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Change in the physical state of platelet plasma membranes upon ionophore A23187 activation. A fluorescence polarization study

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Human platelets were isolated and fluorescence-labelled by 1,6-diphenylhexatriene. Diphenylhexatriene was essentially localized in the plasma membrane, as indicated by trinitrobenzenesulfonate-quenching experiments. A decrease of the fluorescence polarization of diphenylhexatriene was observed upon ionophore A23187 addition in the absence of aggregation. 0.3 μM ionophore allowed to reach the maximum rate of the decrease of fluorescence polarization; it also maximally stimulated the light transmission change, the serotonin release and the thromboxane B_2 synthesis. The amplitude of the fluorescence polarization decrease was maximum at platelet concentrations between $4 \cdot 10^7$ and $7 \cdot 10^7$/ml. The presence of Ca^{2+} in the medium increased the rate constant of the polarization change. Chlorpromazine (60 μM) completely inhibited this transition, but at 30 μM its inhibitory effect was reversed by Ca^{2+}. The membrane events implied in platelet activation very likely lead to fluidization of the plasma membrane, perhaps by its fusion with the membranes of internal granules which are relatively depleted of cholesterol. Ca^{2+} plays a central role in the triggering of the observed effects at the membrane level.

Introduction

In the absence of external Ca^{2+}, ionophore A23187 can penetrate internal membranes and mobilize Ca^{2+} stores in organelles [1].

In 1982, we have suggested that platelet activation was triggered by internal Ca^{2+} fluxes: this was corroborated by the use of some phenothiazine derivatives like chlorpromazine. Further, the observed inhibition of the ionophore-induced activation was partially reversed by the addition of external Ca^{2+}, again suggesting that these changes are calcium-dependent [2]. Nathan et al. [3–4] have reported that membrane microenvironmental changes during thrombin-induced platelet activation can be probed by measuring 1,6-diphenylhexatriene fluorescence polarization. We have used the same technique to investigate membrane changes in the case of ionophore-induced platelet activation in the absence of aggregation. This is a more favourable experimental condition for measuring fluorescence polarization as the platelet suspension does not undergo major changes of light diffusion due to aggregation. Aggregation per se could introduce interactions between the aggregated membranes which could conceal membrane events occurring in isolated platelets. Moreover, we found that about 95% of the diphenylhexatriene was localized within the plasma membrane of platelets.

In this case, ionophore stimulation induces modifications in the diphenylhexatriene fluores-

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cence polarization which are closely related with morphological and biochemical changes previously observed [2]. Ca^{2+} enhanced the rate of all these phenomena, whereas chorpromazine inhibited them, thus emphasizing the close relationship between the membrane dynamics and platelet activation.

Materials and Methods

1,6-Diphenylhexatriene was purchased from Sigma. It was dissolved in dimethylformamide at a concentration of 2 mM and stored at 4°C. It was diluted to 2 μM in the buffer prior to use. Metrizamide is a triiodinated benzamido-derivative of glucose from Nyegaard Laboratories. Ionophore A23187 was obtained from Calbiochem, France. It was dissolved in ethanol and used at a final concentration of 0.06–0.3 μM. The volume of ionophore which was used did not exceed 1/100 of the platelet volume. Chlorpromazine was a gift of Rhône Poulenc, France. 2,4,6-Trinitrobenzenesulfonate (from Serva) was dissolved in chloroform and neutralized with an equal amount of sodium bicarbonate. Chloroform was evaporated under dry nitrogen and the remaining powder dissolved in the buffer (final concentration $1 \cdot 10^{-2}$ M).

Preparation of washed platelet suspensions using metrizamide gradients. Nine parts of blood were anticoagulated with one part of 0.077 M EDTA in saline and centrifuged for 15 min at $120 \times g$ and at room temperature to obtain platelet-rich plasma. The metrizamide gradient platelet were prepared according to the method described by Lévy-Tolédano et al. [5]. 5 ml platelet-rich plasma were layered onto a gradient composed of 1 ml 25% and 1 ml 10% metrizamide and centrifuged for 15 min at $1000 \times g$ and at 18°C. The platelet layer was resuspended in 4 or 5 ml calcium-free buffer (pH 6) (140 mM NaCl, 1.45 mM NaH_2PO_4, $2H_2O$, 6.85 mM Na_2HPO_4, 11 mM glucose in twice-distilled water) and deposited on a new metrizamide gradient at the same concentrations. After centrifugation, the platelet layer was resuspended in the same calcium-free buffer before further use.

