LOCAL ANESTHETIC-INDUCED INHIBITION OF COLLAGEN SECRETION IN CULTURED CELLS UNDER CONDITIONS WHERE MICROTUBULES ARE NOT DEPOLYMERIZED BY THESE AGENTS

JOHN H. EICHHORN and BEVERLY PETERKOFSKY

From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. Dr. Eichhorn's present address is the Department of Anesthesia, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02215

ABSTRACT

Tertiary amine local anesthetics previously have been shown to influence some microtubule-dependent cellular functions. Since several cell secretion processes, including secretion of collagen, have been shown to be inhibited by microtubule-disrupting drugs such as colchicine, we determined whether local anesthetics affect collagen secretion. Six local anesthetics inhibited collagen and non-collagen protein secretion (up to 98%) into the extracellular medium of 3T3 cells and human fibroblasts, an effect apparently independent of influences on proline transport and total protein synthesis.

A combination of colchicine and cytochalasin B did not duplicate the effects of local anesthetics. The effects of subsaturating concentrations of colchicine and procaine on secretion were additive, suggesting that both drugs act on the secretory pathway at the level of microtubules, but other effects of the two types of drugs were strikingly different.

In comparing the mechanisms of action of colchicine and local anesthetics, it was seen that, in contrast to colchicine, radioactive procaine and lidocaine were slowly transported into 3T3 cells, did not bind to the tubulin-containing TCA-insoluble fraction, and did not bind to purified tubulin in vitro. The fraction of cellular tubulin present as microtubules (47% in normal cells) was determined by measuring tubulin in stabilized, sedimentable microtubules compared to total tubulin, using a [^{3}H]colchicine binding assay. Pretreatment of cells in the cold or with colchicine led to depolymerization of microtubules, but pretreatment with five local anesthetics tested did not. Therefore, in contrast to colchicine, local anesthetics in concentrations that inhibit secretion do not directly interact with or depolymerize microtubules. These drugs, however, do affect a microtubule-dependent process and may do so by detaching the microtubular system from the cell membrane.

KEY WORDS local anesthetics · protein secretion collagen · microtubules · tubulin · tubulin polymerization · colchicine

Local anesthetics have many effects on cellular function other than inhibiting membrane excitability and thus blocking nerve conduction. Tertiary amine local anesthetics interact with membrane lipids (26, 27) and the actions of these drugs on membranes have been extensively studied (50). Local anesthetics can cause alteration of prostaglandin-stimulated increases in adenylcylase (2), inhibition of exchange transport of glucose (20), inhibition of cell spreading (38) and adhesion (39), change in osmotic fragility (46), inhibition of cell exocytosis and fusion (35), inhibition of Semliki forest virus production (44), and enhancement of concanavalin A-induced cell vacuolization (41). Cell surface receptors are altered by local anesthetics in that there is enhancement of concanavalin A-induced cell agglutination (36, 37) and inhibition of ligand-induced immunoglobulin receptor capping (37, 48, 49).

Marked changes in cell shape seen with exposure to local anesthetics (40) correlate with the suggestions that these agents disrupt the microtubule and microfilament cellular cytoskeleton. Such suggestions were based on electron micrographic studies (7, 12, 25) or experiments in which a combination of colchicine and cytochalasin B duplicated the effects of local anesthetics (36, 37, 24, 9), although one study reported no change in microtubules from exposure to a local anesthetic alone (15).

Secretion or release of substances synthesized by several cell types (21, 55, 34, 14, 43, 17) has been assumed to be dependent on microtubules because these processes are inhibited by the drugs colchicine or vinca alkaloids (such as vinblastine) which disrupt microtubules (54). Secretion of collagen from embryonic chick bones and from cultured cells is inhibited by colchicine and vinblastine (11). The inhibition of secretion by colchicine has been assumed to be caused by the colchicine-induced depolymerization of microtubules (53, 54). Recent studies have suggested that colchicine binds to soluble tubulin dimers which are then added to the growing ends of microtubules during normal assembly and block further assembly (22).

On the basis of the above findings and assumptions and recent reports that local anesthetics inhibit secretion of plasma proteins in rat liver slices (3) and release of histamine in rat mast cells (18), we undertook a series of experiments to investigate whether these drugs would influence collagen secretion by cultured cells and found that, like colchicine, they inhibit collagen secretion independent of decreases in amino acid transport and total protein synthesis. These observations led us to compare the mechanisms of action of the two types of drugs.

Several studies have utilized various techniques to quantitate the relationship of polymerized tubulin (assembled microtubules) and depolymerized (soluble) tubulin (19, 33, 42, 28, 47). Insulin secretion by pancreatic islet cells has been shown to vary parallel to changes in the total amount of tubulin and its degree of polymerization (32). Because it is difficult to secure quantitative data from electron micrographs, we utilized these biochemical techniques to evaluate the effects of colchicine and local anesthetics in concentrations which inhibit secretion on the degree of tubulin polymerization into microtubules. To determine further whether the mechanism of action of local anesthetics in BALB 3T3 cells is analogous to that of colchicine, we also evaluated the possible interaction of local anesthetics with tubulin in cells and in vitro.

MATERIALS AND METHODS

Reagents

Uniformly labeled L-[^{14}C]proline (255 mCi/mmol), [carboxyl-^{14}C]procaine HCl, 46.8 mCi/mmol, and [carbonyl-^{14}C]lidocaine HCl, 30 mCi/mmol, were purchased from New England Nuclear, Boston, Mass. [Ring A-4-^{3}H]colchicine, 7.6 Ci/mmol, was purchased from Amersham Corp., Arlington Heights, Ill. Procaine HCl, tetracaine HCl, colchicine, guanosine triphosphate (GTP) and cytochalasin B were purchased from Sigma Chemical Co., St. Louis, Mo. Dibucaine HCl was purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio, lidocaine base from ICN-K & K Laboratories, Inc., Plainview, N. Y., glycerol from J. T. Baker Chemical Co., Phillipsburg, N. J., and dimethylsulfoxide (DMSO) from Fisher Scientific Co., Pittsburgh, Pa. Cocaine HCl (Merck and Co., Inc., Rahway, N. J.) was a gift from Dr. Irwin Kopin. Mepivicaine HCl was a gift from the Sterling-Winthrop Research Institute. Chromatographically purified collagenase was purchased from Worthington Biochemical Corp., Freehold, N. J., and further purified as previously described (31). Purified bovine brain tubulin was a gift from Drs. Martin Flavin and Jayasree Nath and had been prepared by three cycles of assembly (51) modified in that glycerol was present only during the first assembly.

Cell Culture

Cell culture medium was prepared by the National Institutes of Health (NIH) medium section. Cells were cultivated in a humidified atmosphere of 95% air-5% CO_2 at 37°C in Falcon Plastics (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) tissue culture flasks or plates using Eagle's minimal essential medium containing 10% fetal calf serum (North American Biologicals, Inc., Miami, Fla.) with gentamycin (50 μg/ml) and amphotericin B (1 μg/ml) and modified by reducing the $NaHCO_3$ concentration to one-half normal (0.013 M) and adding Tricine buffer, pH 7.4, 0.025 M. If serum and amphotericin B were omitted, the medium is designated MEM-O. We used a subclone (P-3) of mouse BALB 3T3 A-31 cells isolated in this laboratory which exhibits contact inhibition at confluence and a line of human embryonic fibroblasts (Microbiological Associates, Walkersville, Md.) (MAHE).

