Functional Water Channels and Proton Pumps Are in Separate Populations of Endocytic Vesicles in Toad Bladder Granular Cells†

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ABSTRACT: Functional water channels are retrieved by endocytosis from the apical membrane of toad bladder granular cells in response to vasopressin [Shi, L.-B., & Verkman, A. S. (1989) *J. Gen. Physiol. 94*, 1101-1115]. To examine whether endocytic vesicles which contain the vasopressin-sensitive water channel fuse with acidic vesicles for entry into a lysosomal pathway, ATP-dependent acidification and osmotic water permeability were measured in endosomes from control bladders and bladders treated with vasopressin (VP) and/or phorbol myristate acetate (PMA). Endosomes were labeled with the fluid-phase markers 6-carboxyfluorescein or fluorescein–dextran. Osmotic water permeability (P_f) was measured by stopped-flow fluorescence quenching and proton ATPase activity by ATP-dependent, *N*-ethylmaleimide-inhibitable acidification. In a microsomal pellet, P_f was low (<0.002 cm/s, 20 °C) in labeled endocytic vesicles from control bladders but high (0.05–0.1 cm/s) in a subpopulation (50–70%) of vesicles from VP- and PMA-treated bladders. Following ATP addition, the average drop in pH was 0.1 (control), 0.3 (VP), and 0.2 (PMA) unit. Measurement of pH in individual endocytic vesicles by quantitative image analysis showed that <20% of vesicles from VP-treated bladders acidified by >0.5 pH unit. To examine whether water channels and proton pumps were present in the same endocytic vesicles, the pH of endosomes with high and low water permeability was measured from the effect of ATP on the amplitude of the fluorescence quenching signal in response to an osmotic gradient. ATP did not alter the pH of endosomes with high P_f but decreased by >0.3 unit the average pH of endosomes with low P_f. We conclude that endocytic vesicles from toad bladder granular cells that contain the vasopressin-sensitive water channel *selectively* do not contain functional proton pumps. The data suggest that trafficking of the vasopressin-sensitive water channel retrieved by endocytosis does not involve fusion with acidic intracellular vesicles.

There is strong morphological and functional evidence that regulation of toad bladder water permeability by vasopressin involves the trafficking of vesicles containing water channels between an intracellular location and the apical plasma membrane [for recent reviews, see Brown (1989), Handler (1988), and Verkman (1989)]. Although vasopressin-dependent exocytic fusion of aggrephores (Muller et al., 1980, 1984) and endocytic retrieval of functional water channels (Shi & Verkman, 1989; Shi et al., 1990a,b) have been demonstrated, little is known about the intracellular processing of water channels from endocytic vesicles to vesicles destined for exocytosis. It is not known whether endocytic vesicles enter a lysosomal pathway to undergo progressive acidification and cellular processing or whether they bypass the acidification pathway to remain intact for exocytic recycling at a later time. The mechanism of recycling without lysosomal fusion has been described for vesicles containing the transferrin receptor (Mellman et al., 1986).

We have recently developed functional assays for water permeability in endosomes from vasopressin-sensitive epithelia. In kidney collecting duct, vasopressin acting at a V_2 receptor induced the formation of endosomes with high water permeability (Verkman et al., 1988; Lencer et al., 1990a). Retrieval of water channels in kidney probably occurs by clathrin-mediated endocytosis (Brown & Orci, 1983; Strange et al., 1988); purified clathrin-coated vesicles from kidney contained water channels, whereas vesicles with identical appearance from brain did not (Verkman et al., 1989). In the toad urinary bladder, vasopressin induced the apical endocytosis of functional water channels (Shi & Verkman, 1989). Endocytic vesicles which contain the vasopressin-sensitive water channel had low urea and passive proton permeabilities (Shi et al., 1990a), indicating that the retrieval mechanism for water channels is highly selective.

We examine here whether endocytic vesicles which contain the vasopressin-sensitive water channel have functional ATP-dependent proton pumps that cause intravesicular acidification. It was found that vasopressin does induce the appearance of a population of endocytic vesicles from toad bladder granular cells which acidify; however, the water channels were present in a separate population of endocytic vesicles which did not acidify. The results provide support for a specialized recycling mechanism for the vasopressin-sensitive water channel in toad bladder.

