BINDING OF MICROTUBULES TO PITUITARY SECRETORY GRANULES AND SECRETORY GRANULE MEMBRANES

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ABSTRACT

Microtubules assembled in vitro were bound to purified porcine pituitary secretory granules and to isolated granule membranes. The interaction between microtubules and whole secretory granules was demonstrated by alteration in the sedimentation properties of the microtubules. Incubation of secretory granules with microtubules resulted in pelleting of microtubules which increased as a function of the number of granules added. Binding was quantitated by measurement of the tubulin remaining in the supernate after centrifugation. The interaction of secretory granules and microtubules was inhibited by nucleoside triphosphates and augmented by adenosine 5'-monophosphate and adenosine. When depolymerized protein from microtubules was incubated with secretory granules, the granules did not appear to bind the soluble tubulin dimer present in these preparations. However, the high molecular weight protein associated with microtubules was adsorbed by secretory granules during the binding process. Incubation of isolated secretory granule membranes with microtubules followed by centrifugation to density equilibrium in a discontinuous sucrose density gradient caused pelleting of the membranes, which otherwise banded higher in the gradient. The visible alteration in membrane sedimentation was confirmed by measurements of the membrane-associated magnesium-ATPase activity and by a shift in radioactivity in iodinated membrane preparations. Our data suggest a role for microtubules in the intracellular movement of secretory granules; this movement is perhaps brought about by dynein-like cross bridges which link the tubulin backbone and granule surface.

In recent years, numerous studies have suggested that microtubule integrity is essential for normal secretion to occur (8, 11, 16, 22-24, 27, 28, 42, 43, 48). The bulk of the evidence for this proposition rests upon the ability of the microtubule-disruptive agents vinblastine and colchicine (49) to block the release of a variety of secretory products. Despite the general agreement that microtubules are necessary for secretion, their exact role is unclear.

Those cells which release proteins, peptides, and neurotransmitters package the product in the Golgi apparatus within membrane-bounded vesicles or granules. Upon the appropriate stimulus, the granule membrane fuses with the cell membrane and exocytosis occurs. Movement of secre-

tory granules from their site of packaging in the Golgi apparatus to the periphery of the cell subjacent to the plasma membrane is a necessary prerequisite to secretion. Microtubules might facilitate this interior-to-surface movement either actively, providing the energy for such movement as well as giving it direction, or passively, by providing a pathway or track along which granules could move. Either hypothesis requires that there be intimate contact between granules and microtubules, something which has been only infrequently observed. The difficulty in defining the exact relationship between these two organelles in the intact cell has led us to study their interaction in the test tube.

MATERIALS AND METHODS

Materials

Colchicine, 2(N-morpholino) ethane sulfonic acid (MES), ethylene-glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP), uridine 5'-triphosphate (UTP), inosine 5'-triphosphate (ITP), thymidine 5'-triphosphate (TTP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphoric acid (AMP), and adenosine were purchased from Sigma Chemical Co., St. Louis, Mo. [3][H]colchicine was purchased from New England Nuclear, Boston, Mass.

Preparative Procedures

Highly purified, large secretory granules enriched in growth hormone and prolactin were prepared from porcine anterior pituitary glands by the method of Jacobs et al. (19), modified so that the last purification step was the discontinuous sucrose gradient procedure described by Poirier et al. (34). These granules are similar to rat pituitary secretory granules (13). Granules were stored until use at 4°C in 0.3 M sucrose containing 50 mM Tris, pH 7.4.

Granule membranes were prepared by hypotonic lysis. Purified granules were suspended in a 15-fold excess of distilled water and, after 60 min of stirring at 4°C, the solution was centrifuged for 1.5 h at 25,000 rpm (SW 27.1 rotor [Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.]) in a discontinuous sucrose gradient. Granule membranes were concentrated at the water −1.14d sucrose and 1.14d-1.18d sucrose interfaces. These interfaces were carefully removed, the samples were diluted 1:4 in water, and the membranes were collected by sedimentation at 45,000 g.

