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Receptor Capping in Mouse T-Lymphoma Cells: A Ca^{2+} and Calmodulin-Stimulated ATP-Dependent Process

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Summary. The roles that Ca^{2+}, calmodulin, and ATP play in the redistribution of conconavalin A (Con A) binding sites on the surface of mouse T-lymphoma cells were examined. Membranes of cells labeled with fluorescein-conjugated Con A (Fl-Con A) were made permeable ("skinned") to ions and proteins by incubation in a solution containing no added Ca^{2+}, 7 mM EGTA, and ATP. The intracellular ionic and protein concentrations could then be varied, and the degree of Con A receptor capping monitored simultaneously. A graded increase (9.0 to 30%) was found in the number of capped cells with increasing Ca^{2+} concentration from 10^{-6}–$10^{-4.9}$ M. Increasing concentrations of trifluoperazine, chlorpromazine, and promethazine (1.5×10^{-6} to 1.0×10^{-4} M) in cell suspensions containing 10^{-4} M Ca^{2+} produced graded inhibition of capping in the same order that the drugs bind to calmodulin. Removal of extracellular Ca^{2+} dissociated (reversed) some of the caps into patches, thus reducing their number (12%). ATP was required for either capping or cap dissociation to occur. Addition of calmodulin (3.9×10^{-8}–6.3×10^{-7} M) to the cell suspension increased the Ca^{2+} sensitivity. These results provide direct evidence that capping of Con A receptors is a reversible process (i) regulated by intracellular Ca^{2+} concentration, (ii) requiring ATP as an energy source, and (iii) susceptible to the influence of calmodulin. These findings are consistent with the hypothesis that the collection of surface receptor patches into cap structures is controlled by the interaction of actomyosin filaments, which in turn is regulated by a Ca^{2+}-calmodulin-activated control system.

Key Words T-lymphoma cells · capping · calmodulin · Ca^{2+} · concanavalin A · ATP

Introduction

The aggregation (capping) of the receptors upon binding to multivalent ligands, such as lectins and specific antibodies, on the surface of lymphoma cells is a two-step process. A rapid, energy-independent cross-linking of the receptors into small *patches* occurs, followed by an energy-dependent collection of the patches into a *cap* at one pole of the cell (Taylor, Duffus, Raff & de Petris, 1971; Schreiner & Unanue, 1976). In previous work (Bourguignon & Singer, 1977; Bourguignon, Tokuyasu & Singer, 1978; Butman, Bourguignon & Bourguignon, 1980) by the double-immuno-fluorescence staining technique, showed that increased concentrations of actin and myosin were invariably found directly under the capped receptors. It was thus proposed that a "transmembrane interaction" between the receptors and intracellular actin and myosin-containing structures enables their collection into a cap, by a mechanism analogous to the sliding-filament mechanism responsible for muscle contraction (Bourguignon & Singer, 1977). Studies on both smooth muscle (Chacko, Conti & Adelstein, 1977; Sobieszek, 1977) and non-muscle cells (Adelstein & Conti, 1975) suggest that the interaction between thick and thin filaments (myosin and actin-tropomyosin) is regulated by the myosin light-chain kinase, and it in turn is activated by calmodulin in the presence of Ca^{2+} (Dabrowska & Hartshorne, 1978). Anti-psychotic drugs, such as chlorpromazine (thorazine) and trifluoperazine (stelazine), which inhibit the biological function of calmodulin (Weiss & Levine, 1978), also inhibit receptor mobility or capping, so that lymphocyte capping may be Ca^{2+}- or calmodulin-dependent (Bourguignon & Balazovich, 1980; Salisbury, Condeelis, Maihle & Satir, 1981). Although removal of extracellular Ca^{2+} will not inhibit capping, increasing it will overcome the inhibitory effects of antipsychotic drugs; thus, Ca^{2+} may play a role in receptor capping (Braun, Fujiwara, Pollard & Unanue, 1978). Recent studies show that the binding of anti-immunoglobulin (anti-Ig) antibodies to B-lymphoma cells causes an increase in intracellular Ca^{2+} (Pozzan, Arslan, Tsien & Rink, 1982). To test the hypothesis that Ca^{2+}, calmodulin, and contractile proteins are involved in capping, we made use of "skinning" solutions to render the membranes of the lymphocytes permeable to ions and proteins (Hoar, Kerrick & Cassidy, 1979; Kerrick & Krasner, 1975), and then monitored the effect of varying intracellular Ca^{2+} on degree and extent of capping. We report direct

functional evidence that capping is stimulated by micromolar Ca^{2+} and calmodulin and is dependent on ATP as an energy source, and that the cap structure is reversibly dissociated in the absence of Ca^{2+}. Our findings strongly support the hypothesis that a Ca^{2+}-calmodulin-regulated contractile mechanism is responsible for receptor capping in lymphocytes.

