Platelets and Microtubules

EFFECT OF COLCHICINE AND D_2O ON PLATELET AGGREGATION AND RELEASE INDUCED BY CALCIUM IONOPHORE A23187

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ABSTRACT We examined the role of microtubules in platelet aggregation and secretion (release reaction) induced by the calcium ionophore A23187 (0.8–5 μM). At these concentrations, platelet aggregation was preceded by a lag period of ~1 min. Colchicine (an agent that disrupts microtubule assembly-disassembly) was shown to bind to platelet microtubules by employing [^{3}H]colchicine at a concentration that is specific for microtubules in other tissues (10 nM). Colchicine prolonged the lag period, inhibited the secondary wave of platelet aggregation, and inhibited the release reaction (release of [^{14}C]serotonin). Platelets were next incubated with 20–60% D_2O, an agent that stabilizes microtubules. D_2O overcame colchicine-induced inhibition of the lag period, aggregation, and release. D_2O alone enhanced platelet aggregation by 59±14% (SEM) and shortened the lag period by 43±10%. We conclude that functioning microtubules are required for platelet aggregation and release, and that microtubules of platelet preparations are functioning submaximally.

INTRODUCTION

The platelet is a cell whose major physiologic function, formation of a firm hemostatic plug, requires primary platelet aggregation by physiologic aggregating agents (collagen, ADP, thrombin, epinephrine); secretion of platelet contents (release reaction); and the irreversible secondary wave of platelet aggregation. This is accompanied by a change in platelet shape from disk to sphere (1). The secondary wave of platelet aggregation is preceded by, or associated with (2), the secretion of various platelet constituents such as ADP, Ca^{++}, and serotonin, which contribute to further propagation of the platelet plug. In other tissues, secretory events have been closely associated with microtubules (3, 4). Little is known of this association in platelets.

The role of microtubules has been postulated to be that of a supporting structure that helps maintain the platelet discoid shape (5). Its possible role in platelet secretion can be inferred from other studies using platelet aggregating agents and electron microscopy (6). A centripetal movement of circumferential microtubules and platelet granules before the release reaction has been described. Irreversible secondary platelet aggregation and release were associated with the apparent disappearance of centrally located microtubules (7). The secondary wave of platelet aggregation (induced by ADP or epinephrine) could be inhibited by "toxic" concentrations (1 mM) of anti-microtubule agents that cause the apparent disappearance of microtubules under electron microscopy (7, 8), and that have additional properties other than their inhibition of microtubule assembly (9–16).

The purpose of this investigation was to study the role of microtubules in platelet aggregation induced by the Ca^{++} ionophore A23187, as well as other aggregating agents, with concentrations of antimicrotubule agents that are specific for microtubules. The Ca^{++} ionophore was employed because internal as well as external translocations of calcium are thought to trigger internal contractile events, as well as platelet release and aggregation (17, 18), and serotonin release from platelets by the Ca^{++} ionophore has some characteristics of the physiologic release reaction (17).

METHODS

Platelet-rich plasma (PRP)[1] *for platelet aggregation studies.* PRP was obtained from laboratory volunteers who had not

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[1] *Abbreviation used in this paper*: PRP, platelet-rich plasma.

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ingested any drugs for at least 1 wk. Whole blood was collected into 3.8% trisodium citrate (nine parts plus one part) in plastic tubes and centrifuged at 150 *g* for 5 min at room temperature in a desktop International centrifuge (International Equipment Co., Boston, Mass.). PRP was kept at room temperature for 30 min in tightly capped plastic tubes and then equilibrated at 37°C for 3 min before testing. Platelet-poor plasma was obtained by centrifuging the remainder of the blood at 2,000 *g* for 20 min.

Aggregating agents. The aggregating agents used were dissolved in saline and diluted with 0.03 M veronal buffer and 0.13 M NaCl, pH 7.4, in a volume of 50 μl. Final concentrations were as follows: ADP disodium salt, 50 μM; epinephrine hydrochloride, 10 μM; connective tissue suspension prepared by the method of Zucker and Borrelli (19) and henceforth referred to as "collagen" at dilutions of 1:8,000 to 1:16,000; Ca^{++} ionophore A23187, 0.8–5 μM.

