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EFFECT OF COLCEMID ON THE WATER PERMEABILITY RESPONSE TO VASOPRESSIN IN ISOLATED PERFUSED RABBIT COLLECTING TUBULES

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SUMMARY

1. The effect of the microtubule-disruptive agent, colcemid (*N*-deacetyl-*N*-methylcolchicine), on the water permeability response to vasopressin has been investigated in isolated cortical collecting tubules from the rabbit kidney perfused *in vitro*.

2. Pretreatment of collecting tubules with colcemid inhibited the increase in water permeability elicited by vasopressin, 50 μU ml^{-1}, in a time- and dose-dependent manner. After 75 min exposure to the drug, inhibition of the response to the hormone averaged 72$\pm$6% ($n = 4$, $P < 0{\cdot}01$) at a colcemid concentration of $7{\cdot}2 \times 10^{-5}$ M. Inhibition was estimated to be half-maximal at a colcemid concentration of $1{\cdot}9 \times 10^{-6}$ M.

3. Colcemid, $2{\cdot}7 \times 10^{-7}$ to $7{\cdot}2 \times 10^{-5}$ M, had no effect on basal water permeability nor on the increase in lumen negative potential difference (PD) induced by the hormone.

4. Lumicolcemid, an isomer of colcemid that does not disrupt microtubules, had no influence on the water permeability response to vasopressin.

5. Pretreatment with colcemid, $2{\cdot}7 \times 10^{-5}$ M, for 45 min inhibited the water permeability response to 8-CPT-cAMP, $1{\cdot}8 \times 10^{-5}$ M, by 38$\pm$4% ($n = 5$, $P < 0{\cdot}01$).

6. When collecting tubules were exposed to colcemid, $5{\cdot}5 \times 10^{-5}$ M, for 45 min after the hydrosmotic response to vasopressin had been established, the drug had no influence on the maintenance of the raised water permeability.

7. The results provide further evidence that cytoplasmic microtubules play a role in the initiation of the hydrosmotic response to vasopressin in the mammalian collecting tubule at a site distal to the generation of cyclic AMP.

INTRODUCTION

Vasopressin promotes the osmotic movement of water across responsive epithelial tissues by increasing the water permeability of the rate-limiting apical plasma membrane of its target cells. In amphibian urinary bladder, the increase in water permeability induced by the hormone involves the addition of new membrane components to the apical membrane of the granular epithelial cells (Wade, 1989). Following vasopressin stimulation, intramembranous particle aggregates appear in the granular cell apical membrane; these particle aggregates, which have been

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visualized by freeze-fracture electron microscopy, are thought to represent transmembrane proteins containing specific water channels (Kachadorian, Wade & DiScala, 1975; Bourguet, Chevalier, Parisi & Gobin, 1981). In unstimulated tissues, the particle aggregates are present in the limiting membranes of tubulovesicles located in the apical cytoplasm of the granular cells (Muller, Kachadorian & DiScala, 1980). The aggregate-containing tubulovesicles are evidently translocated towards the apical cell surface prior to their exocytotic incorporation into the apical plasma membrane under the influence of vasopressin (Muller *et al.* 1980). The particle aggregates (putative water channels) are thus transferred from cytoplasmic membranes to the apical membrane, and the water permeability of the latter is hence dramatically increased. Following hormonal stimulation the particle aggregates are retrieved by endocytosis (Coleman, Harris & Wade, 1987) and may be recycled (Wade, 1989).

A similar cycle of exo- and endocytosis is thought to be involved in the water permeability response to vasopressin in the mammalian collecting tubule (Brown, 1989). Following hormone administration, intramembranous particle clusters (similar, but not identical, to the particle aggregates in amphibian bladder) have been visualized in the apical membranes of the principal cells of collecting tubules from Brattelboro rats (Harmanci, Stern, Kachadorian, Valtin & DiScala, 1978). As in amphibian bladder, correlative evidence suggests that these intramembranous structures contain the putative water channels (Harmanci, Stern, Kachadorian, Valtin & DiScala, 1980). Increased endocytotic activity has been observed in the principal cells of rabbit cortical collecting tubules during exposure to and/or removal of vasopressin (Strange, Willingham, Handler & Harris, 1988). Moreover, endocytotic vesicles prepared from rat renal medulla have been shown to have vasopressin-dependent water permeability properties consistent with the presence of water channels (Verkman, Lencer, Brown & Ausiello, 1988). By analogy with the situation in amphibian bladder, these water channels may exist preformed in cytoplasmic vesicles in the principal cells, and may be delivered to the apical membrane by an exocytotic process in response to vasopressin (Brown, 1989).

In amphibian bladder, it is well established that the vasopressin-stimulated delivery of water channels to the apical membrane of the granular cells depends on an intact cytoskeleton. Thus colchicine and related drugs that disrupt cytoplasmic microtubules inhibit the magnitude and rate of onset of vasopressin-stimulated water flow (Taylor, Mamelak, Golbetz & Maffly, 1978; Parisi, Pisam, Merot, Chevalier & Bourguet, 1985), the hormone-induced appearance of particle aggregates in the apical membrane (Kachadorian, Ellis & Muller, 1979), and also the associated increase in apical membrane fusion events (Muller *et al.* 1980). These various inhibitory effects are manifest only if the tissue is exposed to the drugs prior to hormonal stimulation (Kachadorian *et al.* 1979; Muller *et al.* 1980; Parisi *et al.* 1985). Combined functional, biochemical and ultrastructural studies have provided evidence that the inhibitory effect of colchicine is related to its interaction with tubulin and consequent disruption of assembled microtubules in the hormone-responsive epithelial cells (see Taylor *et al.* 1978). These various findings have led to the notion that microtubules are involved in the translocation of the aggregate-containing tubulovesicles towards the apical surface of the granular cells prior to

their exocytotic incorporation into the apical plasma membrane. According to this view, microtubules play an integral role in the initiation of the water permeability increase induced by the hormone (Kachadorian *et al.* 1979; Muller *et al.* 1980; Parisi *et al.* 1985; reviewed in Pearl & Taylor, 1985).

