Spin-labeling studies of the conformation of the Ca^{2+}-regulatory protein calmodulin in solution and bound to the membrane skeleton in erythrocyte ghosts: Implications to transmembrane signaling

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ABSTRACT Electron paramagnetic resonance (EPR) studies of the Ca^{2+}-regulatory protein calmodulin (CaM) have been performed. The conformation of CaM in solution changes upon binding of Ca^{2+} allowing the protein to bind to target proteins existing in the red blood cell membrane. In this study a maleimide spin label, covalently attached to the single cysteine residue of CaM located in the first Ca^{2+}-binding domain, was used to monitor allosteric conformational changes induced by interaction of CaM with Ca^{2+} and subsequently with the red blood cell membrane. The results show, relative to apo-CaM, a significant increase in the apparent rotational correlation time, τ, of the spin label when Ca^{2+} was present in solution ($P < 0.001$). When apo-CaM was exposed to red blood cell membrane ghosts in the absence of Ca^{2+}, no significant difference in spin label motion was seen relative to solution, consistent with the idea that Ca^{2+} is required for CaM to bind to skeletal proteins. When Ca^{2+} was added to CaM which was then exposed to ghosts, a highly significant increase in τ (decrease in motion) ($P < 0.000001$) relative to apo-CaM exposed to ghosts was found. This latter increase in τ is significantly greater than that produced by the addition of Ca^{2+} to CaM in solution ($P < 0.001$). The major interaction sites of CaM were found by photoaffinity labeling and autoradiography on SDS-PAGE to be on the principal skeletal protein, spectrin. EPR was also used to investigate the biophysical correlates of transmembrane signaling. Spin-labeled CaM was bound to the membrane skeleton in the presence of Ca^{2+}. On the opposite side of the erythrocyte membrane a lectin was bound to the external glycoconjugate of Band 3, the major transmembrane protein of the erythrocyte. A highly significant increase in τ of the maleimide spin probe was found relative to the control system in which the lectin was absent. ($P < 0.00001$). These results suggest that electron paramagnetic resonance spectra of spin-labeled CaM can provide useful information about protein structure and function when in solution and when bound to membranes.

INTRODUCTION

Protein–protein interactions in the erythrocyte membrane skeleton are the origin of cell shape, and it is believed that the regulation of these cytoskeletal associations allows the cell its deformability and its strength (for review see reference 1). One of the potential regulators of these associations is calmodulin (CaM), a 17-kD protein with two globular domains connected by an α-helix. CaM has four Ca^{2+}-binding sites which, when filled, elicit a conformational change in the protein causing an increase in the α-helical content. This Ca^{2+}-induced change in conformation exposes a hydrophobic region that allows CaM to interact with the membrane (Fig. 1) (2). When a red blood cell is exposed to shear forces as it is in arterial turbulent flow, under great pressure as it is when entering and exiting capillaries, or subjected to other stimuli, intracellular levels of Ca^{2+} can increase (3). In experimental studies an increase of Ca^{2+} of one- to threefold over normal intracellular levels caused a decrease in cell deformability which can be restored by the addition of a CaM antagonist such as trifluoperazine (4). CaM can, under certain conditions, bind to the skeletal protein network; thus, CaM is likely to mediate the effect of Ca^{2+} on the membrane.

The crystal structure of CaM has been determined (5) and subsequently refined at 2.2 Å resolution (6). More recently, differences in CaM conformation have been suggested between the crystal structure and the solution structure (7–10). The conformation of CaM changes even further upon binding to inhibitors such as mastoparan (10) and chlorpromazine (11). With the knowledge that the central α-helix, exposed when CaM binds three to four Ca^{2+} ions, is flexible (12) allowing CaM manifold conformations in solution, it might be expected that the structure upon binding to red blood cell membranes would be different from the structure when free in solution or different even from the structure when bound to inhibitors (11) or other target proteins.

Spectrin, the major membrane skeletal protein, associates with itself and actin, with the help of Band 4.1 and other minor proteins, to form a latticelike network which laminates the interior of the cell membrane (4). Two major attachment sites of the skeleton to the bilayer are Band 2.1 (ankyrin), which in turn is bound to a fraction of the Band-3 molecules, and Band 4.1, which is thought to be bound to glycophorin. Band 3 is the major transmembrane glycoprotein of the erythrocyte membrane, whereas the glycophorins are the major sialoglycoproteins.

