TWO DISTINCT MECHANISMS FOR REDISTRIBUTION OF LYMPHOCYTE SURFACE MACROMOLECULES

II. Contrasting Effects of Local Anesthetics and a Calcium Ionophore

JONATHAN BRAUN, KEIGI FUJIWARA, THOMAS D. POLLARD, and EMIL R. UNANUE

From the Departments of Pathology and Anatomy, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

In the previous study, lymphocyte surface molecules were separated into two subsets depending on whether capping was associated with redistribution of cytoplasmic myosin. In the present study, the effects of the local anesthetic chlorpromazine and of the Ca\(^{2+}\) ionophore A23187 were compared. Both drugs affected the surface redistribution of immunoglobulin (Ig), Fc receptors, and the TL antigen—molecules that appear to cap by association with microfilaments—but had no effect on the Thy.1 (\(\theta\)) and H2 antigens—molecules that cap slowly, apparently unlinked to microfilament function. The capping of Ig, Fc receptor, and TL was inhibited while that of H2 and \(\theta\) was not. Both drugs reversed the Ig, Fc receptor, and TL caps but not the H2 and \(\theta\) caps. In the former group, the reversal of caps was accompanied by a parallel reversal of the myosin segregated to the cap area. The appearance of myosin after drug treatment varied: chlorpromazine resulted in a diffuse pattern similar to that of normal lymphocytes, whereas A23187 produced an array of aggregates and coarse filaments. The results are compatible with the view that two mechanisms for capping exist in the lymphocyte. The Ca\(^{2+}\) ionophore may affect capping of microfilament-dependent caps by producing a systemic activation of contractile proteins while chlorpromazine may act by disrupting a Ca\(^{2+}\)-dependent link between surface complexes and the contractile proteins.

KEY WORDS capping • cell motility • lymphocyte • myosin • surface molecules

The evidence presented in the accompanying paper (1) suggested that lymphocyte membrane molecules fall into two distinct classes with respect to their spontaneous or ligand-induced redistribution into polar caps. Antibodies to immunoglobulin (Ig), antibodies to thymic leukemia antigen (TL), and immune complexes which bind to Fc receptors all form caps associated with a concentration of myosin in the underlying cytoplasm. On the other hand, antibodies against the histocompatibility antigen (H2) or to theta antigen (\(\theta\)) form caps without associated cytoplasmic myosin. To gather further information about the mechanisms of these surface molecule redistributions, we now have studied the effects of the Ca\(^{2+}\) ionophore A23187 and the local anesthetic chlorpromazine on the capping of the various surface molecules.
and the distribution of cytoplasmic myosin. Both drugs were known to inhibit Ig capping and to disrupt previously formed Ig caps (2-5). Given circumstantial evidence that Ca$^{2+}$ entry during ionophore treatment might cause a generalized, disorganized cortical contraction (4) and that the anesthetic might disrupt links between contractile proteins and the plasma membrane (2, 5), we predicted that the drugs might perturb the capping of only those molecules which depend on contractile proteins. Such a differential effect of the drugs was found, providing further evidence for two distinct capping mechanisms in the lymphocyte. In passing, we also have evaluated the effects of sodium azide, a metabolic inhibitor known to inhibit capping (7), on the distribution of cytoplasmic myosin.

MATERIALS AND METHODS
Preparation of cells and antisera and methods for studying capping were identical to those reported in the accompanying paper (1). The only new reagent was a goat anti-rabbit IgG. This was an IgG fraction conjugated to fluorescein at a molar ratio of 8.4, used at a concentration of 290 μg/ml. In one experimental protocol (that of Fig. 6), we utilized an unlabeled rabbit antimouse Ig. This was from the same batch as the fluorescein-conjugated reagent described before (1).

Drugs
Chlorpromazine (Smith, Kline, and French Laboratories, Philadelphia, Pa.) and sodium azide (Fisher Chemical Company, Fair Lawn, N. J.) were freshly prepared in 0.15 M phosphate-buffered saline, pH 7.2. A23187 was a gift from Eli Lilly Company, Indianapolis, Ind. It was dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml and stored at 4°C. Serial dilutions of A23187 were made in medium immediately before use. Corresponding dilutions of DMSO alone had no effect on capping.

