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EFFECT OF NOCODAZOLE ON THE WATER PERMEABILITY RESPONSE TO VASOPRESSIN IN RABBIT COLLECTING TUBULES PERFUSED *IN VITRO*

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SUMMARY

1. The effect of the microtubule-disruptive agent, nocodazole (methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate), on the water permeability response to vasopressin or the synthetic cyclic AMP analogue, 8-parachlorophenylthio-cyclic AMP (8-CPT-cAMP), has been investigated in isolated cortical collecting tubules from rabbit kidneys, perfused *in vitro*.

2. Pre-treatment with nocodazole, 1–4 μg ml^{-1}, had no significant effect on basal water permeability, but inhibited the increase in hydraulic conductivity elicited by vasopressin, 50 μU ml^{-1}, in a dose-dependent manner. Inhibition of the response to the hormone averaged 65 $\pm$ 6% ($n = 8$, $P < 0{\cdot}001$) at a nocodazole concentration of 4 μg ml^{-1}.

3. Nocodazole, 1–4 μg ml^{-1}, had no effect on the increase in lumen-negative potential difference (PD) induced by the hormone.

4. Pre-treatment with nocodazole, 4 μg ml^{-1}, inhibited the development of the water permeability response to 8-CPT-cAMP, $1{\cdot}8 \times 10^{-5}$ M, by 45 $\pm$ 7% ($n = 7$, $P < 0{\cdot}001$).

5. When collecting tubules were exposed to nocodazole, 4 μg ml^{-1}, after the hydrosmotic response to vasopressin had been fully established, the drug had no inhibitory effect on the maintenance of a high water permeability.

6. The results are consistent with the view that cytoplasmic microtubules play a role in the initiation of the water permeability response to vasopressin in the mammalian cortical collecting tubule at a cellular site beyond the generation of cyclic AMP.

INTRODUCTION

The neurohypophyseal hormone, vasopressin, promotes the absorption of water and stimulates net sodium transport in amphibian urinary bladder and mammalian cortical collecting tubules via the mediation of cyclic AMP. Vasopressin and its second messenger induce specific increases in the water and sodium permeability of the plasma membrane at the apical (luminal) surface of the hormone-responsive epithelial cells (Ausiello & Orloff, 1982).

The dramatic increase in water permeability elicited by vasopressin is believed to depend on the addition of new membrane components to the apical membrane of the

target cells. Freeze-fracture electron microscopy has revealed that the water permeability response is associated with the appearance of aggregates or clusters of intramembranous particles in the apical membrane of the granular cells of amphibian bladder (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & DiScala, 1975) and the principal cells of mammalian collecting tubules (Harmanci, Stern, Kachadorian, Valtin & DiScala, 1980). The incidence of the particle aggregates/clusters correlates with the increase in transepithelial water flow/urine osmolality, and these intramembranous structures are believed to represent the sites of transmembrane water movement, i.e. the putative water channels (Wade, Stetson & Lewis, 1981). In the toad bladder, the particle aggregates exist preformed in membrane vesicles in the apical cytoplasm of the granular cells (see Wade *et al.* 1981). The aggregate-containing vesicles are incorporated into the apical plasma membrane by an exocytotic fusion event under the influence of vasopressin (Muller, Kachadorian & DiScala, 1980) and are retrieved by endocytosis following hormone removal (Coleman, Harris & Wade, 1987). The particle clusters in the principal cells of the collecting duct may also be inserted into and removed from the apical membrane by a cycle of exo- and endocytosis under the influence of the hormone. Stimulation of endocytosis during exposure to and/or removal of vasopressin has now been documented in rabbit cortical collecting tubules (Strange, Willingham, Handler & Harris, 1988), as well as in toad urinary bladder (Masur, Holtzmann, Schwartz & Walter, 1971; Gronowicz, Masur & Holtzmann, 1980; Wade *et al.* 1981; Harris, Wade & Handler, 1986). In a recent study, endocytotic vesicles derived from renal medulla were shown to have permeability properties consistent with the presence of vasopressin-sensitive water channels (Verkman, Lencer, Brown & Ausiello, 1988).

In amphibian bladder, the initiation of the water permeability response to vasopressin is dependent on an intact cytoskeleton. Pharmacological agents that disrupt cytoplasmic microtubules (e.g. colchicine, nocodazole) inhibit the development of the hydrosmotic response to vasopressin or exogenous cyclic AMP (Taylor Mamelak, Golbetz & Maffly, 1978; Brady, Parsons & Coluccio, 1981). Such agents also interfere with the hormone-induced increase in apical membrane area (Gronowicz *et al.* 1980; Palmer & Lorenzen, 1983), and with the appearance of the particle aggregates in the apical plasma membrane (Kachadorian, Ellis & Muller, 1979). The available data are consistent with the view that microtubules in the apical cytoplasm are involved in the translocation of the aggregate-containing vesicles towards the apical surface of the granular cells, prior to their exocytotic insertion into the apical plasma membrane (Kachadorian *et al.* 1979; Muller *et al.* 1980; reviewed in Pearl & Taylor, 1985).

