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Effect of Mercurial Compounds on Net Water Transport and Intramembrane Particle Aggregates in ADH-Treated Frog Urinary Bladder

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Summary. It has been suggested that during the oxytocin-induced hydrosmotic response, water crosses the luminal membrane of urinary bladder epithelium cells through membrane-spanning proteins. Although specific inhibitors of osmotic water transport have not been found, certain sulfhydryl reagents such as mercurial compounds may help to identify the proteins involved in this permeation process. We tested the effects of *p*-chloromercuribenzene sulfonate (PCMBS) and of fluoresceinmercuric acetate (FMA) on the net water flux, the microtubule and microfilament structures of the frog urinary bladder, and the distribution of intramembrane particle aggregates in the luminal membrane.

We observed that: (*i*) 5 mM PCMBS at pH 5 and 0.5 mM FMA at pH 8 added to the mucosal bath at the maximum of the response to oxytocin partially inhibited the net water flux. Inhibition then increased progressively when the preparation was repeatedly or continuously stimulated, until it reached a maximal inhibition at 120 min. This inhibition was not reversed even when cystein was added in the mucosal bath. PCMBS and FMA effects were also observed when cyclic AMP (3′,5′ cyclic adenosine monophosphate) was used to increase water permeability. (*ii*) PCMBS mucosal pretreatment did not modify the basal water flux but potentiated the inhibitory effect of PCMBS or FMA on the hydrosmotic response to oxytocin. (*iii*) Microtubule and microfilament network, visualized in target cells by immunofluorescence, was not affected by PCMBS. (*iv*) The maximal PCMBS or FMA inhibition was not associated with a reduction of aggregate surface area in the apical membrane.

The persistence of the intramembrane particle aggregates associated with the oxytocin-induced hydrosmotic response during the net water flux inhibition by PCMBS, suggests that the PCMBS effect occurs possibly at the level of sulfhydryl groups of the water channel itself.

Key Words water transport · SH reagents · PCMBS · amphibian urinary bladder · oxytocin

Introduction

According to current models of antidiuretic hormone (ADH) action, the hormone attaches to its receptor, stimulates the production of 3′,5′-cyclic adenosine monophosphate (cyclic AMP) and through a subsequently initiated sequence of events, induces a significant increase in water permeability in the apical membrane (for review *see* Handler & Orloff, 1973).

Morphological studies using freeze-fracture electron microscopy reveal that ADH stimulation also leads to the appearance of organized arrays of aggregated intramembrane particles in this same membrane (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & DiScala, 1975; Bourguet, Chevalier & Hugon, 1976; Wade, 1978; Muller, Kachadorian & DiScala, 1980).

Strong experimental evidence indicates that intramembrane particle aggregates are closely related to the ADH-induced water pathway (Kachadorian et al., 1981; Brown, Grosso & De Sousa, 1983). They appear to be inserted into the apical membrane from preformed cytoplasmic vesicles in response to the hormonal stimulus (Humbert et al., 1977; Wade, 1980; Wade, Stetson & Lewis, 1981). The transfer and the insertion of aggregates involve the participation of microtubules and microfilaments (Kachadorian, Ellis & Muller, 1979; Muller et al., 1980). Although there is much evidence that the aggregates are specific markers of apical membrane water permeability, the actual sites for water pathways have not yet been identified.

Sulfhydryl reactive compounds have been useful in the characterization of water pathways in red cells (Naccache & Sha'afi, 1974), kidney proximal tubules (Whittembury et al., 1984) and urinary bladders (Adragna & Bourguet, 1987). In the frog urinary bladder, using sulfhydryl reagents that do not contain mercury (5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) iodoacetamide (IAM) and N-ethylmaleimide (NEM)), it has been shown that a low concentration of NEM, added to the mucosal bath at the peak of hormonal stimulation, is able to stabi-

lize high net water flux with the presence of intramembrane particle aggregates. On the contrary, a high concentrations of NEM do inhibit water transport and the ADH-induced aggregation phenomenon (Adragna & Bourguet, 1987).

The aim of the present work was to characterize water permeability inhibition in the frog urinary bladder (Ibarra et al., 1988) by sulfhydryl reagents that contain mercury: *p*-chloromercuribenzene-sulfonic acid (monosodium salt) (PCMBS) and fluorescein mercuric acetate (FMA) and to correlate this effect with the presence, the distribution and the size of intramembrane particles in the apical membrane. We chose PCMBS and FMA because of their higher specificity of action, compared to other sulfhydryl reagents, in preparations such as red cells (Naccache & Sha'afi, 1974; Benga et al., 1982).

Our results show that PCMBS and FMA inhibit the water permeability and increase the aggregate density in the apical membrane of ADH-stimulated bladder. These findings show for the first time a situation in which an increase of the aggregates surface in the apical membrane was associated with a low water permeability.

A promising line of research could be the study of a possible binding of mercurial compounds to label these aggregates.

Materials and Methods

Frogs (*Rana esculenta*) originating from Central Europe were kept at 20°C in running tap water for at least five days before sacrifice. The urinary bladders were isolated and mounted between two lucite chambers, the serosal face of the tissue being bathed with a buffer solution containing (mM): NaCl 112; KCl 5; $CaCl_2$ 1; $NaHCO_3$ 2.5; pH 8.1. The mucosal bath was made hypotonic by reducing the NaCl concentration to 5.6 mM.

The mercurial compounds were added only to the mucosal bath. FMA was used at 0.5 mM, pH 8 in hypotonic Ringer solution (35 mOsm/Kg H_2O). PCMBS was applied at a concentration of 5 mM in a MES (2(N-morpholino)ethanesulfonic acid) 20 mM-Tris (Tris(hydroxymethyl) aminomethane) buffer, pH 5 (36 mOsm/kg H_2O). In this case, the mucosal medium of control hemibladder was also replaced by MES-Tris solution, pH 5 (26 mOsm/kg H_2O).

