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REVIEW

Membrane lipids and proteins as modulators of urothelial endocytic vesicles pathways

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Abstract The increased studies on urinary bladder umbrella cells as an important factor for maintaining the permeability barrier have suggested new pathways for the discoidal/fusiform endocytic vesicles which is one of the main features of the umbrella cells. The biological role of these vesicles was defined, for many years, as a membrane reservoir for the umbrella cell apical plasma membrane which are subject to an increased tension during the filling phase of the micturition cycle and, therefore, the vesicles are fused with the apical membrane. Upon voiding, the added membrane is reinserted via a non-clathrin or caveolin-dependant endocytosis thereby restoring the vesicle cytoplasmic pool. However, in the last decade, new evidence appeared indicating alternative pathways of the endocytic vesicles different than the cycling process of exocytosis/endocytosis. The purpose of this review is to analyze the molecular modulators, such as membrane lipids and proteins, in the permeability of endocytic vesicles, the sorting of endocytosed material to lysosomal degradation pathway and recycling of both membrane and fluid phases.

Keywords Urothelium · Endocytic vesicles · Fatty acids · Endocytosis/exocytosis · Membrane recycling · Lysosomal degradation · Membrane permeability

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Introduction

A traffic model of urothelial endocytic vesicles was proposed (Hicks 1975) that basically supports the increase and decrease of urothelial luminal surface during filling and voiding of the micturition cycle, respectively. According to it, the vesicles located beneath the apical plasma membrane are exocytosed in response to the increase of membrane tension during the filling phase (Lewis 2000; Apodaca 2004). In the voiding phase or micturition, the added membrane is internalized by endocytosis, thus restoring the vesicles pool. Nevertheless, in the last decade, new evidences have demonstrated alternative endocytotic pathways of vesicles and their content. The most studied was the lysosomal degradative pathway (Zhang and Seguchi 1994; Truschel et al. 2002; Guo et al. 2009; Khandelwal et al. 2010; Kreft et al. 2009a, b; Grasso and Calderón 2013). This degradation correlated with de novo synthesis of vesicles (Kreft et al. 2010b). Besides, the recycling and leakage of fluid phase of the endocytic vesicles were also described (Truschel et al. 2002; Grasso and Calderón 2009, 2013).Within this context, we propose to summarize all alternative pathways of endocytic vesicles but with an emphasis on membrane lipid composition and proteins that somehow modulates the sorting of endocytosed material into different intracellular fates.

General aspects of the urothelium

The urothelium is a specialized epithelium that covers the mucosa surface of urinary tract from the renal pelvis to the proximal urethra (Jost et al. 1989) and prevents the unregulated exchange of substance between urine and blood (Kreft et al. 2010a), Fig. 1a. This stratified epithelium is composed of three cell types: basal, intermediate

and superficial or umbrella cells (Hicks 1975; Zancanaro et al. 1993). Basal cells are small, having 10 μm in diameter, and they are in direct contact with the basal membrane. Intermediate cells have 10–25 μm in diameter with a pyriform shape and in some species they have long, thin cytoplasmic processes that connect them to the basal membrane (Petry and Amon 1966). The external cell layer which is in direct contact with the urine is composed of large polyhedral cells denominated umbrella cells with diameters of 25–250 μm. In some species, such as rats and guinea pigs, they present several nuclei. As the intermediate cells, umbrella cells can extend thin projections connecting the upper cell layer with the basal membrane, (Petry and Amon 1966). Umbrella cells have a long life with a turnover rate of ~200 days (Hicks 1975), but when damaged, they are rapidly regenerated. The cell replacement is primarily the result of the division and differentiation processes of basal cells but also, according to Martin, by the fusion of intermediate cells resulting in a new generation of umbrella cells (Martin 1972).

The primary function of urothelium is to form a barrier that prevents the entry of pathogens microorganisms (bacteria, fungi and viruses) and selectively controls the passage of water, ions, non-charged solutes, and large macromolecules across the mucosal surface into the underlying tissue (Lewis 2000; Apodaca 2004; Kreft et al. 2010a). The barrier function of urothelium depends on intercellular union integrity and specialized membrane domains that seal the apical plasma membranes of adjacent umbrella cells (Jost et al. 1989). There are high resistance tight junctions that divide the cell surface of umbrella cells into apical and basolateral membrane domains (Lewis 2000). Moreover, these tight junctions confer to the urothelium the highest paracellular resistance of all epithelia measured to date (Lewis and Diamond 1976; Kreft et al. 2010a). Also the apical membrane of umbrella cells has a unique lipid and protein composition which is believed to contribute to the low permeability of this membrane to water and solutes (Chang et al. 1994; Calderon et al. 1998; Calderón and Eynard 2000). On the other hand, the urothelium is part of a sensory web where it receives, amplifies and transmits information about its environment to the nervous and muscular systems (Khandelwal and Abraham 2009). In fact, Khandelwal and Abraham (2009) proposed that through urothelial-associated channels and receptors, the urothelium can "sense" changes in hydrostatic pressure by binding of mediators such as ATP (Wang et al. 2005) and epidermal growth factor (Balestreire and Apodaca 2007; Flores-Benítez et al. 2007). The increment of hydrostatic pressure as a consequence of bladder filling results in a morphological transition that occurs in all three urothelial layers, especially in umbrella cells layer, where a process of endocytosis–exocytosis of vesicles is observed (Hicks et al. 1974; Lewis and de Moura 1982). The movement of vesicles from and towards the apical membrane is in part modulated by the release of ATP during the filling phase and its binding to P2X and possible P2Y receptors in umbrella cells (Wang et al. 2005).

Asymmetric unit membrane

The surface of the umbrella cells appears pleated (Fig. 1b) and each cell is surrounded by a tight junction ring when they are examined by scanning electron microscopy (Lewis

