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Calmodulin modulates protein 4.1 binding to human erythrocyte membranes

Christian R. Lombardo [1], Philip S. Low *

Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

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Abstract

Calmodulin, an abundant protein in the red cell cytosol, exerts its effects on erythrocyte membrane properties via interactions with numerous proteins. To evaluate whether calmodulin might regulate association of protein 4.1 with one of its integral membrane protein anchors, protein 4.1 binding to inside-out erythrocyte membrane vesicles (IOVs) in the presence and absence of calmodulin and Ca^{2+} was examined. Ca^{2+} plus calmodulin was found to competitively inhibit protein 4.1 association with IOVs with a K_i of 1.4 μM and a maximal inhibition of 83%. In the absence of Ca^{2+}, calmodulin still reduced protein 4.1 binding by 43%, consistent with the known Ca^{2+} independent association of calmodulin with protein 4.1. Ca^{2+} alone had no effect on protein 4.1-membrane interactions. Digestion studies revealed that both band 3 and glycophorin sites were similarly affected by calmodulin competition, suggesting all major protein 4.1 anchors are potentially regulated. In light of other data showing regulation of the same interactions by phosphoinositides, protein kinases, and the concentration of free cytosolic 2,3-diphosphoglycerate, it can be argued that association of protein 4.1 with integral protein anchors constitutes one of the more sensitively regulated interactions of the membrane.

Keywords: Protein 4.1; Calmodulin; Band 3; Glycophorin; Erythrocyte membrane

1. Introduction

Elevations in intracellular Ca^{2+} concentration have been measured in a variety of erythrocyte pathologies including sickle cell anemia, β-thalassemia, malaria, and the densest (most rapidly cleared) fraction of normal circulating red cells [1–6]. Because such cells also commonly display abnormal membrane properties, the possible involvement of Ca^{2+} in promoting membrane defects has been frequently proposed. Indeed, Ca^{2+} has been found to not only affect erythrocyte shape and stability [1,3,5,7,8] but to also activate certain enzymes [9,10] and modulate binary and ternary interactions within the membrane skeleton. At the skeletal level, Ca^{2+} has been found to interfere with the ability of spectrin to gel F-actin [11] and to weaken the capacity of adducin and protein 4.1 to stabilize spectrin-actin junctions [12–15]. Consistent with these findings is the observation that calmodulin interacts directly with spectrin, adducin, and protein 4.1 under selected conditions [13–20], providing a possible molecular mechanism whereby certain of the cellular effects of elevated intracellular Ca^{2+} might arise.

Because the tensile properties of the erythrocyte membrane were originally thought to derive almost exclusively from interactions within the two-dimensional spectrin-actin meshwork, no attention was initially paid to possible effects of modulators such as calmodulin on linkages between skeletal proteins and the membrane. Recently, however, the importance of membrane-skeletal interactions on overall membrane stability has been documented [7,21], raising the possibility that such linkages might be regulated by calmodulin also. Since the binding site of calmodulin on protein 4.1 is located within the domain that is thought to mediate membrane association [13,22,23], we decided to examine whether calmodulin might modify the interaction of protein 4.1 with either band 3 or a glycophorin, i.e., the major membrane proteins that bind protein 4.1 in vitro [24,25]. We report here that calmodulin

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PBS, phosphate-buffered saline; IOV, inside-out erythrocyte membrane vesicles; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

* Corresponding author. Fax: +1 (317) 4940239.

[1] Present address: Glaxo Inc., 5 Moore Dr., Research Triangle Park, Raleigh, NC 27709, USA.

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inhibits protein 4.1 association with the membrane in both a Ca^{2+} dependent and Ca^{2+} independent manner. We further show that both band 3 and glycophorin interactions are weakened by the Ca^{2+}-calmodulin complex. Taken together with previously established effects of protein 4.1 phosphorylation [24], membrane phosphoinositide levels [26–28], and free 2,3-diphosphoglycerate concentrations [29] on protein 4.1-membrane interactions, the effects of calmodulin on this association argue that the interaction of protein 4.1 with the membrane is one of the more heavily regulated associations in the erythrocyte.