To increase water permeability, the bladders were stimulated with a maximal concentration (2.2×10^{-8} M) of oxytocin (OXY), a synthetic structural analog of the antidiuretic hormone (ADH) or with its intracellular second messenger, cyclic AMP (5×10^{-3} M).

Net water flux was recorded minute by minute as previously described (Bourguet & Jard, 1964). Experiments were performed at room temperature.

For ultrastructural studies, the samples were obtained by fixing the bladders immediately after the water permeability determinations. Preparations were fixed for 15 min with 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, cryoprotected in glycerol Ringer solution 30% vol/vol for 40 min and subsequently frozen in Freon 22 chilled by liquid nitrogen. Samples were then processed using the freeze-fracture technique (Chevalier et al., 1974).

The total cell surface area occupied by the aggregates and the size distribution of the aggregates were calculated using a Kontron IBAS I image analyzing computer from pictures of an apical surface replica at a magnification of 45,500×.

Four to six experiments were performed in each condition. In this work the term "aggregate" refers to units composed of intramembranous particles condensed in closed-space parallel linear arrays. Two groups of particle arrays, at angles to one another, were thus counted as two aggregates according to Chevalier, Parisi and Bourguet (1983).

For fluorescence studies, stretched bladders were cut in thin slices and immediately fixed in cold (−20°C) methanol for 10 min and then in cold (−20°C) acetone for 10 min. Tissues were then immersed overnight in a PIPES (piperazine-N-N′-*bis*(2-ethanesulfonic acid)) buffer pH 7 at 4°C. The mucosal side was gently scraped in Ca^{2+}-free PBS and the epithelial cell sheets obtained were placed on poly-L-lysine coated coverslips. For the observation of microtubules, the cells were postfixed in cold (−20°C) methanol and immersed for 60 min in 1% bovine serum albumine (BSA) in a PBS buffer. They were then incubated at room temperature with a monoclonal antibody against tubuline (Serlabo, Paris, France) at a dilution of 1/1,000 in phosphate buffer saline solution for 120 min, washed 3 × 10 min in 1% rabbit serum and transferred into fluorescein-conjugated rabbit antirat IgG (1/400) (Serlabo, Paris, France) for 30 min (Valenti et al., 1987). For the visualization of the microfilaments, the coverslips plated with the epithelial cell sheets were fixed in 3% formaldehyde for 15 min and incubated in an extraction buffer (Turksen et al., 1983) for 30 min with constant agitation. They were post-fixed in cold (−20°C) acetone for 15 min, allowed to dry and put in a rhodamine-phalloidin solution (10 μl/200 μl) for 120 min, quickly rinsed and mounted with Citifluor (Linh).

The specimens were observed and photographed with an Olympus Vanox T photomicroscope with an epi-fluorescence attachment.

The results are expressed as the mean ± SE. Student's *t* test was used to compare the control and experimental values. For the statistical comparison of frequency distributions of the aggregate sizes obtained from different samples, the Kolmogorov-Smirnov two-sample test was applied (De Laat, Tertoolen & Bluemink, 1981). To this end, the maximum cumulative difference "D_{max}" was determined by the difference between the calculated cumulative fractions of the aggregate number present in control and in experimental bladders. An approximation of the level of significance could be given by

$$P = 2/e^{2ND^2_{max}} \quad (1)$$

with $N = n_1 \cdot n_2/n_1 + n_2$ where n_1 and n_2 are the total number of aggregates of the two compared distributions.

Results

Effects of Mercurial Compounds on Transepithelial Net Water Flux

Basal Net Water Flux

In some series of experiments, net water flux was monitored in resting preparations before and after a

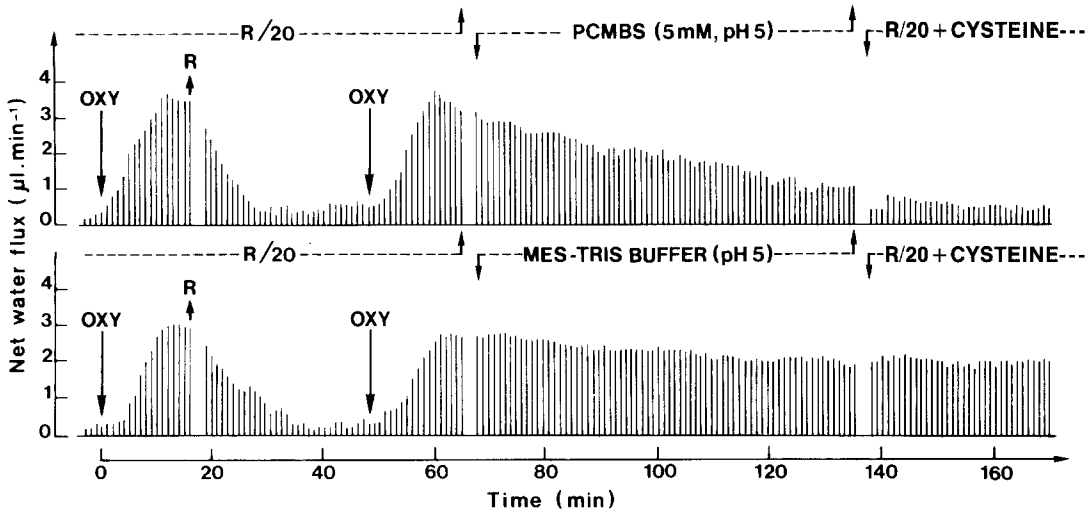

Fig. 1. Effect of PCMBS on the hydrosmotic response to oxytocin in bladders exposed to the hormone. Two fragments of the same bladder were tested in each experiment. PCMBS (5 mM) or MES-Tris buffer was added in the mucosal bath of each fragment at the top of the oxytocin response (arrow). At the maximal inhibition, the mucosal side was rinsed and a hypotonic Ringer solution containing cysteine (10 mM) was added. In this typical experiment, the height of each stroke indicates the 1-min net water flux across 1–6 cm^2

