Association between Human Erythrocyte Calmodulin and the Cytoplasmic Surface of Human Erythrocyte Membranes

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This report describes Ca\(^{2+}\)-dependent binding of \(^{125}\)I-labeled calmodulin (\(^{125}\)I-CaM) to erythrocyte membranes and identification of two new CaM-binding proteins. Erythrocyte CaM labeled with \(^{125}\)I-Bolton Hunter reagent fully activated erythrocyte (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase. \(^{125}\)I-CaM bound to CaM depleted membranes in a Ca\(^{2+}\)-dependent manner with a \(K_a\) of 6 × 10\(^{-8}\) M Ca\(^{2+}\) and maximum binding at 4 × 10\(^{-7}\) M Ca\(^{2+}\). Only the cytoplasmic surface of the membrane bound \(^{125}\)I-CaM. Binding was inhibited by unlabeled CaM and by trifluoperazine. Reduction of the free Ca\(^{2+}\) concentration or addition of trifluoperazine caused a slow reversal of binding. Nanomolar \(^{125}\)I-CaM required several hours to reach binding equilibrium, but the rate was much faster at higher concentrations. Scatchard plots of binding were curvilinear, and a class of high affinity sites was identified with a \(K_a\) of 0.5 nM and estimated capacity of 400 sites per cell equivalent for inside-out vesicles (IOVs). The high affinity sites of IOVs most likely correspond to Ca\(^{2+}\) transporter since: (a) \(K_a\) of activation of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase and \(K_D\) for binding were nearly identical, and (b) partial digestion of IOVs with a-chymotrypsin produced activation of the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase with loss of the high affinity sites. \(^{125}\)I-CaM bound in solution to a class of binding proteins (\(K_D\) ~ 55 nM, 7.5 pmol per mg of ghost protein) which were extracted from ghosts by low ionic strength incubation. Soluble binding proteins were covalently cross-linked to \(^{125}\)I-CaM with Lomant’s reagent, and 2 bands of 8,000 and 40,000 \(M_r\) (\(M_r\) of CaM subtracted) and spectrin dimer were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiography. The 8,000 and 40,000 \(M_r\) proteins represent a previously unrecognized class of CaM-binding sites which may mediate unexplained effects in the erythrocyte.

CaM\(^1\) is a Ca\(^{2+}\)-binding protein (\(M_r = 17,000\)) which is highly conserved among eukaryotic cells (see monograph in Ref. 1). CaM undergoes a conformational change when complexed with Ca\(^{2+}\) permitting it to bind to the regulator domains of certain enzymes reviewed in Refs. 2-4). Binding of Ca\(^{2+}\)-CaM activates CaM-sensitive enzymes in many different cell types including (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase in the erythrocyte (5, 6).

Erythrocytes contain micromolar concentrations of CaM (7). Nanomolar CaM fully activates Ca\(^{2+}\) transporter (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (8) maintaining cytosolic free Ca\(^{2+}\) at <10\(^{-6}\) M (9). Elevation of cytosolic free Ca\(^{2+}\) produces alterations in erythrocyte filterability and shape (10), but it is uncertain if these changes are governed by CaM-sensitive enzymes. It is also unclear what roles, in addition to activation of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase, the large concentration of CaM plays in the erythrocyte.

Radiolabeled CaM has been used to directly investigate CaM interactions in several systems. Radiolabeled CaM binds to synaptosomal membranes (11) and adipocyte membranes (12), binds to both brain phosphodiesterase (13) and inhibitor protein (14), and binds to proteins in post-synaptic densities (15). Radiolabeled CaM binds to erythrocyte spectrin (16), and the CaM concentration, ~2.5 × 10\(^{-6}\) M, is close to both the \(K_D\) of the interaction and to the concentration of spectrin. Radiolabeled CaM also binds to proteins related to spectrin in other tissues (17, 18). Erythrocyte (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase interacts directly with CaM (19, 20). \(^{125}\)I-CaM binding to erythrocyte membranes revealed a high affinity class of sites with positive cooperativity which was interpreted to represent direct binding to (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (21-23).

This report describes detailed studies of \(^{125}\)I-CaM binding to erythrocyte membranes. The data support the concept that CaM binds to a class of sites on the membrane with high affinity (\(K_a = 0.5\) nM) and that these sites represent (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase. In addition two new CaM-binding proteins have been discovered of 8,000 and 40,000 \(M_r\), and these may mediate presently unexplained actions of Ca\(^{2+}\) in erythrocytes.

