EVIDENCE THAT ADH-STIMULATED INTRAMEMBRANE PARTICLE AGGREGATES ARE TRANSFERRED FROM CYTOPLASMIC TO LUMINAL MEMBRANES IN TOAD BLADDER EPITHELIAL CELLS

JACQUELINE MULLER, WILLIAM A. KACHADORIAN, and VINCENT A. DISCALA

From the Membrane Research Laboratory, Renal Service, U. S. Public Health Service Hospital, Staten Island, New York 10304

ABSTRACT

In freeze-fracture (FF) preparations of ADH-stimulated toad urinary bladder, characteristic intramembrane particle (IMP) aggregates are seen on the protoplasmic (P) face of the luminal membrane of granular cells while complementary parallel grooves are found on the exoplasmic (E) face. These IMP aggregates specifically correlate with ADH-induced changes in water permeability. Tubular cytoplasmic structures whose membranes contain IMP aggregates which look identical to the IMP aggregates in the luminal membrane have also been described in granular cells from unstimulated and ADH-stimulated bladders. The diameter of these cytoplasmic structures (0.11 ± 0.004 μm) corresponds to that of tubular invaginations of the luminal membrane seen in thin sections of ADH-treated bladders (0.13 ± 0.005 μm). Continuity between the membranes of these cytoplasmic structures (which are not granules) and the luminal membrane has been directly observed in favorable cross-fractures. In FF preparations of the luminal membrane, these apparent fusion events are seen as round, ice-filled invaginations (0.13 ± 0.01 μm Diam), of which about half have the characteristic ADH-associated aggregates near the point of membrane fusion. They are less numerous than, but linearly related to, the number of aggregates counted in the same preparations ($n = 78$, $r = 0.71$, $P < 0.01$). These observations suggest that the IMP aggregates seen in luminal membrane after ADH stimulation are transferred preformed by fusion of cytoplasmic with luminal membrane.

Freeze-fracture electron microscopy has shown that stimulation of the isolated amphibian urinary bladder with antidiuretic hormone (ADH) results in a structural change in the luminal membrane of granular epithelial cells (1, 3, 11, 12). Whereas in the unstimulated bladder intramembrane particles (IMP) are randomly distributed in an apparently homogeneous matrix, after ADH stimulation IMP aggregates of various sizes are seen at discrete membrane sites on the protoplasmic half-membrane (fracture face P). Complementary areas of the apposed exoplasmic half-membrane (fracture face E) exhibit parallel rows of depressions (grooves), reflecting the linear organization of par-

ticles (11, 12). Both the number of aggregates and the area of luminal membrane they occupy have been shown to be specifically related to the level of ADH-induced toad bladder water permeability (7, 9–12). The constancy of the relationship between osmotic water permeability and aggregates has led to the hypothesis that aggregates are or contain the actual channels for transmembrane water flow (10).

Initially, there appeared to be two possibilities to explain the origin of aggregates: either assembly from IMP already present in the luminal membrane or insertion of new material (preaggregated or not) from the cytoplasm of the granular cell (12). Observations which argued against the former possibility were that: (*a*) ADH stimulation did not change the number or distribution of IMP which were not part of aggregates (12), and (*b*) as early as 2.5 min after ADH stimulation (when the physiologic response to ADH is submaximal), although less numerous, the aggregates already showed the same size distribution as after 30 min of stimulation when both aggregate number and water flow are near a maximum (7). Recently, evidence for the possible cytoplasmic origin of aggregates was provided independently by Humbert et al. (6) and Wade (24), who described, in freeze-fracture preparations of both resting and ADH-stimulated bladders, cytoplasmic structures whose membranes contained linear arrays of aggregated IMP on their P faces and parallel grooves on their E faces, this arrangement being identical to that seen in the luminal membrane after ADH stimulation.

In this paper we confirm the existence of intracellular structures whose membranes contain preformed aggregates and show, with evidence from thin-section and freeze-fracture electron microscopy, that ADH stimulation induces their fusion with the luminal membrane. Morphologic recognition of the fusion event in luminal membrane fractures has enabled us to show a quantitative relationship between the number of fusion events and the number of aggregates added to the luminal membrane. Experiments with colchicine and cytochalasin B are interpreted to suggest roles for microtubules and microfilaments in the process of membrane fusion and aggregate insertion.

MATERIALS AND METHODS

Some experiments analyzed in this study were originally done to assess the relationship between osmotic water flow and aggregates at different times after ADH-stimulation and under different experimental conditions. The time-course data are found in reference 7 and the colchicine and cytochalasin B data in reference 8.

In general, the protocol was as follows. Urinary hemibladders from double-pithed Dominican toads (*Bufo marinus*) were prepared as sacs on the ends of glass tubes. After they were washed inside (mucosal surface) and out (serosal surface) with Ringer's solution (111 mM NaCl, 3.5 mM KCl, 2.5 mM $NaHCO_3$, 1.0 mM $CaCl_2$; pH 7.6–8.2; 220 mOsm/kg H_2O), they were suspended in an aerated Ringer's bath and their mucosal volumes were replaced with Ringer's solution diluted 1:5 with distilled water. During a 30-min equilibration period, transbladder electrical potential (PD) was measured and, if it was found to be <20 mV, the particular experiment was terminated. Bladders were stimulated maximally with ADH (arginine vasopressin, Sigma Chemical Co., St. Louis, Mo., 20 mU/ml serosal solution) for the times indicated in Results and in Figs. 9 and 10. Water flow was measured gravimetrically.

For studies in which colchicine or cytochalasin B treatment preceded vasopressin stimulation, control bladders were put in fresh, aerated Ringer's baths and experimental bladders in similar baths which in addition contained either 0.1 mM colchicine (Sigma) or 0.04 mM cytochalasin B (Sigma). In all of these experiments, after an incubation period of 3 h, both control and experimental bladders were stimulated with vasopressin for 30 min.

For studies in which treatment with either colchicine or cytochalasin B followed vasopressin stimulation, the procedure was first to stimulate paired control and experimental hemibladders with vasopressin for 30 min and then to transfer them to fresh baths containing either vasopressin or vasopressin plus either 0.1 mM colchicine or 0.04 mM cytochalasin B. Measurements of osmotic water flow were made before and during initial vasopressin stimulation and during the final 30 min of a 3.5-h experimental period.

Bladders for freeze fracture were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 15 min and then stored in 0.1 M cacodylate buffer at 4°C. Before fracture, tissues were treated with 25% glycerol in 0.1 M cacodylate buffer for at least 60 min. Freeze fracture was performed with a Balzers freeze-etch unit (BAF 301) (Balzers Corp., Nashua, N. H.).

Bladders for thin-section electron microscopy were fixed with the same glutaraldehyde fixative as described above for 1 h before storage in buffer. Tissue was later postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated with ethanol, and embedded in epoxy resin. Both platinum-carbon replicas from freeze fracture and thin plastic sections were examined in either a Zeiss EM 10A or RCA EMU 4B electron microscope.

Membrane fusion events were counted in the same micrographs from which aggregate frequency had previously been determined. The method of quantitation involved taking a single random micrograph of each of 10–14 randomly selected cells for each bladder studied. The total area examined per bladder varied between 220 and 330 μm^2. Aggregates and membrane fusion events were counted and their frequency per reference area of membrane (235 μm^2) was calculated.