Table 1. Influence of apical PCMBS on the hydrosmotic response to oxytocin

Exptl group	pH	Addition of inhibitor		*n*	Percentage of maximal net water flux	Half time of the oxytocin washout (min)
		Before oxytocin	During oxytocin			
1	5 or 8	NO	NO	20	100	4.8 ± 0.2
2	8	NO	PCMBS, 30 min	3	94 ± 6	3.5 ± 0.5
3	8	NO	PCMBS, 120 min	3	99 ± 1	4.2 ± 0.1
4	5	NO	PCMBS, 30 min	14	79 ± 6[a]	7.5 ± 0.7[a]
5	5	NO	PCMBS, 120 min	8	25 ± 3[a]	9.5 ± 2.4[a]
6	5	PCMBS, 60 min	NO	8	87 ± 11	5.3 ± 0.3
7	5	PCMBS, 60 min	PCMBS, 30 min	8	30 ± 5[a]	7.3 ± 0.9[a]

The net water fluxes are expressed as a percentage of the flux observed in the control fragment from the same bladder in the presence of oxytocin. Mean ± SE.
[a] $P < 0.01$.

treatment by PCMBS or FMA. Results show that the basal net water flux was not affected by a 60 min incubation in either 5 mM PCMBS (pH 5) (*see* Fig. 4*a*) or 0.5 mM FMA (pH 8) (*not shown*).

Hydrosmotic Response to Oxytocin

The effects of mercurial compounds were assessed in different experimental conditions. In the first type of experiments, the mucosal bath was replaced by MES-Tris buffer at pH 5 or 8, in the presence or in the absence of PCMBS 5 mM, once the hydrosmotic response to oxytocin had been fully developed. The preparations were maintained in the presence of the inhibitor for at least 60 min. Some of them were continuously exposed to oxytocin. For some others, the hormone was rinsed after 30 min of inhibitor exposure and repetitive cycles of oxytocin stimulation were performed at 30-min intervals. When PCMBS was tested in MES-Tris buffer at pH 8, no effect was observed (Table 1, Exptl. groups 2 and 3). Nevertheless, when medium pH was reduced to 5, PCMBS was found to inhibit the oxytocin-induced net water flux in both experimental conditions: (*i*) bladders continuously exposed to oxytocin (Fig. 1), and (*ii*) upon repetitive cycles of oxytocin stimulation (Fig. 2). Measurements of the transepithelial osmotic gradient during PCMBS treatment showed no significant alteration up to 120 min (Table 2).

Moreover, it was observed that, in the presence of 5 mM PCMBS at pH 5, the half time of oxytocin

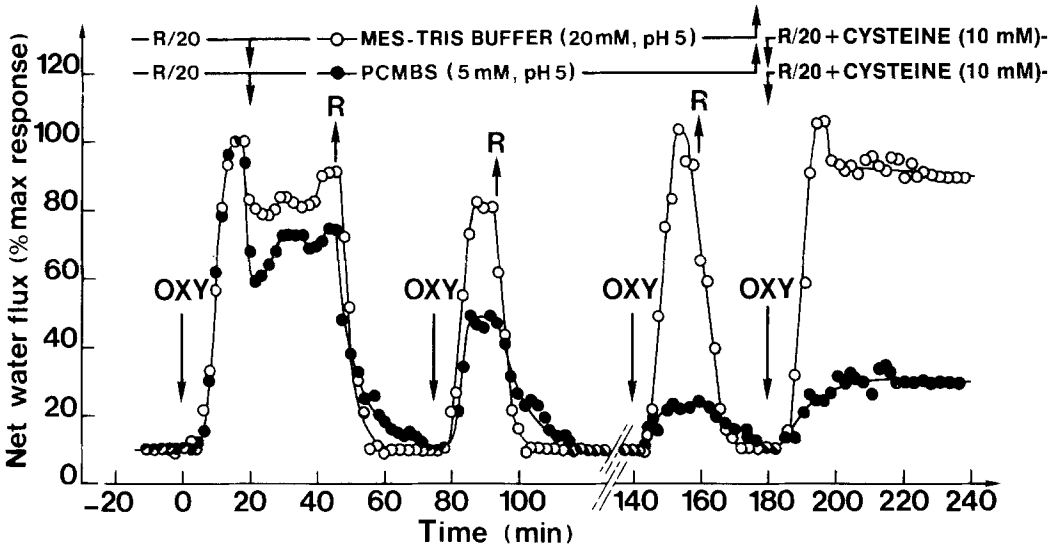

Fig. 2. Effect of PCMBS on the hydrosmotic response to oxytocin in bladder exposed to repeated hormonal stimulations. PCMBS was added in the mucosal bath of the experimental channel (black dots) and MES-Tris buffer in the mucosal bath of the control channel (open dots) as soon as the hydrosmotic response to oxytocin was fully developed. Two other oxytocin challenges were performed in both fragments, 1 and 2 hr after PCMBS addition. Finally, the mucosal bath was replaced by a hypotonic Ringer solution containing cysteine (10 mM) and an additional stimulation by oxytocin took place. Each mean curve represents eight experiments

Table 2. Effect of the PCMBS treatment on the transepithelial osmotic gradient

t (min)	*n*	% of the initial transepithelial osmotic gradient
0	10	100
30	5	99 ± 2
60	5	99 ± 2
90	5	94 ± 3
120	5	83 ± 3[a]

In these series of experiments, the bladders, continuously exposed to oxytocin, were treated with 5 mM PCMBS (pH 5). At different times, the osmotic pressure of mucosal and serosal baths were measured. At $t = 0$: serosal bath (2 ml) osmolality was 225 mOsm/kg H_2O and mucosal bath (2 ml) osmolality was 36 mOsm/kg H_2O.

