CALCIUM UPTAKE ASSOCIATED WITH AN INTRACELLULAR MEMBRANE FRACTION PREPARED FROM HUMAN BLOOD PLATELETS BY HIGH-VOLTAGE, FREE-FLOW ELECTROPHORESIS

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Received 1 March 1982

1. Introduction

Many of the morphological changes and intracellular metabolic events that occur when platelets are activated by haemostatic agents such as ADP, thrombin or collagen are calcium-requiring processes. The dissociation of microtubules, the contraction of actomycin, glycogenolysis, the action of lipases in the release of arachidonic acid for prostaglandin synthesis, the phosphorylation of key substrates by protein kinases are just a few examples of intracellular mechanisms in platelets which are triggered in some way by the surface membrane signals and are believed to be regulated by mobilisation of internal Ca^{2+} stores. An ATP-dependent accumulation of Ca^{2+} by platelet mixed-membrane fractions has been shown in [1–4]. However, interpretation of the localisation and importance of this property in platelet behaviour has been frustrated by the inability to separate discretely from a mixed population of membrane vesicles those intracellular features which may control cytosolic Ca^{2+} levels, from the vesicles of plasma membrane origin that may have both inflow and outflow transport processes for Ca^{2+}. Membrane elements in blood platelets, analogous to the sarcoplasmic reticulum of skeletal or cardiac muscle, have not yet been established, although their presence was postulated in [5].

We have reported a new procedure for the differential separation of highly purified fractions of human platelet surface and intracellular membranes [6]. The origin of these fractions in the whole platelet has been determined by surface probes and marker enzymes.

Here, we present evidence that an ATP-dependent, Ca^{2+}-accumulating process is almost exclusively associated with the intracellular membrane fraction that is derived from features which electron microscopists have clearly distinguished from plasma membrane, and the open canalicular membrane system contiguous with it, which is now commonly referred to as the dense tubular membrane system (DTS) [7].

2. Experimental

2.1. *Platelet isolation*

Platelets were prepared from fresh buffy coats by a differential centrifugation procedure done exactly as in [6].

2.2. *Membrane preparation and isolation of subfractions*

The technical details of the electrophoretic procedure for separating a gradient prepared human platelet mixed membrane fraction into surface and intracellular membranes have been fully reported [6]. Briefly, the method depends upon lowering the electronegativity of the plasma membrane by pretreatment at the whole cell level with neuraminidase and after sonication a granule-free mixed membrane fraction is taken from the low density zone of a 1.0–3.5 M sorbitol gradient. This membrane fraction is then sedimented on to a cushion of 3.5 M sorbitol, buffered with 10 mM Hepes (pH 7.2) and containing 1 mM EGTA. The membrane band was removed and diluted 1:1 with electrophoresis chamber buffer (10 mM triethanolamine–acetate, 1 mM EGTA (pH 7.2) adjusted to 400 mOsmol with sorbitol). The

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

surface and intracellular membranes are then separated by high-voltage electrophoresis using a Bender Hobein/MSE VAP5 apparatus. The peak fractions representing intracellular membranes (N_I) and the two surface membrane peaks (N_{II} and N_{III}) are pooled and concentrated by sedimentation on to a cushion of 3.5 M sorbitol as before. The interface zone of membrane vesicles (~1 mg membrane protein/ml) is taken for the measurement of Ca^{2+} uptake.

2.3. *Measurement of calcium uptake*

The incubation mixture consisted of 120 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 20 mM Tris–HCl buffer (pH 7.0) and 50 μM $CaCl_2$ containing ^{45}Ca (spec. act. ~70 mCi/mmol) and 2.5 mM oxalate. For the Ca^{2+} uptake assays, an aliquot of membrane suspension, between 40 and 50 μl and containing 30–50 μg membrane protein, was added to the assay mixture (final vol. 1.0 ml) and incubated at 21–22°C. For 50 μl aliquots of membrane suspension, EGTA was 50 μM in the assay mixture. Adjustment was made to this concentration when smaller volumes of membrane suspension were used. At 50 μM EGTA, calculated free Ca^{2+} was 3 μM [8,9]. At the times indicated in the experimental protocol 0.9 ml mixture was removed and filtered rapidly through a Millipore membrane filter of type GS, 0.45 μm pore size. After 3 successive washings with 10 ml ice-cold buffer containing 120 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.0) and 50 μM unlabelled $CaCl_2$, the filters were dried and counted in a scintillation counter.

