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LOWERING THE STIMULATED FREE INTRACELLULAR CALCIUM LEVEL IN PLATELETS IN THE REFRACTORY STATE

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ADP is a specific activator of platelets which can induce platelet aggregation both in vitro and in vivo. Preincubation of platelets with ADP lowers their sensitivity to the secondary action of this inducer or, as is usually stated, transition of the platelets into a state of refractoriness [5]. Reduction of aggregation in the refractory state cannot be explained entirely by the formation of aggregation inhibitors, namely adenosine and AMP [8].

On the other hand, we know that platelet activation depends essentially on synthesis and (or) movement of secondary messengers, such as calcium ions, inositol triphosphate, diacylglycerol, and cAMP [6]. The connection between refractoriness and the secondary messenger system has not been adequately studied. Previously the writers found changes in the concentration of calcium ions bound with the platelet membrane corresponding to the state of refractoriness [2]. The aim of this investigation was to study changes in the free Ca^{++} concentration in the cytoplasm during a change in shape and aggregation of platelets in the normal and refractory state.

EXPERIMENTAL METHOD

The following preparations were used: Indo 1-AM, Indo 1, and MOPS from "Calbiochem," apyrase, ADP, and bovine serum albumin from "Sigma," and EGTA from "Fluka." All other reagents were of the chemically pure grade.

A rabbit's blood was stabilized with standard acid citrate–dextrose solution in the ratio of 8.5:1.5. Platelets were washed by the method in [3]. MOPS–NaOH was used as the buffer. After centrifugation of the platelet plasma and removal of the supernatant the platelets were resuspended in 1% Tyrode solution, incubated with 10 μM Indo 1-AM for 30-45 min at 37°C, and washed twice.

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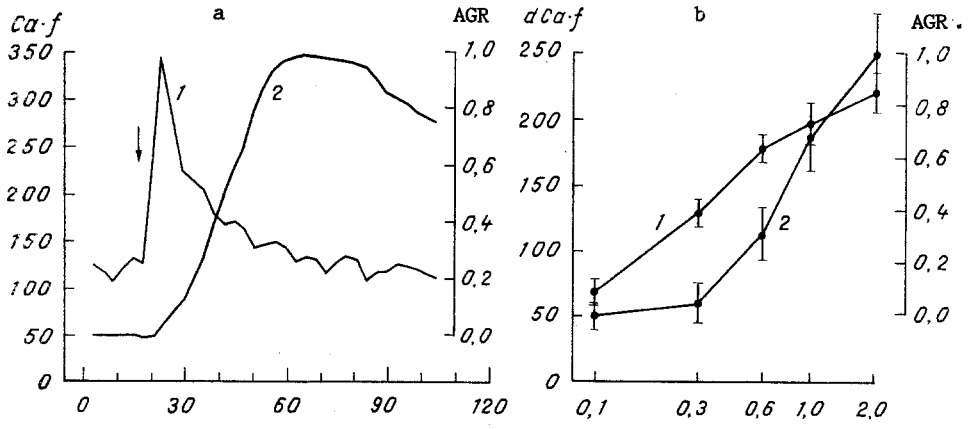

Fig. 1. Stimulation of free intracellular Ca^{++} level (1) and of platelet aggregation (2) by ADP. a) Kinetics of change in free intracellular Ca^{++} level (K_d = 250 nM) and aggregation. Abscissa, time (in sec), AGR) platelet aggregation (in relative units). Arrow indicates time of addition of ADP (2 μM); b) dependence of aggregation of platelets and elevation of their free Ca^{++} level on ADP concentration (in μM). Abscissa, ADP concentration, dCa·f) greatest rise of free Ca^{++} level in platelets (in nM). Mean results of four experiments.

Aggregation and fluorescence were measured simultaneously in the same sample of platelet suspension, introduced into a standard cuvette of the aggregometer (1 ml). Aggregation was measured by means of a photometer at a wavelength of 860 nm [1].