The volume density of granules in unstimulated vs. ADH-stimulated bladders was assessed in a separate series of six experiments. Paired hemibladders were prepared as described above except that bladder sacs were filled with full-strength Ringer's so as to minimize cell swelling due to osmotic water flow. One of each pair was stimulated maximally with ADH. PD and short-circuit current (SCC) were measured at 5-min intervals. ADH-treated bladders showed the characteristic changes in PD

and SCC. After 30 min, both bladders were fixed for thin-section electron microscopy. Twenty small pieces were cut from each hemibladder and processed until Epon infiltration; ten of these pieces were then chosen at random for final embedding. Of the ten resulting blocks from each hemibladder, five were selected at random for sectioning. One minimally-obstructed section from each of the five blocks was photographed at ×20,000 according to a predetermined pattern; ~10 micrographs/block were obtained. Micrographs, printed at a final magnification of ×55,000, were analyzed by the point-counting method of Weibel (26). A 0.25-in lattice was used for counting the number of points on granules (Pg) and a 0.50-in lattice for the number of cytoplasmic points (Pc). In our sac preparations the epithelium is sufficiently stretched so that the micrograph contained the entire width of the granular cell. Volume density of the granules was calculated for each block as Pg/Pc × 100. An overall mean for each hemibladder was then derived, giving equal weight to the value for each block.

The statistical methods used in this work include: Student's *t* test for comparison of means for paired or unpaired observations,

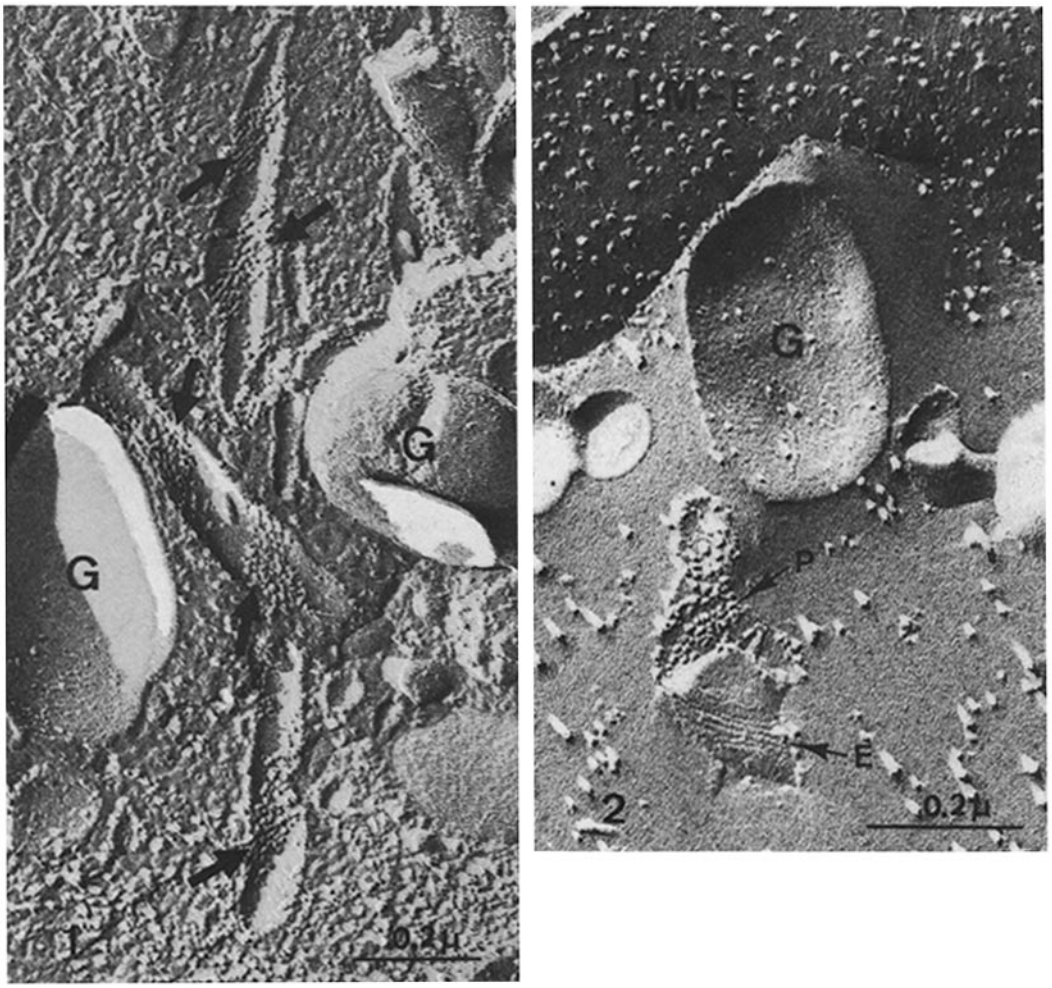

FIGURE 1 Cross fracture of a granular epithelial cell from an unstimulated control bladder. The membranes of certain intracellular tubular structures contain parallel arrays of intramembrane particles (IMP aggregates) on their P fracture faces (arrows) that are identical to the IMP aggregates seen on the P face of the luminal membrane after ADH stimulation (cf. Fig. 8 *a*). Smooth areas of membrane separate IMP aggregates. *G*, granule. × 85,000.

FIGURE 2 An intracellular, aggregate-containing membrane fractured such that both P and E faces are revealed. The parallel grooves seen on the E face are the impressions of linear IMP arrays (aggregates) on the apposed P face. From an ADH-stimulated bladder. *G*, granule. *LM-E*, E face of the granular cell luminal membrane. × 102,000.

regression analysis by the method of least squares, and calculation of the correlation coefficient (r) (23). To judge statistical significance, the 95% confidence limit was used.

RESULTS

Intracellular, Aggregate-containing Membrane Structures

Cross fractures of granular cells from both unstimulated (Fig. 1) and ADH-stimulated (Figs. 2 and 3) bladders reveal structures in the cytoplasm whose membranes have parallel arrays of IMP on their P faces and parallel grooves on their E faces. That the parallel grooves are the impressions left by the closely spaced particles can be inferred from the occasional fracture in which both the P and E faces of the same structure are seen (Fig. 2). Wade, moreover, has shown this directly with complementary replicas (24). These structures have a tubular shape with a relatively uniform diameter of ~0.11 μm. Profiles resembling vesicles are also seen, but these may represent partial fracture of a longer, tubular structure. The overall length of such tubular membrane structures was not determined, but lengths up to 1.12 μm were measured. The parallel linear arrays of IMP (or grooves) appear to be disposed at an angle to the long axis of the tubule, which perhaps imparts structural stability and accounts for their uniform diameter (Figs. 1, 2, and 3). IMP are not the subunits from which the tubule is constructed, however, since variable smooth spaces are often seen between groupings of aggregates (Fig. 1). Such membrane structures were not found to have a preferential distribution within the granular cell.