[a] $P < 0.001$.

washout response was greatly slowed down (Fig. 2, Table 1, Exptl. groups 4 and 5). This inhibition was not reversed by mucosal addition of 10 mM cysteine, a sulfhydryl reagent known to get a high affinity for PCMBS (Figs. 1 and 2).

Another mercurial compound, FMA (0.5 mM), at physiological pH, was also added to the mucosal bath when the oxytocin response was developed; an inhibition of the water flux comparable to the inhibition obtained with PCMBS was observed. The results are summarized in Table 3 (Exptl. groups 2 and 3). The time course of the inhibitory effects of PCMBS and FMA on the net water fluxes are shown in Fig. 3. Both curves show that the inhibition depends on the time of exposure of the bladders to mercurial compounds during the oxytocin stimulation. At 120 min, maximal inhibition of ADH-induced net water flux was observed and in some bladders, this inhibition was as much as 100%.

In another series of experiments, after a first stimulation by oxytocin and washout, bladders at rest were mucosally incubated for 60 min with 5 mM PCMBS (pH 5). After that, in some bladders the inhibitor was washed whereas in some others, it was not. Thereafter in both conditions, a second stimulation by oxytocin was achieved. The data show that, when PCMBS was washed out, the second hydrosmotic response to oxytocin was similar to that observed before the PCMBS treatment (Fig. 4*a*; Table 1, Exptl. group 6). On the contrary, when PCMBS was maintained in the mucosal bath, a clear inhibition of the second oxytocin response at 30 min was observed (Fig. 4*b*; Table 1, Exptl. group 7). This inhibition was more important compared with that found after 30 min of PCMBS incubation without a preincubation (Table 1, *compare* Exptl. groups 4 and 7). The inhibitory effect on the hydrosmotic response by FMA was also potentiated by the preincubation with PCMBS, 60 min (Table 3, *compare* Exptl. groups 2 and 5). Therefore, a 60-min pretreatment of bladders with PCMBS at rest results in a greater degree of inhibition of the hydrosmotic response to oxytocin by both mercurial compounds. Finally, FMA does not affect the oxy-

Table 3. Influence of apical FMA on the hydrosmotic response to oxytocin

Exptl group	pH	Addition of inhibitor		*n*	Percentage of maximal net water flux	Half time of the oxytocin washout (min)
		Before oxytocin	During oxytocin			
1	8	NO	NO	16	100	4.8 ± 0.2
2	8	NO	FMA, 30 min	9	68 ± 5[a]	10.2 ± 2.2[a]
3	8	NO	FMA, 120 min	4	30 ± 4[a]	8.0 ± 0.7[a]
4	8	FMA, 60 min	NO	4	92 ± 5	5.6 ± 0.7
5	8	PCMBS, 60 min	FMA, 30 min	4	24 ± 2[a]	8.3 ± 1.2[a]

The net water fluxes are expressed as a percentage of the flux observed in the control fragment from the same bladder in the presence of oxytocin. Mean ± SE.
[a] $P < 0.01$.

Table 4. Influence of apical PCMBS and FMA on the hydrosmotic response to cyclic AMP

Addition of inhibitor at the maximal response to cyclic AMP	*n*	Percentage of maximal net water flux
NO	7	100
PCMBS, 60 min	3	49 ± 5[a]
FMA, 60 min	4	27 ± 7[a]

In these series of experiments, the bladders were first allowed to develop a maximal response to cyclic AMP. Then, the mucosal bath was replaced by 5 mM PCMBS (pH 5) or 0.5 mM FMA (pH 8) for 60 min. Mean ± SE.
[a] $P < 0.01$.

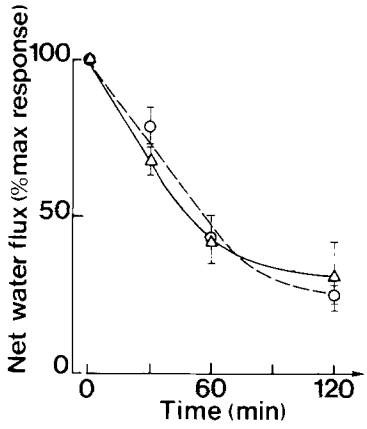

Fig. 3. Evolution of the inhibitory effect of 5 mM PCMBS at pH 5 (circles) and 0.5 mM FMA at pH 8 (triangles) on the oxytocin-induced net water flux, during repeated challenges by oxytocin

tocin-response when it is added in bladders at rest and washed out before the hormonal stimulation (Table 3, Exptl. group 4).

Hydrosmotic Response to Cyclic AMP

Cyclic AMP is the accepted second messenger for the hydrosmotic response to ADH. Table 4 shows that the hydrosmotic response elicited by cyclic AMP is also inhibited by 5 mM PCMBS (pH 5) or by 0.5 mM FMA (pH 8). The inhibition produced by both mercurial compounds had similar characteristics to those observed during the response to oxytocin.

Fluorescence Studies of Microtubules and Microfilaments in Bladders Treated with PCMBS

The presence of microtubules in isolated cells or in epithelial sheets was determined by using an immunofluorescence technique. Two fragments of the same bladder were incubated with or without 5 mM, PCMBS (pH 5) for 60 min, fixed and processed as previously described (*see* Materials and Methods). Although the resolution of the technique employed is low, a clear microtubular network in epithelial cells of control bladders was observed. The microtubular network had the same appearance in both control and PCMBS-treated cells (Fig. 5*a*,*b*).

The cellular distribution of microfilaments in bladders incubated with or without 5 mM PCMBS (pH 5) were also observed. No difference between experimental and control preparations was detected (Fig. 5*c*,*d*).