Microtubule protein was prepared from rat brain through two cycles of polymerization-depolymerization (36) and was stored in buffer W (47) (100 mM MES, 0.5 mM MgCl$_2$, 1 mM GTP, 1 mM EGTA, pH 6.4, at 4°C)

in 1-ml aliquots (~3 mg protein/ml) at −80°C until use. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis revealed only tubulin (90% of the protein) and a high molecular weight (HMW) doublet (5-10% of the protein). 1 mol of tubulin (assumed mol wt 110,000) from this preparation bound approximately 0.55 mol of colchicine. Microtubules were made by adding 1 ml of buffer W containing 8 M glycerol (W + 8G) to 1 ml of microtubule protein and incubating for 1 h at 37°C. The solution was then centrifuged at 100,000 g for 1 h at 25°C and the tubule pellet, which contained 60% of the original starting material, was resuspended in 1.2 ml of buffer W containing 4 M glycerol (W + 4G) at 25°C by homogenizing with 30 strokes in a 2-ml Kontes hand homogenizer (Kontes Co., Vineland, N. J.). Tubules could be stored in this buffer at room temperature for at least a week with only minimal (<10%) loss of colchicine-binding capacity or ability to bind to granules.

Incubation

Incubations were carried out in 6 × 50 mm disposable glass test tubes. A complete reaction mixture contained secretory granules (240-350 μg of protein), microtubules (20-40 μg of protein), and other agents as indicated, in 60 μl of W + 4G. After incubation for 5 min at 37°C, the tubes were centrifuged to sediment the secretory granules.

In preliminary experiments (not shown) it was determined that, in the 4 M glycerol used, a force of 10,000 g for 10 min was sufficient to pellet secretory granules completely, whereas less than 10% of microtubules were pelleted under these conditions.

Binding Assay

These observations provided a basis for detecting and quantitating an interaction between microtubules and secretory granules. Microtubules were incubated in the presence or absence of granules, then centrifuged as described. Duplicate 20-μl samples of each supernate from this centrifugation were diluted 1:6 in buffer W containing 1% bovine serum albumin (W + 1% BSA), incubated for 1 h at 4°C to depolymerize the microtubules, then assayed for tubulin by [3][H]colchicine binding as previously described (37). The sedimented pellets were gently rinsed with 50 μl of W + 4G, resuspended in 300 μl of W + 1% BSA, and incubated for 60 min at 4°C to depolymerize microtubules. After resedimentation of granules, tubulin was assayed in the supernate by colchicine binding.

No colchicine binding was detected in supernates or pellets of tubes incubated with granules but without added tubules. In control tubes lacking granules, essentially all the expected colchicine-binding activity was detected when correction was made for incomplete microtubule depolymerization (65-70% depolymerized) under these experimental conditions. In any given experiment, the range of triplicate determinations of tubulin by colchicine binding was within 5% of the mean value.

Iodination of Secretory
Granule Membranes

100 μg of secretory granule membrane protein were iodinated with 1 mCi ^{125}I-Na and lactoperoxidase (44) in 0.5 m NaPO$_4$ at pH 7.6; 100 ng of H$_2$O$_2$ were present in the reaction mixture. After 60 s reaction time at room temperature, 40% of the iodide was incorporated into membranes. Dialysis against a 100-fold excess of water (three changes in 12 h) was used to remove unreacted iodide. The labeled membranes were then removed from the dialysis bag, and 100 μl of diluted labeled membranes were mixed with an equal volume of unlabeled membranes before incubation with preformed microtubules.

Protein was determined by the method of Lowry et al. (25). SDS-polyacrylamide gel electrophoresis was carried out by the method of Neville (32). The significance of differences between means was determined by Student's *t*-test.

RESULTS

When microtubules and granules were incubated together as described, granules were found to adsorb a significant fraction of the total tubulin, and therefore of microtubules, contained in the mixture (Fig. 1). The amount of tubulin adsorbed by the granules is equal to the difference between the quantity of tubulin in the granule pellet and that pelleted in the absence of granules. This value is equal to the difference in tubulin content between the two supernates. Since the presence of granules altered only the partition of tubulin between the pellet and supernate and did not affect total tubulin recovery, adsorption was computed in subsequent studies from the difference between supernates, as this determination, although indirect, was technically somewhat simpler. The amount of tubulin bound as a function of the amount of granules added is shown in Fig. 2.