Measurement of Free Proline Pool and Collagen Synthesis and Secretion

3T3 cells were inoculated into 100 × 20 mm culture plates at 2×10^5 cells/plate on day 0, refed on day 2, and used on day 3 in late logarithmic phase at 3×10^6 cells/plate. MAHE cells were plated at 1.2×10^5, refed on day 3, and used on day 4. Duplicate samples of two plates of cells each were used for each experimental point with the replicate variation <10%. At the beginning of each [^{14}C]proline incorporation procedure, the growth medium was removed and the cells were washed with MEM-O containing 0.1 mM ascorbic acid. 3.0 ml of MEM-O with ascorbate plus the drug to be tested, if any, was added to each plate, which was then incubated for 30 min at 37°C, after which 3.0 μCi of [^{14}C]proline (5 μCi/μmol) were added. After a 3-h incubation at 37°C, the medium (containing secreted [^{14}C]proline-labeled procollagen) was collected and the cell layer was washed twice with ice-cold MEM-O. Ice-cold buffered saline (0.11 M NaCl, 0.05 M Tris-HCl, pH 7.4) containing 1 mM proline was added to the plate, and the cells were harvested with a rubber policeman and then stored in polycarbonate tubes at −20°C.

Before assay, the medium fractions were dialyzed twice vs. 30 vol of 0.01 M Tris, pH 7.6, lyophilized and dissolved in H_2O to which 1.5 mg of carrier chick embryo protein was added. The cells were sonicated for 20 s with a Bronwill Biosonik IV (Bronwill Scientific, Rochester, N. Y.), using a needle probe at 20% of maximum voltage. To both cell and medium fractions, trichloroacetic acid (TCA) containing L-proline was added to a final concentration of 10% TCA and 2 mM proline. Samples were centrifuged at 1000 *g* for 5 min, and the radioactivity in the TCA-soluble fraction of the cell samples was measured in New England Nuclear Formula 950A scintillation fluid to determine the intracellular pool of free [^{14}C]proline. All precipitates were washed three times in 5% TCA and then dissolved in 0.2 N NaOH and assayed for [^{14}C]collagen with the collagenase-digestion assay previously described (31, 29), except that the ribonuclease step was omitted. DNA was determined by the method of Burton (6), and all samples contained 75–100 μg of DNA. The amount of [^{14}C]collagen in the medium compared to the sum of collagen in the cells plus medium for a given sample represents the percent collagen secreted. Radioactivity in non-collagen protein was measured by dissolving the final pellets from the collagenase-digestion assay in 0.1 M Tris, 0.5% sodium dodecyl sulfate, 0.005 M dithiothreitol (DTT), heating at 100°C for 5 min, cooling, and then counting an aliquot as above. The sum of radioactive collagen plus non-collagen protein yields total protein synthesized, and the relative rate of collagen synthesis was determined by comparing collagen to total protein synthesis, correcting for the difference in relative proline contents (10). The rate of protein synthesis independent of the rate of amino acid transport was calculated by determining the fraction of total [^{14}C]proline taken up into the cell (TCA-soluble plus TCA-precipitable) which had been incorporated into protein (TCA-precipitable fraction).

Prolyl Hydroxylase Assay

Prolyl hydroxylase activity in aliquots of cell sonicates prepared in 0.05 M Tris, 0.1 mM DTT was measured as previously described (30).

Binding and Transport of Radioactive Colchicine and Local Anesthetics in Intact Cells

An assay procedure was developed to study transport and binding of colchicine and the two representative local anesthetics procaine and lidocaine in intact cells. Single 60 × 15 mm plates of cells, in duplicate, were washed twice with and then incubated in 2.0 ml of MEM-O containing 0.1 mM ascorbic acid to which the radioactive test drug was added for the specified time. The incubation medium was removed and the cells were washed twice with buffered saline, after which 0.5 ml of deionized water was added and the cells were harvested with a rubber policeman. The plate was washed with another 0.5 ml of water, and then the 1.0-ml cell suspension was allowed to stand for 30 min at 4°C. Preliminary experiments had shown that this osmotic shock lysed all the cells as effectively as sonication. After lysis, 110 μl of 50% TCA was added and, after 5 min, the samples were filtered through a Millipore filter (Millipore Corp., Bedford, Mass.), 0.45 μm, in a device that allowed the filtrate to drain directly into a scintillation vial. The tube was washed with 1.0 ml of 5% TCA which was then passed through the filter. The scintillation vial was removed, 12 ml of New England Nuclear Formula 950A scintillation fluid was added and the

radioactivity, representing intracellular TCA-soluble (unbound) drug, was measured. The filter was washed with 5% TCA three times, dissolved in 6 ml of 950A, and the radioactivity representing TCA-insoluble (bound) drug was determined. In all such experiments, "zero time" points were determined by adding radioactive drug to the incubation medium and then immediately removing the medium and processing the sample as described above to determine how much drug would adhere to the cells on contact. These values were subtracted from all results of incubations for the various times. Solutions of the radioactive drugs alone were applied to filters to determine adherence to the filters, and these values were also subtracted from the experimental results.

In Vitro [^{3}H]Colchicine-DEAE Filter Binding Assay

The tubulin assay used in drug-binding experiments and the experiments measuring the proportion of intact microtubules was a modification of the diethylaminoethyl (DEAE)-cellulose filter assay (52, 5). To study drug binding to tubulin, purified bovine brain tubulin was incubated with radioactive drug in concentrations shown to inhibit protein secretion, the reaction was stopped with saturating amounts of ice-cold nonradioactive drug, and the mixture was then applied to 2.5-cm discs of Whatman DE81 filter paper (Whatman, Inc., Clifton, N. J.) in a filter manifold. PM buffer (10 mM sodium phosphate, 10 mM $MgCl_2$, pH 6.95) 10 ml was added, and then filtration was allowed to proceed by gravity to allow time for the tubulin to bind to the DEAE cellulose, after which vacuum was applied and the filter was washed four times with 10 ml of PM buffer. The filter was dried and placed in 6 ml of 950A, and the radioactivity representing drug bound to the purified tubulin was determined. In each case, the drugs alone were applied to the filters and the radioactivity retained was subtracted from the experimental value.

Measurement of Pool of Intact Microtubules in Cells

To measure the fraction of the total intracellular pool of tubulin present as polymerized microtubules, a microtubule-stabilizing medium (MTM) (13, 47) consisting of 50% glycerol, 10% DMSO, 5 mM sodium phosphate, 5 mM $MgCl_2$, pH 7, was used. Two 100 × 20 mm plates of 3T3 cells (4×10^6 each) in duplicate were incubated in MEM-O with test drugs for 60 min at 37°C, after which the medium was drained and the cells were carefully washed twice with buffer as above. Then, 0.75 ml of MTM was applied to each plate and, after 7 min at 22°C, the cells were harvested with a rubber policeman, transferred to a 10 × 75 mm polycarbonate tube, and sonicated at 22°C with a Branson Sonifier Cell Disruptor at 25 W for 20 s using a needle probe (Branson Sonic Power Co., Danbury, Conn.). Samples were then spun at 43 kG for 1 h at 22°C to sediment the intact microtubules and the supernate was removed. The recovered pellet was resuspended in 1.0 ml of ice-cold PM buffer with 1 mM GTP and 0.5% Triton X-100 and then resonicated at 0°C to insure disruption of the microtubules and any attachment of microtubules to membranes. A 0.1-ml portion of this tubulin-containing solution was used in the colchicine-binding assay in which the reaction mixture was brought to 2.5 μM colchicine (molar excess to tubulin) by addition of 0.5 μCi of [^{3}H]colchicine (2 μCi/nmol) and incubated at 37°C for 90 min. The reaction was stopped with 0.5 ml of ice-cold 0.5 mM colchicine, and the DEAE-cellulose filter paper assay was carried out as described above. The radioactivity recovered represented tubulin polymerized into microtubules. It was not possible to measure the free, soluble tubulin in the 43 kG supernate because the MTM inhibited the colchicine-binding reaction. Therefore, cell samples treated identically as indicated above, except that PM buffer was substituted for MTM and sonication was performed at 0°C to insure that all microtubules would depolymerize into soluble tubulin, were prepared. Portions of these sonicates were assayed to determine the total amount of tubulin present in the cells, thus allowing a comparison with the MTM-treated samples to yield the percent of total tubulin polymerized into microtubules. Experiments were done twice, on separate days, and the results did not vary.

