Participation of Calmodulin in Immunoglobulin Capping

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ABSTRACT When mouse B lymphocytes are incubated with antibodies against their surface immunoglobulin (Ig), patching and capping occur in a process that involves the action of the actomyosin cytoskeleton and the mobilization of cell calcium. Calmodulin (CaM) plays a central role in the Ca++ regulation of many cellular structures and processes, including the cytoskeleton and membrane-bound enzymes, and therefore was investigated for its role in capping. CaM was isolated from mouse lymphocytes by affinity chromatography on Flu Phenazine-Sepharose. Lymphocyte CaM co-migrates with calf brain CaM on SDS polyacrylamide gels, where its Rf is Ca++-dependent. It stimulates the activity of the CaM-dependent cyclic AMP phosphodiesterase (PDE) of bovine heart. Several phenothiazine and thioxanthene compounds as well as the drugs W7, W5, and R24571 inhibit CaM in in vitro enzyme assays with ID50's of from 1 μM to >1 mM. These were tested for their effects on capping of Ig and were found to inhibit capping in dose-dependent fashions with ID50's that corresponded to their anti-CaM potencies. The drugs also disrupted preformed caps and were all reversible. CaM was localized in lymphocytes by staining with a highly fluorescent trifluoperazine derivative (TFP*) produced by photo-oxidation. TFP* staining was diffuse in untreated lymphocytes but stained under caps and in uropods in cells capped with anti-Ig antibodies. Staining of cells with antibodies against calf brain and rat testis calmodulin gave similar staining patterns. Staining of patched cells with either antibodies or TFP* showed patched distributions of CaM, but submembranous CaM "patches" did not map one-on-one with respect to Ig patches. These observations suggest that calmodulin participates in the latter stages of ligand-induced Ig redistribution probably by regulating the interaction of the cytoskeleton with the membrane.

The capping of surface immunoglobulin on B lymphocytes is a dramatic example of surface receptor redistribution mediated by components of the underlying cytoskeleton (5, 21, 32, 53, 57). The arrangement and regulation of the cell's actomyosin-based motility apparatus and the means by which surface receptors are connected to this machinery through the lipid bilayer are two of the central issues in understanding patching and capping. Cross-linking of receptors by multivalent ligands is required to initiate the energy-independent patching of receptors that must occur before the energy-dependent, cytoskeleton-mediated capping process gathers the patched receptors to one pole of the cell (40, 57, 58, 59). It is during the patching phase of receptor movement that initial connections to the cytoskeleton are made (3, 5, 14, 18, 20, 71). The cell's motility machinery then coordinates the movement of receptor aggregates. These two physiologically and structurally distinct steps may be regulated in similar or dissimilar ways.

One known regulatory species whose local distribution is altered during cross-linking and patching is the calcium ion. Cross-linking of surface immunoglobulin (Ig) by anti-Ig antibodies induces movement of protein-associated Ca++ to lipid regions of the membrane, as reported by the fluorescent probe chlorotetracycline (32). 45Ca++ efflux from intact lymphocytes occurs subsequent to cross-linking (7). Studies of the effects of free-fatty acids on membrane structure and capping have led to the description of membrane domains and implicate changes in local Ca++ distribution as occurring concomitantly with the cross-linking of immunoglobulin receptors (32, 33).
Calcium plays a regulatory role in the capping of receptors as well as in their patching. In the presence of external calcium, the calcium ionophore A23187 is a potent inhibitor of capping (but not patching) and will even disperse preformed caps in an energy dependent process (6, 53). Cis-unsaturated free fatty acids will inhibit capping (but not patching) and can induce changes in the arrangement of the cytoskeleton—these effects are antagonized by high concentrations of extracellular calcium (29, 32). A variety of tertiary amine anesthetics and tranquilizers act as capping inhibitors and in some cells have been reported to dissociate microfilaments from the cell membrane (2, 6, 42, 48, 51, 72). Their anti-capping affects are also antagonized by high extracellular calcium. These observations indicate a role for calcium in maintaining the connection of the cytoskeleton to the membrane. The regulation by calcium of microtubule polymerization, actomyosin-based contraction and cell motility is well documented (11, 23, 24, 26, 28, 41, 43, 62) and must also be considered in the context of capping.

The common theme of calcium control unites these observations and recent studies suggest that the protein calmodulin may provide the key to understanding the molecular basis of this calcium control. Calmodulin (CaM) is a ubiquitous calcium-binding protein that has been found to regulate a variety of cellular processes in eukaryotes; many of these processes are directly relevant to capping (10, 11). Calmodulin regulates the Ca\(^{++}\)/Mg\(^{++}\) ATPases of cell membranes and thereby the internal ionic states of cells (34, 44). It regulates the polymerization of microtubules in vitro and is associated with microtubules in vivo (13, 41, 68). Actomyosin in smooth and skeletal muscles is regulated via the calmodulin dependence of myosin light chain kinase (23, 24, 28, 62). The shapes and ligand binding properties of platelets are altered by calmodulin inhibitors (31). Immunocytochemical localization of calmodulin shows it to be associated with stress fibers or microfilament bundles and the mitotic spindles of fibroblasts as well as with postsynaptic densities of nerve membranes (13, 25, 63, 68, 70). Finally, several of the drugs that are known to inhibit capping have been shown to be potent specific inhibitors of calmodulin in vitro and in vivo (22, 34, 36, 56, 60, 61, 64, 66, 67). These observations prompted us to investigate directly whether calmodulin plays a role in lymphocyte capping.

In this paper we identify and characterize a calmodulin antibody that may regulate capping of surface immunoglobulin and other surface receptors.

