pH GRADIENT ON MEMBRANES OF PLATELET SEROTONIN GRANULES: PRINCIPLES OF STUDY WITH THE AID OF FLUORESCENT AMINO DERIVATIVES OF ACRIDINE

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Platelets from different species of animals contained granules with the property of accumulating cationic amino derivates of acridine. These organelles are serotonin (5-HT) granules and the number of them in the cell is 6.3 ± 0.1 in man, 17.0 ± 0.6 in the rabbit, 8.8 ± 0.2 in the guinea pig, and 5.6 ± 0.3 in the rat [6]. Fluorescent properties of acridine derivatives are used to study the role of intracellular granules in the platelet activation test [1, 2] and also to count the granules [5].

It was shown recently that accumulation of amines takes place through the presence of a proton gradient (ΔpH) on the granule membranes. The interior of the granules is acid (in the case of 5-HT granules of pig platelets pH 5.74) whereas the cytoplasm has a pH of 7.2 [4]. Fluorescent amines can be used to study the proton gradient on membranes of platelet 5-HT granules intravitally.

EXPERIMENTAL METHOD

Rabbit platelets were studied. Citrated, platelet-enriched plasma (PEP) was obtained in the usual way [1]. A platelet suspension (PS) was made up from acid PEP. Acid citrated PEP was obtained by centrifuging blood stabilized with acid sodium citrate (0.085 M Na citrate and 0.065 M citric acid) in the ratio of 9:1. The cells were sedimented from acid PEP at 800g for 40 min and resuspended in a specially chosen medium of the following composition: 0.658 g NaCl, 0.949 g Na citrate, 0.451 g glucose, 0.433 g $MgSO_4$, 0.092 g $CaCl_2$, 750 i.u. heparin, and 50 ml 0.01 M phosphate buffer, pH 7.2, to 250 ml of solution. Fluorescence of the acridine orange solutions was measured on the ÉF-3 MA fluorometer; a 350-480 nm filter was used for excitation and 510-650 nm filters as cut off filters. Fluorescence and transmission of the PEP samples were recorded simultaneously on an apparatus described previously [2]. The pH of the solutions was determined electrometrically. All concentrations of added substances are given as final concentrations in the sample.

EXPERIMENTAL RESULTS

Method of Determination of ΔpH on Membranes of 5-HT Granules of Native Platelets by Fluorometric Titration. The ability of some amines to accumulate in cell organelles with an acid medium was used for quantitative determination of their pH. For instance, by measuring the distribution of methylamine-^{14}C the pH of the intracellular platelet granules can be determined [4] and fluorescent amines have been used to measure the pH of chloroplasts [8]. These methods are based on the observation that amines can pass through biomembranes in the neutral form ($R{-}NH_2$) but do not pass through in the charged form ($R{-}NH_3^+$). For amines with a sufficiently high pK_a (K_a — dissociation constants; $pK_a \geqslant$ pH inside and pH outside). ΔpH on the membrane can be calculated from concentrations of the amine inside and outside the granules. The equation $C^+_{inside}/C^+_{outside} = H^+_{inside}/H^+_{outside}$ (1), in which C^+ denotes the concentration of $R{-}NH_3^+$, is satisfied under these circumstances.

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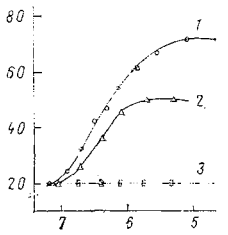

Fig. 1. Determination of pH on membranes of platelet 5-HT granules by fluorometric titration. 1) Native PS; 2) PS incubated with serotonin (0.0154 mM); 3) PS incubated with NH_4Cl (15 mM). Incubation for 30 min at 22°C. Abscissa, pH; ordinate, intensity of fluorescence (in %).

This method is highly accurate but does have some shortcomings: First, the determination is carried out only on a suspension of organelles isolated from cells; second, the internal volume of the granules must be determined.

