Modulation of Erythrocyte Membrane Material Properties by Ca^{2+} and Calmodulin

Implications for Their Role in Regulation of Skeletal Protein Interactions

Yuichi Takakuwa and Narla Mohandas
Department of Laboratory Medicine and Cancer Research Institute, University of California, San Francisco, California 94143; and Department of Biochemistry, School of Medicine, Hokkaido University, Sapporo, Japan 060

Abstract

Skeletal proteins of the red blood cell apparently play an important role in regulating membrane material properties of deformability and stability. However, the role of various intracellular constituents in regulating membrane properties has not been clearly defined. To determine whether Ca^{2+} and calmodulin might play a role in this regulation, we measured the membrane stability and deformability of resealed ghosts prepared in the presence of varying concentrations of Ca^{2+} and calmodulin (CaM). For membranes resealed in the presence of Ca^{2+} and physiologic concentrations of CaM (2–8 μM), membrane stability decreased with increasing Ca^{2+} concentrations (> 1.0 μM). Moreover, Ca^{2+} and CaM-induced alterations in membrane stability were completely reversible. In the absence of CaM, an equivalent decrease in membrane stability was seen only when Ca^{2+} concentration was two orders of magnitude higher (> 100 μM). Calmodulin did not alter membrane stability in the absence of Ca^{2+}. Compared with these changes in membrane stability, membrane deformability decreased only at Ca^{2+} concentrations > 100 μM, and calmodulin had no effect on Ca^{2+}-induced decrease in membrane deformability. Examination of the effects of Ca^{2+} and CaM on various membrane interactions have enabled us to suggest that spectrin-protein 4.1-actin interaction may be one of the targets for the effect of Ca^{2+} and CaM. These results imply that Ca^{2+} and calmodulin can regulate membrane stability through modulation of skeletal protein interactions, and that these protein interactions are of a dynamic nature on intact membranes.

Introduction

Calmodulin (CaM)[1] is a highly conserved calcium-binding protein that is present in a wide variety of eukaryotic cells (1–3). CaM has previously been shown to mediate a number of Ca^{2+}-dependent enzyme and cellular functions in a wide variety of cell types (1–3). The human erythrocyte contains micromolar concentrations of CaM (4, 5). One important identified function of this protein in red cells is the regulation of the activity of membrane-bound Ca^{2+}-ATPase (6, 7). CaM and Ca^{2+}-ATPase working in concert are able to maintain extremely low levels of free Ca^{2+} (< 1 μM) in the red cell cytosol against a massive calcium concentration gradient across the membrane (plasma Ca^{2+} concentrations: 2–3 mM) (8, 9). However, it should be pointed out that only a small fraction (< 5%) of CaM present in the cell is necessary for complete activation of Ca^{2+}-ATPase (10), and the function of the remaining CaM is not known. At present, it is not clear if Ca^{2+} and CaM can alter membrane material properties through their interaction with skeletal proteins.

Evidence suggesting a role for Ca^{2+} in regulating skeletal protein interaction was provided by earlier studies of Fowler and Taylor (11) and Cohen et al. (12). These investigators showed that low shear viscosity of mixtures of spectrin, actin, and protein 4.1 are dependent on the Ca^{2+} concentration. The low shear viscosity of spectrin, actin, and protein 4.1 mixtures increased as the Ca^{2+} concentration was increased from 0.01 to 0.5 μM. Surprisingly, the viscosity decreased with further increases in Ca^{2+} concentration. A possible role for CaM in regulating skeletal protein interactions was also suggested by studies that showed that this protein can bind to the erythrocyte membrane (13, 14) as well as to various skeletal proteins (spectrin monomer [5, 15, 16], $\alpha\beta$ spectrin dimer [14, 17], and protein 4.1 [18]). Gardner and Bennett (19) also recently purified a CaM-binding protein from the erythrocyte membrane, and suggested that this protein is associated with the membrane skeleton. Together, these observations suggest that Ca^{2+} and CaM have the potential to modulate skeletal protein interactions.

In this study, we have attempted to determine if Ca^{2+} and CaM could modulate material properties of intact erythrocyte membranes through their interaction with the skeletal protein network. Using an ektacytometer, we measured membrane stability and deformability of white ghosts prepared in the presence of physiologic concentrations of CaM and various concentrations of Ca^{2+}. Our data show that Ca^{2+} in the concentration range of 1.0 to 100 μM can markedly decrease membrane stability only in the presence of CaM, but not in its absence. Examination of the effects of Ca^{2+} and CaM on various membrane protein interactions that have previously been shown to be important in regulating membrane stability has enabled us to suggest that the observed changes in membrane stability may be partly related to the effects of Ca^{2+} and CaM on spectrin-protein 4.1-actin interaction.

These data identify an important new functional role for Ca^{2+} and CaM, that of regulating erythrocyte membrane mechanical stability through modulation of skeletal protein interactions.

Address reprint requests to Dr. Narla Mohandas, Cancer Research Institute, Box 0128, M-1282, University of California, San Francisco, Third and Parnassus, San Francisco, CA 94143-0128.

Received for publication 22 October 1986 and in revised form 26 January 1988.

1. *Abbreviations used in this paper:* CaM, calmodulin; DI, deformability index.

J. Clin. Invest.

0021-9738/88/08/0394/07 $2.00
Volume 82, August 1988, 394–400

Methods

Materials. Calmodulin purified from bovine brain (20) was obtained from Sigma Chemical Co. (St. Louis, MO). Calmidazolium was purchased from Boehringer Mannheim Diagnostics, Inc. (Indianapolis, IN). Protein 4.1 and spectrin dimer were purified according to the method of Tyler et al. (21), with some minor modifications. These purified proteins were also labeled with ^{125}I using Bolton-Hunter reagent (2,000 Ci/mmol) (22), purchased from New England Nuclear (Boston, MA), for use in some experiments. CaM-agarose was purchased from Bio-Rad Laboratories (Richmond, CA). Purified rabbit muscle G actin was provided by Dr. R. Takashi of the University of California, San Francisco.

