ALTERATIONS IN MEMBRANE-ASSOCIATED PARTICLE DISTRIBUTION DURING ANTIDIURETIC CHALLENGE IN FROG URINARY BLADDER EPITHELIUM

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ABSTRACT Frog urinary bladder epithelium has been examined by freeze-fracture electron microscopy of preparations previously fixed by glutaraldehyde either at rest or during antidiuretic challenge. All the agonists tested were observed to induce membrane particle clustering in the A face of the apical plasma membrane of granular cells. This was the case for the natural hormone (hypophysial extracts) and its presumed cellular mediator, adenosine 3',5'-monophosphate. Particle clustering was observed both in the presence and in the absence of water net flow and is thus independent of these movements. Clusters were also observed during hydrosmotic challenge by hypertonic serosal media, a condition which depresses transepithelial sodium transport. No complementary patterns of these A face clusters could be found on the B face. The significance of these membrane-associated particle clusters is discussed in terms of membrane structure and function.

INTRODUCTION

The introduction of freeze-etching technique in biology (Moor and Muhlethaler, 1963) has led to considerable progress in our knowledge of biological membranes. This technique affords a direct insight of the interior of the membrane. At low temperature, the fracture concerns preferentially the lipid phase of the membrane (Branton, 1966, 1971), and the replicas obtained provide thus a view of the inner face of the two membrane leaflets.

Replicas show two major constituents. The overall background is smooth and, most probably, corresponds to the hydrophobic acyl chains of the phospholipids forming the two leaflets of the membrane bilayer. Numerous particles (membrane associated particles, MAP) are observed, protruding from that smooth background.

The density of these particles is extremely variable from one preparation to the other and this variability appears to be related to the metabolic activity of the tissue. Myelin, which behaves as a rather inert insulator, presents very few particles, while metabolically active membranes may exhibit as many as two to three thousand par-

ticles per square micron (Branton, 1969). Particle density has also been shown to vary during the life cycle of the cell (Scott et al., 1971) and to differ widely from one place to the other on the membrane of the same cell (Friend and Fawcett, 1974).

Typical particle organization has been described in connection with various cellular structures or processes: pinocytosis vacuoles (Orci and Perrelet, 1973), ciliae (Gilula and Satir, 1972; Bergstrom et al., 1973), mucocysts (Satir et al., 1972), etc. Some structure, such as gap junctions which are involved in cell to cell communication (Loeweinstein, 1967) present a very high particle density (Kreutziger, 1968; Satir and Gilula, 1973; Staehelin, 1974) while other regions involved in membrane to membrane fusion are, on the contrary, free of any particle (Ahkong et al., 1975). Thus particle distribution appears to be closely linked to membrane function and can be taken as an index of its activity. (See also the review of Branton and Deamer, 1972). Two types of information have recently been obtained on the distribution of MAP in the epithelia of the amphibian urinary bladder, a useful model in the study of the mechanism by which hormones control membrane permeability.

It has been shown, in that tissue, that the particle partition coefficient (Satir and Satir, 1974) between A and B faces is considerably lower for the physiologically limiting membrane—the apical or luminal membrane—than for the laterobasal membranes of the epithelial cells (Chevalier et al., 1974*a*; Wade et al., 1975). Furthermore, a significant clustering of the A face particles of the apical membrane has recently been reported during oxytocin challenge (Chevalier et al., 1974*a*).

The aim of the present work was to study the relationship between the cluster formation and the alteration of transepithelial permeability elicited by the neuropeptide. Our observations show that clusters are also present in preparations exposed to: (*a*) neurohypophysial extracts (the natural antidiuretic hormone in *Rana esculenta* is arginine-8-vasotocin); (*b*) cyclic adenosine 3′,5′-monophosphate (cAMP), the presumed intracellular mediator of the hormonal actions; (*c*) serosal hyperosmolar solutions, a condition which mimics the hydrosmotic effect of antidiuretic hormone but depresses the transepithelial sodium transport.

We also observed that during antidiuretic challenge, clusters were formed both in the presence and the absence of a transepithelial osmotic gradient. This observation suggests that they are not due to the establishment of a net water flow, but to the hormone action. Finally, we observed in very rare cases, elongated particles on the A face of both apical and laterobasal membranes. Different considerations suggest that they are membranes of "mitochondria-rich" cells.