Cytoplasmic microtubules may also play a role in the water permeability response to vasopressin in the principal cells of the mammalian collecting tubule, although evidence for their involvement is as yet scanty. Colchicine has been shown to impair urinary concentrating ability in Sprague–Dawley (Dousa & Barnes, 1974) and Brattelboro rats (Hall, Taylor & Maffly, 1974), and to inhibit vasopressin-stimulated tritiated water uptake in renal medullary slices (Iyengar, Lepper & Mailman, 1976). According to a preliminary report (Abramow, 1976), pretreatment of isolated collecting tubules with colchicine (10^{-4} M) can interfere with the hydrosmotic response to the hormone. In our recent study on isolated collecting tubules from rabbit kidneys, perfused *in vitro*, we have demonstrated that the fast-acting microtubule-disruptive agent, nocodazole, specifically inhibits the initiation of the water permeability increase induced by vasopressin (Phillips & Taylor, 1989).

In the present study we have used the isolated perfused tubule technique to examine the effects of another microtubule-disruptive agent, colcemid (a colchicine derivative, *N*-deacetyl-*N*-methylcolchicine), and its inactive isomer, lumicolcemid, on the response of the rabbit collecting tubule to vasopressin. Colcemid was used in this study in preference to colchicine because of its relative rapidity of action (Schliwa, 1986). The results of these investigations provide further support for the view that cytoplasmic microtubules play a role in the initiation of the water permeability response to vasopressin in the mammalian nephron, as in amphibian bladder. The findings have been published in preliminary form (Phillips & Taylor, 1987, 1988).

METHODS

In vitro perfusion

Cortical collecting tubules were isolated from New Zealand White rabbits and perfused *in vitro* using the technique originated by Burg (Grantham & Burg, 1966), as previously employed in this laboratory (Phillips & Taylor, 1989). Rabbits of both sexes (weighing between 1 and 1·5 kg) were maintained on a diet containing 0·34 % sodium and 1·78 % potassium (Maintenance Diet SDS). The rabbits were killed by dislocation of the neck and the left kidney rapidly removed and placed in cooled dissection medium (see Table 1). Transverse (coronal) tissue slices of about 1 mm thickness were cut and transferred to fresh dissection medium. A wedge-shaped section of a suitable slice was 'teased' out and transferred to a Petri dish containing dissection medium cooled on a chilled metal tray; this cooling tray kept the medium at a temperature of around 4 °C for the course of the subsequent fine dissection. Cortical collecting tubules were identified and dissected free; tubules were usually between 1 and 3 mm in length. The total time taken for tubule isolation from the death of the animal was ~ 15–30 min. After isolation, a single tubule was transferred in a Pasteur pipette to a thermoregulated perfusion chamber mounted on the stage of a Nikon Diaphot Inverted microscope, and containing bathing medium at room temperature (Table 1).

The tubule was mounted between two sets of concentric micropipettes (made from precision bore, borosilicate glass tubing) (Drummond Scientific Company, PA, USA) and prepared with a Stoelting (Chicago) Pipette Puller and Microforge). The pipettes were assembled on a conventional microperfusion apparatus (J. P. White, Bethesda, MD, USA). The perfusion system consisted of an outer tubule holding pipette, a perfusion pipette and an inner exchange pipette. The collection system was composed of an outer tubule holding pipette and an inner volumetric constriction pipette. A 95 % O_2–5 % CO_2 gas mixture was bubbled into the bathing medium in the perfusion

chamber via a fine plastic tube, ensuring adequate oxygenation and stirring. The temperature of the bathing medium was raised over ~ 15 min to 37 °C, and thereafter maintained constant by a temperature controller (model 73ATD Yellow Springs Instruments, Yellow Springs, OH, USA). After raising the temperature, the tubule was allowed to equilibrate for ~ 30 min prior to the start of the experimental protocol. The bathing medium was changed periodically (at ~ 10 min

TABLE 1. Composition of solutions used in the experiments

	Dissection	Bathing	Perfusion
NaCl	141	116	35
$NaHCO_3$	0	25	25
K_2HPO_4	2·5	2·5	2·5
$MgSO_4$	1·2	1·2	1·2
D-Glucose	5·5	5·5	5·5
DL-Lactate	4·0	4·0	4·0
L-Alanine	6·0	6·0	6·0
$CaCl_2$	2·0	2·0	2·0
Osmolality	290	290	145

Values are in mM except for osmolality, which is in mosmol kgH_2O^{-1}.

intervals) during the course of each experiment to minimize changes in osmolality. The length of perfused tubule was measured using an eye-piece micrometer. This length ranged from 0·9 to 3·0 mm with a mean of $1·7 \pm 0·1$ mm. The mean internal diameter was assumed to be 20 μm (Frindt, Windhager & Taylor, 1982).

All tubules were perfused by gravity at a rate of ~ 20–30 nl min^{-1}. The perfusate solution (see Table 1) had an osmolality of a half that of the bathing solution; an osmotic gradient of 145 mosmol kgH_2O^{-1} was thus imposed across the tubule wall to provide a driving force for transepithelial water flow. The perfusate contained [^{14}C]methoxy inulin (Amersham) at a concentration of ~ 5 μCi ml^{-1} as a volume marker. The rate of collection of tubule fluid was determined by measuring the time required to fill a calibrated volumetric pipette; collections were usually made at 3–7 min intervals. The perfusion rate was calculated from the rate of appearance of the marker in the collected fluid and its measured concentration in the perfusate.

In each experiment the system was checked for potential leaks by periodically measuring the [^{14}C]inulin in the bathing medium. The mean leak was $1·4 \pm 0·2$% ($n = 48$) of the perfusion rate. The radioactivity of all samples was measured in a liquid scintillation counter (Nuclear Chicago, Isocap/300).

The transepithelial potential difference (PD) was measured by means of two saline bridges (containing 4% agar in 0·15 M-NaCl) in contact with the bathing and perfusion fluids and each connected to a 3 M-KCl reservoir and a calomel half-cell. These cells were connected to a Keithley Digital Electrometer.

Solutions, hormone agonists and drugs

The composition of the experimental solutions is given in Table 1. All solutions were passed through a Millipore filter (pore size 0·45 μm), and their osmolality was checked by means of a vapour pressure osmometer (Wescor 5100C) prior to use. Bovine serum albumin was added to the dissection fluid (0·5 gm%) to prevent the tubule sticking to the dissecting instruments or to the pipette during transfer to the perfusion chamber.