EXPERIMENTAL PROCEDURES

Materials—\(^{125}\)I-Bolton Hunter Reagent (2200 Ci/mmol) and a \(^{125}\)I-CaM radioimmunoassay kit were from New England Nuclear. [\(^{35}\)S]PMSF, phenylmethysulfonyl fluoride; NaEGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)\(-N,N,N',N'\)-tetraacetic acid, sodium salt; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IOVs, spectrin/actin-stripped inside-out vesicles; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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washed again before lysis in 15 liters of 7.5 mM NaP04, pH 7.5, and
associated counts were determined (see under "Methods").

2.486 mM CaC12 (pCa2+ 5.2), pH 7.3, and Ca2+-dependent membrane-
creasing concentrations of erythrocytes

Hepes, 0.25 mg/ml of gelatin, 2.50 mM NaEGTA, with or without
Solid NaCl was added to the lysate (to 0.15 M) and 40 ml of DE52
duplicate determinations.

subtracting the amount of P, hydrolyzed in the absence of membranes.

A, and applied to an AcA 54 Ultrogel column (2.6

5% w/v, concentration 0.4 mM) and MgCl2 (final concentration 0.8 mM) were

4.5 mol per liter packed cells

4.5, basal, Fig. 1). These erythrocytes in this study were prepared in buffers containing EGTA and

RESULTS

Radiolabeled binding proteins must retain biological activity
if physiologic conclusions are to be made. CaM radiola-
beled with 125I-Bolton Hunter reagent retained full ability to
activate brain phosphodiesterase (29), although another re-
port noted such preparations had reduced biological activity
(22). The CaM in this report was purified from erythrocytes and
radiolabeled with 125I-Bolton Hunter reagent to 1.05 mol
of 125I per mol of CaM. 125I-CaM and native CaM activated
erthrocyte membranes (Ca2 + + Mg2+)-ATPase identically (K0.5
0.3 ml, Vmax = 4.5 X 10-9 mol/min). Fresh ghosts in this study were prepared in buffers containing EGTA and
retained less than 0.1% of native erythrocyte CaM (Table 1). These
ghosts were permeable to large molecules such as Ficoll

FIG. 1 (left). Effect of increasing concentrations of 125I-CaM (C)
and of unlabeled CaM (O) on the activation of erythrocyte
membrane-associated (Ca2 + + Mg2+)-ATPase. Various concen-
trations of 105 CaM (0.15-105 mol of CaM) were added to 1.5 ml of
CaM (were incubated with erythrocyte ghosts (16 ug of membrane
protein) for 2 h at 0°C and then 1 h at 24°C in 0.2 ml of 30 mM
KCI, 50 mM Hepes, 80 mM NaCl, 0.5 mM MgCl2, 20 mM NaEGTA,
2.486 mM CaCl2 (pCa 5.2), 0.1 mM ouabain, 0.25 mg/ml of gelatin, pH
7.5 (modified from Ref. 44, [1-25P]ATP (7700 cpn/nmol) (final
concentration 0.4 mM) and MgCl2 (final concentration 0.8 mM) were
added for an additional hour at 24°C. Trichloroacetic acid (1.0 ml,
5% w/v, 0°C) and Norit A (0.2 ml, 5% w/v) were added and
free P1 in the supernatant was determined by counting Cerenkov
radiation after centrifugation for 15 min at 4000 g. Basal and CaM-
activated (Ca2 + + Mg2+) -ATPase activity were calculated after sub-
tracting the amount of P1 hydrolyzed in the absence of membranes.
Free 125I-CaM concentrations were determined in parallel as described
(see under "Methods"). The data are expressed as the average of
duplicate determinations.

Fig. 2 (right). Ca2+-dependent binding of 125I-CaM to in-
creasing concentrations of erythrocytes (C), ghosts (O), and
IOVs (4). Erythrocytes, ghosts, or IOVs were incubated with 125I-
CaM (7.2 nM, 44,000 cpn/pmol) for 2 h at 24°C in 0.2 ml of 0.1 M
Hepes, 0.25 mg/ml of gelatin, 2.50 mM NaEGTA, with or without
2.486 mM CaCl2 (pCa 5.2), pH 7.5, and Ca2+-dependent membrane-
associated counts were determined (see under "Methods").

TABLE I

Measurement of residual native CaM in CaM-depleted erythrocyte
ghosts and IOVs

Erythrocyte ghosts and IOVs were prepared as described under
"Methods." After the final wash in 10 mM Hepes, pH 7.3, the following reagents were added making final concentrations: 1 mM
dithiothreitol, 1 mM NaEGTA, 1 mM NaCl, and 2 mM MgCl2. Samples were boiled for 10 min and then centrifuged (130,000 X g for
30 min). Supernatants were assayed with a commercial CaM radioim-
omunoassay kit exactly as directed with controls for buffer effects.