Preparation of ghosts. Ghosts were prepared by lysing washed red blood cells with hypotonic buffer (5 mM Tris, 7 mM KCl, and 0.5 mM EGTA, pH 7.4). White ghosts were obtained by multiple washing of the ghosts with the same buffer. Repeated washing with the buffer containing EGTA enabled almost complete removal of native cytoplasmic and membrane-bound erythrocyte CaM (13). The membranes were subsequently resealed in the isotonic buffer (20 mM Tris, 130 mM KCl, 10 mM NaCl, and 0.5 mM EGTA, pH 7.4) with various concentrations of $CaCl_2$ and CaM. In this mixture, Ca^{2+} concentration was regulated by the use of Ca^{2+}-EGTA buffer (23). The mixture was incubated at 37°C for 30 min to allow ghosts to reseal. To evaluate the effect of CaM antagonists, various concentrations of these reagents were included in the resealing buffer.

Measurement of membrane stability and deformability. The resealed ghosts were suspended in dextran (40,000 mol wt, 35% wt/vol) and examined by the ektacytometer, a laser diffraction method, as previously described (24, 25). Briefly, suspended ghosts were subjected to a constant shear stress of 750 dyn/cm^2 and the change in their laser diffraction pattern was measured by recording a signal designated as the deformability index (DI) as a function of time. The DI provides a measure of the ellipticity of the deforming ghosts in the flow field. When the shear stress is applied, ghosts are deformed into ellipsoids and produce a narrow elliptical pattern that generates a high value of DI. With time, as the ghosts are unable to withstand the large value of applied shear stress, they begin to fragment. The resultant loss of membrane surface produces decreasing DI values. The rate at which the DI decreases is a measure of the rate of membrane fragmentation, and hence provides us with a quantitative measure of membrane stability. The time required for the DI to reach half the maximum value initially attained is designated $t_{1/2}$. To evaluate relative changes in membrane stability, we compared $t_{1/2}$ values for the various membrane preparations.

The property of membrane deformability that determines the extent of membrane deformation that can be induced by a defined level of applied force was also measured by the ektacytometer. For resealed membranes, the shear stress required to obtain a defined value of DI is determined by the property of membrane deformability, without contributions from either internal viscosity or cell volume (26). There is a correlation between changes in deformability measured by this technique and those measured using the micropipette (27, 28). To measure this property, resealed ghosts were suspended in Stractan (22 centipoise viscosity, 290 mosM). The ghosts were then subjected to a linearly increasing shear stress in the ektacytometer. The DI was measured as a function of applied shear stress. When the DI is plotted on a linear scale and the shear stress is plotted on a logarithmic scale, a linear relationship is seen between these two variables for normal membranes. For membranes with decreased membrane deformability, the linear relationship still exists; however, there is a parallel displacement of the lines to higher values of shear stress. By determining the magnitude of increase in shear stress required to obtain equivalent DI along these lines, a value for relative membrane deformability is derived (29).

Red cell membrane protein analysis. To assess the effect of Ca^{2+} and CaM on spectrin self-association, nondenaturing PAGE (30) was performed on spectrin extracted from variously treated membranes. Spectrin from membranes prepared in the presence of either 100 μM Ca^{2+} alone, 2 μM CaM alone, or both 100 μM Ca^{2+} and 2 μM CaM was extracted by dialysis against hypotonic buffer (0.3 mM Na phosphate, 0.1 mM EDTA, and 0.1 mM PMSF, pH 8.0) at 4°C for 17 h. The percentage of spectrin dimer (expressed as dimer/dimer plus tetramer) was determined by densitometric analysis of the nondenaturing gels.

For analysis of the association of glycophorin β (glycophorin C) with the membrane skeleton, the sialoglycoprotein content of Triton shells was determined. Membranes that had been prepared in the presence of either 100 μM Ca^{2+} alone, 2 μM CaM alone, or both 100 μM Ca^{2+} and 2 μM CaM were treated with 1% Triton X-100 in 5 mM Na phosphate and 1 mM DTT, pH 7.4. The resultant Triton shells were analyzed by SDS-PAGE and Western blot analysis (31) using an MAb (NBTS/BRIC-10) that recognizes an epitope on the NH_2-terminus of β-sialoglycoprotein (32).

Binding studies. Protein 4.1-stimulated spectrin to F actin binding was measured by cosedimentation assay (33). G actin was polymerized at 3.5 mg/ml by incubation in 0.2 mM ATP, 0.5 mM DTT, 50 mM NaCl, 2 mM $MgCl_2$, and 2 mM Tris (pH 8.0). 192 μg of F actin was mixed with 10 μg of ^{125}I-spectrin and 42 μg of protein 4.1 in 130 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT, and 20 mM Tris (pH 7.4) in the presence of varying concentrations of Ca^{2+} and calmodulin. The protein mixture was incubated at room temperature for 1 h. A 120-μl aliquot of the mixture was then layered over 200 μl of 5% sucrose in incubation buffer, the spectrin-actin complex was sedimented by centrifugation at 30,000 *g* for 3 h at 4°C, and the amount of ^{125}I-spectrin in the pellet was quantitated.

Results

The effect of added Ca^{2+} and CaM on the membrane stability of ghosts from which endogenous CaM has been removed is shown in Fig. 1. When resealed membranes were subjected to an applied shear stress of 750 dyn/cm^2, membrane fragmentation occurred over a period of time. The extent of membrane fragmentation, which is a measure of mechanical stability, was quantitated by the rate of decrease in DI. To compare changes

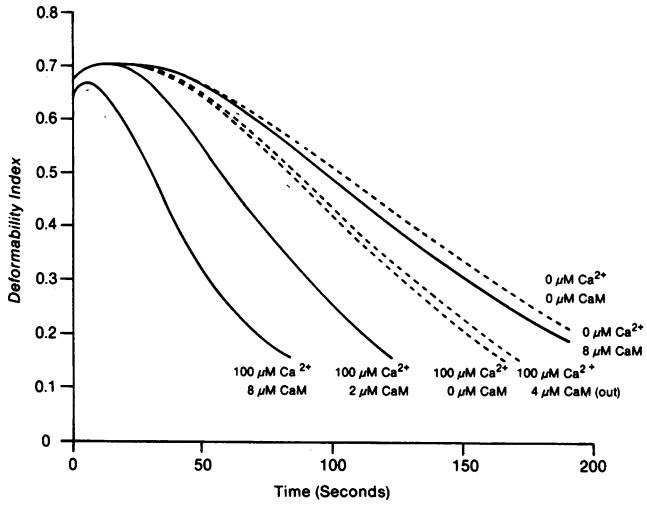

Figure 1. Ca^{2+}-CaM-induced change in erythrocyte membrane stability. Resealed ghosts were prepared in the presence or absence of Ca^{2+} (0 or 100 μM) and various concentrations of CaM (0, 2, 4, and 8 μM) and exposed to 750 dyn/cm^2 in the ektacytometer. In one experiment, 4 μM CaM was added to membrane first resealed in the presence of 100 μM Ca^{2+} (designated by 4 μM CaM [out]). This maneuver allowed the CaM to have access to the outer surface of the membrane, and not to the cytoplasmic side, as in all other experiments. The decline of the DI was measured as a function of time to quantitate membrane mechanical stability.