Equation (1) is valid for solutions separated by one membrane (for example, a suspension of isolated granules). In case of a suspension of native cells, we can write: for the outer cell membrane: $C^+_{cell}/C^+_{outside} = H^+_{cell}/H^+_{outside}$ (2), and for the membrane of 5-HT granules: $C^+_{gran}/C^+_{cell} = H^+_{gran}/H^+_{cell}$ (3), where C^+_{cell} is the $R{-}NH_3^+$ concentration inside the cell and C^+_{gran} its concentration inside the granules. Expressing C^+_{cell} from Eq. (2) and substituting in (3), we finally obtain:

$$C^+_{gran}/C^+_{outside} = H^+_{gran}/H^+_{outside}. \quad (4)$$

Equation (1) is thus satisfied also for granules located inside native cells. It is therefore suggested that the value of pH of granules in native cells be determined by the method of fluorometric titration, as follows: Cells are sedimented from acid PEP and resuspended in a volume of medium equivalent to the volume of PEP (the number of cells must be 300,000-800,000/mm³); 2 ml of the resulting PS is diluted with 6 ml 0.85% NaCl; the fluorescent indicator (acridine orange, final concentrations $3 \cdot 10^{-6}$ M) is added and the mixture incubated for 15 min (the incubation time depends on the species of the source of platelets; for human platelets it is 40 min); the solution is put into the fluorometer and 0.1 N HCl is added in doses of 15 µl, the fluorescence and pH of the solution being determined 2 min after each addition of acid. A typical curve obtained by this method is given in Fig. 1. A stepwise increase in $H^+_{outside}$, caused by addition of HCl, according to Eq. (4) leads to an increase in $C^+_{outside}$, expressed as intensification of the fluorescence of the mixture. It will easily be seen that the curve flattens out on a plateau at the moment when $H^+_{gran} = H^+_{outside}$.

Incubation of platelets with NH_4Cl and serotonin is known to lead to alkalification of the matrix of 5-HT granules. These observations were tested by the suggested method on a suspension of native cells and similar results were obtained (Fig. 1).

Method of Investigation of ΔpH on Membranes of 5-HT Granules and Exocytosis Reactions to Platelet Activation. The phenomenon of liberation of fluorescent marker from intracellular granules of platelets [1, 2] can be explained by two types of reactions: first, by a decrease in pH on the granule membranes; second, by liberation of the contents of the matrix by exocytosis. The first reaction can take place with preservation of the morphological integrity of the granules, whereas the second involves adhesion of the granules to the outer membrane. The available experimental data suggests that during activation of cells by proteolytic enzymes the process of outflow of the label takes place through exocytosis [1, 2]. Meanwhile liberation induced by compounds accumulating in the matrix and membranes of platelet 5-HT granules [2] evidently takes place through a decrease in pH on the granule membranes.

The kinetics of the change in pH on membranes of 5-HT granules and of the exocytosis reaction to activation of platelets in suspension were recorded fluorometrically [1, 2]. By recording fluorescence and transmission of light by the sample by means of a special device (a fluonephelometer [2]) the course of the reactions of the pH change and exocytosis could be compared with cell aggregation and clot formation. As an example let us consider experiments to study the phenomenon of "platelet sensitization" which the writers have discussed.

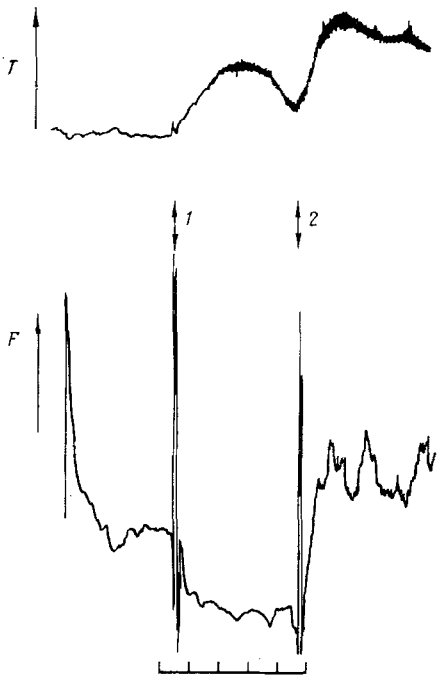

Fig. 2. Phenomenon of "sensitization" of platelets. Recording made by apparatus for simultaneous recording of fluorescence (F) and transmission (T) of PEP sample. Acridine orange ($3 \cdot 10^{-6}$ M) used as marker of 5-HT granules. 1) Addition of ADP ($0.9 \cdot 10^{-6}$ M); 2) addition of $(NH_4)_2SO_4$ (9 mM); one division corresponds to 1 min.

