A monoclonal antibody (PL/IM 430) to human platelet intracellular membranes which inhibits the uptake of Ca^{2+} without affecting the $Ca^{2+}+Mg^{2+}$-ATPase

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To probe the structure–function relationships of proteins present in the endoplasmic reticulum-like intracellular membranes of human blood platelets a panel of monoclonal antibodies have been raised, using as immunogen highly purified platelet intracellular membrane vesicles isolated by continuous flow electrophoresis [Menashi, Weintroub & Crawford (1981) J. Biol. Chem. **256**, 4095–4101]. Four of these antibodies recognize a single 100 kDa polypeptide in the platelet membrane by immunoblotting. One antibody PL/IM 430 (of IgG_1 subclass) inhibited ($\sim 70\%$) the energy-dependent uptake of Ca^{2+} into the vesicles without affecting the $Ca^{2+}+Mg^{2+}$-ATPase activity or the protein phosphorylation previously shown to proceed concomitantly with Ca^{2+} sequestration [Hack, Croset & Crawford (1986) Biochem. J. **233**, 661–668]. The inhibition is independent of ATP concentration over a range 0–2 mM-ATP but shows dose-dependency for external $[Ca^{2+}]$ with maximum inhibition of Ca^{2+} translocation at concentrations of $Ca^{2+} > 500$ nM. This capacity of the antibody PL/IM 430 functionally to dislocate components of the intracellular membrane Ca^{2+} pump complex may have value in structural studies.

INTRODUCTION

Resting blood platelets maintain their cytosolic $[Ca^{2+}]$ within a narrow range (50–100 nM; Rink *et al.*, 1983) despite a 10000:1 gradient of this cation across the platelet plasma membrane. The precise mechanisms whereby Ca^{2+} homoeostasis is achieved are by no means well characterized, although it is generally agreed that the platelet's endoplasmic reticulum-like intracellular membrane structures contribute substantially to the sequestration and mobilization of cytosolic Ca^{2+}. The first indirect evidence for this role came from the studies of Statland *et al.* (1969) which led them to draw an analogy between the platelet intracellular membranes, referred to by electron microscopists as the dense tubular system (DTS), and muscle sarcoplasmic reticulum. Some years later the uptake of Ca^{2+} by isolated platelet membrane fractions was investigated by Robblee *et al.* (1973), Käser-Glanzmann *et al.* (1977, 1978) and Enouf *et al.* (1984). Although most of these studies were performed with platelet mixed membrane, which contained vesicle elements of both plasma membrane and intracellular membrane origin, all these reports provided evidence for platelet membrane translocation of Ca^{2+}. In no case, however, was it possible to identify with certainty the subcellular localization of this ATP-dependent Ca^{2+} uptake. More recent studies by Brass (1984), using ^{45}Ca to identify, in whole platelets, the intracellular exchangeable and non-exchangeable Ca^{2+} pools, led to the concept that whilst Ca^{2+} influx across the plasma membranes can occur in platelets, and particularly in response to certain platelet activators, the increase in cytosolic free $[Ca^{2+}]$ which follows agonist-induced activation is, at least in part, due to the release

of the cation from a non-mitochondrial intracellular store. During the last few years, through the introduction in our laboratory of high-voltage continuous-flow electrophoresis procedures for membrane subfractionation and purification, the differential separation of human platelet surface and intracellular membranes with little cross-contamination has become possible (Menashi *et al.*, 1981; Lagarde *et al.*, 1982). Using these highly purified membrane fractions we have identified and partially characterized an ATP-dependent Ca^{2+} accumulating property in platelet intracellular membrane vesicles and this is linked kinetically to a high-affinity $Ca^{2+}+Mg^{2+}$ATPase activity (Hack *et al.*, 1986). In this study we also observed, during the Ca^{2+}-uptake process, phosphorylation of two membrane polypeptides of size similar to those phosphorylated in muscle sacroplasmic reticulum during Ca^{2+} translocation (90 and 95 kDa). These intracellular membranes have been confirmed to be endoplasmic reticulum-like by the absence of plasma membrane markers at the whole cell level before fractionation, and by the exclusive localization of an antimycin-insensitive NADH:cytochrome *c* reductase activity (Menashi *et al.*, 1981). Later the presence of phospholipase A_2 and the complete enzyme sequence for converting arachidonic acid to thromboxanes in these intracellular membranes was established (Authi *et al.*, 1985; Carey *et al.*, 1982). Surprisingly, a highly purified platelet surface membrane vesicle population, also isolated by the continuous-flow electrophoresis technique, showed no Ca^{2+} uptake and no $Ca^{2+}+Mg^{2+}$-ATPase activity (Hack *et al.*, 1986), unlike the plasma membrane of erythrocytes and other blood cells. This finding has been substantiated by others using different procedures for fractionating surface and intracellular membranes

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Abbreviations used: mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; e.l.i.s.a., enzyme-linked immunosorbent assay; PAGE, polyacrylamide-gel electrophoresis.