in membrane stability, the time required for the DI to decrease to half the maximum value ($t_{1/2}$) was determined. A decrease in membrane stability was reflected by a decrease in the measured value of $t_{1/2}$. For white ghosts resealed in the absence of both Ca^{2+} and CaM, the $t_{1/2}$ was 145 s. The incorporation of 8 μM CaM into these membranes did not alter their membrane stability, whereas incorporation of 100 μM Ca^{2+} resulted in a small decrease in mechanical stability ($t_{1/2}$ = 116 s). However, when the membranes were resealed in the presence of both 2 μM CaM and 100 μM Ca^{2+}, the decrease in membrane stability was more pronounced ($t_{1/2}$ = 83 s), and increasing the concentration of CaM to 8 μM, whereas maintaining the Ca^{2+} concentration at 100 μM resulted in even greater decrease in membrane stability ($t_{1/2}$ = 46 s). These data imply that CaM by itself does not affect membrane stability, but that in conjunction with Ca^{2+}, it can markedly decrease membrane stability.

To establish that CaM must have access to the cytoplasmic side of membrane to affect membrane stability, we added CaM to membranes that had been previously resealed in the presence of 100 μM Ca^{2+}. As shown in Fig. 1, the addition of CaM to the outside of ghosts after resealing with 100 μM Ca^{2+} did not decrease their membrane stability, implying that the effect of CaM on membrane stability is due to its effect on the cytoplasmic side of the membrane.

To determine if the decreases in membrane stability induced by the combined effect of Ca^{2+} and CaM can be reversed, the following series of experimental manipulations were performed; the data from these experiments is shown in Fig. 2. Ghosts, first resealed in the presence of either EGTA, Ca^{2+}, or both Ca^{2+} and CaM were relysed in 100 vol of lysing buffer containing EGTA and at the second step were resealed in the presence or absence of Ca^{2+} and CaM. As can be seen in Fig. 2, only membranes that contained Ca^{2+} and CaM during the second step of resealing showed decreased membrane stability. Membranes resealed with Ca^{2+} and CaM during the first step, but when subsequently resealed with EGTA had the same stability as membranes that were not exposed to CaM during both steps of resealing. These data imply that the effect of Ca^{2+} and CaM on membrane stability is indeed reversible and that this decrease in stability is not due to proteolysis of skeletal proteins by some Ca^{2+}-dependent proteases, a process that would lead to irreversible changes in membrane stability.

To further document an important role for CaM in these observed changes in membrane stability, we quantitated Ca^{2+}-CaM-induced alterations in membrane stability in the presence of calmidazolium (previously referred to as R24571), a potent CaM antagonist (34, 35). As shown in Fig. 3, this CaM antagonist was able to completely inhibit Ca^{2+}-CaM-induced change in membrane stability. Complete inhibition was seen at a calmidazolium concentration of 5 μM when the membranes were resealed in the presence of 2 μM CaM and 100 μM Ca^{2+}. Increasing the CaM concentration to 4 μM required higher calmidazolium concentration to inhibit the observed change in membrane stability, suggesting a competitive inhibition process. Calmidazolium alone up to concentrations of 5 μM did not alter stability of membranes resealed in the absence of Ca^{2+} and CaM or in the presence of 100 μM Ca^{2+} alone (data not shown). This complete inhibition of Ca^{2+}-CaM-induced change in membrane stability by a CaM antagonist further supports the contention that alteration in membrane stability is the result of the interaction of both Ca^{2+} and CaM with the membrane. Another CaM antagonist, trifluo-

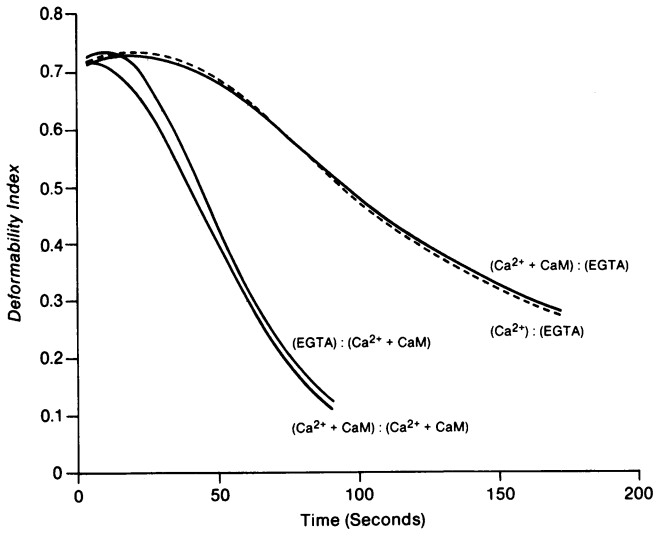

Figure 2. Reversibility of Ca^{2+}-CaM-induced decrease in erythrocyte membrane stability. Resealed ghosts prepared in the presence of 100 μM Ca^{2+} and 4 μM CaM were relysed and then resealed for the second time in the presence and absence of 100 μM Ca^{2+} and 4 μM CaM (designated [Ca^{2+} + CaM]:[Ca^{2+} + CaM] and [Ca^{2+} + CaM]:[EGTA], respectively). One control consisted of ghosts first resealed with EGTA and relysed and then resealed in the presence of 100 μM Ca^{2+} and 4 μM CaM ([EGTA]:[Ca^{2+} + CaM]). The second control consisted of ghosts first resealed in the presence of 100 μM Ca^{2+} and no CaM and relysed and then resealed with EGTA in the absence of Ca^{2+} and CaM ([Ca^{2+}]:[EGTA]). The decline of DI for the resealed ghosts at the second stage was measured as a function of time to quantitate membrane stability.