Platelet aggregometry. Aggregometry was performed with platelet aggregating agents in a Bio-Data Aggregometer (Bio-Data Corp., Willow Grove, Pa.) at 37°C, using 0.50 ml combined volume of PRP and reagents in a cylindrical glass cuvette (0.8 × 5 cm) under constant stirring with a magnetic stirring bar. Platelet aggregation was measured turbidometrically by recording changes in the light transmission of PRP during aggregation. As platelets coalesce into larger aggregates, there is greater transmission of light, which is recorded by a downward deflection of the recording pen on a chart recorder (driven at a constant speed of 2.5 cm/min). Platelet aggregation was quantified by measuring the slope of the aggregation curve in units of percent transmission per minute (%T/min). The aggregometer was standardized automatically with platelet-poor plasma and PRP on a scale of 0–100% light transmission. Maximal percent transmission change, at 4 min, was also measured on some occasions.

Serotonin release. [^{14}C]Serotonin was dissolved in 70% ethanol to a concentration of 8 μCi/ml. PRP was prepared as above and incubated with [^{14}C]serotonin at 37°C for 5 min, 1 μl [^{14}C]serotonin/ml PRP. This PRP was then used for platelet aggregometry and for determination of percent serotonin release (platelet release reaction). Serotonin release was determined after addition of the aggregating agent for 4 min. The PRP was centrifuged at 2,000 *g* for 10 min at 4°C. A portion of the platelet-poor plasma obtained (100 μl) was assayed for radioactivity in a Beckman LS100 scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.), and compared with the radioactivity obtained from a similar aliquot of uncentrifuged PRP. Blank radioactivity was obtained by centrifuging PRP that had not been exposed to the aggregating agent. This was subtracted from the platelet-poor plasma radioactivity obtained after platelet aggregation. Percent release was calculated by dividing the released radioactivity by the total PRP radioactivity and multiplying by 100.

Incubation of platelets with [^{3}H]colchicine. 1 U of human PRP, collected in acid citrate dextrose, was obtained from the New York Blood Center. Platelets were sedimented at 2,500 *g* for 15 min at 4°C, resuspended in 30 ml of 1% ammonium oxalate, and allowed to sit at room temperature for 10 min. The platelets were then resedimented and washed with 10 ml of Tris-buffered saline, pH 7.4, containing 0.14 M NaCl, 0.015 M tris-HCl, and 5.5 mM glucose (20); resuspended in 1 ml of buffer (5–10% volume suspension); and incubated in 17 × 100-mM polypropylene tubes (Falcon Labware, Div. of Becton, Dickinson, and Co., Oxnard, Calif.) at 37°C, in the presence of [^{3}H]colchicine, 1 × 10^{6} cpm/ml, for 60 min.

Preparation of platelet cytoplasm. The platelets were sedimented at 2,000 *g* for 15 min at 4°C and washed three times with 10 ml of buffer. Platelets were resuspended in 1 ml of buffer and sonicated at 0°C with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at maximal intensity for two 15-sec periods. The sonicated platelets were then centrifuged at 100,000 *g* for 1 h at 4°C, using a Beckman model L Ultracentrifuge.

Molecular weight determination of protein bound to tritiated colchicine. The extract prepared above was applied to a calibrated Sephacryl S-200 gel filtration column (Pharmacia Diagnostics, Div. of Pharmacia, Inc., Piscataway, N. J.) and the molecular weight of the tritiated protein peak was determined with protein molecular weight standards (21): immunoglobulin (155,000), bovine serum albumin (68,000), ovalbumin (43,000), and carbonic anhydrase (29,000). Protein was monitored by 280 μm absorbance. [^{3}H]Colchicine was assayed for radioactivity by scintillation counting.