Evidence for Fusion of Intracellular, Aggregate-containing Membrane Structures with the Luminal Membrane after ADH Stimulation

In freeze-fracture preparations of ADH-treated bladders, continuity between the luminal membrane and the type of intracellular membrane structure described above is seen. Fig. 4 shows the E face of the luminal membrane continuous with the E face of an aggregate-containing intracellular membrane. Both membranes have similar parallel grooves (arrows). Such images led to the hypothesis that in response to ADH treatment the previously separate intracellular membranes fuse with the luminal membrane.

Favorable fractures showing membrane continuity are rare. Supportive evidence for fusion of cytoplasmic with luminal membrane was provided by thin-section electron microscopy. In unstimulated bladders there are intracellular membrane structures which correspond morphologically to the cytoplasmic aggregate-containing structures seen in freeze-fracture preparations. As seen with thin-section electron microscopy, these structures have tubular or vesicular profiles with diameters of ~0.10 μm and appear devoid of internal content. Moreover, they may be found in any part of the granular cell and, in the absence of ADH, are not continuous with the luminal membrane. This latter situation changes dramatically after ADH stimulation (Figs. 5 and 6). Membrane profiles of this type are then often found near the luminal membrane, and many instances of continuity are seen, even in random sections. The invaginations of the luminal membrane seen after ADH treatment are thought to represent fusion with intracellular membranes rather than luminal membrane deformation because membrane structures of this type are seen in the cytoplasm before ADH and because the invaginations are relatively long. The one shown in Fig. 5 is ~0.68 μm and others up to 1.7 μm have been measured.

When the electron-opaque extracellular marker lanthanum is added to the mucosal solution during ADH stimulation many labeled tubular and vesicular profiles are seen in random thin sections (unpublished observations). Similar observations were made by Masur et al., using horseradish peroxidase (15). Serial sections, however, show that these labeled structures often represent section of a longer tubular structure which is, in many cases, continuous with the luminal membrane.

Evidence That These Fusion Events Do Not Involve Granules

Exocytosis of granules has been reported in toad bladder stimulated with oxytocin or dibutyryl cyclic AMP for 2–5 h (16). In random thin sections, intracellular membrane structures fused with the luminal membrane sometimes have vesicular profiles suggesting emptied granules. Other observations indicate, however, that these are not granules. First, a comparison of these structures with granules in thin section shows some basic differences in size and shape. Granules are membrane-bounded, ovoid, flattened structures with a variably dense content which sometimes appears to have a crystalline substructure. Although thin sec-

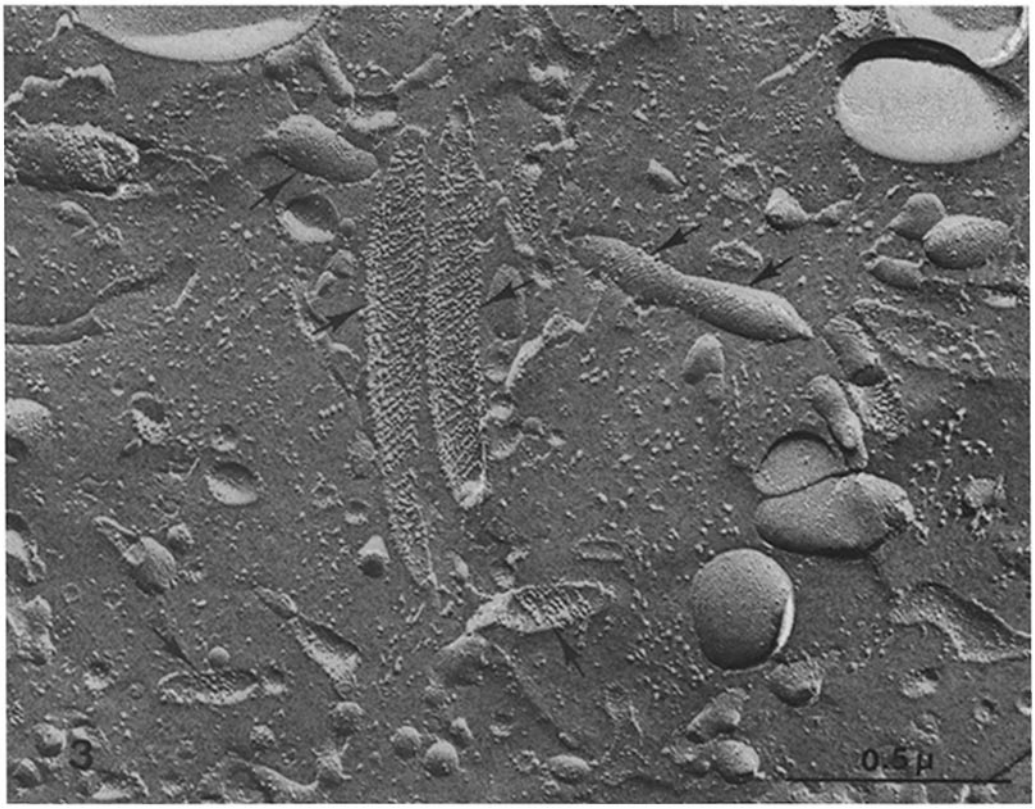

FIGURE 3 Granular cell of an ADH-treated bladder. The P and E faces of aggregate-containing membranes are indicated (arrows). Such membranes are most often seen as tubular structures in the cell. × 57,500.

tions through such a shape can produce profiles of varying dimensions, the short diameter of granules was found to be 0.22 ± 0.01 μm and the long diameter 0.36 ± 0.01 μm. Luminal membrane invaginations on the other hand had a fairly consistent diameter of 0.13 ± 0.005 μm. Similar differences are also seen in the freeze-fracture preparations where intracellular, aggregate-containing membrane structures had a diameter of 0.11 ± 0.004 μm as compared with the short diameter of granules which was 0.24 ± 0.01 μm. Second, granule membranes are characterized by their lack of IMP (Figs. 1 and 2). All cases of continuity between the luminal membrane and cytoplasmic membrane structures involved intracellular membranes with linear arrays of particles or grooves. Finally, stereologic measurements done on thin sections of paired hemibladders, which were either unstimulated or stimulated for 30 min with ADH in the absence of an osmotic gradient, showed no difference in granule content. The volume density of granules in the unstimulated bladder was 2.45 ± 0.42% and that of the ADH-stimulated bladder was identical (2.26 ± 0.36%). Furthermore, there was no difference between stimulated and unstimulated hemibladder pairs in either Pg, total number of granule points (443 ± 91 vs. 527 ± 110) or Pc, total number of cytoplasmic points (19.4 ± 1.5 × 10^3 vs. 20.1 ± 1.7 × 10^3). The same lack of effect of ADH on granule content has also been reported by others (22, 25).

Quantitation of Fusions of Intracellular, Aggregate-containing Membrane Structures with Luminal Membrane

P and E fracture faces of granular cell luminal membrane of unstimulated bladders are shown in Fig. 7 *a* and *b*, respectively. In the well-stretched bladder, microvilli are not as prominent as in unstretched preparations. Each fracture face has randomly distributed IMP of a characteristic size.

