Interaction of Calmodulin with the Red Cell and Its Membrane Skeleton and with Spectrin†

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ABSTRACT: The binding of calmodulin to red cell membrane cytoskeletons and to purified spectrin from red cells and bovine brain spectrin (fodrin) has been examined. Under physiological solvent conditions binding can be measured by ultracentrifugal pelleting assays. The membrane cytoskeletons contained a single class of binding sites, with a concentration similar to that of spectrin dimers and an association constant of 1.5×10^5 M^{-1}. Binding is calcium dependent and is suppressed by the calmodulin inhibitor trifluoperazine. The binding showed a marked dependence on ionic strength, with a maximum at 0.05 M, and a steep dependence on pH, with a maximum at pH 6.5. It was unaffected by 5 mM magnesium. An azidocalmodulin derivative, under the conditions of our experiments, did not label the spectrin-containing complex, although it could be used to demonstrate binding to fodrin. Binding of calmodulin to spectrin tetramers and fodrin in solution could be demonstrated by a pelleting assay after addition of F-actin. Calculations (which are necessarily rough) suggest that at the free calcium concentration prevailing in a normal red cell about 1 in 20 of the calmodulin binding sites in spectrin will be occupied; this proportion will rise rapidly with increasing intracellular calcium. To determine whether inhibition of calmodulin binding to red cell proteins disturbs the control of cell shape, as has been suggested, calcium ions were removed from the cell by addition of an ionophore and of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid to the external medium. This did not affect the discoid shape. Trifluoperazine still induced stomatocytosis, exactly as in untreated cells. These results suggest that trifluoperazine and related substances exert their effects on cell shape by association with the membrane bilayer.

Calmodulin is present in the red cell (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977) and is known to bind to and regulate the membrane (Ca^{2+} + Mg^{2+})-ATPase (Larsen & Vincenzi, 1979; Graf & Penniston, 1981). This enzyme probably accounts for the small number (ca. 10^3) of strong calmodulin binding sites present per cell (Graf et al., 1980; Agre et al., 1983). There appears also to be a population of much weaker sites, corresponding in number to one of the more abundant proteins, conjectured to be spectrin. However, the affinity of spectrin for calmodulin has been a matter of considerable dispute. Thus, Glenney et al. (1982a), using a gel overlay technique, obtained evidence of binding to the α-chain of chicken red cell spectrin, as well as to those of the spectrin-like proteins TW240/260 and fodrin (brain spectrin), and Bartelt et al. (1982, 1984) obtained a similar result for chicken and dogfish spectrin and for fodrin. On the other hand, both these authors and Glenney et al. (1982a) reported a negative result for human red cell spectrin, and Bartelt et al. (1982, 1984), both by gel overlay and cross-linking with a photoactivated calmodulin derivative, likewise found no binding. Binding of calmodulin to human red cell spectrin was first reported by Sobue et al. (1981a) to occur only in a medium containing 8 M urea [in which spectrin is fully unfolded (Calvert et al., 1980)]; Sears et al. (1982) found binding to the β-chain of human spectrin by affinity chromatography in 6 M urea, and Boivin & Galand (1984) also observed binding by a similar procedure, only in 6 M urea, and both to the α-chain and to the β-chain. These results led to the suggestion of cryptic binding sites. By contrast Berglund et al. (1984) found relatively weak but undoubted binding in aqueous solution using both affinity chromatography and partition in a two-phase solvent system, and Husain et al. (1984) found even weaker association by a sedimentation equilibrium technique. The reasons for these discrepancies are at present obscure.

Whether and with what affinity spectrin binds calmodulin may not be a trivial issue, for the concentration of calmodulin in the red cell is high (Penniston et al., 1980) and, given the small number of strong binding sites, could be sufficient, if the binding constant is even moderate, to occupy a significant fraction of the available spectrin sites. Moreover, it has been inferred from the stomatocytogenic effect of all 39 different calmodulin inhibitors on intact red cells that calmodulin is an agent of shape control [Nelson et al., 1983; see also Kidoguchi et al. (1982)]. If this is so, the probable implication would be that it acts by way of its attachment to spectrin, since, besides the (Ca^{2+} + Mg^{2+})-ATPase, there is no other known membrane constituent capable of binding calmodulin.