2. Materials and methods

2.1. Materials

Human blood was purchased from the Central Indiana Regional Blood Center and used within one week of the drawing date. Diisopropylfluorophosphate and bovine serum albumin were obtained from Sigma. Bovine brain calmodulin was purchased from Calbiochem and was devoid of major contaminants as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis [30]. Accell QMA anion exchange resin and a tangential flow filtration apparatus with membrane filters were obtained from Millipore. Calmodulin-Sepharose and Dextran-T-500 were obtained from Pharmacia. PMSF and DTT were from Research Organics. Sucrose was obtained from Schwarz-Mann, chymotrypsin and trypsin from Worthington Biochemical, and Bolton-Hunter reagent was purchased from ICN.

2.2. Methods

Protein purification

Human erythrocyte protein 4.1 was purified by a modification of a previously published procedure [31]. Unless otherwise noted, all steps in the protocol were performed at 0–4°C. Human blood was washed in PBS (135 mM NaCl, 1 mM EDTA, 5 mM Na_2HPO_4, 50 μg/ml PMSF, pH 7.5) three times to remove leukocytes and serum proteins. Complete removal of leukocytes was then accomplished by allowing the erythrocytes to settle through 0.75% Dextran T-500 in PBS (4 l) supplemented with 0.5 mM diisopropylfluorophosphate overnight on ice. The erythrocytes were washed three additional times with PBS, and white ghosts were prepared by repeated washing with lysis buffer (5 mM Na_2HPO_4, 1 mM EDTA, 1 mM NaN_3, 20 μg/ml PMSF, pH 8.0) in a Pellicon tangential flow filtration apparatus until the membranes were white. The resulting ghosts were treated with 2 mM diisopropylfluorophosphate on ice for 30 min and then depleted of band 6 by incubating in 2.5 l of PBS on ice for 30 min, centrifuging, and washing again in PBS. The resulting membranes were converted to inside out vesicles (IOVs) by washing in 0.2 mM EDTA, 0.2 mM DTT, 0.2 mM diisopropylfluorophosphate, pH 9.0, incubating at 37°C for 40 min in 40 vol of the same buffer, and after sedimenting, washing once more in the same buffer. The resulting IOVs were extracted in a final volume of 200 ml with 1.0 M KCl, 25 mM Na_2HPO_4, 1 mM EDTA, 1 mM DTT, 2 mM diisopropylfluorophosphate, pH 7.7 at 37°C for 40 min. The suspension was then centrifuged at 45 000 rpm in a Beckman 45 Ti rotor, and the supernatant was dialyzed overnight against 4 liters of 25 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 50 μg/ml PMSF, pH 7.8 (column buffer). The dialyzed extract was loaded onto a 1×15 cm QMA column, washed with column buffer containing 100 mM KCl, and eluted with a linear KCl gradient from 100 mM to 250 mM in column buffer. Fractions containing purified protein 4.1 were pooled and loaded onto a 5 ml calmodulin-Sepharose column equilibrated in 100 mM KCl, 25 mM Tris, 1 mM EDTA, pH 7.4, and protein 4.1 was eluted with a linear gradient of KCl from 100 to 1000 mM. Protein 4.1 was then radioiodinated for 4 h at 4°C with Bolton-Hunter reagent in 100 mM NaCl, 20 mM Hepes, 1 mM EDTA, 1 mM NaN_3, pH 8.0, and unreacted reagent was removed by dialysis [32]. pH 11 stripped IOVs were prepared as described [24]. Prior to performing experiments, proteins and stripped IOVs were exhaustively dialyzed against buffer A (130 mM KCL, 20 mM NaCl, 10 mM Hepes, 1 mM NaN_3, pH 7.5).

