COLCHICINE BINDING IN TISSUE SLICES

Decrease by Calcium and Biphasic Effect of Adenosine-3',5'-Monophosphate

ELIZABETH GILLESPIE. From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

INTRODUCTION

In the past few years microtubules have been implicated in an ever increasing number of functions. In addition to their roles in the mitotic spindle and in cilia and flagella, evidence has accumulated suggesting their involvement in (a) the aggregation of platelets (24), (b) degranulation and secretion (11, 14, 15, 19), (c) melanin granule movement (18), (d) chemotaxis (5), and (e) axonal flow (6). Many of these functions are affected by cyclic adenosine-3',5'-monophosphate (cAMP).[1] Direct or indirect evidence suggests that cAMP (a) inhibits platelet aggregation (20), (b) disperses melanin granules (3), and (c) causes the release of several compounds stored in granules (2, 9, 13, 17).

This juxtaposition of cAMP and microtubules suggested that possibly cAMP might affect the functions in question by acting on the motile system which involves microtubules. To test this idea, a biochemical approach to the study of microtubules in situ has been developed. It is based on the fact that formed microtubules are in equilibrium with depolymerized subunits (see below), and uses colchicine, a drug known to bind to microtubule subunits (4, 23), to sample the size of the subunit pool in tissue slices incubated in

vitro. Under appropriate conditions changes in colchicine binding should reflect changes in the microtubule-subunit equilibrium. The effects of both cAMP and calcium on colchicine binding were examined. Calcium was included in this study, for two reasons. First, it is necessary for some of the functions that have been linked with microtubules, and second, cAMP is known to alter calcium metabolism (21). The results obtained show that both cAMP and calcium alter colchicine binding to soluble material in liver, pancreas, and spleen. A preliminary account of a portion of this work has appeared (10).

MATERIALS AND METHODS

Female Sprague-Dawley rats (180–220 g) from Charles River Breeding Laboratories, Wilmington, Mass. were guillotined, and either their liver, spleen, or pancreas was removed. Liver and spleen slices approximately 0.5 mm thick were obtained with the use of a Stadie-Riggs apparatus. Pancreatic tissue was cut into thin strips under 1 mm in width. Tissues were incubated in a medium composed of: NaCl, 115 mM; KCl, 6.0 mM; MgSO$_4$, 1.2 mM; glucose, 10 mM; and N-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid (TES buffer), pH 7.4, 10 mM. The CaCl$_2$ concentration ranged from 0 to 20 mM. Choline chloride was used to maintain isosmolarity. Incubations were carried out in 4-ml volumes at 37°C under oxygen in Erlenmeyer vessels equipped with sidearms. 60–100 mg tissue was used per vessel. Total incubation times were 45 min for spleen and pancreas and 75 min for liver. Calcium was added at the beginning of the incubation period and cAMP and N^{6}O^2-dibutyryl adenosine-3',5'-monophosphate

---

[1] *Abbreviations used in this paper:* cAMP, cyclic adenosine-3',5'-monophosphate; DBC, N^{6}O^2-dibutyryl adenosine-3',5'-monophosphate; TES buffer, N-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid.

(dibutyryl cAMP) were added 15 min later. Colchicine-^{14}C (2.5×10^{-6} M) was tipped from the sidearm 15 min before the end of the incubation period. Incubations were terminated by transferring the contents of each vessel on to squares of nylon stocking. Tissues were rinsed in cold 0.24 M sucrose containing 1 mM $MgCl_2$ and 10 mM sodium phosphate buffer, pH 7.0, and homogenized in 1.5 ml of the same mixture. Following the procedure developed by Borisy and Taylor (4), the homogenates were centrifuged at 100,000 g for 1 hr and portions of the clear supernatants were applied to 1×15 cm Sephadex G-50 columns. Elution was carried out with 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$. Four fractions were collected: bound colchicine, marked by dextran blue (2.5–3.0 ml), two 1.5 ml fractions, and a 20 ml fraction. Portions of each fraction were counted in a liquid scintillation counter. Radioactivity associated with the particulate fraction was determined after resuspending the material in water.