Labelling of platelets by diphenylhexatriene. 1 vol. platelet suspension ($3 \cdot 10^8$/ml) was added to 1 vol. 2 μM diphenylhexatriene solution. The incubation was carried out for 1 h at room temperature and in the dark. Platelets were then diluted three times with the same buffer before centrifugation for 15 min at $1000 \times g$ at room temperature. After one more washing, they were finally resuspended in the same buffer at pH 7.4. Platelet concentrations were adjusted as indicated in Results.

Fluorescence measurements – quenching by trinitrobenzenesulfonate. These measurements were made in a Jobin-Yvon JY3C spectrofluorometer equipped with excitation (360 nm) and emission (430 nm) polarizers in a quartz cell (1×1 cm) in a thermostated holder. The emission intensities (I) were recorded in the vertical (v) and horizontal (h) positions of the emission polarizer. Polarization (P) of the fluorescence light was calculated as $P = (I_v - I_h)/(I_v + I_h)$ without correction of the apparatus-induced polarization shift [6].

Kinetics of platelet activation were recorded in the following way: 10 μl of the effector solution (usually ionophore A23187) were added at time zero after temperature equilibration at 37°C and under stirring at 1100 rpm with a magnetic bar in the cell. Vertical and horizontal intensities were recorded by rotating the emission polarizer of 90° every 6–10 s for 3 min and polarization was calculated from these records. Apparent first-order rate constants of polarization changes were determined by semilogarithmic plotting of the experimental values.

Quenching experiments were performed as described by Grunberger et al. [7]. Diphenylhexatriene-labelled platelets were kept for various times at room temperature and then incubated at 4°C with 2,4,6-trinitrobenzenesulfonate at the desired final concentration for 1 h. Fluorescence intensities were measured and reported for control cells incubated in the same conditions without trinitrobenzenesulfonate.

Change in light transmission, serotonin release and thromboxane synthesis. The change in light transmission was measured in a Payton aggregometer at 37°C according to the method of Born and Cross [8]. To 0.5 ml platelet suspension was added ionophore A23187 at different concentrations. The results are given in terms of the change in light transmission 3 min after the addition of the ionophore and are expressed as a percentage of the

difference in light transmission between washed platelets and suspending buffer.

Platelet count were performed on washed platelets by phase-contrast microscopy. The non-aggregated platelets were counted in the supernatant after addition of the ionophore. Results were refered to the initial number of platelets in the assay tube, which was considered as 100%. Serotonin release and thromboxane synthesis were measured in the same samples as the light transmission change 3 min after the addition of ionophore [2]. After prelabelling the EDTA-platelet-rich plasma with 0.5 μM [^{14}C]serotonin (48 mCi/mmol) (from CEA, Saclay, France), the experiment was carried out as described previously [2]. The results were expressed as percent release of total [^{14}C]serotonin in the platelets.

Thromboxane B_2 was measured by radioimmunoassay according to the procedure of Granstrom et al. [9] using an iodinated tracer of thromboxane B_2.

Results

Localization of diphenylhexatriene in the plasma membrane

2,4,6-Trinitrobenzenesulfonate is a nonpermeant anions which can only bind to the outer-half layer of plasma membrane of intact cells and which can quench diphenylhexatriene fluorescence if the two molecules are close enough together within the same membrane medium [7]. As it can be seen in Table I, the intensity of diphenylhexatriene emission at 430 nm is decreased down to 7 and 5% by trinitrobenzenesulfonate incubation at $1 \cdot 10^{-2}$ M, indicating that very likely about 93–95% of diphenylhexatriene is localized within the plasma membrane of platelets.

Effect of increasing concentrations of ionophore on platelet activation and on the rate constant of fluorescence polarization change

Ionophore activated platelets in the absence of aggregation. Table II shows that the change in light transmission, the release of [^{14}C]serotonin as well as the thromboxane B_2 synthesis were increased with increasing concentrations of ionophore from 0.06 to 0.3 μM. The increase in light transmission occurred in the absence of platelet aggregation, as estimated by the platelet count in the supernatant which remained the same as in the absence of ionophore (Table II). The labelling of platelets by diphenylhexatriene cause no measurable damage to the platelets, as evaluated by the same criteria of functions as well as leakage of insignificant amounts of lactate dehydrogenase, the cytoplasmic marker.

