Capping of Cholera Toxin-Ganglioside GM_1 Complexes on Mouse Lymphocytes Is Accompanied by Co-capping of α-Actinin

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ABSTRACT We used cholera toxin, which binds exclusively and with a high affinity to the ganglioside GM_1, as a probe to investigate the distribution of this glycolipid on the surface of mouse lymphocytes. When lymphocytes are incubated with cholera toxin (or its B subunit) and then sequentially with horse anti-toxin and FITC-swine anti-horse Ig at 37°C, the cholera toxin-ganglioside GM_1 complex is redistributed to a cap at one pole of the cell. The capping of cholera toxin-GM_1 complexes is slower than the capping of surface-Ig complexes, requires two antibodies, and is inhibited at high toxin concentrations. Cholera toxin-GM_1, like surface-Ig capping, is an energy-dependent process and is inhibited by sodium azide, low temperatures, or cytochalasin B, but is unaffected by demecolcine. An affinity-purified antibody against α-actinin was used to examine the distribution of this cytoskeletal component during the capping process. 88% of the cells that had a surface Ig cap displayed a co-cap of α-actinin, and 57% of the cells that had a cholera toxin-GM_1 cap displayed a co-cap of α-actinin. Time course studies revealed similar kinetics of external ligand cap formation and the formation of α-actinin co-caps. We conclude that capping of a cell-surface glycolipid is associated with a reorganization of the underlying cytoskeleton. The implications of such an association are discussed in the context of current models of the mechanism of capping.

Although improvements in analytical procedures have led to the characterization of a large number of new glycosphingolipids in animal cells in recent years, surprisingly little is known about the function and organization of glycosphingolipids in membranes (1). The complexity of membrane glycolipid profiles suggest that they are likely to fulfill other than a purely structural role, and evidence that glycolipids can act as cell surface receptors for *Escherichia coli* and cholera enterotoxins, tetanus toxin, the glycoprotein hormones, interferon, macrophage migration inhibition factor, fibronectin, and Sendai virus has been presented (reviewed in reference 1), although in some cases this has been disputed (2, 3).

The conclusion that the high-affinity binding of cholera toxin to the cell surface is mediated by the sialic acid-containing glycolipid ganglioside GM_1[1] is supported by a wide variety of experimental approaches, including the isolation of the cholera toxin-ganglioside GM_1 complex from the surface of several different cell types by immune precipitation (4–6). Because of this, cholera toxin has been widely used as a probe to investigate the levels and organization of ganglioside GM_1 in membranes. Interestingly, binding of cholera toxin to lymphocytes leads to the subsequent redistribution of the toxin-ganglioside GM_1 complex to one pole of the cell, the so-called capping phenomenon (7, 8). A similar response can be induced with anti-glycolipid antibodies (9). Capping of the toxin-ganglioside GM_1 complex is temperature dependent, requires energy, and is inhibited by cytochalasin B (7, 8). The results are analogous to those obtained in experiments on capping of surface Ig in lymphocytes (reviewed in reference 10).

Two main models have been proposed to explain the capping of cell surface receptors. Bretscher (11) suggested that there was a directed flow of membrane lipids towards one pole of the cell, and that monomeric proteins could resist this flow by Brownian motion, whereas large cross-linked aggregates would be swept along to one pole of the cell. The alternative hypothesis proposes that the cross-linking of cell

[1] Ganglioside nomenclature is according to L. Svennerholm, 1963, *J. Neurochem.* 10:613–623.

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surface receptors triggers a transmembrane linkage of the ligand-receptor complex to the microfilament system, which then directs it to one pole of the cell (12). This latter model is supported by experiments that show that capping of a number of surface proteins is associated with co-capping of components of the microfilament system such as actin (13–15), myosin (15–17), and α-actinin (18–19). In addition, a detailed analysis of ligand-induced redistribution of surface Ig, thymus leukemia antigen, and Fc receptors on mouse lymphocytes showed that the kinetics of the capping process was essentially similar to that of the co-capping of myosin (17). These results appear to provide strong, though circumstantial evidence for a direct involvement of the microfilament system in the capping of membrane proteins. The finding that Thy1 capping in mouse lymphocytes induces conversion of G to F actin is also consistent with the above interpretation (20).

No such evidence is available, however, to support the idea that the microfilament system is involved in ligand-induced capping of glycolipids. In the present study, we have therefore attempted to clarify the role of the cytoskeleton in capping of membrane glycolipids by making a detailed comparison of cholera toxin-ganglioside GM_1 capping with that of surface Ig capping in mouse lymphocytes. In particular, we have compared the distribution of α-actinin in cells exhibiting cholera toxin-ganglioside GM_1 caps with those displaying surface Ig caps.

MATERIALS AND METHODS

Preparation and Characterization of an Affinity-purified Antibody against α-Actinin: α-Actinin was isolated from chicken gizzard as described by Feramisco and Burridge (21), and further purified using preparative SDS PAGE (22) followed by electroelution (23). An antibody to α-actinin was produced in rabbits by subcutaneous injection of 500 μg of the purified protein in Freund's complete adjuvant, followed by intramuscular injections of the same dose in incomplete adjuvant. Injections were repeated at 21-d intervals until an antibody was detectable using a solid phase radioimmune assay. A crude Ig fraction of the antiserum was then prepared by ammonium sulphate precipitation, and pure antibodies to α-actinin isolated by affinity chromatography on a 1-ml column of Sepharose 4B (Pharmacia, Hounslow, Middlesex, England) coupled with α-actinin (0.5 mg of protein per ml of Sepharose). Bound IgG was eluted with 0.1 M glycine pH 2.8 (24).

