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THE PARTICIPATION OF ADENYLATE CYCLASE IN LYMPHOCYTE CAPPING

LILLY Y.W. BOURGUIGNON * and YUE-CHANE HSING **

Department of Anatomy and Cell Biology, School of Medicine, University of Miami, Miami, FL 33101 (U.S.A.)

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In this study, we have observed that cells increase their intracellular cAMP to relatively high levels during receptor capping induced by either ligand-dependent (anti-Thy-1 antibody) or ligand-independent (colchicine) treatment. In addition, we have found that under capping conditions, membrane-bound adenylate cyclase is induced to co-cap with independent membrane molecules such as Thy-1 antigens. These findings suggest that the binding of anti-Thy-1 to its receptors or treatment with colchicine induces the molecular reorganization of membrane-bound adenylate cyclase which may be responsible for activating the contractile machinery required for the collection of surface receptors into a cap structure.

Introduction

Redistribution of lymphocyte surface receptors, so-called capping, is known to be induced either with or without the addition of external ligands. In the case of ligand-dependent capping, the ligand (e.g., antibodies against specific surface receptors or lectins) and its receptors appear to be clustered by a cross-linking event at the cell surface that is highly dependent on the valency of the ligand [1–5]. During ligand-independent capping, a number of reagents such as colchicine, hypertonic media, mitogens, trypsin and cholinergic drug each induce surface receptors to form cap structures in the absence of any externally added ligand [6–9]. The molecular mechnisms underlying both ligand-dependent and ligand-independent capping are not yet understood. However, it is now generally agreed that actin and myosin participate in the redistribution of surface receptors, as demonstrated by the preferential accumulation of both actin and myosin directly underneath the receptor cap structures induced by the two different treatments [6,10–13]. In this study, we have begun to explore the regulatory mechanism(s) which may be responsible for activating the contractile machinery which is needed for both antibody- (ligand-dependent) and colchicine- (ligand-independent) induced capping events.

Cyclic adenosine monophosphate (cAMP) has been implicated as an important regulatory molecule in a variety of cellular events [14,15]. Recently, we have observed that agents known to elevate intracellular levels of cAMP, such as N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid (dibutyryl cAMP) and theophylline, cause a remarkable stimulation of lymphocyte receptor capping phenomenon [16,17]. Using radioimmunoassay we have observed that cells increase their intracellular cAMP to relatively high levels during receptor capping induced by either ligand-dependent (e.g., anti-Thy-1 antibody) or ligand-independent (e.g., colchicine) treatment. Since this observation implies that cAMP is involved in the receptor capping process, we have examined the

* To whom reprints request should be addressed.
** Present address: Department of Microbiology and Immunology National Yang-Ming Medical School, Taipei, Taiwan, China.

distribution of adenylate cyclase during surface receptor capping.

Material and Methods

Mouse T-lymphocytes including T-lymphoma cells, thymocytes and splenic T-lymphocytes were used in all experiments. Mouse T-lymphoma cells (BW5147, an AKR/J lymphoma line obtained from Dr. R. Hyman, The Salk Institute) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (GIBCO) at 37°C in 5% CO_2/95% air. Mouse thymocytes and splenic T-lymphocytes [11] were obtained from the thymus and spleen of C57BL/6J strain mice.

Induction of receptor capping was carried out as follows. (1) Ligand-dependent capping: unfixed cell suspensions ($1 \cdot 10^7$ cells/ml) were treated with primary antibody (monoclonal rat anti-Thy-1, generously provided by Dr. I. Trowbridge, The Salk Institute) at 0°C for 30 min, followed by secondary antibody (fluorescein-conjugated rabbit anti-rat immunoglobin, Miles) at 37°C for 15 min. The capped cells were then fixed with 1% paraformaldehyde. (2) Ligand-independent capping: unfixed cell suspensions ($1 \cdot 10^7$ cells/ml) were treated with 0.1 mM colchicine (Sigma) at 37°C for 15 min. Cells were then fixed with 1% paraformaldehyde and labeled with primary antibody (monoclonal rat anti-Thy-1) plus secondary antibody (fluorescein-conjugated rabbit anti-rat immunoglobulin). In order to demonstrate the initial distribution of surface molecules, cells were prefixed with 1% paraformaldehyde and then treated with rat anti-Thy-1 antibody and fluorescein-conjugated rabbit anti-rat immunoglobulin as described above.

