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Evidence for Involvement of Microtubules in the Action of Vasopressin in Toad Urinary Bladder

I. Functional Studies on the Effects of Antimitotic Agents on the Response to Vasopressin

Ann Taylor, Mortimer Mamelak, Helen Golbetz, and Roy Maffly

Department of Medicine, Stanford University School of Medicine, Stanford, California 94305, the Veterans Administration Hospital, Palo Alto, California 94304, and the Department of Physiology, Cornell University Medical College, New York, N.Y. 10021

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Summary. The antimitotic agents colchicine, podophyllotoxin, and vinblastine inhibit the action of vasopressin and cyclic AMP on osmotic water movement in the toad urinary bladder. The alkaloids have no effect on either basal or vasopressin-stimulated sodium transport or urea flux across the tissue. Inhibition of vasopressin-induced water movement is half-maximal at the following alkaloid concentrations: colchicine, 1.8×10^{-6} M; podophyllotoxin, 5×10^{-7} M; and vinblastine, 1×10^{-7} M. The characteristics of the specificity, time-dependence and temperature-dependence of the inhibitory effect of colchicine are similar to the characteristics of the interaction of this drug with tubulin *in vitro*, and they differ from those of its effect on nucleoside transport. Inhibition of the vasopressin response by colchicine, podophyllotoxin, and vinblastine is not readily reversed. The findings support the view that the inhibition of vasopressin-induced water movement by the antimitotic agents is due to the interaction of these agents with tubulin and consequent interference with microtubule integrity and function. Taken together with the results of biochemical and morphological studies, the findings provide evidence that cytoplasmic microtubules play a critical role in the action of vasopressin on transcellular water movement in the toad bladder.

Vasopressin (antidiuretic hormone) is known to have two major physiological actions. Vasopressin induces the contraction or relaxation of certain types of smooth muscle [23, 29] and promotes the movement of water (and, in some instances, of sodium and urea) across responsive epithelial tissues—most notably, amphibian skin [18] and urinary bladder [2, 19, 21, 25] and the mammalian distal renal tubule [11].

The mode of action of vasopressin on water movement across epithelial tissues has been extensively investigated; however, the cellular

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mechanisms involved in this action of the hormone remain to a large extent unknown [13]. It is generally accepted that the hormone interacts with specific receptors in the baso-lateral cell membranes, leading to activation of adenylate cyclase and the generation of cyclic 3′5′ adenosine monophosphate (cyclic AMP) [1, 12, 13, 30]. The cellular events that intervene between the generation of cyclic AMP at the level of the baso-lateral cell membranes and the increase in permeability of the apical cell membrane have not yet been defined. Phosphorylation [16, 17, 30] and dephosphorylation [5, 7] systems have been implicated in the action of vasopressin, but their precise functional role in the physiological response to the hormone remains to be established.

The possibility that the actions of vasopressin on smooth muscle contraction and on transepithelial water movement involve analogous cellular and molecular mechanisms is intuitively appealing. Recent evidence indicates that many types of cellular function involving the translocation of cell constituents in nonmuscle cells depend on mechanisms similar to those involved in muscle contraction [9]. The cytoskeletal elements, microtubules and microfilaments, appear to play an integral role in such processes [9, 14, 27, 32]. These considerations led us to investigate the possibility that microtubules are involved in the action of vasopressin.

The antimitotic agents colchicine, podophyllotoxin, and vinblastine are known to exert disruptive effects on microtubules *in vivo* [6] and to interact with tubulin, the major protein subunit of microtubules, *in vitro* [3, 38, 40]. This paper describes the effects of these agents on the response to vasopressin in the toad urinary bladder. Biochemical and electron microscopic studies have been carried out in parallel with the functional studies described here and are reported in two accompanying papers [28, 42]. A systematic attempt has been made in these studies to relate the functional effects of the antimitotic agents to their locus and mode of action in the tissue. The results of the combined studies support the view that cytoplasmic microtubules participate in the action of vasopressin on transcellular water movement in the toad bladder.[1]

Materials and Methods

Colombian toads *(Bufo marinus)* were obtained from Tarpon Zoo, Tarpon Springs, Florida; they were sacrificed by pithing.

1 Portions of this work have been published in preliminary form [35, 36].

Measurement of Osmotic Water Movement

Osmotic water movement across isolated urinary bladders was measured by a modification of the method of Bentley [2]. Paired hemibladders were excised and mounted as bags on a short length of glass tubing. After being rinsed out, each hemibladder bag was filled with 5 ml of distilled water and suspended in a covered bath containing 50 ml of aerated full-strength frog Ringer's solution (Na 111.2, Cl 113.0, K 5.4, Ca 1.78, HPO_4 4.8, H_2PO_4 0.6 mEq/liter; pH 7.3; 220 mOsm/kg H_2O). Water movement out of the paired hemibladders was measured gravimetrically; at intervals each hemibladder bag was removed from its bath, gently blotted, and weighed on a Mettler scale. After an initial period of equilibration, the test alkaloid was added to the serosal bathing medium (except when otherwise specified) of one of each pair of hemibladders while its pair served as a control. Weight loss of each hemibladder was measured hourly during the period of preincubation with the test alkaloid (0–6 hr). Vasopressin (or cyclic AMP) was subsequently added to the serosal medium of both members of each pair of hemibladders and weight loss measured at 30-min intervals for 2 hr. In order to minimize differential changes in the volume of fluid within the bags, water lost from the individual hemibladders after addition of vasopressin (or cyclic AMP) was replaced at the end of 1 hr by refilling each hemibladder bag to its initial volume. At the end of the experiment, the osmolality of the solutions bathing the mucosal and serosal surfaces of each hemibladder was determined using a Fiske osmometer.

Measurement of Sodium Transport

Transepithelial sodium transport was measured in separate experiments in freshly-isolated paired hemibladders using the short circuit current (scc) technique of Ussing and Zerahn [34, 37]. The test alkaloid was added to the serosal bathing medium of one of each pair of hemibladders; the scc was monitored for 2 to 4 hr, and vasopressin 20 mU/ml was then added to the serosal medium of both members of each pair of hemibladders.

Measurement of Urea Permeability

Measurement of urea permeability of the bladder was carried out using the method of Maffly *et al.*, [21] employing ^{14}C-urea.

All studies were carried out at room temperature (24 ± 2 °C), except where specified.

Values for P were calculated for paired data, or for unpaired data where specified, using Student's "t" test.

