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The role of microtubules and microfilaments in the hydrosmotic response to antidiuretic hormone

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To test the effects of colchicine and cytochalasin B on the ADH-induced response, unidirectional and net water fluxes were measured at one or two minutes intervals in frog urinary bladder. The action of these agents on the appearance of intramembrane particles aggregates in the luminal membrane of target cells under oxytocin stimulation and the changes in the tissue ultrastructure induced by cytochalasin B were also studied. It was observed that: (1) the time-course of the response to oxytocin was strongly slowed by colchicine while the washout was not affected; (2) the time-course of the 'on and off' of the response to oxytocin was not modified by cytochalasin B; (3) cytochalasin B pretreatment proportionally reduced unidirectional and net water fluxes measured after glutaraldehyde fixation; (4) the combined action of colchicine and cytochalasin B proportionally reduced the net water flux and the number of intramembrane particles aggregates, observed in freeze-fracture studies; (5) after cytochalasin B action the dilation of intercellular spaces classically observed under oxytocin stimulation is strongly reduced. It is concluded that: (1) microtubules probably play an important role in the water channels plug-in, but not in their removal; (2) microfilaments integrity is necessary for the mechanisms inducing intercellular space dilation and (3) the observed results confirm that water permeability is controlled by the number of permeation units present in the luminal border of granular cells and probably represented by the intramembrane particle aggregates.

Introduction

After the initial report showing that drugs interacting with microtubules and microfilaments inhibit the action of antidiuretic hormone (ADH) and cyclic AMP on osmotic water movement across the toad bladder [1] work has been directed to inscribe these observations into the frame of current views on the mechanism regulating water permeability in ADH sensitive cells. Progress came from freeze-fracture studies showing that ADH induces the appearance of intramembrane particle aggregates in the apical membrane of target cells [2,3]. These aggregates, that probably contain water channels, would be transferred from cytoplasmic vesicles to luminal membranes under hormonal stimulation [4,5]. Microtubules and microfilaments would play a crucial role in this 'membrane traffic' process [6–8] and an elaborated hypothesis has been proposed to explain their mechanism of action [4,9].

We have now re-examined the effects of colchicine and cytochalasin B in frog urinary bladder. Water net and unidirectional fluxes were recorded at one or two minute intervals. To test the 'non osmotic' routes, sucrose permeability was also simultaneously measured, in some experiments. These observations were correlated, in a quantitative morphofunctional study, with the structural changes induced by colchicine and cytochalasin B. They are compatible with previous observations coming from ultrastructural and biophysical stud-

ies [10,11] indicating that water permeability is controlled by water channels present in the apical membrane of bladder epithelial cells and probably induced in the intramembrane particle aggregates. The obtained results give also additional information on the effects of agents interacting with microtubules and microfilaments on the mechanism controlling the insertion and removal of the ADH-induced water channels in the apical membrane.

Methods

Frogs (*Rana esculenta*) originating from Central Europe were kept at 20°C in running tap water for at least 5 days before the experiments. The urinary bladders were removed from pithed frogs and mounted as flat sheets, horizontally between two twin lucite chambers, with the serosal border facing the upper solution. The employed buffer contained (mM): NaCl 112; KCl 5; $CaCl_2$ 1; $NaHCO_3$ 2.5 (pH 8.1 when bubbled with air). An osmotic gradient was imposed in some experiments by reducing the NaCl concentration in the mucosal solution to 5.2 mM. To increase water permeability, urinary bladders were stimulated with a maximal concentration of oxytocin ($2.2 \cdot 10^{-8}$ M) (Syntocinon, Sandoz Pharmaceuticals). The term ADH is used throughout the text as a generic expression covering the hydrosmotic action of oxytocin and other neurohypophyseal peptides.

Net water fluxes. The net water fluxes were measured with a modification of a previously described technique [12]: water was automatically injected into the lower chamber to maintain a constant volume and the magnitude of this fluid movement, equivalent to the net flux, was recorded every minute.