3. Results and discussion

The free flow electrophoresis procedure results in the mixed membrane fraction separating discretely into intracellular (N_I) and two surface membrane vesicle subfractions (N_{II} and N_{III}). The identification of their site of origin in the whole cell has been established on the basis of the localisation of ^{125}I-labelled *Lens culinaris* applied as a probe to the surface of the intact platelets, the presence of adenylate cyclase activity and vulnerability to neuraminidase for the two surface membrane fractions N_{II} and N_{III} and the exclusive localisation in the intracellular membrane fraction, N_I, of the endoplasmic reticulum marker enzyme antimycin-insensitive NADH cytochrome *c*-reductase [6]. The surface and intracellular membranes also differed markedly in polypeptide, cholesterol and phospholipid composition, microviscosity, content of actin and myosin and presence of marker enzymes. Other distinguishing features of the full phospholipid profiles and the fatty acid content of all the phospholipid classes present have appeared in [10]. Why the surface membrane of platelets consistently separates into 2 discrete components N_{II} and N_{III} by this technique is not yet known. Their polypeptide profiles are essentially the same, both label well with ^{125}I-lectins and their analytical compositions are closely similar. At present it is considered that they may represent two different surface domains perhaps with differing content of sialic acid and/or vulnerability to neuraminidase. Alternatively they could also be an expression of whole platelet subpopulation heterogeneity in the circulating pool. The orientation of the electrophoretically separated membrane vesicles has been established as normal, i.e., outside-out, on the basis of three criteria; the further removal of sialic acid from the vesicles and consequent reduction in electronegativity by neuraminidase treatment, the binding to the separated vesicles of ^{125}I-*Lens culinaris* and also the binding of a monoclonal antibody to a known surface-oriented sialoglycoprotein complex, the glycoprotein 11b 111a. This antibody was kindly provided by Dr S. Shapiro of the Cardeza Foundation, Philadelphia.

Fig.1a shows that Ca^{2+} accumulation is confined to the vesicle subpopulation earlier established to be of intracellular origin [6]. With platelet membrane fractions from many different donors the uptake of Ca^{2+} by the intracellular membranes gave values of 40–150 nmol . mg protein^{-1} . h^{-1}. In contrast, the surface membrane vesicles gave values for Ca^{2+} uptake between 5–10% of the rates recorded for intracellular membranes from the same platelet preparation. Fig.1b shows that the uptake is ATP-dependent and in all intracellular membrane preparations studied the uptake in the absence of ATP was very low or negligible. Fig.1 also shows the effect of adding the Ca^{2+} ionophore A23187 to the incubation mixture 45 min after commencement of the uptake assay. There is an immediate loss of calcium from the loaded vesicles and it is clear that almost all of the ATP-dependent accumulated calcium is liberated by the action of the ionophore which destroys the vectoral properties of the Ca^{2+} pump. This indicates too that binding of Ca^{2+} to the vesicle membrane is not a significant feature. The Ca^{2+} accumulating property of the intracellular

