Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1938–1941, April 1983
Cell Biology

Cells injected with guanosine 5′-[α,β-methylene]triphosphate, an α,β-nonhydrolyzable analog of GTP, show anomalous patterns of tubulin polymerization affecting cell translocation, intracellular movement, and the organization of Golgi elements

(microtubule/nucleotide/cell movement/microinjection)

JURGEN WEHLAND*† AND IGNACIO V. SANDOVAL‡§

*Laboratory of Molecular Biology, National Cancer Institute; and ‡National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Communicated by George H. Hitchings, December 30, 1982

ABSTRACT **Injection of the α,β-nonhydrolyzable GTP analog, guanosine 5′-[α,β-methylene]triphosphate (pp[CH_2]pG) into PtK2, A549, and Swiss 3T3 cells produced dramatic changes in the normal pattern of long radiating microtubules displayed by the cells before injection. Injection of pp[CH_2]pG into cells growing in normal medium resulted in the formation of microtubule bundles resistant to depolymerization by Colcemid and calcium. Cells injected with pp[CH_2]pG after incubation with Colcemid for 2 hr showed polymerization of tubulin into long wavy ribbons within 2 hr after injection. Removal of Colcemid 1 hr after the injection of pp[CH_2]pG resulted in assembly of tubulin into short single randomly oriented microtubules. All cells injected with pp[CH_2]pG showed impeded translocation and restriction or absence of intracellular movement. pp[CH_2]pG also prevented the fragmentation of Golgi elements in A549 cells treated with Colcemid. Cells first treated with Colcemid and then injected with pp[CH_2]pG failed to reassemble the Golgi elements after the removal of Colcemid. Cells in intimate membrane contact with cells injected with pp[CH_2]pG showed similar changes in microtubule polymerization, cell movement, and organization of Golgi elements.**

Tubulin binds 2 mol of GTP per mol (1). One GTP does not exchange with exogenous GTP and is bound to the N (nonexchangeable) nucleotide site of tubulin (2). The second GTP exchanges relatively quickly with added GTP and is bound to the E (exchangeable) nucleotide site of tubulin (2). The first step in microtubule polymerization *in vitro* is the condensation of tubulin into microtubule nuclei (3) and requires both the binding of GTP to the E site of tubulin (4, 5) and a minimum tubulin concentration (i.e., critical tubulin concentration) (3). Microtubules assembled *in vitro* show continuous head-to-tail polymerization of tubulin, resulting from hydrolysis of the GTP bound to the E site of tubulin to GDP (6). In contrast, little is known about microtubule polymerization *in vivo*. Polymerization *in vivo* is organized by microtubule organizing centers (7) but it is not clear whether this requires the formation of nuclei. It is also not known whether microtubules polymerized *in vivo* show the continuous head-to-tail polymerization characteristic of microtubules polymerized *in vitro* (6). We have reported that the (α,β)-nonhydrolyzable analog of GTP guanosine 5′-[α,β-methylene]triphosphate (pp[CH_2]pG) specifically enhanced microtubule nucleation and inhibited the head-to-tail polymerization of microtubules *in vitro* (8). Microtubules polymerized by pp[CH_2]pG were more resistant to low temperatures (4°C), colchicine, podophyllotoxin, and millimolar concentrations of Ca^{2+}, which readily depolymerized microtubules polymerized by GTP (9). In addition, pp[CH_2]pG promoted polymerization of colchicine–tubulin and podophyllotoxin–tubulin complexes into characteristic ribbons of three or four protofilaments (10).

We have studied the polymerization of tubulin in normal PtK2, A549, and Swiss 3T3 cells injected with pp[CH_2]pG in both the presence and absence of Colcemid. These studies show that cells injected with pp[CH_2]pG undergo dramatic changes in the patterns of tubulin polymerization and microtubule organization. The microtubule changes produced by pp[CH_2]pG result in suppression of cell translocation and restriction or absence of intracellular movement (i.e., saltatory movement) and have profound effects on the integrity and location of the Golgi elements.

