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Topical Review

The Role of Membrane Turnover in the Water Permeability Response to Antidiuretic Hormone

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Introduction

Antidiuretic hormone (ADH)[1] regulates the permeability of cells in certain tight epithelia such as mammalian renal collecting duct and anuran skin and urinary bladder. The binding of ADH to its receptor on the basolateral membrane of these epithelia stimulates adenylate cyclase activity. The increase in intracellular cAMP concentration, in turn, produces permeability changes in the apical plasma membrane that result in the transfer of water and solutes across the epithelium [35]. Apparently, independent changes in the apical membrane are responsible for the increase in permeability to sodium, urea and water [42].

Research by many laboratories has established several fundamental facts regarding the responsive cells: 1) in the unstimulated state, the apical membranes of these cells have an extremely low permeability to water [32, 43, 74, 94, 102]; 2) the dramatic increase in permeability occurs within minutes of the addition of ADH to the basolateral side of the epithelium [34, 80, 94]; 3) when ADH is withdrawn, the reversal of permeability also occurs within minutes [34, 80, 94]; and 4) the increase in apical membrane water permeability is in all likelihood due to pores or channels which are highly selective, admitting water but excluding small nonelectrolytes and ions [4, 25, 26, 45, 59, 60, 79] (reviewed recently in reference [27]).

This review will focus on the dynamics of apical membrane turnover in the ADH-elicited water permeability response with special reference to the toad urinary bladder. First, we discuss evidence relating the ADH-elicited increase in apical membrane water permeability to the insertion of water-permeable membrane units identified as membrane particle aggregates on freeze-fracture electron microscopy. After considering the specific nature of apical membrane turnover in the toad bladder, we present a strategy for the identification of components of the ADH-elicited water channel, and discuss the problems inherent in that strategy. Finally, we speculate briefly regarding the evolutionary origin of the putative water channel.

Evidence that Membrane Particle Aggregates are the ADH-Regulated Water Channel

About 95 percent of the urinary surface of toad and frog urinary bladders is made up of the apical plasma membrane of granular cells, so designated because of the distinctive carbohydrate rich granules clustered in the cytoplasm below the apical membrane. The water permeability response to ADH occurs in the granular cells [19].

Following the observations of Chevalier et al. [14], freeze-fracture electron microscopy of the apical membrane of frog and toad bladder granular cells has yielded a host of data correlating membrane particle aggregates with the ADH-elicited water permeability response. Prior to ADH stimulation, the outer half of the granular cell apical plasma membrane lipid bilayer (E-face) contains abundant intramembrane particles, whereas the inner half (P-face), which is in contact with the cytoplasm, is sparsely populated with intramembrane particles [98]. Intramembrane particles in both leaflets of the lipid bilayer are distributed in an apparent random

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[1] The abbreviation ADH is used to indicate: arginine vasopressin, oxytocin and arginine vasotocin that have been used to elicit the ADH water permeability response.

array. This random arrangement is altered minutes after stimulation by ADH, when distinctive clusters of ordered intramembrane particles appear in the P-face, while a set of matching grooves appears in the corresponding area of the E-face [11, 14, 54]. Accordingly, these clusters of particles were named membrane particle aggregates. Initially, they were attributed to the aggregation of intramembrane particles that were present in the apical membrane under basal conditions. More recent information indicates that, at least for toad skin [7] and toad and frog urinary bladders [15, 77], these particle aggregates originate from cytoplasmic vesicles.

Mammalian cortical [65] and medullary [8, 36] collecting duct respond to ADH with an increase in water permeability. ADH stimulation of these tissues also results in the appearance of membrane particle aggregates whose structure is similar to the aggregates in the toad bladder. In both anuran and mammalian epithelia, the number of apical membrane particle aggregates per unit area of apical membrane is proportional to the concentration of ADH [7, 12, 36, 54, 55, 65]. Both the incidence and cumulative area of apical membrane particle aggregates are correlated with the level of ADH-stimulated water permeability [7, 48, 51] except under certain circumstances [20, 48, 62]. These exceptions, however, may be due to unidentified permeability barriers beyond the apical membrane [49, 52, 53]. In the toad bladder, agents which inhibit [51, 61, 64] or enhance [63] ADH-stimulated water flow cause a decrease or increase, respectively, in the number of membrane particle aggregates per unit membrane area. Inhibitors of ADH-stimulated sodium or urea transport that have no effect on water permeability also have no effect on particle aggregates [58]. Mutant strains of mice which suffer from nephrogenic diabetes insipidus also lack particle aggregates [10]. Few particle aggregates are visualized in the renal papillas of Brattleboro rats until these animals are infused with exogenous ADH [8, 21]. Based on the foregoing, it is widely believed that particle aggregates constitute or are closely associated with ADH-elicited water channels [41, 97].

