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Ultrastructural Characterization of Cholesterol Distribution in Toad Bladder Using Filipin

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Summary. The polyene antibiotic filipin was used to characterize the cholesterol distribution in the membranes of the toad bladder epithelium in freeze-fracture replicas. The apical membranes of granular and mitochondria-rich cells incorporate moderate amounts of filipin while the basolateral membranes of both cell types incorporate substantially greater amounts. Intracellular membranes, in general, take up very little filipin. The major exception to this is the granule membrane, which appears to be rich in cholesterol. An inverse correlation was found between the density of filipin-sterol complexes in the apical membrane and the incidence of granules in the cytoplasm. This suggests that fusion of granules with the apical membrane may be responsible for variation in the concentration of cholesterol in the apical membrane. Thirty minutes following vasopressin exposure, there is no consistent change in the cholesterol content of the apical membrane of granular cells as measured by the incidence of filipin-sterol complexes. The lack of change in the amount of membrane cholesterol indicates that the vasopressin-induced increase in transepithelial water permeability is not mediated by a change in cholesterol content of the apical membrane.

Key Words filipin · 3β-hydroxysterols · cholesterol · toad bladder · antidiuretic hormone

Introduction

A principal feature of the response of the amphibian urinary bladder epithelium to vasopressin stimulation is the appearance of distinctive intramembrane particle aggregates in the apical plasma membrane of granular cells (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & DiScala, 1975). These aggregates exist preformed in tubular vacuoles within the granular cell cytoplasm (Humbert et al., 1977; Wade, 1978; Dratwa et al., 1979). The vacuoles appear to fuse with the apical membrane upon vasopressin stimulation Muller, Kachadorian and DiScala, 1980; Wade, 1980; Kachadorian, Muller & Finkelstein, 1981). The occurrence of intramembrane particle aggregates in the apical membrane has been related, quantitatively and temporally, to changes in water permeability induced by vasopressin (Kachadorian et al., 1977*a*, *b*; Kachadorian, Casey & DiScala, 1978; (Ellis, Kachadorian & DiScala, 1980).

Recent measurements of capacitance in the toad bladder indicate that a change in luminal membrane area of 20 to 36% is associated with the action of vasopressin (Warncke & Lindemann, 1981; Stetson et al., 1982). Capacitance measurements, however, cannot distinguish between membrane containing intramembrane aggregates and other intracellular membranes which may fuse with the apical membrane in response to vasopressin. It has been suggested that vasopressin stimulates the exocytosis of the granules which are characteristic of this cell type, and that this response may contribute in some way to the increase in transepithelial hydraulic conductivity (Masur, Holtzman & Walter, 1972; Gronowicz, Masur & Holtzman, 1980). While this observation has not been confirmed by other studies (Reaven, Maffly & Taylor, 1978; Muller et al., 1980; Wade, 1980), the functional role of the granules remains obscure. Because one consequence of granule exocytosis might be an alteration in the composition of the apical membrane, it is of interest that granule membranes have been reported to be extremely rich in cholesterol (Orci, Montesano & Brown, 1980). Any change in the cholesterol content of the apical membrane might alter its water and solute permeability (Finkelstein & Cass, 1967; Papahadjopoulos, Nir & Ohki, 1971; Demel, Bruckerdorfer & Van Deenen, 1972).

Recently, the antibiotic filipin has been introduced as a probe for the localization of cholesterol because it binds stoichiometrically to unesterified 3β-hydroxyl sterols (Bittman & Fischkoff, 1972;

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Norman et al., 1972), and because it creates large (~25 nm) intramembrane bulges when viewed in freeze-fracture replicas (Tillack & Kinsky, 1973; Verkleij et al., 1973; Elias, Friend & Goerke, 1979; Montesano et al., 1979; Robinson & Karnovsky, 1980). Friend and Bearer (1981) have shown that the incorporation of filipin into biological cell membranes is directly proportional to the amount of cholesterol in those membranes. Thus, the relative abundance of cholesterol in membranes can be assessed from the density of filipin-sterol complexes in freeze-fracture electron microscopy.

This study uses filipin to examine the distribution of cholesterol in toad bladder epithelial cells. The incidence of filipin-sterol complexes observed following vasopressin stimulation is evaluated in order to assess possible sources of newly added luminal membrane.