To determine whether microtubule integrity is required for adsorption to occur, depolymerized microtubule protein was incubated with secretory granules and the percent adsorption of tubulin (measured by colchicine binding activity) was compared to that of intact microtubules formed from the same batch of protein. While there was some adsorption to secretory granules of tubulin from the depolymerized microtubule preparation (12 ± 3%), it was considerably less than that which occurred when intact tubules were used (30 ± 4%). In the above experiment the granules and depolymerized microtubule protein were incubated together under conditions ideal for microtu-
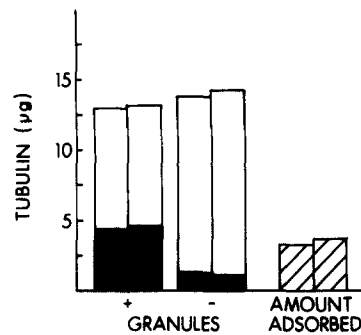

FIGURE 1 Adsorption of microtubules to porcine pituitary secretory granules. Tubules, with (+) or without (−) granules, were incubated and centrifuged as described in Materials and Methods. Open bars represent the amount of tubulin remaining in the supernate. Closed bars represent the amount of tubulin pelleted. Cross-hatched bars represent the amount of tubulin pelleted in the presence of granules minus the amount pelleted in their absence.
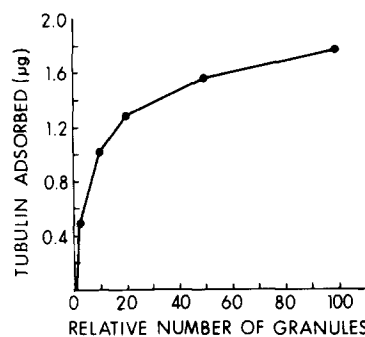

FIGURE 2 Dose response of microtubule adsorption by granules. Microtubules (8 μg of protein) and granules (100 = 225 μg of protein) were incubated and centrifuged as described in Materials and Methods. The difference in tubulin content of the supernate in the absence and presence of granules was taken as the amount of tubulin adsorbed by the granules.

bule polymerization. It seemed possible, therefore, that adsorption of tubulin from a solution of soluble microtubule protein might in fact have been due to adsorption of microtubules which had formed during the incubation period. To test this hypothesis, granules were incubated with microtubule protein in the presence and absence of 6 mM calcium. Since the W + 4G buffer contains 1 mM EGTA, a free calcium concentration of approximately 5 mM resulted. This concentration should be more than sufficient to block microtubule formation (47). It can be seen from Fig. 3 that, while
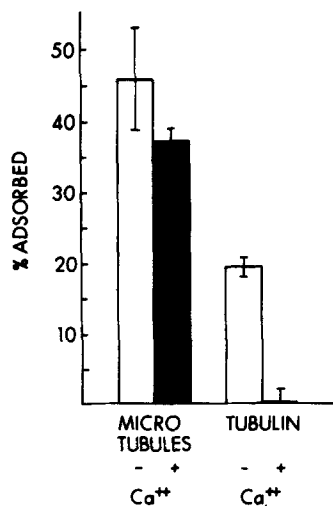
FIGURE 3 Effect of calcium on microtubule and tubulin adsorption by granules. An equal amount of microtubule protein in the polymerized (microtubules) or depolymerized (tubulin) state was incubated with granules in the absence (open bars) or presence (closed bars) of 6 mM Ca^{++}. Tubulin adsorption was determined as described in Materials and Methods and in the legends to Figs. 1 and 2.

Granules did not detectably adsorb tubules in buffer W with or without 0.3 M sucrose, or in Tris buffer alone. Glycerol was required during the binding interaction since granules preincubated with W + 4 M glycerol, centrifuged, then resuspended in W did not bind tubules during a subsequent incubation in the absence of glycerol. Since glycerol has been recently reported to bind tubulin (10), it seemed possible that this binding during the preparative procedure either might modify tubulin or might select for isolation classes of microtubules which require glycerol for some of their
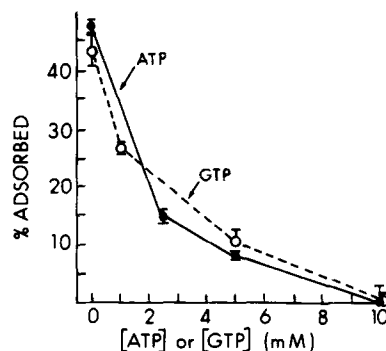

FIGURE 4 Inhibition of granule-microtubule interaction by ATP and GTP. Granules and tubules were incubated together, and tubulin adsorption was determined as described in Materials and Methods and in the legends to Figs. 1 and 2. Stock ATP and GTP solutions (30 mM) in buffer W were readjusted to pH 6.5 with 10 N NaOH before use.

calcium had little or no effect on the adsorption of preformed tubules by granules, it completely prevented adsorption of tubulin from a solution of soluble microtubule protein, supporting the hypothesis that depolymerized tubulin is not adsorbed to secretory granules in vitro.