The affinity-purified antibody was characterized by immunoblotting (25) and found to be monospecific for both chicken and mouse α-actinin. The antibody also (*a*) specifically immune precipitated α-actinin from detergent extracts of [^{35}S]methionine-labeled BALB/c 3T3 mouse fibroblasts, (*b*) stained only the Z-line of murine myofibrils, and (*c*) stained the microfilament bundles of BALB/c 3T3 cells in the characteristic punctate manner, with concentrations near the membrane described by Lazarides and Burridge (26).

Other Antibody Preparations: Horse anti-cholera toxin was a gift from Dr. R. O. Thomson, Wellcome Research Laboratories, Beckenham, Kent, England. Rabbit anti-cholera toxin was prepared as previously described (4). Fab_2 fragments of the rabbit anti-toxin were prepared by pepsin digestion of the IgG fraction followed by protein A-Sepharose (Sigma Chemical Co., Poole, Dorset, England) affinity chromatography (27). SDS PAGE of the preparation showed that it contained no detectable intact Ig heavy chains.

Fluorescein-labeled Fab_2 fragments of affinity-purified goat anti-rabbit IgG was a gift from R. Sutherland, Imperial Cancer Research Fund Laboratories, London, England. The IgG fraction of a goat anti-rabbit IgG (Miles Laboratories, Slough, Bucks, England) was labeled with tetramethyl rhodamine isothiocyanate (Miles Labs) according to the method of Brandtsaeg (28), and affinity-purified on a rabbit Ig-Sepharose column (1.8 mg protein per ml of Sepharose) as described previously.

Cells: 2- to 3-mo-old C57BL mice were sacrificed by cervical dislocation and the inguinal, axillary, and mesenteric lymph nodes were excised and pooled. The lymph nodes were gently teased apart in ice-cold PBS/BSA[2] to produce a single cell suspension of lymph node lymphocytes, which was then washed three times in PBS containing 0.1% BSA and 5 mM glucose, and the cells suspended to a concentration of 2×10^7 cells/ml in ice-cold PBS/BSA. This procedure results in a suspension of cells with a viability of >90% of which >94% are small or medium lymphocytes (29).

Immunofluorescent Staining of Cells: Cholera toxin (Schwarz-Mann, Becton Dickinson, Wembley, Middlesex, England) was bound to lymphocytes by incubating 0.1 ml of a cell suspension (2×10^6 cells in PBS/0.1% BSA) with 100 ng/ml cholera toxin for 30 min on ice. After washing three times in PBS/BSA, the cells were incubated with horse anti-cholera toxin (diluted 1:10) followed by a 1:50 final dilution of a fluorescein-labeled IgG fraction of a swine anti-horse Ig (Nordic Immunological Labs, Maidenhead, England). Incubations were for 30 min on ice, with three washes between incubations. Both antisera were diluted with an equal volume of normal mouse serum at least 1 h before use. The cholera toxin-antibody complex was then capped by raising the temperature to 37°C for the times specified, and the process stopped by adding 2 volumes of ice-cold PBS/BSA. 50 μl aliquots were plated onto ice-cold coverslips and the cells allowed to attach to the glass for 5 min at 4°C. The cells were then fixed in 3.8% formaldehyde in PBS for 30 min at room temperature. At this stage, the cells could either be examined directly by mounting coverslips in PBS/50% vol/vol glycerol, or treated for the visualization of α-actinin.

Redistribution of membrane-bound IgG was effected by incubating cells with a 1:40 dilution of a fluorescein-labeled IgG fraction of a goat anti-mouse Ig (Nordic) for 30 min on ice, washing, then raising the temperature to 37°C.

For visualization of α-actinin, the fixed cells were washed thoroughly in PBS, rinsed briefly in 0.1 M glycine in PBS, and extracted for 5 min in 0.2% vol/vol Triton X-100 in PBS. After thorough washing, the coverslips were overlaid sequentially with 50 μg/ml of the rabbit anti-α-actinin in PBS/BSA then 100 μg/ml of an affinity-purified, rhodamine-labeled goat anti-rabbit Ig, with extensive washing between incubations. The coverslips were washed for 1 h in repeated changes of PBS/BSA, mounted in PBS/glycerol, and examined using a Zeiss Standard 16 microscope equipped with epifluorescence. Photographs were taken on Ilford HP5 400 ASA film. Quantitative results were obtained by examining the fluorescence distribution on at least 150 cells directly from the microscope slides. These counts were confirmed using photographs of random fields of view.

Identification of the Receptor for Cholera Toxin on Mouse Lymphocytes: The cholera toxin receptor in these cells was partially characterized by an adaptation of the method of Burridge (30), exactly as described previously by us (5). Briefly, lymphocyte whole cell protein (75 μg per track) was run in a 7.5% SDS-polyacrylamide gel, and after fixation the gel was overlaid with ^{125}I-labeled cholera toxin. After extensive washing, the gel was dried, and bound toxin was detected by autoradiography.

RESULTS

Identification of the Cholera Toxin Receptor in Mouse Lymphocytes

We have previously provided extensive evidence that the cholera toxin receptor in BALB/c 3T3 cells and a mouse lymphoid cell line is exclusively glycolipid in nature (4, 5). To establish that this was also true for normal mouse lymphocytes, cell proteins were resolved in SDS polyacrylamide gels, and the gels were overlaid with ^{125}I-labeled cholera toxin. Fig. 1 shows that cholera toxin bound only to material running at the dye front where we have previously shown glycolipids to travel (4), and there was no evidence for binding to any of the cellular proteins. This binding was totally eliminated when the gel was overlaid with ^{125}I-labeled toxin that had been preincubated with ganglioside GM_1 (Fig. 1*c*), and when the gel was first overlaid with nonradioactive toxin (data not shown), confirming the specificity of the labeling procedure. The result is consistent with the conclusion that the cholera toxin receptor in mouse lymphocytes is exclusively glycolipid in nature.

