ANTAGONISM BY DIBUTYRYL ADENOSINE CYCLIC 3′,5′-MONOPHOSPHATE AND TESTOLOLACTONE OF CONCANAVALIN A CAPPING

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ABSTRACT

Exposure of CHO-K1 cells in vitro to dibutyryl adenosine cyclic 3′,5′-monophosphate (DBcAMP) plus testololactone produces a rapid, reversible antagonism of ligand-induced collection of initially dispersed concanavalin A (Con A) binding sites into a caplike mass. Morphologically, as Con A capping occurs, the cells become less spread and then round completely. With prolonged Con A exposure, cells cultured in either the absence or the presence of DBcAMP plus testololactone cap and round. Capping is blocked by cold treatment and respiratory inhibitors. Colcemid at concentrations >1 μM promotes both Con A capping and cell rounding. Cytochalasin B at similar concentrations inhibits both capping and cell rounding. Treatment of cells with Con A has little effect on intracellular cAMP concentration. Possible mechanisms by which cAMP may modulate the movement of Con A binding sites are discussed.

Treatment of Chinese hamster ovary cells in vitro with 3', 5'-cyclic AMP (cAMP) analogues such as dibutyryl cyclic AMP (DBcAMP) or 8-bromo-cAMP, either alone or in synergistic combination with testosterone, theophylline, or other specific agents, converts cells from an epithelial-like to a fibroblast-like morphology (10–12, 20, 21).[1] This conversion is preceded by a rapid, reversible change in cell surface properties (10, 28).[1] Cells cultured on solid substrate in the presence of DBcAMP plus testosterone become rapidly resistant to rounding by removal of divalent cations (28) or brief exposure to the lectin, concanavalin A (10, 28). Basically similar observations have been reported with other cell lines (15, 22, 34), suggesting that cAMP may be an important general agent in regulating the surface properties of mammalian cells.

The differential response of Chinese hamster ovary cells grown in the absence or presence of DBcAMP plus testosterone to the rounding action of concanavalin A (Con A) is not absolute (28). After a time lag suggestive of an autocatalytic or cooperative process, cells treated with DBcAMP plus testosterone also round. The amount of Con A bound to cells cultured in the absence or presence of DBcAMP is equal (29). The process of Con A cell rounding is temperature dependent and blocked by inhibitors of cellular respiration such as azide, cyanide, or iodoacetate. One known cooperative interaction of multivalent lectins or antibodies with a similar dependence on temperature

[1] Storrie, B., L. Wenger, and T. T. Puck, 1975. The role of butyrate in the reverse transformation reaction in mammalian cells. Submitted for publication.

and respiration is the phenomenon of ligand-induced cap formation (9, 13, 30, 36).

In the present work, the distribution of Con A bound to Chinese hamster ovary cells cultured in the absence or presence of DBcAMP plus testololactone, a testosterone derivative, has been investigated. An association between differential Con A capping and cell rounding has been found.

MATERIALS AND METHODS

The Chinese hamster ovary cell clone CHO-K1 (16) was used in all experiments. Cells were routinely cultured on solid substrate as described previously (28). Unless otherwise indicated, cells were normally exposed to DBcAMP plus testololactone at a concentration of 0.3 mM and 33 μM respectively, for 16–20 h after plating. Cells were treated with Con A, and rounded cells were scored as described (28).

For assay of the distribution of fluorescent Con A bound to cells, 1×10^4 cells were plated on 22-mm square glass coverslips in the absence or presence of DBcAMP plus testololactone and challenged 16–20 h later by addition of fluorescein isothiocyanate conjugated Con A (fl-Con A, Yeda Research and Development Co., Ltd., Rehovoth, Israel) at a concentration of 40 μg/ml (unless otherwise indicated) to the preexisting serum-containing medium. Cells were incubated with fl-con A for 30 min at 37°C in a humidified CO_2 incubator (unless otherwise indicated). Incubations were terminated by rinsing the coverslips briefly three times in phosphate-buffered saline (5) containing DBcAMP plus testololactone as appropriate, at room temperature, followed by fixation for two min in 10% Carnoy's fixative. Coverslips were mounted in Elvanol (E. I. du Pont de Nemours & Co. Inc., Wilmington, Del.) and observed with a Leitz Orthoplan microscope fitted with a vertical Ploem illuminator. Between 100 and 120 cells were scored to assay frequency of Con A capped or rounded cells. The specificity of fl-Con A binding was tested by pretreating cultures with α-methylmannopyranoside (26) at a concentration of 0.05 M. Staining is inhibited completely by α-methylmannopyranoside at this concentration.

In experiments to test the effect of temperature on fl-Con A capping, cultures were preincubated for 5 min with cooled medium (2°C) minus or plus DBcAMP and testololactone and with 20 mM Hepes buffer (pH 7.2) added to control medium pH outside the CO_2 incubator. Cultures were then incubated with either 40 μg/ml or 100 μg/ml fl-con A (to compensate for the decreased Con A binding at 2°C [29]). In inhibitor experiments, cultures were pretreated for 30 min with agent(s) before Con A addition. In some experiments, cells were fixed in freshly prepared 1% formaldehyde in phosphate-buffered saline for 10 min at 37°C (32) before exposure to fl-Con A.

