Studies on the bivalent-cation-activated ATPase activities of highly purified human platelet surface and intracellular membranes

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Membrane-bound Ca^{2+} -ATPases are responsible for the energy-dependent transport of Ca^{2+} across membrane barriers against concentration gradients. Such enzymes have been identified in sarcoplasmic reticulum of muscle tissues and in non-muscle cells in both surface membranes and endoplasmic-reticulum-like intracellular membrane complexes. In a previous study using membrane fractionation by density-gradient and free-flow electrophoresis, we reported that the intracellular membranes of human blood platelets were a major storage site for Ca^{2+} and involved in maintaining low cytosol $[\text{Ca}^{2+}]$ in the unactivated cell. In the present report we demonstrated that the intracellular membranes also exhibit a high-affinity Ca^{2+} -ATPase which appears to be kinetically associated with the Ca^{2+} -sequestering process. We found that both the surface membrane and the intracellular membrane exhibited a basal Mg^{2+} -ATPase activity, but Ca^{2+} activation of this enzyme was confined only to the intracellular membrane. Use of Ca^{2+} -EGTA buffers to control the extravesicle $[\text{Ca}^{2+}]$ allowed a direct comparison of the Ca^{2+} -ATPase and the Ca^{2+} -uptake process over a Ca^{2+} range of 0.01 μM to 1.0 mM, and it was found that both properties were maximally expressed in the range of external $[\text{Ca}^{2+}]$ 1–50 μM , with concentrations > 100 μM showing substantial inhibition. Double-reciprocal plots for the Ca^{2+} -ATPase activity and Ca^{2+} uptake gave apparent K_m values for Ca^{2+} of 0.15 and 0.13 μM respectively. However, similar plots for ATP with the enzyme revealed a discontinuity (two affinity sites, with K_m 20 and 145 μM), whereas plots for the Ca^{2+} uptake gave a single K_m value for Ca^{2+} , 1.1 μM . Phosphorylation studies during Ca^{2+} uptake using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ revealed two components of 90 and 95 kDa phosphorylated at extravesicle $[\text{Ca}^{2+}]$ of 3 μM . The Ca^{2+} -ATPase activity, Ca^{2+} uptake and phosphorylation were all almost completely inhibited in the presence of 500 μM - Ca^{2+} . Similar studies using mixed membranes revealed four other phosphoproteins (50, 40, 20 and 18 kDa) formed in addition to the 90 and 95 kDa components. The findings are discussed in the context of platelet Ca^{2+} mobilization for function and the mechanisms whereby Ca^{2+} homeostasis is controlled in the unactivated cell.

INTRODUCTION

A wide variety of platelet metabolic and mechanochemical processes are regulated by the concentration of free (ionized) calcium, $[\text{Ca}^{2+}]$, in the cytosol of the cell. These processes include glycogen metabolism, prostanoid synthesis, phospholipid modifications, protein phosphorylation, secretion, membrane transport, surface receptor revelation and configuration, microtubule assembly/disassembly and the maintenance of integrity and interactive functions of the platelet's membrane-associated cytoskeletal networks.

Quiescent (unstimulated) platelets maintain their cytoplasmic $[\text{Ca}^{2+}]$ within the range 10–100 nM (Rink *et al.*, 1983), despite a 10000:1 gradient of the cation across the plasma membrane with respect to millimolar concentrations in plasma, and there is now substantial evidence to indicate that activation of the cell by excitatory agonists is mediated through, or accompanied by, an elevation of cytosol $[\text{Ca}^{2+}]$. In many activatable

cells, much attention has been focused on mitochondria for their known capacity to store Ca^{2+} , but there are now compelling arguments for the view that the major contributors towards the maintenance of platelet Ca^{2+} homeostasis are (a) the plasma membrane, with its capacity to efflux Ca^{2+} to an unlimited plasma sink, and (b) the platelet's intracellular (endoplasmic-reticulum-like) membrane, with its ability to sequester Ca^{2+} by uptake from the cytosolic compartment (Brass, 1984).

The first report which suggested that the platelet intracellular membranes (at that time referred to by electron microscopists as the dense tubular membrane system, or DTS) may have a sarcoplasmic-reticulum-like Ca^{2+} -storing property was made by Statland *et al.* (1969). Later studies by Robblee *et al.* (1973), Käser-Glanzmann *et al.* (1977), Fox *et al.* (1979) and Enouf *et al.* (1984), all using mixed membrane fractions prepared from platelet homogenates, which contained both surface and intracellular membrane elements, confirmed the presence of a Ca^{2+} -ATPase activity which was thought to be either a

Abbreviation used: Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate

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plasma-membrane Ca^{2+} -extrusion pump, as is present in erythrocyte surface membranes, or an intracellular-membrane energy-dependent Ca^{2+} -sequestering process, or perhaps both. More recently Dean and his colleagues (Dean, 1984) have isolated and partially characterized a Ca^{2+} -ATPase from platelet membranes, and Brass (1984), who examined the kinetics of Ca^{2+} exchange, using steady-state influx and efflux data in whole unstimulated platelets, identified two distinct Ca^{2+} pools, one in the cytosol and another at an internal site believed to be the 'dense tubular membrane system' (DTS). In Brass's (1984) studies he was unable to identify the action of a plasma-membrane Ca^{2+} extrusion pump, but concluded that low cytosol $[\text{Ca}^{2+}]$ was largely maintained by (a) limiting Ca^{2+} entry to the cell from the plasma milieu, (b) a Ca^{2+} -efflux mechanism which may or may not be energy-dependent, and (c) a Ca^{2+} -ATPase-associated uptake of Ca^{2+} by intracellular membranes (DTS).

