Biochimica et Biophysica Acta 901 (1987) 138–146
Elsevier

BBA 73612

Parallel investigation of exocytosis kinetics and membrane fluidity changes in human platelets with the fluorescent probe, trimethylammonio-diphenylhexatriene

Martial Kubina [a], François Lanza [b], Jean-Pierre Cazenave [b], Gilbert Laustriat [a] and Jean-Georges Kuhry [a]

[a] *Laboratoire de Biophysique, U.A. 491 du CNRS, Université Louis Pasteur, Strasbourg and* [b] *INSERM U 311, Biologie et Pharmacologie des Interactions du Sang avec les Vaisseaux et les Biomatériaux, Centre de Transfusion Sanguine, Strasbourg (France)*

(Received 24 November 1986)

Key words: Exocytosis; Membrane fluidity; Fluorescence; Trimethylammonio-diphenylhexatriene; Secretion; Shape change; (Human platelet)

A simple, flexible and sensitive fluorescence method is described, which, from the same experiment, provides coupled quantitative informations on membrane fluidity changes and exocytosis, and reliable kinetic analyses of these effects, in intact cell suspensions. The method is based on the features peculiar to trimethylammonio-diphenylhexatriene (TMA-DPH), a fluorescent hydrophobic probe, which, in intact cells, is incorporated specifically into the plasma membranes, according to an instantaneous partition equilibrium. The method was tested on human platelets upon stimulation with various agents, such as human α-thrombin, adenosine diphosphate (ADP), adrenalin and ionomycin, which act through different types of mechanism. The experimental conditions were chosen to allow platelet shape change and exocytosis, but no aggregation. The kinetics and the dose-dependence of the changes in TMA-DPH fluorescence intensity and anisotropy were compared to the simultaneous physiological responses of platelets to the same stimuli, under the same conditions. Quantitative correlations were established between serotonin secretion and the increase in fluorescence intensity, whereas fluorescence anisotropy, which monitors membrane fluidity changes was associated with platelet shape change. The specificity of the effects was confirmed with appropriate antagonistic or modulating agents.

Introduction

1-[4-(Trimethylammonio)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) is a fluorescent hydrophobic probe, introduced by Prendergast et al. [1], for fundamental purposes, in membrane fluidity studies of phospholipidic bilayers by fluorescence anisotropy. Unlike its previously used parent molecule, diphenylhexatriene (DPH), it was assumed to have a well defined 'wobbling in cone' motion, according to the theory of Kinosita et al. [2], presumably remaining anchored to the phospholipid polar heads by its positively charged group. We have demonstrated [3], that for this very reason, in intact living cells, TMA-DPH remains specifically in the plasma membranes after very rapid incorporation (1 s). Further studies in our laboratory [4,5] confirmed that it was a most suitable tool for plasma membrane fluidity studies in cell systems, unlike DPH and other commonly

Correspondence: M. Kubina, Laboratoire de Biophysique, U.A. 491 du CNRS, Université Louis Pasteur, B.P. 10, 67048 Strasbourg cedex, France.

used fluorophores, which label all hydrophobic regions of the cells uniformly and so do not provide specific information.

Furthermore, this TMA-DPH molecule has been described as a sensitive and reliable monitor of exocytosis processes [6], due to the remarkable features of its incorporation into the membranes: when interacted with a cell suspension, TMA-DPH is subject to instantaneous partition equilibrium between the membranes, where it is fluorescent, and the aqueous buffer, where it is not. So, in secretory cells, the fluorescence intensity of TMA-DPH increases upon exocytosis, due to its supplementary incorporation into granule membranes newly fused with the plasma membrane. The response is proportional to the granule surface involved.

It seemed particularly worthwhile to use these properties conjointly, to investigate the membrane dynamics of human blood platelets, which display several types of response (shape change, aggregation, secretion) to a great variety of stimuli [7,8]. Earlier membrane fluidity studies on platelets have used fluorescence anisotropy [9–11], with DPH, a non-specific probe or with other probes, presumed to be non-permeant [12], but which called for very high concentrations. Spin label results have been reported [13], but without any kinetic development. In addition, fluorescence methods have been proposed to follow platelet exocytosis on the basis of the reduction of fluorescence quenching of amine dyes such as mepacrine [14], quinacrine [15], and acriflavine [16]. Those methods require long incubation to load the cells with the marker, whereas the method with TMA-DPH, presented in this paper, can be used immediately, in purified cells. In addition, it provides simultaneous information on exocytosis and membrane fluidity from fluorescence intensity and anisotropy measurements, respectively. Using both results, several aspects of the complex behaviour of human platelets in response to a selection of stimuli can be revealed.

Materials and Methods

Chemicals

The chemicals used in this work were all of first grade purity and were used without further purification. All products were controlled for possible interference with the photophysical properties of the fluorescent labels.