Although there is compelling evidence (albeit indirect) that microtubles play a role in the water permeability increase induced by vasopressin in amphibian bladder, there is little information regarding their involvement in the action of the hormone on the renal collecting tubule. Colchicine administration has been shown to impair urinary concentrating ability in conscious Sprague–Dawley rats (Dousa & Barnes, 1974) and to blunt the antidiuretic response to exogenous vasopressin in Brattelboro rats (Hall, Taylor & Maffly, 1974). Iyengar, Lepper & Mailman (1976) have reported that colchicine inhibits the vasopressin-stimulated uptake of tritiated water in slices

from dog renal medulla. Abramow (1976), in a brief preliminary study on isolated perfused collecting tubules from rabbit kidneys, demonstrated that pre-incubation with colchicine (10^{-4} M) can interfere with the development of the water permeability response to vasopressin.

In the present study we have used the fast-acting microtubule-disruptive agent, nocodazole, to investigate the possible role of microtubules in the response of isolated cortical collecting tubules of the rabbit to vasopressin or exogenous cyclic AMP. The results support the view that cytoplasmic microtubules play a critical role in the initiation of the water permeability increase induced by vasopressin in the mammalian nephron, as in amphibian bladder. Preliminary reports of the findings have been published in abstract form (Phillips & Taylor, 1986, 1987*a*).

METHODS

In vitro perfusion

Cortical collecting tubules were isolated and perfused *in vitro* according to the method described by Grantham & Burg (1966). New Zealand White rabbits (weighing 1–1·5 kg) of both sexes were maintained on a special diet (Maintenance Diet SDS) containing 0·34% sodium and 1·78% potassium. They were killed by dislocation of the neck and the left kidney was removed. Transverse tissue slices of about 1 mm thickness were cut and transferred to chilled dissection medium (see Table 1). A wedge-shaped section of one slice was dissected out and transferred to a Petri dish containing fresh dissection medium cooled on a chilled metal tray. Cortical collecting tubules of approximately 1–3 mm length were dissected free. (This procedure usually took 15–30 min from the death of the animal; very occasionally the dissection period was longer but this did not seem to have a deleterious effect on the tissue.) 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 on glass micropipettes (made from soft fine glass (Drummond Scientific Company, PA, USA) and prepared with a Stoelting (Chicago) Pipette Puller and Microforge). The pipettes were set up on a conventional microperfusion apparatus (J. P. White, Bethesda, MD, USA). The bathing medium was stirred and oxygenated with a 95% O_2, 5% CO_2 gas mixture and the temperature of the fluid gradually raised over about 15 min to 37 °C, and thereafter maintained with a temperature controller (model 73ATD Yellow Springs Instruments, Yellow Springs, OH, USA). After raising the bath temperature, the tubule was allowed to equilibrate for ~ 30 min prior to the start of the experimental protocol. The bathing medium was changed periodically during each experiment.

The tubules were perfused by gravity at a rate of about 20–30 nl min^{-1}. The perfusate solution (Table 1) had an osmolality of half that of the bathing solution. It contained [^{14}C]methoxyinulin (Amersham) at a concentration of about 5 μCi ml^{-1}. The tubule fluid collection rate was measured by determining the time required to fill a calibrated volumetric pipette; collections were usually made every 3–7 min. The perfusion rate was calculated from the rate of appearance of the marker in the collected fluid and its measured concentration in the perfusate. The radioactivity of perfused and collected fluids was measured in a liquid scintillation counter (Nuclear Chicago; Isocap/300).

The tubule length was measured using an eyepiece micrometer. The length of the perfused segments ranged from 0·5 to 2·5 mm with a mean of $1·3 \pm 0·1$ mm. The mean internal diameter was assumed to be 20 μm (Frindt, Windhager & Taylor, 1982).

In each experiment the system was checked for potential leaks by measuring the [^{14}C]inulin in the bathing solution. The mean leak of inulin into the bath was $2·1 \pm 0·2$% ($n = 34$) of the perfusion rate.

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 calomel half-cell. These cells were connected to a Keithley Digital Electrometer.

Solutions

The composition of the experimental solutions is given in Table 1. Both the bathing and perfusion solutions were passed through a Millipore filter (pore size, 0·45 μm), and their osmolality was checked prior to use by means of a vapour pressure osmometer (Wescor 5100C).

Bovine serum albumin (0·5 g 100 ml^{-1}) was added to the dissection fluid. This helped to prevent the tubule 'sticking' to the dissecting instruments and the Pasteur pipette, thus facilitating transfer to the perfusion chamber.

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
$CaCl_2$	2·0	2·0	2·0
Osmolality	290	290	145

Values are in mM except for osmolality which is mosmol (kg H_2O)$^{-1}$.

Calculation of hydraulic conductivity

The hydraulic conductivity, L_p (10^{-7} cm s^{-1} atm^{-1}), was calculated using the equation of Dubois, Verniory & Abramow (1976).

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

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

Experimental protocols

Repeated challenge with vasopressin. Throughout this study each tubule served as its own control. It was therefore first necessary to show that the tissue would respond reproducibly to two successive challenges with vasopressin and the following protocol was adopted. After a period during which samples were collected for estimation of baseline water permeability, vasopressin (Sigma) was added to the bathing solution at a concentration of 50 μU ml^{-1} (this maximal dose (Grantham & Burg, 1966) was used in all subsequent experiments). The tissue was exposed to vasopressin for 30 min at which point the hormone was removed by replacing the bathing solution with hormone-free medium. The tubule was then allowed to recover for 30 min prior to a second 30 min exposure to vasopressin.

