antagonists of allergy mediators with ability to inhibit their secretion in the absence of HRA, and provided that the inhibitory effect of the compound is preserved after its removal from the extracellular medium.

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INHIBITORY ACTION OF ANTIARRHYTHMIC PHENOTHIAZINE DRUGS ETHMOZINE AND ITS DIETHYLAMINO ANALOG ON PLATELET AGGREGATION AND METABOLISM OF ENDOGENOUS ARACHIDONIC ACID

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The new phenothiazine derivatives ethmozine and its diethylamino analog (DAAE) are the first compounds of the phenothiazine series which have marked antiarrhythmic activity under both experimental [1, 2] and clinical conditions [1, 3]. The psychotropic phenothiazines, namely trifluoperazine and chlorpromazine, are known to inhibit platelet aggregation [7, 14] and the formation of proaggregant metabolites of arachidonic acid (AA) [13]. These effects of the phenothiazine are due to blocking of activity of the Ca^{++}-binding regulatory protein, calmodulin [8, 12].

In the investigation described below, to obtain further information on the spectrum of action of cardiotropic phenothiazines, the effect of ethmozine and DAAE on aggregation of platelets and on the formation of AA metabolites in them was studied.

EXPERIMENTAL METHOD

To obtain platelet-rich plasma, blood from the cubital vein of healthy blood donors was collected into 3.8% sodium citrate (ratio of anticoagulants to blood 1:9) and centrifuged at

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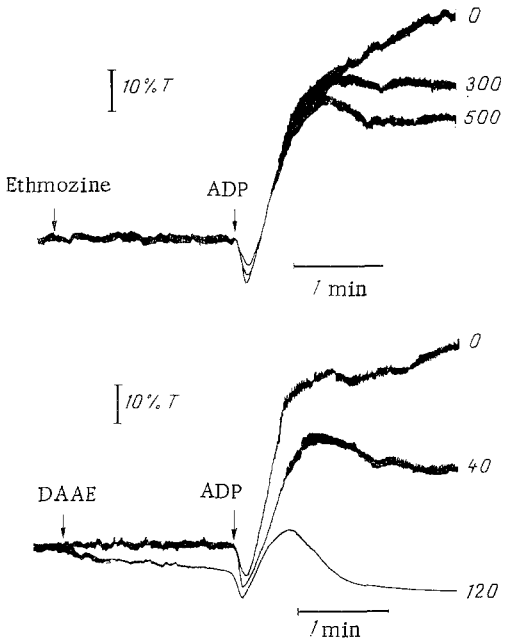

Fig. 1. Inhibition of ADP-induced platelet aggregation in platelet-rich plasma by ethmozine and DAAE. Platelet-rich plasma preincubated in aggregometer cuvette for 1 min with rate of mixing 900 rpm and at 37°C, ethmozine or DAAE added, and incubated for 2 min. Platelet aggregation induced by 10 μM ADP and aggregation curves recorded during next 5 min. Numbers near curves represent final concentrations of ethmozine and DAAE (in μg/ml). The rapid decrease in the amplitude of the oscillations and light transmission after addition of ADP corresponds to change in shape of platelets (conversion of discoid platelets into spherical), and subsequent increase in amplitude of oscillations and light transmission correspond to first and second waves of aggregation.

200g for 12.5 min at 20°C. Platelet-free plasma was obtained by recentrifuging the residue at 1000g for 20 min at 20°C. The platelets were washed to remove plasma by the method in [10], using acid citrate–dextrose (ratio 1:9) as the anticoagulant. After the last (3rd) resedimentation the platelets were suspended in Tyrode solution containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.35% bovine serum albumin, and 10 mM HEPES, pH 7.4. The platelet concentration was determined by means of the PL-100 TOA automatic platelet counter (Medical Electronics, Japan).