Experimental Protocols
Two experimental protocols were used: one tested the effect of drugs on cap formation; another tested the effect of drugs on caps which had already formed. To study the influence of the drug on cap formation, cells were incubated on ice with antisera, rinsed, and aliquoted into tubes as described above but were settled in the absence of drugs. The cells were then incubated at the appropriate temperature and time interval, allowing normal cap formation. When the capping interval ended, drugs were added; the cells were further incubated (10 min with A23187; 20 min with other drugs) and finally fixed with paraformaldehyde.

In both cases, the cells were rinsed twice in medium after fixation, resuspended in fetal calf serum, smeared on cover slips, and air dried. The cover slips were then stained with rhodamine antimyosin and examined with a Leitz Ortholux II microscope using fluorescent epillumination. The appropriate excitation and barrier filters were used to detect in the same cell the surface complexes tagged with the fluorescein label and the myosin labeled with the rhodamine-conjugated antibody. Cells were considered capped when the fluorescent reaction covered less than half the cell surface. At least 200 stained cells were scored for each experimental point. Except for the experiments with anti-TL and anti-H2, which were done only once, the remaining studies reported here were done at least on three different occasions with identical results.

RESULTS
Effects of Chlorpromazine and A23187 on Myosin Distribution in Untreated Lymphocytes
Chlorpromazine had no effect on the diffuse antimyosin staining of the lymphocyte cytoplasm (at 10$^{-4}$-10$^{-5}$ M) (Fig. 1, panels A and B). A23187 at 0.01-0.1 μg/ml in medium containing Ca$^{2+}$ caused the antimyosin staining to aggregate into coarse, irregular clumps. In most cells the antimyosin clumps formed a network of coarse bands, sometimes centering on one point (Fig. 1, panels C and D). The effects of the ionophore were not observed in Ca$^{2+}$-depleted medium. In cells treated with ionophore in regular Ca$^{2+}$-containing medium and then fixed, subsequent reaction with ligands revealed that ~10% of the surface Ig and Fc receptors were redistributed into coarse patches, most of which appeared to be closely associated with the myosin aggregates in the cytoplasm (Fig. 2, panel A). Patching of θ was not observed in T cells treated with A23187. (H2 and TL were not tested.)
**FIGURE 1** Fluorescence micrographs of splenic lymphocytes stained with rhodamine antimyosin for cytoplasmic myosin. Treatments (A) none; (B) $10^{-4}$ M chlorpromazine for 30 min at 37°C; (C and D) 0.1 µg/ml A23187 for 10 min at 37°C. Note the coarse filamentous pattern produced by A23187 in some cells (arrowhead). Similar patterns were observed on lymphocytes treated with ligands before or after addition of the drugs.

**FIGURE 2** Fluorescence micrographs of splenic lymphocytes stained with (left) fluorescein anti-Ig for surface Ig and (right) with rhodamine antimyosin for cytoplasmic myosin. (A) splenic lymphocytes were exposed to A23187 (0.1 µg/ml) for 30 min, then fixed and stained. (B) splenic lymphocytes were bound with anti-Ig at 0°-4°C, rinsed, and incubated at 37°C in the presence of sodium azide (10 mM). After 30 min, the cells were fixed to terminate the reaction and stained with antimyosin. Note the correspondence of many surface Ig patches with myosin aggregates.

**Effects of Chlorpromazine or A23187 on the Capping of Ig, Fc Receptors, and TL and on the Redistribution of Cytoplasmic Myosin**

Chlorpromazine inhibited in a concentration-dependent fashion the capping of Ig, Fc receptors, and TL and the redistribution of cytoplasmic myosin which usually takes place when these molecules cap. The antimyosin staining remained diffuse as in Fig. 1, panel B. The inhibition of anti-Ig capping was 70–75% at $10^{-3}$ M and 90–100% at $10^{-4}$ M (see also reference 3 and the experiment of Fig. 7, described later). Fig. 3, upper left panel, shows the results with Fc receptors. In a single experiment, capping of TL was inhibited by 95% at $10^{-4}$ M chlorpromazine. A23187 also inhibited the capping of Ig, Fc...
receptors, and TL and the cytoplasmic redistribution of myosin. The inhibition of Ig capping was well detailed before (4) and was confirmed now using concentrations of 0.01-0.1 \( \mu g/ml \). The effects on Fc receptor capping are shown in Fig. 3, upper right panel. In the single experiment done, capping of TL was inhibited by 90% at a dose of 0.1 \( \mu g/ml \). All these effects of the ionophore required \( Ca^{2+} \) in the medium. The distribution of myosin was as depicted in Fig. 1, panel C and D, in coarse aggregates.