Membranes

<table>
<thead>
<tr>
<th>CaM Content</th>
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<tbody>
<tr>
<td>Ghosts</td>
</tr>
<tr>
<td>IOVs</td>
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<tr>
<td>Intact erythrocytes</td>
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*This value is corrected to the volume of the original erythrocytes.

*These values are from the literature (16, 23) and were determined
due by enzyme activation not radiomunoassay.
and did not reseal during the CaM binding assay (see under "Methods").

Characteristics of Ca\textsuperscript{2+}-dependent \textsuperscript{125}I-CaM Binding to Erythrocyte Membranes—Ca\textsuperscript{2+}-dependent binding (see below) occurred at intracellular sites and increased linearly with increased concentrations of membranes. Intact erythrocytes failed to bind \textsuperscript{125}I-CaM (Fig. 2) indicating that binding was restricted to the cytoplasmic membrane surface. Binding to ghosts and IOVs increased linearly up to 0.06 mg of ghost protein per assay, so all studies were conducted in the linear range. IOVs bound less \textsuperscript{125}I-CaM than ghosts suggesting a loss of binding sites during preparation (see below).

\textsuperscript{125}I-CaM binding depended upon the free Ca\textsuperscript{2+} concentration. Membranes bound negligible \textsuperscript{125}I-CaM at pCa\textsuperscript{2+} 8.0 but reached maximum at pCa\textsuperscript{2+} 6.4 (Fig. 3). Ca\textsuperscript{2+}-independent binding was subtracted from all data since it was judged to be nonspecific. Ca\textsuperscript{2+}-independent binding did not saturate at increasing concentrations of \textsuperscript{125}I-CaM (Fig. 8, A and B), was not reduced by trifluoperazine (Fig. 3), and was not displaced by excess unlabeled CaM (Fig. 4). Furthermore, Ca\textsuperscript{2+}-independent binding was not time dependent.

\textsuperscript{125}I-CaM binding was determined (see under "Methods"). Other samples were incubated identically except that various concentrations of trifluoperazine were added after 60 min and reversal (0) of Ca\textsuperscript{2+}-dependent binding was determined after an additional 60 min of incubation.

**Characteristics**

**Fig. 3.** Ca\textsuperscript{2+} dependence of \textsuperscript{125}I-CaM binding to erythrocyte ghosts (○) and IOVs (△). Ghosts (0.13 mg of membrane protein/ml) or IOVs (0.12 mg/ml) were incubated with \textsuperscript{125}I-CaM (30 nM, 27,000 cpm/pmol) in 0.1 M Hepes, 0.25 mg/ml of gelatin with 2.50 mM NaEGTA, pH 7.3, for 2 h at 24 °C. In addition most of the samples contained varying concentrations of CaCl\textsubscript{2} (0.399–2.486 mM giving a final pCa\textsuperscript{2+} of 6.0–5.2). Ca\textsuperscript{2+}-dependent binding was determined (see under "Methods").

**Fig. 4.** Inhibition of Ca\textsuperscript{2+}-dependent binding of \textsuperscript{125}I-CaM to erythrocyte ghosts by increasing concentrations of unlabeled CaM. \textsuperscript{125}I-CaM (1.5 nM, 20,000 cpm/pmol) was incubated for 2 h at 24 °C with ghosts (0.18 mg of membrane protein/ml) in the presence of various concentrations of unlabeled CaM, and Ca\textsuperscript{2+}-dependent binding was determined (see under "Methods").

**Fig. 5.** Inhibition (●) and reversal (○) of Ca\textsuperscript{2+}-dependent binding of \textsuperscript{125}I-CaM to erythrocyte ghosts with increasing concentrations of trifluoperazine. \textsuperscript{125}I-CaM (12 nM, 45,000 cpm/pmol) was incubated for 90 min at 24 °C with ghosts (0.2 mg of membrane protein/ml) in 0.1 M Hepes, 0.25 mg/ml of gelatin, 2.50 mM NaEGTA, with or without 2.486 mM CaCl\textsubscript{2} (pCa\textsuperscript{2+} 5.2), pH 7.3, which also contained various concentrations of trifluoperazine, and inhibition (●) of Ca\textsuperscript{2+}-dependent binding was determined (see under "Methods"). Other samples were incubated identically except that various concentrations of trifluoperazine were added after 60 min and reversal (○) of Ca\textsuperscript{2+}-dependent binding was determined after an additional 60 min of incubation.

