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Evidence for Permanent Water Channels in the Basolateral Membrane of an ADH-Sensitive Epithelium

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Summary. The transepithelial water permeability in frog urinary bladder is believed to be essentially dependent on the ADH-regulated apical water permeability. To get a better understanding of the transmural water movement, the diffusional water permeability (P_d) of the basolateral membrane of urinary bladder was studied. Access to this post-luminal barrier was made possible by "perforating" the apical membrane with amphotericin B. The addition of this antibiotic increased P_d from $1.12 \pm 0.10 \times 10^{-4}$ cm/sec ($n = 7$) to $4.08 \pm 0.33 \times 10^{-4}$ cm/sec ($n = 7$). The effect of mercuric sulfhydryl reagents, which are commonly used to characterize water channels, was tested on amphotericin B-treated bladders. $HgCl_2$ (10^{-3} M) decreased P_d by 52% and *para*-chloromercuribenzoic acid (*p*CMB) (1.4×10^{-4} M) by 34%. The activation energy for the diffusional water transport was found to increase from 4.52 ± 0.23 kcal/mol ($n = 3$), in the control situation, to 9.99 ± 0.91 kcal/mol ($n = 4$) in the presence of 1.4×10^{-4} M *p*CMB. Our second approach was to measure the kinetics of water efflux, by stop-flow light scattering, on isolated epithelial cells from urinary bladders. *p*CMB (0.5 or 1.4×10^{-4} M) was found to inhibit water exit by $91 \pm 2\%$. These data strongly support the existence of proteins responsible for water transport across the basolateral membrane, which are permanently present.

Key Words frog urinary bladder · water permeability · *p*CMB · water channels · basolateral membrane · amphotericin B

Introduction

Amphibian urinary bladder has been widely used as a model for the kidney collecting duct to study the mechanism of action of the antidiuretic hormone (ADH). It was shown that ADH induces an increase of the apical membrane water permeability (*see* for review Handler & Orloff, 1973), accompanied by the appearance of linearly arranged intramembrane particles in the apical membrane of granular cells (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & Di Scala, 1975; Bourguet, Chevalier & Hugon, 1976; Wade, 1978; Muller, Kachadorian & Di Scala, 1980) which seem closely related to the ADH-induced water pathway (Kachadorian et al., 1981; Brown, Grosso & De Souza, 1983). Whereas the permeability of the apical membrane has been greatly studied, the resistance offered by the basolateral membrane during the transepithelial water movement has scarcely been investigated. The only experimental evidence for the existence of a barrier distal to the luminal membrane under ADH stimulation was reported by Kachadorian, Casey & Di Scala, 1978; Kachadorian, Sariban-Sohraby & Spring, 1985; Kachadorian, Coleman & Wade, 1987). They observed, on toad urinary bladder, that the maximal hydrosmotic response to ADH was followed by a decrease in the water flow (Kachadorian et al., 1978) without any change in the surface area covered by intramembranous aggregates. They therefore proposed the existence of a post luminal barrier and later suggested (Kachadorian et al., 1985, 1987) that the water movement through this barrier was regulated by ADH or prostaglandins. But whether the large amount of water, that penetrates the cell through the apical membrane under ADH stimulation, crosses the basolateral membrane by diffusion through the lipid bilayer or by a facilitated pathway is yet unknown.

The present work reports evidence for permanent water channels in the basolateral membrane of the frog urinary bladder. The protocol we proposed to measure the diffusional water permeability (P_d) of the basolateral membrane consists in "perforating" the apical membrane with the polyene antibiotic amphotericin B. With this experimental device, the biophysical properties of basolateral water transport could be studied. The obtained results showed an increase in transepithelial diffusional water permeability upon antibiotic incorporation thus implying a high basolateral P_d at rest. The activation energy for diffusional transport was low and increased in the presence of mercurial agents. In a second series of experiments, the osmotic water permeability of isolated epithelial cells from urinary bladders, at rest, was measured

by stop-flow light scattering. Kinetics of water efflux from the cells were affected by mercurials thereby confirming the existence of proteins responsible for water transport.