Unidirectional fluxes. Unidirectional water fluxes were measured as previously described [13]. In these experiments the volume of the lower chamber was 12 ml, and the volume of the upper one was 2 ml. Both solutions were vigorously stirred with a magnetic bar (lower chamber) and a teflon helix (upper chamber, see Fig. 1 in Ref. 14). ^{3}HOH was added to the lower bath up to a final concentration of 10 $\mu Ci \cdot ml^{-1}$. The solution in the upper chamber was then completely removed every two minutes and refilled with unlabelled solution. The ^{3}HOH activity of the sample was determined and the unidirectional flux expressed in $\mu l \cdot h^{-1} \cdot cm^{-2}$ (The exposed area was 3.14 cm^2). The specific activity in the inferior chamber was recalculated for each period, taking into account the previous transfer of radioactivity. [^{14}C]Butanol and ^{3}HOH unidirectional fluxes were simultaneously determined in some experiments, to test the unstirred layers importance. As previously reported [11], the unstirred layers thickness (280 μm in our experimental conditions) was mainly determined by the chamber geometry and stirring rate. Unidirectional ^{3}HOH fluxes were subsequently corrected by applying the equation.

$$1/P_{obs} = 1/P_m + 1/P_{unst}$$

where P_{obs} is the observed water permeability, P_{unst} the unstirred layers permeability and P_m the corrected water membrane permeability [15]. To test the existence on 'non-specific' pathways, [^{14}C]sucrose unidirectional permeability was simultaneously determined with unidirectional ^{3}HOH movements in most experiments.

Ultrastructural studies. As soon as the appropriated permeability level had been reached preparations were fixed for 20 min in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate buffer at pH 7.2. Samples for electron microscopy studies were postfixed for 2 h at room temperature in a mixture of osmium tetroxide and potassium ferrocyanide according to the method of Karnovsky [16]. The total surface area between the basal and apical membrane of the bladder cells (see Fig. 7) and the fraction of this area occupied by intracellular vacuoles or intercellular spaces were measured using an image analysing computer on pictures at a final magnification of 3500 ×. Seven different experiments were performed in each tested condition. On the average, 300 different cells, taken at random, were analyzed in each case.

Samples for freeze-fracture were cryoprotected, after fixation, in glycerol buffer solution 30% (v/v) for 40 min and subsequently frozen in Freon 22 chilled by liquid nitrogen. They were then processed as usual [17]. The density and total cell surface area occupied by intramembrane particle aggregates were calculated with the image analysing computer at a final magnification of 45 000 ×. At least four different experiments were performed

in each condition. On the average, 11 different cells and an apical surface of 490 ± 40 μm^2, taken at random, were analyzed in each case.

Colchicine and cytochalasin B were purchased from Sigma. In studies involving cytochalasin B, dimethyl sulfoxide (DMSO) was used as a vehicle and it was both present in control and experimental preparations at a final concentration of 0.2%.

Results

Colchicine effects on the oxytocin-induced hydrosmotic response

Fig. 1 shows the effects of preincubation with colchicine (0.1 mM, serosal side) on the oxytocin hydrosmotic response (mean curves for 12 experiments). Two fragments of the same bladder were simultaneously tested. After a first stimulation and washout colchine was added (and maintained through the experiment) on the experimental fragment. The basal net water flux did not change during six hours while the time-course of the onset of the response to oxytocin was strongly slowed (Table I). At the same time the half-time of the offset of the hydrosmotic response was not af-

TABLE I

EFFECTS OF PREINCUBATION WITH COLCHICINE AND CYTOCHALASIN B ON THE OCYTOCIN-INDUCED HYDROSMOTIC RESPONSE

Condition	*n*	Net water flux *	Half-time of the response (min)	
			onset	washout
Oxytocin	12	100	7.0 ± 0.5 (7.1 ± 0.4)	4.9 ± 0.3 (4.8 ± 0.4)
+ colchicine 10^{-4} M, 3 H	12	78 ± 6 **	10.0 ± 1.0 ** (6.1 ± 0.4)	5.0 ± 0.3 (5.1 ± 0.4)
+ colchicine 10^{-4} M, 6 H	12	61 ± 8 **	16.1 ± 2.3 ** (6.2 ± 0.5)	5.0 ± 0.3 (5.0 ± 0.3)
+ cytochalasin B 10^{-5} M, 3 H	8	32 ± 15 **	7.6 ± 0.6 (6.1 ± 0.4)	5.1 ± 0.4 (4.9 ± 0.2)

* Means ± S.E. The net water fluxes are expressed as % of the net water flux observed in the control fragment from the same tissue that was tested under similar time-course evolution (fig. 1).

** Differences with the corresponding control value were statistically significant ($p < 0.01$). Numbers in parenthesis are the mean half-times values in the control fragment.

