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Membrane Structural and Functional Responses to Vasopressin in Toad Bladder

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Summary. Freeze-fracture electronmicroscopy demonstrates that vasopressin stimulation of isolated toad bladder results in a striking morphologic alteration of epithelial membrane structure. This alteration is characterized by the aggregation of intramembranous particles in orderly linear arrays at multiple sites in the luminal membranes of granular cells specifically. The size of these aggregates varies considerably, in terms of area, over a range from 0.5 to 70×10^{-3} μm^2. The median aggregate size is about 10.5×10^{-3} μm^2. Since the extent of vasopressin-associated particle aggregation, in terms of frequency of sites per area of membrane or cumulative area of membrane occupied by them, closely correlates with induced changes in transport function, as measured by osmotic water flow, the aggregates themselves appear to be of physiologic significance in the mechanism of action of vasopressin. This hypothesis is supported by the observations that sites of aggregation occur (a) in response to serosal exposure to hormone specifically, (b) independently of an osmotic gradient, and (c) following stimulation with cyclic adenosine monophosphate.

The isolated toad urinary bladder has been used extensively to investigate the mechanism by which vasopressin acts to enhance the transepithelial movement of water and many solutes, especially sodium and urea. It has been established that the permeability barrier in toad bladder which is altered by vasopressin stimulation to permit this effect on these substances is located within its epithelial luminal membrane [7, 14, 16, 19, 22, 24]. In addition, considerable evidence indicates that the pathways by which these different substances traverse the luminal barrier are selective and distinct [2, 3, 12, 20, 21, 27, 31, 34, 39]. In the case of water, the luminal permeability barrier which is altered by vasopressin stimulation appears specifically confined to granular cells [6, 8, 11, 33], but for the other substances comparable information is not available.

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The effects of vasopressin stimulation on transport activity across the toad bladder appear to be mediated by elevated levels of intracellular adenosine 3′,5′ monophosphate (cAMP) and subsequent alteration of the luminal membrane [15, 26]. The nature of the specific changes in the luminal membrane which might explain induced changes in permeability, however, remains unknown. On biochemical grounds protein membrane components have been implicated in vasopressin-induced movement of water, sodium, and urea [9, 10, 13, 31, 38]. Although correlated structural evidence for these components has not been reported, recent freeze-fracture observations by Chevalier *et al.* [5] and us [18] may be pertinent. These observations indicate that in response to oxytocin or vasopressin stimulation intramembranous particles (presumably representing intramembranous proteins [23, 30, 35, 36]) aggregate in organized arrays in epithelial cell luminal membranes of amphibian bladders.

In the present investigation, we have used freeze-fracture electronmicroscopy to study the effect of vasopressin stimulation on the morphology of epithelial membranes from toad urinary bladders. Some of the data of this investigation have been reported in a preliminary publication [18]. The purpose here is to elaborate our preliminary observations by additional quantitative characterization of vasopressin-induced aggregates and to report other lines of evidence which suggest that these aggregates are of functional significance in the mechanism of action of vasopressin.

Materials and Methods

For this investigation paired urinary hemibladders from double-pithed female Dominican toads (*Bufo marinus*) were used; for every pair one served as an experimental bladder, the other as a reference-control. Each was mounted as a sac on the end of a glass tube with the serosal surface facing outward. They were bathed at room temperature (22–24 °C) with aerated Ringer's solution (111 mM NaCl, 2.5 mM KCl, 2.5 mM $NaHCO_3$, 1.0 mM $CaCl_2$; pH 7.6–8.2; 220 mOsm/kg H_2O) on the serosal surface and Ringer's solution diluted 1:5 (except as otherwise indicated) with distilled water on the mucosal surface. Their surface areas were estimated from measurements of mucosal volumes. For every experiment the spontaneous potential difference across each of the paired hemibladders was measured at the end of a 30 min stabilization period. If for either hemibladder transepithelial voltage was less than 20 mV, the experiment was terminated; otherwise a 30 min pre-experimental baseline period began during which water movement was measured gravimetrically [1].

Specific Experimental Protocols

In this investigation 4 different types of experiments were conducted. In the first experimental series, paired hemibladders from 8 toads were studied in relation to different levels of vasopressin (Pitressin, Parke-Davis) stimulation. After the 30 min pre-experimental period, reference-control bladders for each pair of hemibladders were stimulated with 20 mU vasopressin/ml serosal bathing solution to induce maximal or near-maximal osmotic water flow, which was thereafter measured for a 30 min experimental period. The paired experimental bladders were either not stimulated with vasopressin, stimulated with levels of vasopressin which induce submaximal rates of osmotic water flow relative to those achieved with the concentration used to stimulate the reference-controls, or stimulated with a level of vasopressin which was equal to or 10 times greater than that used to stimulate the reference-controls. For all of these experimental bladders osmotic water flow for 30 min was also measured, and the relative effects of vasopressin stimulation were evaluated by expressing the rate of osmotic water flow in these bladders (per unit area of membrane surface) as a percentage of that for the corresponding reference-controls. The purpose of these experiments was to investigate the extent to which vasopressin-associated intramembranous particle aggregation relates to induced changes in function (i.e., water flow), and therefore quantitation of the aggregation response was necessary. As we have indicated in our preliminary studies, aggregated intramambranous particles observed subsequent to vasopressin stimulation of toad bladder are found only on the inner fracture face of luminal membranes of granular cells [18]. Our approach to the quantitation of this morphologic phenomenon was therefore to randomly photograph a standard area (22 μm^2) of this membrane fracture face from each of at least 10 different granular cells for each hemibladder studied. Thereafter, at 45,000×, the number of aggregates was counted and the surface area of each aggregate was evaluated by planimetry, using an Elograph graphical digitizer (E241) and a Wang programmable calculator (720C).