[e.g. Steiner & Lüscher (1985), who used lectin chromatography, and Fauvel et al. (1986), who used Percoll density gradients]. The contribution of the platelet plasma membrane to the maintenance of Ca^{2+} homoeostasis remains to be clarified, although there is some evidence for a Na^+/Ca^{2+} counter-ion-exchange process which could become operationally significant in the stimulated cell (Brass, 1984).

The platelet surface membrane has been the focus of a considerable amount of research, particularly with respect to integral glycoproteins, mobilizable phospholipids and receptor status for platelet activating ligands, but less is known about the polypeptide composition of the intracellular membranes. In two-dimensional isoelectric focusing–SDS/PAGE run under reducing conditions over 30 polypeptides can be visualized by Coomassie Blue staining and many more by high-sensitivity silver stains (Hack & Crawford, 1984). Few, however, have been characterized and little is known about the structure/function relationship of the proteins present in these intracellular membranes. As an initial approach to this problem we prepared a panel of monoclonal antibodies (mAbs) to a highly purified intracellular membrane fraction and of these mAbs four recognized a single 100 kDa polypeptide in SDS gels of the intracellular membrane by immunoblotting techniques. One of these antibodies, PL/IM 430, was found to inhibit strongly the capacity of intracellular membrane vesicles to sequester Ca^{2+} (around 70–80 % inhibition) but without any measurable effects upon the $Ca^{2+} + Mg^{2+}$-ATPase activity. We report here the preliminary characterization of this PL/IM 430 antibody.

MATERIALS AND METHODS

Materials

ATP (dipotassium salt) and the proteinase inhibitors aprotinin, phenylmethanesulphonyl fluoride and pepstatin A were all obtained from Sigma. [γ-^{32}P]ATP (sp. radioactivity 5000 Ci/mmol) and $^{45}CaCl_2$ (sp. radioactivity 70 mCi/mmol) were purchased from Amersham International or NEN. Polyacrylamide-gel-electrophoresis reagents were purchased from BDH, as also was the AnalaR water ([Ca^{2+}] $\sim$ 2 μM) used throughout these studies. Protein 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), except FITC (Sigma).

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 membrane fractions

The procedure used to isolate membrane fractions, mixed membrane, intracellular membrane and surface membrane was essentially as reported by Menashi et al. (1981), the only difference being the use of a VAP 22 free flow machine (Bender Hobein, Munich, Germany) to separate the intracellular membrane from the surface membrane.

Measurements of $^{45}Ca^{2+}$ 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$_2$, 1 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–0.01 μM by the use of Ca^{2+}–EGTA buffers (Portzehl et al., 1964). Appropriate proportions of $^{45}CaCl_2$ were included in the unlabelled $CaCl_2$ solutions when added to the suspension 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.

Measurements of $Ca^{2+} + Mg^{2+}$-ATPase activities

The procedure was essentially that of Chamberlain et al. (1984) using [γ-^{32}P]ATP in the reaction mixture at a concentration of 2 mM unless otherwise stated. Routinely, the membrane vesicles were incubated at 37 °C in the buffer used for the Ca^{2+} uptake experiments for the required time and the reaction was terminated by adding 2.5 ml of Norit-A (25 μg/ml in 0.1 M-phosphoric acid) to a 500 μl aliquot of the incubation mixture. This 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 used for scintillation counting. The basal Mg^{2+}-ATPase activity determined in the presence of 5 mM-EGTA was subtracted to give the Ca^{2+}-stimulated activity.

Measurement of membrane phosphorylation

This procedure was carried out essentially as described earlier (Hack et al., 1986). The membranes were incubated in the presence of [γ-^{32}P]ATP (250 μM) and 3.0 μM external Ca^{2+} for 5 min. The reaction was stopped by the addition of 25 % (w/v) trichloroacetic acid containing 4.0 % sodium pyrophosphate and 100 mg of BSA/ml. The mixture was filtered through Whatman GF/F microfibre filters and the filters were washed three times with 10 ml of 5 % (w/v) trichloroacetic acid/2.0 % (w/v) sodium pyrophosphate and counted for radioactivity in a liquid-scintillation cocktail (Beckman).