Vesicle Fusion with the Apical Membrane Mediates the ADH-Stimulated Water Permeability Response

The general concept that the ADH-mediated water permeability response in the toad urinary bladder results from the fusion of vesicles with the granular cell apical membrane is not of recent origin. In 1971, Masur et al. [69, 70] proposed that ADH increased toad bladder water permeability by the addition of new membrane to the granular cell apical plasma membrane. As the water permeability declined, this additional membrane was removed via apical membrane pinocytosis. These studies [33, 69, 70] quantified the number of endocytic vesicles, using electron-dense markers such as horseradish peroxidase, and focussed on the ADH-stimulated exocytosis of vesicles called granules which contain a carbohydrate-rich material within their lumen. Granules are located immediately beneath the granular cell apical plasma membrane and are structurally different from membrane particle aggregate-containing aggrephores (*see below*). ADH induces the exocytosis of granules and the fusion of granule membrane with the apical plasma membrane [33, 69–71, 82]. The role of the granules in the ADH water permeability response has been disputed. Some laboratories have found that granule exocytosis is not proportional to the increase in water permeability [77, 93, 100, 101]. Exocytosis of granules does not explain the appearance of particle aggregates in the apical membrane, for particle aggregates are not present in granules [77, 93, 101]. The ADH-responsive cells of the toad skin have particle aggregates but lack granules [7], whereas granules, like those in the toad urinary bladder, are found in the urinary bladder of other anurans, such as *Xenopus laevis*, which do not have a water permeability response to ADH [5]. In our opinion, the function of the granules remains to be determined.

The independent observations of Humbert et al. [47], Wade et al. [101] and Muller and Kachadorian [77] have demonstrated that particle aggregates originate from intracellular vesicles which fuse with the apical membrane of anuran bladders. Under basal conditions (no ADH), membrane particle aggregates reside in elongated tubular vesicles within the cytoplasm of granular cells [47, 77]. These vesicles, later termed aggrephores [75], are: large (average diameter 0.1 μm with a length of 1–1.5 μm) [75], occur in groups within the cytoplasm [96] and contain membrane particle aggregates arranged in a right-handed helix within the aggrephore limiting membrane [42, 101]. Aggrephores appear to have substructure; these are constrictions or segmentations along the tubular portion [86, 93]. There is a spherical "head" at one end of the aggrephore [44, 86, 93] that is coated with a fuzzy material that may be clathrin [29]. Definitive immunolocalization will be required to establish that the fuzzy material is clathrin.

Upon stimulation with ADH, aggrephores fuse with the apical plasma membrane to create long finger-like invaginations in the apical membrane. After fusion, a portion of the aggrephore's complement of particle aggregates is apparently translocated into

the lipid bilayer of the apical membrane [75]. Removal of ADH causes the detachment of aggrephores from the apical plasma membrane and a rapid decrease in the tissue water permeability [75, 76]. Considerable evidence supports Wade's proposal [96, 101] that aggrephores shuttle particle aggregates into and out of the otherwise water-impermeable granular cell apical membrane. Similar vesicles containing membrane particle aggregates have also been discovered in granular cells of the toad skin [7] (D. Brown, *unpublished observations*), but aggrephores have not been visualized in principal cells of the mammalian kidney.

The details of the ADH-elicited apical membrane events in the mammalian collecting duct are less clear. Brown et al. [8, 10] found an increase in the number of both clathrin-coated pits (thin-section electron microscopy) and patches of apical membrane containing membrane particle aggregates (freeze-fracture electron microscopy) during ADH stimulation. Both coated pits and membrane particle aggregates excluded the membrane-disrupting antibiotic filipin. Since both membrane structures excluded filipin, they concluded that membrane particle aggregates are located within coated pits in the apical membrane of principal cells [8]. Clathrin-coated pits appear to be involved in the reversal of permeability when ADH is withdrawn. In whole animals [9] and isolated perfused cortical collecting tubules [95], withdrawal of ADH induces a wave of principal cell apical membrane retrieval in the form of coated pits.