Effect of nocodazole pre-treatment on the response to vasopressin or 8-parachlorophenyl-thio cyclic AMP (8-CPT-cAMP). The general protocol was similar to that described above. Since nocodazole was dissolved in DMSO (dimethyl sulphoxide), this solvent was included in the bathing solution during the first (control) challenge with vasopressin. (The concentration of DMSO was 0·02% (v/v); in a trial series of four experiments this concentration of DMSO was shown to have no effect on the response of the tissue to vasopressin.) The initial 30 min period of exposure to hormone (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 30 min. Half-way through the latter, the bath was changed to a solution containing nocodazole (Janssen Pharmaceuticals) in DMSO, 0·02%, and 15 min later the tubule was challenged with a second dose of vasopressin or cyclic nucleotide analogue in the presence of the drug; after a further 30 min both agonist and drug were washed out.

Effect of nocodazole added after the vasopressin response was established. In the final series of experiments, because of the length of the procedure, the sequence of control and experimental periods was reversed. To avoid any uncertainties about the viability of the tissue it was decided that the second hormonal challenge should be the control period and the first challenge should be the experimental period. In these studies, exposure to vasopressin (50 μU ml^{-1}) was maintained for 75 min during both the control and experimental periods. Thirty minutes after the initial challenge with hormone, the tubule was exposed to nocodazole (4 μg ml^{-1} in 0·02% DMSO) in the continued presence of vasopressin. Both the 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. The tubule was exposed to DMSO (0·02%) 30 min after the second hormonal challenge.

Statistics

Measurements are expressed as the mean (±S.E. of the mean). The significance of the difference between the means for the control and experimental periods was determined by a paired t test.

RESULTS

Response to successive challenges with vasopressin

In initial experiments we examined the effect of two consecutive doses of 50 μU ml^{-1} vasopressin on hydraulic conductivity, L_p. Although there was considerable variation in the magnitude of the peak hydrosmotic response to vasopressin between different tubules (as noted by other investigators, see Jones, Frindt & Windhager, 1988) there was no difference in the magnitude of the response to the first and second hormonal challenge in individual tubules. Figure 1 shows the response of a representative tubule to two successive doses of vasopressin. Initially, after a period of equilibration, the resting L_p was close to zero. After the first challenge with vasopressin there was a rapid rise in hydraulic conductivity to a peak, followed by a slight decline over the next 15 min; L_p fell rapidly towards zero when the hormone was washed out. A second dose of vasopressin again led to a rapid increase in L_p, the peak value being of similar magnitude to the first. The results from five such experiments are shown in Fig. 2. The mean peak L_p responses to the two doses of vasopressin were $400(\pm 24) \times 10^{-7}$ cm s^{-1} atm^{-1}and 396 $(\pm 27) \times 10^{-7}$ 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 an increase in the lumen-negative transepithelial PD as illustrated in Fig. 1. In this experiment, the PD increased by 3 mV in response to both the first and second hormonal stimulations. The PD response was found to be variable in magnitude and sometimes transient, but as with the stimulation of L_p by vasopressin, there was no significant difference between the peak responses to the two successive hormonal challenges. In five experiments, the mean increase in PD was 4 ± 1 and 3 ± 2 mV, respectively, following the two doses of vasopressin (n.s.).

Effect of nocodazole pre-treatment on the vasopressin response

Figure 3 illustrates the result of an experiment designed to test the effect of pre-treatment with nocodazole on the vasopressin response. The figure shows the changes in both hydraulic conductivity and transepithelial PD following hormonal stimulation. After the initial period of equilibration, L_p and PD were both low. After stimulation with the control dose of vasopressin, L_p rose and reached a peak of

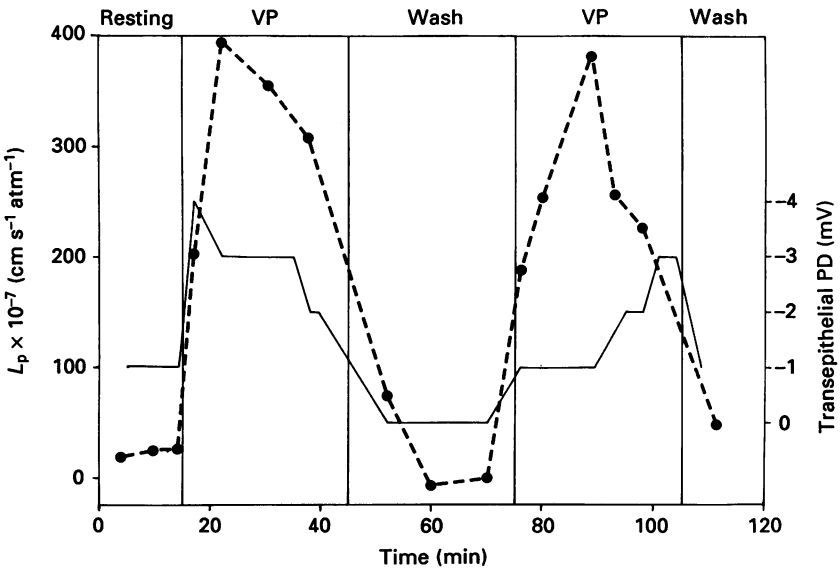

Fig. 1. Time course of a representative experiment showing the response of a single cortical collecting tubule to two consecutive doses of vasopressin (VP, 50 μU ml^{-1}). The dashed line represents hydraulic conductivity, L_p, and the continuous line transepithelial PD. The lines drawn between the points are for clarity and have no theoretical significance.