In view of these considerations we have attempted to resolve whether indeed calmodulin binds to spectrin, and especially to the membrane skeletal complex, and we have reexamined the basis of the stomatocytogenic effect using a single well-characterized calmodulin inhibitor, trifluoperazine (TFP). We conclude that there is weak but specific binding of calmodulin to spectrin in the cytoskeletal complex, as well as in isolation, that bovine fodrin is similar in this respect to spectrin, and that the action of TFP on the cell is unrelated to any association with intracellular calmodulin.

Materials and Methods

Preparation of Calmodulin. Calmodulin from frozen bovine brain was prepared according to the procedure of Gopalakrishna & Anderson (1983).

Preparation of ^{125}I-Calmodulin. Purified calmodulin was radioiodinated to a specific activity of 0.8×10^5–3×10^5

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cpm/μg by using the procedure of Bolton & Hunter (1973). Free reagent remaining after iodination was removed by making the sample 5 mM in Ca^{2+} and applying it to a 2 × 1 cm phenyl-Sepharose column. The column was washed with buffer, containing 0.1 mM calcium chloride, 1 mM 2-mercaptoethanol, and 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, and the eluant was monitored until the radioactivity reached background level. The ^{125}I-calmodulin was then eluted with the same buffer but with 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) in place of calcium.

Preparation of Spectrin and Fodrin. Spectrin was prepared from membrane cytoskeletons of red cells by dissociation with 1 M Tris followed by chromatography on Sepharose 6B in the same medium (Ohanian & Gratzer, 1984). Fodrin was prepared from bovine brains by a modification of the method of Bennett et al. (1982), described earlier (Burns et al., 1983).

Preparation of Membrane Cytoskeletons. Triton-insoluble red cell cytoskeletons were prepared, using high ionic strength, essentially as described by Sheetz (1979). A 50% (v/v) suspension of washed red cells in 0.15 M sodium chloride and 5 mM sodium phosphate, pH 7.6, was mixed with an equal column of a solution containing 15% (w/v) Triton X-100, 0.15 M sodium chloride, 0.2 mM ATP, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol, 3 mM phenylmethanesulfonyl fluoride, and 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.0. The suspension was then centrifuged for 20 min at 18000 rpm (Beckman SW 28 rotor) through a sucrose column, consisting of a cushion of 60% (w/v) sucrose, containing 0.6 M potassium chloride, 0.2 mM ATP, 0.2 mM dithiothreitol, 0.2 mM magnesium chloride, 1 mM EGTA, and 20 mM HEPES, pH 7.0, surmounted by a column of 30% (w/v) sucrose in the same buffer. The cytoskeletons collected in an opalescent layer at the top of the cushion.

Binding of Calmodulin to Membrane Cytoskeletons. Cytoskeletons at a protein concentration of about 1 mg/mL were mixed with varying proporitons of labeled calmodulin in 0.15 M sodium chloride, 10 mM HEPES, and 0.1 mg/mL sodium azide, pH 7.6. The samples were allowed to come to equilibrium on ice, and aliquots were centrifuged at 22 psi for 10 min in a Beckman airfuge. The supernatants were carefully removed with a fine Pasteur pipet, and the radioactivity in the small pellets was counted. In all cases paired experiments were performed, with calcium and EGTA in the buffer.

Azidocalmodulin and Cross-Linking. Azidocalmodulin was prepared by reaction with methyl 4-azidobenzimidate (MABI) following the procedure of Andreasson et al. (1981). Cross-linking was performed with either cytoskeletons (1.5 mg/mL) or fodrin (ca. 0.2 mg/mL) in 100 mM borate buffer, pH 7.8. For activation the sample in a glass tube was placed in a beaker of ice and left for 80 s at a distance of 2 cm from the envelope of a low-pressure mercury lamp. The azidocalmodulin concentration in the mixture was 1.3 mg/mL.

