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HETEROGENEITY OF TOAD BLADDER GRANULAR CELL LUMINAL MEMBRANES

DISTRIBUTION OF FILIPIN-STEROL COMPLEXES IN FREEZE-FRACTURE

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Summary

Glutaraldehyde-fixed toad urinary bladders were incubated with the sterol specific antibiotic, filipin, and the distribution of the resulting filipin-sterol complexes was studied in freeze-fracture replicas. While the luminal membrane of some cells had a heavy, homogeneous labelling, most had clusters of filipin-sterol complexes scattered on an otherwise poorly labelled membrane. These clusters were seen to overlie cytoplasmic granules, which are found just beneath the luminal plasma membrane of this cell type, and which were always heavily labelled. These results suggest that (a) the granule membranes have a high cholesterol content and (b) sites of interaction between granule and luminal membranes might be enriched in cholesterol. In addition, filipin-sterol complexes were absent from vasopressin-induced particle aggregates on the granular cell luminal membrane.

Introduction

The urinary bladder of amphibians is widely used as a model for the collecting duct of mammalian kidney, since it responds to antidiuretic hormone by increasing active sodium transport in a mucosal to serosal direction and also by increasing its permeability to water flow along an osmotic gradient [1,2]. The

increase in epithelial water permeability is thought to be a result of a change in the properties of the luminal membrane of the granular cells [3]. There is now a great deal of evidence showing that the appearance of this membrane changes during increased water flow, both in scanning electron microscopy [4,5] and in freeze-fracture studies of the bladder. The changes seen in freeze-fracture involve an aggregation of many of the intramembrane particles which are normally distributed at random on the cytoplasmic leaflet (P-face) of the apical membrane [6,7]. The development and extent of the aggregation phenomenon are closely correlated with the magnitude of the increased water flux [8]. It is possible, therefore, that the aggregates of intramembrane particles may represent specific sites in the membrane which, following an alteration of the protein-lipid ratio in these regions, may be highly permeable to water.

In addition, it has been reported that vasopressin stimulation of the bladder results in the exocytosis of granules which are normally found aligned just beneath the apical membrane of the granular cells [9].

Although the relationship of the granules to aggregate formation and the increase in water permeability is not clear, the granules are known to contain some lysosomal enzymes, including cathepsin B, the inhibition of which has been reported to markedly reduce the vasopressin-induced stimulation of trans-epithelial water flux [10,11]. It has also been suggested that incorporation of granule membrane into the luminal membrane of the granular cell may influence the properties of the latter [12,13].

The important role of cholesterol, a major component of cell membranes, in modulating their fluidity [14–16] and permeability [17–20] is well known. Cholesterol can be detected in freeze-fractured membranes by the addition of the antibiotic, filipin before [21,22], during [23], or after [24–26] fixation of the tissue with glutaraldehyde. Filipin specifically binds to cholesterol and related 3β -hydroxysterols [27,28] and resulting filipin-sterol complexes are clearly visible in freeze-fracture replicas. We, therefore, made use of this technique in order to examine the distribution of filipin-sterol complexes in the apical and secretory granule membranes of the toad bladder granular cell in the non-stimulated state. Furthermore, the distribution of filipin-sterol complexes within vasopressin-induced particle aggregates, which are potential sites of high membrane water permeability, was also examined.

Materials and Methods

The toads used in this study were *Bufo bufo*, maintained at room temperature on damp soil, with free access to water. The abdominal cavity was opened following decapitation and pithing of the animals, and the two lobes of the urinary bladder were exposed. Bladders not treated with vasopressin were fixed by direct injection of fixative (2% glutaraldehyde in 0.05 M cacodylate buffer) into the lumen, whilst simultaneously dripping fixative onto the serosal surface. In this way, the bladder was well distended during the process of fixation. When the bladder was full of urine, this was first removed prior to fixation. For treatment with vasopressin, a solution of 50 mU/ml vasopressin in amphibian Ringer solution was dripped onto the serosal surface, in situ, for either 5, 10 or 15 min prior to fixation as described above.