RESULTS

Effect of Local Anesthetics on Proline Transport and Protein Synthesis and Secretion

In experiments carried out to determine the effect of local anesthetics on protein synthesis and secretion, cultured cells were exposed to six local anesthetics at concentrations having approximately equal potency with respect to clinical anesthetic activity. These concentrations are at the extreme lower end of the range of concentrations used to produce regional anesthesia in humans. As shown in Table I, all the local anesthetics significantly inhibited collagen secretion into the culture medium. In both types of cells, 10 mM mepivicaine was most effective (98% inhibition in 3T3 cells and 84% inhibition in MAHE cells). Cocaine at 2 mM inhibited collagen secretion 48% in 3T3 cells while 10 mM cocaine killed the cells during the 3-h incubation. In this experiment, total incorporation into collagen was decreased by 35–75%. However, this largely reflects the decrease in proline transport (47–56%) shown in Table II. The relative rates of collagen synthesis

TABLE I

Effect of Local Anesthetics on Collagen Synthesis and Secretion

Cells and drug		Collagen synthesis: relative rate	Total collagen synthesized	Collagen in medium	Fraction secreted into medium	Inhibition of collagen secretion
	mM	%	*DPM/μg DNA*	*DPM/μg DNA*	%	%
3T3 Cells						
None		1.2	391	235	61	–
Dibucaine	0.2	1.2	147	30	20	67
Procaine	5	1.8	255	29	11	82
Tetracaine	0.5	1.2	99	8	8	87
Lidocaine	5	1.2	127	8	6	90
Mepivicaine	10	1.2	120	1	1	98
3T3 Cells						
None		1.9	337	211	63	–
Procaine	5	2.5	372	43	11	83
Cocaine	2	2.2	256	87	33	48
Cocaine	10	–	0	–	–	–
Human fibroblasts (MAHE)						
None		5.9	1278	622	49	–
Dibucaine	0.2	4.1	454	133	29	41
Procaine	5	5.0	567	93	16	68
Tetracaine	0.5	3.8	332	58	17	65
Lidocaine	5	4.1	562	132	23	53
Mepivicaine	10	4.7	403	34	8	84

Procedures and calculations are described in Materials and Methods.

compared to total protein synthesis did not change in local anesthetic-treated 3T3 cells and were slightly decreased in MAHE cells (Table I), showing no selective inhibition of collagen synthesis, but, rather, that changes in collagen synthesis paralleled the general changes in the entire protein synthetic mechanism. The inhibition of overall (collagen plus non-collagen) protein synthesis measured independent of changes in proline transport into the cell was 27–45% (Table II). Secretion of non-collagen protein into the medium (Table II) was also inhibited by these drugs, but to a lesser extent than collagen secretion. The relative inhibitory effectiveness of the drugs was essentially the same in both cases.

Failure of Local Anesthetics to Inhibit Prolyl Hydroxylase

Since inhibition of hydroxylation of nascent procollagen molecules is known to inhibit collagen secretion (8), we investigated the possibility that the inhibition of collagen secretion by local anesthetics could be caused by interference with proline hydroxylation. Such inhibition can be caused by Fe^{++} chelators such as α,α'-dipyridyl since Fe^{++} is required for the hydroxylation reaction (8, 30). Cells were treated with α,α'-dipyridyl, colchicine, procaine, and lidocaine under conditions which inhibit collagen secretion and then were assayed for prolyl hydroxylase activity (Table III). As expected, α,α'-dipyridyl treatment inhibited the enzyme while colchicine did not, in agreement with previous results (11). The local anesthetics also did not significantly inhibit prolyl hydroxylase, indicating that their inhibition of secretion is achieved by another mechanism.

Morphological Effects of Local Anesthetics at Concentrations That Inhibit Secretion

Striking morphologic changes occurred when cultured cells were exposed to local anesthetics. Fig. 1 shows the changes seen in 3T3 cells, and there were similar changes in the human fibroblasts. The cells tended to round up, withdraw their processes, and swell. Nuclei became contracted and more dense and were surrounded by many clear vacuoles, especially with procaine, lidocaine, and mepivicaine treatment. While there were general patterns of changes among the cells

TABLE II

Effect of Local Anesthetics on Proline Transport, Protein Synthesis, and Non-Collagen Protein Secretion

Cells and drug		Proline transport	Protein synthesis	Non-collagen protein secretion	
		[^{14}C]Proline in cell (free plus in protein)	Fraction of total [^{14}C]proline incorporated into protein	Fraction secreted into medium	Inhibition of secretion
	mM	*DPM/μg DNA*	%	%	%
3T3 Cells					
None	–	39,915	84	4.7	–
Dibucaine	0.2	20,372	61	2.5	47
Procaine	5	21,722	60	1.9	60
Tetracaine	0.5	18,504	46	1.9	60
Lidocaine	5	17,250	60	1.5	68
Mepivicaine	10	19,335	54	1.2	75
3T3 Cells					
None	–	27,777	69	6.0	–
Procaine	5	22,718	66	2.4	60
Cocaine	2	17,492	69	4.8	20
Cocaine	10	7,221	6	–	–
Human fibroblasts (MAHE)					
None	–	40,425	57	8.1	–
Dibucaine	0.2	25,483	45	5.6	31
Procaine	5	28,137	43	4.6	43
Tetracaine	0.5	23,933	38	3.7	54
Lidocaine	5	31,119	46	4.5	45
Mepivicaine	10	21,031	42	3.1	62

Procedures and calculations are described in Materials and Methods.

TABLE III

Effect of Local Anesthetics and Colchicine on Prolyl Hydroxylase Activity

Drug		Prolyl hydroxylase activity	Inhibition
		DPM [^{3}H]H_2O released/μg protein	%
None		1,401	–
Lidocaine	2 mM	1,458	0
Procaine	2 mM	1,162	17
Colchicine	1 μM	1,392	1
α,α′-dipyridyl	0.5 mM	469	67

0.03-ml aliquots of sonicated cell suspensions were assayed as described in Materials and Methods.

treated with the various drugs, each different local anesthetic consistently produced unique changes in 3T3 cells that made unknown culture plates or photographs readily identifiable as to which drug had been used. The morphologic changes were fully reversible, and drug-treated cells returned to normal morphology after a change to drug-free medium. This occurred in approximately the same amount of time that it took for the drug-induced changes to appear.