The ionophore-induced platelet activation was accompanied by a decrease of fluorescence polarization of diphenylhexatriene-labelled platelets. By raising the ionophore concentration from 0.06 to 0.3 μM, we observed an increase in the rate constant of polarization change (Fig. 1). Metrizamide did not intefere with the probe as platelets washed

TABLE I

QUENCHING OF DIPHENYLHEXATRIENE-LABELLED PLATELETS BY INCREASING CONCENTRATIONS OF 2,4,6-TRINITROBENZENE SULFONATE (TNBS)

Platelets were labelled at time 0 with diphenylhexatriene (DPH) and incubated at time 0, 1 and 2 h with TNBS at 4°C for 1 h before measurements of diphenylhexatriene fluorescence intensities. Values are relative to those obtained with control cells incubated without TNBS.

TNBS (mM)	Fluorescence intensity at time after DPH labelling (h)		
	0	1	2
0	1	1	1
1.2	0.43	0.41	0.39
2.5	0.24	0.25	0.24
5	0.13	0.14	0.10
10	0.07	0.07	0.05
20	0.06	0.07	0.05

TABLE II

EFFECT OF INCREASING CONCENTRATIONS OF IONOPHORE ON PLATELET ACTIVATION

LT, light transmission; 5HT, serotonin; TXB_2, thromboxane B_2.

	Ionophore (μM)		
	0.06	0.15	0.30
LT change (%)	18 ± 4	47 ± 5	55 ± 10
[^{14}C]5HT release (%)	32 ± 10	75 ± 5	70 ± 12
TXB_2 synthesis (ng/ml)	25 ± 8	400 ± 220	600 ± 180
Platelet count in the supernatant (%)	95	98	100

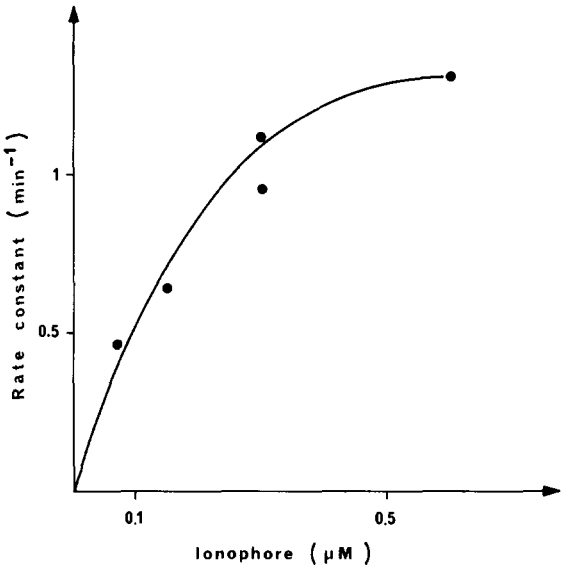

Fig. 1. Effect of different concentrations of ionophore on the rate constant of the decrease in fluorescence polarization of diphenylhexatriene-labelled platelets (platelet concentration 50 $\cdot 10^6$/ml).

by a different procedure which avoids the gradient gave the same decrease of fluorescence polarization in the presence of ionophore. As for the other activation parameters, a plateau level was reached beyond 0.3 μM. The same values of the rate constants were found for fluorescence intensity changes (results not shown). Ionophore stimulation induced a decay of fluorescence intensity.

Effect of the platelet concentration on the fluorescence polarization change

The fluorescence polarization change induced by 0.3 μM ionophore was investigated in the presence of different platelet concentration. It can be seen in Fig. 2 that the maximum was observed when platelet dilution approximated $45 \cdot 10^6$ to $70 \cdot 10^6$ platelets/ml.

At higher concentration (up to $150 \cdot 10^6$/ml), the observed decrease of amplitude might be attributed to artefactual reasons, as it is known that light diffusion at high particle concentrations depolarizes the emitted fluorescent beam [10]. At lower platelet concentration (from $45 \cdot 10^6$ to $16 \cdot 10^6$/ml), the diminution of the amplitude cannot be attributed to the previously cited reasons but