Materials and Methods

Bufo marinus were maintained on moist peat moss and fed meal worms (*Tenebrio* larvae) weekly. Portions of bladders were removed from doubly pithed toads, mounted as sacs on acrylic tubes, and suspended in and filled with full-strength amphibian Ringer's solution. The composition of this solution is as follows (in mM/liter): NaCl, 110; KCl, 3.5; $NaHCO_3$, 2.5; $CaCl_2$, 1.0. The pH of this solution is about pH 7.8 when bubbled with air.

Filipin Treatment

For each experiment, paired bladder sacs were removed from the animal and allowed to equilibrate for 30 min. One sac of a pair was then exposed on the serosal side to 20 mU/ml of arginine vasopressin (AVP, Sigma Chemical Co., St. Louis, Mo.) for 30 min. The paired control sac was not stimulated by vasopressin, but otherwise was treated in an identical manner. The sacs were then fixed for 30 min in 1.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.05% $CaCl_2$ and 2% sucrose at pH 7.5. In one experiment, the fixative also contained 300 μM filipin (Upjohn, obtained through Polysciences). The filipin had been dissolved in dimethyl sulfoxide (DMSO) before addition. In all other experiments the filipin was added to a 30-min 0.1 M cacodylate rinse following a 15-min fixation. Both techniques yielded identical results with respect to epithelial filipin distribution and density. Other controls evaluated were: 1) the addition of DMSO without filipin to the fixative or rinse; and 2) AVP exposure of the bladder without subsequent filipin treatment. Typical filipin-induced intramembrane particles were never observed in the membranes of these control bladders.

Electron Microscopy

Small pieces of tissues were soaked in 25% glycerol in 0.1 M cacodylate buffer for at least two hours before being frozen in Freon 22 slush. Freeze-fracture replicas were produced in a Balzers BAF 301 freeze-fracture plant using a double-replica device, EVM 052 electron evaporation guns, and a QSG quartz crystal thin-film monitor. Replicas were cleaned overnight in Chlorox®, rinsed with several changes of distilled water, and examined using a Zeiss EM 10B electron microscope.

Quantitation of filipin-sterol complexes was accomplished on micrographs at 25,000×. The number of particles in five separate randomly selected 1 cm^2 patches on each print was determined. Vasopressin-induced aggregates were counted and areas were determined from the entire face of the same prints.

Morphometric analysis of thin-sectioned material was performed on micrographs of cross-sectioned epithelium at 12,500× using a square grid of 1 cm spacing. Volume-percent (V_v) of granular cell granules was determined as described by Weibel and Bolender (1973). Cell height was assessed from granular cell area divided by the straight-line length of the apical aspect of the granular cells.

Significance levels were determined using the paired Student's *t*-test. Reported *P* values are for two-tailed tests. All results are reported as the mean ± standard error of the mean.

Results

Localization of Filipin-Sterol Complexes

In all cases, filipin has access to all membranes of the bladder. In bladders which had been exposed to filipin on one side only, the membranes of the entire mucosal epithelium, muscle layers, and the mesothelium on the serosal surface contained filipin-sterol complexes. However, the incidence of filipin complexes varied significantly between different membranes (Fig. 1). The basolateral membranes of all epithelial cells, and the apical membranes of basal cells displayed dense filipin-sterol complexes, usually too dense to allow reliable quantitation. However, the basal membrane of the basal cells, that membrane which is in contact with the basal lamina, showed very little filipin uptake. The apical membranes of granular cells showed moderate filipin incorporation, with particles at a density of 180 ± 20 per μm^2. The filipin-sterol complexes in these membranes were usually evenly distributed across entire cells but occasionally were clumped to various degrees (Fig. 2*A*, *B*). Mitochondria-rich cells also displayed moderate apical filipin incorporation, while the goblet cell apical membranes and the mucous granule membranes showed extensive incorporation. The intercellular junctions, especially desmosomes and zonulae occludentes, contained no filipin-sterol complexes (Fig. 3).