**Parallel Disruption of Ig, Fc Receptors, and TL Caps by Chlorpromazine and A23187**

Chlorpromazine dispersed the formed caps of Ig, immune complexes, and TL and their underlying concentrations of antimyosin staining material. With chlorpromazine, the capped complexes redistributed into a random, finely granular pattern. The antimyosin staining pattern was diffuse, as in Fig. 1 B.

Treatment with A23187 in \( Ca^{2+}\)-containing medium caused both the capped complexes and the cytoplasmic antimyosin staining material to reorganize into coarse, irregular patches (Fig. 1, C and D). Figs. 3 and 4 show the results of Fc receptor and Ig cap disruption. Table I summarizes the effects of A23187 on Ig capping.

**Effects of Chlorpromazine or A23187 on Cap Formation and Cap Disruption of \( \theta \) and H2**

Chlorpromazine slightly enhanced, rather than inhibited, the capping of anti-\( \theta \) (Fig. 5). A23187 had little effect except at high concentrations. Neither drug disrupted previously formed \( \theta \) caps. Similar results were obtained in a single experiment with anti-H2.
**TABLE 1**

*Summary of A23187 Effects on the Distribution of Surface Ig and Myosin*

| Experimental manipulation | Distribution of surface Ig | Distribution of myosin |
|---------------------------|---------------------------|------------------------|
| Fix → Anti-Ig             | Diffuse                   | Diffuse                |
| Anti-Ig → 37°C → Fix      | Caps in more than 90% of cells | Segregated to the area of the cap in more than 50% of cells |
| A23187 → Fix → Anti-Ig    | Patches in 10% of cells   | Random aggregates and coarse filaments in all cells |
| A23187 → Anti-Ig → 37°C → Fix | Capping inhibited; patches | Polar segregation inhibited; random aggregates and coarse filaments |
| Anti-Ig → 37°C → A23187 → Fix | Caps disrupted; patches | Polar segregation inhibited; random aggregates and coarse filaments |

**FIGURE 5**

- **θ CAP FORMATION**
- **θ CAP DISRUPTION**

**FIGURE 6**

Patches of H2. T cells with patches of H2 (A) show diffuse staining of myosin (B). The plane of the photograph of myosin is focused on the edge to demonstrate the lack of patching of myosin, which was diffuse.

**Effects of Sodium Azide on Ig, H2, and θ Capping**

The metabolic inhibitor sodium azide had no influence on the distribution of myosin in untreated lymphocytes but inhibited both the capping of Ig and altered the redistribution of cytoplasmic myosin which usually accompanies capping. In the presence of azide (10 mM), the anti-Ig formed patches on the outside of the cells, and the myosin formed aggregates in the cytoplasm, but azide prevented a subsequent step in which both types of molecules become concentrated at one pole to form a cap. In many instances, the surface patches of Ig appeared to be close to the cytoplasmic myosin aggregates (Fig. 2, panel B). T cells treated with either anti-H2 or anti-θ (and anti-Ig) in the presence of azide did not cap and had a diffuse distribution of myosin (Fig. 6).

**Effects of Chlorpromazine on Anti-Ig Capping Induced by Two Ligands**

The capping of θ and Ig seem to be fundamentally different (Table II) but, in the presence of chlorpromazine, Ig could be induced to cap much like θ; the rate was slow, two ligands were required (rabbit antimouse Ig plus a goat anti-rabbit Ig), and there was no redistribution of cytoplasmic myosin (Figs. 7 and 8). Specifically, if B lymphocytes were incubated in the cold with unlabeled
TABLE II
Redistribution of Two Sets of Surface Molecules

| Molecule | Cells | Rate | Number of ligands | Myosin segregation | Motility | A23187 | Local anesthetic | Spontaneous capping |
|----------|-------|------|-------------------|--------------------|----------|--------|-----------------|---------------------|
| Ig       | 80–100| Fast  | One               | Yes                | Stimulated| Prevents | Prevents or      | Yes: to uropod       |
|          |       |       |                   |                    |          | or breaks caps | breaks caps     |                     |
| Fc        | 80–100| Fast  | One               | Yes                | Stimulated| Prevents | Prevents or      | Yes: to uropod       |
| receptors|       |       |                   |                    |          | or breaks caps | breaks caps     |                     |
| TL       | 90    | Fast  | One               | Yes                | Stimulated| Prevents | Prevents or      | Yes                 |
| Con A    | 40–50 | Intermediater | One—very dose dependent | ± ±                  | Not done | Not done | Not done      | No                  |
| θ        | 30–50 | Slow  | Two               | No                 | No effect | No effect | Slight enhancement; no disruption | No: partial exclusion from uropod |
| H2       | 30–50 | Slow  | Two               | No                 | No effect | No effect | Slight enhancement; no disruption | No: partial exclusion from uropod |

