SOME MECHANISMS OF THE EFFECT OF EDTA ON PLATELET AGGREGATION

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Interest of research workers in the biochemistry and physiology of platelets has shifted clearly in the last decade toward the study of intracellular mechanisms of regulation of their function. In many publications on this subject [3, 12] the key role is ascribed to calcium ions and cyclic nucleotides. An increase in the Ca^{++} concentration in the cytoplasm of platelets caused, in particular, by ionophore A 23187 [15] can stimulate most types of platelet function (stimulation of aggregation, triggering of the liberation reaction, stimulation of secretion by the cells and their degranulation). It has also been shown that the action of a powerful inducer of aggregations such as ADP on platelets is mediated through Ca^{++}-dependent processes [13]. Regulation of the activity of most of the principal platelet enzyme systems – phosphorylase b [5], phospholipase A_2 [3], phosphodiesterases of cyclic nucleotides [12], and adenylate and guanylate cyclases [3] – also is known to take place with the participation of intracellular Ca^{++}.

The role of cyclic AMP in the regulation of platelet activity is no less important. It is well known that triggering of platelet aggregation is accompanied by a fall in the intracellular cyclic AMP concentration [14]. Substances increasing the cyclic AMP concentration in the platelets are inhibitors of platelet function. Cyclic AMP alters the permeability of the plasma membrane of platelets and can take part in restoration of the disturbed membrane structure of the platelets [11]. The action of both intermediaries on the dynamic properties of the platelets are interdependent and interconnected. For instance, Ca^{++} can inhibit adenylate cyclase, i.e., can reduce cyclic AMP formation in the platelets [3]. Cyclic AMP can regulate Ca^{++} liberation from the functionally active pool in the cytoplasm of platelets [12].

Chelating agents of bivalent cations EDTA (ethylenediaminetetraacetic acid) and EGTA – ethyleneglycol-bis-(β-aminoethyl ester)-M,N'-tetraacetic acid – are known to affect platelet function. They can inhibit their aggregation, depress the liberation reaction [15], and reduce the intensity of active serotonin assimilation [7]. The mechanism of these effects of chelating agents has not yet been fully explained, for EDTA can block the sensitivity of platelets to ADP even after addition of an excess of $CaCl_2$ to the incubation medium [16]. It can accordingly be postulated that the effect of chelating agents on platelet function is mediated through changes in intracellular regulation.

The object of this investigation was to study the action of EDTA on ADP-induced aggregation and on the intracellular regulatory mechanisms of platelets.

EXPERIMENTAL METHOD

Platelet-enriched plasma (PEP), obtained from citrated rabbit blood (3.8% citrate solution, blood:citrate ratio 9:1) was used. The aggregating power of the platelets was determined by the method in [1]. ADP (from Reanal, Hungary), in a final concentration of 10 μM, was used to induce aggregation. The ADP was always added after incubation of the PEP with the agents to be tested. The cyclic AMP content in the platelets was increased by incubating the PEP with N^6,$N^{2'}$-dibutyryl-cyclic AMP (dibutyryl-cyclic AMP), from Sigma, USA, in a final concentration of 0.56 mM, or with theophylline (a phosphodiesterase inhibitor), from Sigma, in a final concentration of 0.25 mM. The disodium salt of EDTA (from Biochemical Lab. Inc., USA), EGTA (from Sigma), and verapamil·HCl (izoptin, from LEK, Yugoslavia), and tetracaine (amethocaine) were used.

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TABLE 1. Effect of EDTA, EGTA, Theophylline, and Dibutyryl-Cyclic AMP on ADP-Induced Platelet Aggregation

Index	EDTA (1)	Theophylline (2)	EDTA + theophylline	EDTA (1)	Dibutyryl-cyclic AMP (3)	EDTA + dibutyryl-cyclic AMP	EGTA (4)	Dibutyryl-cyclic AMP (3)	EGTA + dibutyryl-cyclic AMP
Percent inhibition of aggregation	14,4	3,2	26,8 $P_1<0,01$ $P_2<0,001$	18,1	11,7	34,8 $P_1<0,001$ $P_3<0,001$	17,2	18,5	39,0 $P_4<0,001$ $P_3<0,01$

The incubation time (at 37°C) for PEP with EDTA and EGTA was 4 min. During combined incubation the agents were added 1 min after the chelating agent. With tetracaine the incubation time was 4 min, and with the agents to be studied it was 3 min. The results were expressed as percentages of change in amplitude (A) of platelet aggregation relative to the corresponding control (in which the reagents were replaced by the same volume of physiological saline): $(A_{exp}/A_{control}) \times 100$.

EXPERIMENTAL RESULTS

In the experiments of series I the effect of EDTA in a final concentration of 0.55 mM and of theophylline or EDTA in the same concentration and of dibutyryl-cyclic AMP on the aggregating power of the platelets was studied separately or in combination (Table 1).