**Fig. 6.** Reversal of Ca\textsuperscript{2+}-dependent binding of \textsuperscript{125}I-CaM to erythrocyte ghosts at various times after reduction of the free Ca\textsuperscript{2+} concentration. \textsuperscript{125}I-CaM (14 nM, 45,000 cpm/pmol) was incubated for 2 h at 24 °C with ghosts (0.14 mg of membrane protein/ml) in 0.1 M Hepes, 0.25 mg/ml of gelatin with 2.50 mM NaEGTA, 2.376 mM CaCl\textsubscript{2}, pH 7.3 (pCa\textsuperscript{2+} 6.00, △), or with 2.50 mM NaEGTA, 2.376 mM CaCl\textsubscript{2}, pH 7.30 (pCa\textsuperscript{2+} 7.61, ○), with 2.50 mM NaEGTA, 2.376 mM CaCl\textsubscript{2}, pH 7.30, and 2.50 mM NaEGTA, 2.376 mM CaCl\textsubscript{2}, pH 7.30, or with 2.50 mM NaEGTA, 2.376 mM CaCl\textsubscript{2}, pH 7.30, but with addition of NaEGTA at 120 min to a final concentration of 7.50 mM (pCa\textsuperscript{2+} 6.00 → 7.61, △). Aliquots were removed at various times thereafter and Ca\textsuperscript{2+}-dependent binding was determined (see under "Methods").

Ca\textsuperscript{2+}-dependent binding was not time dependent. Ca\textsuperscript{2+}-dependent binding of \textsuperscript{125}I-CaM was inhibited by both unlabeled CaM and trifluoperazine. Binding of 1.5 nM \textsuperscript{125}I-CaM was 50% displaced by 2 nM unlabeled CaM and 99% displaced by 112 nM (Fig. 4). Phenothiazines such as trifluoperazine are antagonists of CaM (30). \textsuperscript{125}I-CaM binding was 50% inhibited by ~15 μM trifluoperazine and at 50 μM complete inhibition was observed (Fig. 5).

Membrane-bound \textsuperscript{125}I-CaM was dissociated by lowering the free Ca\textsuperscript{2+} concentration or by adding trifluoperazine (Figs. 5 and 6). Maximum high affinity binding occurred at pCa\textsuperscript{2+} 6.0. Subsequent addition of EGTA reduced the free Ca\textsuperscript{2+} concentration to pCa\textsuperscript{2+} 7.61, and binding was slowly reversed (Fig. 6). The reversal was biphasic on a semi-log scale with the first
T_{1/2} \sim 18\text{ min} \text{ and second } T_{1/2} \sim 80\text{ min}.\text{ Dissociation of membrane-bound }^{125}\text{I-CaM required 4-fold greater concentrations of trifluoperazine than required for inhibition of binding. The reversibility is further evidence that the binding is specific and does not represent trapping.}

Binding was slow at low concentrations of 

$^{125}$I-CaM (Fig. 7). Binding of 1.5 nM 

$^{125}$I-CaM (a concentration near the $K_a$ for activation of (Ca$^{2+}$ + Mg$^{2+}$)-ATPase), was still increasing slightly after 4 h of incubation. Slow association and slow dissociation indicate that the sites are in slow equilibrium with CaM. The slow off-rate could also influence the extent of extraction of CaM from membranes during ghost preparation (Table I). The on-rate was driven much faster at 60 nM 

$^{125}$I-CaM (Fig. 7). Erythrocytes contain \( >10^{-5}\) M CaM (7), so binding may be extremely rapid in vivo.

**Analysis of Membrane-binding Affinities and Capacities**

Binding of $^{125}$I-CaM to ghosts and IOVs was measured as a function of CaM concentration (Fig. 8, A and B). Scatchard plots were curvilinear at equilibrium (Fig. 8C) indicating for activation of (Ca$^{2+}$ + Mg$^{2+}$)-ATPase was measured at various times (see under "Methods").

**Fig. 7.** Time course of Ca$^{2+}$-dependent binding of $^{125}$I-CaM to erythrocyte ghosts. $^{125}$I-CaM (1.5 nM (●) or 60 nM (○), 44,300 cpm/pmol) was incubated at 24° C with ghosts (0.17 mg of membrane protein/ml) and Ca$^{2+}$-dependent binding was measured at various times (see under "Methods").

**Fig. 8.** Effect of increasing $^{125}$I-CaM concentrations on Ca$^{2+}$-dependent binding to erythrocyte ghosts (●, A) and IOVs (△, B). Various concentrations of $^{125}$I-CaM were incubated for 180 min at 24° C with ghosts (0.15 mg of membrane protein/ml, A) and IOVs (0.28 mg of membrane protein/ml, B), and Ca$^{2+}$-dependent binding was determined (see under "Methods"). Binding in 0.1 M Hepes, 0.25 mg/ml of gelatin, 2.50 mM NaEGTA with 2.486 nM CaCl$_2$ (pCa$^{2+}$ 5.2), pH 7.3, O - - - O, specific binding in 0.1 M Hepes, 0.25 mg/ml of gelatin 2.60 mM NaEGTA, pH 7.3, x - - - x, V - - - V. The difference represents Ca$^{2+}$-dependent binding, ●, ○, △, and binding to IOVs was corrected to the original ghost protein concentration. Ca$^{2+}$-dependent binding is presented in C according to the Scatchard equation (54): $B/F = N/K - B/K$ where $B = pmol of {^{125}}$I-CaM bound per mg of membrane protein, $F = $ unbound (nanomolar); $K = $ dissociation constant, and $N = $ capacity (pmol/mg).
the IOV membranes after low ionic strength extraction. These conditions most likely remove a different class of CaM-binding proteins and also remove (or damage) a different class of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase which is inactive in solution.