Electron microscope visualization of Con A bound to glutaraldehyde-prefixed cells was carried out by slight modifications of the peroxidase-diaminobenzidine procedure (17). In brief, 8×10^5 cells were plated per 60-mm plastic petri dish (Lux Scientific Corporation, Thousand Oak, Calif.) in the absence or presence of DBcAMP plus testololactone as appropriate, then rinsed, and exposed to 40 μg/ml Con A for 30 min at 37°C in serum-containing medium. After a second fixation, peroxidase was added, and the culture was rinsed, and treated with diaminobenzidine. Cells were post-fixed with 1% osmium tetroxide in 0.1 M collidine buffer for 1 h at 22°C, dehydrated, and detached from the petri dish in sheets with amyl acetate (23). Pellets were embedded in Epon 812 (Shell Chemical Co., New York, N. Y.) and gold-to-silver sections were cut with a diamond knife. Sections were examined with a Philips 200 electron microscope set at 60 kV. Peroxidase-diaminobenzidine staining was completely prevented by the addition of 0.05 M α-methylmannopyranoside during Con A and peroxidase binding.

Intracellular cAMP levels were measured by the protein kinase binding assay as described previously[1]. Parallel cultures were fixed and stained for assay of rounded cells as described previously (28). Cell number was assayed with a Coulter counter. For total cell counts of cultures incubated with Con A for several h, cells were detached by 0.25% trypsin after exposing cultures to 0.05 M α-methylmannopyranoside for 15 min at 37°C in serum-containing media.

RESULTS

Effect of DBcAMP plus Testololactone on Con A Distribution

When CHO-K1 cells grown on solid substrate in the absence or presence of 0.3 mM DBcAMP plus 33 μM testololactone are fixed and then incubated with 40 μg/ml Con A at 37°C, the distribution of fl-Con A bound to cells under either culture regime is identical. Prefixed cells of either epithelial-like or fibroblast-like morphology treated with fl-Con A stain diffusely and, essentially, uniformly over the entire cell under both culture conditions (Fig. 1 A,B). Cells prefixed with glutaraldehyde and stained by the peroxidase-diaminobenzidine method for bound Con A show a correspondingly uniform distribution of Con A, as indicated by the electron-dense deposit, about the cell periphery (Fig. 1 C,D).

If, however, cells cultured in basal medium or medium supplemented with DBcAMP plus testololactone are incubated with Con A before fixation, the distribution of Con A bound to either cell population during brief Con A exposures is markedly dissimilar (Fig. 2). After a 30-min exposure to fl-con A, a high proportion of cells in