In the second series of experiments paired hemibladders from 3 toads were studied in a basically similar manner as in the previous experiments and the intent was to determine whether vasopressin-associated intramembranous particle aggregation would occur in the absence of an osmotic gradient. During the 30 min stabilization period mucosal and serosal surfaces of these bladders were bathed with undiluted Ringer's solution. Thereafter, the mucosal solution of the reference-controls was replaced with Ringer's solution diluted 1:5, that of the experimentals with undiluted Ringer's solution, and after the 30 min pre-experimental period a 30 min experimental period began during which 20 mU vasopressin/ml serosal solution was used to stimulate both.

In the third series of experiments paired hemibladders from 3 toads were again used. The purpose was to determine whether stimulation with cAMP was qualitatively similar to that with vasopressin in affecting intramembranous particle aggregation. The protocol followed was similar to that followed in the first series except that during the experimental period the reference-controls were not stimulated with any agent and the paired experimentals were stimulated with 2 mM of the monosodium salt of N^6, O^2-dibutyryl cAMP (dibut cAMP).

For the final experimental situation considered in this investigation paired hemibladders from 3 toads were used to determine whether the aggregation of intramembranous particles would occur with the addition of vasopressin to the mucosal, rather than serosal, bathing medium of the preparation. Accordingly, the hemibladders which served as the reference-controls were stimulated with 20 mU vasopressin/ml serosal solution for 30 min, as in the first experimental series, and the experimental bladders were treated in a similar fashion as the reference-controls except that during the experimental period vasopressin stimulation (20 mU/ml) was attempted from the mucosal, rather than serosal surface.

Procedures for Freeze-Fracture Electronmicroscopy

Immediately after the experimental period, bladders were fixed for 15 min in 2.5% glutaraldehyde containing 0.1 M cacodylate buffer (pH 7.4), and then stored under refrigeration in 0.1 M cacodylate buffer. In preparation for freeze fracture, tissues were soaked for at least 90 min in 25% glycerol containing 0.1 M cacodylate buffer. Thereafter, they were frozen at −150 °C in liquid Freon 22, which was cooled by liquid nitrogen, and then fractured and replicated in a Balzers freeze-etch unit (BAF 301) which was equipped with a mechanism to yield complementary membrane fracture faces (DA 300), an electron beam evaporation device (EVM 052), and a quartz thin film monitor (QSG 201). After tissue digestion with bleach and cleaning, complementary replicas were examined with an RCA electronmicroscope (EMU 4B) at 75 kV.

In every case the identity of a bladder studied in this investigation was coded and freeze fracture, electronmicroscopy, and quantitative evaluation of electronmicrographs were done without knowledge of tissue status.

Results

The Effect of Vasopressin Stimulation on Toad Bladder Epithelial Membrane Morphology

For comparative purposes, luminal membrane fracture faces of granular cells from a toad bladder not stimulated with vasopressin are illustrated in Figs. 1 and 2. On the inner fracture face P^1 (Fig. 1) and on the complementary fracture face *E* (Fig. 2), intramembranous particles appear rather uniformly distributed. As is obvious by comparison of these fracture faces and in accord with previous observations of unstimulated bladders [37], intramembranous particles which fracture with face *E* appear larger in diameter and more densely distributed than those which fracture with face *P*.

Figs. 3 and 4 illustrate luminal membrane fracture faces of granular cells from a toad bladder stimulated for 30 min with 20 mU vasopressin/ml serosal bathing solution. Associated with vasopressin stimulation, a striking alteration of membrane structure is observed. On fracture face *P* (Fig. 3) intramembranous particles are aggregated at multiple sites which are between or near the bases of (but, in our experience, not on) microvilli. That some of these aggregated particles are often not seen distinctly appears to be a consequence of an overshadowing effect which we presume to result from the particles being closely packed and of different sizes. On fracture face *E* (Fig. 4) separate distinctive

1 In accord with the nomenclature recently proposed by Branton *et al.* [4], fracture face *P* refers to the half-membrane closer to the protoplasm and fracture face *E* to the half-membrane closer to the extracellular space.

Fig. 1. Fracture face *P* of the luminal membrane of a granular cell from toad urinary bladder not stimulated with vasopressin. Ridge-like microvilli are prominent and intramembranous particles are distributed randomly over the entire membrane face. ×47,500

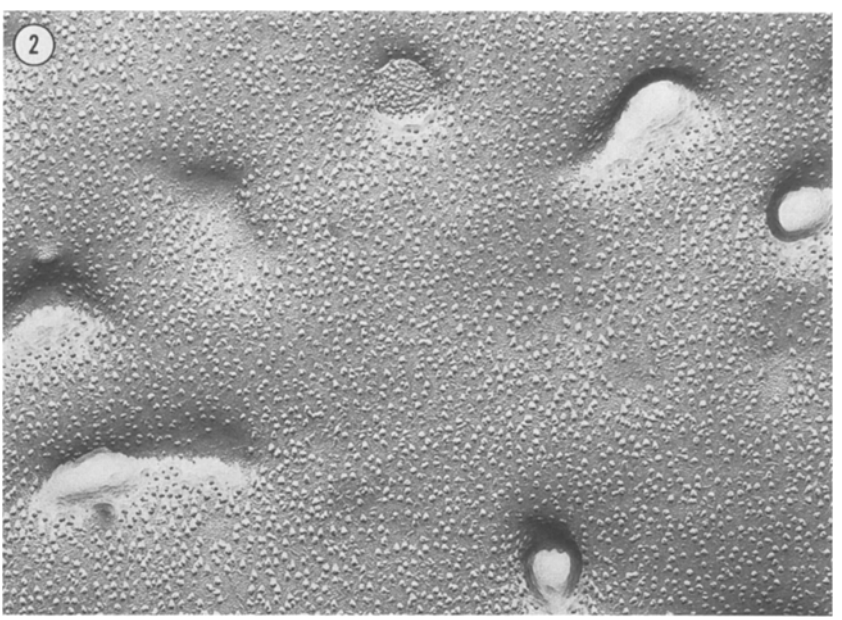

Fig. 2. Fracture face *E* of the luminal membrane of a granular cell from the same (unstimulated) toad bladder as shown in Fig. 1. Microvilli are seen on this fracture face as large depressions and intramembranous particles, which tend to be larger and more densely distributed than those on the apposed fracture face, are randomly distributed. ×47,500

