MICROTUBULES AND CYCLIC AMP IN HUMAN LEUKOCYTES:
ON THE ORDER OF THINGS

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ABSTRACT
We have shown previously that the β-agonist isoproterenol (2 μM) and the phosphodiesterase inhibitor isobutylmethylxanthine (1 mM) produce a much greater increase in cyclic AMP in human leukocytes that have been pretreated with colchicine (or with other agents that affect microtubule assembly) than in control leukocytes. The effects of colchicine were both time- and dose-dependent. These and other data suggested that the generation of cyclic AMP is normally restricted by an intact system of cytoplasmic microtubules. If so, then the same time and dose dependencies might apply to other colchicine-induced changes in leukocyte function.

We have now assayed the distribution of concanavalin A (Con A)-receptor complexes on the leukocyte membrane, taking into account that leukocytes competent to assemble microtubules show a uniform distribution of surface-bound Con A whereas microtubule-deficient cells accumulate Con A in surface caps. We have found that the effect of colchicine on capping is also both time- and dose-dependent, and that the dose-response relationships conform to those required to increase cyclic AMP levels. These findings provide further evidence that both colchicine-induced Con-A capping and colchicine-induced cyclic AMP generation depend upon the relaxation of constraints normally imposed by cytoplasmic microtubules upon the plasma membrane, which limit, respectively, lateral mobility of the lectin-receptor complexes, and expression of hormone-sensitive adenylate cyclase.

Moreover, colchicine-induced Con-A cap formation is not affected even by very large changes in leukocyte cyclic AMP levels. Thus, elevated cyclic AMP levels do not appear to promote the dissolution of microtubules; rather, the dissolution of microtubules permits the generation of increased amounts of cyclic AMP.

KEY WORDS human leukocytes · microtubule assembly (disassembly) · 3',5'-cyclic adenosine monophosphate (cyclic AMP) · concanavalin A (Con A)-receptor complex · Con-A · capping · colchicine · isoproterenol

The existence in polymorphonuclear leukocytes (PMN) of colchicine-sensitive microtubules (6)
that may in part regulate lysosomal degranulation during phagocytosis (7, 8) was first established a decade ago. Other colchicine-sensitive (presumably microtubule-dependent) PMN functions have since been described. For example, colchicine inhibits the segregative movement of membrane proteins (16, 10) and lipids (2) during phagocytosis and enhances the movement of concanavalin A (Con A)-receptor complexes into caps (11). These studies suggest that microtubules may interact with the plasma membrane to restrain or direct the topographical distribution of membrane components.

Despite increasing insight into likely functions of microtubules, the mechanism(s) of microtubule assembly and disassembly in PMN has remained obscure. Weissman, Ignarro, and others (18, 5, 17) consider that microtubule assembly-disassembly is modulated by cyclic nucleotides. Thus, like colchicine, both cyclic AMP and compounds that inhibit the segregative movement of membrane proteins (16, 10) and lipids (2) during phagocytosis (7, 8) was first established a time- and dose-dependent, (b) the colchicine doses required to induce capping conform to those required to increase leukocyte cyclic AMP levels, but (c) Con-A cap formation is not affected by very large changes in cyclic AMP levels.

MATERIALS AND METHODS

Human leukocytes were obtained from freshly drawn, heparinized blood from healthy adult donors, either inuffy coats (for capping) or after dextran sedimentation (for cyclic AMP levels), as described previously (11, 14), and suspended in a modified Krebs-Ringer phosphate buffer, pH 7.4 (7). The leukocyte population consisted of ~70-80% neutrophils and 20-30% lymphocytes with 1-4% monocytes and eosinophils. Platelets were rare. For capping experiments, 0.5 ml of the leukocyte suspensions (~0.5 × 10^6 cells) were incubated for 5 min at 37°C with 14 μg/ml fluorescein isothiocyanate-conjugated Con A (FITC-Con A) prepared as described previously (11). The cells were then fixed in an equal volume of 4% paraformaldehyde, resuspended in buffer, and examined by combined phase fluorescence microscopy with a Zeiss fluorescence microscope. At least 100 cells were counted under each experimental condition. Capped cells showed intense, polarized fluorescence. Cells without caps showed either diffuse membrane fluorescence or additional fluorescent patches, the latter indicating internalization of Con A (11). Patching occurred in only ~10% of cells and was not influenced consistently by the various drugs employed. For cyclic AMP determinations, 0.2-ml aliquots of the leukocyte suspensions (~5 × 10^6 cells), after incubations as described in the text, were placed in a boiling water bath for 5 min. After freezing, thawing, and the removal of particulate matter (14), cyclic AMP was determined in triplicate by an isotope dilution assay (4). For the effects of Con A on cyclic AMP levels, the same preparation of FITC-Con A used in the capping assay was incubated with leukocytes for an additional 5 min before boiling.
RESULTS

The effect of colchicine on Con-A cap formation is illustrated in Fig. 1a. With the usual 30-min preincubation period, colchicine has a maximal effect on Con-A cap formation at $1 \times 10^{-6}$ M, and no effect at $1 \times 10^{-7}$ M (11). However, with variation in the time of preincubation, a series of dose-response curves were generated. Thus, colchicine at $1 \times 10^{-6}$ M, $5 \times 10^{-7}$ M, $2 \times 10^{-7}$ M, and $1 \times 10^{-7}$ M produced Con-A capping that was at least half-maximal after preincubation.