Reagents

The following compounds were obtained commercially: colchicine (Sigma Chemical Co., Cal Biochem); vinblastine sulphate ("Velban", Eli Lilly & Co.); podophyllotoxin (Aldrich Chemical Co.); vasopressin ("Pitressin", Parke-Davis and Co.); cyclic 3′5′-adenosine monophosphoric acid (Sigma Chemical Co.); ^{14}C-urea (Amersham/Searle). Lumicolchicine (prepared by irradiation of colchicine with ultra-violet light [41] was kindly provided by Dr. L. Wilson.

Fresh solutions of colchicine, podophyllotoxin, and vinblastine were made daily; colchicine and podophyllotoxin were dissolved in distilled water, and vinblastine in Ringer's solution. Lumicolchicine was dissolved in 95 % ethanol and was added to the

bathing medium of the experimental hemibladders to give a final concentration of ethanol of 0.1 %; a comparable amount of ethanol was added to the medium bathing the control hemibladders.

Results

I. Studies of Osmotic Water Movement

Effect of Colchicine on Osmotic Water Movement

The basal rate of water movement across isolated hemibladders in the absence of vasopressin was not influenced by exposure of the tissues to colchicine in the range 2×10^{-7} to 8×10^{-4} M; thus, even at the highest concentration of colchicine tested (8×10^{-4} M) the mean weight loss of hemibladders exposed to the alkaloid over a 4 hr period was 0.7 ± 0.1 mg/min, while that of their paired controls was 0.6 ± 0.1 mg/min ($n = 5$, NS).

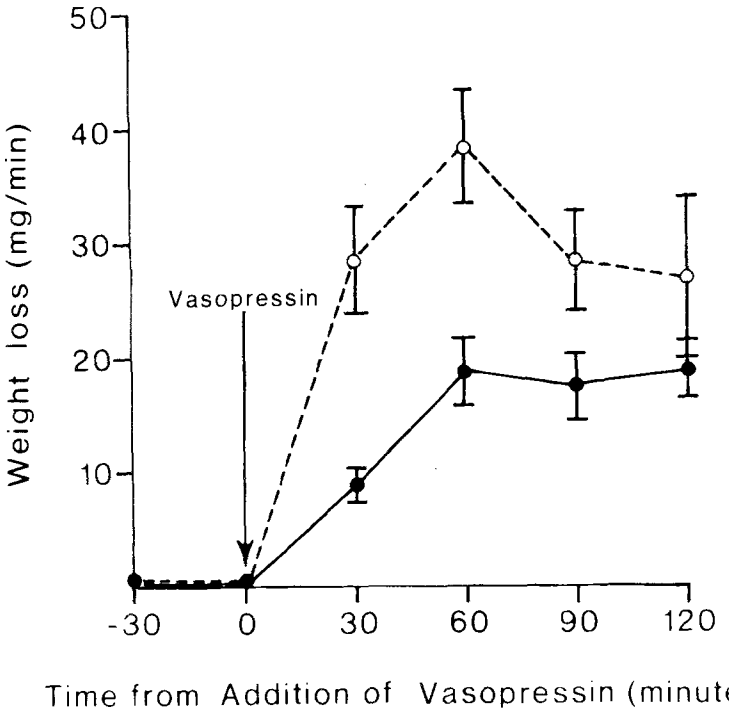

Fig. 1. The effect of colchicine on vasopressin-induced osmotic water movement. One member of each pair of hemibladders was exposed to colchicine 2×10^{-5} M for 4 hr prior to addition of vasopressin 20 mU/ml to the bathing medium of the experimental (●——●) and control (o-----o) hemibladders. Each point represents the mean ±SE of 7 paired experiments

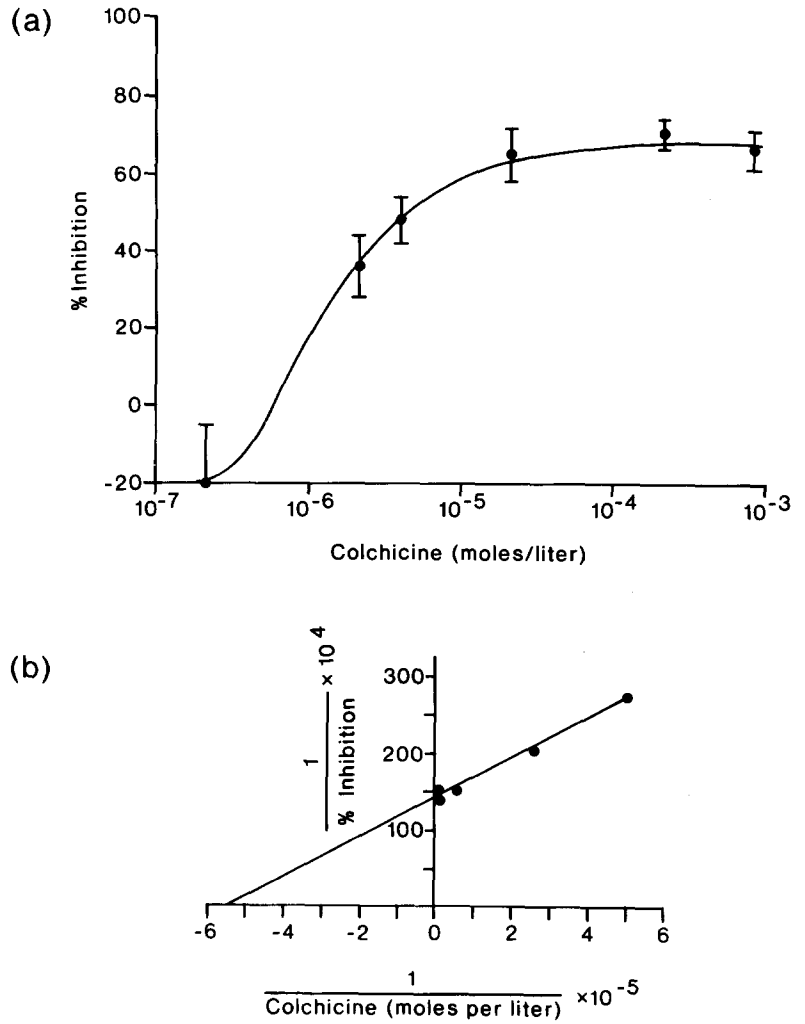

Fig. 2. (*a*): Dose-response relationship of the inhibition of vasopressin-induced water movement by colchicine. Colchicine was added 4 hr before the addition of vasopressin 20 mU/ml. Percent inhibition of the vasopressin response was calculated from the difference in weight loss of the experimental hemibladders and that of their paired controls over the 30-min period following addition of the hormone. Each point represents the mean ± SE of 5–12 experiments. (*b*): Double-reciprocal plot of inhibition of vasopressin-induced water movement by colchicine