**Preparation of Calf Brain Calmodulin for Antibody Elicitation and as a Biochemical Standard**

Purified calmodulin was prepared from calf brain by the method of Wasterson et al. (65) or by a modification of the Fluphenazine-Sepharose affinity chromatography method of Charbonneau and Cormier (9) with similar results and yields of 23-27 mg calmodulin per 6 calf brains (~1 kg wet weight). Fluphenazine-Sepharose 4B was prepared according to the protocol of Charbonneau and Cormier (9).

**Purification of Calmodulin from Lymphocytes**

Lymphocytes from 35 B6AF1/J males were isolated as for capping experiments. 1.6 x 10\(^6\) cells were suspended in 16 ml of 50 mM HEPES, 0.1 M NaCl, 0.1 mM PMSF, pH 7.0 and lysed by nitrogen cavitation (equilibration for 400 psi for 5 min followed by slow exhaust) in a Parr model 4635 cell disruption bomb (Parr Instrument Co., Moline, IL). The lysate was cleared by centrifugation for 30 min at 100,000 g and the supernatant brought to 0.5 M in NaCl and 10 mM Ca\(^{++}\). This material was applied to a 15 ml Fluphenazine-Sepharose 4B affinity column and calmodulin was eluted with 10 mM EGTA, pH 7.8 as described by Charbonneau and Cormier (9).

**Preparation of Antisera and Affinity Purification**

Four New Zealand albino rabbits were inoculated with 0.5 mg of dimethylphenylated calmodulin (DNB-CaM) in Freund’s complete adjuvant (Cappel Laboratories Inc.). DNB-CaM was prepared according to Wallace and Cheung (63). Rabbits were boosted on day 32 with 0.5 mg of DNB-CaM and on days 44, 50, and 65 with 0.5 mg of native calmodulin in incomplete adjuvant. Sera were collected 7 d after injections and screened for activity by indirect immunofluorescence. Sera that gave the brightest staining were further characterized and affinity purified by chromatography on calmodulin-Sepharose. Calmodulin-Sepharose was prepared according to Niggli et al. (44), 10 mg of calmodulin was coupled to 2.5 ml of CNBr-activated Sepharose 4B. 12 ml of serum 115 (46 mg/ml protein) was incubated with CaM-Sepharose as a slurry for 1 h at 4°C in phosphate buffered saline, loaded into a 10 ml CNBr cage barrel and washed. CaM binding antibodies were eluted with 0.2 M glycine pH 2.8 and protein peak fractions were pooled, dialyzed against PBS plus 0.01% NaN\(_3\) and stored frozen in aliquots at 0.13 mg/ml.

**Staining of Lymphocytes with Anti-Calmodulin Antibodies**

Anti-CaM antibodies were used to stain lymphocytes in various states of Ig receptor distribution by indirect immunofluorescence. Unchallenged, patched, and capped lymphocytes (patched and capped with FITC-GaM-Mg) were fixed with 3.7% formaldehyde plus 1% methanol in PBS and allowed to settle onto polyethyleneimine coated coverslips at 1 g. The cells were incubated with 0.1 M L-lysine pH 7.5 to block unreacted aldehydes and permeabilized by immersion for 3-5 min in acetone cooled on dry ice. The cells were rehydrated in PBS and used for staining. Coverslips were inverted over 1% BSA + 1% normal goat serum, 3% fetal calf serum and 3.7% formaldehyde plus 1% methanol in PBS and incubated for 45 min. The coverslips were rinsed for 3-5 min in acetone cooled on dry ice. The cells were rehydrated in PBS and used for staining. Coverslips were overcoated with 1% BSA and 1% goat serum in PBS for 1 h to block nonspecific binding of protein to the cells and coverslips stained with 25 μl of anti-CaM antibodies at 1:10 dilution in PBS plus 1% BSA and 1% goat serum overnight at 4°C. The coverslips were rinsed three times for 30 min in PBS then incubated with absorbed RITC-GaM-Rg at 1:100 dilution in PBS plus 1% BSA and 1% goat serum for 2 h at 23°C, rinsed several times with PBS and mounted over 50% glycerol in PBS plus 0.01% NaN\(_3\). Absorption of RITC-GaM-Rg was done by incubating the antibodies overnight at 4°C with an acetic precipitate of mouse splenic lymphocytes that had been fixed with 2% glutaraldehyde for 30 min and blocked with 0.1 M L-
lysine, pH 7.5, followed by several PBS washes. After incubation the absorbant was spun out for 3 min in a Beckman microcentrifuge (Beckman Instruments, Inc., Fullerton, CA) and the soluble RITC-GAR IgJ used as above to label the bound rabbit anti-CaM antibodies. Control staining consisted of the second serum alone, which gave very low background stain, or of primary and then secondary antibody where primary staining was conducted in the presence of soluble CaM; the latter gave significantly reduced intensity. Identical procedures were used to stain cells with sheep anti-rat testis calmodulin (CAABCO, Inc., Houston, TX) using FITC-Rabbit anti-sheep antibodies (Cappel Laboratories, Inc.) in 1% serum plus 1% BSA as secondary serum.

Indirect immunofluorescence of cultured cells grown on glass coverslips was performed according to Fujisawa and Pollard (19) using rabbit antibodies against calmodulin, as well as fish muscle actin and chicken gizzard myosin antibodies generously supplied by Dr. Keiji Fujisawa. In all cases the cells were fixed in 3.7% formaldehyde, acetic-permeabilized and stained with antibodies as above in the presence of 1% BSA and 1% goat serum. Goat anti-rabbit Ig antibodies were preabsorbed with glutaraldehyde-fixed acetone precipitates of the target cells.