Previous studies in our laboratory have investigated the biophysical and biochemical consequences of transmembrane signaling, those processes by which a stimulus on one side of the membrane leads to a response on the opposite side (for a review, see reference 13). We have shown that it is possible to alter the physical state of one side of the membrane by modulating the physical state of the opposite side. For example, the polyamine,

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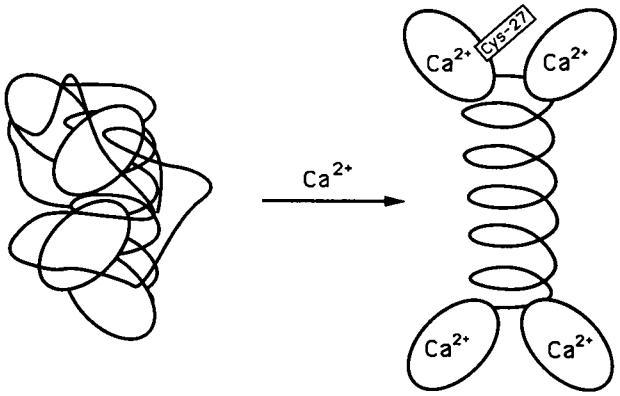

FIGURE 1 Schematic drawing of CaM showing exposure of a hydrophobic domain upon addition of Ca^{2+}. Cys-27 in the first Ca^{2+}-binding domain, the only cysteine in CaM and the binding site for MAL-6, is also indicated.

spermine, was shown to increase protein–protein interactions in the erythrocyte membrane skeleton on the cytoplasmic side of the membrane by bridging spectrin to Band 3 (14) and consequently significantly increasing motion of cell-surface sialic acid, 70% of which is on glycophorin on the opposite side of the membrane. In contrast, hemin was shown by us (15) to decrease skeletal protein–protein interactions while decreasing the motion of cell-surface sialic acid.

In the current study, electron paramagnetic resonance (EPR) was used to investigate the difference in the physical state of CaM in solution and when bound to its target in red blood cell ghosts. EPR, employing a spin probe covalently attached to CaM's only sulfhydryl group (Cys-27) located in the first Ca^{2+}-binding domain, was used for this investigation. Further, this paper reports the results of a study to determine whether a lectin bound to the external glycoconjugate of the major transmembrane protein would elicit a change in the physical state of CaM which was bound to the skeleton on the cytoplasmic side of the erythrocyte membrane.

MATERIALS AND METHODS

Preparation of ghosts

Whole blood was drawn from human donors into heparinized tubes and used within 30 min. 5-mL aliquots of the whole blood were placed in plastic tubes, diluted to 50 mL with PBS (5 mM phosphate buffer/150 mM NaCl/pH 8), centrifuged at 2,420 *g*, 4°C for 5 min. The supernatant was discarded and the buffy coat carefully removed. This procedure was repeated for a total of three washes. The packed cells were diluted to 50 mL in a hypotonic buffer, 5P8 (5 mM phosphate buffer/pH 8), and were allowed to osmotically lyse for 15 min at 4°C. The cells were centrifuged at 27,000 *g*, 4°C for 10 min. The supernatant was discarded. The 5P8 washes were repeated five to six times or until ghosts were white and hemoglobin-free. The membrane ghosts were then diluted to 50 mL with buffer (5 mM Tris-base/0.5 mM EGTA/pH 7.0), and centrifuged at 27,000 *g*, 4°C for 10 min. The supernatant was discarded. This was repeated for a total of three washes to remove any native CaM bound to the membrane (16). Protein concentration of ghosts was determined by method of Lowry et al. (17).

Chemical modification of calmodulin

CaM was isolated from wheat germ (Con Agra, New Prague, Minnesota) and purified as previously described (7). The SH-group specific spin label 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6) was covalently and specifically attached to Cys-27 in the first Ca^{2+} binding domain of CaM also as previously described (7).

