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FURTHER CHARACTERIZATION OF CALCIUM-ACCUMULATING VESICLES FROM HUMAN BLOOD PLATELETS

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Summary

Human blood platelets are capable of removing Ca^{2+} from the cytoplasm by means of an active, ATP-dependent and cyclic AMP-stimulated transport system. Calcium-accumulating vesicles are obtained by sonicating platelets. On density gradient centrifugation, this activity is found in the heavier of two membrane fractions. Concentrated in this fraction are also the Ca^{2+}-stimulated Mg^{2+}-ATPase and glucose-6-phosphatase, believed to be a marker for internal membrane systems.

When the isolated vesicles are loaded with Ca^{2+}, a third band separates from the two vesicular fractions in the density gradient. This band C contains virtually all the Ca^{2+}-accumulating activity. Evidence that this activity is due to an active uptake and not to surface binding or adsorption is presented. Whereas electron microscopy does not reveal striking differences between active and inactive fractions, differences in protein composition are revealed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Furthermore, this band contains an enzyme system which converts arachidonic acid to malondialdehyde and therefore this fraction must be the site of prostaglandin synthesis.

Membranes prepared by loading platelets with glycerol, followed by osmotic lysis are unable to accumulate calcium. In sodium dodecyl sulphate-polyacrylamide gel electrophoresis such membranes show significant differences in their protein pattern as compared to the actively Ca^{2+}-accumulating vesicular membranes of band C.

All preparations with Ca^{2+}-accumulating activity also contain markers for plasma membranes and the question whether this activity is due exclusively to an intracellular structural element equivalent to the sarcoplasmic reticulum of muscle or whether an "extrusion pump" expelling Ca^{2+} to the outside of the cell is also involved, cannot yet be answered.

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Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid; DD-ISA, diazotized diiodosulfanilic acid.

Introduction

Blood platelets are activated by a wide variety of agents, ranging from low molecular weight substances such as ADP and biogenic amines, to proteases and high molecular weight complexes such as aggregated IgG and collagen. In every case the same manifestations of the activated state are observed; they consist of aggregation, the selective release of material from storage organelles, as well as morphological changes and manifestations of contractile activity. This suggests that independent of the inducing agent a common pathway of reactivity is induced and more recent observations suggest that the decisive event is the mobilization of Ca^{2+} from intracellular storage sites [1—4]. Accordingly, the resting state of the platelet would be characterized by a low concentration of cytoplasmic Ca^{2+} and the reversal of activation corresponds to the removal of the cation from the cytoplasmic matrix [5]. Such a calcium-accumulating system which is comparable to the "relaxing factor" or sarcoplasmic reticulum of muscle has in fact been demonstrated in blood platelets [6—8]. We have recently shown that the ATP-dependent, active uptake of Ca^{2+} by a vesicular fraction isolated from homogenized platelets is considerably stimulated by the presence of cyclic 3′:5′-adenosine monophosphate (cyclic AMP) and a protein kinase preparation of platelet origin [9]. It is well established that any measure taken to increase intracellular cyclic AMP levels will lead to inhibition of platelet activity; in fact, some of the most powerful known inhibitors of platelet activity, such as the prostaglandins E_1 and I_2 (prostacyclin), are potent activators of adenylate cyclase [10]. In view of this it must be assumed that the cyclic AMP-dependent calcium-accumulating system occupies a key role in the control of platelet function and further studies of its characteristics appeared appropriate.

Materials and Methods

A crude calcium-accumulating vesicular fraction, "40000 × *g* sediment" (designated here as membrane vesicles), was prepared from washed human platelets by sonication and differential centrifugation as described earlier [9].