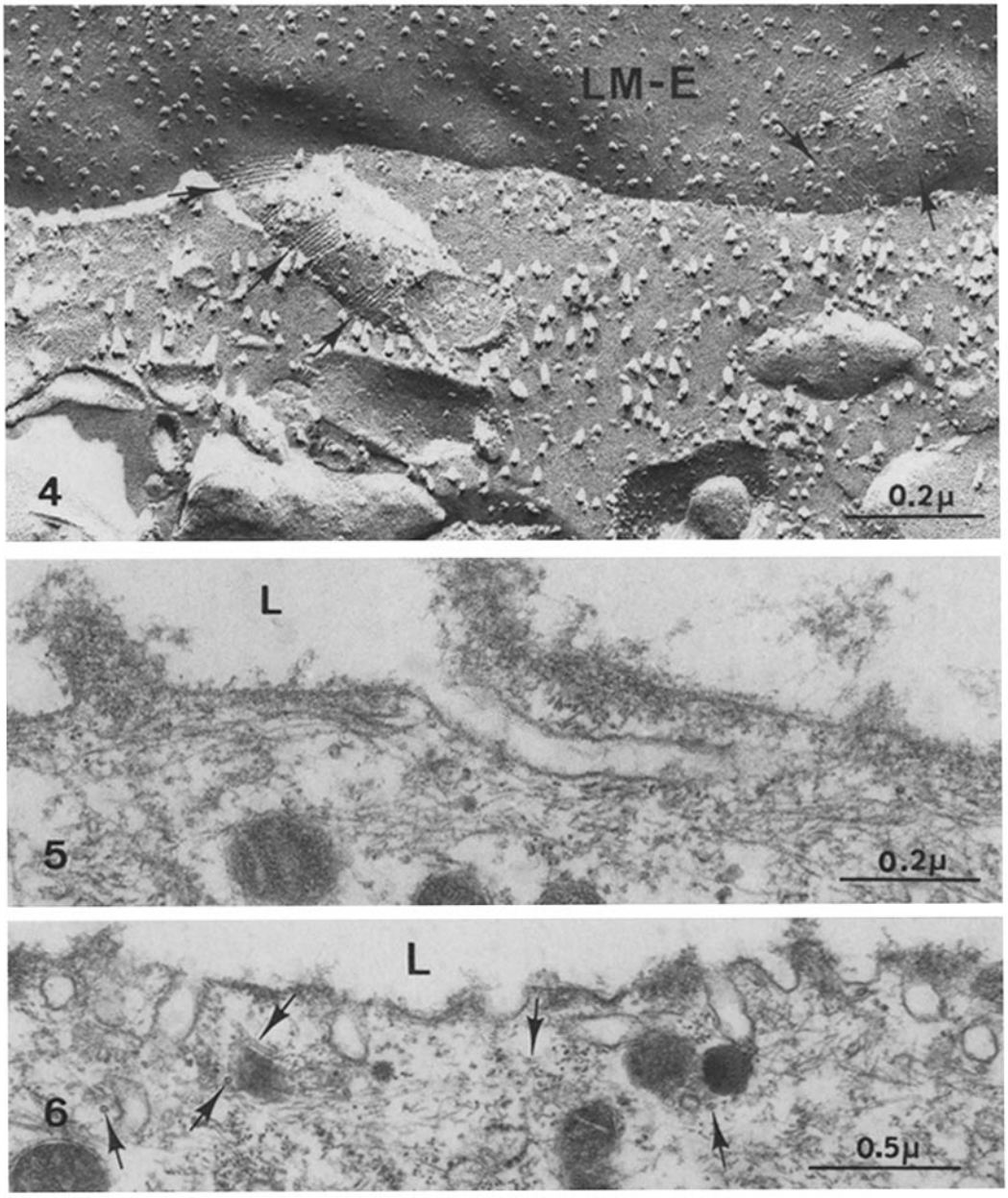

FIGURE 4 Intracellular, aggregate-containing, tubular membrane structure fused with the luminal membrane (*LM-E*) in an ADH-treated bladder. In this fracture, the E faces of the cytoplasmic and luminal membranes are continuous. The parallel grooves on the luminal membrane and those on the cytoplasmic membrane (arrows) are similar. × 95,000.

FIGURE 5 A tubular, cytoplasmic structure fused with the luminal membrane. The overall length of such intracellular membrane structures is not known. In thin section, lengths up to 1.7 μm have been measured. Granular cell from a bladder stimulated with ADH for 30 min. *L*, bladder lumen. × 67,000.

FIGURE 6 Granular cell from an ADH-stimulated bladder. In random sections, cytoplasmic membrane structures close to or fused with the luminal membrane are frequently seen. In serial sections, many of the vesicular profiles were found to be sections of longer, tubular structures in continuity with the luminal membrane. Arrows indicate microtubules. × 42,500.

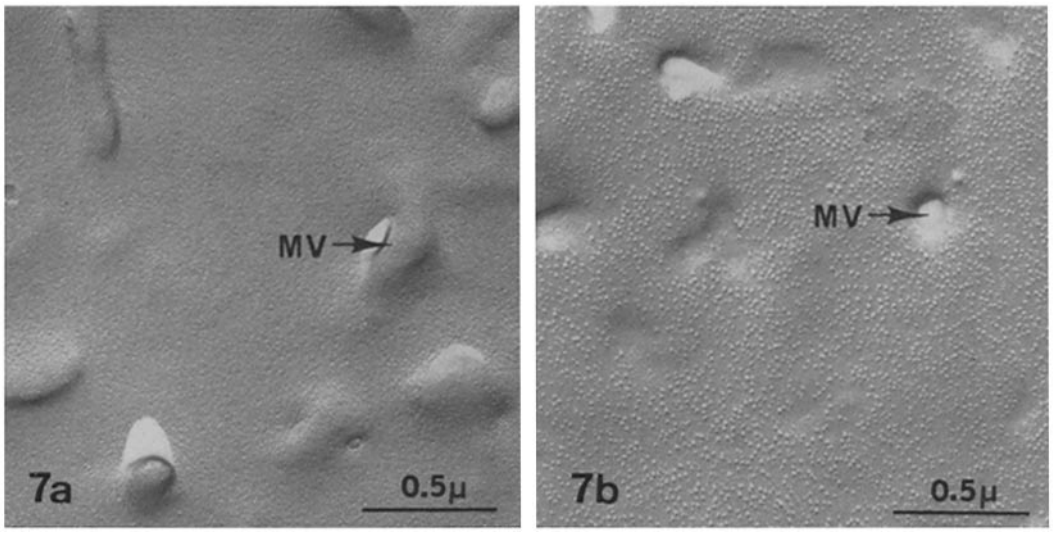

FIGURE 7 Luminal membranes of unstimulated control bladders. IMP are randomly distributed. *MV*, microvillus. (*a*) P fracture face. × 35,500. (*b*) E fracture face. × 35,000.

They appear larger and more numerous on the E face. Aggregated IMP are almost never observed in the isolated, unstimulated bladder.