The cytoplasmic membranes of granular cells, especially nuclear envelope, Golgi, and mitochondria, rarely contained filipin-sterol complexes. A major exception to this observation was the membrane of the granule characteristic of granular cells. As previously reported by Orci et al. (1980) a high density of filipin-sterol complexes is observed in the membranes of these structures, even to the extent that no free membrane surface can be seen (Fig. 1). Thus, it is impossible to quantify

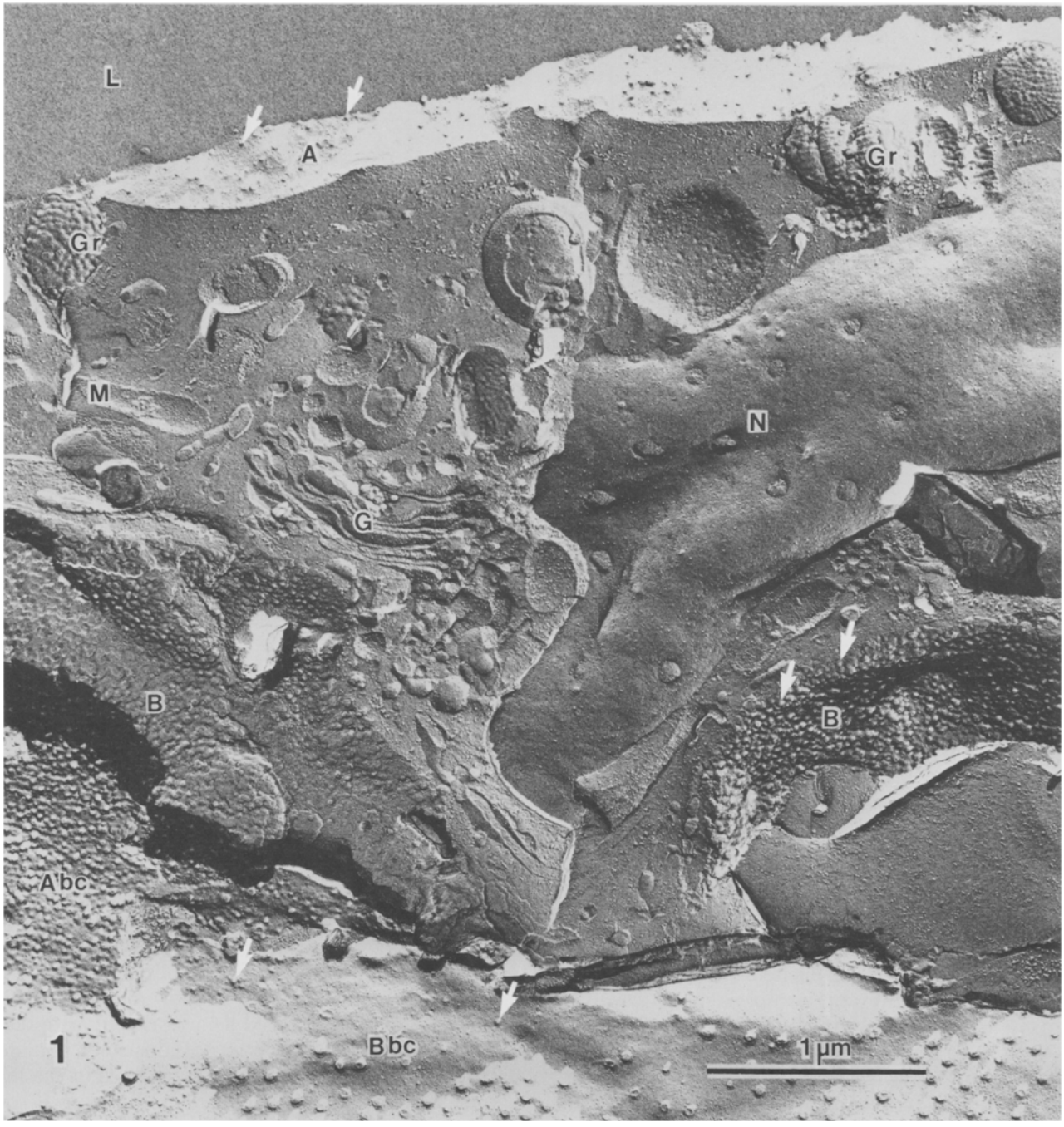

Fig. 1. A cross-fracture of a filipin-treated granular cell and basal cell. The *P* fracture faces of the apical (*A*) and basolateral (*B*) membranes of the granular cell and the apical (*Abc*) membrane and *E* fracture face of the basal (*Bbc*) membrane of the basal cell display the typical pattern of filipin incorporation: moderate in the granular cell apical membrane, rare in the basal cell basal membrane, and dense in the basal cell apical and the granular cell basolateral membranes. The granules (*Gr*) take up large quantities of filipin, in contrast to the membrane of the nucleus (*N*), mitochondria (*M*), and golgi (*G*), which show no filipin incorporation. *L*, lumen; arrows, filipin cholesterol complexes

the complexes in this membrane. In contrast, the distinctive cytoplasmic vacuoles with aggregates were never observed to have filipin-sterol complexes in their membranes (Fig. 4).