Actin Pelleting Assays. The ability of tetrameric spectrins to bind to and cross-link F-actin was exploited to facilitate the pelleting of purified spectrin and fodrin. Iodinated calmodulin at about 35 μg/mL (3×10^5 cpm/mg) was added to F-actin at 0.1 mg/mL, and spectrin and fodrin were added at concentrations ranging up to about 1 and 0.3 mg/mL, respectively. After the samples were allowed to come to equilibrium on ice, aliquots were centrifuged in the Beckman airfuge for 30 min at 24 psi; the supernatants were removed, and the radioactivity in the pellets was counted. For each concentration a pair of determinations with calcium and EGTA in the buffer was performed. Spectrin alone, in the absence of actin, was partly pelleted under these conditions; to detect binding of calmodulin in the absence of actin, the mixture was centrifuged for 15 min at 26 psi, and the radioactivity in the pellet was determined.

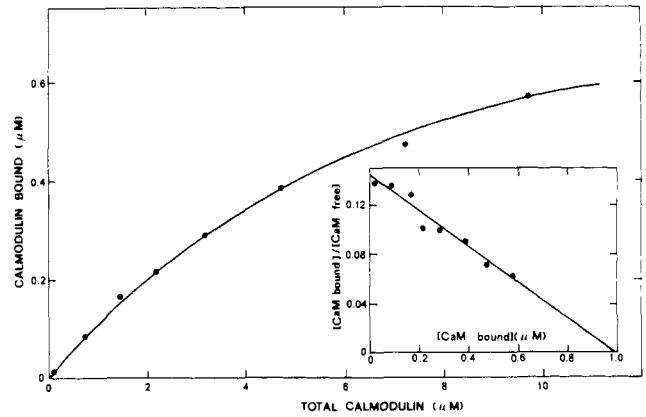

FIGURE 1: Binding of radioiodinated calmodulin to purified red cell membrane cytoskeletons. Concentration of total cytoskeleton protein was 0.9 mg/mL. Calmodulin bound was determined from the radioactivity pelleted in the ultracentrifuge, making correction for the background obtained with EGTA in place of calcium in the medium. Inset: corresponding Scatchard plot.

Red Cell Shape Observations. All experiments were performed on freshly drawn heparinized human blood. Cells were washed 3 times with isotonic saline (0.14 M sodium chloride, 5 mM potassium chloride, 1 mM magnesium sulfate, 1 mM sodium phosphate, 5 mM glucose, and 10 mM Tris, pH 7.6). The cells were then resuspended at 10% haematocrit in this buffer. The buffer was made 1 mM in calcium or EGTA, and the calcium ionophore A23187 was added to a final concentration of 1 μM. After incubation at room temperature for periods between a few minutes and 12 h, samples of the cell suspension were fixed with 1% glutaraldehyde in the same buffer. TFP was added to the remaining suspension at a concentration of 4 μM, and further samples were again fixed.

Fixed cells were allowed to settle on polyamide filters of pore size 0.6 μm (Sartorius) and dehydrated with successive washes (2 × 5 mL) of 20%, 40%, 60%, 80%, and finally 100% (v/v) acetone. The cells were sputter coated with gold and examined in a Jeol JSM-T20 scanning electron microscope.

RESULTS

Binding of calmodulin to purified membrane cytoskeletons was studied by a pelleting assay. The cytoskeletons, prepared as described, contain no significant protein components besides spectrin, actin, and the two other structural components, protein 4.1 (M_r ~78000) and the relatively minor species protein 4.9 (M_r ~50000). The calmodulin was radioiodinated, and the preparation was diluted with unlabeled protein as required. Data obtained with mixtures in different proportions lay on the same binding profile. Binding equilibrium was reached within the time of mixing and pelleting. The calcium-dependent binding was obtained from the difference in the amounts of calmodulin carried down in the presence and absence of calcium. The blank value obtained in EGTA could be largely or entirely accounted for by the holdup volume of the pellet.