Staining of Cells with Photo-oxidized Tryptifluorperazine

Live cells or cells preserved by a variety of methods were used for staining with trifluoperazine (TFP) and nearly identical results were obtained with all methods of preservation. Cells fixed for 10 min in 3.7% formaldehyde in PBS gave the best staining. Cold acetone permeabilization alone or in combination with formaldehyde fixation and antibody staining produced similar staining patterns but were somewhat reduced in intensity. Live cells were distinguishable from formaldehyde-fixed cells when stained for 1 or 2 min and immediately observed, but TFP can induce morphological changes in cells after only a few minutes. The most convenient staining method was to mount coverslips with attached cells on a slide containing a drop of 3 × 10^−8 M to 1 × 10^−7 M TFP in PBS plus 1 mM CaCl_2. The coverslips are then rinsed in PBS before mounting. Rinsing can sometimes be eliminated as background fluorescence is quite low and no obvious differences in staining pattern are obtained. The slide is then UV-irradiated directly for 10 min using a mineral light or mounted on the unfiltered UV or the filtered light from the Leitz filter combination #513410 used for observation on a Leitz Ortholux II microscope equipped for epifluorescence. Yellowish fluorescence appears almost immediately and intensity stabilizes after a minute or less depending upon the drug concentration used. When using double fluorescent staining with fluorescein-tagged antibodies it was a good practice to observe and photograph the fluorescein (as it bleaches) before irradiating for photo-oxidized TFFP observation.

Phosphodiesterase Measurements

Assays of 3':5'-cyclic AMP phosphodiesterase from bovine heart were by a modification of the procedure of Butcher and Sutherland (9). Briefly, 60 μl of sample (containing CaM) was added to 420 μl of 0.1 M glycine, 2.86 mM CaCl_2, 1.43 mM MgSO_4, pH 7.5, 60 μl of phosphodiesterase (0.02-0.04 U, Sigma Chemical Co., St. Louis, MO) in 20 mM imidazole, 0.1 M glycine, pH 7.5, from frozen aliquots was then combined. The reaction was started by adding 60 μl of 6 mM cyclic AMP in 0.1 M glycine, pH 7.5 (made fresh or stored at −70°C), and incubating for 30 min at 37°C. The first reaction was stopped by boiling and flocculant material, if present, was spun out at 2,000 g for 5 min. 0.1 unit of S' nucleotidase (Crotalus atrox, Sigma Chemical Co.) in 100 μl of 3.3 mM MnCl_2, 0.1 M glycine, pH 7.5, was added to initiate the cleavage of phosphate from AMP and incubated for 15 min at 37°C. The S' nucleotidase reaction was stopped with 200 μl of 0.25 M ZnSO_4 pH adjusted to 2.5 with HCl and phosphate measured in duplicate 0.3-ml aliquots from the above solution according to Ebel and Lands (16).

Gel Electrophoresis

SDS PAGE was processed according to Laemmli (35). Samples sometimes included 10 mM CaCl_2 or 10 mM EGTA for assaying conformational changes in CaM. 2 mM EGTA was included in the resolving gel buffer when it was necessary to suppress smearing or heterogeneity of CaM antibodies in samples in which calcium concentration was not controlled. Isoelectric focusing was according to the first dimension protocol of O'Farrel (46) substituting an ampholine mixture of pH 4.0-7.0 for the fixative used. Transition fractions, CaM migrated as a doublet. With silver staining, proteins vary in staining color with calcium concentration from M_\text{app} = 17,900 + or − 1400 (N = 4) in the presence of 10 mM CaCl_2 to M_\text{app} = 20,500 + or − 1100 (N = 3) when the sample contained 10 mM EGTA. The protein stained 3':5'-cyclic AMP phosphodiesterase from bovine heart in a calcium-dependent fashion and had an isoelectric point of pH 3.9 with a minor component at pH 4.0 (Fig. 1). Elution of the protein from a Fluphenazine-Sepharose affinity column with EGTA demonstrated the calcium-dependent binding of CaM to the phenothiazine.

RESULTS

Purification and Characterization of Brain Calmodulin

Calmodulin (CaM) from calf brain was purified for use as a biochemical standard and for eliciting anti-CaM antibodies in rabbit by two standard methods, each of which gave essentially the same results. CaM was judged to be pure by SDS PAGE and alkaline urea gels and ran as a single electrophoretic species. The apparent molecular weight (M_\text{app}) in SDS gels varied with calcium concentration from M_\text{app} = 17,900 + or − 1400 (N = 4) in the presence of 10 mM CaCl_2 to M_\text{app} = 20,500 + or − 1100 (N = 3) when the sample contained 10 mM EGTA. The protein stimulated 3':5'-cyclic AMP phosphodiesterase from bovine heart in a calcium-dependent fashion and had an isoelectric point of pH 3.9 with a minor component at pH 4.0 (Fig. 1). Elution of the protein from a Fluphenazine-Sepharose affinity column with EGTA demonstrated the calcium-dependent binding of CaM to the phenothiazine.

