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SIMULTANEOUS ISOLATION OF TWO PLATELET MEMBRANE FRACTIONS : BIOCHEMICAL, IMMUNOLOGICAL AND FUNCTIONAL CHARACTERIZATION

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Simultaneous isolation of two platelet membrane subfractions was achieved by centrifugation on 40 % sucrose from a 100.000g crude membrane fraction. Characterization of both types of membranes was carried out by different biochemical and immunological markers. Using a surface label, ^{3}H Concanavaline A (^{3}HCon A), a marker enzyme, phosphodiesterase, and lipid analysis, one of the fraction has been identified as external or plasma membranes, the other consists of intracellular membranes. Further two specific antibodies directed against external membrane antigens (LeK^{a} and IgG L) react almost exclusively with the external membranes. Finally both kinds of membranes were able to uptake calcium but the affinity for this cation was higher for the internal than for the external membranes. This suggests that both membranes are implicated in the regulation of the cytoplasmic calcium concentration and that the internal membranes (dense tubular system) play the major part in this regulation.

Calcium is recognized as an important messenger in the platelet activation. Both the external and internal membranes are considered to play an important role in the Ca^{++} homeostasis of the cell. Isolated and purified external and internal membrane preparations would allow the investigation of the biochemical processes underlying the platelet activation ; this should increase our understanding of the regulation of the cytoplasmic free Ca^{++} concentration by both kinds of membrane preparations. However, the major problem in the work dealing with the platelet membranes is to avoid cross contamination between the external and intracellular isolated fractions. Recently different approaches were used by several investigators. Some authors (1,2) isolated from a crude platelet microsomal fraction those microsomes which are able to take up Ca^{++} in the presence of ATP by loading them with calcium oxalate. However this approach suffered from major disadvantages namely that the membrane vesicles which were heavily loaded with calcium oxalate could not be used to study subsequently the major function of

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these membranes i.e. calcium transport. Other authors (3) have used the free flow electrophoresis to isolate and characterize platelet surface and intracellular membranes. However, the pretreatment by neuraminidase at the whole cell level before submitting the platelet homogenate to high voltage free flow electrophoresis makes difficult the interpretation of the results obtained for the external membranes.

We have developed a simple and rapid procedure for the simultaneous isolation of an external and internal membrane preparation using a centrifugation on a sucrose cushion from a 100.000g crude membrane fraction. The purity of the fractions obtained was characterized enzymatically, by lipid analysis and by the use of specific antibodies and a surface label. Furthermore the calcium uptake function which has been investigated emphasizes the major part in the regulation of calcium transport for internal membranes (dense tubular system) but does not rule out a possible role of the external membranes.

MATERIAL AND METHODS

The 100.000 g crude membrane fraction was prepared using a modification of the method of Kaser Glanzmann et al (1) as described by Le Peuch et al. (4). The fraction was characterized by Le Peuch et al. (4) and by Enouf and Levy-Toledano (5). The membranes were resuspended in an incubation medium containing potassium oxalate 10mM, KCl, 100mM, $MgCl_2$ 10mM, Hepes 20mM, ATP 5mM pH 7.55. This 100.000 g fraction (1 ml at 1 mg) was layered over 4 ml 40 % (w/v) sucrose solution containing ClNa, 8.1 g, NaH_2PO_4, $2H_2O$, 0,219 g, $Na_2H\ PO_4$ 1.22 g and glucose 2 g in 1 000 ml twice-distilled water, pH 6.5, followed by centrifugation in a swinging bucket rotor (Beckman SW 55 Ti) for 90 min at 95 000 g and 4°C. A band at the sample sucrose interface and a pellet were recovered, washed in the previously described buffer (1 vol for 9 vol), and centrifuged at 100 000 g for 60 minutes in the same rotor. The two membrane subfractions were then resupended in the same buffer. The protein concentration of the 100.000 g fraction and the two membrane subfractions was determined by the Bradford method (6) using gamma globulins as standard.

Phosphodiesterase activity was measured by the procedure of Koerner and Sinsheiner (7) using bis p nitrophenyl phosphate as a substrate. Labeling of the 100 000 g fraction with the ^3H concanavalin A (^3H Con A) was carried out after Rendu et al (8).