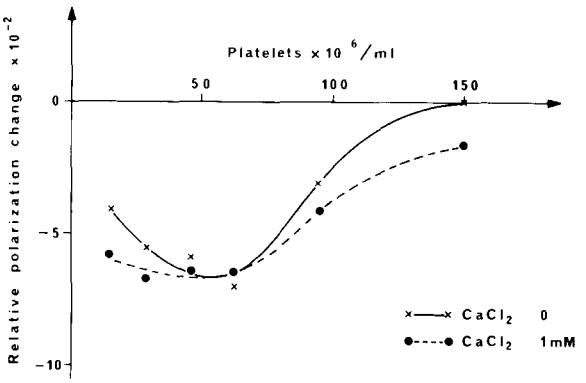

Fig. 2. Effect of the platelet concentration on the fluorescence polarization change in the absence (× ——— ×) or in the presence (●— — —●) of 1 mM $CaCl_2$.

rather to the decreased interaction between platelets at this low cell concentration. In the presence of 1 mM Ca^{2+}, the maximum amplitude is not modified at optimal platelet concentration ($45 \cdot 10^6$ to $70 \cdot 10^6$/ml). However, out of this concentration range, the presence of Ca^{2+} increased the amplitude of the polarization change. The Ca^{2+}-induced increase of the amplitude at elevated platelet concentration could be attributed to the presence of aggregates, the large size of which would induce less light diffusion than in the absence of $CaCl_2$ where no aggregates were observed.

Taking this result into account, we have chosen the platelet concentration of $50 \cdot 10^6$/ml for further experiments.

Effect of Ca^{2+} concentration on polarization changes induced by ionophore

The stimulating role of $CaCl_2$ observed in Fig. 2 led us to investigate the effect of increasing concentrations of $CaCl_2$ on polarization changes at a constant concentration of ionophore. Fig. 3 indicates that $CaCl_2$ from 50 to 1000 μM increased the rate of the polarization changes in the presence of 0.3 μM ionophore when compared with the curve in the absence of $CaCl_2$ (inset), without changing the total amplitude.

Effect of chlorpromazine on ionophore-induced polarization change: competition with Ca^{2+}

Chlorpromazine from 30 to 90 μM inhibits the polarization changes induced by 0.15 μM iono-

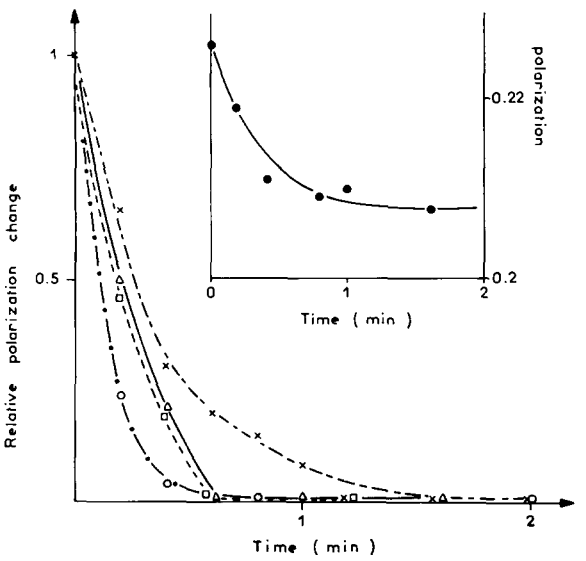

Fig. 3. Effect of Ca^{2+} concentrations: ×— — —×, 50 μM; △———△, 100 μM; □— — —□, 500 μM; ○·-·-·○, 1000 μM, on the rate of diphenylhexatriene polarization decrease in the presence of 0.3 μM ionophore (platelet concentration $50 \cdot 10^6$/ml). Experimental values are given relative to the total amplitude change. Inset: time-course of the decrease of fluorescence polarization of diphenylhexatriene-labelled platelets induced by 0.3 μM ionophore.

phore (Fig. 4). A complete inhibition was reached at 60 μM (Fig. 4A). However, when chlorpromazine was incubated with platelets in the absence of ionophore, an increase in the value of fluorescence polarization was observed mainly at 60 and 90 μM. This suggests that the rigidifying role of