MATERIALS AND METHODS

Swiss 3T3 mouse fibroblasts and A549 (human lung carcinoma) cells were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum. PtK2 cells were grown in Ham's F12 medium/5% fetal calf serum. Solutions (0.1 M) of pp[CH_2]-pG (ICN), GTP, and guanosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppG) were prepared in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 7.2) immediately prior to use. Microinjection of cells was carried out as described (11). The volume of nucleotide solution injected into the cells was 5–10% of the cell volume, making the concentration of nucleotide inside the cell 5–10 mM. Rhodamine-labeled goat anti-guinea pig IgG (10 mg/ml) (Cappel Laboratories, Cochranville, PA) was added to the injection solution to mark the injected cells. Rat monoclonal anti-yeast α-tubulin antibodies (clone YL 1/2) were a gift of J. V. Kilmartin (12). Affinity-purified rabbit anti-human galactosyltransferase polyclonal antibodies to label the Golgi elements were a gift of E. G. Berger (13). Fluorescein- and rhodamine-labeled goat anti-rat and goat anti-rabbit IgGs (Cappel Laboratories) were affinity purified on IgG-Sepharose columns. At different times after nucleotide injection, the cells were fixed and permeabilized with

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: pp[CH_2]pG, guanosine 5′-[α,β-methylene]triphosphate; p[CH_2]ppG, guanosine 5′-[β,γ-methylene]triphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

† Present address: Max-Planck-Institute of Biophysical Chemistry, Dept. of Biochemistry I, D-3400, Federal Republic of Germany.
§ To whom correspondence should be addressed.