Materials and Methods

Unidirectional Water Fluxes

Frogs (*Rana esculenta*) were kept in running tap water for five days before the experiments. The urinary bladders were mounted horizontally as a flat sheet between two Lucite chambers with the serosal side upwards. The tissue was maintained against a nylon mesh placed on its serosal side by a slight hydrostatic pressure applied on the mucosal bath. The exposed tissue area was 3.14 cm^2. The two chambers were filled with a potassium Ringer solution containing (in mM): KCl 100, NaCl 5, $CaCl_2$ 0.5, sodium phosphate 5, pH 7.8. The volume of the lower compartment was 12 ml. Mixing in the chambers was ensured by two magnetic bars.

The unidirectional water transfer was estimated from ^{3}HHO fluxes in the absence of osmotic gradient as previously described (Parisi, et al., 1979). ^{3}HHO was added to the lower chamber (1–2 μCi/ml). The serosal solution (2 ml) was completely removed every minute and replaced with unlabeled buffer. ^{3}HHO transfer, under control conditions, was measured for 10 consecutive periods. Amphotericin B was then added to the mucosal bath (12.5 μg/ml) and samples were removed over at least 20 periods before the addition of mercurial compounds to the serosal side. The action of the drugs was then examined for 15 to 40 min.

The thickness (δ) of the "equivalent" unstirred layers was estimated from ^{14}C-butanol permeability experiments:

$$\delta = D_{but}/P_{but}$$

where D_{but} is the diffusion coefficient in water and P_{but} the observed permeability of butanol. ^{14}C-butanol was also added to the mucosal side and appropriate corrections for double labeling were performed to calculate butanol and water permeabilities. Corrections for unstirred layers were made in every experiment for each individual sample.

Each 2-ml aqueous sample was mixed with 7 ml scintillation solution of Ultima Gold (Packard Instrument), and radioactivity was counted in a liquid scintillation counter with an external standardization. The specific activity in the lower chamber was recalculated for each period taking into account the previous serosal-to-mucosal transfer of radioactivity (Parisi et al., 1979).

Amphotericin B and *para*-chloromercuribenzoic acid (*p*CMB) were prepared in dimethyl sulfoxide at concentrations of 35 and 25 mg/ml, respectively, before each experiment and were kept screened from light. It was verified in preliminary experiments that dimethyl sulfoxide 1/500, the maximal solvent concentration used, did not affect the unidirectional water flux.

For ultrastructural studies, bladders were submitted for 20 min to apical amphotericin B and then fixed for 30 min with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. Pieces of tissue were washed in cacodylate buffer, cryoprotected in 30% vol/vol glycerol-sodium Ringer solution (NaCl 111 mM, KCl 5 mM, $CaCl_2$ 1 mM, $NaHCO_3$ 2.5 mM, pH 8.00) for 40 min, frozen in Freon 22, chilled by liquid nitrogen and then processed using the freeze-fracture technique as described by Chevalier et al. (1974).

Unless otherwise specified, experiments were performed at 20°C by imerging the Lucite chambers in a temperature-controlled water bath.

Isolated Cell Preparation

The bladder was first rinsed of its blood by perfusing the heart of the animal with 20 ml Ca^{2+}-free Ringer buffer (NaCl 111 mM, KCl 5 mM, $MgCl_2$ 1 mM, $NaHCO_3$ 2.5 mM, glucose 0.9 mM, ethylene glycol-*bis*(β-amino-ethyl ether) N,N,N′,N′-tetraacetic acid 1 mM, pH 8.00). The epithelial cells were then isolated as described by Pisam and Ripoche (1976). The bladders were filled to capacity with the Ca^{2+}-free Ringer solution, tied as closed sacs and suspended in a bath of Ca^{2+}-free Ringer for 20 min. Each sac was then rubbed gently between the thumb and the forefinger for 2 min, in order to release the epithelial cells from the basal membrane. The sac was then excised and the cells were collected in a beaker. The cell suspension was dispersed by 100 passages through a Pasteur pipette and filtered on a nylon gauze (mesh area: 5.29×10^3 μm^2). An aliquot was immediately fixed for freeze-fracture experiments. The absence of erythrocytes was checked by light microscopy (less than 1%), and cell viability was estimated by determining the fraction of the population able to exclude 0.2% trypan blue (more than 80%). The remaining of the preparation was centrifuged at $600 \times g$ for 5 min at 4°C. The pellet was resuspended in Ca^{2+}-free Ringer solution to reach a 3×10^6 cells/ml concentration for stop-flow experiments which were performed within 1 hr after cell isolation.