Binding assays

Binding of protein 4.1 to IOVs was assayed in buffer A [25] supplemented with various ratios of Ca^{2+}/EDTA to buffer Ca^{2+} concentration [33]. Assay tubes were coated with 10 mg/ml BSA in buffer A and then rinsed with buffer A prior to performing each binding assay [25]. ^{125}I-protein 4.1 was preincubated with calcium and/or calmodulin for 15 min before addition of stripped IOVs. Binding was initiated by incubation of the desired components for 60 min at 25°C, after which bound protein 4.1 was separated from free protein 4.1 by centrifuging (19 000 rpm, 30 min, Sorvall SS-34 rotor) the IOVs through a 20% sucrose cushion in buffer A containing 2 mg/ml BSA. Tubes were frozen in liquid nitrogen, the tips containing the pelleted IOVs were cut off with a heated razor, and the radioactivity in the severed tip was measured in a gamma counter. Binding assays were performed in duplicate or triplicate.

3. Results

3.1. Calmodulin competitively inhibits protein 4.1 binding to erythrocyte membranes

In view of the proximity of the calmodulin binding site on protein 4.1 to its site of interaction with the membrane [14], it was reasoned that calmodulin might regulate asso-

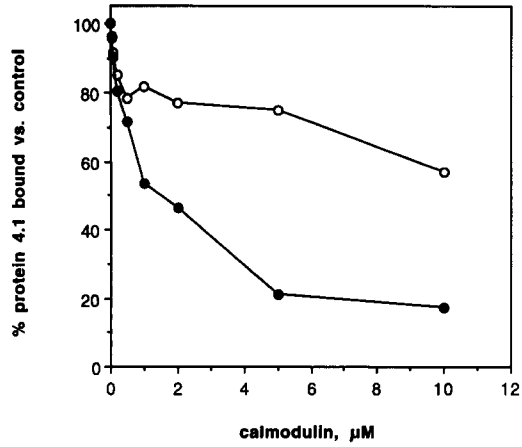

Fig. 1. Calmodulin blocks the association of protein 4.1 with stripped IOVs-^{125}I-protein 4.1 (12 μg/ml) was incubated with increasing concentrations of calmodulin in the absence (○) or presence (●) of 200 μM calcium for 15 min at room temperature. Stripped IOVs (20 μg/ml) were added and binding was allowed to proceed for 60 min, and membrane bound ^{125}I-protein 4.1 was then separated and quantitated as described in Materials and methods.

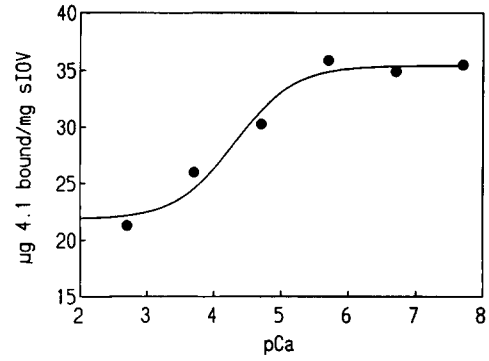

Fig. 3. Calcium dependency of the calmodulin inhibition of protein 4.1 binding-^{125}I-protein 4.1 (12 μg/ml) was incubated with increasing concentrations of calcium in the presence of 5 μM calmodulin. Stripped IOVs were added (20 μg/ml) and after 60 minutes incubation, bound and free ^{125}I-protein 4.1 were separated and quantitated as described in experimental procedures. Note that at physiological calcium concentrations, binding of protein 4.1 is already reduced due to the Ca^{2+}-independent component of calmodulin inhibition. The protein 4.1 concentration used in this study was sufficient to occupy ~30% of available sites.

ciation of protein 4.1 with integral membrane proteins. The results of Fig. 1 confirm this hypothesis. Pre-incubation of protein 4.1 with calmodulin in the absence of calcium reduced its ability to bind stripped IOVs in a concentration dependent manner. By 10 μM calmodulin, protein 4.1 binding was diminished 43%. Inhibition of protein 4.1 binding was even more pronounced in the presence of 200 μM calcium, where only 17% of normal binding was retained. Half maximal calcium-dependent inhibition was obtained at 2 μM calmodulin. Protein 4.1 binding measured in the presence of calcium and calmodulin at different protein 4.1 concentrations indicated that calmodulin inhibition was probably competitive in nature, with a K_i of 1.4 μM calmodulin (Fig. 2). Unfortunately, the Dixon plot cannot unequivocally distinguish competitive from noncompetitive inhibition. Both the concentration of calcium-dependent half maximal inhibition and the derived K_i are well within the physiological range of erythrocyte calmodulin concentrations [34].