Freeze-Fracture of Preparations Treated with PCMBS

Morphological Aspects of Intramembranous Particles

Both control and experimental bladders were stimulated with oxytocin. At the peak of the hydrosmotic response, mucosal baths were replaced by MES-

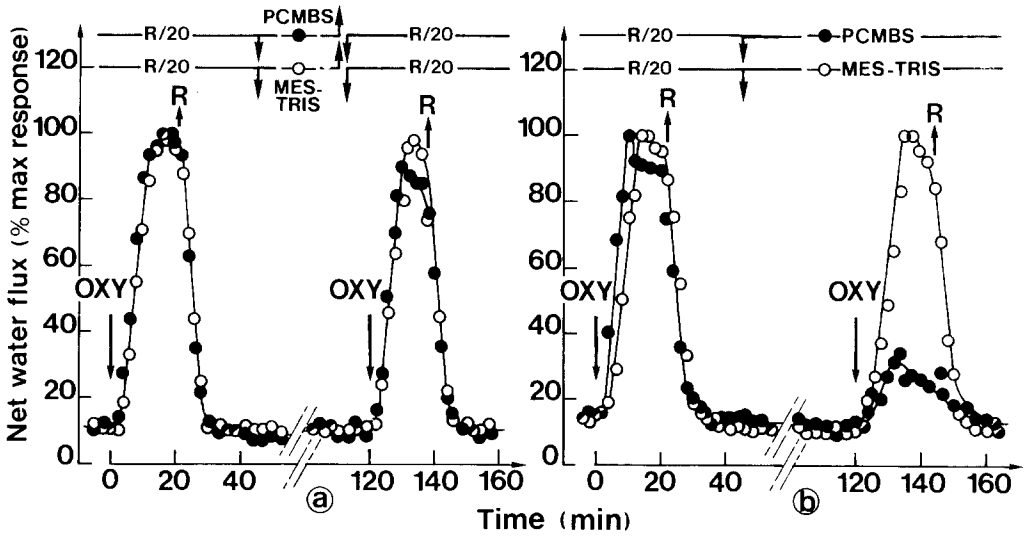

Fig. 4. Effect of preincubation with PCMBS (mucosal side) on the hydrosmotic response. After an initial stimulus by oxytocin and washout, 5 mM PCMBS (pH 5) and MES-Tris buffer pH 5 alone were added in two fragments of the same bladder for 60 min. After that, PCMBS was (*a*) washed out or (*b*) maintained in the mucosal bath during the whole experiment. An additional stimulation by oxytocin was performed. Each mean curve represents eight experiments

Tris buffer (pH 5) for controls, and 5 mM PCMBS (pH 5) for the experimental bladders. Once maximal inhibition by PCMBS was observed, the bladders were fixed for freeze-fracture electron microscopy. At least five pairs of bladders were studied for each experimental condition. The results show that the intramembranous particles present in the apical membrane of oxytocin-stimulated cells exhibited the same appearance in control (Fig. 6*a*) as well as in the PCMBS-treated bladders (Fig. 6*b*). In both cases, the aggregates were observed on the P face, whereas the complementary groove networks were visible on the E face. In some cases, a well-defined orthogonal pattern was clearly visible inside these groove networks.

The distribution of aggregate sizes from both experimental conditions were analyzed by histograms. Figure 7 shows the results obtained and suggests that there is a tendency for the aggregate size to be larger in PCMBS-treated bladders. Application of the Kolmogorov-Smirnov two-sample test to the maximum cumulative difference between the aggregate number fractions present in both distributions revealed that the aggregates from PCMBS-treated preparation are significantly larger than those from control samples (Fig. 7 inset).

Correlation Between the Net Water Flux and the Presence of Aggregates in Control and PCMBS-Treated Bladders

Two fragments of the same bladder were stimulated by oxytocin and the net water flux was recorded. At the peak of the hormonal action, the mucosal bath was replaced by a MES-Tris buffer pH 5 with or without 5 mM PCMBS, pH 5. When the inhibition induced by PCMBS was maximal, both fragments were fixed and processed for freeze fracture. Other bladders, treated or not by PCMBS, but at rest, were also fixed and observed.

The fraction of the cell surface area occupied by the aggregates and the corresponding net water flux are given in Table 5. Although water permeability was strongly reduced, the aggregate surface area was significantly increased. Thus, a clear dissociation between water flux and the aggregate surface area in oxytocin-stimulated bladders was observed.

In the absence of oxytocin, the surface area covered by the aggregates remained low and comparable for bladders incubated with or without PCMBS (Table 5).

Discussion

Previous works have shown that sulfhydryl reagents that do not contain mercury (DTNB, IAM, NEM) have variable and multiple effects on the water permeability (Rasmussen et al., 1960; Bentley, 1964; Brown, Feinstein & Sha'afi, 1975; Solomon et al., 1983; Adragna & Bourguet, 1987) while sulfhydryl blocking agents that do contain mercury (PCMBS, $HgCl_2$, FMA) are more specific as inhibitors of water transport (Macey & Farmer, 1970; Naccache & Sha'afi, 1974; Klip et al., 1980; Benga et al., 1983; Whittembury et al., 1984). Comparison

