THE PLATELET REVERSIBLE ENDOCYTOSIS APPARATUS

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The function of granules in rabbit platelets participating in the reversible endocytosis reaction was investigated. The reaction is induced by two types of inducers: proteolytic enzymes and compounds accumulating inside the granules (biogenic amines, dyes, anesthetics, phenothiazines). Heparin inhibits exocytosis induced by thrombin. In the case of exocytosis induced by accumulation of compounds within the granules of the platelets the cells lose their ability to maintain the aggregated state. The nature of the platelet granules participating in the reversible endocytosis reaction is discussed.

KEY WORDS: reversible endocytosis; fluorescent labeling; platelets.

By fluorescent labeling with acridine orange (AO), granules occupying a considerable part of the intracellular space have been discovered in platelets. Fluorescent microscopy enables granules of this type to be observed intravitally [5], and the recently suggested fluorometric method can be used to study quantitatively processes taking place with the granules in the course of clot formation [2]. The object of the present investigation was to study the function of granules of this type under different conditions. The results obtained suggest that the platelets contain a special intracellular "reversible endocytosis apparatus."

EXPERIMENTAL METHOD

Platelet-enriched citrated rabbit blood plasma (PEP) was obtained by the usual method [2, 3]. Aggregation of the platelets was recorded nephelometrically on a platelet aggregation meter (EEL, U.K.). The reversible endocytosis reaction of the platelets was studied by means of the fluorometric system described previously [2]. To reduce the volume of PEP, glass cuvettes 100 mm in diameter were used. The working mixture contained the following components: 0.5 ml PEP (containing 300,000-400,000 cells/m^3); 1.5 ml physiological saline; 0.05 ml AO solution (final concentration in the mixture $3.7 \cdot 10^{-6}$ M). Recalcification was carried out by the addition of 0.2 ml of 1.29% calcium chloride solution. The substances for testing were dissolved in physiological saline and added in volumes of 0.04 to 0.1 ml. Final concentrations in the mixture are indicated.

To record the reversible endocytosis reactions of the platelets and their aggregation simultaneously a modified fluorometric system was used. The following modifications were introduced into the system described previously [2]: The cuvette block was modified for cylindrical cuvettes 11 mm in diameter and 50 mm long; the temperature of the block in the modified system was maintained constantly by circulating water in the jacket of the block (working temperature 22°C); the mixer was similar to that used in the aggregation meter with a controllable number of revolutions (recording was carried out at 800 rpm); of the two F-14 photoelectric cells used in the ÉF-3 MA apparatus, one was left in its original position but the other was moved in order to measure the intensity of light passing through the specimen (opposite the source); a filter, similar to the exciting filter (350-480 nm) was placed in front of this photoelectric cell; the photoelectric cells were connected to separate amplifiers from ÉF-3 MA apparatuses; KSP-4 automatic writers were connected to the outputs of the amplifiers. By means of this modified system it was possible to record fluorescence and light transmission of the mixture independently and simultaneously. By recording fluorescence, it is possible to investigate the kinetics of the reversible AO endocytosis reaction by platelets

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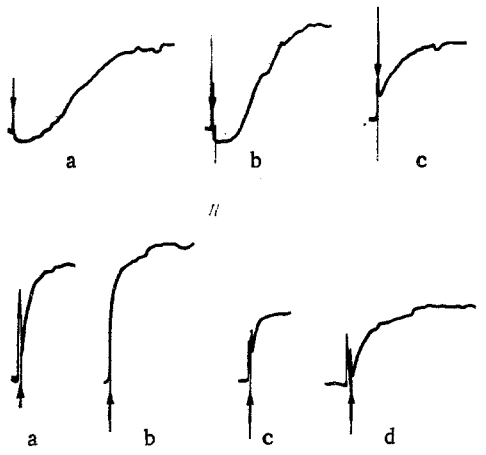

