CALCIUM-SENSITIVE MODULATION OF Ig CAPPING: EVIDENCE SUPPORTING A CYTOPLASMIC CONTROL OF LIGAND-RECEPTOR COMPLEXES*

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The binding of anti-immunoglobulin (Ig) to the surface Ig receptors of the B lymphocyte sets into motion a train of events: the complexes of membrane-bound Ig and anti-Ig antibody rapidly form disseminated patches which then aggregate into a cap at a polar end of the cell in an energy-requiring step (1–4). The complexes are subsequently endocytosed, an interval of a few hours elapsing before the cell synthesizes and replaces new molecules on its surface (5). Unique to the anti-Ig ligand is the parallel stimulation of translational motility shortly after the cap has formed (6, 7). This motility is susceptible to modulation by cyclic nucleotides and cholinergic and adrenergic agents (8).

The nature of the energy-dependent event involved in capping has not been elucidated but is thought to be related to the activation of intracellular structures involved in this process. A series of studies on various cells suggest a relationship between surface interactions, or ligand-receptor complexes, and cytoplasmic structures (9, 10). For example, morphological studies on macrophages have suggested a role for microfilaments in membrane movement and endocytosis (discussed in Reference 11). In capping of lymphocytes, the adverse effects of cytochalasin, albeit variable and not extensive, have suggested involvement of the microfilament system in the phenomenon (10). Also, there is morphological evidence of increased microfilaments in the area of the cap (10, 12). Colchicine-sensitive structures, microtubules, likewise, have been thought to play some regulatory role in surface modulatory events (9, 13). Microtubules appear to affect capping of some surface complexes, implying some form of direct control by these structures (9, 12, 13), the nature of which is unclear.

The rapid onset of the phenomena of capping and stimulated movement by anti-Ig antibodies in B lymphocytes led us to a consideration of a possible role for ions as stimulators or modulators in capping and motility. The inhibitory effects on cap formation of microfilament-affecting drugs, such as the cytochalasins, and the postulated role of microfilaments in cell motility led our initial investigations into the examination of the effects of calcium modulation on cap formation. We have utilized an experimental antibiotic, A-23187, that forms lipid-soluble complexes with divalent cations, facilitating their transmembrane diffusion (14,

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15). Its calcium transporting property has been shown to stimulate a variety of calcium-dependent cell processes (16–19).

In this report, we describe the role of calcium and an apparent calcium-sensitive mechanism for cytoplasmic control of seemingly membrane-restricted events. We show that capping and motility in the lymphocyte do not require extracellular calcium. More importantly, we describe the disruption of the complexes in the cap by the calcium ionophore A-23187 in a calcium- and energy-requiring process. This latter effect focuses on the possibility of a direct link between surface complexes and the contractile elements of the cell.

Materials and Methods

Cell Preparation. Lymphocytes were obtained by teasing cells from the spleens of A/St mice (West Seneca Laboratories, Buffalo, New York), purifying them in Ficoll-Hypaque gradients, and collecting only the interface. The collected cells, over 98% viable lymphocytes, were washed twice. The medium was usually Hanks' balanced salt solution with 10 mM n-2-hydroxyethylpiperazine-N-2 ethane sulfonic acid (HEPES) (Microbiological Associates, Bethesda, Md.) and 2% fetal calf serum (Associated Biomedic Systems, Inc., Buffalo, New York). Experiments with calcium-free medium employed minimal essential medium without calcium with 10 mM HEPES and 2% fetal calf serum dialyzed against phosphate-buffered saline (PBS)[1] for 3 days.

Antiserum. The preparation and specificity of both the rabbit antimouse Ig (anti-Ig) and its fluorescent conjugate have been described in previous experiments (7, 8). They contain antibodies to all mouse Fc classes and to Fab determinants. One set of experiments used a fluorescent conjugate of goat antiserum to rabbit Ig (GARG), whose preparation has also been previously described (8).

Analysis of Capping of Ig-Anti-Ig Complexes. Unless the use of anti-Ig is noted specifically, fluorescein-conjugated rabbit anti-Ig (FITC-anti-Ig) was added to lymphocytes at a concentration of 50 μg per 5 × 10⁶ cells (in 0.5 ml) at 4°C (unless otherwise noted) for 20 min. The cells were then washed once and resuspended at a concentration of 10⁷ cells per ml. For the experiments on the morphology of capping, the cells were brought at 37°C for varying lengths of time before fixation by the addition of an equal volume of 2% paraformaldehyde. The fixed cells were then washed twice and resuspended in PBS.

Two kinds of experiments were done; one was to test the effects of calcium deprivation, A-23187, etc., on capping and a second was to test the effects on the already formed cap. In experiments on the ionophore's effects on capping, 5 × 10⁶ lymphocytes in 0.5 ml were incubated for 5 min at room temperature in medium containing the drug. After preincubation, 50 μg of FITC-anti-Ig was added and the reaction was maintained at 20° C for another 20 min before the addition of paraformaldehyde. In experiments with colchicine, lymphocytes were preincubated in colchicine (10⁻⁴ M) for 1 h at 37°C, cooled to 20°C for 10 min, exposed to FITC-anti-Ig, and allowed to cap for 20 min.