In previous reports from our laboratory, we described a combined density-gradient/free-flow-electrophoresis procedure for the differential isolation of human platelet surface and intracellular membranes (Menashi *et al.*, 1981; Lagarde *et al.*, 1982; Hack *et al.*, 1984). These membrane subfractions were of high purity, with little cross-contamination, and in later studies we identified and characterized an ATP-dependent Ca^{2+} -uptake property of the intracellular membrane vesicles in which the classical endoplasmic-reticulum marker enzyme, NADH:cytochrome *c* reductase, was exclusively localized (Menashi *et al.*, 1982, 1984). More recently we have demonstrated that the intracellular membrane vesicles, preloaded in the presence of ATP to steady-state concentrations with $^{45}\text{CaCl}_2$, release approx. 50% of the sequestered cation in 15–30 s in response to $\text{Ins}(1,4,5)\text{P}_3$ (Authi & Crawford, 1985). After this $\text{Ins}(1,4,5)\text{P}_3$ -induced release, a re-uptake occurs rapidly until the former steady-state values are almost fully restored. This release/re-uptake sequence, we believe, is due to firstly the binding of the $\text{Ins}(1,4,5)\text{P}_3$ to a specific receptor in the intracellular membrane, triggering Ca^{2+} release, followed by the breakdown of the $\text{Ins}(1,4,5)\text{P}_3$ *in situ* by a membrane phosphomonoesterase (K_m for $[\text{Ins}(1,4,5)\text{P}_3]$ approx. $10\text{ }\mu\text{M}$; N. Hack & N. Crawford, unpublished work}. This allows a Ca^{2+} -ATPase-associated re-accumulation of Ca^{2+} to take place.

In the present paper we provide further information on the Ca^{2+} -ATPase and basal Mg^{2+} -ATPase activities of these electrophoretically prepared human platelet membrane subfractions. Kinetic parameters are presented for the ATPases, and additionally we have identified in the intracellular membranes two major phosphopolypeptides formed during the Ca^{2+} sequestration, which are in some respects analogous to those believed to be associated with the Ca^{2+} -pump complex of muscle sarcoplasmic reticulum.

MATERIALS AND METHODS

Chemicals

ATP (dipotassium salt), hydroxylamine and the proteinase inhibitors aprotinin, phenylmethanesulphonyl fluoride and pepstatin A were all obtained from Sigma (Poole, Dorset, U.K.). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 5000 Ci/mmol) and $^{45}\text{CaCl}_2$ (sp. radioactivity

70 mCi/mmol) was purchased from Amersham International (Amersham, Bucks., U.K.) or NEN (Dreieich, West Germany). Polyacrylamide-gel-electrophoresis reagents were purchased from BDH (Poole, Dorset, U.K.), as also was the Analar water ($[\text{Ca}^{2+}] \sim 1\text{ }\mu\text{M}$) used throughout these studies. Protein M_r standards for polyacrylamide-gel calibrations (94–14 kDa) and β -galactosidase (116 kDa) were obtained from Pharmacia.

All other reagents were of Analar grade (BDH) wherever possible.

Isolation of platelets

Human platelets were isolated either from whole-blood packs or buffy-coat-residue packs provided by the Blood Transfusion Service Laboratories, Tooting, London S.W.17, U.K. Processing in the laboratory was carried out with plastic vessels throughout, usually within 2–3 h of donation. The method for the preparation of platelet-rich plasma, its adjustment to pH 6.4 (procedure of Lagarde *et al.*, 1980) and the subsequent isolation and washing of the platelets have been described in detail by Menashi *et al.* (1981). The resuspension buffer for the final washed platelet pellet contained Hepes (10 mM), NaCl (150 mM), EDTA (3 mM) and KCl (4 mM), and was adjusted to pH 7.2.

Preparation of homogenate, mixed membrane fractions and the isolation of surface and intracellular membrane subfractions

The procedures for preparing the mixed membrane fraction and the free-flow electrophoresis were carried out essentially by the experimental protocols reported by Menashi *et al.* (1981), as also was the prior treatment of the whole cells with neuraminidase to decrease the electronegativity of the surface-membrane-derived vesicles and improve the electrophoretic resolution of the subfractions. After short neuraminidase treatment (Sigma Type X; 0.05 unit/ml; 30 min at 37 °C) the platelets were washed twice in the resuspension buffer and suspended (1 g of cells/4 ml) in a sonication buffer containing 0.3 M-sorbitol and 10 mM-Hepes, pH 7.2. A proteinase-inhibitor cocktail containing 0.1 unit of aprotinin/ml, 10 μg of pepstatin A/ml and 400 μM -phenylmethanesulphonyl fluoride was then added to the cell suspension. The platelet suspension was sonicated (Dawes Sonifier, position 6, max. setting) for 10 s, and a portion of the homogenate layered on to a 1.0–3.5 M-sorbitol linear density gradient and centrifuged at 42000 *g* for 90 min. The mixed membrane fraction, containing both surface and intracellular membrane vesicles, located in the low-density region of the gradient, was removed with a plastic Pasteur pipette and centrifuged at 90000 *g* for 60 min on to a cushion of 3.5 M-sorbitol. The upper mixed-membrane band was removed and used directly for some of the studies or diluted with an equal volume of electrophoresis chamber buffer, containing triethanolamine (10 mM) and sorbitol (0.3M), adjusted to pH 6.8 with acetic acid and then to pH 7.2 with NaOH. This latter procedure ensures sufficient ions for the electrophoretic separation. This diluted vesicle suspension was applied to the chamber of a free-flow-electrophoresis unit (VAP5; Bender and Hobein, Munich, Germany), and the electrophoretic separation of the surface and intracellular membranes carried out at 140 mA, 100 V/cm, with buffer flow conditions as reported by Menashi *et al.* (1981).