The concentration of intracellular free calcium (Ca·f) was determined by the method described in [7], with the aid of the Ca-sensitive fluorescent probe Indo 1 [4]. The method consists essentially of distinguishing the spectrum of the probe contained in the platelets, which decomposes into components corresponding to the free form of the probe $F_i^- \times In{\cdot}f$ (where F_i^- is the fluorescence spectrum of 1 μM of the free probe and In·f the concentration of the free probe) in the complex of the probe with calcium $F_i^+ \times In{\cdot}b$ (where F_i^+ denotes the fluorescence spectrum of 1 μM of the complex of Indo 1 with calcium, and In·b denotes the concentration of that complex). The concentration of free intracellular Ca^{++} (Ca·f) is determined by the equation

$$Ca{\cdot}f = K_d \times In{\cdot}b / In{\cdot}f$$

where K_d is the dissociation constant of the complex of Indo 1 with Ca^{++}. In cases when the exact absolute value of Ca·f was not necessary, only the value of the ratio In·b/In·f was determined, so as not to introduce any error connected with inaccuracy of determination of K_d. The value of the ratio In·b/In·f is directly influenced by a change in shape of the platelets, by their aggregation, or even by the escape of some cells from the mixed volume in the cuvette (i.e., sedimentation of aggregates to the bottom and adhesion of platelets and aggregates to the mixer and the walls of the cuvette. The content of the probe in the cells in the optical channel is characterized by the value of In·t = In·b + In·f. The concentration of the probe in the cells is equal to the ratio of In·t to the specific volume of the platelets.

The cAMP concentration in the platelets was measured by radioimmunoassay, using kits from "Amersham International."

EXPERIMENTAL RESULTS

Addition of ADP to a suspension of washed platelets, loaded with the probe Indo 1 (50 μM) and resuspended in medium containing $CaCl_2$ (1 mM) caused an extremely rapid increase in the intracellular Ca^{++} level. The maximal value of the Ca^{++} concentration was reached not later than after 3 sec (3 sec being the time between two consecutive measurements of the spectra). This was followed by a gradual decrease in the Ca^{++} concentration (Fig. 1a, curve 1). ADP also induced platelet aggregation (Fig. 1a, curve 2). A characteristic feature in this case is that the aggregation process was significantly slower than the change in the intracellular Ca^{++} level. Platelet aggregation reached a maximum at a time when the Ca^{++} concentration was not yet very much higher than the level characteristic of unactivated cells. With

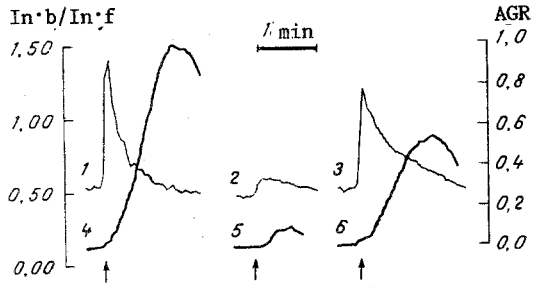

Fig. 2

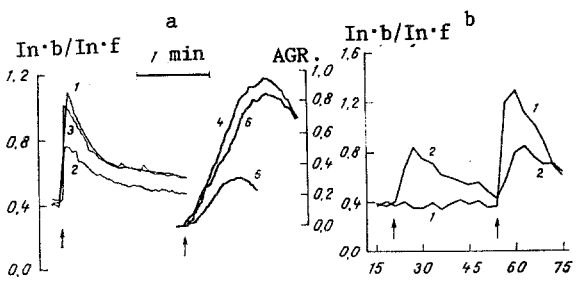

Fig. 3

Fig. 2. Repeated stimulation of platelets by ADP. Curves 1, 2, and 3 show values of In·b/In·f, proportional to free Ca^{++} level in platelets (explanation in text); platelet aggregation — curves 4, 5, and 6 respectively; 1, 4) after 1st dose of ADP (0.6 μM), 2, 5) after repeated dose of ADP (interval 5 min), 3, 6) after 1st dose of ADP added 5 min after beginning of mixing of suspension. Remainder of legend here and in Fig. 3 as to Fig. 1.

Fig. 3. Refractory state of platelets not taking part in aggregation. a) Extracellular Ca^{++} concentration 1 mM. Curves 1, 2, and 3 denote values of In·b/In·f, platelet aggregation — curves 4, 5, and 6 respectively. Curves 1, 4 — native platelet suspension, with addition of 0.6 μM ADP. Suspension of platelets treated beforehand with 0.1 μM ADP (aggregation absent), 0.6 μM ADP (curves 2, 5) or 1.0 μM ADP added (curves 3, 6); b) extracellular Ca^{++} concentration lowered to 30 nM by EGTA. Physiological saline (curve 1) or 0.1 μM ADP (curve 2) added initially to platelet suspension, followed 35 sec later by 0.1 μM ADP. Abscissa, time (in sec).

an increase in concentration of the inducer, there was an increase both in the rise of the free Ca^{++} concentration in the cytoplasm of the platelets, and also of their aggregation (Fig. 1b). Platelets were aggregated in response to addition of ADP in cases when the increase in the Ca^{++} concentration exceeded 80% of its initial level.

