Stopped-flow spectrophotometry monitoring the initial processes of platelet activation by platelet-activating factor

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The initial processes of platelet activation by platelet-activating factor (PAF) were observed by stopped-flow light scattering and fluorometry. The binding of PAF to rabbit platelets did not change the membrane fluidity, though it caused the removal of calcium from internal stores and induced concomitantly platelet shape changes. These results were quite in contrast to those by thrombin and ADP activation, where the membrane fluidity increased before the calcium release from the internal stores. The increased membrane fluidity in the latter system seemed to be used to transmit an external signal to a GTP-binding protein.

Platelet *Platelet-activating factor* *Stopped-flow* *Membrane fluidity* *Pyrene* *Light scattering*

1. INTRODUCTION

In various cells, the binding of ligands to their specific receptors induces an increase in cytosolic calcium concentration. This rise in cytosolic calcium is suggested to follow the breakdown of phosphatidylinositol bisphosphate into diacylglycerol and inositol trisphosphate. The latter is known to cause release of calcium from intracellular stores [1–4]. The increase in the intracellular level of calcium and the released diacylglycerol, which stimulates protein phosphorylation, are known to mediate the subsequent physical responses [1–4].

To understand the physical aspects of such kinds of signal transfer, we have recently studied the initial transmembrane events involved in platelet activation by thrombin and ADP using stopped-flow photometry [5,6]. From these experiments we found that the binding of the ligands to their membrane receptors increased the platelet membrane fluidity before the release of calcium from intracellular stores [5,6]. Therefore, it seems that the rapid rise of the membrane fluidity is used for initial signal transduction, i.e. to transmit a signal to a GTP-binding protein [7,8] or to a phospholipase C which breaks down phosphatidylinositol bisphosphate into diacylglycerol and inositol trisphosphate [1].

Here, we have studied the initial processes of platelet activation by a platelet-activating factor (PAF) using stopped-flow spectrophotometry. The binding of PAF to rabbit blood platelets induced the release of calcium from intracellular stores as did the binding of thrombin (or ADP). Contrary to thrombin (or ADP), however, PAF did not change the platelet membrane fluidity at all. These observations lead us to discuss the relationship between the initial membrane fluidity change and cell signaling.

2. MATERIALS AND METHODS

Washed platelet samples were prepared from rabbit blood according to Ardlie et al. [9]. Thrombin, ADP, chlortetracycline and pyrene were purchased from Sigma. 1-*O*-Octadecyl-2-*O*-acetyl-

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sn-glycero-3-phosphorylcholine (PAF) was kindly provided by Professor Keizo Inoue (University of Tokyo).

Chlortetracycline was incorporated into platelets by incubating 9×10^8 cells/ml platelet suspension with 10 μM chlortetracycline for 30 min at 25°C. Pyrene was incorporated into platelets by incubating 1.4×10^8 cells/ml platelet suspension with 10 μM pyrene for 30 min at 25°C. The platelets were then washed with Ca^{2+}-free Tyrode solution (10 mM Tris-HCl, 137 mM NaCl, 2.6 mM KCl, 0.1% glucose, pH 7.3) and resuspended in the same buffer for fluorescence measurements.

Fluorescence spectra were observed using a Hitachi model 650-10S fluorescence spectrophotometer. The time courses of fluorescence and light scattering were traced with a Union Giken RA-401 stopped-flow spectrophotometer in combination with an RA-450 microcomputer system [10,11]. The dead time of this instrument is 0.5 ms. We used a Hoya Y46 cut filter (which allows the emitted light of wavelength longer than 460 nm to enter the detector) in the chlortetracycline and pyrene fluorescence measurements. Light scattering measurement was made at an angle of 90° from the incident beam, whose wavelength was set at 500 nm.

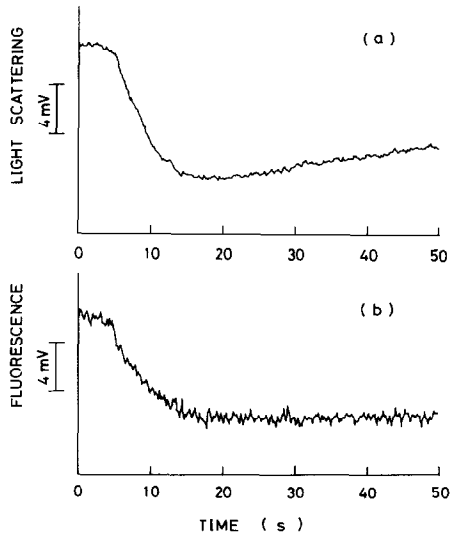

Fig.1. Stopped-flow spectrophotometry traces indicating effects of platelet-activating factor on rabbit platelets. Platelet concentration was 7.0×10^7 cells/ml and PAF concentration was 8.0 nM after mixing. Temperature was 25°C. (a) Light scattering intensity (90°) at 500 nm. (b) Fluorescence from chlortetracycline-labeled platelets was monitored on exciting at 400 nm and by observing through a cut filter which allows light of wavelength longer than 460 nm to enter the detector.