Following an initial fixation * period of 10 min the bladders were removed from the animal and cut into squares about 3–4 mm in size. These squares were then immersed either in the same fixative, or in fixative containing 300 μ M filipin for at least 1 h at room temperature. The glutaraldehyde-filipin solution was prepared by dissolving filipin in a small volume of DMSO which was then added to the fixative to obtain the desired concentration. The final solution contained 1% DMSO. Tissue for freeze-fracture was then soaked in 30% glycerol in phosphate buffer for 1 h, frozen in Freon cooled with liquid nitrogen and fractured at -115°C in a Balzers freeze-etch device as previously described [29]. Platinum-carbon replicas were cleaned by sequential treatment with sodium hypochlorite, chloroform-methanol and dimethylformamide, rinsed in distilled water and collected on copper grids. For thin sections, glutaraldehyde-fixed bladders were post-fixed in 1% OsO_4 , stained en bloc with 5% aqueous uranyl acetate, dehydrated in ethanol and embedded in Epon 812. They were examined in a Philips EM 300 electron microscope.

Results

Control bladders

Large areas of apical plasma membrane were revealed in fractures of the luminal surface of the bladder. As in other tissues, filipin-sterol complexes were visible either as raised protuberances, 30 nm in diameter, (usually on the P-face) or large pits (usually on the E-face). It was immediately apparent that the degree of labelling of the apical membrane by these complexes was not the same in all cells. Whilst some cells were heavily-labelled others had only a light labelling (Fig. 1). In the latter cells, the filipin labelling often took the form of discrete clusters of complexes which appeared to be present on bulges in the membrane. The membrane between these labelled bulges was relatively free of complexes (Figs. 1 and 4). In some cells, areas of the plasma membrane having either both of these labelling patterns or intermediate patterns were found.

When the plane of the fracture passed from the apical membrane into the cytoplasm, the membranes of intracellular organelles, including the granules which characterise this cell type (Fig. 2) were revealed. In this case, the limiting membrane of these granules was heavily labelled with filipin-sterol complexes, regardless of the density of labelling of the apical membrane (Fig. 3).

In many instances, the fracture plane stepped from the apical membrane just overlying a granule to the membrane of the granule itself. In these cases, it could be established beyond doubt that, in lightly-labelled granular cells, the discrete clusters of complexes represent those regions of the plasma membrane which overlie granules (Figs. 3 and 5). The exact relationship between the plasma membrane and the granule membrane could not be determined, since details were obscured by the presence of numerous filipin-sterol complexes in those regions. However, in non-filipin-treated, stretched bladders, where the relationship between granule and plasma membranes could be clearly seen, numerous granules were found in various stages of exocytosis (Brown, D.,

* Glutaraldehyde fixation is required to prevent the redistribution of intramembrane particles and the cell lysis resulting from the action of filipin on living cells [30].