MATERIAL AND METHODS

Experiments were run on isolated frog urinary bladders. Animals (*Rana esculenta*, L.) were kept in running tap water at 2°C until their sacrifice. Whole bladder or isolated epithelium (Bourguet et al., 1975) was employed.

In most experiments, the preparations were incubated in Ringer's solution on their both sides, so that no net transepithelial water flow could take place (Na^+, 114.5 meq; K^+, 5 meq; Ca^{2+}, 1 meq; Cl^-, 119 meq; HCO_3^-, 2.5 meq; pH 8.1 when bubbled with air). In other experi-

ments, the preparations were incubated in the presence of a transepithelial osmotic gradient, the apical solution being made hypotonic by reducing the NaCl concentration to 5.6 mM. Net water flow was recorded according to Bourguet and Jard (1964) for the whole bladder and according to Bourguet et al. (1975) for the isolated epithelium.

Preparations were then kept for 20 min either at rest (Ringer solution on the serosal side) or in experimental series in the presence of either hypophysical extracts (1% of hypophysis per ml), or synthetic oxytocin (Syntocinon, Sandoz Pharmaceuticals, Basel, Switzerland) or cyclic adenosine 3',5'-monophosphate (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.).

In some experiments an increase in water permeability was elicited by making the serosal solution hypertonic through the addition of 220 mosmol of mannitol (Bentley, 1964; Ripoche and Pisam, 1973). At the peak of the hydrosmotic response, preparations were fixed for 15 min in 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The samples were immersed in a 30% glycerol solution for 20 or 30 min and quickly frozen in Freon 22 chilled by liquid nitrogen. The specimens were freeze-cleaved or -etched at −100°C for 20 s to 2 min in a Balzers freeze-etch unit BA 500 (Balzers High Vacuum Corp., Santa Ana, Calif.) according to the method of Moor and Muhlethaler (1963). The shadowing was performed by a Ta-W film using an electron beam evaporation source (E.V.M. 052, Balzers). Replicas were washed for 24 h in trypsin and then in sulfochromic solutions, mounted on 300 mesh grids, and examined in a Philips E.M. 200 electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, N.Y.).

OBSERVATIONS

Ultrastructural alterations occurring during antidiuretic challenge interest mainly the luminal border of the granular cells, which represent the most frequent cell type in *Rana esculenta.*

Observations on the Apical Plasma Membrane A Face

Preparations at Rest. Present observations show, in agreement with our previous data (Chevalier et al., 1974*a*) that the apical A face is particularly low in particle density (Fig. 1). This is at variance with the high particle density observed on the laterobasal membranes and on the A face of most cell membranes.

The particles appear to be spherical. They are generally randomly dispersed but some small clusters can be observed. Although no quantitative estimations were made they appear to be significantly smaller than particles observed on the B face of the same cells (see Fig. 7 for comparison).

Preparations Exposed to Oxytocin in the Presence or in the Absence of a Transepithelial Osmotic Gradient. In order to determine if cluster formation is not linked to transepithelial water movements, preparations were examined after incubation either in Ringer's solution or in the presence of a transepithelial osmotic gradient. In the latter case, water net flow was recorded and preparations were fixed at the maximum of the response to oxytocin.

Clusters similar to those described in our previous paper were repeatedly observed in both experimental conditions (Figs. 2 and 4). They ranged from a dozen to a few hundred particles. These particles appear to be larger than isolated particles present at rest (compare Figs. 1 and 2), but evaluation of their dimensions is difficult due to the existence of some fusion between particles (see inset, Fig. 4). Low magnification views