The experiments on the effect of either the ionophore or the cytochalasins on the formed cap were carried out by warming the FITC-anti-Ig-bound cells, after washing, to 20°C in a water bath (Forma Scientific, Inc., Marietta, Ohio). (We chose this temperature for several reasons. At 37°C, capping and subsequent endocytosis of complexes is extremely rapid [Fig. 1]. Cells cap well at 20°C but more slowly, an experimental condition that may help to minimize such factors as the rate of diffusion of a drug into a cell, which may occur too slowly to affect the capping phenomenon at 37°C. Also, as has been noted, endocytosis appears to be preferentially inhibited for a long period of time when the cell is maintained at 20°C [12], although it ultimately occurs [in about 1 h]; thus, the surface molecules are retained longer on the membrane for experimental manipulation.) By 15–20 min at 20°C, approximately 55–90% of the Ig-bearing cell are capped. After 15–25 min, specified in the text, reagents were added to the reaction maintained at 20°C for another 15–25 min

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[1] *Abbreviations used in the paper:* DMSO, dimethylsulfoxide; EGTA, ethyleneglycol-bis (β-amino-ethyl ether)N,N'-tetra-acetic acid; FITC, fluorescein isothiocyanate; GARG, goat anti-rabbit Ig; PBS, phosphate-buffered saline.

before fixation by the addition of paraformaldehyde. In experiments to determine whether ionophore-disrupted caps remained on the surface, cells were capped by adding nonlabeled anti-Ig in the same protocols as above. After fixation and washing, the cells were then exposed to FITC-GARG (100 μl per 10^7 cells for 30 min at 4°C) and washed again. In some experiments cells were examined directly under the microscope, as detailed before (8).

Lymphocytes were examined at a 400 magnification on a Leitz microscope with fluorescent epiillumination. In several experiments, caps were graded according to the fraction of the cell surface stained by the fluorescent ligand. In experiments reporting results only in terms of caps, we have defined a cap as fluorescent staining covering less than one-half the cell surface. Unless otherwise specified, results are percentages of a minimum of 200 positively stained cells. Viability of lymphocytes was estimated by exclusion of trypan blue.

Reagents. The ionophore A-23187 was a gift from the Eli Lilly Co., Indianapolis, Ind. It was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mg per ml, and serial dilutions in PBS were made from this stock. Appropriate dilutions of DMSO alone had no effect on capping. (The experiments reported in this paper were all done with a single batch of ionophore. A second batch, received just recently, gives us identical results but requires a dose about four times higher.) Cytochalasin D was a kind gift from Dr. Gabriel Godman (Columbia University College of Physicians and Surgeons, New York). Cytochalasin B, obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., was dissolved in DMSO at a concentration of 1 mg per ml and stored frozen; serial dilutions in medium were made as needed. Cytochalasin D was dissolved in PBS with 10% DMSO at a concentration of 100 μg per ml, stored at 4°C, and added directly to the reaction as needed. Sodium azide (Na azide), sodium cyanide (NaCN), and oligomycin were obtained from Matheson, Coleman, & Bell, East Rutherford, N.J., Fisher Scientific Co., Pittsburg, Pa., and Sigma Chemical Company, St. Louis, Mo., respectively. Colchicine and ethyleneglycol-bis(β-amino-ethyl ether)N,N'-tetra-acetic acid (EGTA) were obtained from Sigma Chemical Company and diluted in medium to a final concentration of 10^{-4} M and 2×10^{-3} M, respectively.

Results

Relationship Between Capping and Changes in Cell Shape. We first report on a time-sequence study on the development of the cap and its relationship with the change in cell shape. FITC-anti-Ig antibody was added to murine splenic lymphocytes at 4°C and allowed to bind for 20 min. The cells were spun, washed, and portioned in several tubes, then allowed to settle and brought to 37°C for varying lengths of time before fixation in 2% paraformaldehyde. Fig. 1 shows the results of one such experiment. After binding in the cold, all the Ig-bearing cells were diffusely stained, appearing as rings whose surface was covered with small patches of fluorescence. The subsequent reorganization of these surface patches at 37°C was extremely rapid. After 1 min at 37°C the number of diffusely stained cells dropped to 36% and thence to 4% after 2.5 min as the complexes redistributed into caps. In the caps, the patches aggregated in an orderly fashion, covering successively less of the cell's surface. 17% of the cells after 1 min had caps covering one-half of the cell's surface; this percentage peaked at 31% after 1.5 min and the dropped as the caps progressively coalesced into tight caps covering less than one-fourth of the cell membrane. The percentage of tightly capped cells rose rapidly to 60% after 2 min and was virtually complete at 90% after 4 min. Changes in cell shape took place in a high percentage of cells after the development of caps. The most striking change was a constriction taking place in the cytoplasm just below the area of the cap (see also Fig. 3 A, 3 B, and 5). Note in Fig. 1 that by 2 min most cells have tight caps and yet the constriction has not developed until about 30 s later. A second early change in shape was a small cytoplasmic and transient protrusion in the form of one or two
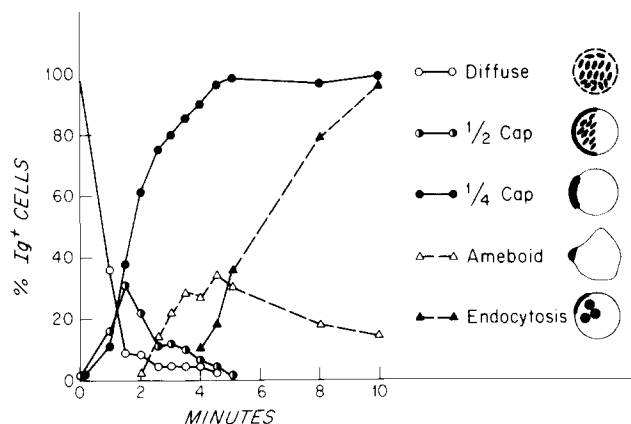
FIG. 1. The explanation of this experiment is in the text. The capping of cells with FITC-anti-Ig was stopped by fixation at the times given and the extent of the cap (read as percent of the cell surface covered by fluorescent staining) and the associated changes of morphology were evaluated microscopically. 200 positively stained cells were counted at each time.

or three small spikes always opposite the cap. The thin spike was seen in cells with or without constriction at 2–3 min in time. After the constriction, other changes in cell shape, notably, pseudopod formation, took place. These have been described in past studies in more detail and correlated with a true, albeit limited, translatory motion (6–8). The direction of cytoplasmic flow into a pseudopod was always opposite to the cap. Similar sequence of changes was observed by direct microscopic examination of cells mounted in slides and examined under a curtain of 37°C air. Finally endocytosis followed cap formation starting at about 4 min. In essence, this kind of detailed study reaffirmed previous conclusions of two interrelated but separated events: the cap and the changes in shape (6, 7). We emphasize here the first evidence of gross contraction in the cell occurring past the capping event and in a topography close to the cap area.