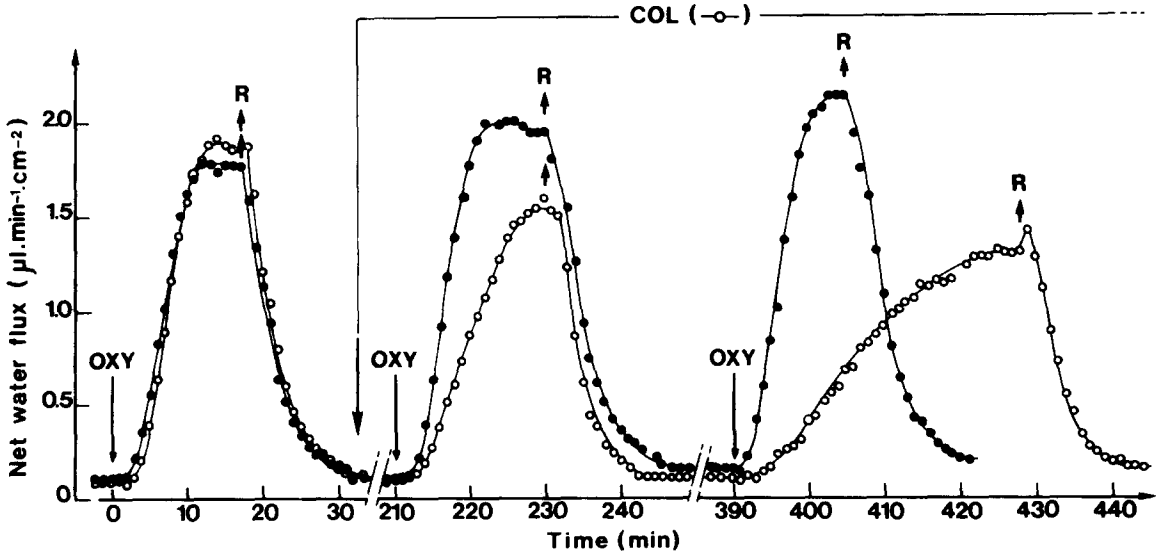

Fig. 1. Effect of preincubation with colchicine (COL, 10^{-4} M serosal side) on the hydrosmotic response (R) to oxytocin (OXY). Two fragments of the same bladder (black and open dots) were tested in each experiment. After an initial stimulus and wash out colchicine was added in the experimental channel (open dots). Two other oxytocin challenges were performed on both fragments, 3 and 6 h after colchicine addition to the experimental one. (Mean curves for 12 experiments. See Table I for statistical analysis of differences in maximum values and half-times).

fected by colchicine action. As a consequence of this a strong assymetry in the on-off of the response was observed. Colchicine effect developed quite slowly, as can be observed when effects after three and six hours of incubation are compared. After six hours the response was reduced to 61% of the control (Table I). It must be stressed here that if water fluxes had been measured with the 'sac weighing technique' during 30 min after oxytocin addition, as it was the case in previous studies, the effect of colchicine on the magnitude of the response would be exagerated: after six hours of colchicine incubation, the net water flux would be considered as 'inhibited' to $44 \pm 6\%$ of the control value, because the observed loss of weight represents the mean permeability value within the 30 min period.

The increase in water permeability induced by oxytocin was not modified when colchicine was added at the maximum of the hormonal action (Fig. 2). The net water flux was followed during 180 min after the alkaloid addition to the serosal bath.

Cytochalasin B effects on the oxytocin-induced hydrosmotic response

Preincubation with cytochalasin B (10^{-5} M, 3 h) significantly increased the basal net water flux (Fig. 3) and the subsequent hydrosmotic response

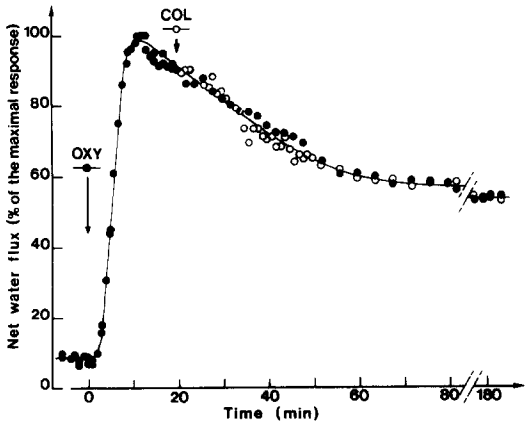

Fig. 2. Effect of colchinine addition (COL, 10^{-4} M serosal side) at the top of the oxytocin (OXY) response. Two fragments of the same bladder were tested in each experiment. Colchicine was added (arrow) on the experimental channel (open dots). (Mean curve for six experiments).