Samples for spectral analysis

0.25 mg of MAL-6-labeled CaM in 24 μL buffer (10 mM Tris-base/1 mM EGTA/pH 8.4) was delivered to each tube. Enough buffer (5 mM Tris-base/0.5 mM EGTA/pH 7.0) was added to give a final volume of 300 μL. For those samples containing Ca^{2+}, 13.6 μL of 20.5 mM $CaCl_2$ in buffer (5 mM Tris-base/0.5 mM EGTA/pH 7.0) was added to give a final free Ca^{2+} concentration of 350 μM in 300 μL. The CaM and Ca^{2+} were allowed to equilibrate and freshly made ghosts were added to give a total protein concentration (due to ghosts) of 2.5 mg/mL in 300 μL. Control samples contained no ghosts. The pH of all samples was adjusted to 7.0 with small amounts of 1.0 M HCl and 1.0 M KOH. EPR data presented were obtained with a minimum of five separate CaM preparations.

Photolabeling of membranes

CaM, functionalized with succinimidyl *N'*-[*N''*-(3-azido-5-nitrobenzoyl)tyrosyl]-β-alanate and radioiodinated (18), was used to photolabel the red blood cell membrane. The CaM sample was prepared as before. 0.25 mg ^{125}I-labeled monoadduct of CaM in 24 μL (10 mM Tris-base/1 mM EGTA/pH 8.4) was placed in an Eppendorf tube. Enough buffer (5 mM Tris-base/0.5 mM EGTA/pH 7.0) was added to give a final volume of 300 μL. 13.6 μL of 20.5 mM $CaCl_2$ in buffer (5 mM Tris-base/0.5 mM EGTA/pH 7.0) was added to give a final free Ca^{2+} concentration of 350 μM in 300 μL. The CaM and Ca^{2+} were allowed to equilibrate and freshly made ghosts were added to give a total protein concentration (due to ghosts) of 2.5 mg/mL in 300 μL. The pH was adjusted to 7.0 with small amounts of 1.0 M HCl and 1.0 M KOH. The sample was then irradiated for 2 min (λ = 254 nm) which led to the gel and autoradiogram in Fig. 4 after separation on 7.5% SDS-PAGE and exposure to film.

Lectin studies

A stock solution of Concanavalin A (CON-A) was prepared by dissolving 10.8 mg of the lectin in 1 mL of buffer (5 mM tris-base/0.5 mM EGTA/pH 8.2). 50 μL of the CON-A stock solution was added to appropriate tubes for a final concentration of 1.8 mg/mL. CON-A was the last constituent to be added before checking the pH. The pH of the samples was adjusted to 7.0 with small amounts of 1.0 M HCl and 1.0 M KOH.

All reagents except $CaCl_2$ were essentially Ca^{2+} free, containing a maximum of 0.0005% Ca^{2+} or less in the original stock solution or solid. Data were derived from several batches of CaM separately isolated, purified, and labeled with MAL-6.

EPR spectral acquisition

EPR spectra were obtained at room temperature on a Varian E-109 X-band spectrometer interfaced to a computerized data acquisition system and located in a room controlled for temperature and humidity. Constant temperature was maintained for each sample by allowing N_2 to flow through the cavity. Spectrometer conditions were: field sweep: 50 Gauss; modulation amplitude: 0.32 Gauss; modulation frequency: 100 kHz; microwave power: 16 mW.