The P and E faces of luminal membrane shown in Fig. 8 *a* and *b* are from ADH-stimulated bladders. The internal structure of the membrane is now seen to include discrete areas of aggregated IMP which fracture with the P face (Fig. 8 *a*, arrows). The parallel grooves which are seen on the apposed E face are impressions left by the linear arrays of particles (11, 12) (Fig. 8 *b*, arrows). In addition, the P face now has round-to-oval, ice-filled depressions which are sometimes associated with aggregates (Fig. 8 *a*, asterisks). On the complementary E face these areas appear as similarly shaped, ice-filled elevations, sometimes associated with parallel grooves (Fig. 8 *b*, asterisks). These images were interpreted to reflect an *en face* view of the fusion of intracellular aggregate-containing membrane structures with the luminal membrane. The reason why they appear as ice-filled depressions on the P face or slight elevations on the E face rather than as deep holes or projections is probably that the fracture plane cannot follow a membrane set at sharp angles to its preferred direction. Many microvilli are truncated for the same reason. As invaginations of the luminal membrane, they are filled with aqueous mucosal solution. The mean diameter of these depressions was 0.13 ± 0.01 μm, which is close to that observed for intracellular aggregate-containing structures in freeze fracture (0.11 ± 0.004 μm) as well as to that measured in thin section for intracellular structures in continuity with the luminal membrane (0.13 ± 0.005 μm).

The number of presumptive membrane fusion events was quantitated for different times of ADH stimulation and hormonal washout (Fig. 9). Osmotic water flow and aggregates for each of these bladders were known. Because these fusion events were thought to involve intracellular aggregate-containing membrane structures, note was also made of the presence or absence of aggregates touching the circumference of the depression, which would be the approximate point of fusion of the two membranes. In the unstimulated bladder, where aggregates are not present by random sampling, there were rare images which resemble membrane fusions. An example is shown in the lower right of Fig. 7 *a*. They were not associated with aggregates. At 2.5 min of ADH stimulation the number of fusion events increased, ~73% of them being associated with aggregates. The response is maximal in bladders stimulated for 10 min and does not differ with 20, 30, or 60 min of stimulation. Between 10 and 60 min, ~50% of fusion events are associated with aggregates. With 5 and 15 min of hormonal washout, the number of fusions, as well as the portion associated with aggregates, progressively decreases.

The time course relationship for membrane fusion events closely parallels that for aggregates

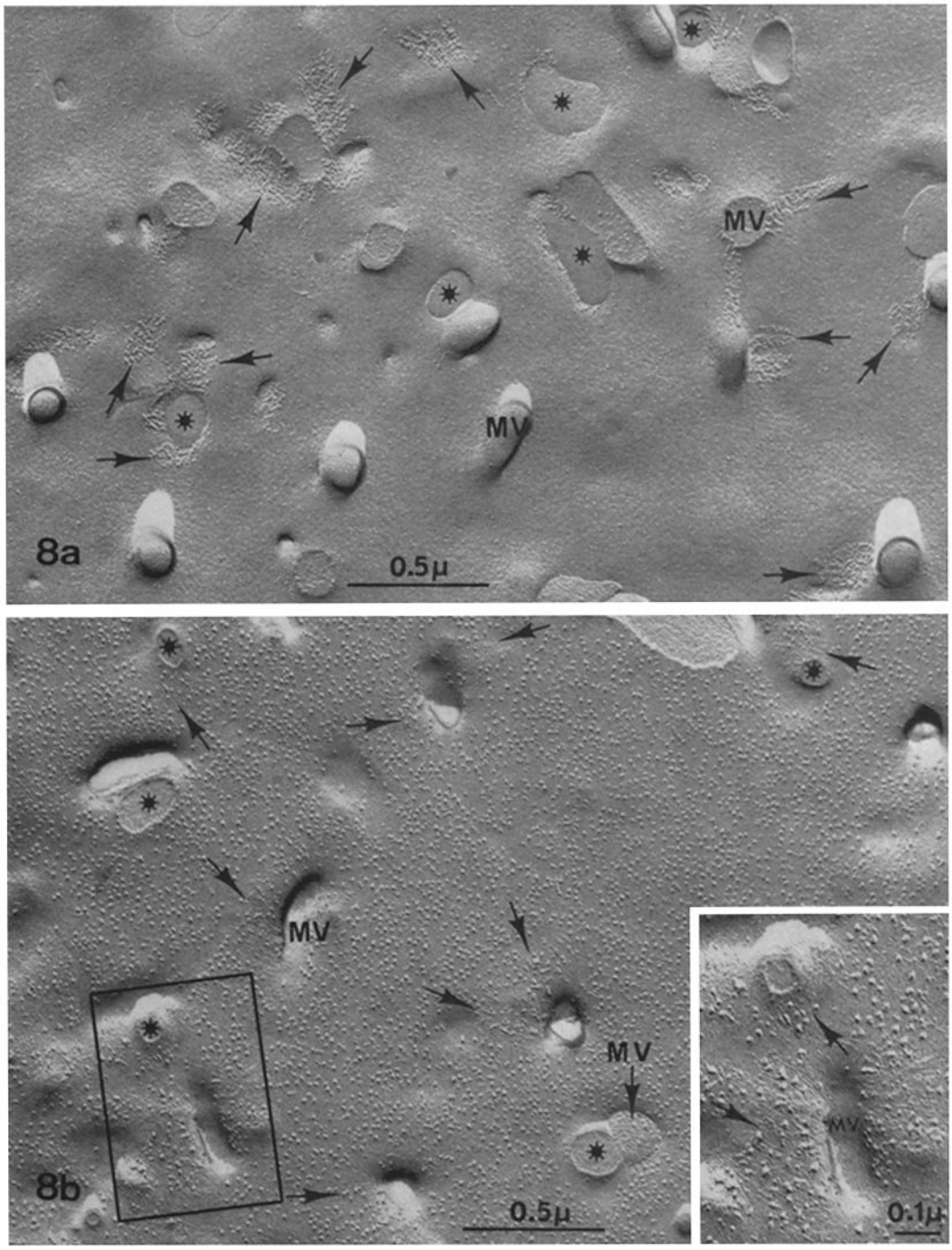

FIGURE 8 Luminal membrane of bladders stimulated with ADH for 30 min. *MV*, microvillus. (*a*) P fracture face. Compare with Fig. 7*a*. Numerous IMP aggregates are present on the P face (arrows). Asterisks indicate some of the ice-filled depressions that result from the fusion of cytoplasmic tubular membrane structures with the luminal membrane. Aggregates are sometimes associated with the membrane fusion sites. × 42,500. (*b*) E fracture face. Compare with Fig. 7*b*. Parallel grooves reflect the linear organization of IMP in aggregates (arrows). Asterisks indicate the ice-filled elevations that result from the fusion of cytoplasmic tubular membrane structures with the luminal membrane. Grooves are sometimes associated with these fusion sites. × 42,500. *Inset* is a higher magnification of the enclosed rectangular area. × 66,000.