Sucrose density gradient centrifugation. The density gradient was prepared by layering sucrose solutions in 0.01 M Tris · HCl, pH 7.4, containing 2 mM ATP of the following concentrations (w/v) and volumes: 60% (0.5 ml), 40% (0.25 ml), 38% (0.25 ml), 37% (0.5 ml), 35% (1 ml), 30% (0.5 ml), 27% (1 ml) and 22% (0.25 ml). On this discontinuous gradient was layered 1 ml of sample containing about 10 mg of protein in "homogenizing medium" (cf. ref. 9) and it was then centrifuged for 90 min in a Beckman SW 50.1 rotor at 45000 rev./min. The gradient was then either separated into 0.2-ml fractions or the visible, turbid bands were separated, generally pooled from 3 to 6 simultaneously prepared gradients, diluted with 0.005 M Tris · HCl, pH 7.4, to 13.5 ml and sedimented in a Ti 50 rotor for 30 min at 35000 rev./min. The pellets obtained were taken up in "homogenizing medium" with or without addition of oxalate, resuspended carefully with a glass pestle homogenizer and adjusted to a protein content of about 0.5%. The apparent densities of the isolated fractions of the sucrose gradient were determined by refractometry.

Protein was estimated by the Biuret method [11].

Uptake of $^{45}Ca^{2+}$ by membrane vesicles was determined as described earlier [9].

(Ca^{2+} + Mg^{2+})-stimulated ATPase was estimated by incubating a sample containing 0.5 mg protein for 30 min at 37°C in a medium composed of 100 mM KCl, 5 mM $MgCl_2$ 0.1 mM $CaCl_2$, 2 mM ATP, and 20 mM Tris · HCl, pH 7.0. From the value obtained for liberated P_i (estimated according to ref. 9) was subtracted the P_i content of a similarly treated sample containing 1 mM EGTA instead of $CaCl_2$.

Glucose-6-phosphatase was estimated according to Aronson and Touster [12], whereby P_i was measured as described in ref. 9.

Malondialdehyde production: To 0.2 ml of a membrane fraction containing 0.5% protein was added 0.01 ml of 2 mM arachidonic acid (dissolved in 0.1 M Tris · HCl, pH 8.0) and the mixture was stirred for 5 min at 37°C. Protein was then precipitated by the addition of 0.2 ml of 20% (w/v) trichloroacetic acid and separated by centrifugation after standing in ice for 10 min. To 0.2 ml of the supernatant 0.4 ml of 0.1 M thiobarbituric acid, pH 9, were added and the covered test tubes were kept for 7.5 min in a boiling water bath. After cooling in ice, the solutions were extracted with 1.5 ml of acidified butanol (cf. ref. 13). The extinction of the butanol phase was read at 533 nm against a blank of reagents without sample. Evaluation was done with the aid of a calibration curve established by measuring samples prepared from different concentrations of malondialdehyde.

Bis-(*p*-nitrophenyl)-phosphate phosphodiesterase and 5′-dTMP-*p*-nitrophenylester-phosphodiesterase were determined according to the modification by Taylor and Crawford [14] of the method of Koerner and Sinsheimer [15], where 2 M NaOH instead of 1 M NH_4OH is used to dissolve the proteins.

^{125}I surface labeling of platelets: Intact, washed platelets were labelled using the lactoperoxidase technique [16] or with diazotized [^{125}I]diiodosulfanilic acid (DD-ISA) according to George et al. [17]. DD-ISA was synthesized by Dr. D.A. Sears, San Antonio, Texas, by the method of Helmkamp and Sears [18].

Loading of membrane vesicles with Ca^{2+} was done under conditions comparable to those used for studying Ca^{2+} uptake (cf. ref. 9), i.e. by stirring the preparation for 60 min at room temperature in the presence of either Ca^{2+} or $^{45}Ca^{2+}$, followed by centrifugation for 15 min in a Ti 50 rotor at 45000 rev./min. The pellet was resuspended by careful homogenization in a ATP-containing buffer as described above. In some experiments, the ionophore A 23187 was present in the incubation mixture at an end concentration of 3.6 μM.

Electron microscopy was done as described earlier [9] with a Siemens Elmiscope S300.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed in 6 × 100 mm cylindrical gels of 5% acrylamide and 0.14% *N*,*N*′-methylene bis-acrylamide in 0.1 M sodium phosphate buffer, pH 7.0, with 0.1% SDS. The samples were solubilized in a boiling water bath for 10 min with 2% SDS and 2% dithiothreitol. Staining of the gels was done with Coomassie Blue or for carbohydrate with periodic acid-Schiff's reagent according to the method of Fairbanks et al. [19].

Mechanical homogenization of platelets was done in the same medium as the

one used for sonication for 8 min with an MSE blendor-type homogenizer running at 14000 rev./min.