Dynamics of Apical Membrane Turnover in Toad Urinary Bladder

Since aggrephore fusion with the apical membrane in response to ADH and detachment when ADH is withdrawn are critical events in the water permeability response, characterization of the dynamics of apical membrane turnover involving particle aggregates is fundamental in understanding the dramatic change in membrane water permeability induced by ADH. The task is greatly impaired, unfortunately, by the lack of a specific marker for particle aggregates other than their appearance in freeze-fracture electron microscopy. Until recently, characterization of membrane turnover has depended on indirect techniques such as: 1) examination of the fate of fluid-phase markers (marking vesicles containing solution derived from that bathing the bladder's apical membrane) and inferring the fate of particle aggregates (for example refs. [33, 40, 75]); 2) treatment of granular cell apical membranes with polycations which induce endocytosis and effect ADH-stimulated water flow [3]; or 3) inhibitors such as cytochalasin b or colchicine have been used to disrupt cellular processes involved in membrane turnover and their effects on water permeability and particle aggregates examined (for example *see* ref. [50]). A more direct approach has been developed recently by Coleman et al. [16] who modified the technique of label-fracture electron microscopy to obtain simultaneous visualization of the fluid-phase marker in a vesicle and the particle aggregates in that vesicle's limiting membrane. This technique should allow integration of data from the large number of studies using fluid phase tracers with information regarding the location of particle aggregates retrieved concurrently from the apical membrane.

Plasma membrane turnover is usually measured either by quantitation of the uptake of a fluid-phase marker from the solution on the exterior of the cell which marks the contents of vesicles formed at the plasma membrane, or by tracing the vesicular pathway taken by a specific membrane receptor which serves as a marker for the vesicle's membrane component. Both nonpolar (fibroblast and macrophage) [90] and polarized epithelia (hepatocyte) [18], internalize and recycle plasma membrane components at a rapid rate. Cultured fibroblasts, for example, internalize and recycle one-half of their entire surface area each hour [90]. In contrast, under basal conditions the apical plasma membrane of toad bladder granular cells internalize electron-dense [75, 86, 101], enzymatic [16, 33, 40, 69, 70, 77] and fluorescent [39] fluid-phase markers at an extremely low rate. Both fluorescent and thin-section electron microscopy show essentially no internalization of granular cell apical membrane over a 30-min period. It is possible that low rates of constitutive membrane internalization contribute to the low water permeability exhibited by toad bladder in its baseline state. The relationship between rates of apical membrane internalization and water permeability have not been explored in epithelia other than that of the toad bladder.

ADH elicits dramatic changes in apical membrane turnover in granular cells. Aggrephore fusion and apical membrane retrieval during ADH stimulation are dependent on several parameters, including the concentration of ADH, the duration of stimulation and the magnitude of transepithelial water flow. Figure 1 displays data of Ellis et al. [24] who simultaneously measured the number of aggrephore fusions, apical membrane particle aggregate area and water permeability in toad bladders stimulated for various intervals with a high concentration of ADH in the presence or absence of a 175 mOsm gradient. During the initial 10 min of ADH stimulation, water permeability, aggrephore fusion events and apical

