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Membrane Structural Specialization of the Toad Urinary Bladder Revealed by the Freeze-Fracture Technique

III. Location, Structure and Vasopressin Dependence of Intramembrane Particle Arrays

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Summary. Examination of the toad urinary bladder by freeze-fracture electron microscopy reveals intramembrane particle arrays at a number of membrane sites. An array in which particles are aggregated into closely apposed parallel rows is found in the granular cell luminal membrane of dehydrated toads fixed *in situ*. These aggregates are structurally indistinguishable from those previously associated with vasopressin exposure *in vitro*. Aggregates are not found in granular cell luminal membrane in the case of hydrated toads fixed *in situ*. However, structurally similar arrays are found at low frequency in the membrane of cytoplasmic vacuoles in granular cells and in the plasma membrane of basal cells in both hydrated and dehydrated toads. Aggregates are also present at these sites in control and vasopressin-treated bladders from *in vitro* experiments. Particle arrays characteristic of gap junctions, desmosomes and hemidesmosomes also occur in the plasma membrane of basal cells. In addition, distinctive square arrays of particles exist in the plasma membrane of the bladder's mesothelium. Although a variety of intramembrane particle arrays exist in the toad urinary bladder, only the occurrence of organized particle aggregates in the luminal membrane of granular cells appears to be associated with vasopressin exposure.

Several years ago we began an effort to use the freeze-fracture technique to characterize the structural specialization of different membranes of the toad urinary bladder. Actually, this work began first as a test of the freeze-fracture technique. It has been clear since the early work of Koefoed-Johnsen and Ussing (1958) that in epithelia the permeability of luminal membranes differs from that of basal membranes, but it was not obvious that the freeze-fracture technique would be capable of distinguishing the structural specialization of one type of membrane from that of another. Although the significance of these specializations remains

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uncertain, it is now clear that by using the freeze-fracture technique we are able in amphibian urinary bladder to not only distinguish between the luminal membrane and the basal membrane (Chevalier, Bourguet & Hugon, 1974; Orci *et al.*, 1975; Wade, DiScala & Karnovsky, 1975), but also between membrane of the granular cell type and membrane of the mitochondria-rich cell type (Orci *et al.*, 1975; Bourget, Chevalier & Hugon, 1976; Wade, 1976).

While these observations are consistent with the possibility that freeze-fracture can reveal structural details relevant to membrane permeability, much more convincing evidence of this came from studies which showed that occurrence of intramembrane particle aggregates in granular cell luminal membrane is closely associated with the change in membrane permeability induced by neurohypophysial hormones (Chevalier *et al.*, 1974; Kachadorian, Wade & DiScala, 1975). The same structural organization is also observed when bladders are stimulated by cyclic AMP (Bourguet *et al.*, 1976; Kachadorian *et al.*, 1977b). In bladders exposed to different concentrations of vasopressin, both the number of sites and the area of luminal membrane covered by these aggregates are proportional to the level of water permeability measured (Kachadorian *et al.*, 1977*b*). In addition, the anesthetic methohexital was found to reduce significantly both the number of aggregates and their total area in vasopressin treated bladders while at the same time inhibiting the water permeability response (Kachadorian *et al.*, 1977*a*). Since methohexital did not significantly alter the increase in short-circuit current nor the increase in urea permeability that occurs with vasopressin, it appears that aggregates may be specifically related to the increase in water permeability that occurs with vasopressin.

The present work deals with three issues related to the significance of these membrane specializations. First, what are the membrane structural features of the bladder *in situ* when a natural stimulus such as dehydration causes neurohypophysial hormone release? Are these features the same as those seen when the bladder is exposed *in vitro* to vasopressin? The second issue is whether structurally similar aggregates can occur in any membranes other than the granular cell luminal membrane. If so, are such aggregates associated with exposure to vasopressin? Finally, there is a brief description of several other types of intramembrane particle arrays which exist in the toad bladder. Although these other arrays do not appear to be related to the action of vasopressin, they do provide a further indication of the degree of membrane specialization present in the toad bladder.

Materials and Methods

For these studies, tissue was obtained from pithed female toads, *Bufo marinus*, from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.). In one series of experiments, bladders from dehydrated toads (denied access to water for 48 h, urine osmolality greater than 200 mosmol) and hydrated toads (toads allowed free access to water, urine osmolality less than 100 mosmol) were fixed *in situ* by dripping 2.5% glutaraldehyde (buffered by 0.1 M sodium cacodylate at pH 7.4) onto the exposed serosal surface of the bladder, while at the same time bladder urine was exchanged for fixative by using a syringe. Total time of fixation was 15 min.