On the other hand, the rate of water movement in response to vasopressin was consistently reduced in hemibladders previously exposed to colchicine 2×10^{-6} to 8×10^{-4} M. Figure 1 depicts the response to vasopressin in 7 pairs of hemibladders in which one member of each pair had been exposed to colchicine 2×10^{-5} M for 4 hr. Inhibition of the vasopressin response, calculated as the percent difference between the

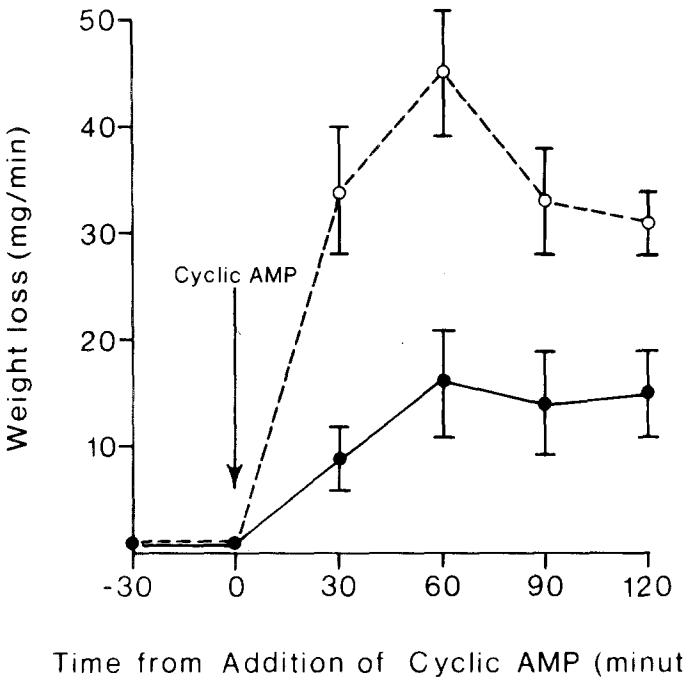

Fig. 3. The effect of colchicine on vasopressin-induced osmotic water movement. One member of each pair of hemibladders was exposed to colchicine 2×10^{-5} M for 4 hr prior to addition of cyclic AMP 2 mM to the bathing medium of the experimental (●——●) and control (o-----o) hemibladders. Each point represents the mean ± SE of 6 experiments

weight loss of the experimental hemibladders and that of their paired controls, was maximal in the first 30-min interval after addition of the hormone; the relative degree of inhibition decreased progressively in successive 30-min intervals thereafter. This relative decrease was a consequence of the decline in the rate of water loss from the control hemibladders during the second and/or subsequent 30-min intervals after addition of vasopressin (a decline that was not seen in the drug-treated pairs) (*see* Fig. 1). Accordingly, quantitative estimates of the relative inhibitory effect of colchicine, as well as the other alkaloids, reported in this paper were calculated from the data obtained over the first 30-min interval following addition of the hormone.

The dose-response relationship of the effect of 4-hr prior exposure to colchicine on the response to vasopressin is depicted in Fig. 2*a*. At the lowest concentration of colchicine tested (2×10^{-7} M), no inhibition was seen; rather a slight although statistically insignificant enhancement of the vasopressin response was apparent. Inhibition of the hormone response approached a maximum of 70 % at a colchicine concentration of

2×10^{-5} M. Half-maximal inhibition, estimated as an apparent inhibition constant from a double reciprocal plot of the data (Fig. 2*b*) [10], occurred at a colchicine concentration of 1.8×10^{-6} M.

The increase in water movement elicited by cyclic AMP was also consistently inhibited by colchicine (Fig. 3). The degree of inhibition of the response to cyclic AMP was maximal in the first 30-min interval after addition of the nucleotide, and it did not differ significantly from that of the response to vasopressin; thus, following exposure to colchicine 2×10^{-5} M for 4 hr, the response to cyclic AMP 2 mM was inhibited by $76 \pm 4\%$ ($n=6$) and that to vasopressin 20 mU/ml by $65 \pm 7\%$ ($n=7$) (unpaired difference $= 11 \pm 8$, NS).

The osmolality of the solutions bathing the hemibladders was not altered as a result of exposure to the alkaloid. Thus, the reduced response to vasopressin and to cyclic AMP in colchicine-treated hemibladders was not due to a reduction in the osmotic gradient across the bladder wall.

Characteristics of the Inhibitory Effect of Colchicine on the Vasopressin Response

In an attempt to define the mechanism of action of colchicine in the toad bladder the effect of colchicine on the vasopressin response was further characterized.

Specificity. To test the specificity of the effect of colchicine, the effect of lumicolchicine – its structural isomer which lacks antimitotic activity [40] – was investigated. As shown in Fig. 4, neither the basal rate of water movement nor the response to vasopressin was influenced by 4-hr exposure to lumicolchicine 2×10^{-5} M.

Time-dependence. The binding of colchicine to tubulin *in vitro* is a slow process, requiring as much as 6–8 hr to reach equilibrium [40]. The time-dependence of the inhibitory effect of colchicine on the vasopressin response was therefore determined in a series of experiments in which hemibladders were exposed to colchicine 2×10^{-5} M for varying time periods, prior to addition of the hormone. As seen in Fig. 5, no inhibition of the hormonal response was apparent in the absence of a period of preincubation with colchicine (rather a slight but statistically insignificant enhancement of the response was seen); the degree of inhibition of the hormonal response increased curvilinearly as the period of preincubation with colchicine was increased from 1 to 6 hr.