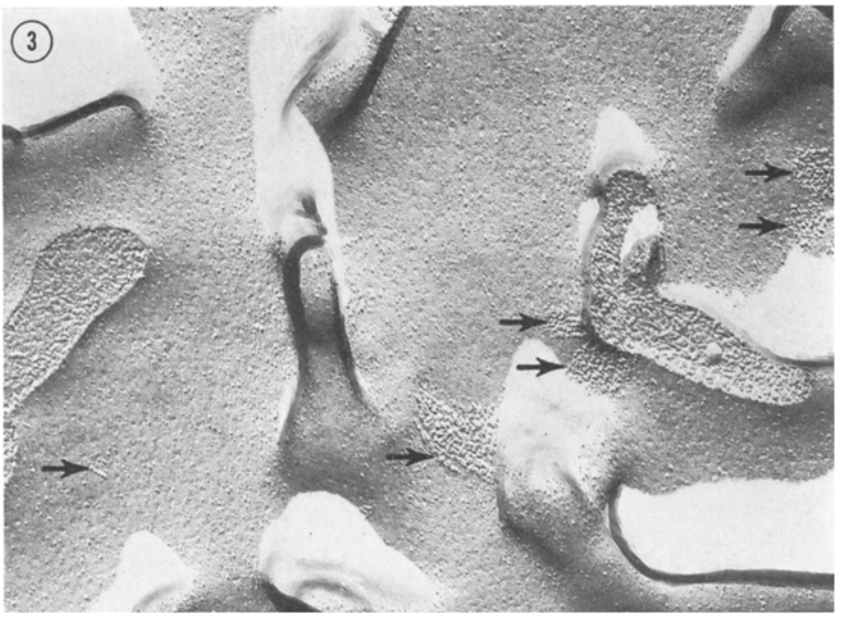

Fig. 3. Fracture face *P* of the luminal membrane of a granular cell from toad urinary bladder stimulated with 20 mU vasopressin/ml serosal bathing solution. At multiple sites between or near bases of microvilli intramembranous particles are aggregated (arrows). ×47,500

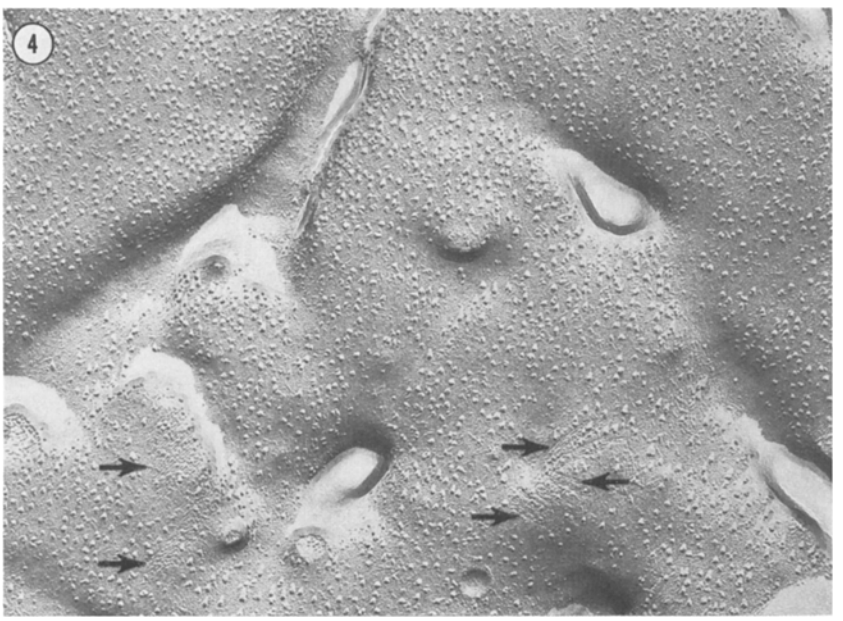

Fig. 4. Fracture face *E* of the luminal membrane of a granular cell from the same (vasopressin stimulated) toad bladder as shown in Fig. 3. Distinctive sites, each consisting of linearly organized depressions, are observed (arrows). In areas remote to these sites particle density appears similar to that for unstimulated controls (Fig. 2), but within these distinctive sites and also in their immediate periphery particle density appears reduced. ×47,500

sites are found, each characterized by rows of depressions arranged in linear arrays. Not only is there a remarkable reduction in the number of particles within these sites, but in the immediate periphery of the sites, relative to areas which are more remote, there appears also to be a reduction. It is clear from the exact complementary replicas shown in Fig. 5 that sites of aggregated intramembranous particles on fracture face *P* (Fig. 5*a*) relate spacially to areas of organized depressions on fracture face *E* (Fig. 5*b*). Indeed, the organized depressions within these areas must themselves be interpreted to represent impressions made by the intramembranous particles which are aggregated.