Arginine vasopressin (vasopressin), 8-(4-chlorophenylthio)-adenosine 3′, 5′-cyclic monophosphate (8-CPT-cAMP), and *N*-deacetyl-*N*-methylcolchicine were purchased from Sigma Chemical Company. (*N*-deacetyl-*N*-methylcolchicine, or demecolcine, is commonly known as colcemid (Dustin, 1984) and this name is employed throughout this paper.)

Colcemid was prepared as a stock solution ($2·7 \times 10^{-2}$ M) in distilled water; this solution was divided into aliquots and stored at −4 °C. Lumicolcemid was prepared by irradiation of colcemid with ultraviolet light (Aronson & Inoué, 1970). A $2·7 \times 10^{-5}$ M solution of colcemid was exposed to a UV light source (Agar Aids) at 366 nm, in a darkened cold room, for 24 h prior to use. The absorbance of the irradiated sample was compared to that of a similar solution of colcemid at

355 nm, using a Perkin Elmer Lambda Spectrophotometer. In all samples thus prepared there was a large decrease in absorbance at 355 nm (see Fig. 1). Based on comparison with published UV absorption spectra, it was assumed that the irradiated solution contained lumicolcemid (cf. Aronson & Inoué, 1970; Wilson, Bamburg, Mizel, Grisham & Creswell, 1974).

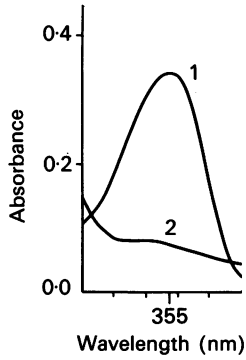

Fig. 1. Change in absorbance at 355 nm following 24 h ultraviolet irradiation (366 nm) of a solution of colcemid ($2{\cdot}7 \times 10^{-5}$ M). Curve 1 before irradiation; curve 2 after irradiation.

Calculation of hydraulic conductivity

The equation of Dubois, Verniory & Abramow (1976) was used to calculate hydraulic conductivity, L_p (10^{-7} cm s^{-1} atm^{-1}):

$$L_p = \frac{1}{RTSC_b^2}\{C_b(V_i - V_f) + C_i V_i[\ln(C_b - C_i)V_i - \ln(C_b V_f - C_i V_i)]\},$$

where C_b and C_i are the bath and perfusate fluid osmolalities, respectively; V_i and V_f are the perfusion and collection rates, respectively; R is the gas constant, T is the absolute temperature, and S is the luminal surface area.

Experimental protocols

Successive challenges with vasopressin. After a 'baseline' collection period, vasopressin was added to the bath at a final concentration of 50 μU ml^{-1}. (This dose was used in all subsequent experiments since it is thought to induce a maximal water permeability response; Grantham & Burg, 1966). The period of exposure to vasopressin lasted 30 min, at which point the hormone was removed by replacing the bathing medium with hormone-free solution. The tubule was then allowed to recover for ~ 90 min before a second 30 min exposure to vasopressin followed by hormone wash-out. (An interval of 120 min between vasopressin additions was chosen for these studies to allow adequate time for colcemid binding to tubulin (see Discussion); this interval represents the maximum time between successive hormonal stimulations in the experimental protocols described below.)

Effect of colcemid pretreatment on the response to vasopressin or 8-(4-chlorophenylthio)-adenosine 3', 5'-cyclic monophosphate (8-CPT-cAMP). The general protocol was similar to that described above. The initial 30 min exposure to vasopressin (50 μU ml^{-1}), or 8-CPT-cAMP ($1{\cdot}8 \times 10^{-5}$ M), was followed by a wash-out and recovery period of 60–90 min (see Results). Colcemid was added to the bath 10–15 min after hormonal wash-out. The second challenge with hormone, or nucleotide analogue, followed 45–75 min later. After a further 30 min both agonist and drug were washed out.

Effect of lumicolcemid pretreatment on the response to vasopressin. The protocol followed in these experiments was identical to that described above, employing a 60 min wash-out and recovery period. Lumicolcemid was added to the bath 10–15 min after wash-out of the first dose of vasopressin.

Effect of colcemid added after the vasopressin response was established. The sequence of control and experimental periods was reversed in this final series of experiments because of the increased length of the procedure. The first period of exposure to vasopressin constituted the experimental period,

and the second, the control period (this reversed protocol avoided any uncertainties about the viability of the tissue). In these studies, exposure to vasopressin (50 μU ml^{-1}) was maintained for 75 min during both the experimental and control periods. Thirty minutes after the initial challenge with hormone, the tubule was exposed to colcemid ($5{\cdot}5 \times 10^{-5}$ M) in the continued presence of vasopressin. Both hormone and drug were washed out 45 min later, and the tubule was allowed to recover for 30 min prior to the second addition of vasopressin.

Statistics

Measurements are expressed as the mean ($\pm$S.E. of the mean). The significance of the difference between the means for the control and experimental periods was determined by a Student's paired t test.

RESULTS

Response to successive challenges with vasopressin

Throughout this study, each tubule served as its own internal control, as in our previous experiments with nocodazole (Phillips & Taylor, 1989). For the purpose of the present study with colcemid (which binds to tubulin more slowly than nocodazole), it was necessary to demonstrate that the tissue would respond reproducibly to two successive 30 min exposures to vasopressin when separated by a time interval of 120 min. Figure 2 summarizes the results from four control

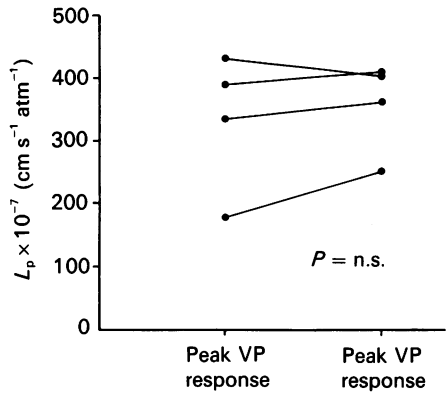

Fig. 2. Peak L_p response to two successive 30 min exposures to vasopressin (VP, 50 μU ml^{-1}) separated by an interval of 120 min. Lines join data from four individual collecting tubules.

