# BINDING OF $^{125}$I-CALMODULIN TO PLATELET α-GRANULES

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## 1. Introduction

There is convincing evidence indicating that several aspects of platelet activation, including the release reaction, are regulated by alterations in cytoplasmic [$Ca^{2+}$]. Release can be triggered by $Ca^{2+}$ ionophores [1] and inhibited by agents that interfere with $Ca^{2+}$ mobilization in the cell [2]. Moreover, increased transmembrane fluxes of $^{45}Ca^{2+}$ upon activation have been reported [3].

Many of the effects of $Ca^{2+}$ on enzymes and other proteins are mediated by calmodulin, a ubiquitous $Ca^{2+}$-binding regulator protein. Calmodulin has been detected in platelets of several species [4–6]. The protein was found mainly in the cytoplasm, but a significant amount (20%) cosedimented with the particulate fraction [5].

Based on the inhibitory effects of phenothiazines (a group of calmodulin-antagonists) it has been suggested that calmodulin is involved in the reactions leading to granule exocytosis in platelets [7,8]. The aim of this study was to determine whether platelet secretory granules possess calmodulin-binding sites. α-Granules were selected among the different types of platelet secretory organelles, since they are more abundant and comparatively easy to isolate.

## 2. Methods

α-Granules were isolated from 4 l fresh porcine blood following VanderMeulen, J. et al. (submitted). Briefly, the method involves platelet isolation, followed by ATP depletion with deoxyglucose and rotenone and a short incubation with chymotrypsin to prevent aggregation [9]. Lysis was performed by nitrogen cavitation [10] in 250 mM sucrose, 1 mM Tris–HCl (pH 7.4) (sucrose–Tris) containing 1 mM EGTA. Granule isolation was achieved by differential and sucrose-gradient (1.2–2.0 M) centrifugation. Purified α-granules were resuspended in sucrose–Tris at ~10 mg/ml.

Calmodulin (Calbiochem) was iodinated in 40 mM phosphate buffer (pH 8.0) as in [6,11]. For binding assays, α-granules (1–2 mg/ml) and $^{125}$I-calmodulin were incubated in a medium containing 8 mg bovine albumin/ml, 6 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 250 mM sucrose and 25 mM Hepes (pH 7.4) in the presence or absence of $CaCl_2$ (0.8 mM), EGTA (1 mM), phenothiazines or non-radioactive calmodulin (20 μg/ml). Incubation was at 22°C for 1 h followed by removal of unbound $^{125}$I-calmodulin by centrifugation.

Identification of calmodulin binding polypeptides was made by the overlay technique [12] as modified in [6]. α-Granules (200 μg/lane) were run in 7.5% polyacrylamide gels [13]. These were overlaid with $^{125}$I-calmodulin solutions containing either 1 mM $CaCl_2$, 1 mM EGTA or 1 mM $CaCl_2$ plus 0.2 mM chlorpromazine. For comparison equivalent lanes were stained with Coomassie blue.

Turbidity was measured at 540 nm in a Gilford spectrophotometer with plotter attachment. Protein was measured as in [14]. The phenothiazines were a gift from Smith, Kline and French and Hoffman-LaRoche laboratories.

## 3. Results and discussion

Fig.1 shows a typical preparation of porcine α-granules. A small number of dense (bull's eye) granules are the only detectable contaminant, in agreement with enzymatic marker studies which show an enrichment in the fribrinogen content of this fraction, and reduced activities of plasma membrane, mitochon-

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drial, lysosomal and endoplasmic reticulum markers (W. F., submitted). The majority of the α-granules retain their electron-dense protein-rich core, suggesting that they are sealed to macromolecules.