Comparison of Effects of Local Anesthetics on Secretion and Morphology with Those of Colchicine and Cytochalasin B

To compare the inhibition of collagen secretion by local anesthetics with that by colchicine, a series of secretion experiments was carried out to establish dose-response curves for this inhibition, using procaine as a representative local anesthetic (Fig. 2). Colchicine was inhibitory in the μM range with a maximum inhibition of 50% achieved by 10 μM colchicine. In contrast, procaine was inhibitory in the mM range with a maximum inhibition of 92% at 10 mM. Concentrations of procaine above 10 mM inhibited total protein synthesis to such an extent as to make evaluation of secretion difficult. The pattern of inhibition of non-collagen protein secretion was the same, except that the magnitude of inhibition was less for both drugs (data not shown).

Because of previous findings that a combination of the microtubule-disrupting drug colchicine and the microfilament-disrupting drug cytochalasin B

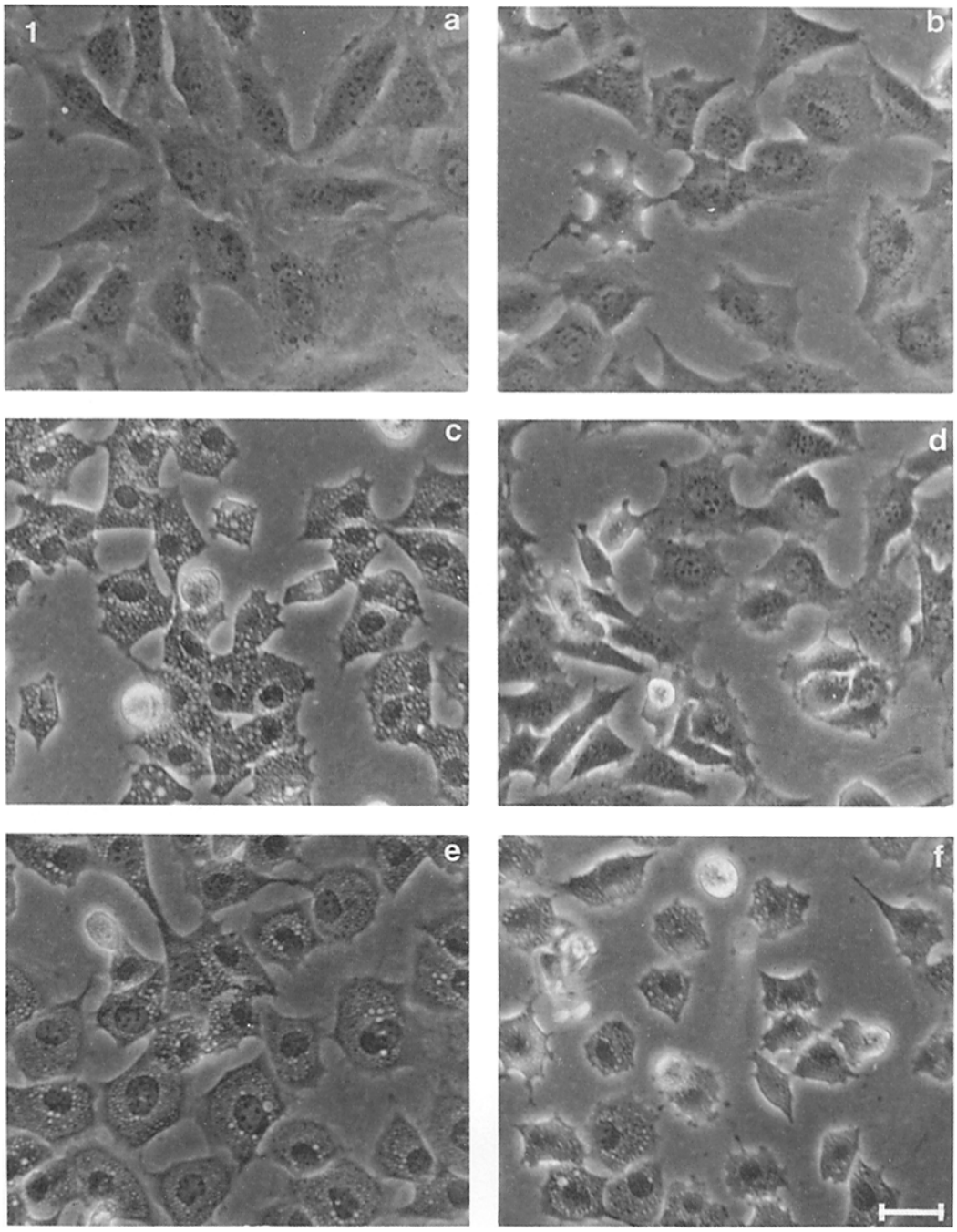

FIGURE 1 Morphology of 3T3 cells after treatment with various local anesthetics. Cells were incubated for 3 h at 37°C in MEM-O containing 0.1 mM ascorbic acid and: (*a*) no drug, (*b*) dibucaine 0.2 mM, (*c*) procaine 5 mM, (*d*) tetracaine 0.5 mM, (*e*) lidocaine 5 mM, and (*f*) mepivicaine 10 mM. Phase contrast microscopy. Bar, 50 μm. × 200.

duplicated the cellular effects of local anesthetics (36, 37, 24, 9), and since maximal inhibition of secretion by colchicine was significantly less than by the local anesthetics, the effect of colchicine and cytochalasin B together was tested for inhibition of collagen secretion (Table IV, exp. 1). Cytochalasin B alone at the same concentration used in previous work (37, 24), did not significantly inhibit collagen secretion (9%), as observed previously (11), and the addition of cytochalasin B to maximally inhibitory colchicine (10 μM) did not increase the inhibition of collagen secretion produced by colchicine alone (49 vs. 50%). Similar results were obtained for the inhibition of secretion of non-collagen protein (17 vs. 16%, Table V, exp. 1), again the magnitude of inhibition of secretion being less than that for collagen. In both cases, the combination of colchicine plus cytochalasin B produced much less inhibition of secretion than that seen with the representative local anesthetic procaine. Also, colchicine plus cytochalasin B inhibited overall protein synthesis less than procaine (Table V, exp. 1).

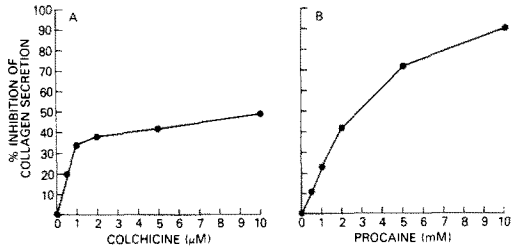

FIGURE 2 Dose-response curves for inhibition of collagen secretion by colchicine (*A*) and procaine (*B*). Collagen secretion was measured as described in Materials and Methods.

To further compare the cellular effects of the local anesthetics with those of the microtubule- and microfilament-disrupting drugs, the morphologic changes induced by these various agents were studied (Fig. 3). The dramatic changes induced by colchicine (Fig. 3*b*) and by cytochalasin B (Fig. 3*e*) were quite different, and the appearance of cells treated with a combination of the two (Fig. 3*f*) did not at all resemble the appearance of cells treated with procaine (Fig. 3*c*). Cells treated with cytochalasin B, either alone or in combination with colchicine, became small, round, and refractile with poorly defined nuclei. These cells contrasted sharply with the swollen, vacuolated cells with well-defined nuclei seen after exposure to procaine.