Materials and Methods

Cells: Mouse T-lymphoma cells from the line BW 5147, a AKR/J lymphoma line (gifts from Dr. R. Hyman, The Salk Institute), were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% heat-inactivated horse serum (Gibco) at 37 °C in 5% CO_2/95% air. Concanavalin A (Con A)-induced capping: Cell suspensions (approximately 3×10^7 cells/ml were washed in DME medium three times and then treated with 50 μg/ml fluorescein-conjugated Con A (Fl-Con A) (Sigma) for 60 min at 0 °C. Cells were then rinsed with DME medium twice for removal of unbound Fl-Con A (Bourguignon et al., 1978).

Solutions

The EGTA-buffered Ca^{2+} solutions used in capping measurements were similar to those used by Kerrick and Krasner (1975) as modified for skinning smooth muscle fibers (Hoar et al., 1979). These solutions render the membranes of muscle cells permeable to ions and proteins and are referred to as *skinning solutions*. They contain K^+, 85 mM; EGTA, 7 mM; Mg^{2+}, 1 mM; $MgATP^{1-}$, 2 mM; Ca^{2+}, 10^{-8} to 10^{-3} M. Ionic strength is adjusted to 0.15 with imidazole propionate and pH adjusted to 7.0. The major anion is propionate. The amounts of the chemical constituents needed for the given ionic conditions were determined by computer from the ionic-equilibrium equations with binding constants from the literature (Kerrick & Donaldson, 1972). Calmodulin was prepared from bovine brain by the method of Wang and Desai (1977). Trifluoperazine, chlorpromazine and promethazine were gifts of Smith, Kline, and French Laboratories.

Protocol

Cell suspensions (1×10^7 cells/ml) were twice in our skinning solutions with Ca^{2+} in low concentration (1×10^{-8} M Ca^{2+}) at 0 °C. Aliquots of cells (2×10^6) were resuspended in 0.5 ml of different test solutions and incubated for various lengths of time as indicated in the Table. Samples of cells were then fixed in 2% paraformaldehyde. The fluorescent-labeled samples were examined with a Zeiss photomicroscope at 40× under an epi-illuminator. Cells were photographed on Kodak plus X film.

Criteria for Capping

Cells were scored as capped when most of the fluorescently labeled receptors formed a large aggregate at one pole of the cell surface, occupying less than half of it (as previously described by Schreiner & Unanue, 1976). Examples of capped cells are indicated by arrow in Fig. 1.

The fields scored for capping contained from 21 to 300 cells, and every cell was scored for capping. The number of fields varied, depending on the total number of cells in a field.

Table. The effects of Ca^{2+}, trifluoperazine (TFP), ATP, and calmodulin (CaM) on the percentage of cells capped[a]

Samples	Conditions	Percent cells capped
Cont	DME (incubation medium)	16
A	10^{-8} M Ca^{2+}, +2 mM ATP	9
B	10^{-4} M Ca^{2+}, +2 mM ATP	31
C	Sample B transferred to 10^{-8} M Ca^{2+}, +2 mM ATP for 20 min	12
D	Sample B transferred to 10^{-8} M Ca^{2+}, no ATP for 20 min	33
E	Sample D transferred to 10^{-8} M Ca^{2+}, +2 mM ATP for 20 min	11
F	10^{-4} M Ca^{2+}, no ATP	10
G	10^{-4} M Ca^{2+}, +2 mM ATP, +5 μM TFP	10
H	Sample G transferred to 10^{-4} M, Ca^{2+}, +5 μM calmodulin	38

[a] All incubations were 10 min at room temperature (21 °C) except where indicated

