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 Fluphenazine-Sepharose. Lymphocyte CaM co-migrates with calf brain CaM on SDS polyacrylamide gels, where its R_f 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 ID_{50}'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 ID_{50}'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). $^{45}Ca^{++}$ 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 occuring concomitantly with the cross-linking of immunoglobulin receptors (32, 33).

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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 in vitro enzyme systems (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 protein from murine splenic lymphocytes. The preferential distribution of calmodulin under immunoglobulin caps is mapped with anti-calmodulin antibodies and a fluorescent calmodulin-binding drug. The dose-dependent inhibition of capping by a variety of calmodulin-specific inhibitors is then demonstrated. Finally, these observations are all interpreted along with earlier observations in terms of how calmodulin may regulate capping of surface immunoglobulin and other surface receptors.

MATERIALS AND METHODS

Lymphocyte Isolation and Capping Protocol

Spleens were dissected from B6AF1/J mice (Jackson Laboratories, Bar Harbor, ME) into phosphate buffered saline (PBS) and teased to release cells from the capsule. Lymphocytes were washed and suspended in cold PBS following hypotonic lysis of contaminating erythrocytes. 3×10^6 lymphocytes were suspended in 250 μl of buffer and incubated with 12–15 μl of fluorescently tagged antibodies (fluorescein isothiocyanate labeled goat anti-mouse Immunoglobulin G heavy and light chains [FITC-GAMIg], Bionetics Laboratory Products, Litton Bionetics, Inc., Kensington, MD, or rhodamine isothiocyanate-labeled rabbit anti-mouse immunoglobulin G heavy and light chains [RITC-RAMIg], Cappel Laboratories Inc., Cochranville, PA). The cells were washed with 15 ml of PBS and resuspended in a volume of 0.5 ml. For patching, cells were incubated for 20–30 min at 23°C in PBS containing 10 mM NaN_3. For capping, the lymphocytes were warmed to 23°C for 30 min. Reactions were stopped by fixing the cells in excess 3.7% formaldehyde 1% methanol in PBS. For drug inhibition studies capping and patching were performed as above, except that all solutions contained the drug and the cells were incubated with the drug for 20 min on ice before addition of the antisera. For cap dispersal tests, the lymphocytes were capped in PBS as above. Then 0.5 ml of 2 × concentration of drug was added and the cells were incubated for an additional 15 min at 23°C followed by fixation. Reversibility of the drugs was measured by incubating the lymphocytes in drug solutions at their 50% and minimum 100% effective doses (for capping inhibition) for 20 min at 0°C. The cells were then washed four times in PBS for 5 min at 0°C and warmed for 15 min to recover. They were then processed for capping as above.

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

Purified calmodulin was prepared from calf brain by the method of Watterson 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. 1.6×10^9 cells were suspended in 16 ml of 50 mM HEPES, 0.15 M NaCl, 0.1 mM PMSF, pH 7.0 and lysed by nitrogen cavitation (equilibration at 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 in $CaCl_2$. 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 dinitrophenylated 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 immunofluorescent staining of cultured cells and serum from the rabbit that gave the brightest staining was 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 syringe 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 + 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-GAMIg) 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 in PBS for 1 h to block nonspecific absorption of protein to the cells and coverslips and 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-GARIg antibodies 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-GARIg was done by incubating the antibodies overnight at 4°C with an acetone 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-GARIg 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% rabbit serum plus 1% BSA as secondary serum.