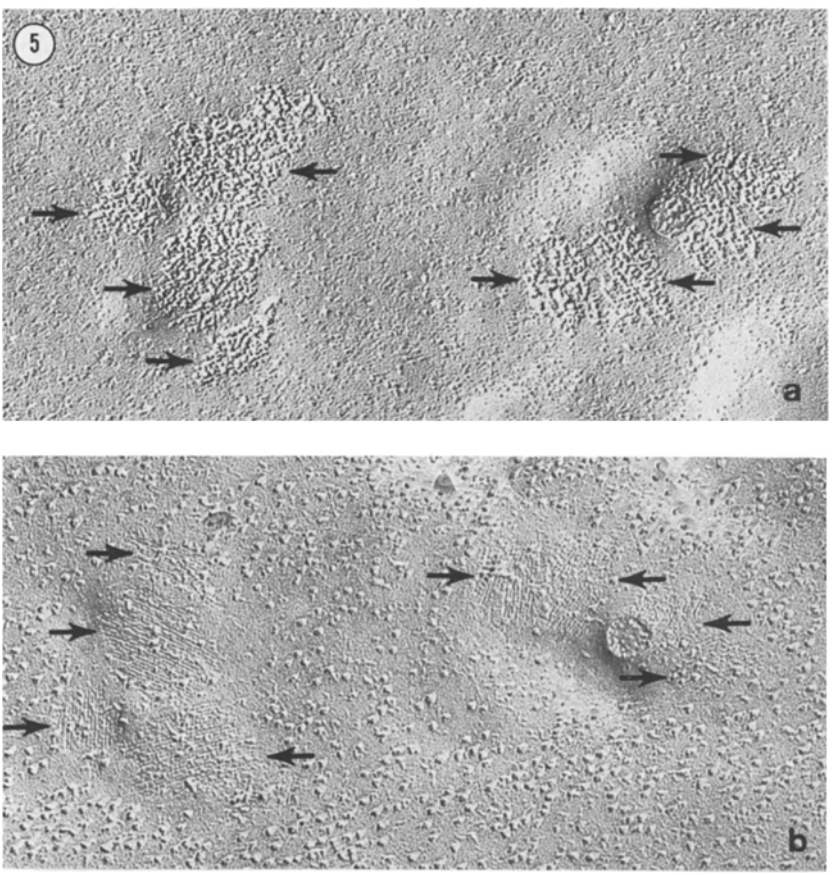

Fig. 5. Complementary replicas of sites of intramembranous particle aggregation and linearly organized depressions. (*a*). Separate aggregates are emphasized (arrows) on fracture face *P*. In some cases the linear organization of aggregated particles can be seen. (*b*) The distinctive areas of fracture face *E* that are emphasized (arrows) are apposed to the aggregation sites shown in *a* and consist of rows of depressions organized in linear arrays. Clearly, these distinctive areas on fracture face *E* confirm the linear organization of the aggregated particles of fracture face *P*. ×63,500

The observation of vasopressin-associated intramembranous particle aggregation in our experience is restricted specifically to the luminal membranes of granular cells. Basolateral membranes of granular cells and the membranes of other epithelial cell types of toad bladder (i.e., mitochondria-rich, goblet, and basal cells) appear not to be similarly affected by vasopressin stimulation.

Correlation of Vasopressin-associated Intramembranous Particle Aggregation with Vasopressin-induced Osmotic Water Flow

To deal with the physiologic significance of aggregated intramembranous particles, the frequency of aggregation sites and the cumulative area occupied by them were correlated with measured rates of osmotic water flow. As illustrated in Fig. 6, the frequency of aggregation sites per standard area of membrane fracture face for the experimental bladders studied in the first series of experiments, as a percentage of that found per standard area of membrane fracture face in reference-control bladders, increases linearly with relative increases in osmotic water flow. The correlation coefficient (r) for these data is 0.95, which is highly

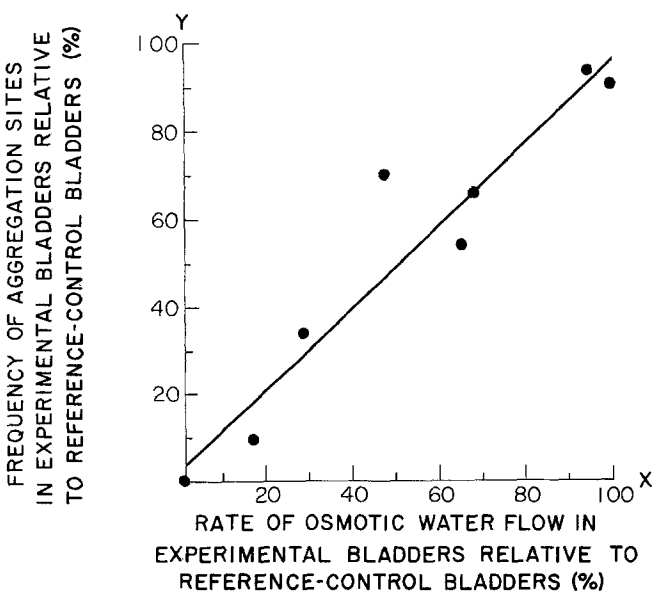

Fig. 6. Relationship between the relative frequency of vasopressin-associated sites of intramembranous particle aggregation and the relative rate of vasopressin-associated osmotic water flow. Least squares regression line: $y=3.631+0.937x$ ($r=0.95$; $P<0.005$)

Table 1. Quantitative Physiologic and Morphologic Responses to Vasopressin

Hemibladder identity	Vasopressin concentration in serosal bath (mU/ml)	Vasopressin-induced osmotic water flow		Frequency of aggregation sites		Cumulative area occupied by aggregation sites	
		Actual (mg/30 min/cm^2)	Relative[a] (%)	Actual (#/220 μm^2 membrane)	Relative[a] (%)	Actual (μm^2/220 μm^2 membrane)	Relative[a] (%)
A–Exp	0	0	0	0	0	0	0
A–R-C	20	121	–	219	–	2.561	–
B–Exp	0.01	11	16.7	9	9.7	0.082	4.1
B–R-C	20	66	–	93	–	1.981	–
C–Exp	0.1	28	28.0	53	34.0	0.553	22.5
C–R-C	20	100	–	156	–	2.453	–
D–Exp	0.1	50	46.7	110	70.5	1.463	74.8
D–R-C	20	107	–	156	–	1.955	–
E–Exp	1	59	64.8	94	54.3	1.168	50.8
E–R-C	20	91	–	173	–	2.298	–
F–Exp	1	72	67.9	83	66.4	1.264	125.8
F–R-C	20	106	–	125	–	1.005	–
G–Exp	20	90	98.9	199	90.5	3.061	86.4
G–R-C	20	91	–	220	–	3.543	–
H–Exp	200	88	93.6	138	93.9	1.803	97.6
H–R-C	20	94	–	147	–	1.848	–