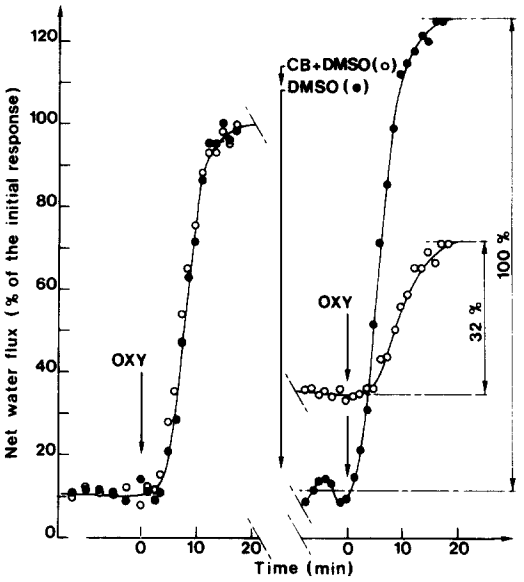

Fig. 3. Effect of preincubation with cytochalasin B (CB, 10^{-5} M serosal side) on the hydrosmotic response to oxytocin. Two fragments of the same bladder (black and open dots) were tested in each experiment. After an initial stimulus cytochalasin B was added in the experimental channel (open dots). The employed solvent, dimethylsulfoxide (DMSO) was present at the same concentration on control and experimental sides. Three hours later both fragments were tested again. (Mean curves for eight experiments. See Table I for statistical analysis of differences in maximum values and half-times).

to oxytocin was strongly depressed (Fig. 3, mean curves for six experiments, and Table I). Nevertheless, the time-course of the remaining response was not significantly affected (Table I). When cytochalasin B was added at the maximum of the response an inhibitory effect began to develop immediately (Fig. 4). A small 'solvent (DMSO) effect' was observed in both control and experimental fragments (initial transient increase in the net water flux). Twenty minutes after cytochalasin B addition the net water flux was reduced to 65% of the control.

Comparison of osmotic and diffusional permeabilities in cytochalasin B treated frog urinary bladders

To compare the effects of cytochalasin B on osmotic and unidirectional permeabilities both parameters were measured in the same preparations, after glutaraldehyde fixation, in the presence and in the absence of an osmotic gradient. It is known that the fixation procedure 'freezes' the

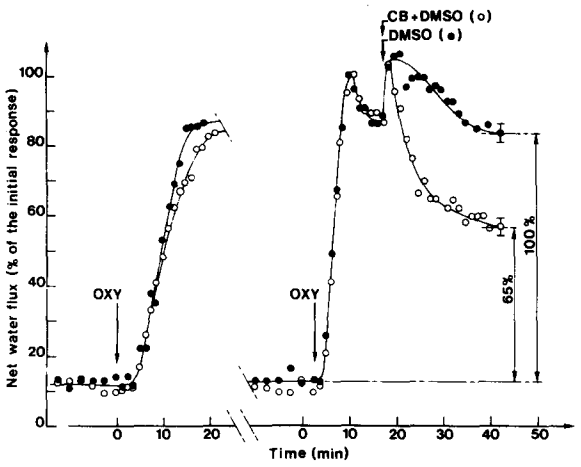

Fig. 4. Effect of cytochalasin B addition (CB, 10^{-5} M serosal side) at the top of the response to oxytocin. Two fragments of the same bladder were tested in each experiment. After an initial stimulation and washout, cytochalasin B was added (arrow) on the experimental channel (open dots).

