Characterization of the calcium-sequestering process associated with human platelet intracellular membranes isolated by free-flow electrophoresis

Suzanne MENASHI,*† Kalwant S. AUTHI,* Frank CAREY† and Neville CRAWFORD*§

*Department of Biochemistry, Royal College of Surgeons of England, 35/43 Lincoln's Inn Fields, London WC2A 3PN, U.K., and †I.C.I., Pharmaceuticals Division, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.

(Received 6 March 1984/Accepted 3 May 1984)

By using density-gradient fractionation and high-voltage free-flow electrophoresis, human platelet membranes were separated into highly purified subfractions of surface (SM) and intracellular (IM) origin. Associated exclusively with the IM fraction is an ATP-dependent Ca^{2+} uptake that, in the absence of oxalate, reaches steady-state levels in 5–10 min. When Ca^{2+} –EGTA buffers were used to control the external Ca^{2+} concentrations (range 0.1–50 μM) there was an increase in the intravesicle steady-state level of Ca^{2+} up to 10 μM external Ca^{2+} concentration. Above this level the intravesicle space becomes saturated at a concentration between 10 and 20 nmol of Ca^{2+} ·(mg of protein) $^{-1}$. The ionophore A23187 promotes a rapid and almost total release of the sequestered Ca^{2+} (>90%, t_1 1–2 min). The presence of oxalate in the external medium greatly enhances the Ca^{2+} accumulation to levels as high as 200 nmol·(mg of protein) $^{-1}$, but the uptake process is more variable and rarely reaches steady-state level even after 2 h incubation. Moreover, accumulation in the presence of oxalate effects ionophore release with less than 80% depletion in 45–60 min. These findings, taken together with the known presence in the platelet of a wide variety of functional and metabolic processes triggered by this cation, suggest that the platelet IM has a key role in controlling cytosolic Ca^{2+} concentrations.

In a previous report (Menashi *et al.*, 1982) we presented some evidence for an ATP-dependent Ca^{2+} -sequestering process associated with human platelet intracellular membranes. The membranes had been isolated from freshly sonicated platelets and purified free from surface-membrane elements by high-voltage free-flow electrophoresis (Menashi *et al.*, 1981). In order to isolate well-sealed and functionally competent vesicles, capable of accumulating Ca^{2+} , fresh platelets were required, and the membrane vesicles also needed a short exposure to slightly hyperosmolar conditions during the isolation. In addition to ATP-dependence, the uptake of Ca^{2+} into these intracellular membrane

vesicles was substantially enhanced by including oxalate in the incubation mixtures.

In the present paper we have further characterized this Ca^{2+} -uptake property, particularly with respect to the rate of uptake and the steady-state levels of intravesicular Ca^{2+} reached in the presence and absence of oxalate. We have also investigated the effect on the uptake process of a range of different concentrations of Ca^{2+} outside the vesicles and also the action of the calcium ionophore A23187 on the sequestered Ca^{2+} at steady-state levels.

Other fairly recent papers from this group (Lagarde *et al.*, 1981, 1982; Carey *et al.*, 1982) reported studies that have established that platelet intracellular membranes are the predominant site for the phospholipid-modifying enzymes that liberate arachidonic acid and the synthetases for cyclic endoperoxide and thromboxane production. These enzymic data, together with the present report, support the concepts put forward by Gerrard and his colleagues (Gerrard *et al.*, 1982) that the internal membrane complexes involved in

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IM, intracellular membrane(s); SM, surface membrane(s).

† Present address: Bone and Joint Research Unit, London Hospital Medical College, Arthritis and Rheumatism Council Building, 25–29 Ashfield Street, London E1 2AD, U.K.

§ To whom requests for reprints should be addressed.

controlling platelet cytosol levels of Ca^{2+} and the sites for production of pro-aggregatory metabolites of arachidonic acid may be structurally associated.