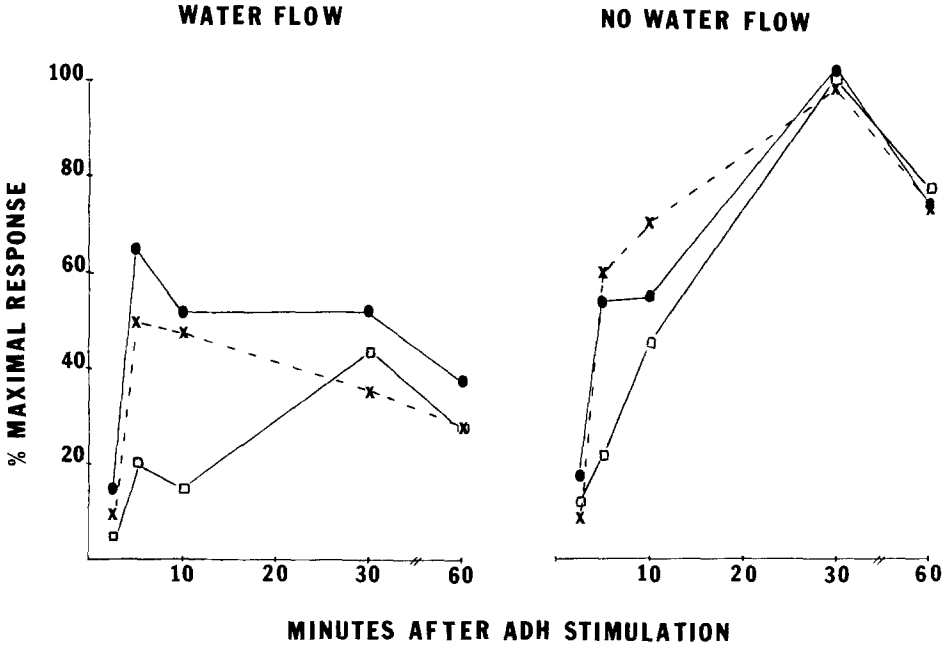

Fig. 1. Data from Ellis et al. [24] showing water permeability, apical membrane particle aggregate area, and the number of aggrephore fusion events in toad bladders stimulated for various intervals with ADH. The graph on the left displays values obtained in the presence of water flow (175 mOsm gradient), whereas the graph on the right shows those recorded without water flow (no gradient). All values are plotted as percent maximal response of the largest value which occurs at 30 min in the absence of water flow. Water permeability was measured after brief glutaraldehyde fixation of the bladder's apical surface [23] which fixed its water permeability state, then the fixative was removed and the permeability of all bladders measured with the same osmotic gradient. In both graphs the ×'s represent water permeability, the filled ○'s the area of apical membrane occupied by membrane particle aggregates and the □'s the number of aggrephore fusion events with the apical membrane expressed as percent maximal response

membrane aggregate area rose rapidly in the presence and absence of water flow. After 30 min of ADH stimulation, however, all values in bladders with water flow were approximately half those in the absence of water flow (no osmotic gradient). Calculation of additional membrane surface area added to the apical plasma membrane based on the observed number of aggrephore fusion events revealed an increase of approximately 8% in the presence of water flow as compared to a 25% increase in the absence of water flow (for calculations *see* ref. [78]). Both values are in close agreement with estimates for the change in apical membrane surface area based on capacitance measurements [78, 91].

Our recent studies [40] combined with those of others [16, 22, 23, 67, 76, 81] show that the decrease in aggrephore fusion events, apical membrane particle aggregates, and water permeability in bladders exposed to large osmotic gradients is due to water flow-stimulated retrieval of vesicles containing particle aggregates. When a large osmotic gradient is imposed, the water flow across the cell causes endocytosis of apical membrane which contains membrane particle aggregates and water permeability falls. To test the proposal that water flow → endocytosis → reduced water permeability, we induced maximal water permeability in a series of bladders by exposing them to a high concentration of ADH in the absence of an osmotic gradient. When high rates of water flow were initiated by imposition of an osmotic gradient, water permeability fell rapidly, and there was considerable endocytosis of apical fluid phase marker. When endocytosis was inhibited by chilling the bladder, the fall-off in permeability was also inhibited. Finally, the amount of fall-off in permeability and the amount of endocytosis were proportional to the magnitude of water flow (Fig. 2). The vesicles retrieved from the apical membrane in response to high rates of water flow contain particle aggregates in their limiting membrane [16]. Thus, regulation of ADH-elicited apical membrane water permeability includes a feedback loop in which high rates of water flow induce selective retrieval of apical membrane containing permeability units.

