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CALMODULIN BINDING TO PLATELET PLASMA MEMBRANES

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Calmodulin copurifies with platelet plasma membranes isolated by glycerol-induced lysis and density gradient centrifugation. These membranes also bind ^{125}I-labeled calmodulin in vitro in the presence of Ca^{2+}. Binding is largely reduced by replacing Ca^{2+} by Mg^{2+} or by addition of an excess unlabeled calmodulin. The specific component of binding is saturable, with an apparent K_d of 27 nM and a maximum of 15.9 pmol binding sites per mg of membrane protein. This is equivalent to approx. 4100 binding sites per platelet. Binding was inhibited by addition of phenothiazines, a group of calmodulin antagonists. Half-maximal inhibition was attained with approx. 20 μM trifluoperazine or 50 μM chlorpromazine. In contrast, chlorpromazine-sulfoxide which is inactive towards calmodulin, did not affect the binding. Calmodulin binding polypeptides of the plasma membrane were identified by a gel-overlay technique. A major calmodulin-binding component of molecular weight 149000 was detected. Binding to this band was Ca^{2+}-dependent and inhibited by chlorpromazine. The molecular weight of this polypeptide is similar to that of glycoprotein I and also that of the red cell ($Ca^{2+}+Mg^{2+}$)-stimulated ATPase, which is known to bind calmodulin. The possible role of calmodulin in platelet activation is analysed.

Introduction

A variety of experimental approaches have provided evidence for the involvement of cytoplasmic Ca^{2+} in platelet activation. LeBreton and collaborators [1,2] obtained spectroscopic evidence for an increase of free cytoplasmic Ca^{2+} during stimulation by ADP, and suggested that membrane-bound cation was the primary source of the released Ca^{2+}. Charo et al. [3] found that compounds such as 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate, which interfere with Ca^{2+} redistribution in muscle cells also inhibit the release reaction in platelets. Importantly, many aspects of platelet activation can be triggered by the addition of Ca^{2+}-specific ionophores such as A23187 and ionomycin [4,5]. More recently, platelets permeabilized by the application of high voltage electric fields have been used to assess the role of Ca^{2+} in platelet function [6]. In that study, serotonin release could be initiated by simply increasing the Ca^{2+} concentration of the medium bathing the leaky platelets.

In the last few years, it has become increasingly clear that many of the regulatory effects initially attributed directly to Ca^{2+} are in fact modulated by a heat-stable, low molecular weight, ubiquitous protein: calmodulin. A variety of Ca^{2+}-activated phenomena (ranging from kinase activation to neurotransmitter release and microtubule disassembly) have been found to be regulated by calmodulin in vitro, and presumably in vivo as well (for review, see Refs. 7 and 8). Along these lines, calmodulin has been suggested as a mediator

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

of the action of Ca^{2+} during platelet activation. The evidence is largely indirect, based mostly on the inhibitory effects of two calmodulin antagonists, trifluoperazine [9] and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide [10], on platelet aggregation and secretion.

The plasma membrane is central to all facets of platelet activation. It is involved not only in signal transduction and messenger generation, but also in the manifestation of many of the responses, such as adhesion, aggregation and the release reaction. Thus, if calmodulin plays an active role in platelet stimulation, the plasma membrane would be a preferred target for the Ca^{2+}-dependent modulator. We have tried to determine whether isolated platelet plasma membranes contain calmodulin, and whether additional binding sites for this protein can be detected in vitro.

Materials and Methods

(1) Membrane isolation. 72-h old platelet concentrates were obtained from the Red Cross blood bank. The concentrates were spun 25 min at $100 \times g$ to remove contaminating red cells, and then for 15 min at $1000 \times g$ to sediment the platelets. These were then washed twice in a modified Ca^{2+}-free Tyrode's solution and resuspended in a 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM EDTA (sucrose-Tris buffer). The platelets were lysed by the glycerol-loading method of Barber and Jamieson [11] at 0°C, and the membranes isolated by centrifuging the homogenate over a 28% sucrose cushion for 4 h at $65000 \times g$. The overlaying membrane fraction was then washed once in sucrose-Tris buffer without EDTA. The purity of the membrane preparations was routinely assessed by their enrichment in phosphodiesterase activity, measured as in Ref. 12, compared to the homogenate. It must be pointed out that membranes prepared by this method are likely contaminated with internal membranes [13]. However, for simplicity, this material will be referred to hereafter as 'plasma membranes'.