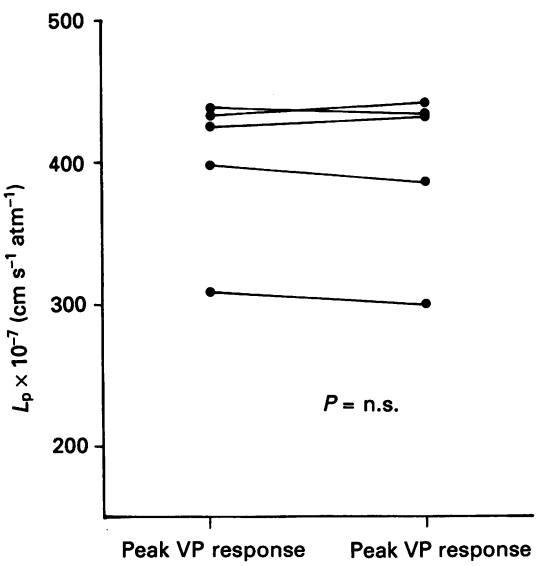

Fig. 2. Peak L_p response to two consecutive doses of vasopressin (VP, 50 μU ml^{-1}). Lines join data from five individual collecting tubules.

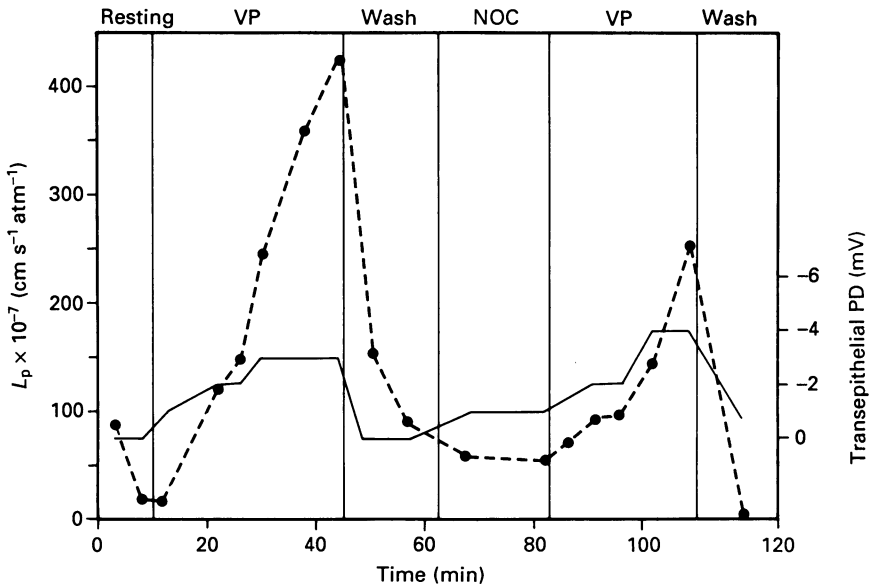

Fig. 3. Time course of a representative experiment showing the effect of nocodazole (NOC, 4 μg ml^{-1}) on the response of a single tubule to vasopressin (VP, 50 μU ml^{-1}). The dashed line represents L_p and the continuous line transepithelial PD.

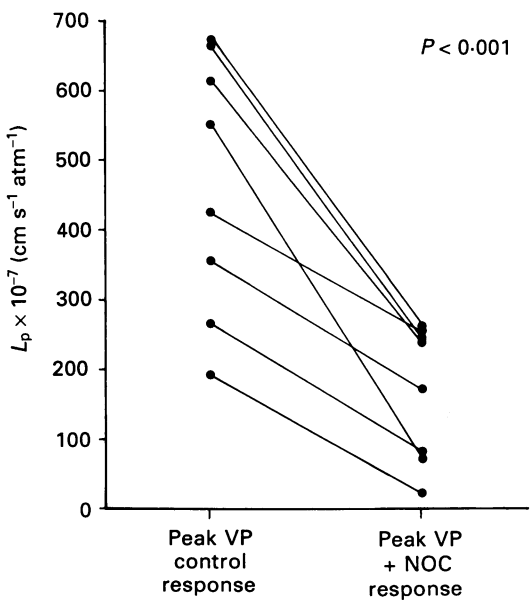

Fig. 4. Comparison of peak L_p response to vasopressin (VP, 50 μU ml^{-1}) before and after exposure to nocodazole (NOC, 4 μg ml^{-1}). Lines join data from eight individual collecting tubules.

425×10^{-7} cm s^{-1} atm^{-1} after 30 min; the transepithelial PD rose to -3 mV. When the hormone was washed out both L_p and PD returned towards resting levels. Following exposure to nocodazole, 4 μg ml^{-1}, L_p was unchanged and PD rose slightly. The second challenge with vasopressin in the presence of nocodazole caused a similar increase in the transepithelial PD as seen in the control period, but the increase in L_p was reduced relative to the first challenge, reaching only 256×10^{-7} cm s^{-1} atm^{-1} after 30 min. In this experiment, the peak L_p response to vasopressin was