Capping of Cholera Toxin in Mouse Lymphocytes

Cholera toxin binding to mouse lymphocytes was visualized using a double antibody technique as described in the Materials and Methods. After binding the toxin and antibodies to

[2] *Abbreviations used in this paper:* BSA, bovine serum albumin; cAMP, 3′,5′-cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; NP40, Nonidet P-40; PBS, Dulbecco's complete phosphate-buffered saline.

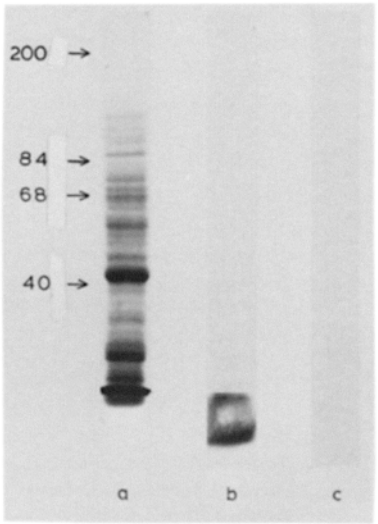

FIGURE 1 Identification of cholera toxin binding molecules in mouse lymphocytes. Total cellular proteins (150 μg/track) were resolved in 7.5% SDS polyacrylamide gels, and the cholera toxin binding components identified by overlaying gel tracks with ^{125}I-labeled toxin (3×10^6 cpm in 0.75 ml of PBS/0.1% BSA specific activity 22 μCi/μg) in conjunction with autoradiography, as described in Materials and Methods. (*a*) Coomassie Blue-stained total cell protein. (*b*) Track overlaid with ^{125}I-labeled cholera toxin. (*c*) As in Fig. 2 *b*, except the ^{125}I-labeled cholera toxin was preincubated with 50 μg/ml of bovine brain mixed gangliosides (Sigma Chemical Co., Poole, Dorset, England) for 1 h at 0°C.

cells kept on ice, the toxin was distributed as small aggregates or patches over the surface of the cell, (Fig. 2 *a* and *b*). However, when the cells were subsequently incubated at 37°C for 30 min, many of the cells displayed an accumulation of toxin at one pole of the cell, the so-called capping phenomenon (Fig. 2 *c* and *d*). Capping of surface Ig in response to anti-mouse Ig is shown for comparison (Fig. 2 *e* and *f*). Cholera toxin could be capped only when the double antibody method was used. If the toxin was bound at 0°C, and the cells incubated at 37°C for 30 min before fixation and the subsequent addition of the two antibodies, there was no evidence of any redistribution, and the toxin was found uniformly distributed over the cell surface (Fig. 2 *g*). When both cholera toxin and the anti-toxin were bound and the cells incubated at 37°C, then fixed before addition of a fluorescent second antibody, capping also failed to occur although there was evidence of patching in many of the cells (Fig. 2 *h*).

To check the specificity of the antibodies used, cholera toxin was omitted from the system and the cells incubated with the two antibodies alone. No detectable fluorescence was observed, showing that the antibodies were not cross-reacting with any molecules on the lymphocyte cell surface (Fig. 2 *i*). The possibility that the toxin-antibody complex was capping through interaction of the antibodies with the Fc receptor was excluded, since the toxin could be capped using Fab_2 fragments of a rabbit anti-cholera toxin plus FITC-labeled Fab_2 fragments of an affinity-purified goat anti-rabbit Ig (Fig. 2 *j*).

Agents that disrupt microfilament organization (cytochalasin B), or the energy supply of the cell (sodium azide), were potent inhibitors of cholera toxin capping at all the times investigated (Fig. 3). Conversely an inhibitor of microtubule organization, demecolcine, was totally ineffective, but interestingly led to much brighter and more distinct caps. Quantitative analysis showed that each of these agents affected cholera toxin capping in a manner similar to Ig capping (data not shown). The results confirm earlier observations of other workers (8), and point to the involvement of the microfilament system in capping of the cholera toxin-ganglioside GM_1 complex.

Comparison of the Kinetics of Cholera Toxin Capping with Ig Capping

The capping of cholera toxin occurred only at concentrations between 0.01 μg/ml and 0.2 μg/ml. Below 0.01 μg/ml of toxin the level of fluorescence fell below detectable limits. Interestingly above 0.2 μg/ml there was a marked reduction in capping of the toxin, an observation previously reported by Revesz and Greaves (8). Binding of cholera toxin through its B-subunit to cell surface ganglioside GM_1 subsequently leads to activation of adenylate cyclase by the A-subunit of the toxin (31). We therefore considered the possibility that the above phenomenon was a consequence of a massive increase in intracellular levels of cAMP produced when high concentrations of the toxin were used. This possibility was, however, excluded by the observation that capping of cholera toxin B-subunit alone was also markedly reduced at higher concentrations (data not shown).

The rate of cholera toxin capping was slow compared with Ig capping (Fig. 4). Over 90% of cells within the population were able to bind toxin. Capping was maximal after 30–40 min, but only 50–60% of positively stained cells exhibited a cap of bound toxin. In contrast only 50% of the cells displayed surface Ig, but of these 80–90% were able to form caps within 7–10 min. Capping of cholera toxin B-subunit showed identical kinetics to the intact toxin, again excluding the possibility that elevated levels of cAMP were reducing the rate of capping. The slow rate of toxin capping was also apparently unrelated to the need for a double antibody layer since the kinetics of surface Ig capping was the same using a single or double antibody technique (Fig. 4).