[a] Calculated for experimental bladders relative to paired reference-controls.

significant ($P<0.005$). A similar data plot in which absolute (rather than relative) values for osmotic water flow and aggregate frequency per standard area of membrane were used (*see* Table 1), revealed a similar relationship between them ($r=0.89$; $P<0.005$).

In Fig. 7 the cumulative surface area of membrane fracture face *P* occupied by sites of aggregated intramembranous particles in the experimental bladders, again as a percentage of that for corresponding reference-controls, is shown also to increase linearly with increments in osmotic water flow. For these factored data *r* is 0.84 which is highly significant ($P<0.005$). As before, if absolute values for the cumulative area of membrane fracture face *P* occupied by aggregates and osmotic water flow (*see* Table 1) are plotted, the relationship between them ($r=0.77$; $P<0.025$) is similar.

Aggregate Size and Its Relationship to Vasopressin Stimulation

As a measure of the size of an aggregate, the area of fracture face *P* occupied by the aggregated particles was used. In Fig. 8 is histographically illustrated the size distribution of aggregates found in the reference-control bladders of the first series of experiments. For each reference-control bladder the frequency of aggregates within each of the aggregate size categories (as labeled in the histogram) was calculated on a proportional basis, and corresponding data for all 8 bladders studied were used to calculate the mean proportional frequency of aggregates (and standard error) in each aggregate size category. As is clearly illustrated, the frequency distribution of aggregate size is asymmetrical and follows a smooth, continuous curve whose range is from about 0.5 to 70×10^{-3} μm^2. Overall, for the 1536 separate aggregates measured to prepare the histogram, the mean value for aggregate surface area was 14.0×10^{-3} μm^2 and the median value (which in this case is perhaps a better measure of central tendency than the mean) was 10.5×10^{-3} μm^2.

On the basis of the data shown in Figs. 6 and 7 which indicate that the frequency of aggregates and the cumulative area occupied by them relate linearly to osmotic water flow, it appears reasonable that these variables should relate linearly to each other. As shown in Fig. 9 this is the case ($r=0.92$; $P<0.005$). This relationship suggests that by the 30th min of vasopressin stimulation mean (or median) aggregate size does not obviously vary with different levels of vasopressin stimulation (as indicated by data for osmotic water flow in Table 1 which corre-

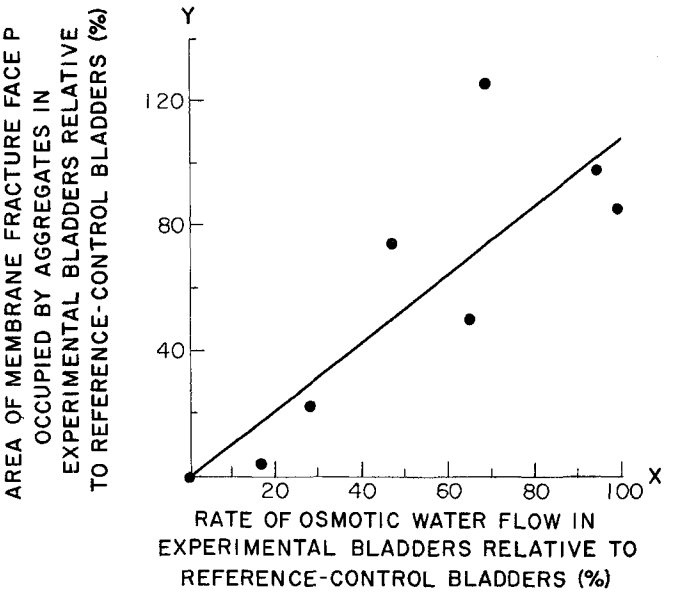

Fig. 7. Relationship between the relative area of membrane occupied by vasopressin-associated sites of intramembranous particle aggregation and the relative rate of vasopressin-stimulated osmotic water flow. Least squares regression line: $y=1.310+1.083x$ ($r=0.84$; $P<0.005$)

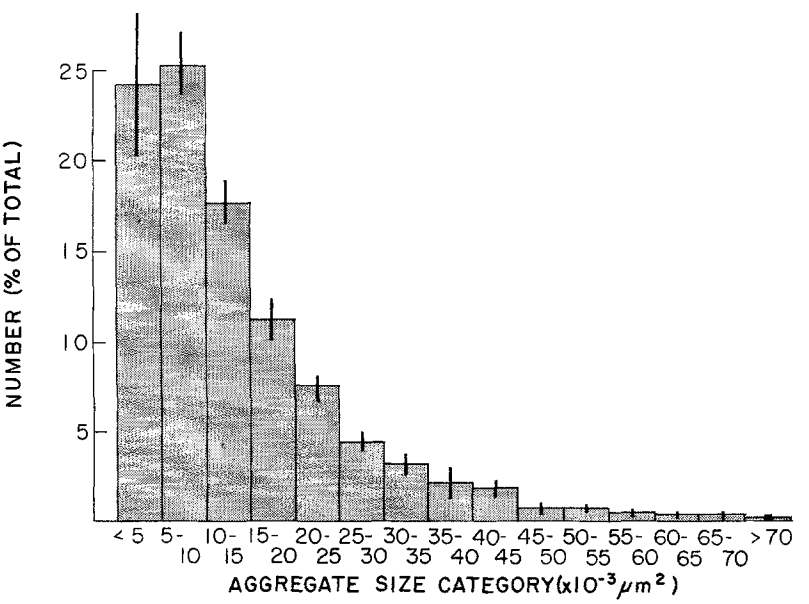

Fig. 8. Frequency histogram of aggregate size following stimulation with 20 mU vasopressin/ml serosal bathing solution for 30 min. For this histogram the size of 1536 separate aggregates from bladders of 8 toads was measured