Lipids were extracted by the procedure of Bligh and Dyer (9). Phospholipids were determined by phosporus measurement according to Bottcher et al (10) while cholesterol was determined according to Lieberman (11).

Two antibodies were used for the immunological tests : the IgG L which occurred in a polytransfused thrombasthenic patient, was largely described as recognizing an antigenic determinant absent in thrombasthenic platelets (12). The anti LEK^a antibody has been detected in the serum of a patient who had developped a post transfusion purpura. The absence of reactivity of this antibody with the thrombasthenic platelets suggested that this new platelet antigen could be beared also on the glycoprotein complex IIb-IIIa (13).

The anti LEK^a serum and the IgG L were incubated with the different subfractions during 1 hour at 37°C. 0.1 ml of the 100.000 g fraction or the

external or internal membranes either at 2 mg/ml or 1,5 mg/ml were incubated with 0.1 ml of the anti LEK^a or IgG L antibodies. The mixture was then centrifuged for 15 minutes at 15 500 g and the supernate tested in quantitative immunofluorescence. For the platelet immunofluorescence test, normal platelets isolated from EDTA-anticoagulated blood (13) were treated with 1 % paraformaldehyde solution. After washing, platelets were incubated with the different supernatants for 30 min at 37°C, then washed and tested with the immunofluorescent antisera (FITC labelled rabbit antihuman IgG (Organon Teknica France) and examined with a Zeiss vertical fluorescent microscope. For some experiments we have quantified the fluorescence with a cytofluorograph "cell sorter" Ortho Instrument 50 Fc 210. The results were expressed as arbitrary units of fluorescence (AUF) as defined as : total fluorescence of platelets/number of platelets

The calcium uptake measurement were performed at 37°C using the millipore filtration technique (pore size 0,45 uM Type HA) according to the method of Kaser-Glanzmann (14) slightly modified (5). Calcium uptake was expressed as nmoles per mg protein. For Km determinations, incubation media containing concentrations of Ca^{2+} between 0.5 x 10^{-9} to 10^{-3}M were employed. To obtain the required free Ca^{2+}-concentration,a fixed EGTA concentration (0,5 mM) was added to variable $CaCl_2$ concentrations from 0.084 to 1.68 mM. Calculations were as reported by Lotersztajn et al (15) using apparent association constants of 6.02 x 10^8, 9.7 x 10^2, 9.55 x 10^3, 4.75 x10^4 M^{-1} for Ca EGTA, Mg EGTA, Ca ATP and Mg ATP respectively.

RESULTS AND DISCUSSION

The procedure used to isolate the two membrane fractions is similar to that described by Barry Pitts (16) for the preparation of cardiac sarcolemmal vesicles, but the composition of the buffer and the sucrose concentration have been altered in order to improve the yield and the specific activity of the calcium uptake. External membranes (E.M.) remained at the sample sucrose interface while intracellular membranes (I.M.) entered the sucrose layer and formed a pellet.

The protein recovery, a specific enzyme activity as well as a lectin labeling were estimated in both kinds of membrane fractions by comparison with the 100 000g fraction (Table I). It can be seen that after centrifugation of the 100 000g on the sucrose cushion, 60 ± 10 % of the total protein are recovered in both subfractions. The protein recovery for the external membranes reaches 53 ± 9 %, this belonging to internal membranes reaches 6.7 ± 0.9 %. The phosphodiesterase (PDE) activity (88 ± 38 pm/mg /h) and the ^3H Con A label (49 ± 20 x 10^3cpm/mg protein) are predominantly associated with external membranes while intracellular membranes show a very low enzymatic activity (2.9 ± 1.3 pm/mg/h) as well as a low ^3H con A labeling as compared with the internal membranes. These two markers were considered to be external membrane markers. Thus the PDE enzyme was found enriched in the external membranes of pig (17) and human (18) platelets, while the ^3H con A

Table I

Protein recovery, enzyme activity and bound lectin for the homogenate (100.000 g) and two membrane fractions : external (EM) and intracellular (IM) membranes

Fraction	Recovery %	P D E pmol/mg/h	Ratio	^{3}H Con A labelling cpm x 10^3/mg protein	Ratio
100.000 g	100	71 ± 24		95 ± 37	
E M	53 ± 9	88 ± 38	1.2	49 ± 20	0.52
I M	6.7 ± 0.9	2.9 ± 1.3	0.04	13 ± 7	0.12

Values are expressed as mean ± SEM from five individual membrane fractions.

lectin was found to bind to specific sugar from external membranes (19). The cholesterol and phospolipid contents, and cholesterol/phospholipid ratios help us to better discriminate both subfractions.