Polyacrylamide gel electrophoresis of tritiated colchicine peak fraction. The fraction containing the peak radioactivity was concentrated by ultrafiltration under vacuum and electrophoresed under nondenaturing conditions. The gel was 7 cm long, 0.6 cm in diameter, and composed of 10% polyacrylamide with 0.28% cross-linkage in 0.1 M phosphate buffer, pH 7.0. The procedure of Weber and Osborn (22) was followed, except for the absence of sodium dodecyl sulfate, using a Pharmacia gel electrophoresis apparatus,

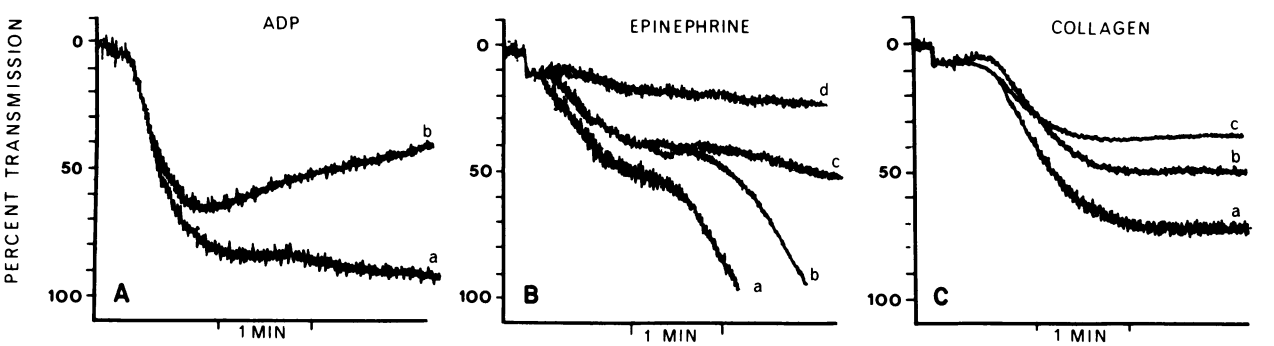

FIGURE 1 Effect of colchicine on platelet aggregation induced by ADP, epinephrine, or collagen. 0.4 ml of PRP was incubated with varying concentrations of colchicine in a volume of 50 μl for 2 min at 37°C, followed by stirring with a magnetic stirring bar for 1 min and addition of 50 μl of aggregating agent. (A) ADP, 0.05 mM; *a* and *b* refer to 0 and 1 mM colchicine, respectively. (B) Epinephrine, 0.01 mM; *a*, *b*, *c*, and *d* refer to 0, 0.5, 1, and 2 mM colchicine, respectively. (C) Collagen, 1:8,000 dilution; *a*, *b*, and *c* refer to 0, 0.25, and 0.5 mM colchicine, respectively.

TABLE I

*Additive Effect of Colchicine and Vinblastine on Platelet Aggregation Velocity Induced with Ca^{++} Ionophore A23817**

Experiment	1	2	3
		% inhibition	
Colchicine, 1 mM	19	20	20
Vinblastine, 0.06 mM	31	18	12
Colchicine and vinblastine	54	36	24
Theoretical	50	38	32

* 0.35 ml of PRP was mixed with 50 μl of veronal buffer or used undiluted, and then incubated with either 50 μl of colchicine, vinblastine, buffer, or colchicine plus vinblastine (100 μl) for 8 min at 37°C. This was followed by 1 min of stirring with a magnetic stirring bar, then the addition of 50 μl of Ca^{++} Ionophore at a final concentration of 2 μM. Percent inhibition refers to inhibition of control aggregation (buffer addition) performed in the absence of colchicine or vinblastine.

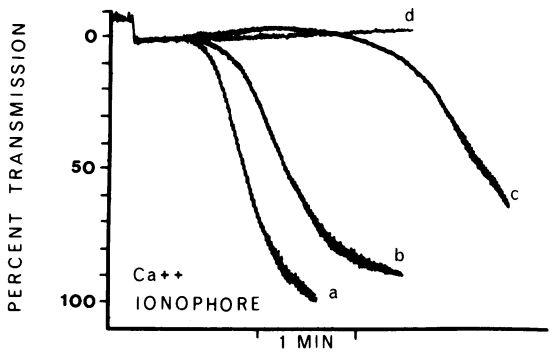

FIGURE 2 Effect of vinblastine on Ca^{++} ionophore-induced platelet aggregation. PRP was diluted 1:2 in veronal buffer and 0.4 ml of this suspension was incubated at 37°C for 4 min with 50 μl of buffer solution containing indicated final vinblastine concentration. *a*, *b*, *c*, and *d* refer to 0, 0.04, 0.08, and 0.16 mM vinblastine, respectively. The suspension was then stirred with a magnetic stirring bar for 1 min, followed by addition of 50 μl of Ca^{++} ionophore at final concentration, 2 μM.