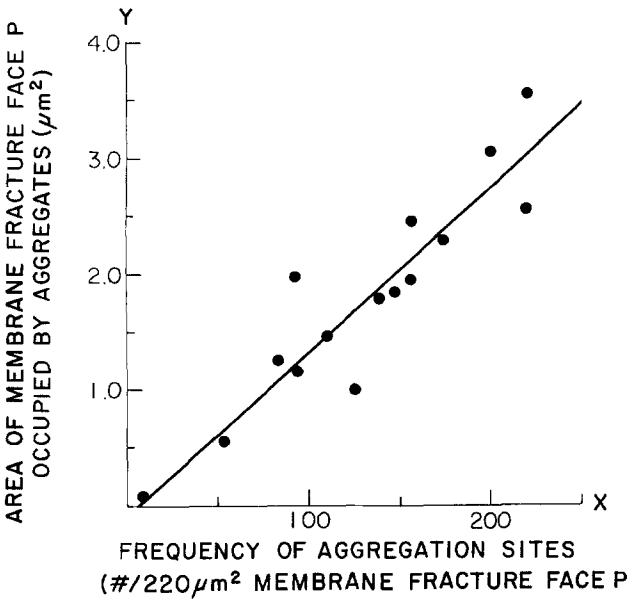

Fig. 9. Relationship between the frequency of vasopressin-associated aggregates per area of membrane and the cumulative area of membrane occupied by them. Least squares regression line: $y = -0.063 + 0.014\,x$ ($r = 0.92$; $P < 0.005$)

Table 2. Level of Vasopressin Stimulation and Aggregate Size

Vasopressin concentration in serosal bath (mU/ml)	Total no. aggregates observed	No. toad bladders	Aggregate size		
			Mean ± SD ($\times 10^{-3}\,\mu m^2$)	Median ($\times 10^{-3}\,\mu m^2$)	Interquartile range ($\times 10^{-3}\,\mu m^2$)
200	138	1	13.1 9.8	10.8	6.8–16.1
20	1536	8	14.0 12.5	10.5	5.4–18.8
1	185	2	13.8 14.0	9.9	4.9–18.5
0.1	163	2	12.2 12.1	9.1	4.2–15.9

spond to aggregate frequency and area data), but remains about constant. In Table 2 are listed data which not only substantiate this interpretation, but further suggest that the variability in data for aggregate size is also independent of the vasopressin concentration used for stimulation.

Vasopressin-associated Alteration in Epithelial Membrane Structure in the Absence of an Osmotic Gradient

In this series of experiments, as before, membranes of all epithelial cell types were examined. For each of the bladders not subjected to an osmotic gradient transepithelial water movement was negligible, but nevertheless vasopressin stimulation was associated with the aggregation

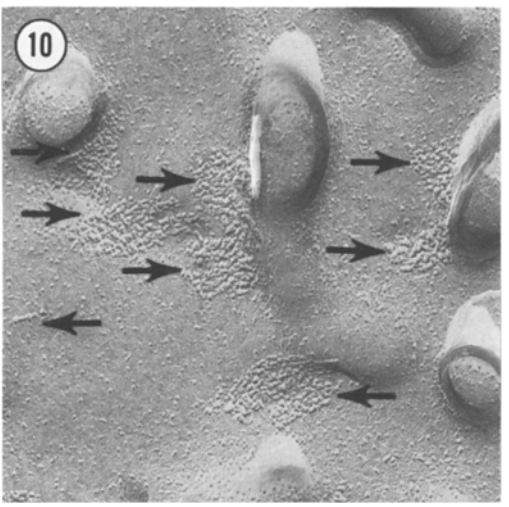

Fig. 10. Fracture face *P* of granular cell luminal membrane from toad bladder stimulated with vasopressin (20 mU/ml) in the absence of an osmotic gradient. Aggregates (arrows) appear identical to those found after vasopressin stimulation in the presence of an osmotic gradient (*compare* with Fig. 3). ×60,000

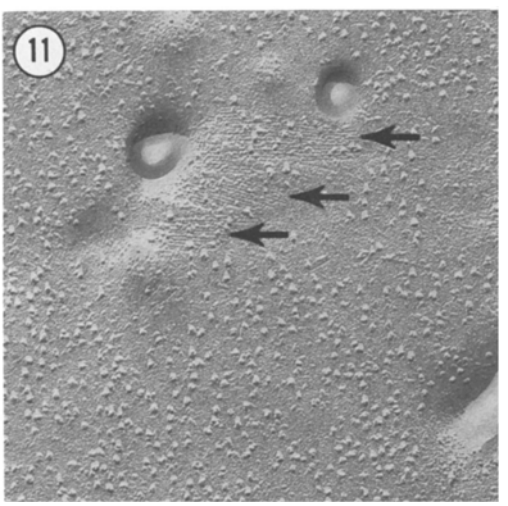

Fig. 11. Fracture face *E* of granular cell luminal membrane from the same bladder as shown in Fig. 10. Distinctive areas of linearly organized depressions (arrows) are observed in the absence of an osmotic gradient, which appear identical to those found in the presence of an osmotic gradient (*compare* with Fig. 4). ×60,000

of intramembranous particles on the inner fracture face *P* of granular cell luminal membranes specifically, while on fracture face *E* of these membranes areas were observed in which depressions were organized