The high affinity binding sites remaining on IOVs most likely represent binding of \(^{125}\)I-CaM directly to the Ca\(^{2+}\) transporter. Estimates of the \(K_D\) and \(K_C\) were nearly identical (Fig. 9). IOVs were estimated to retain \(-400\) nonextractable high affinity binding sites per cell equivalent which is the number of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase copies per erythrocyte estimated from studies of phosphorylated intermediates (32).

This value is much lower than estimates based on turnover number (33), photoaffinity labeling (34), or direct binding to ghosts and IOVs is approximately 0.3 mM and the \(K_D\) for binding is approximately 0.5 mM.

FIG. 9. Effect of increasing CaM concentrations on (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity (A) and Ca\(^{2+}\)-dependent \(^{125}\)I-CaM binding (B). Various concentrations of CaM were incubated for 2 h at 0 °C and then for 1 h at 24 °C with ghosts (●, 0.06 mg of membrane protein/ml) or IOVs (▲, 0.09 mg of membrane protein/ml) in 30 mM KCl, 30 mM Hepes, 80 mM NaCl, 0.5 mM MgCl\(_2\), 0.1 mM ouabain, 0.25 mg/ml of gelatin, 2.50 mM NaEGTA, with or without 2.486 mM CaCl\(_2\) (pCa 5.2), pH 7.30. In A [\(^{32}\)P]ATP (1600 cpm/nmol) and MgCl\(_2\) were then added (final concentrations of 0.2 mM and 0.4 mM) for an additional hour at 24 °C. (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity was calculated from the free P\(_i\) determinations (see Fig. 1). B was identical except that the CaM was \(^{125}\)I-CaM (400,000 cpm/ml), the ATP was unlabeled, and Ca\(^{2+}\)-dependent \(^{125}\)I-CaM binding was determined (see under "Methods"). The \(K_D\) for the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase for both ghosts and IOVs is approximately 0.3 mM and the \(K_D\) for binding is approximately 0.5 mM.

FIG. 10. Effect of mild \(\alpha\)-chymotrypsin digestion of IOVs on Ca\(^{2+}\)-dependent binding of \(^{125}\)I-CaM (top) and (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (bottom). IOVs were incubated with various concentrations of \(\alpha\)-chymotrypsin (0–8.6 \(\mu\)g/ml) in 0.5 ml of 10 mM Hepes, 1 mM NaCl, 0.5 mM dithiothreitol, pH 7.3, for 45 min at 0 °C. The IOVs were then diluted in 20 volumes of 10 mM Hepes, 50 \(\mu\)g of PMSF/ml, and centrifuged (25 min at 44,000 \(\times\) g). The digested IOVs still contained at least 83% of the original protein when resuspended in the original volumes. In the top panel, \(^{125}\)I-CaM (14.4 nM, 45,000 cpm/pmol) was incubated for 90 min at 24 °C with the various vesicle pellets (0.1 mg of membrane protein/ml) and Ca\(^{2+}\)-dependent \(^{125}\)I-CaM binding was determined (●) (see under "Methods"). The inset contains a Scatchard plot (52) from a similar experiment showing Ca\(^{2+}\)-dependent \(^{125}\)I-CaM binding was determined (●) (see under "Methods"). The \(K_D\) for the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase for both ghosts and IOVs is approximately 0.3 mM and the \(K_D\) for binding is approximately 0.5 mM.