tubule perfusions (see Methods for details). As in our earlier *in vitro* studies, we found considerable variability in the magnitude of the peak hydrosmotic response to vasopressin in different collecting tubules (loc. cit.; see also Jones, Frindt & Windhager, 1988). However, in individual tubules there was very little difference between the L_p responses to the first and second hormonal challenges. The mean peak L_p response to the two successive doses of vasopressin were $335(\pm 55) \times 10^{-7}$ and $358(\pm 35) \times 10^{-7}$ cm s^{-1} atm^{-1}, respectively; the mean difference between the two responses in each experiment was not significantly different from zero. Vasopressin also caused a small transient increase in the lumen negative transepithelial PD (reflecting stimulation of net sodium absorption; Frindt & Burg, 1972); as with the stimulation of L_p, there was no significant difference between the peak increment in

PD in response to the two successive hormonal challenges. In the four experiments the mean increases in lumen negative PD were 2 ± 1 and 3 ± 1 mV, respectively, following the two doses of vasopressin (n.s.).

These results, together with those of our previous study (Phillips & Taylor, 1989), indicate that isolated perfused cortical collecting tubules will respond reproducibly to two successive challenges with a maximal dose of vasopressin separated by a time interval of 60–120 min.

Effect of colcemid pretreatment on the hydrosmotic response to vasopressin

This series of experiments was designed to test the effect of colcemid pretreatment on the water permeability response of the collecting tubule to vasopressin. Figure 3 shows the time course of a typical experiment in which the period of drug pretreatment was 45 min. After a period of equilibration the resting L_p was close to zero. On

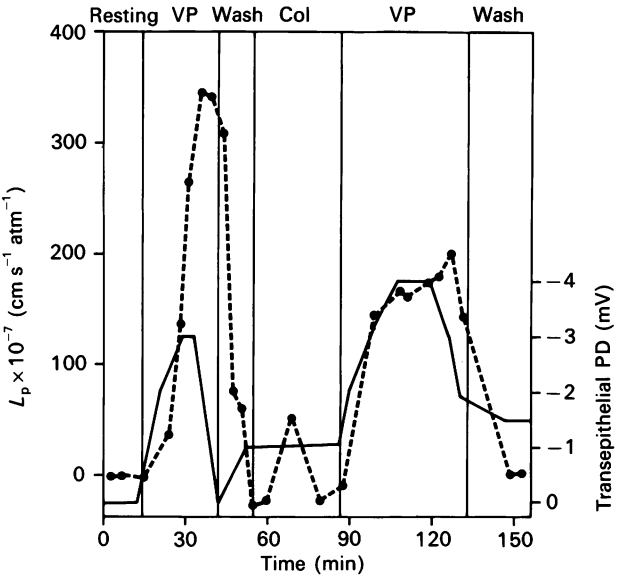

Fig. 3. Time course of a representative experiment showing the effect of 45 min pretreatment with colcemid (Col, $2{\cdot}7 \times 10^{-5}$ M) on the response of a single tubule to vasopressin (VP, 50 μm ml^{-1}). The dashed line represents hydraulic conductivity, L_p, and the continuous line transepithelial PD. The lines drawn between the points are for clarity and have no theoretical significance.

addition of the first control dose of vasopressin, L_p rose rapidly reaching a peak of 344×10^{-7} cm s^{-1} atm^{-1} within 20 min. The transepithelial PD also rose to -3 mV (lumen negative); this effect was more rapid and was transient. When the hormone was washed out, L_p fell back towards resting levels and the PD, which had already returned to the resting value, increased slightly. The addition of colcemid, $2{\cdot}7 \times 10^{-5}$ M, during the recovery period had no clear cut effect on L_p or PD. The second challenge with vasopressin, in the continued presence of colcemid, caused an increase in lumen negative PD similar to that seen in the control period; however the

increase in L_p was reduced relative to the first challenge, reaching a maximum of only 205×10^{-7} cm s^{-1} atm^{-1}. In this tubule the peak L_p response to vasopressin was inhibited by 40% after 45 min pretreatment with colcemid. The results from seven such experiments using this dose of colcemid ($2{\cdot}7 \times 10^{-5}$ M) are summarized in Fig. 4.

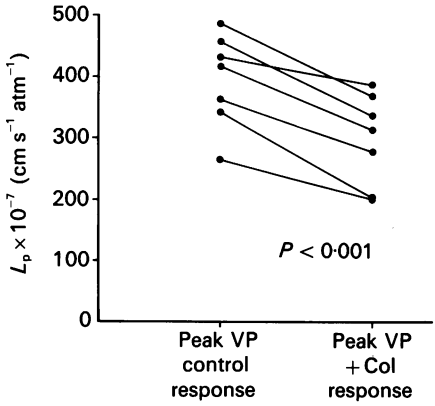

Fig. 4. Comparison of peak L_p response to vasopressin (VP, 50 μU ml^{-1}) before and after 45 min exposure to colcemid (Col, $2{\cdot}7 \times 10^{-5}$ M). Lines join data from seven individual collecting tubules.

The inhibition of vasopressin-stimulated L_p averaged $25(\pm 3)$%; the mean peak L_p values for the control and experimental hormone challenges were $396(\pm 29) \times 10^{-7}$ and $300(\pm 28) \times 10^{-7}$ cm s^{-1} atm^{-1}, respectively ($P < 0{\cdot}001$).

TABLE 2. Inhibitory effect of colcemid pretreatment on hydrosmotic response to vasopressin: time dependence

Period of drug exposure (min)	Colcemid concentration (M)	n	Inhibition (%)
45	$2{\cdot}7 \times 10^{-5}$	7	25 ± 3
	$7{\cdot}2 \times 10^{-5}$	7	49 ± 5
75	$2{\cdot}7 \times 10^{-5}$	4	67 ± 6
	$7{\cdot}2 \times 10^{-5}$	4	72 ± 6

Individual tubules were stimulated with two successive doses of vasopressin (50 μU ml^{-1}) separated by a 90 min wash-out period; prior to the second (experimental) hormone challenge, the tubules were exposed to the stated concentration of colcemid for either 45 or 75 min. Percentage inhibition was calculated from the peak L_p responses to the control and experimental hormone challenges; values represent means $\pm$ S.E.M. of n paired observations.