Indirect immunofluorescence of cultured cells grown on glass coverslips was performed according to Fujiwara and Pollard (19) using rabbit antibodies against calmodulin, as well as fish muscle actin and chicken gizzard myosin antibodies generously supplied by Dr. Keigi Fujiwara. In all cases the cells were fixed in 3.7% formaldehyde, acetone-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 Trifluoperazine

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 indistinguishable 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^{-5} M to 1×10^{-4} 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 microscope, and selected fields are irradiated individually for 10–30 s with 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 TFP* 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 5′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 5′nucleotidase reaction was stopped with 200 μl of 0.25 M $ZnSO_4$ pH adjusted to 2.5 with H_2SO_4 and phosphate measured in duplicate 0.3-ml aliquots from the above solution according to Eibl 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 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 1:1 pH 2.5–4: pH 3.5–10 (LKB Instruments, Inc., Rockville, MD). Alkaline urea gels were run according to Head and Perry (26) using 3.5 M urea instead of 6 M.

Coomassie Blue staining for SDS PAGE was according to Fairbanks et al. (17) and silver staining was according to Oakley et al. (45). Isoelectric focussing (IEF) gels were stained with (*a*) 50% trichloroacetic acid, 0.1% Coomassie Blue and destained in 10% isopropanol, 10% acetic acid or (*b*) fixed in 50% trichloroacetic acid followed by several washes in 10% ethanol, 10% acetic acid and processed according to Oakley et al. pH gradients were measured by slicing frozen IEF gels into half cm sections, incubating the sections for 2 h in 1 ml of distilled water and measuring the pH of the resulting solutions.

Sources of Materials

Trifluoperazine·2 HCl, trifluoperazine sulfoxide·2HCl, chlorpromazine·HCl, chlorpromazine sulfoxide·HCl and prochlorperazine·2 HCl were gifts from Smith, Kline and French Laboratories, Philadelphia, PA. Trifluopromazine·2 HCl and Fluphenazine·2 HCl were obtained from E. R. Squibb & Sons, Inc., Princeton, NJ. Thiothixene·HCl was purchased from Pfizer, Inc., New York, NY. Chlorprothixene·HCl was purchased from Hoffmann-LaRoche, Nutley, NJ and R24571, an analogue of the antiseptic Sepazonium, was obtained from Janssen Pharmaceutica, Beerse, Belgium. Propranolol was purchased from Sigma Chemical Co., St. Louis, MO and compounds W7 and W5 were from Rikaken Co., Ltd., Nagoya, Japan. Acrylamide and N,N′-methylene bis-acrylamide were purchased from Bio-Rad Laboratories, Richmond, CA and glutaraldehyde was obtained from Alpha Products, Danvers, MA. All other materials were from Sigma Chemical Co., or Fisher Scientific Co., Pittsburgh, PA.

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_{app}) in SDS gels varied with calcium concentration from M_{app} = 17,900 + or − 1400 (N = 4) in the presence of 10 mM $CaCl_2$ to M_{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

Calmodulin was isolated from 100,000 *g* supernatant of murine splenic 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_{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 smokey 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 them to have little or no intrinsic PDE activity, but they stimulated exogenously added PDE from bovine heart. Peak PDE stimulatory activity and intensity