The minimum total number of cells counted was 150. In six experiments the largest standard error of the mean was 1.67% for $p\mathrm{Ca}=4.0$ and 0.9% for $p\mathrm{Ca}=8.0$ ($p\mathrm{Ca}=-\log_{10}[Ca^{2+}]$)

Results

Skinned Cells

Skinned lymphoma cells are cells which have been made permeable to ions and proteins. To show that the cells are permeable, the following experiment was carried out. A batch of cells was incubated in the culture medium for 10 min in the presence of 70 μM fluorescein-conjugated calmodulin. The cells were then fixed as described in the methods. Another set of cells from the same batch was first skinned in the skinning solution and then exposed to the same concentration of fluorescein-conjugated calmodulin for 10 min in the skinning solution and fixed. Figure 2 shows that cells treated with calmodulin in the culture medium did not bind calmodulin (Fig. 2*A*) but that the cells which had been exposed to the skinning solution did bind calmodulin (Fig. 2*B*). Therefore, this data in combination with the functional evidence that follows strongly supports our contention that the cells are permeable to ions and proteins.

Ca^{2+}-activated Capping

A batch of skinned cells suspended in a solution containing low Ca^{2+} ($p\mathrm{Ca}=8.0$), showed only 9% caps (Figs. 1*A* and Table Sample A). Transferring the cells from this solution to one with high Ca^{2+}