Colchicine-methoxy-^{14}C (SA 20 mCi/g) was obtained from New England Nuclear Corp., Boston, Mass. and cAMP and its dibutyryl derivative from Schwarz Bio Research Inc., Orangeburg, N. Y.

RESULTS

Initial experiments determined the effects of incubation time and of drug concentration on colchicine binding. Radioactivity associated with high molecular weight material in the soluble fraction (protein-bound colchicine), associated with the low molecular weight material in the same fraction (free colchicine), and associated with the precipitate obtained upon centrifugation (particulate-bound colchicine) was measured. The effect of time on the accumulation of colchicine in these three pools is shown in Fig. 1.

The effects of colchicine concentration were studied after a 15 min incubation period. Protein-bound colchicine was directly proportional to concentration over the range $1–5 \times 10^{-6}$ M but was below proportionality at higher concentrations ($5 \times 10^{-6}–5 \times 10^{-4}$ M). The free pool remained proportional to drug concentration at concentrations from 1×10^{-6} M to 5×10^{-4} M. The binding of colchicine to the particulate fraction fell below proportionality with increasing drug concentration (5×10^{-6} M–5×10^{-4} M) markedly in the spleen, moderately in the pancreas, and only slightly in the liver. At low concentrations ($1–5 \times 10^{-6}$ M) it was directly proportional to drug concentration in all three tissues.

The above experiments led to the adoption of a colchicine concentration of 2.5×10^{-6} M and an incubation time of 15 min as standard conditions. Under these conditions the total amount of protein bound to colchicine represents, at maximum, 2, 4, and 5% of the total microtubule subunits in liver, pancreas, and spleen, respectively.

Effects of Calcium and cAMP on Protein-Bound Colchicine

When the calcium concentration of the medium was increased, the amount of protein-bound colchicine in liver and spleen gradually decreased (Fig. 2). Calcium had no effect on colchicine binding in pancreas unless a portion of the sodium chloride in the medium was replaced with choline chloride.

The effects of cAMP and its dibutyryl derivative on colchicine binding were studied at three different calcium concentrations, 0, 2, and 20 mM. In many cases a biphasic response to cAMP was seen, lower cAMP levels decreasing binding and higher levels increasing it again. In the pancreas (Fig. 3) a biphasic response was observed at all three calcium concentrations. In the spleen (Fig. 4) there was a clear biphasic effect only in the presence of 2 mM calcium, while in the liver (Fig. 5) a biphasic response was seen in the presence of 2 mM calcium and also in the absence of calcium.

Control Experiments

The effects of calcium and cAMP on the levels of free and particulate-bound colchicine were measured throughout this study. In most cases these levels did not vary. When they did, the changes noted could not in any way explain the observed effects on protein-bound colchicine levels.

Experiments were carried out to determine if either calcium or cAMP and its dibutyryl derivative affected (a) the initial uptake of colchicine into tissue, (b) the binding of colchicine to soluble protein in a cell-free system, and (c) any possible metabolism of colchicine during the incubation period. No effects of calcium or cAMP were noted on these three parameters.