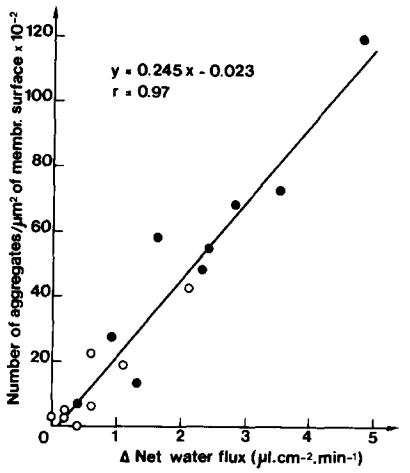

Fig. 5. Observed correlation between the net water flux induced by oxytocin and the number of aggregates in the luminal membrane of granular cells. Two fragments of the same bladder were tested in each experiment. Black dots: control; open dots: fragments pretreated with 10^{-4} M colchicine and 12.5 μg/ml cytochalasin B, during 2 h.

structure in the 'open' condition (after ADH action) or in the 'closed' condition (in the absence of the hormonal stimulus) [18,19]. The following protocol was employed: two fragments of the same bladder were mounted in the absence of an osmotic gradient and the experimental side exposed to cytochalasin B ($5 \cdot 10^{-5}$ M) during 30 min. Both fragments were then stimulated with oxytocin and 20 min later fixed with 2% glutaraldehyde. Water and sucrose unidirectional movements were then tested during ten consecutive 2-min periods. Subsequently, the net water flux was first determined in the presence and then in the absence of an osmotic gradient. In this last situation the (non osmotic) net water flux was drived by the hydrostatic pressure applied by the measurement device (10 cm of water on the mucosal side). Table II summarizes the obtained results: in five paired experiments, after correction for unstirred layers and non specific pathways, the osmotic and diffusional permeabilities were similarly reduced by 30 min preincubation with cytochalasin B. The ratio between the increase in the osmotic and diffusional permeability coefficients induced by ADH

TABLE II

EFFECT OF CYTOCHALASIN B ON DIFFUSIONAL (P_d) AND OSMOTIC (P_f) WATER PERMEABILITIES (10^{-4} cm·s^{-1}) MEASURED IN THE SAME BLADDER AFTER GLUTARALDEHYDE FIXATION

P_d values were corrected for unstirred layers and non-specific pathways. P_f values were corrected for the 'non osmotic pathways' estimated in the absence of an osmotic gradient. oxy, oxytocin; oxy + cyto, ocytocin after 10^{-4} M cytochalasin.

Expt. No.	P_d		Diff. (%)	P_f		Diff. (%)
	oxy	oxy + cyto		oxy	oxy + cyto	
1	7.91	6.96	−12	66	60	−9
2	7.73	6.09	−21	74	50	−32
3	14.22	10.42	−27	104	81	−22
4	9.09	7.97	−12	45	39	−13
5	11.22	7.73	−13	89	64	−28
Means ± S.E.	10.03		−17.0 ±2.7	75.6		−20.8 ±3.9