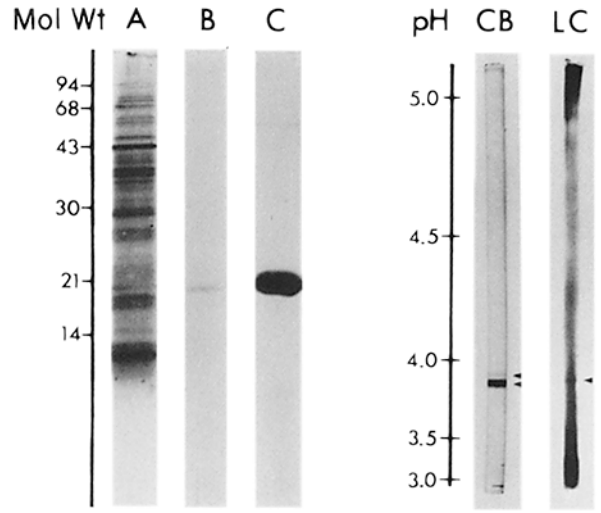

FIGURE 1 Properties of lymphocyte calmodulin. Plate at left shows the purification of lymphocyte calmodulin by Fluphenazine-Sepharose affinity chromatography as analyzed by SDS PAGE on a 12.5% acrylamide gel, silver stained. (*A*) 100,000 *g* supernatant of lymphocytes. (*B*) Pooled elution fractions containing lymphocyte calmodulin. (*C*) Calf brain calmodulin used as a standard (2 μg). Plate at right shows isoelectric focussing gels of calf brain calmodulin (*CB*) stained with Coomassie Blue, and lymphocyte calmodulin (*LC*) stained with silver. Both proteins have isoelectric points of pH 3.9. A minor component of the brain protein runs with a pI of pH 3.95. Dense staining of the extremities of the silver stained gel is due to ampholines.

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 calcium corresponded precisely to that of brain CaM. Isoelectric focusing of the pooled protein gave an isoelectric point of pH 3.9 (Fig. 1). From protein determinations and densitometry of SDS PAGE bands, we made the crude estimate that CaM isolated by affinity chromatography from soluble cell protein comprises of the order of 0.1% of soluble cell protein or 50–60 μg per 10^9 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), trifluopromazine (TPZ), fluphenazine (FPZ), prochlorperazine (PCP), chlorprothixene (CPT), and thiothixene (TT). All of the phenothiazines and thioxanthenes except two inhibited capping at micromolar concentrations with ID_{50}'s of 20–40 μM. Two analogues, TFPSO and CPZSO, were ineffective inhibitors; for these, ID_{50}'s were >1 mM. D,l-propranolol was a weak inhibitor with ID_{50} = 550 μM. R24571 was the most potent inhibitor with ID_{50} = 1 μM. W7 was 50% effective at 20 μM whereas its weaker analogue, W5, had an ID_{50} of 170 μM. ID_{50}'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 TFPSO and CPZSO, which were indistinguishable from controls. Dispersal was effective within 15 min at room temperature even in the presence of 3–10 mM sodium azide (Fig. 3). Calmodulin inhibitors were reversible and cells recovered well from exposure to the drugs following several brief washes and a 15-min recovery period at room temperature (Fig. 3).

Controls for General Membrane Perturbation

Because phenothiazine tranquilizers and tertiary amine anesthetics are thought to be membrane-active substances, we tested several of the analogues to find out whether their anti-capping effects might be due to a general change in membrane structure or activity. TFP, CPZ, and their sulfoxide derivatives were tested for their ability to perturb the fluorescence polarization of the probe diphenylhexatriene (DPH) (31, 48). 10^7 lymphocytes were treated with phenothiazines for 30 min at room temperature, washed three times in PBS, and suspended in 2 μM DPH in PBS for fluorescence depolarization measurements using an Elscint model MV1A 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 perfunctory increases in lateral diffusion of Fab'-labeled 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

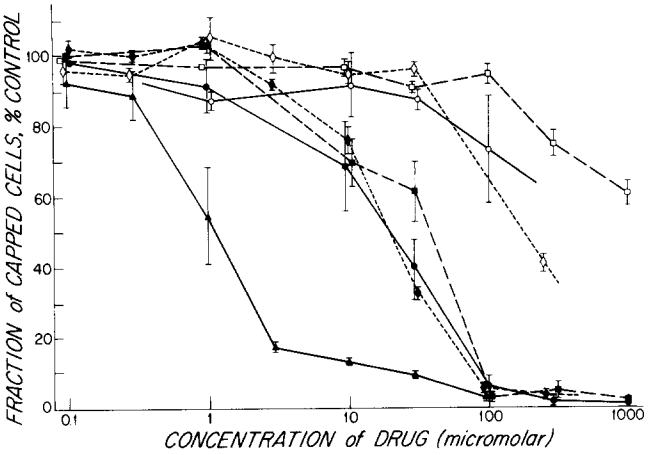

FIGURE 2 Calmodulin inhibitors are inhibitors of lymphocyte capping. Dose-response curves for capping inhibition were obtained with the CaM-inhibiting drugs *R24571* (▲), *W7*(♦), *W5*(◇), the phenothiazines trifluoperazine (*TFP*, ●), Chlorpromazine (*CPZ*, ■), and their sulfoxide derivatives (*TFP-SO*, ○ and *CPZ-SO*, □). ID_{50}'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 after a further 20-min incubation at 0°C were washed and warmed to room temperature for 30 min to cap. The reaction was stopped by fixation with formaldehyde. Error bars show mean and standard deviation for two or more determinations on at least 250 cells.