DISCUSSION

The present study demonstrates that calcium and cAMP influence the binding of colchicine to high molecular weight material in the soluble fraction of liver, spleen, and pancreas. The effect of calcium is to lower binding, while cAMP has a biphasic effect that is modified by calcium. Before consid-
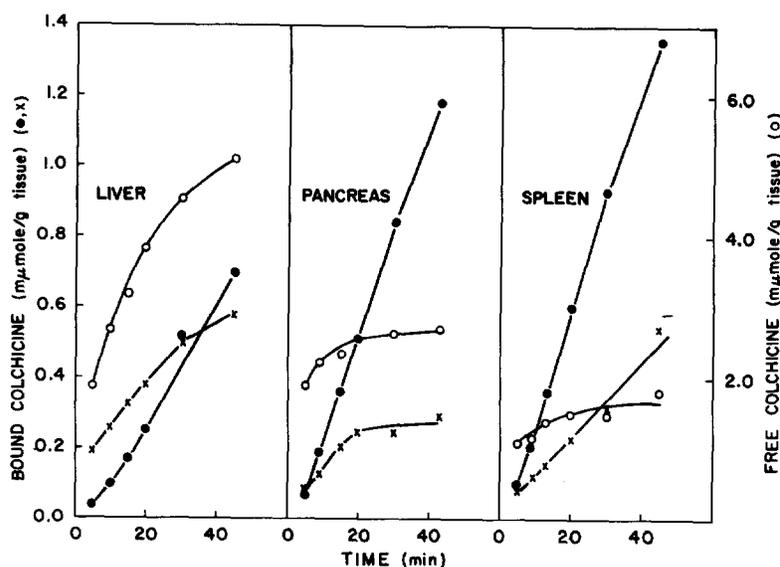
FIGURE 1 Effects of time on the binding and uptake of colchicine in slices of rat liver, pancreas, and spleen. ●, Colchicine bound to high molecular weight material in the soluble fraction; ×, colchicine associated with particulate material; O, free colchicine.
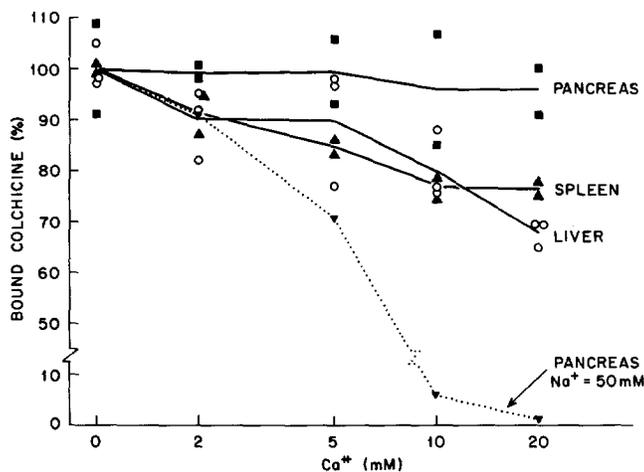

FIGURE 2 Effects of calcium on the binding of colchicine to high molecular weight material in the soluble fraction of rat liver, pancreas, and spleen slices.

ering the possible implications of these findings it is pertinent to first discuss the mechanism of action of colchicine. The studies of Taylor and his colleagues (4, 23) make it clear that colchicine binds to the subunit protein of microtubules. However, it is uncertain whether it binds to preexisting subunits, to subunits as they become available, or initially to the formed microtubule. Available evidence favors the first of these possibilities. Micro-

tubules are known to be labile upon either cooling (12), for example, or upon exposure to high pressure (25). Also, studies on the regeneration of cilia and flagella (1, 22) show that a substantial subunit pool is present in some cell types at least.