Although faster than that of colchicine, the binding of colcemid to tubulin *in vitro* is a relatively slow process requiring at least 45 min to reach equilibrium (Banerjee & Bhattacharyya, 1979; see Discussion). The time dependence of the inhibitory effect of colcemid on the hydrosmotic response to vasopressin was therefore examined in a series of experiments in which tubules were exposed to the drug at two different concentrations ($2{\cdot}7$ and $7{\cdot}2 \times 10^{-5}$ M) for either 45 or 75 min, prior to hormonal stimulation. The extent of the inhibition caused by colcemid in these experiments is summarized in Table 2. At both concentrations tested, the extent of

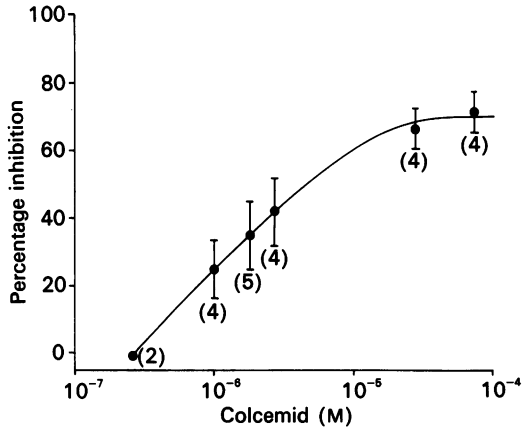

Fig. 5. Inhibition of vasopressin-stimulated water permeability by colcemid: dose–response relationship. Points represent mean inhibition of peak L_p at each drug concentration ± S.E.M.

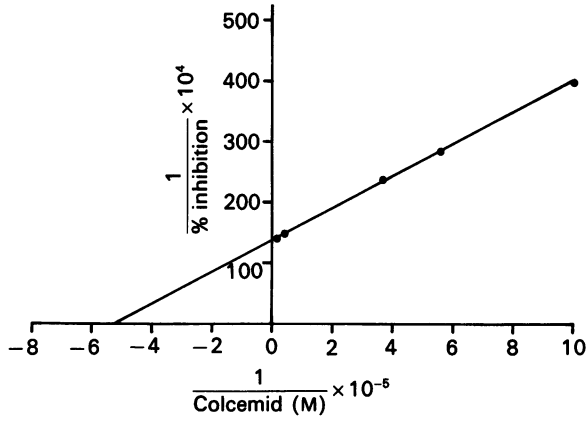

Fig. 6. Double reciprocal plot of inhibition of vasopressin-stimulated water permeability by colcemid.

the inhibition of the hydrosmotic response was substantially greater ($P < 0{\cdot}02$) following a 75 min, *versus* 45 min, period of drug pretreatment; with the longer period of exposure, the degree of inhibition observed at the two drug concentrations did not differ significantly.

The effect of colcemid on the water permeability response to vasopressin was accordingly examined at a number of different drug concentrations using a 75 min period of drug pretreatment. The mean peak L_p responses to the control and experimental hormone challenges at the different concentrations of colcemid are shown in Table 3. The relationship between the concentration of colcemid and the extent of inhibition of vasopressin-stimulated L_p is depicted in Fig. 5. At the lowest colcemid concentration tested ($2{\cdot}7 \times 10^{-7}$ M), no inhibition was seen. Inhibition of the

TABLE 3. Effect of colcemid pretreatment on hydrosmotic response to vasopressin: dose dependence

Colcemid concentration (M)	n	Peak L_p response	
		Control	Experimental
$2{\cdot}7 \times 10^{-7}$	2	360	360
$1{\cdot}0 \times 10^{-6}$	4	456 ± 65	359 ± 64
$1{\cdot}8 \times 10^{-6}$	5	385 ± 57	251 ± 55
$2{\cdot}7 \times 10^{-6}$	4	373 ± 52	215 ± 44
$2{\cdot}7 \times 10^{-5}$	4	325 ± 48	102 ± 20
$7{\cdot}2 \times 10^{-5}$	4	409 ± 84	105 ± 27

Individual tubules were challenged with two successive doses of vasopressin (50 μU ml^{-1}) separated by a 90 min wash-out period; prior to the second hormonal challenge, the tubules were exposed to colcemid for 75 min. Values for L_p(cm s^{-1} atm^{-1}) are means $\pm$ S.E.M. of observations in n tubules.

hormone response approached a maximum of approximately 70% at a colcemid concentration of $2{\cdot}7 \times 10^{-5}$ M. Half-maximal inhibition, derived as an apparent inhibition constant from a double reciprocal plot of the data (Fig. 6) is estimated to occur at a colcemid concentration of $1{\cdot}9 \times 10^{-6}$ M.

Colcemid had no significant effect on basal (hormone-independent) water permeability at any of the concentrations tested. As an example, in the series of tubule perfusions in which $7{\cdot}2 \times 10^{-5}$ M-colcemid was employed (the highest drug concentration tested), the mean baseline L_p prior to the control challenge with vasopressin was $27 \pm 15 \times 10^{-7}$ cm s^{-1} atm^{-1} and the mean L_p averaged over the 75 min period when colcemid was present in the bath prior to the experimental hormone challenge was $34(\pm 11) \times 10^{-7}$ cm s^{-1} atm^{-1} ($n = 4$, n.s.).

Colcemid also had no significant effect, at the concentrations employed, on the hormonal stimulation of transepithelial PD. Thus, after 75 min exposure to colcemid, $7{\cdot}2 \times 10^{-5}$ M, the mean peak increment in lumen negative PD in response to vasopressin did not differ from that following the control challenge (4 ± 1 *vs.* 3 ± 1 mV, $n = 4$, n.s.).

Effect of lumicolcemid pretreatment on the hydrosmotic response

A further series of experiments was designed to examine the specificity of the inhibitory action of colcemid on the L_p response to vasopressin. The effect of lumicolcemid, a photoisomer of colcemid, which does not bind to tubulin and does not disrupt microtubules (Aronson & Inoué, 1970), was examined using a protocol identical to that in the previous section, employing a 45 min period of drug exposure and substituting lumicolcemid, $2{\cdot}7 \times 10^{-7}$ M, for colcemid. A typical experiment is shown in Fig. 7. The results of six such experiments are summarized in Fig. 8; the mean peak L_p values for the control and experimental challenges with vasopressin were $287(\pm 41) \times 10^{-7}$ and $306(\pm 45) \times 10^{-7}$ cm s^{-1} atm^{-1}, respectively ($n = 6$, n.s.). Thus lumicolcemid, unlike colcemid, had no significant effect on the stimulation of L_p by the hormone.