In another series of experiments, paired bladders were mounted as sacs tied to tubes and bathed *in vitro* with a Ringer's solution consisting of (mM): 111 NaCl, 3.5 KCl, 2.5 $NaHCO_3$ and 1 $CaCl_2$. Vasopressin treated half-bladders were exposed to 20 mU/ml vasopressin (arginine vasopressin, Sigma Chemical Co., St. Louis, Mo.) in the serosal bathing solution for 30 min. Bladders were fixed for 15 min as in previous reports (Kachadorian *et al.*, 1977*b*) by immersion of bladder sacs in 2.5% glutaraldehyde (buffered by 0.1 M sodium cacodylate at pH 7.4) and substitution of fixative for mucosal Ringer's by using a syringe. Several bladders were also fixed by simultaneous addition of neutralized 25% glutaraldehyde directly to both mucosal and serosal Ringer's solutions, resulting in a final solution of 2.5% glutaraldehyde. No differences could be identified between bladders fixed by these two methods.

In all cases, tissue was washed and stored in 0.1 M sodium cacodylate buffer after fixation. Prior to freezing, tissue was soaked in 25% glycerol in 0.1 M sodium cacodylate. This concentration of glycerol was reached by drop-wise addition of glycerol to the tissue in a known volume of buffer over a period of 10 min. Tissue was frozen as previously described using undercooled liquid nitrogen (Wade *et al.*, 1975). A Balzers freeze-etch unit BAF 301 (Balzers High Vacuum, Liechtenstein) was used, equipped with mirror image replica device (DA 300), electron beam evaporation device (EVM 052), and quartz crystal thin film monitor (QSG 201). Replicas were examined with a Zeiss 10B electron microscope and quantitation performed without prior knowledge of the tissue's status.

Results

Granular Cell Luminal Membrane of Toad Bladders Fixed in Situ

The luminal membrane of a granular cell from a toad deprived of water for 48 hr is shown in Fig. 1. The membrane has aggregates (arrows, Fig. 1) which appear to be structurally indistinguishable from those found in bladders exposed *in vitro* to vasopressin or cyclic AMP (Kachadorian *et al.*, 1977*b*). The characteristic organization of this type of intramembrane array consists of closely aggregated linear rows of particles on fracture face *P* (insert *P*, Fig. 1) and a distinctive series of parallel grooves on fracture face *E* (insert *E*, Fig. 1). A range of 20–40 aggregation sites per 100 μm^2 have been identified in bladders from dehydrated toads. Although a low frequency, this is comparable to the range of 42–100 aggregation sites per 100 μm^2 previously found in bladders maximally