(2) Chymotrypsin treatment. Membranes (100 μg protein) were incubated at room temperature in 5 ml of sucrose-Tris buffer containing 1μg/ml of α-chymotrypsin (Sigma) for variable periods of time. Digestion was terminated by addition of 200 μM (final concentration) of phenylmethylsulfonyl fluoride (Sigma), and the samples sedimented at $48000 \times g$ for 30 min. The pellets were dissolved and electrophoresed as described below. Human red blood cell ghosts were used as molecular weight standards.

(3) Purification of erythrocyte calmodulin. Calmodulin was purified by a combination of the methods of Jarrett and Penniston [14] and Mualem and Karlish [15]. Recently outdated blood was obtained from The Hospital for Sick Children Blood Bank. The blood was centrifuged at $4000 \times g$ for 6 min and the plasma and buffy coat were removed. A total of 150 ml packed red cells were washed four times in 172 mM Tris-HCl (pH 7.6) and lysed in 3 liter of ice-cold water. Membranes were sedimented at $23000 \times g$ for 30 min. The hemolysate was added to 15 g of precycled Cellex D (BioRad), the pH adjusted to 6.5 with 1 M acetic acid and the mixture stirred for one hour at room temperature. The resin was then separated by filtration and washed repeatedly with 10 mM imidazole (pH 6.5) until the A_{280} of the washes dropped below 0.1. The resin was resuspended in 10 mM NaCl (pH 6.5) and packed into a column (2.5 cm internal diameter). The column was washed with 10 mM imidazole, 0.3 M NaCl (pH 6.5) and finally eluted with 0.6 M NaCl, 10 mM imidazole (pH 6.5) as in Ref. 14. The protein peak eluted by the latter treatment was pooled and dialyzed overnight against distilled water. The sample was then heated at 70°C for 30 min and centrifuged for 10 min at $45000 \times g$. A Diaflo apparatus (PM 10 filter) was used to concentrate the supernatant down to 4–6 ml. This concentrate was then applied to a 1.2×16 cm DE-52 (Whatman) column, that had been preequilibrated with 10 mM imidazole (pH 6.5). The column was washed first with 0.2 M NaCl, 10 mM imidazole and then with 0.15 M NaCl, 10 mM imidazole, 5 mM $CaCl_2$ as in [15]. The calmodulin-rich fraction was eluted with 0.15 M NaCl, 10 mM imidazole, 20 mM $CaCl_2$ (pH 6.5), the peak was pooled and the rest of the purification carried out as described [15]. The purified calmodulin was dialyzed, concentrated and stored frozen. The purity of the preparation was routinely checked by gel electrophoresis (15% polyacrylamide).

Calmodulin was iodinated with the Bolton-

Hunter reagent (Amersham) in 40 mM phosphate buffer pH 8.0 as described by Chafouleas et al. [16].

(4) ^{125}I-Labeled calmodulin binding assay. Binding was measured by a method similar to that of Graf et al. [17]. A given amount of membranes and ^{125}I-labeled calmodulin, with or without non-radioactive calmodulin, Ca^{2+} or one of several inhibitors were suspended in a final volume of 0.5 ml of a medium containing (final concentrations): 4 mg/ml bovine serum albumin, 100 mM NaCl, 20 mM KCl, 0.5 mM EGTA, 25 mM Hepes sodium salt (pH 7.4). The concentrations of free Ca^{2+} were calculated as in Ref. 18. The reaction mixture was incubated for 60 min at 37°C in a shaking water bath, and then 1 ml of ice-cold buffer was added to each tube. The tubes were spun at 48000 $\times g$ for 30 min at 4°C, the supernatants aspirated off and the pellets and sides of the tube were carefully rinsed three times with 0.5 ml of buffer, care being taken not to resuspend or dislodge the pellet. The tubes were finally counted in a Nuclear Chicago Gamma Counter.

(5) ^{125}I-Labeled calmodulin overlay technique. Platelet plasma membranes (150 μg protein per lane) were solubilised without boiling and electrophoresed in 7.5% gels by the method of Laemmli [19]. The gels were then processed by the method of Glenney and Weber [20] which involves fixation, detergent removal and overlay with ^{125}I-labeled calmodulin in the presence of either 1 mM $CaCl_2$, 1 mM EGTA or 1 mM $CaCl_2$ plus 0.1 M chlorpromazine. After removal of unbound ^{125}I-labeled calmodulin, the gels were dried and used for autoradiography. One lane of each gel was stained with Coomassie blue, for comparison.