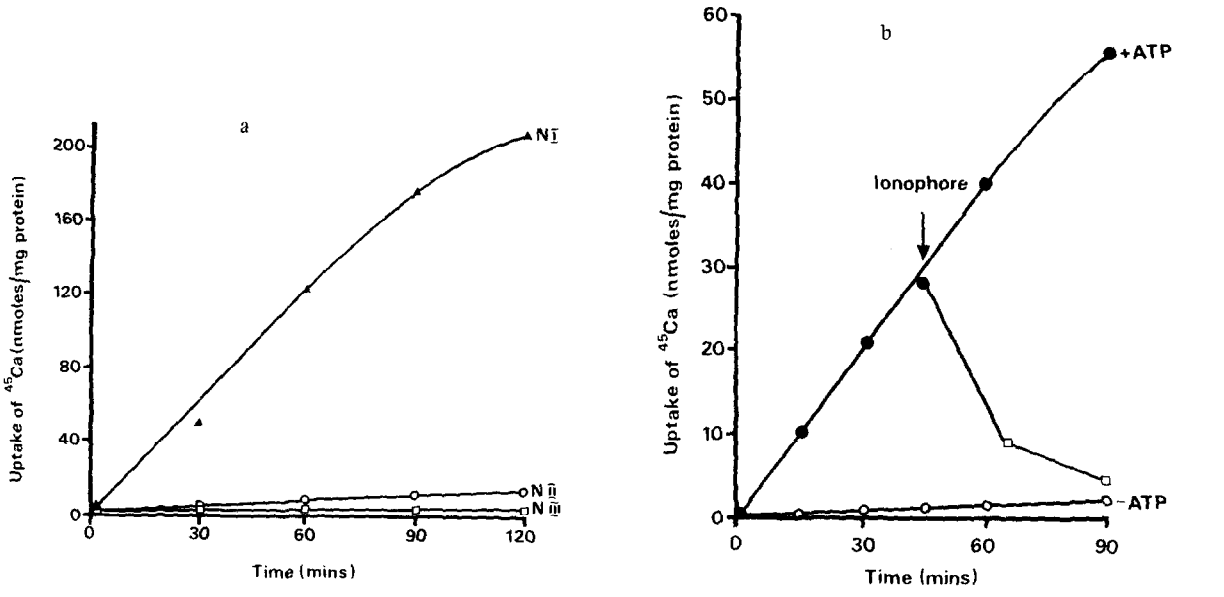

Fig.1.(a) Uptake of calcium by the intracellular membrane fraction N_I (▲——▲) and the surface membrane fractions N_{II} (○——○) and N_{III} (□——□). (b) Uptake of calcium by the intracellular membrane fraction N_I in the presence (●——●) and absence (○——○) of ATP. After 45 min incubation a sample was removed to which the divalent cation ionophore A23187 was added to 5.4 μM final (□——□).

membrane was also dependent upon the presence of oxalate (table 1). It is clear that in all the fractions the absence of either ATP or oxalate results in low or negligible accumulation of Ca^{2+}, indicating that an energy-dependent transport process enhanced by the presence of oxalate is the major mechanism involved. To date we have been unable to demonstrate any significant increase in Ca^{2+} accumulation by the addition of either dibutyryl cAMP, purified platelet calmodulin or aliquots of platelet cytosol to the assay mixture.

Since the electrophoretic procedure depends upon pretreatment of the intact cells with neuraminidase we investigated the Ca^{2+} uptake of mixed membrane fractions prepared from untreated and neuraminidase-treated platelets. These mixed membrane fractions taken from the sorbitol gradient were assayed after sedimentation on to a cushion of high molarity sorbitol as earlier described. The results indicate that similar values for Ca^{2+} accumulation are obtained with mixed membranes from untreated and treated cells (table 2). The Ca^{2+} uptake of intracellular membrane vesicles prepared from neuraminidase-treated platelets are compared with an aliquot of the same vesicles pre-

Table 1
The effect of ATP and oxalate on the Ca^{2+} uptake of intracellular (N_I) and surface membranes (N_{II} and N_{III}) (nmol . mg protein^{-1} . h^{-1})

Additions to assay medium		Calcium uptake		
ATP (2 mM)	Oxalate (2.5 mM)	N_I	N_{II}	N_{III}
–	+	1.2	0.9	2.5
+	–	5.4	2.4	2.3
+	+	38.6	4.8	3.6

Table 2
Effect of neuraminidase-treatment on Ca^{2+} uptake with and without ATP in the assay (nmol . mg protein^{-1} . h^{-1})