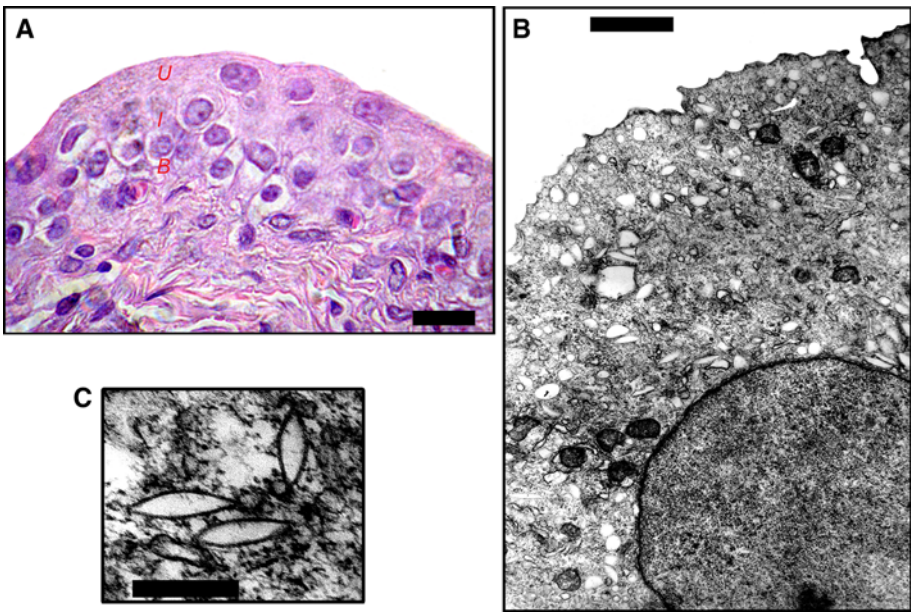

Fig. 1 Urothelium. **a** Urothelium from rat urinary bladder stained with Hematoxylin and Eosin. *U*, *I* and *B* correspond to umbrella, intermediate and basal cells, respectively. Magnification ×1,000. *Scale bar* 10 μm. **b** TEM image of umbrella cell from rat bladder. Note beneath the scalloped apical plasma membrane the pool of cytoplasmic vesicles. Magnification ×21,000. *Scale bar* 2 μm. **c** TEM image with a higher magnification of rat urothelial vesicles. *Scale bar* 0.5 μm. The images **b** and **c** are a courtesy of Dr. Mirta Valentich

2000; Apodaca 2004, Kreft et al. 2010a). When high magnification is used, the umbrella cells show raised ridges, also called hinges (Yu et al. 1992; Lewis 2000; Apodaca 2004; Kreft et al. 2010a), which surround areas called plaques. The association of hinges and plaques results in the characteristic scalloped appearance of the umbrella apical plasma membrane, which is well apparent when the apical surface of cross-sectioned umbrella cells is viewed by transmission electron microscopy (TEM) (Lewis 2000; Apodaca 2004; Kreft et al. 2010a), Fig. 1b.

Apical plasma membrane of umbrella cells contains specific lipids and proteins (Hicks 1975; Wu et al. 1990; Yu et al. 1990; Calderon et al. 1998). The protein constituents of this membrane are the uroplakins, a group of at least five proteins including the tetraspan family members UPIa (27 kDa) and UPIb (28 kDa), and the type I single-span proteins UPII (15 kDa), UPIIIa (47 kDa) and UPIIIb (35 kDa) (Wu et al. 1994). All uroplakins are integral membrane proteins with high degree of conservation during mammalian evolution (Firth and Hicks 1973; Wu et al. 1994; Garcia-España et al. 2006). Uroplakins Ia and Ib have four α-helixes and play multiple roles in cell migration, cell signaling, viral infection and membrane architecture (Maecker et al. 1997). The UPIa also acts as a receptor for the uropathogenic *Escherichia coli* (Min et al. 2002) which represent more than 90 % of the urinary tract infections. Uroplakin IIIa has a 50-aminoacid-long cytoplasmic single domain, which is believed to mediate the interaction between the urothelial plaques and underlying cytoskeleton (Wu et al. 1995). Uroplakin IIIb is a minor protein found only in urothelial plaques (Deng et al. 2002).

Liang et al. (1999) using a harsh detergent, sarkosyl, obtained from the urothelium apical plasma membrane a fraction containing plaques and the hinge areas. The highly detergent insolubility of this luminal membrane may reflect its unusual lipid composition, which is enriched in cholesterol, phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cerebroside (Cb), a lipid profile similar to myelin (Chang et al. 1994; Calderon et al. 1998; Calderón and Eynard 2000; Bongiovanni et al. 2005). Ketterer et al. (1973) reported the presence of saturated and polyunsaturated n-6 derivative fatty acids mainly and high levels of one eicosatrienoic (a marker of essential fatty acid deficiency).

The most important morphological characteristic of the apical plasma membrane of umbrella cells is that the outer leaflet appears to be twice thicker as the inner leaflet, thus forming an asymmetric unit membrane (AUM) (Walz et al. 1995). When negative staining solubilized membranes of AUM were observed by high resolution microscopy and by quick freeze-deep etch techniques, a well-developed paracrystalline array of 16 nm diameter AUM particles was observed. This paracrystalline array presents sixfold symmetry, forming a twisted ribbon structure composed of an inner ring containing six large particles and an outer ring containing six small particles (Walz et al. 1995), Fig. 2a. Min et al. (2002) suggested a possible association of the uroplakin pair Ia/II and Ib/III with the inner and outer subdomains, respectively. The images resulting from studying the electron micrographs not only revealed a striking two-dimensional (2D) organization but also the presence of stain-excluding areas of a likely lipid nature. Kachar et al. (1999), based on a combination of freeze/deep etch images and a negative staining data, suggested a close head-to-head interaction between neighboring particles and that the particle structure can change its shape and size as a response to mechanical factors. The model proposed by those authors emphasizes the preferential head-to-head interaction as a determinant factor of plaque size and hinge formation of this unique urothelial luminal surface. A projection map generated by electron cryo-microscopy of the urothelial plaques showed several differential extensive connections between inner and outer subdomains raising the possibility of a flexible urothelial particle (Kachar et al. 1999; Min et al. 2003).

We described for the first time the dependence of AUM particles association with the membrane lipid composition. By modifying the membrane fatty acid composition by dietary treatments, we observed relative changes in uroplakins dimers (Bongiovanni et al. 2005). In fact it has been shown by chemical "cross-linking" an increase of the heterodimer UPIb/UPIII and a decrease of the homodimer UPIII/UPIII in the oleic acid-derived urothelium. From those observations, we inferred that a possible lipid-protein alteration may be the cause of the altered uroplakin dimerization observed (Bongiovanni et al. 2005). In agreement with those data, we observed, by morphometric analyses of AUM, a significant increase of the particle size from 15 nm (control particles, Fig. 2b) to 17 nm center-to-center in oleic acid-derived particles (Fig. 2b, Calderon and Grasso 2006). With these results, we developed a hypothetical model (Calderon and Grasso 2006) that assumes: (1) the extracellular part of the particles may be allowed to expand by changing the ''twist'' according to the ribbon model (Walz et al. 1995; Min et al. 2002), in a manner that the angle between the inner and outer subdomains of each subunit is increased, thus, leading to an increased total particle size. This ''twist'' change may be accompanied by a decreased separation between the UPIb/UPIII heterodimers as mentioned above (Bongiovanni et al. 2005; Calderon and Grasso 2006).