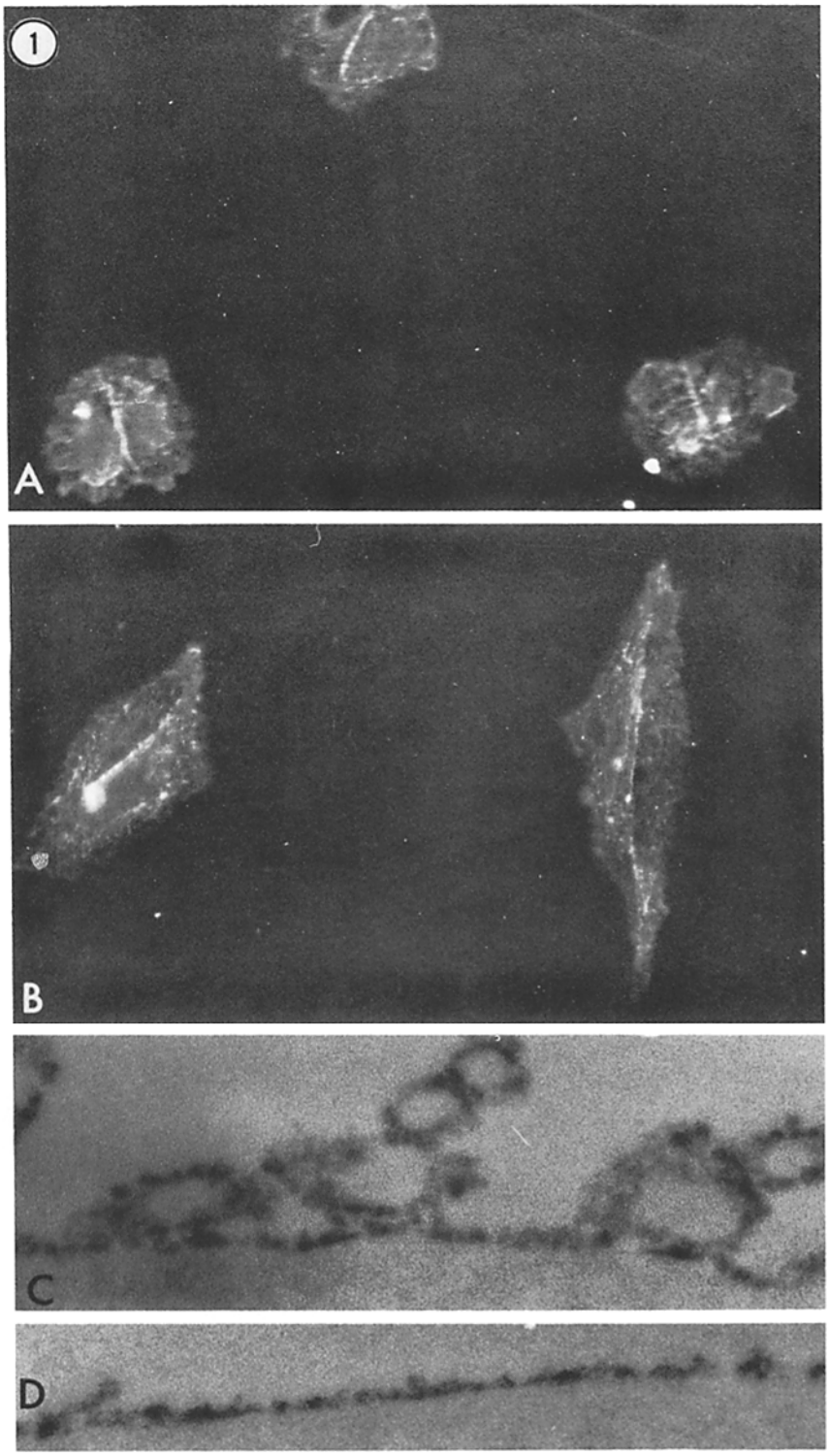

FIGURE 1 Distribution of Con A bound to formaldehyde-prefixed (A,B) or glutaraldehyde-prefixed (C,D) cells cultured in the absence (A,C) or presence (B,D) of DBcAMP plus testololactone. (A,B) × 675; (C,D). × ~60,000.

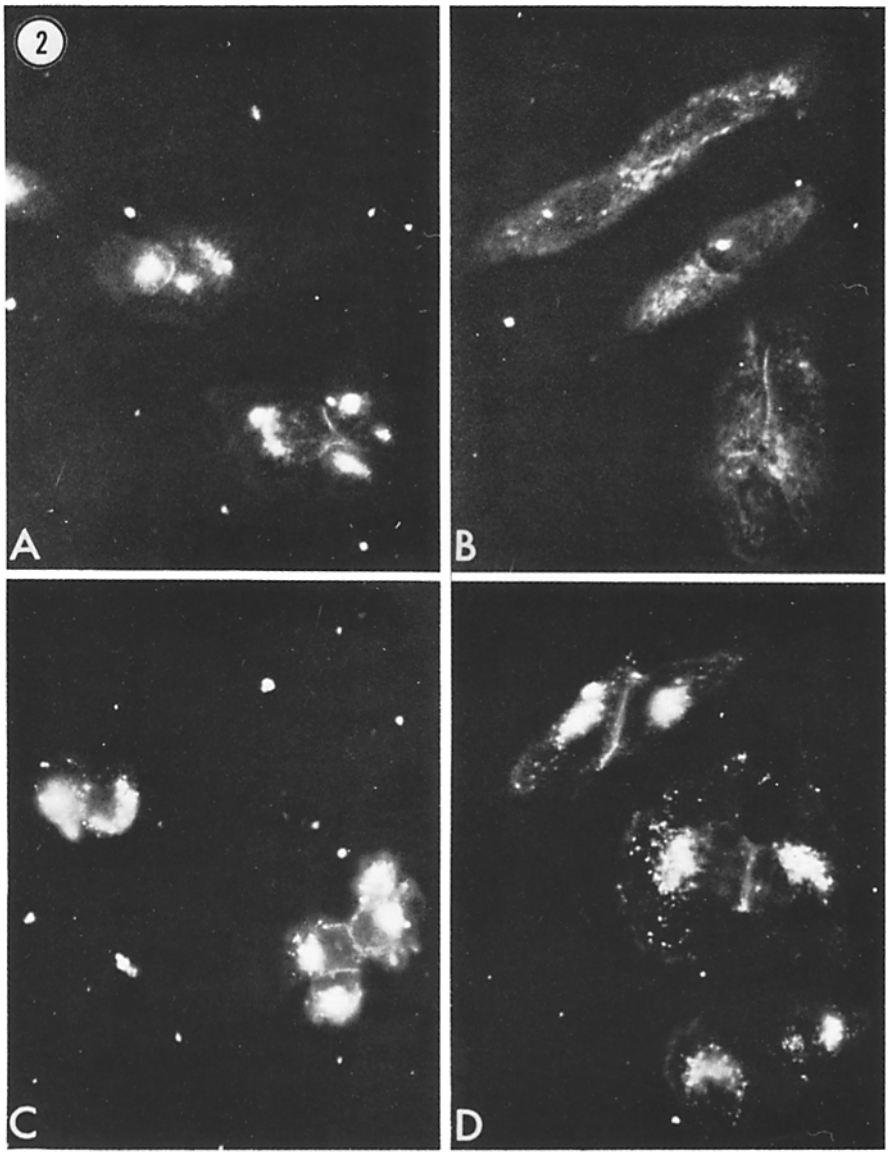

FIGURE 2 Distribution of fl-Con A bound to cells exposed to 40 μg/ml fl-Con A for 30 min (A,B) or 4 h (C,D) before fixation. Cells were cultured in the absence (A,C) or presence (B,D) of DBcAMP plus testololactone for 18 h before fl-Con A addition. (A,B) × 500; (C,D) × 675.

basal medium is stained only over a small portion of the cell in a cap-like mass located perinuclearly on the top side of the cell (Fig. 2 A). In contrast, cells in DBcAMP plus testololactone-containing medium treated identically with fl-Con A for 30 min are stained in a somewhat patch-like distribution over the entire cell (Fig. 2 B). However, if the duration of Con A exposure is prolonged, cells in the presence of DBcAMP plus testololactone also cap. Thus, after 4 h, a large fraction of either population exposed to fl-Con A is capped, and the cells in basal medium are largely rounded (Fig. 2 C,D).

