MICROTUBULES AND AXOPLASMIC TRANSPORT

Inhibition of Transport by Podophyllotoxin: an Interaction with Microtubule Protein

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ABSTRACT

Pharmacological evidence is presented for the involvement of microtubules in the process of fast axoplasmic transport. A quantitative measure of the inhibition of axoplasmic transport in an in vitro preparation of rat sciatic nerve is described. The alkaloids colchicine, podophyllotoxin, and vinblastine, which are known both to disrupt microtubules and to bind to the protein subunit of microtubules, are inhibitors of axoplasmic transport. Lumicolchicine and picropodophyllin, unlike their respective isomers colchicine and podophyllotoxin, are poor inhibitors of axoplasmic transport. The dissociation constants for the binding of colchicine, lumicolchicine, podophyllotoxin, and picropodophyllin to purified microtubule protein from rat brain have been measured. Inhibition of axoplasmic transport by these drugs correlates favorably with their affinities for microtubule protein.

The movement of material from a neuronal cell body through its axon toward nerve endings is called axoplasmic transport. This process is characterized in mammalian nerves by a slow rate of 1-3 mm/day and a fast rate of 100-400 mm/day. Ochs and Smith (15) have used inhibitors of glycolysis and oxidative phosphorylation to demonstrate that energy for fast axoplasmic transport is generated locally along the nerve. Little else is known concerning the molecular mechanics of the transport process.

Schmitt (20) has suggested that microtubules might be involved in the process of fast axoplasmic transport. Microtubules, which are found in all axons, are thought to run in a continuous unbranched fashion from the nerve cell body to the nerve ending. The primary evidence for the involvement of microtubules in axoplasmic transport is the inhibition of the transport process by colchicine and vinblastine (See review, reference 12). These two drugs are known to disrupt microtubules in axons (30) and to bind to tubulin, the protein subunit of microtubules (3, 26). Colchicine and vinblastine, however, interact with proteins other than microtubule protein, and inhibit processes not mediated by microtubules (9, 11, 14, 25, 27). Furthermore, electron micrographic studies of nerves in which transport had been inhibited by colchicine reveal microtubules which appear normal (10). Finally, silver grains in electron microscope autoradiographs of axons through which tritiated proteins were being carried by rapid axoplasmic transport lie near the axonal membrane (6, 4). In contrast, microtubules are distrib-

uted more randomly throughout the cytoplasm.

In view of the observations cited above, we wished to examine further the hypothesis that microtubules are involved in the process of axoplasmic transport. To test whether inhibition of transport by colchicine and vinblastine is mediated by a specific action on microtubules, we have considered three additional pharmacological agents: lumicolchicine, podophyllotoxin, and picropodophyllin. Lumicolchicine, a structural isomer of colchicine, has been shown to have little or no affinity for microtubule protein (3, 29). Lumicolchicine does not inhibit the action of vasopressin, while colchicine is a potent inhibitor (24). Nucleoside uptake in HeLa cells, however, is inhibited just as effectively by lumicolchicine as it is by colchicine (14). Differential activity of these two drugs in the first case suggests the involvement of microtubules, while a lack of differential activity in the second case may represent an action of colchicine not related to binding to microtubules. Podophyllotoxin and its diastereomer, picropodophyllin, may also be used as probes of microtubule function. Podophyllotoxin has been shown to compete with colchicine for binding to microtubule protein (3, 29). Picropodophyllin is less effective as a competitive inhibitor of colchicine binding (13, 29). These two pairs of isomers have been studied as inhibitors of fast axoplasmic transport in an in vivo system employing the rat optic nerve (18). Inhibition of transport by colchicine and podophyllotoxin, and lack of inhibition by lumicolchicine and picropodophyllin, is consistent with the involvement of microtubules in axoplasmic transport.

In this paper we have extended the pharmacological evidence for the involvement of microtubules in fast axoplasmic transport. In order to allow quantitative comparison of inhibitors, an in vitro system with which to study axoplasmic transport has been used. Several agents have been tested both as inhibitors in this in vitro system and, in a companion study (13), as ligands for binding to isolated microtubule protein from rat brain. Inhibition of axoplasmic transport by these agents reflects their relative affinity for microtubule protein.