On the other hand, the destructuring effects of oleic acid derivatives on lipid bilayers were extensively studied. Oleic acid derivatives are capable to induce a marked decrease of the gel to liquid crystalline phase transition temperature, as demonstrated by Marsh (1999). This effect

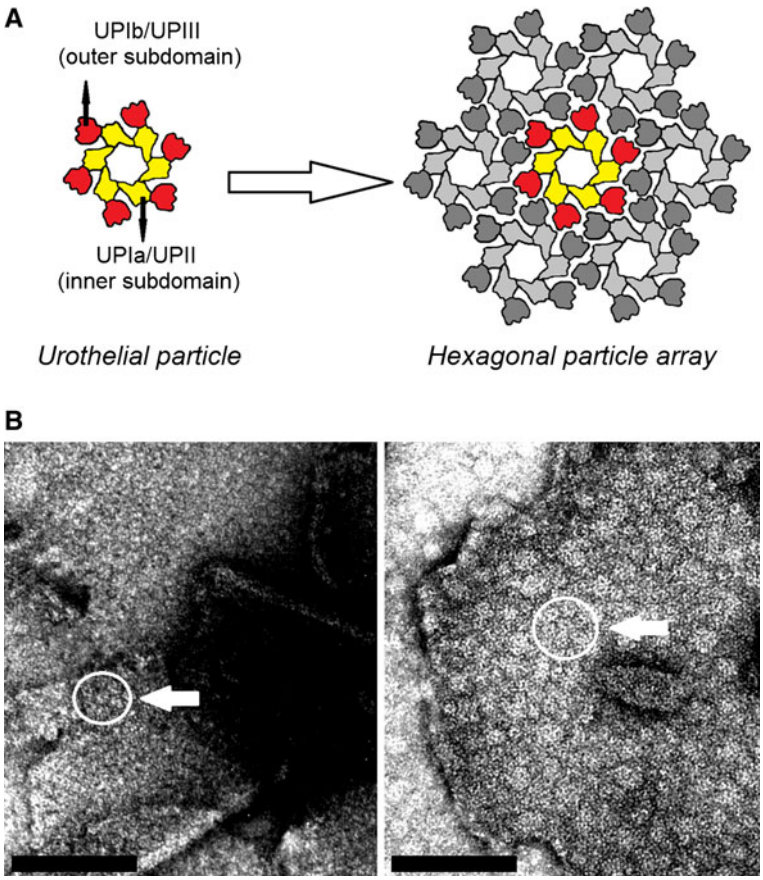

Fig. 2 **a** Schematic figure of an urothelial particle. The inner subdomain is composed of UPIa/UPII heterodimers. The outer subdomain is composed of UPIb/UPIII heterodimers. The urothelial particles associated with each other in a paracrystalline array present sixfold symmetry and form a hexagonal structure. The urothelial particle model was adapted from Wang et al. (2009). **b** The luminal surface of mouse urinary bladder urothelium. TEM micrographs of apical plasma membrane from control (*left panel*) and oleic acid diet treated mice (*right panel*), respectively. Note the increased particle size of oleic acid-derived membrane. Circles delimit a hexagonal particle array, also indicated by *arrows*. Magnification ×160,000. *Scale bars* 150 nm

was adjudicated to the double bond position near the geometric center of the acyl chain and it is related to lipid packing and Van der Waals interactions in the gel phase (Wang et al. 1995). The area occupied by a molecule of dipalmitoylphosphatidylcholine (DPPC) in a lipid bilayer in liquid crystalline phase at 50 °C is 64 $Å^2$ while the area occupied by a molecule of dioleoylphosphatidylcholine (DOPC) is 72.5 $Å^2$ (Nagle and Tristram-Nagle 2000). This same effect of oleic acid was demonstrated by computer simulations (Martinez-Seara et al. 2007). Also oleic acid (18:1) induces important concentration-dependent alterations in the supramolecular organization of PE derivatives, whereas the closely related fatty acids, elaidic (18:1 trans) and stearic (18:0) acid, did not (Funari et al. 2003). Oleic acid probably exerted a lateral pressure on PE fatty acids moieties, favoring a negative-curvature strain. This effect induced the formation of inverted tubular micelles, which are the basic supramolecular units of the H_{II} phase (inverted hexagonal) lattice. This hypothesis is consistent with the marked decrease of the liquid crystalline-to-H_{II} phase transition temperature induced by oleic acid derivatives (Funari et al. 2003). The H_{II} phase has an important role in membrane fusion events, protein control, ultrastructural organization and crystallization of membrane integral proteins (de Kruijff 1997). Curiously, we observed a high content of PE in urothelial oleic acid-derived vesicles (Bongiovanni et al. 2005). PE, associated to the highest content of oleic acid, could induce the formation of H_{II} phase in these membranes. This hypothesis seems to have support by the increment of the bilayer disorder degree in oleic acid-derived membranes as determined by fluorescence anisotropy measurements (Calderón and Eynard 2000).

It is possible to analyze the physio-pathological meaning of particles expansion. As mentioned above, the uropathogenic *E. coli* has, as preferential binding site, the inner subdomain of urothelial particles. Thus, we previously suggested that the motion of the outer subdomain, in the oleic acid-derived membranes, may lead to an "opening" of the extracellular part of the particle, thus, facilitating the access of the bacteria to reach the inner subdomain (Calderon and Grasso 2006).

Urothelial vesicles

As it is known, the umbrella cells contain a high density of vesicles, Fig. 1c. These vesicles are composed of two opposing plaques joined together by hinge membrane (Lewis 2000). Hicks (1965) reported that the vesicles are

associated with intracellular filaments. Staehelin et al. (1972) observed that the cell cytoplasm of umbrella cells contained a dense network of filaments and proposed that the plaques are connected to these filaments by short, cross-linking filaments. Coincidently, Minsky and Chlapowski (1978) proposed that the surface plaques and vesicles are interconnected via the filaments. Moreover, they proposed that these filaments attach to the tight junctions between the lateral and apical membrane and to the desmosomes in the basolateral membrane of the umbrella cells. Urothelial vesicles are intimately associated with cytokeratins, which form a dense subapical network (Veranic and Jezernik 2002). The constituents of the cytokeratin network include cytokeratins-7 and-20 (Veranic and Jezernik 2002, 2006), but may also include other members of this family.

At least two kinds of endocytic vesicles have been characterized: the discoidal/fusiform vesicles (FVs) and the peripheral junction-associated apical endosomes (Khandelwal et al. 2010). It has long been thought that FVs undergo fusion with the apical plasma membrane, thus delivering crystalline plaques to the surface. The population of FVs can be restored by retrieval of membrane from surface of the umbrella cells. Thus, this mechanism has been related to the increase–decrease of hydrostatic pressure in the urinary bladder during the filling–voiding phases of the micturition cycle (Lewis 2000; Apodaca 2004). However, the membrane recycling of FVs has been questioned on the basis of the results suggesting that these vesicles can be regarded as exocytic rather than endocytic delivering uroplakins (the major proteins of the surface membrane) to the apical plasma membrane (Guo et al. 2009; Kreft et al. 2009a, b). The peripheral junction-associated vesicles proceed from an apical membrane compensatory endocytosis (Khandelwal et al. 2010) and represent an integrin-regulated and RhoA- and dynamin-dependent pathway.