Experimental	ATP	Ca^{2+} uptake	
1. Mixed membrane fraction	–	1.6	–
from untreated platelets	+	151.0	151.2
2. Mixed membrane fraction from	–	2.0	–
neuraminidase-treated platelets	+	174.0	150.0
3. Intracellular membrane from			
neuraminidase-treated platelets	–	2.4	–
(control N_I fraction)	+	146.4	157.0
4. Intracellular membrane from			
neuraminidase-treated platelets			
incubated with neuraminidase	–	2.6	–
(treated N_I fraction)	+	136.8	161.0

exposed to neuraminidase for 15 min at 37°C at a similar concentration as was used for the whole platelets (table 2). Although the electrophoretic mobility of these treated vesicles is very significantly reduced when passed through the electrophoresis chamber a second time, neither the neuraminidase treatment nor the re-electrophoresis affected the Ca^{2+}-accumulating property. It is unlikely therefore that the low values for this property in the surface membrane fractions represent a loss of activity due to exposure to enzyme at the whole cell level. Although an ATP-dependent accumulation of Ca^{2+} could be demonstrated in mixed membrane fractions taken from the sorbitol gradient, this property was substantially diminished during washing of the membranes preparatory to electrophoresis (not shown). Although all buffer solutions were adjusted to 290–310 ideal mOsmol with sorbitol, vesicle permeability or unsealing appeared to be a major problem. By exposing the gradient-separated vesicles to a higher osmolarity through sedimentation onto a cushion of 3.5 M sorbitol before resuspension and raising the electrophoresis chamber buffer to 400 ideal mOsmol the Ca^{2+} accumulating property was fully maintained. Using these conditions serial studies have shown that Ca^{2+} accumulation decreases ~10%/day when the membrane vesicles are stored at 4°C. Others forms of storage have not yet been investigated.

In studies with human platelets [3], an active Ca^{2+} uptake by a mixed membrane vesicle fraction isolated by differential centrifugation (a 40 000 × *g* sediment prepared from platelet sonicate) was shown. A wide range of values with maximum uptakes of 70–150 nmol Ca^{2+} . mg protein^{-1} . h^{-1} was also observed [3]. Similar values were obtained for calf platelet mixed membranes in [2]. In all these studies, however, an appreciation of the role of the Ca^{2+} accumulating property in platelet function has been difficult because of the morphological complexity of the platelet membrane systems and the lack of certainty about the cellular origin of membrane fractions isolated by conventional procedures. The relationship between the various platelet membrane systems has been well reviewed in [7] and the analogy between platelet intracellular membrane (DTS) and muscle sarcoplasmic reticulum has been made [11]. Our separation of surface from intracellular membranes substantially removes this uncertainty about their origin since these data clearly show that the ATP-dependent Ca^{2+} accumulation is mainly associated with intracellular vesicles. In [12,13] we demonstrated that this same subpopulation of membrane vesicles is also substantially enriched, with respect to homogenate levels, in the activities of a sequence of enzymes known to liberate arachidonic acid from membrane phospholipids and subsequently convert it to endoperoxides and thromboxanes (phospholipase A_2 diglyceride lipase, cyclooxygenase and thromboxane synthetase). Since phospholipase A_2, and probably other enzymes in the sequence too, have a requirement for calcium it seems possible that this Ca^{2+} accumulating property of the intracellular membranes and their capacity to produce prostaglandin endoperoxides and thromboxanes are in some way mechanistically linked. Attention was drawn to such a possible association in [14] and it was demonstrated that thromboxane A_2 has ionophoric properties. From [14], which included some theoretical considerations of Ca^{2+}–TXA_2 complex formation, it was postulated that thromboxane A_2 may be involved in transporting Ca^{2+} out of the intracellular storage site and into the cytoplasm. The mobilisation of platelet intracellular Ca^{2+} through the counteraction of the Ca^{2+} accumulating property of these membranes and some internally generated ionophore involved in its release, could constitute a dynamic system central to the regulation of platelet shape change, pseudopod formation, secretion processes and other motile events known to be triggered by haemostatic agents acting at the plasma membrane.

Acknowledgements

We are grateful to the British Heart Foundation and the Thrombosis Research Unit of Ciba-Geigy, Horsham, for their generous support of our research.

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