Figure 1 shows the profile of calcium-dependent binding of calmodulin to membrane cytoskeletons, and the inset gives the corresponding Scatchard plot, which delivers a binding constant of 1.5×10^5 M^{-1}, and a number of sites that must correspond to a major protein. Thus, the assay shown in Figure 1 leads to a binding site concentration of 1.0 μM. Taking the proportions of constituent proteins determined earlier (Pinder

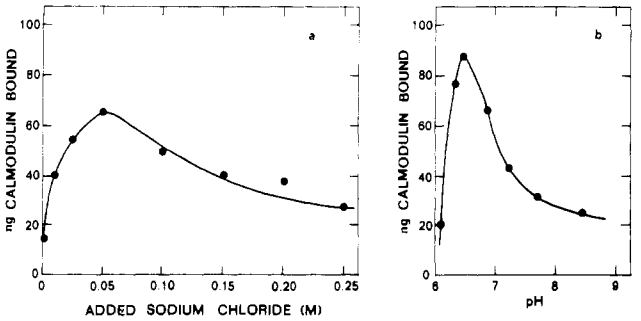

FIGURE 2: Dependence of calmodulin binding to purified red cell membrane cytoskeletons on ionic strength at pH 7.6 (a) and on pH at an ionic strength of 0.16 M (b). Total calmodulin concentration was 1.6 μg/mL. The assay mixture (180 μL) contained 0.16 mg of total membrane cytoskeleton protein.

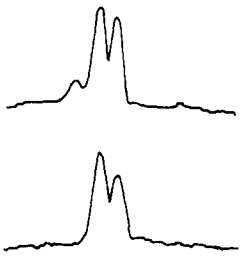

FIGURE 3: Densitometer traces of stained electrophoretic SDS gels, showing the result of photoactivation of a mixture of azidocalmodulin and fodrin in the presence (upper trace) and absence (lower trace) of calcium ions. Migration is from left to right. Note appearance of a new component of higher molecular weight in the presence of calcium, corresponding to an α-chain–calmodulin complex (for experimental conditions see text).

et al., 1981) for membrane skeleton preparations of the same kind, this corresponds to some 75% of the concentration of spectrin, reckoned as dimer, in the assay. The much smaller population of strong binding sites observed in intact membranes (Graf et al., 1980; Agre et al., 1983) was not deteted in the cytoskeletons.

The addition of 4 μM TFP brought the calmodulin detected in the cytoskeleton pellet down to the base-line level, i.e., to the value measured in the absence of calcium ions. The binding of calmodulin showed a considerable dependence on ionic strength (Figure 2a), with a maximum binding at 0.05 M. Magnesium at 5 mM had no detectable effect. There was also a striking pH dependence with a fourfold increase in binding when the pH was reduced from 8.5 to 6.5; the midpoint was pH 7.1. Below pH 6.5 there was a precipitate drop (Figure 2b).

An attempt was made to identify the binding site directly by photoaffinity labeling by using the procedure of Andreasson et al. (1981) to introduce an azido group into the calmodulin. The calmodulin thus derivatized was equilibrated with the cytoskeletons and photoactivated. No new electrophoretic component was detected in the cytoskeletons, solubilized in sodium dodecyl sulfate (SDS). Thus, the azidocalmodulin had not bound, or the reactive nitrene group was unfavorably located for reaction with side chains on the binding protein.

By contrast, azidocalmodulin in association with fodrin in the presence of calcium gave rise to a new electrophoretic zone in SDS gels, resolved from the α-subunit (Figure 3). The mobility increment is consistent with the attachment of a single calmodulin molecule to the α-chain. This is in accord with the observations of Bartelt et al. (1982).

An additional assay for binding of calmodulin to spectrin was devised. This consisted of using F-actin to carry down the spectrin in the tetrameric form in the ultracentrifuge.

