AGGREGATORY REACTIONS OF BLOOD PLATELETS IN UNSTIRRED DILUTE SUSPENSIONS AND THEIR MONITORING BY SPECTROPHOTOMETRY

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ABSTRACT

A double beam recording spectrophotometer was used to trace agonist-induced turbidity changes in unstirred dilute suspensions of gel-filtered calf platelets ($2–6 \times 10^7$ per ml) differentially against a sample of platelets in the reference beam. Aggregatory reactions were revealed by phase-contrast microscopy of glutaraldehyde-fixed samples, and were manifested in the tracings as turbidity decreases. The slope of the initial turbidity decrease varied with platelet and agonist concentrations and could be useful to characterize dose-response behaviour of agonists.

INTRODUCTION

Two conditions widely believed to be essential for agonist-induced aggregation of isolated blood platelets are high platelet concentrations (3×10^8 per ml, or more)[1] and a 'collision force' of stirring. These conditions appeared unrealistic to us because the specific intercellular interactions, of which platelet aggregation is an example, are mediated by cell surface-bound specific biochemical species[2] interacting through molecular forces[3]. Hence only rates may vary with platelet concentration or by stirring. We show here that various agonists induce aggregation of low concentrations of gel-filtered calf platelets without having recourse to a 'collision force' and that the aggregation can be readily detected and its rate measured employing an ordinary double beam recording spectrophotometer.

MATERIALS AND METHODS

ADP (sodium salt), thrombin (human plasma), arachidonic acid (99% pure), the calcium ionophore, A23187, dimethylsulphoxide, glutaraldehyde (25% solution) and Tris base were purchased from Sigma Chemical Co., St. Louis, U.S.A.

Collection, centrifugation and storage of platelets were done in polypropylene tubes. The platelet preparations were pipetted employing automatic pipettes and polypropylene tips. All operations were carried out at ambient temperature (31 ± 1°C).

Preparation of platelets

Calf (male) blood was collected by jugular venic puncture into 1/10th volume of 0.13 M trisodium citrate and platelet-rich plasma (PRP) was obtained by centrifuging at $70 \times g$ (r_{av}. 8.5 cm) for 30 min. The PRP was centrifuged again at $24 \times g$ to remove residual erythrocytes and leukocytes. Gel-filtered platelets (GFP) were obtained according to Tangen *et al*[4] using their noncolloidal buffer system (column buffer): glucose (5.5×10^{-3} M), $CaCl_2$ (5.0×10^{-5} M), $MgCl_2$ (9.8×10^{-4} M) and KCl (5.4×10^{-3} M) in 142 mM NaCl–145 mM Tris–HCl buffer, pH 7.4, in the volume proportion 9 : 1.

Only those preparations in which at least 90% of platelets appeared as single platelets in the microscope were used after diluting with column buffer to an A_{540} of 0.5 which corresponded to a platelet concentration of $6 \pm 0.06 \times 10^7$ per ml.

Spectrophotometric and phase-contrast microscopic detection of aggregation

The absorbance A and turbidity, τ, of a dilute platelet suspension in an optical path of a given wavelength, are related by the equation:

$$A = \log (I_o/I) = 0.434\, \tau\, l, \qquad (1)$$

where I_o and I are respectively the intensities of the incident and transmitted beams and l is the optical pathlength. τ depends on the concentration, optical properties, shape and orientation of platelets[5]:

$$\tau = \sum_{n=1}^{\infty} C_n R_n\,, \qquad (2)$$

where C_n is the number of n-mers ($n = 1, 2$ etc) per ml and R_n the scattering cross-section of a particular n-mer.

The scattering cross-section of an *n*-mer of platelets is much less than that of *n* monomers[5]. As a result R_n decreases drastically during aggregation when C_n also decreases. Thus turbidity (absorbance value) should decrease sharply during aggregation of platelets in dilute suspensions. This could be detected in a spectrophotometer but the sensitivity of monitoring could be improved by using a double beam spectrophotometer and placing a cuvette containing platelets in the reference beam also. We have used a Shimadzu UV-240 recording spectrophotometer. Platelet suspensions (1 ml) were taken in a pair of a siliconized glass cuvettes. After temperature equilibration the instrument, with the cuvettes in position, was set to read zero at 540 nm. Platelet activation was initiated by adding agonist to the sample cuvette and mixing. The turbidity changes that ensued were recorded as a function of time.

For phase-contrast microscopy the contents of the reference and sample cuvettes were treated as described in figure 2 (see legend).

RESULTS AND DISCUSSION

Representative recorder response patterns obtained in experiments employing various agonists are shown in figure 1. On mixing with the agonist in the sample cuvette the absorbance value (turbidity) at first increased above the zero setting (OA) owing to platelet shape-change[6,7]. The increase was followed, after a lag period, by a decrease. The rate of turbidity decrease slowed down faster in the case of ADP or thrombin than in the case of A23187 or arachidonic acid. In the case of arachidonic acid rate acceleration often occurred as the reaction progressed. That the platelets had indeed been aggregated during the spectrophotometrically recorded turbidity decrease was clear from microscopic examination of glutaraldehyde-fixed platelet samples (figure 2).