Fig. 1

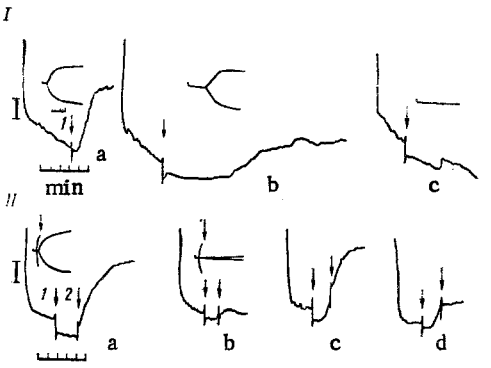

Fig. 2

Fig. 1. Initiation of exocytosis of contents of granules in rabbit platlets. I) Proteolytic enzymes: trypsin, $2.4 \cdot 10^{-6}$ M (a); $4 \cdot 10^{-6}$ M (b); papain, $6 \cdot 10^{-5}$ M (c); II) compounds interacting with platelet granules; chlorpromazine $2.8 \cdot 10^{-4}$ M (a); nonachlazine $1.4 \cdot 10^{-4}$ M (b); procaine $5 \cdot 10^{-4}$ M (c); serotonin $12 \cdot 10^{-4}$ M (d). Arrow indicates time of addition of inducing agent.

Fig. 2. Inhibition of reaction of exocytosis of contents of platelet granules and of clot formation by heparin. I) Heparin added to sample before AO and Ca^{++}. Arrow 1) addition of Ca^{++}. Fluorogram and thromboelastogram of same mixture. a) Control; b) 0.1 unit/ml; c) 0.5 unit/ml mixture; II) heparin added after Ca^{++}. Arrow 1) Ca^{++}; arrow 2) heparin. a) 1.5 unit, b) 4 units, c) 4 units (added later), and d) 6 units/ml mixture.

[2], and the light transmission during mixing gives an estimate of aggregation of the cells in the sample.

EXPERIMENTAL RESULTS

1. Inducers of Liberation of the Contents of Granules Detectable on Intravital Treatment of Platelets with AO. It was shown previously that the contents of the granules are liberated upon the addition of thrombin to PEP [2]. Proteolytic enzymes such as trypsin and papain have also been found to induce this liberation reaction, although in higher concentrations (Fig. 1). Besides proteolytic enzymes, at least two other types of compounds have been shown to induce AO liberation from platelet granules in concentrations of 10^{-4} M or higher: These are substances affecting membrane structure (phenothiazines) and compounds accumulating in the granules (biogenic amines, local anesthetics) (Fig. 1). Similar information is given in the literature. It has been shown, for instance, that serotonin is liberated from platelets by the action of phenothiazines [4, 9], local anesthetics [4], dyes such as quinacrine (mepacrine) [9] and methylene blue [11], and also on the addition of biogenic amines [9].

It has been suggested that platelets contain receptors specific for thrombin, and that the reaction of thrombin with the platelets is similar to the hormone–cell reaction [8] and that it has a similar course to exocytosis mediated by Ca^{++} cations [7]. Proteolytic enzymes react in the same way with platelets. Liberation induced by compounds accumulating in the granules follows a rather different course. Two mechanisms have been postulated: 1) During accumulation of compounds localized in the inner part of the organelles, the contents are displaced by competition for the combining site (biogenic amines, dyes); 2) during accumulation of contents localized in the membranes (phenothiazines) a change in permeability takes place [9].

2. Interconnection between the Exocytosis Reaction and Clot Formation. The fluorometric method of investigation of the reversible endocytosis reaction of platelets has two main advantages over other methods of studying the liberation reaction: The kinetics of the process can be recorded and the liberation of the contents of the granules can be observed in a sample of clotting PEP. Consequently, intraconnection between this reaction and clot formation can be

Fig. 3. Deaggregation of platelets. a) Nonachlazine ($5 \cdot 10^{-4}$ M); b) serotonin ($6 \cdot 10^{-3}$ M); c) procaine ($1.2 \cdot 10^{-3}$ M); d) AO ($9 \cdot 10^{-4}$ M). Arrow 1) Addition of ADP ($9 \cdot 10^{-6}$ M); arrow 2) addition of nonachlazine; original ADP-aggregation omitted from other traces.