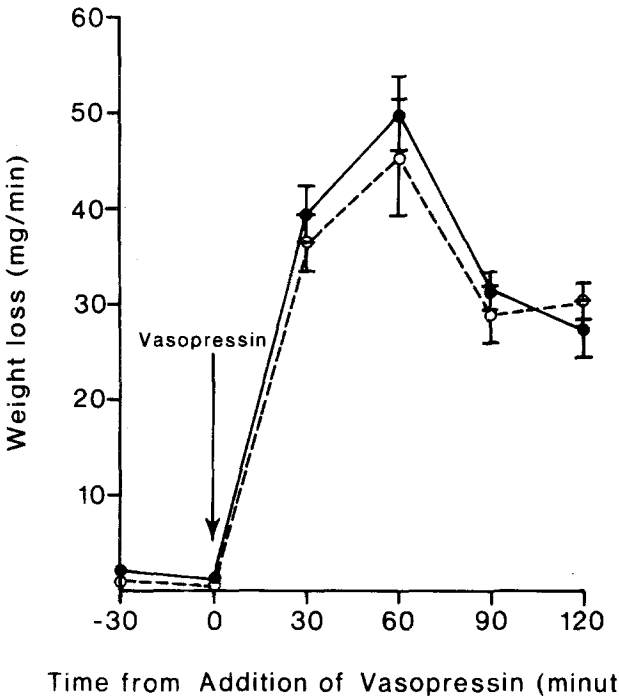

Fig. 4. The effect of lumicolchicine on vasopressin-induced osmotic water movement. One member of each pair of hemibladders was exposed to lumicolchicine 2×10^{-5} M for 4 hr, prior to addition of vasopressin 20 mU/ml to the bathing medium of the experimental (●——●) and control (o-----o) hemibladders. Each point represents the mean ± SE of 6 experiments

Temperature-dependence. The rate of binding of colchicine to tubulin *in vitro* is markedly reduced at lower temperatures and essentially nil at 0 °C [3, 40]. Accordingly, the temperature-dependence of the inhibitory effect of colchicine on the vasopressin response was examined. The response to the hormone was itself found to be temperature-dependent, being negligible or slight and variably delayed at 0 °C [31]; the studies were therefore carried out over the range 5 to 35 °C. At the lower temperatures there was considerable variability, both in the response to vasopressin and in the relative inhibitory effect of colchicine; however, as shown in Table 1, the degree of inhibition of the hormone response following exposure to colchicine 2×10^{-5} M for 1 hr increased roughly twofold with every rise of 10 °C.

Reversibility. The binding of colchicine to tubulin is only very slowly reversible *in vitro* [8, 40]. In our initial studies the inhibitory effect of colchicine on the vasopressin response appeared to be readily reversed

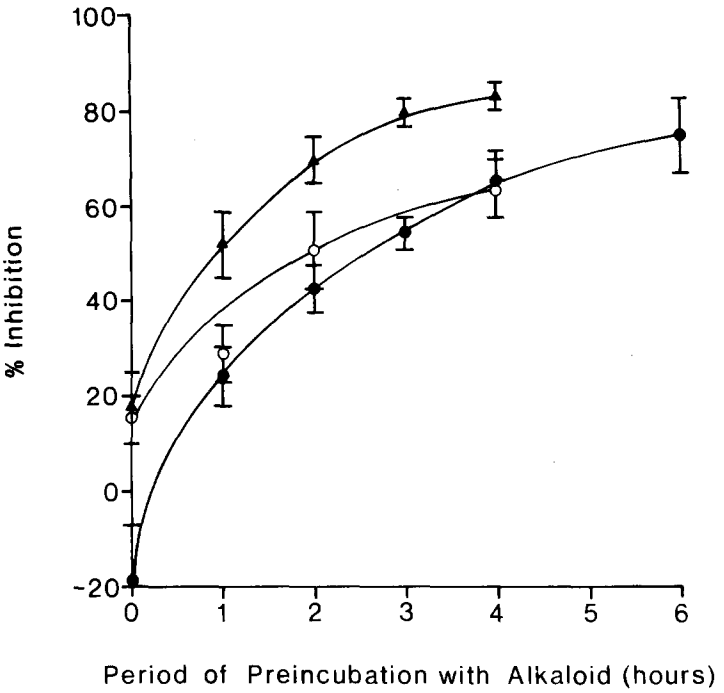

Fig. 5. Time-dependence of the inhibition of vasopressin-induced water movement by colchicine 2×10^{-5} M (o——o), podophyllotoxin 4×10^{-6} M (●——●), and vinblastine 2×10^{-5} M (▲——▲). One member of each pair of hemibladders was exposed to alkaloid for varying time periods prior to addition of vasopressin 20 mU/ml. Each point represents the mean ± SE of 5–12 paired experiments

Table 1. Temperature-dependence of the inhibitory effect of colchicine on vasopressin-induced water movement

Temperature (°C)	n	Inhibition (%)
5	14	6± 9
15	6	15±14
25	6	24± 6
35	5	39± 7

For the studies at 5, 15, and 35 °C, paired hemibladders were suspended in beakers of Ringer's solution immersed in a water bath at the appropriate temperature. In all studies, one member of each pair of hemibladders was exposed to colchicine 2×10^{-5} M for 1 hr prior to addition of vasopressin 20 mU/ml. (A short period of preincubation was employed in order to minimize possible tissue damage at the extremes of temperature).

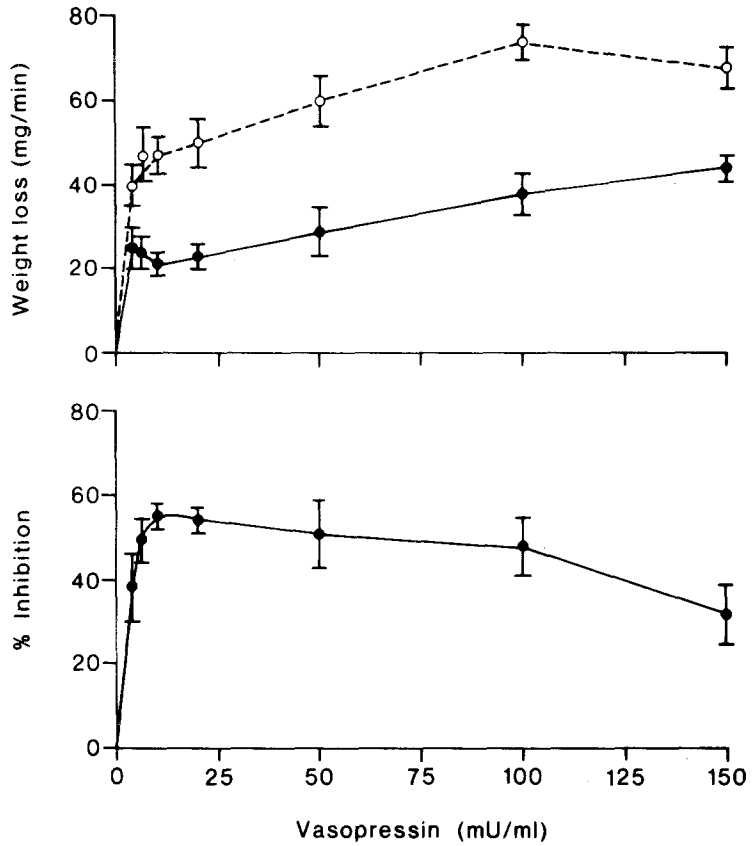

Fig. 6. Inhibition of vasopressin-induced water movement by colchicine; relation to hormone concentration. Upper panel: One member of each pair of hemibladders was exposed to colchicine 2×10^{-5} for 3 hr prior to addition of vasopressin 4–150 mU/ml to the bathing medium of the experimental (●——●) and control (○------○) hemibladders. *Lower panel:* percent inhibition of the vasopressin response. Each point represents the mean ± SE of 4–14 paired experiments