Osmotic lysis of the platelets was effected after glycerol loading according to Barber and Jamieson [20]. The membranes thus obtained were isolated either by centrifugation against a sucrose density barrier or by differential centrifugation, as after sonication (cf. ref. 9).

Results

Density gradient separation of calcium-accumulating membrane vesicles

The fractionation on a sucrose density gradient of a membrane preparation obtained by differential centrifugation from homogenized platelets led to the formation of two bands with apparent densities of 1.09 and 1.12 (band A and band B). Some properties of these bands are shown in Table I. Band B contains 76% of the total calcium-accumulating capacity and 80% of the total Ca^{2+}-stimulated Mg^{2+}-ATPase activity. In both instances, the relative specific activities were in band B 3—4 times higher as compared to band A. Also in band B was found 73% of the total glucose-6-phosphatase activity with a specific activity which was higher by a factor of 2.3 than that found in band A and the same distribution and ratio of specific activity was also established for the production of malondialdehyde upon incubation of both bands with arachidonic acid.

After surface labeling of intact platelets, by either the [^{125}I]DD-ISA or by the lactoperoxidase technique, band B contained 40% of the introduced ^{125}I. On the other hand, bis-(*p*-nitrophenyl)-phosphate phosphodiesterase, a marker enzyme for plasma membranes was evenly distributed between bands A and B. Another enzyme, 5′-dTMP-*p*-nitrophenylester-phosphodiesterase, described for pig platelets by Taylor and Crawford [14] as a marker enzyme for internal

TABLE I

SOME PROPERTIES OF THE BANDS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION OF MEMBRANE VESICLES OBTAINED WITH AND WITHOUT PRELOADING WITH Ca^{2+}

r.s.a., relative specific activity, i.e., percent of total activity per percent of total protein content. %, activity found in each band expressed as percent of total activity of all two, or three bands. All values are means from double or triple determinations of each of 2—4 independent experiments.

	No Ca^{2+}-preloading				With Ca^{2+}-preloading					
	Band A		Band B		Band A		Band B		Band C	
	r.s.a.	%	r.s.a.	%	r.s.a.	%	r.s.a.	%	r.s.a.	%
$^{45}Ca^{2+}$ uptake	0.5	24	1.6	76						
$^{45}Ca^{2+}$ distribution					0.05	1	0.15	3	4.4	96
$(Ca^{2+} + Mg^{2+})$-ATPase	0.4	20	1.5	80						
Glucose-6-phosphatase	0.6	27	1.4	73	0.3	10	0.8	27	1.9	63
Malondialdehyde	0.6	28	1.3	72	0.3	7	0.9	37	1.6	56
^{125}I distribution	1.5	60	0.7	40	0.8	30	0.9	42	1.3	28
bis-(*p*-nitrophenyl)-phosphate phosphodiesterase	1.0	53	1.0	47	0.9	32	0.8	31	1.2	37