From the data in Table II we can see that the two membrane fractions differ significantly : cholesterol content and phospholipid amounts are respectively 5 times and 3.6 times higher in external membranes than in intracellular membranes, giving a cholesterol/phospholipid ratio somewhat lower in intracellular membranes. Similar differences between extracellular (plasmatic) and intracellular membranes have been reported in liver, muscle (20, 21).

The immunological characterization of both kinds of membranes which is achieved thanks to two antibodies emphasize the efficiency of the isolation procedure. Using the quantitative immunofluorescence technique and the anti LEK^a antibody we have shown in Fig. 1 that the 100 000g fraction reacted with the anti LEK^a antibody and the supernatant inhibited completely the immunofluorescence test with normal platelets until the titer of 1/8. In the meantime, the external

Table II

Cholesterol and phospholipid content of the 100.000 g and membrane subfractions : external (EM) and internal (IM)

Fractions	Cholesterol	Phospholipids	Cholesterol / Phospholipids
	umol/mg protein		Molar ratio
100.000 g	0.57 ± 0.025	1.09 ± 0.11	0.53 ± 0.006
EM	0.9 ± 0.13	1.52 ± 0.18	0.59 ± 0.049
IM	0.18 ± 0.036	0.42 ± 0.07	0.42 ± 0.045

Values are expressed as mean ± SEM from four experiments.

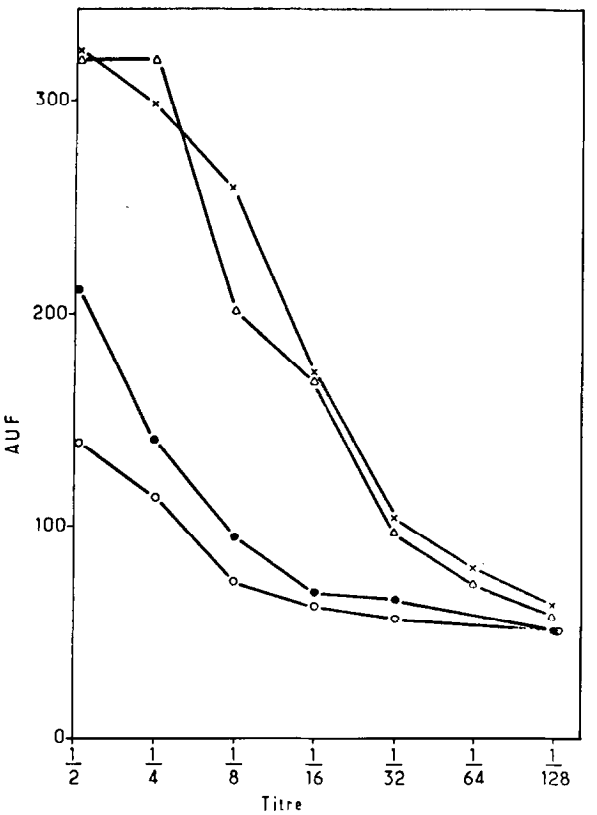

Figure 1 : Quantitative immunofluorescence reactivity of anti Lek^a serum with noraml platelets after incubation with buffer (x-x) and the different subfractions : 100.000g (●-●), external (o-o) and internal (Δ-Δ) membranes.
Results are expressed in arbitrary units of fluorescence (AUF).

membrane fraction exhibited the same reactivity as the 100 000g fraction against the antiserum while the internal membrane fraction did not react at all with the anti LEK^a antiserum and gave the same results as the antibody alone. When the anti Lek^a serum dilution reached 1/2, the quantity of fluorescence of 100 000g fraction expressed in AUF, was 65 % of the control while this from the external membranes was 43 % of the control. Values obtained in two sets of experiments were nearly identical. The anti LEK^a antibody is specific to platelet antigen which is found in 98.18 % of the normal population (13) ; furthermore this antigen as PLA_1 or Bak^a antigens, (22, 23) is not represented in thrombasthenic platelets and can be carried by the glycoprotein IIb-IIIa complex (13). Similar results were found using the same technique in the presence of IgG L (anti IIb, IIIa complex), (10) (Table III). The absorption of the IgG L by internal membranes was significantly

Table III

Quantitative immunofluorescence reactivity of various dilutions of IgG L after incubation with the 100.000 g and the subfractions : external (EM) and internal (IM) membranes. The control represents the antibody alone.