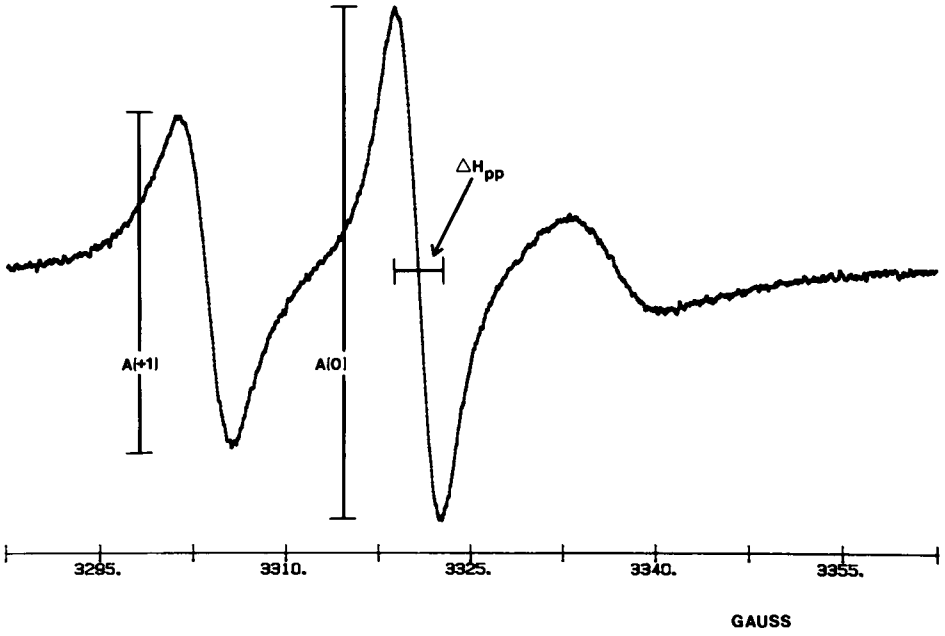

FIGURE 2 A typical EPR spectrum of MAL-6 covalently attached to Cys-27 of CaM in solution. EPR spectrometer conditions are given in Methods.

Statistical analysis

Rotational correlation times were calculated for all samples, and their means were compared for each group by a two-tailed Student's *t*-test.

RESULTS

Wheat germ CaM has a single cysteine (Cys-27) in the first Ca^{2+}-binding domain. Previous studies in our laboratory showed that MAL-6 covalently binds exclusively to the S-H group of Cys-27 in a 1:1 molar ratio (7). No difference of the ability of spin-labeled CaM from nonlabeled CaM to activate phosphodiesterase activity could be demonstrated (7), showing that the presence of MAL-6 does not perturb the structure or function of CaM, a result also consistent with the finding that spin labeled CaM binds CaM inhibitors (11). Fig. 2 gives a typical spectrum of CaM spin labeled with MAL-6.

The motion of the MAL-6 spin label on Cys-27 in the first Ca^{2+}-binding domain of CaM was estimated by an apparent rotational correlation time (τ) employing a well-established, composite equation according to Kivelson (19), Eq. 1.

$$\tau = 6.5 \times 10^{-10}\, \Delta H_{PP}\left[\left(\frac{A(0)}{A(+1)}\right)^{1/2} - 1\right], \qquad (1)$$

where ΔH_{PP}, $A(0)$, $A(+1)$ are, respectively, the peak-to-peak width of the $M_I = 0$ central EPR resonance line of MAL-6, and the peak-to-peak amplitude of the $M_I = 0$ and $M_I = +1$ lines (Fig. 2). Anisotropy values of the *g*- and *T*-tensors of 2,2,6,6-tetramethylpiperidine-1-oxyl-4-one were used in determining the constant in Eq. 1 (20). Tau can be envisaged as the time necessary for the spin label to rotate through an angle of one radian. Larger values of tau suggest slower rotational motion of MAL-6 bound to the first Ca^{2+}-binding domain. We previously showed (7) that addition of Ca^{2+} to spin labeled CaM at low ionic strength, the conditions used in the studies to be described in this report, led to a significant increase in τ (slower motion) of MAL-6 bound to Cys-27.

Fig. 3 shows that the spin label motion slows significantly when Ca^{2+} is bound to CaM relative to apoCaM both in solution and when exposed to ghosts. In the absence of Ca^{2+} (Fig. 3, *white columns*) there is no significant difference ($P < 0.2$) in spin label motion whether the CaM is in solution or exposed to ghosts. This result is consistent with the requirement of Ca^{2+} for CaM interaction with the erythrocyte membrane (21, 22). In contrast, when Ca^{2+} is added at the low ionic strength used in these studies to both the solution and ghost systems, the spin label motion is significantly slowed in each case ($P < 0.001$ and $P < 0.000001$, respectively). Further, statistical analysis suggests that in the presence of Ca^{2+}, the motion of MAL-6 on CaM is significantly slower when CaM is bound to the membrane than when in solution (Fig. 3, patterned columns, $P < 0.001$). That is, when Ca^{2+} is added to spin labeled CaM in solution, spin label motion is slowed; when Ca^{2+} is added to spin labeled CaM, which then binds to the red blood cell ghost membrane, motion of the spin label is slowed to an even greater extent.