The scheme that explains the present results best is illustrated in Fig. 6. It suggests that calcium acts to promote the formation of microtubules from available subunits and thereby lower colchicine
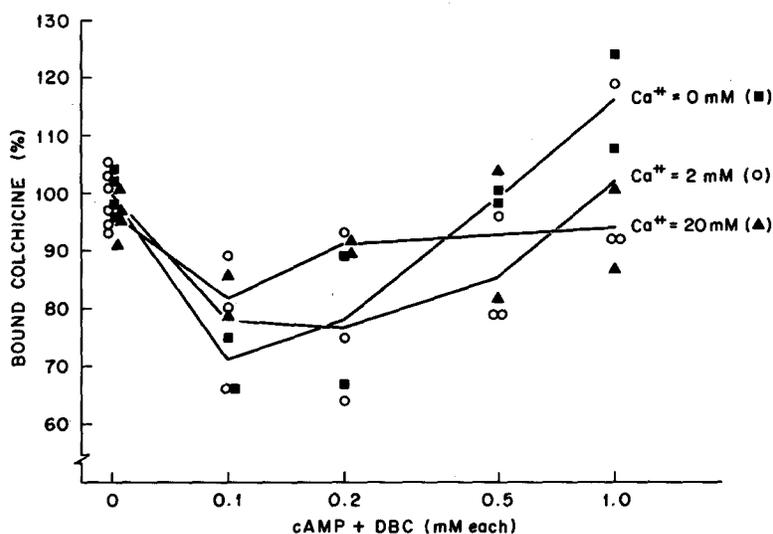
FIGURE 3 Effects of cAMP and dibutyryl cAMP (*DBC*) on the binding of colchicine to high molecular weight material in the soluble fraction of rat pancreatic strips.
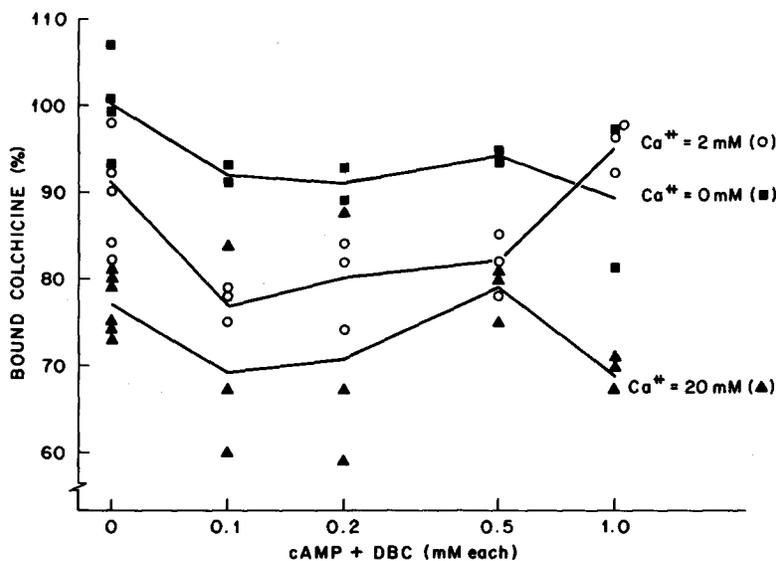

FIGURE 4 Effects of cAMP and dibutyryl cAMP (*DBC*) on the binding of colchicine to high molecular weight material in the soluble fraction of rat spleen slices. Control values in the presence of 2 and 20 mM calcium have been lowered to correspond to the decrease in binding caused by these concentrations of calcium (Fig. 2).

binding, while cAMP has two effects. At lower concentrations cAMP presumably acts mainly to mobilize calcium and thereby favor microtubule formation, while at higher concentrations it acts in the opposite direction to increase the size of the subunit pool. This scheme has the novel feature of permitting cAMP, in the presence of calcium, to increase the over-all rate of interconversion between subunit and microtubule.

While the above scheme fits the experimental data it does not relate the present findings to the control of microtubule activity. A modified scheme
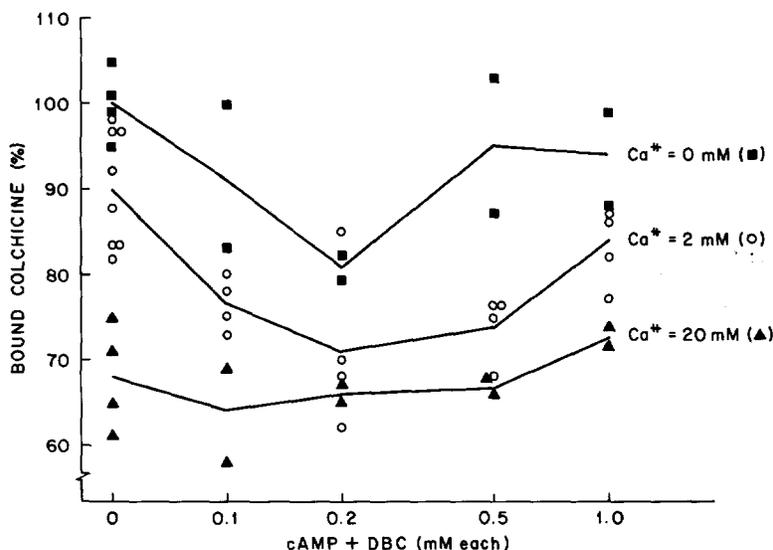
FIGURE 5 Effects of cAMP and dibutyryl cAMP (*DBC*) on the binding of colchicine to high molecular weight material in the soluble fraction of rat liver slices. Control values in the presence of 2 and 20 mM calcium have been lowered to correspond to the decrease in binding caused by these concentrations of calcium (Fig. 2).
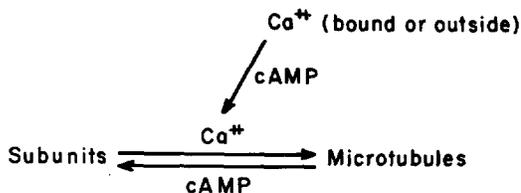