Methods and materials

Platelets were isolated by a differential-centrifugation procedure from fresh buffy-coat pools supplied by the National Blood Transfusion Service Laboratories, Tooting, London S.W.17, U.K. (Menashi *et al.*, 1981). The final washed platelet suspension, which was free from red-cell contamination, was treated with neuraminidase (Sigma grade X; affinity-purified) at a concentration of $0.03 \text{ unit} \cdot \text{ml}^{-1}$ for 20 min at 37°C . This treatment decreased the surface-membrane electronegativity by removal of sialic acid moieties, and after sonication a mixed membrane fraction, well separated from, and uncontaminated by, granular elements, could be harvested by sorbitol-density-gradient centrifugation (linear gradient of 1.0–3.5M-sorbitol). The mixed membrane fraction, which was located in the low-density region of the gradient, contained both surface and intracellular membranes. The fraction was removed from the gradient and concentrated by sedimentation on to a cushion of 3.5M-sorbitol buffered with Hepes to pH 7.2. The membranes were removed from the cushion interface and diluted 1:1 (v/v) with electrophoresis-chamber buffer (10mM-triethanolamine/acetate, pH 7.2) and adjusted to 400 mosM by addition of sorbitol. This mixed membrane fraction was applied to the chamber of a Bender Hobein VAP5 electrophoresis unit operating at $110 \text{ V} \cdot \text{cm}^{-1}$ and 140 mA with an injection flow rate of 2 ml/h. Two major vesicle subfractions were resolved; the most electronegative peak, which had been unaffected by the neuraminidase treatment at the whole-cell level, represented intracellular membranes, and the least electronegative fraction represented plasma-membrane vesicles modified by the removal of sialic acid. Analytical enzymic data have allowed us to characterize definitively these two major membrane fractions and establish their site of origin in the whole cell (Menashi *et al.*, 1981; Lagarde *et al.*, 1982). Pools of the two major fractions, IM and SM, were concentrated by again centrifuging on to a sorbitol cushion, and the interface layer was removed and resuspended, as described above, to a concentration approximating $1 \text{ mg of protein} \cdot \text{ml}^{-1}$ for the Ca^{2+} -uptake experiments. The precise technical details of these isolation, purification and characterization procedures were described previously (Menashi *et al.*, 1981, 1982).

For the measurements of Ca^{2+} uptake, the incubation mixture contained 120mM-KCl/5mM- MgCl_2 /2mM-ATP in 20mM-Tris/HCl buffer,

pH 7.0. Ca^{2+} was added at a concentration of 50 μM - CaCl_2 (or as indicated) and containing $^{45}\text{Ca}^{2+}$ of specific radioactivity 70 mCi/mmol. External Ca^{2+} levels were controlled in the range 10 μM –10 nM by the use of Ca^{2+} -EGTA buffers made up as described by Portzehl *et al.* (1964). A portion of the membrane vesicle suspensions (50–100 μg of membrane protein) was added last to start the reaction and the mixtures incubated at 21 – 22°C . At the times indicated in the experimental protocols, 0.9 ml of the incubation mixture was removed and filtered rapidly through a Millipore membrane (type GS; pore size 0.45 μm), followed by three washings with 10 ml volumes of ice-cold buffer containing 120mM-KCl, 5mM- MgCl_2 , 50 μM unlabelled CaCl_2 and 20mM-Tris/HCl, pH 7.2. The filter membranes were carefully removed with forceps, well dried and counted for radioactivity in a liquid-scintillation counter.