Materials and Methods

Endosome Isolation. Dominican toads (*Bufo marinus*, National Reagents, Bridgeport, CT) were maintained on wet peat moss. Excised urinary hemibladders were mounted serosa side out and rinsed with toad Ringer solution (buffer A) containing 110 mM NaCl, 2.5 mM $NaHCO_3$, 3 mM KCl, 2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, and 5 mM glucose, pH 7.8 at 23 °C. Hemibladders were vigorously

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aerated and incubated for 30 min in buffer A in the presence of serosal vasopressin (Pitressin, Parke-Davis, Morris Plains, NJ; 0 or 50 milliunits/mL) and/or mucosal phorbol myristate acetate (PMA, 0 or 1 μM). Endosomes were labeled by replacement of the mucosal solution with an isosmolar solution of buffer A (containing PMA if present initially) containing 15 mM 6-carboxyfluorescein (6CF) or 25 mg/mL FITC–dextran (Sigma, 9 kDa, dialyzed against buffer A for >24 h) at pH 7.8. After a 10-min incubation, bladders were washed extensively with buffer B (100 mM mannitol and 5 mM potassium phosphate, pH 8.5) at 0–2 °C, and cells were scraped with a glass slide. Subsequent procedures were performed at 0–2 °C.

To remove externally bound fluorescein, cells were suspended in 30 mL/bladder of buffer B and pelleted at 100g for 3 min. The pellet was homogenized with a Dounce homogenizer (15 strokes) and a Potter–Elvehjem homogenizer (4 strokes) in buffers C (100 mM KCl, 2 mM $MgCl_2$, and 5 mM potassium phosphate) or D (30 mM mannitol, 20 mM KCl, 2 mM $MgCl_2$, and 5 mM potassium phosphate). The pH of buffers C and D was 7.5 for FITC–dextran labeling and 8.5 for 6CF labeling. The pH of the final wash buffer was always 7.5. A heavy pellet (P_1) was obtained by centrifugation at 1000g for 10 min. A microsomal pellet (P_2) containing the majority of labeled endosomes was obtained by centrifugation of the supernatant at 100000g for 30 min. A very light pellet (P_3) was obtained by centrifugation of the remaining supernatant at 200000g for 2 h. In addition, the final supernatant (S_3) was saved. Pellets were washed once with >50 volumes of buffers C or D, homogenized by passage through a 23-gauge steel needle, and used within 1 h. Protein concentration was measured by the Bradford assay, and total entrapped 6CF or FITC–dextran was measured by steady-state fluorescence intensity (excitation 480 nm, emission 520 nm) in the presence of a purified polyclonal anti-fluorescein antibody (anti-FITC; Molecular Probes, Junction City, OR) to quench external fluorescence.

ATP-Dependent Acidification. Pellets (0.1–0.2 mg of protein/mL) were suspended in buffers C or D at pH 7.5 and stirred continuously at 23 °C in an SLM 48000 fluorometer. Anti-FITC was added to eliminate external fluorescence; further addition of anti-FITC at the end of the experiment caused no signal change. In some experiments, valinomycin (5 μM) or *N*-ethylmaleimide (NEM, 0.5 mM) was present. Fluorescence was excited at 490 ± 4 nm and detected at >515 nm using an OG515 cut-on filter (Schott Glass, Duryea, PA). Data were averaged over 1-s time intervals.

To initiate inward proton pumping, ATP (0–2 mM, 100 mM stock titrated to pH 7.5) was added. After the internal pH was stabilized (5–10 min), nigericin (5 μM) was added to collapse the pH gradient to demonstrate that the decrease in fluorescence was due to internal acidification. One hundred microliters of 1 M HCl was added at the end of the experiment to eliminate the fluorescein fluorescence to give background signal (<5% of maximal signal). The average pH was determined by using the fluorescence vs pH relation and a two-point calibration (initial and final fluorescence) as described previously (Ye et al., 1989).

Osmotic Water Permeability. Osmotic water permeability was measured by a stopped-flow fluorescence quenching technique (Chen et al., 1988). Pellets were suspended in buffer D to a final concentration of 0.2–0.5 mg of protein/mL and subjected to a 100 mM inwardly directed sucrose gradient in a Hi-Tech SF-51 stopped-flow apparatus (Wiltshire, England). In some experiments, 2 mM ATP and/or 5 μM nigericin were present. In some experiments, external pH was decreased to 6.0 at specified times before the stopped-flow measurement.

The sucrose gradient caused osmotic water efflux and a decrease in 6CF fluorescence due to endosome shrinkage and fluorescence self-quenching. Fluorescence was excited at 490 ± 5 nm and detected by a photomultiplier through an OG515 cut-on filter. Intensities were acquired at a maximum rate of 5 points/ms with an instrument dead time of 1.7 ms and an electronic response time of 0.1 ms. Sample temperature was maintained at 23 °C by a circulating water bath. Each kinetic experiments (512 data points) was repeated 5–10 times for signal averaging prior to curve fitting. Osmotic water permeability coefficients (P_f) (cm/s) were calculated from time constants of fitted exponential functions by using an endosome surface-to-volume ratio of 6×10^5 cm^{-1} (Shi & Verkman, 1989).