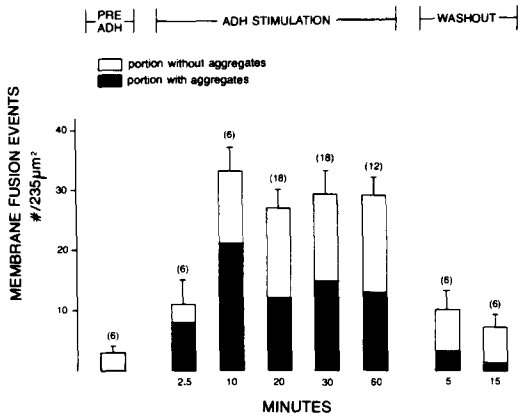

FIGURE 9 The number of membrane fusion events per 235 μm^2 area of luminal membrane in the unstimulated condition, at various times after a standard maximal dose of ADH (20 mU/ml, arginine vasopressin), and after 5 and 15 min of hormonal washout. The number of bladders examined for each case is given in parentheses. The height of each bar shows the total number ±SEM and is shaded to indicate the number of fusions associated with aggregates (dark) and the number not associated with aggregates (light).

assessed in the same bladders. In a plot of fusion events vs. aggregate frequency (Fig. 10), fusions are much less numerous than, but linearly related to, the number of aggregates counted in the same preparations ($r = 0.71$, $n = 78$, $P < 0.01$).

Evidence That Microtubules Are Involved in Membrane Fusion Events

Treatment of bladders with colchicine decreased the number of formed microtubules in granular epithelial cells by ~63% (8). When colchicine treatment precedes ADH stimulation, osmotic water flow is inhibited and aggregates are less numerous. If, on the other hand, fully stimulated bladders are treated with colchicine, although about the same decrease in microtubule content occurs (73%), neither osmotic water flow nor the number of aggregates is affected (8). These findings suggest that microtubules are involved in the initiation, but not the maintenance, of both phenomena.

Membrane fusion events were quantitated in the same pre- or post-ADH colchicine-treated bladders which were used to measure water flow and aggregates (Table I). Colchicine pretreated bladders (upper pair) had significantly fewer membrane fusion events after ADH-stimulation than the untreated ADH-stimulated controls. When colchicine treatment followed the establishment of a full ADH response (lower pair), however, the number of fusion events was not different in bladders exposed to colchicine vs. controls. These data suggest that intact microtubules normally function to promote the fusion of cytoplasmic structures with the luminal membrane and that this is one of the initiating events which leads to the appearance of aggregates. Once fusion has occurred, however, microtubules do not seem to be necessary for the maintenance of membrane continuity.

In thin-section, microtubules are frequently found near these intracellular membrane structures and in the vicinity of the luminal membrane (Fig. 6). Several instances of apparent attachment

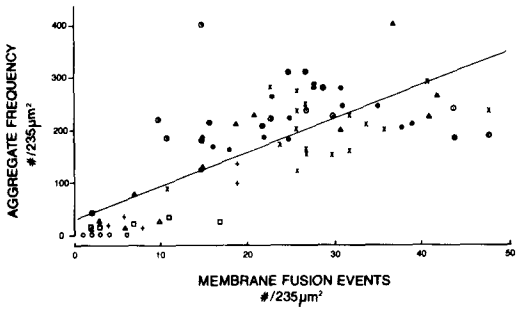

FIGURE 10 Relationship between the numbers of aggregates and membrane fusion events per standard 235 μm^2 area of luminal membrane. Same bladders as in Fig. 9. Each point represents data from a single bladder ($n = 78$). $y = 30.4 + 6.3+$; $r = 0.71$; $P < 0.01$. ○, no ADH; +, 2.5 min; ▲, 10 min; ⊙, 20 min; ×, 30 min; ●, 60 min; △, 5-min wash; □, 15-min wash.

TABLE I

Effects of Pre- or Post-ADH Treatment with Colchicine (COL) on the Number of Membrane Fusion Events per 235 μm^2 of Granular Cell Luminal Membrane

	Membrane fusion events per 235 μm^2			
	With aggregates	Without aggregates	Total	*P*
ADH	18 ± 4	15 ± 1	33 ± 4	<0.01
COL + ADH	9 ± 2	11 ± 2	20 ± 4	
ADH	10 ± 5	26 ± 6	36 ± 10	NS
ADH + COL	11 ± 4	21 ± 4	32 ± 7	

The time of ADH stimulation was 30 min for the upper pair and 4 h for the lower pair. Colchicine treatment in both sets of experiments was 3.5 h.

Values represent the mean ±SEM; $n = 6$ for each case. *P*, probability of a statistical difference in total number of fusion events.

of microtubules to the cytoplasmic structures (Fig. 11) and the luminal membrane (Fig. 12) were noted.

Evidence That Microfilaments Are Not Involved in Membrane Fusion

Treatment of cells with cytochalasin B reportedly disrupts actinlike microfilaments (2, 20). Pretreatment of bladders with cytochalasin B significantly inhibits subsequent ADH-stimulated osmotic water flow and aggregates (8). The number of membrane fusion events measured in these same bladders, however, is not different in the control vs. the cytochalasin B-treated bladders (Table II, upper pair). When cytochalasin B treatment follows maximal ADH stimulation, osmotic water flow and aggregates are also significantly inhibited (8). In this case as well, the number of membrane fusion events is not altered by treatment with cytochalasin B (Table II, lower pair). These data suggest that microfilaments are not involved either in the initial step of fusion between cytoplasmic and luminal membrane or in the maintenance of membrane continuity once fusion has occurred.

DISCUSSION

The finding that variations in a membrane function accompany variations in the internal structure of that membrane argues for the fundamental importance of the structural changes involved. For the isolated amphibian urinary bladder, such a relationship exists between ADH-stimulated luminal membrane osmotic water permeability and the presence of characteristic aggregates of IMP. That aggregates are not the result of transmembrane water flow has been shown by the fact that they are found in the absence of an osmotic gradient where hormonally stimulated water flow is negligible (1, 9, 12).

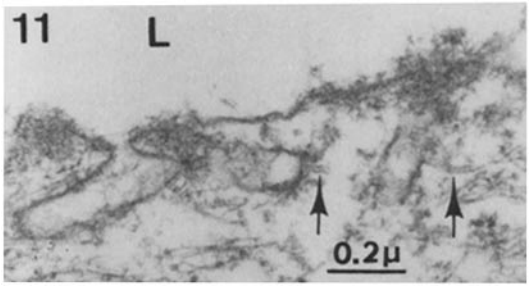

FIGURE 11 Tubular membrane structure fused with the luminal membrane in an ADH-stimulated bladder. Arrows show microtubules associated with the intracellular membranes. *L*, bladder lumen. × 61,500.

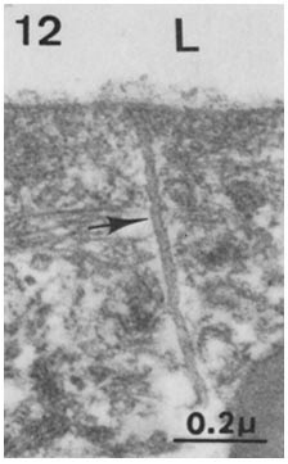

FIGURE 12 Arrow shows a microtubule associated with the luminal membrane. From a bladder stimulated with ADH for 20 min. *L*, bladder lumen. × 60,000.

In other systems, factors such as cold (19), acidity (18), and glycerol treatment without glutaraldehyde fixation (14) can cause IMP to cluster. Although IMP thus become aggregated, we would stress the difference between this type of association and what we have called "ADH-stimulated aggregates." Clustering as a result of changes in electrical charge or lipid environment involves IMP already present in the membrane. The groups so formed lack any apparent organization. In contrast, ADH-stimulated aggregates are groups of organized IMP whose special characteristics appear to be responsible for the measured changes in membrane water permeability. Moreover, in our recent studies, unstimulated bladders did not show a clustering response when exposed to moderate cold (10).