Quantitatively >50% of the cells in basal medium cap during a 30-min exposure to fl-con A (Fig. 3). Only 3% of the cells in the presence of DBcAMP plus testololactone cap during a 30-min exposure, but by 2 h of exposure >50% of cells in media supplemented with DBcAMP plus testololactone are also capped. Kinetically, Con A capping precedes cell rounding by several min in the case of cells in basal medium, and by hours in

the case of DBcAMP plus testololactone-cultured cells (Fig. 3). Cells appear to become first less spread and then round as the Con A caps.

Effect of Synergistic Agents and Respiratory Inhibitors on Con A Distribution

Cyclic AMP antagonism of Con A cell rounding demonstrates synergism with testololactone, a steroid hormone analogue, or theophylline, a phosphodiesterase inhibitor, occurs rapidly, and is readily reversible (28). Antagonism by DBcAMP plus testololactone of Con A capping, if it is a cAMP effect, should behave similarly, and, in fact, it does. Complete to nearly complete antagonism of Con A capping can be produced synergistically by 0.3 mM DBcAMP plus testololactone or theophylline or by DBcAMP alone at a concentration of 1.0 mM (Table I). Addition or deletion of DBcAMP plus testololactone elicits an essentially complete alteration in the Con A-capping properties of CHO-K1 cells in a 1 h period (Table II). This is a much more rapid event than the gross conversion of CHO-K1 cells from epithelial-like to fibroblast-like morphology after drug addition or the converse change after drug removal which requires 8 or 4 h, respectively (28).

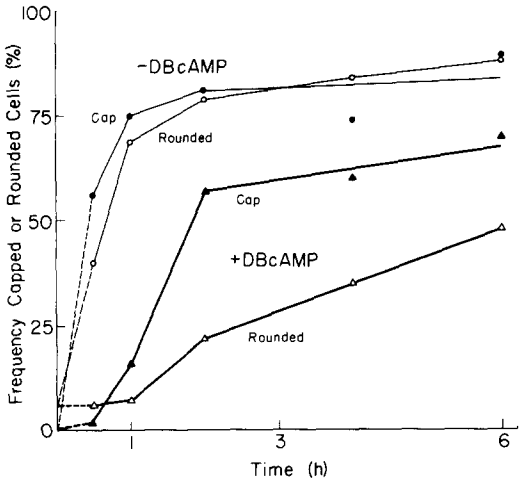

FIGURE 3 Kinetics of Con A capping and cell rounding in the absence or presence of DBcAMP plus testololactone. Cells were cultured in the absence or presence of drug for 18 h before fl-Con A addition. ●——●, frequency of cells capped in absence of DBcAMP plus testololactone; ○——○, frequency of cells rounded in absence of DBcAMP plus testololactone; ▲——▲, frequency of cells capped in presence of DBcAMP plus testololactone; and △——△, frequency of cells rounded in presence of DBcAMP plus testololactone.

TABLE I

Synergistic Antagonism by Testololactone or Theophylline and DBcAMP of Con A Capping

Agents added	Frequency of cells capped by 30-min challenge with 40 μg/ml fl-Con A
	%
None	61
Testololactone (33 μM)	60
Theophylline (0.3 mM)	56
DBcAMP (0.3 mM)	53
(1.0 mM)	9
DBcAMP (0.3 mM) plus testololactone (33 μM)	7
DBcAMP (0.3 mM) plus theophylline (0.3 mM)	20

Cells were plated in basal medium in the absence or presence of agents as indicated. 17 h postplating fl-Con A was added to the preexisting medium.

TABLE II

Rapidity and Reversibility of DBcAMP plus Testololactone Antagonism of Con A Capping

Treatment	Frequency of cells capped by 30-min challenge with 40 μg/ml fl-Con A
	%
Continuous exposure	
No addition	54
+ DBcAMP + testololactone	5
1 h post addition of DBcAMP plus testololactone	9
1 h post deletion of DBcAMP plus testololactone	46

Cells were plated on cover slips in the absence or presence of DBcAMP plus testololactone and transferred 17.5 h postplating to fresh warm medium containing DBcAMP plus testololactone as appropriate.

Con A cell rounding is blocked by low temperature and is sensitive to inhibitors of cellular respiration. Con A capping similarly is blocked by low temperature and is retarded by azide and iodoacetate, inhibitors of cellular respiration (Table III).

Effect of Colcemid and Cytochalasin B on Con A Cell Rounding and Capping

Colcemid, an agent known to disrupt microtubules (33), and cytochalasin B, a presumed

TABLE III

Effect of Cold Treatment and Inhibitors of Respiration on Con A Capping

Treatment	Frequency of cells capped by 30-min challenge with 40 μg/ml fl-Con A	
	−DBcAMP	+DBcAMP
	%	%
No addition	67	3
2°C*	0	0
+ 10 mM sodium azide, 10 μM iodoacetate	29	0

Cells were plated on cover slips in the absence or presence of DBcAMP plus testololactone. 19 h postplating cells were pretreated with inhibitor and then exposed to fl-Con A in the presence of inhibitor.

