Probes of Transmembrane Potentials in Platelets: Changes in Cyanine Dye Fluorescence in Response to Aggregation Stimuli

By William C. Horne and Elizabeth R. Simons

A noncovalent fluorescent probe that responded to changes in transmembrane potential was used to study the response of washed human platelets to aggregating agents. Concentration-dependent changes in the fluorescence were observed in response to ADP and to thrombin. No such changes were observed in response to collagen fibrils. Thus there was an indication that platelet membrane potential changed in response to aggregating stimuli, supporting the hypothesis that the mechanism of platelet aggregation resembled the mechanisms of other systems that show stimulus-response coupling (e.g., muscle, adrenal chromaffin cells). The different responses to specific agents indicate that the agents may trigger platelet aggregation through different mechanisms.

The mechanism of platelet response to stimulation by aggregating agents has been compared to the coupling between a stimulus and its response, as found in muscle and in secretory cells. Since alterations in the transmembrane potential are known to be involved in the mechanism of both excitation-contraction coupling in muscle and in stimulus-secretion coupling in the adrenal, we investigated the possibility that a change in the membrane potential might play a role in the response of platelets to specific stimuli (e.g., ADP, thrombin) of platelet aggregation.

Since direct measurement of the membrane potential of platelets with microelectrodes is not feasible owing to the size of the platelet, we applied an indirect method of measurement in which the transmembrane potential was determined by using a lipophilic cation shown to be distributed between the cells and the medium in response to the potential. One method of this type, which employs a fluorescent dye of the cyanine series, has been applied by other investigators, as well as ourselves, to study the membrane potentials of red blood cells, lymphocytes, and platelets. Another, similar method employs a radioactively labeled probe. The times involved in sampling and measuring the radioisotopes are long enough (15-30 sec) so that the technique might not allow measurement of rapid, possibly transient, changes in the potential. We therefore used, and here report on, measurements using a fluorescent lipophilic cation.

We found that the fluorescence of the cyanine dye 3,3'-dipropthiodicarbo-cyanine [dIS-C1-(5)] changes in response to specific stimuli (e.g., ADP, thrombin) of platelet aggregation. We believe that these changes in fluorescence are at least in part a reflection of the change in the transmembrane potential of the platelet membrane.

---

From the Boston University School of Medicine, Boston, Mass.

Submitted May 3, 1977; accepted October 31, 1977.

Supported by NIH Grants HL 15335 and 1 F32 HL 05252-01.

Address for reprints: Elizabeth R. Simons, Ph.D., Boston University School of Medicine, 80 E. Concord St., Boston, Mass. 02118.

© 1978 by Grune & Stratton, Inc. ISSN 0006-4971/78/5104-1003$01.00/0
platelet in response to interactions between platelet membrane receptors and the specific stimuli recognized by these receptors. The change in fluorescence is accompanied by release of serotonin, does not require external Ca$^{2+}$, and precedes aggregation, since it can be observed even when the aggregation step itself is inhibited.

Changes that occur in the transmembrane potential of red blood cells, lymphocytes, and platelets upon treatment with K$^+$ and Ca$^{2+}$ ionophores have been measured with fluorescent dyes of the cyanine series.\textsuperscript{6-9} They have been shown to be inversely proportional to changes in the fluorescence intensity of the dyes, i.e., hyperpolarization of the membrane leads to decreased fluorescence, while depolarization leads to increased fluorescence. Two cyanine dyes have now been used in our laboratory to investigate the changes in the transmembrane potential of gel-filtered human platelets upon exposure to aggregating agents. One, diS-C$_2$(-5), partitions across the platelet membrane in response to changes in the transmembrane potentials. The second, a sulfonated analogue of diS-C$_2$(-5), 3,3'-dipropylsulfonatethiodiicarbocyanine [diS-C$_2$-SO$_3$(-5)], does not bind to the cell and thus can be used to determine if any of the observed change in the signal is an artifact due to changes in the light-scattering properties of the sample.