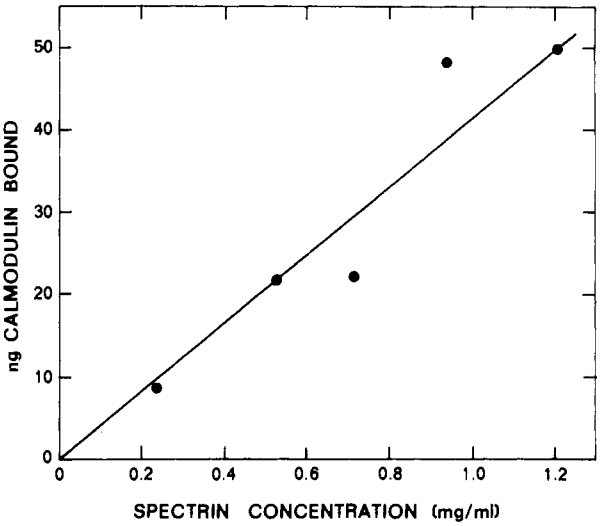

FIGURE 4: Binding of radioiodinated calmodulin to red cell spectrin, measured by ultracentrifugal pelleting of tetrameric spectrin bound to muscle F-actin. Binding was obtained from radioactivity in the pellet, corrected for background level determined with EGTA in place of calcium in the medium. The calmodulin concentration was constant at 33 μg/mL.

Whereas the binding of spectrin dimer to actin in the absence of protein 4.1 is weak (Ohanian et al., 1984), that of the tetramer, which attaches at both ends to actin filaments, is sufficient for the present purpose. The amount of actin pelleted is constant: a dependence of critical concentration on spectrin is seen only in the presence of protein 4.1 (Pinder et al., 1984). The ^{125}I-labeled calmodulin was carried down to an increasing extent with rising spectrin concentration in the mixture, correction being again made for the holdup volume of the pellet and for nonspecific binding from parallel experiments with EGTA in place of calcium chloide. Binding was observed in this way of calmodulin to spectrin (Figure 4). Qualitatively similar results were obtained with fodrin (data not shown).

Actin has not been seen to interact to any detectable extent with calmodulin (Howe et al., 1980; Sobue et al., 1981b). However, a potentiating effect of actin on calmodulin binding by the spectrin could be envisaged. We have attempted to eliminate such a possibility by partially pelleting the spectrin (2 mg/mL) in the mixture with calmodulin directly in the airfuge. Calmodulin alone does not pellet under these conditions. The spectrin pellet contained calmodulin, and the amount of this was reduced when trifluoperazine was present. This affords qualitative evidence of calmodulin binding in the absence of actin, but our results do not allow us to exclude an actin-dependent change in affinity. That this is unlikely, however, is strongly suggested by the remoteness (nearly 100 nm) of the known calmodulin binding site on the analogous fodrin molecule from the actin-binding site (Glenney et al., 1983; Tsukita et al., 1983).

The only (putative) assay for membrane cytoskeleton function is the phosphorylation-dependent gelation under the action of the endogenous cAMP-independent membrane kinase (Pinder et al., 1983). The introduction of calmodulin into this system caused no perceptible change in the appearance of the gel or the time required for gelation.

In view of the reports by Nelson et al. (1983) and Kidoguchi et al. (1982) that calmodulin inhibitors cause stomatocytosis, and the implication that calmodulin is involved in shape control of the intact red cell, we examined the basis of this effect using the well-characterized calmodulin inhibitor TFP. We confirm that this does indeed cause rapid and extensive stomatocytosis (Figure 5). To determine whether it functions by combining