The Role of Calcium in Capping. The role of calcium was studied in two ways. Firstly, cells underwent ligand binding and capping in an environment depleted of calcium either through the use of calcium-free medium or by the addition of varying concentrations of EGTA, a specific calcium-chelating agent which, at 2 mM, remains extracellular (20). Secondly, cells were preincubated with varying doses of A-23187. The results are shown in Table I and Fig. 2. Depleting the extracellular environment of calcium had no inhibitory effect on the cell's ability to cap. The introduction of calcium into the cytoplasm by low doses of the ionophore produced a striking inhibition of capping. Cells to which as little as 0.1 μg per ml of A-23187 was added manifested diffusely scattered patches of fluorescence over the entire cell surface throughout the course of the experiment. A-23187 inhibited capping at doses not toxic to the cells which remained perfectly viable. With this batch of A-23187, toxic effects were seen at doses of 2 μg per ml or more (approximately 30% death). Calcium-free medium or the presence of EGTA in calcium-containing medium completely reversed the inhibitory effect of the ionophore, allowing the cell to cap the complexes

TABLE I
Effects of Calcium on Capping

Media	Percent of caps			
	Experi-ment 1	Experi-ment 2	Experi-ment 3	Experi-ment 4
Control—no treatment	76	79	65	76
Calcium-free medium	—	80	—	76
2 mM EDTA	88	68	—	—
A-23187				
0.002 μg per ml	—	—	50	54
0.01 μg per ml	—	—	25	32
0.10 μg per ml	—	—	6	7
A-23187 (0.10 μg per ml) plus 2 mM EDTA	—	—	—	68
A-23187 (0.10 μg per ml) in calcium-free medium	—	—	—	67

5×10^6 lymphocytes in 0.5 ml were incubated for 5 min at room temperature in the different media. After 5 min at room temperature, 50 μg of FITC-anti-Ig were added and the reaction allowed to proceed a further 20 min at room temperature before termination with paraformaldehyde. In experiments with A-23187, the ionophore was added during the preincubation and remained throughout the reaction. In experiments with EGTA, EGTA was also present throughout the preincubation and incubation periods. Results are expressed as percent of positively stained cells with caps.

(Fig. 2). The ionophore also prevented the changes in shapes of the B lymphocyte, maintaining it in a rounded, immobile position. This inhibition, too, could be reversed by chelating extracellular calcium. In experiments to be published we have confirmed the entrance of calcium into the lymphocyte by A-23187 using ^{45}Ca.

The Effect of Calcium Influx on the Formed Cap. If the influx of calcium was acting to functionally immobilize molecules presumably floating in a lipid matrix, it was possible to hypothesize some potential cytoplasmic link, activated by calcium, to those molecules in whatever pattern they are organized on the cell membrane. Hence, we investigated the effects of an ionophore-mediated calcium influx on Ig caps.

As outlined in Materials and Methods, cells exposed to FITC-anti-Ig were allowed to form caps for 25 min at 20°C. The ionophore A-23187 was then added for another 15 min, at which time the reaction was ended by the addition of paraformaldehyde. Table II contains the representative results of two of seven experiments.

In Experiment 1, 89 and 91% of the control lymphocytes formed caps covering one-half or less of the cell surface after 25 min at 20°C. In Experiment 2, 76% of
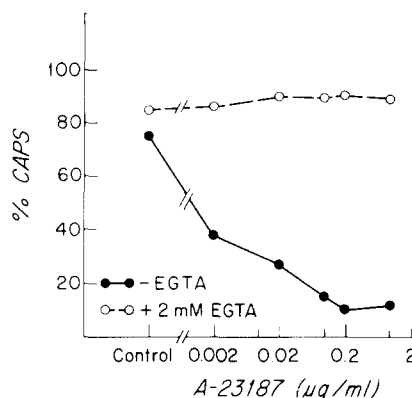
FIG. 2. FITC-anti-Ig ws added to lymphocytes at room temperature in calcium-containing medium after preincubation for 5 min at room temperature. To half the cells, A-23187 (●- - -●) was added during the preincubation and was present throughout the reaction. To the other half, A-23187 was added to lymphocytes in medium plus 2 mM EGTA (○- - -○); the rest of the protocol was the same. The reaction was terminated 20 min after the addition of anti-Ig. Results are expressed as percentage of capped cells in 200 positively stained lymphocytes.

the control cells capped under the same conditions. In both experiments, the percentage of capped cells rose slightly after a further 15-min incubation at 20°C. The addition of A-23187 not only stopped this slight rise, but it also actively disrupted the caps, dispersing fragments of the cap over the entire cell surface. In both experiments, 60–80% of the cells became diffusely stained. The ionophore broke the cap into irregularly sized fragments covering 75–100% of the cell surface (Fig. 3). An occasional cell showed partial fragmentation of the cap. The cells that remained capped in the presence of the ionophore were those that had formed very tight caps that were beginning to undergo endocytosis. In further experiments, we found that, under conditions that stimulated endocytosis, the disruptive effect of the ionophore, though present, was muted. In one of three such experiments, we allowed the cell to cap at 25°C for 15 min, at which point 92% of the Ig-bearing cells capped. The ionophore was added at a 0.2 μg per ml dose and the reaction taken to 37°C for 2 min. In the presence of the ionophore, 61% of the cells remained tightly capped, and 39% were diffused stained. Most of the tightly capped cells were in the midst of actively endocytosing their complexed Ig receptors. Allowing the reaction to proceed longer at 37°C did not markedly enhance the ionophore effect. Thus, there appears to be a temperature-sensitive end stage of cap formation in which the surface molecules are no longer sensitive to an influx of calcium.