MATERIALS AND METHODS

Qualitative studies of the drug-induced inhibition of rapid axoplasmic transport in rat sciatic nerve have been described (5, 19). A sciatic nerve with the L_6 and L_7 dorsal root ganglia attached is dissected from a female rat anesthetized with pentobarbital and urethane. The nerve is placed on the bottom of a rectangular box separated into three cells by slotted partitions (Fig. 1). By means of grease seals built up around the nerve at the partitions, the cells are isolated from each other. After covering the nerve with TC medium 199 or Krebs-Ringer solution (supplemented with glucose, 1 g/liter), the box is incubated at 37°C in 95% O_2-5% CO_2. L-[^{3}H]Proline is introduced into the first cell, which contains the two ganglia. Proteins labeled with tritium are synthesized by the nerve cell bodies, transported down the nerve, and accumulate at a ligation placed on the nerve in the third cell. The second cell (of about 1 ml volume) is continuously perfused with fresh medium (4 ml/h) to wash out any free L-[^{3}H]proline that might leak past the grease seal. After 12–16 h, the nerve is fixed in 5% trichloroacetic acid to remove any free tritiated proline (8) and is cut into 1-mm sections. Since treatment with trichloroacetic acid shrinks the nerve by 50%, 1-mm sections of the treated nerves represent 2 mm of the fresh tissue. Sections were solubilized with Soluene-100 and counted in a Beckman LS-230 liquid scintillated spectrometer after addition of scintillation cocktail (1).

Materials

Female rats of the NLR strain were procured from National Laboratories, St Louis, Mo. L-[2,3-^{3}H]proline · HCl was obtained from New England Nuclear, Boston, Mass. at a specific activity of 35 Ci/mmol and a concentration of 10 mCi/ml. The tissue solubilizer, Soluene-100, was purchased from Packard Instrument Co., Downers Grove, Ill. In vitro nerve chambers were cast from Sylgard 185 (Dow Corning, Midland, Mich.). TC medium 199 was obtained from Difco Laboratories, Detroit, Mich. Vinblastine sulfate was a gift of Eli Lilly and Co., Indianapolis, Ind. Picropodophyllin was kindly donated by Prof. E. Smissman. Podophyllotoxin was obtained from Delta Scientific Corp., Lindenhurst, N. Y., and colchicine from Sigma Chemical Co., St. Louis, Mo. The picropodophyllin and podophyllotoxin used in this paper have chemical structures defined in a series of papers by Schrecker and Hartwell (21), and should not be confused with podophyllin, which is crude extract from which both compounds can be isolated. A mixture of β- and γ-lumicochicine (referred to in this paper as lumicolchicine) was prepared from colchicine by the method of Wilson and Friedkin (28). Characterization of lumicolchicine is presented elsewhere (13).

RESULTS

Using an in vitro preparation of rat sciatic nerve, a typical transport profile of protein labeled with tritiated proline is presented in Fig. 1. A prominent peak of accumulated radioactivity appears proximal to a ligation (arrow) which is placed on the

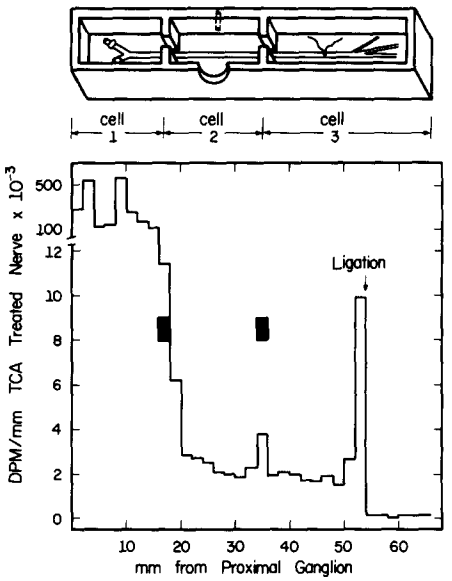

FIGURE 1 In vitro axoplasmic transport through sensory fibers of the rat sciatic nerve. Profile of transported radioactivity from a nerve is plotted as dpm/mm of nerve vs. distance from the L_6 ganglion. Solid blocks represent the position of the grease seals placed between cells. A ligation (arrow) is placed on the nerve in the third cell. No drugs were present. Details of the experiment are given in Methods.