![Figure 1a](image1.png)

**Figure 1** Time and dose dependencies of the effects of colchicine on Con-A cap formation in human leukocytes. (a) Cells were preincubated at 37°C with the concentrations of colchicine indicated; the means of percent capping in three experiments are shown. In one of those experiments, 1 mM IBMX ("X") was added to separate aliquots for an additional 2 min; in another, both IBMX and 2 μM d,l-isoproterenol ("X + I") were added. Then, Con-A cap formation was determined as described in the text. Although the data displayed here are for PMN, lymphocytes and monocytes behaved in a qualitatively similar fashion. (b) The dose-response relationships from (a) are compared to those seen in the generation of cyclic AMP (dotted lines, replotted from reference 14). The design of the latter experiment is like that for Con-A cap formation except that after the 2-min incubation, which included both IBMX and isoproterenol, cyclic AMP levels were determined as in the text.

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times of 15, 30, 60, and 90 min, respectively, and maximal after 30, 60, 120, and 120 min, respectively. Furthermore, the data in Fig. 1a show that, after preincubation with colchicine, incubation for an additional 2 min with 1 mM of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX. "X") or with IBMX + 2 μM isoproterenol ("X + I") had no effect on the capping response to Con A. Lymphocytes and monocytes in the mixed leukocyte suspensions showed surface behavior qualitatively similar to that measured for PMN.

Unlike the effects on Con-A cap formation, preincubation with colchicine alone has no consistent effect on leukocyte cyclic AMP levels (14). However, when a 30-min preincubation was followed by incubation for an additional 2 min with 1 mM IBMX, maximally effective concentrations of colchicine caused a more than twofold increase in cyclic AMP levels. Moreover, when 2 μM isoproterenol or 10 μM prostaglandin E₁ was added with the IBMX, the colchicine effect was markedly potentiated. As noted earlier, the effect of colchicine on the generation of cyclic AMP is also both time- and dose-dependent. In Fig. 1b, the dose-response curves are seen to conform to those for Con-A cap formation. Mononuclear cells isolated from Hypaque (Winthrop Laboratories, New York)-Ficoll (Pharmacia Fine Chemicals Inc., New Market, N. J.) gradients showed similar increases in cyclic AMP qualitatively to those reported here for predominantly PMN.

The addition of Con A itself did not reverse the accumulation of cyclic AMP induced by any of the experimental conditions employed (Table I).

DISCUSSION

The colchicine effect on Con-A cap formation is both time- and dose-dependent, and is not affected by the addition of IBMX, alone or with isoproterenol (Fig. 1a). The colchicine effect on cyclic AMP levels has similar time and dose dependency (Fig. 1b), but requires the addition of IBMX and is markedly potentiated by isoproterenol (14). Thus, the results indicate that the disassembly of microtubules which precedes colchicine-induced Con-A capping is not influenced by large fluctuations in cyclic AMP levels. To make this point unequivocally, it is still necessary to show that treatment with Con A itself does not drive elevated cyclic AMP levels back down. From the data shown in Table I, it is clear that Con A did not substantially decrease cyclic AMP levels either in control cells or in cells treated with colchicine, IBMX, or IBMX and isoproterenol. We propose, therefore, that increased cyclic AMP does not induce Con-A capping. Rather, microtubule disassembly leads simultaneously to increased cyclic AMP generation and to surface cap formation.

The molecular basis of colchicine-induced Con-A capping and of colchicine-induced cyclic AMP generation may in fact be similar. When colchicine causes the disruption of microtubules, constraints on the lateral mobility of Con A-receptor complexes are thought to be relaxed, enabling the complexes to migrate from homogeneous surface distribution into surface caps from which they will eventually be internalized (15, 1). The effect of colchicine on cyclic AMP levels may depend on a similar relaxation of constraints normally imposed by cytoplasmic microtubules on the expression of hormone-sensitive adenylate cyclase in the plasma membrane (14). Consistent with this view are the observations that (a) a phosphodiesterase inhibitor (IBMX) is required to see the effects of colchicine, suggesting increased production of cyclic AMP rather than decreased hydrolysis, (b) potentiation of the colchicine effect is seen with agents that act through three separate hormone receptors in membranes—-isoproterenol, prostaglandin E₁, and histamine, 1 (c) similar effects are seen with other agents that interfere with micro-

The histamine data are unpublished.
tubule assembly—vinblastine, vincristine, podophyllotoxin, oncolazole—but not with lumicolchicine, which does not interfere, and (d) the colchicine effects disappear when the cells are broken, even though the hormone effects persist in membrane preparations (14).

Ordering the relationship between microtubule disassembly and cyclic AMP generation in leukocytes is helpful in defining the specific pathways by which these discrete but overlapping events affect cell structure and function. For example, the leukocytes in patients with the Chediak-Higashi syndrome have various functional defects, deficient microtubule assembly (associated with spontaneous Con-A cap formation), and markedly elevated cyclic AMP levels, all of which approach normal with appropriate therapy (3, 12). The present results indicate that the cyclic AMP elevation in these genetically abnormal cells may be a consequence and not a cause of the defect in microtubule assembly.

More generally, our findings suggest that some colchicine-sensitive cellular functions now thought to be microtubule-dependent may in fact be cyclic AMP-dependent in PMN and in other cells. For example the therapeutic anti-inflammatory action of colchicine in acute gouty arthritis and other disorders may be mediated in part through interference with microtubule assembly and function (9) and in part through cyclic AMP generated when microtubules disassemble. If effects of cyclic AMP occur, they are most likely submaximal and may be potentiated by the addition of other agents that raise cyclic AMP levels. The demonstration in appropriate cell systems of functional synergism between these two classes of well-established therapeutic agents—that affect microtubule assembly and those that stimulate adenylate cyclase activity—could be applied to a wide variety of therapeutic problems, of which the most immediately apparent are in inflammation, allergy and hypersensitivity, and neoplasia.

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