The calcium specificity of the ionophore effect is demonstrated in Fig. 4. The cells were exposed to A-23187 as before, but in the presence or absence of EGTA. In the presence of 2 mM EGTA, the ionophore did not disrupt the caps at any of the dosages studied. The cells were able to maintain their caps for the duration of the experiment.

To be sure that the complexes disrupted by the ionophore were on the cell surface, we carried out experiments using nonlabeled anti-Ig, detecting it by use

TABLE II

The Effect of A-23187 on Capped Cells

Experiment no.	Extent of cap*	Untreated cells				Cells after addition of A-23187		
		25 min		40 min		0.1 µg/ml		0.2 µg/ml
		%				%		%
1	One-fourth	86	82	91	96	11	17	
	One-half	5	7	4	0	5	4	
	Three-fourths	4	4	2	0	10	4	
	No cap	5	7	3	4	74	75	
2	One-fourth	68		75		25		24
	One-half	8		4		7		4
	Three-fourths	6		3		4		2
	No cap	18		18		64		70

* Expressed as fraction of cell surface stained by fluorescent ligand. 50 µg FITC-anti-Ig were added to 5×10^6 lymphocytes in 0.5 ml at 4°C for 20 min. The cells were then washed, warmed to 20°C, and allowed to cap for 25 min. A-23187 was then added and the reaction allowed to proceed a further 15 min. Each result represents one tube when 200 positive cells were counted.

of a fluorescent GARG. That is to say, cells treated with rabbit anti-Ig, capped, and exposed to the ionophore were then stained with the labeled GARG in the cold. The experiments verified that the complexes of anti-Ig–Ig were on the cell surfaces, inasmuch as they could be detected by the labeled antibody in a sandwich reaction (Table III).

The Effect of Metabolic Inhibitors on the Formation and Maintenance of the Cap and on the Calcium-Sensitive Disruption of the Cap. We investigated the effects of metabolic inhibitors on the formation of the cap, the maintenance of the formed cap, and on the disruptive effect of the ionophore-mediated calcium influx. One of three experiments is presented in Fig. 5. Fig. 5 *A* demonstrates the effect of these metabolic inhibitors on the formation of the cap. FITC-anti-Ig was added to the cells in the cold. After washing, the cells were brought to 20°C at the same time that the metabolic inhibitors were added to the concentrations shown. The reaction was terminated after 20 min by the addition of paraformaldehyde, at which time 71% of cells not treated with any drug had capped their surface Ig receptors. The presence of sodium azide, sodium cyanide, or oligomycin blocked cap formation in agreement with past experimental results (1, 2, 4). In Fig. 5 *B*, after binding FITC-anti-Ig and being washed, cells were allowed to cap for 20 min at 20°C; metabolic inhibitors were added at 20 min and the reaction allowed to proceed for another 20 min. To cells with and without the drug present, 0.05 µg of the ionophore A-23187 was added at 25 min and was present for the last 15 min of incubation at 20°C. The presence of metabolic inhibitors alone did not affect the capped cells, which, in other experiments, remained intact after more than an hour's incubation in these compounds. Most interestingly, cells to which metabolic inhibitors were added 5 min before the
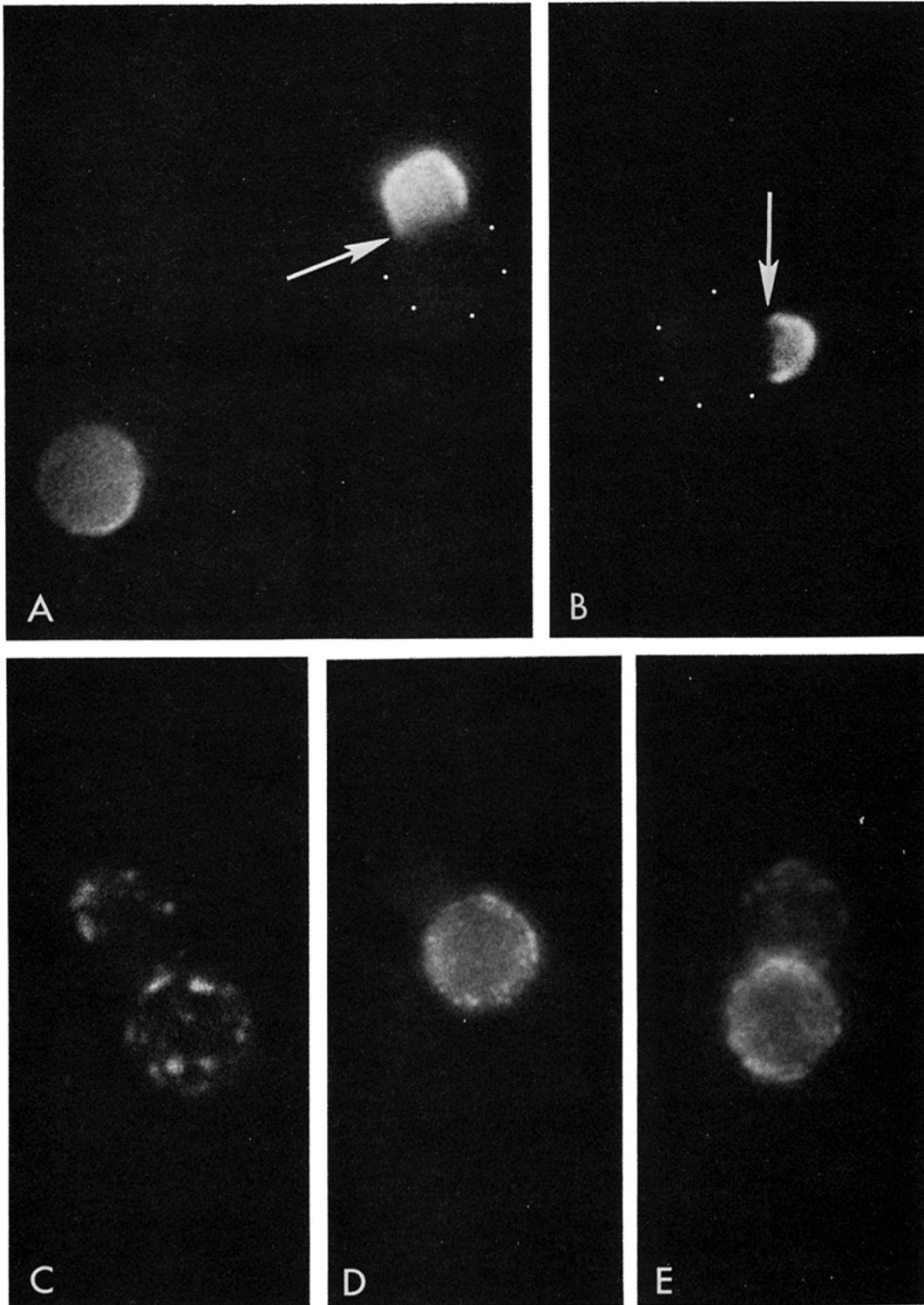
addition of the ionophore experienced little or no disruption of their capped complexes. We concluded that the calcium-mediated reversion of Ig caps is dependent upon metabolic energy.