Measurements of ^{45}Ca uptake into membrane vesicles

For the measurements of Ca^{2+} uptake by the membrane vesicles, an incubation mixture containing the following was routinely used: 120 mM-KCl, 5 mM- MgCl_2 , 2 mM-ATP in 20 mM-Tris/HCl buffer adjusted to pH 7.2. The free Ca^{2+} concentrations in this solution were controlled in the range 1.0 mM to 0.01 μM by the use of Ca^{2+} -EGTA buffers (Portzehl *et al.*, 1964). Appropriate proportions of $^{45}\text{CaCl}_2$ were included in the unlabelled CaCl_2 solutions when added to the suspending medium. Samples of the membrane vesicle suspension (50–100 μg of membrane protein) were always added last to start the reaction, and at the times indicated in the experimental protocols 0.9 ml of the suspension was removed and transferred to a Millipore membrane (type CS, pore size 0.45 μm). After three washings of the filtered vesicles each with 10 vol. of ice-cold buffer containing 120 mM-KCl, 5 mM- MgCl_2 , 50 μM unlabelled CaCl_2 and 20 mM-Tris/HCl, pH 7.2, the cellulose filter membranes were carefully removed with forceps, well dried and counted for radioactivity in a liquid-scintillation counter.

ATPase measurements

When these measurements were made under the conditions of the Ca^{2+} -uptake experiments, [γ - ^{32}P]ATP was substituted for the unlabelled ATP in the vesicle-suspending medium. The incubations were allowed to proceed for the required time and the reaction was terminated by adding 100 μl of 6.0% (v/v) SDS to a suspension volume of 500 μl (Seals *et al.*, 1978) or by the addition of 2.5 ml of Norit-A (25 mg/ml in 0.1 M-phosphoric acid) to a 500 μl portion of suspension (Chamberlain *et al.*, 1984). With the former procedure the liberated P_i forms a phosphomolybdate complex, which is extracted with xylene/2-methylpropan-1-ol (13:7, v/v), and a sample of the solvent phase is taken for ^{32}P counting. In the Norit A procedure the activated-charcoal-vesicle suspension was centrifuged at 2000 g for 10 min at 4 °C, and 1.0 ml of the upper aqueous phase was removed for radioactivity counting. For the determinations of K_m and V_{max} for Ca^{2+} and ATP, the vesicle suspensions were adjusted to different substrate and cation concentrations and incubations allowed to proceed for 10 min before the reaction was stopped by one of the above methods. Both phosphate-assay procedures gave values in close agreement. For time-course measurements of the basal Mg^{2+} -ATPase, the vesicle suspension was added to a mixture consisting of 120 mM-KCl, 5 mM- MgCl_2 , 2 mM-ATP and 5 mM-EGTA with 20 mM-Tris/HCl buffer, pH 7.2. Control values for assays measured in the absence of MgCl_2 but in the presence of EGTA were subtracted throughout the time course (see Table and Figure legends for full details).

Protein phosphorylation

In order to provide sufficient protein-bound ^{32}P radioactivity for autoradiography, the suspension medium for the Ca^{2+} -uptake studies was adjusted to 3.0 μM external Ca^{2+} and 250 μM -ATP containing [^{32}P]ATP, giving a total radioactivity of 200 d.p.m./pmol. This latter substrate concentration was well above the apparent K_m values for ATP for both the ATPase activity and Ca^{2+} sequestration. At steady-state concentrations of sequestered Ca^{2+} (approx. 15 min incubation), the vesicle proteins were precipitated from a 500 μl sample of

suspension by the addition of 50 μl of a mixture containing 50% (w/v) trichloroacetic acid, 1.0 mM-ATP and 100 mM- K_2HPO_4 . The precipitated proteins were pelleted by centrifugation (10000 g for 10 min) and the pellet was washed three times with 5% trichloroacetic acid containing 0.1 mM-ATP and 1 mM- K_2HPO_4 . The trichloroacetic acid precipitate was dissolved in a buffered SDS solution, containing 62.5 mM-Tris, 10% (w/v) glycerol, 5.0% (v/v) β -mercaptoethanol, 2.3% (w/v) SDS and 0.001% Bromophenol Blue, by heating for 30 min at 100 °C. Samples were neutralized with 0.1 M-NaOH and subjected to gel electrophoresis (Hack & Crawford, 1984), followed by staining in 0.1% Coomassie Brilliant Blue R250 in 40% (v/v) methanol/10% (v/v) acetic acid. The gel was destained in 50% methanol/10% acetic acid, dried and autoradiographed by using a Kodak X-Omat AR X-ray film in a Kodak X-ray cassette with regular intensifying screens for 14 days at -70 °C.