To investigate whether the local anesthetics may have the same site of action as the known microtubule disruptor colchicine, cells were incu-

TABLE IV

Effect of Various Drugs and Drug Combinations on Collagen Synthesis and Secretion in 3T3 Cells

Drug treatment		Collagen synthesis: relative rate	Total collagen synthesized	Collagen in medium	Fraction secreted into medium	Inhibition of collagen secretion
		%	*DPM/μg DNA*	*DPM/μg DNA*	%	%
Exp. 1						
None		1.3	323	207	64	–
DMSO*	1 %	1.3	371	243	65	0
Procaine‡	5 mM	1.6	346	66	19	70
Colchicine‡	10 μM	1.2	337	111	33	49
Cytochalasin B‡	20 μM	1.1	218	126	58	9
Colchicine	10 μM					
plus		1.0	209	67	32	50
Cytochalasin B‡	20 μM					
Exp. 2						
None		2.2	338	223	66	–
Colchicine	1 μM	2.1	307	126	41	38
Procaine	2 mM	2.6	568	193	34	48
Colchicine	1 μM					
plus		2.5	578	64	11	84
Procaine	2 mM					

Procedures and calculations are described in Materials and Methods.

* Dimethylsulfoxide (DMSO) was required as solvent for cytochalasin B.

‡ In 1% DMSO.

TABLE V

Effect of Various Drugs and Drug Combinations on Proline Transport, Protein Synthesis, and Non-Collagen Protein Secretion in 3T3 Cells

Drug treatment		Proline transport	Protein synthesis	Non-collagen protein secretion	
		[^{14}C]proline in cell (free plus in protein)	Fraction of total [^{14}C]proline incorporated into protein	Fraction secreted into medium	Inhibition of secretion
		DPM/μg DNA	%	%	%
Exp. 1					
None		30,786	82	5.8	–
DMSO*	1%	36,520	78	5.8	0
Procaine‡	5 mM	32,845	64	2.7	53
Colchicine‡	10 μm	41,417	67	4.8	17
Cytochalasin B‡	20 μM	26,085	77	5.8	0
Colchicine	10 μM				
plus		28,012	74	4.9	16
Cytochalasin B‡	20 μM				
Exp. 2					
None		21,003	76	6.7	–
Colchicine	1 μM	19,269	76	4.6	34
Procaine	2 mM	27,623	80	5.1	24
Colchicine	1 μM				
plus		29,146	81	2.7	60
Procaine	2 mM				

Procedures and calculations described in Materials and Methods.

*, ‡ See Table IV.

bated with subsaturating concentrations of each type of drug either separately or in combination to determine whether their effects were additive. The effect of the two drugs on the inhibition of collagen secretion (Table IV, exp. 2) was almost exactly additive (38 plus 48% vs. 84%), suggesting that the drugs act at the same site, whereas, if two separate steps in the secretory pathway were affected, one would expect 68%. The same pattern was seen for the inhibition of non-collagen protein secretion (34 plus 24% vs. 60%, Table V, exp. 2). This experiment is representative of four experiments with essentially the same results.

Also of interest is the observation that low concentrations of procaine which partially inhibit secretion (Tables IV and V and similar data, not shown, but obtained from the procaine dose-response curve of Fig. 2) appear to stimulate rather than inhibit proline transport into the cell. This effect on transport, coupled with a slight increase in total protein synthesis and also in relative rate of collagen synthesis, yielded a significant increase (68%) in total incorporation of radioactivity into collagen. This stimulation occurred independent of the inhibition of secretion or the presence of colchicine, which acted to further inhibit secretion. These results demonstrate that the inhibition of secretion by the local anesthetics can occur independent of any inhibition of transport or protein synthesis.

The morphologic changes in the cells seen in these experiments are shown in Fig. 3. Colchicine treatment (Fig. 3*b*) produced swollen, less dense cells with ruffled edges and pronounced peripheral densities. While this appearance differed markedly from that of procaine-treated cells described above (Fig. 3*c*), both drugs induced the retraction of cell processes. Cells incubated with a combination of the two drugs (Fig. 3*d*) distinctly showed a combination of both sets of characteristics: vacuolated cells with distinct nuclei and with ruffled edges and peripheral densities.

Uptake of Colchicine and Local Anesthetics in Intact Cells

To measure the transport and binding of colchicine and local anesthetics in intact BALB 3T3 cells, the TCA-precipitation filter assay was used. Although in this assay colchicine was not bound to the same degree as in the DEAE-cellulose filter assay, it was used because transport as well as