We noted that some of the cells with caps treated with metabolic inhibitors (in the absence of A-23187) had a marked exaggeration of their constriction ring. Fig. 6 b and c demonstrate the appearance of capped cells which have been incubated in oligomycin, 2 μg per ml, after cap formation. The cells resemble an "apple core," with a severely constrictive band directly in front of the cap that appears to be forcing the cytoplasm in front to splay out. Similar observations were made on cells in azide and cyanide. We explain this phenomenon to be a form of rigor mortis analogous to an ATP-deprived actomyosin system.

The Effect of Cytochalasins on the Formed Cap.   We have investigated the effect of cytochalasins B and D on the disruption of the caps. Table IV contains the results of two experiments in which cells were allowed to cap for 20 min at 20°C before the addition of the cytochalasins for the last 20 min of the reaction, also at 20°C. One set of cells was preincubated with colchicine, 10^{-4} M, for 1 h before the addition of the ligand and the cytochalasins. Cytochalasin B had significant effects on inducing cap reversion, effects that were enhanced in the
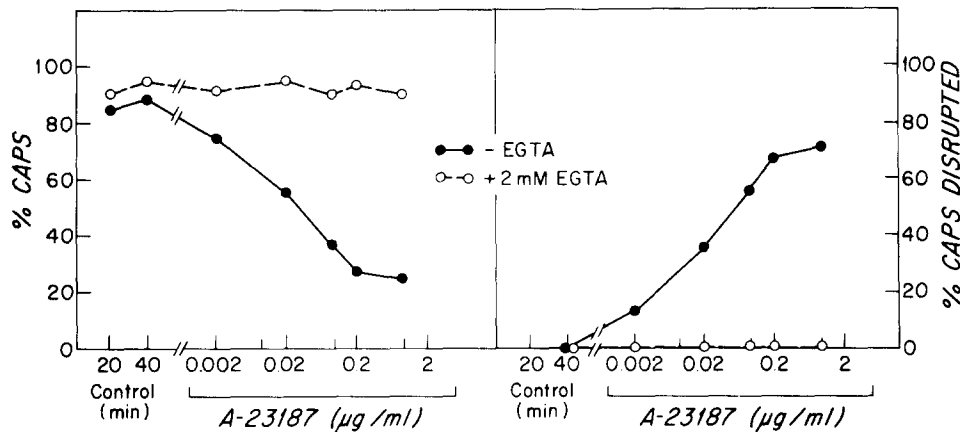

Fig. 4.   Cells exposed to FITC-anti-Ig were allowed to cap for 20 min at 20°C in the absence or presence of 2 mM EGTA, which had no effect on cap formation by itself. A-23187 was added at 20 min, and the reaction was terminated 20 min later. In the left panel, the results are expressed as percentage of 200 positively stained cells manifesting caps at the end of the reaction. In the right panel, the same data has been expressed as percentage of capped cells at 20 min that have undergone cap disruption (fluorescent staining covering 75–100% of the cell surface) by the end of the reaction.

Fig. 3.   (A and B) These figures show positively stained cells from the control group allowed to cap a total of 40 min at 20°C. The lower left-hand cell in A is a cell that never capped; note the diffuse staining organized in micropunctate patches. The upper right-hand cell in A and the cell in B have capped. The arrow points to the constriction frequently seen encircling the cell and delimited by the cap. The small white dots mark the edges of the unlabeled membranes. (C, D, and E) These figures show cells that were allowed to cap for 25 min and to which the ionophore A-23187 (0.1 μg per ml) was added for a further 15 min at 20°C. Cells with disrupted caps manifest diffuse staining in irregularly sized fragments covering most of the cell surface.