Stop-Flow Measurements

Stop-flow experiments were performed on a temperature-controlled Bio-Logic SFM2 (Echirolles, France). A 150-W halogen lamp was used for maximal stability of the light source. Experiments were carried out at 18°C.

Urinary bladder cells (0.1 ml), suspended in the Ca^{2+}-free Ringer buffer, were mixed with an equal volume of a hyperosmotic solution in order to reach an inwardly directed 200-mOsm sucrose gradient. The time course of 90° scattered light intensity (λ = 400 nm) was measured and recorded on a AT Tandon Personal computer for subsequent analysis. One thousand data points were acquired at each run with a maximal acquisition rate of 20 MHz.

All values are expressed as mean ±SEM.

Chemicals

Amphotericin B and *p*CMB were purchased from Sigma (St. Louis, MO), tritiated water was provided by the Commissariat à l'Energie Atomique (France) and ^{14}C-butanol by New England Nuclear (Boston, MA).

Results

Measurements of the Diffusional Water Permeability of the Basolateral Membrane

In order to have access to the basolateral membrane, the apical membrane was treated with 12.5 μg/ml amphotericin B. In preliminary experiments, ^{14}C-

Table. Diffusional water permeability of frog urinary bladder[a]

Condition	P_d (cm/sec · 10^4)
Control	1.12 ± 0.10 (n = 7)
+ Amphotericin B	4.08 ± 0.33 (n = 7)
+ Amphotericin B	
− pCMB (control)	4.26 ± 0.30 (n = 4)
+ pCMB	2.85 ± 0.34 (n = 4)
% Inhibition	34 ± 7 (n = 4)

[a] Net water fluxes were measured in the absence of any osmotic gradient. Twenty min after addition of amphotericin B to the mucosal bath (12.5 μg/ml), pCMB was added to the serosal bath (1.4×10^{-4} M). The observed water permeabilities were corrected for unstirred layer effects. All experiments were performed at 20°C.

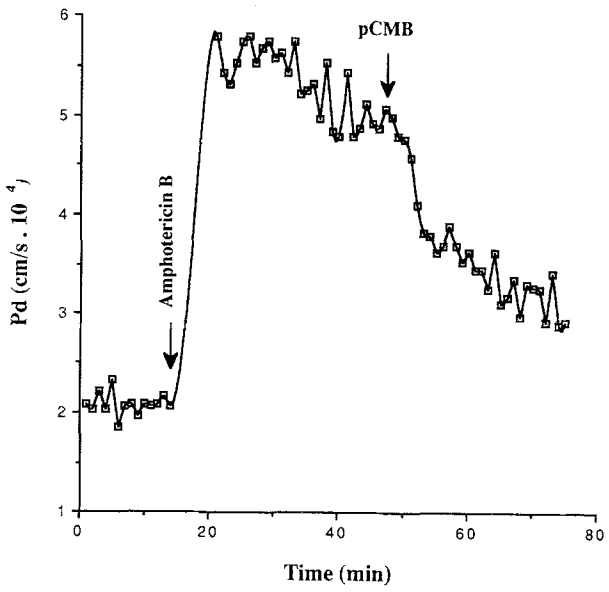

Fig. 1. Effect of amphotericin B and pCMB on the diffusional water permeability of frog urinary bladder. The curve represents a typical experiment of perforation of the apical membrane with amphotericin B (12.5 μg/ml) and the inhibition of the basolateral membrane water permeability by 1.4×10^{-4} M pCMB

mannitol permeability experiments were performed on amphotericin-treated bladders to verify that the antibiotic had not originated any large nonspecific leaks.

Upon insertion of the polyene antibiotic into the apical membrane, the transepithelial P_d was increased nearly fourfold from $1.12 \pm 0.10 \times 10^{-4}$ cm/sec to $4.08 \pm 0.33 \times 10^{-4}$ cm/sec (n = 7) (Table). The effect of amphotericin B was completed within 10 min and water permeability then remained steady, when using a potassium Ringer solution. The use of a classical sodium Ringer solution was discovered to be inappropriate in this type of experiment as it induces a decrease in P_d, after the transient high P_d due to amphotericin B incorporation.