In cell preparation and conditioning. Anticoagulants: heparin (Roche), acid-citrate-dextrose; the components of Tyrode's buffer solution, among them 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) (Sigma), human serum albumin (Centre Régional de Transfusion Sanguine, Strasbourg) and bovine serum albumin (Sigma); stabilizing agents: prostacyclin (Upjohn), in Tris buffer (Sigma) and potato apyrase [17] prepared in INSERM U 311 (one of our labs).

In cell labelling. TMA-DPH (Molecular Probes), DPH (Kochlight) and dimethylformamide (Merck) as solvent.

In platelet activation and physiology. Adenosine diphosphate (ADP) (Sigma), human α-thrombin (3000 U/mg), kindly purified by Dr. J.M. Freyssinet, Strasbourg and prepared according to Ref. 18, adrenalin (Sigma), calcium ionophore ionomycin (Calbiochem) with dimethylsulphoxide (DMSO) as solvent, cytochalasin B (Aldrich), ethylene-glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) (Sigma), diisopropylfluorophosphate (Sigma).

In titrating the mediators released. [^{3}H]Serotonin (New England Nuclear).

Preparation of washed human blood platelets

Human platelets were isolated from acid-citrate-dextrose anticoagulated blood by differential centrifuging and washed at 37 °C according to the method of Cazenave et al. [17]. The platelets were washed twice in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose) containing 5 mM Hepes, 0.25% (w/v) human serum albumin, 1 μM prostacyclin (pH 7.3; 295 mosmol/kg). They were finally suspended at 37 °C in Tyrode's buffer with 0.1% bovine serum albumin and apyrase, and adjusted to a concentration of $3 \cdot 10^8$ cells/ml. Bovine serum albumin was preferred to human serum albumin in the suspending buffer, because of lower interaction with the fluorescent probes. The platelets were counted with a Baker A 810 platelet impedance counter (Baker Instruments). Phase contrast-microscopy showed that under these con-

ditions they remained discoid and functional, being sensitive to low concentrations of aggregating agents for at least 8 h.

Platelet labelling with fluorescent probes

To a 4 ml quartz fluorescence cuvette containing 2 ml Tyrode's buffer, without bovine serum albumin, at 37 °C, first TMA-DPH from a $5 \cdot 10^{-4}$ M stock solution in dimethylformamide, was added under moderate stirring, to give a final concentration of $5 \cdot 10^{-7}$ M, and, after temperature equilibrium, an aliquot of the platelet suspension was added to give a final concentration of 10^7 platelets/ml. The remaining bovine serum albumin concentration was 0.0033%. The sample was then ready for use. Turbidity was less than 0.2 absorbance units at 350 nm, allowing satisfactory fluorescence measurements [4].

DPH, used in control experiments, took a long time (30 min at 37 °C) for incorporation and so was added already during the platelet preparation process, after the first wash. The probe was taken from a 10^{-3} M stock solution in dimethylformamide, to give a final concentration of 10^{-6} M in the sample.

Platelet activation

Platelet activation conditions were exactly the same for shape change, secretion studies and fluorescence measurements. In both cases, the samples contained $5 \cdot 10^{-7}$ M TMA-DPH and 10^7 platelets/ml in Tyrode's buffer at 37 °C, and were stirred at 200 rpm, to avoid platelet aggregation. The absence of aggregation was checked with phase-contrast microscopy and by single platelet counting. The platelets were activated with: human α-thrombin (0.002 to 5 U/ml), ADP (0.05 to 50 μM), adrenaline (0.5 to 50 μM) and ionomycin (0.01 to 10 μM). The effectors were mixed rapidly in the suspension by a short increase in stirring speed. Care was taken to avoid any significant change in volume.

Platelet shape change and secretion measurements

Mean platelet volume, as an indicator of shape change, was measured with a Baker A810 platelet impedance counter, and serotonin release was determined in parallel on the same platelet preparation, at various intervals after stimulation. The cells were preliminarily loaded with [^{3}H]serotonin (1 μCi/ml) by 15 min incubation at 37 °C during the first washing step. Paraformaldehyde (0.5%) was added to block instantaneously (1 s) secretion or re-uptake of [^{3}H]serotonin, and glutaraldehyde (0.5%) was used for better fixation of the cells and to ensure that they retained their original shape [16]. After rapid mixing, the suspension was transferred to an ice bath. A 10 μl aliquot was counted in a Baker A 810 platelet impedance counter. This enabled parallel counting and sizing of the platelets. Mean platelet volume was automatically calculated from the size distribution graphs and expressed in μm^3. For the titration of serotonin released, 900 μl of the suspension were centrifuged for 1 min at $8000 \times g$. 800 μl of the supernatant were removed and mixed with 5 ml of Ready Solve-EP scintillation fluid (Amersham). Radioactivity was measured with a Minibeta 1211 scintillation counter (LKB). Secretion was expressed as a percentage of the total radioactivity contained in non-stimulated platelets.