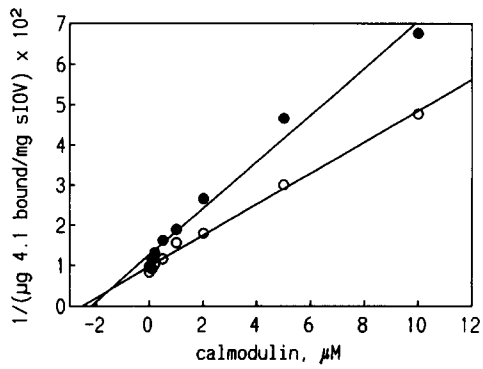

Fig. 2. Dixon Plot of Ca^{2+}/calmodulin inhibition of protein 4.1 binding-^{125}I-protein 4.1 at 12 μg/ml (●) or 20 μg/ml (○) was incubated with increasing concentrations of calmodulin in the presence of 200 μM calcium. Stripped IOVs were added (20 μg/ml), and after 60 min, bound and free ^{125}I-protein 4.1 were separated and quantitated as described in Materials and methods.

3.2. Calcium dependency of inhibition

While it was possible to obtain partial inhibition of protein 4.1 binding in the absence of calcium, even greater reductions in binding were obtained in the presence of 200 μM (Fig. 1). To learn which Ca^{2+} concentrations were most effective in enhancing calmodulin's inhibitory potency, protein 4.1 association with IOVs was assayed in the presence of 5 μM calmodulin and various concentrations of calcium. At normal intracellular calcium levels, inhibition of protein 4.1 binding was comparable to the inhibition seen with calmodulin alone (Fig. 3). However, as Ca^{2+} concentration rose to levels reported in pathological erythrocytes [1,3,5], blockade of protein 4.1 binding also rose significantly. The pCa for half-maximal inhibition was 4.3, i.e., ~50 μM Ca^{2+} (Fig. 3). The effect of calcium in the absence of calmodulin on protein 4.1-membrane interactions was also studied and found to be negligible (Fig. 4), with only 13% inhibition achieved at 2 mM calcium, the highest concentration tested.

3.3. Effects of calmodulin antagonists

The ability of three calmodulin antagonists to reverse the inhibition of protein 4.1 binding to stripped IOVs was studied. Surprisingly, none of the antagonists were capable of blocking calmodulin dependent inhibition, either in the presence or absence of Ca^{2+} (Fig. 5). This behavior may suggest that the calmodulin-protein 4.1 interaction is structurally and functionally different from most other calmodulin-protein complexes. Indeed, Tanaka et al. [13] also

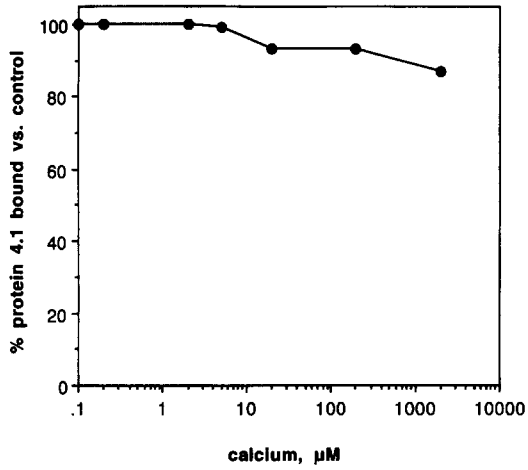

Fig. 4. The calcium dependency of protein 4.1 binding in the absence of calmodulin-^{125}I-protein 4.1 (12 μg/ml) was incubated with increasing concentrations of calcium in the absence of calmodulin. Stripped IOVs were added (20 μg/ml), and after 60 min incubation, bound and free ^{125}I-protein 4.1 were separated and quantitated as described in Materials and methods.

report that calmodulin association with protein 4.1 is Ca^{2+} independent.