* Cells were incubated with 100 μg/ml fl-Con A to compensate for the decreased Con A binding that occurs at 2°C. No capping is observed with 40 μg/ml fl-Con A at 2°C.

TABLE IV

Effect of Colcemid and Cytochalasin B on Con A Capping

Treatment	Frequency of cells capped by 30-min challenge with 40 μg/ml fl-Con A	
	−DBcAMP	+DBcAMP
	%	%
No addition	65	2
Colcemid		
1 μM	71	10
10 μM	77	33
Cytochalasin B		
0.84 μM	66	2
10.00 μM	2	1

Cells were plated on cover slips in the absence or presence of DBcAMP plus testololactone. 17 h postplating cells were pretreated with inhibitor and then exposed to fl-Con A in the presence of inhibitor.

inhibitor of microfilament function (25,27), at concentrations ≤ 1 μM completely block the cAMP-induced conversion of CHO-K1 cells from an epithelial-like to fibroblast-like morphology (11). At these concentrations, the drugs have marginal-to-negligible effects on Con A cell rounding (28, Fig. 4) and little, if any, effect on Con A capping (Table IV).

However, at concentrations >1 μM, colcemid promotes Con A cell rounding (Fig. 4) and Con A capping (Figs. 5 and 6, Table IV). With increasing concentrations of Colcemid, cells grown in the presence of DBcAMP plus testololactone become increasingly sensitive to the cell rounding action of Con A. At a Colcemid concentration of 20 μM, these cells become as sensitive to Con A as cells grown in basal medium. Treatment of cells grown in basal medium with concentrations of Colcemid to 20 μM causes a small increase in Con A cell rounding which is only slightly greater than the accumulation of rounded mitotic cells (~7%) observed in parallel non-Con A-treated cultures. Colcemid treatment causes cells cultured in the presence of DBcAMP plus testololactone to assume a more epithelial-like morphology. Colcemid (10 μM) causes a definite increase in the frequency of cells in basal medium which cap during a 30-min exposure to fl-Con A and a 15-fold increase in the frequency of cells in DBcAMP plus testololactone medium that form caps.

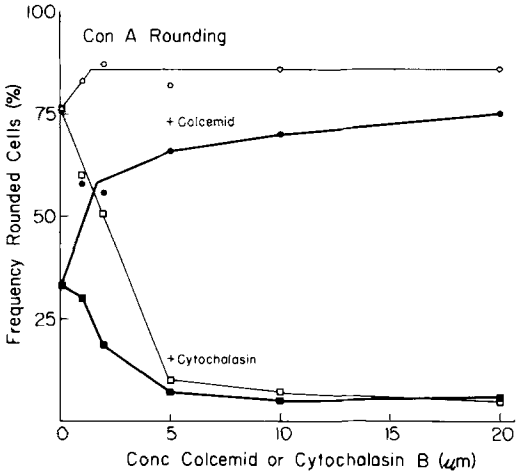

FIGURE 4 Effect of Colcemid and cytochalasin B on Con A cell rounding. Cells were grown in the absence or presence of DBcAMP plus testololactone, pretreated with Colcemid or cytochalasin B, and exposed to 40 μg/ml Con A for 2 h in the presence of inhibitor. O——O, dose-effect relationship for cells cultured in basal medium and treated with Colcemid; □——□, dose-effect relationship for cells cultured in basal medium and treated with cytochalasin B; ●——●, dose-effect relationship for cells cultured in DBcAMP plus testololactone-containing medium and treated with Colcemid; and ■——■, dose-effect relationship for cells cultured in DBcAMP plus testololactone-containing medium and treated with cytochalasin B.

Cytochalasin B at 5 μM blocks almost completely Con A cell rounding of cells in either the absence or the presence of DBcAMP plus testololactone (Fig. 4). Cytochalasin B (10 μM) prevents the formation of a centrally located Con A cap in the case of cells in either the absence or the presence of DBcAMP plus testololactone (Table IV). Con A collection does occur, though, at this concentration of cytochalasin B. At the end of a 30-min exposure to fl-Con A in the presence of cytochalasin B, fl-Con A is found scattered in large clusters or minicaps in the case of cells cultured in either basal medium or in medium containing DBcAMP plus testololactone. (Figs. 5 and 6).