A number of substances were found to influence the interaction between microtubules and secretory granules. Both ATP and GTP (Fig. 4) inhibited binding 50% at a concentration between 1 and 2 mM, and other nucleoside triphosphates were almost as effective (Table I). In contrast, ADP had no significant effect and both AMP and adenosine augmented the interaction (Table II). NaCl and KCl had no effect in concentrations up to 50 mM. The inhibitory effect of ATP on the granule-tubule interaction was found to occur even in the absence of magnesium. ATP at 2.5 mM reduced control adsorption from 22.7 ± 1.14% to 8.7 ± 3.4% in buffer W containing 1 mM ethylenediamine tetraacetic acid (EDTA) and no added magnesium. The nonhydrolyzable ATP analogue AMP-P-N-P (5 mM) inhibited tubulin adsorption from 44.8 ± 1.39% in the absence of nucleotide to 17.9 ± 2.9%; in the same experiment, ATP (5 mM) caused more profound inhibition, to 4.37 ± 1.33%.

TABLE I

Effect of Nucleoside Triphosphates on Secretory Granule-Microtubule Interaction*

Nucleoside triphosphate	Tubulin adsorbed
	% ± SEM
None	65.4 ± 6.58
ATP	17.9 ± 0.47
GTP	20.5 ± 4.30
CTP	21.0 ± 2.77
UTP	24.6 ± 3.32
ITP	24.5 ± 3.61
TTP	32.7 ± 5.81

* Granules and tubules were incubated together, and tubulin adsorption was determined as described in Materials and Methods and in the legends to Figs. 1 and 2. All stock nucleoside triphosphate solutions (15 mM) in buffer W were readjusted to pH 6.5 with 10 N NaOH before use. Final concentration of nucleoside triphosphates during incubation was 5 mM. All incubations were done in triplicate.

Nucleotide	Tubulin adsorbed
	% ± SEM
None	34.1 ± 1.87
ATP	6.7 ± 1.96
ADP	31.6 ± 1.01
AMP	45.7 ± 1.33
Adenosine	52.6 ± 1.50

* Granules and tubules were incubated together, and tubulin adsorption was determined as described in Materials and Methods and in the legends to Figs. 1 and 2. All stock solutions in buffer W were readjusted to pH 6.5 with 10 N NaOH before use. All incubations were done in triplicate.

functions. We therefore prepared microtubules which had never been exposed to glycerol, in order to test their ability to bind secretory granules in the absence of glycerol. Although both the yield and the colchicine-binding activity of tubulin prepared from rat brain without glycerol were approximately an order of magnitude less than what we had observed when glycerol was used in the preparative procedure, we were able nonetheless to establish that addition of secretory granules to these microtubules in the absence of glycerol resulted in reproducible adsorption of colchicine-binding activity to the granules. Adsorption of 25 and 40% was noted in two experiments. Thus, microtubules prepared in the absence of glycerol were capable of binding secretory granules without glycerol. Nevertheless, these experiments must be interpreted with caution. The total colchicine binding was small, and in order to draw definitive conclusions, it will be necessary to characterize much more extensively the interaction between secretory granules and microtubules prepared without glycerol.

Presumably, any in vivo interactions between microtubules and granules as well as the interactions we have demonstrated in vitro are mediated via surface contact involving the granule membranes; therefore, it was important to see if adsorption of microtubules to isolated granule membranes could take place in vitro. To detect such an interaction, a sucrose gradient system had to be devised which would separate the two components when each was applied alone. Microtubules applied to the top of a 10–30% discontinuous sucrose gradient in W + 4G and centrifuged at 25°C