The inhibitory effect of two mercurial reagents, $HgCl_2$ and pCMB was tested on bladders that had been treated with amphotericin B on the mucosal side for 20 min. Figure 1 shows a typical experiment where 1.4×10^{-4} M pCMB was added to the serosal bath after amphotericin B incorporation. The maximal inhibition, reached after 20 min, was 34 ± 4% (n = 4) for pCMB (1.4×10^{-4} M) and 52 ± 7% (n = 4) for $HgCl_2$ (10^{-3} M).

It was checked, by freeze-fracture electron microscopy, on at least three bladders treated with amphotericin B (but without pCMB), that intramembranous particles aggregates, which could have been responsible for the observed increase in P_d, did not appear in the apical membrane. Modifications in the apical membrane were however observed in the amphotericin B-treated bladder (Fig. 2). Raised protuberances were visible on the E face and pits on the P face which were not present in the control bladder; the density of these anomalies was different from one cell to the other. This same morphological aspect of the apical membrane was observed by Orci, Montesano and Brown (1980) on toad bladder treated with filipin, a sterol-specific antibiotic.

Finally, the effect of 1.4×10^{-4} M pCMB on the temperature dependence of P_d in the amphotericin B-treated bladder was investigated. A typical experiment is illustrated Fig. 3. For each studied bladder, the activation energy was calculated from the Arrhenius plot for P_d. Activation energy (E_a) was systematically increased in the presence of pCMB, from 4.52 ± 0.23 (n = 3), in the control condition, to 9.99 ± 0.91 kcal/mol (n = 4).

Isolated Cell Experiments

Freeze-fracture microscopy, carried out on three different preparations, showed that the isolation procedure was not accompanied by the appearance of intramembranous particle aggregates in the cell membranes confirming the observations of Cabana et al. (1977).

Isolated urinary bladder epithelial cells, suspended in a Ca^{2+}-free Ringer solution, were exposed to an inwardly directed 200-mOsm sucrose gradient in a stop-flow apparatus. The hyperosmotic challenge induced cell water efflux, cell shrinkage and thus an increase in scattered light intensity (Fig. 4,

