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Effects of Mitosis Inhibitors on Respiration and Fast Axonal Transport in Frog Sciatic Nerves

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SUMMARY

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The effects of mitosis inhibitors on fast axonal transport of ^{3}H-labeled proteins and $^{14}CO_2$ production from radioactive glucose were studied *in vitro*. Colchicine and lumicolchicine depressed respiration to the same extent but only the former inhibited axonal transport. Nocodazole had effects similar to those of colchicine, whereas podophyllotoxin affected only transport. A stimulation of respiration was found with vinblastine at transport-inhibitory concentrations. None of the drugs affected protein synthesis in the dorsal ganglia or the amplitude and propagation of the compound action potential. The results do not suggest a close coupling and feedback between axonal transport and respiration. Alternatively, the energy requirements of axonal transport are too small to be detectable with the present system. The drug-induced changes in respiration were probably not caused by effects on motility-related functions of microtubules and were not sufficient to depress or stimulate two other energy-requiring processes, protein synthesis and electrical activity.

INTRODUCTION

Mitosis inhibitors have been used extensively to study the mechanisms of AXT[1] (for a review see ref. 1) and other MT-dependent processes (2). Unspecific effects have been reported for the most-used mitosis inhibitors (1) and, especially when used at high concentrations, such effects must be considered. For some of the drugs, non-MT binding analogues have been prepared and shown to have small effects on AXT (3, 4).

The only known common property of the antimitotic substances is their ability to bind to tubulin, the subunits of MTs, whereas their unspecific effects vary. Therefore their inhibitory effects on AXT have been considered evidence for the involvement of MTs in AXT. AXT is dependent on a continuous supply of energy, probably in the form of ATP (5), and the effects of drugs on its energy metabolism are difficult to distinguish from those on MTs (6). As a measure of unspecific effects by various transport inhibitory drugs, we have previously studied how they affect protein synthesis in the dorsal ganglia (7), the levels of high-energy phosphates (8), and the amplitude and propagation of the compound action potential in frog sciatic nerves (6). In the present study we have measured the production of $^{14}CO_2$ from radioactive glucose at concentrations of colchicine, vinblastine, podophyllotoxin, and nocodazole which have been shown to inhibit AXT. Lumicolchicine was included in the study to determine whether the effects on respiration were related to the motile functions of MTs.

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[1] The abbreviations used are: AXT, axonal transport; MT, microtubule; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Axonal transport. Frogs (*Rana temporaria*) were used in this investigation. They were kept at 4° before experimentation. A section consisting of the 8th and 9th dorsal ganglia, the sciatic nerve, and the gastrocnemius muscle (optional) was dissected from decapitated frogs. Two preparations from the same frog were used for experimental and control procedures. The tissue was incubated in oxygenated frog Ringer's solution, pH 7.4, in an apparatus which permitted the isolation of the dorsal ganglia from the rest of the preparation by silicone grease barriers (9). L-[4,5-^{3}H]leucine (105 Ci/mmole, 1 mCi/ml; Radiochemical Centre, Amersham, England) was added to the Ringer's solution in the ganglionic compartments and the drug to be tested to the nerve compartment of th experimental nerve. Lumicolchicine and nocodazole were added from stock solutions in ethanol and dimethylsulfoxide, respectively, and the corresponding amount of vehicle was added to the control nerves. Colchicine-treated, lumicolchicine-treated, and podophyllotoxin-treated nerves were incubated in the presence of the drugs at 6° for 12 hr before the experiments were begun (6).

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AXT in the nerves was assessed by measuring the amount of TCA-insoluble radioactivity accumulating in front of a ligature placed 30 mm from the ganglia. After incubation for 17 hr at 18°, the nerves were cut into 4-mm pieces and each piece was treated with 5% TCA at 80° for 1 hr. After rinsing in water, the ganglia and nerve pieces were solubilized in Soluene-350 (Parkard Instrument Company, Detroit, Mich.). The protein-incorporated radioactivity was measured with a Packard 3375 liquid scintillation spectrophotometer in a 0.55% Permablend III solution (Packard Instrument Company) in toluene.

The effects of the drugs on protein synthesis in the dorsal ganglia was assessed under conditions identical with those used for AXT except that the drugs were present in the ganglionic compartment instead of the nerve compartment. The sciatic nerve compound action potential was recorded at room temperature by placing the nerves on silver electrodes in a moist chamber. The nerves were stimulated at supramaximal voltage at 10 Hz and 0.1-msec duration, and the action potential was displayed on an oscilloscope.