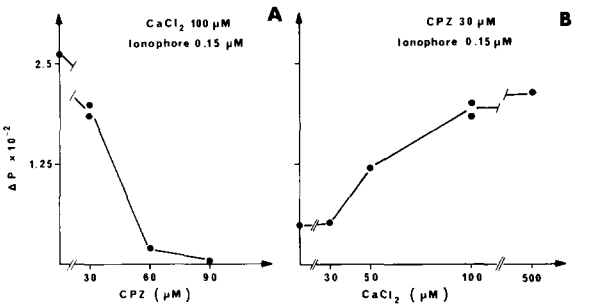

Fig. 4. Effect of chlorpromazine (CPZ) and Ca^{2+} on the amplitude of the fluorescence polarization change (ΔP) of diphenylhexatriene-labelled platelets induced by 0.15 μM ionophore (platelet concentration $50 \cdot 10^6$/ml). (A) Inhibition by chlorpromazine in the presence of 100 μM Ca^{2+}. (B) Reversion of 30 μM chlorpromazine-induced inhibition by increasing Ca^{2+} concentrations.

chlorpromazine could take part in its inhibiting effect on the ionophore-induced polarization decrease.

As it was found in previous results [2], $CaCl_2$ antagonized the inhibitory effect of chlorpromazine on the ionophore-induced platelet activation (i.e., [^{14}C]serotonin, thromboxane). Fig. 4B shows that increasing concentrations of Ca^{2+} between 30 and 500 μM progressively reversed the inhibitory effect on the polarization changes induced by 30 μM chlorpromazine.

Discussion

Nathan et al. [4] have observed membrane microenvironmental changes associated with thrombin-induced platelet activation. They reported an increase in the values of fluorescence polarization in diphenylhexatriene-labelled platelets. At $300 \cdot 10^6$ platelets/ml, we have observed the same increase in polarization when platelets were activated in the presence of ionophore. However, it seems that these results might be questionned because of the high light diffusion induced by the high platelet concentration.

In order to avoid this pitfall, we have investigated the optimum platelet concentrations ($(45–70) \cdot 10^6$/ml) where the polarization change was maximum and paralleled the other changes in platelet activation ([^{14}C]serotonin release, thromboxane B_2 synthesis). In these conditions, the ionophore induced a decrease in polarization and intensity of fluorescence. Both variations suggest a fluidization of the plasma membrane, since diphenylhexatriene was found to be mainly incorporated into the plasma membrane. Indeed, a decrease in fluorescence intensity may be associated with an increase of dynamical quenching of the excited probe [11].

Ionophore is known to liberate Ca^{2+} from internal or plasmatic membranes. Mobilization of Ca^{2+} previously bound to the plasma membrane would be one of the factors responsible for the observed fluidization. Indeed, the addition of external Ca^{2+}, far from inducing an increase of rigidity, elicits an acceleration in the decrease of polarization. In the absence of external Ca^{2+}, only high concentrations of ionophore are able to liberate enough platelet Ca^{2+} to reach a maximum rate of the polarization.

This phenomenon was accompanied by a maximum release of [^{14}C]serotonin and thromboxane B_2 synthesis.

Chlorpromazine was shown to inhibit platelet functions [12–14]. On the other hand, different authors have reported that there is a competition between chlorpromazine and Ca^{2+} regarding membranes [15–17]. Our results show that chlorpromazine inhibits the membrane changes in a dose-dependent manner. Furthermore, calcium is able to counteract this inhibition at the membrane level. The inhibition induced by chlorpromazine on platelet activation was also reported to be reversed by the addition of calcium [2].

The membrane phenomena reported here appear then to be tightly correlated with platelet activation. Several factors can modify the membrane physical state towards fluidization: release of Ca^{2+} from phospholipid headgroups [16], phospholipase activation [18], liberation of unsaturated fatty acids as revealed by thromboxane B_2 synthesis [19] and fusion of the membrane of granule with the plasma membrane. This last possibility appears as particularly relevant: indeed, the plasma membrane in platelets contains more cholesterol than the granule membranes. The fusion of these membranes during the activation lowers the relative cholesterol content of the plasma membrane and is thus responsible for its fluidization.

As the sequence of events occurs very rapidly, it is difficult to say whether the membrane changes precede the whole platelet activation or occur simultaneously. However, as well as stimulation of enzymes can modify the membrane physical state, this fluidization in turn can modulate the activity of proteins and enzymes implied in the activation process as it has been seen for other enzymes such as adenylate cyclase [20–22] or ATPase [23–24]. Changes in protein conformation or protein embedding in the bilayer [25] may be responsible for altered rates of enzyme activation [26] or for modified interactions between proteins [27].

The variation of the membrane fluidity can modulate the platelet activation and/or be modulated by the stimulation of enzymes. The use of specific inhibitors of different steps of platelet activation will help in delineating this mechanism.

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