Aggregation could be detected even at 2×10^7 platelets per ml as already found by Gear[8] for human platelet-rich plasma employing vastly more sophisticated methodology. We employed gel-filtered platelets in this study to avoid problems of protein-mediated cross-linking during glutaraldehyde fixation. But aggregation of calf as well as human PRP could be detected spectrophotometrically (after diluting to reduce platelet concentration); much higher concentrations of agonists were, however, required except in the case of ADP.

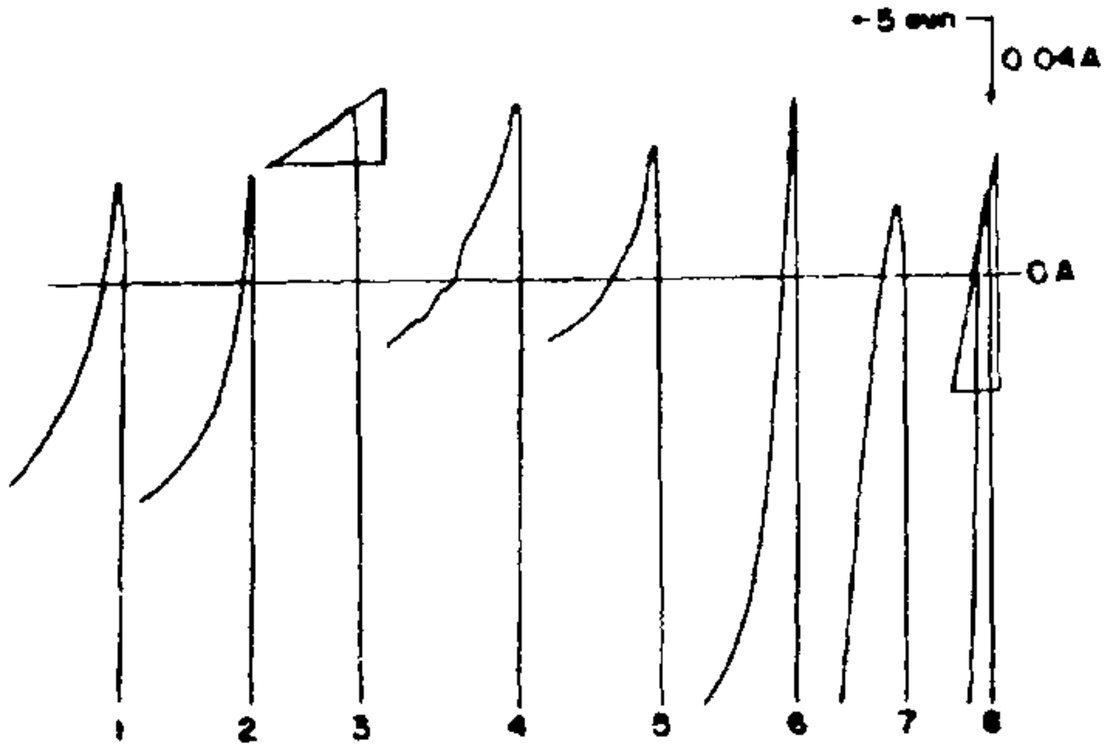

Figure 1. Representative tracings of spectrophotometric recorder response obtained in experiments using different agonists and the manner of measuring rates. Experimental details are given in the text. The agonists and their concentrations (with tracing number in parantheses) were: thrombin, 0.2 unit per ml (1) and 0.6 unit per ml (2); ADP, 2.8 μM (3) and 6.6 μM (4); A23187, 5 nM (5) and 10 nM (6); and arachidonate 10 μM (7) and 40 μM (8). The time and absorbance scales were as indicated at the top right hand corner. The initial setting was at 0 A. The tracings began from below, going up on starting the recorder after agonist addition to the sample cuvette and its keeping in position. The reaction progressed from right to left. The manner of measuring rates is exemplified in tracings 3 and 8. Tracings obtained with different platelet preparations are shown.

The foregoing results clearly showed that platelet aggregation had taken place at low platelet concentrations without the intervention of a 'collision force'. Several empirical observations, in the literature, also argue against the notion that platelet aggregation is in need of a collision force: (i) spontaneous aggregation occurs, infrequently, if at all, in stirred concentrated suspensions of platelets in the absence of an agonist[9]; (ii) even on stirring with an agonist aggregation begins only after a lag period which is related inversely to agonist concentration[8,10], and (iii) aggregation then increases with time[11] depending on the formation of pseudopods by shape-change of platelets[12]. What impels platelets to aggregate then is not so much a collision force as the force(s) generated in the wake of shape-change and other reactions induced by the agonist. In agreement with these ideas is the elegant demonstration by Chang and Robertson[3] that ADP induces aggregation of rabbit platelets by Brownian