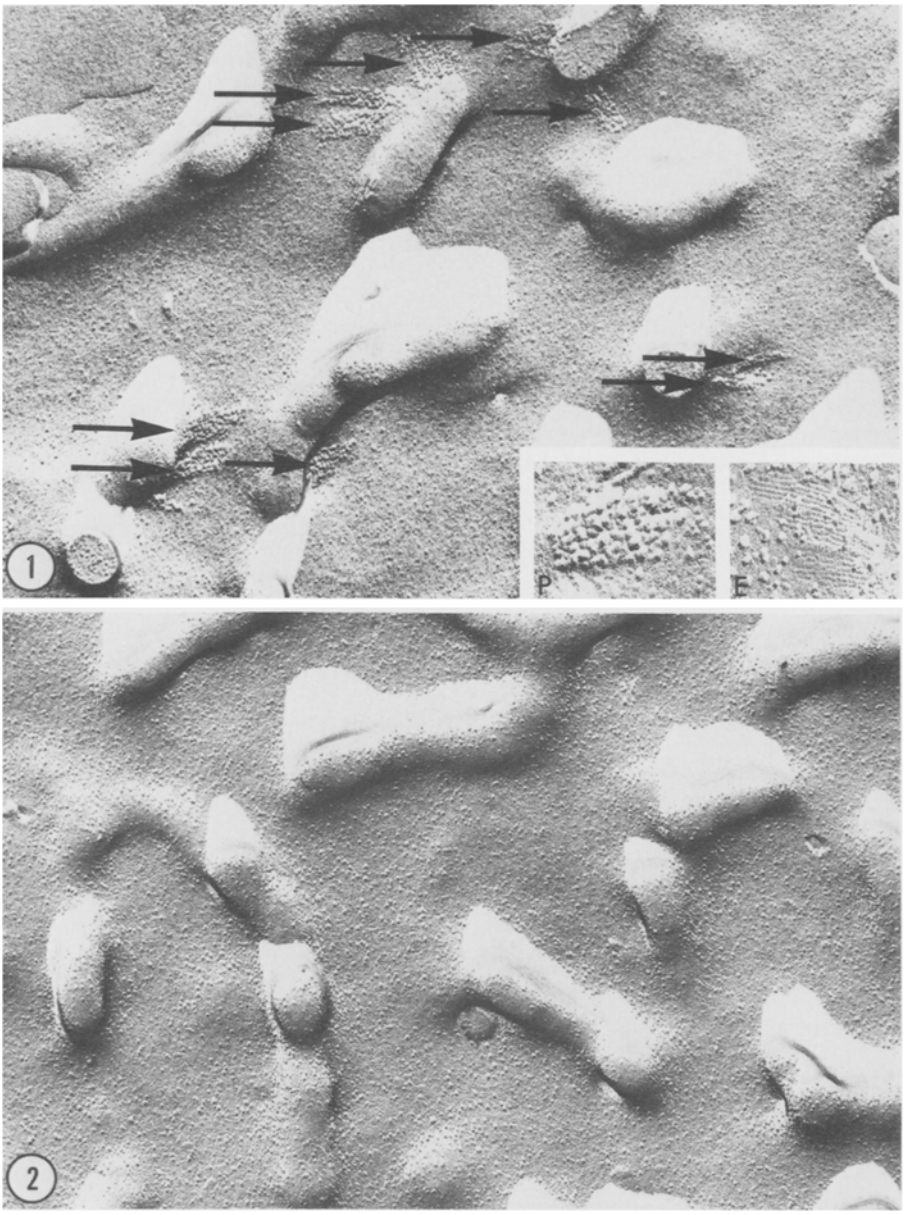


Fig. 1. Granular cell luminal membrane (fracture face *P*) from the bladder of a dehydrated toad fixed *in situ*. Sites of aggregated intramembrane particles are indicated by arrows. Aggregates are characterized structurally by aligned particles in closely associated parallel rows on fracture face *P* (insert *P*) and a corresponding organization of parallel grooves on fracture face *E* (insert *E*) (50,000×; inserts 100,000×)

Fig. 2. Granular cell luminal membrane (fracture face *P*) from the bladder of a hydrated toad fixed *in situ*. No aggregates of intramembrane particles are found (50,000×)

stimulated by vasopressin *in vitro* (Kachadorian *et al.*, 1977*b*). In contrast, no aggregates are found in the luminal membrane of granular cells from bladders of hydrated toads fixed *in situ* by the same procedure (Fig. 2).

Granular Cell Cytoplasmic Vacuoles

Hydrated toads do, however, have intramembrane particle arrays at other membrane sites. Organized arrays structurally similar to those found in the luminal membrane of dehydrated toads are observed in the membrane of vacuoles found in the cytoplasm of granular cells (*V*, Fig. 3). The striking structural similarity between these aggregates and those found in the luminal membrane is shown in complementary replicas (Fig. 4). Closely aggregated particles organized in rows are found on the fracture face *P* of the vacuoles (arrows, Fig. 4*p*), while parallel grooves are found on the fracture face *E* of the vacuoles (arrows, Fig. 4*e*). This fracturing pattern is indistinguishable from that characteristic of the luminal membrane aggregates. Similar cytoplasmic vacuoles with aggregates have also been identified in bladders from dehydrated toads, as well as in both control and vasopressin-treated bladders from *in vitro* experiments.[1] Whether or not the number of these vacuoles varies with the presence of vasopressin has not been established.

Although these vacuoles have only been identified in the cytoplasm of granular cells, they appear to differ significantly from the cytoplasmic granules which characterize this cell type. Vacuoles with aggregates tend to have a more elongate shape than the granules (*G*, Fig. 3). They are also extremely rare in comparison to the number of granules. A preliminary study of eleven granular cells from a hydrated toad found over 400 granules, but only four cytoplasmic vacuoles with aggregates.

Membrane Specialization of Basal Cells

The degree of membrane specialization characteristic of the basal cell is remarkable, considering that these cells do not reach the lumen of the bladder and have no known functional role. There has never

1 The presence of cytoplasmic vacuoles with aggregates in unstimulated bladders has also been observed independently by Humbert *et al.* (F. Humbert, R. Montesano, A. Grosso, R.C. de Sousa & L. Orci (1977). Particle aggregates in plasma and intracellular membranes of toad bladder (granular cell). *Experientia* **33**:1364