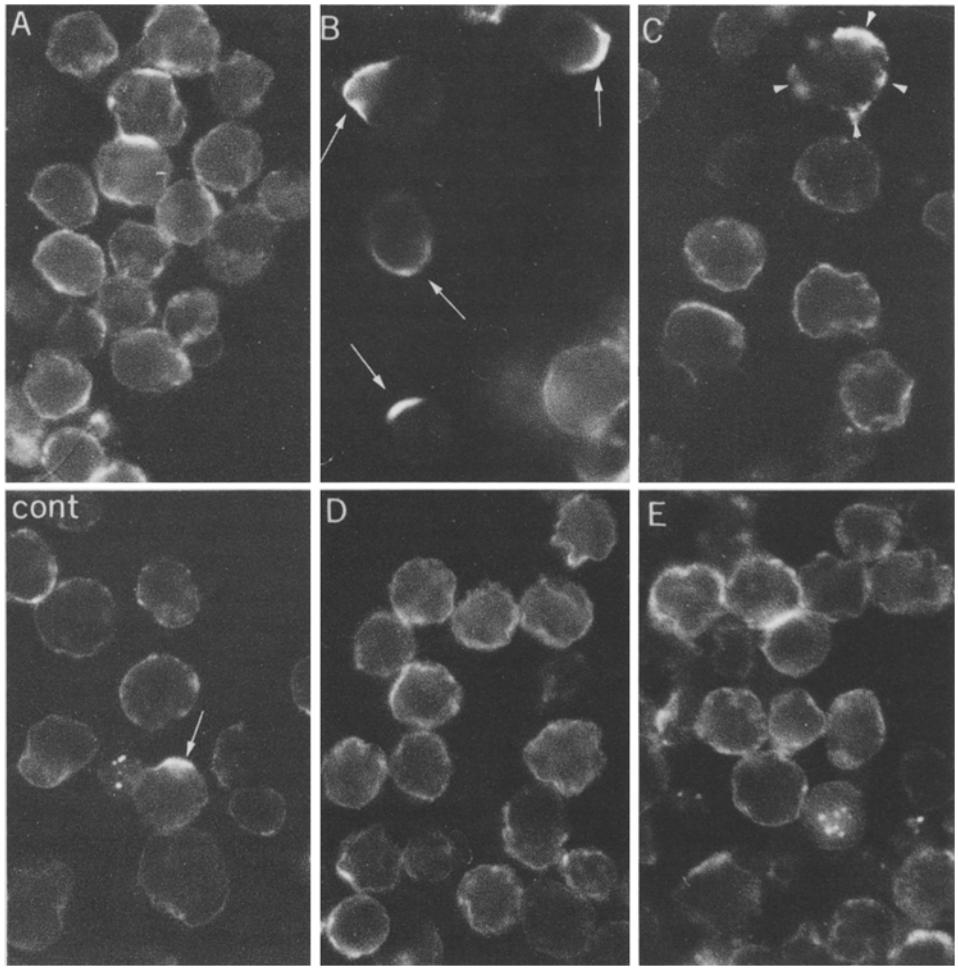

Fig. 1. Composite photograph showing the morphology and extent of cap formation. All solutions used in these experiments were as described in the methods except where noted in the legend. Photographs labeled *A, B,* and *C* represent a sequential exposure of cells to 10^{-8} M Ca^{2+} (10 min), 10^{-4} M Ca^{2+} (10 min), and finally to 10^{-8} M Ca^{2+} (20 min) again. *D* shows cells that had been exposed to solutions containing (pCa = 8.0) no ATP (for removal of ATP) (10 min) and then transferred to a solution containing 10^{-4} M Ca^{2+} and no ATP (10 min). *E* shows cells that had been washed in solutions with 10^{-8} M Ca^{2+} and 50 μM TFP and then transferred to one with 10^{-4} M Ca^{2+} (10 min) and 50 μM TFP. *Cont* shows nonskinned cells that had been incubated in DME medium for 10 min at 21 °C. (↑) indicates surface Con A capped structure; (▲) Shows the reversal of surface Con A structures into patches. (×1,300)

(pCa = 4.0) raised the number of capped cells to 31% (Fig. 1 *B* and Table Sample B). Resuspending the cells in a solution with low Ca^{2+} for 20 min resulted in a reversal of the number of cells capped to 12% (Figs. 1 *C*, and Table Sample C). The sequential procedure was replicated several times, and the results remained approximately the same. Ca^{2+}-activated capping reached its maximum level of 31% within 10 min of transfer of cells from 10^{-8} to 10^{-4} M Ca^{2+} (Fig. 3). Reversal of cap formation took considerably longer, reaching the near-base line capping level of 12% in 20 to 30 min (Fig. 3). In order to determine the concentration of Ca^{2+} which would activate capping, we suspended groups of cells from the same batch in solutions of different Ca^{2+} concentrations for 10 min, subsequently fixed them, and then measured the respective percentages of cells capped. Figure 4 shows that the percentage of caps was regulated by intracellular concentration of Ca^{2+}. Further, these results indicate activation of the capping process at Ca^{2+} concentrations comparable to those required for activation of contraction in smooth and striated muscles (Kerrick et al., 1981).

Role of Calmodulin

Figure 4 shows that addition of 5 μM calmodulin, at just threshold Ca^{2+} concentration (pCa = 6.0) required to activate capping, results in maximum