Experiments with FITC

Reactions with FITC were performed by preincubating aliquots of intracellular membranes (30 μg) with varying amounts of FITC for 2 h at 24 °C in a medium containing 120 mM-KCl, 50 μM-CaCl$_2$, 5 mM-MgCl$_2$ and 20 mM-

Tris/HCl, pH 7.0. At the end of the incubation period [γ-³²P]ATP was added to a concentration of 1.0 mM and after 10 min incubation the Ca²⁺ + Mg²⁺-ATPase activity was determined or Ca²⁺ uptake assayed as described above.

Production of monoclonal antibodies

Female Balb/c mice (~ 10 weeks old) were immunized by footpad injection with 100 μg of the purified intracellular membrane emulsified in complete Freund's adjuvant. Subsequent injections were given intraperitoneally in Freund's complete adjuvant on day 10, intraperitoneally in PBS on day 17 and intraperitoneally and tail vein on day 28. On day 22 samples of blood were removed for screening and on day 31 the mouse was killed and spleen cells fused with SP 2/0 mouse myeloma cells using polyethylene glycol 1500 as described by Galfre *et al.* (1977). Hybrid cells were plated into five 96-well culture plates and after culturing, hybridoma colonies were screened for antibody production against intracellular membranes using an e.l.i.s.a. procedure (see below for details). Hybridoma cells from colonies generating positive supernatants in the e.l.i.s.a assay ($A_{495} > 1.0$) were screened against BSA, and if negative the cells grown up in 24-well plates. The media from these colonies were then screened by immunoblotting against SDS/PAGE separations of platelet intracellular membranes after transfer to nitrocellulose membranes. Hybridoma cells giving positive immunoblotting in the latter procedure were cloned by limiting dilution and injected into female Balb/c mice primed with 2,6,10,14-tetramethylpentadecane (Sigma) for production of ascites.

E.l.i.s.a.

Nunc Immunoplates were prepared by overnight incubation at 4 °C with a solution of 1 μg/well of platelet intracellular membranes dissolved in 0.05 M-sodium carbonate, pH 9.6. They were then washed with PBS containing 0.5% Tween 20 (PBS/Tween). Antibody binding was detected using a horseradish peroxidase-conjugated goat anti-(mouse Ig) antibody (BioRad)

followed by a substrate solution containing 0.4 mg of o-phenylenediamine/ml. The absorbance of each well at 495 nm was measured with a Minireader II (Dynatech).

SDS/PAGE

SDS/PAGE was run in 0.75 mm thick slabs using the method of Laemmli (1970) with a BioRad mini-slab system. All gels were stained with Coomassie Blue.

Electrophoretic transfer and immunostain

Proteins from gels stained with Coomassie Blue were transferred to nitrocellulose membranes by the method of Jackson & Thompson (1984). Immunostaining was carried out either with undiluted culture supernatant, ascites fluid or 10 × culture supernatant diluted in PBS/Tween 20. Specifically bound antibody was detected using horseradish-peroxidase conjugated goat anti-(mouse IgG) (BioRad) diluted in PBS/Tween. Immunostain was developed in a substrate solution containing diaminobenzidine. The immunoblots were photographed directly to show Coomassie Blue stain and also through a Wratten no. 47B filter to show only the immunostain.

Isolation of monoclonal antibodies

Monoclonal antibodies were isolated from ascites fluid or 10 × culture supernatants by the procedure described by Ey *et al.* (1978). Mouse IgG was isolated on a protein A–Sepharose column. Briefly, 1.0 ml of 0.1 M-sodium phosphate buffer, pH 8.0, was added to 1.0 ml of ascites or concentrated culture fluid. The immunoglobulin mixture was then applied to a protein A–Sepharose column (0.6 cm × 6 cm) pre-equilibrated with 0.1 M-sodium phosphate buffer, pH 8.0. The column was then washed with 0.1 M-sodium phosphate buffer at a flow rate of 5 ml/h until tests for protein in the eluate were negative. The immunoglobulin was then eluted with 0.1 M-glycine/HCl, pH 3.2. The protein concentration of the eluate was determined ($A_{280}^{1\%} = 14$) and aliquots frozen at −80 °C until required. The activity is unaffected by cycles of freezing and thawing.
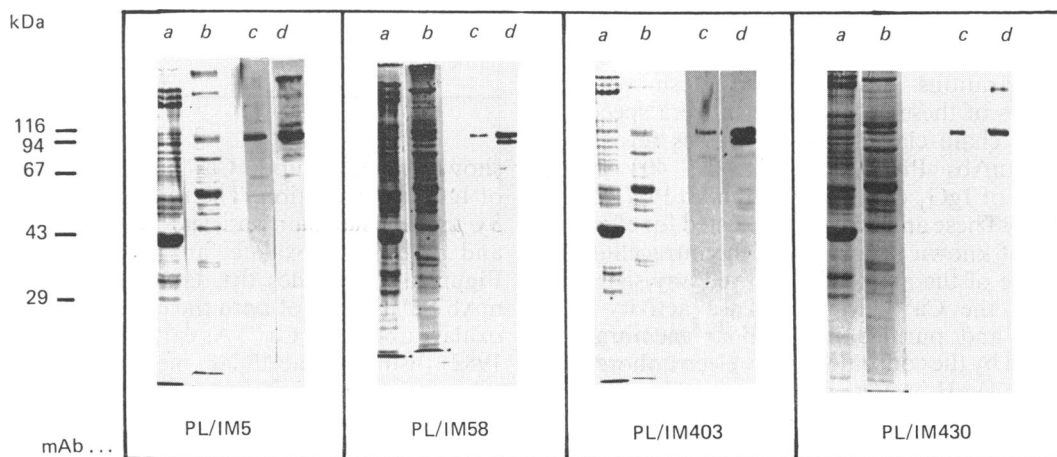