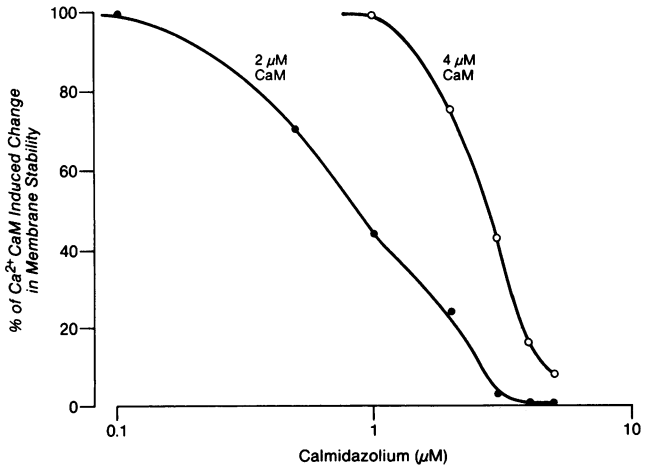

Figure 3. Inhibition of Ca^{2+}-CaM-induced change in membrane stability by CaM inhibitor, calmidazolium. Resealed ghosts were prepared in the presence of 100 μM Ca^{2+} and 2 and 4 μM CaM, together with various concentrations of calmidazolium. Membrane stability of the various preparations was measured and $t_{1/2}$ was derived. The difference in the measured values of $t_{1/2}$ for membranes resealed with Ca^{2+} alone and Ca^{2+} and CaM was defined as 100% induced change in membrane stability and was used to normalize the data obtained after addition of calmidazolium. Note that calmidazolium by itself did not affect stability of the ghosts prepared in the presence of 100 μM Ca^{2+} and no CaM.

perazine, also inhibited the Ca^{2+}-CaM-induced change in membrane stability (data not shown). However, this effect could not be unequivocally evaluated because this drug, by itself, marginally decreased membrane stability in the absence of Ca^{2+}, probably through its intercalation into the lipid bilayer.

Ca^{2+} concentration dependence of this Ca^{2+}-CaM-induced decrease in membrane stability is illustrated in Fig. 4. In the presence of 2 μM CaM, Ca^{2+} concentration-dependent decrease in membrane stability was seen for Ca^{2+} concentration > 1.0 μM. The Ca^{2+}-induced decrease in membrane stability was more pronounced when CaM concentration was increased to 8 μM. In the absence of CaM, a decrease in membrane stability was not seen until Ca^{2+} concentration exceeded 100 μM, and an equivalent decrease in membrane stability was seen only when Ca^{2+} concentration was two orders of magnitude higher (> 100 μM). It should be emphasized that, in the presence of physiologic concentrations of CaM, membrane stability could be modulated by Ca^{2+} in the concentration range of 1.0 to 10 μM. This suggests that the observed change may play a role in altering membrane stability of abnormal red cells in which Ca^{2+} homeostasis is deranged.

The CaM concentration dependence of the Ca^{2+}-CaM-induced decrease in membrane stability is shown in Fig. 5. At fixed Ca^{2+} concentrations of 100 and 500 μM, a CaM concentration-dependent decrease in membrane stability was seen. The maximal effect was seen at a CaM concentration of 8 μM. In the absence of Ca^{2+}, CaM up to a concentration of 16 μM induced only minimal changes in membrane stability.

Having shown that Ca^{2+} and CaM can induce alterations in membrane stability, we then examined if another distinct membrane property, that of membrane deformability, is also altered by the interaction of Ca^{2+} and CaM with the membrane. The relative membrane deformability of resealed ghosts prepared with increasing concentrations of Ca^{2+} in the presence and absence of 2 μM CaM was measured, and the data are shown in Fig. 6. A decrease in relative membrane deformability implies that an increased value of applied shear stress was needed to reach the same extent of membrane deformation. For example, a relative membrane deformability of 0.5 implies that a twofold higher shear stress was needed to obtain the same DI. As can be seen in Fig. 6, membrane deformability was unchanged up to Ca^{2+} concentrations of 100 μM. At higher Ca^{2+} concentrations, the membrane became progressively less deformable. Most importantly, compared with the effect on membrane stability, the presence of CaM had no effect on Ca^{2+}-induced changes in membrane deformability.

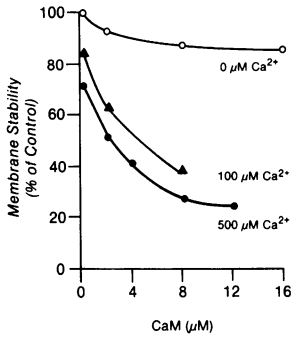

Figure 5. Dependence of membrane stability changes on CaM concentration in the presence and absence of Ca^{2+}. Resealed ghosts were prepared in the presence of 0 (○), 100 (▲), or 500 (●) μM Ca^{2+} and varying concentrations of CaM (0–16 μM). Membrane stability of the ghosts was measured and $t_{1/2}$ was plotted against CaM concentrations used. In these plots, $t_{1/2}$ obtained with control ghosts prepared in the absence of both Ca^{2+} and CaM was used to normalize the stability data.

To obtain insights into the molecular mechanism(s) responsible for Ca^{2+}-CaM-induced decrease in membrane stability, we adopted the following approach. Based on our current understanding of red cell membrane protein organization, at least three different protein interactions (illustrated in Fig. 7) have been shown to play a key role in regulating membrane mechanical stability. These include spectrin dimer-dimer interaction, spectrin-protein 4.1-actin interaction, and glycophorin β-protein 4.1 interaction. As defects involving each of these interactions result in decreased stability (36–41), we examined the effect of Ca^{2+} and CaM on these interactions. Spectrin dimer-dimer interaction was evaluated by determining the spectrin dimer content of low ionic strength extracts of spectrin at 4°C from variously prepared membranes. Spectrin dimer content of untreated membrane was 11%, whereas those treated with 100 μM Ca^{2+} alone, 2 μM CaM alone, and both 100 μM Ca^{2+} and 2 μM CaM were 11, 12, and 12%, respectively. These data suggest that Ca^{2+} and CaM do not influence spectrin dimer-dimer interaction. Since interaction between glycophorin β (glycophorin C) and membrane skeleton is apparently important for normal membrane stability, we next evaluated whether Ca^{2+} and CaM dissociated this important interaction. Glycophorin β association with the membrane skeleton was examined by the extractability of this glycoprotein from untreated and Ca^{2+} and CaM-treated membranes after treatment with Triton X-100. Western blot analysis using an MAb against glycophorin β showed that, after treatment with both Ca^{2+} and CaM, this sialoglycoprotein continued to be associated with the membrane skeleton and was not re-