In each experiment, mean platelet volume and platelet aggregation were measured to check if there was no undesirable activation of the platelets and to test their functionability. For this, turbidity was measured on $3 \cdot 10^8$ platelets/ml samples at 37 °C in a dual-channel Payton aggregometer. The aggregation was tested in the presence of human fibrinogen treated with diisopropylfluorophosphate. When thrombin was used as the aggregating agent, fibrinogen was omitted.

Fluorescence measurements

Fluorescence intensity (I) and anisotropy (r) were measured with an SLM 8000 spectropolarofluorimeter. Samples were illuminated with linear-polarized light (excitation wavelength, 350 nm), at 37 °C under gentle stirring (200 rpm). The components of emitted light intensity (maximum at 435 nm), at 90°, respectively parallel and perpendicular to the excitation polarization direction were measured simultaneously (T configuration detection). The excitation polarizer was switched alternately from vertical (V) to horizontal (H) to allow the recording every 2 s of I_{VV}, I_{VH} and I_{HV}, I_{HH} (the second index referring to the position of the emission polarizer) and of I_C, a control value, for excitation light intensity. I and r were de-

duced from the following expression

$$I = (I_{VV} + 2I_{VH} \cdot I_{HV}/I_{HH})/I_C$$

$$r = \frac{I_{VV}/I_{VH} - I_{HV}/I_{HH}}{I_{VV}/I_{VH} + 2I_{HV}/I_{HH}}$$

where the factors I_{HV}/I_{HH} and I_C take into account, respectively, the asymmetry of the detection system, and the excitation lamp fluctuations.

Other corrections were introduced for scattered excitation light [4] and the fluorescence background resulting from the interaction of TMA-DPH with bovine serum albumin (BSA). To that end, appropriate controls were measured separately. The final values of I and r are then given by

$$I(\text{TMA-DPH}) = I(\text{measured}) - I(\text{scatter.}) - I(\text{BSA})$$

$$r(\text{TMA-DPH}) = \frac{rI(\text{measured}) - rI(\text{scatter.}) - rI(\text{BSA})}{I(\text{TMA-DPH})}$$

All experimental data were fed into a Sil'z 16 microcomputer and then transferred to an IBM 3081 computer, which gave corrected I(TMA-DPH) and r(TMA-DPH) as a function of time before and after platelet stimulation.

Further data processing was done with an SAS® package for best fit of the experimental points, with the following equations for I

$I = I_0 + \delta It$ before activation,

$I = I_0 + \delta' I(t - \delta t) + \Delta I(1 - \exp(-k(t - \delta t)))$ after activation

where I_0 represents the basic fluorescence intensity, δI and $\delta' I$ take into account a small linear intensity shift, which may be due to progressive penetration of TMA-DPH into damaged cells, ΔI is the typical fluorescence intensity increase due to the activation process and δt, the time-lag between adding the inducer and the beginning of the effect. Similar expressions were used for r.

Fluorescence lifetime determinations

The fluorescence decay times of TMA-DPH in resting and activated platelets, were measured with time correlated single-photon-counting equipment. The decay curves were deconvoluted with the method of Grinvald and Steinberg [19] and the autocorrelation function was considered a test of fit.

Results

Preliminary controls

All chemicals used in this study were checked, to ensure that they had no significant absorbance or fluorescence effects in the range of TMA-DPH and DPH spectral properties, and did not modify them. For instance, the calcium ionophore, A23187, was avoided because of its own fluorescence, and ionomycin was used instead. It was also checked that TMA-DPH and DPH did not modify the platelet characteristics: mean platelet volume, aggregation by ADP, α-thrombin and collagen, and serotonin secretion induced by α-thrombin and collagen. DMSO had to be used at concentrations below 0.2%. Bovine serum albumin, added to the Tyrode's physiological solution, interacted with TMA-DPH through its hydrophobic regions; so appropriate corrections had to be made (see Materials and Methods).

Fluorescence lifetime plays an important role in fluorescence data and has to be measured for correct interpretation of fluorescence anisotropy in terms of membrane fluidity [19]. Our results showed that within the limits of experimental error, there were no changes in fluorescence lifetimes upon platelet stimulation; for example, with thrombin (0.2 U/ml), the two lifetimes of TMA-DPH after treatment were 6.5 and 0.4 ns, versus 6.4 and 0.4 ns before.