Polyacrylamide gel electrophoresis of the granules in the presence and absence of $Ca^{2+}$ [6] failed to show significant amounts of intrinsic calmodulin. This finding is not unexpected, since the granules are exposed to EDTA-containing media repeatedly during their isolation. When exposed to radiolabeled calmodulin, however, substantial amounts of the modulator protein were bound by the granules (fig.2). At least

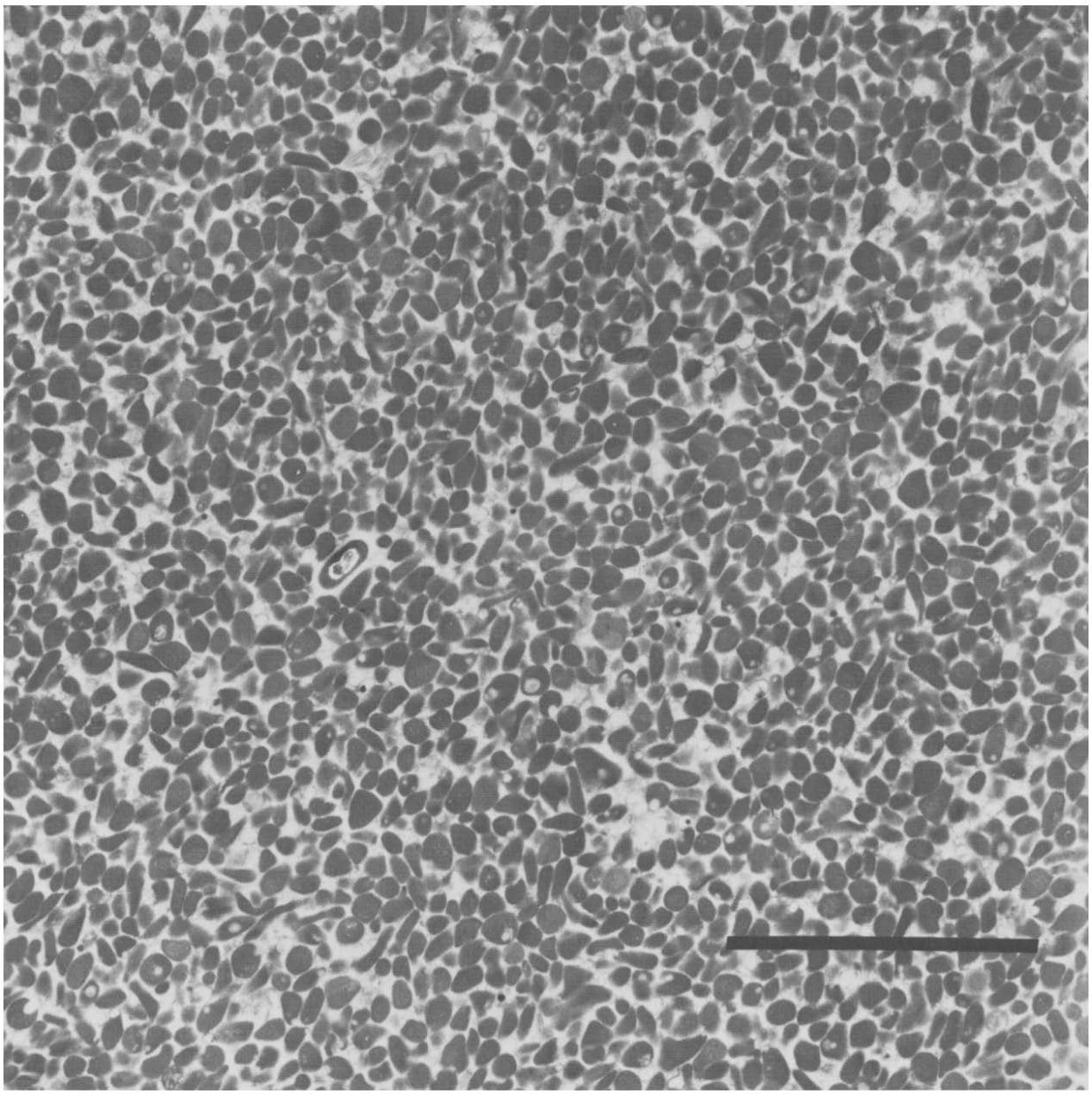

Fig.1. Electron micrograph of a typical α-granule preparation. Samples were fixed in 2.5% glutaraldehyde, postfixed in 1% $OsO_4$ and dehydrated through graded ethanol solutions and into propylene oxide. Embedding was in Araldite-Epon, and staining with uranyl acetate and lead citrate. Bar = 5 μm.