Respiration. Frog sciatic nerves without the ganglia and muscles were incubated in Ringer's solution (containing unlabeled glucose, 1 g/liter), in a gas-tight test tube in the presence of D-[*U*-^{14}C]glucose (333 mCi/mmole, 1 mCi/ml; Radiochemical Centre). Pure oxygen was bubbled first through water and then through two tubes containing the experimental and control nerves in parallel. The evolved carbon dioxide was led through cold traps containing small amounts of acidified water into 1 ml of Carbo-Sorb (Packard Instrument Company). Initially a second CO_2 absorber was coupled in series with the first one, but a control experiment showed that it did not take up any additional CO_2; the Carbo-Sorb was subsequently mixed with 10 ml of scintillation fluid and counted as above.

Lumicolchicine was prepared from colchicine (Sigma Chemical Company, St. Louis, Mo.) by UV irradiation in absolute ethanol (10) and the conversion was controlled with a Perkin-Elmer 554 spectrophotometer. Podophyllotoxin was purchased from Aldrich Chemical Company, Milwaukee, Wisc.; vinblastine sulfate from Eli Lilly and Company, Indianapolis, and nocodazole (methyl-5-(thienylcarbonyl)-1-*H*-benzimidazole-2-yl-carbamate) was obtained from Sigma Chemical Company.

RESULTS

Colchicine, at 5 mM concentration, was found to reduce the accumulation of labeled proteins at the ligature to 19% of control values, whereas lumicolchicine had only a slight effect and reduced the accumulation to 78% of control values (Table 1). Without preincubation, this colchicine concentration gave a less complete transport inhibition, probably because of slow penetration of the drug into the nerve fibers (4, 6). Lower concentrations of colchicine were tested and no concentration was found which significantly depressed AXT but not respiration, or the reverse. The concentrations of colchicine and the other drugs were therefore primarily chosen to give a reliable inhibition of AXT. Podophyllotoxin was also more effective with preincubation, whereas vinblastine and nocodazole did not show potentiation with prolonged incubation. Nocodazole was the most potent transport inhibitor of those studied (Fig. 1), and reduced AXT to the same extent as did colchicine at a concentration 500 times lower (10 μM).

TABLE 1

Mean values and standard errors of the means of TCA-insoluble radioactivity in ganglia and 4-mm segments of nerve proximal to a ligature 30 mm from the ganglia

From each animal one nerve was used as a control. The preparations were preincubated overnight at 6° in the presence of the various drugs. The ganglion compartments contained 20 μCi of [^{3}H]leucine per milliliter of frog Ringer's solution. Mitosis inhibitors were added to the nerve compartments of the experimental nerves and normal Ringer's solution to the other compartments. A two-tailed *t*-test for paired preparations was used for statistical evaluation.

Drug	Concentration	Radioactivity in ganglia	Radioactivity at ligature	*p*	No. of animals
	mM	*% of control*	*% of control*		
Colchicine	5.0	98 ± 8	19 ± 5	<0.005	5
Lumicolchicine	5.0	111 ± 14	78 ± 12	<0.05	6
Vinblastine	0.1	102 ± 7	5 ± 1	<0.005	6
Podophyllotoxin	0.1	87 ± 9	6 ± 1	<0.005	7
Nocodazole	0.01	86 ± 10	23 ± 7	<0.005	8

None of the drugs affected [^{3}H]leucine incorporation into TCA-insoluble proteins in the dorsal ganglia when they were added to the ganglionic compartments in separate experiments (data not shown). The block of export of material from the cell bodies into the axons, obtained

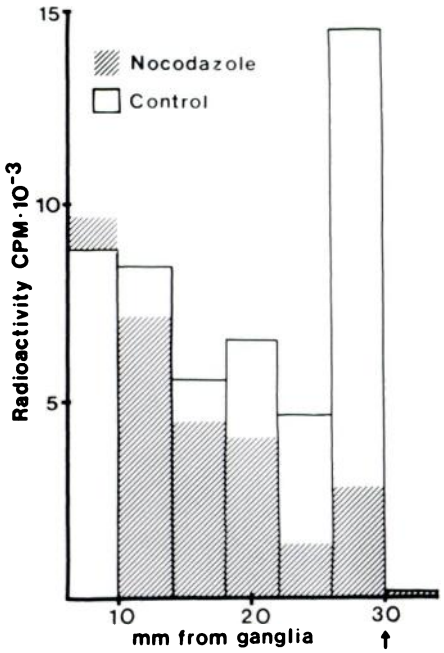

FIG. 1. *Effect of nocodazole on fast axonal transport*

Frog sciatic nerves were incubated for 17 hr at 18° in the presence of 0.01 mM nocodazole or normal Ringer's solution. The drug was prevented from reaching the ganglia by a silicone grease barrier. The ganglionic compartment contained 20 μCi of [^{3}H]leucine in 1 ml of Ringer's solution. The most proximal nerve segments were located in the silicone barriers and partly exposed to leucine. An *arrow* indicates the position of the ligature.