Because filipin clearly labeled the various intracellular membranes, it is likely that filipin gained access to the cytoplasm through the plasma membranes, perhaps through pores formed by filipin-sterol complexes. It is perhaps by this route also that filipin gained access to the submucosa.

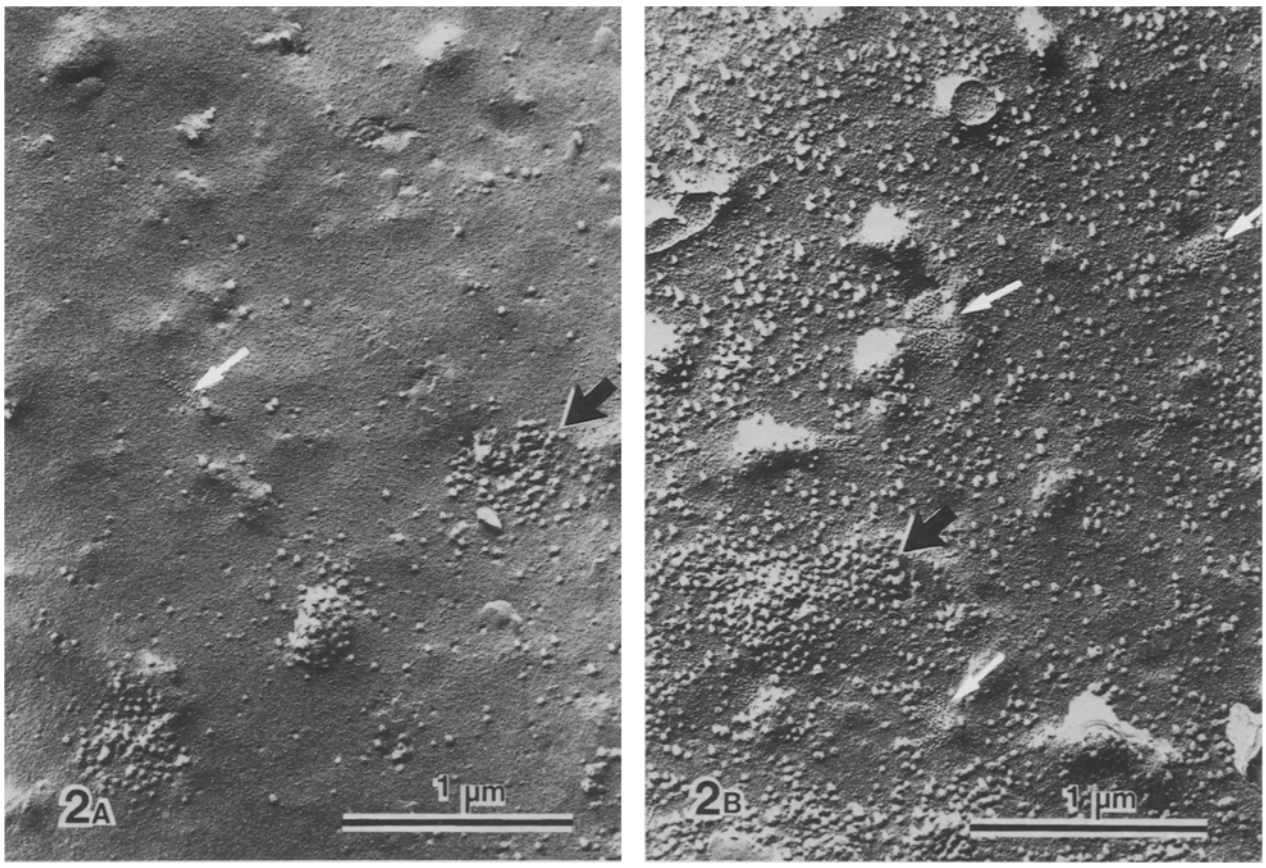

Fig. 2. Patterns of clumping of filipin-sterol complexes in the apical membrane (*P* face) of granular cells. *A*) Three discrete patches (large arrow) in an otherwise relatively bare membrane from a control bladder. Occasional aggregates (small arrow) exist even in control bladders. *B*) Slightly dispersed clumps (large arrow) in a membrane from a vasopressin-treated bladder displaying moderate filipin uptake and frequent intramembrane particle aggregates (e.g., small arrows)

Effect of Vasopressin on the Distribution of Filipin-Sterol Complexes

Vasopressin treatment elicited the usual appearance of intramembrane particle aggregates in the apical membrane. Filipin-sterol complexes could be observed in close association or in direct apposition to the vasopressin-induced aggregates. Clusters of complexes were as frequent in these vasopressin-treated bladders as in controls.