Since it has been demonstrated with the Ca^{2+} + Mg^{2+} -ATPase of muscle sarcoplasmic reticulum that protein phosphorylation involves covalent attachment to the β -carboxyl group of an aspartate residue and that this phosphorylatable amino acid is found in a tripeptide sequence common to both Ca^{2+} - and Na^{+} + K^{+} -activated ATPases, the nature of the platelet-membrane phosphoprotein was investigated by hydroxylamine hydrolysis.

Samples of intracellular-membrane suspension (~0.1 mg of protein) were phosphorylated as described above. The vesicles were pelleted by centrifugation and suspended in 0.1 M-sodium acetate buffer, pH 5.2, containing 1.0 M-hydroxylamine. They were incubated for 1 h at room temperature. Control membrane samples were incubated in buffer without hydroxylamine. The incubation was terminated by addition of trichloroacetic acid (final concn. 5.0%) and the precipitated protein separated by centrifugation at 10000 g for 10 min. The supernatant was removed and three 20 μl portions were counted for ^{32}P radioactivity. The precipitated protein was washed three times with 5% trichloroacetic acid and then dissolved in 0.2 ml of 63 mM-Tris/HCl buffer, pH 6.8, containing 2.0% SDS and 5.0% β -mercaptoethanol. Portions (5 μl) of this solubilized protein solution were taken for radioactivity counting. Samples were also taken of the original membrane suspension and the solubilized membrane protein for protein assay by the Bradford (1976) procedure.

RESULTS

Fig. 1(a) shows the results from six different platelet preparations of the Ca^{2+} -sequestering property of the mixed membrane fractions and the electrophoretically purified surface and intracellular membrane subfractions all measured in the presence and absence of ATP. For these experiments an external $[\text{Ca}^{2+}]$ of 3 μM was used, and the uptake was allowed to proceed for 15 min at 30 °C. It is considered that the small non-ATP-dependent increases in vesicle Ca^{2+} probably represent binding, and these control values have been subtracted from all measurements made in the presence of ATP. In fact, our earlier studies (Menashi *et al.*, 1984) with the ionophore A23187 introduced into the vesicle suspension preloaded to steady-state concentrations with ^{45}Ca revealed that only 90–95% of the sequestered Ca^{2+} could be released by the ionophore. Clearly the surface-membrane vesicles do not sequester Ca^{2+} by an energy-dependent process,

Fig. 1. Uptake of Ca^{2+} by mixed membrane (MM), intracellular membranes (IM) and surface membranes (SM) and the lability of this property and the Ca^{2+} -ATPase activity with time

(a) The uptake values are means $\pm$ S.D. of duplicates from six different membrane preparations. These uptakes were measured on the same preparation from which the ATPase values (Table 1) were derived. Both the Ca^{2+} -uptake and the Ca^{2+} -ATPase activities were determined at $3.0 \mu\text{M}$ external Ca^{2+} . (b) The results are expressed as percentages of the mean of duplicate values of the Ca^{2+} uptake (□) and Ca^{2+} -ATPase activity (●) measured immediately after free-flow electrophoresis. Values for Ca^{2+} uptake and Ca^{2+} -ATPase were determined at $3.0 \mu\text{M}$ external Ca^{2+} .

Table 1. Specific activity of $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase and basal Mg^{2+} -ATPase of mixed membranes and surface and intracellular membrane subfractions

Data are means $\pm$ S.D. of duplicate assays from six different membrane preparations.

Membrane fraction	ATPase activities (nmol/min per mg of protein)		
	Mg^{2+}	$\text{Ca}^{2+} + \text{Mg}^{2+}$	Ca^{2+}
Mixed membranes	31.8 ± 10.1	59.1 ± 14.1	27.3 ± 10.2
Intracellular membranes	38.6 ± 13.2	94.7 ± 16.5	56.1 ± 12.7
Surface membranes	27.5 ± 10.8	26.2 ± 10.2	Nil

the steady-state values reached after 15 min incubation being around 10-fold higher in the intracellular membrane. The determinations of this property and the Ca^{2+} -ATPase activity on the mixed membranes were performed immediately after removal from the gradient, and there was at least a 4–6 h delay between the removal of the mixed-membrane fraction from the sorbitol density gradient and the isolation of the two subfractions from it by the free-flow-electrophoresis procedure. In fact, generally both the ATPase-activity and Ca^{2+} -uptake determinations on the membrane subfractions were carried out on the next day (some 15–20 h later). Membranes could not be stored at temperatures lower than 4°C if the vesicles were to remain sealed.

Fig. 1(b) shows the decrease of both properties in the intracellular membranes with time of storage at 4°C .