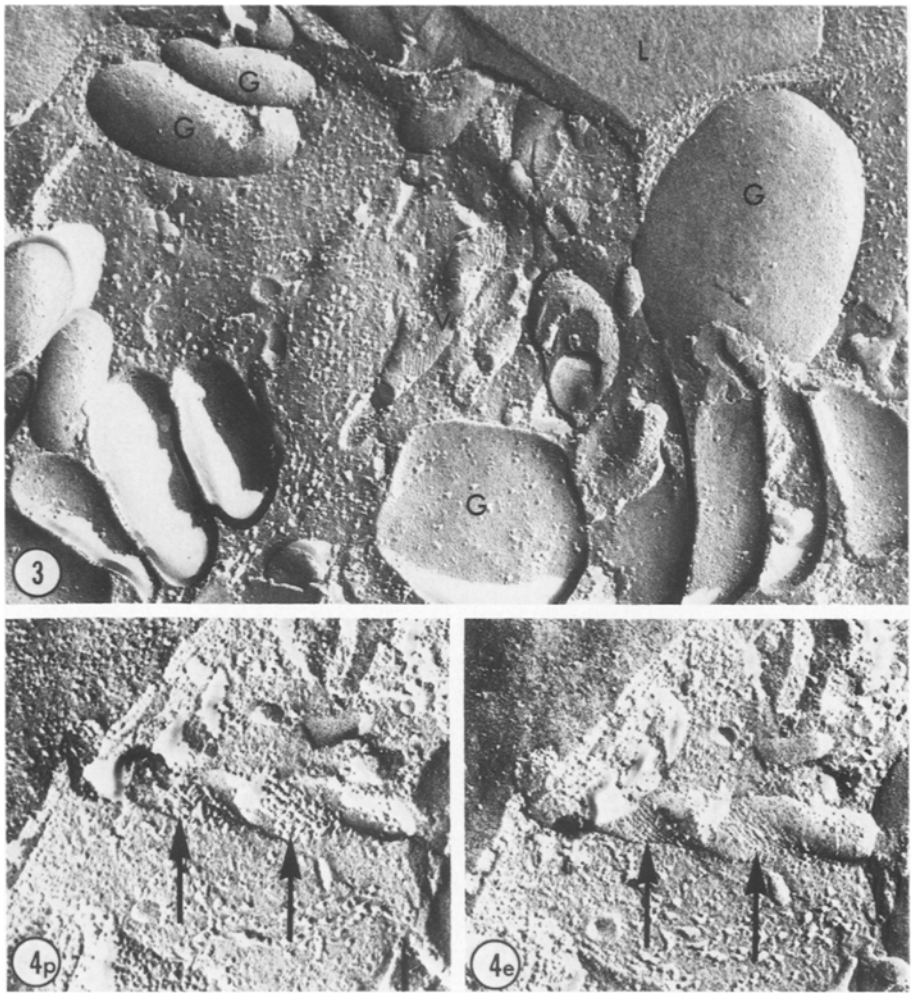


Fig. 3. The apical cytoplasm of a granular cell from the bladder of a hydrated toad fixed *in situ*. Organized arrays are found in the membrane of cytoplasmic vacuoles (*V*). These vacuoles are usually much more elongate in shape than the granules (*G*) which characterize this cell type (65,000 ×)

Fig. 4. Complementary freeze-fracture replicas of a cytoplasmic vacuole from a hydrated toad. One of the replicas was photographed inverted with respect to the other so that the two images would correspond rather than be mirror images of each other. Aggregates (arrows) in this membrane appear to be structurally indistinguishable from those found in the luminal membrane of dehydrated toads (compare with Fig. 1). Parallel rows of particles appear on fracture face *P* (arrows, 4p) while corresponding parallel grooves appear on fracture face *E* (arrows, 4*e*). (80,000 ×)

been any difficulty distinguishing the basal cell membrane from that of granular cells because basal cells, especially on the side adjacent to the basal lamina, display a high degree of pinocytotic activity, represented by many crater-like indentations in the membrane (*PV*, Fig. 5). This membrane also has intramembrane particle aggregates (*Ag*, Fig. 5) that are structurally indistinguishable from the aggregates found in granular cells. Just as in the luminal membrane and the cytoplasmic vacuoles of granular cells, this array is characterized by closely associated parallel rows of particles on fracture face *P* (insert *P*, Fig. 3) and similarly arranged grooves on fracture face *E* (insert *E*, Fig. 5). However, the occurrence of aggregates in basal cells does not appear to be related to vasopressin exposure. These aggregates have been identified in basal cells from both hydrated and dehydrated toads fixed *in situ* as well as in control and vasopressin-treated bladders from *in vitro* experiments. To test the possibility that the number of such aggregates might be altered by vasopressin exposure, random micrographs were taken of basal membrane (plasma membrane adjacent to the basal lamina) from at least twenty basal cells of paired control bladders and bladders exposed to vasopressin (20 mU/ml) *in vitro* for 30 min. For three sets of paired bladders, the number of aggregates found in control bladders was 6 ± 2.8 (mean $\pm$ SEM) aggregates per 100 μm^2, while in vasopressin-treated bladders the number was $2 \pm .4$ (mean $\pm$ SEM) aggregates per 100 μm^2. Thus, there appears to be no indication that vasopressin increases the frequency of aggregates in basal cells. The overall frequency of aggregates in basal cell membrane is much lower than the 73 ± 7 (mean $\pm$ SEM) aggregates per 100 μm^2 (calculated from data of Kachadorian *et al.*, 1977*b*) found in the luminal membrane of granular cells in bladders maximally stimulated by vasopressin.