FIGURE 6 Proposed relationship between microtubules, cAMP, and calcium.

(Fig. 7) indicates a possible control relationship. Several assumptions are made. First, the assumption is made that microtubules exist in two forms (designated *A* and *B*) and that it is the interconversion of these forms that leads to sustained activity of the microtubule system. Second, it is assumed that only one microtubule form breaks down to subunits, and third, it is assumed that cAMP and calcium act on the equilibrium between the two microtubule forms. These two forms can represent, of course, any type of difference either in an entire microtubule, in a portion of one microtubule, or in a single subunit.

The foregoing hypothesis makes it possible to explain in a unified way many diverse observations concerning the effects of calcium and cAMP on microtubule-related functions. While requiring the presence of both substances for microtubule activity, it suggests that the balance between them is of importance. This makes it easy to see that either compound might either stimulate or inhibit microtubule function, depending on existing conditions within the cell. In fact, stimulation and inhibition by both cAMP and calcium have been observed in several cases. One example, that of degranulation and secretion, will be considered here. Microtubules have been implicated in this function in several cell types to date (11, 14, 15, 19), suggesting that their involvement is a general one. At the same time both calcium and cAMP have been shown to cause, be required for, or inhibit many release processes. Calcium appears to be the final mediator of release in some cases (7, 8), while cAMP has been assigned this role in other cases (2, 9, 17). At the same time excess calcium inhibits vasopressin release from the pituitary (7), while theophylline and isoproterenol inhibit histamine release from leukocytes (16).

Co^{++} (bound or outside)

cAMP

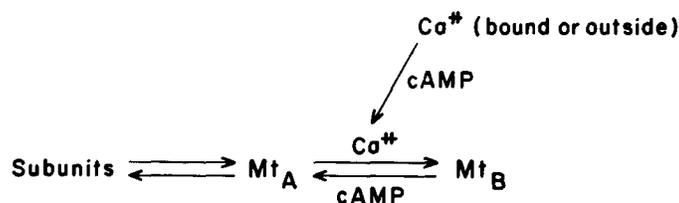Subunits ⇌ Mt$_A$ ⇌ $\dfrac{Co^{++}}{cAMP}$ Mt$_B$

FIGURE 7 Proposed relationship between microtubules, cAMP, and calcium.