nerve in the third cell. Although the slight peak of radioactivity at the grease seal was sometimes seen, in six control nerves it was never larger, with respect to the peak at the ligation, than that depicted in Fig. 1. If a nerve in which the ganglia were removed is incubated in this system, no tritiated proteins are transported past the first grease seal (19). The presence of an inhibitor of protein synthesis (1 mM cycloheximide) in the first cell will also prevent the appearance of any transported radioactivity in the nerve, although the same drug has no effect upon transport when placed in either or both of the two remaining cells (data not shown).

To test an inhibitor of axoplasmic transport, the drug is dissolved in the medium bathing the nerve in the third cell. In this manner, only the nerve in the third cell is exposed to compound being studied. Inhibition of transport is detected by an increase in the tritium which accumulates in the nerve at the entrance to the third cell, and a corresponding decrease in the peak of material at the ligation. Maximal inhibition is observed when no transported radioactivity enters the third cell.

Three plant alkaloids known to disrupt microtubules (26, 30) were studied in this system. Colchicine, vinblastine, and podophyllotoxin were found to be potent inhibitors of axoplasmic transport. Transport profiles demonstrating equivalent, but not complete inhibition are shown (Fig. 2).

Lumicolchicine (3, 29) and picropodophyllin (29) bind less tightly to microtubule protein than do their respective isomers, colchicine and podophyllotoxin. When tested at concentrations near their limits of solubility, these agents gave transport profiles indistinguishible from controls in which no drug was present (Fig. 3).

The preceding experiments may be compared quantitatively by expressing the amount of radioactivity transported into the third cell as a percentage of the total radioactivity transported into the second and third cells. In practice, when an inhibitor was placed in the third cell the peak of

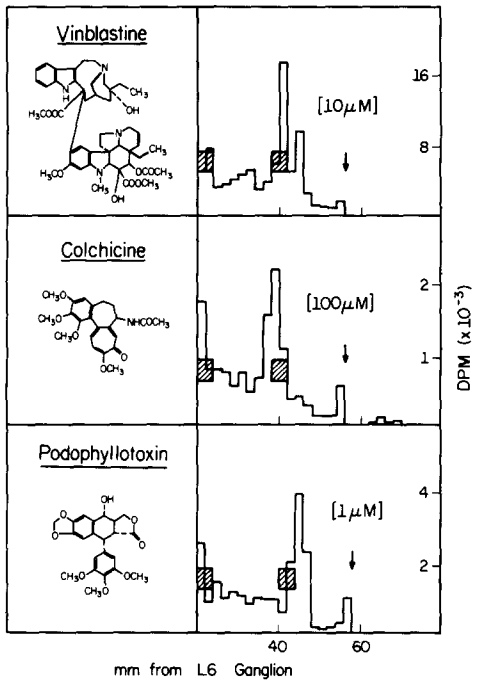

FIGURE 2 Inhibition of axoplasmic transport by microtubule disruptive agents. Examples of transport profiles obtained in the presence of vinblastine (10 μM, top frame), colchicine (100 μM, middle frame), and podophyllotoxin (1 μM, bottom frame) are shown. In each case the drug was dissolved in the medium bathing the nerve in the third cell. Data from the first cell have been omitted as they contain no information relating to the inhibition of transport by a drug placed in the third cell. See Fig. 1.

radioactive material which accumulated at the grease seal extended 4 mm into the third chamber. Therefore, the radioactivity in the two sections distal to the second grease seal was not included as material transported into the third cell. In six control experiments with no test compound in the third cell, the percentage of the radioactivity transported past the second grease seal (%P) was 28.8 ± 8.0. A net transport value (NT) may be calculated for experiments involving inhibitors:

$$NT = \frac{\%P\,(\text{experimental})}{\%P\,(\text{control})}$$

Compounds which did not inhibit would produce an NT value of 1.00, while those giving complete inhibition would produce a value of 0.00. Values between these extremes would indicate partial inhibition.

