cent optical density). The platelet suspensions had an optical density of 70 per cent. Comparative experiments were performed on the same batch of platelets in order to determine the potency of drugs in inhibiting aggregation. Inhibition was always expressed as the percentage of change in optical density between a control sample and the sample to which the drug was added. The log dose–response curves were estimated according to Finney.[22]

RESULTS

Aggregation of rabbit blood platelets by epinephrine, norepinephrine, isoprenaline and serotonin

Aggregation of rabbit blood platelets was induced by epinephrine and by epinephrine

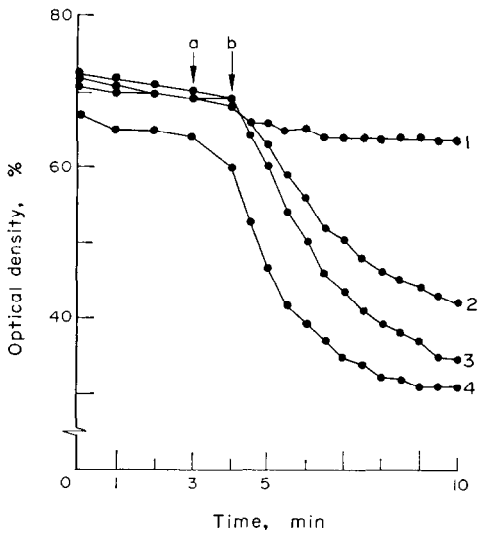


FIG. 1. Aggregation of rabbit blood platelets by (1) serotonin, 10^{-6} M, (2) epinephrine, 5×10^{-6} M, (3) epinephrine, 5×10^{-6} M, after serotonin, 10^{-6} M, and (4) "tendon extract", 0·2 ml/4 ml. Arrow (a) addition of serotonin resp. "tendon extract", arrow (b) addition of epinephrine. Each point represents the average of values obtained from ten experiments.

after pretreatment of platelets with serotonin. The typical slope of the aggregation curves is shown in Fig. 1. For comparison, the curve of aggregation for blood platelets by "tendon extract" is also shown. Serotonin alone did not aggregate blood platelets, but it potentiated the aggregation induced by epinephrine. In 6 of 24 rabbits (25 per cent) the blood platelets did not respond to epinephrine. In contrast, platelets from only 4 of 62 animals (6 per cent) showed no aggregation by epinephrine after incubation with serotonin. The potentiation of the epinephrine-induced platelet aggregation by serotonin can be seen more clearly in Fig. 2. which shows the log dose–response relationships between the concentration of the compounds used for inducing aggregation and the extent of aggregation. Serotonin and isoprenaline did not induce any aggregation at the concentrations tested (10^{-9}–10^{-4} M).

The aggregation of blood platelets induced either by epinephrine and norepinephrine or by combination of either of the drugs with serotonin caused maximal change in optical density at concentrations of about 10^{-6} M. Following a "plateau", the curves decline at concentrations of 10^{-5} M and 10^{-4} M respectively. This means that using the latter concentrations there was no induction of aggregation by either of the compounds.

Influence of inhibitors on the aggregation of blood platelets induced by epinephrine and by epinephrine after pretreatment with serotonin

The effect of a series of antiadrenergic compounds, the antiserotoninergic agent BOL 148, and the β-stimulant isoprenaline was tested on the aggregation of blood platelets induced by epinephrine or by epinephrine after pretreatment with serotonin. The results of these experiments are shown in Figs. 3a and 3b. α-Receptor blocking