Fig. 1. SDS/PAGE (10%) of a 1% SDS extract of whole platelets (lanes *a* and *c*) and platelet intracellular membranes (lanes *b* and *d*)

The protein bands were stained with Coomassie Blue (lanes *a* and *b*) or transferred to nitrocellulose and immunostained with the respective antibody (lanes *c* and *d*).

Table 1. Effect of monoclonal antibodies towards the 100 kDa polypeptide of human platelet intracellular membranes on the membrane $Ca^{2+}+Mg^{2+}$-ATPase activity and Ca^{2+} uptake

$n = 5$ for all experiments with each antibody (20 μg/ml of membrane suspension). Ca^{2+} uptake was measured as the amount of $^{45}Ca^{2+}$ sequestered at steady-state levels ($\sim$ 12 min incubation). The control IgG, raised against rabbit lymphoid cells (L11/135), showed no cross-reaction with human platelets. Significance (Student's t test): $*P < 0.001$.

Monoclonal antibody (purified IgG)	$Ca^{2+}+Mg^{2+}$-ATPase (nmol/min per mg of protein)	Ca^{2+} uptake (nmol/mg of protein)
Control monoclonal IgG_1	101.8 ± 12.3	$14.1 \pm 0.7*$
PL/IM 5 (IgG_{2a})	98.1 ± 10.9	12.2 ± 2.2
PL/IM 58 (IgG_1)	112.7 ± 6.4	12.7 ± 1.3
PL/IM 403 (IgG_1)	116.9 ± 6.2	13.5 ± 0.6
PL/IM 430 (IgG_1)	100.4 ± 6.4	$5.4 \pm 0.3*$

Immunodiffusion for antibody isotype

Double-diffusion (Ouchterlony) precipitation was performed in 1.0% Agar and aliquots of the 10 × culture supernatant were tested against rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA kindly provided by Dr. David Catty, Department of Immunology, University of Birmingham, U.K.

RESULTS

Monoclonal antibody screening

Supernatants from the hybridoma cultures were assayed for antibodies directed towards human platelet intracellular membrane antigens by using the e.l.i.s.a. technique as described by Bird *et al.* (1985). Of 500 wells examined, approximately 80 were positive ($A_{495} > 1.0$). After transfer and further culture, the supernatants were tested by immunoblotting on gel separations of solubilized intracellular membranes as outlined in the Materials and methods section.