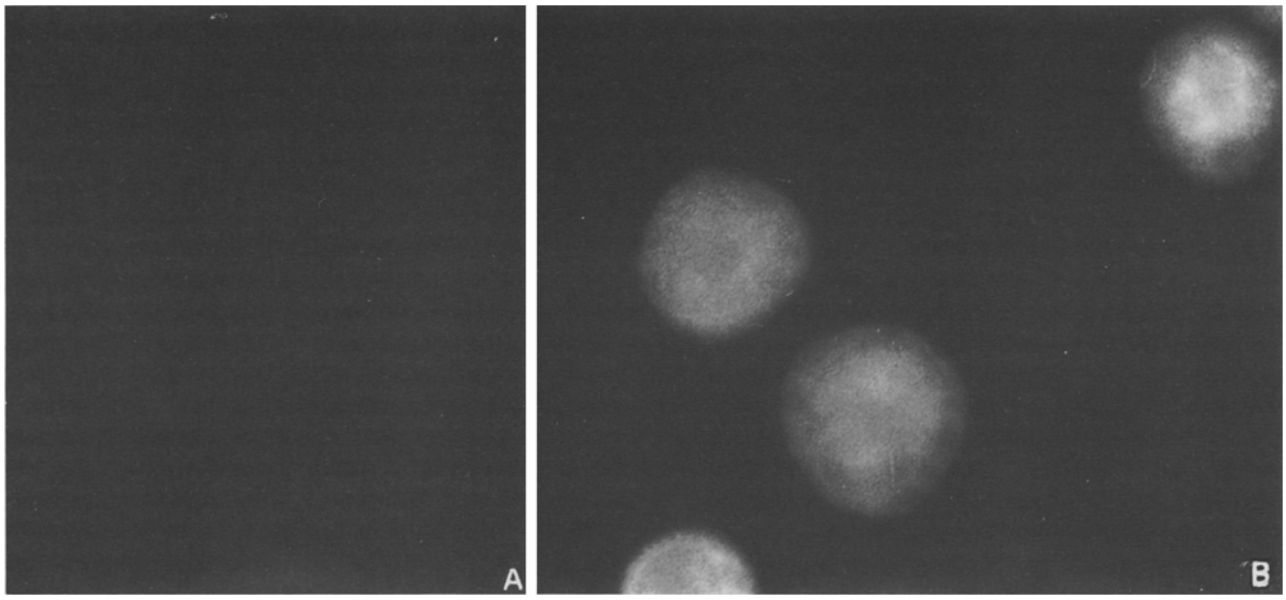

Fig. 2. Photographs showing epi-illumination of cells treated with 70 μM fluorescein-calmodulin (10 min) before and after a 20-min exposure of the cells to the skinning solution. Cells in the left panel show no fluorescent label, whereas cells in right panel are labeled. (×4,630)R

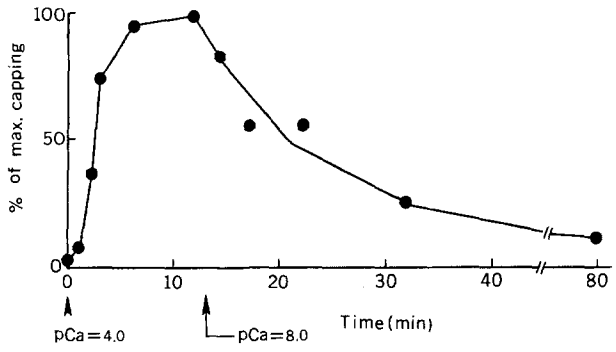

Fig. 3. The time course of capping in skinned mouse T-lymphocytes following exposure to solution with high Ca^{2+} (pCa = 4.0) and reversal of capping following resuspension of the cells in a solution with low Ca^{2+} concentration (pCa = 8.0)

capping but has no effect on capping at very low concentration of Ca^{2+} (pCa = 8.0). These data suggest that a Ca · calmodulin complex is required for the activation. To test this hypothesis we added increasing concentrations of calmodulin to the cells at pCa = 6.0. Figure 5 shows that increasing calmodulin concentration from 3.9×10^{-8}–6.3×10^{-7} M causes a graded increase in percent of cells capped.

Inhibitors of Calmodulin

Antipsychotic drugs such as trifluoperazine and chlorpromazine prevent capping from occurring in cells. It thus seemed possible that calmodulin is the site of action of these drugs. To test this possibility, three antipsychotic drugs (phenothiazines) known to inhibit calmodulin function at different concentrations were used (Weiss & Levin, 1978). Figure 6 shows that these drugs inhibit capping over the expected concentration range and order which they inhibit calmodulin activity. The antipsychotic drug haloperidol, which does not have an effect on calmodulin, has no effect on Ca^{2+}-activated capping Fig. 6. Cells previously inhibited by the phenothiazines in pCa = 4.0 reversibly cap when transfered to solutions with high Ca^{2+} (pCa = 4.0) and calmodulin (Table Sample H).

Role of ATP

It is well established that muscles contract and relax by the cyclic attachment of myosin cross-bridges to actin filaments and that the energy for this process is provided by ATP (Huxley, 1972); therefore, in the instance of lymphocytes no capping should occur in the absence of ATP. Transferring cells suspended in a solution containing low Ca^{2+} and no ATP to a solution containing high Ca^{2+} and no ATP, produced no increase in capping (Fig. 1 *D* and Table Sample F). Depletion of ATP from the myofilaments of muscle produces a noncyclic association of actin and myosin that prevents the sliding of actin and myosin filaments