REFERENCES

1. AUCLAIR, W., and B. W. SIEGEL. 1966. Cilia regeneration in the sea urchin embryo: Evidence for a pool of ciliary proteins. *Science* (*Washington*). 154:913.
2. BDOLAH, A., and M. SCHRAMM. 1965. The function of 3'5' cyclic AMP in enzyme secretion. *Biochem. Biophys. Res. Commun.* 18:452.
3. BITENSKY, M. W., and S. R. BURSTEIN. 1965. Effects of cyclic adenosine monophosphate and melanocyte-stimulating hormone on frog skin *in vitro*. *Nature* (*London*). 208:1282.
4. BORISY, G. G., and E. W. TAYLOR. 1967. The mechanism of action of colchicine. Binding of colchicine-³H to cellular protein. *J. Cell Biol.* 34:525.
5. CANER, J. E. Z. 1965. Colchicine inhibition of chemotaxis. *Arthritis Rheum.* 8:757.
6. DAHLSTROM, A. 1968. Effect of colchicine on transport of amine storage granules in sympathetic nerves of rat. *Eur. J. Pharmacol.* 5:111.
7. DOUGLAS, W. W., and A. M. POISNER. 1964. Stimulus-secretion coupling in a neurosecretory organ: The role of calcium in the release of vasopressin from the neurohypophysis. *J. Physiol.* (*London*). 172:1.
8. DOUGLAS, W. W., and R. P. RUBIN. 1961. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol.* (*London*). 159:40.
9. FLEISCHER, N., R. A. DONALD, and R. W. BUTCHER. 1969. Involvement of adenosine-3',5'-monophosphate in release of ACTH. *Amer. J. Physiol.* 217:1287.
10. GILLESPIE, E., and R. J. LEVINE. 1970. Effects of cyclic adenosine-3',5'-monophosphate (cAMP) and calcium on colchicine binding in tissue slices. *Fed. Proc.* 29:474.
11. GILLESPIE, E., R. J. LEVINE, and S. E. MALAWISTA. 1968. Histamine release from rat peritoneal mast cells: Inhibition by colchicine and potentiation by deuterium oxide. *J. Pharmacol. Exp. Ther.* 164:158.
12. INOUÉ, S., and H. SATO. 1967. Cell motility by labile association of molecules. *J. Gen. Physiol.* 50(6, Pt. 2):259.
13. KULKA, R. G., and E. STERNLIGHT. 1968. Enzyme secretion in mouse pancreas mediated by adenosine-3',5'-cyclic phosphate and inhibited by adenosine-3'-phosphate. *Proc. Nat. Acad. Sci. U. S. A.* 61:1123.
14. LACY, P. E., S. L. HOWELL, D. A. YOUNG, and C. J. FINK. 1968. New hypothesis of insulin secretion. *Nature* (*London*). 219:1177.
15. LEVY, D. A., and J. A. CARLTON. 1969. Influence of temperature on the inhibition by colchicine of allergic histamine release. *Proc. Soc. Exp. Biol. Med.* 130:1333.
16. LICHTENSTEIN, L. M., AND S. MARGOLIS. 1968. Histamine release in vitro: Inhibition by catecholamines and methylxanthines. *Science* (*Washington*). 161:902.
17. MALAISSE, W. J., F. MALAISSE-LAGAE, and D. MAYHEW. 1967. A possible role for the adenyl-cyclase system in insulin secretion. *J. Clin. Invest.* 46:1724.
18. MALAWISTA, S. E. 1965. On the action of colchicine: The melanocyte model. *J. Exp. Med.* 122:361.
19. MALAWISTA, S. E., and P. T. BODEL. 1967. The dissociation by colchicine of phagocytosis from increased oxygen consumption in human leukocytes. *J. Clin. Invest.* 46:786.
20. MARQUIS, N. R., R. L. VIGDAHL, and P. A. TAVORMINA. 1969. Platelet aggregation. I. Regulation by cyclic AMP and prostaglandin E₁. *Biochem. Biophys. Res. Commun.* 36:965.
21. RASMUSSEN, H. 1970. Cell communication, calcium ion and cyclic adenosine monophosphate. *Science* (*Washington*). 170:404.
22. ROSENBAUM, J. L., J. E. MOULDER, and D. L. RINGO. 1969. Flagellar elongation and shortening in *Chlamydomonas*. The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. *J. Cell Biol.* 41:600.
23. SHELANSKI, M. L., and E. W. TAYLOR. 1968. Properties of the protein subunit of central pair and outer-doublet microtubules of sea urchin flagella. *J. Cell Biol.* 38:304.
24. WHITE, J. G. 1969. Effects of colchicine and vinca alkaloids on human platelets. III. Influence on primary internal contraction and secondary aggregation. *Amer. J. Pathol.* 54:467.
25. ZIMMERMAN, A. M., and D. MARSLAND. 1964. Cell division: effects of pressure on the mitotic mechanism of marine eggs (*Arbacia punctulata*). *Exp. Cell Res.* 35:293.