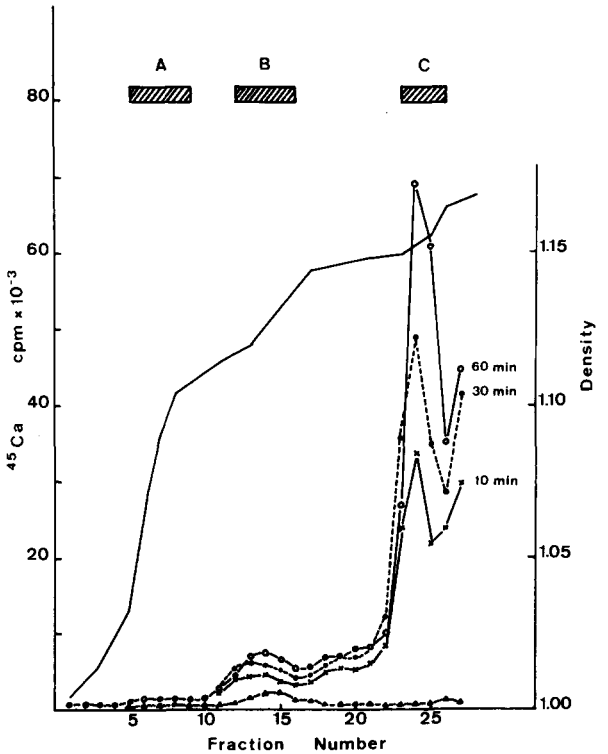

Fig. 1. Appearance of band C in relation to time of incubation with $^{45}Ca^{2+}$ of the crude preparation of membrane vesicles. The membrane vesicles were incubated with $^{45}Ca^{2+}$ and samples were applied to the sucrose density gradient after 10, 30 and 60 min. After centrifugation the gradient was subdivided into 0.2-ml fractions, and the $^{45}Ca^{2+}$ activity of each was determined. Shaded bars at the top of the figure represent the location of the visible bands. In the presence of the ionophore A23187 no band C was formed (▲———▲; 60 min incubation). The single solid line gives the densities of the sucrose gradient.

membranes was not detectable in either band of the human membrane preparation.

These results show that one of two bands obtained by density gradient fractionation is particularly rich in calcium-accumulating activity; however, they allow no clearcut identification of the subcellular localization of this activity.

Further fractionation of calcium-accumulating membrane vesicles

After preloading of the crude membrane preparation for 60 min with Ca^{2+} in the presence of ATP, sucrose density gradient separation reveals a new band with an apparent density of 1.15 (band C). On preincubation with $^{45}Ca^{2+}$, virtually all the isotope was recovered in this band (Table I). In order to establish, whether the calcium was taken up into vesicles or only carried down in an aggregate of membrane fragments, several experiments were performed:

(a) Time dependence of Ca^{2+} loading of band C. As shown in Fig. 1 the amount of Ca^{2+} taken up during incubation of the vesicles in the presence of ATP is directly proportional to the incubation time. This excludes an adsorption or coprecipitation phenomenon.

(b) In the presence of the ionophore A23187 which prevents the storage

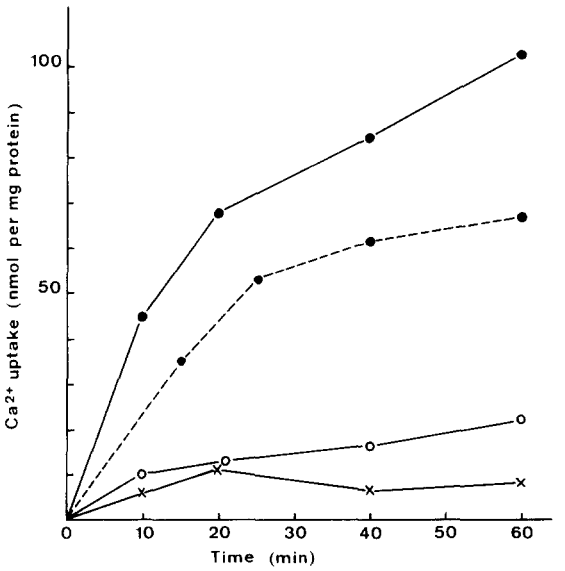

Fig. 2. Comparison of the time course of $^{45}Ca^{2+}$ uptake by the three bands obtained by density gradient centrifugation after partial preloading with Ca^{2+}. Plotted are values of $^{45}Ca^{2+}$ uptake in nmol/mg protein. X———X, band A; o———o, band B; ●———●, band C; ●- - - - -●, vesicular material before separation.

in, but not the adsorption to membrane vesicles, the development, on density gradient centrifugation of band C was not observed (Fig. 1).

(c) Upon incubation for 30 min of crude membrane vesicles in the presence of ATP, but in the absence of added Ca^{2+}, a faint band C was nevertheless formed, indicating that this particular fraction is capable of accumulating endogenous Ca^{2+} from within the incubated mixture. This effect is prevented by the addition of EGTA (2 mM final concentration).

The bands A—C thus obtained were isolated and their Ca^{2+}-accumulating capacity was measured. From Fig. 2 it can be seen that by far the most important calcium uptake is found in band C.