DISCUSSION

Although the cellular mechanisms of action are not entirely known, chlorpromazine and A23187 have been extraordinarily valuable in our analysis of capping because they have allowed us to identify two subsets of lymphocyte surface molecules with different capping properties (Table II).
effects of chlorpromazine and A23187 on Ig, Fc receptors, and TL were striking: cap formation was inhibited; formed caps were disrupted. In marked contrast, θ and H2 cap formation were not inhibited, even slightly enhanced; and cap disruption was minimal or absent. At face value, these results imply that the mechanism subserving capping of each group of molecules must be different. The molecules with drug-sensitive capping are all thought to cap actively, accompanied by a redistribution of cytoplasmic myosin. The molecules with drug-insensitive capping cap slowly, independent of a direct association and/or activation of microfilaments by a progressive formation of large membrane aggregates. Our interpretation of the mechanisms of these two forms of capping was discussed in the accompanying paper.

To summarize the effects of chlorpromazine found in this and previous studies: (a) it stops capping or disrupts formed caps of those molecules that are associated with cytoplasmic segregation of myosin (Figs. 3 and 4); (b) it has no effect or even enhances slightly capping of molecules not associated with myosin redistribution (Fig. 4); and (c) when it disperses caps, it also disperses the cytoplasmic myosin segregated with the cap (Fig. 3); (d) it disrupts caps even in the presence of inhibitors of energy metabolism (5); and (e) its effects on capping are antagonized by high concentrations of extracellular Ca²⁺ (5). All five of these properties are compatible with a mechanism in which the drug (which is known to interact with membrane lipids [6]) in some way severs the link between surface complexes and the contractile apparatus of the cell, most likely by displacement of membrane-bound Ca²⁺. Once this putative link is broken, the complexes are free to diffuse within the plane of the membrane. In accordance, molecules that cap without association with contractile elements (like θ and H2) would not be expected to be affected in a negative way by the anesthetic, which is the case. Rather, because these anesthetics can increase membrane fluidity, it is interesting to note that the latter molecules are slightly enhanced in their extent of capping. Lastly, although Ig capping was inhibited by chlorpromazine, it was possible to induce it in the presence of the drug if large aggregates were formed on the membrane (by using an antibody to anti-Ig). Under these conditions, Ig capping was slow and was not associated with segregation of myosin, as took place under usual circumstances (experiment of Fig. 7). Thus, Ig in the presence of chlorpromazine had the same capping characteristics as θ. Our interpretation of the effects of tertiary amine anesthetics is compatible with that offered by Poste et al., which suggested that the drugs displace microfilaments from the cell surface (2).

The Ca²⁺ ionophore, in turn: (a) also stops cap formation or breaks caps of molecules associated with myosin segregation, but (b) does not affect molecules that do not segregate myosin; (c) produces a redistribution of myosin into a coarse network; and (d) its effects on cap disruption require energy metabolism (4). These effects are compatible with the explanation that the Ca²⁺ influx produced by the ionophore interferes with the organized process of activation of contractile proteins required for formation and maintenance of the cap.

This and the accompanying study (1) support the contention that two forms of capping exist in the lymphocyte. When the capping properties of lymphocyte surface molecules are compared (Table II), two patterns emerge: in one case, the contractile apparatus intimately and selectively interacts with certain surface molecules; in the other case, the role of the contractile apparatus, if any, is indirect. Thus, the molecular basis of these forms of capping may differ fundamentally. The pharmacological approach used here in conjunction with biochemical studies should provide further understanding of the different mechanisms involved.

**Figure 8** Fluorescence micrograph of B lymphocytes with Ig caps in the presence of chlorpromazine. Splenic lymphocytes were exposed sequentially at 0°-4°C to anti-Ig and anti-antibody. The cells were rinsed, incubated at 37°C for 30 min in the presence of 10⁻⁴ M chlorpromazine, then fixed and stained with antimyosin. (A) fluorescein fluorescence of surface Ig; (B) rhodamine antimyosin. Note the absence of myosin segregation beneath the surface Ig caps.
that control the properties of surface molecules in
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