for 2 h in an SW 41 rotor at 20,000 rpm (g_{avg} = 48,000) were recovered in a pellet at the bottom of the tube. Secretory granule membranes centrifuged under identical conditions collected predominantly at the 20–25% interface and 25–30% interface or at the 20–30% interface when larger gradient steps were used. When granule membranes and microtubules were incubated together for 15 min at 37°C in W + 4G and then centrifuged, the membranes and tubules were recovered together in a pellet at the bottom of the tube. The membrane bands previously visible at the 10–20% and 20–30% interfaces were no longer present and the distribution of radioactive membranes in the gradient confirmed that a large increase in sedimentable particles had occurred after incubation with microtubules (Fig. 5). The visible shift in the secretory granule membrane position and the
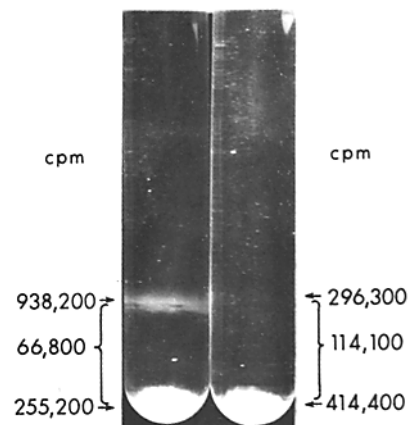

FIGURE 5 Binding of SG membranes by microtubules. Secretory granule membranes were iodinated as described in the text. Iodinated membranes after dialysis were mixed with unlabeled membranes, and aliquots of the mixture were incubated at 37°C for 15 min with or without 200 μg of microtubules. Incubation mixtures were placed on the top of a discontinuous sucrose gradient (10, 20, and 30% sucrose in W + 4G) and centrifuged at 20,000 rpm for 2 h in the SW 41 rotor. The tube on the left shows membranes banded at the 20–30% sucrose interface after control incubation. This band is not present in the tube on the right, and the detected radioactivity reflects this difference. Although reflections at the bottoms of the tubes make it impossible to see it in this photograph, there was a visible pellet in the right-hand tube not present in the control. Cpm = counts per minute of radioactivity. Counts are recorded for the 20–30% sucrose interface, removed with a bent needle, for the remainder of the 30% sucrose, as indicated by the bracket, and for the pellet after removal of all fluid.

observed shift in radioactivity were confirmed by ATPase measurements. The ATPase activity associated with the secretory granule membranes underwent a similar shift in position from the interfaces to the pellet when the membranes were centrifuged with microtubules (Table III).

To obtain qualitative information on the adsorbed protein species, polyacrylamide gel electrophoresis of soluble microtubule protein incubated (in the presence of 6 mM Ca^{++}) with and without granules was carried out. The electropherogram shown in Fig. 6 demonstrates that, while there is some adsorption of tubulin by the granules, there is selective loss of the high molecular weight protein.

DISCUSSION

A central issue raised by all studies of biological phenomena in a noncellular environment is the relationship between the behavior of the system in vitro and the situation in the living cell. This study

TABLE III

Effect of Microtubules on Sedimentation of Magnesium-ATPase Activity of Secretory Granule Membranes *

Fraction	ATPase activity			
	Control	(%)	Microtubules	(%)
1	7.04	(3.8)	9.8	(6.2)
2	2.30	(1.3)	7.4	(4.7)
3	2.06	(1.1)	11.6	(7.4)
4	0	(0)	13.7	(8.7)
5	154.60	(83.0)	60.2	(38.3)
6	20.2	(10.8)	54.6	(34.7)

* Secretory granule membranes were incubated with or without added microtubules as described in the text, then layered onto the top of a discontinuous sucrose gradient (10, 15, 20, 25, and 30% sucrose, all in W + 4G), and centrifuged in the SW 41 rotor at 20,000 rpm for 2 h. Each interface was aspirated sequentially; fractions 1-6 correspond to the 5 interfaces and the pellet, respectively, from top to bottom. ATPase activity was measured using gamma-labeled $AT^{32}P$ with 3.5 mM ATP and 2.0 mM magnesium at pH 8.0 and 37°C for 30 min. The reaction was stopped with SDS at a final concentration of 1%, and the inorganic phosphate extracted into xylene-isobutanol, 65:35 (vol:vol). These conditions extracted 98-100% of P_i, and less than 0.6% of fresh labeled ATP preparations (Bruns, D., R. M. Smith, J. McDonald, and L. Jarett, unpublished method). The results are expressed as micromoles ATP hydrolyzed per total fraction in 30 min. Percentages refer to the proportion of recovered radioactivity found in each fraction.

demonstrates that, under certain in vitro conditions, microtubules and secretory granules are capable of interacting with one another. As such, it may represent a step towards understanding the role of microtubules in secretion. However, before definitive conclusions can be drawn regarding the secretory process, we will need to know how well the present model system mirrors the circumstances inside secreting cells.