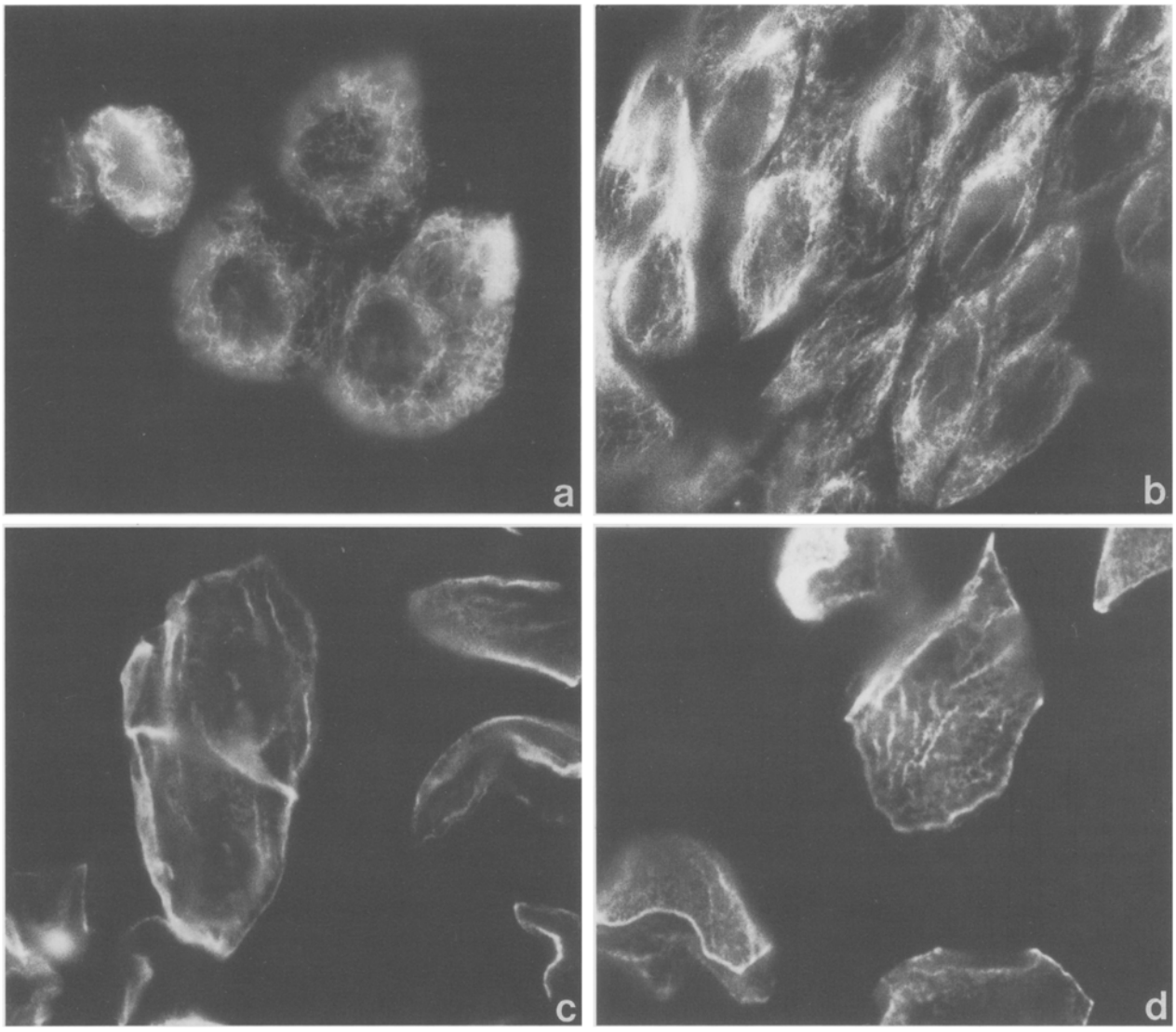

Fig. 5. Effect of PCMBS on the cytoskeleton integrity. Two hemibladders were incubated in MES-Tris buffer pH 5 with or without 5 mM PCMBS on the mucosal side for 2 hr. Then they were treated with Ca^{2+}-free Ringer for 20 min and the isolated cells were processed for fluorescence microscopy. In (*a*) and (*b*), a typical microtubular network in cells treated (*a*) or not (*b*) with PCMBS was observed. In (*c*) and (*d*), cellular distribution of microfilaments in bladders incubated with (*c*) or without (*d*) PCMBS was also observed (1,250×)

of the different types of sulfhydryl blocking agents in frog urinary bladder seems to indicate that sulfhydryl groups involved in the hormonally induced water flow exhibit more specificity to mercurial components than those involved in the basal water transport. For example, PCMBS inhibits the water transport induced by oxytocin but does not modify the basal water flux (Ibarra et al., 1988) while NEM affects the hormone-induced water transport and also the basal net water flux (Adragna & Bourguet, 1987).

In the present study, we show that 5 mM PCMBS at pH 5 or 0.5 mM FMA at pH 8 added in the mucosal bath of frog urinary bladders inhibit the net water flux induced by oxytocin. Both inhibitions were observed in two experimental conditions: (*i*) when the preparations were kept all the time in the presence of the hormone or (*ii*) when the preparations were exposed to repeated hormonal stimulations.

Permeability studies carried out in erythrocyte have shown that the sensitive sites for PCMBS are not accessible on the outer face of the membrane (Solomon et al., 1983), and that the penetration of the drug to reach sensitive sites is slow (Vansteveninck, Weed & Rothstein, 1965). In our studies, PCMBS or FMA induced a slow development of the inhibition, which was irreversible at least 30