CaM binding to the membrane

Spectrin is able to bind CaM (23) and is known to be a major binding site. This finding was confirmed under

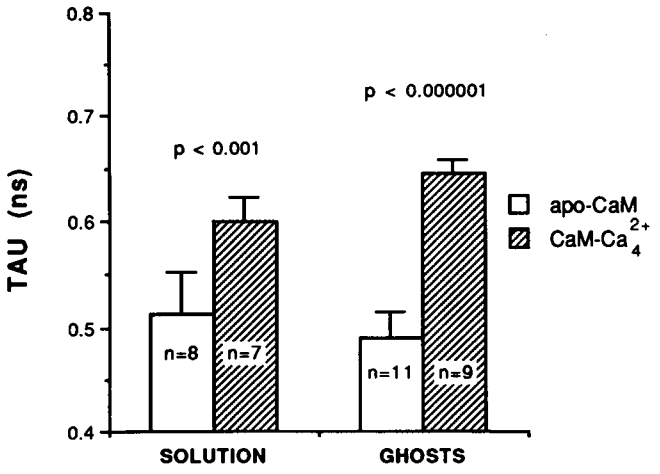

FIGURE 3 Effect of Ca^{2+} on MAL-6 labeled CaM in solution and when bound to ghosts. White bars represent apo-CaM and patterned bars represent CaM-$(Ca^{2+})_4$ complex. The number of replicates for each bar is denoted by *n*. The *P* values (two-tailed Student's *t*-test) are given at the top of the plot. Solution studies were carried out at final concentrations of 50 μM MAL-6 labeled CaM, 5.4 mM Tris-base and 0.5 mM EGTA. For those samples containing Ca^{2+} or ghosts, final concentrations were 350 μM free Ca^{2+} and 2.5 mg/mL ghosts, respectively. The pH was adjusted to 7.0 and the low ionic strength was kept constant. The mean values of tau were obtained from a minimum of five separate CaM preparations.

the conditions of the present study by use of a photoaffinity labeling method. CaM modified with a ^{125}I-labeled, heterobifunctional photoaffinity probe was photo cross-linked to the membrane after incubation of CaM with ghosts in the presence of Ca^{2+}. Fig. 4 shows that most, though not all, of the ^{125}I intensity from CaM is associated with spectrin.

CON-A studies

The columns designated solution in Fig. 5 show that CON-A by itself has no effect on CaM in solution, a not unexpected result because CON-A is known to bind to the external portion of Band 3 (24). This result shows that there is no nonspecific effect of CON-A on spin labeled CaM. In contrast, when CaM is bound to spectrin in the skeleton on the cytoplasmic side of erythrocyte membranes (*ghosts column*, Fig. 5), the addition of CON-A to the external glycoconjugate of Band 3 on the opposite side of the membrane causes a significant increase in the correlation time of MAL-6 labeled CaM. As shown in Fig. 3, relative to CaM in solution, there is an increase in correlation time when MAL-6 labeled CaM binds to membranes in the absence of CON-A; but Fig. 5 (*ghosts column*) shows that the increase in the correlation time seen upon addition of CON-A is augmented further ($P < 0.00001$).

DISCUSSION

Although interaction between CaM and spectrin is weak, with the reported K_d ranging from 2-22 μM (22, 23, 25), due to the relatively high concentration of spectrin near the bilayer it is thought that a substantial fraction of CaM is bound to this major skeletal protein (26). Fig. 4 confirms that this idea has considerable merit. Other skeletal or membrane proteins may also interact with CaM, but spectrin appears to be the predominant interaction site under the conditions in this study.

Because binding of CaM to the skeleton is Ca^{2+}-dependent (21, 22), no difference in conformation of apo-CaM upon incubation with ghosts in the absence of Ca^{2+} is expected. Consistent with this expectation, Fig. 3 shows that when apo-CaM is incubated with ghosts in