GE-14 (Pharmacia Inc.). The gel was equilibrated with buffer for 30 min at 5 mA/gel before adding the sample. The sample was then run at 5 mA/gel for 30 min, to allow protein to penetrate the gel, and the amperage was then raised to 15 mA/gel for 3–4 h. The gel was stained with 0.25% Coomassie Blue (dissolved in 46% methanol-9% glacial acetic acid) by immersing a test tube containing the gel and staining fluid in a boiling water bath for 15 min. The gels were destained in 5% acetic acid-35% ethanol at 56°C overnight. An unstained gel was fixed overnight in 46% methanol-9% acetic acid, frozen with dry ice, and cut into 1-mm slices with a Brinkmann Gel Slicer (Brinkmann Instruments, Inc., Westbury, N. Y.). The gel slices were placed in scintillation vials, and 5 ml of 3% Protosol-0.4% Omnifluor in toluene was added (New England Nuclear, Boston, Mass.). The vials were maintained at 37°C for 3 h and then assayed for radioactivity by scintillation counting.

Materials. All materials were reagent grade. Colchicine, vinblastine, and D_2O were obtained from Sigma Chemical Co., St. Louis, Mo. Lumicolchicine was prepared by exposure

TABLE II

Effect of D_2O on Platelet Aggregation Velocity Induced by Calcium Ionophore A23187 and on Colchicine-induced Inhibition of Aggregation Velocity

Number of experiments	Aggregation velocity					
		D_2O			2 mM Colchicine	
	Control	20%	40%	60%	+Buffer	+30% D_2O
			% Transmission/min			
7	108±4.5	124±4.8 (+15)*				
4	122±9.3		150±2.5 (+23)‡			
7	80±7.6			127±16 (+59)‡		
5	127±6.6				83±6.0 (−35)§	106±9.2 (−16)‖

0.2 ml of veronal buffer made up in 100% D_2O or HOH was mixed with 0.2 ml of PRP to give varying concentrations of D_2O. 50 μl of colchicine or buffer (dissolved in H_2O or D_2O) was then added. The mixture was incubated at 37°C for 8 min and then stirred with a magnetic stirring bar for 1 min, before the addition of 50 μl of Ca^{++} ionophore in H_2O or D_2O at final concentration, 1 μM. Percent change from control velocity is given in parentheses. *P* values were obtained from a matched *t* test.

* $P < 0.01$.

‡ $P < 0.05$.

§ $P < 0.001$.

‖ Difference between colchicine and colchicine plus 30% D_2O. $P < 0.05$.

TABLE III

*Effect of Colchicine and D_2O on [^{14}C]Serotonin Release Induced by Ca^{++} Ionophore A23187**

Experiment	Colchicine	Percent release of serotonin			
		HOH buffer	HOH + alkaloid	D_2O buffer	D_2O + alkaloid
	μM				
1	100	34	7.6	29	35
	200	34	0		17
2	100	24	7.1		20
3	500	62	13.0	42	32
4	300	66	0		20
5	100	42	30.0		
	200	42	27.0		
	300	42	13.0		
	400	42	0		38
6	100	30	7.0	39	
	200	30	0		23
7‡	0.2	76±4.9	5.1±2.8	68±5.4	63±8.4

* 0.2 ml of PRP was diluted 1:1 with veronal buffer in HOH or D_2O (60% final concentration) and preincubated for 4 min (in the presence or absence of 50 μl of buffer or colchicine) before stirring with a magnetic stirring bar for 0.5 min, followed by addition of 50 μl of Ca^{++} ionophore, final concentration 5 μM (experiments 4 and 5, 10 μM).