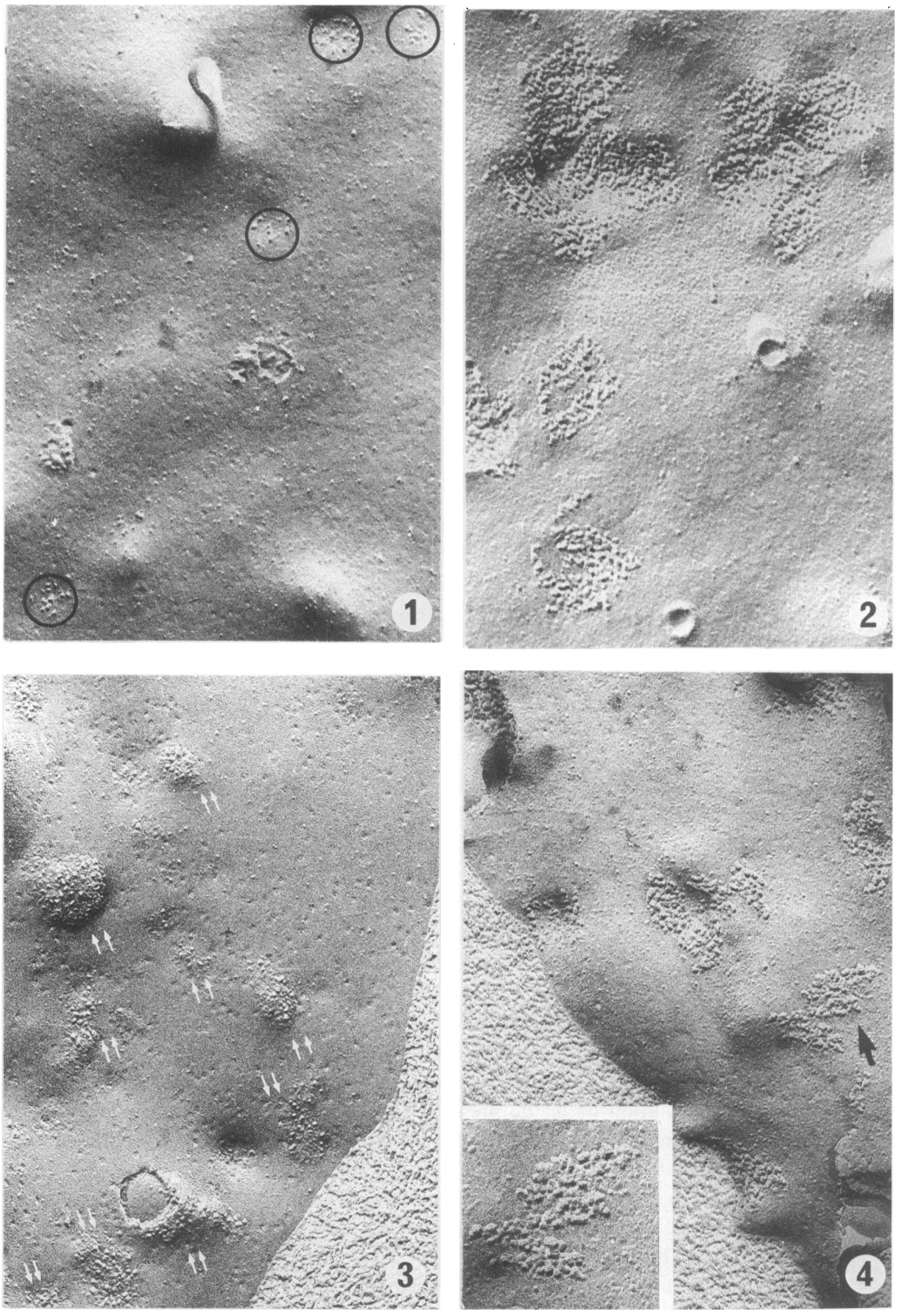


FIGURE 1 Ultrastructure of the apical membrane of a glutaraldehyde-fixed resting bladder: A face (× 70,000). Note the presence of a few randomly dispersed spherical particles. Some very small aggregates are also observed (circles).

FIGURES 2–4 Ultrastructure of the A face of the apical plasma membrane during a challenge by (*a*) neurohypophysial extract (Fig. 3); (*b*) oxytocin in the absence (Fig. 2) or in the presence (Fig. 4) of a transepithelial net water flow. (× 70,000; inset of Fig. 4, × 110,000)

(see Fig. 6) illustrate that the fraction of the apical surface interested by the clusters is always small.

Influence of Neurohypophysial Extracts. Oxytocin is not a natural neuropeptide in *Rana esculenta*, where most of the antidiuretic activity of the hypophysis can be accounted for by arginine-8-vasotocin. Extracts of *Rana esculenta* hypophysis were prepared in Ringer's solution and their hydrosmotic activity tested in separate experiments. Preparations incubated in the presence of maximal concentration of this extract were found to exhibit the same particle clustering on the A face of their apical plasma membrane (Fig. 3).