The values for inhibition of aggregation during incubation of PEP with a combination of agents differed significantly not only from those due to the separate effect of EDTA, theophylline, or dibutyryl-cyclic AMP, but also from the sum of the separate effects of EDTA and theophylline ($P < 0.01$) or of EDTA with dibutyryl-cyclic AMP ($P < 0.01$) obtained in the experiments. It should be noted that dibutyryl-cyclic AMP has no effect on binding of ADP with platelets [13]. The results suggest that the inhibitory effect of EDTA on aggregation is due not only to binding of intercellular Ca^{++} in the citrated PEP, which is known to interfere with the process of platelet aggregation, but also to its action on the intracellular mechanisms of platelets. Potentiation of the effects thus observed may indicate that the cellular action of EDTA is coupled with the effect of the induced increase in the intracellular cyclic AMP concentration in the platelets on their aggregating power.

Table 1 shows the results of a similar experiment in which the EDTA was replaced by EGTA (final concentration 0.3 mM). As the results showed, the characteristics of the observed effects of EGTA agreed with those of EDTA. It can thus be concluded that differences in the ability of these chelating agents to bind bivalent cations do not significantly modify the mechanism of their action on platelet aggregation.

In the experiments of series II, to study the separate and combined effects of EDTA and theophylline or dibutyryl-cyclic AMP, below-threshold concentrations of the agents (i.e., not changing the character of the aggregatogram) were chosen. For EDTA and dibutyryl-cyclic AMP this value was 0.15-0.20 mM in the final concentration, and 0.1-0.125 mM for theophylline. It was shown that an increase in the intracellular cyclic AMP concentration and the action of EDTA on the platelets can mutually potentiate one another. Both these agents, which in the above concentrations do not separately affect aggregating power of the platelets, on combined incubation with PEP caused inhibition of ADP-induced aggregations by about 25%. Consequently, it can be concluded that inhibition of platelet aggregation under the influence of EDTA is due not only to its effect on the composition of the intercellular medium of the PEP, but also to its action on the intracellular mechanisms of regulation of platelet function, which are associated with the intracellular action of cyclic AMP.

Results obtained by a number of workers indicate that EDTA and EGTA can remove Ca^{++} from the plasma membrane of platelets [16], erythrocytes [18], and granulocytes [9]; removal of Ca^{++} from platelets inhibits their aggregating power. It has also been shown that lysophosphatidic acids induce platelet aggregation by stimulating intracellular mobilization of Ca^{++} and that they can liberate Ca^{++} from the plasma membrane fraction of platelets. EDTA inhibited aggregation induced by lysophosphatidic acids [6]. EDTA can also inhibit the uptake of Ca^{++} and disturb active transport of serotonin through the plasma membrane of platelets [7]. It can be tentatively suggested that the action of EDTA and EGTA in inhibiting the aggregating power of platelets is due to their effect on intracellular processes reducing the possibility of Ca^{++} mobilization and (or) reducing the Ca^{++} concentration in the cytoplasm. Cyclic AMP can also activate the calcium pump, which removes the cation from the cytoplasm [12] and can reduce the content of free Ca^{++} by speeding up its binding with proteins of the cytoplasm or membrane [3].

On the basis of data in the literature and the results of the present investigation it can be postulated that the inhibition by EDTA of ADP-induced platelet aggregation observed experimentally, and the potentiation of this effect with an increase in the intracellular cyclic AMP concentration can be explained as follows. EDTA (or EGTA), while binding intercellular Ca^{++}, also removes part of the functionally active intracellular Ca^{++} of the platelets, preventing their aggregation. Lowering the Ca^{++} concentration in the platelets may increase adenylate cyclase activity [3] and, consequently, increase the intracellular cyclic AMP concentration. In that case, the experimentally induced increase in the intracellular cyclic AMP concentration is supplemented by removal of Ca^{++} induced by an increase in its concentration, which leads to potentiation of inhibition of aggregation. In turn, the decrease in the free Ca^{++} concentration in the cytoplasm of the cells under the influence of cyclic AMP can supplement the analogous effect induced by EDTA. Cyclic AMP, which affects the permeability and integrity of the plasma membrane of platelets [11], can also facilitate the removal of Ca^{++} from the platelets under the influence of EDTA.

The additional experiments with verapamil, which blocks calcium channels [10], and with tetracaine, which depresses the intracellular mobilization of Ca^{++} in the platelets [4], showed that, first, verapamil (10^{-5} M) potentiated inhibition of ADP-induced aggregation due to EDTA, dibutyryl-cyclic AMP, EDTA + dibutyryl-cyclic AMP, or EGTA + dibutyryl-cyclic AMP and that, second, disturbance of Ca^{++} liberation from its intracellular storage sites in the platelets, induced by tetracaine (in a final concentration of 0.75 mM) potentiated the inhibition of aggregation induced by EDTA, dibutyryl-cyclic AMP, and EDTA + dibutyryl-cyclic AMP. The combined action of tetracaine and the above agents was weaker than the sum of the separate effects, pointing to interference with the mechanism of action of EDTA and of dibutyryl-cyclic AMP by intracellular mechanisms induced by tetracaine.

In the writers' opinion, the results give grounds for the conclusion that EDTA inhibits platelet aggregation not only by binding intercellular Ca^{++}, but also by reducing its intracellular functional pool. Accordingly, interpretation of the results of a study of the functional properties of platelets, using EGTA as anticoagulants when taking blood, must evidently take account of the possible effects of the chelating agents on the composition and properties of the platelets.

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