Under the influence of small doses of ADP, inducing reversible aggregation of platelets, the cells are known to pass into a "refractory" state. This state is characterized by the fact that on addition of repeated doses of ADP the sensitivity of the cells to this agent is reduced. After ADP-induced aggregation the platelets were found to be in a state of sensitization to the action of agents lowering pH on the membranes of 5-HT granules. As a rule these agents do not themselves induce platelet aggregation, but they induce removal of the fluorescent marker from 5-HT granules, e.g., $(NH_4)_2SO_4$ in a concentration of 9 mM. However, the addition of such an agent after ADP gave rise to distinct aggregation of the cells (Fig. 2). This "sensitized" state continued for 5-10 min after ADP-induced aggregation. It was observed not only with ammonium salts, but also with 5-HT.

Use of the Fluorescent Label for Counting the Number of 5-HT Granules in Platelets. The microfluorometric method of counting the number of granules in platelets is based on the observation that when cells treated with a fluorochrome are irradiated with blue-violet light, "melting" of the granules is observed. If the level of fluorescence of a single cell is measured under these conditions, flashes of secondary radiation can be recorded; the number of peaks corresponds to the number of granules [5, 6].

Study of the Organization of the 5-HT Granule Matrix. The internal organization of the matrix of 5-HT granules in platelets is not yet known. The particular features of interaction between biogenic amines, and also certain synthetic agents, and these granules described above are evidence of the powerful electron-acceptor properties of their contents. It has been suggested that ΔpH existing on the granule membrane is responsible for operation of the mechanism of accumulation of biogenic amines both in platelets and in other cells. The existence of an ATP-dependent H^+-translocase in the membrane of 5-HT granules has been postulated [4]. The experimental data also are evidence of binding of amines inside granules [3, 4], leading to an increase in the ability of the organelles to accumulate. When the electron-acceptor (acid) properties of the matrix of 5-HT granules are characterized, not only the pH value, but also the buffer capacity created by the activity of the translocase and by the presence of combining sites for compounds with electron-donor properties, must be taken into account. The buffer capacity can also be estimated quantitatively by fluorometric titration (but with

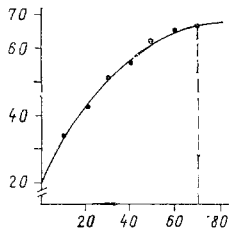

Fig. 3. Determination of buffer capacity of 5-HT granules of platelets by fluorometric titration. Abscissa, volume of NH_4Cl solution (in μl); ordinate, intensity of fluorescence (in %).

constant pH of the medium) by the addition of an agent which penetrates into the granules and lowers their pH. Ammonium salts, which penetrate into granules in the same way as amines, i.e., the equation $H^+_{gran}/H^+_{outside} = NH_4{}^+_{gran}/NH_4{}^+_{outside}$ is satisfied, can be used for this purpose [7].

The method is as follows: A suspension of washed cells is obtained by the method described above for determination of pH inside the granule; it is diluted with buffer to a concentration of 500,000 cells/mm^3; 2 ml of suspension is treated with 6 ml of 0.14 M NaCl and $3 \cdot 10^{-6}$ M acridine orange; after incubation with the indicator titration is carried out with 1.2 M NH_4Cl added in volumes of 10 μl, and after exactly 2 min fluorescence is measured; buffer capacity is expressed in conventional units (volume of 1.2 M NH_4Cl used up in titration).

A curve obtained during determination of the buffer capacity of 5-HT granules of rabbit platelets is shown in Fig. 3.

The electron-acceptor properties of 5-HT granules of platelets can thus be studied quantitatively with the aid of fluorescent amino derivatives of acridine. By this method it is possible to determine the pH and buffer capacity of the granules and also to record the kinetics of changes in ΔpH during activation of the cells. Investigations of this type can be performed on suspensions of different cells possessing granules with similar properties.

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