Heterogeneity in the Cellular Response to ADH

Under certain circumstances, there is considerable heterogeneity among granular cells in response to ADH. Chevalier et al. [15] observed that shortly after exposure to ADH, some granular cells had

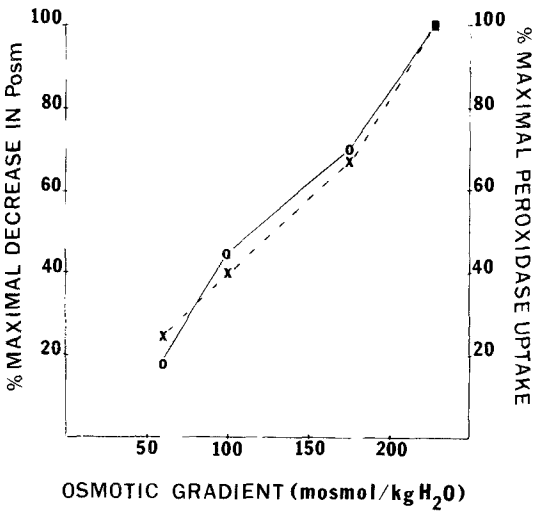

Fig. 2. The relationship between the decrease in ADH-elicited water permeability and apical membrane retrieval in toad urinary bladder. Toad urinary bladders were suspended in amphibian Ringer's solution and stimulated with 50 mU/ml of ADH for 15 min in the absence of a transepithelial osmotic gradient. The isotonic apical solutions were removed and replaced with hypotonic solutions of various osmolalities to establish a different osmotic gradient in each bladder. Osmotic water permeability (P_{osm}) was high immediately after application of an osmotic gradient but declined to different extents after the bladder had been exposed to water flow for 15 min. The difference (ΔP_{osm}) between permeability when first exposed to a gradient and 15 min later is shown as the open circles and plotted as percent maximal change in P_{osm}. Apical membrane retrieval was measured under identical conditions by addition of horseradish peroxidase (HRP) to the various hypotonic apical solutions. After the 15-min period of exposure to a gradient, the apical surfaces of the bladders were rinsed to remove adherent HRP, the epithelial cells were scraped from the bladder and their intracellular HRP content quantitated using a spectrophotometric assay. Peroxidase values (×'s) are expressed as percent of the maximal uptake. For further experimental details *see* reference [40].

many particle aggregates in their apical membrane while others in the same bladder had none. Heterogeneity would not be revealed in the standard morphometric analysis of toad bladder aggregate distribution, which is expressed per unit total area examined. We also observed considerable cellular heterogeneity in apical membrane retrieval in bladders incubated with low concentrations of ADH for brief periods (Fig. 3). Interestingly, freeze-fracture electron microscopy of collecting ducts from mice with hereditary nephrogenic diabetes insipidus shows that affected animals have a greatly reduced number of principal cells manifesting clusters of apical membrane particle aggregates in response to ADH [10]. However, those cells which do respond appear to have a full complement of membrane particle aggregates. Further study is needed to evaluate the role of cellular heterogeneity in determining the overall characteristics of water permeability response to ADH.

Strategy for Identification of Particle Aggregate Components Using Selective Retrieval of Apical Membrane

Until recently, there has been no information about the water-permeable components of the apical membrane in ADH-treated bladders that could lead to their isolation. High-affinity inhibitors or other agents that might specifically label the water-permeable membrane units have not been developed. Particle aggregates can only be identified by freeze-fracture electron microscopy, a complex assay as best. Nonetheless, it may be possible to identify membrane components or even the channel itself by iodinating membrane proteins under selected conditions.

We have taken advantage of the fact that apical membrane retrieval is minimal in the absence of water flow (no osmotic gradient) and that particle aggregates are present in large numbers in the permeable apical membrane of bladders stimulated with ADH and are absent in the water-impermeable membrane of unstimulated bladders. Therefore, apical membrane proteins labeled by membrane-impermeant reagents in ADH-stimulated bladders that are not labeled in control bladders are probably components of particle aggregates and/or aggrephores. We performed a series of lactoperoxidase (LPO)-catalyzed iodinations of the apical surface of ADH-stimulated and control bladders in the absence of apical membrane internalization. The resultant ^{125}I-labeled proteins were separated by SDS gel electrophoresis and detected by autoradiography. The autoradiograms revealed at least three bands of molecular weights, 55, 17-14 and 7 kD, which were present in ADH-treated bladders and not in controls [38].