Results and discussion

The combined procedures of density-gradient centrifugation and free-flow electrophoresis result in the separation of platelet membranes into two well resolved vesicle subfractions. These have been previously characterized analytically and enzymically and identified as of IM and SM origin. With respect to total platelet membrane protein, approximately two-thirds can be accounted for by the SM fraction and one-third by the IM fraction. Initially both fractions were investigated for their capacity to sequester Ca^{2+} , and Fig. 1 shows that this property is exclusively confined to IM and is clearly ATP-dependent. This specific localization, together with results of previous studies using monoclonal antibodies to surface-oriented antigens and lectin labelling before and after fractionation, supports our view that the surface-membrane vesicles are substantially outside-out or normally oriented. The platelet surface membrane is known to have a Ca^{2+} -extrusion pump which would simulate an uptake process if surface-membrane vesicles formed with inside-out orientation. Fig. 2 illustrates the time course of Ca^{2+} uptake and the plateau level at which intravesicle saturation is achieved (or almost achieved) when the experiments were carried out in the presence and absence of oxalate. Uptake of Ca^{2+} in the absence of oxalate is extremely rapid, a steady-state concentration being reached within the vesicles at 5–10 min. In contrast, when oxalate is present in the incubation mixture and diffuses into the vesicles, it acts as a Ca^{2+} sink, and the rate of calcium accumulation is much slower, but reasonably constant, over the first hour. However, it rarely reaches a true steady-state equilibrium even after 2 h incubation, at which time the concentra-

Fig. 1. Uptake of Ca^{2+} by purified surface and intracellular membrane vesicles in the presence or absence of ATP (2mM)

Vertical bars represent S.E.M. values for sequestered Ca^{2+} after 12min incubation. Five different platelet preparations were used.

tion within the vesicles is at least ten times higher than in vesicles exposed to Ca^{2+} in the absence of oxalate. Under these experimental conditions the values for the amount of sequestered Ca^{2+} without oxalate present ranged between 10 and 20 $\text{nmol}(\text{mg of vesicle protein})^{-1}$ for many different platelet membrane preparations. In the presence of oxalate, however, the internal Ca^{2+} concentrations were much higher and varied between 150 and 250 $\text{nmol} \cdot (\text{mg of protein})^{-1}$ after 120min incubation.

When the calcium ionophore A23187 (5.4 μM) was added to the incubation mixture shortly after a steady-state level had been reached, in the absence of oxalate (Fig. 3), the sequestered calcium was rapidly and almost totally released from the vesicles. Over 50% of the stored calcium was liberated in a few minutes and more than 90% within 20min (t_1 1–2min). This finding contrasts markedly with the effect of the same concentration of ionophore A23187 on vesicles pre-loaded with Ca^{2+} in the presence of oxalate (Menashi *et al.*, 1982). Here

Fig. 2. Time course of calcium accumulation by intracellular membrane vesicles in the presence of (○) and absence (●) of oxalate (2.5mM)

Fig. 3. Time course of release of Ca^{2+} from preloaded intracellular membrane vesicles by ionophore A23187 (5.4 μM)

The ionophore was added after steady-state concentration had been reached in the absence of oxalate.

the release is significantly slower, with less than 80% of the stored Ca^{2+} liberated after 45–60min (t_1 15min). It is clear that when an intravesicle Ca^{2+}

sink, such as oxalate anions, is present, although this greatly enhances the uptake, it also results in a less labile pool of Ca^{2+} in which the rate of dissociation of the intravesicle calcium oxalate complex greatly influences the levels of free Ca^{2+} susceptible to ionophore-induced liberation. A series of experiments was then carried out to explore the relationship between the levels of sequestered Ca^{2+} that can be achieved at steady state and the concentrations of free Ca^{2+} present in the incubation mixtures. Ca^{2+} -EGTA buffers were used to control external levels in the range $10\ \mu\text{M}$ – $10\ \text{nM}$. These results are presented in Figs. 4 and 5. It will be seen (Fig. 4) that the maximum uptake levels increased progressively with increasing concentrations of external Ca^{2+} . However, above $10\ \mu\text{M}$ external Ca^{2+} in the incubation mixture, there were no further increases in vesicle Ca^{2+} concentration reached at steady state. In Fig. 5 the values for sequestered Ca^{2+} reached after 12 min incubation have been plotted against the extravesicular Ca^{2+} concentrations in each experiment. It can be seen that the greatest incremental change in steady-state levels of sequestered Ca^{2+} occurs in the range 0.1 – $1\ \mu\text{M}$ external Ca^{2+} . At external concentrations above $10\ \mu\text{M}$ the vesicle space is saturated and no further increase occurs. In the experiments using the calcium ionophore A23187, the release from a non-oxalate system is rapid, and almost total, suggesting that binding of Ca^{2+} to vesicle membranes accounts for only a very small proportion of the stored Ca^{2+} .