Purification of Calmodulin from Lymphocytes

CaM was isolated from 100,000 g supernatant of murine spleenic lymphocytes by Fluphenazine-Sepharose affinity purification. CaM-containing fractions eluted from the affinity column beginning about one bed volume after application of EGTA-containing elution buffer. SDS PAGE of elution fractions showed that CaM in early fractions migrated with an apparent molecular weight of 18,000 but, this increased to M_\text{app} = 20,200 in later fractions containing 10 mM EGTA and these co-migrated with brain CaM (also applied with 10 mM EGTA) (Fig. 1), transition fractions, CaM migrated as a doublet. With silver staining, proteins vary in staining color from reddish brown to smoky gray and both lymphocyte and calf brain CaM stained gray. Occasionally, proteins of molecular weights 28,000, 44,000, and 72,000 eluted with CaM and may represent CaM-binding proteins as their apparent abundance on gels parallels that of CaM for various elution fractions. They may also be phenothiazine-binding proteins in their own right. We have encountered similar contaminants in affinity purifications of CaM from neutrophils and CHI B lymphoma cells. Phosphodiesterase (PDE) measurements on elution fractions showed they had little or no intrinsic PDE activity, but they stimulated exogenously added PDE from bovine heart. Peak PDE stimulatory activity and intensity
of the 18–21,000 mol wt protein band were obtained from the same fractions. Electrophoresis of pooled CaM fractions showed that the change in molecular weight as a function of pH 3.9 (Fig. 1). From protein determinations and densitometry of SDS PAGE bands, we made the crude estimate that CaM comprised of the order of 0.1% of soluble cell protein or 50–60 μg per 10⁶ cells.

Anti-Capping Effects of Calmodulin Inhibiting Drugs

Dose-response curves for capping inhibition were obtained with the new CaM-inhibiting drugs R24571, W7, W5, and a variety of phenothiazines and thioxanthenes (Fig. 2). These include: trifluoperazine (TFP), trifluoperazine sulfoxide (TFPSO), chlorpromazine (CPZ), chlorpromazine sulfoxide (CPZSO), trifluoperazine (TPZ), fluphenazine (FPZ), prochlorperazine (PCP), chlorprothixene (CPT), and thiioxanthenes (TT). All of the phenothiazines and thioxanthenes except two inhibited capping at micromolar concentrations with ID₅₀’s of 20–40 μM. Two analogues, TFPSO and CPZSO, were ineffective inhibitors; for these, ID₅₀’s were >1 mM. β,β-propranolol was a weak inhibitor with ID₅₀ = 550 μM. R24571 was the most potent inhibitor with ID₅₀ = 1 μM. W7 was 50% effective at 20 μM whereas its weaker analogue, W5, had an ID₅₀ of 170 μM. ID₅₀’s for capping inhibition paralleled the inhibitions measured in vitro for CaM-stimulated activity, showing that the specificities are such as would be expected for a CaM-dependent process (22, 27, 60, 64, 66, 67). The ability to disperse preformed caps was obvious for all of the analogues except TFP and CPZ. Thus the rotational diffusion of DPH (a measure of fatty acyl chain ordering) was not affected. Woda et al. (69) have shown that CPZ at similar concentrations leads to only perfunctorial increases in lateral diffusion of Fab'-labelled surface immunoglobulin and the lipid probe Hedaf (5-(N-hexadecanoyl)aminofluorescein).

Fluorescent Localization of Calmodulin with Photo-oxidized Trifluoperazine

Trifluoperazine can be photo-oxidized to highly fluorescent derivative(s) (TFP*) that can be made to bind reversibly or irreversibly to CaM. Dose-response curves for capping inhibition were obtained with the CaM-inhibiting drugs R24571 (▲), W7 (■), W5 (○), the phenothiazines trifluoperazine (TFP, ●), Chlorpromazine (CPZ, □), and their sulfoxide derivatives (TFP-SO, ○ and CPZ-SO, □). ID₅₀’s for capping inhibition paralleled the inhibitory doses measured in vitro for CaM-stimulated enzymes. Lymphocytes were incubated for 20 min on ice with the drugs in PBS. Anti-Ig antibodies were added and cells were washed three times in PBS and suspended in 2 μM DPH in PBS for fluorescence depolarization measurements using an Elscint model MVIA microviscometer. None of the four phenothiazines affected the fluorescence depolarization values for DPH at concentrations from 10⁻⁵ to 10⁻⁴ M (at which concentration total capping inhibition occurs for TFP and CPZ). Thus the rotational diffusion of DPH (a measure of fatty acyl chain ordering) was not affected. Woda et al. (69) have shown that CPZ at similar concentrations leads to only perfunctorial increases in lateral diffusion of Fab'-labelled surface immunoglobulin and the lipid probe Hedaf (5-(N-hexadecanoyl)aminofluorescein).
irreversibly to CaM depending upon the procedure used. This property allows visualization of TFP binding sites (CaM) by fluorescence. O'Kane et al. (47) showed that one of the TFP photo-oxidation products made by UV irradiation of TFP in aqueous solution would bind reversibly to CaM in a Ca⁺⁺-dependent fashion. This product stained CaM in nondenaturing acrylamide gels. The acrosomes of guinea pig sperm perfused with the derivative were also visualized using an image intensification system and the staining pattern corresponded to the staining with anti-CaM antibodies. Prozialeck et al. (50, 66) recently demonstrated that if tritiated CPZ or TFP are irradiated in the presence of CaM or other proteins, irreversible Ca⁺⁺-dependent binding of the tritiated drugs to CaM occurs, but observed no significant binding to other proteins. We have combined the irreversibility and fluorescence properties of TFP to irreversibly stain CaM in lymphocytes and other cells. The irreversible photo-oxidation reaction is rapid (30 s is usually adequate) and can be performed in situ on slide-mounted cells or sections of tissue directly under the microscope. Photo-oxidation occurs with either unfiltered UV light (from a mercury or xenon source) or with the band pass filtered excitation light between 340-380 nm of the Leitz #513410 filter combination. Using epifluorescent illumination the emission is quite bright and does not require image intensification. In agreement with O'Kane et al. (47) excitation maxima at 307 nm (minor) and 336 nm (major) and emission maxima at 416 nm (minor) and 506 nm (major) were measured for the yellow TFP photo-oxidation product that migrates with an Rf of 0.04 in 50:50:1.5 methanol:acetone:triethanolamine on silica gel G (Rf of TFP is 0.48). These parameters were used to choose the above Leitz filter combination. The Zeiss BG3 excitation and 50 barrier filter combination is also acceptable but allows more cross-channel leak when used with fluorescein. Bleaching does not occur with TFP* and, in fact, samples usually increase somewhat in brightness during observation. The excitation and emission spectra of TFP* allow double or triple fluorescent staining with rhodamine- or fluorescein-labeled antibodies. The distribution of TFP* fluorescence was found to be the same in cells that were unfixed, formaldehyde-fixed, acetone-fixed, or both, and to be stable for more than a month in slides stored at 4°C under 50% glycerol. Cells not subjected to acetone extraction were the brightest.