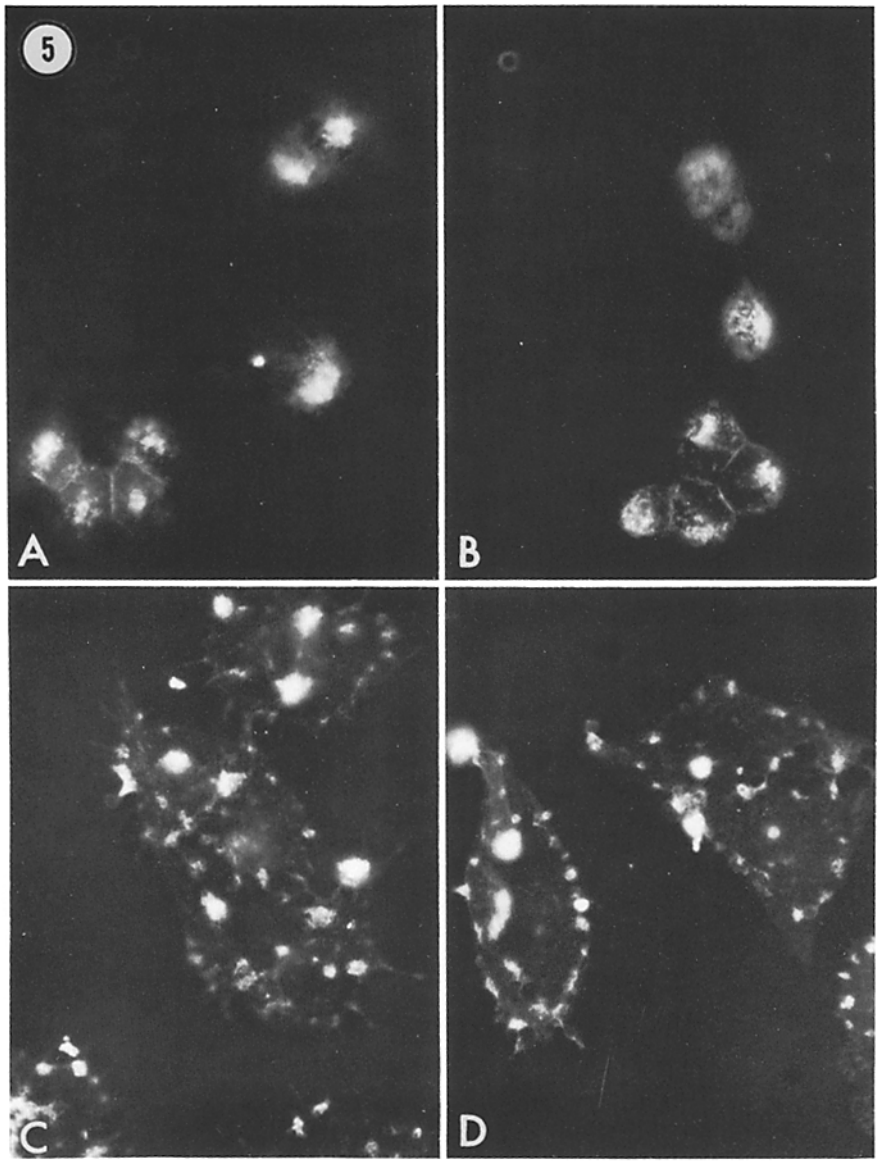

FIGURE 5 Effect of Colcemid and cytochalasin B on the distribution of fl-Con A bound to cells grown in basal medium. Cells were cultured in basal medium for 18 h, pretreated with Colcemid, cytochalasin B, or Colcemid and cytochalasin B together, and exposed to fl-Con A for 30 min in the presence of inhibitor. In (A) no addition; (B) 10 μM Colcemid; (C) 10 μM cytochalasin B; and (D) 10 μM Colcemid plus 10 μM cytochalasin B. $\times$ 675.