The Influence of Cholera Toxin Capping on Cytoskeletal Organization

It has recently been shown that the patching and capping of a number of cell surface proteins is accompanied by co-patching and co-capping of α-actinin (18, 19). The result provides additional evidence for a role of the cytoskeleton in redistribution of components in the plane of the membrane. Since cholera toxin bound to the cell surface through a lipid, ganglioside GM_1, can also be capped, we further examined the role of the cytoskeleton in this process by investigating the distribution of α-actinin in cells following capping of bound toxin. For comparison the distribution of α-actinin with respect to surface Ig caps was also investigated.

Lymphocytes incubated with anti-mouse Ig at 0°C showed substantial redistribution of surface Ig into patches whilst the α-actinin remained diffusely distributed around the cell periphery, although there was some evidence of co-patching (data not shown). Incubation of cells at 37°C for 7.5 min resulted in capping of surface Ig and in the majority of such cells, the α-actinin was concentrated directly under the cap of surface Ig, (Fig. 5 *a* and *b*). Similarly incubation of lymphocytes with cholera toxin and the double antibody layer at 0°C resulted in patching of the toxin and a diffuse peripheral staining of α-actinin. Cells that had been incubated at 37°C for 30 min before visualization of α-actinin showed capping of the toxin and in many cases the α-actinin was again concentrated under the caps of membrane-bound cholera