‡ 1 h preincubation with colchicine, HOH buffer, D_2O buffer, or D_2O buffer plus colchicine at 37°C, before addition of Ca^{++} ionophore (mean of six experiments ±SEM).

of colchicine to UV light. Epinephrine hydrochloride was obtained from Parke-Davis, Detroit, Mich. Ca^{++} ionophore A23187 was a gift from Eli Lilly and Co., Indianapolis, Ind., and was dissolved in dimethyl sulfoxide. [2-^{14}C]Serotonin, 30 mCi/mmol, was obtained from Amersham and Searle, Arlington Heights, Ill. [^{3}H]Colchicine, 10–20 Ci/mmol, was obtained from New England Nuclear, Boston, Mass. All reagents for polyacrylamide gels were obtained from Bio-Rad Laboratories, Richmond, Va. Sephacryl S-200 was obtained from Pharmacia Inc., Piscataway, N. J. Purified human brain tubulin was a gift from Dr. L. Liebes, New York University Medical School.

RESULTS

Effect of colchicine on platelet aggregation induced by ADP, epinephrine, and collagen. Fig. 1*a*, *b*, and *c* demonstrate the inhibitory effect of preincubation of 0.25–2 mM colchicine for 2 min on platelet aggregation induced by ADP, epinephrine, or collagen, confirming initial observations of White (7) as well as others (23, 24). As can be noted, this inhibition affects the secondary wave of platelet aggregation more than the primary wave.

Effect of colchicine or vinblastine on platelet aggregation induced by Ca^{++} ionophore A23187. Calcium ionophore-induced platelet aggregation at <5 μM was preceded by a lag period which in all studies was followed by a single wave of platelet aggregation. Fig. 2 demonstrates the effect of 0.04–0.16 mM vinblastine on Ca^{++} ionophore-induced aggregation. This prolonged the lag phase by 33–72%, and impaired platelet aggregation velocity. Similar results were obtained with colchicine, which was found to be 20- to 40-fold less potent on a molar basis. For example, 1.5 mM colchicine inhibited the aggregation velocity by 31±1.1% (SEM) and increased the lag period by 37±2.7% (12 experiments, $P < 0.001$). Similar results were obtained with 0.04 mM vinblastine, which inhibited the velocity of aggregation by 28±1.5% and

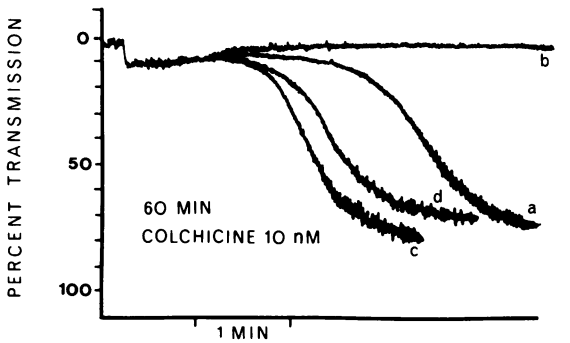

FIGURE 3 Effect of preincubation of platelets with 10 nM colchicine for 1 h on platelet aggregation with veronal buffer. (*a*) 0.4 ml of PRP (diluted 1:1 with veronal buffer) was incubated with 50 μl of veronal buffer for 1 h at 37°C, followed first by stirring with a magnetic stirring bar for 1 min, then the addition of Ca^{++} ionophore in 50 μl at final concentration, 0.8 μM. (*b*) Same as in *a*, except that veronal buffer contained colchicine to yield a final concentration of 10 nM. (*c*) Same as in *a*, except that platelets were preincubated for 1 h with veronal buffer made up in D_2O to yield a final concentration of 50% D_2O, at 37°C. (*d*) Same as in *c*, except that veronal buffer also contained colchicine at 10 nM.