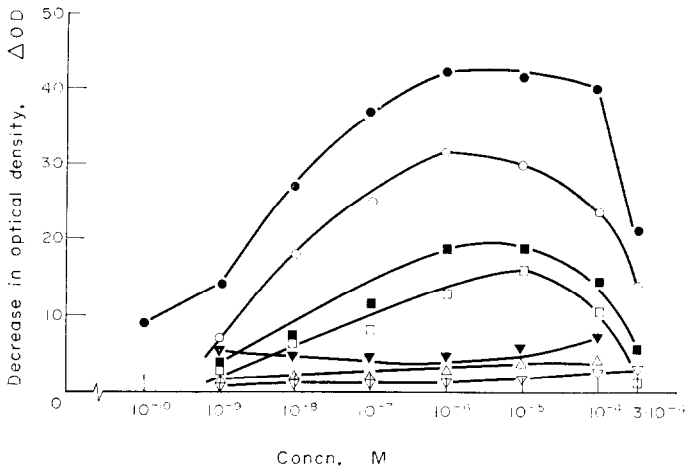


FIG. 2. Dose–response curves for aggregation of rabbit blood platelets by epinephrine (○), norepinephrine (□), serotonin (△), isoprenaline (▽), and by epinephrine (●), norepinephrine (■), and isoprenaline (▼) after pretreatment of platelets with serotonin, 10^{-6} M. Each point represents the average of values obtained from three to four experiments.

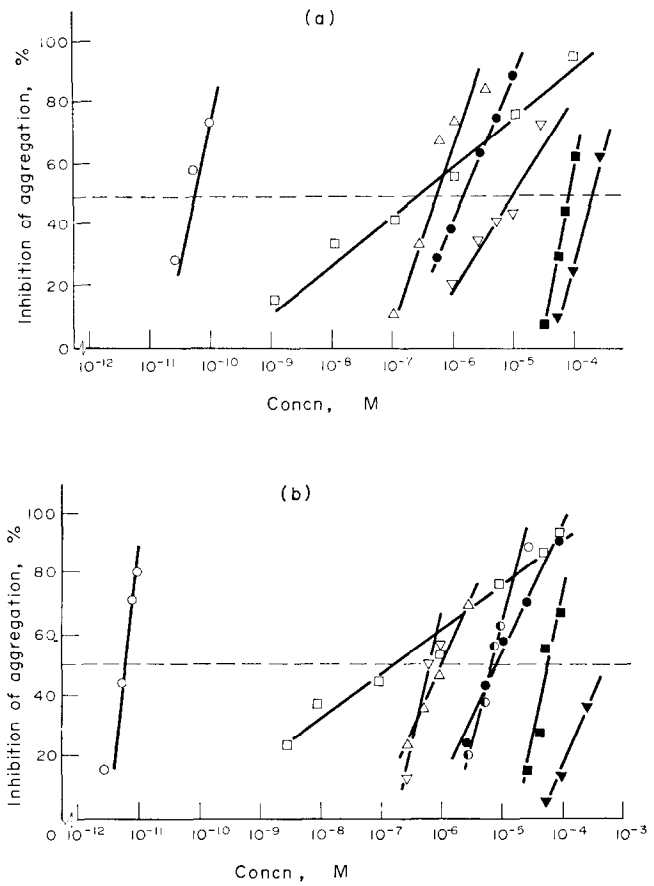
agents were the most effective inhibitors. Besides the regression lines of the log dose–response curves in these figures, the rank order of the compounds tested expressed by their I_{50}-values is also listed. These values were calculated from the regression equations of the log dose–response curves.

The α-receptor blocking agents dihydroergotamine and dibenamine were more effective in inhibiting the aggregation of blood platelets by epinephrine after pretreatment with serotonin, whereas yohimbine and phentolamine showed a stronger activity on aggregation induced by epinephrine alone.

Papaverine which was tested as a reference drug in the experiments with serotonin (Fig. 3b) was as effective as most of the α-receptor blockers. In contrast, the β-receptor blocking agent propranolol and the β-stimulant isoprenaline were far less potent in both the experiments without significant differences in potency. The log dose–response curves of BOL 148 show a very slow incline. This suggests a mechanism of action other than that exerted by the other inhibitors, especially the α-receptor blockers.