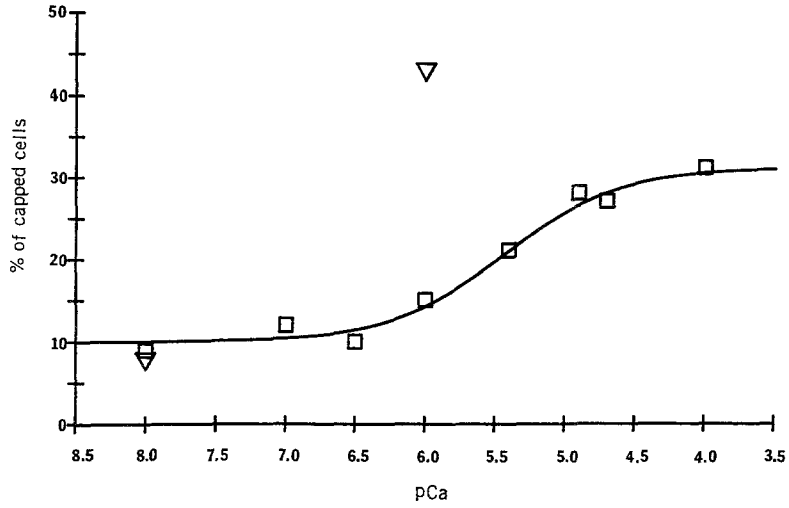

Fig. 4. Relationship between percentage of total cells capped and Ca^{2+} concentration. Solutions were as described in the Methods. (□) represents data with no added calmodulin; (▽) represents data in the presence of 5 μM calmodulin

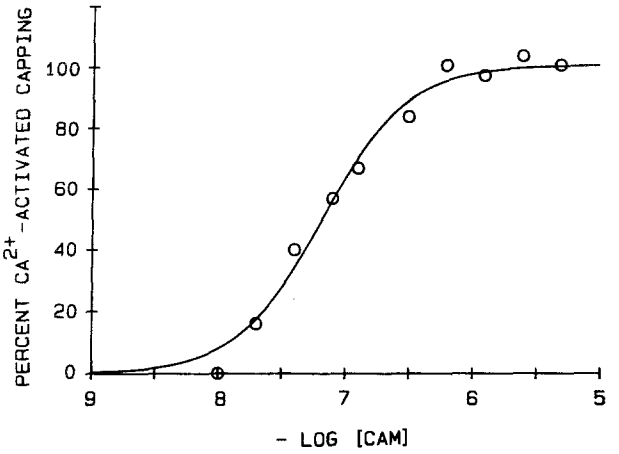

Fig. 5. Relationship between percentage of total cells capped and exogenous calmodulin concentration at (pCa = 6.0). Ca^{2+}-activated capping was normalized to 100% by substracting the percentage of cells capped at pCa = 8.0 from the percentage of cells capped in the test solution and dividing by the difference in the percentage of cells capped between pCa = 8.0 and pCa = 4.0

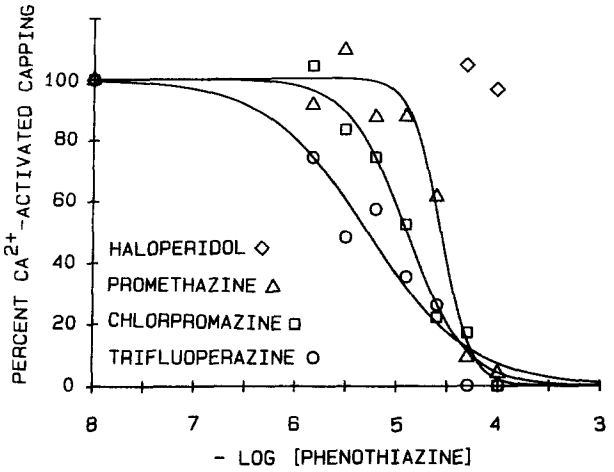

Fig. 6. Relationship between maximal Ca^{2+}-activated capping and inhibitors of calmodulin (phenothiazines). Cells were first treated with the phenothiazines for 10 min at pCa = 8.0 and then transfered to solutions containing phenothiazines at the same concentration and high Ca^{2+}-activated capping was normalized to 100% by substracting the percentage of cells capped at pCa = 8.0 from the percentage of cells capped in the test solution and dividing by the difference in the percentage of cells capped between pCa = 8.0 and pCa = 4.0

(rigor state) even in the absence of Ca^{2+} (Huxley, 1972). If the reversibility of capping that occurs when Ca^{2+} is withdrawn from the lymphoma cells is a result of the dissociation of actin and myosin by ATP, then removal of ATP from the solutions during capping should cause a noncyclic association between actin and myosin and prevent reversibility of capping in the absence of Ca^{2+}. When we transferred capped cells to a solution containing high Ca^{2+} and no ATP to produce such a rigor-like state and then transfered the cells to a solution without either Ca^{2+} or ATP, capping was not reversible (Table Sample D); but when we resuspended the cells in a solution containing ATP and no Ca^{2+} dissociation occurred (Table Sample E). That ATP is required for cap formation and dissociation strongly supports the hypothesis of a mechanism analogous to the sliding-filament mechanism for muscle contraction and relaxation.