For the histochemical localization of membrane-bound adenylate cyclase, a modified enzyme cytochemical staining method [18–22] was used. Cell suspensions, either uncapped cells (prefixed condition) or capped cells (with either antibody or colchicine treatments), were fixed with 1% paraformaldehyde in 0.15 M NaCl for 5 min at 4°C. After rinsing with 0.15 M NaCl twice, cells were incubated with histochemical buffer comprising 8% sucrose/2 mM theophylline/10 mM NaF/2 mM $MgSO_4$/80 mM Tris-malate (pH 7.2)/4 mM $Pb(NO_3)_2$/1 mM 5′-adenylyl imidodiphosphate (Ado*PP*[NH]*P*) (Sigma) for 80 min at 37°C. Control experiments, in which cells were incubated in the complete histochemical reaction buffer except for the omission of substrate, Ado*PP*[NH]*P*, or in which cell suspensions were heated to 70°C for 15 min after fixation but before incubation in the histochemical reaction medium. Finally, 1% ammonium sulfide was added for 1 min at room temperature and washed with 0.15 M NaCl. The cells were examined and photographed with a Zeiss microscope equipped with epifluorescent illumination and filter setting for both bright field and fluorescein fluorescence.

Results

Our results indicate that Thy-1 and adenylate cyclase are evenly distributed either uniformly or in small clusters over the cell surface of untreated (prefixed) cells (Figs. 1a, c and e; 2a and c). Upon treatment of the cells with either antibody against Thy-1 molecules (ligand-dependent process) or colchicine (ligand-independent process), the immunofluorescence staining indicates that surface Thy-1 molecules are induced to form cap structures (Figs. 1b, d and f) as described previously [6,10,23]. We now report that membrane-bound adenylate cyclase is also aggregated into cap-like structures (Figs. 2b and d; 3a and b). We believe our adenylate cyclase staining procedures are specific for the following reasons: (1) omission of substrate, Ado*PP*[NH]*P* or (2) heating the cell suspension to 70°C results in the elimination of reaction products (Figs. 4a and b). During both ligand-dependent and -independent capping using both immunofluorescence and the histochemical staining methods, we have found that Thy-1 antigens and adenylate cyclase co-cap on the surface of T-lymphocytes (Figs. 5a and b). These findings suggest that the continuous production of relatively high levels of intracellular cAMP by membrane-bound adenylate cyclase may be required in the capping process.

Previously, we have reported that there is a close association between intracellular contractile proteins and surface Thy-1 cap structure [6,10]. We have also found that the 20 kDa myosin light chain is both phosphorylated and preferentially

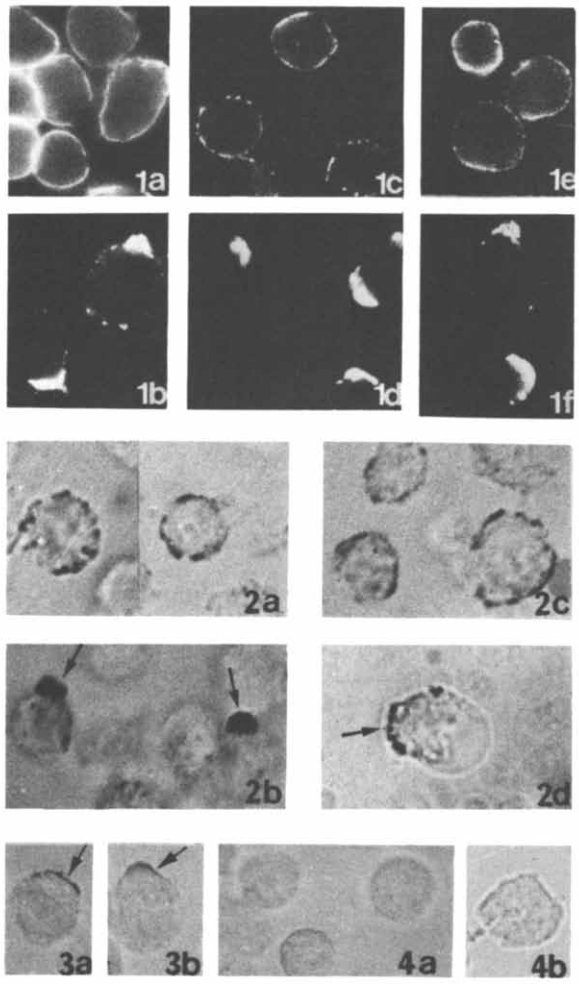

Fig. 1. Redistribution of Thy-1 antigens on the surface of mouse T-lymphoma. Prefixed cells stained with rat anti-Thy-1 antibody plus fluorescein-conjugated rabbit anti-rat immunoglobulin (a, c, e). Unfixed cells were either treated with rat anti-Thy-1 followed by fluorescein-conjugated rabbit anti-rat immunoglobulin or treated with 0.1 mM colchicine, fixed with 1% paraformaldehyde and stained with rat anti-Thy-1 plus fluorescein-conjugated rabbit anti-rat immunoglobulin (b, d, f). a, b: mouse T-lymphoma cells; c, d: mouse thymocytes; e, f: mouse T-lymphocytes.