Kinetic studies

All the results with the fluorescence method were derived from kinetic experiments, in which fluorescence intensity and anisotropy were monitored as a function of time before and after platelet stimulation with different doses of various effectors: α-thrombin, ADP, adrenalin, ionomycin. Human α-thrombin was selected, as the most potent physiological platelet activator, inducing both shape change and extensive secretion, whereas ADP only induced shape change in washed platelets. Although adrenaline binds to specific α_2-adreno-receptors it did not induce any shape change or secretion, despite its potentiating effects

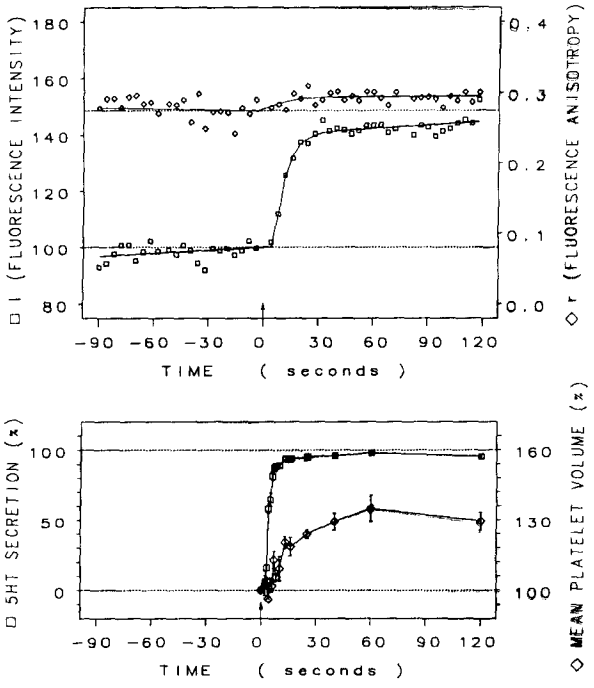

Fig. 1. Comparison of the physiological response parameters: mean platelet volume and serotonin (5HT) secretion (lower part), and of the TMA-DPH corrected fluorescence data: intensity (I) and anisotropy (r) (upper part), upon stimulation of platelets with human α-thrombin (1 U/ml), in relation to time, and under the same experimental conditions. Serotonin secretion and mean platelet volume values (mean $\pm$ S.E., $n = 3$) are expressed, respectively, as % of the total [^{3}H]serotonin content and of the mean platelet volume of unstimulated platelets. I is expressed in arbitrary units and r as its absolute value. Time zero is when the agent was added. Solid lines on fluorescence data represent the best fit to experimental points, computed as described in Materials and Methods.

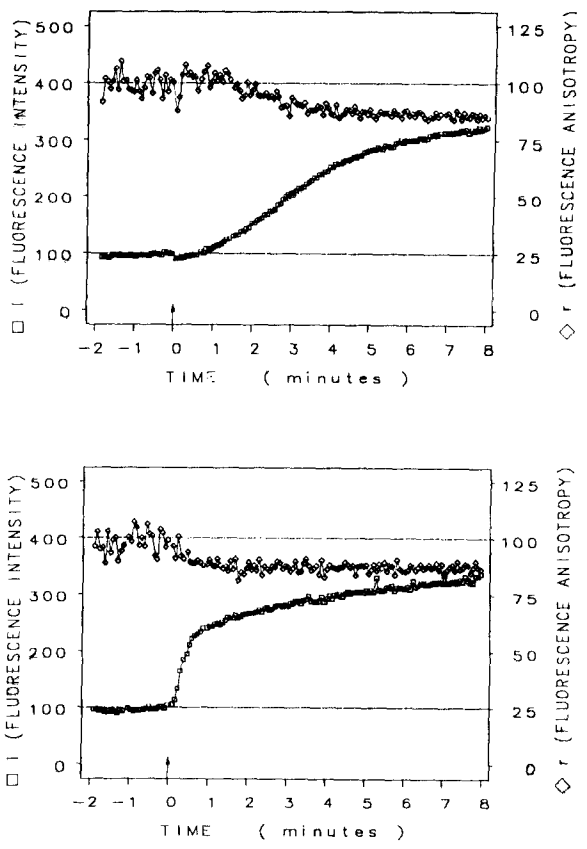

Fig. 2. Kinetics of the TMA-DPH fluorescence intensity and anisotropy change after stimulation of human platelets with ionomycin. The figure emphasizes the difference in behaviour at low doses (0.2 μM) (upper part) (not single exponential) and at higher doses (1 μM) (lower part) (single exponential).

of the other agonists [20,21]. Ionomycin acts through calcium transfer and differs from the other agonists by the absence of any receptor-ligand interactions.