3.4. Preliminary analysis of membrane sites affected by calmodulin binding to protein 4.1

Protein 4.1 has been reported to associate with three distinct erythrocyte membrane-spanning polypeptides [24,25,27]. Recent analysis suggests that ~2/3 of the protein 4.1 sites on IOVs reside on band 3, while the remainder of membrane sites are probably divided between glycophorins A and C [26,35–38]. To learn which of the

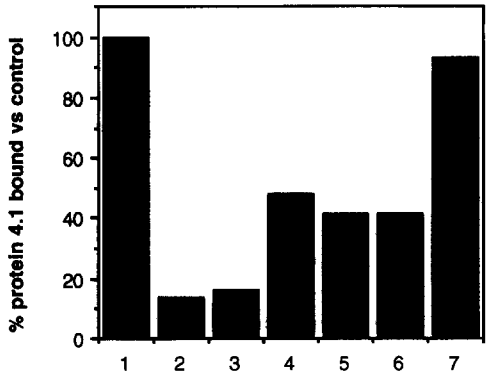

Fig. 5. Effect of calmodulin antagonists on calmodulin inhibition of protein 4.1 binding to IOVs. ^{125}I-protein 4.1 binding to IOV's was assayed as described in Materials and methods. Prior to incubation with the IOVs, ^{125}I-protein 4.1 was equilibrated with: (1) binding buffer (control), (2) 5 μM calmodulin + 200 μM Ca^{2+}, (3) 5 μM calmodulin + 200 μM Ca^{2+} + 5 μM calmidazolium, (4) 5 μM calmodulin + 1 mM EDTA, (5) 5 μM calmodulin + 1 mM EDTA + 100 μM W-13, (6) 5 μM calmodulin + 1 mM EDTA + 100 μM chlorpromazine, (7) 200 μM Ca^{2+}. Binding is indicated relative to the control in lane 1.

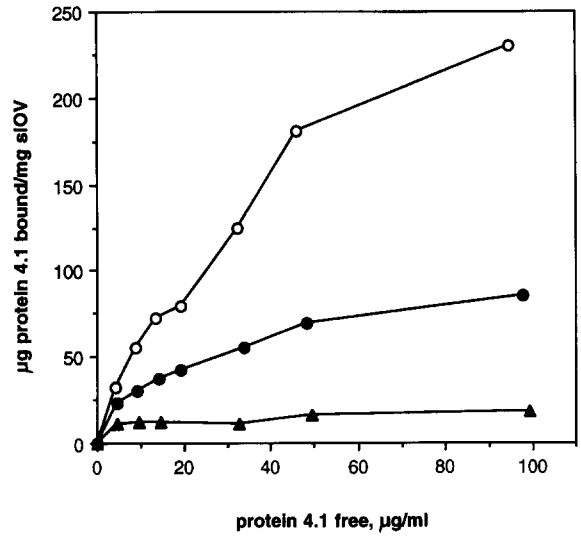

Fig. 6. Effect of calmodulin on binding of protein 4.1 to band 3-deficient IOVs. Increasing concentrations of ^{125}I-protein 4.1 were incubated with unmodified IOVs (○) or trypsin-digested IOVs (● and ▲), and bound protein 4.1 was assessed as described in Materials and methods. To evaluate whether calmodulin might inhibit protein 4.1 association with band 3 depleted IOVs, the depleted IOVs were either incubated with protein 4.1 alone (●) or protein 4.1 that had been pre-equilibrated with 5 μM calmodulin plus 200 μM Ca^{2+} (▲). Tryptic removal of the cytoplasmic domain of band 3 from IOVs was accomplished by digesting stripped IOVs (0.2 mg/ml) with 0.5 μg/ml trypsin for 15 min on ice [23]. Digestions were terminated with 1 mM diisopropylfluorophosphate. The digested membranes were centrifuged and resuspended in buffer A.