Using these dyes we examined the changes in fluorescence resulting from the discharge of a K$^+$ gradient across the membrane by valinomycin and those due to the interaction of ADP, thrombin, or collagen with the platelet. We found that thrombin and ADP, but not collagen, cause a change in the fluorescence and that the nature of the observed change is specific to the aggregating agent added.

**MATERIALS AND METHODS**

All procedures involving whole blood or platelets were performed using plastic or siliconized glass vessels.

**Preparation of Platelets**

Blood was drawn by peripheral venipuncture from normal human volunteers and mixed with 3.8\% sodium citrate (9 vol blood to 1 vol citrate). The citrated blood was centrifuged for 10 min at 126 g using an International HN-S centrifuge. The platelet-rich plasma (PRP) was pipetted off. Platelets were freed from soluble plasma components by the gel-filtration technique of Tangen and Berman.\textsuperscript{21} The PRP (10 ml) was applied to a 1 1 x 2.5 cm Sepharose 2B column equilibrated with 0.1\%, glucose, 5.4 mM KCl, 1.0 mM MgCl$_2$, 0.05 mM CaCl$_2$, and 0.5\%, bovine serum albumin dissolved in one part 0.145 M Tris·HCl, pH 7.6, to nine parts 0.140 M NaCl. Apyrase (final concentration 0.15 U/liter) was also added to the buffer. We determined that concentrations higher than this would lead to reversible aggregation by $10^{-6}$ M ADP. Platelets were eluted using the same buffer. Fractions containing the platelets were pooled. Platelet concentrations were determined by measuring turbidity at 436 nm.

**Aggregometry**

Aggregation of platelets was monitored by measuring the changes in turbidity using a Chronolog aggregometer according to the method of Michal and Born.\textsuperscript{22} Platelet suspensions (100,000-150,000/µl) were aggregated by either thrombin (0.1 U/ml), ADP ($10^{-7}$ M), or collagen (30 µg/ml). For aggregation by ADP or collagen, fibrinogen (Kabi, Stockholm, final concentration 250 mg/dl) was added to the platelets.
**Fluorescence**

Fluorescence measurements were made on a Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with a thermostatted cell holder and a stirring apparatus. The sample was excited at 620 nm (slit, 6 nm) and the emission monitored at 670 nm (slit, 6 nm). The signal was first measured in the absence of probe; then the dS-C$_5$(5) (final concentration $2.0 \times 10^{-6} M$) was added. After the fluorescence had stabilized, the appropriate perturbant was added and the fluorescence monitored until no further change occurred (usually 20 min). The results are reported as ratios $F/F_0$ of observed ($F$) to initial ($F_0$) fluorescence intensities.

**Serotonin Release**

Serotonin release was measured by the method of Jerusalmy and Zucker.$^{13}$

**Determination of K$^+$**

Intracellular and extracellular concentrations of potassium were determined with an Instrumentation Laboratory 243 flame photometer. $^{3}H$-inulin was added to the platelet suspension to allow the determination of extracellular water, and the platelets were then sedimented through silicone oil. To determine extracellular K$^+$, the supernatant was diluted fiftyfold with LiCl (15 meq/liter) before measuring. To determine intracellular K$^+$, the platelet pellet was lysed with acetone and resuspended in 2 ml LiCl (15 meq/liter) using a vortex mixer.

**Collagen Fibril Formation**

For experiments where preformed collagen fibrils were added to the platelets, the fibrils were formed by adding collagen stock solutions to 0.05 $M$ Tris, 0.05 $M$ CaCl$_2$, pH 7.6 (final collagen concentration 0.1 mg/ml) warmed to 30°C. The solution was stirred for 3 min, the time necessary to form fibrils of sufficient size to cause immediate platelet aggregation,$^{15}$ and an aliquot of the resulting suspension of collagen fibrils was added to the platelet suspension.