Because the membrane of the granules incorporated large amounts of filipin and aggregate-containing membranes contained none, the filipin density in the luminal membrane could provide a means to evaluate the possible source of the membrane added to the apical membrane. Therefore, we determined the density of filipin-sterol complexes in replicas of apical membrane from paired vasopressin-treated and control bladders. In the same specimens we determined the density of vasopressin-induced aggregates in the luminal membrane. The incidence and volume density of cytoplasmic granules [$C_v(Gr)$] were also evaluated in thin sections of these specimens and in additional specimens not treated with filipin. As shown in the Table only the density of vasopressin-induced aggregates changed as a result of vasopressin stimulation. There was no consistent change in the density of filipin-sterol complexes in the luminal membrane, nor was the incidence or V_v of granules in the cytoplasm consistently altered by vasopressin.

The incidence of filipin-sterol complexes and granules varies over a wide range in both control and vasopressin-treated bladders. The hypothesis that granule fusion may be responsible for variation in the concentration of cholesterol in the apical membrane (Orci et al., 1980) is supported by the relationship shown in Fig. 5. The filipin-sterol complex density in the apical membrane of the granular cells is inversely related to the V_v of granules in the cytoplasm ($r=0.65$, $P<0.05$).

Brown et al. (1981) have suggested that increases in stretch can induce granule fusion in toad bladder. In order to evaluate the possible role of stretch in the present study, the degree of bladder stretch (measured as the mean epithelial cell height) was compared to V_v of granules and to apical membrane filipin density (Fig. 6). Although the cell height varied from 1.3 to 6.6 μm, there appears to be no relationship between these parameters in this study. Also, vasopressin had no effect on the relationship between stretch and intracellular granule content or apical membrane filipin-sterol complex density. Thus, in the range employed in this study, stretch does not appear to be correlated with granule fusion.

Discussion

The density of filipin-sterol complexes in freeze-fracture replicas is a measure of the density of 3β-OH sterols, most commonly cholesterol, in cell membranes (Tillack & Kinsky, 1973; Verkleij et al., 1973; Elias et al., 1979; Montesano et al., 1979; Robinson & Karnovsky, 1980). Using this tool, a number of conclusions can be drawn concerning the distribution of cholesterol in the membranes of the toad bladder epithelium. 1) Cholesterol exists in higher concentrations in basolateral membranes of granular cells and mitochondria-rich cells than in their apical membranes. In contrast, the apical membrane of basal cells contains much more cholesterol than the membrane adjacent to the basal lamina. These observations imply that movement of cholesterol between apical and basal membranes is restricted even in basal cells where no occluding junction exists. 2) The mem-