The combined evidence from freeze-fracture and thin-section electron microscopy presented here indicates that these specific aggregates exist preformed in the membranes of intracellular structures and that ADH stimulation triggers a mechanism by which they are added to the luminal membrane. This conclusion is based on the observation that aggregates are not present in the luminal membrane before ADH whereas at this same time they are present in certain cytoplasmic membranes. These cytomembranes are reminiscent of a tubulo-cisternal endoplasmic reticulum which has been described in other transporting

TABLE II

Effects of Pre- or Post-ADH Treatment with Cytochalasin B (CB) on the Number of Membrane Fusion Events per 235 μm^2 of Granular Cell Luminal Membrane

	Membrane fusion events per 235 μm^2			
	With aggregates	Without aggregates	Total	*P*
ADH	12 ± 2	14 ± 2	26 ± 3	NS
CB + ADH	10 ± 2	24 ± 3	34 ± 4	
ADH	4 ± 2	23 ± 3	27 ± 5	NS
ADH + CB	4 ± 2	22 ± 4	26 ± 5	

The time of ADH stimulation was 30 min for the upper pair and 4 h for the lower pair. CB treatment in both sets of experiments was 3.5 h.

Values represent the mean ±SEM; $n = 6$ for each case. *P*, probability of a statistical difference in total number of fusion events.

epithelia (17). The entire argument rests on the assumption that the linear arrays of IMP which are in cytoplasmic membranes are in fact the same as the morphologically similar aggregates found in the luminal membrane after ADH. This assumption is strengthened further by findings that ADH induces the fusion of intracellular structures with luminal membrane and that aggregate-containing membranes appear to be exclusively involved in these fusion events. Moreover, when the number of fusion events is quantitated in luminal membrane fractures, a linear relationship is found under normal conditions between the number of fusions and the number of aggregates in the luminal membrane.

The initiation of fusion seems to be dependent on intact microtubules. When the number of formed microtubules is appreciably reduced, the number of fusion events is also significantly decreased. Since in the pre-ADH condition aggregate-containing membranes are found away from the luminal membrane, one likely role for microtubules might be to bring a part of the cytoplasmic tubular structure close enough to the luminal membrane for fusion to occur. The finding that microtubules may be attached to both the luminal membrane and intracellular aggregate-containing membranes provides a physical basis for such a mechanism. Translocation of organelles is a well-documented function of microtubules and, in the case of membrane-bounded secretion products, intact microtubules seem to be required for membrane fusion leading to exocytosis (13, 21).

If aggregates are present exclusively in the membranes of cytoplasmic structures before ADH and in the luminal membrane after ADH, it follows that either new membrane containing aggregates is incorporated into the surface membrane, or that the aggregates move from one position to the other during the period of continuity and that the carrier membrane eventually detaches. Precedents for both mechanisms have been described. The release of secretory products from glandular cells occurs by exocytosis in which the containing membrane is incorporated into the plasmalemma. Excess membrane is subsequently retrieved by pinocytosis to maintain a constant cell volume (5). In the case of the trichocyst membrane of *Paramecium*, on the other hand, continuity between it and the cell membrane lasts only for the time of trichocyst discharge (4). Immediately after discharge the trichocyst membrane detaches from the plasmalemma and disintegrates in the cytoplasm. Wade has found that granular cells from ADH-stimulated bladders have fewer intracellular aggregate-containing membrane structures than cells from unstimulated bladders (25). No conclusion can be reached, from this observation, as to whether the cytoplasmic loss involves the entire membrane or the aggregates alone, since the presence of aggregates is the only way of distinguishing these membranes in freeze-fracture preparations.

It would be difficult to rule out the possibility that some parts of these intracellular membranes are incorporated into the luminal membrane. The following observations, however, favor the hypothesis that once fusion has occurred the membranes remain in continuity as long as ADH is present and that aggregates progressively move from the cytoplasmic portion to and away from the point of fusion. For bladders exposed, under similar experimental conditions, to various times of maximal ADH stimulation, it has been observed that the number of fusion events does not change between 10 min and 4 h (Fig. 9, Tables I and II). A comparison of the control (ADH) bladders in Tables I and II illustrates this point. In each case the control bladders in the upper set were stimulated with ADH for 30 min while those of the lower set were stimulated for 4 h. There is no difference in the total number of fusions between 30 min and 4 h (33 ± 4 vs. 36 ± 10 and 26 ± 3 vs. 27 ± 5, respectively). Evidence that this constancy in the number of fusion sites for the duration of ADH stimulation probably does not represent a dynamic equilibrium comes from the colchicine

experiments (Table I). Pretreatment with colchicine significantly decreases the number of fusion events. If the number of luminal membrane fusion sites were the net result of attachment and detachment of membrane structures, one would expect that colchicine treatment subsequent to full ADH stimulation would also result in a decreased number of fusion sites by interfering with the fusion process. Instead, post-ADH colchicine treatment had no effect on the total number of fusion events, thereby suggesting that, once initiated, the membrane continuity is maintained as a stable conformation. A decrease in the proportion of fusions directly associated with aggregates during the time of ADH stimulation has been interpreted as a movement of aggregates away from fusion sites. At the earliest time studied, 2.5 min, ~73% of fusions have aggregates touching their perimeter at some point (Fig. 9). At 30 min, this value is ~50% and at 4 h ~20% (see Tables I and II).

We have previously shown that bladders treated with cytochalasin B before ADH had significantly fewer aggregates in the luminal membrane than untreated ADH-stimulated controls (8). The present study shows that cytochalasin B pretreatment does not affect the number of ADH-induced fusions (Table II). These results suggest that, while microfilaments do not seem to be involved in the process of membrane fusion, once fusion has occurred they may play a role in the movement of aggregates from the intracellular membranes to the luminal membrane. If aggregates are or contain the transmembrane channels for water passage, as we have proposed (10), it would at first seem that they could be equally effective at either site since both are in contact with the mucosal solution. Further considerations suggest, however, that this may not be the case. Aggregates functioning in deep invaginations would quickly result in the dissipation of any transmembrane osmotic gradient. In the absence of an osmotic gradient, net water flow is negligible. Since the rate of mucosal fluid renewal in such invaginations would be slow, transbladder water movement would probably be limited. To avoid this large "unstirred layer" effect, it may be necessary for aggregates to be in contact with the bulk of the mucosal solution.

While it appears that the foregoing mechanism can account for the addition of aggregates to the luminal membrane after ADH stimulation, the manner of their disappearance during hormonal washout is not yet clear. A simple reversal of the insertion process does not seem to explain the morphology of washout. During washout, aggregates decrease in number and size (7). During this time the number of fusion events is also decreasing and the proportion associated with aggregates falls (Fig. 9). If aggregates were returning to the membranes of cytoplasmic structures, one would expect that the number of fusions would remain high (or perhaps increase), that sites of fusion would be as much (if not more) associated with aggregates (as is the case of 2.5 min when the number of aggregates in the luminal membrane is increasing rapidly), and that the size distribution of the remaining aggregates would not be affected. This is not the case. As to the results when ADH is withdrawn, therefore, the data suggest that the cytoplasmic membrane portion detaches from the luminal membrane and that aggregates break up and disperse in the luminal membrane. The fate of the carrier membrane and possible recycling mechanism of the IMP units await further investigation.