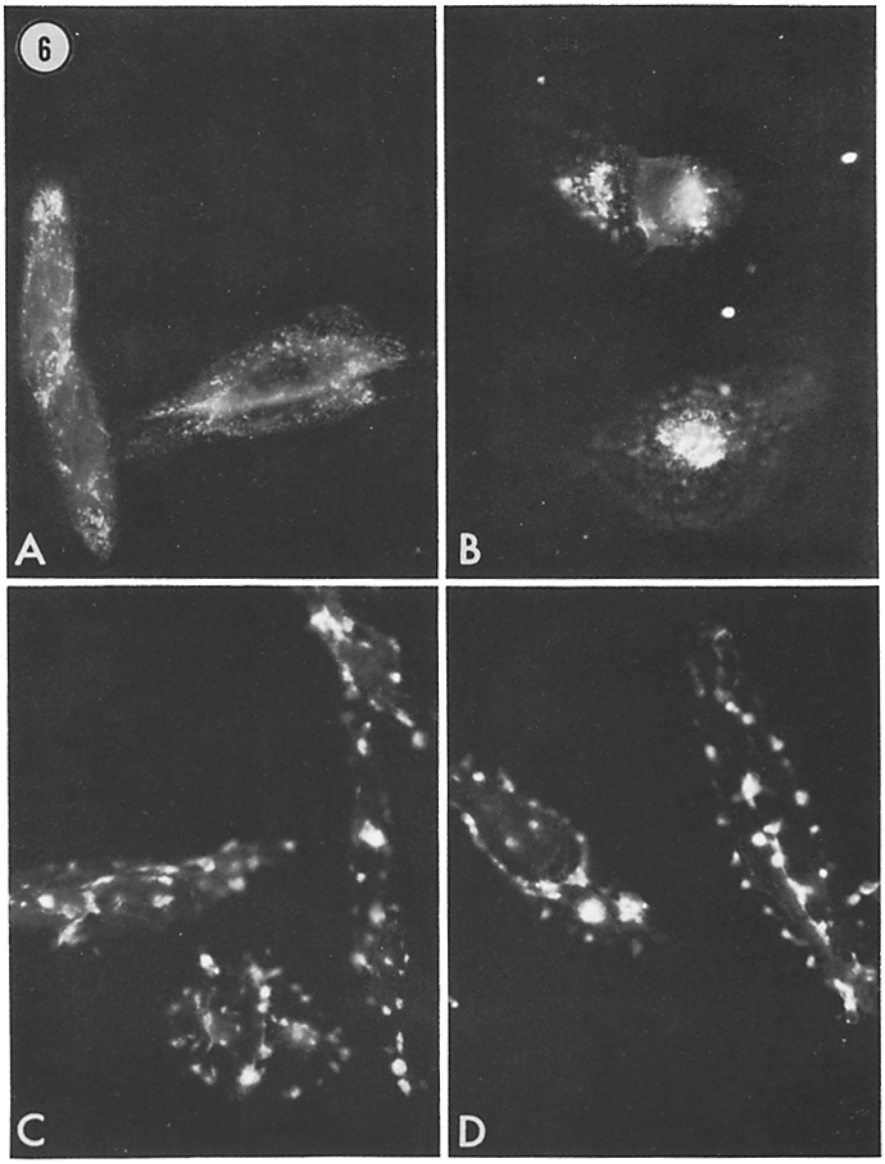

FIGURE 6 Effect of Colcemid and cytochalasin B on the distribution of fl-Con A bound to cells grown in the presence of DBcAMP plus testololactone. Cells were cultured in the presence of DBcAMP plus testololactone for 18 h, pretreated with Colcemid, cytochalasin B, or Colcemid and cytochalasin B together, and exposed to fl-Con A for 30 min in the presence of inhibitor. In (A) no addition; (B) 10 μM Colcemid; (C) 10 μM cytochalasin B, and (D) 10 μM Colcemid plus 10 μM cytochalasin B. × 675.

Treatment of cells with 10 μM Colcemid and 10 μM cytochalasin B together blocks cell rounding, and the formation of a central Con A cap, and results in minicaps that tend to be located about the cell periphery (Figs. 5 D and 6 D). Synergistic interaction between Colcemid and cytochalasin B in inhibiting Con A capping was not observed. If cells which had been exposed for 30 min to fl-Con A are post incubated in Con A-free medium containing cytochalasin B (10 μM) for 30 min, further cap formation is prevented, and a reversal of 50% of the caps present at the start of the post-incubation occurs (Table V) in agreement with recent observations of de Petris (4).

TABLE V

Effect of Colcemid and Cytochalasin B on Preexisting Con A Caps

Treatment	Frequency of cells capped
	%
No postincubation	64
30-min fl-Con A-free medium postincubation	
No addition	90
+10 μM colcemid	85
+10 μM cytochalasin B	32

Cells were plated on cover slips in basal medium, exposed for 30 min to fl-Con A, and then either fixed immediately or transferred to fl-Con A-free medium.

Effect of Con A on Intracellular cAMP Levels

Recent observations by Willingham et al. (34) indicate that a drop in intracellular cAMP levels results in the spontaneous rounding of a temperature-sensitive variant of mouse 3T3 cells. Con A rounding could be due to a Con A-induced depression of intracellular cAMP levels. Such a depression does not occur (Fig. 7). In fact, under conditions in which Con A rounds an appreciable fraction of the cell population, there is a slight elevation in intracellular cAMP levels. In a cell population 94% rounded, cAMP levels are raised by approximately 25% (Fig. 7 b).

DISCUSSION

The difference in the rate of Con A capping between CHO-K1 cells grown in the absence and those grown in the presence of DBcAMP plus synergistic concentrations of testololactone correlates with the previously reported differential Con A-rounding response of such cells (10,28). Morphologically, the centripetal collection of initially disperse Con A-binding sites into a cap-like mass is accompanied by a decrease in cell spreading followed by a complete rounding of the cell. The possibility that Con A capping is a consequence of cell rounding is eliminated by the fact that kinetically, capping precedes rounding.

That Con A capping may be a causal event in Con A cell rounding is suggested by the similar effect of cAMP and inhibitors on both processes. The addition of cAMP analogues rapidly and reversibly antagonizes both Con A capping and cell rounding. Both appear to be the result of active metabolic processes. Capping and cell rounding are both blocked by low temperature and inhibitors of cellular respiration and are similarly affected by inhibitors of microtubule or microfilament function. That the action of Con A in rounding cells could be modulated by a depression in intracellular cAMP levels can likely be excluded by the fact that cAMP levels are actually slightly elevated. The possibility that Con A in binding to the cells causes a transient depression in intracellular cAMP levels cannot, though, be absolutely excluded. While this manuscript was in preparation, other authors (32) reported that cell rounding is accelerated by Con A capping and that under conditions where cell bound Con A remains dispersely distributed, cells do not round.

Possible mechanism(s) by which Con A capping and cell rounding may be related can presently only be speculative. That simple binding of Con A to cells followed by redistribution of Con A binding sites is not sufficient to cause Con A cell rounding is indicated by the inhibition by 10 μM cytochalasin B of Con A cell rounding even though extensive redistribution of Con A binding sites into scattered minicaps does occur under these conditions. The oriented collection of Con A binding sites into a single centrally located cap may cause the disruption or organized reorientation of intracellular components. Transmembrane linkage of lectin-binding sites to intracellular components has recently been reported to occur in erythrocytes (14,19). Other more indirect effects of Con A capping on the small molecule or ionic composition of the cell may be important. More likely, the formation of a centrally located Con A cap may be related to Con A cell rounding by triggering internalization of the plasma membrane and consequent rounding of the cell to minimize cell surface area. A time lag between capping and membrane internalization, particularly in the case of cells cultured in the presence of DBcAMP plus testololactone, could explain the observed delay between Con A capping and cell rounding. Preliminary electron microscope observations indicate that internalization of Con A caps does occur in CHO-K1 populations. This hypothesis is consistent with recently reported observations of Edelson and Cohn (7,8) that Con A treatment of macrophages promotes pinocytosis and results in the formation of metabolically stable Con A pinosomes. This hypothesis is currently being investigated.