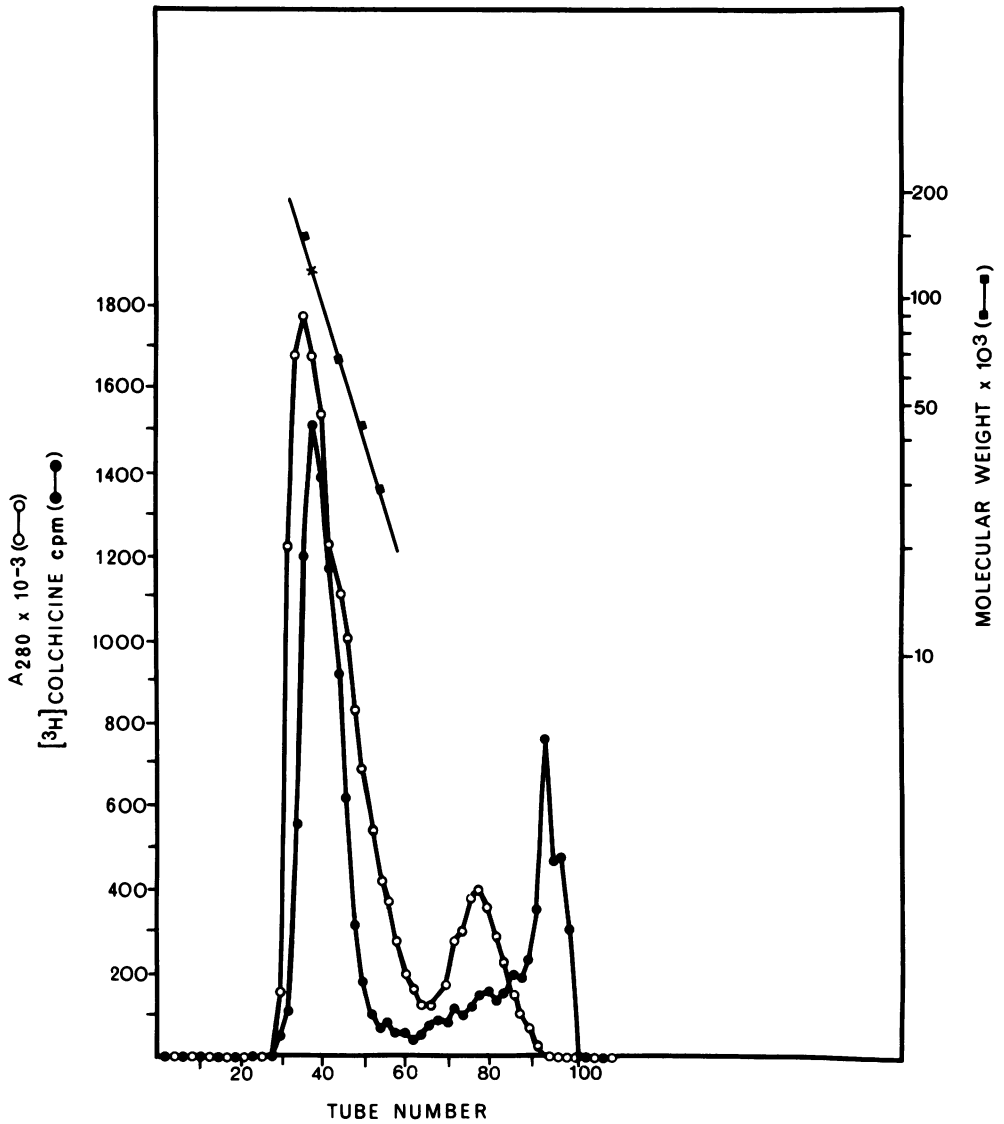

FIGURE 4 Binding of 10 nM colchicine to platelet tubulin. Platelets were incubated in the presence of 10 nM tritiated colchicine, 1 million cpm/ml, for 1 h, and the cell sap was prepared as cited in Methods. 1 ml of the cell sap was applied to a calibrated (■) Sephacryl-200 gel filtration column, 1.0 × 28 cm, and the effluent (0.5-ml fractions) monitored for radioactivity (●) and 280 μm absorbance (○). Peak radioactivity was obtained at an apparent molecular weight of 110,000. Free tritiated colchicine peaked at tube number 94.

increased the lag period by 33±2.5% (Five experiments, $P < 0.001$). The effect of both colchicine and vinblastine was additive (Table I).