**Materials**

The cyanine dyes were the kind gift of Dr. Alan Waggoner. ADP, valinomycin, creatine phosphate, creatine phosphokinase, and bovine serum albumin were obtained from Sigma Chemical, St. Louis. Thrombin (topical) was obtained from Parke-Davis. $^{3}H$-inulin was obtained from New England Nuclear. Collagen was prepared as previously described.$^{14}$ Apyrase was prepared by the method of Molnar and Lorand.$^{16}$ All other chemicals were reagent grade.

All human experimentation was done in accordance with the Helsinki Declaration and with approval of the Human Experimentation Committee.

**RESULTS**

**Dye Concentration**

The optimal dye concentration was determined by monitoring the change in fluorescence induced by $2 \times 10^{-6} M$ valinomycin in the presence of various concentrations of dye (Table 1). The optimal concentration, i.e., the concen-

<table>
<thead>
<tr>
<th>$[dS-C_5(5)] (\times 10^{-6} M)$</th>
<th>$F/F_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.89</td>
</tr>
<tr>
<td>1.0</td>
<td>0.81</td>
</tr>
<tr>
<td>2.0</td>
<td>0.78</td>
</tr>
<tr>
<td>4.0</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Changes in fluorescence recorded after addition of valinomycin (final concentration $2 \times 10^{-6} M$) to cell suspensions equilibrated with dS-C$_5$(5) in a medium containing 5.4 mM K$^+$. Changes are expressed as the ratio of the fluorescence observed after the addition of valinomycin ($F$) to that observed before the addition of valinomycin ($F_0$).
tration at which the largest change was observed when valinomycin was added, was $2.0 \times 10^{-6} M$ diS-C$_3$-(5). diS-C$_3$SO$_3$-·(5) did not bind to the platelets, as shown by the fact that less than 5% of the dye added could be extracted from platelets isolated after incubation with the dye.

**Valinomycin**

To show that the dye responded to changes in the membrane potential of the platelet and to calibrate the fluorescence change in terms of a change in the membrane potential, the change in fluorescence was measured when valinomycin was added to the suspension, increasing the membrane permeability to $K^+$ and discharging the $K^+$ gradient across the membrane. The resulting change in the membrane potential was a function of the $K^+$ gradient before valinomycin was added and therefore of the external $K^+$ concentration (Fig. 1).

The relationship between the change in fluorescence and that in membrane potential was estimated using the method of Hoffman and Laris. It was assumed that the membrane potential of the platelet, like that of the red cell, could be described by the constant field equation

$$E = \frac{RT}{F} \ln \frac{P_{Na}[Na]_o + P_{K}[K]_o + P_{Cl}[Cl]_o}{P_{Na}[Na]_i + P_{K}[K]_i + P_{Cl}[Cl]_i},$$

(1)

where $R$ is the gas constant, $T$ the absolute temperature, $F$ Faraday's constant, $P_{Na}$, $P_{K}$, and $P_{Cl}$ the permeability constants of the respective ions; $o$ and $i$ subscripts refer to the concentrations outside and inside the cell, respectively. We also assumed that the relative values of the permeability constants for platelets were equivalent to those for the erythrocyte. If this assumption is valid, $P_{Na}$ is much smaller than $P_{K}$ or $P_{Cl}$, and the Na term can therefore be neglected, resulting in

![Fig. 1. Changes in fluorescence resulting from exposure of washed platelets to valinomycin (final concentration $2 \times 10^{-6} M$) in the presence of $2.0 \times 10^{-6} M$ diS-C$_3$-(5). Dye added in ethanol (final ethanol concentration 0.2%) to suspensions of washed platelets (55,000/μl) in isosmotic buffers containing the desired $K^+$ concentration (KCl substituted for NaCl). Fluorescence was monitored, when a steady level of fluorescence was observed valinomycin was added. Fluorescence expressed as ratio of observed fluorescence ($F_o$) to fluorescence before addition of valinomycin ($F_o$).](image-url)
PLATELET TRANSMEMBRANE POTENTIALS

\[ E = \frac{RT}{F} \ln \frac{\alpha [K]^n + [Cl]^n}{\alpha [K]^i + [Cl]^i}, \]  

(2)

where \( \alpha = P_K / P_{Cl} \). It follows from Eq. (2) that when \([K], [Cl], [K]_o, [Cl]_o\) the value of \( E \) is independent of \( \alpha \) and no change in potential is expected when the permeability to \( K^+ \) is increased by the addition of valinomycin. For the case where valinomycin produces no charge in the fluorescence, \([K^+]_o\) was determined to be 36.6 meq/liter, \([K^+]_i\) 136.0 meq/liter, and \([Cl^-]_o\) 137.6 meq/liter. \([Cl^-]_i\) can be calculated to be 37.0 meq/liter.