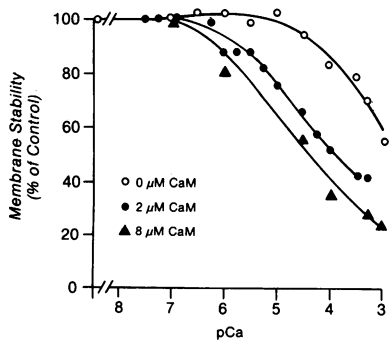

Figure 4. Dependence of membrane stability changes on Ca^{2+} concentration in the presence and absence of CaM. Ghosts were resealed in the presence of 0 (○), 2 (●), or 8 (▲) μM of CaM and various concentrations of Ca^{2+}. Membrane stability of the ghosts was measured and $t_{1/2}$ was plotted against Ca^{2+} concentrations used. In these plots, the membrane stability was normalized using $t_{1/2}$ obtained for control ghosts prepared in the absence of Ca^{2+}. $t_{1/2}$ value for control ghosts (no Ca^{2+}) was taken to be 100%. pCa represents log (Ca^{2+}).

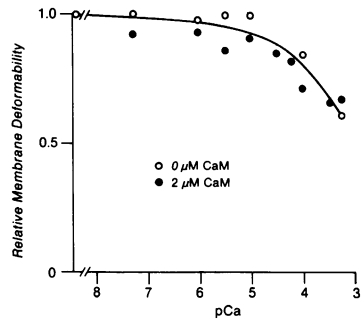

Figure 6. Effect of Ca^{2+} on membrane deformability in the presence and absence of CaM. Resealed ghosts were prepared in the presence (●) or absence (○) of 2 μM CaM and various concentrations of Ca^{2+}. A decrease in relative membrane deformability implies that an increased value of applied shear stress is needed to reach the same extent of membrane deformation.

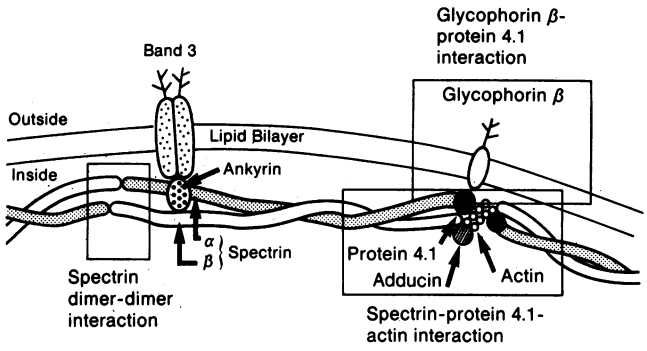

Figure 7. A schematic diagram of the erythrocyte membrane organization based on our current understanding of various protein associations. The three key protein interactions identified to date that are considered to be important in regulating erythrocyte membrane stability are shown in an enclosed rectangle. These include spectrin dimer-dimer interaction, glycophorin β-protein 4.1 interaction, and spectrin-protein 4.1-actin interaction. Adducin, a recently identified membrane skeleton-associated calmodulin-binding protein, promotes binding of spectrin to actin filaments.

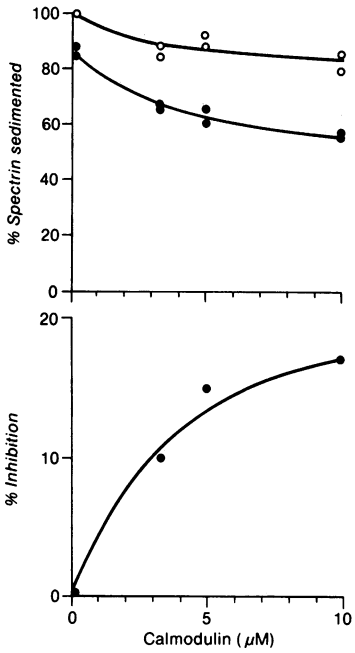

Figure 8. Inhibition of ^{125}I-spectrin binding to F-actin by calcium and calmodulin. (*Top*) ^{125}I-spectrin, F-actin, and protein 4.1 were incubated with various concentrations of CaM in the presence of 100 μM $CaCl_2$ (●) or 1 mM EGTA (○) for 60 min at room temperature. The samples were then sedimented and the amount of ^{125}I-spectrin in the pellet was quantitated. The amount of spectrin pelleted in the presence of 1 mM EGTA with no added CaM was used to normalize the data. (*Bottom*) Calcium-dependent, CaM inhibition of spectrin binding to F-actin.

leased into the supernatant fraction (data not shown). This finding suggests that Ca^{2+} and CaM do not perturb the physical association between glycophorin β and the membrane skeleton, since such a perturbation would have resulted in the release of this glycoprotein from the membrane during Triton extraction.

A series of experiments was performed to examine the effect of Ca^{2+} and CaM on spectrin-actin-protein 4.1 interaction. The effect of exogenously added purified protein 4.1 and spectrin dimer on Ca^{2+}-CaM-induced decrease in membrane stability was examined. In the absence of added protein 4.1, Ca^{2+} and CaM decreased membrane stability by 68% (relative membrane stability = 0.32). With increasing concentration of added protein 4.1 (0.25–2 μM), the Ca^{2+}-CaM-induced decrease in membrane stability was ameliorated. At a concentration of 2 μM added protein 4.1, the stability of the Ca^{2+}-CaM-treated membrane was decreased by only 23% (relative membrane stability = 0.77). We were unable to determine if complete restoration could be achieved, as it was technically difficult to obtain preparations with protein 4.1 concentrations > 2 μM. In the absence of either Ca^{2+} or CaM, added protein 4.1 had minimal effect on membrane stability. Importantly, compared with protein 4.1, spectrin dimer up to a concentration of 2 μM had no ameliorating effect on the Ca^{2+}-CaM-induced decrease in membrane stability.