As discussed above, modified label-fracture analysis [16] has shown that particle aggregates are retrieved from the apical membrane of ADH-stimulated bladders when ADH is removed. If the 55, 17-14 and 7 kD proteins identified by apical surface iodinations are components of particle aggregates and aggrephores, then iodination of the inner surface of vesicles retrieved from ADH-stimulated bladders should detect the same species. Selective iodination of the inner surface of these endocytic vesicles was accomplished by allowing them to endocytose a mixture of LPO, glucose oxidase (GO) and glucose [73]. After removal of all extracellular LPO, GO and glucose from the apical membrane, the cells were incubated with Na^{125}I, which diffuses across cell membranes to reach the interior of the

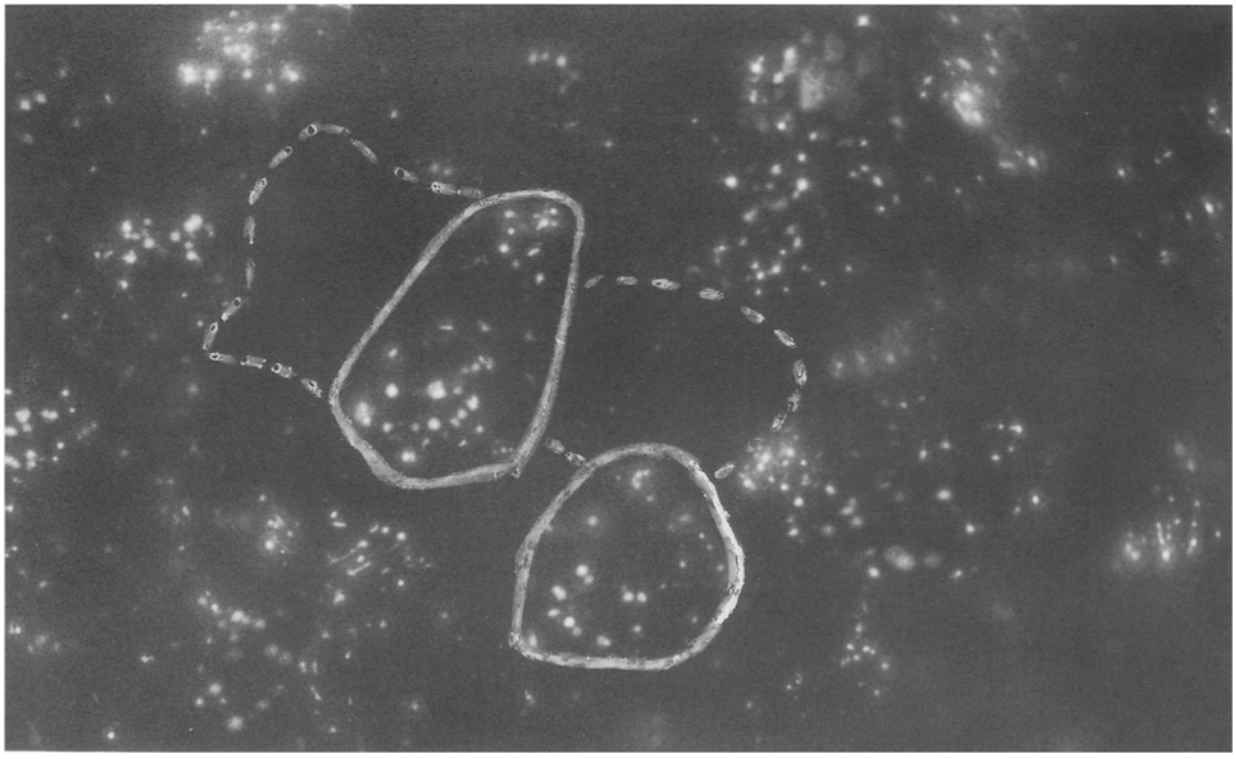

Fig. 3. Cellular heterogeneity of apical membrane retrieval during ADH stimulation. Toad bladders were suspended in amphibian Ringer's solutions so that their serosal surfaces were exposed to a 215 mOsm/kg solution and their apical surfaces were bathed in a 40 mOsm/kg solution to create a 175 mOsm transepithelial gradient. The hypotonic apical solution also contained fluorescein-conjugated dextran (F-dextran) at a concentration of 10 mg/ml. The bladders were stimulated with 2.5 mU/ml of ADH for 5 min. The apical solution was then rapidly removed and the apical surface rinsed to remove adherent F-dextran. Epifluorescence microscopy [39] was performed within 2 min after apical rinsing. This representative photomicrograph was obtained from a homogeneous field of granular cells which show great variability in F-dextran uptake. Examples of granular cells showing uptake are outlined by solid white lines; in contrast, others exhibit little or no F-dextran uptake and are outlined by broken white lines. Granular cell borders were determined by phase-contrast microscopy. Magnification 3200×