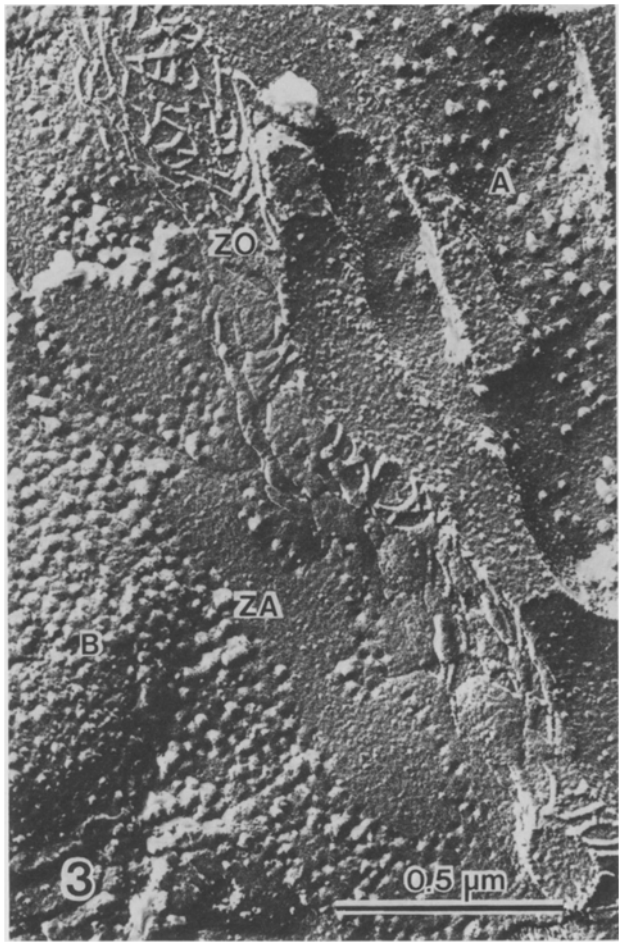

Fig. 3. A fracture through the junctional complex between epithelial cells. The apical (*A*) and basolateral (*B*) membranes show the typical filipin-sterol complex pattern. Both zonula occludens (*ZO*) and the zonula adherens (*ZA*) exclude filipin. Free desmosomes resemble the zonula adherens with respect to filipin incorporation

Table.

Animal	Filipin density (#/μm²)[a]		Aggregate density (#/100 μm²)[a]		$V_v(Gr)$ (%)		Granule number (#/100 μm²)[b]	
	Control	AVP	Control	AVP	Control	AVP	Control	AVP
I	202	291	0	40.2	5.6	2.5	58	37
II	270	239	1.4	77.0	2.8	5.9	86	93
III	172	194	5.1	59.0	5.0	2.3	75	42
IV	151	157	0.1	23.6	4.5	8.9	81	118
V	136	281	0	39.4	10.3	1.9	96	22
VI	146	192	1	19.7	4.3	4.4	73	72
Mean	180	226	1.3	43.2	5.4	4.3	78	64
SEM	±20	±22	±0.8	±8.9	±1.0	±1.1	±5	±15
	NS		$P<0.01$		NS		NS	

[a] μm² of membrane surface using freeze-fracture replicas.
[b] μm² of cell area on micrographs of thin sections.