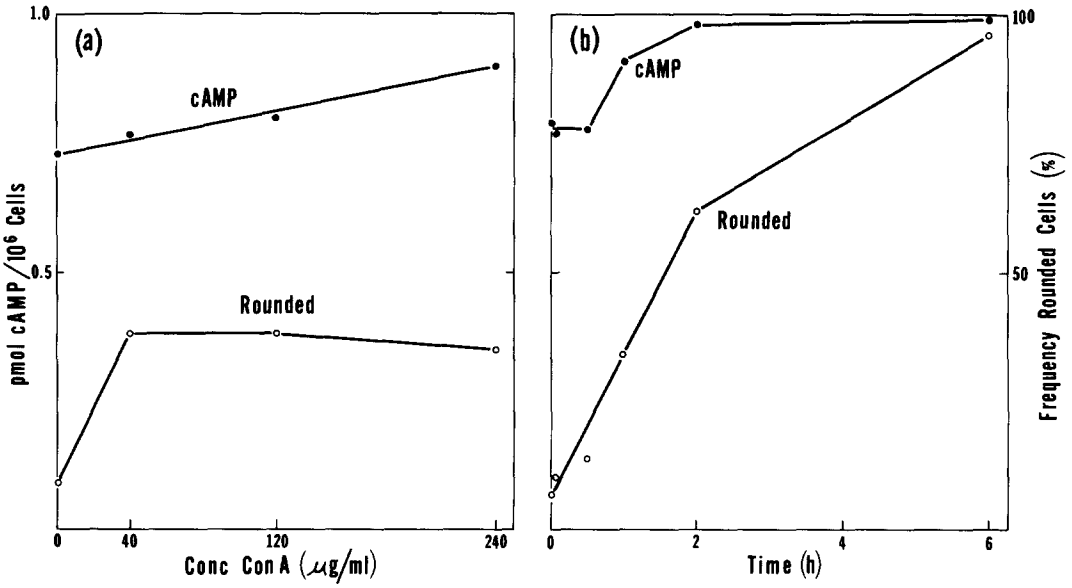

FIGURE 7 Effect of Con A on intracellular cAMP levels. Cells were cultured in basal medium for 18 h before Con A treatment. In (*a*) cells were exposed to Con A for 1 h. In (*b*) cells were incubated with 120 μg/ml Con A. ●——●, intracellular cAMP level; and O——O, frequency of rounded cells.

The mechanism by which cAMP may modulate movement or collection of Con A-binding sites in the cell membrane is obscure. No matter what the actual structure modulated by cAMP in antagonizing Con A capping, the event could result in either the anchoring of the lectin receptor in place or the disengagement of the Con A receptor from the capping process. Since CHO-K1 cells cultured in the absence or presence of DBcAMP plus testololactone are nonmotile, the effect is not related to translational cell motility. The effect of Colcemid in promoting Con A cell rounding and capping, particularly of cells treated with DBcAMP plus testololactone, is consistent with the proposal by other authors (2,6,31,37) that a membrane-associated, drug-sensitive, microtubular network may regulate cell surface receptor mobility. Cytochalasin B, a presumed inhibitor of microfilament function (25,27), has been reported by Ukena et al. (32), in the case of SV-40-transformed 3T3 cells, to inhibit Con A capping with a consequent random distribution over the entire cell surface of large Con A patches being observed. However, the interpretation of these drug effects is not clear. Observations by Sheetz and Singer (24) indicate that direct intercalation in the lipid bilayer of the membrane may be the primary explanation of many drug effects rather than disruption of intracellular structures.

Cyclic AMP modulation of lectin receptor movement may be related to the differences in cell surface and growth properties between normal and transformed cells. In general, transformed cells have lower cAMP levels than normal cells (1) and are more agglutinable by lectins (3). If, as suggested by Nicolson (18), cell agglutinability by lectin reflects receptor mobility, then agglutinability may be a reflection of cAMP levels.

Recently reported Con-A-resistant variants of CHO cells that bind normal amounts of lectin (35) could be variants in Con A capping. If so, such cells, together with the techniques of somatic cell genetics, could provide a genetic approach to analysis of receptor movement.

Cyclic AMP antagonism of Con A capping and cell rounding and the attendant conversion of CHO-K1 cells from an epithelial-like to a fibroblast-like morphology appear to be completely dissociable processes. DBcAMP plus testololactone addition produces a complete antagonism of Con A capping in time intervals during which no appreciable alteration in cell morphology has occurred. A similar difference in kinetics between the onset of cAMP antagonism of Con A cell rounding and the conversion of the cell population to a fibroblast-like morphology has been reported previously (28). Furthermore, Colcemid and cytochalasin B at concentrations of < 1 μM under

conditions similar to those found to prevent cell elongation to a fibroblast form (11) do not noticeably affect capping or cell rounding.

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