endocytic vesicles. Selective iodination is accomplished because only the interior of these endocytic vesicles contains the full complement of reagents necessary for LPO iodination of proteins. When we performed this protocol on bladders containing vesicles retrieved from the apical membrane when ADH was withdrawn, there was ^{125}I-labeling of proteins which have the same molecular weights as those found exclusively on the apical membrane of ADH-stimulated bladders [38].

Courtoy et al. [17] have developed the density-shift technique to isolate endocytic vesicles. Vesicles to be isolated are induced to endocytose horseradish peroxidase (HRP). Then cells are washed free of extracellular enzyme and homogenized. The homogenate is fractionated on a sucrose gradient where its contents are sedimented to their equilibrium densities. Endocytic vesicles containing the HRP form a band at their density along with other contaminating organelles. This fraction is then incubated with diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2), both of which are membrane permeant. In the presence of H_2O_2, HRP catalyzes the polymerization of DAB into a dense, membrane-impermeant polymer. This occurs only in the interior of vesicles containing HRP. After treatment, the fraction is placed on a second sucrose gradient of the same composition as the first, and is again sedimented to equilibrium. Contaminants again form a band at their original density, whereas the vesicles containing the polymerized DAB are now denser and form a band at a higher density within the sucrose gradient.

We have used the density-shift technique to purify apical membrane vesicles endocytosed when ADH is withdrawn. Using a combination of differential and density gradient centrifugation combined with the density-shift technique, we obtained a highly enriched vesicle fraction which contained HRP-loaded vesicles as visualized by electron mi-

croscopy [37]. SDS gel electrophoresis of the density-shifted fraction revealed that proteins of 55, 17, 15 and 7 kD were prominent members of the 20 species present. It is likely these proteins, present in retrieved aggrephores containing membrane particle aggregates, and also present in the apical membrane of ADH-treated bladders, but not in apical membranes of control bladders, are involved in the ADH-stimulated water permeability response. It is intriguing to note that ADH produces changes in the phosphorylation state of 17 and 15.5 kD proteins in intact toad bladders in which endogenous ATP was labeled by incubation with ^{32}P-orthophosphate [57].

Our strategy of apical surface and intracellular iodinations is designed to identify proteins that are components of aggrephores, and depends critically on the specificity of apical membrane turnover in response to ADH and to withdrawal of ADH. The evidence that ADH stimulates quantitative exocytosis of granules is controversial (*see above*) and there is no evidence that when ADH is withdrawn, the endocytic membrane contains retrieved granule membrane. In contrast, the evidence that aggrephore fusion into the apical membrane mediates the insertion of water channels, and that they are retrieved when ADH is withdrawn is, in our opinion, compelling. At present, however, we have no direct way to distinguish between proteins that are components of a water channel and those which have another function but participate in membrane turnover associated with the water permeability response because they are components of aggrephores. These uncertainties are further complicated by data demonstrating that the density-shifting technique may cross-link proteins which are contained within the lumen of the density-shifted vesicles [1]. Although it may be feasible to assay the water permeability of vesicle fractions derived from the apical membrane of toad bladder, a determination of exactly which vesicle components are responsible for water flux will be more difficult. The use of freeze-fracture electron microscopy on purified fractions is also problematic, for particle aggregates may be unstable structures [85].

Speculation

Based as it is on indirect evidence, our concept of the ADH-mediated water channel has changed several times since Koefoed-Johnsen and Ussing [56] first proposed that ADH increased water flow across cell membranes via water-filled pores. Currently, collective evidence from the toad bladder and mammalian collecting duct (recently reviewed by Finkelstein [27]) leads to the concept that water transport occurs through very narrow channels that have the following characteristics: 1) they exclude small nonelectrolytes as though the channels possess a radius of approximately 2 Å at its narrowest point where water flow may occur by single-file diffusion; 2) they exclude ions such as Na or K; and 3) they conduct protons [30].