Discussion

The results reported in this paper provide direct functional evidence that in cap formation and dissociation intracellular Ca^{2+} concentration, ATP, and calmodulin play definite roles.

Ca^{2+}-Activated Capping

Little information exists suggesting that Con A capping in lymphoma cells is a Ca^{2+} calmodulin-activated process. Evidence in favor is the following: (i) Drugs known to inhibit the activity of the Ca^{2+}-binding protein calmodulin (chlorpromazine and trifluoperazine) inhibit or reverse cap formation, and this inhibition can be partially overcome by adding Ca^{2+} to the extracellular medium (Schreiner & Unanue, 1976; Braun et al., 1978; Bourguignon & Balazovich, 1980). (ii) Calmodulin has also been located under the Con A cap structure (Salisbury et al., 1981). Our data with regard to Ca^{2+} calmodulin activation of Con A capping is also in agreement with the following results for Ig capping. For example, the formation of the Ig cap was shown to be sensitive to the phenothiazines and closely associated with intracellular calmodulin (Nelson, Andrews & Karnovsky, 1982). Thus, the involvement of Ca^{2+} and calmodulin during Ig capping is also implicated.

There are apparent discrepancies which still exists against Ca^{2+} being involved in anti-Ig induced capping (Poste & Nicolson, 1976; Schreiner & Unanue, 1976; Pozzan et al., 1982). These discrepancies may be explained as follows: (i) The fact that removal of extracellular calcium does not prevent capping from occurring (Schreiner & Unanue, 1976) may be due to Ca^{2+} released from intracellular stores. (ii) The reason that Ca^{2+} does not facilitate Ig capping (Poste & Nicolson, 1976) in the presence of an ionophore may be because the anti-Ig treatment induced the maximum accumulation of Ca^{2+} required for activation and a further increase in Ca^{2+} with an ionophore could not activate further. (iii) The fact that anti-Ig capping follows a transient increase in intracellular Ca^{2+} (Pozzan et al., 1982) may not be an argument against Ca^{2+} involvement. In smooth muscle Ca^{2+} transients always precede contraction and reach near baseline levels before contraction begins (Fay, Shlevin, Granger & Taylor 1979). (iii) The reversal of Ig caps by an ionophore in the presence of Ca^{2+} (Schreiner & Unanue, 1976) could be because too high an ionophore concentration (10^{-7}–10^{-6} M) acts as a nonspecific detergent and may dissociate the caps because the membrane has been disrupted (Bourguignon & Pressman, 1983). The discrepancies may also be due to different processes between Con A and anti-Ig capping.

It is important to note that capping is activated by Ca^{2+} over the same range (Fig. 4) of Ca^{2+} required for activation of muscle contraction in skinned skeletal and smooth muscle fibers (Kerrick & Krasner, 1975; Hoar et al., 1979). The time course for Ca^{2+}-sensitive activation and dissociation of capping is similar to that observed for smooth muscle (Hoar et al., 1979; Kerrick et al., 1981). These similarities in activation properties suggest that they may both be regulated by a similar mechanism.

Ca^{2+}-Calmodulin

The evidence reported here strongly supports the conclusion that it is a Ca^{2+}-calmodulin complex that is responsible for Ca^{2+}-activated capping. Increasing concentrations of calmodulin at a Ca^{2+} concentration (pCa = 6.0), which is just threshold for activation, increased capping to its maximal value (Fig. 5). Since Ca^{2+} was kept at a constant value, adding calmodulin had the effect of adding a Ca^{2+}-calmodulin complex. This was not just a calmodulin effect since adding high concentration of calmodulin (5.0 μM) at 10^{-8} M Ca^{2+} did not have an effect. Although the concentration of calmodulin required for half maximal activation of capping (Fig. 5) is 2–60 times higher than that required for activation of myosin light-chain kinase (Adelstein & Klee, 1981; Crouch et al., 1981) this can be accounted for by the fact that only a small fraction of Ca^{2+} is bound to the total calmodulin (Crouch & Klee, 1980) at pCa = 6.0.