Four mAbs, PL/IM 5, PL/IM 58, PL/IM 403 and PL/IM 430, strongly and consistently blotted a single 100 kDa polypeptide in whole platelets (Fig. 1, lanes c). In the purified membrane preparations (as prepared by continuous-flow electrophoresis) a number of smaller polypeptides blotted with the antibodies (Fig. 1, lanes d). These are believed to be proteolytic products produced during the membrane isolation and were not present in preparations of solubilized fresh whole platelets. Analyses of these mAbs using antisera specific for mouse heavy chain classes and subclasses revealed that the three mAbs PL/IM 58, PL/IM 403 and PL/IM 430 were of IgG_1 class, whereas mAb PL/IM 5 was of IgG_{2a} class. These antibodies were tested for effects upon a number of known properties of the intracellular membranes. None of the antibodies showed any significant effect upon the $Ca^{2+}+Mg^{2+}$-ATPase activity of freshly prepared and purified intracellular membrane fractions prepared by the continuous-flow electrophoresis technique (Table 1). However, when screened against the Ca^{2+} uptake property of the intracellular membrane vesicles, one mAb, PL/IM 430, gave very significant blocking ($\sim 62\%$) of the translocation of Ca^{2+} into the intracellular membrane vesicles (Table 1). Fig. 2 shows that maximum inhibition ($\sim 82\%$) occurs at antibody concentrations of about 20–30 μg of IgG/ml. A control mAb (IgG_1 class, raised against rabbit lymphoid cells)
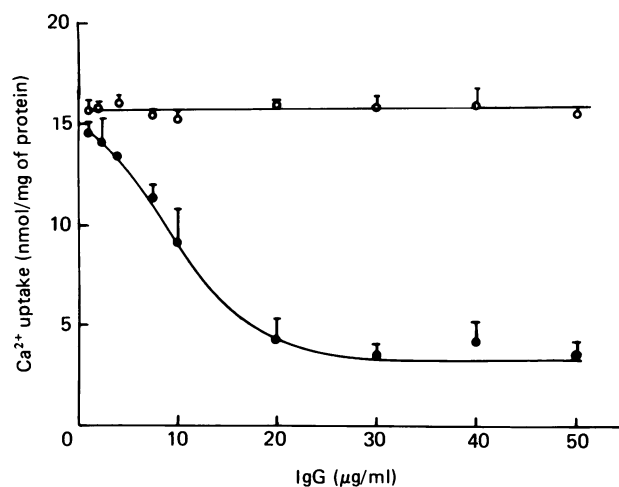

Fig. 2. Effect of mAb PL/IM 430 on Ca^{2+} uptake of human platelet intracellular membrane vesicles at 3 μM external [Ca^{2+}] and 1 mM-ATP

Aliquots (20 μg of membrane) were incubated with various concentrations of mAb PL/IM 430 (●) or with non-immune IgG (○) in buffer containing 120 mM-KCl, 1.0 mM-ATP and 4 mM-$MgCl_2$ in 20 mM-Tris/HCl, pH 7.2. Sequestered $^{45}Ca^{2+}$ was measured after reaching steady-state levels (12–15 min). Data are mean values $\pm$ s.D. for five experiments with different membrane suspensions. For other experimental details see the Materials and methods section.

showed no effect upon Ca^{2+} uptake over a similar range of IgG concentration. The time courses for Ca^{2+} uptake by the intracellular vesicles, measured in the presence and absence of oxalate, are shown in Fig. 3, and the Figure also includes the data for the inhibition by the mAb PL/IM 430 of both the oxalate-enhanced and non-oxalate uptake of Ca^{2+}. As earlier shown (Menashi *et al.*, 1982) using intracellular membrane vesicles, oxalate substantially enhances the Ca^{2+} sequestration. In the present studies both the oxalate-stimulated and non-oxalate uptake were suppressed to approximately the same levels throughout the 30 min time course for measured uptake. Inhibition of ATP-dependent Ca^{2+} uptake was investigated in membrane vesicle suspensions in which the ATP content was varied in the range 0.1–2.0 mM. Control experiments were performed as
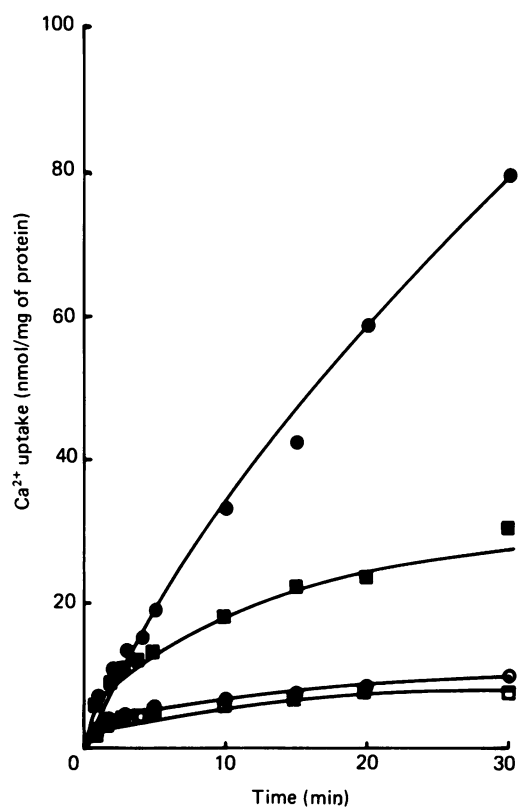
Fig. 3. Time course of the uptake of $^{45}Ca^{2+}$ by human platelet intracellular membrane vesicles at 3 μM external $[Ca^{2+}]$ and 1 mM-ATP in the presence of mAb PL/IM 430 (open symbols) and non-immune IgG (closed symbols) measured in the presence ($\bigcirc$, $\bullet$) and absence ($\square$, $\blacksquare$) of 2.5 mM-potassium oxalate