The urothelial endocytic vesicles can be localized in lysosomes

Vesicular trafficking is an essential cellular process in eukaryotic cells to deliver either membrane proteins or soluble cargos from one compartment to another (Marshansky and Futai 2008). It has been shown that the intracellular traffic can be regulated, among others, by the luminal acidic pH of intracellular organelles established by a transmembrane pH gradient and/or membrane potential (Marshansky and Futai 2008). Moreover, this pH gradient is necessary not only from molecules recycling, such as transferrin (Mayle et al. 2012; Luck and Mason 2012), but also for lysosomal degradative pathway. There are plenty of evidences that demonstrate the degradation of the endocytosed material in umbrella cells. For instance, Khandelwal et al. (2010) demonstrated that both membrane and fluid internalized were targeted to lysosomal degradation. In fact, this fate was not the known lysosomal pathway, since the internalized membrane and fluid probes were delivered to the junction-associated vesicles and not to FVs or classical early endosomes, and the fate of the cargo was the degradation in late endosomes/lysosomes. On the contrary, Kreft et al. (2009a, b) provide evidences that some membrane-bound endocytotic marker is sorted to early endosome compartment which matures in late endosome and lately in lysosome. Zhang and Seguchi (1994) also gave evidences of the vesicle pathway toward the lysosomal degradation demonstrating the surface characteristic AUM structure in multivesicular bodies, autophagosomes, and lysosomes of umbrella cells. Truschel et al. (2002) have also reported that once the vesicles have been endocytosed, their membrane protein content, specifically UPIII, could be degraded via lysosomes. Guo et al. (2009) have recently demonstrated the acidification of the endocytic vesicle interior and its dependence of Vps33a, a Sec-1 related protein implicated in vesicular transport to the lysosomal compartment. We recently corroborated these facts. By entrapping a pH-sensitive fluorescent probe, we determined the luminal pH of endocytic vesicles differentiated in their lipid composition (Grasso and Calderón 2013), Fig. 3. The higher pH of endocytic vesicles was observed in oleic acid-derives vesicles compared to linoleic acid-derived vesicles and control vesicles (Grasso and Calderón 2013). The acidic pH is achieved by an urothelial V-ATPase (Grasso et al. 2011a), composed of two multisubunit domains: the membrane proton channel V_0, responsible for proton translocation, and the peripheral catalytic segment V_1, where ATP hydrolysis occurs, Fig. 4. In fact, the proton translocation efficiency or acidification rate is dependent on the coupling of both domains (Cipriano et al. 2008). Related to this, we described for the first time the correlation between the lipid composition and the V-ATPase function/activity. It was shown how changes in membrane lipid composition (obtained by differential dietary treatments) of endocytic vesicles could induce a functional uncoupling between both V-ATPase domains (Grasso et al. 2011a). We suggested that the uncoupling of V-ATPase domains, observed in oleic acid-derived vesicles, was result of alterations in acyl chain interdigitation and membrane-hydrophobic core thickness. In fact, both parameters were lower in oleic acid-derived vesicles (Grasso and Calderón 2013). This may imply some lipid matrix alterations, leading to a lipid-protein hydrophobic mismatch similar to that described by Lee (2003, 2004). Moreover, Lee proposed that the exposure of the protein hydrophobic fragments to the aqueous phase could induce the stretching of surrounding fatty acyl chains favored by a

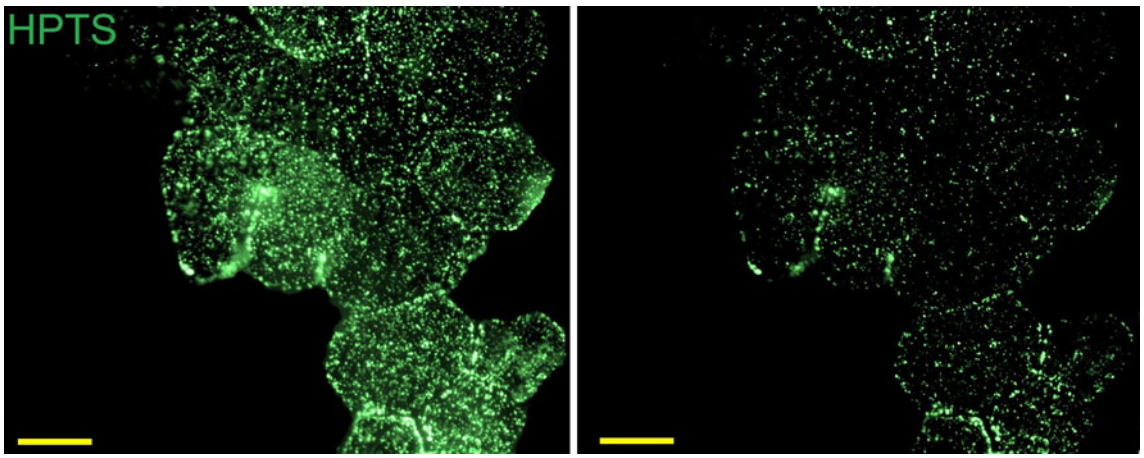

Fig. 3 Epifluorescence pH determination of umbrella cells loaded with HPTS. The fluorescence intensity of live HPTS-loaded rat umbrella cells was determined by fluorescence spectral shifts according to Grasso and Calderón (2013). The fluorescence decrease in *right panel* indicates the probe acidification. *Scale bars* 20 μm