Fractions	Antiserum titer (IgG L)						
	1/2,5	1/5	1/10	1/20	1/40	1/80	1/320
100.000 g	49	25	12	6	0	0	0
EM	8	6	0	0	0	0	0
IM	67	56	27	13	0	0	0
Control	100	99	94	77	45	24	7

Results are expressed in arbitrary units of fluorescence (AUF).

less than the same absorption by external membranes ; the serum dilutions of 1/5 and 1/10 gave respectively 6 and 0 AUF for external membranes as compared with 56 and 27 AUF for internal membranes.

The results obtained with the 100 000g fraction were intermediated with those obtained with external and internal membranes. One can notice the difference of reactivity of the internal membranes with respect to either anti LEK^a or IgG L antibodies : no reactivity between anti LEK^a and internal membranes was detected while the IgG L partly reacted with this fraction. This could be explained by at least two suggestions : either the epitope (s) recognized by IgG L could be accesible both on external and internal membranes, the complex IIb-IIIa being transmembraneous (24) while the blood group antigens as LEK^a are only expressed in the external side of the external membrane ; or internal membranes would contain the antigenic determinants against the IgG L.

Recently, we reported the characterization of the functional activity of the 100 000 g fraction i.e. calcium accumulation, suggesting that the preparation consisted mostly of dense tubular system assimilated to reticulum sarcoplasmic membranes (4,5); After separation by centrifugation on 40 % sucrose, both subfractions were investigated as for their functional activity by comparison with the 100 000 g fraction. Fig. 2A shows that Ca^{2+} uptake occurs in the three fractions. Further the rate of the Ca^{2+} uptake as well as the Ca^{2+} accumulation are increased in external and internal membranes as compared with the crude 100 000 g fraction. This decreased calcium uptake may be due either to unavoidable