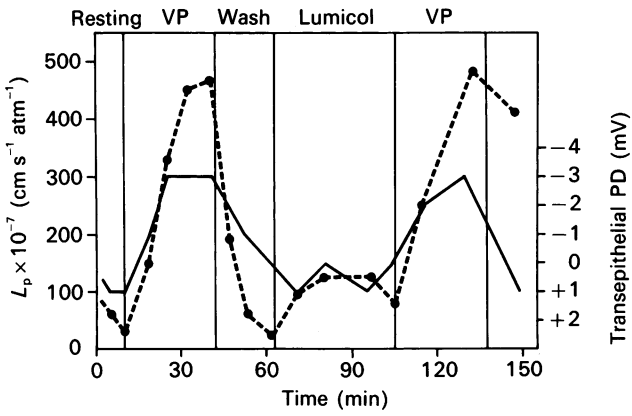

Fig. 7. Time course of a representative experiment showing the effect of 45 min pretreatment with lumicolcemid (Lumicol, $2{\cdot}7 \times 10^{-5}$ M) on the response of a single tubule to vasopressin (VP, 50 μU ml^{-1}). The dashed line represents L_p and the continuous line transepithelial PD.

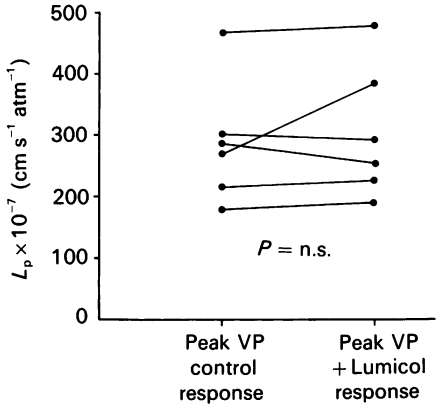

Fig. 8. Comparison of peak L_p response to vasopressin (VP, 50 μU ml^{-1}) before and after 45 min exposure to lumicolcemid (Lumicol, $2{\cdot}7 \times 10^{-5}$ M). Lines join data from six individual collecting tubules.

Effect of colcemid pretreatment on the hydrosmotic response to exogenous cAMP

To ascertain whether the inhibitory effect of colcemid on the vasopressin response is exerted at a pre- or post-cAMP site, the action of the drug on the water permeability response to exogenous cAMP was investigated. The highly permeant cAMP analogue, 8-CPT-cAMP was employed in these experiments, as in our previous studies (Phillips & Taylor, 1989). There was some variability in the response of the tubules to the nucleotide, both in the stimulation of L_p and PD, but the overall pattern of response was very similar to that observed with vasopressin. The effect of 45 min exposure to colcemid, $2{\cdot}7 \times 10^{-5}$ M, on the water permeability response to 8-CPT-cAMP, $1{\cdot}8 \times 10^{-5}$ M, is summarized in Fig. 9. The mean peak L_p

response to the first (control) challenge with the nucleotide analogue was $291(\pm 24) \times 10^{-7}$ cm s^{-1} atm^{-1}, and to the second challenge in the presence of colcemid, $178(\pm 18) \times 10^{-7}$ cm $s^{-1}atm^{-1}$. This represents a mean inhibition of $38 \pm 4\%$ ($n = 5$, $P < 0{\cdot}01$).

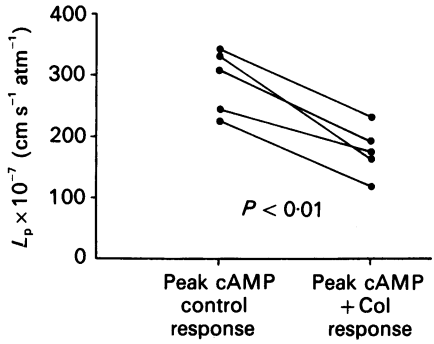

Fig. 9. Comparison of peak L_p response to 8-CPT-cAMP ($1{\cdot}8 \times 10^{-5}$ M) before and after 45 min exposure to colcemid (Col, $2{\cdot}7 \times 10^{-5}$ M). Lines join data from five individual collecting tubules.

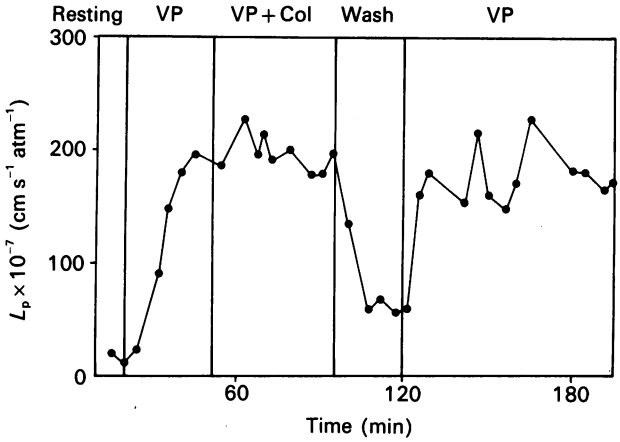

Fig. 10. Time course of a representative experiment showing the lack of inhibitory effect of colcemid (Col, $5{\cdot}5 \times 10^{-5}$ M) on the L_p response of a single tubule to vasopressin (VP, 50 μU ml^{-1}) when added 30 min after hormone addition.