It was not easy to represent all the kinetic curves and it was easier to discuss the results on the basis of dose-response data, which afford a more general and comparative view. However, particular kinetic results are referred to for clarification, whenever appropriate. We merely present here (Fig. 1) the results obtained with purified human α-thrombin (1 U/ml) at 37°C. Fluorescence intensity and serotonin secretion increased rapidly, reaching a maximum in less than 30 s; for instance the time required for the fluorescence intensity effect to reach half maximum was $t_{1/2} = 4.3 \pm 0.7$ s (asymptotic standard error). In fact, the kinetic of serotonin secretion was slightly faster. The kinetics of fluorescence anisotropy and of mean platelet volume change were similar, but rather slower than those of exocytosis: $t_{1/2}$ was 7 ± 7 s for the Δr effect. The variation in fluorescence intensity (ΔI) was $40 \pm 1\%$ and that in fluorescence anisotropy (Δr), $7 \pm 2\%$ (asymptotic standard error); the secretion of preloaded serotonin was $95 \pm 2\%$ and the increase in mean platelet volume, $30 \pm 4\%$ (mean $\pm$ S.E., $n = 3$ separate determinations), 120 s after treatment with α-thrombin (1 unit/ml). The absolute mean platelet volumes were 5.40 ± 0.05 μm^3 before and 7.0 ± 0.2 μm^3 after treatment. Decreasing the temperature to 22°C slowed down in a similar way

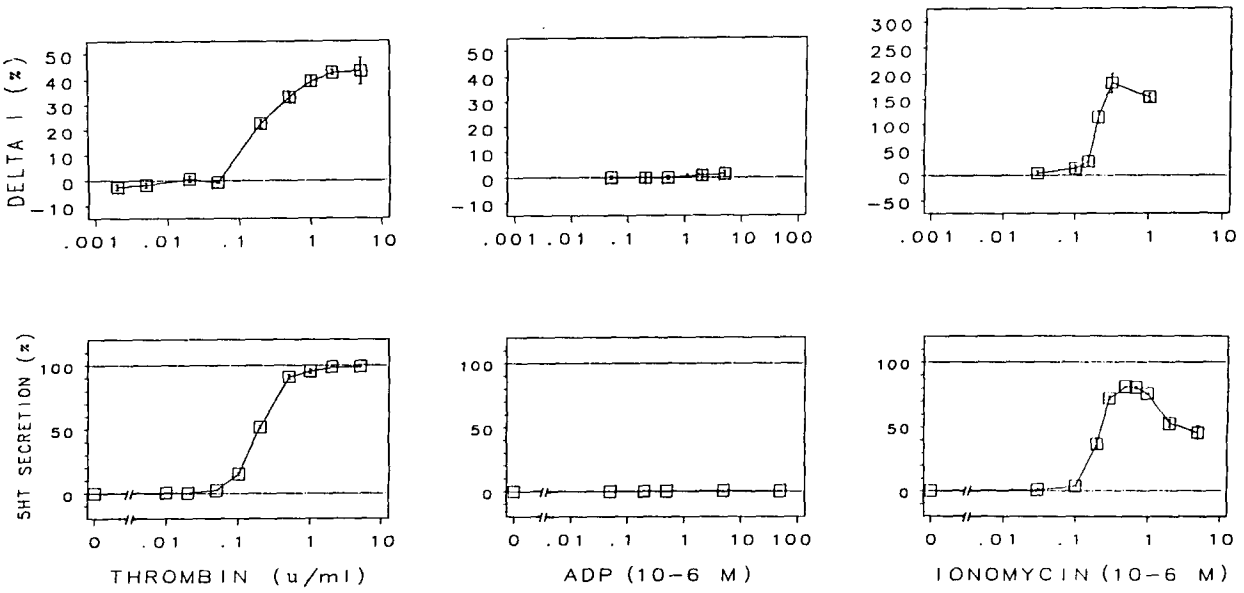

Fig. 3. Comparative dose-response curves for the increase in TMA-DPH fluorescence intensity (ΔI) and for the serotonin (5HT) secretion after stimulation of human platelets with α-thrombin, ADP and ionomycin. TMA-DPH corrected fluorescence intensities were extracted from kinetic curves after data processing using the expressions given in Materials and Methods. These expressions could not be used for ionomycin (no single exponential behaviour). ΔI was taken as the average variation in I between 110 and 130 s after the addition of ionomycin. ΔI (mean ± S.E. from 3 to 10 separate experiments) is expressed as % of I_0, the basic intensity before stimulation. Serotonin (5HT) secretion was measured 2 min after stimulation and is expressed as % of total [^{3}H]serotonin content (mean ± S.E. from three separate experiments).