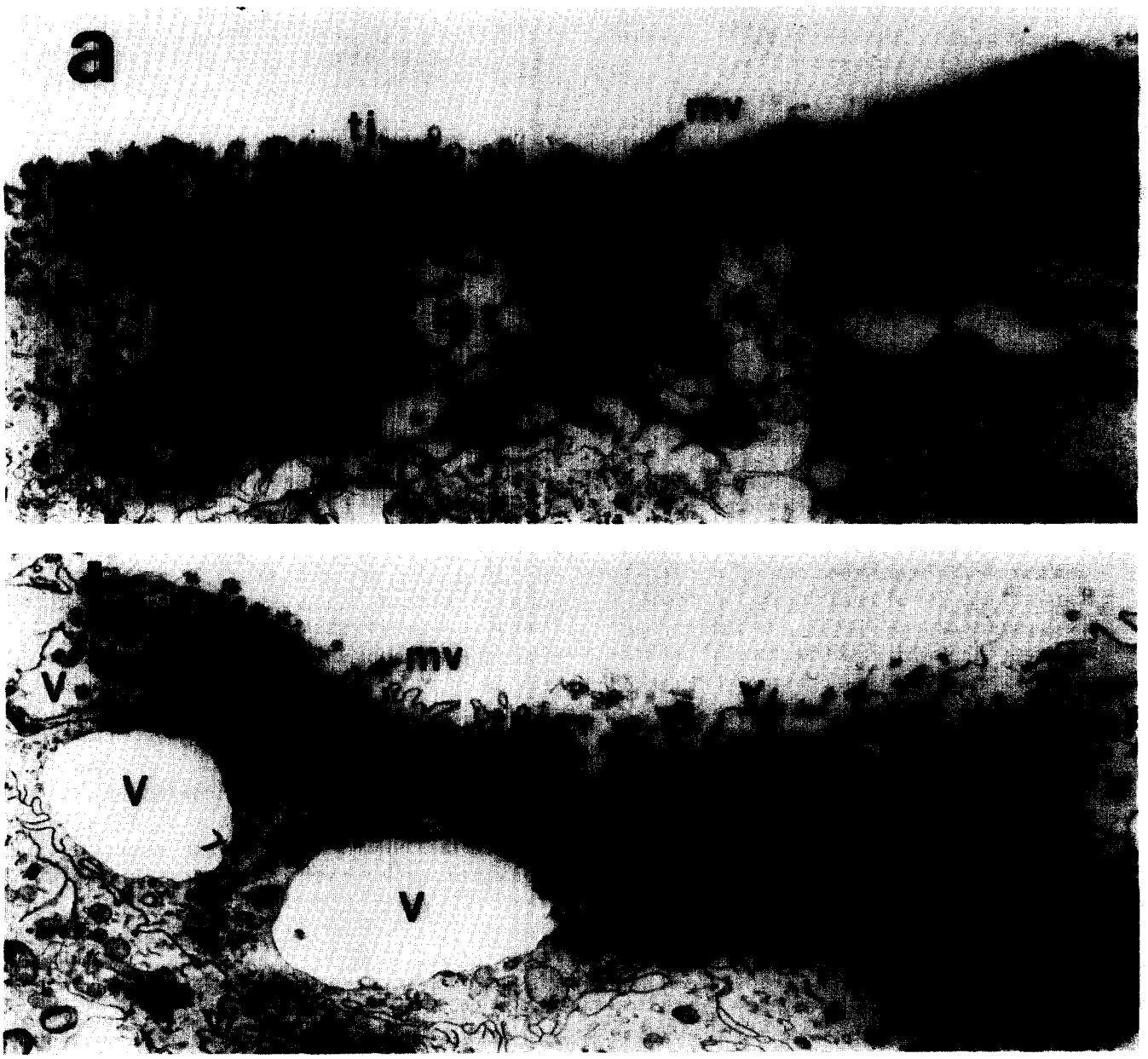

Fig. 6. Electron micrographs of epithelium from frog urinary bladder exposed to an osmotic gradient and stimulated by oxytocin (×7000). (a) Control bladder. The intercellular spaces (IS) are markedly dilated, however the tight junctions (tj) remain closed. The epithelial cells (EC) do not show vacuoles in their cytoplasm. mv, microvilli. (b) Cytochalasin B was added to serosal bath 40 min prior to addition of oxytocin. The epithelial cells (EC) present large vacuoles (V) in their cytoplasm, whereas their intercellular spaces (IS) are closed. Some microvilli (mv) are dilated (white arrows).

($\Delta P_f/\Delta P_d$) were similar in the presence or absence of preincubation with cytochalasin B. The observed value ($\Delta P_f/\Delta P_d \cong 10$) was also similar to the one observed in non fixed bladders [11,20].

Morphofunctional correlations

Pretreatment with colchicine or cytochalasin B proportionally reduced, in toad urinary bladder, the intramembrane particle aggregates density, and

the water hydrosmotic permeability [9]. To obtain a maximum effect we have now combined the action of both poisons of cytoskeleton. After 2 h preincubation with 10^{-4} M colchicine and 10^{-5} M cytochalasin B, one bladder fragment was exposed to oxytocin and the number of aggregates and the net water flux compared to the same parameters measured in a control fragment only exposed to the hormone. Fig. 5 shows the observed correlation between the aggregates density in the luminal membrane of granular cells and the increase in net water flux induced by oxytocin stimulation. It can be observed that pretreatment with cytochalasin B and colchine did not distort the excellent correlation observed between the aggregate density and the net water flux. Table III gives the mean values for net water fluxes, aggregate density and the membrane surface area occupied by the aggregates. Furthermore, the relative frequency and the relative surface area occupied by different size categories of aggregates (not shown) was not modified by the combined action of colchicine and cytochalasin B.

We confirm here the previously reported effects of cytochalasin B on cellular ultrastructure, as observed by electron microscopy: (1) disorganisation of the subcellular structure, specially microfilament orientation [6]; (2) the appearance, under ADH stimulation and in the presence of an osmotic gradient, of an important cytoplasmic vacuolization [21,22]. Nevertheless we report a new observation that we believe of importance when interpreting cytochalasin B effects (Fig. 6): preincubation with this agent prevents the important dilation of intercellular spaces always observed when ADH is added in the presence of an osmotic gradient [23]. These observations were quantified and the obtained results are shown in Table IV. It can be remarked that, in rough, the reduction in the surface occupied by dilated intercellular spaces was compensated by the increase in the intracellular vacuoles surfaces.