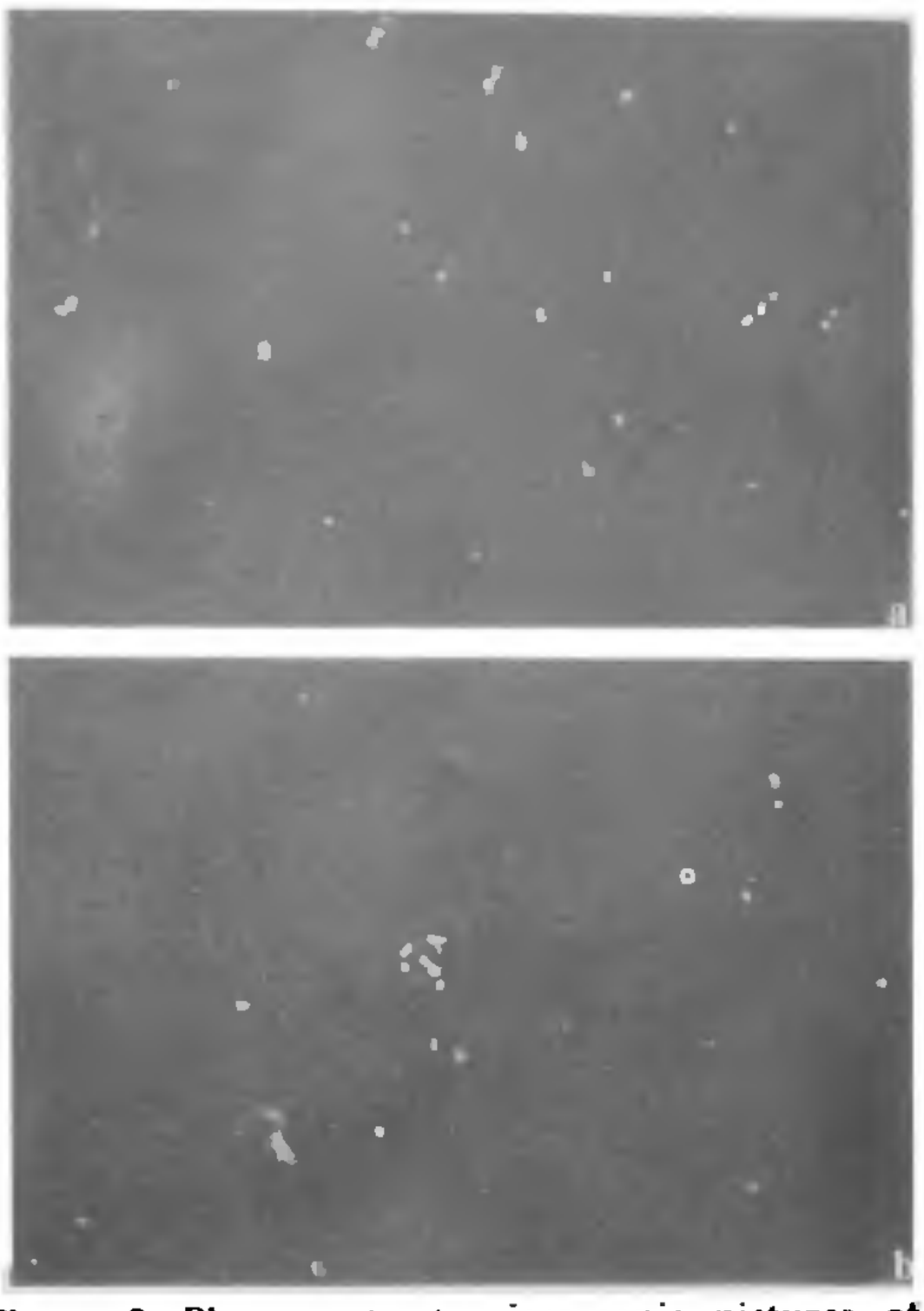

Figure 2. Phase-contrast microscopic pictures of platelet samples in the reference (a) and sample (b) cuvettes 60 s after activation with 40 μM arachidonic acid (tracing 8 in figure 1). The contents of the cuvettes were treated with 80 μl of glutaraldehyde and after gentle thorough mixing, 0.1 ml of the fixed platelets were pipetted into 0.3 ml of column buffer. The diluted platelets were examined in a haemocytometer under a binocular Nikon phase contrast microscope and photographs were taken. Similar results were obtained using other agonists employed in figure 1.

motion alone at 25°C. Bell and Goldsmith[13] showed further that the rate of aggregation of human platelets in Poisuille flow, within 10 sec of mixing with ADP is in fact independent of two-body collisions between platelets. These workers have employed high platelet concentrations. We have demonstrated not only the occurrence of aggregation by Brownian motion at low platelet concentrations but also a simple method of detecting and, possibly, quantitating it. It may be pointed out here that to detect aggregation at platelet concentrations less than 5×10^7 per ml, vastly more sophisticated instrumentation[8] or time-consuming microscopic procedure is needed.

Since a double beam spectrophotometer may be available in many laboratories, and some hospitals, the present method could readily be used for platelet aggregation research, both fundamental and clinical.

ACKNOWLEDGEMENTS

We thank Mira Mohanty for taking microscopic pictures and platelet counting and Jose Jacob for technical assistance. The scheme is supported in part by grants from the Departments of Atomic Energy, and Science and Technology, Government of India.

14 October 1985; Revised 17 September 1986

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