Influence of inhibitors on the aggregation of blood platelets induced by "tendon extract"

In order to demonstrate the specificity of the action of the α-receptor blocking agent dihydroergotamine on epinephrine-induced aggregation of blood platelets its potency in inhibiting the aggregation of blood platelets induced by "tendon extract" was tested (Fig. 4). In these experiments dihydroergotamine was shown to be less effective. Some of the other agents which were tested, such as propranolol and isoprenaline, showed a similar low effectiveness as in the case of epinephrine-induced aggregation. The slope of the dose–response curve for BOL 148 was similar to that in the experiments with epinephrine-induced aggregation but a somewhat weaker potency was shown by the I_{50}-values. Only papaverine proved to be more potent in



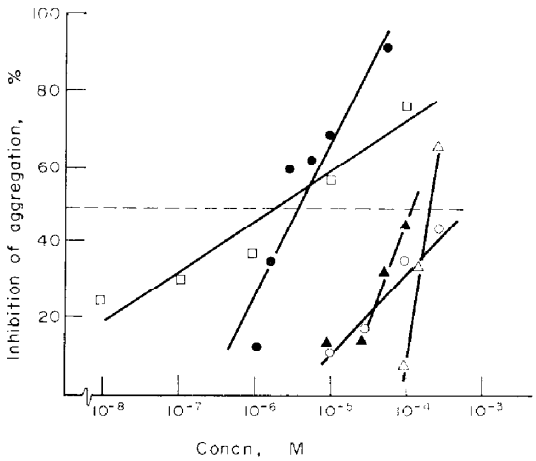
(a) I_{50}-Values		(b) I_{50}-Values	
Dihydroergotamine	(○) $5{\cdot}2 \times 10^{-11}$ M	Dihydroergotamine	(○) $5{\cdot}9 \times 10^{-12}$ M
BOL	(□) $2{\cdot}6 \times 10^{-7}$ M	BOL 148	(□) $1{\cdot}7 \times 10^{-7}$ M
Yohimbine	(△) $4{\cdot}7 \times 10^{-7}$ M	Dibenamine	(▽) $7{\cdot}2 \times 10^{-7}$ M
Phentolamine	(●) $1{\cdot}3 \times 10^{-6}$ M	Yohimbine	(△) $1{\cdot}1 \times 10^{-6}$ M
Dibenamine	(▽) $9{\cdot}8 \times 10^{-6}$ M	Papaverine	(◐) $7{\cdot}6 \times 10^{-6}$ M
Propranolol	(■) $7{\cdot}4 \times 10^{-5}$ M	Phentolamine	(●) $9{\cdot}6 \times 10^{-6}$ M
Isoprenaline	(▼) $1{\cdot}3 \times 10^{-4}$ M	Propranolol	(■) $6{\cdot}3 \times 10^{-5}$ M
		Isoprenaline	(▼) $6{\cdot}0 \times 10^{-4}$ M

FIG. 3 (a) and (b). Dose–response curves for the inhibition of rabbit blood platelet aggregation by drugs. (a) Aggregation induced by epinephrine, 5×10^{-6}M, (b) aggregation induced by epinephrine after pretreatment of platelets with serotonin, 10^{-6} M. Each point represents the average of values obtained from five to six experiments.

inhibiting aggregation of blood platelets induced by "tendon extract" than in epinephrine-induced aggregation.

Type of inhibition exerted by dihydroergotamine and BOL 148

To characterize further the mode of action of dihydroergotamine and BOL 148 in inhibiting epinephrine-induced aggregation of blood platelets, experiments were done



	I_{50}-Values
BOL 148 (□)	$2{\cdot}4 \times 10^{-6}$ M
Papaverine (●)	$3{\cdot}4 \times 10^{-6}$ M
Dihydroergotamine (▲)	$1{\cdot}1 \times 10^{-4}$ M
Propanolol (△)	$2{\cdot}2 \times 10^{-4}$ M
Isoprenaline (○)	$7{\cdot}6 \times 10^{-4}$ M

FIG. 4. Dose–response curves for the inhibition by drugs of rabbit blood platelet aggregation induced by "tendon extract". Each point represents the average of values obtained from three to four experiments.