By this method, data for five compounds have been summarized in Fig. 4. Vinblastine, colchicine, and podophyllotoxin produce a dose-related inhibition of fast axoplasmic transport. In contrast, lumicolchicine and picropodophyllin are not active at the highest doses allowed by the solubility of these compounds.

In the experiments presented above, colchicine never inhibited transport completely. It has been shown that maximal sensitivity of axoplasmic transport to colchicine requires exposures of long duration (10, 18). To establish in this system that colchicine is more active after preincubation, nerves were incubated with the drug in the third cell before introducing L-[^{3}H]proline into the first cell. 6 h of preincubation increase the efficiency of colchicine as an inhibitor of axoplasmic transport by a factor of about 10 (Fig. 5). Under these conditions a concentration of 100 μM produces nearly complete inhibition. A strong block of transport is seen even at 10 μM, a concentration

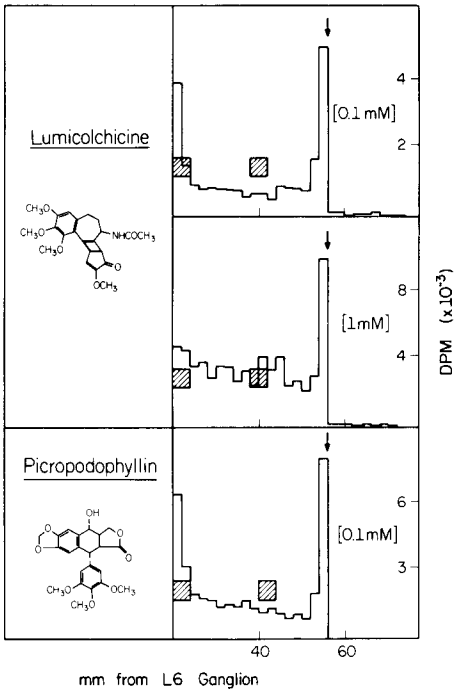

FIGURE 3 Lack of inhibition of transport by lumicolchicine and picropodophyllin. The drugs were present in the third cell, at the given concentrations. For details see the captions to Figs. 1 and 2.

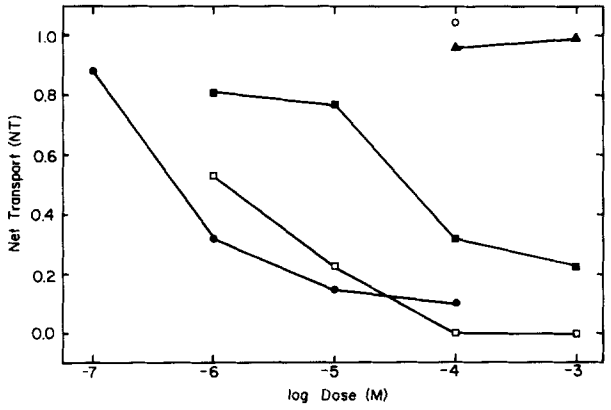

FIGURE 4 Dose response curves of several agents as inhibitors of axoplasmic transport. ●, Podophyllotoxin; ⊙, picropodophyllin; □, vinblastine; ■, colchicine; and ▲, lumicolchicine. Each point is the average of two or three experiments. The range of values about each point is typically ±0.10–0.15. See discussion of net transport value (NT) in Results.