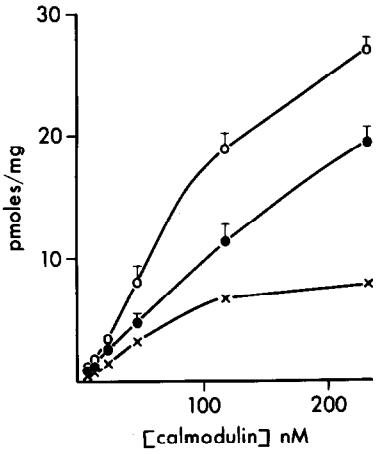


Fig.2. Binding of $^{125}$I-calmodulin to platelet α-granules. The granules were incubated with the indicated concentrations of calmodulin in the presence of 0.8 mM $Ca^{2+}$ (○) or 1 mM EGTA (●), and binding was measured as in section 2. The $Ca^{2+}$-dependent fraction of binding (×) was determined by subtraction. The points are the mean ± SE of ⩾4 detn.

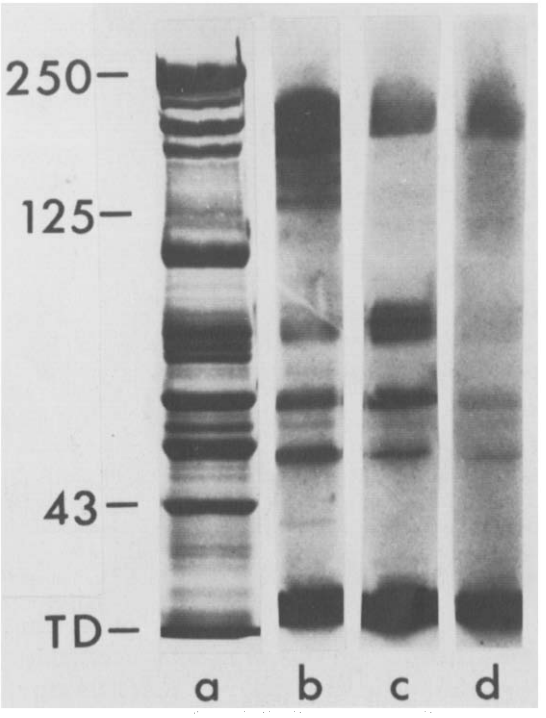


Fig.3. Identification of calmodulin-binding polypeptides of α-granules. Purified α-granules (200 μg protein/lane) were electrophoresed, and after detergent removal overlaid with $^{125}$I-calmodulin: (a) Coomassie blue stained sample; (b–d) autoradiograms – (b) incubation with $^{125}$I-calmodulin was in the presence of 1 mM $CaCl_2$; (c) incubation with $^{125}$I-calmodulin plus 1 mM EGTA; (d) incubation with $^{125}$I-calmodulin and 1 mM $CaCl_2$ plus 0.2 mM chlorpromazine. The $M_r$-values are $\times 10^{-3}$; TD, tracking dye. The radiogram is representative of 3 similar expt.

part of the binding is specific, as defined by the following criteria:

(i) Binding of $^{125}$I-calmodulin (0.1 μg/ml) is largely (77%) prevented by the addition of 20 μg/ml unlabeled calmodulin, whereas much higher concentrations of albumin (8 mg/ml) were not inhibitory;

(ii) A fraction of the binding is $Ca^{2+}$-dependent (fig.2);

(iii) Binding is inhibited by phenothiazines (table 1).