In lymphocytes that have been unchallenged by anti-Ig antibodies, photo-oxidized trifluoperazine fluorescence is found throughout the cell, often concentrated in one or more diffuse areas (Fig. 4). Occasionally, a radiating star-sapphire-like distribution is observed that is reminiscent of the tubulin distribution seen in lymphocytes (29, 71). In patched lymphocytes, Ig patches and large patches of TFP* are both observed, but the two fluorescent species distribute independently of one
myosin is concentrated (23, 24). Brush borders from rat small intestine were labeled heavily in the microvilli and throughout the cytoplasm except for the nucleus and the terminal web (not shown). TFP* stains the tips of secreted trichocysts in Paramecium (39). All of these structures have been shown to be occupied by CaM using immunofluorescence and immunoelectron microscopy techniques (13, 25, 43, 63, 70).

**Antibody staining**

Staining of lymphocytes with rabbit anti-brain calmodulin antibodies (RACaM) revealed staining patterns similar to those

![Image of antibody staining](https://example.com/antibody-staining.png)

...another and do not necessarily map one-on-one. In capped lymphocytes, TFP* is concentrated under the cap or throughout the uropod of the cell. The frequency of co-localization with anti Ig is 82% (N = 56 cells). This is the same region stained in capped lymphocytes by anti-actin, anti-myosin, anti-tubulin, and alpha-actinin antibodies, suggesting an association of TFP* with the cytoskeleton. Non-uniform TFP* fluorescence is also observed in anti-immunoglobulin negative cells (presumably T lymphocytes). In B and C, the top row is fluorescein-labeled goat anti-mouse Ig and the bottom row is TFP*. In C the right hand group of cells was stained after formaldehyde fixation and cold acetone permeabilization, whereas other samples received no acetone treatment. X 450.

![Image of antibody staining](https://example.com/antibody-staining-2.png)

...the uropod of the cell. This co-localization is specific for CaM as fluorescence is inhibited by incubating cells and antibody in the presence of soluble CaM and no staining is observed with secondary antibody alone. Identical staining patterns are observed with rabbit anti-calf brain CaM and sheep anti-rat testis CaM (CAABCO). (A-F) Three groups of capped cells showing co-localization of anti-lg and anti-calf brain CaM. (C-F) Are specificity controls. (A, C, and E) FITC-goat anti-mouse Ig. (B) RITC-goat anti-rabbit Ig plus rabbit anti-calf brain CaM in 1% BSA plus 1% goat serum. (D) As in B, but in the presence of 100 μg/ml CaM. (F) RITC-goat anti-rabbit Ig only (G-I) Patched and capped cells stained with FITC-rabbit anti-mouse Ig (G and I) and RITC-rabbit anti-sheep Ig plus sheep anti-rat testis CaM (CAABCO) (H and J) in the presence of 1% BSA plus 1% rabbit serum. X 700.
Smooth muscle cells stained with anti-CaM were labeled brightly along stress fibers. Diffuse staining was found throughout the cells, but was concentrated in the perinuclear region. Little or no staining was observed in the nuclei and, unlike staining with TFP*, nucleoli were unlabeled, perhaps because of incomplete penetration (Fig. 7).

DISCUSSION

Identification of Calmodulin in Lymphocytes

A protein exhibiting the characteristic properties of calmodulin has been isolated from splenic lymphocytes. Lymphocyte CaM demonstrates calcium-dependent binding to phenothiazines and this feature was used to purify CaM by affinity chromatography on Fluphenazine-Sepharose, to which it binds in the presence of calcium and elutes with EGTA. Lymphocyte CaM co-migrates with calf brain CaM in SDS PAGE and its apparent molecular weight varies as a function of calcium in the sample, reflecting the conformational change induced by calcium binding. The isoelectric point of lymphocyte CaM is the same as that measured for brain CaM (pI = 3.9) and the protein stimulates CaM-dependent cAMP phosphodiesterase. Taken together, these properties define a calmodulin with features of CaM's isolated from other sources (9, 10, 11, 65).

Effects of Calmodulin Inhibitors

Pharmacological experiments indicate that inhibitors of calmodulin are inhibitors of ligand-induced capping (but not patching) of surface immunoglobulin on murine B lymphocytes. Preformed Ig caps are also dispersed by these inhibitors. Therefore the maintenance of caps as well as their formation requires the action of calmodulin. Not only do CaM inhibitors block capping, but they do so with dose responses that closely match their in vitro antagonism of CaM-dependent enzymes such as cAMP phosphodiesterase, myosin light chain kinase, and Ca++ transport ATPase (22, 27, 60, 64, 66, 67). Especially important are the observations that: (a) the inhibitor analogues W5 and phenothiazine sulfoxides, which are inactive or only slightly active against CaM, are also ineffective in blocking capping, and (b) that CaM inhibitors with widely differing chemical structures can block capping. These results argue that the anti-capping effects are tied to the anti-CaM effects and are not due to general toxicity or membrane-perturbing properties (49). Furthermore, no significant alterations in membrane structure were detected with membrane probes DPH and Hediaf (69) in the presence of phenothiazines, nor was the lateral diffusion of Fab'-labeled surface Ig significantly altered (69).