TABLE III

EFFECTS OF THE COMBINED ACTION OF COLCHICINE AND CYTOCHALASIN B ON WATER FLUXES, AND ADH-INDUCED AGGREGATE DENSITY AND SURFACE

Means ± S.E. Net water fluxes in $\mu l \cdot cm^{-2} \cdot min^{-1}$; aggregates density in number per μm^2; aggregates surface in percentage of the total surface apical area.

	Net water flux	Aggregates density	Aggregates surface
Oxytocin ($n = 6$)	2.5 ± 0.5	0.62 ± 0.14	0.99 ± 0.24
Oxytocin + colchicine + cytochalasin ($n = 9$)	0.7 ± 0.3	0.14 ± 0.05	0.35 ± 0.13

TABLE IV

MORPHOMETRIC VARIATIONS INDUCED BY CYTOCHALASIN B AND OXYTOCIN

Means ± S.E. for seven paired experiments. Net water fluxes in $\mu l \cdot cm^{-2} \cdot min^{-1}$; intercellular spaces and vacuoles as percentage of the surface area between the basal and luminal membrane of epithelial cells.

	Net water flux	Intercellular spaces	vacuoles
Oxytocin	1.60 ± 0.22	10.2 ± 2.2	2.24 ± 0.67
Oxytocin + cytochalasin	0.85 ± 0.14	1.4 ± 0.4	13.40 ± 2.50
Differences	0.75 ± 0.27	8.8 ± 0.4	11.50 ± 2.40

Discussion

This work contribution is centered in two points. (1) The time-course study of changes induced by colchicine and cytochalasin B in net and unidirectional water fluxes. Previous work was performed employing the 'sac technique' that gives an average of water flux during relatively long periods so that significant information was lost. Furthermore correction for unstirred layers effects on unidirectional fluxes and estimation of 'non-specific leaks' give a more solid base to our permeability studies. (2) The quantification and morpho-functional correlation of colchicine and cytochalasin B effects on epithelial structure.

On the role of microtubules in the hydrosmotic response

Previous work has shown that the action of colchicine on the ADH-induced hydrosmotic response is probably due to the drug interaction with tubulin [24] that would be the molecular

support of the microtubules present in the tissue [25]. Furthermore this interaction reduces the number of intramembrane particle aggregates transferred to the apical border in toad urinary bladder [9]. As a consequence, water permeability is proportionally reduced. The minute by minute study of water fluxes gave us two new informations on the colchicine action in frog urinary bladder. (1) The time-course of the onset of the response to the hormone was strongly slowed and, as previously reported [1,9], partially inhibited. The magnitude of this inhibition was probably exaggerated in previous studies in which the 'sac technique' was employed. (2) The offset of the response to oxytocin was not affected by colchicine action.

The time-course of the onset and offset of the response to ADH and cAMP are similar [14,26] and also similarly temperature dependent [17,27]. At 20°C and 10°C the onset and reversal of water permeability variation remain rather symmetrical, even when both processes were strongly slowed down [17]. The strong assymmetry observed after colchicine treatment indicates that, at least as regarding microtubules participation, the plug-in and removal of water channels are independent processes. This observation can be associated with previous ones in toad urinary bladder, showing that while microtubules are necessary for the aggregates insertion, they seem not to be required for their maintenance in the apical membrane [9].

Morphofunctional studies have previously shown that the 'rate-limiting step' of the hydrosmotic response is probably situated after cAMP accumulation [26] and that aggregate appearance parallels the increase in water permeability at different temperatures [17]. The here presented observations seem to indicate that this rate limiting step is related to the microtubules function in the hydrosmotic response.

On the role of microfilaments in the hydrosmotic response

Information on the role of microfilaments in the ADH-induced response comes from structural [6,9,21,28], biochemical [7,29] and permeability studies [19,21,22]. Most of the obtained information can be interpreted as the result of a reduction in the number of water channels induced by ADH in the luminal membrane of target cells and we are now adding new information showing that even after the combined action of colchicine and cytochalasin B, the observed correlation between the density of intramembrane particle aggregates and water flux remains significantly high.