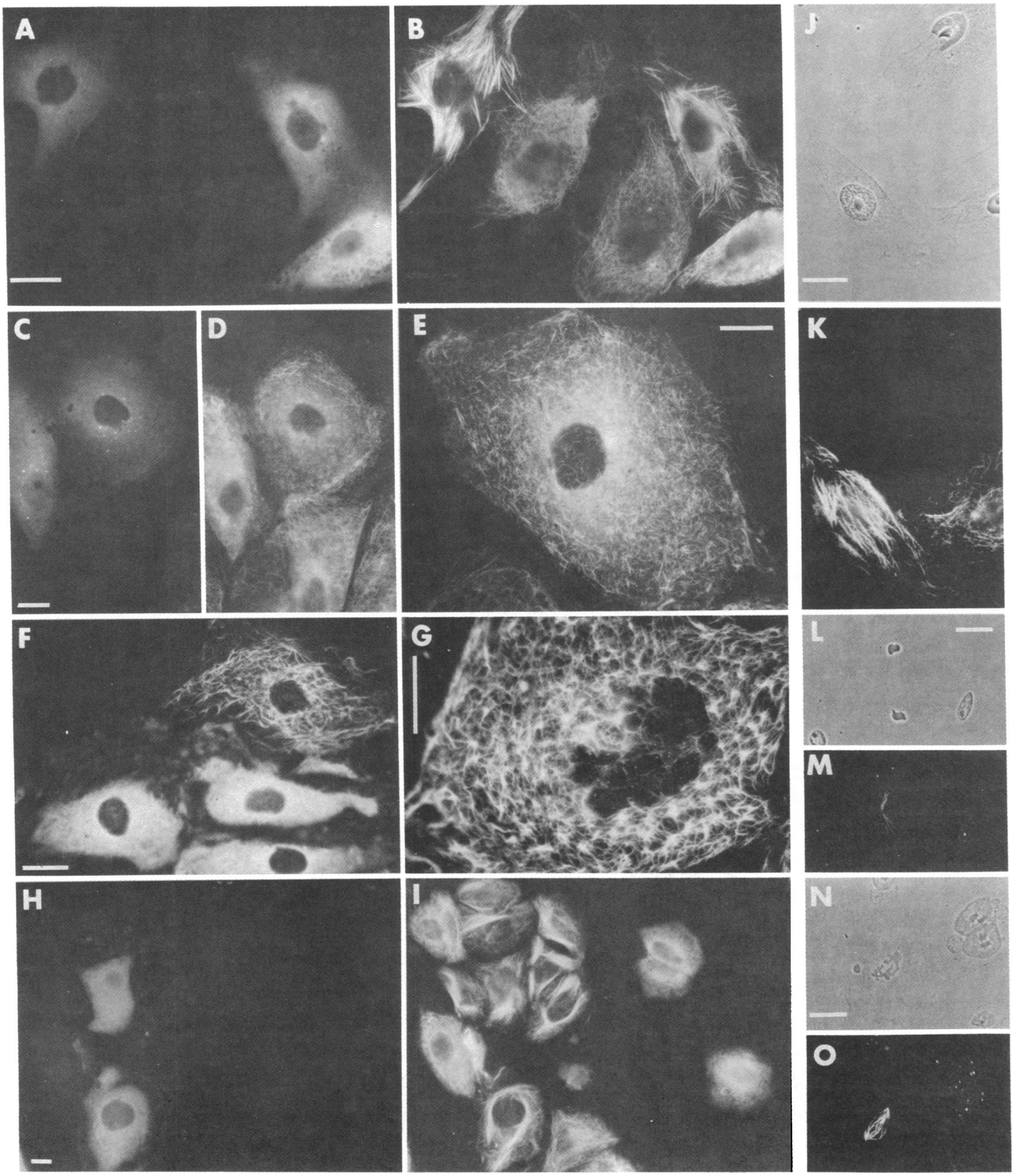

FIG. 1. Regulation of microtubule polymerization in pp[CH_2]pG-injected PtK2 and A549 cells. (*A* and *B*) PtK2 cells growing in normal medium were injected with pp[CH_2]pG and the marker rhodamine-labeled goat anti-guinea pig IgG. The three cells injected with pp[CH_2]pG (*A*, rhodamine channel) showed bundles of microtubules (*B*, fluorescein channel) while the uninjected cells displayed normal microtubules (*B*). (*C–E*) The two PtK2 cells showing rhodamine fluorescence (*C*) were injected with pp[CH_2]pG after 2 hr of incubation with 1 μM Colcemid. One hour later, the Colcemid was removed and the cells were incubated in Colcemid-free medium for 2 hr. Observe the large number of short randomly oriented microtubules polymerized in the pp[CH_2]pG-injected cells (*D* and *E*). (*F* and *G*) As in *C–E* but without the removal of Colcemid from the culture medium after injection of pp[CH_2]pG. Observe the presence of wavy polymers of tubulin in the cells injected with pp[CH_2]pG and the absence of tubulin polymers in the uninjected cells. (*H* and *I*) As in *A* and *B*, except that 2 hr after injection of pp[CH_2]pG, the cells were treated with 1 μM Colcemid for 2 hr. Observe (*H*, rhodamine channel; *I*, fluorescein channel) that uninjected cells having close membrane contact with pp[CH_2]pG-injected cells also displayed Colcemid-resistant microtubules. (*J* and *K*) Cytoskeletons of normal and pp[CH_2]pG-injected cells obtained in the presence of 2 mM $CaCl_2$. The two cells shown in the bottom of the phase-contrast picture (*J*) were injected with pp[CH_2]pG while being incubated in normal medium. Observe the resistance of cytoplasmic microtubules to calcium in the pp[CH_2]pG-injected cells. (*L–O*) Resistance of midbody (*L* and *M*) and spindle (*N* and *O*) microtubules to calcium in cytoskeletons of uninjected PtK2 cells. (Bars = 20 μm.)

cold methanol (−20°C) and then studied by double immunofluorescence with the various antibodies. Cell translocation and saltatory movement were studied by video intensification microscopy using a time-lapse ratio of 1:72 (14). Cytoskeletons were prepared by extracting PtK2 cells with 0.1 M Mes/1 mM $MgCl_2$/1 mM GTP/1 mM EGTA/0.2% Triton X-100, pH 6.8.

RESULTS

Polymerization of Tubulin in Normal and pp[CH_2]pG-Injected PtK2 and A549 Cells. PtK2 and A549 cells growing in normal medium displayed single microtubules extending radially from the vicinity of the nucleus to the plasma membrane. Injection of pp[CH_2]pG into the cells resulted in formation of bundles of microtubules (Fig. 1 *A* and *B*) resistant to depolymerization by Colcemid and calcium (Fig. 1 *H–K*). Microtubules resistant to calcium were also observed in the spindle (Fig. 1 *L* and *M*) and midbody of uninjected cells (Fig. 1 *N* and *O*). Cells incubated with Colcemid for 2 hr and then injected with pp[CH_2]pG showed polymerization of tubulin into randomly oriented wavy polymers, sometimes packed into bundles (Fig. 1 *F* and *G*), that resembled the wavy ribbons polymerized by pp[CH_2]pG from colchicine–tubulin complexes *in vitro* (10). Removal of Colcemid 1 hr after the pp[CH_2]pG injection resulted in assembly of many short single microtubules randomly oriented in the cytoplasm (Fig. 1 *C–E*). Interestingly, uninjected cells in intimate membrane contact with cells injected with pp[CH_2]pG often showed similar ef-