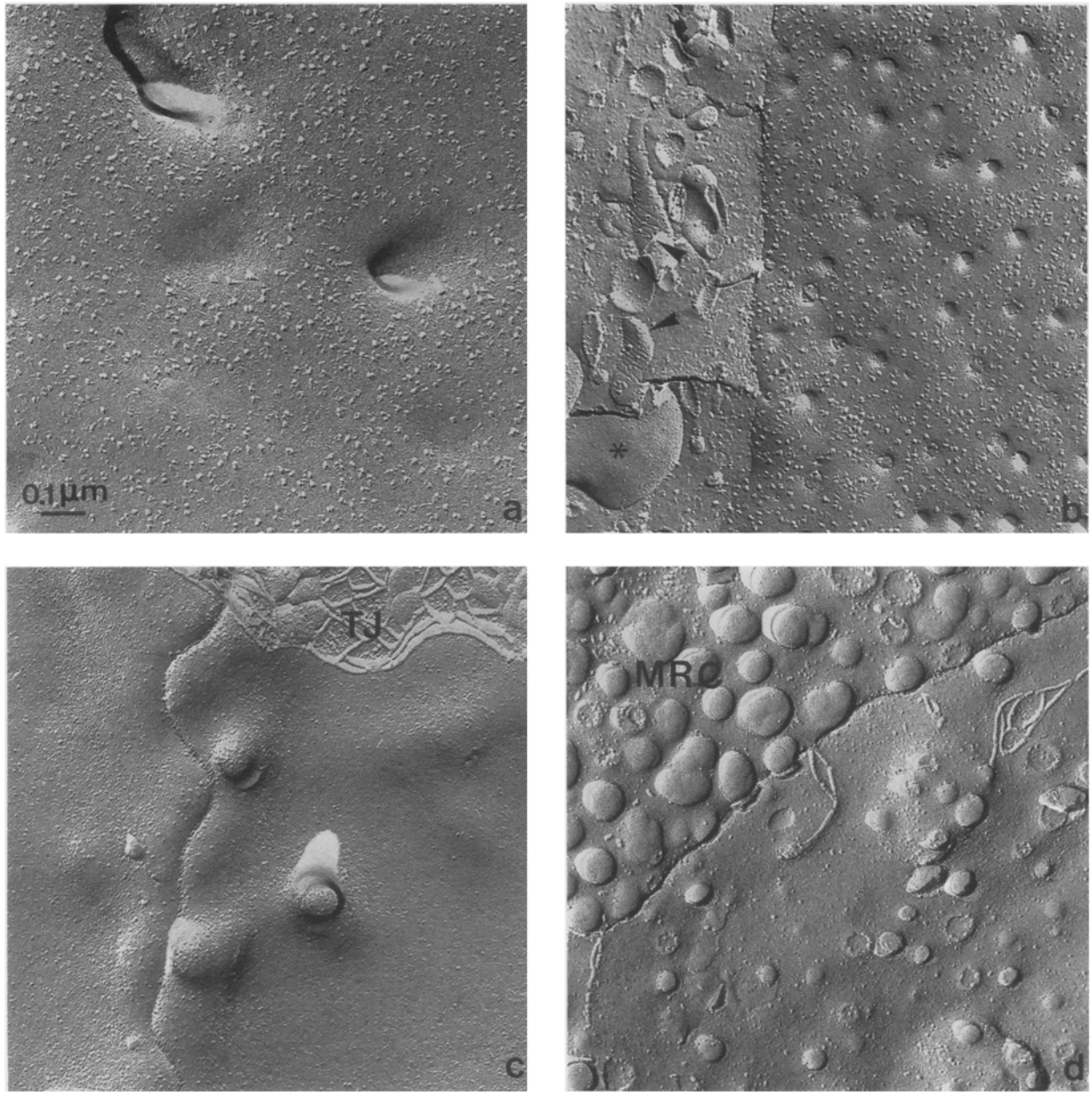

Fig. 2. Fine structure of the apical membrane of control (*a*, *c*) and amphotericin B-treated (*b*, *d*) bladders. As in the control situation (*a*, *c*), no particle aggregates are observed in the bladder fixed 20 min after amphotericin B (12.5 µg/ml) had been added to the mucosal side of the epithelium (*b*, *d*). Pits appeared in the E face (*b*) and raised protuberances in the P face (*d*) of the treated bladders which are not seen in the controls (*a*, E face; *c*, P face). These anomalies are not present in the granules (*) nor in the aggrephores of the treated bladder (arrowheads). *TJ:* tight junction; *MRC:* mitochondria-rich cell. (×63,000)

upper curve). The effect of *p*CMB on osmotic water transport was studied by incubating the cells for 10 min with 5×10^{-5} or 1.4×10^{-4} M (both given maximal inhibition) of the mercurial reagent and then submitting them to a 200-mOsm sucrose gradient as in the control condition. Water flow was drastically inhibited. Whereas the half-time ($t_{1/2}$) of cell shrinkage was of 0.10 ± 0.01 sec in the control situation, $t_{1/2}$ increased to 1.13 ± 0.13 sec in the *p*CMB-treated cells, showing a 91 ± 2% inhibition ($n = 3$).

Discussion

Our data give a new insight in the understanding of the transepithelial water transport mechanisms in ADH-sensitive epithelia. Obtained by two different