TABLE III

Effect of A-23187 on Cells Capped with Anti-Ig or FITC-anti-Ig

Extent of cap*	Untreated cells				Cells + A-23187			
	20 min		35 min		0.1 µg/ml		0.2 µg/ml	
FITC-anti-Ig								
$1/4$	50	51	68	71	12	14	14	8
$1/2$	14	14	12	8	3	5	7	9
$3/4$	6	7	1	1	2	4	4	3
No cap	30	28	19	20	83	77	75	80
Anti-Ig + FITC–GARG								
$1/4$	57	57	72	67	19	19	8	9
$1/2$	17	10	11	10	5	10	4	8
$3/4$	3	5	2	4	7	8	6	1
No cap	23	28	15	19	69	63	82	82

50 µg of either anti-Ig or FITC-anti-Ig were added to 5 × 10^6 lymphocytes at 4°C for 20 min. The cells were washed and warmed to 20°C for 20 min. A-23187 was then added to both preparations at the concentrations noted for another 15 min. After fixation and washing, cells treated with anti-Ig were exposed to FITC-GARG (100 µl/10^7 cells for 30 min at 4°C) and then washed. Results for duplicate tubes are expressed as percentage of 200 positively stained cells.
* Expressed as fraction of cell surface stained by fluorescent ligand.

presence of colchicine, as has been observed (12). Cytochalasin D was a powerful disrupter of Ig caps by itself. Also, the caps disrupted by cytochalasin D were more diffuse than those fragmented by cytochalasin B. Colchicine not only had no synergistic effect, but appeared to inhibit somewhat the extent of the cytochalasin-induced reversion of the cap. Colchicine, alone, had no effect on caps (data not shown).

Discussion

The progression from the aggregation of molecules into a cap through the stage of cell movement to endocytosis and catabolism of the complexes is orderly and rapid. Frequently, visible around the edge of the shrinking cap is an encircling contractile band that ultimately may force the cytoplasm to stream in the direction opposite to that of the now tightly aggregated cap. Thus, the polarity induced by the position of the cap appears in some part to determine the asymmetrical contraction necessary for translational motility. We have observed a calcium-activated cytoplasmic modulation of the aggregation of patches of Ig complexes in the plane of the membrane and their maintenance in cap structure. The ionophore-mediated influx of calcium prevents capping. Furthermore, the calcium ionophore produces a striking effect in that it fragments and disperses the capped complexes across the surface of the cell. While cap formation and lymphocyte motility are not affected by the deletion of calcium from the extracellular medium, the inhibitory and disruptive effects of A-23187 are completely abrogated by the presence of the calcium-chelating EGTA. Once the cap is constituted, its maintenance is not dependent upon further consumption
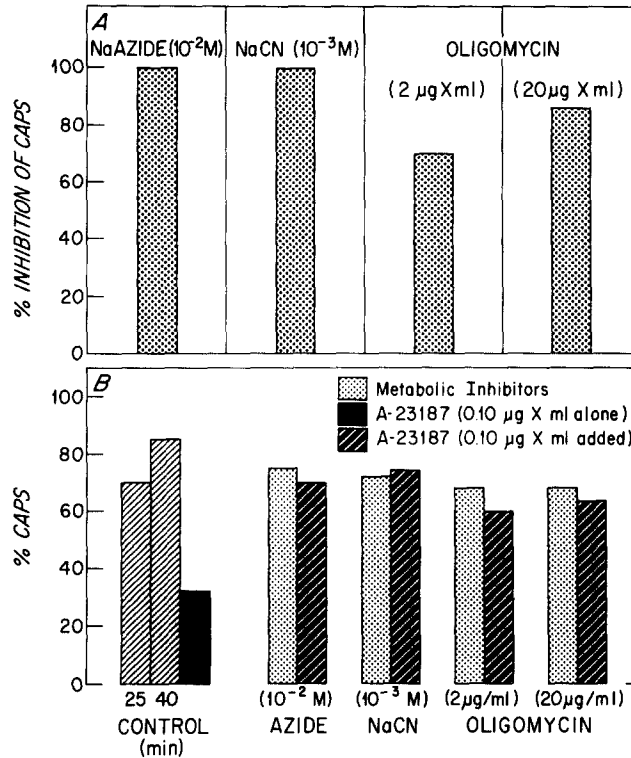
FIG. 5.   (A) The explanation of the experiment is in the text. Na azide, NaCN, or oligomy-
cin were added to FITC-anti-Ig-bound lymphocytes as they were warmed from 4°C to 20°C.
Results are expressed as inhibition of cap formation, in which the number of capped cells in
200 positively stained lymphocytes is calculated as percentage of the capped cells in the
control group (71%) and subtracted from one. (B) The experiment is explained in the text. It
shows the disruption of caps by treatment with ionophore, metabolic inhibitors, or both in
combination. Results are expressed as the percentage of capped cells in 200 positively
stained lymphocytes.

of metabolic energy, although subsequent endocytosis is (5). The disruptive
effect of the calcium influx on capping is apparently dependent upon the
avaliability of ATP.

We feel that the requirements for extracellular calcium and for metabolic
energy for disruption of the cap by the ionophore can best be explained by
postulating an effect on the contractile elements of the lymphocyte. Previous
experiments on the effects of capping by cytochalasin B (1, 10) also suggested the
involvement of microfilaments, but were somewhat inconclusive inasmuch as
the exact mode of action of the drug was not clear, and the effects seen were not
extensive, requiring large doses and the synergistic effects of colchicine (12, 21,
22). Recent studies using cytochalasin D strongly suggest that this drug, in fact,
produces a marked aggregation-contraction of the microfilament network of
the cell cortex in an energy-requiring step (23, 24). This drug inhibits cap
formation[2] and, as was shown herein, also disrupts the cap. Both the effects of A-