How did the ADH-elicited, aggrephore-resident water channel evolve? Finkelstein [27] has contrasted it with the red blood cell water channel which has been intensively studied and notes that although both are extremely narrow pores, 4-chloromercuribenzenesulfonic acid (*p*CMBS) inhibits flow through red cell membranes, but does not inhibit flow through toad bladder membranes (R. Hays and M. Rubin, *unpublished observation* in reference [27]; H.W. Harris, *unpublished observation*). Several diverse facts about the ADH water channel may be unified by considering the hypothesis that the ADH channel may have evolved from some form of H^+-ATPase such as the class FoF1-ATPase. The FoF1-ATPase has been extensively characterized from both bacterial and eukaryotic sources. It is composed of a larger catalytic unit (F1) and a smaller membrane-spanning pore (Fo) [88]. The Fo complex forms a very narrow channel across the lipid bilayer and only allows the transit of water and protons. Maloney and Wilson [66] pointed out that in eukaryotes, the FoF1 pump is restricted to membranes with no permanent external disposition in the cell such as mitochondria and the vacuolar system of cells including coated vesicles, lysosomes and golgi. The pump that acidifies urine in the mammalian collecting duct [6, 31] and the toad and turtle urinary bladders [92] is an FoF1-ATPase. In mitochondria-rich and intercalated cells, it is packaged in the membrane of vesicles which fuse with the apical plasma membrane in response to cellular acidification [87]. Is the ADH water channel an Fo analogue in which the narrow and highly selective membrane-spanning channel serves primarily for conductance of water rather than protons? Thus the packaging of the ADH water channel in aggrephores might reflect its evolution from the FoF1-ATPase located in an intracellular vesicle. The Fo portion of the FoF1-ATPase is basically composed of three components with molecular weights of approximately 7-8, 15-17 and 30 kD. There may be structural analogy between these Fo proteins and those of 7 and 14-17 kD which we have identified in the toad bladder. This hypothesis is testable since antibodies to the FoF1-ATPase are available. Physiological similarities could also be examined. For example, proton translocation through FoF1 is inhibited by dicyclohexylcarbodiimide (DCCD). Does DCCD inhibit proton con-

ductance or water flow in ADH-treated bladders? Do turtle bladders become more permeable to water when acidifying vesicles have fused with the apical plasma membrane?

Concluding Remarks

Ultimately, we will have to identify, isolate and reconstitute the components of the water channel. The water-conducting proteins may prove to be the most difficult epithelial transporter to isolate. Progress in isolating other eukaryotic membrane transporters has been based on specific high-affinity blocking agents [13, 83, 84, 89], specific antibodies [2] and the techniques of molecular genetics [28]. Since none of the foregoing are available for the water channel, we have followed the lead of others [71, 99] and anticipate that isolation of the water channel will probably require the development of a large number of specific antibodies. Standard subcellular fractionation procedures coupled with the density-shifting technique have yielded highly purified aggrephores. Alternatively, implantation of exogenous proteins in the toad bladder apical membrane may facilitate isolation of aggrephores [68]. Antibodies should be raised against proteins such as those we have identified by selective iodination, and tested for co-localization with membrane particle aggregates and for alteration of water permeability. The development of an antibody to any aggrephore component will be extremely useful in a number of ways. These include: 1) as a tool for the affinity purification of particle aggregate-rich vesicles and proteins, similar to the purification performed on brain synaptic vesicles [72]; 2) as a probe to determine whether other ADH-responsive tissues contain similar proteins. This would be a particularly valuable probe for the mammalian collecting duct where aggrephore-like vesicles have not been identified. Similar experiments involving the use of antibodies prepared against vesicles of the *Narcine* electric organ defined distinct classes of vesicles in mammalian nerve terminals [46]; 3) as a specific marker to determine the extent of aggrephore/apical membrane recycling that occurs during ADH stimulation. Toward this goal, we have raised monoclonal antibodies using density-shift purified aggrephores as immunogen. Appropriate antibodies should provide the tools to link the large body of information summarized above with information about the molecular structure of components that are set in motion by ADH.

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