Once the concentrations have been determined or calculated, one can calculate the value of \( \alpha \) in the presence of valinomycin that gives the best fit between the calculated potential change and the observed fluorescence change. For platelets this value of \( \alpha \) was 10, compared to a value of 3 for erythrocytes calculated by Hoffman and Laris \(^7\) under similar conditions. The expected change in membrane potential was then calculated for each \([K^+]_o\), using \( \alpha = 10 \). These calculated values were then plotted against the observed change in fluorescence (Fig. 2); a linear relationship between them is evident. The slope of the line indicates that an increase of 1\(^\circ\) in dye fluorescence under these conditions corresponds to a membrane depolarization of 1.8 mV.

Aggregating Agents

The effect of ADP, thrombin, or collagen was investigated at various concentrations. Experiments were done in the presence of 2 mM ethyleneglycol-bis(\(\beta\)-aminoethyl-\(\gamma\))-N, N'-tetraacetic acid (EGTA) to prevent aggregation, since aggregating platelets adhered to the stirring apparatus, resulting in a decrease in the fluorescence due to the removal of platelet-bound dye from the exciting light beam rather than to a change in the potential.

When EGTA was omitted, aggregation in the presence of dye was indis-

![Fig. 2. Observed changes in diS-C\(_2\)-(5) fluorescence after addition of valinomycin versus changes in membrane potential calculated from K\(^+\) and Cl\(^-\) concentrations (Eq. (2)). Fluorescence experiments carried out as described in Fig. 1.](https://www.bloodjournal.org)
Table 2. Release of ³H-Serotonin: Effect of EGTA

<table>
<thead>
<tr>
<th>Aggregating Agent</th>
<th>³H-Serotonin Released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ EGTA (2 mM)</td>
</tr>
<tr>
<td>ADP ($10^{-5}$ M)</td>
<td>71</td>
</tr>
<tr>
<td>Collagen (25 μg/ml)</td>
<td>94</td>
</tr>
<tr>
<td>Thrombin (0.05 U/ml)</td>
<td>93</td>
</tr>
</tbody>
</table>

tinguishable from that seen with control platelets, demonstrating that the probe did not interfere with the platelet response. The release of ³H-serotonin accompanied the change in fluorescence, indicating that the EGTA prevented only aggregation and that the platelets were otherwise responsive (Table 2).

Under our conditions, ADP caused a rapid, transitory decrease in the fluorescence followed by a slow increase to a final level higher than the intensity before the addition of the perturbant (Fig. 3). The maximum decrease in fluorescence occurred 2 min after addition of the ADP. Increasing concentrations of ADP, up to but not beyond $3 \times 10^{-5}$ M, caused larger changes in the fluorescence. Neither AMP nor adenosine ($3 \times 10^{-3}$ M) caused any change. Addition of $3 \times 10^{-5}$ M ADP to platelets in the presence of diS-C₃-5 (-SO₃) caused no changes in the signal, indicating that the observed effect with diS-C₃-5 (-SO₃) was not caused by changes in the light-scattering characteristics of the system as, for example, might have been caused by platelet shape change. It should be noted in this connection that the fluorescent emission was measured at a wavelength (670 nm) 50 nm longer than that of excitation (620 nm), a separation large enough to avoid most of the Rayleigh scattering from the dilute platelet suspension. No change in fluorescence was observed when $3 \times 10^{-5}$ M ADP was added to day-old, nonaggregable (and therefore nonresponsive) platelets.