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[2] Unanue, E. R., and G. Godman. Unpublished results.
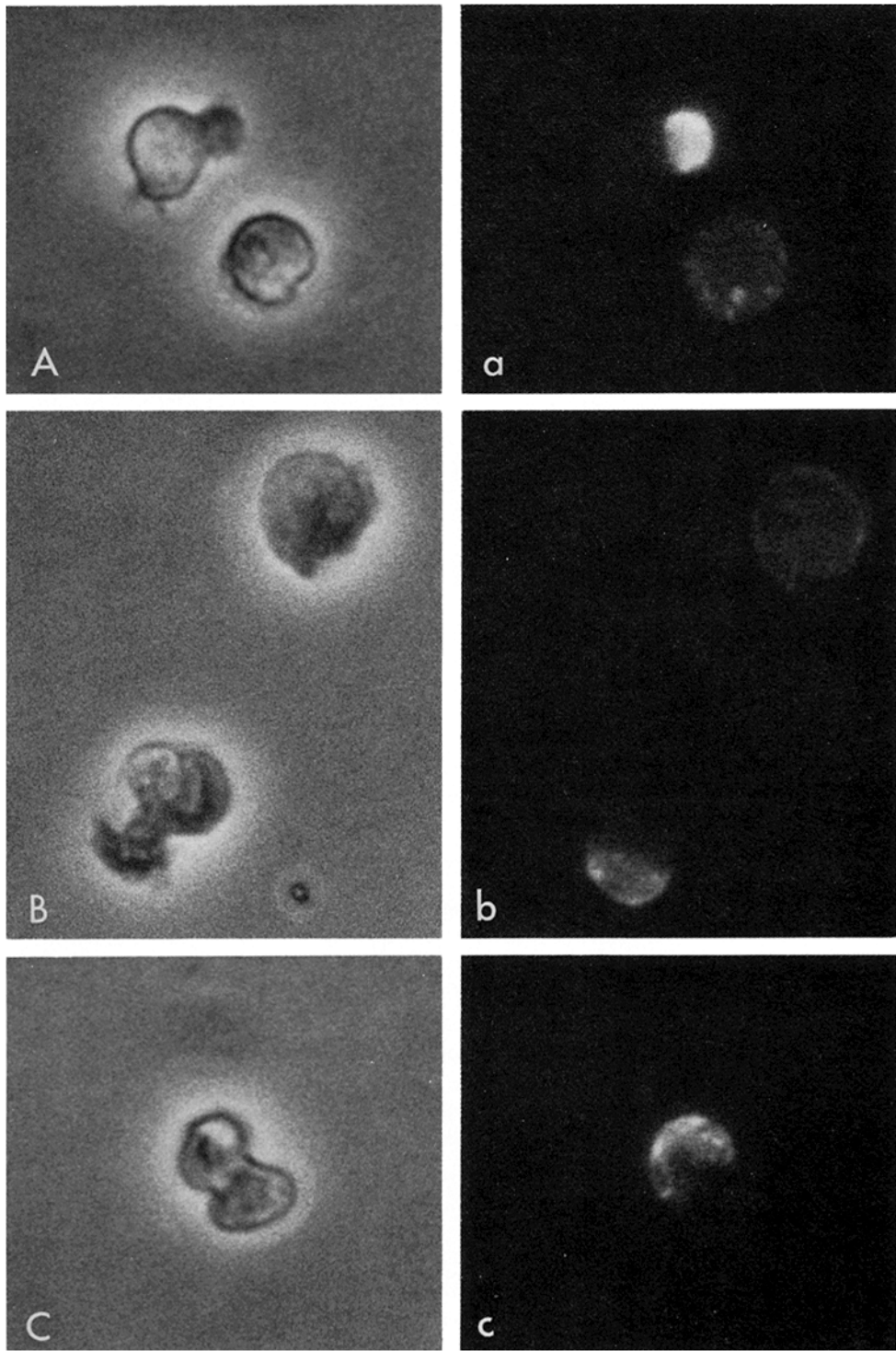
23178 and cytochalasin D could be interpreted on the basis that they produce a systemic hypercontractile state of the microfilaments. An alternative explanation is that both these drugs directly affect the plasma membrane, dislodging in some way the cap. The calcium and energy requirement for A-23187 to break the cap tends to argue against this. The mechanisms of cap disruption described by de Petris (12) and discussed later may involve, however, a different process.

We wish to offer a working hypothesis to explain much of what we and others have described and to provide a guide to further investigation. Available evidence suggests that there is a calcium-responsive system in the cytoplasm that modulates both the formation and the maintenance of the Ig cap. The identification of the microfilament network in the cytoplasm as responsible for the generation of contractile force in many cell systems (11, 23, 25–27) has been

TABLE IV
Effect of Cytochalasins on Caps

	Experiment 1	Experiment 2
	%	%
Untreated cells		
at 20 min	81	82
at 40 min	83	94
Cytochalasin B, 20 μg/ml	52	48
Cytochalasin B, 20 μg/ml + colchicine, 10^{-4} M	19	37
Cytochalasin D, 5 μg/ml	20	30
Cytochalasin D 5 μg/ml + colchicine, 10^{-4} M	42	44

After exposure to FITC-anti-Ig (50 μg/5 × 10^6 cells in 0.5 ml for 20 min at 4°C followed by washing), cells were capped for 20 min at 20°C before the addition of Cytochalasin B or D. Cells were incubated a further 20 min at 20°C before termination of the reaction. One set of cells was incubated with 10^{-4} M colchicine for 1 h at 37°C, cooled to 20°C, and exposed to 50 μg/FITC-anti-Ig/5 × 10^6 cells in 0.5 ml at 20°C. The procedure was then identical to the above. Results are expressed as percentage of 200 positively stained cells with caps covering less than one-half of the cell surface. Colchicine alone has no inhibitory effect on caps.