(6) Partial purification of calmodulin from platelet membranes. Membranes isolated in sucrose-Tris were dissolved in 10 mM imidazole (pH 6.5) containing 0.2% Triton X-100. After removal of insoluble material by centrifugation, the solubilized proteins were chromatographed on Cellex D as described in section 3. The fraction eluted with 0.6 M NaCl, 10 mM imidazole was dialysed, concentrated and used for electrophoresis in 15% polyacrylamide gels. Where indicated, Ca^{2+} (1 mM) or EDTA (10 mM) were added to the sample shortly before electrophoresis.

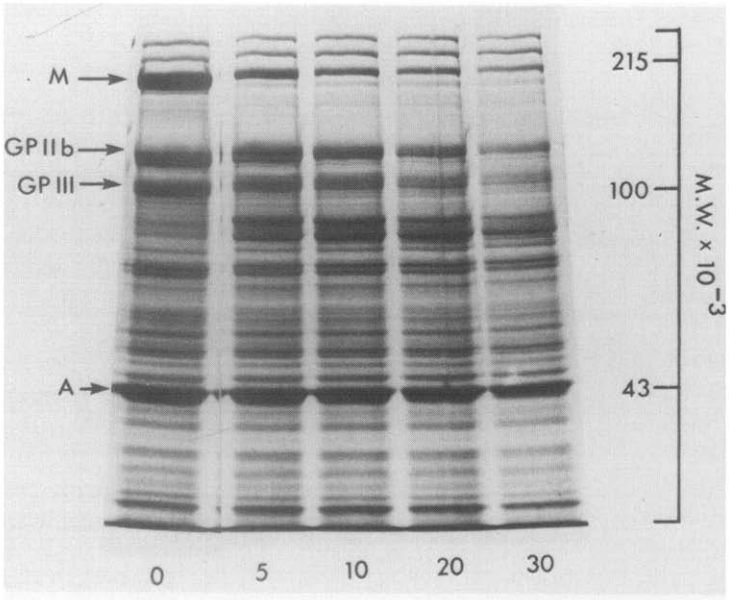
Results

(a) Membrane sidedness

Calmodulin, an intracellular protein, is expected to interact with the cytoplasmic side of the plasma membrane. Therefore, for an adequate assessment of calmodulin binding, this aspect of the isolated membranes must be fully exposed. Membranes prepared by the method used in this report are generally believed to be leaky [11], and evidence is available of the exposure to the medium of externally facing markers, such as acetylcholinesterase [21] or the carbohydrate bearing moieties of the major glycoproteins [22]. Whether the cytoplasmic surface is exposed to the medium is, however, less clear. Fig. 1 presents evidence that this is indeed the case. Isolated membranes were exposed to chymotrypsin and their polypeptide pattern was determined at increasing time intervals. The purpose of this experiment was to establish whether myosin, a major component of isolated platelet membranes which is known to be confined to the cytoplasmic surface [23] was accessible to externally added proteases. As shown in this figure, essentially complete hydrolysis of the 200000 mol. wt. polypeptide, generally accepted to be myosin [13,24] was achieved, indicating that the cytoplasmic side of the membrane vesicles is exposed.

(b) Calmodulin in isolated platelet membranes

Calmodulin has been identified in a variety of plant and animal tissues, including human platelets (Ref. 25 and Furuya, W., unpublished data). To determine whether some of this calmodulin is bound to the plasma membrane, samples of isolated membrane vesicles were electrophoresed in the presence and absence of added Ca^{2+}. Addition of divalent cations is known to affect the migration of calmodulin in polyacrylamide gels [26], which greatly facilitates its identification. Since we anticipated that membranes might contain relatively small amounts of calmodulin, and because this protein is known to stain only poorly with Coomassie blue, we performed a partial purification step prior to electrophoresis. This step, described in detail in Methods, is based on the chromatographic properties of calmodulin in anion exchange resins. The calmodulin-rich fraction



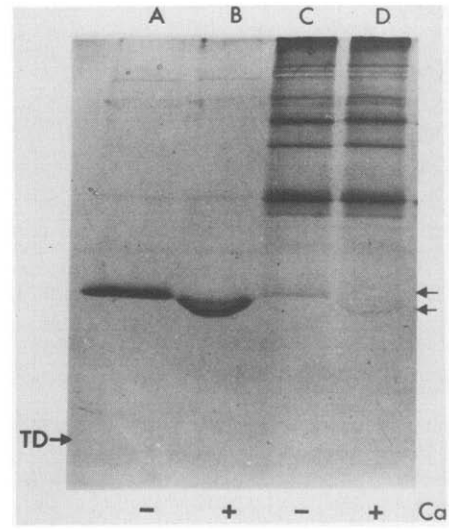


Fig. 1. Proteolysis of isolated platelet plasma membranes. Membranes isolated by the method of Barber and Jamieson [11] were exposed to 1 μg/ml of chymotrypsin as described in Materials and Methods. The reaction was terminated at different times by addition of phenylmethylsulfonyl fluoride and the samples were sedimented. The pellets were dissolved and electrophoresed in polyacrylamide (7.5%) gels by the method of Laemmli [19]. The numbers indicate the length of the proteolytic treatment, in minutes. M, myosin; GP, glycoproteins IIb and III; A, actin.