with the mitosis inhibitors, probably increased the total protein-incorporated radioactivity in the cell bodies, but since the transported fraction represents only a small percentage of the total activity, the increase was not measurable. Colchicine was a more potent transport inhibitor when it was present in the ganglionic compartment than in the nerve compartment, whereas the other drugs were equally effective in either compartment.

The amplitude of the compound action potential was unaffected by all of the drugs tested and ranged from 90% to 108% of control values ($n = 4$, not significant for all drugs). No difference was found between freshly dissected tissues and those preincubated at low temperature.

When [^{14}C]glucose was used as a precursor in the ganglionic compartment instead of [^{3}H]leucine, 10% of the total radioactivity was recovered in the TCA-insoluble fraction, 20% as $^{14}CO_2$ and 70% as TCA-soluble material. A small amount (2%) of the TCA-insoluble radioactivity was exported into the nerve and recovered in front of a ligature 30 mm from the ganglia. This was approximately the same ratio between exported and stationary protein as when leucine was used as a precursor in the ganglionic compartment. However, the activity in front of the ligature with glucose as a precursor was less than 1% of that with leucine. Only the ganglia incorporated glucose into chloroform/methanol-extractable components, and none was recovered at the ligature. TCA-soluble material was present in the most proximal 10–12 mm of the nerve, but did not reach the ligature during the 17-hr incubation period.

The frog nerve preparation can maintain AXT for 1 day at 18° in the absence of exogenous glucose. However, added ratioactive glucose was rapidly taken up locally along the nerve and converted to CO_2. The ratio between labeled and nonlabeled CO_2 increased for at least 7 hr (Fig. 2). A reduced uptake of glucose could therefore affect the amount of $^{14}CO_2$ produced. No detailed study of glucose uptake was made, but the drugs used did not affect the amount of TCA-soluble radioactivity in the nerves after 2.5- and 5-hr incubation periods in the presence of [^{14}C]glucose.

To determine the response time of the respiration chamber, 5 mM NaCN was added after a 3-hr incubation in normal Ringer's solution in the presence of labeled glucose. A reduced $^{14}CO_2$ production was detectable after 15 min and the output after 1 hr was reduced to 25% of its value before the addition of cyanide.

Colchicine reduced to 72% of control the amount of $^{14}CO_2$ produced (Fig. 2, Table 2). Lumicolchicine, unexpectedly, had more effect on respiration than did colchicine and reduced CO_2 production to 55% that of control. Nocodazole inhibited respiration to the same extent as colchicine (67%), whereas podophyllotoxin was without effect. Vinblastine was the only drug of those tested which increased respiration at transport inhibitory concentrations (127% of control).

DISCUSSION

Colchicine and vinblastine are the most frequently used mitosis inhibitors in studies on axonal transport (1). Podophyllotoxin, nocodazole, and other drugs with similar effects on MTs also inhibit AXT (4, 11) but have been less well studied. Lumicolchicine has been used to study non-MT-related effects of colchicine on AXT and has been shown to be slightly inhibitory (3, 4). The consistent inhibitory effects on AXT obtained with mitosis inhibitors strongly suggest that MTs provide the motile machinery or guidelines along which material is conveyed. However, contradictory results have been obtained (12), and other interpretations of the effects of MT-affecting drugs are possible (1).

AXT is a complex process which, apart from MTs and possibly other filamentous proteins, might involve MT-associated proteins (8), energy in the form of ATP, (5) and Ca^{2+} (13). Interference with any of these mechanisms or protein synthesis in the cell bodies will impair trans-

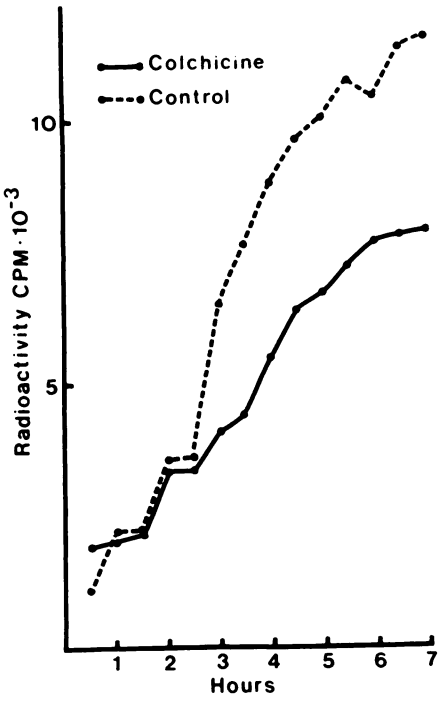

FIG. 2. *Effect of 5 mM colchicine on $^{14}CO_2$ production in frog sciatic nerves*

The nerves were preincubated overnight in the presence of colchicine at 6°. For other experimental details see legend to Table 2.