Previously equilibrated column due to Ca\(^{2+}\)-dependent interaction with soluble binding sites and was not detected in the absence of Ca\(^{2+}\) (lower panel). The affinity of the association was estimated by separation of bound and unbound \(^{125}\)I-CaM by gel filtration over a range of \(^{125}\)I-CaM concentrations (Fig. 12). Scatchard plots were curvilinear and tangential extrapolation along each of three regions suggests that different solubilized binding sites exist with most points falling along tangent \(Y\) (\(K_D\) = 55 nM, \(N\) = 7.3 pmol/mg based upon the original membrane protein). There also appeared to be a very small number of higher affinity sites (slope \(X\)) and another class of sites (Z) which did not approach saturation at 150 nM \(^{125}\)I-CaM.
plotted according to the Scatchard equation which the extract was made. The concentration refers to mg of protein of the original ghosts from volume (cpm = 34,000 cpm/pmol) in 0.1 M Heps, 2.5 mg/ml of gelatin, 0.2 mM dithiothreitol, 2.50 mM NaEGTA, pH 7.3 with (○) or without (□) 2.497 mM CaCl₂ (pCa₄ = 5.0) for 2 h at 4 °C. Volumes of 0.4 ml were loaded into the appropriate number of paired AcA54 Ultragel columns (1 × 25 cm) previously equilibrated with the same buffer (without I²-I-CaM) and the column was eluted at 5 ml/h at 4 °C. Fractions of 0.3 ml were collected and the excluded volume appeared in fraction 30.

**Fig. 11.** Ca²⁺-dependent binding of I²-I-CaM in solution by low ionic strength extract from erythrocyte ghosts. Binding of I²-I-CaM to low ionic strength extract in solution (see under “Methods”) was measured using an adaptation of the gel filtration method (40). Extract (0.3 mg of protein/ml) was incubated with I²-I-CaM (2.5 nM, 34,000 cpm/pmol) in 0.1 M Heps, 2.5 mg/ml of gelatin, 0.2 mM dithiothreitol, 2.50 mM NaEGTA, pH 7.3 with (○) or without (□) 2.497 mM CaCl₂ (pCa₄ = 5.0) for 2 h at 4 °C. Volumes of 0.4 ml were loaded into the appropriate number of paired AcA54 Ultragel columns (1 × 25 cm) previously equilibrated with the same buffer (containing I²-I-CaM), Heps, gelatin, dithiothreitol, NaEGTA with or without CaCl₂, and the column was eluted at 5 ml/h at 4 °C. Fractions of 0.3 ml were collected and the excluded volume appeared in fraction 30.

**Fig. 12.** Effect of increasing concentrations of I²-I-CaM on Ca²⁺-dependent binding to low ionic extract from erythrocyte ghosts. Extract (see under “Methods”) was incubated with varying amounts of I²-I-CaM (2.5-150 nM, 170,000 cpm/pmol) in 0.1 M Heps, 2.5 mg/ml of gelatin, 0.2 mM dithiothreitol, 2.50 mM NaEGTA, 2.497 mM CaCl₂ (pCa₄ = 5.0) for 2 h at 24 °C. Volumes of 0.1 ml were loaded onto AcA54 Ultragel columns (0.5 × 6 cm) previously equilibrated with the same buffer (without I²-I-CaM) and the column was eluted at 9 ml/h at 24 °C while collecting 0.15-ml fractions. The excluded volume (cpm = bound) appeared at 1.3 ml and the retained (cpm = free) appeared at 2.1 ml. Points represent duplicate determinations plotted according to the Scatchard equation (54), and three parameters were estimated by linear extrapolations (x, y, and z). Protein concentration refers to mg of protein of the original ghosts from which the extract was made.

**Fig. 13.** Cross-linking of I²-I-CaM to low ionic strength extract of erythrocyte ghosts. Ghosts were extracted in low ionic strength buffer (see under “Methods”), and supernatant (“extract”, 0.3 mg of protein/ml) or IOVs (1 mg/ml) were incubated with I²-I-CaM (6 nM, 136,000 cpm/pmol) in 0.1 M Heps, 2.50 mM NaEGTA in the presence or absence of 2.497 mM CaCl₂ (pCa₄ = 5.0) for 2 h at 24 °C under various conditions (in the presence of excess unlabeled CaM or trifluoperazine). Dithiobis-N-hydroxysuccinimidylpropionate (a cross-linker, 41) was added (to 0.25 mg/ml) and incubated an additional hour at 4 °C before addition of glycine (to 0.6 mM) to quench the cross-linking. Aliquots were analyzed by SDS-PAGE (Laemmli 65) adapted to include a 7.5-15% acrylamide gradient with autoradiography. Molecular weight standards were determined by a semilog plot of migration distance of erythrocyte ghosts proteins from a corresponding lane which was stained with Coomassie brilliant blue. All lanes contained I²-I-CaM and NaEGTA. In addition: lane 1, no cross-linker and no CaCl₂; lane 2, no cross-linker with CaCl₂; lane 3, cross-linker and CaCl₂ with 12 nM (instead of 6 nM) I²-I-CaM; lane 4, extract, cross-linker, and CaCl₂; lane 5, extract and cross-linker without CaCl₂; lane 6, 20 nM unlabeled CaM, extract, cross-linker, and CaCl₂; lane 7, 100 nM unlabeled CaM, extract, cross-linker, and CaCl₂; lane 8, 500 nM unlabeled CaM, extract, cross-linker, and CaCl₂; lane 9, 0.1 mM trifluoperazine, extract, cross-linker, and CaCl₂; lane 10, IOVs, cross-linker, and CaCl₂; and lane 11, IOVs and cross-linker without CaCl₂.