In Table I some other properties of the three membrane fractions are compared. Band C, which is the most actively calcium-accumulating fraction, contains 63% of the total glucose-6-phosphatase activity and, on incubation with arachidonic acid, 56% of the malondialdehyde-forming activity. However, it is also obvious that band C still contains plasma membrane, since, if prepared from [^{125}I]DD-ISA surface-labelled platelets, 28% of the total radioactivity is found associated with it. The same result is found after ^{125}I labelling of the intact platelets with the lactoperoxidase technique. In accordance with these findings, the activity of bis-(*p*-nitrophenyl)-phosphate phosphodiesterase in band C was also estimated to be 37% of the total activity, with a slightly higher specific activity than in the other fractions.

The electron microscope does not reveal any striking differences between the different membrane bands (Fig. 3). The size of the vesicular structures seems to increase from band A to band C, and also the ratio of vesicles to rod-like structures (perhaps collapsed vesicles) increases. Intact granules or mitochondria

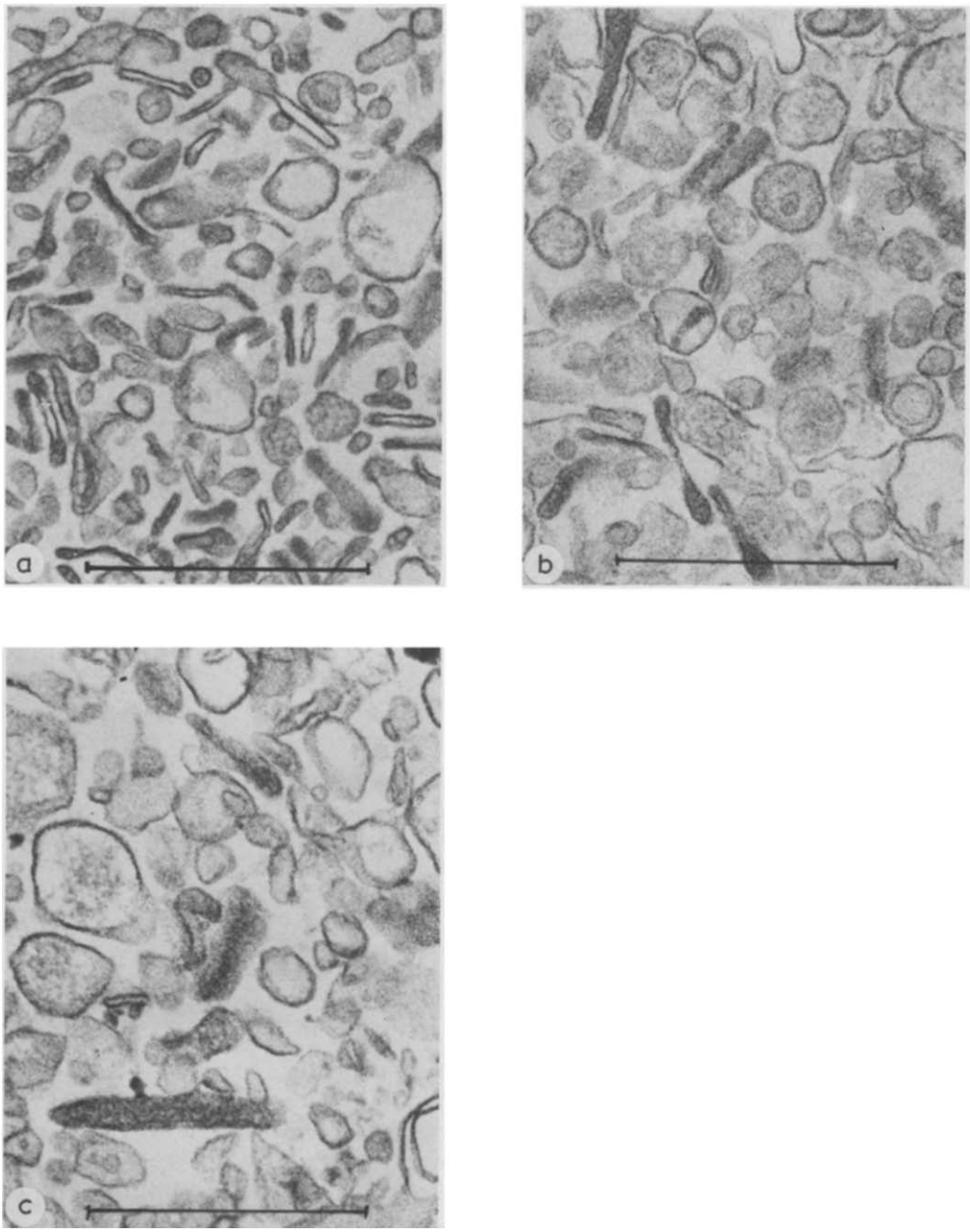

Fig. 3. Electron micrographs of band A (a), B (b) and C (c) as obtained by sucrose density gradient centrifugation of Ca^{2+}-preloaded vesicles. Magnification X 76000, bar 500 nm.