TABLE 2

Mean values and standard errors of the mean for $^{14}CO_2$ production in frog sciatic nerves

The nerves were preincubated overnight at 6° in the presence of the various drugs. Subsequently 5 μCi of [^{14}C]glucose was added to 1 ml of frog Ringer's solution, and O_2, approximately 2 ml/min, was bubbled through the solution. The CO_2 absorber (1 ml) was changed each hour and the total radioactivity released during 7 hr was calculated.

Drug	Concentration	Radioactivity in $^{14}CO_2$	p	No. of animals
	mM	*% of control*		
Colchicine	5.0	72 ± 5	<0.01	6
Lumicolchicine	5.0	55 ± 7	<0.01	8
Vinblastine	0.1	127 ± 4	<0.02	8
Podophyllotoxin	0.1	100 ± 5	NS[a]	8
Nocodazole	0.01	67 ± 6	<0.01	10

[a] NS, Not significant.

port and be difficult to distinguish from direct effects on MTs (6, 7). A reduced energy supply induces both transport inhibition (6) and a loss of MTs (14). The reverse experiment—interference with MT and possible effects on the energy-supplying system or indirectly on other energy requiring processes, protein synthesis, and electrical activity—has been investigated several times (6, 8, 15). None of the mitosis inhibitors has been shown to affect protein synthesis, the electrical activity of nerves, or the levels of high-energy phosphates except at very high concentrations. However, subtler effects might be present, as demonstrated in this study. Colchicine has previously been shown to increase glycolysis in frog muscles (16), to depress it in thyroid cells (17), and to stimulate CO_2 production in the same types of cells in another investigation (18). Most studies indicate that the electrical activity is unaffected by moderate doses of colchicine (1), but some changes can be observed by intracellular recording techniques (19). In the squid giant axon, similar effects were obtained with colchicine and lumicolchicine (19). Lumicolchicine does not affect MT-dependent AXT, but mimics other colchicine effects, e.g., nucleoside transport in non-neural cells (20). However, MTs have been shown to participate in a wider range of cellular activities than previously suspected, and lumicolchicine might have effects on nonmotility-related MT functions.

Podophyllotoxin, which was more effective than colchicine as an inhibitor of AXT in the present study, had no respiratory effects. Podophyllotoxin also inhibits nucleoside transport (21) and binds to the colchicine-binding site on MTs (22), but otherwise little is known about the unspecific effects of this drug. Even less is known about the action of the synthetic mitosis inhibitor nocodazole, but in the present study this drug was the most potent transport inhibitor of all of the drugs and depressed respiration to the same extent as colchicine.

Vinblastine stimulated respiration, and similar effects have been observed in thyroid cells (17, 18). Vinblastine can affect impulse transmission at high concentrations (15), induce changes in ribosome function (23), and inhibit amino acid uptake (24). Some acidic proteins, notably actin, can be precipitated by vinblastine (25). The relationship between unspecific effects of vinblastine and AXT is not known.

The metabolic rate of nerve tissue can be increased by electrical stimulation or high concentrations of K^+ (26, 27). The increase has been attributed in part to elevated Na^+-K^+ pump activity (28). The proportion of available energy which is used for ion pump activity has been estimated to be approximately 26% in brain slices (28). The fate of the remaining 74% is unknown. Some energy is probably required for protein synthesis, and a considerable part might be channeled to intracellular transport processes. Investigations on respiratory enzymes in toad brain have suggested a tight coupling between oxygen utilization and neuronal function (29). The present results do not support such a coupling or the presence of a feedback mechanism between AXT and respiration. Alternatively, the energy requirements of AXT are too small to be detected in changed CO_2 production under conditions of inhibited AXT. The respiratory changes obtained with some of the drugs did not cause corresponding changes in two other energy-requiring neuronal processes, protein synthesis in the dorsal ganglia and electrical transmission, and were probably not related to MT-associated motility functions.

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