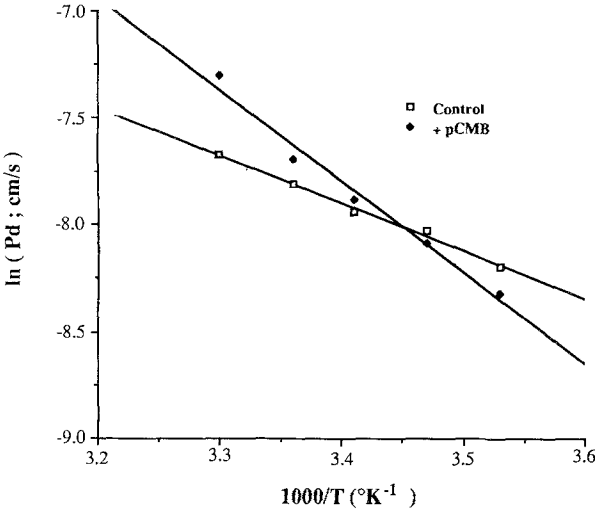

Fig. 3. Representative Arrhenius plot for diffusional water transport across the basolateral membrane of frog urinary bladder. Unidirectional water fluxes were measured over a temperature range of 10–30°C on the same bladder. Each point is the mean of at least four measurements. E_a was determined from the slopes of the linear approximations of the experimental data. The two conditions, control and incubation with *p*CMB 1.4×10^{-4} M were carried out on two different bladders. The mean E_a values were: 4.52 ± 0.23 kcal/mol (n = 3) in the control situation and 9.99 ± 0.91 kcal/mol (n = 4) in the presence of *p*CMB

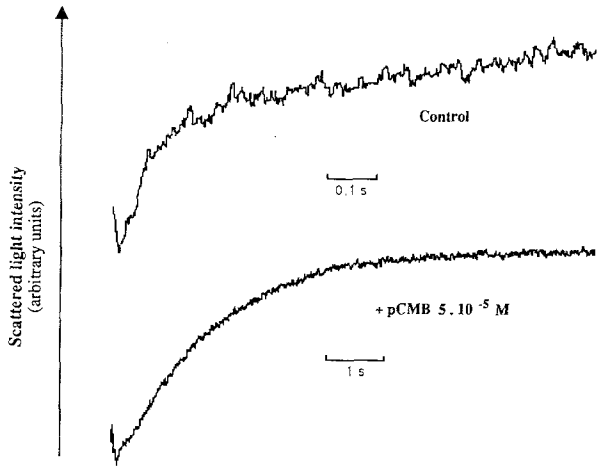

Fig. 4. Influence of *p*CMB of the time course of osmotic water transport of isolated cells from frog urinary bladder. In the control situation, isolated urinary bladder cells suspended in a calcium-free Ringer solution were mixed with a hyperosmotic solution in order to reach a 200-mOsm inwardly directed sucrose gradient. The bottom time course was obtained by preincubating the cells for 10 min with 5×10^{-5} M *p*CMB and then submitting them to a 200-mOsm sucrose gradient

experimental approaches, our results support the existence of constantly present water channels in the basolateral membrane of frog urinary bladder, independently of any transepithelial water permeability stimulation by the antidiuretic hormone.

In our first series of experiments, we developed a protocol enabling the access to the basolateral membrane which consisted in "perforating" the apical membrane with amphotericin B. These antifungal molecules penetrate into membranes by binding to the sterols and form pores (Finkelstein, 1987). As the frog urinary bladder cell membranes contain more than 50% cholesterol (Parisi, Gauna & Rivas, 1976; Mazur et al., 1986), it is very sensitive to amphotericin B action. The diffusional water permeability was thus increased nearly fourfold, as previously described on fixed (Parisi, Mérot & Bourguet, 1985; Hoch et al., 1989) and unfixed tissue (Levine, Jacoby & Finkelstein, 1984*b*). This increase is of the same order of magnitude as that observed after ADH stimulation (Parisi & Bourguet, 1983; Levine, Jacoby & Finkelstein, 1984*a*), but it is not due to the appearance of intramembrane aggregates, as shown by our freeze-fracture experiments.

The underlying idea was then that, if the basolateral membrane contained water channels, by exposing the amphotericin B-treated bladder to water transport inhibitors, the basolateral membrane would become the limiting barrier to transmural water movement. At the present time, mercurial reagents are the only compounds known to inhibit water transport as shown on erythrocytes (Solomon et al., 1983; Macey, 1984; Benga et al., 1986), proximal tubule plasma cell membranes (Whittembury et al., 1984; van Heeswijk & Van Os, 1986; Pratz, Ripoche & Corman, 1986; Meyer & Verkman, 1987; Van Der Goot, Podevin & Corman, 1989), ADH-treated amphibian urinary bladder (Hoch et al., 1989; Ibarra, Ripoche & Bourguet, 1989) and endosomes from kidney collecting duct (Verkman et al., 1988) and from toad urinary bladder epithelial cells (Shi & Verkman, 1989; Harris, Handler & Blumenthal, 1990). Moreover, these mercurial SH blockers do not modify the water permeability of artificial liposomes (Van Der Goot et al., 1989) nor of brush-border membrane vesicles from small intestine (van Heeswijk & Van Os, 1986; F. Van der Goot et al., *unpublished data*) and are thus a good tool to characterize proteic water pathways.