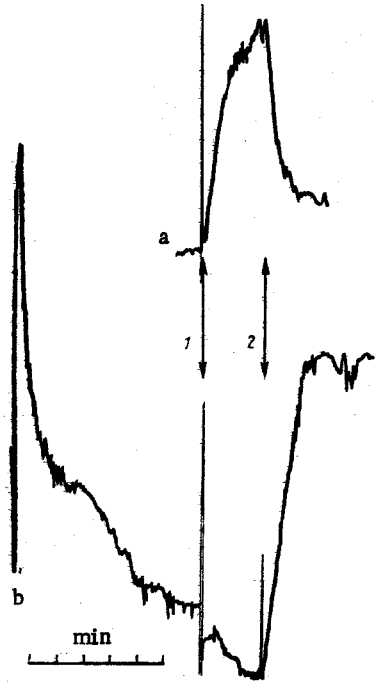

Fig. 4. Simultaneous recording of deaggregation and exocytosis of platelets. a, b) As in Fig. 3.

investigated. It was shown previously [3] that liberation of the contents of the granules takes place simultaneously with formation of the clot and before its retraction. Addition of heparin to a sample of clotting PEP led to the arrest of exocytosis and of clot formation (Fig. 2). In the present writers' opinion this is evidence that fresh amounts of thrombin are constantly being bound in the course of exocytosis. Heparin, which inhibits the enzyme, arrests further liberation of contents of the granules. The later the inhibitor is added, the larger the amounts of it required.

3. Interconnection between the Exocytosis Reaction and Platelet Aggregation. The study of interconnection between the two most important functions of platelets — the aggregation and exocytosis reaction — is particularly interesting. Compounds accumulating in platelet granules and expelling their contents (biogenic amines, local anesthetics, dyes, phenothiazine) were found to have a deaggregating action in concentrations of 10^{-4} M or higher, i.e., they induced the breaking up of aggregates formed previously (Fig. 3). Deaggregation and exocytosis induced by nonachlazine are shown in Fig. 4: clearly the two processes take place simultaneously. It is stated in the literature that on the addition of $4 \cdot 10^{-4}$ M methylene blue, which causes the liberation of serotonin from granules, increased synthesis of membrane phospho-

lipids de novo takes place in the platelets [10]. The possibility cannot be ruled out that the loss of the ability of the cells to maintain their aggregated state may be connected with this phenomenon.

4. Nature of the Granules Detected by Intravital Staining of Platelets with AO. The investigations showed that during formation of a hemostatic clot, simultaneously with polymerization of fibrinogen, the contents of the platelet granules are liberated (exocytosis). Exocytosis is induced by the binding of thrombin with the cell. Other proteolytic enzymes (trypsin, papain) can also interact with platelet receptors. The exocytosis reaction in a sample of recalcified PEP can be arrested by the addition of heparin. Granules taking part in exocytosis can accumulate biogenic amines, dyes, local anesthetics, and phenothiazines. When substances accumulate in the cell by endocytosis in sufficiently high concentrations (10^{-4} M or higher), exocytosis of the substances contained in the granules takes place; the cells thereby lose their ability to remain in the aggregated state. This combination of properties suggests that in the platelets there is a special intracellular apparatus responsible for their specific functions. In view of the now general acceptance of the term "reversible endocytosis reaction" suggested by Stormorken in 1969 [12], we suggest that the system of organelles participating in this reaction be called the platelet reversible endocytosis apparatus.

By fluorometric determination of AO [2] it is possible to make a detailed investigation of the function of the platelet reversible endocytosis apparatus. A microfluorometric method, using another acridine derivative, namely quinacrine (mepacrine), has also been suggested [6].

There is evidence in the literature that fluoroescent acridine derivatives will also accumulate in lysosome-like α-granules of platelets [1, 2] and also in 5-HT-organelles [6]. It is not impossible that the "reversible endocytosis apparatus" includes both types of organelles.

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