Effect of D_2O and colchicine on platelet aggregation and release of serotonin induced by Ca^{++} ionophore A23187. D_2O, an agent known to stabilize microtubules in other tissues, shortened the Ca^{++} ionophore-induced lag period by 43±10% and increased the velocity of platelet aggregation by 59±14% ($P < 0.05$) at a D_2O concentration of 60% (average of seven experiments). Similar changes were noted at 40% ($P < 0.05$) and 20% ($P < 0.01$) D_2O (Table II). The combination of D_2O and colchicine apparently corrected the alkaloid-induced inhibition of platelet aggregation velocity and prolonged the lag period (Table II). For example, 2 mM colchicine inhibited the H_2O control by 35% ($P < 0.001$), whereas inhibition (16%) was negligible in the presence of 30% D_2O ($P < 0.1$). Similar results were obtained with the lag period (data not shown).

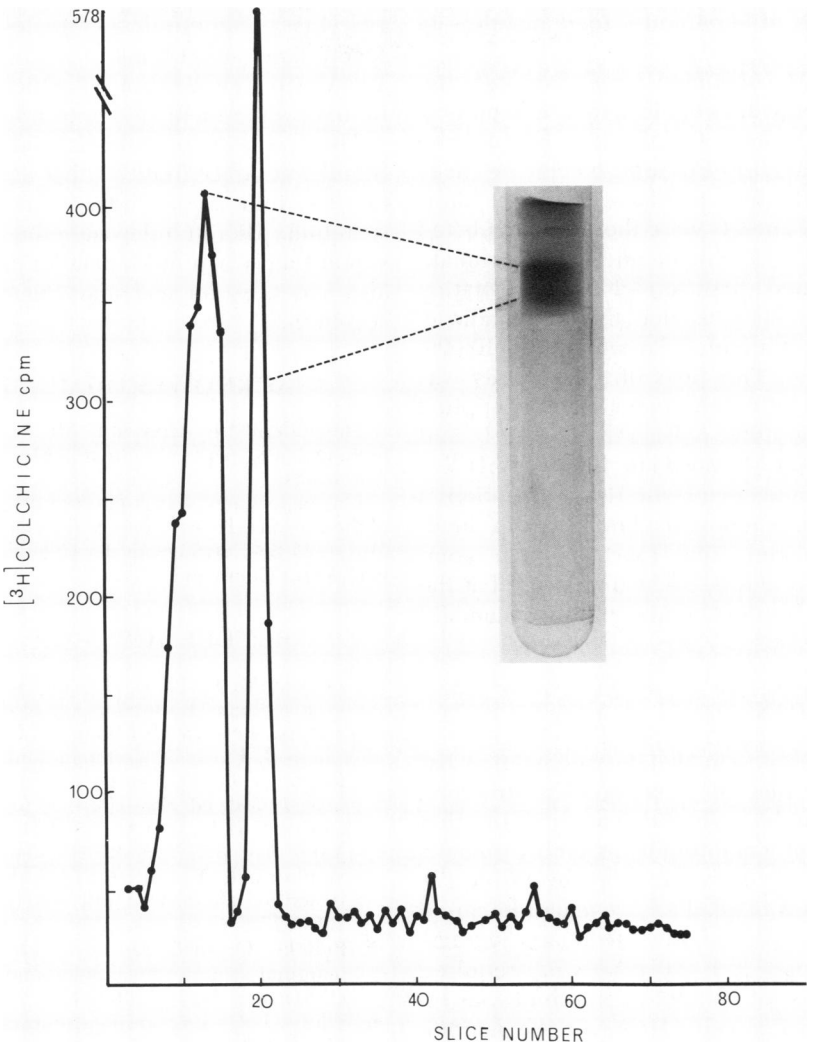

FIGURE 5 Electrophoresis of tritiated colchicine peak fraction from Fig. 4 on 10% polyacrylamide gel in 0.1 M phosphate buffer, pH 7.0. The gel was fixed, frozen, sliced, monitored for radioactivity, and compared with a similar gel on which a purified brain tubulin standard was applied (inset). The two radioactive (●) monomer components of tubulin, α and β, were electrophoresed at the same position as purified brain tubulin standard.