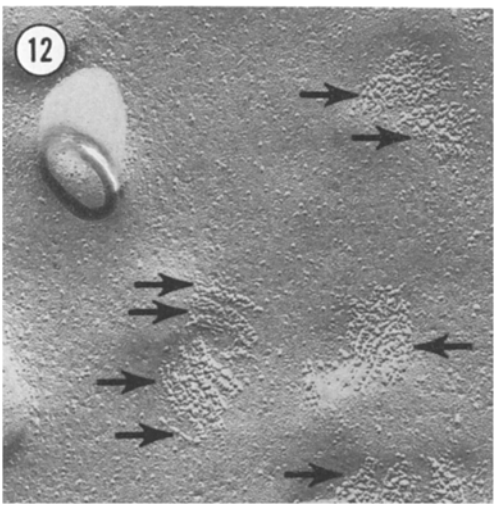

Fig. 12. Fracture face *P* of granular cell luminal membrane from toad bladder stimulated with dibut cAMP (2 mM). Sites of aggregated particles (arrows) appear similar to those found in association with vasopressin stimulation (*compare* with Fig. 3). ×60,000

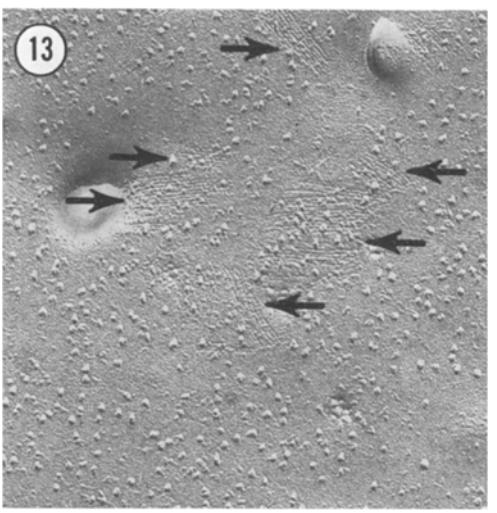

Fig. 13. Fracture face *E* of granular cell luminal membrane from the same toad bladder as shown in Fig. 12. Areas of linearly organized depressions (arrows) are seen with dibut cAMP stimulation which resemble those found with vasopressin stimulation (*compare* with Fig. 4). ×60,000

in rows. These alterations are illustrated in Figs. 10 and 11 and appear qualitatively similar to those found in the paired reference-controls which were subjected to an osmotic gradient (*compare* with Figs. 3 and 4).

The Effect of cAMP Stimulation on the Structure of Epithelial Membranes

With 2 mM dibut cAMP stimulation mean osmotic water flow across the toad bladders studied increased from 3 to 48 mg per 30 min per cm^2 and alterations which resembled those found in association with vasopressin stimulation (Figs. 3 and 4), were found in granular cell luminal membranes. Examples of the alterations associated with dibut cAMP stimulation, as seen on both fracture faces, are illustrated in Figs. 12 and 13. As before, other epithelial membranes were examined, but with respect to paired unstimulated controls, structural alterations associated with dibut cAMP stimulation appeared to be restricted to luminal membranes of granular cells only.

The Effect of Mucosal Exposure to Vasopressin on the Structure of Epithelial Membranes

As expected, for the reference-control bladders which were stimulated in the usual manner by vasopressin addition to the serosal bathing solution, sites of aggregated intramembranous particles in granular cell luminal membranes were seen. In contrast, for the paired experimental bladders which were exposed to vasopressin by addition of the hormone to the mucosal bathing solution, there was neither a physiologic response in terms of osmotic water flow nor any noticeable morphologic alteration of granular cell luminal membrane structure.

Discussion

Morphologic Considerations

It has been previously established that the permeability barriers of the toad bladder which are altered by vasopressin stimulation to permit the transepithelial movement of water and solutes are located in the luminal membranes of the epithelial cells [7, 14, 16, 19, 22, 24]. In the case of water, this barrier has been shown to be specifically localized in the luminal membrane of granular cells [6, 8, 11, 33]. We have shown with freeze-fracture electronmicroscopy that the epithelium of isolated toad urinary bladders stimulated with vasopressin is morphologically altered and that this alteration is restricted to the luminal membrane of granular cells. The morphologic alteration itself consists of multiple sites of aggregated intramembranous particles on the inner fracture face

P and, on corresponding areas of fracture face *E*, distinctive areas of linearly organized depressions representing impressions made by the aggregated particles.

Others have shown that the aggregation of intramembranous particles can be induced by cold [32], acidity [29], or exposure of unfixed tissue to cryoprotectants [25]. None of these factors, however, explains our observation of particle aggregation since with our procedure tissue bathing solutions were not acidic or cold (prior to fixation) and tissues for freeze fracture were fixed in glutaraldehyde before cryoprotection with glycerol. More importantly, bladders which we have studied that have not been stimulated with vasopressin, but otherwise treated in a similar fashion as ones which were, do not exhibit the morphologic changes which occur in vasopressin-stimulated bladders. Accordingly, our conclusion is that the aggregates which are found in association with vasopressin stimulation are indeed specific effects of vasopressin stimulation.

In relation to the sites of intramembranous particle aggregation themselves, it seems reasonable that they should be considered as distinct intramembranous structures because a consistent pattern of particle organization is observed within them. As distinct structures, these sites vary considerably in size, in terms of the area of membrane which they occupy, from about 0.5 to 70×10^{-3} μm^2 (Fig. 8). With maximal vasopressin stimulation the mean and median aggregate size calculate to be 14.0 and 10.5×10^{-3} μm^2, respectively. Since our data show that these values and standard indices of their variability are about constant with concentrations of vasopressin between 0.1 and 200 mU/ml, then it follows that the distribution of aggregates sizes is, in proportional terms, quantitatively the same with each of these levels of vasopressin. Since it is also clear from the data listed in Table 1 that there exists a general relationship between the level of vasopressin used for stimulation and the morphologic response, in terms of the frequency of aggregates and the total area occupied by them (*see* below), then it seems reasonable to suggest that the morphologic response to vasopressin is a standard response whose intensity varies in relation to the level of stimulation induced by vasopressin.