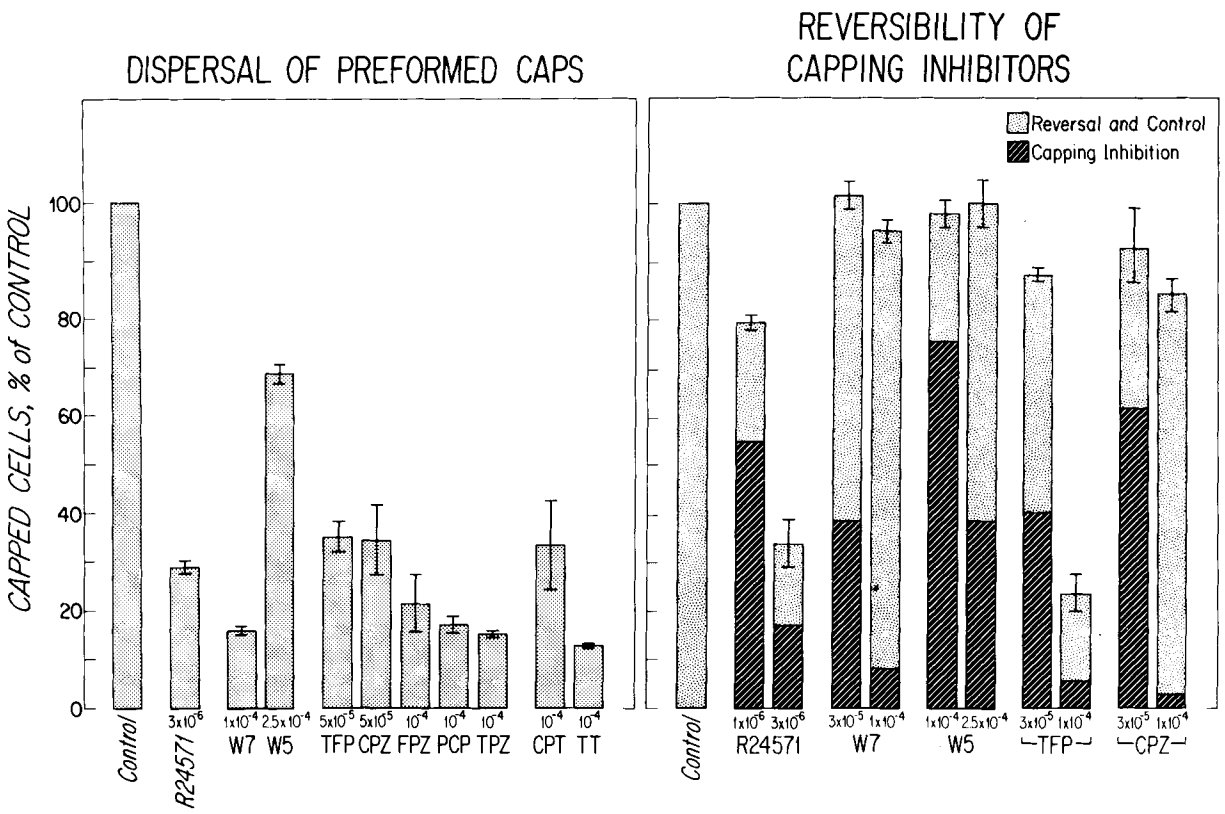

FIGURE 3 Reversibility of drugs and dispersal of preformed caps. The ability to disperse preformed caps was obvious for all of the effective capping inhibitors tested (left panel). Trifluoperazine sulfoxide and chlorpromazine sulfoxide, which are not effective inhibitors, were indistinguishable from controls at up to 100 μM. Dispersal was effective within 15 min at room temperature even in the presence of 3 to 10 mM sodium azide. Cells were capped for 30 min at room temperature in PBS followed by a 15-min exposure to the drug tested. To measure the reversibility of capping inhibitors, lymphocytes were incubated with drugs for 30 min at 0°C, washed four times for 30 min with PBS, and warmed to room temperature for 20 min to allow recovery. The cells were then processed for capping as usual. Right panel shows the degree of capping for lymphocytes processed as above (stippled) and the corresponding degree of capping inhibition obtained with the same drug doses (striped). The difference between the two values reflects the degree of recovery from the drugs. *TFP* and *CPZ* stand for trifluoperazine and chlorpromazine, respectively. Abbreviations for other phenothiazine and thioxanthene compounds are in the text.