Platelet aggregation was recorded photometrically by means of a two-channel Payton (USA) aggregometer, in cuvettes holding 0.5 and 1.0 ml. Platelet-rich plasma was first diluted with platelet-free plasma to a concentration of $2 \cdot 10^8$ platelets/ml, and washed platelets were diluted with Tyrode solution to a concentration of $3 \cdot 10^8$/ml. The aggregometry reaction was carried out at 37°C, by mixing the suspension of platelets at the rate of 900 rpm. ADP, AA, the Ca^{++}-ionophore A 23187, and thrombin were used as aggregation inducers. ADP and thrombin were dissolved in 0.15M NaCl and the A 23187 in 100% ethanol. AA was added to the platelets as the sodium salt. For this purpose, immediately before use, an equimolar quantity of NaOH was added to a solution of AA in 100% ethanol, the ethanol was evaporated *in vacuo* and the residue was dissolved in 0.15M NaCl. ADP, thrombin, and AA were added to the platelet sus-

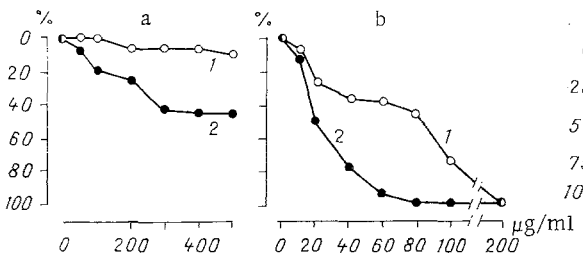

Fig. 2

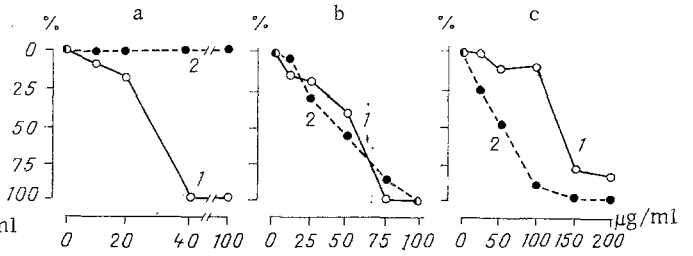

Fig. 3

Fig. 2. Dependence of inhibitory effects of ethmozine (a) and DAAE (b) on first (1) and second (2) waves induced by ADP on concentration. Scheme of experiment described in caption to Fig. 1. Platelet aggregation measured as light transmission (T): for first wave — at maximum corresponding to peak of first wave; for second wave, 5 min after addition of ADP. Inhibition of aggregation calculated by the formula $[(T_o - T_e)/T_o] \times 100\%$, where T_o and T_e represent amplitudes of transmission in the absence and presence of ethmozine or DAAE respectively. Mean results of two experiments shown. Here and in Fig. 3: abscissa, dose of preparation (in µg/ml); ordinate, inhibition (in %).

Fig. 3. Dependence of action of DAAE on aggregation of platelets washed to remove plasma (1), on MDA formation (2), induced by AA (a), the Ca^{++}-ionophore A 23187 (b), and thrombin (c). Scheme of experiment described in caption to Fig. 1. Final concentration of AA 200 µM, of A 23187 2 µM, and thrombin 2 units/ml. Inhibition calculated by measuring amplitude of second aggregation wave (see caption to Fig. 2) and MDA content [11]. Mean results of two experiments given.

pension in a volume of 10 µl, and A 23187 in a volume of 2 µl. Ethmozine and DAAE were dissolved in triple-distilled water and added to the platelet suspension in a volume of not more than 20 µl. The amplitude of the first wave of platelet aggregation was measured by the change in light transmission at the point corresponding to the peak of the first wave, and the amplitude of the second wave was measured as the change in light transmission 5 min after addition of the inducer.

The quantity of malonic dialdehyde (MDA) formed in the platelets was determined 5 min after addition of the inducer by the method in [11].

EXPERIMENTAL RESULTS

Ethmozine and DAAE inhibit ADP-induced platelet aggregation in platelet-rich plasma. Ethmozine inhibits only the second (irreversible) wave of aggregation, whereas DAAE inhibits both the first (reversible) and second waves (Fig. 1). Concentrations leading to 50% inhibition (ID_{50}) of the second wave of ADP-induced aggregation were 300-500 µg/ml for ethomozine and about 20 µg/ml for DAAE (Fig. 2). The first wave of aggregation was inhibited by higher concentrations of DAAE (ID_{50} about 80 µg/ml) and was practically not inhibited at all by ethmozine (Fig. 2, 1).