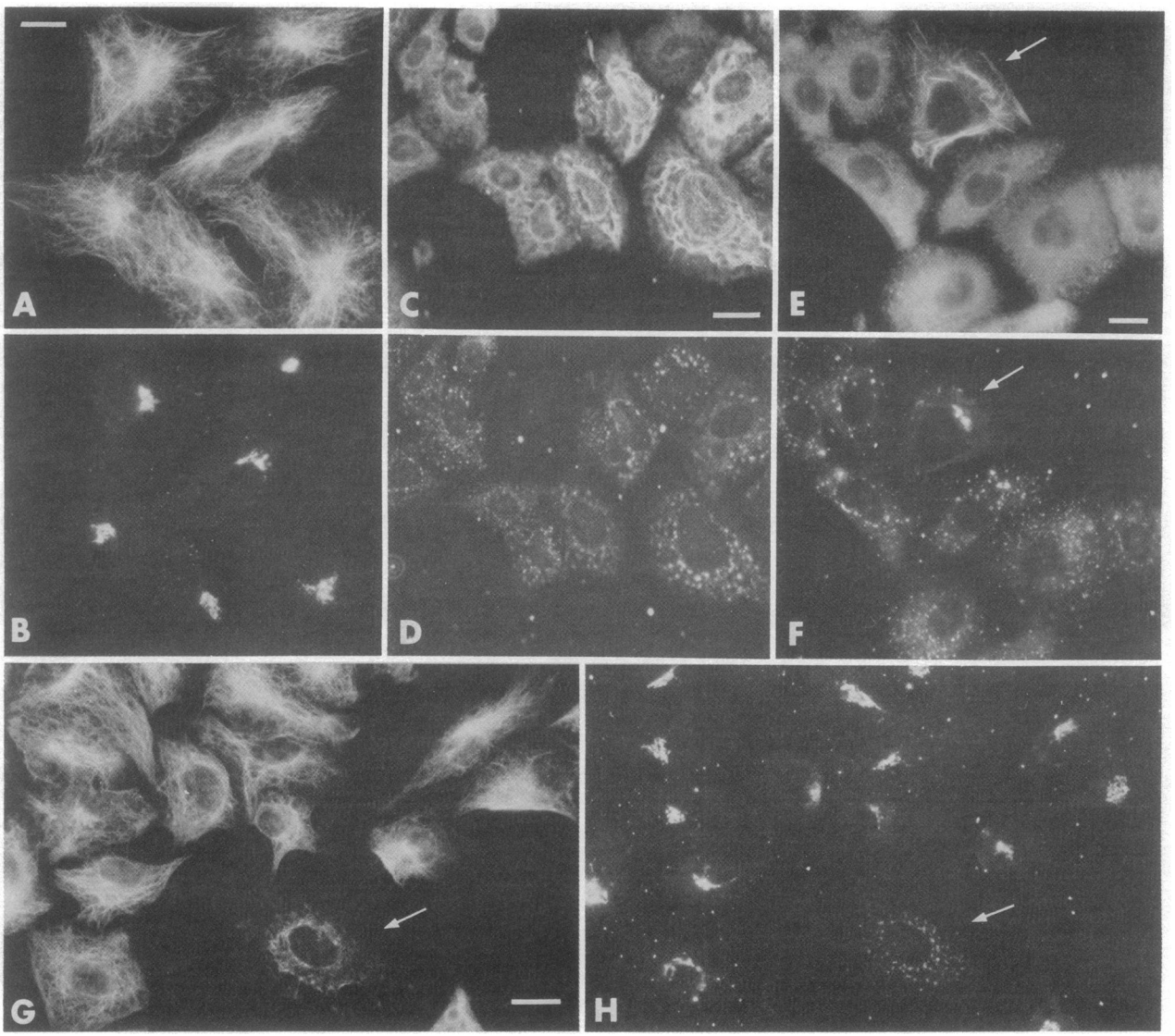

FIG. 2. Effect of pp[CH_2]pG-controlled polymerization of microtubules on the integrity and location of the Golgi complex in A549 cells. (*A* and *B*) Microtubules (*A*) and Golgi complexes (*B*) of A549 cells growing in normal medium. Observe that both the Golgi complex and the microtubule organizing center are localized in the same perinuclear area. (*C* and *D*) Failure of tubulin ribbons polymerized by pp[CH_2]pG in the presence of Colcemid to reassemble the Golgi complex fragmented by Colcemid (see description of the experiment in Fig. 1 *F* and *G*). Observe the tubulin ribbons and the dispersion of the Golgi elements in cells injected with pp[CH_2]pG and the absence of tubulin polymers and dispersion of the Golgi elements in uninjected cells. (*E* and *F*) Resistance of both microtubules and the Golgi complex to Colcemid in cells injected with pp[CH_2]pG (see description of the experiment in Fig. 1 *A* and *B*). Observe the microtubule bundles (*E*) and the perinuclear location of the Golgi complex (*F*) of the pp[CH_2]pG-injected cell (arrows) and the absence of microtubules and the dispersed Golgi complex in the uninjected cells. (*G* and *H*) Reorganization of the Colcemid-dispersed Golgi complex requires assembly of microtubules of the right length, adequate orientation, and proper distribution (see description of the experiment in Fig. 1 *C* and *D*). Observe the large numbers of short randomly oriented microtubules and the dispersion of the Golgi complex in the cell injected with pp[CH_2]pG (arrows) and the display of normal microtubules and Golgi complex in the uninjected cells. (Bars = 20 μm.)

fects in microtubule polymerization and stabilization (Fig. 1 *H* and *I*). Cells growing in normal medium injected with either GTP or p[CH_2]ppG showed normal microtubules that were readily depolymerized by Colcemid and calcium.