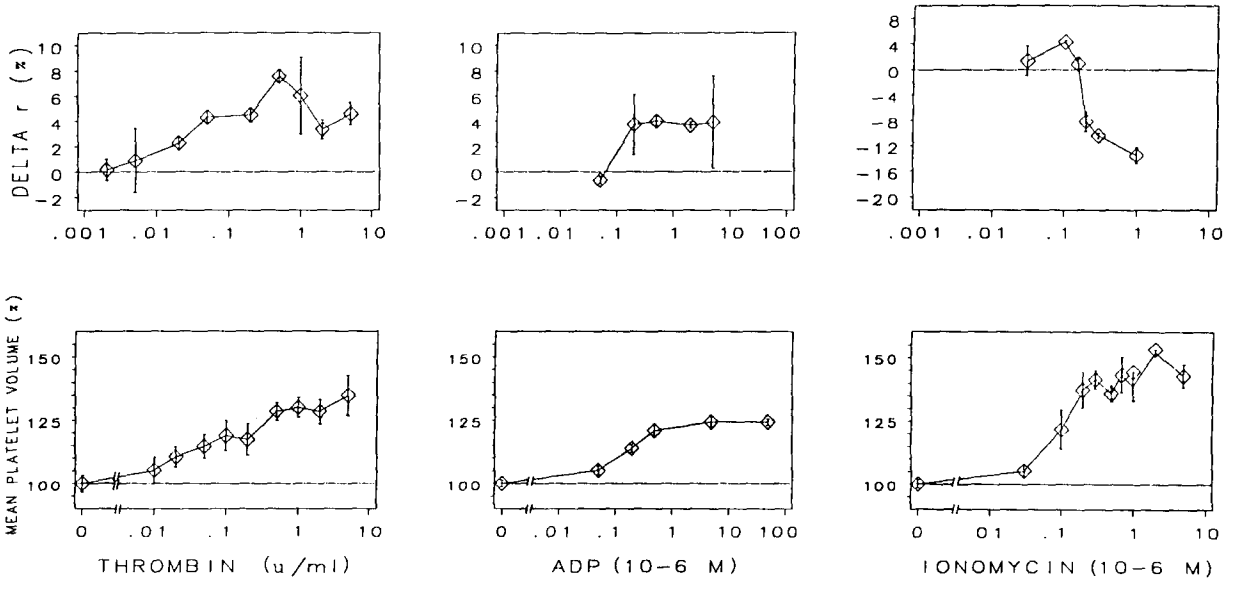

Fig. 4. Comparative dose response curves for variation in TMA-DPH fluorescence anisotropy (Δr) and the change in mean platelet volume after stimulation of human platelets with α-thrombin, ADP and ionomycin. Mean platelet volume was measured 2 min after stimulation and is expressed as % of mean platelet volume of unstimulated platelets (mean ± S.E. from three separate experiments). Corrected Δr values were obtained from the results as described in Materials and Methods, excepted for ionomycin: see remark under Fig. 3.

the increase in fluorescence intensity and the serotonin secretion rate, but did not modify the maxima of the two parameters (data not shown). Specific behaviour was observed after stimulation with ionomycin, where the change in the fluorescence intensity and anisotropy could not be fitted with a single exponential at low inducer doses, as it could at higher doses (Fig. 2). So the data processing technique in Materials and Methods could not be applied. Fluorescence data were standardized taking the average variation in I and r for ΔI and Δr, respectively, between 110 and 130 s after stimulation.

Dose-response curves

Dose-response curves were obtained from the foregoing kinetic data for fluorescence intensity and anisotropy and were compared with serotonin secretion and mean platelet volume changes after stimulation with increasing concentrations of thrombin, ADP, adrenalin and ionomycin. As expected, adrenalin did not affect the parameters measured (data not shown), even at high concentrations (50 μM). The fluorescence intensity results are presented with those for serotonin secretion (Fig. 3), and the fluorescence anisotropy results with those for mean platelet volume changes (Fig. 4).

Almost superimposable curves were obtained for the increase in fluorescence intensity and in serotonin secretion induced by increasing concentrations of α-thrombin (0.001–10 U/ml). Both effects started at the same α-thrombin concentration ($\approx$ 0.1 U/ml) and reached a maximum for 1 U/ml α-thrombin (45% for ΔI and 95% of preloaded serotonin). Dose-response curves were also very similar for both processes after ionomycin stimulation (0.05 to 10 μM). The maximum was obtained with 0.3 μM ionomycin and it corresponded to a 200% increase in fluorescence intensity and to 70% secretion of the preloaded serotonin.

Stimulation of platelets with increasing concentrations of ADP gave no observable secretion or ΔI, but did induce parallel increases in fluorescence anisotropy and in mean platelet volume, which both reached a plateau with 1 μM ADP. Stimulation with human α-thrombin or ionomycin also induced a dose-dependent increase in mean platelet volume; fluorescence anisotropy was biphasic with these agents, with an increase in Δr at concentrations below 1 U/ml (α-thrombin) and 0.1 μM (ionomycin), followed by a decrease in Δr for higher concentrations. The effect was more pronounced with ionomycin; in this case the decreasing part of the curve was prevalent, giving lower r values than in unstimulated platelets.

Discussion

Fluorescence intensity results and serotonin secretion

The fluorescence intensity effect discussed in this section is the specific increase following platelet stimulation, referred to as ΔI. The slight continuous shift, δI, in fluorescence intensity observed throughout the experiments is a trivial effect attributed to the penetration of TMA-DPH into the inner compartments of progressively damaged cells [3,4].