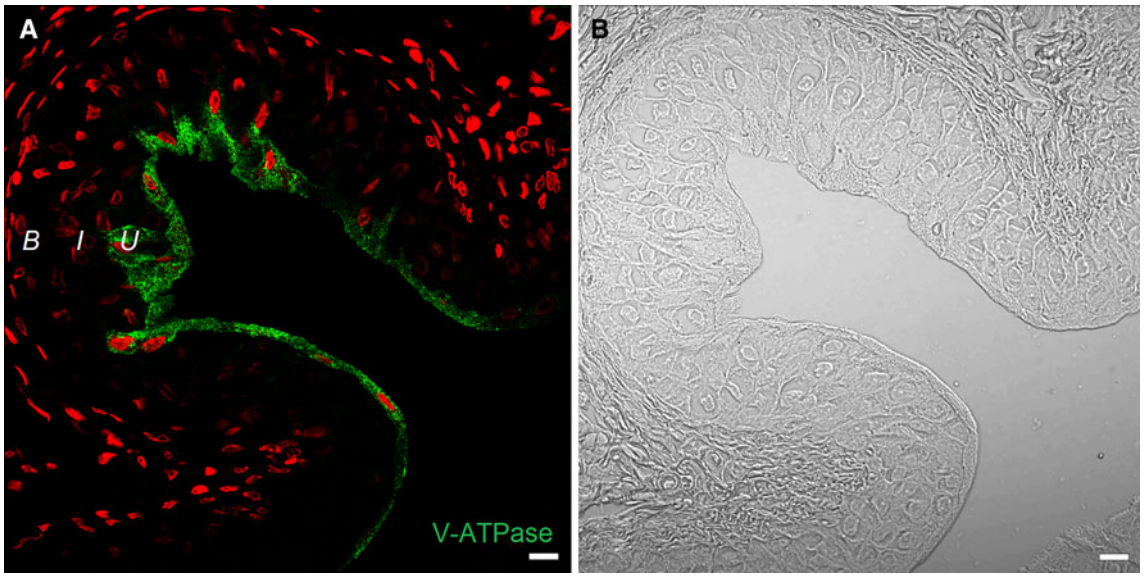

Fig. 4 Detection of urinary bladder V-ATPase by immunofluorescence. **a** The image shows the three layers of rat bladder urothelium: the superficial umbrella cells (*U*), intermediate cells (*I*), and basal cells (*B*). The presence of the V-ATPase (*green positive labeling*) is viewed in the umbrella cell layer. No positive signal was seen in other layers. The nuclei were stained with propidium iodine (*red*), exc 546 nm. **b** DIC image. *Scale bars* 10 μm. Magnification ×1,000

lower interdigitation (Lee 2004). Based on these data, we propose that the hydrophobic mismatch in the oleic acid-derived vesicles may represent an alteration of the lipid matrix surrounding the V-ATPase. This condition could be reflected in the altered capacity of the proton-transporter domain (V_0) to function in phase with the catalytic domain (V_1) and consequently leads to a delayed acidification rate of V-ATPase in oleic acid vesicles (Grasso et al. 2011a). Finally, we suggested that V-ATPase could function as a biochemical switch in the sorting of endocytosed material to the lysosome and, in turn, the enzyme can be regulated by the surrounding lipid matrix (Grasso and Calderón 2013). Besides V-ATPase, some other sorting proteins have been described. The endocytic vesicles have a GTPase, Rab27b, which participates in targeting the endocytic vesicles to lysosomes (Chen et al. 2003). As mentioned before, Guo et al. (2009) demonstrated the presence of Vps33a, a Sec-1 related protein implicated in vesicular transport to the lysosomal compartment. This result was reinforced by studies with Vps33a mutation in mouse which causes the Hermansky–Pudlak syndrome, characterized by oculocutaneuos albinism and bleeding, due to traffic alterations of melanosomes and platelets, respectively, all lysosome-related organelles (Guo et al.

2009; Hurford and Sebastiano 2008). In relation to this, it is important to note that those vesicles are Vps33a positive but CD63 negative (Guo et al. 2009), a tetraspanin protein known as a lysosome-related organelle marker and, therefore, they are not typical lysosome-related structures (Dell'Angelica et al. 2000). On the other hand, UPIII was also suggested to be a possible targeting molecule to the lysosome (Kreft et al. 2009a; Bonifacino and Traub 2003).

Curiously, it was demonstrated that fluid and membrane-bound phases from endocytic vesicles did not follow the same pathway to lysosome (Kreft et al. 2009a, b; Grasso and Calderón 2013). Kreft et al. (2009a, b) proposed a model of internalization where two pathways are possible: one coming from the urothelial plaques, carrying the UPIII as a targeting molecule, (Kreft et al. 2009a; Bonifacino and Traub 2003) and the other coming from the hinge areas, containing most of the fluid phase. Those authors concluded that contrary to the endocytosed membrane, the endocytosed fluid ends up in late endosome–lysosome without passing throughout the early endosomal compartment. Accordingly, results of our group may reflect the dichotomous origin of the vesicles and their internalization pathways, as proposed by Kreft et al. (2009a, b). In fact, we studied the delivery of both fluid and membrane probes to the lysosomal compartment. By fluorescence colocalization analyses, we determined the amount of endocytosed fluid probe and membrane probe in the lysosome (Grasso and Calderón 2013), Fig. 5. We observed that not only the fluid phase was sorted to lysosomes in minor amount than membrane-bound probe, but also it was dependent on membrane lipid composition. In fact, oleic acid-derived vesicles showed the lesser colocalization coefficients of both fluid and membrane-bound probes than the linoleic acid and control derived vesicles (Grasso and Calderón 2013). These results were well correlated with the higher luminal pH and lower proton translocation by the V-ATPase in oleic acid-derived vesicles (Grasso et al. 2011a; Grasso and Calderón 2013).

Finally, Kreft el al. (2009a, b) by performing in vitro studies of constitutive apical endocytosis in live urothelial cells immunolabeled with anti-uroplakins antibodies showed that rare uroplakin-positive membrane regions are internalized in partially, but none in highly differentiated umbrella cells. Moreover, these authors did not find, in lysosome, the uroplakins from the apical plasma membrane. Thus, they suggested that uroplakins found in