following removal of colchicine from the bathing medium [36]. However, the results of more rigorously controlled experiments indicate that this is not the case. When one member of each pair of hemibladders was exposed to colchicine 2×10^{-5} M for 3 hr and both members of each pair were then transferred through four changes of alkaloid-free Ringer's solution over the next hour prior to addition of vasopressin, the rate of water movement in the hemibladders previously exposed to colchicine was inhibited by $61 \pm 6\%$ ($n=6$, $P<0.001$) during the 30-min period following addition of the hormone, *viz.*, to the same extent as that predicted were colchicine still present ($54 \pm 4\%$, $n=10$, $P<0.001$, Fig. 5).

Even when the washout period was extended from 1 to 2 hr, a similar degree of inhibition during the first 30-min interval (53 ± 6 %, $n = 5$, $P < 0.001$) was observed.

Relation to hormone concentration. Colchicine inhibited the response to vasopressin at all hormone concentrations tested (Fig. 6). However, the response to very low concentrations of vasopressin (4 and 6 mU/ml) was apparently slightly enhanced, in relation to higher concentrations, in colchicine-treated tissues. Thus, the relative inhibitory effect of colchicine increased with rising hormone concentration from 4 to 10 mU/ml, but then levelled off and subsequently decreased somewhat with a further rise in concentration from 10 to 150 mU/ml. It is noteworthy that a supramaximal concentration of the hormone (150 mU/ml) did not overcome the colchicine effect; thus, inhibition of the vasopressin response by colchicine does not appear to be competitive in nature.

Lack of effect of mucosal colchicine. Neither the basal rate of water movement nor the response to vasopressin were significantly altered when colchicine was added to the mucosal, as opposed to the serosal, bathing medium. In 9 hemibladders preincubated with colchicine 2×10^{-5} M in the mucosal medium for 3 hr, mean inhibition over the 30-min period following addition of vasopressin 20 mU/ml was 8 ± 6 %, NS. This figure may be compared with the mean value of 54 ± 4 % inhibition ($n = 10$, $P < 0.001$) which was observed when hemibladders were exposed for 3 hr to the same concentration of colchicine in the serosal bathing medium (*see* Fig. 5).

Effect of Podophyllotoxin on Osmotic Water Movement

The effect of podophyllotoxin on osmotic water movement was similar to that of colchicine. Exposure to podophyllotoxin 7.5×10^{-5} M to 1.5×10^{-5} M had no influence on the basal rate of water movement but consistently reduced the response to vasopressin (Fig. 7). The reduced hormonal response was not associated with a decrease in the osmotic gradient across the tissue. The concentration-dependence of the inhibition of the response to vasopressin after 4 hr exposure to podophyllotoxin is depicted in Fig. 8*a*; half maximal inhibition, estimated as an apparent inhibition constant from a double reciprocal plot of the data (Fig. 8*b*) [10], occurred at a podophyllotoxin concentration of 5×10^{-7} M. The response to cyclic AMP was also inhibited by podophyllotoxin. The degree of inhibition of the response to the nucleotide did not differ

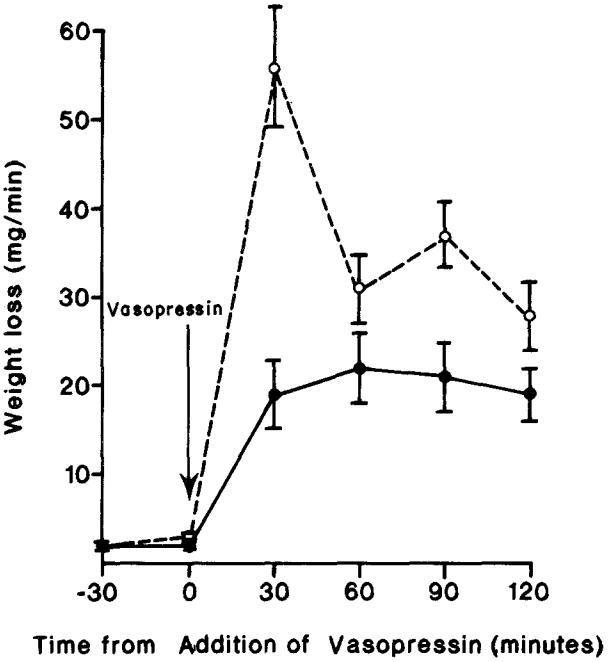

Fig. 7. The effect of podophyllotoxin on vasopressin-induced osmotic water movement. One member of each pair of hemibladders was exposed to podophyllotoxin 4×10^{-6} M for 4 hr prior to addition of vasopressin 20 mU/ml to the bathing medium of the experimental (●——●) and control (o-----o) hemibladders. Each point represents the mean ± SE of 6 experiments

significantly from that of the response to vasopressin: following exposure to podophyllotoxin 4×10^{-6} M for 4 hr the response to cyclic AMP 4 mM was inhibited by 68 ± 4 % ($n = 5$), and that to vasopressin 20 mU/ml by 64 ± 7 ($n = 6$) (unpaired difference $= 4 \pm 8$, NS).

The time dependence of the inhibitory effect of podophyllotoxin on the vasopressin response is shown in Fig. 5. In contrast to colchicine, podophyllotoxin inhibited the vasopressin response significantly even in the absence of a period of preincubation with the alkaloid. As with colchicine, the degree of inhibition increased curvilinearly as the period of preincubation with podophyllotoxin was increased from 0 to 4 hr.