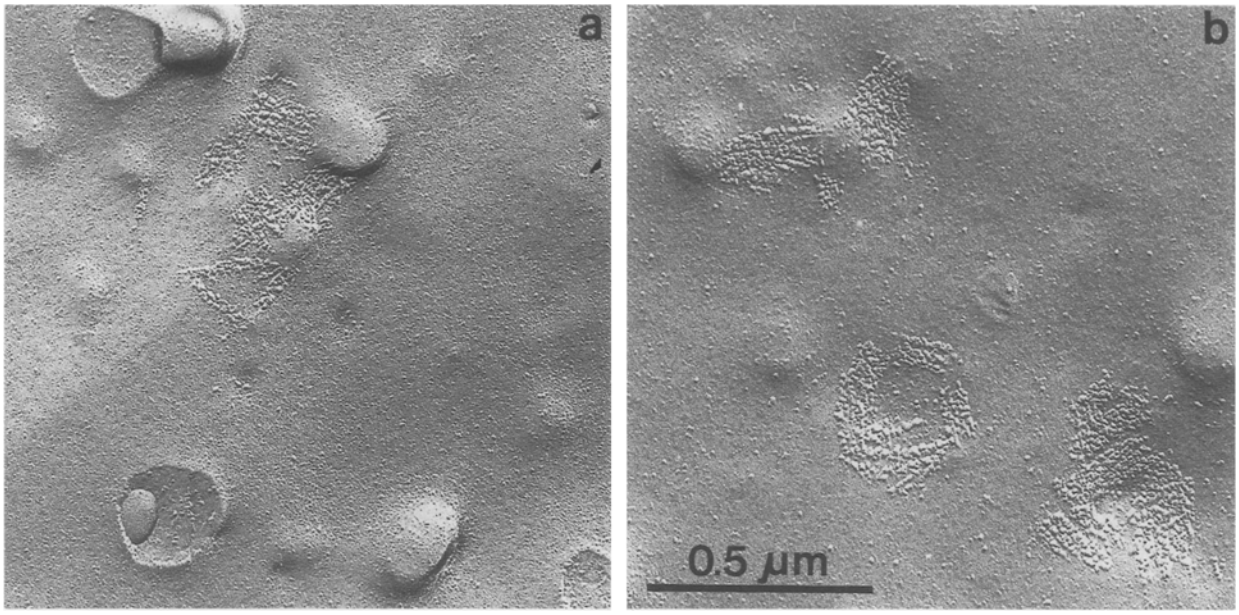

Fig. 6. Fine structure of the apical plasma membrane of (*a*) control and (*b*) PCMBS-treated bladders, in the presence of oxytocin. Typical particle aggregates, similar to those present in control, were observed in bladders treated with PCMBS (5 mM) and fixed after a maximal inhibition. As shown in (*b*), particularly large areas of particle aggregates are observed in this condition (60,000×)

Table 5. Relation between aggregate surface area and net water flow in bladders exposed to apical PCMBS

Inhibition	Condition	A Net water flux ($\mu l \cdot min^{-1} \cdot cm^{-2}$)	B $\frac{\text{Aggregate surface area}}{\text{total surface area}} \times 100$	B/A
Control	at rest ($n = 4$)	0.21 ± 0.10	0.02 ± 0.05	0.09 ± 0.07
	maximal response to oxytocin ($n = 5$)	2.06 ± 0.20	0.37 ± 0.07[a]	0.19 ± 0.04[a]
PCMBS	at rest ($n = 4$)	0.15 ± 0.08	0.01 ± 0.02	0.07 ± 0.04
	maximal response to oxytocin ($n = 6$)	0.64 ± 0.17	0.90 ± 0.05[a]	1.40 ± 0.4[a]

Bladders were first allowed to develop a maximal response to oxytocin. Then, the mucosal solution was replaced by 5 mM PCMBS (pH 5) or MES-Tris buffer pH 5 (control). When the inhibition by PCMBS was maximal, the samples were fixed and processed for freeze fracture. Some bladders at rest were also incubated with or without 5 mM PCMBS (pH 5) for 2 hr, fixed and processed.
[a] $P < 0.01$.

min after the inhibitor washout even in the presence of cysteine. It can be inferred, by analogy with red cells, that in frog urinary bladder these drugs penetrate very poorly and slowly into the cell. Apparent irreversibility under these circumstances is not surprising. The outside concentration driving the penetration of PCMBS is 5 mM, but the concentration near the inhibitory sites, given the high affinity for SH groups, can be very low. The reversal would be expected to be very slow even though the interac-

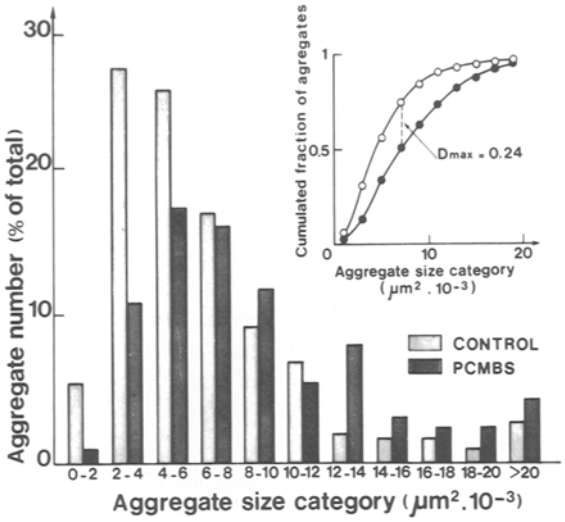

Fig. 7. Aggregate size distribution at the maximum of a response to oxytocin in control (left bars) and PCMBS (5 mM) treated bladders (right bars). For each aggregate size category, bars represent the percentage of aggregate number found in four experiments performed in control and PCMBS conditions. Inset represents statistical comparison between the calculated cumulative fractions of the aggregate number present in control (open dots) and PCMBS-treated bladders (black dots). The Kolmogorov-Smirnov test gave a significant difference between both conditions ($D_{max} = 0.24$; $N = 248$; $P < 0.01$)

tion of PCMBS with SH groups is chemically reversible.

The reaction of PCMBS with sulfhydryl groups is probably affected by the influence of pH on their environment (Cecil & McPhee, 1959; Nacchache & Sha'afi, 1974). The observation that PCMBS affects the net water flow at pH 5 and not at pH 8 could be explained as either an effect of pH on the reactivity of membrane sulfhydryl groups or as a change of the charge of PCMBS that increases its accessibility to sulfhydryl groups. The fact that the same net water flux inhibition was observed with FMA at pH 8 shows that a shift from pH 8 to 5 is important for the charge of PCMBS rather than for the charge of membrane proteins.

In order to determine the site of the action of PCMBS and FMA, experiments were also performed using cyclic AMP as the hydrosmotic agonist. As shown in Table 3, the mercurial compounds also reduced the hydrosmotic response to cyclic AMP. The step sensitive to mercurial drugs is thus located at a post-cyclic AMP site. Similar results were also found by Adragna and Bourguet (1987) using NEM.