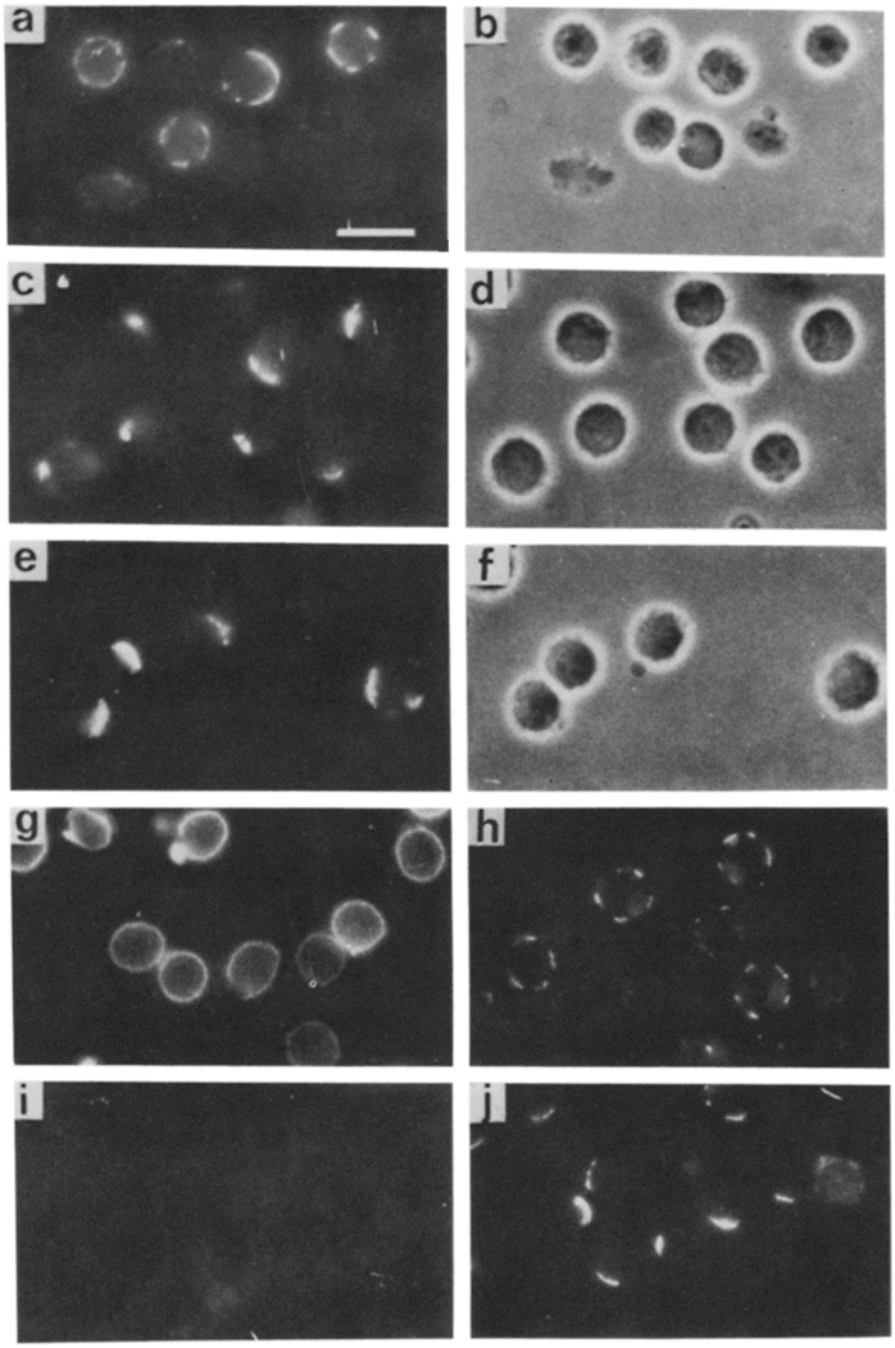

FIGURE 2 Antibody-induced capping of surface Ig and cholera toxin bound to mouse lymphocytes. (*a–d*) Cholera toxin (100 ng/ml), horse anti-toxin (1:10), and FITC-labeled swine anti-horse Ig (1:50) were bound to cells at 0°C, with washing between each step. Cells were then incubated for 30 min at 0°C (*a* and *b*) or 37°C (*c* and *d*). Fluorescence and phase contrast micrographs of the same fields of view are shown. (*e–f*) FITC-labeled goat anti-mouse Ig (1:40) was bound to cells at 0°C, and the temperature raised to 37°C for 7.5 min. Cells were examined by fluorescence (*e*) and phase-contrast (*f*) microscopy. (*g*) Cholera toxin (100 ng/ml) was bound at 0°C, the cells washed and subsequently incubated at 37°C for 30 min, before fixation. Horse anti-toxin and FITC-labeled swine anti-horse Ig were then sequentially bound at 20°C. (*h*) Cholera toxin and horse anti-toxin were bound at 0°C, and the cells then incubated at 37°C for 30 min. Following fixation cells were stained with FITC-labeled swine anti-horse Ig. (*i*) Cells were treated as in c but with the omission of cholera toxin. (*j*) Cells were incubated sequentially with cholera toxin, Fab_2 fragments of rabbit anti-toxin (1.5 mg/ml), and Fab_2 fragments of affinity-purified FITC-labeled goat anti-rabbit (50 μg/ml) at 0°C for 30 min each, with washing between each step. Cells were then incubated at 37°C for 30 min. Bar, 10 μm. × 1,000.

toxin, (Fig. 5 *c* and *d*). The specificity of the α-actinin staining was confirmed by substituting the affinity-purified anti α-actinin with nonimmune Ig (Fig. 5 *e* and *f*) and preabsorbing the antibody with α-actinin (Fig. 5 *g* and *h*).

Quantitative analysis of the results showed that >89% of Ig-positive cells displayed a cap after incubation with fluorescent anti-mouse Ig for 7.5 min at 37°C. Of the cells with an Ig cap, ~88% also showed an exact co-distribution of α-actinin. That is, in only a small percentage (12%) of cells displaying an Ig cap was there no co-distribution of α-actinin. In the case of cholera toxin, ~62% of positively stained cells displayed a cap of bound toxin after incubation for 30 min at 37°C. Of the cells with a cholera toxin cap ~57% also showed an exact co-distribution of α-actinin. Similar results were obtained when B-subunit was used in place of the intact toxin.

If the redistribution of surface Ig and the cholera toxin-receptor complex is directly related to the redistribution of the cytoskeletal protein α-actinin, the kinetics of the two events should be identical. Fig. 6 *a* shows the time course of surface Ig capping and the formation of co-incident α-actinin caps in an experiment carried out at 20°C to decrease the rate of capping and thus improve the accuracy of the analysis. The kinetics of surface Ig capping were almost exactly paralleled by that of α-actinin co-capping. Essentially similar results were obtained in experiments with cholera toxin (Fig. 6 *b*). In both cases the number of cells displaying an α-actinin cap in the absence of ligand also increased with time, but the rate of this so-called spontaneous α-actinin cap formation was much slower than that observed in the presence of added ligand.

DISCUSSION

We have shown that cholera toxin or the toxin B-subunit

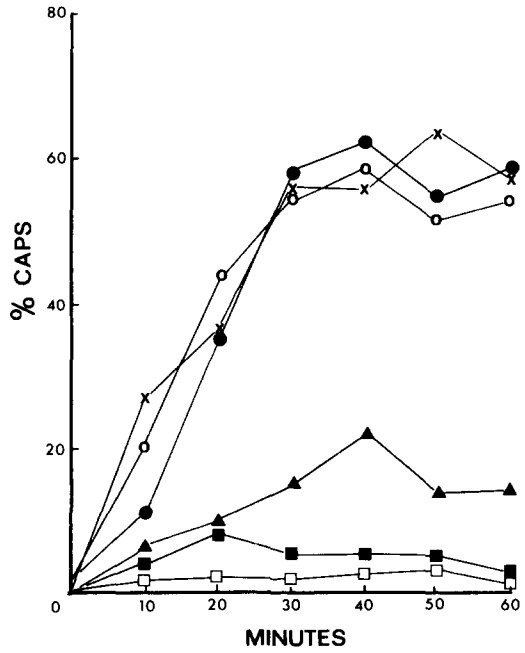

FIGURE 3 Effect of inhibitors of cytoskeletal function on the kinetics of capping of bound cholera toxin. Cholera toxin (100 ng/ml), horse anti-toxin, and FITC-labeled swine anti-horse were bound at 0°C as described in Fig. 2, and the cells then incubated at 37°C for 30 min. Binding and the subsequent incubations at 37°C were performed in the presence of the appropriate inhibitors. O, no addition. □, plus 10 mM NaN_3. ▲, plus 20 μg/ml cytochalasin B (stock solution 2 mg/ml in dimethyl sulfoxide (DMSO). Final DMSO concentration 1%. ●, 1% DMSO alone. ×, plus 10^{-4} M demecolcine. ■, plus 10^{-4} M demecolcine and 20 μg/ml cytochalasin B.

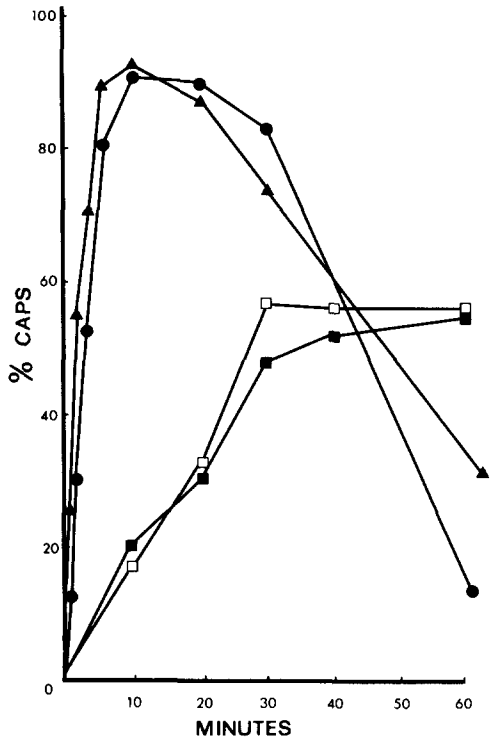

FIGURE 4 Kinetics of capping of surface Ig and cholera toxin bound to mouse lymphocytes. All ligands were bound at 0°C, and the temperature raised to 37°C for the times indicated. After fixing, the number of cells displaying a cap was counted. The results are expressed as a percentage of the ligand-positive cells. ●, FITC-labeled goat anti-mouse Ig (1:40 dilution). ▲, Rabbit anti-mouse Ig (1:30) followed by FITC-labeled goat anti-rabbit Ig (1:50). □, Cholera toxin (100 ng/ml) followed by horse anti-toxin (1:10) and FITC-labeled swine anti-horse Ig (1:50). ■, Cholera toxin B subunit (100 ng/ml) followed by horse anti-toxin (1:10) and FITC-labeled swine anti-horse Ig (1:50).