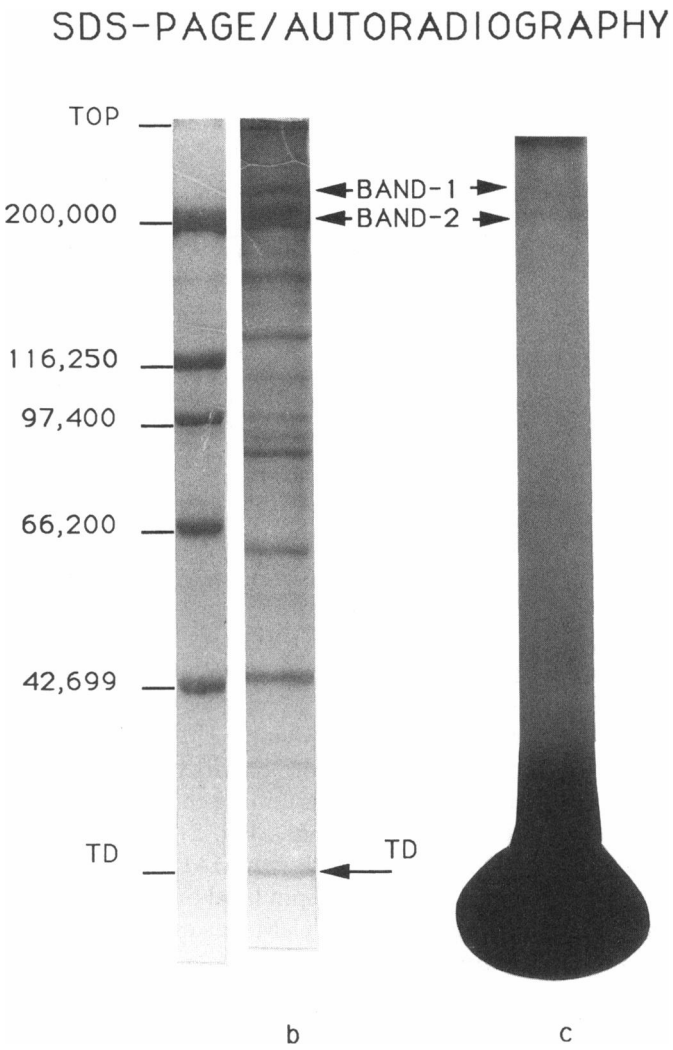

FIGURE 4 Identification of the major binding sites of CaM to the erythrocyte membrane skeleton. An ^{125}I-labeled monoadduct of CaM was bound to ghosts in the presence of Ca^{2+}. The CaM-membrane complex was photolyzed, the proteins separated by SDS-PAGE, and an autoradiogram produced. (*a*) Molecular weight standards in Daltons stained by Coomassie blue dye. (*b*) CaM red blood cell membrane complex stained by Coomassie blue dye. (*c*) ^{125}I-autoradiogram of *b*. Spectrin bands 1 and 2 are the predominantly labeled binding sites for CaM under the conditions of this study. The intensity of the radioactivity at the bottom of the gel is due to the monoadduct of CaM and its dimer, 17 and 34 kD, respectively. TD = tracking dye.

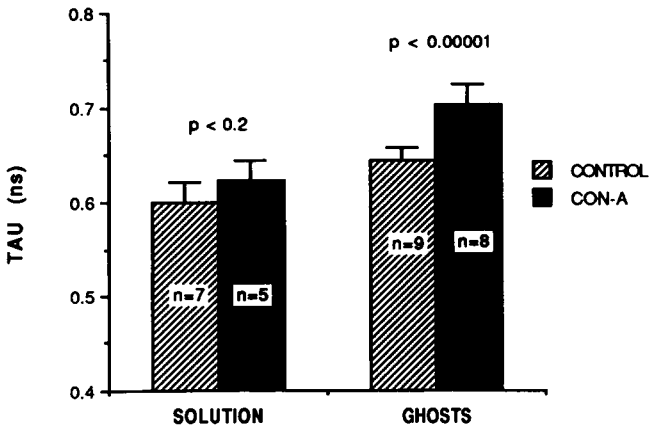

FIGURE 5 Effect of CON-A on MAL-6-labeled CaM in solution and bound to ghosts. Tau is the apparent rotational correlation time. Patterned bars represent those samples without CON-A. Black bars indicate the addition of CON-A to a final concentration of 1.8 mg/mL. Solution studies were carried out at final concentrations of 5.4 mM Tris-base and 0.54 mM EGTA. Those samples containing ghosts were adjusted to a final protein concentration (due to ghosts) of 2.5 mg/mL. The final concentrations of MAL-6 labeled CaM and free Ca^{2+} were 50 μM and 350 μM, respectively. The pH was adjusted to 7.0 and the low ionic strength was kept constant. The mean values of tau were obtained from a minimum of five separate CaM preparations.