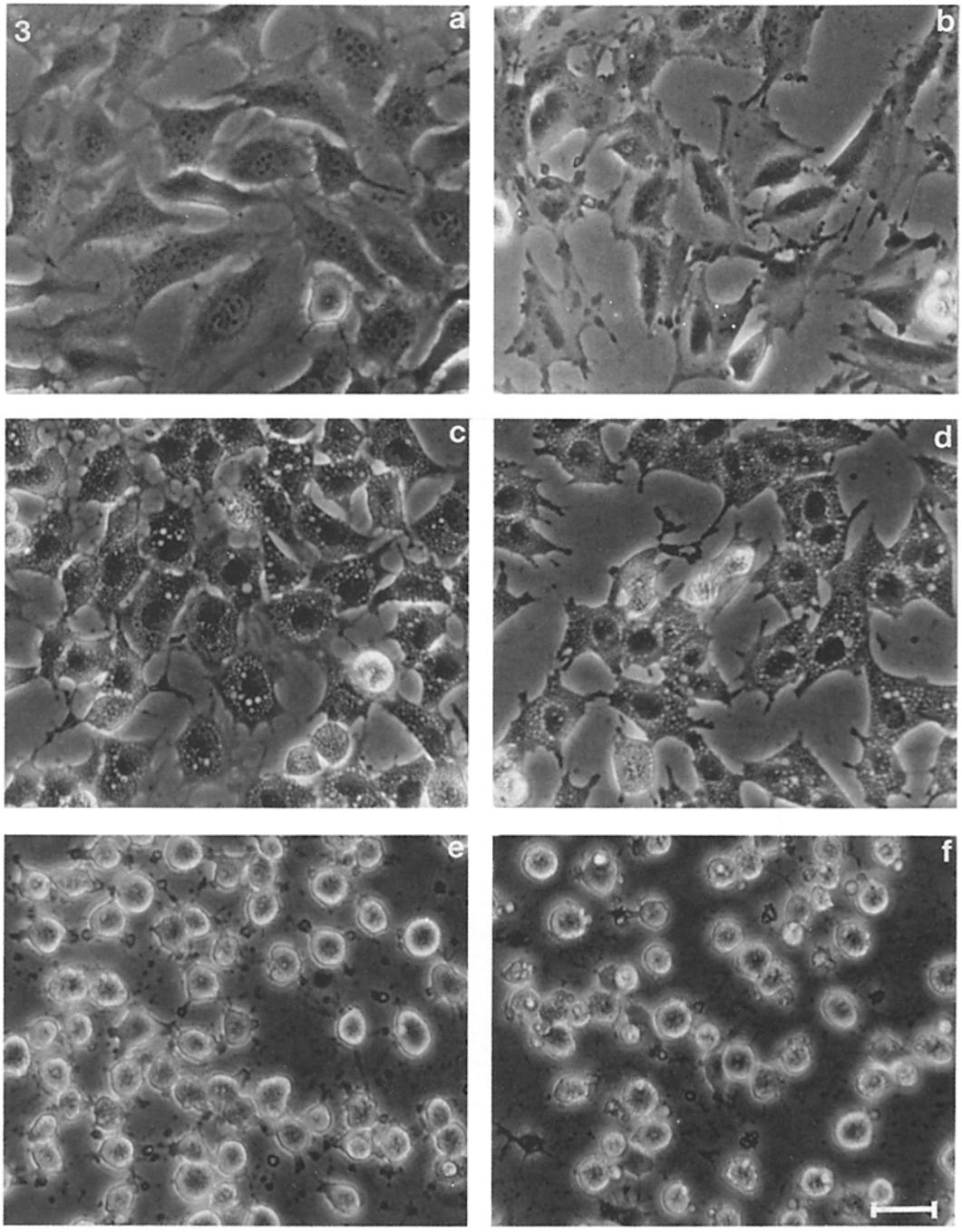

FIGURE 3 Morphology of 3T3 cells after treatment with various agents. Cells were incubated for 3 h at 37°C in MEM-O containing 0.1 mM ascorbic acid and: (*a*) no drug, (*b*) colchicine 1 μM, (*c*) procaine 2 mM, (*d*) colchicine 1 μM plus procaine 2 mM, (*e*) cytochalasin B 20 μM in 1% DMSO, and (*f*) colchicine 10 μM plus cytochalasin B 20 μM. Phase contrast microscopy. Bar, 50 μm. × 200.

binding to any macromolecular cell fraction could be determined. To verify that this assay reflected colchicine binding, binding was measured at varying [^{3}H]colchicine concentrations. The results (Fig. 4) revealed a sharp increase in TCA-precipitable binding occurring above 0.5 μM colchicine, indicating the onset of depolymerization of microtubules into free tubulin, and therefore subsequent uptake experiments were carried out at 1 μM colchicine. These results are the same as data obtained for colchicine binding to tubulin in Chinese hamster ovary cells (47). The sharp increase in binding occurred at the same concentration that produced a sharp increase in the inhibition of collagen secretion (see Fig. 2), suggesting that microtubule depolymerization is associated with inhibition of secretion.

Using this TCA-precipitation assay, a series of experiments to study the uptake and binding of colchicine, procaine, and lidocaine was carried out and the results are shown in Fig. 5. Colchicine at 1 μM was very rapidly taken up into the cell and bound to the TCA-insoluble fraction, presumably reflecting binding to tubulin. Little change was seen after 10 min. Procaine and lidocaine were tested at 2 mM which is a nonsaturating concentration for inhibition of protein secretion, but inhibits to approximately the same degree as 1 μM colchicine (Fig. 2, Table IV, and preliminary data not shown). Procaine and lidocaine were taken up into the cell, as shown by their presence in the TCA soluble fractions, but at a somewhat slower initial rate. The two local anesthetics did not bind to the TCA-insoluble fraction, in striking contrast to the results for colchicine.

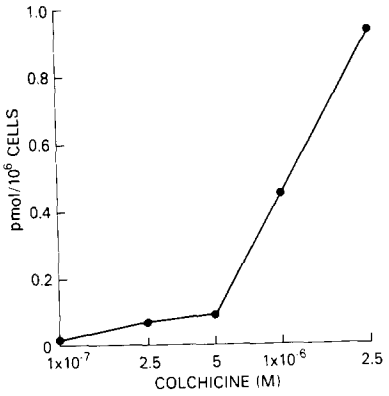

FIGURE 4 Binding of [^{3}H]colchicine to TCA-insoluble fraction of 3T3 cells. Samples contained 1.5 × 10^6 cells and were incubated at 37°C with 1 μCi [^{3}H]colchicine (1.9 Ci/mmol) at the designated concentrations for 10 min and processed as described in Materials and Methods.

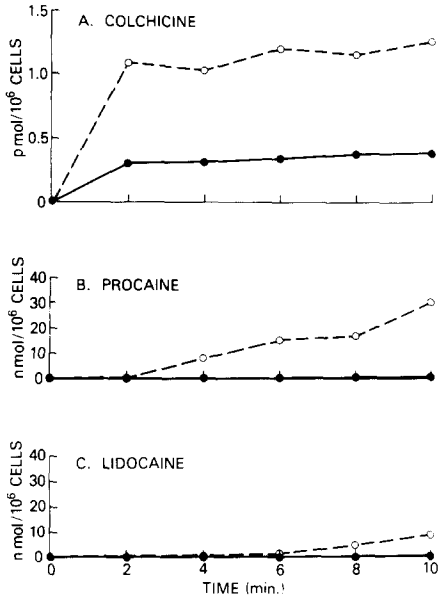

FIGURE 5 Distribution of drugs taken up by 3T3 cells into free and bound fractions. Samples contained 1.5 × 10^6 cells and 2 ml of medium and were incubated at 37°C with (*A*) 1 μM colchicine with 1 μCi [^{3}H]colchicine, (*B*) 2 mM procaine with 0.5 μCi [^{14}C]procaine, and (*C*) 2 mM lidocaine with 0.5 μCi [^{14}C]lidocaine for the indicated times and then processed as described in Materials and Methods. Solid lines represent TCA-insoluble cell fractions and dashed lines represent the TCA-soluble cell fractions.

In Vitro Assay for Binding to Tubulin

To evaluate whether local anesthetics will bind specifically to tubulin, they were tested in an in vitro binding assay using [^{3}H]colchicine binding for comparison. Concentrations of the radioactive drugs which inhibited secretion to the same degree were incubated with purified bovine brain tubulin. The results (Table VI) show the expected binding of colchicine to tubulin and essentially no binding of the local anesthetics. This correlates well with the failure of local anesthetics to bind to the TCA-insoluble fraction in the intact-cell experiments.

Determination of whether Local Anesthetics Depolymerize Microtubules

The fact that we did not detect binding of the anesthetics to tubulin did not rule out the possibil-

TABLE VI

In Vitro Assay for Drug Binding to Purified Bovine Brain Tubulin

Drug		Drug bound
		pmol/pmol tubulin
Colchicine	2.5 μM	0.58
Procaine	5 mM	0.007
Lidocaine	5 mM	0

Reactions were carried out in a final volume of 0.1 ml of PM buffer containing 1 mM GTP, 23 μg tubulin and, where indicated, 1 μCi [^{3}H]colchicine (3.8 Ci/mmol), 0.7 μCi [^{14}C]procaine (1.5 mCi/mmol), or 0.7 μCi [^{14}C]lidocaine (1.0 mCi/mmol), all of which were in molar excess to the tubulin. Samples were incubated for 2 h at 37°C after which the reactions were stopped with 0.5 ml of 0.5 mM colchicine, 250 mM procaine, or 250 mM lidocaine. The assay technique is described in Materials and Methods. The molecular weight of tubulin was taken as 120,000 daltons (52).

ity that these drugs still might disrupt (depolymerize) microtubules, but by a mechanism different from that of colchicine. A series of experiments was undertaken to investigate whether microtubules in anesthetic-treated cells were disrupted. Colchicine-treated cells or cells exposed to 4°C for 3 h were used as controls since both of these treatments should significantly reduce the fraction of cellular tubulin in assembled microtubules recovered in the 43 kG pellet. Preliminary experiments with radioactive colchicine had shown that no colchicine from the pretreatment was carried over into (and thus potentially interfering with) the tubulin assay. The amount of tubulin derived from microtubules in the 43 kG pellet of MTM-treated cells and in cells prepared without MTM (total tubulin) was determined with the in vitro DEAE-cellulose disk colchicine-binding assay. Fig. 6 shows that this assay is linear up to amounts of tubulin well in excess of those assayed in the experimental samples. In Table VII, it may be seen that normal 3T3 cells had 47% of the total cellular tubulin present as sedimentable microtubules. This result correlates well with reported values for the fraction of total tubulin assembled into microtubules in mouse liver (28) and pancreatic islet cells (32) and is somewhat higher than values for rat liver (33, 42), mouse brain (19), or Chinese hamster ovary cells (47). The validity of the tubulin assay was confirmed by adding purified tubulin to diluted control samples; the binding was exactly additive (Table VII).