In reversibility studies, carried out with strictly paired controls as for colchicine, the effect of podophyllotoxin on the vasopressin response was found not to be reversed following removal of the alkaloid from the bathing medium. When one member of each pair of hemibladders was exposed to podophyllotoxin 2×10^{-6} M for 2 hr and both members of each pair then washed in alkaloid-free bathing solution, the hormonal response was inhibited in the hemibladders previously exposed to podophyllotoxin to

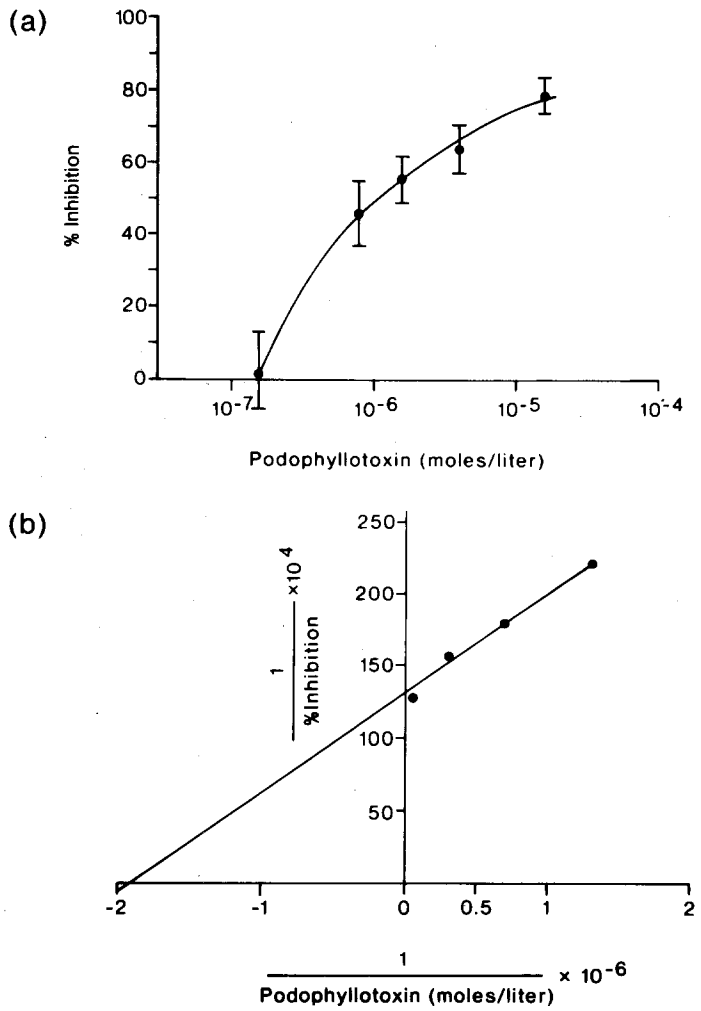

Fig. 8. (*a*): Dose-response relationship of the inhibition of vasopressin-induced water movement by podophyllotoxin. The alkaloid was added 4 hr before the addition of vasopressin 20 mU/ml. Percent inhibition of the vasopressin response was calculated as described in Fig. 2*a*. Each point represents the mean ± SE of 5–6 experiments. (*b*): Double-reciprocal plot of inhibition of vasopressin-induced water movement by podophyllotoxin

the same extent as that predicted were the alkaloid still present, *viz.*, 46 ± 3 % ($n=6$, $P<0.001$) *vs.* 51 ± 8 % ($n=5$, $P<0.01$).

Effect of Vinca Alkaloids on Osmotic Water Movement

The effect of vinblastine on osmotic water movement paralleled that of colchicine and podophyllotoxin. Thus, exposure to vinblastine 2

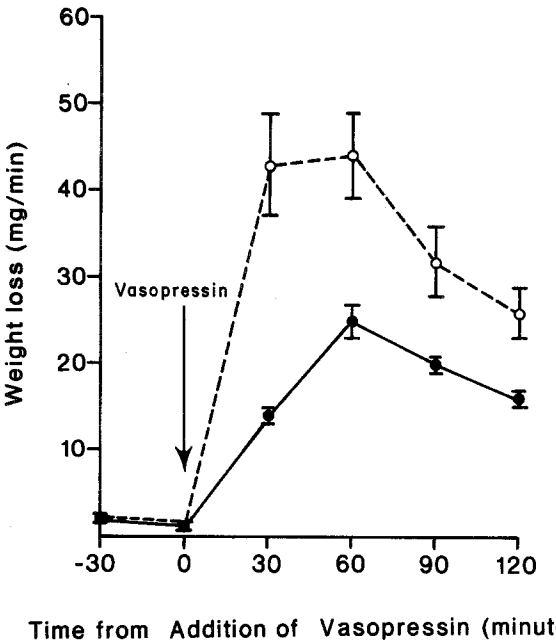

Fig. 9. The effect of vinblastine on vasopressin-induced osmotic water movement. One member of each pair of hemibladders was exposed to vinblastine 2×10^{-6} M for 4 hr prior to addition of vasopressin 20 mU/ml to the bathing medium of the experimental (●——●) and control (o-----o) hemibladders. Each point represents the mean ±SE of 8 experiments

$\times 10^{-7}$ to 2×10^{-5} M had no effect on the basal rate of water movement, but markedly reduced the response to vasopressin (Fig. 9); the reduced response to the hormone was not associated with a decrease in the osmotic gradient across the tissue. The dose-response relationship of the inhibitory effect of 4 hr exposure to vinblastine is shown in Fig. 10*a*; half maximal inhibition, estimated from a double reciprocal plot of the data (Fig. 10*b*), occurred at a vinblastine concentration of 1×10^{-7} M. Vinblastine inhibited the response to cyclic AMP to the same extent as the response to vasopressin; thus following exposure to vinblastine 2×10^{-5} M for 4 hr the response to cyclic AMP 4 mM was reduced by 82 ±4 % ($n=5$), and the response to vasopressin 20 mU/ml by 83 ± 3 % ($n=12$) (unpaired difference = 1 ± 5, NS).

The time dependence of the inhibitory effect of vinblastine on the vasopressin response is shown in Fig. 5. As with podophyllotoxin, and in contrast to colchicine, a significant inhibition of the hormone response was observed even in the absence of a period of preincubation with vinblastine; however, as with both of the other alkaloids, the degree of