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 R_f of 0.04 in 50:50:1.5 methanol:acetone:triethanolamine on silica gel G (R_f 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

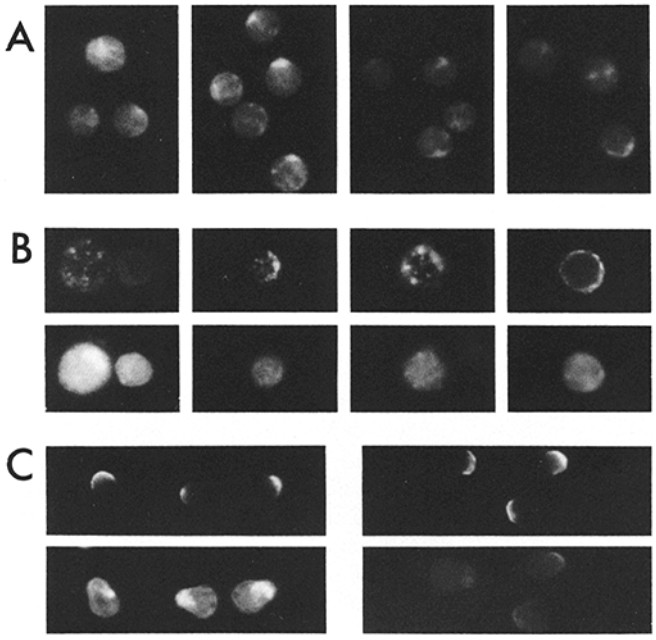

FIGURE 4 Localization of calmodulin in lymphocytes using a fluorescent trifluoperazine derivative. Trifluoperazine (TFP) can be photo-oxidized to a highly fluorescent derivative (TFP*) which binds to calmodulin. In lymphocytes which have been unchallenged by anti-immunoglobulin antibodies (*A*) photo-oxidized trifluoperazine fluorescence is found throughout the cell, often concentrated in one or more diffuse areas. Occasionally, a radiating star-sapphirelike distribution is observed, which is reminiscent of the tubulin distribution seen in lymphocytes. In patched lymphocytes (*B*), surface antibody patches and large patches of TFP* are both observed, but the two fluorescent species distribute independently of one another and do not necessarily map one-on-one. In capped lymphocytes (*C*), TFP* is concentrated under the cap or throughout the uropod of the cell. 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. × 450.