were never observed. Since the addition of NaN_3 (0.5—5 mM, final concentration) to the Ca^{2+}-accumulating system remained without effect, the participation of fragments of vesicular membranes of mitochondrial origin appears unlikely.

On sodium dodecyl sulphate-polyacrylamide gel electrophoresis the three

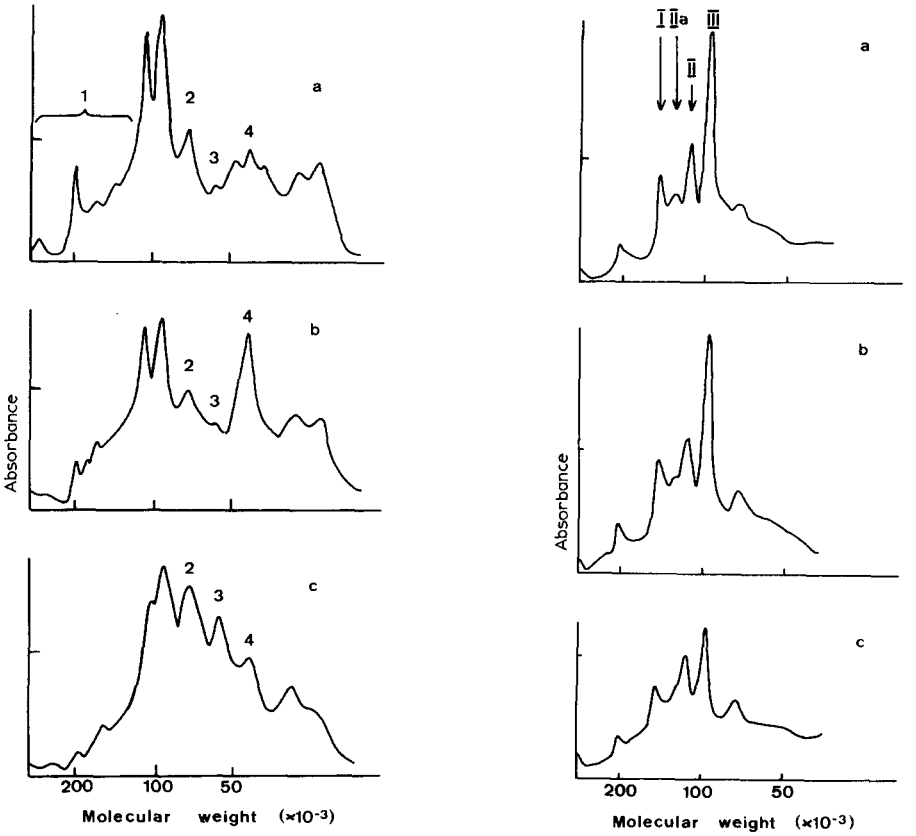

Fig. 4. Densitograms of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of the three bands obtained from Ca^{2+}-loaded vesicles. Coomassie Blue stain. a, band A; b, band B; c, band C; 1, high molecular weight region. Numbers 2, 3 and 4 refer to proteins with apparent molecular weights of 75000, 56000 and 42000, respectively.

Fig. 5. Densitograms of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of the three vesicular bands after staining for carbohydrate with periodic acid-Schiff's reagent. a, band A; b, band B; c, band C. The protein concentration was the same in all three preparations.

bands often show significant differences in their protein composition (Fig. 4). A polypeptide of 42000 molecular weight is most prominent in band B. In band C, which is richest in Ca^{2+}-accumulating vesicles, the zones containing materials with apparent molecular weights higher than 100000 are less pronounced; on the other hand, two polypeptides of 56000 and 75000 are quite prominent. As judged from the staining intensity with periodic acid-Schiff's reagent, the typical membrane glycoproteins I and II, and in particular III are decreased in band C compared to the other fractions: the material of an apparent molecular weight of 75000 remains about constant in all three bands and obviously is also a glycoprotein (Fig. 5).