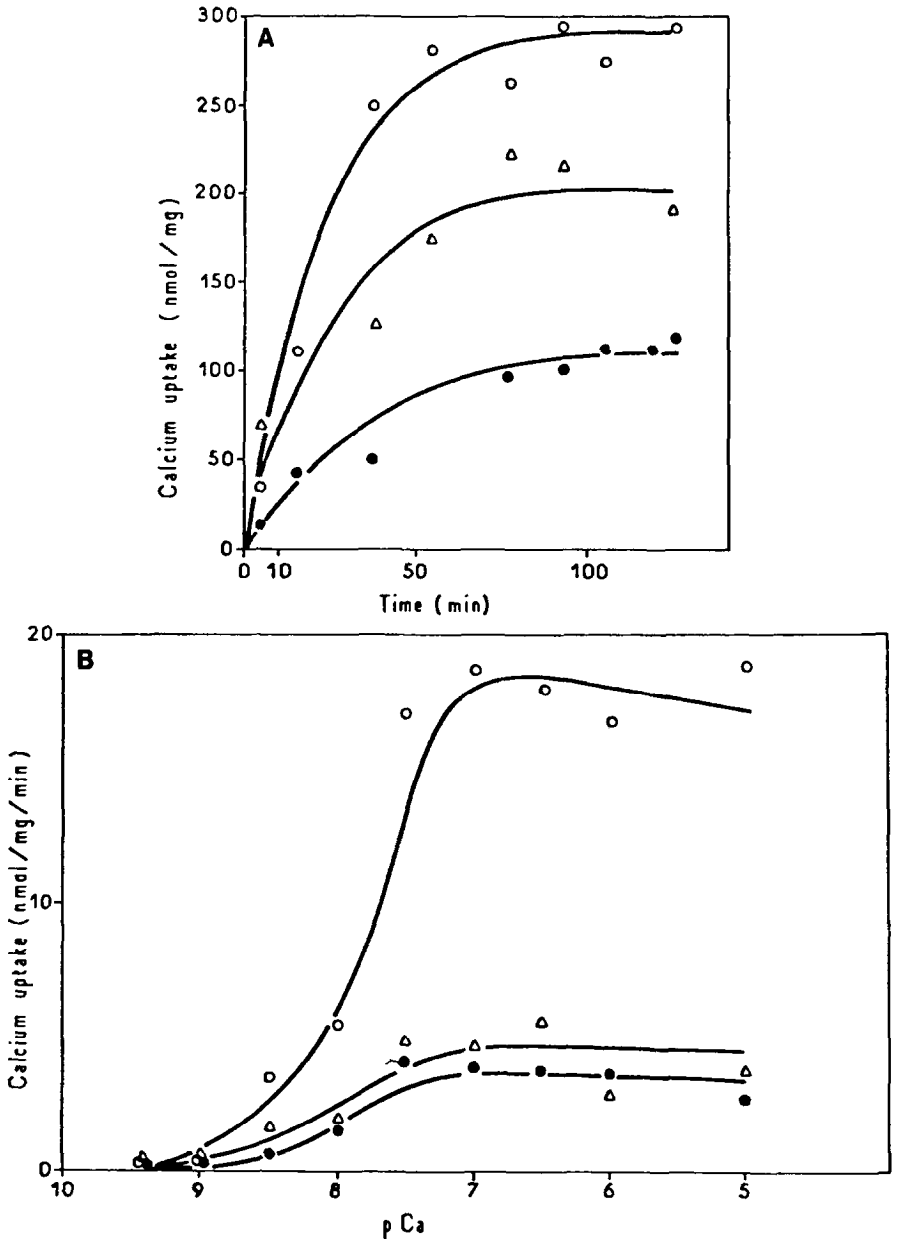

Figure 2 : Calcium uptake measurements
2A Uptake of calcium by the 3 membrane subfractions (150 ug per ml) in the presence of 100 uM $CaCl_2$. 100.000 g fraction (●-●), internal membrane fraction (Δ -Δ) and external membrane fraction (o-o). 2B : Effect of different concentrations of calcium on calcium uptake by the 100.000g fraction (●-●), internal (Δ-Δ) and external (o-o) membrane fractions . Experiments were done in the presence of free Ca^{2+} concentrations as described under "Materials and Methods".

loss of activity during a repeated high speed centrifugation or to the presence of an inhibitor which has been suppressed in both subfractions during the procedure isolation.

The uptake of calcium by external membranes would reflect the presence of a certain percentage of inside-out membrane vesicles formed during the ultrasonication step (25); then an ATPase pump would seem essentially localized in the inner leaflet of the external membranes as it is described in many other kinds of cells (26) ; although the binding of ^{3}H Con A and also the binding of the polyclonal antibodies to a known surface oriented sialoglycoprotein complex (GP IIb-IIIa) are criteria for designating part of the EM as right side-out vesicles. However one cannot rule out that the extracellular membranes can be contaminated by a subfraction of internal membranes, since it has been described recently that sarcoplasmic reticulum from muscle (27) consisted in light and heavy fractions which sedimented differently. In Fig. 2B, Ca^{2+} uptake measurements were determined in the presence of different free Ca^{2+} concentrations.From these results we have estimated the apparent affinity for calcium. The three subfractions have an apparent high affinity for calcium ; however the Km (Ca^{2+}) of the intracellular membranes uptake system is lower (8×10^{-9}M) than that of the extracellular membranes (3×10^{-8} M) and the 100.000 g fraction (1.5×10^{-8} M). This suggests a major role of the dense tubular system in regulating the cytoplasmic Ca^{2+} concentration in the platelets.

In the present study we have attempted to separate plasma membranes from dense tubular membranes and to document this separation by defining biochemical and functional studies. A strenght of this study is that membrane fractions enriched in plasma membranes were isolated in parallel with intracellular membranes enriched in dense tubular system. If we cannot rule out definitely that plasmic membranes are slightly contaminated by internal membranes we can assume however that the high Ca^{2+} affinity of the internal membranes was entirely due to dense tubular system.

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