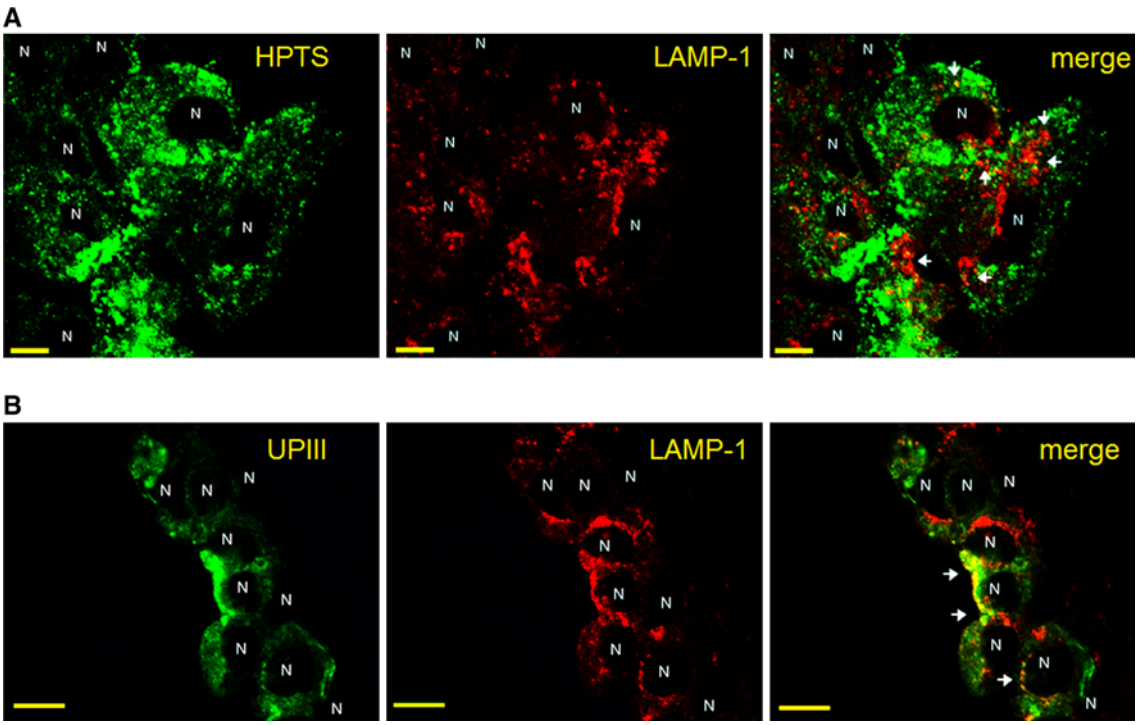

Fig. 5 Colocalization of the endocytosed probes (fluid and membrane-bound) and the lysosome-specific probe in the lysosomal compartment. **a** left panel (*green*) cryosections of rat umbrella cells loaded with HPTS by endocytosis induction for 20 min. Central panel (*red*) umbrella cells immunostained with the anti-lysosome membrane protein (LAMP-1) antibody. *Right panel* merged images. *N* nuclei, *arrows* colocalized structures. *Scale bars* 10 μm. **b** Left panel (*green*) cryosections of rat umbrella cells showing the internalized membrane-bound probe (UPIII-FITC). Central panel (*red*) umbrella cells immunostained with the anti-lysosome membrane protein (LAMP-1) antibody. *Right panels* merged images. Note the higher colocalization of membrane-bound probe when compared to fluid probe. *N* nuclei, *arrows* colocalized structures. *Scale bars* 10 μm

lysosome were originated from other intracellular compartments rather from the apical plasma membrane in highly differentiated umbrella cells (Kreft et al. 2009a, b). However, we observed a higher endocytosis of both fluid and membrane probes in highly differentiated umbrella cells (Grasso and Calderón 2009, 2013; Grasso et al. 2011a, b) than that reported by Kreft et al. (2009a, b), by applying the protocol for endocytosis induction of umbrella cells developed by Lewis and de Moura (1984). Nevertheless, it is likely that the endocytotic compartment studied by us is not fully comparable to that of Kreft et al. (2009a, b), since a stretch-induced endocytosis was used in our experimental system whereas the constitutive apical endocytosis without mechanical stimuli in umbrella cells was studied by Kreft et al. (2009a, b).

Recycling of endocytic vesicles to the bladder lumen

Membrane recycling is a necessary event after any endocytosis process (Perez Bay et al. 2012). In fact, the model of urothelial vesicle traffic, proposed by Hicks, defined the endocytic/exocytic events as a response to changes in hydrostatic pressure during the micturition cycle (Hicks 1975). Exocytosis in umbrella cells has been studied in cell culture (Truschel et al. 1999), in situ (Wang et al. 2005) and in isolated urothelial tissue mounted in modified Ussing chambers (Truschel et al. 2002). Outward bowing of the urothelium mounted in Ussing chambers (which simulates bladder filling) increases exocytosis as measured by surface biotinylation (Truschel et al. 2002; Yu et al. 2009), stereology (Truschel et al. 2002), release of secreted proteins (Khandelwal et al. 2008; Truschel et al. 2002) and transepithelial capacitance (Truschel et al. 2002; Yu et al. 2009). Upon voiding, the membrane added during filling must be rapidly recovered. The mechanisms that govern this process are not well understood.

The endocytosis/exocytosis cycle of urothelial vesicles was related to cytoskeleton integrity. In fact, Sarikas and Chlapowski (1989) have shown that disruption of the intermediate filament network with 5 mM thioglycolic acid resulted in the blocking of vesicles trafficking toward and from the apical plasma membrane. Moreover, they observed, during the bladder contraction, that the interphase between umbrella and intermediate cells unfolded and concomitantly the apical plasma membrane surface ballooned out into the luminal cavity of urinary bladder (Sarikas and Chlapowski 1989). Besides, in expanded bladders, large intracellular spaces were formed in umbrella cells along their lateral and basal margins. From this evidence, it has been suggested that cytokeratin filaments render mechanical support to the plasma membrane against external pressures during the expansion–contraction cycle by forming important linkages in cell–cell adhesion systems (Moll et al. 1982).

We recently have shown that the lower colocalization of both fluid and membrane-bound probes with lysosome in oleic acid-derived vesicles correlated with the higher recycling of both probes (Grasso and Calderón 2013). These results might be considered as a vesicle displacement from lysosomal degradation to the surface recycling. In fact, the lower acidification of the endosomal compartment in oleic acid-derived vesicles, delaying their subsequent transfer to lysosomes, would increase the amount of endosomal cargo at the more apical vesicles just below the membrane, forcing its transfer to exocytosis. Curiously, we also observed that the fluid and membrane-bound probes were not proportionally recycled. In fact, in all three diet-derived bladders, the membrane-bound probe recycled was higher than the fluid probe (Grasso and Calderón 2013). This fact could be associated with the dichotomous origin of vesicles proposed by Kreft et al. (2009a, b). However, Derganc et al. (2011) have analyzed, in a theoretical way, the shape and structure of one kind of vesicles, the FVs. They reported that the FVs, which are compartments with small volume-to-surface area ratio, ensure the membrane reinsertion at the apical surface with the minimal internalization of urine. Thus, recycling of FVs, over other kind of vesicles, may also be an alternative explanation for the difference between recycled fluid and membrane-bound probes observed (Grasso and Calderón 2013).

On the other hand, Kreft et al. (2009a, b) proposed that the fluid phase recycled comes from the recycling endosome and the membrane does not. Thus, these authors proposed that discoidal/fusiform vesicles are not recycling vesicles but are most likely formed de novo along the biosynthetic pathway (Kreft et al. 2009a, b, 2010a, b).

This fact has a strong experimental support and is an evidence of a new alternative pathway for the urothelial vesicles but, again, we remark that the endocytotic compartment studied by our group is not quantitatively comparable to that of Kreft et al. (2009a, b) since, unlike us, they studied the constitutive apical endocytosis without mechanical stimuli in umbrella cells. Moreover, in the absence of mechanical stimulus, the umbrella cells in tissue appear to be quiescent, and there is little evidence of endocytosis or exocytosis (Truschel et al. 2002). This is consistent basically with the fact that constitutive apical endocytosis is significantly diminished in highly differentiated cultures of urothelium (Kreft et al. 2009a). Even more, these authors proposed that the low endocytotic capacity might be an additional protection mechanism against internalization of toxic compounds from urine into the cells (Kreft et al. 2009a). On the other hand, the recycling of those toxic compounds could be another

important mechanism against the development of several pathologies, such as bladder cancer. This hypothesis has a strong experimental support. In fact, in vivo experiments reported from Cohen et al. (2007) and Suzuki et al. (2008) indicated that the reactive metabolite DMAIII (dimethylarsinous) produced in rats treated with sodium arsenite accounts for cytotoxicity and regenerative proliferation observed in the urinary bladder urothelium. An increased incidence of bladder tumors and hyperplasia was observed by Wei et al. (1995, 2002) in F344 rats treated with DMAV (dimethylarsinc) in the drinking water. Similarly, we observed a cellular hyperproliferative response (hyperplasia) in rats receiving arsenic in their drinking water (Grasso et al. 2011b). Thus, this pathological effect generated in in vivo situation could be ascribed to the lack of recycling of toxic compounds, such as arsenic and also, as it was demonstrated by us, the leakage of those compounds to the cytosol, as we shall discuss in the next section. Therefore, it seems to be an association between the pathological effects generated in vivo and the diminished recycling demonstrated in vitro by us. Taken together, these results seem to indicate the possible relevance of in vitro studies for the in vivo situation. Nevertheless, some other experimental support would be necessary to directly demonstrate such suggestion.