Effect of colcemid on the maintenance of the vasopressin response

These experiments were designed to test the effect of colcemid on the maintenance phase of the hormone-induced water permeability response. As described in Methods, for technical reasons the protocol was different to that used in the previous experiments: the experimental challenge with vasopressin was performed first and the control second. Figure 10 illustrates the result of a typical experiment in this series. Following the first (experimental) challenge with vasopressin (50 μU ml^{-1}), L_p rose from its low initial level to around 200×10^{-7} cm s^{-1} atm^{-1} over the first 30 min. On exposure to colcemid ($5{\cdot}5 \times 10^{-5}$ M) this high level of water permeability was

maintained over the next 45 min, L_p averaging $194(\pm 6) \times 10^{-7}$ cm s^{-1} atm^{-1} during this period ($n = 9$). When the hormone and drug were washed out, L_p returned towards baseline. After the second (control) vasopressin challenge, L_p rose to a peak of 215 cm s^{-1} atm^{-1} within 30 min; over the subsequent 45 min of exposure to the

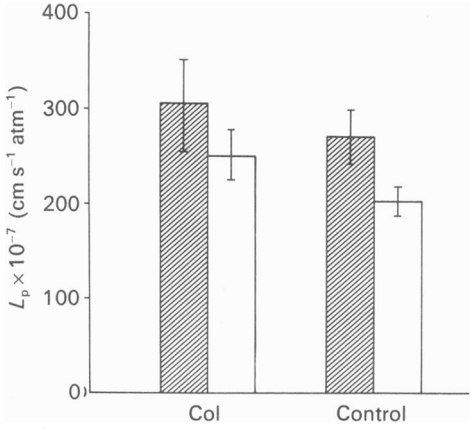

Fig. 11. Water permeability response to vasopressin (50 μU ml^{-1}) when collecting tubules were exposed to colcemid (Col, $5{\cdot}5 \times 10^{-5}$ M) 30 min after the first hormonal challenge compared to that following a second control challenge. The bars represent mean values (±S.E.M.) from five experiments: hatched bars represent peak L_p value attained during the first 30 min of hormonal stimulation and the open bars the average steady-state value during maintained hormonal stimulation.

hormone, L_p fluctuated somewhat, averaging $177(\pm 9) \times 10^{-7}$ cm s^{-1} atm^{-1} ($n = 7$). This control response was similar in magnitude to that seen with the initial challenge, although there was in this case a slightly lower mean L_p during the period of maintained exposure to the hormone. In five experiments, summarized in Fig. 11, addition of colcemid had no significant inhibitory effect on the maintenance phase of the response to vasopressin. The mean steady-state L_p, averaged over the 45 min period after the hormone response was fully established, was $250(\pm 27) \times 10^{-7}$ cm s^{-1} atm^{-1} in the presence of colcemid and $201(\pm 16) \times 10^{-7}$ cm s^{-1} atm^{-1} ($n = 5$, n.s.) in its absence.

DISCUSSION

The present results demonstrate that the colchicine analogue, colcemid, specifically inhibits the initiation, but not the maintenance, of the water permeability increase elicited by vasopressin in isolated cortical collecting tubules from rabbit kidneys. The drug has no effect on the basal level of water permeability of the tissue, nor on the stimulation of transepithelial potential difference by the hormone. The major findings of this study are similar to those obtained in our previous *in vitro* study on the effects of nocodazole on the response of the rabbit cortical collecting tubule to vasopressin (Phillips & Taylor, 1989).

The plant alkaloid and classical antimitotic agent, colchicine, has been extensively employed in previous studies on the role of cytoplasmic microtubules in the cellular

response to vasopressin (see Pearl & Taylor, 1985). Colcemid has been widely used as an alternative to colchicine to explore the properties and biological functions of microtubules in other cellular systems (see Dustin, 1984; Schliwa, 1986). Colcemid binds to tubulin at the same site as colchicine (Ray, Bhattacharyya & Biswas, 1981), thereby interfering with microtubule assembly, and exposure of cells to colcemid results in the disruption of cytoplasmic microtubules (see e.g. Osborn & Weber, 1976). Colcemid was chosen for the present study on isolated collecting tubules because its binding to the microtubule subunit protein, although not rapid, is faster and more readily reversible than that of colchicine (Wilson *et al.* 1974; Banerjee & Bhattacharyya, 1979; Ray *et al.* 1981). The use of a relatively fast-acting and readily reversible drug allowed us to employ an experimental design in which each tubule served as its own control, as in our earlier studies with nocodazole (Phillips & Taylor, 1989).

Colchicine and its analogues, and also other antimitotic agents, may exert inhibitory effects on membrane transport processes that are unrelated to disruption of microtubules (Wilson *et al.* 1974). For example, colchicine is known to inhibit nucleoside uptake by cultured mammalian cells (Mizel & Wilson, 1972). However, photo-inactivated isomers of colchicine and colcemid, which lack the ability to bind to tubulin (Wilson & Friedkin, 1967; Wilson *et al.* 1974) and do not disrupt microtubules (e.g. Kawahara & French, 1990) or block mitosis (Wilson & Friedkin, 1967; Aronson & Inoué, 1970), but do inhibit nucleoside transport (Mizel & Wilson, 1972), can be employed to test the specificity of the inhibitory effects of these drugs. Lumicolcemid, prepared by ultraviolet irradiation of colcemid (Fig. 1), was accordingly employed in the present studies to assess whether the observed inhibitory effect of colcemid is specifically related to its ability to bind to tubulin and disrupt cytoplasmic microtubules.

Our present results demonstrate that colcemid inhibits the development of the hydrosmotic response to vasopressin in isolated cortical collecting tubules in a manner which is both time- and concentration dependent. These findings are similar to those obtained in our studies with colchicine in the toad urinary bladder (Taylor *et al.* 1978). Although the binding of colcemid to tubulin is more rapid than that of colchicine it is still a relatively slow process. Banerjee & Bhattacharyya (1979) reported that the binding of colcemid to tubulin *in vitro* requires a period of 45 min to reach equilibrium; however, this value is almost certainly an underestimate since the spontaneous decay of drug binding activity of tubulin (cf. Wilson *et al.* 1974) was not taken into account in that study. In our present experiments on cortical collecting tubules, we tested the effects on the vasopressin response of two different periods of drug exposure (viz. 45 and 75 min prior to hormone stimulation). At the two relatively high drug concentrations tested (2·7 and $7{\cdot}2 \times 10^{-5}$ M), the degree of inhibition increased significantly with the period of drug pretreatment; however when a 75 min period of drug exposure was employed, there was no significant difference in the magnitude of the inhibition produced at the two drug concentrations (Table 2). A 75 min pretreatment period was therefore chosen for the dose-dependence studies on the assumption that this would allow adequate time for colcemid binding to tubulin in the tubular epithelial cells to reach equilibrium.