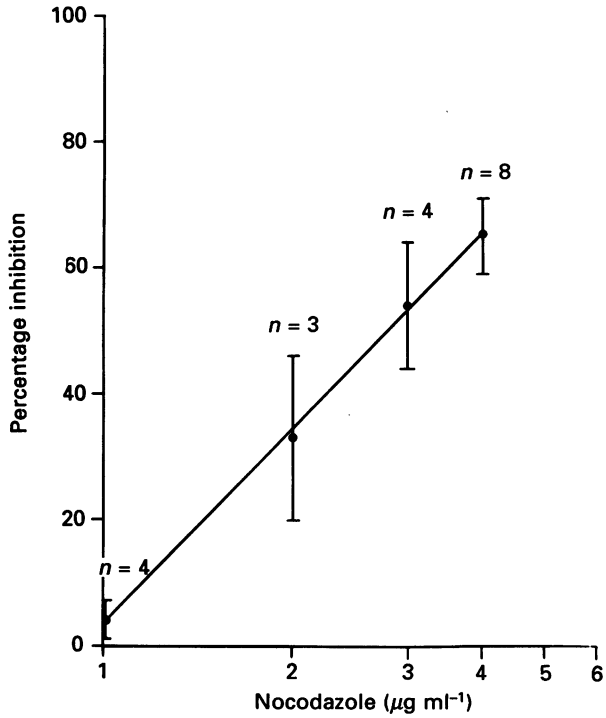

Fig. 5. Inhibition of vasopressin-stimulated water permeability by nocodazole: dose–response relationship. Points represent mean inhibition of peak L_p at each drug concentration tested ±S.E.M.

inhibited by 40% after exposure to nocodazole. The results of eight such experiments with nocodazole, 4 μg ml^{-1}, are summarized in Fig. 4. At this dose of nocodazole, the mean inhibition of the L_p response was 65 ± 6%; mean peak L_p values for the control and experimental periods were 467 (± 66) $\times 10^{-7}$ and 168 (± 33) $\times 10^{-7}$ cm s^{-1} atm^{-1}, respectively ($P < 0{\cdot}001$).

The dose dependence of the effect of nocodazole on the L_p response to vasopressin was determined in several series of tubule perfusions using a single concentration of vasopressin (50 μg ml^{-1}) but different concentrations of the drug. At a dose of 1 μg ml^{-1}, nocodazole had no significant effect on the water permeability response to vasopressin; peak L_p averaged 365 (± 63) $\times 10^{-7}$ and 355 (± 66) $\times 10^{-7}$ cm s^{-1} atm^{-1} during control and experimental periods respectively ($n = 4$, n.s.). At 2 μg ml^{-1} nocodazole, the vasopressin response was reduced by 33 ± 13% (peak L_p averaged 292

(± 84) $\times 10^{-7}$ cm s^{-1} atm^{-1} in the control period and 212 (± 85) $\times 10^{-7}$ cm s^{-1} atm^{-1} after exposure to nocodazole ($n = 3$; $P < 0{\cdot}05$). At a nocodazole concentration of 3 μg ml^{-1}, the water permeability response to vasopressin was inhibited by 54 ± 10% (mean peak L_p decreased from a control value of 390 (± 64) $\times 10^{-7}$ cm s^{-1} atm^{-1} to 195 (± 63) $\times 10^{-7}$ cm s^{-1} atm^{-1} after nocodazole treatment ($n = 4$; $P < 0{\cdot}001$). The relationship between the concentration of nocodazole and the extent of inhibition of the water permeability response to vasopressin is depicted in Fig. 5.

Nocodazole had no significant effect on basal (hormone-independent) water permeability at any concentration tested. For example, in that series of tubules in which 4 μg ml^{-1} nocodazole was employed, mean baseline L_p immediately prior to the control challenge with vasopressin was 22 (± 27) $\times 10^{-7}$ cm s^{-1} atm^{-1}, and the mean L_p during the wash-out period when nocodazole was present in the bath (averaged over 15 min) was 43 (± 35) $\times 10^{-7}$ cm s^{-1} atm^{-1} ($n = 8$, n.s.).

Nocodazole had no significant effect, at any concentration tested, on the increase in transepithelial PD elicited by the hormone. The mean increase in PD following vasopressin was $6 \pm 1{\cdot}2$ mV in the control period and $5 \pm 0{\cdot}9$ mV in the presence of nocodazole, 1–4 μg ml^{-1} ($n = 13$, n.s.).

Effect of nocodazole pre-treatment on the response to exogenous cyclic AMP

To determine whether nocodazole exerts its inhibitory effect at a pre- or post-cyclic AMP site, the effect of the drug on the water permeability response to exogenous cyclic AMP was investigated. In these experiments, the highly permeant cyclic AMP analogue, 8-CPT-cAMP (Hall, Barnes & Dousa, 1977), was employed. As in the studies with vasopressin, there was considerable variability between tubules in the magnitude of the stimulation of both L_p and PD by the nucleotide, but the pattern of each tubule's response to the cyclic AMP analogue was very similar to that with vasopressin.

The effect of nocodazole, 4 μg ml^{-1}, on the hydrosmotic response to 8-CPT-cAMP, $1{\cdot}8 \times 10^{-5}$ M, in seven tubules is summarized in Fig. 6. The mean peak L_p response to the first (control) challenge with the nucleotide analogue was 315 (± 20) $\times 10^{-7}$ cm s^{-1} atm^{-1}, and to the second challenge in the presence of nocodazole, 168 (± 33) $\times 10^{-7}$ cm s^{-1} atm^{-1}. This represents a mean inhibition of 45 ± 7% ($n = 7$, $P < 0{\cdot}001$).

As found in the experiments with vasopressin, nocodazole had no effect on the stimulation of transepithelial PD by 8-CPT-cAMP. The mean difference between the PD responses to the two challenges with the nucleotide was not significantly different from zero (data not shown).