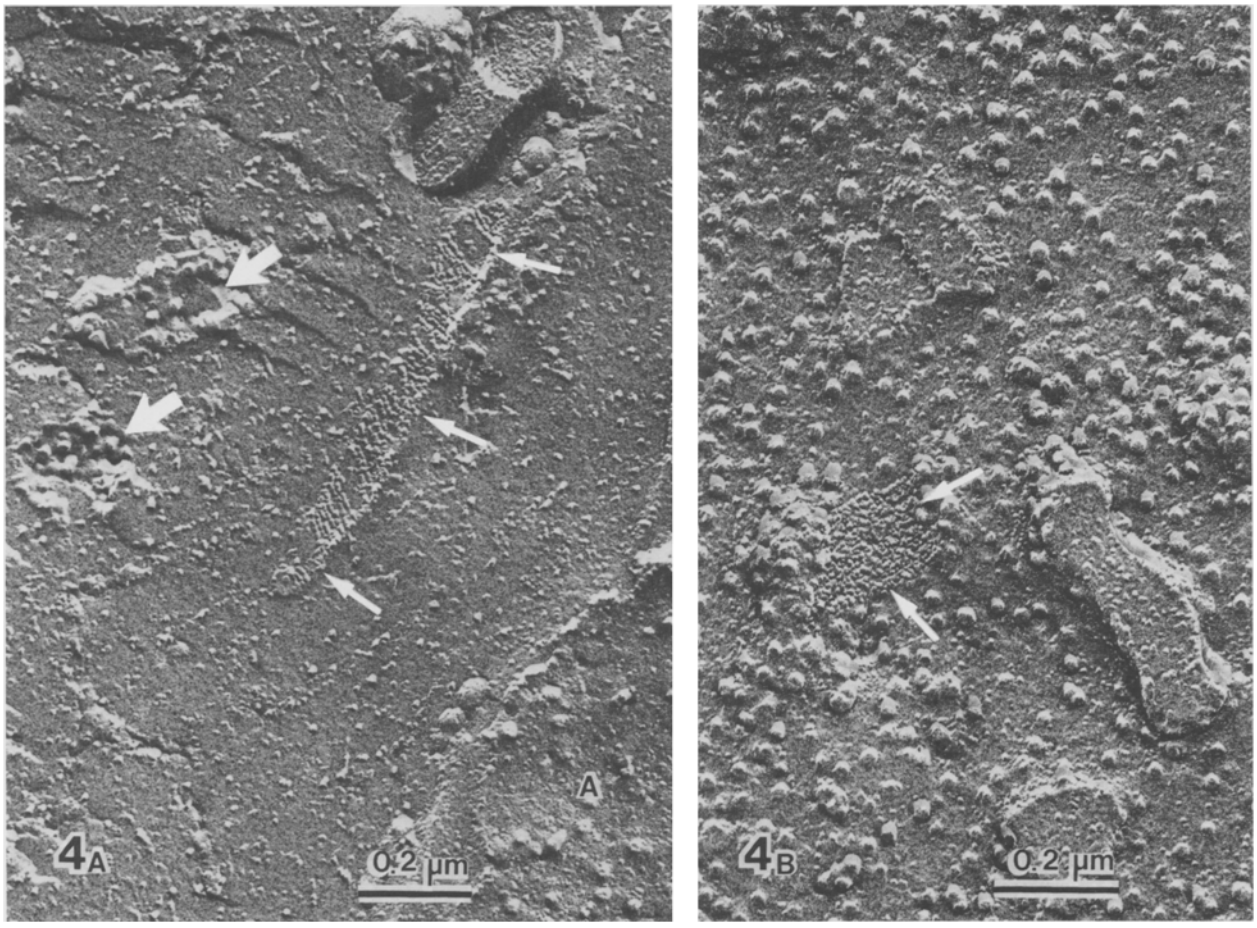

Fig. 4. *A*) A fracture from the apical membrane (*A*) into the cytoplasm of a granular cell. A tubular vesicle that contains particle aggregates (small arrows) shows no filipin uptake, while membranes on either side (*A* and large arrows) show many filipin-sterol complexes. *B*) An aggregate in the apical membrane of a granular cell after vasopressin stimulation. Filipin-sterol complexes have free access to the periphery of the aggregate though no complexes intrude into the aggregate

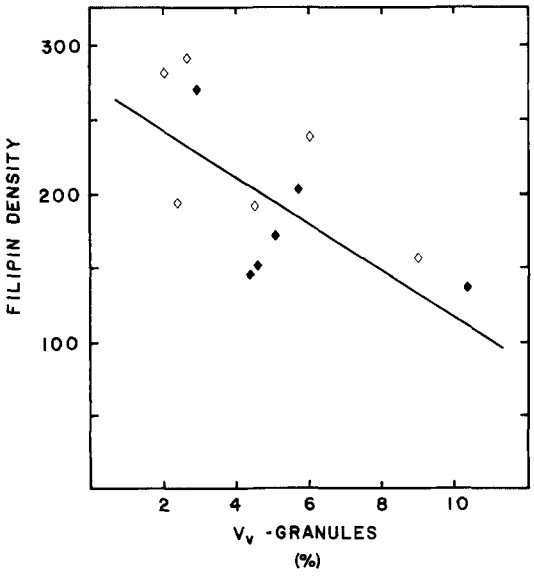

Fig. 5. Filipin-sterol complex density (#/μm²) of the apical membrane *vs.* V_v-granules (%). The equation for the line determined by linear regression is $Y=270-13.8X$, $P<0.05$. Closed symbols, control hemibladders; open symbols, AVP-stimulated

branes of intracellular organelles, e.g. Golgi, endoplasmic reticulum, nucleus, are relatively poor in cholesterol. This is in general agreement with the biochemically measured cholesterol levels for other cell systems (reviewed by Jain, 1975). 3) The limiting membranes of granules are extremely rich in cholesterol as reported by Orci et al. (1980). The presence of discrete clusters of filipin-sterol complexes in the apical membrane of granular cells as well as the inverse correlation between filipin density and V_v granules strongly suggests that granules fuse with the apical membrane and are an important source of cholesterol. The observation that clusters are found in various states of assembly suggests that some diffusion of granule-derived membrane components does occur within the apical membrane. Membranes of cytoplasmic vacuoles that contain organized intramembrane particle aggregates contain little cholesterol. But, upon fusion of the vacuole with the apical membrane following vasopressin exposure, filipin-sterol