In the present study, we mainly used *p*CMB, a sulfhydryl mercurial reagent that has a higher specific action and is more lipophilic than *p*CMBS (*para*-chloromercuribenzene-sulfonic acid) (Ashley & Goldstein, 1981), thereby enabling the use of a far lower dose. *p*CMB decreased P_d in amphotericin B-treated bladders by 34% which is slightly less than the 50% inhibition observed in *p*CMBS-treated erythrocytes (Macey, Karan & Farmer, 1972). This lower percentage could have been explained by the fact that, in our diffusional water permeability experiment, 40 min after the addition of amphotericin B to

the mucosal bath, a slight diffusion of the antibiotic towards the basolateral membrane could have occurred thus creating noninhibitable water pathways in this membrane. But there were no morphological indications of the presence of amphotericin B in basolateral membrane in our experiments, as suggested by the observations of Lichtenstein and Leaf (1965).

As *p*CMB does not affect amphotericin B-elicited water flow (Levine et al., 1984*a*, Hoch et al., 1989), the inhibition must take place on the basolateral membrane. This result suggests a facilitated basolateral water pathway since the subepithelial layers do not hinder water diffusion (Levine et al., 1984*a*).

To confirm the hypothesis of basolateral water channels, we investigated whether the activation energy (E_a) of diffusional water transport would increase after mercurial inhibition, a property not present in artificial liposomes. In the control condition, E_a of the amphotericin B-treated bladder was low and comparable to the activation energy for self-diffusion in water and to E_a measured in erythrocytes (Macey, 1984), kidney proximal tubule (van Heeswijk & Van Os, 1986) and endosomes (Shi & Verkman, 1989). Upon mercurial treatment, E_a was increased to 10 kcal/mol, a value characteristic of liposomes (Fettiplace & Haydon, 1980). This suggests that, after channel "closure", an essentially lipid-mediated component of water permeability remains present.

Our second experimental approach was to measure the effect of *p*CMB on the kinetics of water efflux of isolated epithelial urinary bladder cells. Even if, after cell dissociation, the basolateral water channels had slid towards the apical membrane, as suggested by the mechanism of redistribution of macromolecules described by Pisam and Ripoche (1976), they would still be present and accessible to mercurial action. As expected, the kinetics were slowed down tremendously in *p*CMB-treated cells when compared to the control situation. This effect was observed after *p*CMBS treatment on erythrocytes (Macey & Farmer, 1970) in the same proportion: 90%, and on ADH-stimulated frog urinary bladder (Ibarra et al., 1989) in a lower proportion: 70%.

The data from both of our series of experiments are consistent with the existence of permanent water channels in the basolateral membrane of the epithelium lining the frog urinary bladder. Our results do not exclude an hormonally regulated component of the basolateral water permeability, as suggested by Kachadorian et al. (1978, 1985, 1987), but further studies on the effect of ADH stimulation on amphotericin B-treated bladders are required to reach any conclusions. They also do not exclude the presence, in the basolateral membrane, of F_0–F_1 proton-ATPase-like-channels which are in some conditions shared by water (Harvey, Lacoste & Ehrenfeld, 1989).

It thus appears that the two plasma membranes of the frog urinary bladder have different water permeability properties. Very low at rest, the apical permeability is considerably increased under ADH stimulation, with simultaneous appearance of intramembranous particle aggregates. Inhibition can then be obtained by incubation with *p*CMBS (Hoch et al., 1989; Ibarra et al., 1989). The basolateral membrane, on the other hand, has, even at rest, a very high water permeability, mediated by water channels as evidenced by activation energy and mercurial inhibition. Particle aggregates have never been observed in this membrane (Kachadorian et al., 1975; Bourguet et al., 1976). Thus it seems that the water channels encountered in the urinary bladder basolateral membrane have properties similar to those found in kidney proximal tubule apical and basolateral membranes and in erythrocytes.

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