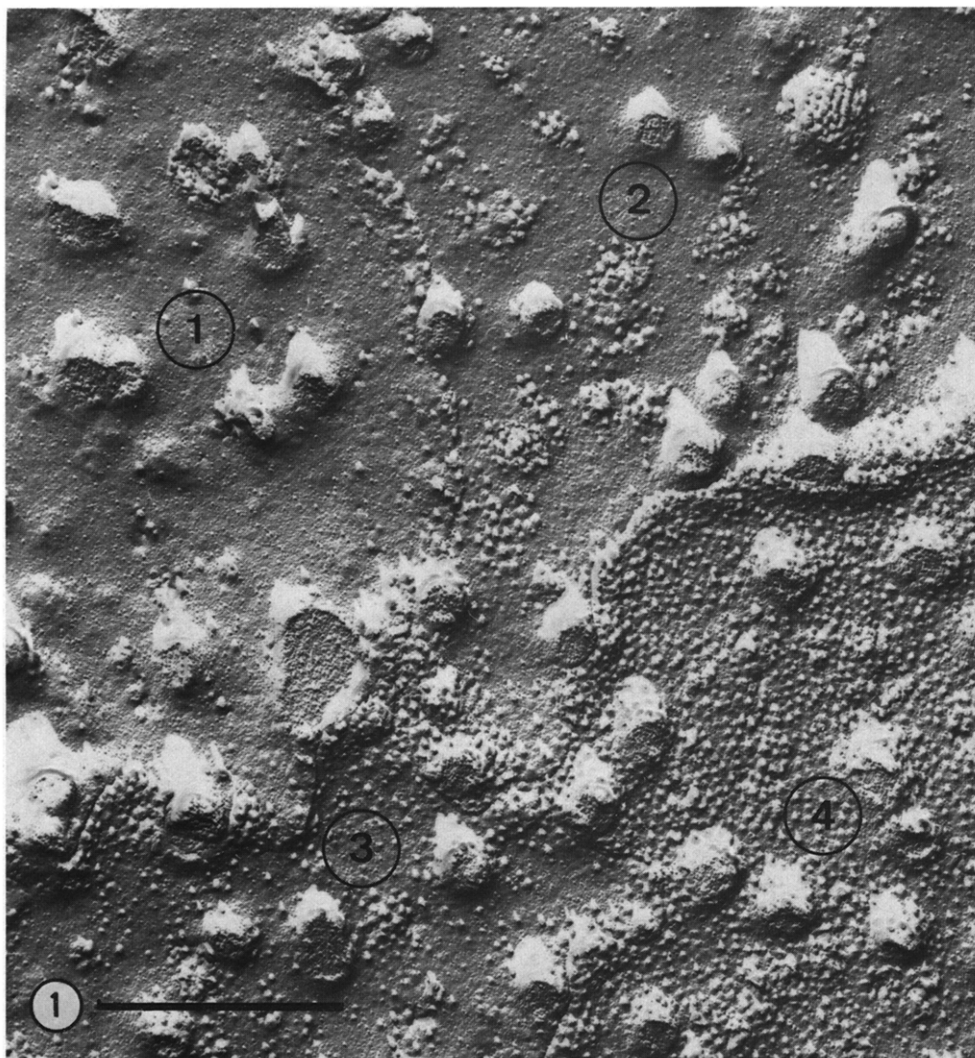


Fig. 1. Part of the mucosal surface of a filipin-treated, freeze-fractured bladder showing regions of the luminal membrane (P-face) from four granular cells. Filipin-cholesterol complexes appear as small, stud-like protuberances on the membrane. The larger projections are cross-fractured microvilli. Cell 1 is lightly labelled, cells 2 and 3 show discrete clusters of complexes on an otherwise lightly labelled membrane and cell 4 is heavily and homogeneously labelled. Magnification: $\times 33\,000$. Bar = $1\ \mu\text{m}$.

Montesano, R. and Orci, L., unpublished results). Thus, the clusters of filipin-sterol complexes may be associated with sites of prospective or ongoing exocytosis.

In the few instances where the baso-lateral membranes of the granular cells were exposed, they did not contain clusters of complexes as found on the luminal plasma membranes. However, the access of filipin to baso-lateral membranes could be restricted by limited diffusion through apical tight junctions or sub-epithelial layers.