With respect to activities measured immediately after harvesting the fractions from the free-flow-electrophoresis unit, some 5–10% of the Ca^{2+} uptake was lost in 24 h, and this continued to decline steadily. The Ca^{2+} -ATPase activity, however, decreased by about 40% in the first 24 h, and around 60% loss occurred after 60 h or storage at 4°C . Accordingly, for these logistic and lability reasons, enrichment and depletion values for enzyme activities and Ca^{2+} sequestration have little meaning. Table 1 shows the bivalent-cation-activated-ATPase activities of the mixed-membrane fractions taken directly from the sorbitol density gradient in six preparations, and for the corresponding surface and intracellular membrane subfractions produced from these mixed membranes by free-flow electrophoresis. The basal Mg^{2+} -ATPase activities were measured in the presence of 5 mM-EGTA. Table 1 shows that the basal Mg^{2+} -ATPase activities of all three membrane fractions were not significantly different. However, although the Ca^{2+} -ATPase activities of the intracellular membrane were always 2–3-fold higher than the basal Mg^{2+} -ATPase activities in the same platelet preparation, these two ATPase activities were essentially the same in the surface membrane fraction, implying the absence of any significant Ca^{2+} -enhanced component from this fraction. In order to exclude the possibility that the neuraminidase treatment of the whole cells before fractionation destroyed the Ca^{2+} -enhanced component of the ATPase selectively, mixed membrane fractions from neuraminidase treated and untreated platelets were compared. In the presence of both Ca^{2+} and Mg^{2+} there was no difference in activity between the two mixed membrane fractions (results not shown). Additionally, a mixed membrane fraction from untreated platelets was divided into two portions, one of which was exposed to neuraminidase (0.05 unit/ml for 30 min at 37°C) and the other acting as control. Again there was no difference in Ca^{2+} -ATPase activity between the two fractions (results not shown). Table 2 shows that the apparent K_m values derived from double-reciprocal plots for Ca^{2+} for the ATPase and the Ca^{2+} uptake were of the same order, $0.15 \mu\text{M}$ and $0.13 \mu\text{M}$ respectively. However, although the plots for ATP from the measurements of Ca^{2+} uptake were linear, those for ATP with the enzyme assay showed discontinuity. Measurements carried out over a range of 5–50 μM -ATP gave a K_m for (ATP) for 20 μM , whereas for measurements made in the range 0.05–10 mM-ATP these gave an apparent K_m for ATP of 145 μM .

Time-course curves for the measured ATPase activities are shown in Fig. 2, showing that linearity extends for at least 60 min. The reaction rates were also proportional to membrane protein concentration over the range 5–20 μg of membrane protein (results not shown).

The Ca^{2+} -dependence of the Ca^{2+} -ATPase activity is shown in Fig. 3(a), with the corresponding values for Ca^{2+} uptake determined under exactly the same experimental conditions also shown (Fig. 3b). Maximum cation activation of the intracellular-membrane enzyme occurs in the range of extracellular Ca^{2+} between 1 and 50 mM. Concentrations of Ca^{2+} of 100 μM and greater significantly inhibited the ATP hydrolysis. Similarly (Fig. 3b) the stimulation of Ca^{2+} sequestration within the intracellular membrane vesicles was maximum at extravesicle $[\text{Ca}^{2+}]$ of 1–50 μM and almost totally inhibited at $[\text{Ca}^{2+}] \sim 100 \mu\text{M}$.

Under the conditions used for the Ca^{2+} -uptake

Table 2. Apparent K_m and V_{max} values for the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase and Ca^{2+} uptake of human platelet intracellular membranes

All data are averages of duplicates from two separate membrane preparations. For both the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase and Ca^{2+} -uptake studies, $[\text{Ca}^{2+}]$ was set at $3 \mu\text{M}$ for the measurement of the ATP parameters and $[\text{ATP}]$ was fixed at 2 mM for the measurement of the Ca^{2+} values. Two different ranges of ATP were used for the ATPase measurements for derivation of K_m and V_{max} for ATP (shown in parentheses); with both ranges $[\text{Ca}^{2+}]$ was $3 \mu\text{M}$.

	$\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase			Ca^{2+} uptake	
	Ca^{2+}	ATP ($5\text{--}50 \mu\text{M}$)	ATP ($0.05\text{--}10 \text{ mM}$)	Ca^{2+}	ATP
Apparent K_m (μM)	0.15	20	145	0.13	1.1
V_{max} (nmol/min per mg)	59	27	49	2.7	2.7

Fig. 2. Time course of basal Mg^{2+} -ATPase and $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activities of human platelet intracellular membranes

Results are typical of two different membrane preparations.

○, $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase at $3.0 \mu\text{M}$ free external Ca^{2+} ; ●, basal Mg^{2+} -ATPase.