Influence of Cyclic Adenosine 3',5'-Monophosphate. Considerable evidence (Orloff and Handler, 1967) indicates that this nucleotide is the intracellular mediator of antidiuretic hormone actions in amphibian urinary bladder. As illustrated by Fig. 5, the nucleotide elicited the same clustering of MAP as observed with natural hormone.

Influence of Incubation in Hyperosmolar Serosal Media. Fig. 6 is a replica of the apical A face as observed in this condition. In this particular experiment, serosal Ringer solution was made hypertonic by the addition of 220 mosmol of mannitol and the transepithelial net water flow exhibited a progressive increase: $+1.5\ \mu l\ min^{-1}$.

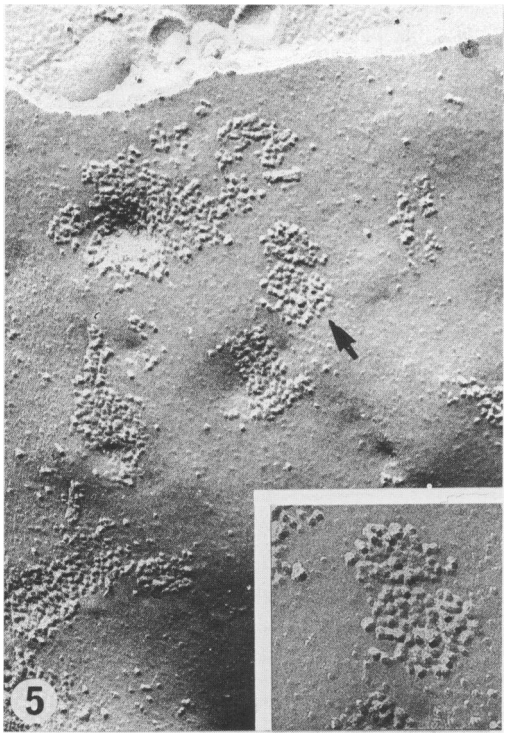
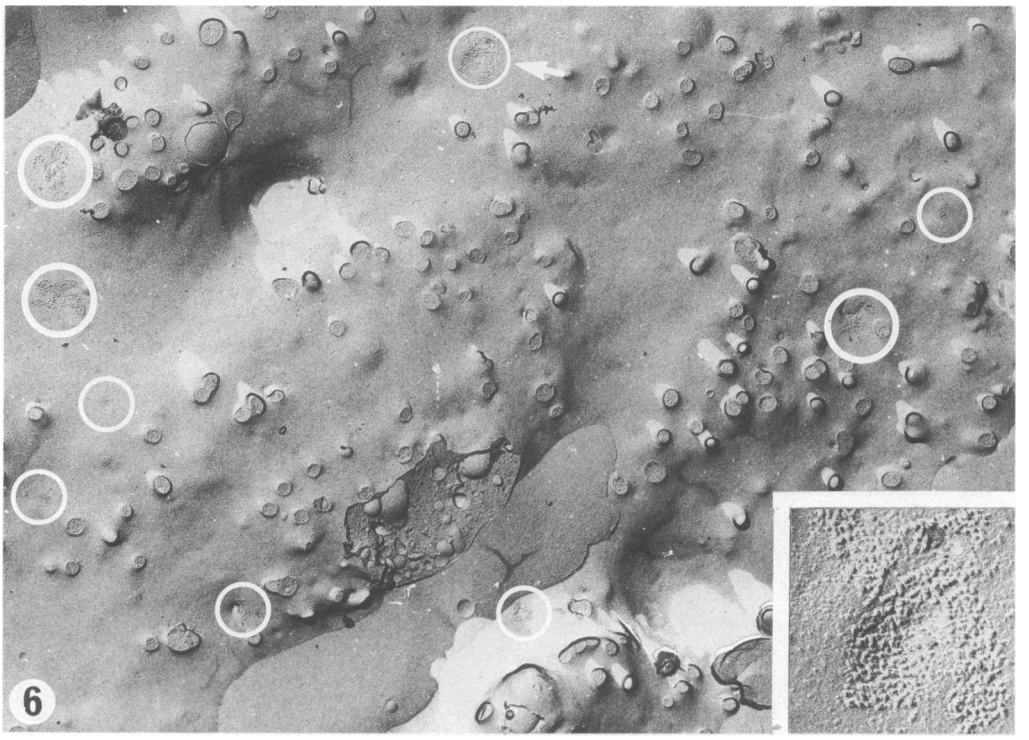
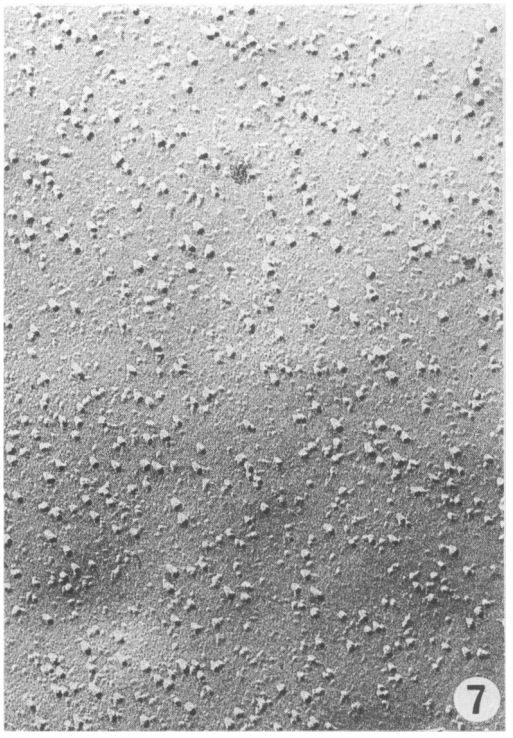