Figs. 2–4. Redistribution of adenylate cyclase on the surface of mouse T-lymphocytes. Prefixed cells were processed through histochemical staining for adenylate cyclase (2a and c). Unfixed cells were either treated with rat anti-Thy-1 followed by rabbit anti-rat immunoglobulin or treated with 0.1 mM colchicine followed by 1% paraformaldehyde and histochemical staining for adenylate cyclase in the presence (2b, d; 3a, b) or in the absence (4a, b) of substrate Ado*PP*[NH]*P*. 2a–d and 4b: mouse T-lymphoma cells; 3a: mouse thymocytes; 3b and 4a: mouse T-lymphocytes. Magnifications for Figs. 1–4: ×1000.

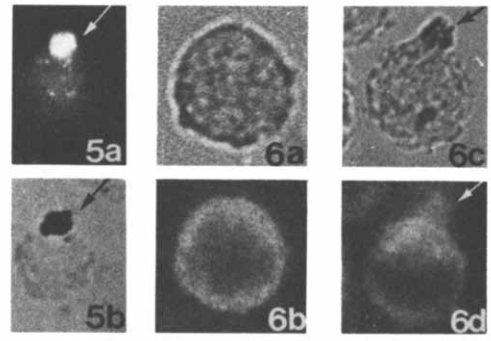

Fig. 5. Simultaneous localization of Thy-1 antigens and adenylate cyclase using immunofluorescence and histochemical staining method. Unfixed mouse T-lymphoma cells were treated with either anti-Thy-1 antibodies or colchicine as described in Fig. 1b to induce Thy-1 capping (a). Subsequently, Thy-1 capped cells were processed through histochemical staining for adenylate cyclase (b). a: immunofluorescence staining for Thy-1 cap. b: histochemical staining for adenylate cyclase cap. Magnifications: ×1000.

Fig. 6. Localization of myosin (or actin) on sections of mouse T-lymphoma cells that had been treated with anti-Thy-1 antibodies or colchicine under uncapped and capped conditions. a, b. Prefixed cells were stained histochemically for adenylate cyclase (a) and labeled immunofluorescently for intracellular myosin (or actin) (b). c, d. Under capping condition (e.g., anti-Thy-1 antibody or colchicine treatment), histochemical staining for adenylate cyclase (c) and immunofluorescence labeling for intracellular myosin (or actin) (d). Magnifications were all ×1500.

accumulated in the plasma membrane of lymphocyte during capping [6]. In this study, cells treated with Thy-1 antibody or colchicine were processed through histochemical reactions required for localizing adenylate cyclase as described in Figs. 2 and 3. Subsequently, these histochemically labeled cells were sectioned and stained with fluorescein-conjugated rabbit anti-human uterus smooth muscle myosin [24] to locate intracellular myosin [6,10–13] using frozen sectioning procedures (infusing cells with 1.0 M sucrose, freezing and sectioning in the frozen state ($-30°C$) to a thickness of approx. 1 μm as described previously [11]). The immunofluorescence data show that in the absence of any treatment, intracellular actin and myosin are distributed uniformly throughout the cytoplasm, while adenylate cyclase is arranged evenly on the cell surface (Figs. 6a and b). However, in the

presence of antibody or colchicine, there is a large accumulation of actin and myosin directly beneath the adenylate cyclase cap formation (Figs. 6c and d). This observation suggests that there may be a close relationship between the accumulation of actin-myosin and the rearrangement of membrane-bound adenylate cyclase during both antibody and colchicine treatment.