when bound to the surface of mouse lymphocytes can be induced to cap by the addition of anti-toxin followed by a second antibody. We were, however, unable to demonstrate capping of bound toxin without the addition of the two antibodies, in contrast to the results of Craig and Cuatrecasas who used FITC-labeled cholera toxin and rat mesenteric lymph node cells (7). Similar difficulties in capping toxin alone have been experienced by others (32), and the discrepancy remains to be explained, although it may be due to differences in the surface density of GM_1 among various cell types (8). Although we have not investigated the structure of the cholera toxin receptor in mouse lymphocytes in detail, our results are consistent with it being a glycolipid, presumed to be ganglioside GM_1, as in all other systems studied so far (4–6, 31). Because binding of cholera toxin to ganglioside GM_1 has a high affinity ($K_D \sim 10^{-10}$ M) and is thought to be essentially irreversible, we assume that the capping process actually involves a toxin-ganglioside GM_1 complex. This interpretation is supported by the observation that the low levels of binding of cholera toxin to human acute leukemia cells can be markedly increased by preincubation of the cells with ganglioside GM_1, and that addition of a double antibody layer subsequently leads to capping (8). Capping of the cholera toxin-ganglioside GM_1 complex was not observed when cells were incubated at high concentrations of the toxin even in the presence of a double antibody layer. The phenomenon has previously been reported by Revesz and Greaves (8), who found that it was only exhibited by cells displaying a high number of toxin binding sites. The significance of the observation remains unclear.

The rate of capping of the cholera toxin-ganglioside GM_1 complex was slower than that of surface Ig capping. This was not simply due to the relatively large toxin-double antibody complex moving more slowly in the plane of the membrane, since surface Ig capped at about the same rate whether a single or a double antibody layer was used. There was no reduction in the number of observable cholera toxin-ganglioside GM_1 caps up to 1 h after raising the temperature to 37°C, whereas surface Ig caps had been largely removed by this time, presumably by endocytosis (33). Interestingly, it has been reported that other cell surface molecules such as histocompatibility antigens, which cap comparatively slowly and require a double antibody layer to induce capping, also remain on the cell surface much longer than surface-Ig complexes (34).

Antibody-induced capping of cholera toxin-ganglioside GM_1 was inhibited by low temperature, azide, and an inhibitor of microfilament but not microtubule organization. These results are essentially similar to those reported by Revesz and Greaves (8) who also used a double antibody system to induce capping of bound toxin, but differ from those of Craig and Cuatrecasas (7) who found that capping of bound FITC-labeled cholera toxin was more sensitive to agents that disrupt microtubule rather than microfilament organization.

As the effects of the above treatments on cholera toxin-ganglioside GM_1 capping were exactly comparable to the effects on surface Ig capping, we decided to extend the com-