Treatment of 3T3 cells in the cold or with 10 μM colchicine reduced the fraction of tubulin polymerized from 47 to 25%, confirming the disruption of microtubules. These results constitute further biochemical evidence that, at concentrations of colchicine which inhibit protein secretion, microtubules are disrupted. Cytochalasin B, which should not influence microtubular polymerization, did not reduce the polymerized fraction. Treatment of the cells with five local anesthetics, in concentrations that inhibit protein secretion to a greater extent than the saturating concentration of colchicine (10 μM), did not result in any reduction of the fraction of tubulin recoverable as sedimentable microtubules, indicating that these drugs, unlike colchicine, do not depolymerize microtubules.

DISCUSSION

Tertiary amine local anesthetics in concentrations lower than those used clinically inhibited significantly (up to 98%) collagen secretion in two different types of cultured mammalian cells. Secretion of non-collagen protein was also inhibited, but to a somewhat lesser degree, a pattern also seen with colchicine. This inhibition of secretion of both types of protein appears to be independent of any effects on proline transport into the cell, the overall rate of protein synthesis, or the rate of collagen synthesis in particular. This conclusion was drawn from the observations that proline

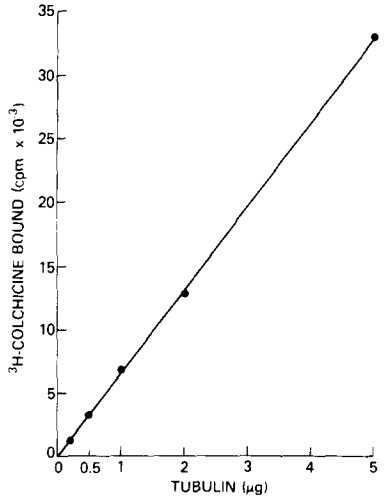

FIGURE 6 Standard curve for the in vitro colchicine-binding tubulin assay. Known amounts of purified bovine brain tubulin were stabilized in 1 mg/ml (final concentration) bovine serum albumin and assayed as described in Materials and Methods.

TABLE VII

Fraction of Total Cellular Tubulin in 3T3 Cells Present as Sedimentable Microtubules

Pre-treatment of cells		[^{3}H]colchicine bound	Fraction of tubulin polymerized
		cpm	%
None		5,865	47
Cold (4°C, 3 h)		3,230	26
Colchicine	5 μM	3,830	30
	10 μM	3,089	25
Procaine	5 mM	5,836	47
	10 mM	6,553	52
Lidocaine	5 mM	5,216	42
Mepivicaine	10 mM	5,957	48
Tetracaine	0.5 mM	6,241	50
Dibucaine	0.2 mM	6,256	50
DMSO	0.5%	7,024	56
Cytochalasin B (in DMSO 0.5%)	20 μM	6,589	53
None (sample diluted 1:1)		2,914	–
Purified bovine brain tubulin 1 μg		7,099	–
None (diluted 1:1) plus purified tubulin 1 μg		10,014	–

Each sample represents 8×10^5 cells incubated with the test drug for 1 h except as noted. Procedures are described in Materials and Methods. Radioactivity represents [^{3}H]colchicine bound to the tubulin in the 43 kG pellet, except in the case of the tubulin standard. In the assay of tubulin recovery, purified tubulin was added to a diluted portion of the resuspended pellet of the sample which received no drug pretreatment. Experimental values were all well within the linear range of the assay (Fig. 3). To determine total cellular tubulin, a replicate untreated cell sample was prepared with buffer alone, without MTM, and a portion equivalent to 8×10^5 cells bound 12,454 cpm of [^{3}H]colchicine. This represented 2.2 μg of tubulin which, in turn, represented ~1.4% of the total cellular protein. The amount of tubulin recovered in the 43 kG pellet after MTM treatment was divided by the total cellular tubulin to determine the fraction of tubulin polymerized.

transport and protein synthesis (both collagen and non-collagen) were, in fact, slightly increased at low concentrations of procaine which still partially inhibit secretion and that the relative rate of collagen synthesis in 3T3 cells was unaffected by local anesthetic treatment. The enhancement of cellular function by a low concentration of anesthetic is unusual and merits further study.

Inhibition of collagen secretion by local anesthetics was not caused by inhibition of proline hydroxylation. It was found that treatment of 3T3 cells with the representative local anesthetics procaine and lidocaine did not result in decreased prolyl hydroxylase activity (Table III). This observation plus the fact that non-collagen protein secretion was also inhibited suggests that the inhibition of secretion of collagen results from a drug influence on the secretory mechanism rather than on some specific step in the modification of procollagen. The lack of effect of local anesthetics on intracellular levels of ATP (3) may further suggest that a "mechanical" aspect of secretion is affected rather than the energy requirement.

The inhibition of protein secretion by local anesthetics independent of protein synthesis seen in this study is consistent with findings for inhibition of secretion of plasma proteins from rat liver slices by local anesthetics (3). The degree of inhibition of protein synthesis seen in the liver slices, however, was much greater than that observed here in cultured cells. In those studies it was observed that there was no difference in the [^{14}C]leucine radioactivity in the TCA-soluble fraction between control and anesthetic-treated slices, and it was concluded that leucine transport was not altered. The amount of leucine in the acid-soluble fraction did not entirely reflect leucine transport, however, because at the same time there was a marked decrease of incorporation of leucine into the TCA-insoluble fraction. Therefore, the total leucine in the cells (free plus incorporated into protein) was significantly decreased, reflecting a decrease in leucine transport and thus corresponding well with the decrease in proline transport seen in the studies reported here (Table II). The mechanism of this inhibition of amino acid transport by local anesthetics has not been defined, but presumably could relate to any of the many membrane effects of these drugs (50).

It has been widely assumed that the inhibition of cellular secretion and release functions by colchicine is directly related to the disruption of the microtubular system by colchicine (21, 55, 34, 14, 43, 17, 11). Confirmatory biochemical evidence reported here shows that the same concentration of colchicine inhibits protein secretion (Fig. 2 and Table V), binds to tubulin (Fig. 4 and Table VI), and depolymerizes microtubules (Table VII). As detailed in the Introduction (7, 9, 12, 24, 25, 36, 37), various studies also have implicated local anesthetics in an interference with microtubule function, possibly by depolymerization. Our stud-

ies and another (3) clearly indicate that local anesthetics inhibit protein secretion and, in addition, our experiments support the suggestion that local anesthetics interfere with microtubule function. When concentrations of colchicine and the local anesthetic procaine which individually were subsaturating with respect to inhibition of both collagen and non-collagen protein secretion were added together to cells, there was an exactly additive effect (Tables IV and V, exp. 2). If the two drugs acted at two different points in the secretory process, it might be expected that a combination of the two drugs would inhibit considerably less than the sum of the two separately, which was not the case.