experiments, but with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ replacing the unlabelled substrate, the rate and extent of protein phosphorylation was studied by polyacrylamide-gel electrophoresis/autoradiography. Fig. 4 shows an autoradiography of a typical gel separation of the solubilized intracellular-membrane vesicles prepared after steady-state $[\text{Ca}^{2+}]$ was reached in a suspending medium containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $3 \mu\text{M}$ extravesicular $[\text{Ca}^{2+}]$. Only two major polypeptides are phosphorylated (90 kDa and 95 kDa) during the Ca^{2+} uptake. The densely labelled phosphorylated components at the leading fronts of the gels have been identified principally as phospholipids of the phosphatidylserine and phosphatidylinositol classes. When the experiments were performed in the presence of $500 \mu\text{M}$ external Ca^{2+} , a concentration close to that required for maximum inhibition of both ATPase activity and Ca^{2+} uptake (see Fig. 3a), the total ^{32}P associated with the 95 kDa and 90 kDa polypeptides was substantially decreased. When mixed membrane vesicles were used in the uptake experiments in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, several additional bands could be identified in the autoradiographs, the most prominent being those of 50, 40, 20 and 18 kDa. Summing the ^{32}P radioactivity in the two major intracellular-membrane polypeptides allowed a determination of the nmol of P_i incorporated into these proteins over a time course of Ca^{2+} uptake between 1 and 40 min. The data from these experiments are shown in Fig. 5, indicating that, at the time when the vesicles have reached steady-state concentrations of intravesicle Ca^{2+}

Fig. 3. Relationship between vesicle stored calcium (a) and Ca^{2+} -activated-ATPase activities (b) measured at different external free calcium concentrations

The sequestered Ca^{2+} was measured after the vesicles had reached steady-state concentrations ($\sim 15 \text{ min}$). The Ca^{2+} -ATPase activities are initial-rate measurements.

(approx. 10 min), only about two-thirds of the potentially phosphorylatable sites in the membrane proteins are occupied by ^{32}P .

Acyl phosphates (phosphoaspartate and phosphoglutamate), unlike other phosphorylated amino acids, are hydrolysed by hydroxylamine, and such acyl phosphate bonds are a known feature of several ATPases (Martonosi, 1969; Inesi *et al.*, 1970; Whittam & Chipperfield, 1975; Pang & Briggs, 1977). The effect of hydroxylamine on the major phosphoproteins of the intracellular membrane was examined after incubation of the membranes for 15 min with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and in the presence of $3 \mu\text{M}$ - Ca^{2+} . During this time 2.5 nmol of P_i had

Fig. 4. Phosphoproteins formed during Ca^{2+} sequestration

Phosphorylation was allowed to proceed as described in the Materials and methods section. The Figure is of an autoradiogram (typical of five such experiments) prepared from a Coomassie-Blue-stained gel. Lane (a) represents mixed membranes at $3.0 \mu\text{M}$ external free Ca^{2+} , lane (b) the intracellular membranes also at $3.0 \mu\text{M}$ external free Ca^{2+} and lane (c) the intracellular membranes at $500 \mu\text{M}$ external free Ca^{2+} . Acrylamide concns. (% w/v) in the gel are shown to the left.

been incorporated per mg of total membrane protein. After 1 h incubation with hydroxylamine at room temperature (see the Materials and methods section), the membrane proteins were precipitated with trichloroacetic acid, well washed and separated by centrifugation. The radioactivity was counted in both the supernatant and the washed precipitate. Of the 2.5 nmol of P_i incorporated per mg of membrane, over 60% was liberated by the hydroxylamine treatment, calculated after subtraction of control values for the amount of P_i liberated by incubation in the presence of only the acetate buffer.

DISCUSSION

The pattern of intracellular responses that are initiated by an agonist/receptor interaction can vary with the stimulating agent, and distinguishing between major processes initiated by the signal and those which may only operate as 'fine tuning' can be a formidable problem. The range of different functional activities in mammalian cells which are known to be regulated in some way by Ca^{2+} is now most extensive, and one feature that has become reasonably well established during the last few years is

Fig. 5. Time course of intracellular-membrane protein phosphorylation during Ca^{2+} uptake

Results are typical of three different membrane preparations. The suspension medium contained $3.0 \mu\text{M}$ - Ca^{2+} and $250 \mu\text{M}$ - $[\text{P}^{32}]\text{ATP}$ (200 d.p.m./pmol). The uptake was terminated at steady-state concentrations of sequestered Ca^{2+} , and the membrane proteins were precipitated with trichloroacetic acid for gel electrophoresis (see the Materials and methods section).

that most cells that are responsive to a surface stimulus maintain in their quiescent (inactivated) state a low (nanomolar) concentration of Ca^{2+} in their cytosolic compartment. This low value is believed to be regulated by efflux of Ca^{2+} to the plasmatic environment or by the uptake of Ca^{2+} into some internal membrane complex (or perhaps by the combined action of both these processes). With respect to the blood platelet, which can be activated by a number of naturally occurring excitatory agonists, investigations using fluorescent Ca^{2+} -binding compounds such as quin-2, chlortetracycline and aequorin have indicated that a many fold increase in cytosol Ca^{2+} concentration (to almost micromolar) can occur in response to the agonist (Le Breton *et al.*, 1976; Cutler *et al.*, 1978; Rink *et al.*, 1983; Johnson *et al.*, 1985). In addition to Ca^{2+} , other second-messenger candidates (e.g. diacylglycerol and cyclic AMP) may also be involved in the full expression of the metabolic and morphological changes initiated by the stimulus, but at present a fully integrated picture of their actions has not yet emerged.