Not only aggregation, but also changes in shape of platelets in suspension can be recorded by the photometric method [4]. ADP-induced conversion of discoid platelets into spherical was accompanied by an instant fall in the amplitude of the oscillations and a decrease in light transmission (Fig. 1). Ethmozine and DAAE did not inhibit ADP-induced changes in platelet shape (Fig. 1). In relatively high concentrations (80-200 µg/ml) DAAE itself caused a gradual decrease in amplitude of the oscillations and a decrease in light transmission (Fig. 1b). These changes, however, were much slower than under the influence of ADP.

The antiaggregation properties of DAAE also were studied by the use of more powerful inducers of aggregation than ADP, namely AA, the Ca^{++}-ionophore A 23187, and thrombin. These inducers, in the concentrations tested (200 and 2 µM, 2 units/ml respectively), induce irreversible platelet aggregation without any distinct first wave. DAAE in concentrations exceeding 40 µg/ml completely prevented AA-induced aggregation of platelets washed to remove plasma, but in concentrations exceeding 75 µg/ml it prevented aggregation induced by A 23187 (ID_{50} values about 30 and 55 µg/ml respectively; Fig. 3: a, b, 1). Aggregation induced by thrombin was inhibited by higher concentrations of DAAE (ID_{50} about 130 µg/ml), and 80-90% inhibition was observed in concentrations of 150-200 µg/ml (Fig. 3c, curve 1).

Synthesis of AA metabolites in platelets was measured as accumulation of MDA [11]. In the absence of DAAE and during stimulation of the platelets by AA, A 23187, and thrombin the quantity of MDA formed was 3.01 ± 0.52, 0.26 ± 0.08, and 0.34 ± 0.06 nmoles/10^8 platelets respectively. DAAE did not reduce the amount of MDA formed in the platelets as a result of metabolism of exogenous AA (Fig. 3a, 2). Total inhibition of aggregation induced by AA thus takes place against the background of a continuously high level of synthesis of its metabolites, and on that account the antiaggregating action of DAAE cannot be explained by inhibition of synthesis of proaggregant prostaglandin endoperoxides and thromboxane A_2. Although DAAE in a concentration of 100 μg/ml does not affect exogenous AA metabolism, it completely inhibits MDA formation induced by A 23187 and thrombin in concentrations of 100 and 150-200 μg/ml respectively (ID_{50} in both cases about 50 μg/ml; Fig. 3b, c, 2). The results suggest that DAAE inhibits release of endogenous AA from membrane phospholipids effected by phospholipase A_2 but does not affect the activity of enzymes catalyzing its subsequent metabolic conversions (lipoxygenase, cyclo-oxygenase, thromboxine synthetase).

The action of DAAE on platelets is similar to the action of trifluoperazine, chlorpromazine, and other phenothiazines, which inhibit platelet aggregation induced by ADP, A 23187, and thrombin, and inhibit platelet phospholipase A_2 [13]. Like trifluoperazine [7], DAAE effects the second (irreversible) wave of ADP-induced aggregation more effectively. DAAE and ethmozine do not inhibit changes in shape of platelets induced by ADP. In relatively high concentrations (80-200 μg/ml) DAAE itself causes a decrease in amplitude of oscillations and a slow decrease in light transmission, possibly connected with changes in platelet shape. Conversion of discoid platelets into spherical under the influence of trifluoperazine has recently been described [6].

Assembly and disassembly of microtubules and contraction of actomyocin microfilaments play a key role in aggregation and changes in shape of platelets and they are dependent on Ca^{++}-calmodulin [5, 9]. Release of AA from membrane phospholipids during activation of platelets also is effected by the Ca^{++}-calmodulin-dependent enzyme phospholipase A_2 [13, 15]. It can be postulated that the action of DAAE and ethmozine on platelets, like the effect of other phenothiazines, is due to binding of these compounds with calmodulin and consequent blocking of Ca^{++}-calmodulin-dependent enzymic reactions and processes.

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