Experiments studying the effects of cytochalasin B indicate that interaction with this drug would result in the inhibition of net water fluxes while not affecting unidirectional water permeability [21,22]. To have a good estimation of the unidirectional water transfer moving through the ADH-induced channels two possible sources of error must be taken into account: (1) the influence of unstirred layers in series with the tested barrier; (2) the eventual development of a 'nonspecific' pathway. We have now estimated the unstirred layers, in our experimental conditions, employing butanol permeability studies (see Refs. 20 and 11 for an exhaustive study of the unstirred layer corrections in amphibian urinary bladder). We have also measured the development of 'non specific pathways' in two different ways: from sucrose permeability and measuring water net flux in the absence of an osmotic gradient. In experiments designed to compare P_d values obtained in 'non gradient' situations with P_f values obtained in 'osmotic gradient' situations an additional problem arises from the modifications in water permeability that can be induced by the osmotic gradient per se [30]. The use of glutaraldehyde fixation prevents any change in the tissue permeability when switching from the 'non osmotic gradient' to the 'imposed osmotic gradient' conditions. In these conditions and when all these corrections were taken into account, we observed that pretreatment with cytochalasin B proportionally reduced the diffusional and osmotic permeabilities. This inhibition was compatible with the reduction in the surface occupied by the intramembrane particle aggregates.

DiBona [31] has recently reviewed and discussed the major structural changes accompanying ADH-induced osmotic flow across toad urinary bladder. Besides the classical swelling of granular cells and distension of intercellular spaces [23] the author suggests that the change in shape of granular cells could be due to a subcellularly localized relaxation of the microfilaments lattice, permitting the assumption of a specialized configuration once

the intracellular volume was increased. This situation would generate a 'preferential route' for water flow and it would be at the origin of the opening of intercellular spaces (Fig. 6a and Fig. 7a). Our results show that after cytochalasin B intercellular spaces are poorly dilated, even in the presence of a significant water flux (Table IV). At the same time the surface occupied by intracellular vacuoles 'compensated' the absence of intercellular distension. These results can be interpretated, as previously proposed [22,31,32] as indicating that cytochalasin B drastically modified the route for water transfer across granular cells. Because the resistance of the basal regions of the cell is reduced, dilation of intracellular spaces is no longer induced (Fig. 6b and Fig. 7b). Nevertheless, this situation does not necessarily imply that water flow through the tissue is more difficult after cytochalasin B. Our results show that, in our experimental conditions, the reduction in either the unidirectional and net water fluxes can be full explained by the reduction in the surface occupied by the intramembrane particle aggregates.

In summary, it seems clear that ADH controls water permeability by modifying the number of channels present in the mucosal border [11,13]. Cytochalasin B-sensitive microfilaments seem to play a role in this regulatory control, as suggested by permeability and freeze-fracture studies. Furthermore, our quantitative morphofunctional studies give additional support to a previously proposed hypothesis [22,31,32]. Cytoplasmic substructure probably 'deviates' the osmotic flux through a particular route. If this mechanism is directly or indirectly controlled by ADH, is a matter of conjecture.

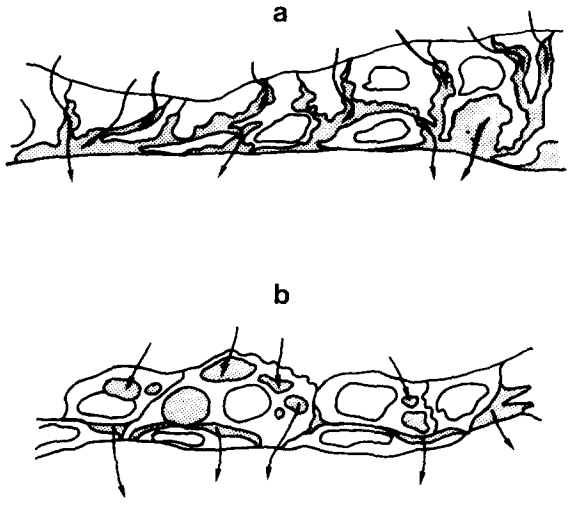

Fig. 7. Schematic views (a from pictures obtained empirically, see Fig. 6) of modifications induced by cytochalasin B on the structural changes accompanying the ocytocin-induced osmotic flow across frog urinary bladder.

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