Unlike the red cell, where a surface-membrane extrusion pump for Ca^{2+} has been well characterized, no similar energy-dependent Ca^{2+} -efflux mechanism has been identified in the blood platelet surface membranes. Many workers have demonstrated Ca^{2+} sequestration and the presence of a Ca^{2+} -ATPase in platelet membranes (Robblee *et al.*, 1973; Käser-Glanzmann *et al.*, 1977; Le Peuch *et al.*, 1983; De Metz *et al.*, 1984), but most studies have been made with crude mixed membrane fractions containing vesicles of both surface and intracellular membrane origin. We reported that an electrophoretically prepared highly purified human platelet intracellular-membrane fraction (in which the classical endoplasmic-reticulum marker enzyme NADH:cytochrome *c* reductase was exclusively localized) actively sequestered Ca^{2+} by an ATP-dependent, non-oxalate-requiring, mechanism (Menashi *et al.*, 1984). The uptake attained steady-state concentrations in 10–12 min and was saturable at external $[\text{Ca}^{2+}]$ of $\sim 10 \mu\text{M}$. Binding of the cation to the membranes was minimal, since over 95% of the Ca^{2+} could be released from the preloaded vesicles in

1–2 min by the ionophore A23187. More recently we have been able to show that the putative second-messenger molecule inositol trisphosphate, derived *in vivo* by the action of phospholipase C on phosphatidylinositol biphosphate, also releases 40–50% of the sequestered Ca^{2+} in 15–30 s. After this induced release, the vesicles can then restore their Ca^{2+} content to the former steady-state values in 30–60 s by an ATP-dependent re-uptake (Authi & Crawford, 1985).

The present investigations were designed to explore further the bivalent-cation-activated ATPase activities in the platelet surface and intracellular membrane sub-fractions harvested in our free-flow-electrophoresis procedure, and particularly to concentrate on the Ca^{2+} -ATPase activity of the intracellular membranes, which appears to be associated with the previously demonstrated Ca^{2+} -sequestering property (Menashi *et al.*, 1984). One aim also was to seek analogies and differences between the platelet's intracellular Ca^{2+} -mobilizing processes and the sarcoplasmic-reticulum membranes of muscle tissues.

The first, and somewhat unexpected, finding was that, whereas a basal Mg^{2+} -ATPase activity was present in all three membrane fractions examined (i.e. the mixed membrane and the subfractions), with little difference in specific activity between them, only the mixed membranes and the intracellular membranes showed any significant enhancement of activity when Ca^{2+} was included in the assay medium. No such enhancement was observed with surface membrane fractions isolated from six different platelet preparations. Our earlier failure to demonstrate any significant ATP-dependent uptake of Ca^{2+} by these surface membrane vesicles (Menashi *et al.*, 1984) to some extent supports the present findings, but does also raise the question of whether the pretreatment at the whole-cell level with neuraminidase could in some way compromise or inhibit a surface-membrane Ca^{2+} -pump complex. Our view that such is not the case is based on comparative studies with neuraminidase-treated and untreated platelets and with mixed membrane fractions prepared from cells not exposed to neuraminidase, which were then divided into two vesicle fractions, one of which was treated with neuraminidase (same conditions as with whole platelets) and the other left untreated as a control. There was no difference in the Ca^{2+} -ATPase activities measured in the control and neuraminidase-treated mixed membrane fractions, whether they were derived from control platelets and those exposed to neuraminidase at the whole-cell level or whether the membranes had been treated with neuraminidase after the density-gradient isolation. We are therefore left with the conclusion that an ATP-associated Ca^{2+} -extrusion pump of the type which has been characterized in red-cell membranes and certain other cells is not a feature of the surface membranes of blood platelets.

With respect to the intracellular membrane fractions, the ATPase activities measured in the presence of both Ca^{2+} and Mg^{2+} were 2–3-fold higher than the basal Mg^{2+} -ATPase of this fraction. Figs. 2(a) and 2(b) show the close parallel between the Ca^{2+} -ATPase activity and the uptake of Ca^{2+} when both properties are measured over an external $[\text{Ca}^{2+}]$ range between 1 nM and 1 mM. High concentrations of Ca^{2+} ($\sim 50 \mu\text{M}$) inhibited both the ATPase and the Ca^{2+} uptake.

From many investigations of the ATPase activity and Ca^{2+} uptake of muscle sarcoplasmic reticulum, it has become widely accepted that a stoichiometry of close to

2:1 exists when the Ca^{2+} transported and ATP hydrolysis are expressed in molar terms. In the present studies, the ATP hydrolysis is substantially activated by Ca^{2+} and the stoichiometry does not approach such a ratio. This discrepancy has been observed, however, in other non-muscle cells (Pershadsingh *et al.*, 1980; Trumble *et al.*, 1981; Ghijsen *et al.*, 1982). In a paper concerned with the mechanisms of Ca^{2+} transport in pituitary-cell membrane vesicles (Barros & Kaczorowski, 1984), it was proposed that the presence of Ca^{2+} -activated phosphatase may result in abnormally high ATP hydrolysis. We have noted with our intracellular membrane preparations that during incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ certain phospholipids (notably phosphatidylinositol) are rapidly phosphorylated, with peak incorporation of ^{32}P occurring in the first 5–10 s. From this peak value, the amount of ^{32}P associated with the phospholipids rapidly decreases and is about 70% lower after 1–2 min incubation. These observations suggest the presence of an active lipid kinase and phosphatase system in the intracellular membranes, with the former enzyme competing with the ATPases for the same substrate.