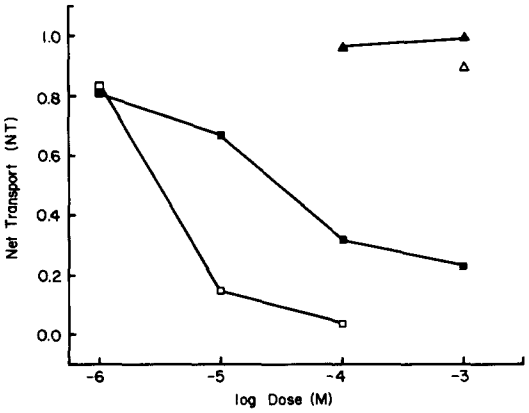

FIGURE 5 Increased inhibition by colchicine and lumicolchicine with 6-h preincubation. Colchicine: ■, no preincubation; □, 6-h preincubation. Lumicolchicine: ▲, no preincubation; △, 6-h preincubation. Each point is the average of two or three experiments. The range of values about each point is typically ±0.10–0.15. For clarity, data with no preincubation are repeated from Fig. 4. See discussion of the net transport value (NT) in Results.

TABLE 1

Correlation for Several Ligands of Affinity for Microtubule Protein and Inhibition of Axoplasmic Transport

Agent	K diss*	Concentration @ NT = 0.5‡
	μM	*μM*
No preincubation		
Colchicine	0.063	36
Lumicolchicine	1,570	No inhibition @ 1,000
Podophyllotoxin	0.28	0.5
Picropodophyllin	37.8	No inhibition @ 1,000
Vinblastine	—	1.1
6-h preincubation		
Colchicine	0.063	2.9
Lumicolchicine	1,570	No inhibition @ 1,000

* Values from reference 13.
‡ Values interpolated from Figs. 4 and 5.

which produced very little effect without preincubation.

In a companion study to this paper we have determined the dissociation constants for the binding of colchicine, lumicolchicine, podophyllotoxin, and picropodophyllin to microtubule protein purified from rat brain (13). Barring problems of permeability into the nerve tissue, inhibition of axoplasmic transport by these drugs might be expected to correlate with their strength of binding to microtubule protein. A comparison of these two parameters is presented in Table I. Colchicine and podophyllotoxin are over 100 times more effective as inhibitors of axoplasmic transport than are their respective isomers, lumicolchicine and picropodophyllin. Colchicine binds microtubule protein with an affinity 25,000 times that of lumicolchicine, and podophyllotoxin binds 135 times more tightly than does picropodophyllin. The differential activity of these two pairs of isomers as ligands for microtubule protein and inhibitors of axoplasmic transport is consistent with the suggestion that colchicine, podophyllotoxin, and vinblastine inhibit transport through their binding to microtubule protein.

DISCUSSION

Evidence presented in this communication suggests that microtubules are in some way required for the process of rapid axoplasmic transport. Colchicine and podophyllotoxin are at least 100 times more active than their respective isomers, lumicolchicine and picropodophyllin, both as inhibitors of axoplasmic transport and as ligands for purified microtubule protein. In addition, vinblastine, which binds porcine brain microtubule protein with an equilibrium constant of 0.17 μM (2, 17, 23), is a potent inhibitor of axoplasmic transport. While a good qualitative correlation exists between the affinity of these agents for microtubule protein and their potency as antitransport agents, the lack of a more quantitative correlation is not surprising. The fact that colchicine is a more potent inhibitor of transport with increasing periods of incubation with the nerve suggests that equilibrium within the cell between colchicine and microtubule protein may not be established during the time of these experiments.

The actual role of microtubules in the molecular mechanism of axoplasmic transport is not clear. As originally suggested by Schmitt and Samson (20), materials carried by axoplasmic flow may be packaged in vesicles which move down the axon through a mechanochemical coupling to microtubules. The only clear demonstration of vesicle-microtubule interaction which has been reported, however, in axons in the spinal cord of *Petromyzon marinus* (22). Recently, it has been shown that movement of protein and particles in membranes is inhibited in a number of systems by colchicine, vinblastine, and podophyllotoxin (7, 16, 31). In one case, inhibition of the movement of concanavalin A receptors by colchicine is not mimicked by an equivalent dose of lumicolchicine

(16). It may prove fruitful to consider the possibility that microtubules, or a protein similar to microtubule protein, play a role in the lateral movement of material in membranes of axons. If so, the movement of materials by fast axoplasmic transport may occur in some type of membrane element which runs continously from the nerve cell body to the synaptic ending. Such a membranous element could be either the axolemma itself, or the smooth endoplasmic reticulum.

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