After α-thrombin stimulation, as shown in Fig. 3, ΔI appears to depend on the effector dose in a very similar way to serotonin release. The kinetics of both effects are also fairly comparable (Fig. 1). The inhibition of the physiological effects of α-thrombin (1 U/ml) by hirudin (1 to 5 U/ml) which forms an inactive complex with thrombin, or by prostacyclin (1 to 1000 nM), which blocks the platelet secretion by increasing the cAMP levels, led to parallel cancellation of the fluorescence intensity effects (data not shown). Moreover, ADP, which induced only shape change but no secretion, did not modify the TMA-DPH fluorescence intensity. (It is generally accepted that ADP does not induce secretion of any granule content in washed human platelets [17]. It has been shown in the INSERM laboratory, that ADP did not induce significant release of platelet factor 4 from α-granules (Hemmendinger, S., unpublished data).) Thus fluorescence intensity increase is secondary to platelet activation and appears to be specifically associated with exocytosis. A similar effect has already been interpreted in a previous work on mast cells and basophil cells [6], where the fluorescence increase was shown to stem from the neo-incorporation of TMA-DPH from the buffer solution into the inner membrane of secretory granules, when they fuse with the plasma membrane as a result of the characteristic parti-

tion equilibrium of TMA-DPH. The effect was not significantly delayed, following the increase in cell surface immediately, as was confirmed in the platelets: the addition of unlabelled cells to a platelet suspension containing TMA-DPH induced an immediate increase in fluorescence intensity. The correlation between exocytosis and ΔI was corroborated by an experiment using platelets labelled with DPH (10^{-6} M) instead of TMA-DPH. No increase in fluorescence intensity was then detected after stimulation with thrombin (1 U/ml), or ionomycin (1 μM); this was to be expected, since DPH, which is a permeant probe, had already labelled the granules inside the cells before treatment with α-thrombin. Finally, according to this interpretation, ΔI corresponds to an increase in the cell membrane surface (40% with thrombin). On this basis, and taking into account the respective dimensions of discoïd platelets (diameter 3 μm; thickness 1 μm [23]) and of spherical granules (diameter 0.3 μm [24]) the number of granules involved in the process may be estimated at roughly 30 to 40. This confirms that the dense granules (seven per platelet [24], i.e. those containing serotonin, are not the only ones involved in thrombin activation, as previously pointed out [22], but also that the exocytosis induced by this agent is not total, since the overall granule count (dense granules, α-granules, lyzosomes) is higher than 100, as inferred from electron microscope data [25].

The results after activation with ionomycin are consistent with these assumptions, although they are complicated by the presence of a peak, probably due to an inhibitory effect at the higher doses, as already observed with the calcium ionophore A 23187 [26]. Maximum fluorescence for $3 \cdot 10^{-7}$ M ionomycin ($CaCl_2$ 2 mM) was about 5-times higher than after α-thrombin stimulation. On the contrary, the serotonin level was lower (70% instead of 95% of total content with thrombin). This indicates that ionomycin is a less efficient secretagogue than thrombin for serotonin and, conversely, a stronger inducer for the exocytosis of other granules (α-granules and/or lysosomes). The present work was not intended to go deeper into such particular behaviour, but these results do emphasize how fruitful the fluorescence method can be in investigating exocytosis phenomena. The fluorescence response to 1 μM ionomycin was examined as a function of external calcium ions using the calcium chelator EGTA. EGTA reduced the fluorescence effect in a dose-dependent way and almost suppressed it for a 5 mM concentration (data not shown). This confirms that the effect on fluorescence intensity is significant and linked to the ionomycin induced influx of Ca^{2+} into platelets.

Membrane fluidity and mean platelet volume modifications

The fluorescence lifetime of TMA-DPH did not vary upon platelet stimulation, so the fluorescence anisotropy changes could be interpretated in terms of membrane fluidity modifications, as already mentioned. A positive Δr (where r is the fluorescence anisotropy corrected for scattering and background fluorescence) corresponds to a decrease in membrane fluidity and vice versa. Again, the results with ADP (Fig. 4) are easier to discuss: only shape change is induced, and this obviously brings about a parallel increase in membrane rigidity, as observed in earlier studies [9–12] with other probes.

The effect of ADP on membrane fluidity was completely abolished by preincubation of the cells, for 1 min at 37 °C, with cytochalasin B (60 μM), a cytoskeleton depolymerizing agent, which under these conditions, suppresses the platelet shape change [23,27] (data not shown).

With α-thrombin, fluorescence anisotropy, as a function of the dose evolved in two phases: an increase in Δr at doses below 0.2 U/ml and a decrease in Δr at higher doses. The former effect was sensitive to cytochalasin B and so was probably correlated to shape change, whereas the latter was assumed to result from granule fusion with the plasma membrane during exocytosis, since a similar effect was not observed with ADP. Specific granule membrane rigidity should then be significantly lower than that of the plasma membranes. The important contribution of the platelet shape change to the modification in membrane fluidity is supported by the kinetics of the phenomenon (Fig. 1), which correspond to that of mean platelet volume change and are somewhat slower than those of exocytosis.