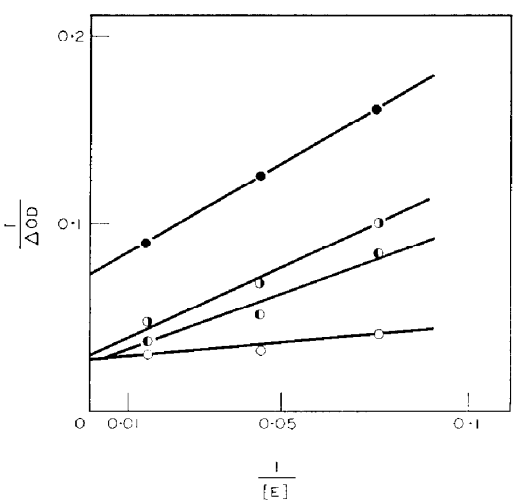


FIG. 5. Lineweaver–Burk-plot of the inhibition of epinephrine-induced platelet aggregation by dihydroergotamine and BOL 148. (○) Control; (◐) dihydroergotamine, 6×10^{-12} M; (◑) dihydroergotamine, 10^{-11} M; and (●) BOL 148, 10^{-7} M. 1/ΔO.D. represents the reciprocal of the change in optical density (per cent), 1/[E] the reciprocal of epinephrine concentration (ng/ml). Each point represents the average of values obtained from four to eight experiments.

to estimate the type of inhibition. For this purpose a Lineweaver–Burk analysis was performed using the values of platelet aggregation induced by epinephrine in the concentration range between 6×10^{-8} M and 3×10^{-7} M under the influence of dihydroergotamine and BOL 148 (Fig. 5). As a result of this analysis the inhibitory effect of dihydroergotamine was found to be of competitive nature, whereas the effect of BOL 148 was non-competitive.

DISCUSSION

In the present study it could be shown that epinephrine caused an aggregation of washed rabbit blood platelets in Tyrode's solution, whereas serotonin and isoprenaline were ineffective. Thus, rabbit blood platelets behave differently in Tyrode's solution and in plasma. Epinephrine does not induce aggregation in platelet-rich citrated plasma, while serotonin causes slight aggregation.[17] This may be caused by the epinephrine- or ADP-destroying activity of the rabbit plasma. Serotonin potentiated epinephrine-induced aggregation of rabbit blood platelets in Tyrode's solution. This corresponds to the potentiation of serotonin-induced aggregation of rabbit platelets by epinephrine in plasma.[17,23]

High concentrations of epinephrine and norepinephrine did not induce aggregation of rabbit blood platelets in Tyrode's solution. Human and rabbit platelets behave similarly at high concentrations of serotonin.[16,17,23] This was interpreted as an imipramine-like membrane-stabilizing effect of serotonin.[16]

Human blood platelets show a biphasic aggregation response to epinephrine. The first phase is directly caused by epinephrine,[24] and the second phase of aggregation follows the release reaction of endogenous adenosine diphosphate (ADP).[25,26] In contrast to human platelets, rabbit blood platelets do not show a biphasic response to epinephrine.[27,28]

The mechanism by which epinephrine induces aggregation of blood platelets is still not fully understood. Recent communications propose that the site of action of epinephrine is the adenyl cyclase of platelet membranes for epinephrine causes a decrease in the content of cyclic 3′,5′-AMP of intact platelets and of platelet lysates.[29–32] This effect is inhibited by phentolamine.