another and do not necessarily map one-on-one. In capped lymphocytes, TFP* is concentrated under the cap or throughout the uropods of motile cells; 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-Ig negative cells (presumably T lymphocyte). That TFP seems to be associated with the cytoskeleton and other known sites of CaM localization is supported by staining of aortic smooth muscle cells from calf and rat where TFP* brightly stains stress fibers (see Fig. 7). Diffuse staining is seen in a perinuclear halo usually concentrated on one end of the nucleus. The intensity there and in other diffusely stained sites matches that of anti-smooth muscle myosin antibodies in the same cells. The pattern of TFP* fluorescence is the same whether or not the cells are acetone permeabilized following fixation. Similar staining occurs in 3T3, PtK, and aortic endothelial cells although stress fiber staining is not as intense as in smooth muscle. At high concentrations TFP* stains nucleoli where myosin light chain kinase has recently been demonstrated (24). Glycerinated chicken skeletal muscle myofibrils are stained in the A bands where 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

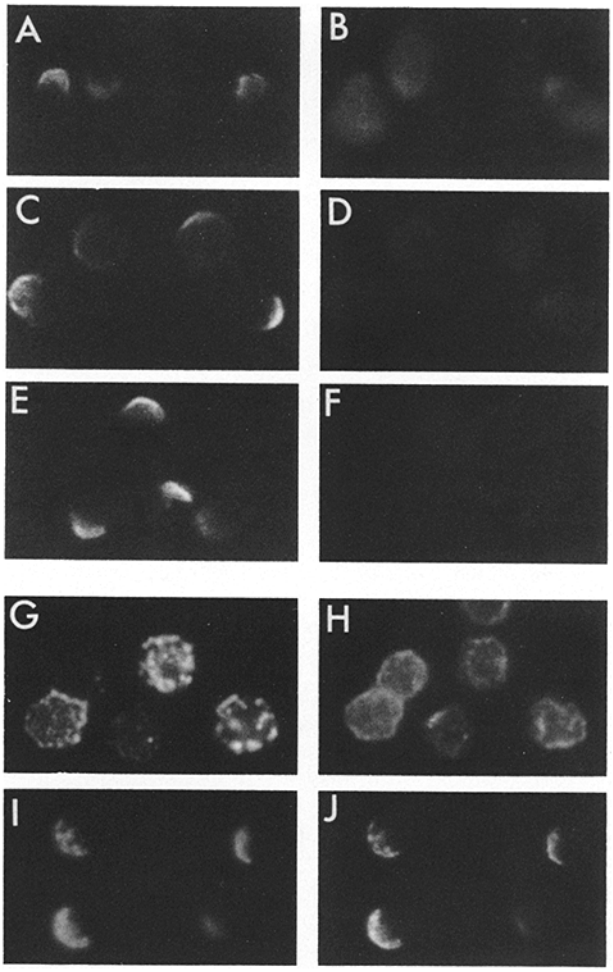

FIGURE 5 Staining of lymphocytes with anticalmodulin antibodies. Staining of lymphocytes with anti-calmodulin antibodies shows diffuse and somewhat patchy distribution in cells that are either untreated or are covered with anti-Ig patches. Anti-CaM "patches" are more frequent in Ig-patched cells, but do not necessarily lie directly under each Ig patch. In capped cells, however, calmodulin is preferentially located under caps and in uropods. This staining 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-Ig 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–J*) 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. × 700.