Both non-oxalate and oxalate-enhanced uptake are inhibited to approximately the same levels in the presence of PL/IM 430. For other experimental details see the Materials and methods section.
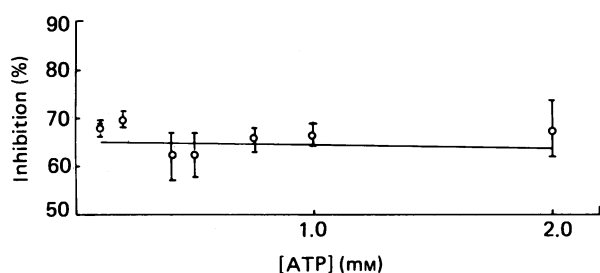

Fig. 4. Effect of varying the ATP concentration (0.1–2.0 mM) on the inhibitory action of mAb PL/IM 430 towards intracellular membrane Ca^{2+} uptake

Membranes (20 μg) were incubated with 10 μg of mAb PL/IM 430 with various concentrations of ATP in the presence of 3.0 μM external $^{45}Ca^{2+}$. Uptake was allowed to proceed for 15 min. Data shown are means $\pm$ S.D. for five preparations.

described above. Fig. 4 shows that under all conditions of ATP concentration the inhibition by the antibody PL/IM 430 lay in the range 60–70 %. In a similar series of experiments the Ca^{2+} uptake was measured with and without the addition of the mAb PL/IM 430 and in
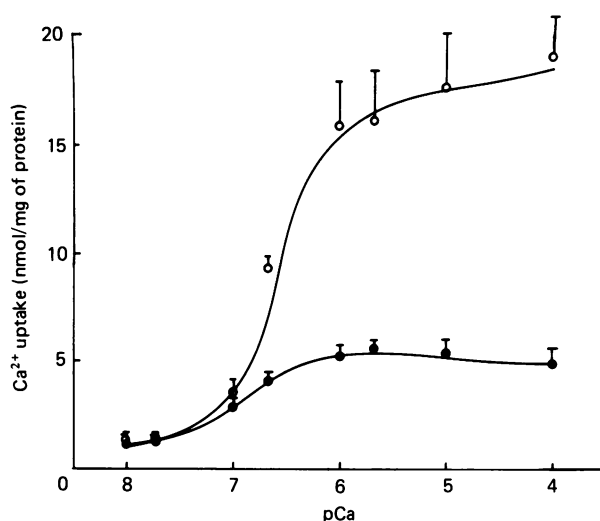

Fig. 5. Ca^{2+} uptake by platelet intracellular membrane vesicles measured in the presence of various concentrations of external Ca^{2+} in the range 10^{-8}–10^{-4} M

The Figure shows uptake measured after 15 min (steady-state levels) in the presence of mAb PL/IM 430 ($\bullet$) and non-immune IgG_1 ($\bigcirc$). Values are means $\pm$ S.D. for five vesicle preparations.

Table 2. Effects of monoclonal antibodies recognizing the 100 kDa polypeptide on intracellular membrane protein phosphorylation

Membranes were incubated for 5 min in the presence of mAb (purified IgG, 20 μg/ml), 50 μM external Ca^{2+} and 250 μM-[γ-^{32}P]ATP and the extent of protein phosphorylation was measured as described in the Materials and methods section.

Antibody (purified IgG)	Phosphorylation (nmol of P_i incorporated/mg of membrane protein)
None	3.8
Non-immune IgG_1	3.0
mAb PL/IM 58	3.2
mAb PL/IM 430	3.3

the presence of various concentrations of external Ca^{2+} (10^{-8}–10^{-4}). The uptake was measured in all cases after reaching steady-state intravesicle $[Ca^{2+}]$. Fig. 5 shows that the inhibitory effect of the antibody PL/IM 430, like the uptake, is Ca^{2+}-sensitive with little or no inhibition of Ca^{2+} uptake below 100 nM external $[Ca^{2+}]$ and that at $[Ca^{2+}]$ > 500 nM maximal inhibition occurred.

Another possible site of action of mAB PL/IM 430 was the phosphorylation domain of the 100 kDa polypeptide. However, experiments to examine the effect of PL/IM 430 on protein phosphorylation showed that the antibody had no effect (Table 2).