Effect of nocodazole on maintenance of the vasopressin response

Figure 7 illustrates the result of a typical experiment designed to test the effect of adding nocodazole after the water permeability increase induced by vasopressin had been established. In these experiments the protocol was varied; for technical reasons the experimental challenge was performed first and the control second, as described in Methods. In response to the first (experimental) challenge with hormone (50 μU ml^{-1}), L_p rose to a peak value of 174×10^{-7} cm s^{-1} atm^{-1}. On exposure to nocodazole

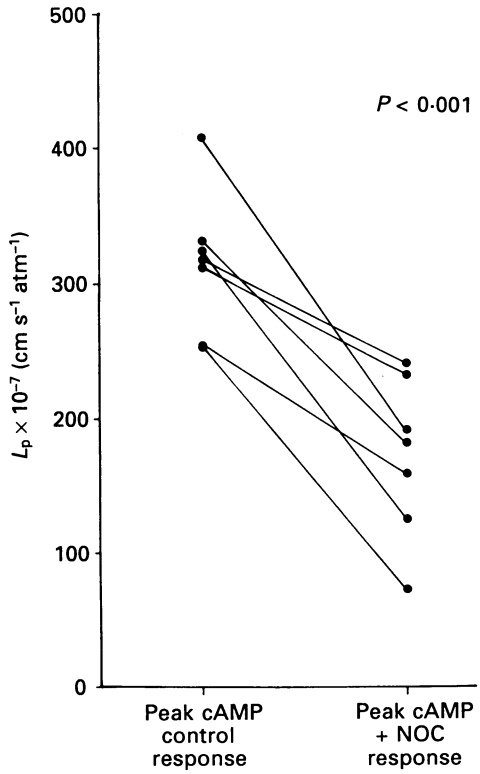

Fig. 6. Comparison of peak L_p response to a control dose of 8-CPT-cAMP ($1{\cdot}8 \times 10^{-5}$ M) and to a second dose in the presence of nocodazole (NOC, 4 μg ml^{-1}). Lines join data from seven individual collecting tubules.

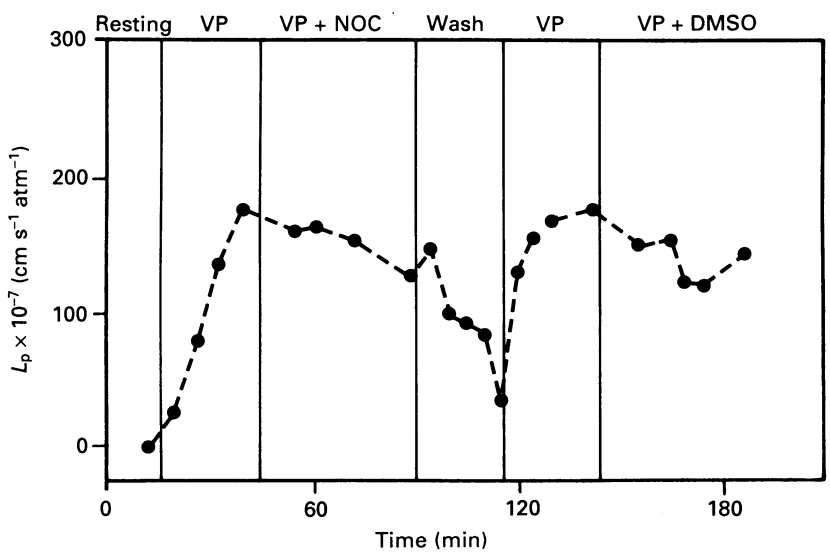

Fig. 7. Time course of a representative experiment showing the lack of effect of nocodazole (NOC, 4 μg ml^{-1}) on the L_p response of a single tubule to vasopressin (VP, 50 μU ml^{-1}) when added 30 min after hormone addition.

(4 μg ml^{-1}), 30 min after addition of vasopressin, the high level of water permeability was maintained; L_p fell only slightly over the next 50 min, averaging 144 (± 8) $\times 10^{-7}$ cm s^{-1} atm^{-1} ($n = 8$) over this period. (In our experience, this is the typical pattern of response seen on prolonged exposure to hormone; see also Frindt *et al.* 1982; Jones *et al.* 1988.) When the hormone and drug were washed out, L_p returned towards baseline. During the second (control) challenge, L_p rose to a peak

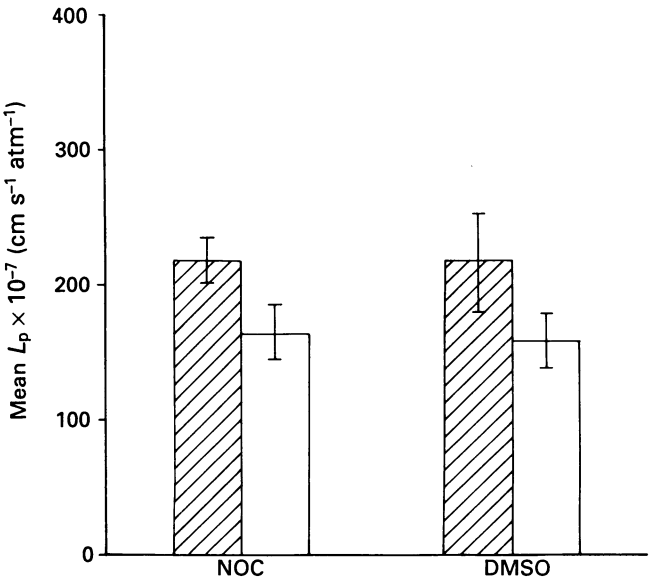

Fig. 8. Comparison of water permeability response to vasopressin (50 μU ml^{-1}) when collecting tubules were exposed to nocodazole (NOC, 4 μg ml^{-1} in DMSO), or DMSO alone, 30 min after hormonal challenge. The bars represent mean values ($\pm$S.E.M.) from five experiments; hatched bars represent peak L_p value attained during the first 30 min of hormonal stimulation and the open bars the average steady-state value during maintained stimulation after exposure to nocodazole or DMSO.

of 176 $\times 10^{-7}$ cm s^{-1} atm^{-1}, and this was again followed by a slight decline over the subsequent 50 min period of exposure to vasopressin; the mean L_p value during this period was 132 (± 6) $\times 10^{-7}$ cm s^{-1} atm^{-1} ($n = 6$). In five experiments, summarized in Fig. 8, there was no significant difference in the steady-state L_p response to maintained vasopressin stimulation in the presence and absence of nocodazole.