It has been proposed by Gerrard *et al.* (1982) that the mobilization of free Ca^{2+} in the platelet cytosol by an energy-dependent membrane uptake process, such as demonstrated here, may be counter-balanced *in vivo* by the releasing action of naturally occurring ionophores. Thromboxane, certain endoperoxides and phosphatidic acid have all been suggested as likely candidates for this ionophoric role, but we have been unable to demonstrate such an ionophore action for any of these three physiologically occurring compounds (F. Carey, S. Menashi & N. Crawford, unpublished work). Other mechanisms of membrane Ca^{2+} -flux control may have to be explored before a clear understanding of the role of the platelet intracellular membranes in controlling cytosol levels of Ca^{2+} emerges.

In conclusion, we have presented evidence for a Ca^{2+} -sequestering mechanism associated with highly purified human platelet intracellular membranes and included some kinetic characterization. This uptake mechanism, which is in some ways analogous to the sarcoplasmic-reticulum Ca^{2+} pump of muscle tissues, is ATP-dependent, operates efficiently in the micromolar range of extravesicular Ca^{2+} and is saturable. The Ca^{2+}

Fig. 4. Ca^{2+} accumulation by intracellular membrane vesicles in the absence of oxalate and at different levels of external Ca^{2+} (range 0.1 – $50\ \mu\text{M}$)

Fig. 5. Relationship between vesicle stored Ca^{2+} levels reached after 12 min incubation with different external Ca^{2+} concentrations

Oxalate was absent from the incubation mixtures.

sequestered at steady state can be rapidly and almost totally released by the ionophore A23187, suggesting that binding of the cation to membrane proteins or phospholipids is negligible.

The presence of this Ca^{2+} pump in platelet intracellular membranes suggests that these membranes may be involved in the fine regulation of cytosolic Ca^{2+} concentrations in the submicromolar range. Fairly recent studies by Rink *et al.* (1983) with the fluorescent indicator quin 2 showed changes in platelet cytosolic Ca^{2+} concentrations in response to thrombin stimulation from less than $0.1\ \mu\text{M}$ to approx. $0.6\ \mu\text{M}$. These intracellular membrane complexes may therefore be of central and critical importance in the many different Ca^{2+} -sensitive processes which are triggered by haemostatic activators at the platelet surface.

We thank the British Heart Foundation for the provision of Fellowships (to S. M. and K. S. A.) and also I.C.I. (Pharmaceuticals Division), for their generous financial support of part of these studies.

References

- Carey, F., Menashi, S. & Crawford, N. (1982) *Biochem. J.* **204**, 847–851
- Gerrard, J. M., Petersen, D. A. & White, J. G. (1982) in *Platelets in Biology and Pathology*, vol. 2 (Gordon, J. L., ed.), pp. 407–436, North-Holland Biomedical Press, Amsterdam
- Lagarde, M., Menashi, S. & Crawford, N. (1981) *Colloq.-Inst. Natl. Sante Rech. Med.* **100**, 89–94
- Lagarde, M., Authi, K. S. & Crawford, N. (1982) *Biochem. Soc. Trans.* **10**, 241–243
- Menashi, S., Weintroub, H. & Crawford, N. (1981) *J. Biol. Chem.* **256**, 4095–4101
- Menashi, S., Davis, C. & Crawford, N. (1982) *FEBS Lett.* **140**, 298–302
- Portzehl, H., Caldwell, P. C. & Ruegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–589
- Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) *Nature (London)* **305**, 317–319