Influence of the homogenization technique on the calcium-accumulating capacity of the vesicular membrane fractions

Three different techniques were used for the homogenization of the plate-

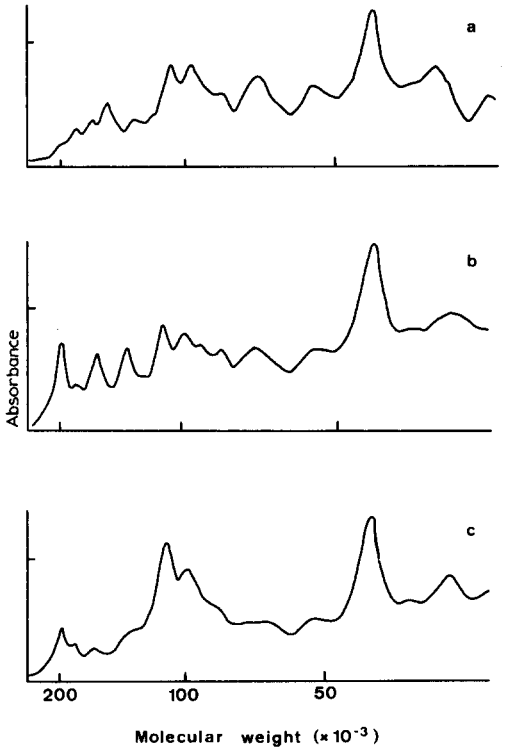

Fig. 6. Densitograms of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of crude membrane fractions prepared with different homogenization techniques, namely: a, sonication; b, blendor technique; c, osmotic lysis after glycerol loading. Coomassie Blue stain.

lets, namely sonication, treatment with a blendor-type, high-speed homogenizer, and osmotic disruption after glycerol loading according to Barber and Jamieson [20]. From all homogenates crude membrane vesicles were prepared by differential centrifugation and tested for calcium-accumulating capacity. The sonicated material is by far the most active (70 nmol Ca^{2+}/mg protein per 60 min); some activity is discernible in the blendor-preparation (27 nmol Ca^{2+}/mg protein per 60 min), whereas the osmotically lysed material is inactive. When examined by disc electrophoresis in the presence of sodium dodecyl sulphate, these three preparations showed distinct differences in their protein composition (Fig. 6). Blendor-prepared vesicles contained larger amounts of polypeptides with apparent molecular weights of 200000, 135000 and 42000 than those obtained from sonicated platelets. This might indicate the presence of myosin, the myosin rod portion, and actin, implying a pronounced contamination with platelet actomyosin. In membranes prepared according to Barber and Jamieson a polypeptide with molecular weight 75000 as well as another one with 56000 are considerably diminished. It is of interest that these two components are particularly prominent in the polypeptide pattern of band C separated by density gradient centrifugation, i.e., the band with the highest calcium-accumulating capacity.

Discussion

With respect to the subcellular localization of an active calcium-accumulating membrane system, several possibilities must be considered:

(a) The dense tubular system, a vesicular or tubular membrane system, supposed to be derived from the endoplasmic reticulum and in many respects comparable to the sarcoplasmic reticulum of muscle [21] has been proposed several times for such a role [22—24].

(b) The plasma membrane could contain a Ca^{2+} extrusion pump, removing the cation from the cytoplasm to the external medium as has been described for erythrocytes [25] or muscle cells [26—28]. Calcium-accumulating membrane vesicles derived from the plasma membrane must then have an inside-out orientation.

(c) Mitochondria are known to be involved in intracellular calcium regulation, as shown for cardiac muscle [29] and liver cells [30].

(d) The dense bodies contain the major part of platelet calcium [31] and it must be considered that the membranes of these organelles contain a Ca^{2+}-accumulating system which up to now has escaped detection because of the lack of adequate markers.