neurin by chemical cross-linking (14). I²-I-CaM and solubilized binding proteins were covalently cross-linked with Lomant’s reagent (41) and studied with SDS-PAGE autoradiography (Fig. 13). I²-I-CaM migrated as a single band of ~80,000 even after cross-linking in the presence or absence of Ca²⁺. Two discrete bands were found when I²-I-CaM was cross-linked to solubilized binding proteins in the presence of Ca²⁺ (lane 4). A Mᵦ = 40,000 protein was prominent (Mᵦ = 57,000 when cross-linked to I²-I-CaM). There was also a smaller amount of Mᵦ = 8,000 protein (Mᵦ = 25,000 when cross-linked to I²-I-CaM). The binding was Ca²⁺-dependent (lanes 2 and 3) and inhibited by trifluoperazine (lane 9). The interaction appeared saturable since 20–100 nM unlabeled CaM inhibited I²-I-CaM binding by 50% (lanes 6–7), so the Mᵦ = 8,000 and 40,000 proteins may correspond to class Y sites (Fig. 12). Some radioactivity appeared on top of the lanes. This consisted of I²-I-CaM bound to spectrin dimer (Mᵦ = 466,000) and large I²-I-CaM aggregates which were separated on more porous gels (not shown). The low affinity large capacity sites (class Z, Fig. 12) probably correspond to spec-
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trin, since purified spectrin dimer will cross-link to 125I-CaM in the presence of Ca\(^{2+}\) (not shown). Neither \(M_r = 8,000\) nor 40,000 protein was found free in the cytosol (not shown). Little \(M_r = 40,000\) protein remained on IOVs while about half of the \(M_r = 8,000\) band remained (Fig. 13, lane 10). If estimates of \(R_o\) from Fig. 12 apply to IOVs, it is likely that these contribute to the lower affinity sites (Fig. 8C). It is unlikely that the \(M_r = 8,000\) and 40,000 proteins are degradation products of the Ca\(^{2+}\) transporter since they appear without variation under a variety of extraction conditions, and there is no evidence of proteolytic degradation of ankyrin (42) or protein 4.1 (not shown). Also, there are too many copies of these proteins for them to be derived from the Ca\(^{2+}\) transporter, and their appearance is not accompanied by activation of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase. Cross-linking of 125I-CaM to the \(M_r \sim 150,000\) Ca\(^{2+}\) transporter was inefficient under these conditions but has been accomplished with a photoaffinity label (34). Altogether these observations are most consistent, with the high affinity sites on IOVs corresponding to the Ca\(^{2+}\) transporter and the \(M_r = 8,000\) and 40,000 proteins representing a new class of CaM-binding proteins.

**DISCUSSION**

This report describes detailed studies of binding of 125I-CaM to sites in human erythrocyte membranes which include the Ca\(^{2+}\) transporter as well as two new CaM-binding proteins. The two high affinity CaM-binding proteins of \(M_r = 8,000\) and 40,000 are not likely to be structural proteins since they make up <5 pmol/mg of ghost protein (~1700 copies per cell). These proteins might be CaM-sensitive enzymes or regulatory subunits of enzymes, and it would not be surprising if the erythrocyte should have multiple CaM-dependent enzymes. Micromolar concentrations of CaM would be sufficient to drive several enzyme systems in addition to the Ca\(^{2+}\) transporter, and it is quite possible that other phenomena such as Ca\(^{2+}\)-induced K\(^+\) efflux could be CaM mediated (43). Azido-125I-CaM has been employed under conditions which optimized photoaffinity labeling of the Ca\(^{2+}\) transporter, yet much of the label was associated with other proteins in the IOVs including a band of \(M_r \sim 40,000\) (34). The present study describes extraction and chemical cross-linking methods permitting more direct evaluation of two CaM-binding proteins of \(M_r = 8,000\) and 40,000 with conditions producing only minimal cross-linking to the Ca\(^{2+}\) transporter of IOVs (Fig. 13). Elimination of the function of these two new proteins may provide insight into additional roles of CaM in the erythrocyte, and this is currently under investigation.