3. RESULTS

3.1. *Platelet shape change by PAF*

When a platelet suspension (final 7×10^7 platelets) was mixed with PAF (final 8.0 nM) by a stopped-flow spectrophotometer, the time-dependent light scattering (90°) was observed as shown in fig.1a. This kind of change in light scattering was also found in the binding of thrombin (1 U/ml) or ADP (0.1 mM) to platelets [5,6]. The later phase of the light scattering changes described here is consistent with the results of conventional light scattering experiments [12,13]. Our new finding is the occurrence of a lag period. That is, after a short lag period (5 s at 25°C), the light scattering was abruptly decreased which reflected the platelet shape changes [5,6,12,13].

3.2. *Intracellular calcium release by PAF*

On mixing with PAF, the fluorescence intensity of the chlortetracycline-labelled platelets decreased after a 5 s lag period at 25°C (fig.1b), which was similar to what was observed in the light scattering measurement. The chlortetracycline fluorescence decrease was considered to be caused by calcium release from internal stores into the cytoplasm [5,6,14,15]. When 5 mM EDTA was added to the external medium to prevent calcium influx into platelets, the chlortetracycline fluorescence decrease was still observed. This indicates that the chlortetracycline fluorescence change in fig.1b was attributable to intracellular calcium mobilization and not to calcium influx [16]. The release of calcium from internal stores occurred concomitantly with the platelet shape changes. Here, thrombin or ADP also induced similar changes in chlortetracycline fluorescence in platelets [5,6].

3.3. *Effect on membrane fluidity*

During the 5 s lag period (at 25°C), a rapid increase in platelet membrane fluidity was observed by the use of pyrene excimer fluorescence when platelets were mixed with thrombin (or ADP)

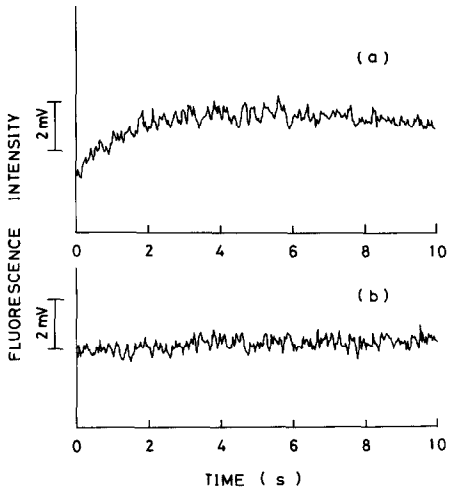

Fig.2. Stopped-flow fluorometry traces indicating effects of ligands on platelet membrane fluidity. Rabbit platelet concentration was 7.0×10^7 cells/ml. Temperature was 25°C. Fluorescence from pyrene-labeled platelets was monitored on exciting at 340 nm and by observing through a cut filter which allows light of wavelength longer than 460 nm to enter the detector. (a) Effects of thrombin (1 U/ml) on the fluorescence of pyrene-labeled platelets. (b) Effects of platelet-activating factor (8.0 nM) on the fluorescence of pyrene-labeled platelets.

[5,6]. Fluorescence spectra of pyrene embedded in platelets membranes showed monomer fluorescence peaks around 390 nm and a broad excimer fluorescence peak around 480 nm [5,6,17,18]. After binding of thrombin (1 U/ml), the excimer fluorescence in platelets increased gradually without a lag period (fig.2a) [5,6]. However, the binding of PAF to platelets did not change the membrane fluidity at all as seen in fig.2b, although PAF induced both the removal of the intracellular calcium and platelet shape changes.

4. DISCUSSION

The present experiment showed that the binding of PAF to platelets did not change the platelet membrane fluidity, although it caused the removal of calcium from the internal stores. However, the binding of thrombin or ADP increased the platelet membrane fluidity before the calcium release from internal stores. In addition, our recent experiments showed that ligand binding to specific cell surface receptors, for example interleukin 2 receptors, also increased the membrane fluidity before the calcium release from internal stores [9]. Thus, the increased membrane fluidity now in question may be used for transmembrane signaling, i.e. to transmit a signal from the cell surface receptors to a GTP-binding protein or to perturb the membrane sufficiently to make phosphatidylinositol bisphosphate accessible to phospholipase C. We cannot say which process really occurred at present. It is plausible, however, that the membrane fluidity increases are used to transmit a signal to the GTP-binding protein, because PAF which did not cause an increase of platelet membrane fluidity is known to induce phospholipase C activation [20].

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