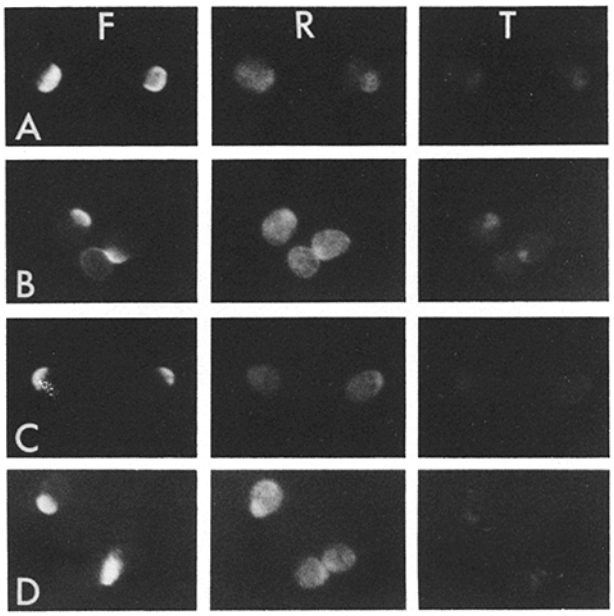

FIGURE 6 Correlation of TFP* and anti-CaM staining. In capped lymphocytes stained with both anti-CaM antibodies and TFP* there was excellent correspondence in staining pattern between fluorescein-tagged anti-Ig caps, and submembranous rhodamine-labeled anti-CaM and TFP* fluorescence. The figure shows four sets of capped cells (*A-D*) stained with fluorescein-labeled goat anti-mouse Ig (column *F*), rhodamine-labeled goat anti-rabbit Ig plus rabbit anti-calf brain calmodulin (column *R*), and photo-oxidized trifluoperazine (column *T*). × 450.

described for TFP* (Figs. 5 and 6). Unchallenged lymphocytes were diffusely stained, but a fine reticular pattern was often superimposed on a diffuse background and nuclei were not brightly stained. Patched lymphocytes were similar to unchallenged cells and we could not determine whether there was co-localization of anti-CaM with anti-Ig at this stage of receptor redistribution. Capped cells were preferentially stained in uropods and under caps with anti-CaM; 61% (N = 66) of cells with Ig caps had submembranous anti-CaM fluorescence. When soluble CaM was included with the primary antibody, staining was significantly reduced and the secondary antibody alone (RITC-GARIg) gave little or no staining. These observations demonstrate the specificity of the affinity-purified antiserum. In capped cells stained with all three reagents (anti-Ig, anti-CaM, and TFP*, Fig. 6) there was excellent correspondence of staining pattern; 73% (N = 30) of cells with TFP* and Ig "caps" were also positive for anti-CaM antibody "caps". As with TFP*, Ig negative cells (T lymphocytes) were occasionally asymmetrically stained with anti-CaM (14%, N = 50). Identical staining patterns were obtained with sheep anti-rat testis calmodulin (CAABCO) and 35 out of 50 cells with Ig caps contained submembranous calmodulin "caps" (Fig. 5). The observation that 61–70% (not 100%) of cells with surface Ig caps also displayed submembranous CaM accumulations suggests that a transient association of CaM with cap components occurs. CaM may be recruited from a total intracellular pool to different sites of action in the lymphocyte much as it moves between I and A bands in skeletal muscles in different states of contraction (25) or as it moves from the cytosol to the plasma membrane of rat pituitary cells after the binding of gonadotropin releasing hormone which, like Ig, is patched and capped (12).

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 maintainance 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 Hedaf (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, phenacaine, 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,