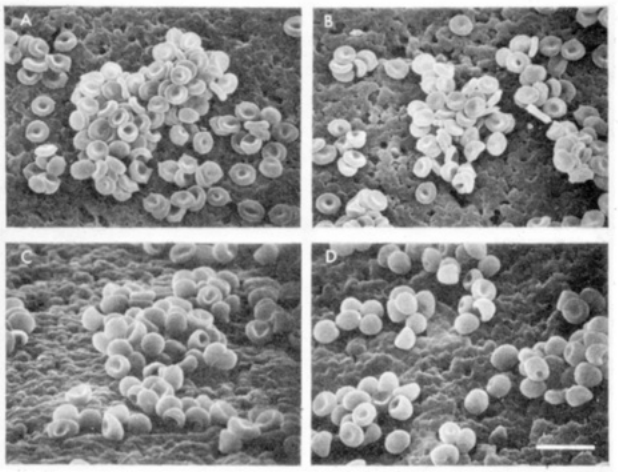

FIGURE 5: Scanning electron microscopy of red cells: (A) untreated, (B) incubated with ionophore A23187 and 1 mM EGTA, (C) incubated with 4 μM trifluoperazine, and (D) incubated with trifluoperazine, following ionophore A23187, and in the presence of 1 mM EGTA. Bar: 10 μm.

with calmodulin or merely by interaction with the membrane bilayer, we depleted the cells of calcium by introducing the ionophore A23187 into the membrane and making the external isotonic medium 1 mM in EGTA. When calcium was present in the external medium, echinocytosis ensued, as expected (Weed et al., 1969). With EGTA outside the cell, withdrawal of intracellular calcium had no effect on the shape (Figure 5). This indicates that, insofar as the action of calmodulin at the membrane must be presumed to be a function of calcium concentration, the perturbation of cell shape does not depend on calmodulin. Moreover, whereas addition of TFP to the cell suspension caused rapid and extensive stomatocytosis (Figure 5), exacty as described by Nelson et al. (1983), this effect was undiminished in cells from which the calcium had been removed in the manner described. In the micromolar range of free calcium concentration the binding of TFP to calmodulin in essentially eliminated (Levin & Weiss, 1977).

Discussion

Our results show that calmodulin is capable of binding to red cell membrane cytoskeletons and to purified spectrin. The association with the latter is of comparable affinity to that with fodrin. The negative results obtained by earlier workers by cross-linking with azidocalmodulin (Bartelt et al., 1982) are explained in terms of the effect of the derivatization itself on the binding process. Since binding to fodrin remains unaffected, this must reflect a steric difference between the calmodulin binding sites. The reason for the failure of several earlier attempts to demonstrate binding of calmoduln to human red cell spectrin is difficult to analyze, but given the low association constant, it may be supposed that experiments at low protein concentrations, or those that rely on transport methods, in which a rapid association–dissociation equilibrium can operate, may give negative results. It may also be recalled (Klee et al., 1973) that gel overlay binding assays not infrequently give spurious results, probably as a result of the failure of proteins, especially of high molecular weight, to refold after exposure to SDS. The reported appearance of binding only under conditions in which spectrin is more or less fully denatured (Sobue et al., 1981) is especially hard to understand, however. Very recently Husain et al. (1984) also obtained evidence of binding of calmodulin to spectrin, from its distribution in a sedimentation equilibrium experiment. With the assumption of one site per dimer an estimated association constant some 10 times lower than ours was obtained.

Our evidence, especially when taken together with that of Berglund et al. (1984), and earlier work on nonerythrocyte spectrins, indicates then that spectrins contain a single weak but specific binding site for calmodulin on each dimer unit, which is conserved throughout this class of proteins. It is not clear what function, if any, such a site might have. Certainly calmodulin is abundant in the cytoplasm, which is in contact with both red cell spectrin and fodrin. Binding, however, is calcium dependent. In the red cell the concentration of spectrin (Steck, 1974; Pinder & Gratzer, 1983) overall is about 3 μM, and that of calmodulin is comparable (Penniston et al., 1980). Various estimates of the calcium concentration in the red cell cytoplasm exist in the literature, generally in the micromolar range. Wiley & Schaller (1977) give a value of 6 μM for total calcium. The binding curves of Ferreira & Lew (1976) would suggest a corresponding value for the free calcium concentration of 1–2 μM. More recently, Simons (1982) has obtained a value of 0.4–0.7 μM, depending on the metabolic state of the cells. To determine the extent of binding of calmodulin to spectrin, the calmodulin–calcium equilibrium must be taken into consideration. The strong calmodulin binding sites (Graf et al., 1980; Agre et al., 1983) make up a total concentration of less than 0.1 μM and can be disregarded for purposes of calculation.