Fig. 2. Evidence for the existence of plasma membrane-bound calmodulin in platelets. Isolated plasma membranes were dissolved in Triton X-100 and chromatographed on Cellex-D as described in detail in Materials and Methods. The calmodulin-rich fraction of the eluate was subjected to electrophoresis (15% acrylamide) in the presence or absence of 1 mM Ca^{2+}, and compared to purified calmodulin, isolated from red cells as described in Materials and Methods. Lanes A and B: purified calmodulin; Lanes C and D: Cellex D-column fraction eluted with 0.6 M NaCl. The signs at the bottom of the gels indicate the presence or absence of Ca^{2+} during electrophoresis. TD, tracking dye (Bromphenol blue). The unlabeled arrows indicate the approximate position of calmodulin (top) and the calcium-calmodulin complex (bottom) in the gel.

eluted from the Cellex-D column loaded with Triton X-100 solubilized membranes, was electrophoresed and the results are illustrated in Fig. 2. Clearly, a band that comigrates with purified calmodulin can be detected in the membrane fraction. Moreover, in the presence of Ca^{2+} this band undergoes a shift that is identical in direction and magnitude to that of the purified erythrocyte calmodulin. Hence, Fig. 2 provides qualitative evidence that calmodulin is a normal constituent of human platelet membranes, as isolated by the method of Ref. 11. The detection method used, though not quantitative, represents a simple and inexpensive alternative to radioimmunoassay for the assessment of the presence of calmodulin.

(c) Binding of ^{125}I-labeled calmodulin to isolated membranes

Radioactively labeled calmodulin was used to detect possible binding sites on platelet plasma membranes. Fig. 3 shows that ^{125}I-labeled calmodulin bound to isolated membrane vesicles in the complete incubation mixture (see Materials and Methods). The figure also shows that binding was dependent on the presence of both membranes and Ca^{2+}. The specificity of the binding is indicated by the fact that an excess unlabeled calmodulin largely prevented radioactively labeled ligand binding. Moreover, chloropromazine, an inhibitor of calmodulin-mediated activation in a variety of systems [27], substantially reduced binding (Fig. 3 and Table I).

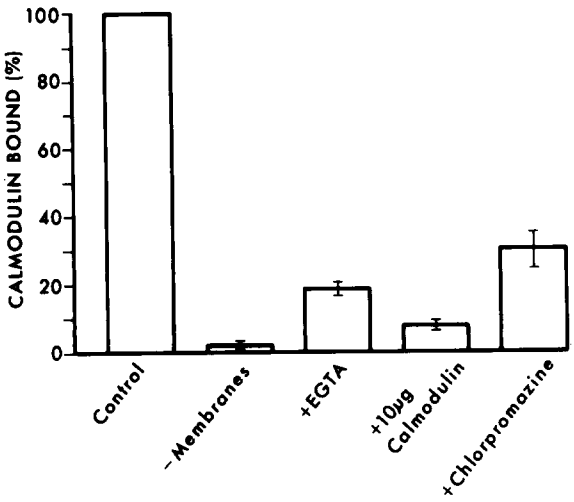


Fig. 3. ^{125}I-Labeled calmodulin binding to isolated platelet plasma membranes. Membranes were incubated under various conditions to define the requirements and the specificity of the observed binding. The complete system (control bar) contained 100 μg membrane protein, 100 ng ^{125}I-labeled calmodulin and 0.8 mM Ca^{2+}, in addition to the standard suspending buffer (see Materials and Methods). Percent binding represents a fractional portion of the binding determined under these conditions. Omission of membranes (second bar) or Ca^{2+} (third bar) from the otherwise complete system largely diminished binding. Addition of 10 μg non-radioactive calmodulin (fourth bar) or 200 μM chlorpromazine hydrochloride (fifth bar) also drastically reduced binding. The data are the mean ± S.E. of four to six determinations.