In attempts to explore similarities and differences between properties of the platelet intracellular membrane Ca^{2+} pump enzyme and those reported for muscle sarcoplasmic reticulum, some preliminary experiments
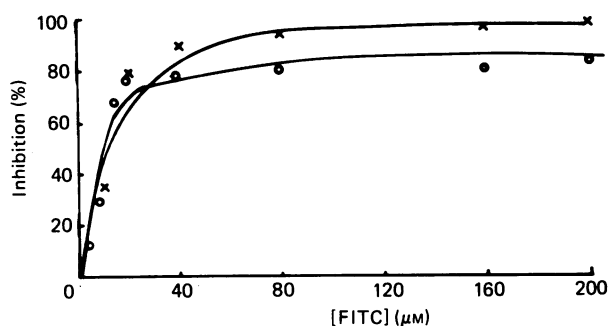
Fig. 6. Inhibition of purified platelet intracellular membrane $Ca^{2+} + Mg^{2+}$-ATPase (X) and Ca^{2+} uptake (O) by FITC

Intracellular membranes (30 μg of protein) in 20 mM-Tris/HCl, pH 7.0, containing 120 mM-KCl, 4 mM-$MgCl_2$ and 50 μM external free calcium was preincubated with FITC for 2 h at 24 °C. The membranes were then assayed for $Ca^{2+} + Mg^{2+}$-ATPase enzyme activity and Ca^{2+} uptake as described in the Materials and methods section. Results are expressed as a percentage of a control which was also preincubated for 2 h but without FITC.

were made with FITC, which has been reported (Pick, 1981a,b; Mitchinson et al., 1982) to inhibit the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum without affecting the Ca^{2+}-independent enzyme activity. This inhibition is believed to be due to its reaction with a single lysine residue in the nucleotide binding fold of the ATPase (Mitchinson et al., 1982). Fig. 6 shows the inhibitory action of increasing amounts (1–100 nmol of FITC/30 μg of membrane protein) of FITC preincubated for 2 h with the vesicle suspension before the Ca^{2+} uptake and $Ca^{2+} + Mg^{2+}$-ATPase measurements. The $Ca^{2+} + Mg^{2+}$-ATPase was inhibited with maximal effects (> 90 %) at concentrations of FITC greater than 40 μM. Little further inhibition occurred at higher FITC concentrations. The relationship between the amount of FITC in the preincubation medium and the extent of inhibition of the Ca^{2+}-ATPase was linear in the range 0–20 μM. The Ca^{2+} uptake process was similarly inhibited.

DISCUSSION

There are numerous well-characterized mAbs now available directed towards human platelet surface membrane antigens and particularly to a number of externally oriented surface membrane glycoproteins believed to have important receptor functions in the platelet's haemostatic behaviour. To our knowledge, however, few mAbs have been reported that show specificity towards proteins of the human platelet intracellular membranes, even though a number of important enzymes and membrane transport functions have been identified within these membranes.

Using highly purified human platelet intracellular membranes isolated by continuous-flow electrophoresis (Menashi et al., 1981) as antigens we have prepared a panel of mAbs to a number of intracellular membrane proteins and these are undergoing detailed molecular and functional characterization. Within the panel of mAbs, four have been identified with strong reactivity towards a single 100 kDa polypeptide present in platelet

intracellular membrane preparations. All these mAbs give strong immunoblotting reactions with this 100 kDa component. In investigating the effects of these four mAbs on human platelet intracellular membrane functions, one mAb, designated PL/IM 430, gave significant and consistent inhibition of the uptake of Ca^{2+} into freshly prepared platelet intracellular membrane vesicles. This inhibition was Ca^{2+}-dependent with maximal effects at > 10^{-6} M external [Ca^{2+}] and the extent of inhibition at optimal [Ca^{2+}] varied little in the presence of a range of ATP concentrations from 100 μM to 2.0 mM. In fact, with all platelet intracellular membrane preparations studied, we have been unable to demonstrate any effect upon the $Ca^{2+} + Mg^{2+}$-ATPase activity as measured by the release of P_i. PL/IM 430 inhibited the Ca^{2+} uptake by the membrane vesicles to the same basal level even if a Ca^{2+} sink such as oxalate was included in the external medium. Additionally, the mAb showed no effect upon the protein phosphorylation process which has previously been shown to proceed concomitantly with Ca^{2+} translocation (Hack et al., 1986).