DISCUSSION

These studies demonstrate that pre-treatment with the microtubule-disruptive drug, nocodazole, results in inhibition of the water permeability response to vasopressin or exogenous cyclic AMP in isolated cortical collecting tubules of the rabbit. In contrast, nocodazole has no effect on the increase in transepithelial potential difference elicited by the hormone.

Nocodazole is a synthetic anticancer drug which interferes with the polymerization of tubulin *in vitro* (Hoebeke, Van Nijen & De Brabander, 1976) and rapidly disrupts

cytoplasmic microtubules in cultured cells (De Brabander, Van de Veire, Aerts, Borgers & Janssen, 1976). Nocodazole appears to interact with the colchicine binding site on tubulin (Hoebeke *et al.* 1976); however, its action is more rapid than that of colchicine, and its effects are more rapidly reversible (De Brabander *et al.* 1976).

Colchicine, the classical antimitotic agent, has been used extensively in studies on the role of microtubules in the vasopressin response in amphibian urinary bladder (reviewed in Pearl & Taylor, 1985). Correlative functional, biochemical and morphological evidence indicates that the inhibition of vasopressin-stimulated water flow by colchicine is due to its interaction with tubulin and the consequent disruption of cytoplasmic microtubules in the hormone-responsive granular epithelial cells (Taylor *et al.* 1978; Wilson & Taylor, 1978; Reaven, Maffly & Taylor, 1978). Because colchicine binds slowly to tubulin (Wilson & Taylor, 1978), a lengthy period of pre-incubation (2–4 h) is required in experiments with this drug. In a more recent study in the toad bladder, Brady *et al.* (1981) employed the faster-acting drug, nocodazole, and demonstrated that it markedly inhibits the onset of the vasopressin response after only 30 min pre-incubation; in their experiments, inhibition of vasopressin-stimulated water flow averaged some 68% at a nocodazole concentration of 10 μg ml^{-1}. Using immunofluorescence microscopy, this concentration of nocodazole has been shown to disrupt cytoplasmic microtubule networks in the vasopressin-sensitive granular epithelial cells of the toad bladder (Pearl & Taylor, 1987).

Nocodazole was chosen for the present study because of its known pharmacological properties; in particular, in view of its rapidity of action, this agent seemed more suitable than colchicine for studies on isolated tubules perfused *in vitro*. The use of a fast-acting drug allowed us to employ an experimental design in which each tubule served as its own control; thus we were able to compare the effects of hormonal stimulation in the absence and presence of nocodazole in a single-nephron preparation. In initial experiments we confirmed that cortical collecting tubules will respond to two successive challenges with vasopressin (separated by a wash-out period) with reproducible increases in hydraulic conductivity (cf. Lorenzen, Taylor & Windhager, 1983; Jones *et al.* 1988); further, we have shown that two consecutive doses of hormone elicit comparable changes in transepithelial PD.

The results demonstrate that nocodazole has no effect on basal (hormone-independent) water permeability, but inhibits the development of the hydrosmotic response to vasopressin in a dose-dependent manner. The effective inhibitory concentrations (in the range 1–4 μg ml^{-1}) are within the dose range reported to interfere with mitosis in cultured mammalian cells (De Brabander *et al.* 1976), and to disrupt cytoplasmic microtubules (Pearl & Taylor, 1987) and inhibit vasopressin-stimulated water flow (Brady *et al.* 1981) in the toad bladder.

The lack of effect of nocodazole on the hormone-induced increase in transepithelial PD (lumen negative) indicates that the inhibitory effect of the drug is specific for the water permeability response of the collecting tubule to vasopressin. The transient increase in lumen-negative PD caused by the hormone is paralleled by an increase in isotopic sodium flux (lumen to bath) and is generally attributed to stimulation of net sodium absorption by the tubule (Frindt & Burg, 1972; Holt & Lechene, 1981). The present results are thus similar to those previously reported for the toad bladder; in that tissue, colchicine and other microtubule-disruptive agents had no effect on

either basal or vasopressin-stimulated transepithelial PD or net sodium transport at concentrations that inhibited the hydrosmotic response to the hormone (Taylor *et al.* 1978).

Since nocodazole does not interfere with the normal stimulation of transepithelial PD, inhibition of the hydrosmotic response to vasopressin cannot be attributed to interference with hormone–receptor interaction or with cyclic AMP generation, or to a reduction in energy metabolism. Moreover the lack of an effect of nocodazole on the maintenance phase of the vasopressin response implies that its inhibitory effect on the initiation phase is not due to a drug-induced decrease in the transepithelial osmotic gradient. The results appear consistent with the view that the inhibitory effect of nocodazole is a consequence of its interaction with tubulin and the disruption of cytoplasmic microtubules. This view receives support from our recent studies with colcemid, a fast-acting colchicine derivative; colcemid (but not its isomer, lumicolcemid, which does not interact with tubulin or disrupt microtubules) also specifically inhibits the hormone-induced water permeability increase in rabbit cortical collecting tubules (Phillips & Taylor, 1987*b*).