Analysis of the dose–response data for colcemid inhibition of the water

permeability increase induced by vasopressin in the collecting tubule indicates that the effective drug concentration range is consistent with the known potency of colcemid as an antimitotic agent (Aronson & Inoué, 1970) and with its ability to disrupt microtubules *in vivo* (Osborn & Weber, 1976; Spiegelman *et al.* 1979). Moreover, the effective potency of colcemid in the collecting tubule is very similar to that previously observed for colchicine in the toad bladder. The concentration of colcemid required for half-maximal inhibition of the vasopressin response in the collecting tubule is estimated to be $1{\cdot}9 \times 10^{-6}$ M; this value is essentially identical to the concentration of colchicine required for half-maximal inhibition of vasopressin-induced water flow ($1{\cdot}8 \times 10^{-6}$ M; Taylor *et al.* 1978) and half-maximal disruption of assembled microtubules in the granular epithelial cells of the toad bladder ($1{\cdot}4 \times 10^{-6}$ M; Reaven, Maffly & Taylor, 1978). (On the other hand, it is one order of magnitude lower than the concentration of colcemid reported to cause 50% inhibition of nucleoside transport in cultured cells, 4×10^{-5} M; Mizel & Wilson, 1972). Furthermore, the concentration of colcemid required for half-maximal inhibition of the hydrosmotic response in the collecting tubule ($1{\cdot}9 \times 10^{-6}$ M) corresponds very closely to the values, estimated from apparent binding constants, for half-saturation of colchicine binding to tubulin derived from brain (2×10^{-6} M; Wilson *et al.* 1974) and toad bladder epithelium (1×10^{-6} M; Wilson & Taylor, 1978), and is slightly lower than that reported for half-saturation of colchicine binding to tubulin from bovine renal medulla ($5{\cdot}9 \times 10^{-6}$ M; Barnes & Roberson, 1979). Finally, the finding that lumicolcemid, which does not interfere with mitosis or disrupt microtubules (Aronson & Inoué, 1970), had no effect on the development of the water permeability response of the collecting tubule to vasopressin supports the view that the inhibitory effect of colcemid is specifically related to its tubulin-binding capacity. Taken together these results strongly suggest that inhibition of the vasopressin response by colcemid depends on interaction of the drug with tubulin and consequent disruption of cytoplasmic microtubules. The findings provide indirect but compelling evidence that the normal development by the hydrosmotic response to vasopressin depends on the integrity of cytoplasmic microtubules in the hormone-sensitive renal tubular epithelial cells.

Consistent with our earlier observations with nocodazole in the collecting tubule (Phillips & Taylor, 1989) and with findings in the toad bladder (e.g. Taylor *et al.* 1978), colcemid pretreatment resulted in inhibition of the water permeability response of the collecting tubule to exogeneous cAMP as well as that to vasopressin. In the present studies, in contrast to those with nocodazole, the drug-induced inhibition of the response to the cyclic nucleotide was quantitatively greater than the inhibition of the response to the hormone. Taken together with the lack of effect of colcemid on vasopressin-stimulated PD, the results indicate that the inhibitory effect of the drug cannot be ascribed to interference with hormone–receptor interaction, or with cAMP production or accumulation, in the collecting tubule cells. Thus the effect of colcemid is evidently exerted at an intracellular site beyond the generation of cAMP. The data are accordingly consistent with the view that cytoplasmic microtubules are involved in the development of the hormone-induced water permeability increase in the principal cells of the collecting tubule at a post-cAMP step (Phillips & Taylor, 1989).

Like nocodazole, colcemid had no effect on the maintenance of the increased water permeability elicited by prior stimulation of the collecting tubule by vasopressin (cf. Phillips & Taylor, 1989). This finding is consistent with the results of a number of studies with microtubule-disruptive agents in amphibian bladder (reviewed in Pearl & Taylor, 1985). For example, in the toad bladder, when colchicine was applied after the water permeability response to vasopressin was already established, the drug had no effect on the rate of osmotic water flow (Kachadorian *et al.* 1979), nor on the incidence of apical membrane particle aggregates or membrane fusion sites (Muller *et al.* 1980), nor on the associated hormone-dependent increase in apical membrane area (Palmer & Lorenzen, 1983). These findings have been interpreted to indicate that microtubules are involved only in the initiation of the hormonal response (Kachadorian *et al.* 1979). The present results in the collecting tubule are consistent with this notion. Alternatively, it is possible that the failure of colcemid to influence the maintenance phase of the vasopressin response reflects incomplete disruption of microtubules in the apical region of the hormone-sensitive epithelial cells.

In summary, the present findings with colcemid, taken together with our earlier results with nocodazole, point to the conclusion that intact microtubules play a role in the normal development of the water permeability response to vasopressin in the principal cells of the rabbit cortical collecting tubule. As yet, definite evidence regarding the origin of the particle clusters (presumed to contain the putative water channels) that appear in the apical plasma membrane of the principal cells after vasopressin stimulation, is lacking (Brown, 1989). However, such particle clusters have been seen in the limiting membranes of vesicles in the apical cytoplasm of the principal cells of rabbit cortical collecting tubules, and such vesicles have been observed in fusion with the apical membrane (Kubat, Lorenzen & Reale, 1989; B. Kubat, personal communication). By analogy with events known to occur in amphibian bladder, it is tempting to speculate that water channels are stored preformed in cytoplasmic vesicles, and that these are incorporated into the apical membrane of the principal cells by an exocytotic fusion event under the influence of vasopressin; the water channels may subsequently be retrieved by endocytosis and recycled (Brown, 1989). It is now widely recognized that the directed movement of intracellular organelles occurs along assembled cytoplasmic microtubules, the motive force for such movement being generated by motor proteins that exhibit microtubule-activated ATPase activity (Shroer & Sheetz, 1991). Our results are consistent with the view that cytoplasmic microtubules in the apical region of the principal cells of the collecting tubule are involved in vesicle translocation towards the apical cell surface, and thus participate in the delivery of water channels to the apical plasma membrane during the initiation of the water permeability increase induced by vasopressin.

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