Because the drugs are reversible and wash out, their action is not likely to be simply a toxicity artifact. The pharmacological data presented here are in good agreement with previous studies from this laboratory and others that have examined the effects of phenothiazines and local anesthetics on capping of B lymphocytes as well as B and T lymphoma cells (2, 42, 48, 51). In earlier reports, the anti-calmodulin action of the drugs was not recognized. In light of what is now known, however, we may add the following compounds to the list of surface Ig capping inhibitors which are also known calmodulin inhibitors: dibucaine, tetracaine, lidocaine = xylocaine, procaine, phencaine, and vinblastine; many of their structural analogues are also anti-capping agents (42, 48, 51; our unpublished observations). Calmodulin inhibitors also block capping of Fc receptors and TL antigen on B lymphocytes, but not H2 or Thy 1 antigens on T cells (6). In the T lymphoma line BW 5147,
Bourguignon (2) found that the antigens T200, gp69/71, and Thy-1 are inhibited by trifluoperazine and speculated that this was related to the drug's anti-CaM activity. In WiL2, a human lymphoma cell line, Con A capping was not inhibited by trifluoperazine but the distribution of calmodulin itself was altered (52).

**Intracellular Localization of Calmodulin**

Localization of calmodulin in lymphocytes in various stages of surface Ig redistribution suggests that this protein associates with the cytoskeleton. Staining with three independent CaM-labeling reagents demonstrated nearly identical calmodulin distributions. In untreated lymphocytes, rabbit anti-calf brain CaM, sheep anti-rat testis CaM (CAABCO) and photo-oxidized trifluoperazine all show diffuse fluorescence throughout the cytoplasm with common local accumulations. In Ig patched cells, CaM distribution is also more patchy but intracellular CaM "patches" do not necessarily lie directly beneath each surface Ig patch. In capped cells there is a dramatic change in the CaM distribution and intense fluorescence is found under caps and in uropods. This is remarkably similar to the behaviors observed for actin, myosin, tubulin, and alpha-actinin, which, although their initial distributions vary, all become concentrated under caps and often are found underneath Ig patches (3, 5, 20, 71). This co-localization suggests that the action of CaM in capping is via the contractile or gelation-solvent activities of the cytoskeleton which is especially important in the later stage of Ig redistribution. Salisbury et al. (52) have also demonstrated the concentration of CaM under Con A caps in the WiL2 human lymphoma cell line. The same correlation between CaM and cytoskeleton has been described by others for a variety of tissues and cultured cells using antibody-labeling techniques and this is true for our antiserum as well.

The specificity of the anti-calf brain calmodulin antibody is indicated by (a) the electrophoretic purity of the antigen, (b) the affinity purification of the antibody on immobilized pure antigen, (c) the observation that staining intensity is significantly reduced in the presence of soluble CaM, but not 1% bovine serum albumin or 1% goat serum, (d) lack of staining by secondary antibody alone, (e) similarity of staining pattern to that of sheep anti-rat testis CaM (CAABCO), and (f) correspondence of staining patterns observed with other anti-CaM antibodies in lymphoma and other cultured cells and Paramecium.
The specificity of photo-oxidized trifluoperazine staining is verified by its co-localization with anti-CaM antibodies and its biochemical binding properties. Known intracellular concentrations of calmodulin which are labeled by TFP* are: under lymphocyte caps, along stress fibers, in the microvilli and cell body, but not in the terminal web of isolated brush border cells, on skeletal muscle A bands, on secreted trichocysts of Paramecium (59) and staining of nuclei that contain the CaM-dependent myosin light chain kinase (13, 24, 25, 63, 70). In the case of skeletal muscle, calmodulin is known to be located on either the I or A band depending on the state of contraction (25). Here, it is significant that TFP* preferentially stains one discrete region as the contractile state of the fiber was not controlled.

The actual mode of in situ labeling is not precisely known, but there are two likely mechanisms. First, in situ labeling would generate the highly fluorescent photo-oxidation product described by O'Kane et al. (47), which would bind to CaM in a calcium-dependent fashion. Formaldehyde fixation and/or acetone extraction may leave calcium-loaded CaM in place and active following rehydration so that it could bind TFP* in this way. Alternatively, a highly reactive photo-oxidation product such as described by Prozialeck et al. (50, 66) may covalently label via a free radical addition to CaM to which the unactivated precursor, trifluoperazine, was bound. The TFP* might also be subject to nucleophilic attack by the CaM to which TFP was bound at the time of illumination. When EGTA-containing buffer was used to rehydrate or wash following fixation and/or acetone extraction, no obvious decrease in TFP* labeling occurred. Similarly, illumination in the presence of nonfluorescent phenothiazines was not effective in competing TFP* from its binding sites and multiple washes and extensive storage over 50% glycerol failed to significantly reduce staining. This favors a covalent mechanism of labeling.

Summary

From the evidence presented above and from what is known about the actions of calmodulin in other systems, we propose that calmodulin is required for the energy-dependent capping of surface immunoglobulin. Calmodulin may be a component of patches as well but is not required for their formation.