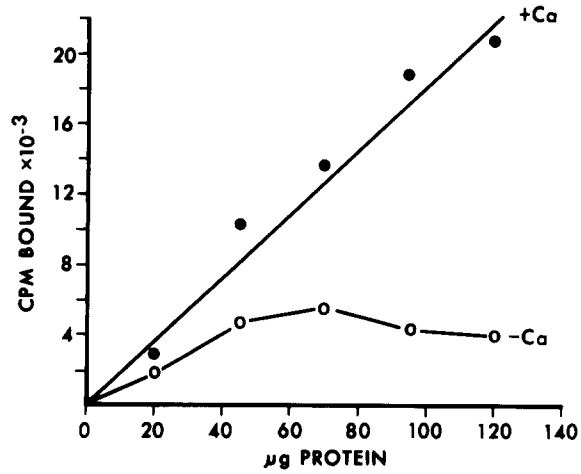


Fig. 4. Calmodulin binding as a function of plasma membrane concentration. A mixture containing 0.5 ml of the basic incubation buffer, with or without added Ca^{2+} (0.8 mM) was incubated with 200 ng ^{125}I-labeled calmodulin and increasing concentrations of plasma membranes. A minimal amount of 'binding' observed in the absence of membranes, probably due to ^{125}I-labeled calmodulin adherence to the tubes, was subtracted in all cases. The straight line was drawn by eye. The presence or absence of Ca^{2+} in the incubation medium is indicated. The Ca^{2+}-independent component of binding has been subtracted from the upper curve. The results are representative of three similar experiments.

Specific (i.e. Ca-dependent) binding, calculated by subtracting nonspecific from total binding, increased with increasing membrane concentration (Fig. 4). In the presence of an excess ^{125}I-labeled calmodulin, the increase was approximately linear within the range analysed. In contrast, unspecific binding was considerably lower, which made the determinations more erratic.

Binding of ^{125}I-labeled calmodulin to platelet membranes as a function of calmodulin concentration is illustrated in Fig. 5A. The Ca^{2+}-dependent and independent fractions of the binding are plotted separately. Ca^{2+}-dependent binding was found to be saturable, whereas the unspecific component showed no evidence of saturation in the range analysed. The specific binding sites displayed relatively high affinity, comparable to that described for other systems [17,28,29]. A Scatchard plot of the specific binding curve is shown in Fig. 5B. A straight line can be drawn through the points with a correlation coefficient of 0.951. Though more than one type of independent or interacting sites probably exist, they cannot be adequately resolved with the present data. Thus, at the risk of oversimplification, a single class of noninteracting sites is taken to explain the observations. This population has an apparent K of 27 nM and a maximum number of 15.9 pmol binding sites per mg membrane protein.

Unlabeled calmodulin competes for the binding sites with ^{125}I-labeled calmodulin. A curve demonstrating the displacement of a fixed amount of radioactively labeled calmodulin by the unlabeled protein is shown in Fig. 6. A concentration of 38 nM calmodulin reduced by 50% the binding of isotopic calmodulin. For comparison, it is worth recalling that the normal incubation mixture con-

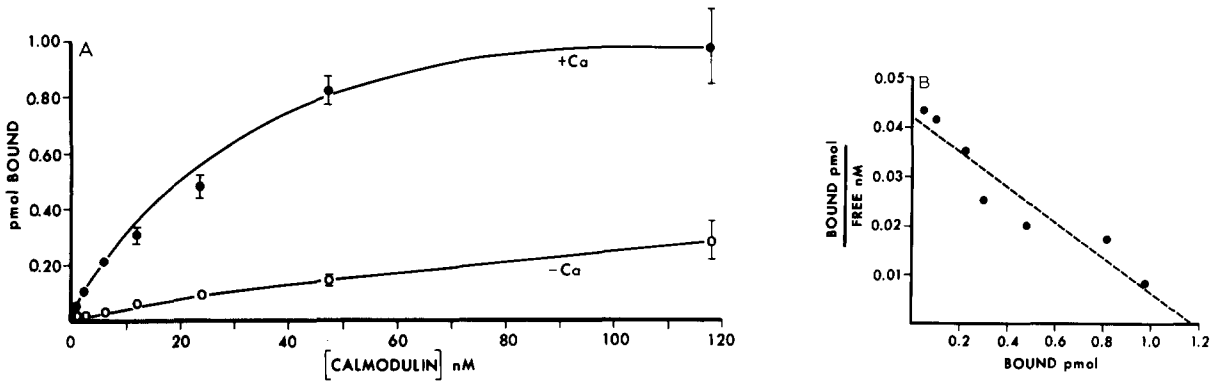


Fig. 5. (A) Concentration dependence of the binding of ^{125}I-labeled calmodulin to plasma membranes. The assay mixture contained 75 μg of membrane protein with or without 0.8 mM Ca^{2+}. The Ca^{2+}-independent component of binding was subtracted from the upper curve. The points are the mean ± S.E. of three experiments each with duplicate determinations. When not indicated, the error bars were equal to or smaller than the symbols. A molecular weight of 17000 was used for the calculation of calmodulin concentrations. (B) Scatchard plot of the Ca^{2+}-dependent binding of ^{125}I-labeled calmodulin to plasma membranes. The data of (A) were replotted as a Scatchard plot. Abscissa: calmodulin bound, in pmol. Ordinate: ratio of bound to free calmodulin. The concentration of free calmodulin (in nM) was assumed to be unaffected by the amount bound, which was always less than 10% of the total added. The line drawing is fit by a linear least squares regression.