Table III demonstrates the effect of colchicine and D_2O on the platelet release reaction. Thus, [^{14}C]-serotonin release was inhibited by colchicine at 0.1–0.5 mM (experiments 1–6), after addition of Ca^{++} ionophore at 5–10 μM. D_2O (40%) partially to completely prevented the colchicine-induced inhibition of [^{14}C]serotonin release.

Effect of 10 or 100 nM colchicine on platelet aggregation and release. Since colchicine at 0.1 mM does not inhibit platelet aggregation after 2 min of preincubation, we examined the possible effect of longer preincubation at lower colchicine concentration on platelet aggregation and release. Fig. 3 demonstrates that after preincubation for 1 h, 10 nM colchicine does inhibit platelet aggregation, compared with a control incubation.[2] D_2O alone shortened the lag period and enhanced aggregation velocity. D_2O plus colchicine completely overcame the inhibition of 10 nM colchicine (representative of five experiments). In similar experiments, low concentration of colchicine (0.1 μM) also inhibited platelet release of serotonin.(Lumicolchicine an agent that does not bind tubulin, at 0.1 μM had no effect). D_2O plus colchicine overcame this inhibition (Table III, experiment 7). Lysis of serotonin-loaded control platelets in D_2O buffer gave similar

[2] It is of interest to note that 0.5 μM colchicine has been shown to inhibit platelet spreading on glass slides (25).

release, as did lysis of serotonin-loaded platelets in H_2O buffer.

Binding of 10 nM colchicine to platelet tubulin. Since 10 nM colchicine does not lead to disassembly of microtubules in platelets, as indicated by electron microscopy, whereas 0.1–1.0 mM colchicine does (7), we examined whether colchicine at 10 nM binds to platelet tubulin. Platelets were therefore incubated in the presence of [^{3}H]colchicine and the cell sap applied to a calibrated Sephacryl-200 gel filtration column. Fig. 4 demonstrates that the peak radioactivity was obtained at a molecular weight of 110,000, that of tubulin dimer. This peak of radioactivity was then concentrated by filtration under vacuum, applied to a nondenaturing 10% polyacrylamide gel, and electrophoresed in 0.1 M phosphate buffer at pH 7.0. The gel was frozen and sliced, and the distribution of radioactivity was compared with a brain tubulin standard simultaneously run. The two monomer components of tubulin, α and β, coelectrophoresed at the same position as purified brain tubulin standard (Fig. 5).

DISCUSSION

The role of microtubules in platelet aggregation has been relegated to that of a supporting structure, maintaining the platelet's discoid shape (5). In other tissues, inhibition of microtubule assembly impairs secretory events (3, 4). Previous work by White (7), employing toxic doses of alkaloids, revealed impaired platelet aggregation, particularly secondary aggregation. These toxic doses (0.1–1.0 mM) were required for the disappearance of platelet microtubules, as indicated by electron microscopy. However, the pharmocologic dosage of colchicine used in the treatment of gout is in the 1-μM range, and concentrations of 10 μM are fatal (26). Furthermore, in vitro concentrations of colchicine in the 10–1000 μM-range are known to affect other cellular processes (9–16).

It was therefore necessary to determine whether colchicine impairs platelet aggregation by reacting with microtubules, and whether colchicine also inhibits platelet secretion. This was accomplished by three approaches. First, concentrations of colchicine were employed that were fairly specific for microtubules in other tissues (27, 28). Platelets were incubated with colchicine at 10 and 100 nM for 60 min before addition of the aggregating agent, Ca^{++} ionophore A23187. This dose was also noted to inhibit secretion of platelet serotonin. Second, D_2O, a known stabilizer of microtubules, nullified the colchicine-induced inhibition of platelet aggregation and secretion. Furthermore, 60% D_2O alone also enhanced by 59% platelet aggregation induced by Ca^{++} ionophore and reduced the lag period by 43%. These data suggest that microtubules of platelet preparations are functioning submaximally. Third, [^{3}H]colchicine at 10 nM was shown to bind specifically to platelet microtubules when incubated with intact platelets.

Thus, colchicine, which binds to platelet microtubules at concentrations that are specific for microtubules, inhibits secondary platelet aggregation and secretion. Microtubules therefore not only provide a supporting structure for platelet discoid shape, but are also necessary for secondary platelet aggregation and secretion.

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