In spite of the fact that the additive experiment described above suggests that local anesthetics and colchicine both inhibit secretion at the level of microtubules, many differences between the actions of colchicine and the local anesthetics were observed in these experiments. The dose-response curves for the inhibition of collagen secretion by colchicine and procaine were significantly different (Fig. 2). Procaine nearly completely inhibited collagen secretion while a saturating concentration of colchicine only inhibited secretion ~50%. Comparison of characteristics of colchicine and local anesthetics at concentrations which inhibit secretion showed further differences. The morphologic changes induced by the local anesthetics did not resemble at all those seen with colchicine treatment of the cells, although both types of drugs induced retraction of cell processes. Colchicine was rapidly transported into the cell (<2 min, Figure 5), bound to tubulin both in the cell and in vitro, and caused disruption of microtubules in cells, as reflected by a decrease in the fraction of total cellular tubulin sedimentable in a microtubule-stabilizing medium (Table VII). In contrast, the representative local anesthetics procaine and lidocaine were more slowly transported into the cell and did not bind to any TCA-precipitable cellular macromolecule, including tubulin, in the cell or to tubulin specifically in vitro. The five local anesthetics tested did not cause depolymerization of cellular microtubules into their component soluble tubulin subunits.

One laboratory has reported electron micrographic studies suggesting that concentrations of lidocaine much higher than that needed to inhibit protein secretion in 3T3 cells and which are, in fact, lethal to 3T3 cells (17–26 mM) caused disruption of microtubules in rabbit vagus nerves in vitro (7), and also reported studies suggesting that lidocaine can inhibit microtubule polymerization in vitro (16). In the results reported here, lidocaine induced a slight decrease in tubulin polymerization, but this was not the pattern seen for the five anesthetics as a group in this cell system at these concentrations.

Because the tertiary amine local anesthetics do not induce depolymerization of microtubules analogous to that caused by colchicine in 3T3 cells, it must be concluded that the local anesthetics have a different mechanism of action in inhibiting protein secretion in these cells. In a study which showed that local anesthetics inhibit secretion of plasma protein from rat liver slices (3), procaine, unlike colchicine, did not cause proteins to accumulate in Golgi-derived secretory vesicles, leading to the suggestion that the two drugs inhibit secretion at different cell sites. Another cellular process which is inhibited by both colchicine and the local anesthetic dibucaine is the production of extracellular Semliki forest virus by infected baby hamster kidney cells (44). In this case, too, it was reported that while colchicine induced depolymerization of microtubules in these cells, dibucaine did not.

One possible mechanism by which local anesthetics might influence the microtubular system without depolymerizing microtubules is through the known multiple interactions of these drugs with membranes (50). It has been reported that microtubules interact with membranes (4), and it has been suggested that the cytoskeleton is associated with membrane components such as surface receptors (37). Local anesthetics, by their action on the membrane, may influence the attachment or anchoring of the microtubular system to the plasma membrane. If the microtubular system were detached from the cell membrane and contracted, the microtubules themselves could remain intact, as was seen here, but microtubule-dependent secretion could be blocked in a manner analogous to that caused by colchicine. There is precedent for such a suggestion.

For example, electron micrographs of tetracaine-treated 3T3 cells (37) show cytoskeletal disruption only immediately beneath the cell membrane. These authors remark on the contraction of organelles to the nuclear area and state that it was impossible to determine whether the cytoskeletal elements were disrupted or merely disorganized. Another electron micrographic study of anesthetic-treated 3T3 cells (25) shows absence of microtubules immediately beneath the

cell membrane. Electron micrographs of human polymorphonuclear leukocytes (15) showed no effect of local anesthetics on the microtubular system in cells unstimulated with respect to exocytosis but the drugs prevented the increase in microtubules asociated with stimulation. The conclusions of that study included the suggestion that the cell membrane was the primary target for the local anesthetics with subsequent influence on the cytoskeleton, possibly through its membrane attachments. Finally, studies on morphological differentiation of mouse neuroblastoma cells showing similar morphologic and cytoskeletal effects of colchicine and local anesthetics led to the suggestion that the cell membrane was the actual target for both types of drugs (12).

It has been suggested that the displacement of Ca^{++} from cell membranes caused by local anesthetics may influence the attachment of cytoskeletal elements to the cell membrane or may even raise the intracellular Ca^{++} concentration sufficiently to induce depolymerization of microtubules (24, 25). Local anesthetic treatment did not lead to microtubule depolymerization in our studies, and there exist no data regarding the possible role of Ca^{++} in the attachment of the cytoskeleton to the cell membrane. One alternative hypothesis regarding the inhibition of secretion by both local anesthetics and colchicine is that both may act on the membrane and that the microtubular system is not involved in the inhibition of secretion. Colchicine can influence membrane phenomena such as nucleotide transport (23), and appears to interact with membrane protein (1). If inhibition of secretion were unrelated to colchicine's actions on the microtubular system, then possibly both colchicine and the local anesthetics cause inhibition by effects on the cell and/or Golgi membranes that might, for example, prevent fusion of secretory granules with the plasma membrane. This seems unlikely because of the strong association of microtubule depolymerization with inhibition of secretion by colchicine as detailed above.

The combination of the microtubule-disrupting drug colchicine and the microfilament-disrupting drug cytochalasin B did not duplicate the effects of local anesthetics on collagen secretion (Table IV, exp. 1), contrary to what has been found in cell receptor systems (36, 37, 24). In those systems (37), colchicine alone had little, if any, effect on the measured parameters while cytochalasin B alone had intermediate effects and the effect of the combination of the two exceeded the sum of the two used separately. The opposite was seen here with respect to collagen secretion: a saturating concentration of colchicine had an intermediate inhibitory effect and cytochalasin B almost none. Combining the two did not increase the effect of colchicine alone. This suggests that the actions of local anesthetics in inhibiting collagen secretion are not completely analogous to those producing alterations in the control of cell surface receptors. Similarly, there are marked differences in the morphologic changes induced by local anesthetics compared to colchicine and cytochalasin B, either separately or in combination (Fig. 3).

That local anesthetics inhibit collagen secretion leads to consideration of possible clinical applications of these drugs where collagen secretion is pathologic. Fibrosis from excessive collagen deposition is a component of several disease processes, cirrhosis of the liver being a prime example. Liver collagen synthesis in vivo in rats with carbon tetrachloride-induced cirrhosis was reduced by administration of colchicine, and this drug has been used to treat human patients with cirrhosis (45). Treatment with local anesthetics in such situations might be tested, since relatively low concentrations of these drugs inhibit collagen secretion to a greater degree than does colchicine. Procainamide, an analogue of procaine in which the central ester linkage is replaced by an amide, is routinely administered to humans on a chronic basis for control of cardiac arrhythmias. Studies of procainamide or other local anesthetics or their analogues in model systems will evaluate the potential usefulness of these drugs in preventing and treating destructive fibrosis resulting from excessive collagen deposition.

In summary, the experiments reported here demonstrate that tertiary amine local anesthetics inhibit secretion of collagen and non-collagen protein in cultured mammalian cells and do so independent of effects on amino acid transport and protein synthesis. The tertiary amine local anesthetics do not bind to tubulin and do not depolymerize microtubules as does colchicine, and the local anesthetics at saturating concentrations inhibit protein secretion to an even greater degree than saturating colchicine. Their action may, however, be related to microtubular function because the effects of subsaturating concentrations of the two types of drugs are additive. We suggest that the local anesthetics inhibit secretion by influencing the microtubular system through an effect on

the cell membrane, possibly by detaching it from the cell membrane.

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