Effect of pp[CH_2]pG-Controlled Tubulin Polymerization *in Vivo* on Cell Translocation and Intracellular Movement. Cells containing bundles of microtubules induced by pp[CH_2]pG showed impeded cell translocation (Swiss 3T3 fibroblasts) and restriction of saltatory movement (PtK2, Swiss 3T3 fibroblasts) to areas containing microtubule bundles. Cells containing tubulin ribbons polymerized by pp[CH_2]pG in the presence of Colcemid showed neither translocation nor saltatory movement. Both translocation and saltatory movement were also absent in cells containing large numbers of short single randomly oriented microtubules polymerized by pp[CH_2]pG from soluble tubulin.

Effect of pp[CH_2]pG-Controlled Tubulin Polymerization *in Vivo* on the Integrity and Location of the Golgi Complex in A549 Cells. Incubation of A549 cells with Colcemid for 2 hr resulted in both complete microtubule depolymerization and dispersion of the Golgi elements (compare Fig. 2 *A* and *B* with Fig. 2 *C* and *D*). Injection of pp[CH_2]pG into cells prevented the dispersion of both microtubules and the Golgi complex by Colcemid (Fig. 2 *E* and *F*). However, dispersion of the Golgi complex by Colcemid was not reversed by the polymerization of tubulin into ribbons on injection of pp[CH_2]pG into cells incubated with Colcemid (Fig. 2 *C* and *D*). Moreover, although uninjected cells treated with Colcemid recovered their normal microtubules and reassembled the Golgi complex near the nucleus after the removal of Colcemid, cells injected with pp[CH_2]pG assembled large numbers of short single randomly oriented microtubules but failed to reorganize the dispersed Golgi complex (Fig. 2 *G* and *H*). Cells injected with either GTP or p[CH_2]ppG showed intact Golgi complexes in typical perinuclear position. The Golgi complexes of these cells were fragmented into vesicles on incubation of the cells with Colcemid and reassembled in the vicinity of the nucleus after removal of the drug.