The concentration dependence of $^{125}$I-calmodulin binding is illustrated in fig.2. The total and $Ca^{2+}$-independent components of binding were measured separately, and the $Ca^{2+}$-dependent fraction was calculated by subtraction. The $Ca^{2+}$-dependent component appears to be saturable, whereas the 'unspecific' ($Ca^{2+}$-independent) binding showed no evidence of saturability in the range studied. Under these conditions, the maximum specific binding corresponded to 9 pmol calmodulin/mg protein and half-maximal binding occurred at 49 nM calmodulin. The latter value is consistent with values reported for calmodulin binding to red cell [15], platelet [6] and synaptic membranes [16], and for activation of several calmodulin-sensitive functions [17].

A group of antipsychotic drugs, the phenothiazines, has been found to interact strongly with a hydrophobic site of the $Ca^{2+}$-activated form of calmodulin [18]. Because the latter site is thought to be essential for the interaction of calmodulin with target proteins, addition of phenothiazines is expected to prevent $^{125}$I-calmodulin binding to α-granules. The results of such experiments are shown in table 1. Both trifluoperazine (Stelazine) and chlorpromazine diminished calmodulin binding in a concentration-dependent manner. The concentration of the phenothiazines required for half-inhibition is higher than their reported $K_d$ for binding to calmodulin in vitro [18]. This is probably due to partitioning of some of the drug into the granule membrane and/or binding to albumin (which is present at a high concentration in the medium to prevent unspecific binding of calmodulin to α-granules and to the tube). The specificity of the inhibitory effect of the phenothiazines was demonstrated using chlorpromazine sulfoxide. This

Table 1
Inhibition of calmodulin binding by phenothiazines

| Inhibitor | μM | Binding (%) |
|---|---|---|
| Trifluoperazine | 20 | 88.1 ± 6.5 |
| | 70 | 59.1 ± 1.2 |
| | 130 | 30.2 ± 3.5 |
| Chlorpromazine | 50 | 85.9 ± 0.7 |
| | 200 | 49.3 ± 5.7 |
| | 500 | 20.5 ± 1.8 |
| Chlorpromazine-sulfoxide | 50 | 97.6 ± 8.7 |
| | 200 | 97.1 ± 7.2 |
| | 500 | 101.4 ± 5.1 |

Granules were incubated in media containing 0.8 mM $Ca^{2+}$ and varying amounts of the indicated inhibitors. Binding is expressed as a fraction of the control value. The data are the mean ± SE of ⩾4 detn

compound, a close structural analog of chlorpromazine, is incapable of interacting with calmodulin [18]. As shown in table 1, the sulfoxide derivative failed to inhibit binding at all the concentrations tested.

The gel overlay technique [12] was used to identify the α-granule components which interact with $^{125}$I-calmodulin. A typical radiogram is shown in fig.3. Two major (one of $M_r$ 175 000–185 000, the other comigrating with the tracking dye) and several minor ($M_r$ 130 000–115 000, 65 000, 57 000 and 49 000) $^{125}$I-calmodulin-labeled bands were detected in the presence of $Ca^{2+}$. Labeling of the larger polypeptides ($M_r$ 185 000–115 000) was substantially reduced if incubation was done in EDTA medium, whereas the 65 000 $M_r$ band intensified and the remaining bands were essentially unaffected (fig.3c). A more generalized reduction in band intensity was noted when binding was inhibited by chlorpromazine (fig.3d).

The precise role(s) of calmodulin in exocytosis are not yet known. However, synaptic vesicles can be aggregated by the addition of $Ca^{2+}$ and calmodulin [19]; the modulator could regulate vesicle–membrane interactions [19]. We performed similar experiments with α-granules in different media and at various $Ca^{2+}$ and calmodulin concentrations. Aggregation was only observed at high (mM) $Ca^{2+}$ levels, regardless of the presence or absence of calmodulin.

Porcine platelet α-granules were found to bind calmodulin in vitro. Calcium-dependent binding was half-maximal at calmodulin concentrations similar to or lower than those reported in the cytoplasm of several tissues [20,21], so that substantial occupancy of binding sites will occur at the $Ca^{2+}$ concentrations attained during stimulation [3]. Similar results were reported with secretory granules from chromaffin cells [22].

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