Two general mechanisms can be envisaged to explain calmodulin's activity and one or both may be operating. Either CaM regulates the contractile or gelation/solation activity of the actomyosin-based cytoskeleton, or it regulates the attachment of the cytoskeleton to membrane-bound immunoglobulin and its complexes with other membrane proteins. In favor of the first mechanism are the observations that (a) CaM colocalizes with the cytoskeleton in lymphocytes (5, 20, 21, 71) as well as with actomyosin-rich structures in other cells such as stress fibers (13), microvilli (43), skeletal muscle “A” bands (25), and the ends of mitotic spindles (13, 25, 64); (b) CaM regulates the action of myosin via its CaM-dependent myosin light chain kinase (24, 62); (c) myosin light chains are phosphorylated during capping of T lymphoma cells (2); (d) CaM inhibitors cause relaxation of smooth muscle cells and block the superprecipitation of their crude extracts (28); (e) muscle cell energy reserves are regulated by CaM via glycerone synthetase (10, 11) and (f) CaM inhibitors prevent the spreading of cultured fibroblasts (60, 66).

The second mechanism is supported by a variety of findings. Platelet shape changes and the binding of von Willebrand factor VIII to platelet surface receptors are specifically altered by CaM inhibitors (31). The binding of gonadotropin-releasing hormone to pituitary cells (which is followed by patching and capping) causes the translocation of calmodulin from the cytosol to the plasma membrane (12). The shapes of intact erythrocytes and erythrocyte ghosts are altered by calmodulin inhibitors, specifically they cause “cupping” (15; our unpublished observations). The shape of erythrocytes is controlled by the peripheral membrane protein spectrin, which binds calmodulin and is phosphorylated by a CaM-dependent kinase (30, 55). Actin is known to bind to the erythrocyte membrane via a complex of proteins including spectrin, ankyrin, and bands 3 and 4.1 (4). Recently, it has become evident that proteins chemically and immunologically related to spectrin and ankyrin have been found in other cell types, which suggests that actin may attach to their membranes by mechanisms analogous to that in the erythrocyte (1, 32, 38). One of these, fodrin, redistributes under the membrane of lymphocytes capped with anti-H2 and anti-lg antibodies (38). Furthermore, microfilaments have been observed to be displaced from the membranes of cultured cells by CaM-inhibiting drugs which are also capping inhibitors (48).

Therefore, the action of calmodulin in the capping of lymphocyte surface immunoglobulin may be a specific instance of a general calmodulin activity in regulating cell membrane topography and cell shape. These functions would be regulated by the action of calmodulin on the cytoskeleton and the latter’s sites of interaction with the cell membrane.

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REFERENCES

1. Bennett, V. 1979. Immunoreactive forms of human erythrocyte ankyrin are present in diverse cells and tissues. Nature (Lond.) 281:597-599.
2. Bourguignon, L. Y. W., and K. G. Balazs. 1980. Effect of the antidepresant drug stelazine on lymphocyte capping. J. Cell. BioL 86:947-952.
3. Bourguignon, L. Y. W., K. T. Takoyasu, and S. J. Singer. 1978. The capping of lymphocytes and other cells. Studied by an improved method for immunofluorescence staining of frozen sections. J. Cell Physiol. 95:2309-2318.
4. Braunton, D. C., M. Cohen, and T. Tyler. 1981. Interaction of cytoskeletal proteins on the human erythrocyte membrane. Cell. 24:24-32.
5. Braun, J., K. Fujiwara, T. D. Pollard, and E. R. Unanue. 1978. Two distinct mechanisms for redistribution of lymphocyte surface macromolecules. I. Relationship to cytoplasmic myosin. J. Cell Biol. 79:401-415.
6. Braun, J., K. Fujiwara, T. D. Pollard, and E. R. Unanue. 1978. Two distinct mechanisms for redistribution of lymphocyte surface macromolecules. II. Contrast effects of local anesthetics and a calcium ionophore. J. Cell Biol. 79:410-420.
7. Braun, J., R. I. Sha'afl, and E. R. Unanue. 1979. Cross-linking by ligands to surface immunoglobulins triggers mobilization of intracellular “Ca2+” in B lymphocytes. J. Cell Biol. 82:735-746.
8. Butcher, R. W., and E. W. Sutherland. 1962. Adenosine 3',5'-phosphate in biological materials. 1. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to determine adenine 3',5'-phosphate in human urine. J. Biol. Chem. 237:1244-1250.
9. Charbonneau, H., and M. J. Cormier. 1979. Purification of plant calmodulin by butyrylarnine-sepharose affinity chromatography. Biochem. Biophys. Res. Commun. 103:1039-1047.
10. Cheng, W. Y. 1979. Calmodulin plays a pivotal role in cellular regulation. Science (Wash. D. C.) 207:19-27.
11. Cheng, W. Y. editor. 1980. Calcium and Cell Function. 1. Calmodulin. Academic Press, New York.
12. Croft, P. M., J. G. Chaikusas, D. Rogers, and A. R. Means. 1981. Gonadotropin releasing hormone stimulates calmodulin redistribution in rat pituitary. Nature (Lond.) 290:264-265.
13. Dedura, J. R., M. L. Lewis, and A. R. Means. 1978. Ca2+ -dependent regulation. Production and characterization of a monospecific antibody. J. Biol. Chem. 253:7515-7521.
14. De Petris, S. 1974. Inhibition and reversal of capping by cytochalasin B, vinblastine and colchicine. Nature (Lond.) 245:54-56.
15. Deuticke, B. 1968. Transformation and restoration of bicornate shape of human erythrocytes induced by amphiphilic agents and changes of tone environment. Biochem. Biophys. Acta 163:494-500.
Eibl, H., and W. E. M. Lands, 1969. A new, sensitive determination of phosphate. Anal. Biochem. 50: 43-50.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1975. Electrophoretic analysis of the major polypeptides of human erythrocyte membrane. Biochemistry. 14:2647-2667.