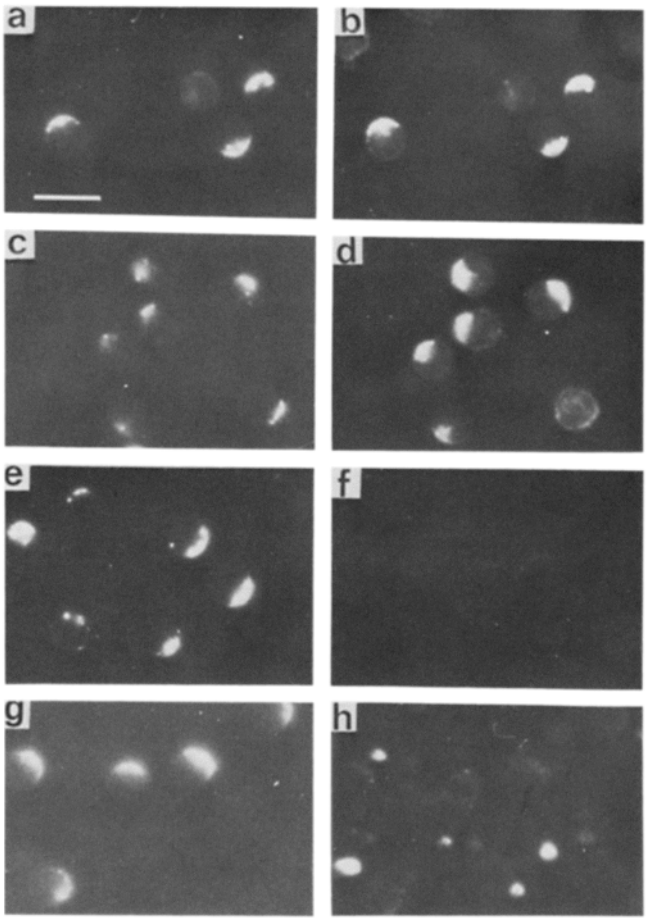

FIGURE 5 Distribution of α-actinin after capping of surface Ig or cholera toxin bound to mouse lymphocytes. Surface Ig was capped by binding FITC-labeled goat anti-mouse Ig, at 0°C, followed by incubation at 37°C for 7.5 min. Cholera toxin was capped by binding toxin, horse anti-toxin, and FITC-labeled swine anti-horse Ig at 0°C, followed by incubation at 37°C for 30 min. After capping the cells were fixed, permeabilized, and stained with rabbit anti-α-actinin (50 μg/ml) and rhodamine-labeled goat anti-rabbit Ig. Paired micrographs show FITC-labeled ligand (left column) and rhodamine-labeled α-actinin (right column). (*a* and *b*) Three lymphocytes bearing capped Ig (*a*) and co-caps of α-actinin (*b*). (c and *d*) A group of six lymphocytes bearing cholera toxin caps (c), five of which show co-caps of α-actinin (*d*). (e and *f*) Cells bearing Ig caps (e) stained with the rabbit IgG fraction which did not bind to the α-actinin affinity column (*f*). (*g* and *h*) Cells bearing cholera toxin caps (*g*) stained with anti-α-actinin preadsorbed with 100 μg/ml α-actinin (*h*). *e–h* clearly establish the specificity of the staining procedure for α-actinin. The brightly fluorescent particles seen in *h* do not coincide with cells and are presumably immune complexes. Bar, 10 μm. × 1,000.

parison of the two processes further. Capping of surface Ig in lymphocytes is accompanied by the co-capping of actin (14, 15), myosin (15–17) and α-actinin (18, 19). We chose to investigate the possible association of α-actinin with cholera toxin-ganglioside GM_1 caps because α-actinin is (*a*) thought to be concentrated at the internal face of the plasma membrane (35), (*b*) can be demonstrated to co-cap with surface Ig using conventional immunofluorescent techniques (18–19), and (*c*) has been shown to co-cap with ligand-induced caps of a variety of other surface components in mouse lymphocytes such as Thy-1 antigen (19), H_2 antigen (18), and concanavalin A receptors (18). We found clear evidence that antibody-induced caps of surface Ig and cholera toxin-ganglioside GM_1 were associated with co-caps of α-actinin. However quantitative analysis showed that less cholera toxin-ganglioside GM_1 caps were associated with an α-actinin co-cap than was the case with surface Ig. Nevertheless >50% of the cells displaying a toxin-ganglioside GM_1 cap showed co-capping of α-actinin. The kinetics of antibody-induced cap formation for both cholera toxin-ganglioside GM_1 and surface Ig was paralleled by the rate of formation of α-actinin co-caps, and there was little or no increase in α-actinin cap formation in the absence of added ligand. In addition, treatments (low temperature, azide, and cytochalasin B) that inhibited capping of the surface components also inhibited the formation of α-actinin caps. The results show that the redistribution of α-actinin is not only associated with capping of surface Ig as previously reported, but also with the capping of the cholera toxin-ganglioside GM_1 complex.

Despite intensive efforts, details of the molecular events involved in the capping process are not understood. Based on a series of elegant experiments, Braun et al. (17) have proposed that capping of lymphocyte surface molecules can be divided into two classes. They found that surface Ig, thymus leukemia antigen, and Fc receptors cap rapidly upon binding of a single ligand, and capping in these cases was associated with co-capping of myosin. In contrast capping of H_2 and Thy-1 antigens was slow, only occurred if a double antibody system was used, and was not accompanied by co-capping of myosin. It was hypothesized that the first type of capping involved a ligand-induced reversible link between the surface component and the microfilament system, which then directed the complex to one pole of the cell. It was proposed that the second type of capping was due to the formation of large cross-linked aggregates at the cell surface that subsequently fused into caps as a result of some other event, such as cell motility or directed flow of membrane lipid (11).

Whilst the idea that membrane proteins can be directly or indirectly linked to the cytoskeleton has widespread appeal (12, 36–38) the idea of two different classes of capping has been disputed. Firstly capping of H_2 and Thy-1 antigens as well as concanavalin A receptors has been shown to be associated with the co-capping of α-actinin (18, 19) or actin (13) by other workers. Secondly it has recently been claimed that given optimal conditions, all ligands able to cross-link surface components will induce capping at a rate similar to that of surface Ig (39). It is also difficult to accommodate our own results on capping of the cholera toxin-ganglioside GM_1 complex into the above proposal. The capping is slow, requires two antibodies yet is clearly associated in many instances, with the co-capping of α-actinin. It should be noted however, that quantitative analysis revealed that many caps of cholera toxin-ganglioside GM_1 had no apparent α-actinin subcap. Reference to the data on co-capping of myosin with surface Ig, thymus leukemia antigen, and Fc receptors show similar discrepancies (17), making the interpretation of this type of experiment less clearcut than might appear when photographic evidence alone is presented (12–16, 18, 19).

If the microfilament system is responsible for the antibody-induced capping of the cholera toxin-ganglioside GM_1 complex as our co-capping experiments would suggest, the interaction must be indirect since the hydrophobic region of the glycolipid is not thought to span the membrane. At present little is known about the organization of glycolipids in mem-