The plasma membrane of basal cells also has three types of membrane particle arrays which almost certainly represent sites of membrane attachment and cell-to-cell communication. One of these arrays has the hexagonal packing of particles characteristic of gap junctions (insert *G*, Fig. 7). This hexagonal packing clearly distinguishes the organization of gap junctions from that of aggregates. In addition, the particles of gap junctions are distinct and uniform in size, while those of aggregates frequently cannot be visualized as distinct particles and appear less uniform in size. Another type of intramembrane particle array found in the plasma membrane of basal cells is an array characteristic of the regions of membrane associated with desmosomes (*D*, Fig. 7). These regions have radially oriented low profile strands which frequently give them a charac-

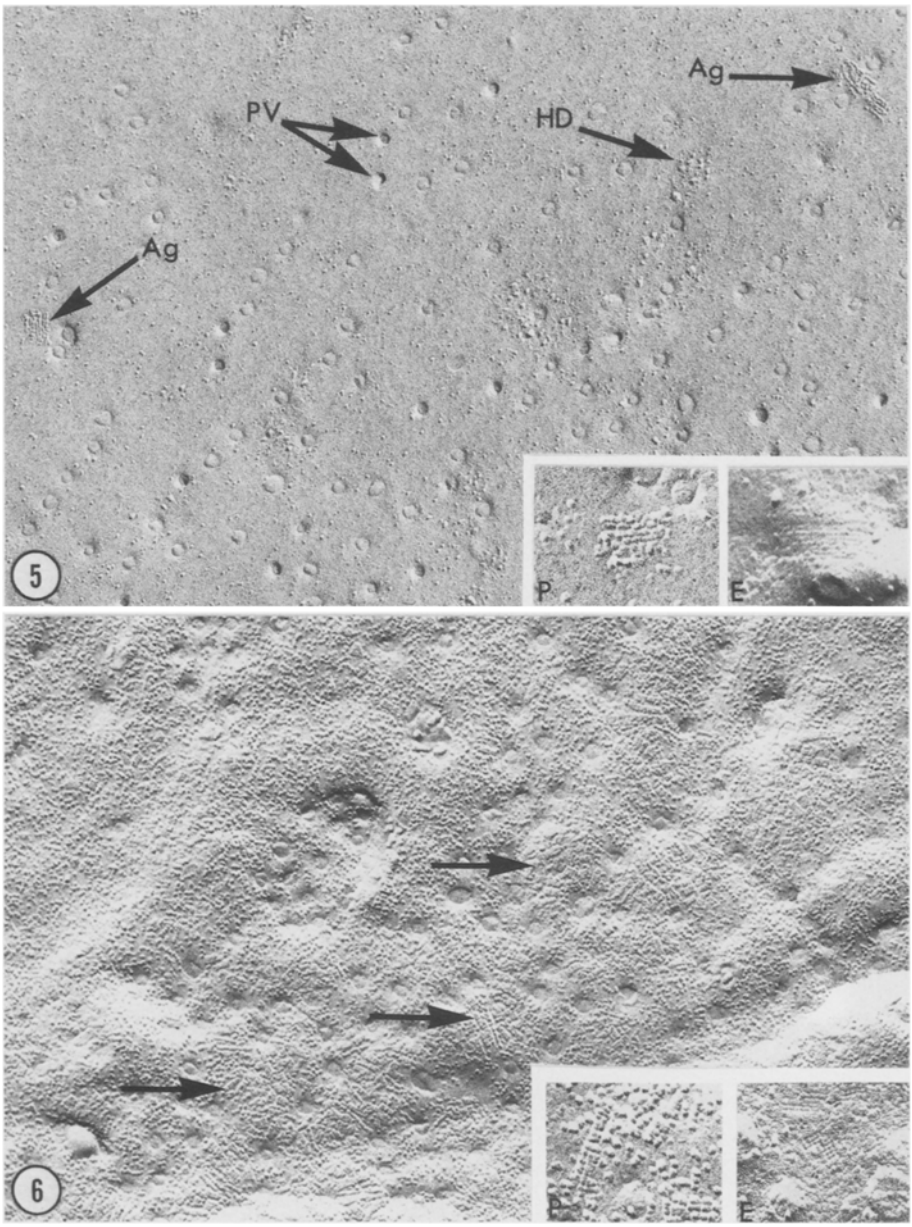


Fig. 5. Basal cell plasma membrane (fracture face *P*) from a toad bladder not exposed to vasopressin. Membrane adjacent to the basal lamina is characterized by a large number of pinocytotic invaginations (*PV*) and loose clusters of very large particles at sites of hemidesmosomes (*HD*). In addition, there are sites of aggregated intramembrane particles (*Ag*). The structure of these aggregates as seen on fracture face *P* (insert *P*) and fracture face *E* (insert *E*) appears to be indistinguishable from that of aggregates which occur in granular cells. (50,000 × ; inserts 100,000 ×)