In our earlier studies of the hydroxylamine-labile phosphopolypeptide of the platelet intracellular membrane we identified two labelled SDS/PAGE components of ~ 90 kDa and ~ 95 kDa (Hack et al., 1986). We now know that the larger phosphopolypeptide is identical with that revealed by immunoblotting procedures using the mAb PL/IM 430 (~ 100 kDa). Using the PL/IM 430 antibody on an affinity column we have purified to reasonable homogeneity a 100 kDa polypeptide from solubilized platelet intracellular membranes. Under the conditions for optimal ATPase activity and Ca^{2+} transport the 100 kDa polypeptide is phosphorylated (N. Hack & N. Crawford, unpublished work). Since all Ca^{2+}-translocating enzymes of the P-class, of which those of sarcoplasmic and endoplasmic reticular origin are examples (Pedersen & Carafoli, 1987), consist of a single polypeptide chain we tentatively conclude that our earlier reported 90 kDa phosphorylated component was a proteolytic product of the ATPase polypeptide. These P-class pump ATPases are also believed to have a single nucleotide binding site on each polypeptide chain, the terminal phosphate of the ATP being transferred to an aspartate residue on the same monomeric unit. However, to date none of these Ca^{2+}-pump enzymes has been fully characterized by crystallography, but the topographical relationship of the nucleotide binding site and the phosphorylatable amino acid residue has been presented conceptually for the sarcoplasmic reticulum from sequence studies and tryptic cleavage data (Green et al., 1986). One concept is that the binding of Ca^{2+} to the enzyme promotes spatial changes such that the bound ATP and the phosphorylation domain approach a reactive configuration. Phosphorylation of the aspartate residue then initiates the translocation of Ca^{2+} from higher to lower affinity sites finally culminating in its liberation in the lumen of the vesicles. The apparent discriminatory property of the mAb PL/IM 430 in inhibiting the intravesicle sequestration of the cation without affecting the $Ca^{2+} + Mg^{2+}$-ATPase activity and the phosphorylation suggests that its site of action may lie either at an extramembranous Ca^{2+}-translocating site or at what has been termed for sarcoplasmic reticulum the transduction domain in close proximity to it. An alternative explanation is the possible action of the antibody on some closely associated site involved in a

charge-compensating counter-ion transport (K^+ being a likely candidate). Studies with reconstituted sarcoplasmic reticulum vesicles have suggested that the pump is electrogenic (Tanford, 1984). It may be of significance, with respect to the action of our antibody, that in reconstitution studies with a Ca^{2+}-ATPase incorporated into a non-fluid phospholipid, Ca^{2+} translocation is blocked through constraints on channel movement, whereas the Ca^{2+}-dependent phosphorylation of the enzyme is not affected. Caution should be exercised in considering analogies between the sarcoplasmic reticulum Ca^{2+}-pumping enzyme complex and the Ca^{2+} + Mg^{2+}-ATPase of a cellular endoplasmic reticulum complex such as the platelet since, for example, the former is considered to be substrate- and product-regulated, whereas the platelet enzyme has been reported to show cyclic AMP-dependent protein kinase modulation (Käser-Glanzmann et al., 1978). This latter property, however, was examined only with relatively crude membrane preparations and cytosol containing the kinase activity. In a study of the Ca^{2+}-ATPase from human platelets, purified by Sepharose 4B and hydroxyapatite chromatography, Dean (1984) compared its kinetic properties with the Ca^{2+}-ATPase of sarcoplasmic reticulum and of skeletal muscle and emphasized their many similarities. More recently, Fischer et al. (1985) have produced evidence that the platelet and sarcoplasmic reticulum Ca^{2+}-pumping enzymes are structurally distinct based upon differences in the cleavage products after tryptic digestion and time course anomalies in the correlation of loss of functions during proteolysis. In the context of FITC inhibition, the platelet enzyme shows similarities with the sarcoplasmic reticulum Ca^{2+} pump (Mitchinson et al., 1982). At optimal concentrations of FITC the Ca^{2+} + Mg^{2+}-ATPase was inhibited by over 90 % and the Ca^{2+} uptake by over 80 %. However, in the commonly accepted reaction sequence for sarcoplasmic reticulum Ca^{2+} translocation, release of phosphate is believed to occur concomitantly with the liberation of Ca^{2+} intralumenally. The action of our antibody might suggest that the reaction cycle differs in the platelet enzyme complex.

In conclusion the mAb antibody PL/IM 430 is to our knowledge the first to be described with a reaction towards an intracellular membrane function. It significantly inhibits both the oxalate-dependent and -independent sequestration of Ca^{2+} into membrane vesicles without any apparent effect upon either the Ca^{2+} + Mg^{2+}-ATPase activity or the associated phosphorylation of a 100 kDa polypeptide.

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