The apparent K_m values for Ca^{2+} of 0.15 and 0.13 μM for the Ca^{2+} -ATPase and the Ca^{2+} uptake respectively are within the ranges quoted for these properties measured in skeletal-muscle sarcoplasmic reticulum (0.1–0.3 μM ; Yamamoto *et al.*, 1979), indicating high-affinity binding sites for Ca^{2+} on the transport-protein complex. The double-reciprocal plots for Ca^{2+} -ATPase activity measured at a fixed concentration of Ca^{2+} (3 μM) over the ATP range 5 μM –10 mM showed a discontinuity. Data for the ATP range 5–50 μM gave an apparent K_m value of 20 μM , whereas over the range of ATP concentration 50 μM –10 mM the K_m for (ATP) was much higher (145 μM). We do not know at this stage if this non-linear behaviour represents a second, low-affinity, site or if it may be due to negative co-operativity of a multimeric ATPase molecule, as suggested by Dean & Sullivan (1982) from their studies of the purified enzyme. At extra-vesicle Ca^{2+} concentrations higher than 50–100 μM , both the ATP hydrolysis and Ca^{2+} sequestration are totally inhibited. The reasons for this phenomenon, which is not observed if oxalate or phosphate is introduced as a Ca^{2+} sink, is not clear, but Ikemoto (1982) noted a similar inhibition with sarcoplasmic-reticulum membranes and suggested that it was due to the binding of Ca^{2+} to low-affinity sites on the internal face of the membrane vesicles and inhibiting the Ca^{2+} -transport complex.

As with sodium-pump-linked $\text{Na}^+ + \text{K}^+$ -ATPases, the energy-dependent transport of Ca^{2+} across the platelet membranes also proceeds through one or more phosphorylated intermediates. One concept is that this phosphorylation of a series of enzyme intermediates is associated with a transmembrane decrease in affinity for Ca^{2+} on the transport-protein complex such that the free ion can eventually be liberated into the intravesicle space (Ikemoto, 1982). During Ca^{2+} uptake with the platelet intracellular membranes, two major polypeptides became progressively phosphorylated. Their mobility characteristics in calibrated polyacrylamide-gel electrophoresis gave values of 90 kDa and 95 kDa. The quoted molecular size in the literature for the analogous phosphopolypeptides of muscle sarcoplasmic reticulum is 100–115 kDa, and, although some workers have observed two closely adjacent components, the possibility, as in our studies, that one is a proteolytic product of the other cannot yet

be determined. The time course of the phosphorylation occurring during Ca^{2+} sequestration in the platelet intracellular membranes is substantially slower than the aforementioned lipid phosphorylation and continues to increase after steady-state concentrations of accumulated Ca^{2+} have been reached. Under steady-state conditions for Ca^{2+} uptake (i.e. measured at 10–12 min), only about 50% of the potentially phosphorylatable sites in the 90 kDa and 95 kDa polypeptides are occupied with ^{32}P . We have no satisfactory explanation for this discrepancy, but, since phosphorylation is also substantially inhibited at high $[\text{Ca}^{2+}]$, as also are the Ca^{2+} -uptake and ATPase properties, further studies of the kinetics of the protein kinase and phosphatase present in the intracellular membranes may shed some light on the problem.

A substantial proportion of the phosphoprotein bonds formed in the platelet membranes are readily cleaved by hydroxylamine. This is a feature of the phosphorylation occurring in muscle sarcoplasmic reticulum, where hydroxylamine lability is due to their acyl phosphate characteristics (aspartate or glutamate phosphorylation).

In conclusion, the highly purified human platelet intracellular-membrane vesicles sequester Ca^{2+} by an ATP-dependent mechanism which, as with muscle sarcoplasmic reticulum, seems to be kinetically linked with a Ca^{2+} -ATPase. This Ca^{2+} -transport complex has a high affinity for Ca^{2+} and, in a way not yet elucidated, appears mechanistically to involve the formation of phosphoprotein intermediates. These phosphoprotein intermediates may be concerned with a transmembrane decrease in the affinity of the transport complex for Ca^{2+} allowing the free ion to be released into the vesicle space. Binding of the cation to the membrane vesicles is minimal, since the Ca^{2+} is rapidly liberated by the ionophore A23187 (Menashi *et al.*, 1984) and also by the action of $\text{Ins}(1,4,5)\text{P}_3$ (Authi & Crawford, 1985). The various mechanistic events involved in translocation of the cation across the vesicle membrane appear to be operationally optimum at $[\text{Ca}^{2+}]$ known to prevail in platelet cytosol and are collectively and substantially inhibited by extra-vesicle $[\text{Ca}^{2+}]$ around $50\ \mu\text{M}$ and greater. The Ca^{2+} -mobilizing properties of platelet intracellular membranes show both analogies and differences when compared with the sarcoplasmic-reticulum membranes of muscle tissues, but they clearly have a role in both maintaining Ca^{2+} homeostasis in the resting cell and initiating some of the Ca^{2+} -regulated metabolic and mechanochemical processes which are a feature of the platelet's behavioural changes during haemostatic functions. A greater understanding of this Ca^{2+} -sequestering system at the molecular level may lead to the identification of intracellular targets for anti-platelet or anti-thrombotic drugs and other agents.

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