teristic "sunburst" appearance. In addition, there are in basal cell membrane adjacent to the basal lamina loose clusters of very large particles (200–300 Å) which appear identical to those recently identified as associated with hemidesmosomes (Shienvold & Kelly, 1976). As would be expected, the occurrence of gap junctions, desmosomes, hemidesmosomes and pinocytotic invaginations does not appear to be influenced by vasopressin exposure.

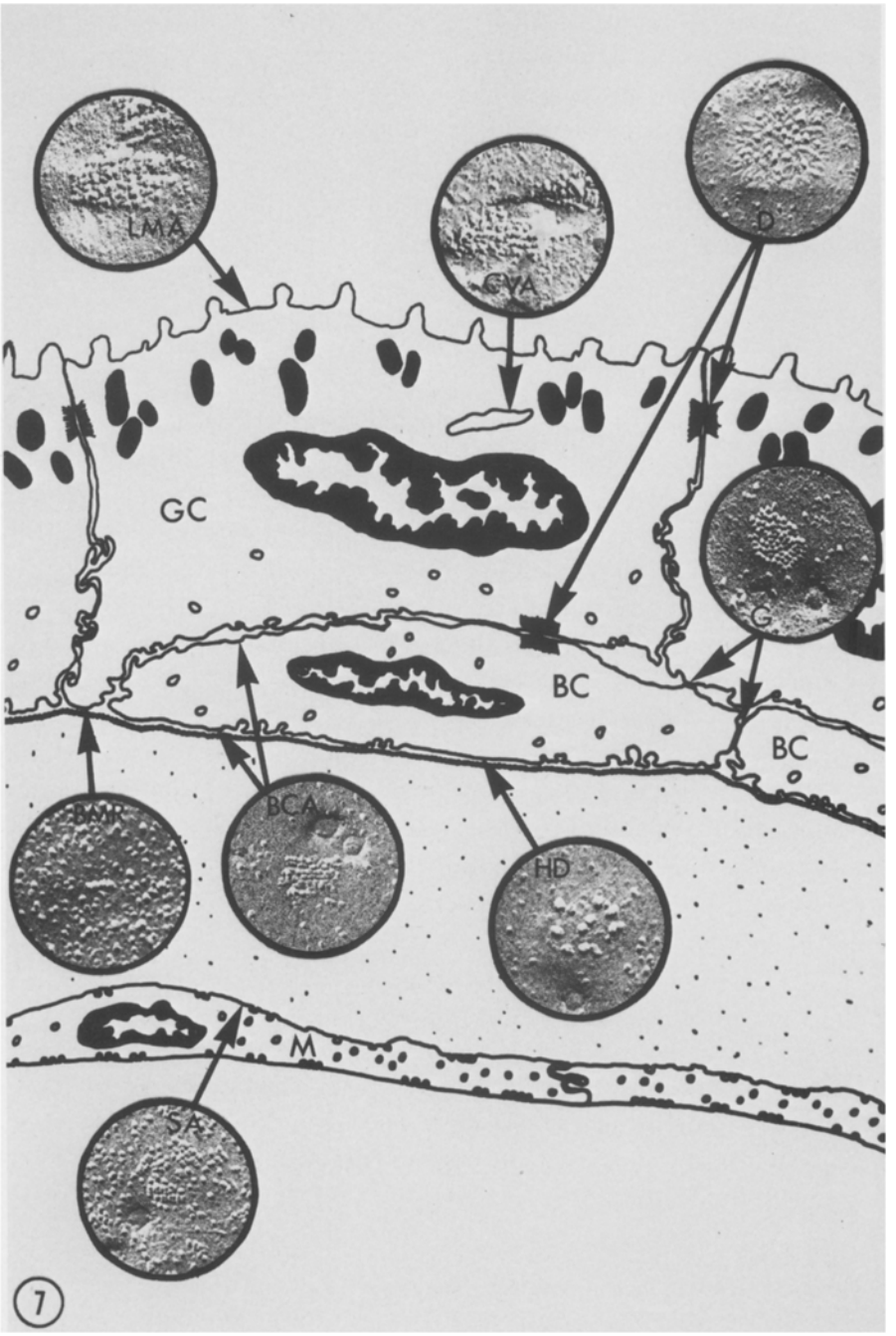
Membrane Specialization of the Mesothelium

Yet another type of membrane particle array is characteristic of the toad bladder's mesothelium, the thin layer of cells which lines the serosal surface of the bladder. This type of array is found in irregular plaques (arrows, Fig. 6) and is structurally similar to the intramembrane particle aggregates found in the epithelium. However, in this array, the particles appear to be more uniform in size (insert *P*, Fig. 6) and there is usually an alignment perpendicular to the rows such that there is a definite square packing of the particles (insert *E*, Fig. 6). Although an indistinct periodicity perpendicular to the rows can occasionally be discerned in aggregates of the epithelium, the prominence of this square packing serves to distinguish mesothelial intramembrane arrays from those found in the epithelium. In addition, the occurrence of mesothelial square arrays does not appear to be detectably influenced by the presence of vasopressin.