tains 4 mg/ml of bovine serum albumin, a concentration several orders of magnitude higher than that of the added nonradioactive calmodulin. This comparison stresses the specific nature of the membrane-calmodulin interaction.

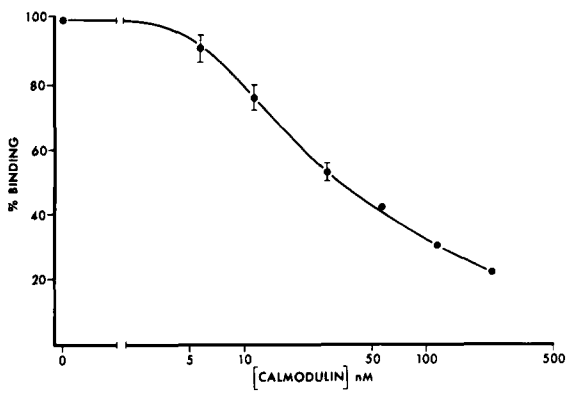


Fig. 6. Competition for ^{125}I-labeled calmodulin binding by unlabeled calmodulin. Membranes (75 μg protein) and ^{125}I-labeled calmodulin (100 ng) were incubated in the presence of 1 mM Ca^{2+} with increasing concentrations of non-radioactive calmodulin. Binding is expressed as percent of the maximum, i.e. that obtained when no unlabeled calmodulin was added. The data are the mean ± S.E. of six determinations. When not indicated, the error bars are equal to or smaller than the symbols. Abscissa (log scale): added non-radioactive calmodulin. Ordinate: fractional binding.

As pointed out above, binding of ^{125}I-labeled calmodulin to the membranes is a Ca^{2+}-dependent process. An excess of EGTA (Fig. 3) or EDTA (not illustrated) markedly diminished the binding. As expected [8], the Ca^{2+}-induced increase in binding reached a maximum in the μM range but, surprisingly, a slight decrease was observed at Ca^{2+} concentrations above 100 μM (not illustrated). No explanation is available to account for this observation. The divalent cation specificity of binding is demonstrated by the fact that Mg^{2+}, in up to mM concentrations, was unable to produce specific interaction.

(d) Inhibition of ^{125}I-labeled calmodulin binding

A series of antipsychotic drugs have been found to interact strongly with a hydrophobic site on the Ca^{2+}-activated form of calmodulin [27]. This site is believed to be involved in the interaction of calmodulin with its physiological target proteins. If so, addition of trifluoperazine or chlorpromazine, two of the calmodulin-specific inhibitors, would be expected to prevent binding of the Ca^{2+}-activator to the membranes. Table I reports the results of these experiments.

Both drugs, at the concentrations reported to inhibit other calmodulin-activated phenomena [27], diminished ^{125}I-labeled calmodulin binding to

TABLE I

INHIBITION OF ^{125}I-LABELED CALMODULIN BINDING BY PHENOTHIAZINES

Membranes (100 μg protein) were incubated in the complete assay buffer (see Materials and Methods) 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 ± S.E. of six determinations.

Inhibitor	Concn. (μM)	Binding (%)
Trifluoperazine	0	100
	20	51.4 ± 1.1
	75	24.6 ± 2.8
	200	11.3 ± 1.6
Chlorpromazine	50	49.1 ± 4.6
	100	36.6 ± 4.6
	200	27.5 ± 2.2
Chlorpromazine sulfoxide	50	89.6 ± 5.1
	100	96.6 ± 3.8
	200	89.5 ± 3.4

platelet membranes. Half inhibition of binding was achieved with approx. 20 μM trifluoperazine and 50 μM chlorpromazine. The specificity of this inhibitory effect was examined using chlorpromazine sulfoxide, a close structural analog of chloropromazine, which is however incapable of interacting with calmodulin. When used at equimolar concentrations, chlorpromazine is considerably more effective than its sulfoxide derivative, which did not significantly inhibit binding.