The response to thrombin was more complex (Fig. 4). A concentration of 0.01 U/ml gave a response similar to that observed with $3 \times 10^{-5}$ M ADP. Higher levels of thrombin, however, resulted in rapid, transitory increases in fluorescence, followed by slow decreases to final levels that were of greater intensity than the initial fluorescence before addition of the thrombin. Experiments using diS-C₃-5 (-SO₃) paralleled those for ADP (see above). Unlike ADP
and thrombin, the addition of preformed collagen fibrils (final collagen concentration 0.03 mg/ml) caused no change in the fluorescence. To ensure that this was not due to the presence of the EGTA and the resulting absence of extracellular Ca^{2+}, a second series of experiments with collagen was performed, using creatine phosphate and creatine phosphokinase to remove released ADP and prevent aggregation. Again, no change in the fluorescence was observed during these experiments, indicating that unlike ADP or thrombin collagen did not cause the changes in the platelet that were monitored by diS-C_{3}(5) fluorescence.

**DISCUSSION**

It has been demonstrated previously that the directly measured membrane potential changes observed with microelectrodes are fully equivalent to those measured indirectly with cyanine dyes when the cell in question is large enough to permit microelectrode measurements. On the basis of these results, changes in fluorescence of diS-C_{3}(5) have been interpreted as changes in the membrane potential of cells that are not amenable to microelectrode techniques, such as erythrocytes, lymphocytes, platelets, and bacteria. This technique has been applied to platelets in other laboratories as well as in our own.

We have attempted to calibrate the dye response in terms of actual membrane potential change by using the type of valinomycin experiment by which such calibrations have been made for red cells and for bacterial vesicles. In applying the Goldman equation to the platelet membrane potential several assumptions have been made:

1. We must assume that the membrane potential of platelets can be described by the Goldman equation. The same assumption has been made for the red cell and for bacterial vesicles, since direct measurement of cell membrane potentials, the sole true verification of this assumption, can be made only by microelectrodes in large cells such as *Amphiuma* red cells.
(2) As a first approximation we assumed that the relative values of the permeability for Na\(^+\), K\(^+\), and Cl\(^-\) of the platelet membrane are equivalent to those of the red cell membrane. The values of the platelet permeability constants will have to be determined before an absolute value of the platelet membrane potential can be calculated.

Using these assumptions we have been able to show (Fig. 2) that upon addition of valinomycin the observed changes in fluorescence are linearly related to the changes in membrane potential as calculated by Eq. (2).

The data presented here show that a change in fluorescence occurs when platelets are exposed to thrombin or to ADP in the presence of diS-C\(_3\)(5). The magnitude of the change is dependent upon the aggregate concentration. The direction of the change, under the conditions used here, is dependent upon the aggregate itself. These data, together with our observation that collagen produces no change in fluorescence of the dye, may indicate that the mechanisms whereby these agents stimulate platelets are different in some ways.

Some caution is advisable in interpreting these results in terms of change in membrane potential alone. Our results with valinomycin clearly show that the dye does respond to changes in the transmembrane potential of platelets. When the platelets are exposed to an ionophore, no inherent change in the membrane itself occurs. When a platelet responds to an aggregating agent, however, intramembrane chemical changes (release of arachidonic acid by phospholipase A\(_2\) for example\(^3\)) are known to occur. In such a case it is possible that the dye responds to the chemical alteration of the membrane as well as to changes in the transmembrane potential. At this time we can report that the dye’s fluorescence responds in a dose-dependent manner to certain platelet aggregation stimuli, but we cannot state that the response reflects solely a change in the transmembrane potential.

We have described here a sensitive and reproducible method for monitoring the interaction of some aggregating agents with the platelet. The method can also be applied to perturbants of the platelet response to aggregants and should make possible investigations of the role of the platelet membrane in platelet aggregation.

REFERENCES


Probes of transmembrane potentials in platelets: changes in cyanine dye fluorescence in response to aggregation stimuli

WC Horne and ER Simons