For example, the apparent requirement for a high concentration of glycerol during microtubule-granule interaction may indicate that the two organelles will adhere only under highly artificial conditions. On the other hand, glycerol is known to facilitate electrostatic interactions as a consequence of the lower dielectric constant of solutions containing glycerol in high concentrations. Since the precise physicochemical state of the environment surrounding tubules within the cell is unknown, it seems possible that glycerol may in fact be more "physiologic" than an entirely aqueous medium. The requirement for the actual presence of glycerol, not substituted by pretreatment and then removel of the glycerol, suggests that the effect of glycerol is to provide a hospitable environment for granule-tubule interaction and not to damage or distort the secretory granules so as to render them capable of binding microtubules.

The mechanism by which nucleoside triphosphates inhibit the interaction of granules and tubules is unclear, although there may be some analogy with the effect of ATP to dissociate actomyosin. A number of nucleoside triphosphates other than ATP can dissociate actomyosin (17, 45). Similarly, GTP (Fig. 4) as well as other triphosphates (Table II) are effective inhibitors of the granule-tubule interaction. The ability of AMP-P-N-P to inhibit adsorption of microtubules to granules indicates that hydrolysis of the terminal phosphate is not required for inhibition of microtubule adsorption to granules. Similarly, actomyosin dissociation does not require such hydrolysis (50, 51). Although magnesium appears to be an absolute requirement for the dissociation of actomyosin induced by ATP, it is not required for inhibition of tubule-granule interaction. In the muscle system, even calcium-ATP cannot substitute, though it binds to actomyosin (46). The dose of ATP required for half-maximal actomyosin dissociation (~0.05 mM at physiological ionic strength) is considerably lower than that required for half-maximal inhibition of the adsorption of tubules to granules (~2 mM, see Fig. 4).
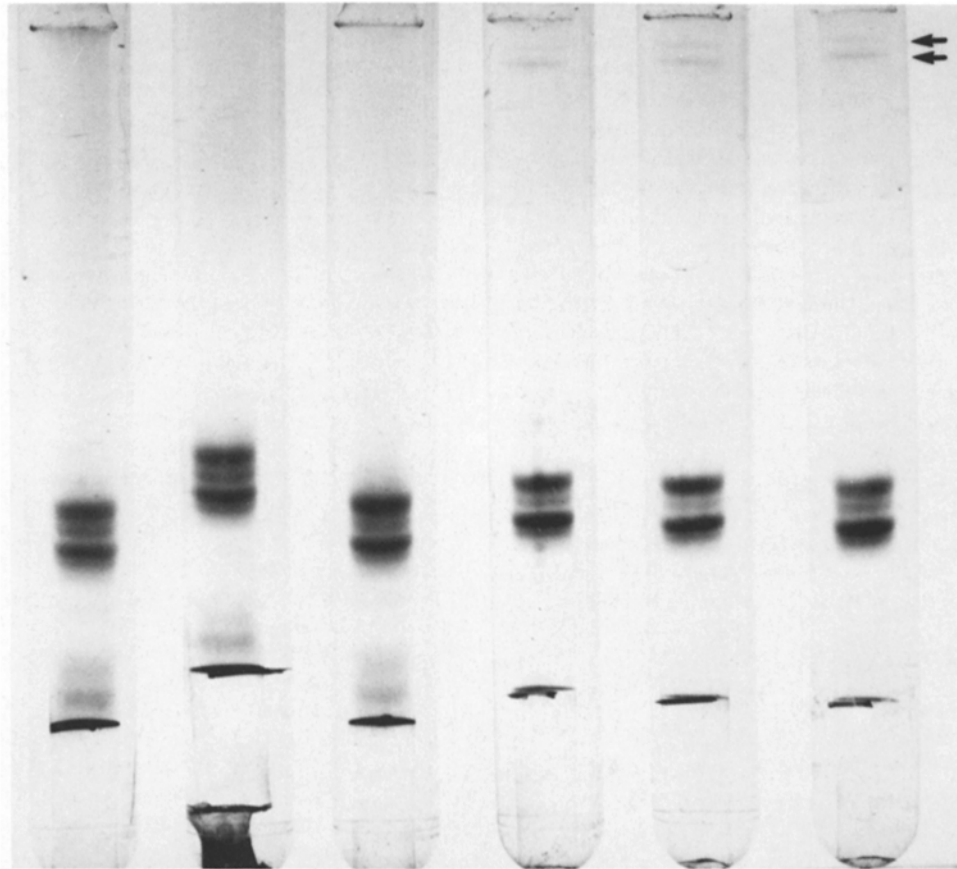
FIGURE 6 Polyacrylamide gel electrophoresis of microtubule protein. These gels show the microtubule protein remaining in the supernate after incubation and centrifugation in the presence (three gels on the left) or absence (three gels on the right) of granules. In order to prevent microtubule assembly, 6 mM Ca^{++} was present during incubation. Note the absence of the high molecular weight bands (arrows) in the gels of protein from the three tubes which contained granules. The bands migrating ahead of tubulin in these gels represent granule proteins.