We gratefully acknowledge the skill and devoted technical assistance of Mrs. Kristine Olsen and Mr. Stelio Fantoli and the secretarial assistance of Ms. Alice Barry. We would also like to thank Drs. S. W. Rudich and M. C. Harmanci for many helpful discussions.

This research was supported by grant STI-78-08 from the Central Clinical Investigation Committee of Public Health Service, Bureau of Medical Services, Division of Hospitals and Clinics and grant AM-18710 from the National Institutes of Health. This work was presented in part at the 11th Annual Meeting of the American Society of Nephrology, New Orleans, La., 1978.

Received for publication 12 April 1979, and in revised form 12 October 1979.

REFERENCES

1. BOURGUET, J., J. CHEVALIER, and J. S. HUGON. 1976. Alterations in membrane-associated particle distribution during antidiuretic challenge in frog urinary bladder epithelium. *Biophys. J.* **16**:627–639.
2. CARASSO, N., P. FAVARD, and J. BOURGUET. 1973. Action de la cytochalasine B sur la réponse hydrosmotique et l'ultrastructure de la vessie urinaire de la grenouille. *J. Microscopie.* **18**:383–400.
3. CHEVALIER, J., J. BOURGUET, and J. S. HUGON. 1974. Membrane associated particles. Distribution in frog urinary bladder epithelium at rest and after oxytocin treatment. *Cell Tissue Res.* **152**:129–140.
4. HAUSMANN, K., and R. D. ALLEN. 1976. Membrane behavior of exocytic vesicles. II. Fate of the trichocyst membranes in *Paramecium* after induced trichocyst discharge. *J. Cell Biol.* **69**:313–326.
5. HERZOG, V., and M. G. FARQUHAR. 1977. Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. *Proc. Natl. Acad. Sci. U. S. A.* **74**:5073–5077.
6. HUMBERT, F., R. MONTESANO, A. GROSSO, R. C. DESOUSA, and L. ORCI. 1977. Particle aggregates in plasma and intracellular membranes of toad bladder (granular cell). *Experientia.* **33**:1364–1367.
7. KACHADORIAN, W. A., C. CASEY, and V. A. DISCALA. 1978. Time course of ADH-induced intramembranous particle aggregation in toad urinary bladder. *Am. J. Physiol.* **234**:F461–F465.
8. KACHADORIAN, W. A., S. J. ELLIS, and J. MULLER. 1979. Possible roles for microtubules and microfilaments in ADH action on toad urinary bladder. *Am. J. Physiol.* **236**:F14–F20.

9. KACHADORIAN, W. A., S. D. LEVINE, J. B. WADE, V. A. DISCALA, and R. M. HAYS. 1977. Relationship of aggregated intramembranous particles to water permeability in vasopressin-treated toad urinary bladder. *J. Clin. Invest.* **59**:576–581.
10. KACHADORIAN, W. A., J. MULLER, S. W. RUDICH, and V. A. DISCALA. 1979. Temperature dependence of ADH-induced water permeability and intramembranous particle aggregates in toad bladder. *Science (Wash. D. C.).* **205**:910–913.
11. KACHADORIAN, W. A., J. B. WADE, and V. A. DISCALA. 1975. Vasopressin: induced structural change in toad bladder luminal membrane. *Science (Wash. D. C.).* **190**:67–69.
12. KACHADORIAN, W. A., J. B. WADE, C. C. UITERWYK, and V. A. DISCALA. 1977. Membrane structural and functional responses to vasopressin in toad bladder. *J. Membr. Biol.* **30**:381–401.
13. MALAISSE, W. J., F. MALAISSE-LAGAE, E. VAN OBBERGHEN, G. SOMERS, G. DEVIS, M. RAVAZZOLA, and L. ORCI. 1975. Role of microtubules in the phasic pattern of insulin release. *Ann. N. Y. Acad. Sci.* **253**:630–652.
14. MCINTYRE, J. A., N. B. GILULA, and M. J. KARNOVSKY. 1974. Cryoprotectant-induced redistribution of intramembranous particles in mouse lymphocytes. *J. Cell Biol.* **60**:192–203.
15. MASUR, S. K., E. HOLTZMAN, I. L. SCHWARTZ, and R. WALTER. 1971. Correlation between pinocytosis and hydroosmosis induced by neurohypophyseal hormones and mediated by adenosine 3′,5′-cyclic monophosphate. *J. Cell Biol.* **49**:582–594.
16. MASUR, S. K., E. HOLTZMAN, and R. WALTER. 1972. Hormone-stimulated exocytosis in the toad urinary bladder. Some possible implications for turnover of surface membranes. *J. Cell Biol.* **52**:211–219.
17. MØLLGÅRD, K., and J. ROSTGAARD. 1978. Morphologic aspects of some sodium transporting epithelia suggesting a transcellular pathway via elements of endoplasmic reticulum. *J. Membr. Biol.* Special Issue. 71–89.
18. PINTO DA SILVA, P. 1972. Translational mobility of the membrane intercalated particles of human erythrocyte ghosts. pH-dependent, reversible aggregation. *J. Cell Biol.* **53**:777–787.
19. SPETH, V., and F. WUNDERLICH. 1973. Membranes of *Tetrahymena*. II. Direct visualization of reversible transitions in biomembrane structure induced by temperature. *Biochim. Biophys. Acta.* **291**:621–628.
20. SPUDICH, J. A. 1973. Effects of cytochalasin B on actin filaments. *Cold Spring Harbor Symp. Quant. Biol.* **37**:585–593.
21. REAVEN, E. P., AND G. M. REAVEN. 1975. A quantitative ultrastructural study of microtubule content and secretory granule accumulation in parathyroid glands of phosphate- and colchicine-treated rats. *J. Clin. Invest.* **56**:49–55.
22. REAVEN, E., R. MAFFLY, and A. TAYLOR. 1978. Evidence for involvement of microtubules in the action of vasopressin in toad urinary bladder. III. Morphological studies on the content and distribution of microtubules in bladder epithelial cells. *J. Membr. Biol.* **40**:251–267.
23. SNEDECOR, G. W., AND W. G. COCHRAN. 1968. Statistical Methods, 6th Edition. Iowa State University Press, Ames, Iowa.
24. WADE, J. B. 1978. Membrane structural specialization of the toad urinary bladder revealed by the freeze-fracture technique. III. Location, structure and vasopressin dependence of intramembranous particle arrays. *J. Membr. Biol.* Special Issue. 281–296.
25. WADE, J. B. 1980. Hormonal modulation of epithelial structures. *Curr. Top. Membr. Transp.* **13**:123–147.
26. WEIBEL, E. R. 1973. Stereological techniques for electron microscopic morphometry. *In* Principles and Techniques of Electron Microscopy. Vol. 3. M. A. Hayat, editor. Van Nostrand Reinhold Co., New York. 238–313.