It was also suggested that there is a relationship between the aggregation of blood platelets induced by epinephrine and serotonin and the uptake of these biogenic amines. Epinephrine taken up by platelets would be involved therefore in the aggregation by binding to a specific receptor like that for ADP.[33] This was concluded from experiments in which phentolamine and dihydroergotamine in low concentrations partially inhibited the uptake of epinephrine by platelets.[33,34] Such a correlation between the uptake of epinephrine and epinephrine-induced aggregation, however, was not confirmed by other authors.[12,35] The serotonin-induced aggregation of rabbit blood platelets is accompanied by the uptake of serotonin by a specific carrier system.[17,36] It was suggested that aggregation brought about by serotonin is mediated by ADP which is released from blood platelets as a consequence of ATP utilization in the active transport of serotonin.[37]

Our experiments confirm the idea that epinephrine-induced aggregation of blood platelets is mediated by a stimulation of α-receptors, as aggregation was inhibited by α-receptor blocking agents. Among the inhibitors tested in the present study, the α-receptor blocking agent dihydroergotamine proved to be the most effective.

In comparison with our findings, ADP-induced aggregation of human platelets in plasma is influenced only by dihydroergotamine at a concentration of 10^{-7}–10^{-6} M.[5] Furthermore, dihydroergotamine (1–2 μmole/l.) prevents the second phase of thrombin induced aggregation. This is discussed as evidence for a release of epinephrine from blood platelets which potentiates the aggregation.[11,38] Another hydrated ergot alkaloid, dihydroergotoxin, completely inhibits epinephrine-induced aggregation at a concentration of 5×10^{-6} M.[39]

Among the α-receptor blocking agents, dihydroergotamine is the most potent inhibitor in other systems[40] and the inhibition is also competitive. The blockade of α-receptors by dihydroergotamine is more complete and longer lasting than that caused by the other competitive inhibitors, e.g. phentolamine. In this respect it is of interest that there is a competitive inhibition of epinephrine-induced aggregation of human blood platelets by phentolamine.[41] Dibenamine is transformed into an unstable cyclic ethylenimonium intermediate which is bound to tissue constituents and plasma proteins.[40,42] This would explain its ineffectiveness in inhibiting epinephrine-induced aggregation of human blood platelets in plasma[8] in contrast to the results found in the present study.

Dihydroergotamine has antiserotoninergic activity in addition to α-receptor blocking activity which could explain its very strong inhibitory effect on epinephrine induced platelet aggregation. BOL 148, which like dihydroergotamine has a lysergic acid nucleus, is distinct from dihydroergotamine, in that it exerts only an antiserotoninergic effect. However, from the present experiments it can be concluded that this antiserotoninergic action is not important for the inhibition of epinephrine-induced aggregation, not even in the presence of serotonin, because BOL 148 inhibits aggregation induced by epinephrine alone, by epinephrine after pretreatment with serotonin, and by "tendon extract". In contrast to dihydroergotamine, the inhibition of epinephrine-induced aggregation is non-competitive. Therefore, BOL 148, unlike the α-receptor blocking agents, shows an unspecific inhibitory effect on the aggregation of blood platelets induced by epinephrine.

Isoprenaline, 0·1–1 μM, influences the aggregation of blood platelets induced by collagen, thrombin or ADP.[5,43,44] Since this effect is abolished by inhibitors of β-receptors, it was suggested that stimulation of β-adrenergic receptors on the platelet surface would cause a disaggregation of blood platelets, concomitantly there may be an increase in the content of platelet cyclic 3′,5′-AMP. Our finding that very high concentrations of isoprenaline are necessary to inhibit epinephrine-induced aggregation of blood platelets does not support this concept.

Papaverine, however, leads to an augmentation of cyclic 3′,5′-AMP in platelets by an inhibition of platelet phosphodiesterase.[45,46] This effect explains at least in part, the inhibitory action of this drug on platelet aggregation. β-Receptor blocking agents inhibit epinephrine-induced platelet aggregation only at very high concentrations. As other authors have stated, this suggests an unspecific effect of these compounds on the platelet membrane.[12,35]

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