FIGURE 5 Ultrastructure of the A face of the apical plasma membrane in the presence of cyclic adenosine 3',5'-monophosphate. Note the presence of clusters similar to those of Figs. 2–4. (× 70,000; inset, × 110,000).





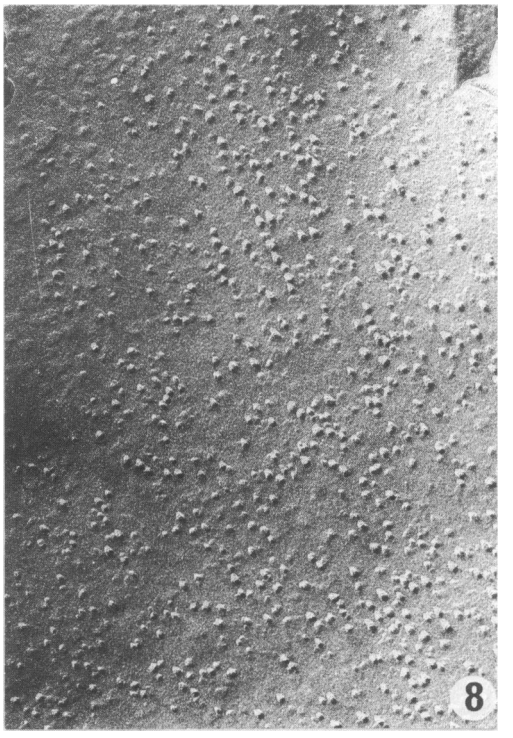


FIGURE 6 A face of the apical plasma membrane of an isolated epithelium exposed to an hypertonic serosal medium and subsequently fixed with glutaraldehyde. Clusters are circled. The low magnification (× 15,000) is intended to show the relatively low fraction of the membrane involved in the clustering phenomenon. (Inset, × 70,000)

FIGURES 7-8 B face of the apical plasma membrane at rest (Fig. 7) and during antidiuretic challenge (cAMP, 2.10^{-3} M, Fig. 8); see text. (× 70,000)

cm^{-2}. The figure shows that typical clustering can be observed after this hypertonic stimulation.

Particle and Cluster Density in Apical Plasma Membrane during Antidiuretic Challenge. Although a definitive estimation of these parameters would require additional observations, an attempt has been made to quantify the particle density and the relative area involved in clusters in the apical plasma membrane. All numerations have been made on preparations previously fixed by glutaraldehyde.

Compared with the low particle density observed on the A face at rest (46 MAP/μm^2) and in area of challenged cells where clusters were not observed (55, 52, 36 MAP/μm^2 in three experiments) particle density was clearly higher (164, 119, 112 MAP/μm^2 in three experiments) on challenged cells in the vicinity of the clusters.