Whereas these data suggest a possible role for protein 4.1 in accounting for Ca^{2+}-CaM-induced alterations in mechanical stability, it does not imply that alterations in spectrin-actin-protein 4.1 interaction may be responsible for observed membrane changes. To address this issue directly, the effect of Ca^{2+} and CaM on protein 4.1-stimulated binding of spectrin to actin was examined, and the data is shown in Fig. 8. Increasing concentrations of CaM in the presence of 100 μM Ca^{2+} reduced the amount of spectrin cosedimenting with F actin. 100 μM Ca^{2+} alone mildly suppressed the protein 4.1-stimulated binding of spectrin to actin; however, in the presence of CaM, a more marked decrease in spectrin binding was noted. In the presence of 1 mM EGTA and varying concentrations of CaM, the amount of spectrin cosedimenting with F actin was only marginally reduced. The extent of Ca^{2+}-specific inhibition of protein 4.1-stimulated binding of spectrin to actin in the presence of CaM is also shown in Fig. 8. It can be seen that at 10 μM CaM and 100 μM Ca^{2+}, spectrin binding to F actin is inhibited by ~ 20%. These data suggest that CaM in concert with Ca^{2+} can alter the protein 4.1-modulated interaction of spectrin with actin.

Discussion

These studies show that Ca^{2+} in the concentration range of 1.0 to 100 μM, in conjunction with physiologic concentrations of CaM, can modulate the mechanical stability of the erythrocyte membrane. As this material property of the membrane is regulated by skeletal protein interactions (28, 29), this finding implies that the observed change in membrane stability is likely to be a consequence of Ca^{2+} and CaM regulating the dynamics of the skeletal protein interactions. Furthermore, the observation that Ca^{2+} by itself in this concentration range (1.0–100 μM) did not alter membrane stability implies that CaM is an important cofactor in modulating this membrane function. Further support for a key role for CaM can be inferred from the finding that CaM inhibitors can block the Ca^{2+}-CaM-induced decrease in membrane stability. Note, however, that concentrations of calcium required to induce changes in membrane stability (half-maximal activity at 10–20 μM Ca^{2+}) are higher than that required for other Ca^{2+}-CaM-dependent processes (1–5 μM).

The properties of membrane deformability and stability are principally regulated by membrane protein interactions (42, 43). Our finding that Ca^{2+} and CaM can reversibly alter membrane stability suggests that some of these protein associations may be modulated by the interaction of Ca^{2+} and CaM with these protein components. Previous studies using biochemically perturbed normal erythrocytes (29, 44) and various pathologic red cells with defined skeletal protein and sialoglycoprotein abnormalities (40, 41) have allowed the identification of at least three major protein associations involved in regulating membrane stability, and these have been schematically identifed in Fig. 7. Defective spectrin dimer-dimer associ-

ation as a result of sulfhydryl blockage of spectrin in normal erythrocytes and a number of different molecular defects in the αI domain of spectrin in hereditary elliptocytosis (36, 38, 39) lead to a marked decrease in membrane stability (29, 44). Glycophorin β (glycophorin C) deficiency also leads to decreased membrane stability, and this finding has been interpreted to suggest a role for glycophorin β interaction with membrane skeleton in regulating membrane stability (40). Finally, defective spectrin-protein 4.1-actin association, either due to a quantitative deficiency of protein 4.1, due to a quantitative defect in β spectrin, or due to perturbation of the interaction by polyphosphates has been shown to result in decreased membrane stability (29, 45, 46). Our finding that Ca^{2+} and CaM had no effect on either spectrin dimer-dimer association or glycophorin β association with membrane skeleton suggests that the effects we have observed are not due to Ca^{2+}-CaM-induced perturbation of these two protein interactions.

The finding that exogenously added protein 4.1 can inhibit Ca^{2+}- and CaM-induced decrease in membrane stability and our observation that CaM in concert with Ca^{2+} decreases protein 4.1-stimulated binding of spectrin to actin suggests that the observed alterations in membrane stability may in part be mediated by reversible perturbations of spectrin-actin-protein 4.1 interaction by Ca^{2+} and CaM. Some recent studies on the effects of Ca^{2+} and CaM on red cell skeletal protein interactions are also relevant to this hypothesis (33, 47, 48). Anderson and Morrow (33) have shown that CaM inhibits protein 4.1-stimulated spectrin-actin interaction in a Ca^{2+}-dependent fashion, findings similar to those we have described. Gardner and Bennett (47) have shown that micromolar concentrations of Ca^{2+} and CaM inhibit the ability of adducin (a newly identified red cell membrane skeleton-associated calmodulin-binding protein) to promote interaction of spectrin and actin. Mische et al. have shown that binding of adducin to actin and its ability to stimulate spectrin-actin binding is down-regulated by calmodulin in a calcium-dependent fashion. Together, these findings suggest that Ca^{2+}- and CaM-induced decrease in membrane mechanical stability is most likely the result of perturbation of spectrin-actin interaction (Fig. 7), and that this process may be mediated by both protein 4.1 and adducin. From the data presently available, it is not possible to quantitatively establish the relative contributions of protein 4.1 and adducin to Ca^{2+}-CaM-induced decrease in membrane stability.

Our finding that Ca^{2+} in the concentration range of 1.0 to 100 μM can induce marked decrease in membrane stability implies that, for the red cell to maintain its normal membrane stability, intracellular Ca^{2+} concentrations must be maintained at levels < 1.0 μM. The remarkably efficient Ca^{2+}-ATPase system in conjunction with CaM is indeed able to maintain these low levels of intracellular Ca^{2+} in normal red cells. However, failure to maintain low normal levels of intracellular Ca^{2+} would result in decreased membrane stability, which in turn can result in red cell fragmentation during circulation. Two instances in which this may occur are sickle cells and thalassemic cells. In both instances, elevation of Ca^{2+} concentration (up to ~ 200 μM) and decrease in membrane stability have been documented (49–51). It is also interesting to note that young reticulocytes that have previously been shown to have high concentrations of CaM (52) have decreased membrane stability (unpublished data).

Recent studies indicate that proteins immunologically related to the membrane skeletal proteins of red cells, such as spectrin and protein 4.1, are present in many nonerythroid cells (53–57). However, the function of these nonerythroid skeletal proteins has not yet been defined. In some of these cells, Ca^{2+} appears to play an important role as a second messenger in regulating various cell functions. From these observations in red cells, the role of Ca^{2+}-CaM-induced modulation of the interaction of protein 4.1 and spectrin-like proteins in regulating calcium-dependent cell functions in other somatic cells needs to be considered.