DISCUSSION

Tubulin can be induced to polymerize into microtubules or ribbons and the distribution and size of the microtubules can be controlled *in vivo* by injection of pp[CH_2]pG into cells incubated in the absence or presence of Colcemid. This manipulation of tubulin polymerization *in vivo* has allowed us to study the role of microtubules in cell translocation, in intracellular movement, and in the organization of Golgi elements containing the enzyme β-galactosyltransferase. We observed that both the loss of microtubule orientation, when polymerization occurred away from the microtubule organizing center (i.e., short randomly oriented microtubules), and the substitution of tubulin ribbons from microtubules resulted in suppression of cell translocation and saltatory movement. Similarly, the change in the distribution of cytoplasmic microtubules that followed the bundling of microtubules in normal cells injected with pp[CH_2]pG resulted in suppression of cell translocation as well as of saltatory movement in areas of the cell devoid of microtubules. These results indicate that properly oriented microtubules of the right size and distribution are required for normal cell translocation and saltatory movement. It is noteworthy that cells having Colcemid-resistant bundles of microtubules polymerized by pp[CH_2]pG showed active saltatory movement in the vicinity of the microtubule bundles. The resistance of microtubules to Colcemid has been ascribed to suppression of microtubule treadmilling (6) and both have been shown to be properties of microtubules polymerized *in vitro* by pp[CH_2]pG (8). Our results suggest that at least some saltatory movement can occur in the absence of microtubule treadmilling.

The dispersion of the Golgi complex that followed the depolymerization of microtubules by Colcemid has led to the assumption that microtubules play a role in the organization and location of the Golgi complex (15). We have observed that, although Colcemid-treated normal cells reconstitute the normal microtubule network and reassemble the Golgi complex in a perinuclear position after removal of the Colcemid, cells induced by pp[CH_2]pG to assemble short randomly oriented microtubules away from the microtubule organizing center were unable to reassemble the Golgi complex. Also interesting was the inability of tubulin ribbons to reconstitute the dispersed Golgi complex in Colcemid-treated cells. These results indicate that both the integrity and the location of the Golgi complex in interphase cells are maintained by microtubules organized by the perinuclear microtubule organizing center(s).

In respect to the mechanism of microtubule polymerization *in vivo*, it is important to note the correlation between the ability of pp[CH_2]pG to specifically enhance microtubule nucleation *in vitro* (8) and the assembly of large numbers of short microtubules away from the microtubule organizing center in cells injected with pp[CH_2]pG. This result supports the hypothesis that in interphase cells the polymerization of microtubules other than at the microtubule organizing center might be prevented by maintaining the cytoplasmic concentration of tubulin below the minimum (i.e., critical tubulin concentration) required to nucleate microtubules in the presence of GTP (16).

We wish to thank Drs. I. Pastan and R. Klausner for their support, Drs. J. V. Kilmartin and E. G. Berger for providing antibodies, and Dr. C. A. L. S. Colaco for critical reading of the manuscript. J.W. was supported by a fellowship from the European Molecular Biology Organization.

1. Weisenberg, R. C., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry* **7**, 4466–4479.
2. Jacobs, M., Smith, H. & Taylor, E. W. (1974) *J. Mol. Biol.* **89**, 455–468.
3. Oosawa, F. & Kasai, M. (1962) *J. Mol. Biol.* **4**, 10–21.
4. Weisenberg, R. C. (1972) *Science* **177**, 1104–1105.
5. Penningroth, S. M., Cleveland, D. M. & Kirschner, M. W. (1976) *Cell Motility*, eds. Goldman, C. R., Pollard, T. D. & Rosenbaum, J. L. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 1233–1257.
6. Margolis, R. L. & Wilson, L. (1980) *Cell* **13**, 1–8.
7. Brinkley, B. R., Fuller, G. M. & Highfield, D. P. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4981–4985.
8. Sandoval, I. V. & Weber, K. (1980) *J. Biol. Chem.* **255**, 6966–6974.
9. Sandoval, I. V., MacDonald, E., Jamesson, J. L. & Cuatrecasas, P. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4881–4885.
10. Sandoval, I. V. & Weber, K. (1979) *J. Mol. Biol.* **134**, 159–172.
11. Wehland, J., Osborn, M. & Weber, K. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5613–5617.
12. Kilmartin, J. V., Wright, B. & Milstein, C. (1982) *J. Cell Biol.* **93**, 576–582.
13. Berger, E. G., Mandel, T. & Schilt, U. (1981) *J. Histochem. Cytochem.* **29**, 364–370.
14. Willingham, M. C. & Pastan, I. (1978) *Cell* **13**, 501–507.
15. Thyberg, J., Piasek, A. & Moskalewski, S. (1980) *J. Cell Sci.* **45**, 42–58.
16. Kirschner, M. W. (1980) *J. Cell Biol.* **86**, 330–334.