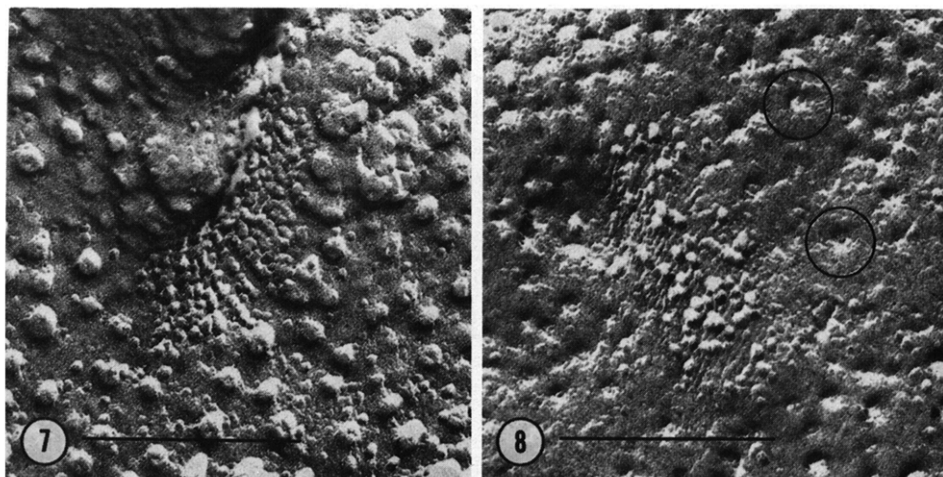


Fig. 2. Thin section showing the characteristic granules (SG) which are often found in close proximity to the luminal plasma membrane (PM) of the granular cell. In stretched bladders, they have a content of variable electron density. Magnification: $\times 94\,500$. Bar = $0.25\ \mu\text{m}$.

Fig. 3. Freeze-fracture of filipin-treated bladder showing the abundance of filipin-sterol complexes on the granule membranes (asterisks) in comparison to the poorly-labelled luminal plasma membrane (PM). Note that in the latter, most of the complexes are present in those regions of the membrane which overlie granules. Magnification: $\times 65\,000$. Bar = $0.25\ \mu\text{m}$.

Vasopressin-treated bladders

In vasopressin-treated bladders, many aggregates of intramembrane particles were found on the apical membrane (P-face) of the granular cells, as previously described [6,7]. The aggregates seemed to be present in both lightly- and heavily-labelled cells, but in both cases, filipin-sterol complexes were absent from the sites of particle aggregation (Figs. 6, 7). On the luminal E-face, complementary patterns of grooves were found as previously described [6,7], and filipin-sterol complexes were also absent from these regions (Fig. 8). Whether aggregates are more common on cells with a greater or lesser degree of filipin labelling is currently being evaluated.

Discussion

The main points to emerge from this study are: (1) Granular cell apical membranes are not labelled homogeneously with filipin-sterol complexes. (2) In many cells, the apical membrane contains discrete clusters of complexes which overlie cytoplasmic granules. (3) The limiting membranes of the cytoplasmic granules are densely labelled with complexes, probably reflecting a high cholesterol content. (4) Complexes are absent from vasopressin-induced sites of particle aggregation.

The heterogeneous labelling of apical membranes was probably not due to problems of filipin penetration (as seen in compact blocks of tissue) since the antibiotic had free access to the apical membranes of the bladder. It is, however, possible that the thickness of the cell coat varies from cell to cell and hinders filipin penetration, although the secretory granule membranes were