the absence of Ca^{2+} there is no significant difference in spin label motion. In contrast, several investigations, including our own (2, 7, 27), have shown that Ca^{2+} affects the conformation of CaM in solution. It is generally accepted that binding of three or four Ca^{2+} ions by CaM exposes a central α-helix which separates two globular regions of the protein each containing two Ca^{2+}-binding domains; this conformational change allows CaM to interact with its target proteins in the membrane. The spin label, located in the first Ca^{2+}-binding domain, experiences slowed motion due to increased steric hindrance upon binding of Ca^{2+} in solution (Fig. 3). When this CaM-$(Ca^{2+})_4$ complex is incubated with ghosts the spin label motion is even slower than that of the complex in solution, suggesting that the environment of the spin label is even more hindered when CaM is bound to the skeleton. The motion of MAL-6 attached to CaM in the first Ca^{2+}-binding domain is relatively insensitive to ionic strength in the presence of Ca^{2+} (7). Thus, this result is expected even at physiological ionic strength. The molecular basis for decreased motion of the spin label in CaM's first binding domain upon binding of CaM to spectrin relative to CaM-$(Ca^{2+})_4$ complex in solution is not known at present. The most obvious ideas are: (*a*) the spin label binding site is simply hindered in motion due to the proximity of the membrane to the MAL-6 site of CaM upon binding to the membrane; and (*b*) allosterism, i.e., when CaM binds to its target (in this case primarily spectrin) often through the hydrophobic domain, the conformation of CaM changes such that this change is reflected in the physical state of the first Ca^{2+}-binding domain. This last idea is not unprecedented. As mentioned earlier, when mastoparan (a small, naturally-occurring peptide) binds to CaM, ^{111}Cd NMR showed conformational changes in both halves of the molecule (the NH_2-terminus and COOH-terminus globular regions) (10). Also, when MAL-6 labeled CaM binds chlorpromazine in the presence of Ca^{2+}, presumably to the hydrophobic region of the molecule, an allosteric-induced slowing of spin label motion was also observed, although not to the extent that was seen when CaM was bound to the skeleton (11). This last observation would lead one to believe that CaM is capable of manifold conformations in solution which may change, depending upon the target of CaM binding. This idea is consistent with the concept that CaM can regulate the activity of several different membrane enzymes and transport proteins.

The lectin, CON-A, has a specific binding site on the glycoconjugate of Band 3 (24), a site located on the exterior side of the erythrocyte membrane. Upon addition of CON-A to erythrocyte membranes (Fig. 5, *ghosts column*) to which spin-labeled CaM is bound, MAL-6 motion is slowed to an even greater extent than simply binding CaM to the membrane. This result suggests that the spin label is experiencing increased steric hindrance, and conceivably could be explained in one of two ways: (*a*) CON-A bound to Band-3 causes a conformational change in this protein which via Band-2.1 is transmitted to spectrin. This putative change in this skeletal protein to which CaM is bound could thereby increase the steric hindrance about the spin label; or (*b*) the conformational change in Band 3 induced by CON-A could cause a conformational change in Band 2.1, which then changes spectrin, which then causes a change in CaM itself. This last change would then hinder the spin label motional freedom. In either case, the information inherent in the binding of CON-A to one side of the membrane has been translated to the skeleton and to CaM. Said another way, a transmembrane signaling event has occurred. Although we have no direct proof, we favor this second alternative because previous EPR studies could not detect gross changes in the physical state of spectrin upon addition of CON-A to ghosts (28). Only a slight change in the conformation of spectrin might be necessary to evoke a larger change in an associated protein, such as CaM. This is reasonable when it is recalled that spectrin, the major protein of the erythrocyte membrane, is thought to be the major contributor to the morphology of the erythrocyte. Consideration of the role of the membrane skeleton in red cell physiology suggests that prudence is called for in the modulation of spectrin itself. For example, when spectrin is altered even by one amino acid, as in hereditary pyropoikilocytosis, severe changes in red cell morphology and protein–protein interactions occur (29). Furthermore, it is desirable that a slight change in spectrin elicited by CON-A, through Band 3 and Band 2.1, would be "amplified" in its associated proteins, thus, leaving the morphology of the erythrocyte

intact but allowing for a message to the interior of the cell.

The research reported here is consistent with a cardinal hypothesis of transmembrane signaling processes: namely, a change in the physical or chemical state of one side of the membrane may elicit a change in the physical or chemical state of the opposite side. Continued studies of the molecular changes involved in transmembrane signaling mechanisms may lead to a better understanding of these important cellular communication processes.

We thank Con Agra for donating the wheat germ and Dr. Lori Dwyer for assistance with the photoaffinity labeling studies.

This research was supported in part by National Science Foundation grants (RII-86-10671 and EHR-9108764).

Received for publication 22 November 1991 and in final form 9 March 1992.

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