Specificity of Intramembrane Particle Arrays

Considering the variety of toad bladder membranes in which intramembrane particle aggregates have been identified, one might be led to the conclusion that there is no real membrane specificity to this type of intramembrane structure. This, however, does not appear to be the

Fig. 6. Mesothelial cell plasma membrane (fracture-face *P*) from the bladder of a hydrated toad fixed *in situ*. This membrane has an irregular distribution of an organized particle array. Sites where the organization is especially evident are indicated by arrows. Although the appearance of these arrays is similar to that of the aggregates found in the epithelium, it differs in that the particles appear more uniform in size (insert *P*) and usually have a square packing which is especially evident on fracture face *E* (insert *E*) (50,000 × ; inserts 100,000 ×)


LMA
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case. Aggregates have not been found in the basolateral membrane of granular cells nor in any membrane of mitochondria-rich cells. Loose clusters of rod-shaped particles are sometimes observed in the luminal membrane of mitochondria-rich cells (Bourguet *et al.*, 1976; Wade, 1976) and, in rare instances, particles are found in the basolateral membrane of granular cells which are aligned into a single short row (insert *BMR*, Fig. 7). Although it is possible that such limited associations represent important membrane specializations with specific function, it is also possible that they occur occasionally simply by chance.

Discussion

Over the last ten years freeze-fracture studies have demonstrated that organized structures exist at a great variety of membrane sites. The sites of intramembrane arrays thus far identified in the toad urinary bladder are summarized in Fig. 7. We are all familiar at this stage with the structural appearance of gap junctions in freeze-fracture replicas. It is now clear that characteristic arrays of particles also exist at the site of desmosomes (McNutt & Weinstein, 1973; Staehelin, 1974) and hemidesmosomes (Shienvold & Kelly, 1976) in a variety of tissues. So it is not surprising that these structural specializations are found in the toad urinary bladder. What is somewhat surprising is that there are sites where we have not found gap junctions. The toad bladder was one of the first epithelia in which cell-to-cell ionic coupling was demonstrated (Loewenstein *et al.*, 1965), and the gap junction structure has been clearly associated with cell coupling (Payton, Bennett & Pappas, 1969; Revel, Yee & Hudspeth, 1971). It is remarkable that all of the gap junctions which we have come across, after years of studying the toad bladder, have been associated with basal cells and we have never observed gap junctions between adjacent granular cells or between granular cells and mitochondria-rich cells. Since basal cells appear to make

Fig. 7. Summary indicating the location of intramembrane particle arrays which have been identified in the toad urinary bladder (all inserts 80,000×).

LMA: Luminal membrane aggregates
CVA: Cytoplasmic vacuole aggregates
D: Desmosomes
G: Gap junctions
BMR: Basal membrane rows
BCA: Basal cell aggregates
HD: Hemidesmosomes
SA: Square arrays
GC: Granular cell
BC: Basal cell
M: Mesothelium

gap junctions with granular cells as well as with each other, it may be that ionic coupling of other cell types is achieved via the basal cells.

Of special interest in the last few years has been the observation that organized arrays of particles can exist at nonjunctional membrane sites and that the occurrence of this type of array in the luminal membrane of granular cells is closely correlated with the action of neurohypophysial hormones (Chevalier *et al.*, 1974; Kachadorian *et al.*, 1975). The finding that structurally similar aggregates exist at the same site in bladders from dehydrated toads fixed *in situ*, while not surprising, indicates that the phenomenon does occur in the living toad and is not strictly limited to bladders studied *in vitro*.