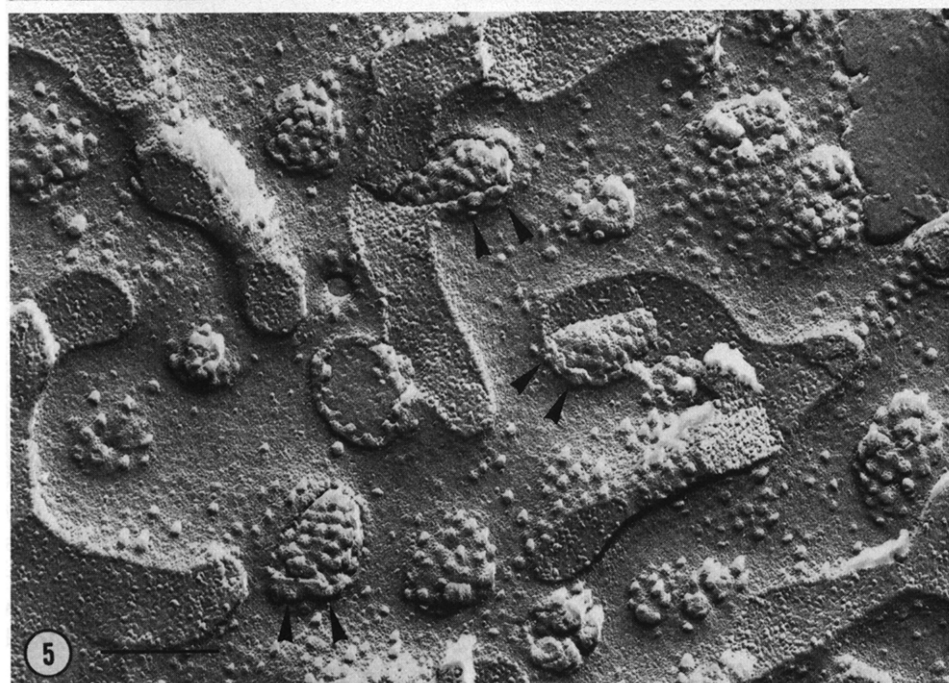
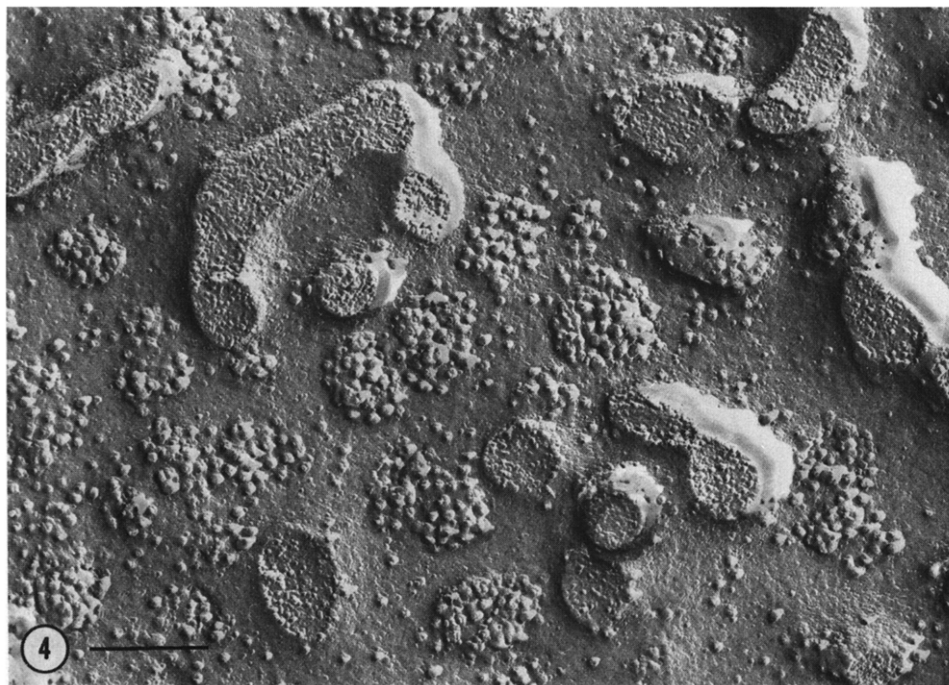


Fig. 4. Luminal membrane of a filipin-treated granular cell showing numerous clusters of complexes on an otherwise poorly-labelled membrane. Magnification: $\times 58\,500$. Bar = $0.25\,\mu\text{m}$.

Fig. 5. Luminal membrane of a filipin-treated granular cell showing clusters of complexes. In some areas the plane of fracture has passed into the cytoplasm underlying a cluster, revealing the heavily-labelled membranes of granules in register with the clusters of complexes on the luminal membrane (arrow heads). Magnification: $\times 58\,500$. Bar = $0.25\,\mu\text{m}$.

always heavily labelled whatever the state of labelling of the apical membrane.