The lack of requirement for magnesium and the fact that all of the nucleoside triphosphates tested inhibit to about the same degree may indicate that the blocking phenomenon is a nonspecific charge effect. Against this, however, is the lack of effect of up to 50 mM sodium or potassium chloride and the relatively physiological concentrations of nucleoside triphosphates required to demonstrate inhibition. The lack of effect of ADP tends to support the argument that the inhibition is specific. The mechanism by which adenosine and, to a lesser extent, AMP augment granule-tubule adherence is not understood.

Since isolated granule membranes bind to tubules, it is likely that the tubules are binding to the granule-membrane surface in the experiments in which intact granules were used. Microtubules have been observed in association with a wide variety of membrane-bounded structures including, in addition to secretory granules and chromosomes, synaptic vesicles (1, 20), mitochondria (35, 38, 39), melanosomes (3, 30), endoplasmic reticulum (38), cell membranes (2, 38), nuclei (18), and viruses (26). The extensive evidence that microtubules are involved in so many different cell processes (33) also tends to support the idea (albeit indirectly) that they may be adapted to interact with many cell structures under appropriate conditions.

The finding that the HMW proteins associated with microtubules (5, 9) are preferentially adsorbed by secretory granules is of great interest

especially in the light of recent studies which indicate that these proteins are located on the surface of microtubules assembled in vitro (9) and form periodic arms similar to those seen on ciliary and flagellar outer doublet microtubules (31). The preferential adsorption of the HMW proteins by granules and their arrangement on the surface of the tubule suggest that they provide the link between the tubule backbone and secretory granule. If this is indeed the case, it would imply that tubulin would sediment with granules only insofar as it is associated with the HMW proteins. In preparations of depolymerized microtubule protein, where most of the tubulin presumably exists as a soluble dimer (21), little or no adsorption would be expected. Interestingly, while no tubulin adsorption whatsoever is detectable by the colchicine-binding assay, a small but reproducible diminution of the tubulin bands is seen on gels of microtubule protein after exposure to granules. This may be explained by the existence of ring forms of tubulin (4, 5, 21) with which the HMW protein may be associated. Tubulin within these rings apparently does not bind colchicine (21) and thus its depletion would only be detectable on gels.

It has been suggested that the HMW proteins associated with cytoplasmic tubules may be similar to flagellar dynein although the proteins from the two sources are said to have slightly different electrophoretic mobilities on SDS gels (5, 9, 29). The work of Gibbons and co-workers has shown that the dynein ATPase provides the energy for sliding between adjacent outer doublet microtubules which results in flagellar bending (6, 14, 15, 40, 41). It is unclear at present whether the HMW protein associated with cytoplasmic tubules has ATPase activity (7, 9, 12, 48). A key question in intracellular organelle translocation is the source of the power for movement. If the HMW protein is truly homologous with dynein, perhaps it not only links tubules with other organelles but powers their motion relative to the tubule as well.

In conclusion, we have demonstrated the in vitro interaction of microtubules with adenohypophysial secretory granules. These data support the concept that microtubules may play an important role in the secretion of growth hormone and prolactin. Further progress towards the in vitro reconstitution of the elements of the secretory machinery should help us to understand secretion as well as other biological processes involving the movement of subcellular organelles.

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