Although our observations demonstrate that an alteration in membrane structure accompanies vasopressin stimulation, the process involved in aggregate formation is not clear. Aggregates might arise by a reorganization of intramembranous particles already present in the membrane and/or by an insertion of new material into the membrane. Although prior to vasopressin treatment intramembranous particles are found uniformly distributed on fracture face *E*, Fig. 5 demonstrates that

few particles are found in areas on fracture face *E* which correspond to sites of aggregation. This suggests the possibility that vasopressin stimulation may alter the fracturing properties of the particles at aggregation sites such that they fracture with fracture face *P* instead of fracture face *E*. Furthermore, it is also possible to implicate local translational movement of particles in the formation of sites of aggregation because (a) the aggregated particles on fracture face *P* appear more densely packed than can be accounted for by simply a change in fracturing properties, (b) the aggregated particles are uniquely aligned in linear arrays, and (c) the number of *E* face particles in the immediate periphery of aggregation sites seems notably diminished compared to the number in areas of membrane more remote from these sites. Overall, our observations are consistent with the possibility that sites of aggregation result from organized translational movement and alignment of intramembranous particles together with an alteration in their fracturing properties.

An alternative explanation for vasopressin-associated aggregation of intramembranous particles might involve the insertion of new material into the membrane. If this were the case, areas remote from sites of aggregation might show an altered frequency of intramembranous particles as compared with that of bladders unexposed to vasopressin. We have made such a comparison in a separate experiment and have been unable to find any such differences. For a control bladder not stimulated with vasopressin the frequency of intramembranous particles on fracture face *P* was $637 \pm 25/\mu m^2$ and on fracture face *E* $1040 \pm 82/\mu m^2$. For the paired vasopressin-stimulated bladder the frequency of particles in areas remote from sites of particle aggregation was $609 \pm 33/\mu m^2$ on fracture face *P* and $935 \pm 71/\mu m^2$ on fracture face *E*. Of course, this approach has limited sensitivity since as demonstrated by the data in Table 1, the area of granular cell luminal membrane affected by the aggregation response after maximal vasopressin stimulation is only about 1% of the total membrane.

At this point the processes involved in intramembranous particle aggregation are unknown. Our current data permit us only to raise some obvious possibilities.

Physiologic Considerations

The findings that both the frequency of vasopressin-associated sites of particle aggregation per area of membrane and cumulative area of membrane occupied by them relate linearly with osmotic water flow

suggest that these sites are of physiologic significance. That these systematic alterations of membrane structure are not simply effects of vasopressin concentration *per se* is suggested by data listed in Table 1. These indicate that stimulation with a level of vasopressin equal to 200 mU/ml serosal solution is quantitatively similar to stimulation with 20 mU/ml in affecting both osmotic water flow and intramembranous particle aggregation.

It is well known that under ordinary physiologic conditions the functional response of toad bladder to vasopressin stimulation occurs only with serosal exposure to hormone. That our observations demonstrate that the same specificity is required for aggregation supports the hypothesis that the aggregates relate to the mechanism of action of vasopressin.

For the experiments performed without an osmotic gradient, morphologic alterations of the granular cell luminal membranes were found which were qualitatively similar to those found in association with an osmotic gradient. It is to be expected that if the morphologic alterations associated with vasopressin stimulation relate mechanistically to the action of vasopressin then they should occur in relation to the permeability change which accompanies vasopressin stimulation and be independent of the presence of an osmotic gradient to the extent that the permeability change itself is independent of an osmotic gradient. Although we did not measure the permeability change associated with vasopressin stimulation in bladders not exposed to an osmotic gradient, others have clearly shown that it is enhanced (e.g., [16, 28]). Our finding of granular cell luminal membrane alterations with vasopressin stimulation for bladders unexposed to an osmotic gradient represents additional support for the hypothesis that these alterations are significant in the mechanism of action of vasopressin.

It seems clear that the vasopressin-induced changes in transport activities across toad bladder, including osmotic water movement, are mediated by cAMP [12, 26]. Our observations which demonstrate that the morphologic alterations associated with vasopressin stimulation also occur following cAMP stimulation suggest again that these alterations relate to the mechanism through which vasopressin acts.

Although our results indicate that vasopressin-associated intramembranous particle aggregation in granular cell luminal membranes is related to the induced change in the water permeability of toad bladder, it should not be concluded at this point that this relationship is necessarily specific. For example, sodium and urea permeability functions of toad bladder are enhanced with vasopressin stimulation (e.g., [7, 24]) and

may have been as closely correlated with changes in morphology as was osmotic water flow had they been measured. We have subsequently considered this problem by using the anesthetic methohexital (Brevital, Eli Lilly) to specifically inhibit vasopressin-induced osmotic water flow without affecting urea permeability or sodium transport (as measured by short-circuit current) [17]. For vasopressin-stimulated bladders treated with methohexital we found that osmotic water flow, aggregate frequency, and the area of membrane fracture face occupied by aggregates were all reduced to about the same extent relative to corresponding measures for vasopressin-stimulated controls. The results of the present investigation and our more recent findings [17] suggest that the morphologic changes in granular cell luminal membranes which are induced by vasopressin stimulation relate mechanistically to the action of vasopressin and specifically to induced changes in water permeability. Whether these morphologic changes reflect a change in membrane fluidity which in toad bladder has been associated with vasopressin stimulation [28] and/or whether they are actual sites for water movement remain to be determined.

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