All these assumptions were strongly corrobo-

rated by the results with ionomycin, where the decreasing part of the curve (Fig. 4) largley predominated, which would indicate a major exocytosis contribution, which of course tallies entirely with the conclusions based on fluorescence intensity.

In conclusion, the results presented in this paper show that a single fluorescent probe, TMA-DPH, can rapidly provide fruitful information on membrane fluidity effects and exocytosis kinetics in living cell systems. In human platelets the method brought out a great difference in the responses to ionomycin and α-thrombin stimulation, not hitherto reported, and gave evidence for the correlation between platelet membrane rigidification, shape change and cytoskeleton assembly.

Acknowledgments

This work was supported by a grant from Rhône-Poulenc Santé and Centre National de la Recherche Scientifique. We are grateful to Dr. J.L. Zundel (Rhône-Poulenc Santé) for numerous fruitful discussions. Another grant was obtained by the Institut National pour la Santé et la Recherche médicale, No. 831016. F. Lanza has a studentgrant from the Ministère de l'Industrie et de la Recherche.

References

1 Prendergast, F.G., Haugland, R.P. and Callahan, P.J. (1981) Biochemistry 20, 7333–7337
2 Kinosita, K., Kawato, S. and Ikegami, A. (1977) Biophys. J. 20, 289–299
3 Kuhry, J.G., Fonteneau, P., Duportail, G., Maechling, C. and Laustriat, G. (1983) Cell Biophys. 5, 129–140
4 Kuhry, J.G., Duportail, G., Bronner, C. and Laustriat, G. (1985) Biochim. Biophys. Acta 845, 60–67
5 Deguercy, A., Schrevel, J., Duportail, G., Laustriat, G. and Kuhry, J.G. (1986) Biochem. Int. 12, 21–31
6 Bronner, C., Landry, Y., Fonteneau, P. and Kuhry, J.G. (1986) Biochemistry 25, 2149–2154
7 Zucker, M.B. and Nachmias, V.T. (1985) Arteriqsclerosis 5, 2–18
8 Holmsen, H. (1985) Semin. Hematol. 22, 219–240
9 Nathan, I., Fleischer, G., Livne, A., Dvilansky, A. and Parola, A.H. (1979) J. Biol. Chem. 254, 9822–9828
10 Nathan, I., Fleischer, G., Dvilansky, A., Livne, A. and Parola, H. (1980) Biochim. Biophys. Acta 598, 417–421
11 Boudet, G., Lévy-Tolédano, S., Maclouf, J., Rendu, F. and Salesse, R. (1985) Biochim. Biophys. Acta 812, 243–248
12 Steiner, M. and Lüscher, F. (1984) Biochemistry 23, 247–252
13 Kowalska, M.A. and Cierniewski, C.S. (1983) IRCS Med. Sci. 11, 112–113
14 Lorez, H.P., Da Prada, M., Rendu, F. and Pletscher, A. (1977) J. Lab. Clin. Med. 89, 200–206
15 Costa, J.L., Fay, D.D. and Kirk, K.L. (1984) Res. Commun. Chem. Pathol. Pharmacol. 43, 25–41
16 Wüthrich, C., Deranleau, D.A., Dubler, D. and Lüscher, F. (1984) Biochemsitry 23, 1224–1229
17 Cazenave, J.P., Hemmendinger, S., Beretz, A., Sutter-Bay, A. and Launay, J. (1983) Ann. Biol. Clin. 41, 167–179
18 Owen, W.G. and Jackson, C.M. (1973) Thromb. Res. 3, 705–714
19 Grinvald, A. and Steinberg, I.Z. (1974) Anal. Biochem. 59, 583–598
20 Lanza, F. and Cazenave, J.P. (1985) Thrombos. Hameostas. 54, 402–408
21 Lanza, F., Cazenave, J.P., Beretz, A., Sutter-Bay, A., Kretz, J.G. and Kiney, R. (1986) Agent Action 18, 586–595
22 Verhoeven, J.M., Gorter, G., Mommersteeg, E. and Akkerman, J.W.N. (1985) Biochem. J. 228, 451–462
23 Frojmovic, M.M. and Milton, J.G. (1982) Physiol. Rev. 62, 185–261
24 Holmsen, H. and Weiss, H.J. (1979) Annu. Rev. Med. 30, 119–134
25 Weiss, H.J., Witte, L.T., Kaplan, K.L., Lages, B.A., Tchernoff, A., Nossel, H.L., Goodman, D.S. and Baumgartne, H.R. (1979) Blood 54, 1296–1319
26 Massini, P. and Lüscher, E.F. (1974) Biochim. Biophys. Acta 382, 109–121
27 Kometani, M., Sato, T. and Fuji, T. (1986) Thromb. Res. 41, 801–809