Acknowledgments

The authors would like to acknowledge Dr. Marion Reid, who performed the immunochemical analysis using monoclonal glycophorin β antibodies, and Ms. Terri Iarocci, who performed the spectrin oligomerization assays. The authors would also like to acknowledge the expert assistance of Mr. James Harris in the preparation of this manuscript.

This work was supported in part by grants DK-26263 and DK-32094 from the National Institutes of Health.

References

1. Cheung, W. Y. 1980. Calmodulin plays a pivotal role in cellular regulation. *Science (Wash. DC).* 207:19–27.

2. Means, A. R., and J. R. Dedman. 1980. Calmodulin: an intracellular calcium receptor. *Nature (Lond.).* 258:73–77.

3. Van Eldik, L. J., and D. M. Watterson. 1985. Calmodulin structure and function. *In* Calcium and Cell Physiology. D. Marme, editor. Springer-Verlag, Berlin/Heidelberg. 105–126.

4. Jarrett, H. W., and J. T. Penniston. 1978. Purification of the Ca^{2+}-stimulated ATPase activator from human erythrocytes. Its membership in the class of Ca^{2+} binding modulator proteins. *J. Biol. Chem.* 253:4676–4682.

5. Sobue, K., Y. Muramoto, M. Fujita, and S. Kakiuchi. 1981. Calmodulin-binding protein of erythrocyte cytoskeleton. *Biochem. Biophys. Res. Commun.* 100:1063–1070.

6. Gopinath, R. M., and F. F. Vincenzi. 1977. Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of [Ca^{2+}-Mg^{2+}] ATPase. *Biochem. Biophys. Res. Commun.* 77:1203–1209.

7. Jarrett, H. W., and J. T. Penniston. 1977. Partial purification of the Ca^{2+}-Mg^{2+} ATPase activator from human erythrocytes: its similarity to the activator of 3′,5′-cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* 77:1210–1216.

8. Schatzman, H. J. 1975. Active calcium transport and Ca^{2+}-activated ATPase in human red cells. *Curr. Top. Membr. Transp.* 6:125–168.

9. Lew, V. L., R. Y. Tsien, and C. Miner. 1982. Physiological $[Ca^{2+}]_i$ level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. *Nature (Lond.).* 298:478–481.

10. Larsen, F. L., and F. F. Vincenzi. 1979. Calcium transport across the plasma membrane: stimulation by calmodulin. *Science (Wash. DC).* 204:306–309.

11. Fowler, V., and D. L. Taylor. 1980. Spectrin plus band 4.1 cross-link actin: regulation by micromolar calcium. *J. Cell Biol.* 85:361–376.

12. Cohen, C. M., and C. Korsgren. 1980. Band 4.1 causes spectrin-actin gels to become thixotropic. *Biochem. Biophys. Res. Commun.* 97:1429–1435.

13. Agre, P., K. Gardner, and V. Bennett. 1983. Association between human erythrocyte calmodulin and the cytoplasmic surface of human erythrocyte membranes. *J. Biol. Chem.* 258:6258–6265.

14. Burns, N. R., and W. B. Gratzer. 1985. Interaction of calmodulin with the red cell and its membrane skeleton and with spectrin. *Biochemistry.* 24:3070–3074.

15. Sears, D. E., J. S. Morrow, and V. T. Marchesi. 1982. Identification of the calmodulin-binding domain of human erythrocyte spectrin. *J. Cell Biol.* 95:251*a*. (Abstr.)

16. Boivin, P., and C. Galand. 1984. Is spectrin a calmodulin-binding protein? *Biochem. Int.* 8:231–236.

17. Berglund, A., L. Backman, and V. P. Shanbhag. 1984. Calmodulin binding to human spectrin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 172:109–113.

18. Husain, A., G. J. Howlett, and W. H. Sawyer. 1985. The interaction of calmodulin with erythrocyte membrane proteins. *Biochem. Int.* 10:1–12.

19. Gardner, K., and V. Bennett. 1986. A new erythrocyte membrane-associated protein with calmodulin binding activity: identification and purification. *J. Biol. Chem.* 261:1339–1348.

20. Gopalakrishna, R., and W. B. Anderson. 1982. Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.* 104:830–836.

21. Tyler, J. M., W. R. Hargreaves, and D. Branton. 1979. Purification of two spectrin-binding proteins: biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1. *Proc. Natl. Acad. Sci. USA.* 76:5192–5196.

22. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins in high specific radioactivities by conjugation to a ^{125}I-containing acylating agent: application to the radioimmunoassay. *Biochem. J.* 133:529–539.

23. Sillen, L. G., and A. E. Martell. 1971. *In* Stability Constants of Metal-ion Complexes, Suppl. 1. The Chemical Society, London.

24. Bessis, M., and N. Mohandas. 1977. A diffractometric method for the measurement of cellular deformability. *Blood Cells (Berl.).* 3:209–221.

25. Mohandas, N., M. Clark, B. P. Heath, M. Rossi, L. C. Wolfe, S. E. Lux, and S. B. Shohet. 1982. A technique to detect reduced mechanical stability of red cell membranes: Relevance to elliptocytic disorders. *Blood.* 59:768–774.

26. Heath, B. P., N. Mohandas, J. L. Wyatt, and S. B. Shohet. 1982. Deformability of isolated red blood cell membranes. *Biochim. Biophys. Acta.* 691:211–219.

27. Chasis, J. A., N. Mohandas, and S. B. Shohet. 1985. Erythrocyte membrane rigidity induced by glycophorin A-ligand interaction: evidence for a ligand-induced association between glycophorin A and skeletal proteins. *J. Clin. Invest.* 75:1919–1926.

28. Evans, E. A. and A. Leung. 1984. Adhesivity and rigidity of erythrocyte membrane in relation to wheat germ agglutinin binding. *J. Cell Biol.* 98:1201–1208.

29. Chasis, J. A., and N. Mohandas. 1986. Erythrocyte membrane deformability and stability: two distinct membrane properties that are independently regulated by skeletal protein associations. *J. Cell Biol.* 103:343–350.