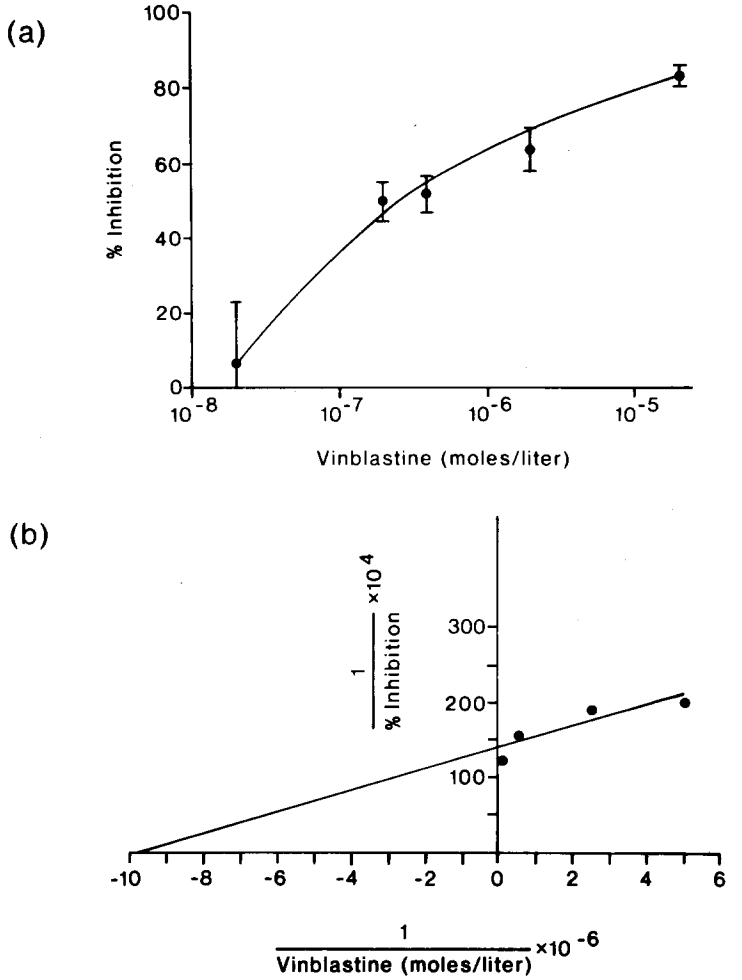

Fig. 10. (*a*): Dose-response relationship of the inhibition of vasopressin-induced water movement by vinblastine. The alkaloid was added 4 hr before the addition of vasopressin 20 mU/ml. Percent inhibition of the response to vasopressin was calculated as described in Fig. 2*a*. Each point represents the mean ±SE of 6–12 experiments. (*b*): Double reciprocal plot of inhibition of vasopressin-induced water movement by vinblastine

inhibition increased curvilinearly as the period of preincubation was increased from 0 to 4 hr.

As observed with both colchicine and podophyllotoxin, the effect of vinblastine on the vasopressin response was not readily reversed following removal of the alkaloid from the bathing medium. When one member of each pair of hemibladders was exposed to vinblastine 2 $\times 10^{-5}$ M for 2 hr and both members of each pair then washed in alkaloid-free bathing solutions, the hormonal response was inhibited in

Table 2. Effects of colchicine, podophyllotoxin, and vinblastine on active sodium transport

Alkaloid	%Δ in scc[a] after addition of alkaloid							Δ in scc[b] after vasopressin[c]		
	n	15 min	30 min	60 min	120 min	180 min	240 min	*n*	Absolute (μA)	%
Colchicine 2×10^{-5} M	27	+2±3	+3± 7	−4± 8	−18± 9	−26±10	−34±10	15	+23± 4	+48±11
Control		0±4	+3± 8	−1± 9	−18±10	−28±10	−33±10		+21± 3	+38± 7
Podophyllotoxin 1×10^{-6} M	6	+2±4	0± 7	−5±10	−9±14	−17±10	−22± 9	6	+18± 7	+19± 8
Control		−4±3	−7± 5	−11± 7	−3±16	−1±20	−13±16		+16± 6	+15± 4
Vinblastine 2×10^{-5} M	6	+5±4	+2±10	−8±13	−27±13	–	–	6	+53±10	+87±15
Control		+5±3	+6± 9	0± 9	−20±12	–	–		+43±11	+67± 8

P values were calculated from paired data; there was no significant difference between the experimental and control values throughout.

[a] A quantitative index of the effect of the alkaloids on the basal rate of sodium transport was obtained by dividing the value of the scc recorded at time *t* after addition (scc_t) by the value recorded just before addition (scc_o); results are expressed as the percent deviation of this value from unity ±SE.

[b] Values were estimated from the peak rise in scc following addition of hormone.

[c] Vasopressin concentration 20 mU/ml; similar results were obtained with colchicine and vasopressin 100 mU/ml.

the hemibladders previously exposed to vinblastine to the same extent as that predicted were the alkaloid still present, *viz.*, $72 \pm 4\%$, ($n=6$, $P<0.001$) *vs.* $70 \pm 5\%$ ($n=6$, $P<0.001$).

Vincristine 2×10^{-7} to 2×10^{-5} M, like vinblastine, had no effect on the basal rate of water movement across the bladders but markedly reduced the response to vasopressin. The inhibitory potency of vincristine was approximately one-fourth that of vinblastine, and its effect was slower in onset.

II. Studies of Active Sodium Transport

The epithelial cells of the toad bladder actively transport sodium ions from their mucosal to their serosal surface [19]. Vasopressin elicits a transient increase in the rate of sodium transport across the tissue [13, 19]. The effects of colchicine, podophyllotoxin, and vinblastine on basal and vasopressin-stimulated sodium transport were studied in separate experiments, using the short circuit current (scc) as a measure of the rate of net sodium transport [37]. The alkaloids had no effect on the basal scc (Table 2), nor on the electrical resistance of the tissue. Furthermore, the alkaloids had no effect on the rise in scc induced by vasopressin (Table 2).

III. Studies of Urea Permeability

Vasopressin enhances the permeability of the toad bladder to urea and to certain other small solutes [21]. The effect of colchicine on the

Table 3. Effect of colchicine on the permeability to urea

	Permeability coefficient ($cm/sec \times 10^{-7}$)		
	Colchicine	Control	Difference
Vasopressin			
Absent	115 ± 33	128 ± 29	-13 ± 13
Present	424 ± 28	468 ± 38	-44 ± 31
Increment	309 ± 39	340 ± 52	-31 ± 29

Colchicine 2.5×10^{-5} M was added to the serosal medium of one of each pair of hemibladders. One hr later unlabelled urea 2 mM was added to both serosal and mucosal media and ^{14}C-labelled urea was added as a tracer to either the mucosal ($n=8$) or serosal bath ($n=6$); measurements commenced after a further 1 hr. Values are means for 2 successive 30-min periods before and after addition of vasopressin 50 mU/ml; differences are based on paired experiments.

permeability of the bladder to urea was examined in the absence and in the presence of the hormone. In hemibladders which had been exposed to colchicine 2×10^{-5} M for 3 hr, permeability to urea both in the absence and presence of vasopressin was not significantly different from that in paired control hemibladders not exposed to the alkaloid (Table 3).

Discussion

These studies demonstrate that colchicine, podophyllotoxin and the vinca alkaloids inhibit the action of vasopressin on transcellular water movement in the toad urinary bladder [35, 36]. In contrast, these agents have no effect on either basal or vasopressin-stimulated sodium transport or urea flux across the tissue. Such a dissociation between vasopressin-induced water and solute movement in the toad bladder has been observed previously with other agents [20, 26].