Among these different possibilities and for our system, mitochondrial membranes appear the least likely, since the calcium-accumulating vesicles of our preparations are not affected by NaN_3, a powerful inhibitor of mitochondrial calcium transport [8,32]. Of the two fractions obtained by density gradient centrifugation the heavier one ($d = 1.12$) shows a higher Ca^{2+}-accumulating activity and at the same time a higher Ca^{2+}-stimulated ATPase activity than its counterpart with $d = 1.09$. This heavier fraction is also rich in glucose-6-phosphatase which is a marker enzyme for the endoplasmic reticulum [33] and most likely is typical for internal membrane systems of the platelet [34]. On incubation with arachidonic acid, the same fraction shows the most active production of malondialdehyde: according to Gerrard et al. [35] this activity is linked to the dense tubular system. All these observations suggest that inner membrane systems and in particular the dense tubular system may be involved in the observed Ca^{2+}-accumulating activity.

In analogy to the sarcoplasmic reticulum of muscle [36,37], the platelet membrane vesicles can be loaded with Ca^{2+} and on density gradient centrifugation then form a third zone with an apparent density of 1.15 which is characterized by a still higher Ca^{2+}-accumulating activity. It appeared important to verify whether this third band contains the looked-for calcium-accumulating structures. The following observations are in favor of this assumption: The rate of formation of band C is in direct relationship to the uptake of Ca^{2+} by the vesicles and in the presence of the ionophore A23187 the band is not formed. Whereas these results leave little doubt that band C at least contains a high proportion of the Ca^{2+}-accumulating vesicles, they allow no final decision as to the subcellular localization of these structures, since markers for both, dense tubular system and plasma membrane are present in this preparation.

Electron microscopy of the three bands, which differ widely in their activities, does not reveal significant differences: in each case vesicular structures are observed, more or less contaminated with rod-like material which may repre-

sent collapsed vesicles. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis shows some differences: thus, band B is particularly rich in material with characteristics of actin. This corroborates the findings of Taylor and Crawford [14], who also found actin in the heavier membrane fraction which supposedly consists of inner membranes. This is rather surprising, because the submembranous actin filaments are found in close affiliation with the plasma membrane and consequently also with the open canalicular system [38]. The possibility that this fraction also contains resealed fragments of this system should be seriously considered. A comparison of the periodic acid-Schiff's reagent stains shows again that the glycoprotein pattern of the three bands is significantly different; this may be interpreted to mean that different membrane systems are involved.

Recently a "high-affinity calcium-binding protein" from sarcoplasmic reticulum of muscle has been described [39] and Solomon [40] attributes Ca^{2+}-binding properties to tubulin from mouse brain. It is of interest that the molecular weights of both these proteins are in the vicinity of 56000. Whether either one of them accounts for the prominent zone in the region of this apparent molecular weight in our band C remains at present an open question.

The homogenization technique used is decisive for the Ca^{2+}-accumulating properties of the preparations. In view of the fact that active vesicles form by resealing of fragments of the original, most likely tubular structures, this is not unexpected. Excessive trapping of proteins will give raise to lower specific activities, although fully active vesicles have formed; this might in part, but certainly not completely explain the reduced activities of blendor-homogenized material. Preparations obtained by the glycerol lysis technique [20] were totally inactive. Surface labels are evenly distributed in the two bands obtained by this method and the authors of this procedure claim that both these bands contain predominantly plasma membranes [41,42]. It seems premature, though, to conclude from this that the plasma membrane is incapable of active Ca^{2+} transport. In order to demonstrate such a transport, the presence of inside-out vesicles would be necessary; furthermore it may well be that glycerol lysis removes essential components of the transport system from the membrane.

In conclusion, the present work does not yet give a final answer as to the subcellular localization of the Ca^{2+}-accumulating system of human platelets. However, the concentration, in the most active fraction, of markers of the dense tubular system points to a major participation of this system; the persistent finding of markers for the plasma membrane in these preparations may either be interpreted to mean that this material is a notorious, inactive contaminant, or that platelets have also a Ca^{2+} extrusion pump, which, under in vitro conditions, would accumulate the cation in inside-out vesicles derived from the plasma membrane. It is quite obvious that more work is needed in order to differentiate between these possibilities. Finally, it must be mentioned that in the intact platelet, mitochondria may also contribute to the control of the cytoplasmic calcium level: however, in the in vitro studies presented here, their participation has been excluded.

Acknowledgements

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