The clustering of filipin-sterol complexes on areas of the apical membrane overlying these granules was a consistent and reproducible observation and so even if it was a result of cholesterol redistribution during fixation (which cannot be completely excluded) it still reflects some underlying difference in the membrane organisation which results in the formation of clusters in these specific regions. This apparent matching between granule membrane and plasma membrane suggests, therefore, an interaction between the two membranes which may be linked to granule exocytosis induced by stretching the bladder during fixation (Brown, D., Montesano, R. and Orci, L., unpublished results). Whether it represents an enrichment of cholesterol at prospective sites of exocytosis or is the result of incorporation of cholesterol-rich granule membrane into the apical membrane (possibly leading to a change from lightly- to heavily-

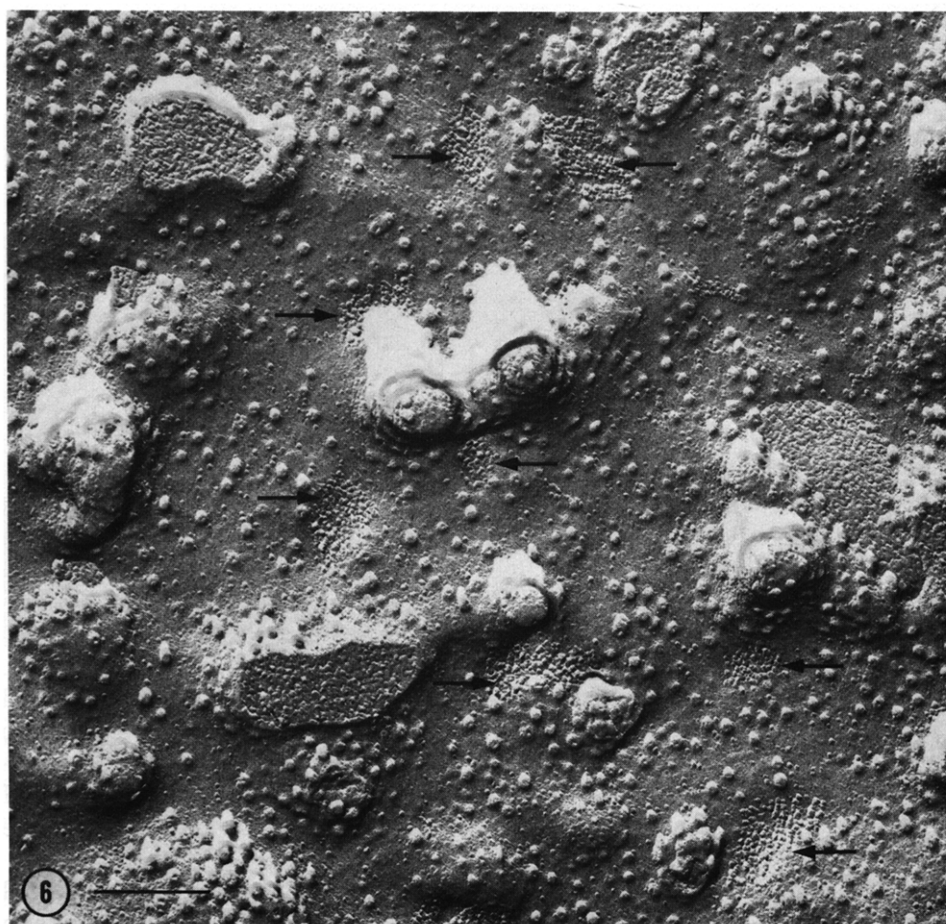


Fig. 6. Luminal plasma membrane P-face of a vasopressin-treated granular cell exposed to filipin. In addition to the filipin-cholesterol complexes, there are numerous typical particle aggregates (arrows). Even though the membrane is relatively lightly labelled, it is clear that there are no filipin-sterol complexes in any of the particle aggregates. Magnification: $\times 58\,500$. Bar = $0.25\ \mu\text{m}$.