Membrane binding of 125I-CaM was very slow at concentrations near 1 mM where binding to the Ca\(^{2+}\) transporter is predominant and required several hours to reach equilibrium (Fig. 7). CaM activation of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (44) and binding of 125I-CaM to erythrocyte ghosts (21, 22) were both interpreted as positively cooperative interactions. Both phenomena might be explained by incomplete binding at the lowest CaM concentrations, for neither were observed in this study when sufficiently long incubations were employed. However, both were observed after short incubations (data not shown). CaM at 1 nM activated (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase after a lag period, but this was eliminated by preincubating membranes with CaM (45). The binding rate, as expected for a bimolecular reaction, was driven much faster at higher concentrations of CaM (Fig. 7). Experimental observations of high affinity interactions require unphysiologic dilutions of CaM (10\(^{-9}\) M), and nonequilibrium experiments are vulnerable to artifacts resembling positive cooperativity due to the slow rate of binding. Also, extraction of native CaM from erythrocyte ghosts may be incomplete due to slow reversal of binding. It was found that the ghosts and IOVs used in this study retained <0.1% of basal erythrocyte CaM (Table I), while a 10- to 20-fold higher level of residual CaM was reported with high basal (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activity (46).

Binding of 125I-CaM to erythrocyte membranes increased as free Ca\(^{2+}\) rose from pCa\(^{8}\) to pCa\(^{6}\). Erythrocyte cytosolic free Ca\(^{2+}\) concentrations were thought to be \(\approx 10^{-6}\) M (9), but free Ca\(^{2+}\) is difficult to measure. Nondisruptive introduction of an intracellular chelator has shown the resting erythrocyte free Ca\(^{2+}\) to be approximately 2 \(\times 10^{-8}\) M (47). Physiological shear stresses have been found to greatly enhance Ca\(^{2+}\) influx (48). Therefore, it is likely that the Ca\(^{2+}\) transporter must respond to a sudden influx of Ca\(^{2+}\) during turbulent arterial flow, pump out Ca\(^{2+}\) until the free concentration is \(\approx 10^{-8}\) M, and then switch off. Ca\(^{2+}\) is considered an essential intracellular signal (49), and it is likely that the shear related influx of Ca\(^{2+}\) produces other CaM-mediated physiologic effects, perhaps a reversible contraction of the membrane skeleton mediated by the \(M_r = 8,000\) or 40,000 CaM-binding proteins. A temporary contraction should help the cell survive rapid flow related stresses and is probably distinct from the pathological Ca\(^{2+}\) effects produced by 10\(^{-3}\) M Ca\(^{2+}\) introduced with ionophores. The high affinity binding of CaM to membrane Ca\(^{2+}\) transporter would also be expected to rise dramatically as free Ca\(^{2+}\) rises above pCa\(^{8}\) (Fig. 3) and would fall off the membrane as the free Ca\(^{2+}\) is reduced (Fig. 6). The ATPase activity of the Ca\(^{2+}\) transporter, however, is negligible below pCa\(^{7}\) and rises to maximum activity near pCa\(^{5}\) (20). Thus there appears to be a discrepancy between the free Ca\(^{2+}\) range required for high affinity CaM binding (pCa\(^{7} - 6.4\)) and the concentration range required for activation of (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (pCa\(^{7} - 5.5\)).

The discrepancy in Ca\(^{2+}\) requirements for membrane binding and (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase activation suggests that two steps are involved. CaM is known to have four different Ca\(^{2+}\) binding sites with micromolar affinities which fill in a pre-ferred sequence, and probably all sites need not be filled in order for the complex to activate some enzymes (50). At submicromolar Ca\(^{2+}\) concentrations it is possible that CaM occupied by a single Ca\(^{2+}\) ion could bind to the (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase which could shift it to a potentially activated form, and a second step would be required for final activation. Perhaps CaM occupied by only one Ca\(^{2+}\) ion will bind to the enzyme but the CaM must be occupied by 2 or 3 additional Ca\(^{2+}\) ions in order for it to completely activate the enzyme. Alternatively, once CaM has bound to the regulator site on the enzyme, additional Ca\(^{2+}\) ions may activate the enzyme directly by binding to the catalytic site of the enzyme as substrate. This hypothesis is likely since partial proteolysis removes the CaM binding regulator site of the enzyme. The digested enzyme is no longer dependent upon CaM but is still dependent upon free Ca\(^{2+}\) very much like the CaM-activated enzyme (20, 36). The \(K_o\) of CaM for Ca\(^{2+}\) and the \(K_M\) of Ca\(^{2+}\) transporter for Ca\(^{2+}\) are both in the micromolar range which is consistent with the Ca\(^{2+}\) concentration being rate limiting for both steps.

Measurement of 125I-CaM binding to erythrocyte ghosts and IOVs may be useful in evaluating clinical disorders such as Duchenne muscular dystrophy (51, 52) or sickle cell anemia.
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REFERENCES