Area involved in the clusters was found to vary widely from one place to the other for the same cell and was measured only in two favorable cases where a sufficient area of membrane was exposed by the fracture. In one case, 118 μm^2 were exposed on the same cell and the cluster surface was 0.700 μm^2, i.e. 0.6% of the total membrane area. In the other case a total membrane area of 943 μm^2 was exposed on four adjacent cells and the cluster surface was 2.5 μm^2, i.e. 0.26% of the total surface.

Observations on the B Face of the Apical Plasma Membrane

Membrane-associated particles appearing on the B face were usually larger (Fig. 7) than particles observed on the A face (see Fig. 1) and their density was greater (see Chevalier et al., 1974*a*). Although this face is complementary to the A face studied in the preceding section, no complementary patterns of the A face clusters, induced during antidiuretic challenge, could be found on this B face. Small areas of variable frequency were free of particles but no correlation with clusters could be made.

Observations on Membrane with Elongated Particles

Spherical-shaped particles studied in the two preceding sections are typical of the major cell type in frog urinary bladder: the granular cells. A few other cells presented elongated particles. Some were dumbbell shaped and apparently formed by juxtaposition of two spherical particles whose dimensions were comparable to those of granular cells. These elongated particles were observed on the A face of both the apical and the laterobasal membranes. On the laterobasal membrane (one observation) particles were randomly distributed with a density of 460 particles/μm^2 (not illustrated).

In two other cases, these elongated particles were localized on an apical plasma membrane, clearly recognizable by the presence of numerous microvilli, protruding into the lumen of the bladder, and by the presence of tight junctions. Clusters of elongated particles were observed in this membrane (Fig. 9). They were surrounded by smooth areas devoid of particles, while, in other regions where clusters were not present (not shown), particles were randomly distributed with a density comparable to that observed on laterobasal membranes. As discussed below, cells with elongated particles are most probably "mitochondria-rich" cells.

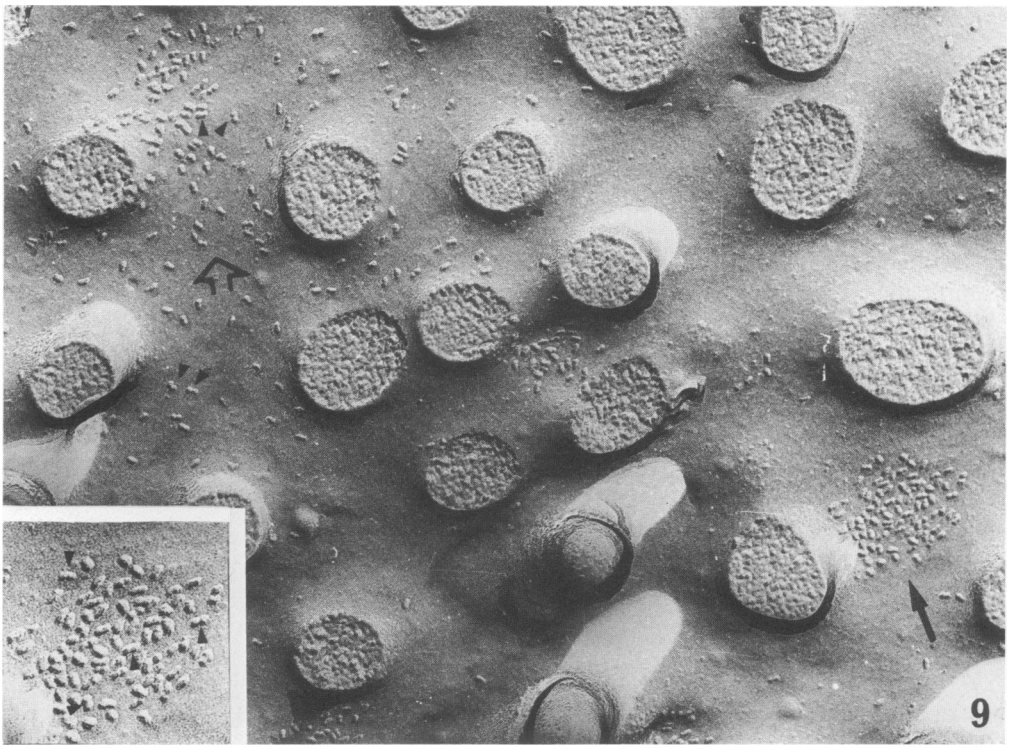


FIGURE 9 A face of an apical plasma membrane showing elongated particles. Particles are frequently dumbbell shaped as if they were formed by the juxtaposition of two subunits (arrows) with a size comparable to the spherical-shaped particles observed in other cells. Note the presence of "loose" clusters surrounded by area free of any particles, and the numerous sectioned microvilli indicative of a "mitochondria-rich" cell. (× 70,000; inset, × 110,000)