(e) Identification of calmodulin binding proteins

By using a gel overlay technique, we have attempted to define which proteins of the platelet plasma membrane interact with calmodulin. This method, developed by Glenney and Weber [20], involves separation of the proteins by gel electrophoresis in the presence of sodium dodecyl sulfate, removal of detergent and an incubation period with ^{125}I-labeled calmodulin. After removing unbound ^{125}I-labeled calmodulin, those bands retaining the radioactive label can be identified by autoradiography. One series of such radiograms was scanned and is depicted in Fig. 7. Gels were overlayed with ^{125}I-labeled calmodulin under three different conditions: (a) in the presence of Ca^{2+};

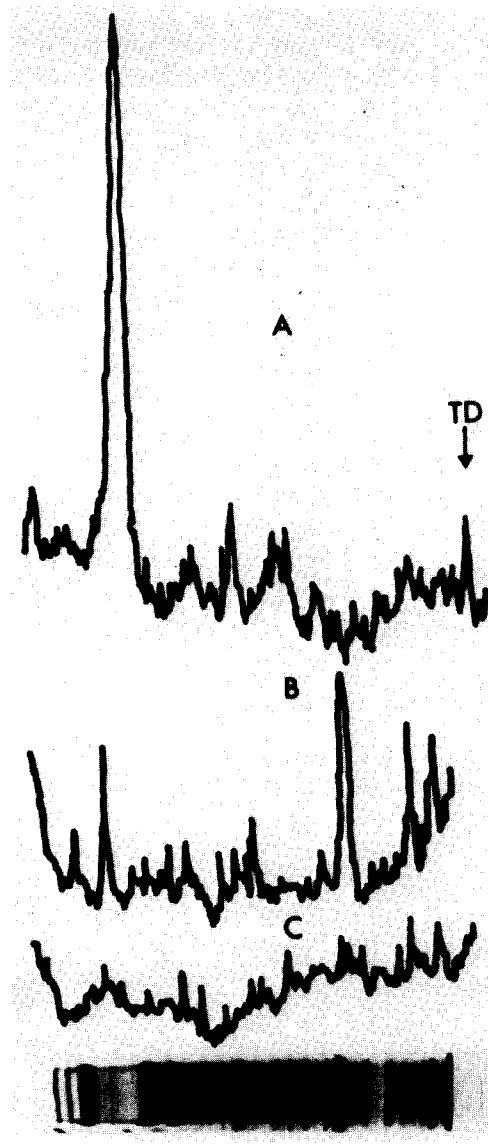


Fig. 7. Identification of ^{125}I-labeled calmodulin-binding polypeptides of platelet plasma membranes. Purified plasma membranes (150 μg protein per lane) were electrophoresed in 7.5% polyacrylamide gels and after detergent removal, they were overlaid with ^{125}I-labeled calmodulin by the method of Glenney and Weber [20]. The resulting gels were dried and used for radioautography, and the radiograms were scanned at 600 nm. Lane A: incubation with ^{125}I-labeled calmodulin was in the presence of 1 mM Ca^{2+}. Lane B: incubation with 1 mM EGTA. Lane C: incubation was in the presence of both Ca^{2+} and chlorpromazine. A Coomassie-blue-stained gel is shown for comparison. TD, tracking dye. The results are representative of three similar experiments.

(b) in Ca^{2+}-free media containing EGTA and (c) in the presence of the binding inhibitor chloropromazine. One major and several minor binding components were detected in the complete system (Fig. 7A). The major band corresponds to a molecular weight of 149000 whereas the minor bands range in molecular weight from 86000 to 30000. Binding to the M_r 149000 band and to most of the minor polypeptides was markedly diminished by omission of Ca^{2+} from the incubation medium (Fig. 7B). Interestingly, a M_r 38000 band is more heavily labeled in Ca^{2+}-free media. This was consistently found in three separate preparations. Other polypeptides which display enhanced calmodulin binding in the presence of EDTA have been reported [20,30]. Chlorpromazine drastically reduced binding to all polypeptides to near background levels (Fig. 7C). These data indicate that binding detected in the gel system is analogous to the one measured with intact membranes.

Discussion

Evidence for the involvement of calmodulin in several aspects of platelet activation is mostly indirect, and stems from experiments in which calmodulin-inhibitors were found to prevent ADP [9] or thrombin [10] induced aggregation and secretion. The concentrations of the drugs required for half-inhibition are similar, though not identical, to the reported affinity of calmodulin for these substances measured in vitro [27]. The disagreement could reflect restricted diffusion of the phenothiazines into the compartments where calmodulin is located, but could also be an indication of an unspecific mode of inhibition. Indeed, the amphiphilic nature of these drugs enables them to interact extensively with membranes and hydrophobic sites in general [31], resulting in eventual cell death and lysis [32].