The inhibition is dependent not only on the duration of the exposure of bladders to PCMBS or FMA during the hormonal stimulation, but also on the preincubation of the tissue with the first compound before the hormonal stimulation. Maximal inhibition by PCMBS and FMA (around 70%) in the presence of oxytocin was reached after 120 min. But, when PCMBS was also added before oxytocin, the same inhibition was observed after 30 min of incubation with the inhibitor in the presence of the hormone. A possible explanation of this potentiation is that an important number of nonspecific sulfhydryl groups are irreversibly blocked during preincubation before hormonal stimulation, so that only the sulfhydryl groups involved in the hydrosmotic response are now accessible to the inhibitor and so a greater degree of inhibition at shorter times can take place. A similar potentiation effect with sulfhydryl groups was reported in red blood cells for NEM (Ralston & Crisp, 1981; Benga et al., 1986) and in the frog urinary bladder for DTNB (Adragna & Bourguet, 1987).

In the present study, we found an inhibition of the hormone-induced water flux associated to an increase of the aggregate surface in the apical membrane of bladders exposed to 5 mM PCMBS at pH 5. If we accept that at the maximum of the response to ADH, the net water flux is moving across specific water channels in the apical border, a reduction in this parameter can result from: (*i*) a reduction in the number of permeability units, (*ii*) a reduction in the permeability of each channel and (*iii*) a reduction in the driving force for net water flow.

Experimental evidence shows that the aggregates are closely related to the water permeability in response to oxytocin (Chevalier et al., 1974; Kachadorian et al., 1975; Bourguet et al., 1976; Wade, Kachadorian & DiScala, 1977; Wade, 1978). Thus, the reduction in the number of permeability units could imply a reduction in the aggregate number. As we have observed an increase of the aggregate appearance in the presence of PCMBS, the first possibility could not be considered.

The effects of PCMBS or FMA could be due to a reduction in the permeability of the channel itself. A similar pattern of action was observed by Parisi and Bourguet (1984) during the cellular acidification of frog bladders. These authors showed that at low pH a water permeability inhibition with the presence of aggregates is observed and they suggested that this condition induced an impairment of the aggregate function. In our studies, the presence of aggregates concomitant with the low water permeability in oxytocin-stimulated bladders treated with mercurial compounds could be due to a modification of the constitutive proteins of the aggregates, which in turn alterate their function.

The inhibition of the net water flux by PCMBS or FMA could also result from a reduction of the driving force for the water permeation. In frog urinary bladder, a hydrosmotic response to oxytocin

could be falsely decreased if the membrane permeability to different solutes were to increase after PCMBS treatment. It has been reported that PCMBS causes a massive cation leak in red cells (Solomon et al., 1983) and also produces the stimulation of the Na^+ transport in toad bladder (Spooner & Edelman 1976), while it does not affect the Na^+ permeability in kidney proximal tubules (Whittembury et al., 1984). Although, we do not know whether PCMBS altered the permeability to other solutes in our preparation, we have observed that the transepithelial osmotic gradient was not significantly modified up to 90 min after PCMBS addition in the mucosal bath. At 120 min a reduction of 17% in the osmotic gradient was observed. But this alteration cannot obviously explain an inhibition of 75% found at this time by PCMBS treatment. On the other hand, measurements of unidirectional water flux in PCMBS-treated bladder show a significant inhibition even in the absence of an osmotic gradient (*in preparation*). Thus, the possibility that the inhibition of the osmotic permeability of PCMBS could be the result of an alteration of the osmotic gradient can be ruled out.

In relation to the mechanisms of ADH onset and offset in the presence of mercurial compounds, an inhibition of the oxytocin response concomitant with the retardation of its washout could be due to an eventual reduction of the osmotic gradient across the apical membrane (Muller & Kachadorian, 1984) or to a direct or indirect reduction of the endocytic process. Under these circumstances, an alteration of the retrieval of the aggregates could explain the amplification of the phenomenon of aggregation observed by us.

Although the role of the cytoskeleton in the aggregate movements to and from the apical membrane upon hormonal stimulation is not yet clear, several studies have shown that the disruption of the microtubule network induces an inhibition of hormone-stimulated water transport (Hardy, Montoreano & Parisi, 1973; Taylor et al., 1973; Kachadorian et al., 1979; Pearl & Taylor, 1985; Valenti, Hugon & Bourguet, 1988) and a delay in the time-course of the onset of the hydrosmotic response to oxytocin, leaving the offset unchanged (Brady, Parsons & Coluccio, 1981; Parisi et al., 1985; Valenti et al., 1987). Moreover, information on the role of microfilaments in the ADH-induced response suggest that these elements also participate in the mechanisms of insertion (Muller et al., 1980) and/or retrieval of the aggregates (Muller & Kachadorian, 1984; Parisi et al., 1985). As PCMBS-induced alterations in the aggregate distribution, it is possible that this effect may be the consequence of microtubule or microfilament modification. Kunimoto, Shibata and Miura (1987) have recently reported that another mercurial compound *p*-chloromercuribenzoate (PCMB), induces a dissociation of microfilament proteins and an aggregation of intramembrane particles in red blood cells. They suggested that this drug may dissociate transmembrane proteins involved in water transport from the peripheral cytoskeletal network modifying the functions of these proteins. Although, we found that PCMBS does not modify the structure of the cytoskeleton, the resolution of the technique employed is, however, low and our observations are not able to discriminate alterations in the organization of microfilaments and their relationship to microtubules or to the apical membrane. Thus, the possibility that the increase in aggregate density upon PCMBS or FMA treatments may be a consequence of alterations in the organization of microfilaments requires further study.

In conclusion, the present work shows that when the conditions of a maximal inhibition of ADH-stimulated net water flux in the presence of mercurial compounds are observed, the surface area occupied by the aggregates in the apical membrane is significantly increased. Thus two effects of the sulfhydryl mercurial reagent can be observed in the ADH-stimulated frog urinary bladder: an inhibition of the water flux across the water channels and an alteration of the relation between apical membrane and aggregates. If we continue to accept that water permeation through the aggregates and not through a barrier in series is the rate-limiting step of its transepithelial water transfer, we can conclude that PCMBS induces the closure of the water channels.

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