above potential membrane anchors might be modulated by cytoplasmic calmodulin, the band 3 sites were selectively removed by tryptic digestion and the effect of 5 μM calmodulin plus 200 μM Ca^{2+} on protein 4.1 binding to residual sites was examined. As shown in Fig. 6, association of protein 4.1 with unmodified IOVs was biphasic, presumably because of its occupancy of both higher affinity/lower abundance glycophorin sites and lower affinity/higher abundance band 3 sites [23]. Removal of the cytoplasmic domain of band 3 by tryptic cleavage then eliminated the majority of low affinity sites as seen by the disappearance of binding manifested mainly at protein 4.1 concentrations greater than 30 μg/ml. Importantly, addition of Ca^{2+} plus calmodulin to these digested IOVs still caused a dramatic reduction in the number of protein 4.1 sites on the membrane. Although such cleavage studies do not allow differentiation of sites on glycophorins A and C, they do at least rule out that only the band 3 linkage is modulated.

4. Discussion

We have demonstrated that interaction of protein 4.1 with the cytoplasmic surface of inside out erythrocyte membrane vesicles is inhibited partially by calmodulin and

more extensively by the Ca^{2+}-calmodulin complex. Membrane binding, however, is not diminished by free Ca^{2+}. The fact that reduction in protein 4.1 binding was observed at normal intracellular calmodulin concentrations suggests the calmodulin effect may be physiologically relevant, especially since other known calmodulin interactions do not require the high calmodulin content seen in normal erythrocytes [34,39,40]. However, whether obstruction of the protein 4.1 interaction with the membrane contributes to calmodulin's effect on membrane stability is questionable, since unligated calmodulin can partially inhibit protein 4.1 binding to IOVs even though it has no effect on membrane stability [12]. This result is consistent with the recent observation that only the spectrin/actin binding domain of protein 4.1 (~ 60 amino acids) is required to restore normal stability to protein 4.1 deficient erythrocytes [41], i.e., suggesting the membrane association of protein 4.1 may have a function that is not readily detected by ektacytometry. Unfortunately, since this other possible function has not yet been resolved, the significance of its modulation by calmodulin must also remain undefined.

Calmodulin has been reported to interact with protein 4.1 at its 30 kDa N-terminal chymotrypsin-resistant domain [13,38]. The fact that calmodulin association blocks binding to both band 3 and one or more glycophorins suggests that the 30 kDa domain may mediate the major protein 4.1-membrane interactions. Earlier studies with *N*-ethylmaleimide-modified protein 4.1 alluded to this same conclusion, since the site of derivatization was similarly localized to the 30 kDa domain and the derivatized protein 4.1 exhibited reduced membrane binding [23].

Protein 4.1 is present in a diversity of cell types [42–47]. As a consequence of alternative mRNA splicing, it also exists in multiple isoforms of differing masses and properties [8,48]. Importantly, none of the splicing isoforms lacks the calmodulin binding domain, suggesting that protein 4.1 in all cell types may be subject to calmodulin regulation. Obviously, until the functions of protein 4.1 in nonerythroid cells are also understood, the significance of this conserved feature must remain similarly obscure.

Although the role of protein 4.1-membrane interactions in maintenance of red cell membrane architecture is still a matter of conjecture, if its level of regulatory control provides an indication of its significance to the cell, the interaction must constitute a very important junction. Thus, in addition to its inhibition by calmodulin, protein 4.1-membrane interactions are regulated by phosphorylation of protein 4.1 by both protein kinase C and casein kinase [34,49] by the degree of phosphorylation of phosphatidylinositols in the membrane [28,34] and by the changing levels of intracellular free 2,3-diphosphoglycerate [29]. Based on information available to date, it can be reasonably argued that no other erythrocyte membrane interaction is more diversely regulated. Perhaps the most critical function of the protein 4.1-membrane association still remains to be described.

Acknowledgements

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