DISCUSSION

Since the pioneering work of Sawyer and Schissgall (1956) and Bentley (1958) the amphibian urinary bladder has frequently been used as an experimental model in epithelial permeability studies (see for example Leaf, 1965). Although simpler than the kidney, this model remains complex. At least, three different cell types are observed. Their relative frequency varies according to the animal species considered. At the present time, however, the specificity of their function, if any, has not been determined. Some alterations occurring during hormonal challenge appear to be limited to one cell type. For instance, this is the case for cell volume increase during hydrosmotic challenge observed in granular cells (but see also Scott et al., 1974). It is clear, however, that this effect is not directly related to the primary action of antidiuretic hormone but is a mere consequence of water movements. Also, at least two barriers in series apparently located at the apical and serosal borders of the epithelial cells are encountered in the model.

From a functional point of view, the limiting barrier appears to be located at the apical border of these cells. This border involves two components: (*a*) the apical plasma membrane and (*b*) the most apical part of the complex of intercellular junctions, the so-called "tight junction." It is presently not known if the hormonal control involves one or the other or both of these components. It has recently been suggested, from a study of a variety of tight and leaky epithelia (Claude and Goodenough, 1973), that functional transepithelial tightness is associated with a high degree of complexity of the fibril network of the tight junctions. Other studies have shown that the tight junction can undergo structural alterations in some experimental conditions (Wade et al., 1973; Ripoche and Pisam, 1973; Chevalier et al, 1974*b*). However, although studies are still in progress, it can be mentioned that no statistically significant alterations of the tight junction ultrastructure have yet been observed during antidiuretic challenge in frog urinary bladder (Chevalier and Bourguet, 1975). On the contrary, structural differences have recently been reported to exist between apical and laterobasal membranes in the urinary bladder epithelium of the frog (Chevalier et al, 1974*a*) and of the toad (Wade et al., 1975). It has been observed that the particle partition coefficient between A and B faces is some seven to eight times lower in the apical plasma membrane than in laterobasal ones, where the high particle density of the A face is similar to that observed in most tissues.

A similar particle distribution, with particle density higher on B face than on A face, has also been observed in unfixed endothelial cells (Dempsey et al., 1973) and in the apical membrane of the acinar epithelial cells of pancreas (de Camilli et al., 1974). The interpretation of this ultrastructural specialization in terms of membrane permeability or function would, however, require additional information. A second ultrastructural characteristic is the presence of particle clusters, on the A face of the apical plasma membrane of preparations exposed to oxytocin (Chevalier et al., 1974*a*). As shown in this previous paper, neither glutaraldehyde fixation nor glycerol treatment can explain such a particle aggregation. Present experimental series have repeatedly confirmed the existence of such clusters. They show also that clusters can be observed in the presence as well as in the absence of a transepithelial osmotic gradient and, consequently, in the absence of any significant transepithelial net water flow. Thus, particle clusters do not result from water movements. They are in this respect at variance with most of the structural alterations (such as the dilatation of the intercellular spaces or the cell swelling and vacuolization) which are not observed in isosmotic media and are consequently linked to the net water flow and not to the primary membrane action of the hormone (Carasso et al., 1966). Our observations establish that clusters observed in the presence of oxytocin, a synthetic structural analog of arginine-8-vasotocin, the antidiuretic hormone in *Rana esculenta,* are also observed in the presence of hypophysial extracts eliciting a maximal increase in net water flow. It is thus likely that cluster formation is a physiological event.