FIG. 6.   Fig. 6 A and a are the phase contrast and fluorescent micrographs, respectively, of a normally capped cell after 40 min at 20°C. Note the ameboid morphology with a rounded pseudopod and an apparent constriction occurring under the cap, in contrast to the adjacent, round lymphocyte. Fig. 6 B and C are cells in phase contrast that were allowed to cap normally for 20 min at 20°C, but which were then exposed to oligomycin, 2 μg per ml. Note the exaggerated morphology with a severely constricting band encircling the cell and bounded by the capped complexes, seen in fluorescent staining in Fig. 6 b and c. The cytoplasm in front of the cap appears to be splayed out. Cells exposed to metabolic inhibitors before addition of FITC-anti-Ig remain in a round configuration and are unable to cap (5).

accompanied by investigations suggesting this system to be actomyosin-like in its ATP-dependent, calcium-mediated mechanisms for the development of shearing force (11, 22, 28–30). We suggest that, in ligand-stimulated capping, there is a continuous, physical relationship between surface Ig, or transmembrane molecules linked with surface Ig, and calcium-responsive cytoplasmic microfilaments that underlie and perhaps attach to the membrane.

Two possibilities exist with respect to this link over time. Either the surface Ig molecules are already bound to the filaments or some component of them or this linkage occurs subsequent to the stimulation of complexing with a ligand. The mass of data underlying Singer and Nicholson's hypothesis (31) concerning the random nature of protein molecules in biological membranes and the lack of associated molecules on Ig isolated from the surface of lymphocytes (reviewed in Reference 32) weighs against this possibility. One can make a stronger case for the existence of a cytoplasmic-surface point linkage occuring after the binding of a ligand to that point, represented here by Ig. This concept offers the advantage of allowing nonrandomness to occur over time and not just in space. The ligand-receptor complex on the surface linked to the assembled microfilament network of the cell cortex is then displaced as the latter system contracts. For the contraction to be effective in producing a cap, we suggest that it necessitates both the relaxation of adjacent filaments as well as a mechanism for orientation and focusing of the contraction to one area or point of the membrane. Underlying the cap should be an area of activated microfilaments which retains its intercalations to the surrounding network of relaxed elements. We interpret the potent inhibition of capping by a calcium influx due to the stimulation of a generalized contraction forcing the cell to remain round and functionally immobilizing the surface complexes by preventing the simultaneous development of areas of contraction and relaxation necessary for net displacement of the complexes. Similarly, such a calcium-stimulated contraction would put tension on the formed cap, stabilized by underlying, attached, activated microfilaments intercalated with a surrounding network of heretofore relaxed filaments, and pull it in all directions.

To envision a chain of specialized microfilament contractions and relaxations moving a molecule within and through the membrane, we must postulate a sequence of activation, an activated contraction running beneath the membrane along the path of the moving cluster of molecules. What would be the source of activation, in light of the fact that extracellular calcium is apparently not required for either capping or movement? Ongoing experiments in this laboratory have demonstrated the triggering of an efflux of calcium upon the addition of anti-Ig to the cell. One could conclude that the cell has intracellular sites for storage of calcium to be released in the proper circumstances (33, 34). Alternatively, calcium strongly bound to the membrane could be released into the cell by membrane depolarization, as has been suggested, for smooth muscle (35). Thus, a role for calcium release at a local spot may be operative in activating the driving force for capping.

In studies emphasizing the capping of Con A (12, 21), de Petris first called attention to the phenomenon of cap disruption. He has envisioned Con A capping to involve a countercurrent effect with noninvolved areas moving

actively away from the fixed complexes. Extrapolating from the data on Con A caps and on the synergistic effects of cytochalasin B and colchicine on Ig caps (12), de Petris has concluded that the disruption of both Con A and Ig caps involves the retraction of the actively moving part of the cell into the capped zone, with elements of the cap mixing with unlabeled membrane. We agree fully with his interpretation of Con A capping, but feel that the evidence suggests that the formation and disruption of Con A and anti-Ig caps involve quite different processes: (a) Con A caps can be disrupted by drugs that affect energy metabolism (12, 21), while these drugs will not affect the anti-Ig caps; (b) capping of Con A is highly sensitive to cytochalasin B in contrast to the anti-Ig capping (12); and (c) time-sequence morphological studies differ, Con A caps necessarily form in cells in movement (12, 21, 22), while, as shown herein, anti-Ig caps form first, before the establishment of gross cell contractions. With its multivalent binding and strict dosage requirements, Con A could be inducing any number of effects: locking of surface complexes bound to microfilaments, altering calcium physiology, preventing the establishment of a focusing point for filamentous contractions, or enhancing microtubular functions (9). We suggest, however, that Ig cap formation is fundamentally different and that, reflecting this difference, disruption of the Ig caps, whether by A-23187 or by ctyochalasin D, is not a passive consequence of inhibiting intracellular elements (21), but rather, is an active phenomenon requiring energy and continuous interaction between contractile elements in the cytoplasm and the complexed Ig receptors in the membrane.

Summary

Capping of anti-Ig–Ig complexes was studied in murine B lymphocytes. Morphological studies indicated that caps formed rapidly on cells before any changes in shape. The first changes in cell shape were evident as a contraction right under the cap area. The removal of extracellular calcium had no effect on cap formation. Furthermore, the introduction of calcium by the ionophore A-23187 stopped capping. The ionophore by itself in the absence of extracellular calcium had no effect. Caps were found to be disrupted, the complexes scattering over the entire cell surface if the cells were treated by A-23187 after the caps had formed. The disruptive effect of A-23187 as dependent on extracellular calcium and could be stopped by drugs that affected energy metabolism. The cytochalasins also disrupted the formed caps. Drugs that affect energy metabolism by themselves did not disrupt the caps. We interpret the effects of the ionophore as resulting from a systemic hypercontractility of microfilaments. A theory for explaining the formation and disruption of capping is discussed.

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