Inhibition of capping by the phenothiazines over the expected concentration range for inhibition of calmodulin activity (Weiss & Levin, 1978) also supports our contention that a Ca^{2+}-calmodulin complex is responsible for the activation of capping (Fig. 6). This conclusion is also supported by the observation that these drugs in the same concentration range inhibit cap formation in intact lymphocytes (Nelson et al., 1982). Like skinned lymphocytes, Ca^{2+}-activation of skinned smooth muscle fibers are inhibited by the same three phenothazines in the same order they bind to calmodulin (Cassidy et al., 1980; Kerrick, Hoar & Cassidy, 1980). This inhibition cannot be due to nonspecific effects since in skinned skeletal muscle fibers which are not regulated by calmodulin the same concentration of phenothiazines has no effect upon Ca^{2+}-activated tension (Kerrick et al., 1981). Skinned smooth muscle has been shown to be regulated by a Ca^{2+}-calmodulin-activated myosin light-chain kinase/phosphatase system (Kerrick, 1981). Calmodulin has also been shown to be localized in the cap structure (Salisbury et al., 1981). In mouse T-lymphocytes our data suggest Ca^{2+} in-

volvement in capping but does not rule out the possibility of involvement of other Ca^{2+} control mechanisms.

Role of ATP

As mentioned earlier an actomyosin sliding filament mechanism has been suggested as a possible mechanism for aggregating receptors into cap structures (Bourguignon & Singer, 1977). During such an event, bipolar myosin filaments would bridge F-actin filaments from adjacent patches, enabling the collection of patches into a cap by the cyclic interaction of myosin cross-bridges with actin and the sliding of actomyosin filaments. In muscle ATP is required for the cyclic interaction of myosin and actin and for contraction to occur (Huxley, 1972). The Table shows that lymphocyte capping also requires ATP. In muscle removal of ATP in the presence or absence of Ca^{2+} results in a noncyclic interaction of actin and myosin and prevents contraction or relaxation of muscle (rigor state) (Huxley, 1972). This state can only be overcome by the re-addition of ATP. Like muscle relaxation, lymphocyte cap dissociation is prevented in the absence of ATP (Table Sample D). This unique interaction between capping and ATP is consistent with the hypothesis that capping is the result of actomyosin interaction.

Skinned Lymphocytes

It is known that the skinning solutions used in this study make muscle cells permeable (Kerrick & Krasner, 1975; Hoar et al., 1979). These same solutions render mouse T-lymphoma cells permeable to ions and proteins for the following reasons: (i) In intact cells (cells not treated with skinning solutions) extracellular Ca^{2+} has no effect on capping (Schreiner & Unanue, 1976), whereas cells in our skinning solutions responded to Ca^{2+}. (ii) It is known that ATP does not freely cross the cell membrane and its absence from the culture medium has no effect on capping, yet in our solutions it was an absolute requirement for capping. (iii) Calmodulin is known to be an intracellular protein (Klee, Crouch & Richman, 1980) and not to act extracellularly, yet in our experiments calmodulin exerted an effect only when the cells have been made permeable with our skinning solutions and in the presence of Ca^{2+}. (iv) Finally, direct observation of fluorescein-conjugated calmodulin in the cells was only observed when the cells had been skinned (Fig. 2). Thus direct as well as functional evidence support our contention that the cells are permeable to both ions and small proteins like calmodulin.

In summary, the data presented in this paper show that capping is Ca^{2+}-activated, reversible, dependent on ATP as an energy source, and affected by calmodulin. These results are consistent with a well-known model for Ca^{2+}-activation, consisting of a Ca^{2+}-calmodulin-activated myosin light-chain kinase/phosphatase system that regulates the interaction of actomyosin filaments. In this example the interaction is associated with muscle tension and relaxation; in ours, it is responsible for receptor cap formation and dissociation.

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