Permeability properties of urothelial vesicles

The permeability barrier of the urothelium has stimulated interest in the role of the luminal membrane as such permeability barrier. As mentioned above, the luminal surface of this epithelium is covered by seemingly rigid plaques (Lewis 2000; Apodaca 2004). A complete disappearance of both, the AUM and the symmetric hexagonal pattern, together with a reduced junctional complex, was observed after a direct exposure of the urothelial luminal surface to carcinogens (Hainau 1979). These membrane changes implicate increased permeability and less adaptability of urothelial function to mechanical stress caused by bladder volume changes (Hainau 1979). Moreover, knockout animals lacking the UPII or UPIIIa (major luminal surface proteins) showed and increased permeability to urea and water supporting the idea that luminal surface is a component of the permeability barrier (Hu et al. 2000, 2001, 2002; Kong et al. 2004; Schönfelder et al. 2006; Sun 2006; Aboushwareb et al. 2009). However, the permeability of the endocytic vesicles was scarcely studied. To our knowledge, there are only two works where it is demonstrated that the endocytic vesicles can release their content into the cytosol (Grasso and Calderón 2009; Grasso et al. 2011b). By inducing the endocytosis of a fluorophorer and its quencher (hydroxypyrene-1,3,6-trisulfonic acid or HPTS and p-xylene-bis-pyridinium or DPX, respectively), we determined both the relative released material to the cytosol and the leakage mechanisms from urothelial endocytic vesicles differentiated in their membrane lipid composition induced by dietary treatments or after arsenic exposure (Grasso and Calderón 2009; Grasso et al. 2011b). For leakage determinations, we used the requenching method developed by Wimley et al. (1994) and widely extended by Ladokhin et al. (1995). This method is based on a simple titration of fluorescence fraction released from the endocytic vesicles to determine the internal quenching of remaining fluorescent molecules in the interior of the vesicles. The internal quenching dependence on the fluorescence fraction released allowed us to define two possible mechanisms of leakage: (a) All-or-None, where the internal quenching is independent on the released fluorescence fraction and (b) Graded, where changes of the internal quenching are dependent on the released fluorescence fraction. In the first case, a population of endocytic vesicles releases all of their content and others do not; in the second case, the released fluorescence from the endocytic vesicles is partial (Wimley et al. 1994; Ladokhin et al. 1995). Our results have shown not only the existence of leakage in urothelial endocytic vesicles, but also the differential mechanisms of release dependent on membrane lipid composition (Grasso and Calderón 2009). Control and linoleic acid-derived vesicles showed a graded leakage mechanism with preferential release of cationic DPX. In the case of oleic acid-derived vesicles, we could not distinguish between the All-or-None leakage mechanism and the highly graded preferential release of HPTS (Grasso and Calderón 2009). As mentioned in a previous section, we observed a high content of PE in oleic acid-derived vesicles (Bongiovanni et al. 2005). It is known that dioleoylphosphatidylethanolamine (DOPE) promotes a non-bilayer phase which favors the formation of inverted hexagonal structures (H_{II}) that confer membrane instability (Seddon 1990; Wasungu et al. 2006). This fact may probably be applied to oleic acid-derived vesicles where the high content of oleic acid may be associated with the increased amount of PE. Thus, this lipid interaction may promote the appearance of non-bilayer structures and, consequently, a vesicular membrane destabilization leading to the non-differential release of its luminal content (Grasso and Calderón 2009). On the other hand, we also described that umbrella cells exposure to inorganic arsenic resulted in an increased permeability of the urothelial endocytic vesicles (Grasso et al. 2011b). This fact was, in part, adjudicated to the arsenic oxidative capacity on biomolecules, such as fatty acids and DNA (Wang et al. 2007). In relation to this, we observed a decrease of the unsaturated double bond index due to the

decrease in the quantity of unsaturated fatty acids, determined by gaseous chromatography (Grasso et al. 2011b). Thus, it is possible that the formation of transient pores, by cleaving the unsaturated fatty acid hydrocarbon backbone, may contribute to increase vesicle permeability (Heuvingh and Bonneau 2009). Besides, the endocytosis of arsenic compounds may be facilitated by the well-known ability, if these molecules to bind to sulphydryl groups of proteins (Cohen et al. 2007) such as those present in uroplakins, affecting the hexagonal symmetric array of the particles covering the urothelial plaques. Taken together, our results related to membrane alteration of the urothelium either due to differentiated fatty acid-diets or arsenic exposure strongly suggest the existence of a "bypass route" to the urothelial permeability barrier leading to the internalization of undesirable molecules transported by the urine (Grasso and Calderón 2009; Grasso et al. 2011b).

Based on all cited evidences, we developed a hypothetical model for the urothelial endocytic vesicles pathways (Fig. 6).

Endocytotic capability of umbrella cells

We conclude this review with an analysis of the endocytotic capability of umbrella cells that will determine the amount of endocytosed material distributed in all intracellular compartments. Kreft et al. (2009a, b) demonstrated that in highly differentiated umbrella cells (high uroplakin labeling), the constitutive endocytosis of fluid phase probe was 32 % lower than membrane-bound probe. In partially differentiated umbrella cells (low uroplakin labeling), the difference was even greater: the endocytosis of fluid phase probe was 84 % lower than membrane-bound probe (Kreft et al. 2009a). These authors suggested that arrested fluid phase endocytosis is a necessary physiological adaptation of bladder urothelial cells, especially for the differentiated umbrella cells with weak uroplakins expression to maintain the permeability barrier (Kreft el al. 2009a, b). Truschel et al. (2002) reported that the mechanical stretch induced the maximum endocytosis as early as 5 min, and this value did not change up to 120 min even if exocytosis was occurring simultaneously. Furthermore, these authors demonstrated that membrane capacitance changes, as a function of time, showed a biphasic tracing with faster changes up to 20 min followed by a remarkable slow trend from 40 to 300 min (Truschel et al. 2002). A similar result was observed by us (Grasso and Calderón 2013). When induced endocytosis, for 20 and 40 min, by an osmotic shock in umbrella cells differentiated in their lipid membrane composition, we did not observe changes in the quantity of endocytosed probes, at both times of endocytosis induction. Nevertheless, oleic acid-derived vesicles endocytosed a lower amount of both fluid and membrane-bound probes in relation to linoleic acid-derived and