30. Liu, S.-C., and J. Palek. 1980. Spectrin tetramer-dimer equilibrium and the stability of erythrocyte membrane skeletons. *Nature (Lond.).* 285:586–588.

31. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.

32. Anstee, D. J., S. F. Parsons, K. Ridgwell, M. J. A. Tanner, A. H. Merry, E. E. Thomson, P. A. Judson, P. Johnson, S. Bates, and I. D. Fraser. 1984. Two individuals with ellipsolytic red cells apparently lack three minor erythrocyte membrane sialoglycoproteins. *Biochem. J.* 218:615–619.

33. Anderson, J. P., and J. S. Morrow. 1987. The interaction of calmodulin with human erythrocyte spectrin. Inhibition of protein 4.1-stimulated actin binding. *J. Biol. Chem.* 262:6365–6372.

34. Van Belle, H. 1981. R24571: a potent inhibitor of calmodulin-activated enzymes. *Cell Calcium.* 2:483–494.

35. Gietzen, K., A. Wuthrich, and H. Bader. 1981. R24571: a new powerful inhibitor of red blood cell Ca^{2+}-transport ATPase and of calmodulin-regulated functions. *Biochem. Biophys. Res. Commun.* 101:418–425.

36. Liu, S. C., J. Palek, J. Prchal, and R. C. Castleberry. 1981. Altered spectrin dimer-dimer association and instability of erythrocyte membrane skeletons in hereditary pyropoikilocytosis. *J. Clin. Invest.* 68:597–605.

37. Wolfe, L. C., K. M. John, J. C. Falcone, A. M. Byrne, and S. E. Lux. 1982. A genetic defect in binding of protein 4.1 to spectrin in a kindred with hereditary spherocytosis. *N. Engl. J. Med.* 307:1367–1374.

38. Knowles, W. J., J. S. Morrow, D. N. Speicher, H. S. Zarkowsky, N. Mohandas, S. B. Shohet, and V. T. Marchesi. 1983. Molecular and functional changes in spectrin from patients with hereditary pyropoikilocytosis. *J. Clin. Invest.* 71:1867–1877.

39. Mentzer, W. C., T. Turetsky, N. Mohandas, S. Schrier, C. S. Wu, and H. Koenig. 1984. Identification of the hereditary pyropokilocytosis carrier state. *Blood.* 63:1439–1446.

40. Mueller, T. J., and M. Morrison. 1981. Glycoconnectin (PAS 2), a membrane attachment site for the human erythrocyte cytoskeleton. *In* Erythrocyte Membranes 2: Recent Clinical and Experimental Advances. W. C. Kruckeberg, J. W. Eaton, and G. J. Brewer, editors. Alan R. Liss, Inc., New York. 95–112.

41. Reid, M. E., J. A. Chasis, and N. Mohandas. 1987. Identification of a functional role for human erythrocyte sialoglycoproteins β and γ. *Blood.* 69:1068–1072.

42. Evans, E. A., and R. M. Hochmuth. 1977. A solid-liquid composite model of the red cell membrane. *J. Membr. Biol.* 30:351–362.

43. Mohandas, N., J. A. Chasis, and S. B. Shohet. 1983. The influence of membrane skeleton on red cell deformability, membrane material properties, and shape. *Semin. Hematol.* 20:225–242.

44. Smith, D. K., and J. Palek. 1983. Sulfhydryl reagents induce altered spectrin self-association, skeletal instability, and increased thermal sensitivity of red cells. *Blood.* 62:1190–1196.

45. Sheetz, M. P., and J. Casaly. 1980. 2,3-Diphosphoglycerate and ATP dissociate erythrocyte membrane skeletons. *J. Biol. Chem.* 225:9955–9960.

46. Tchernia, G., N. Mohandas, and S. B. Shohet. 1981. Deficiency of skeletal membrane protein band 4.1 in homozygous hereditary elliptocytosis: implications for erythrocyte membrane stability. *J. Clin. Invest.* 68:454–460.

47. Gardner, K., and V. Bennett. 1987. Modulation of spectrin-actin assembly by erythrocyte adducin. *Nature (Lond.).* 328:359–362.

48. Mische, S. M., M. S. Moosekar, and J. S. Morrow. 1987. Erythrocyte adducin: a calmodulin-regulated actin-bundling protein that stimulates spectrin-actin binding. *J. Cell Biol.* 105:2837–2845.

49. Eaton, J. W., T. D. Skelton, H. S. Swofford, C. E. Kolpin, and H. S. Jacob. 1973. Elevated erythrocyte calcium in sickle cell disease. *Nature (Lond.).* 246:105–106.

50. Lew, V. L., A. Hockaday, M.-I. Sepulveda, A. P. Somlyo, A. V. Somlyo, O. E. Ortiz, and R. M. Bookchin. 1985. Compartmentalization of sickle-cell calcium in endocytic inside-out vesicles. *Nature (Lond.).* 315:586–589.

51. Shalev, O., S. Mogilner, E. Shinar, E. A. Rachmilewitz, and S. L. Schrier. 1984. Impaired erythrocyte calcium homeostasis in β-thalassemia. *Blood.* 64:564–566.

52. Warth, J. A., G. J. Brewer, M. E. Gnegy, G. Treisman, and K. Near. 1983. Calmodulin level in whole blood correlates with the percentage of reticulocytes. *Am. J. Hematol.* 15:153–157.

53. Lazarides, E., and W. J. Nelson. 1982. Expression of spectrin in non-erythroid cells. *Cell.* 31:505–508.

54. Cohen, C. M., S. F. Foley, and C. Korsgren. 1982. A protein immunologically related to erythrocyte band 4.1 is found on stress fibers of non-erythroid cells. *Nature (Lond.).* 299:648–650.

55. Spiegel, J. E., D. S. Beardsley, F. S. Southwick, and S. E. Lux. 1984. An analogue of the erythroid membrane skeletal protein 4.1 in non-erythroid cells. *J. Cell Biol.* 99:886–893.

56. Aster, J. C., M. J. Welsh, G. J. Brewer, and H. Maisel. 1984. Identification of spectrin and protein 4.1-like protein in mammalian lens. *Biochem. Biophys. Res. Commun.* 119:726–734.

57. Baines, A. J., and V. Bennett. 1985. Synapsin I is a spectrin-binding protein immunologically related to erythrocyte protein 4.1. *Nature (Lond.).* 315:410–413.