Calmodulin contains four calcium binding sites, of which the strongest has been assigned an association constant of 2×10^5 M^{-1} and the weakest 2.5×10^4 M^{-1} (Crouch & Klee, 1980). More recently Burger et al. (1984) have noted that an adequate fit to the calcium binding profile can also be obtained with four independent sites each with the same binding constant, close to 1×10^5 M^{-1}. It cannot be stated which of the ligation states of calmodulin bind to spectrin, although Burger et al. note that there is no further change in calmodulin conformation after three calcium ions have been bound, and both the complexes with three and four calcium ions appear to interact with known target proteins. If the calcium binding constant to generate a conformation that will bind to spectrin is K, and that for binding of this form to spectrin is L, then assuming the free calcium concentrations, c(say), to be buffered, and denoting the overall calmodulin and spectrin concentrations as $\bar{C}$ and $\bar{S}$, we have that

$$[\mathrm{CaC}] = K[\mathrm{C}]c$$

$$D = L[\mathrm{CaC}][\mathrm{S}]$$

where [C] is the concentration of free calmodulin, [CaC] that of its relevant calcium complex, [S] that of free spectrin, and D that of the complex, containing calmodulin, calcium, and spectrin. Moreover

$$\bar{C} = [\mathrm{C}] + D + [\mathrm{CaC}]$$

$$\bar{S} = [\mathrm{S}] + D$$

From these equations, putting the fractional saturation $\alpha = D/\bar{S}$, we obtain

$$\alpha^2\bar{S} - \alpha[\bar{S} + \bar{C} + (1 + Kc)/(KLc)] + \bar{C} = 0$$

For nominal values of $L = 2 \times 10^5$ M^{-1}, $K = 1 \times 10^5$ M^{-1}, $c = 1 \times 10^{-6}$ M, and $\bar{S} = \bar{C} = 3 \times 10^{-6}$ M, α emerges as 0.05. In various pathological states of the red cell much higher calcium concentrations are encountered, and the fractional binding of calmodulin to spectrin will then be expected to increase greatly; for example, a free calcium concentration of 10 μM would lead to a fractional occupancy of binding sites of 0.3. Similar considerations may be supposed to apply to fodrin in neurons, where much higher calcium concentrations occur than in the normal red cell.

The function of the calmodulin binding site on spectrin and related proteins is a matter of conjecture. Nelson et al. (1983) [see also Kideguchi et al. (1982)] have suggested on the basis of the stomatocytogenic effects of a range of calmodulin inhibitors on red cells that calmodulin may be implicated in shape control. Our observations tend to oppose this view, for under conditions of very low free calcium concentration (in equilibrium with EGTA) there is no distortion of discocytic shape. Moreover, there is no reduction in stomatocytosis under the action of the best characterized calmodulin inhibitor, TFP, when calcium ions are removed. Even if the TFP concentration were sufficient to allow some binding to calmodulin even in the absence of calcium ions, it is hard to envisage any satisfactory mechanism by which a calmodulin-dependent preservation of the discoid shape could operate when all calcium has been removed from the cell. It seems most probable that the calmodulin inhibitors, all of which are predominantly hydrophobic in character, and some cationic, associate with the inner leaflet of the bilayer and cause stomatocytosis in accordance with the principles of the bilayer couple model [Deuticke, 1968; Sheetz & Singer, 1974; see also Ferrell & Huestis (1984)] for a recent formulation of the mechanism of red cell shape control in related terms).

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