Discussion

Previously, the term 'lateral membrane protein interactions' has been used to describe the so-called co-capping phenomenon in which two surface proteins are observed to redistribute together [25–28]. For example, in mouse T-lymphoma cells, we have reported that upon the treatment of cells with anti-H-2 antibody a variety of molecularly independent cell surface components such as Thy-1, T-200, gp69/71 and TL antigens co-cap with H-2 antigen [23]. Most recently, we have shown that colchine induces the co-capping of at least three different T-lymphoma surface antigens (e.g., Thy-1, T-200 and gp69/71) in the absence of any external ligand [6]. All the aforementioned studies with regard to co-capping of any two surface molecules have been carried out with double immunofluorescence techniques [6,19,25–28]. Considerably less effort has been devoted to studying the co-capping of fluorescence-labeled antigens and membrane-bound enzymes during either antibody or colchicine treatments. Recently, it has been reported that two membrane-associated enzymes, 5′-nucleotidase and ATPase, apparently co-cap with surface immunoglobulin in B-lymphocytes [29]. However, the distribution of membrane-bound adenylate cyclase during lymphocyte capping was not examined. In this work, we have shown that during anti-Thy-1 antibody or colchicine treatment, adenylate cyclase is clearly induced to co-cap with Thy-1 molecules (Figs. 5a and b).

The simultaneous rearrangement of two molecularly independent membrane molecules, such as Thy-1 and adenylate cyclase, implies that there may be a common regulatory mechanism responsible for the collection of the many diverse, independent surface proteins into a cap. Furthermore, the fact that the intracellular cAMP concentration increases as early as 5 min after the addition of either antibody or colchicine to the cells (Table I) strongly suggests that cAMP is involved in the regulatory mechanism of receptor capping. These ideas are supported by the fact that cAMP itself has been found concentrated preferentially underneath anti-Ig induced surface Ig capping in B-lymphocytes [30]. The rearrangement of adenylate cyclase which takes place during both ligand-dependent and ligand-independent capping suggests that the membrane-bound enzyme may be activated by either the binding of ligand or by the colchicine treatment. Subsequently, the elevated

TABLE I

RADIOIMMUNOASSAY OF INTRACELLULAR cAMP DURING LIGAND-DEPENDENT AND LIGAND-INDEPENDENT CAPPING

Samples of $1 \cdot 10^6$ cells were chosen at various time intervals during the receptor capping response ($t = 0$, 0.5, 5, 15 min) induced by either antibody or colchicine, frozen immediately in liquid nitrogen, thawed at room temperature, and solubilized in 6% trichloroacetic acid. Diethyl ether-extracted residues were then assayed in duplicate for their cAMP content using the acetylation modification [36,37] of the Steiner radioimmunoassay [38] with ^{125}I-labeled cAMP (Collaborative Res. Inc.). For each experiment, a standard curve was generated using unlabeled cAMP ranging in concentration from 2.5 to 1000 pmol. All samples were counted using a Searle 1185 automatic gamma counter. Relative cAMP levels for unknown samples were extrapolated from the standard curve.

Experimental conditions	cAMP accumulation (% of control)
Ligand-dependent:	
0 min (no treatment, 37°C) [a]	100 ± 6 [a]
0.5 min (rat anti-Thy-1 plus rabbit anti-rat immunoglobulin, 37°C) [b]	100 ± 8 [b]
5 min (rat anti-Thy-1 plus rabbit anti-rat immunoglobulin, 37°C) [b]	201 ± 6 [b]
15 min (rat anti-Thy-1 plus rabbit anti-rat immunoglobulin, 37°C) [b]	224 ± 3 [b]
Ligand-independent:	
0 min (no treatment, 37°C)	100 ± 5 [a]
5 min (colchicine, 37°C)	120 ± 6
15 min (colchicine, 37°C)	150 ± 3

[a] The amount of cAMP formed under the 'no treatment' (37°C) condition was approx. 20 pmol per 10^6 cells.

[b] The time quoted for the antibody-induced capping measured from the addition of second antibody (rabbit anti-rat immunoglobulin).

levels of cAMP may then cause the activation of a cAMP-dependent protein kinase [31]. It is speculated that this cAMP-dependent kinase could phosphorylate the appropriate cytoskeleton proteins and related regulatory molecules (e.g., microtubules, intermediate filaments, myosin light chain kinase, etc.); plus membrane proteins or other metabolically important cytoplasmic soluble proteins [6,32–35]. It is conceivable that the cAMP-mediated phosphorylation of the specific membrane proteins and cytoskeleton proteins could then induce the association of actomyosin filaments with certain receptors and thereby trigger the collection of the surface receptors into a cap structure via a sliding filament mechanism analogous to muscle contraction [10]. The identification and characterization of such cAMP-dependent biochemical events are currently under investigation.

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