Flanagan, J., and G. L. E. Koch. 1978. Cross-linked surface Ig attaches to actin. Nature (Lond.). 273:378-218.

Fusco, D., and L. P. Poind. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. J. Cell Biol. 71:848-875.

Gabbiani, G., C. Chaponnier, A. Zumbo, and P. Basallie. 1979. Actin and tubulin co-cap microtubules in the mouse B lymphocyte. Nature (Lond.). 280:697-701.

Geiger, B., and J. S. Singer. 1979. The participation of alpha-actinin in the capping of cell membrane components. Cell 16:213-222.

Goldman, R. T., and J. Rosenbaum. editors. 1976. Cell Motility. Vol. 1-3 Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Guerrero, V. I., K. C. Rowley, and A. R. Means. 1981. Production and characterization of an antibody to myosin light chain kinase and intracellular localization of the enzyme. Cell 27:449-458.

Harter, J. F., M. Y. Cheung, R. W. Wallace, S. N. Levine, and A. L. Steinor. 1980. Immunocytochemical localization of calmodulin in rat tissue. J. Cell and Cell Function I. Calmodulin. Wai Yiu Cheung, editor. Academic Press, New York. 237-290.

Hidaka, H., and S. V. Perry. 1974. The interaction of the calcium-binding protein (troponin C) with bivalent cations and the inhibitory protein (troponin I). Biochem. J. 137:145-154.

Hidaka, H., Y. Sasaki, T. Tanaka, T. Endo, S. Obno, Y. Fuyu, and T. Nagata. 1981. (G-Aminoethoxy-S)-Schior-1-naphthylene sulfonamide, a calmodulin antagonist, inhibits cell proliferation. Proc. Natl. Acad. Sci. U.S.A. 78:4354-4357 and erratum 7844.

Hidaka, H., T. Yamaki, T. Totoroku, and M. Asano. 1979. Selective inhibitors of Ca2+-binding modulator of phosphodiesterase produce vascular relaxation and inhibit arin-myosin interaction. Mol. Pharmacol. 15:49-59.

Hoover, R. L., K. Fujiwara, R. D. Klausner, D. K. Bhalla, R. Tucker, and M. J. Karnovsky. 1980. Aminothiazole antipsychotic drugs interact with bivalent cations and the inhibitory protein (troponin I) with bivalent cations and the inhibitory protein (troponin I). Biochem. J. 137:145-154.

Kao, K-J., J. R. Sommer, and S. V. Pizzo. 1981. Modulation of platelet shape and enolase P-reactivity by phenothiazine antipsychotics. Mol. Pharmacol. 19:264-269.

Kan, G. R., E. R. Unanue, and M. J. Karnowsky. 1974. Inhibition of surface capping of macromolecules by local anesthetics and tranquilizers. Nature (Lond.). 250:56-57.

Kopecky, K. M., R. H. Feldman, and G. L. Nicolson. 1979. Local anesthetics affect membrane cytoskeletal control of motility and distribution of cell surface receptors. Proc. Natl. Acad. Sci. U.S.A. 76:1991-1995.

Krause, W., P. H. Atwood, and J. W. Vail. 1975. Effects of local anesthetics on membrane properties I. Changes in the fluidity of phospholipid bilayers. Biochim. Biophys. Acta 394:506-511.

Krause, W., C. M. Cimino, and B. Weiss. 1981. Photoaffinity labeling of calmodulin by phenoxybenzamidine antipsychotics. Mol. Pharmacol. 19:264-269.

Levine, J., and M. Willard. 1979. The localization of the calcium-binding proteins A and C in the lens of the eye. Exp. Cell Res. 120:354-368.

Lewis, L. M., K. Fujiwara, and G. Nelson. 1981. Factors affecting trichocyst secretion in Paramecium. J. Cell Biol. 91:401-419.

Lewis, L. M., and B. R. Brinkley. 1979. Calcium-dependent regulation of tyrosine phosphorylating activity. J. Biol. Chem. 254:1286-1292.

Loor, F., L. Forni, and B. Pernis. 1972. The dynamics of the lymphocyte membrane. Exp. Hematol. 1: (Suppl. 5):529-534.

Molineux, I. G., and R. A. King. 1978. Calcium-dependent cytoskeletal changes in B lymphocytes induced by ligand-surface immunoglobulin interaction. Adv. Immunol. 24:38-135.

Niggli, V., J. T. Peniston, and J. Carmeliet. 1977. Purification of the (Ca2+,Mg2+)-ATPase from human erythrocyte membrane using a calmodulin affinity column. J. Biol. Chem. 254:9955-9958.

Nunnally, W. S. R., D. K. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasonic method for detecting proteins in polyacrylamide gels. Anal. Biochem. 105:361-363.

O’Farrel, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4012.

O’Kane, D. L., R. W. Lenz, G. W. Schmidt, B. A. Palevitz, and M. J. Cormier. 1980. Binding of photo-oxidized thiol reagents to calmodulin. J. Cell Biol. 87:199 (Abstr.).

Ono, T., D. Papahadjopoulos, and G. L. Nicolson. 1975. Local anesthetics affect membrane cytoskeletal control of motility and distribution of cell surface receptors. J. Cell Physiol. 91:2607-2617.

Pouget, G., D. Papahadjopoulos, and G. L. Nicolson. 1975. Local anesthetics affect membrane cytoskeletal control of motility and distribution of cell surface receptors. J. Cell Biol. 87:199 (Abstr.).