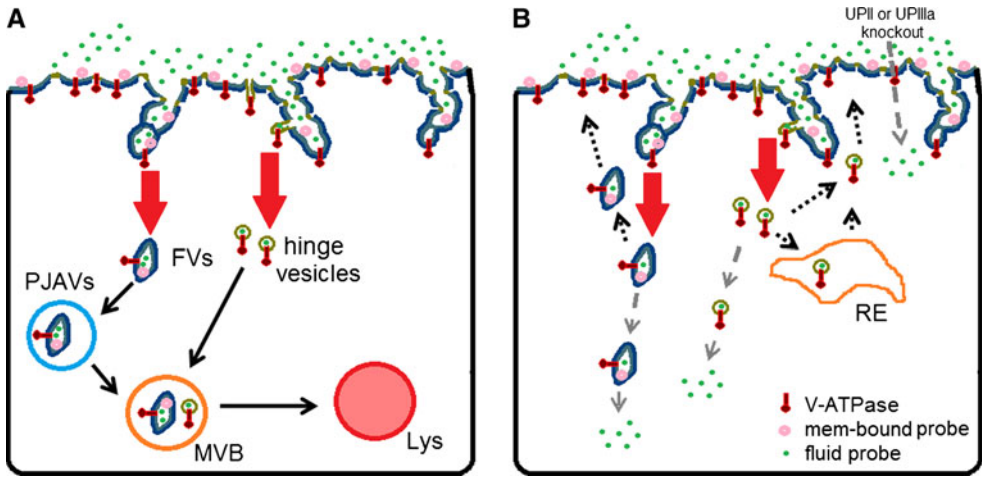

Fig. 6 Hypothetical model of urothelial vesicles pathways. **a** *Thick red arrows* RhoA- and dynamin-dependent endocytosis (Khandelwal et al. 2010). *Thin black arrows* lysosomal degradative pathway. Fusiform vesicles (FVs) Vps33a positive, CD63 negative (Guo et al. 2009) and Rab27 positive (Chen et al. 2003) are sorted to perijunction associated vesicles (PJAVs, Khandelwal and Abraham 2009), ZO-1 and occluding positive, and then directed to multivesicular bodies (MVB, Zhang and Seguchi 1994) and lately to lysosome (Lys, Zhang and Seguchi 1994; Khandelwal and Abraham 2009; Kreft et al. 2009a, b; Grasso and Calderón 2013). The hinge originated vesicles may be sorted to MVBs and lysosomes (Grasso and Calderón 2013). **b** *Dotted black arrows* recycling pathway of endocytics vesicles. Once endocytosed, hinge vesicles could be sorted to recycling endosome (RE, EEA1 positive) and then recycled to the apical membrane (Kreft et al. 2010a, b). However, we showed that also FVs are recycled to the apical membrane (Grasso and Calderón 2013). *Dashed gray arrows* permeability of apical membrane and endocytic vesicles membrane. Knockout animals lacking the UPII or UPIIIa showed and increased permeability to urea and water (Hu et al. 2000, 2001, 2002; Kong et al. 2004; Schönfelder et al. 2006; Sun 2006; Aboushwareb et al. 2009). Furthermore, we demonstrated that, once endocytosed, the endocytic vesicles can release their content to the cytosol, depending on their differential lipid membrane composition (Grasso and Calderón 2009; Grasso et al. 2011b)

control vesicles. The lower endocytosis in oleic acid-derived vesicles was related to an altered structural organization of urothelial plaques, demonstrated by morphometric analyses of negatively stained TEM images (Calderon and Grasso 2006), Fig. 2. Besides, we reported that although the average molecular rotational diffusion (mobility) of oleic acid-derived urothelial membrane is lower than that of the control and linoleic acid-derived membrane, a decreased mobility in the half of the hemilayer was determined in oleic acid-derived membranes (Calderón and Eynard 2000) when analyzing the degree of freedom along the acyl chain depth (Baenziger et al. 1991). Moreover, an increased membrane saturation degree was also observed in oleic acid-derived vesicles concomitant with the lower endocytosis capability (Grasso and Calderón 2013). A similar result was observed in mouse peritoneal macrophages where an increased membrane saturation was related to a diminished endocytosis (Mahoney et al. 1977). In summary, the lipid composition determines the lipid organization, membrane rigidity and membrane saturation degree which are all parameters related to membrane structure, as demonstrated in synthetic lipid systems (Nagle and Tristram-Nagle 2000) that could regulate membrane dynamics involved in endocytosis. Therefore, we conclude, based on the cited evidence, that not only the differentiation stages, measured by the protein composition (Kreft et al. 2009a, b), but also the lipid composition of apical plasma membrane, in totally differentiated umbrella cells, regulates endocytosis events.

On the other hand, it was demonstrated that during the filling phase a stretch regulated compensatory endocytosis is possible (Khandelwal et al. 2010). Because the apical plasma membrane of differentiated umbrella cells lacks coated pits, caveolae or flotilin-positive carriers, a compensatory endocytosis in umbrella cells is apparently a pathway for bulk retrieval of apical plasma membrane and is unlikely to require multiple mechanisms for cargo-selective endocytosis (Khandelwal et al. 2010). The physiological stimulus for this event is increased basolateral tension, which through or in conjunction with β1 integrin stimulates PI-3K and focal adhesion kinase (FAK)-dependent activation of RhoA. In turn, RhoA recruits Rho-associated coiled coil-containing protein kinases (ROCK), which may act by promoting alterations in the actin cytoskeleton that in conjunction with myosin motors could help to pull apical membranes inwards as well as generate tension in these membranes as a preamble to scission (Khandelwal et al. 2010).

Conclusions

The original traffic model proposed for the urothelial endocytic vesicles satisfied for many years the understanding of urothelial biology. Without the addition of membrane by exocytosis, the AUM will eventually disrupt due to the increment of membrane tension during the filling phase of the micturition cycle. Following, during the voiding phase, the added membrane will be reinternalized and then restoring the initial pool of vesicles. Nevertheless, incrementing evidences of alternative pathways of endocytosed material basically leaves this model at least incomplete. New questions are beginning to arise not only from physiological aspects but also from urothelial pathology. The formation of urothelial plaques and apical plasma membrane trafficking in umbrella cells is important for the formation and maintenance of the blood-urine permeability barrier. In many cases of urinary disease, this permeability barrier is altered and toxic compounds present in urine, such as arsenicals, are internalized producing cellular toxicity. Thus, the knowing of fundamental aspects in the intracellular distribution and its regulation of both urothelial endocytic vesicles and their fluid contents is the initial step to understand urinary bladder pathology.

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