With an average intracellular concentration of the probe in the platelets within the range 20-70 μM the kinetics of aggregation of the probe-loaded platelets did not differ significantly from the kinetics of aggregation of the control platelets, not containing the probe; in other words, the probe did not introduce any appreciable change into platelet activation.

A change in the scattering of light of the sample of the platelet suspension after the addition of ADP to it because of a change in shape of the cells and their aggregation not only did not affect the kinetics of the change in free Ca^{++} concentration in the platelets, but actually had no effect on the average concentration (per volume of the cuvette) of the In·f probe. The absence of change in its value is evidence that the mean mass of the platelets in the optical channel remained the same, and it indicated absence of adhesion of the cells to the walls of the cuvette and to the mixer, as well as absence of sedimentation of platelet aggregates to the bottom of the cuvette. Thus the value of In·b/In·f during platelet aggregation characterizes the average concentration of free cytoplasmic Ca^{++} in the whole platelet population.

A second addition of ADP also caused a marked increase in the Ca^{++} concentration in the cytoplasm (Fig. 2, curve 2). However, the rise of the Ca^{++} concentration was significantly lower than that after the first addition of ADP. Repeated addition of ADP caused aggregation of the cells, but this also was of lower amplitude (Fig. 2, curve 4). It must be pointed out that inhibition of aggregation was actually more marked than inhibition of the rise of Ca^{++}. This reduction of the increase in the free Ca^{++} concentration and of platelet aggregation was not connected with prolonged mechanical action on the cells during continuous mixing of the sample of the platelet suspension. Th the control sample of platelets, instead of the first dose of ADP, the same volume of physiological saline was added (Fig. 2, curves 3, 6). Long-term incubation of platelets with constant mixing reduced aggregation and the rise of the Ca^{++} concentration a little. However, these reactions of the cells were much stronger than those of platelets in a refractory state.

Inhibition of platelet aggregation described above was not directly connected with the aggregation process arising from the first dose of ADP. Reduction of the first dose of ADP

to an amount unable to induce aggregation did not prevent the inhibition of the rise of Ca^{++} or aggregation, induced by the second dose of ADP (Fig. 3a, curves 1, 2, 4, 5). With an increase in the second dose of ADP, both the rise of Ca^{++} and aggregation of the platelets increased (Fig. 3a, curves 3, 6). Platelets suspended in medium not containing Ca^{++} were unable to aggregate under the influence of ADP; however, an increase in the Ca^{++} concentration in the cytoplasm of the cells took place and depended on the concentration of inducer. In this case also preliminary incubation of platelets with low doses of ADP caused a reduction of the increase in Ca^{++} concentration in the cytoplasm, induced by the second dose of ADP (Fig. 3b).

We showed that incubation of platelets with low doses of ADP did not cause any marked increase in the total cAMP concentration in the platelets by the time of addition of the second dose of ADP.

Inhibition of the rise of Ca·f under the influence of ADP found in the refractory state may actually be the cause of the decrease in platelet aggregation in the refractory state. Similar inhibition in the presence and absence of extracellular Ca^{++} evidently indicates a common mechanism of inhibition of the rise of Ca·f in these cases.

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CARDIAC OUTPUT OF CONSCIOUS RATS MEASURED BY AN ULTRASONIC DOPPLER TECHNIQUE

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KEY WORDS: cardiac output; ultrasonic Doppler technique; labeled microspheres.

To measure cardiac output in small laboratory animals the methods most widely used are the isotope-labeled microspheres method [3] and the thermodilution method [5], but a defect common to both of them is the discreteness of measurement of cardiac output and the fact that they can be correctly used only under steady-state conditions of function of the cardiovascular system.

In the present investigation the cardiac output of conscious rats was determined by an ultrasonic Doppler technique, the working principle of which is measurement of changes in the frequency of ultrasound reflected from the moving blood cells. The main advantage of the Doppler method is absence of zero line drift, a matter of particular importance when measurements are to be made under chronic experimental conditions.

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