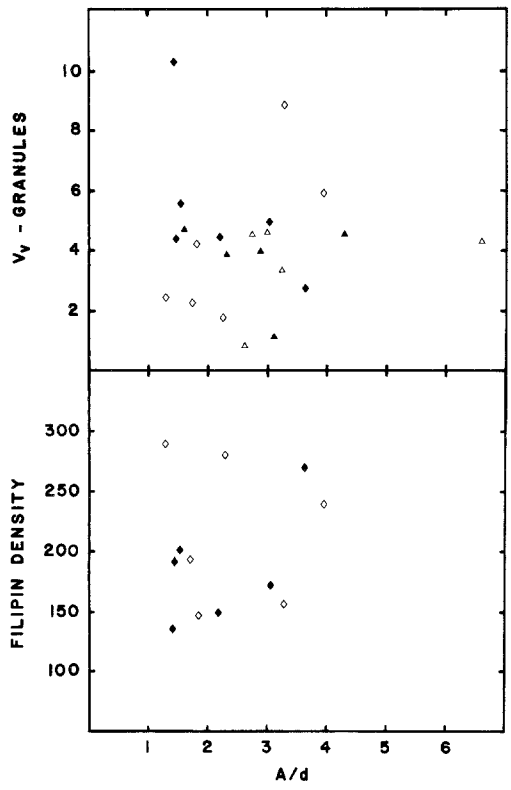

Fig. 6. V_v-granules (%) and filipin density *vs.* mean cell height (A/d, μm). Diamonds, experiments in which filipin distribution was determined; triangles, additional experiments without filipin exposure. Closed symbols, control hemibladders; open symbols, AVP-stimulated

can be found in direct contact with the aggregates. This also suggests diffusion of membrane components within the plane of the membrane. 4) The amount of cholesterol in the apical membrane of granular cells does not change consistently in response to vasopressin. This result argues against the possibility that a change in the proportion of cholesterol in that membrane may modulate the water permeability of the epithelium. 5) Because of the inconsistency of the vasopressin-induced changes in filipin incorporation, this result also argues against the likelihood that granule fusion is exclusively responsible for the consistent increase in membrane area observed with vasopressin stimulation (Warncke & Lindemann, 1981; Stetson et al., 1982). These results do not provide a firm indication for the source of the membrane added to the apical surface during the vasopressin response. If only incorporation of aggregate containing membranes which lack cholesterol occurred, one might expect a small decrease in the density of filipin-sterol complexes in the apical membrane. However, if cells can add aggregates to the apical membrane while maintaining their cytoplasmic carrier membrane as a separate domain (Muller et al., 1980), no decrease in apical membrane cholesterol need occur. These changes are likely too small to be detected in this study. In fact, some bladders display increases in filipin incorporation (4 of 6), but other bladders show no change or even decreases in response to ADH (*see* Table). The cause of these differences is not known.

Although our observations differ in some respects from those of Masur et al. (1972) and Gronowicz et al. (1980), they are also consistent with the concept that some exocytosis of cholesterol-rich granules may occur in vasopressin-stimulated bladders. It should be noted that bladders from occasional animals (e.g. Animals I and V of the Table) do display a striking decrease in the incidence of granules with vasopressin exposure. The failure of this study and previous studies from other laboratories (Reaven et al., 1978; Muller et al., 1980) to confirm a consistent relationship between vasopressin stimulation and granule exocytosis indicates that exocytosis of the granules can be influenced by some unknown variation in methodology. Our analysis suggests that the degree of stretch employed in the present study cannot account for the observed differences in V_v of granules and in filipin-sterol complex density. Moreover, the recent work of Kachadorian and Levine (1982) indicates that the water permeability of the apical membrane is not affected by stretch. Thus, even if extreme changes in stretch do result in fusion of granules, this effect does not appear to be important in regulating the hydroosmotic permeability of the apical membrane.

The failure of a number of independent studies to detect a consistent relationship between changes in the number of granules and water permeability indicates that exocytosis of granules is probably not an important feature in the mechanism of action of vasopressin. In contrast, the relationship between intramembrane particle aggregates and vasopressin action has been noted in numerous studies by many independent laboratories (Chevalier et al., 1974; Kachadorian et al., 1975; Humbert et al., 1977; Dratwa et al., 1979).

In summary, filipin was used to characterize the 3β-OH sterol distribution in membranes of toad bladder epithelial cells. The membranes of the characteristic granular cell granules were found to be particularly rich in sterols. Upon comparing the filipin incorporation into the apical membranes of granular cells from vasopressin-stimulated and unstimulated bladders, we could find no evidence to suggest that the cholesterol content of the apical membrane is altered dramatically as a specific response to vasopressin stimulation.

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