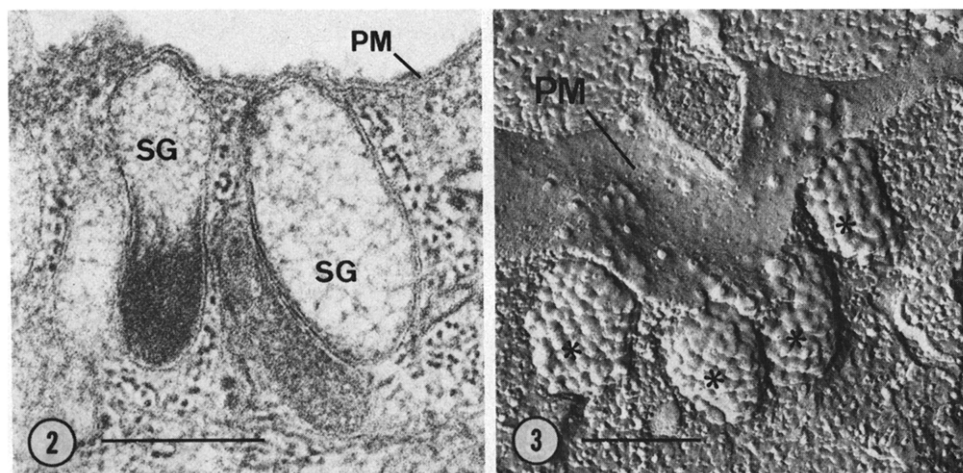


Fig. 7. Detail of the plasma membrane (P-face) from a vasopressin-treated bladder. Although the membrane is heavily labelled with filipin-sterol complexes, they are absent from the particle aggregate. Magnification: $\times 111\,600$. Bar = $0.25\,\mu\text{m}$.

Fig. 8. Detail of the luminal plasma membrane (E-face) from a vasopressin-treated bladder. In this case, the filipin-sterol complexes are represented as pits in the membrane (circles). They are absent from the region of aggregation which is seen as a series of parallel grooves with some associated particles. The particles and pits can be distinguished by comparing their shadowing pattern. The direction of shadowing was from the bottom of the picture and, whereas particles have a dark platinum deposit facing the bottom of the picture and a white shadow towards the top, the situation is reversed for pits in which the white shadow is towards the bottom and the dark platinum deposit towards the top of the picture. Magnification: $\times 111\,600$. Bar = $0.25\,\mu\text{m}$.

labelled membrane) is a crucial point and is the subject of current investigations (see Note added in proof).

In this respect, it is known that the addition to or removal of lipid components from membranes can affect their fluidity and permeability [14–20] and it has been suggested that incorporation of secretory granule membrane into the apical plasma membrane may alter the properties of the latter [12,13]. Although this study does not unequivocally demonstrate such an incorporation, it provides strong evidence suggesting that the granule membrane is cholesterol-rich compared to much of the apical plasma membrane of granular cells.

Finally, the absence of filipin-sterol complexes from vasopressin-induced intramembrane particle aggregates could be interpreted as evidence that these regions have a low cholesterol content and may, therefore, be sites with specialised permeability characteristics. However, in such tightly-packed aggregates of particles the formation of visible complexes may conceivably be impeded due to steric hindrance.

In conclusion, although it is not yet possible to attribute a precise functional role to the observed variations in cholesterol distribution, this work provides a framework necessary for future investigations concerning the role of this sterol in membrane permeability changes in the toad urinary bladder. Some of the results presented here have previously appeared in abstract form [31,32].

Note added in proof (Received July 23rd, 1980)

In a further series of experiments, bladders fixed and stretched simultaneously as in the present study (see Materials and Methods) were compared to bladders pre-stretched for 1 or 5 min prior to fixation. Those stretched during fixation or pre-stretched for 1 min showed the same filipin-labelling pattern as described above, whereas following a 5 min stretching period before fixation, most apical membranes were homogeneously labelled and clusters of complexes were seen only rarely. This difference in labelling pattern suggests that stretching the bladder initiates a chain of events which ultimately effects the organisation of granular cell apical membranes.

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