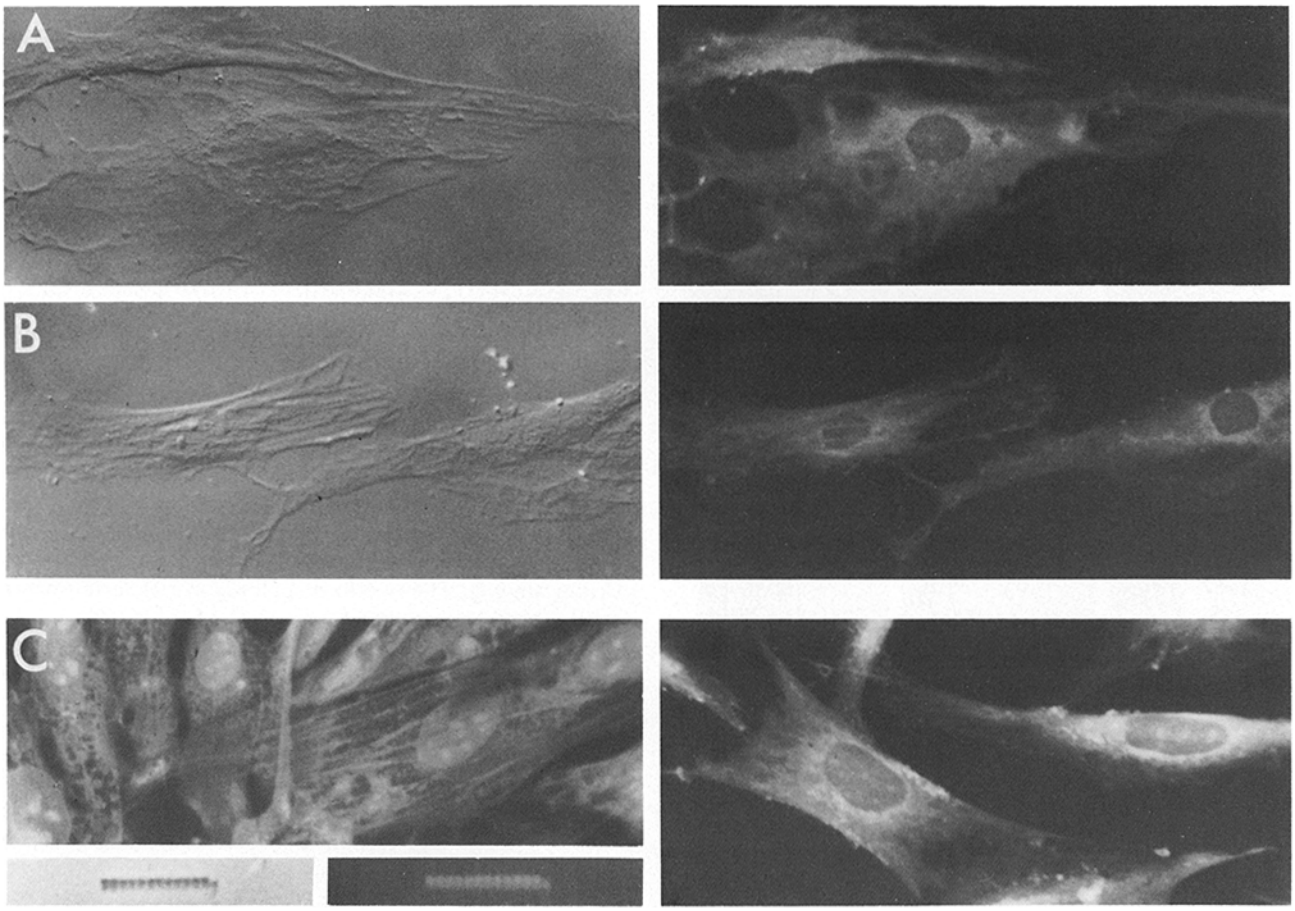

FIGURE 7 Localization of calmodulin in cultured cells and myofibrils. Rat aortic smooth muscle cells were stained by indirect immunofluorescence with anti-CaM antibodies (*A* and *B*) and with photo-oxidized trifluoperazine (*C*). With the antibody, diffuse staining is found throughout the cells and is concentrated in the perinuclear region. Stress fibers are also labeled, but little staining is observed in nuclei. TFP* also stains perinuclear regions of cells and along stress fibers. When used at high concentrations, nuclei are diffusely fluorescence and nucleoli are brightly stained. Similar staining occurs in 3T3, PtK, and aortic endothelial cells (not shown). Glycerinated chicken skeletal muscle fibrils are stained in the "A" bands by TFP*. (*A* and *B*) Nomarski differential-interference contrast/immunofluorescence pairs of rat smooth muscle cells stained with anti-CaM antibodies. (*C*) Fluorescence photos of calf (left, formaldehyde-fixed and acetone permeabilized) and rat (right, formaldehyde-fixed) aortic smooth muscle stained with photo-oxidized trifluoperazine. *Inset*: glycerinated chicken myofibril in phase contrast and stained with TFP*. × 750.

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-solation 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* (39) and staining of nucleoli 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 glycogen 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-Ig 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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