Colchicine, podophyllotoxin and vinblastine also inhibit the increased rate of water movement induced by cyclic AMP; in the case of all three alkaloids the inhibition of the response to cyclic AMP is quantitatively similar to the inhibition of the response to vasopressin. Thus, the effect of the drugs cannot be ascribed to interference with hormone-receptor interaction or to interference with cyclic AMP synthesis. Since the alkaloids have no influence on the rate of active sodium transport across the bladder – a function known to be highly sensitive to metabolic inhibitors – it seems unlikely that the inhibition of hormone or nucleotide-induced water movement is secondary to an effect on energy metabolism.

It is well established that the antimitotic agents bind with high affinity to tubulin [40], and that they interfere with microtubule assembly *in vitro* [24, 39] and exert disruptive effects on cytoplasmic microtubules *in vivo* [6]. However, relatively low concentrations of colchicine and podophyllotoxin inhibit nucleoside uptake in cultured cells [22]; this effect does not appear to be due to binding of the drugs to tubulin and accordingly has been attributed to interaction with a non-tubulin cell membrane component [22]. Since the effect of vasopressin on water movement depends ultimately on a change in membrane function, it was clearly important to establish the locus and mode of action of the antimitotic agents in the toad bladder. The inhibitory effects of these agents on the vasopressin response were therefore characterized in some detail. The results of the present studies indicate that the

characteristics of the functional effects of the drugs in fact correlate closely with the known characteristics of their binding to tubulin, and differ from those of their effects on nucleoside transport.

Analysis of the data obtained from the dose-response studies reveals a striking similarity between the effective concentrations of the various alkaloids in the toad bladder and the affinity with which they are known to interact with tubulin. The concentrations of alkaloid required for half-maximal inhibition of the vasopressin response, estimated as apparent inhibition constants from double-reciprocal plots of the respective dose-response data, are 1.8×10^{-6} M for colchicine, 5×10^{-7} M for podophyllotoxin, and 1×10^{-7} M for vinblastine. The effective concentrations of the three alkaloids are consistent with their known potency as antimitotic agents [4, 38], and with their ability to inhibit microtubule assembly *in vitro* [24, 39]. The value for colchicine differs by more than one order of magnitude from the corresponding value for colchicine inhibition of nucleoside transport (5×10^{-5} M) [38]. The values for both colchicine and podophyllotoxin correspond closely to values for half-saturation of the binding of these drugs to brain tubulin *in vitro*, as estimated from binding constants reported in the literature [40]. Furthermore, there is a striking correlation between the effective inhibitory potency of colchicine and podophyllotoxin, as indicated by the above values, and the affinity with which these agents bind to tubulin obtained from toad bladder epithelial cells [42]. Finally, in the case of colchicine, inhibition of the vasopressin response is paralleled by a proportional dose-dependent reduction in the content of assembled microtubules in the granular epithelial cells of the bladder [28].

The correspondence between the specificity, time-dependence and temperature-dependence of the inhibitory effect of colchicine on the vasopressin response and the known characteristics of the binding of this drug to tubulin *in vitro* [3, 40] also supports the view that the effect of colchicine depends on its interaction with tubulin in the bladder epithelial cells. Thus, the finding that lumicolchicine – which does not disrupt microtubules *in vivo* or bind to tubulin *in vitro* [40], but inhibits nucleoside uptake [22] – has no effect on vasopressin-induced water movement strongly suggests that inhibition of the vasopressin response by colchicine is specifically related to its tubulin-binding capacity. The finding that inhibition of the vasopressin response by colchicine does not occur in the absence of a period of preincubation with the drug, and that the degree of inhibition varies with temperature, correlates with the known time- and temperature-dependence of the reaction of colchicine

with both brain tubulin [3, 40] and toad bladder epithelial cell tubulin [35, 42]. In contrast, the inhibition of nucleoside uptake by colchicine has been shown to be neither time- nor temperature-dependent, at least at drug concentrations similar to those used in our study [22].

The time-dependence of the inhibitory effect of podophyllotoxin and vinblastine on the vasopressin response differs from that of colchicine in that a significant degree of inhibition is apparent in the absence of a period of preincubation with these agents; this finding is consistent with the fact that both podophyllotoxin and vinblastine are known from *in vitro* studies to bind to brain tubulin very rapidly [40]. Otherwise, the time-dependence of the inhibitory effect of the three drugs shows a remarkable parallelism; as with colchicine, the inhibitory effect of podophyllotoxin and vinblastine increased substantially as the period of preincubation with the alkaloids was increased from 0–4 hr (Fig. 5). This time-dependence may well reflect the rate of depolymerization of assembled microtubules within the bladder epithelial cells (*see* [42]).

The finding that the inhibitory effects of podophyllotoxin and vinblastine on the vasopressin response are not readily reversed is somewhat unexpected, since in this respect the functional effects of the drugs do not correlate with their known tubulin-binding properties *in vitro*. Thus, while the binding reaction between colchicine and brain tubulin is known to be only slowly reversible [8, 40], the reaction between both podophyllotoxin and vinblastine and brain tubulin has been shown to be rapidly reversible [38, 40]. This lack of correlation might reflect differences in the drug-binding properties of tubulin *in vivo* and *in vitro*, or perhaps loss of the functional capacity of tubulin following exposure to the drugs *in vivo*. Alternatively, the lack of reversal of the effects of all three alkaloids may be taken to indicate that the effect of the hormone is essentially dependent on the functional integrity of previously assembled microtubules, rather than on the process of microtubule assembly *per se* [33]. This interpretation is consistent with the dose-dependence and time-dependence of the effects of the alkaloids, with the finding that inhibition of the vasopressin response by colchicine is paralleled by a dose-dependent reduction in the content of assembled microtubules in the granular epithelial cells, and furthermore with the observation that exposure to the hormone results in only a modest increase in cytoplasmic microtubule content [28, 42].

In sum, the results of these studies support the view that the inhibitory effects of the antimitotic agents on vasopressin-induced water movement in the toad urinary bladder are dependent on the interaction

of these agents with tubulin. The findings are consistent with the concept that inhibition of the vasopressin response by the alkaloids is the result of interference with the integrity and functional activity of microtubules in the bladder epithelial cells. Taken together with the results of biochemical and morphological studies reported in the two accompanying papers [28, 42], the findings provide evidence that assembled cytoplasmic microtubules play a critical role in the action of vasopressin on transcellular water movement in the toad bladder.

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