Since nocodazole inhibits the water permeability response to 8-CPT-cAMP, as well as that to vasopressin, the effect of the drug must be exerted, at least in part, at a cellular site beyond the generation of cyclic AMP. However, at a nocodazole concentration of 4 μg ml^{-1}, the response to the nucleotide analogue ($1{\cdot}8 \times 10^{-5}$ M) was decreased by a mean of $45 \pm 7\%$ ($n = 7$), while that to vasopressin (50 μU ml^{-1}) was reduced by $65 \pm 6\%$ ($n = 8$). The difference between these two values is just statistically significant (unpaired difference = 20%, $t = 2{\cdot}169$, $P < 0{\cdot}05$). These results raise the possibility that nocodazole acts at a pre-cyclic AMP, as well as post-cyclic AMP, site. However, it should be noted that the peak L_p value achieved in the control period in the experiments with 8-CPT-cAMP averaged $315\ (\pm 20) \times 10^{-7}$ cm s^{-1} atm^{-1}, whereas in the series with vasopressin mean peak L_p in the control period was $467\ (\pm 66) \times 10^{-7}$ cm s^{-1} atm^{-1} (unpaired difference = 152×10^{-7} cm s^{-1} atm^{-1}, $t = 2{\cdot}203$, $P < 0{\cdot}05$). Thus, inasmuch as the dose of exogenous nucleotide employed may have been submaximal whereas the dose of vasopressin was presumed to be maximal (Grantham & Burg, 1966), the conditions in the two experimental series may not have been strictly comparable. The relatively greater effect of nocodazole observed in the experiments with vasopressin might be related to stimulation of prostaglandin synthesis which, in turn, could lead to interference with cyclic AMP generation. Burch & Halushka (1982) have concluded that colchicine may interfere with the hydrosmotic response to vasopressin at both pre- and post-cyclic AMP steps in the toad bladder. In their studies, the effect of colchicine on the response to a submaximal dose of hormone was partially blocked by inhibitors of prostaglandin synthesis and therefore appeared to be mediated by stimulation of prostaglandin production (the mechanism whereby this alkaloid may stimulate prostaglandin synthesis is unknown (Burch & Halushka, 1982) and may or may not be related to its tubulin-binding capacity). However, as noted above, the lack of an effect of nocodazole on the hormone-induced increase in lumen-negative PD would argue against the possibility that the drug interferes with the production of cyclic AMP in the collecting tubule.

The inhibition of the hydrosmotic response to both vasopressin and cyclic AMP

following pre-treatment with nocodazole is consistent with the concept that cytoplasmic microtubules are involved in the development of the hormone-dependent increase in water permeability in the principal cells of the cortical collecting tubule at a step beyond cyclic AMP. This interpretation is similar to that reached on the basis of studies of the effects of a variety of antimitotic agents on the hydrosmotic response to vasopressin or exogenous cyclic nucleotides in amphibian bladder (see Pearl & Taylor, 1985).

In the toad bladder, Kachadorian *et al.* (1979) first noted that colchicine had no effect on either transepithelial water flow or the appearance of particle aggregates in the apical cell membrane when added after the hormone-induced increase in water permeability had been established (in spite of a concomitant reduction in total granular cell microtubule content). Parisi, Pisam, Merot, Chevalier & Bourguet (1985) confirmed these observations in experiments on frog urinary bladder. Brady *et al.* (1982) have obtained similar results in functional studies on the toad bladder using nocodazole. These findings have been interpreted to indicate that microtubules play a role in the onset of the water permeability response to vasopressin but not in its maintenance. In the present study on cortical collecting tubules we have likewise shown that the maintenance of increased water permeability in the presence of vasopressin is not influenced by nocodazole. The results suggest that in the mammalian collecting tubule, as in amphibian bladder, microtubules are involved only in the events leading up to the increase in apical membrane water permeability.

Our results are consistent with the possibility that cytoplasmic microtubules play a role in the processes whereby the particle clusters (assumed to represent or contain water channels) appear in the apical plasma membrane of the principal cells of the collecting tubule under the influence of vasopressin. As yet, there is no clear-cut evidence regarding the origin of the particle clusters that are seen in groups in the apical membrane after vasopressin stimulation. By analogy with events known to occur in amphibian bladder (Muller *et al.* 1980), it seems likely that these structures exist preformed in cytoplasmic vesicles and are incorporated into the apical membrane as a result of an exocytotic fusion event. Tubulovesicles containing intramembranous particles have been visualized in the apical cytoplasm of the principal cells, and such vesicles have been observed fused to the apical plasma membrane following vasopressin stimulation (B. A. Kubat, personal communication). It is now generally accepted that cytoplasmic microtubules play an integral role in the directed movement of cellular organelles (Vale, Scholey & Sheetz, 1986). Thus, on the basis of the available evidence, we suggest that microtubules in the principal cells are involved in the translocation of particle-containing tubulovesicles towards the apical cell surface prior to their incorporation into the apical plasma membrane. According to this view, microtubules would participate in the delivery of the putative water channels to the membrane at the luminal surface of the collecting tubule cells during the initiation of the vasopressin response.

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