At the same time, we have made the observation that aggregates do exist at two membrane sites, regardless of vasopressin exposure. Of course, it is not certain that these are exactly the same membrane structures with exactly the same function as those found in the luminal membrane of vasopressin-treated bladders. It can only be said that they have the same appearance in freeze-fracture replicas. But since the closely apposed parallel rows of particles have a rather distinctive structure, it is likely that they are related. The aggregates that exist in the membrane of vacuoles might actually be the source of the luminal membrane specialization. These aggregates might be inserted into the luminal membrane if in some way a rise in the concentration of cytoplasmic cyclic AMP leads to a fusion of these vacuoles with the luminal membrane. This is a variation on a model originally proposed by Masur, Holtzman and Walter (1972) and later used by Taylor, Mamelak, Reaven and Maffly (1973) to explain how microtubules and microfilaments might be involved in the response to vasopressin. The original suggestion was that it is the granules which fuse with the luminal membrane (Masur *et al.*, 1972). It is clear, however, that aggregates do not exist in the membrane of the granules which are so common in the cytoplasm of this cell type. The vacuoles which have aggregates appear quite different from granules, at least in freeze-fracture. Of course, it is possible that these vacuoles look similar to granules in thin section. If they also have lysosomal enzymes and, in addition, require microtubules and microfilaments for fusion with the luminal membrane, the involvement of these vacuoles in the vasopressin response could tie together a number of observations (Masur *et al.*, 1972; Taylor *et al.*, 1973, Pietras, Seeler & Szego, 1975). However, caution is warranted since we have not yet seen one of these vacuoles in the process of fusing with the luminal membrane. This is not terribly surprising because these vacuoles are rather rare and we have not yet

had time to look at bladders fixed during the initiation of the vasopressin response which is when one would expect such an event, if this model is correct. There are two reasons why these vacuoles might be rare, even supposing they are the sole source of luminal membrane aggregates. First, it should be kept in mind that the luminal membrane aggregates are relatively rare, representing only about 1% of the luminal membrane area in bladders maximally stimulated with vasopressin (Kachadorian *et al.*, 1977*b*). Second, since these vacuoles have membranes with sharp curvature, relatively large areas are often obscured by shadows in freeze-fracture. This means that in some cases we may not recognize such vacuoles because the aggregates happen to be obscured by shadows.

It is difficult to speculate on the significance of finding aggregates in the plasma membrane of basal cells since the function of basal cells is unknown. The fact that their number at this membrane site is very low and does not appear to be increased by exogenous vasopressin may mean that their occurrence at this site does not have important physiological significance. If basal cells differentiate into granular cells, the possession of this membrane specialization at some stage may have some developmental significance.

One can only make similar speculative statements regarding the possible significance of the square arrays found in the mesothelium. Because the two arrays differ in structure, it is unlikely that the square array is related to the aggregates which occur in the epithelium. It may, however, be related to similar "square arrays" of unknown function which have been described in a variety of tissues including brain astrocytes (Landis & Reese, 1974), muscle (Ellisman *et al.*, 1976), intestine (Staehelin, 1972), hepatoma cells (Porvaznik, Johnson & Sheridan, 1976), and the basolateral membrane of light cells in the rat kidney (Humbert *et al.*, 1975).

Although the freeze-fracture technique clearly has limitations (Wade, Kachadorian & DiScala, 1977), there seem to be two important benefits which have come from freeze-fracture studies of the toad bladder. First, it is now apparent that most of the membranes have distinctive structural features when examined by freeze-fracture electron microscopy. These features may serve to identify membranes in isolated preparations and help us in the future to determine which proteins are associated with different membranes and possibly to relate these proteins to specific membrane specializations. Second, the technique has revealed organized regions of membrane that appear to be closely related to vasopressin-induced changes in membrane permeability. Clearly, the most intriguing

prospect is that these sites of particle aggregation may represent regions of membrane with increased permeability. The fact that they occur in the luminal membrane of the epithelium and can be induced by cyclic AMP is consistent with evidence demonstrating that the site of the vasopressin-induced permeability change is the luminal membrane (DiBona, Civan & Leaf, 1969) and that the response is mediated by cyclic AMP (Handler *et al.*, 1965). Also, as would be expected, the occurrence of aggregates at this site is quantitatively proportional to the level of permeability observed both when submaximal concentrations of vasopressin are used (Kachadorian *et al.*, 1977*b*) and when the response is inhibited by methohexital (Kachadorian *et al.*, 1977*a*). The observation that aggregates can exist at membrane sites other than the luminal membrane in the absence of vasopressin is not inconsistent with the possibility that aggregates represent regions of increased permeability. But it does indicate that we do not fully understand the significance of this membrane structural specialization and what, in addition to vasopressin, may influence its occurrence.

Also relevant to the significance of this specialization are recent freeze-fracture studies of the rat renal collecting duct by Harmanci, Kachadorian, Valtin and Di Scala (1977). In this tissue they find that loose clusters of intramembrane particles occur in the luminal membrane of light cells in association with antidiuresis. However, the appearance of these clusters differs significantly from that of the aggregates found in the toad bladder. This indicates that the organization of particles which characterizes these aggregates may not necessarily be a universal feature of vasopressin-induced changes but rather that structurally different intramembrane particle arrays may be associated with the change in permeability in other responsive tissues.

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