Clearly, alternative means of establishing the participation of calmodulin in platelet activation are desirable. As an essential preliminary step, we have established that plasma membranes, which are central to the activation mechanism, are capable of binding calmodulin in a specific, Ca^{2+}-dependent manner. Moreover, calmodulin was found to copurify with plasma membranes following glycerol-induced lysis and density gradient centrifugation. It must be pointed out that the protocol used for membrane isolation involves extensive washes in EDTA-containing media, which are expected to displace most of the membrane-bound calmodulin *. Thus, the amount of the Ca^{2+}-activator originally bound to the membranes was potentially much larger. Other systems have been found to contain Ca^{2+}-independent calmodulin binding sites [20,29,30], and in some of these the 'unspecific' (i.e. Ca^{2+}-independent) fraction can be a significant part of the total binding [29] or even the majority [20].

Platelet plasma membranes were found to bind up to 15.9 pmol of ^{125}I-labeled calmodulin per mg protein, although this number probably underestimates the maximum number of binding sites, since it does not take into account the nonradioactive calmodulin that copurified with the preparation. Assuming that each platelet has $4.3 \cdot 10^{-10}$ mg of membrane protein [33] the maximum binding corresponds to about 4100 calmodulin molecules per platelet. The apparent K_d found for this binding (27 nM) is consistent with the values for half-maximal activation of a variety of calmodulin-sensitive functions [19,29,30,34,35], which range from 4 to 50 nM. These include binding to plasma membranes of other tissues, such as human red blood cells (K_d = 30 nM, [17]) or guinea pig synaptic membranes (K_d = 39 nM, [28]).

The reasonable agreement obtained between the apparent K_d, measured with ^{125}I-labeled calmodulin, and the concentration needed for half-maximal displacement of ^{125}I-labeled calmodulin by nonlabeled calmodulin, indicates that the biological activity of the protein was preserved after iodination with the Bolton-Hunter reagent. This is in agreement with the findings of Chafouleas et al. [16] whose method was employed in this report, but differs from the results of Graf et al. [17] who found a marked inactivation upon Bolton-Hunter iodination.

The results of the gel-overlay technique indicate that one major and at least three minor bands can interact with ^{125}I-labeled calmodulin, and none of these components corresponds to major Coomas-

* Inclusion of EDTA in the isolation media is essential due to the abundance of both intraplatelet Ca^{2+} and Ca^{2+}-activated proteases, which are released upon homogenization.

sie stained polypeptides. The main peak of radioactivity is centered around a molecular weight of 149000. The molecular nature of this binding component remains unclear, but some insight can be gained by comparison with other membrane-related proteins capable of interacting with calmodulin. These include clathrin, M_r 180000 [29], various brush-border core polypeptides, M_r 280000, 140000 and 110000 [20], and the red cell membrane ($Ca^{2+} + Mg^{2+}$)-ATPase, whose molecular weight has been reported to be between 125000 and 145000 [36,37] a value very similar to that of our major band. In this regard, White [38] measured the Ca^{2+}-accumulating properties of a platelet microsomal fraction in response to added calmodulin or antisera to calmodulin. Neither of these additions affected Ca^{2+}-transport, but antagonists of calmodulin such as trifluoperazine and $ZnCl_2$ produced a dose dependent inhibition. One must bear in mind, however, that plasma membranes probably constitute only a fraction of the preparation used. Efforts in our laboratory to detect a calmodulin-sensitive Ca^{2+}-dependent ATPase activity in the purified plasma membrane preparation have been unsuccessful (Furuya, W., unpublished data). The molecular weight of the major calmodulin binding polypeptide also coincides with that of glycoprotein I [39] a major component of the membrane which is, however, not intensely stained with Coomassie blue. This glycoprotein is believed to play a pivotal role in thrombin binding and aggregation [39] and might also be involved in calmodulin binding.

The precise role of calmodulin in platelet activation remains unclear but several possible sites of action have been suggested. It could be involved in prostaglandin metabolism [35], and in platelet contractile activity, as it is needed for myosin light chain kinase activation [10,34]. As pointed out previously, intracellular Ca^{2+} metabolism could also be regulated by calmodulin [17,37]. Moreover, because the molecular details of the aggregation and release reactions are yet to be unraveled, other as yet unidentified sites of calmodulin action are likely to exist. Basic information on calmodulin binding, of the type presented in this report, will hopefully contribute to their eventual identification.

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