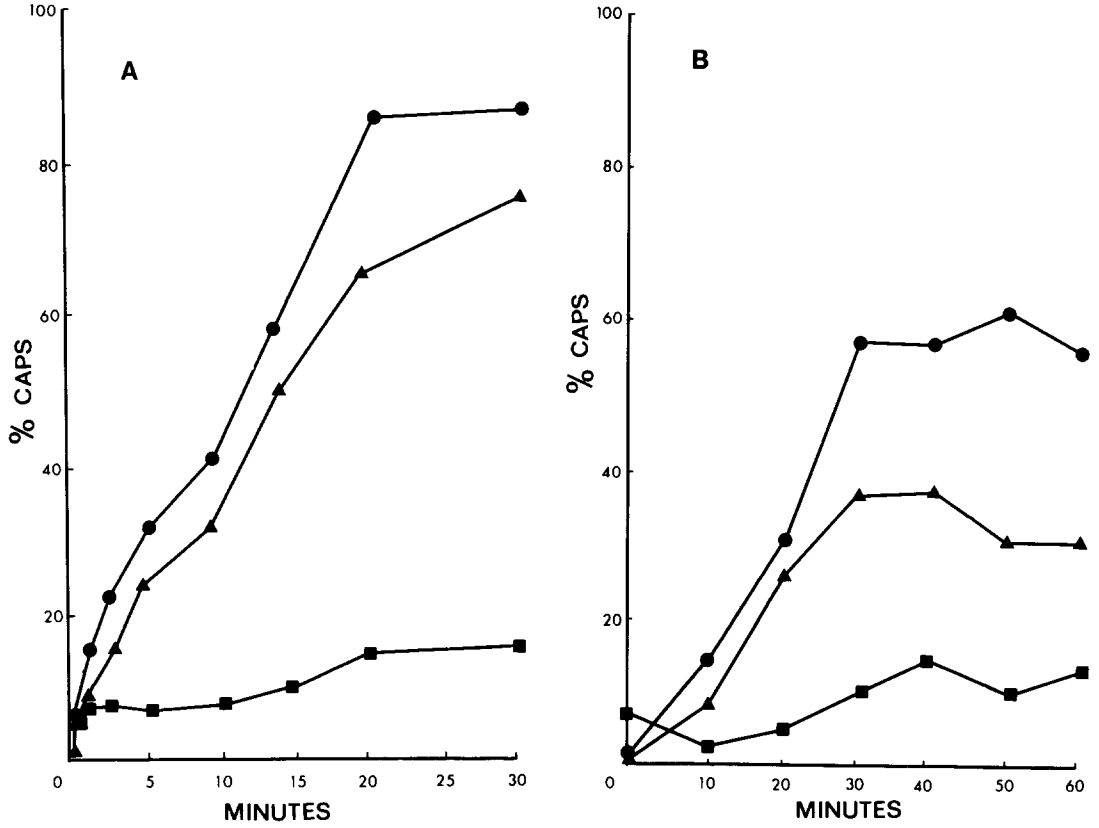

FIGURE 6 Comparison of the kinetics of capping with that of the formation of α-actinin co-caps. (*A*) Antibody-induced capping of surface Ig. Following binding of FITC-labeled goat anti-mouse Ig to cells at 0°C, cells were incubated at 20°C, instead of 37°C to reduce the rate of the capping process. ●, Cells with a cap of surface Ig. ▲, Cells with an Ig cap that also displayed an α-actinin co-cap. Both are expressed as a percentage of Ig-positive cells. ■, Cells displaying an α-actinin cap in the absence of anti-mouse Ig. This so-called spontaneous α-actinin cap formation is expressed as percentage of cells staining positively for α-actinin. (*B*) Antibody-induced capping of bound cholera toxin. ●, Cells with a cholera toxin cap. ▲, Cells with a cholera toxin cap that also displayed an α-actinin co-cap. Both are expressed as a percentage of toxin-positive cells. ■, Cells displaying an α-actinin cap in the absence of bound toxin, expressed as a percentage of cells staining positively for α-actinin.

branes (1). Preliminary evidence that a specific glycolipid could be cross-linked to an erythrocyte membrane protein has been presented (40), and a low molecular weight amphipathic protein with Paul Bunnell antigen activity isolated from bovine erythrocytes, has been shown to have a specific affinity for ganglioside GM_3 (41). Interestingly, we have recently found that cholera toxin-ganglioside GM_1 complexes remain associated with the Triton X-100 insoluble cytoskeleton of BALB/c 3T3 cells, although the molecular basis of the observation remains unexplained (42). Similar results have been obtained by others (43), and Sayhoun et al. (44) have provided some evidence that the cholera toxin-ganglioside GM_1 complex isolated from rat erythrocytes by extraction with Triton X-100 at 37°C is associated with cellular proteins, possibly of cytoskeletal origin.

Arguments against a direct involvement of the microfilament system in the capping process have also been presented. Stern and Bretscher (45) found that Forssman antigen (a glycolipid) inserted into the plasma membrane of a number of cell lines lacking the antigen, could be induced to cap using a double antibody system. Capping of the glycolipid was inhibited by dinitrophenol and cytochalasin B, but not colchicine. Exactly analogous results have recently been reported using trinitrophenyl (tnp)-derivatized phosphatidyl ethanolamine, and anti-tnp antibodies (46). Because glycolipids are not thought to span the lipid bilayer, Stern and Bretscher (45) concluded that capping of Forssman antigen, and therefore other membrane components must occur by a mechanism not directly involving the microfilament system. As an alternative explanation they proposed that membrane lipids are in a continual state of directed flow from a point of insertion to a point of internalization, and that aggregated molecules unable to resist this flow would simply accumulate at one pole of the cell. The hypothesis is partly based on the lipid insertion experiments, and is dependent on the assumption that such molecules do not become associated with membrane proteins that might then provide a link to the cytoskeleton. Since we have now shown that capping of an endogenous glycolipid, ganglioside GM_1, is accompanied by co-capping of α-actinin, it would be of interest to examine the distribution of cytoskeletal components during capping of inserted glycolipids (45, 47) and such studies are under way in our laboratory.

Finally, co-patching of cholera toxin-GM_1 complexes with α-actinin might be explained if GM_1 were to be concentrated in microvilli, areas of the cell surface likely to be enriched in cytoskeletal components. Indeed such a nonuniform distribution of surface Ig has been described (48). How such co-patching might be related to the development of surface caps with associated cytoskeletal material remains unclear.

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