The same particle clustering is also induced by cAMP, the presumed intracellular mediator of the hormone. In frog urinary bladder, cAMP apparently mediates two

effects: (*a*) an increase in sodium transepithelial transport (natriferic effect) and (*b*) an increase in water permeability (hydrosmotic effect). At the present time, there is no evidence that particle clustering could be preferentially linked to one or the other of these effects. The fact that clusters are observed during the hydrosmotic challenge by hyperosmolar media—a condition in which sodium transport is depressed—suggests however that clusters could be related to water permeability modifications. It can be mentioned in this respect that previous observations by Pinto da Silva (1973) suggest that areas with increased particle density have a high water permeability. The interpretation of particle aggregation in terms of membrane structure is still entirely hypothetical. According to the fluid mosaic model, membrane associated particles correspond to hydrophobic segments of the integral proteins which are embedded in the fluid matrix of the lipidic bilayer. Hydrophilic segments of the proteins protrude either into cytoplasmic or into external medium or, in some cases, into both. Repartition of both the lipidic and proteic components of the membrane is asymmetrical. This is demonstrated by the differences in chemical composition of the two leaflets (Bretscher, 1972) and by the asymmetrical distribution of hydrophilic proteins such as the concanavalin A receptors for example (Nicolson and Singer, 1971). Asymmetry is assumed to be maintained by the lipidic barrier which prevents the polar ends from flipping from one side of the membrane to the other.

Two kinds of mechanisms have been proposed to account for membrane permeation in permeability phenomena. The first one assumes the rotation of a proteic carrier in the membrane, its hydrophilic part making alternatively contact with the external and the cytoplasmic faces of the membrane. This hypothesis is, however, difficult to reconcile with the presence of the central hydrophobic barrier. Moreover, recent experiments by Kyte (1974) demonstrating that the fixation of specific antibody on the outer part of an ATPase does not inhibit the sodium transport, makes such a rotation very unlikely. The second mechanism (Singer, 1974) assumes that the ligand transfer results from conformational changes of proteic channels preexisting in the membrane.

None of these mechanisms apparently requires structural alterations such as the MAP aggregates observed in our experiments. Such aggregates, which could result either from the migration of preexisting particles or from the insertion of new particles in the membrane suggest a third mechanism involving the creation of junctions between outer and inner hemichannels located in the outer and inner leaflets of the membrane. Hemichannels in this hypothesis would be somehow linked to the membrane associated particles. They could for instance be located in the hydrophilic core of the particle or result from dislocations of the hydrophobic lipid monolayer created by the presence of the particles.

The low density of particles observed on the apical A face at rest would correspond to a low hemichannel density of the inner leaflet. This would result in a low water permeability of the whole membrane despite a high hemichannel density in the outer leaflet (B face). On the contrary, the presence of areas with a high hemichannel density in the inner leaflet would make more probable a junction between outer and inner hemichannels and result in a higher permeability. Permeability and ultrastructural

alterations would thus be limited to the only inner leaflet directly accessible from the cytoplasm.

Areas free of particles observed earlier on the B face of challenged bladders (Chevalier et al., 1974*a*) were also observed in the present series. Their low frequency (see for examples Figs. 7 and 8) is in agreement with the low frequency of the clusters on the A face but our data do not show a clear complementarity between the two arrangements.[1]

As far as the origin of the clusters is concerned the higher density of particles observed in the area where clusters are formed is suggestive of an insertion of particles in the inner leaflet of the membrane. Rare cells present typical elongated particles probably identical to rod-shaped particles previously observed in laterobasal membranes of mitochondria-rich cells (Wade, et al., 1974). In our material, elongated particles were observed both on laterobasal and on apical plasma membranes. In the latter case, the fracture plane happened to interest only the apical pole of the cell, so that mitochondria which are usually located in the basal part of the cell could not be identified. However, the presence of numerous microvilli was typical of a mitochondria-rich cell. Moreover, the low frequency of membrane with elongated particles is in agreement with the low frequency of mitochondria-rich cells in *Rana esculenta* (ca. 8%). It was also observed that the two adjacent cells presented spherical particles and clusters typical of the granular cells. It is clear, from the examination of this particular preparation, which was exposed to hypophysial extracts, that clusters of elongated particles can be formed in some circumstances.

At variance with the particle arrangement inside granular cells clusters, particles remain separated one from the other in the aggregates, and a large area free of any particles is found around the clusters. This last point could indicate a translational migration of the particles as a mode of cluster formation in this particular cell. Definitive conclusions concerning the distribution of these elongated particles will naturally require additional observations.

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