pH Measurement in Individual Endocytic Vesicles. The fluorescence of individual endosomes was measured by quantitative imaging microscopy as described previously (Shi et al., 1991); 18-mm round glass coverslips were coated with 0.02 mg/mL poly(L-lysine) and washed with buffer C. Vesicles were immobilized on the coated coverslip by incubation with a 0.05–0.1 mg of protein/mL vesicle suspension for 15 min. The coverslip was washed and mounted in a perfusion chamber. The fluorescence was imaged by a microchannel plate intensifier and CCD camera (Videoscope, Washington, DC) through a Nikon 100× fluortar objective (oil immersion, N.A. 1.30, 0.17-mm working distance) in an inverted epifluorescence microscope (Diaphot, Nikon). Image analysis hardware and software were described by Shi et al. (1991).

For measurement of ATP-dependent acidification, 6CF-labeled endosomes were excited at 490 ± 5 nm using a six-cavity interference filter (Omega Optical, Brattleboro, VT) and 510-nm dichroic mirror, and detected through an OG515 cut-on filter. In double-label studies (Lencer et al., 1990b) with 6CF and rhodamine–dextran (9 kDa), vesicles were imaged with FITC (excitation 460 nm, dichroic 510 nm, emission 520 nm) and rhodamine (excitation 560 nm, dichroic 580 nm, emission >595 nm) filter sets.

Results

ATP-Dependent Acidification in Endocytic Vesicles. It was first determined whether fluid-phase markers of apical membrane endocytosis in toad bladder were detectable in acidic membrane compartments. Endocytic vesicles were labeled by incubation of bladders with mucosal 6CF or FITC–dextran and fractionated as described under Materials and Methods. Intravesicular acidification was measured from the time course of fluorescence in response to ATP addition; anti-FITC was present throughout the experiment to eliminate fluorescence from the external solution.

Figure 1 shows that ATP-dependent acidification was measurable in fluorescently labeled vesicles prepared from vasopressin-treated bladders. ATP caused a time-dependent decrease in fluorescence which was reversed by collapse of the pH gradient with the K/H ionophore nigericin. The decrease in fluorescence was blocked by NEM (0.5 mM), but not by vanadate (0.5 mM) or oligomycin (10 μM) (not shown). These results indicate the presence of a vacuolar-type proton ATPase (Forgac, 1989) which pumped protons inwardly. The proton ATPase was detected in labeled vesicles in the low-speed and microsomal pellets and using the fluorophores 6CF or FITC–dextran.

Further experiments were carried out to determine the membrane origin of the ATP-dependent acidification in the

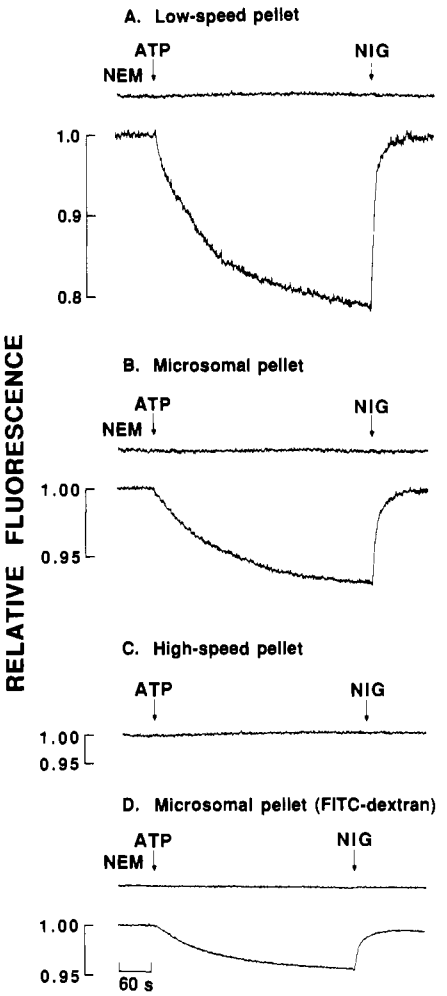

FIGURE 1: ATP-dependent acidification in endocytic vesicle fractions labeled with 6CF (A–C) or FITC–dextran (D). Bladders treated with serosal vasopressin (50 milliunits/mL) were labeled from the mucosa with fluorescent markers for 10 min as described under Materials and Methods. Fluorescence was monitored in a cuvette containing the labeled endocytic vesicles in buffer C. Valinomycin was present throughout the experiment. Where indicated, ATP (2 mM) and nigericin (5 μM) were added. When present, NEM (0.5 mM) was incubated with vesicles for 1 min prior to ATP addition. Results of a series of measurements are summarized in Table I.

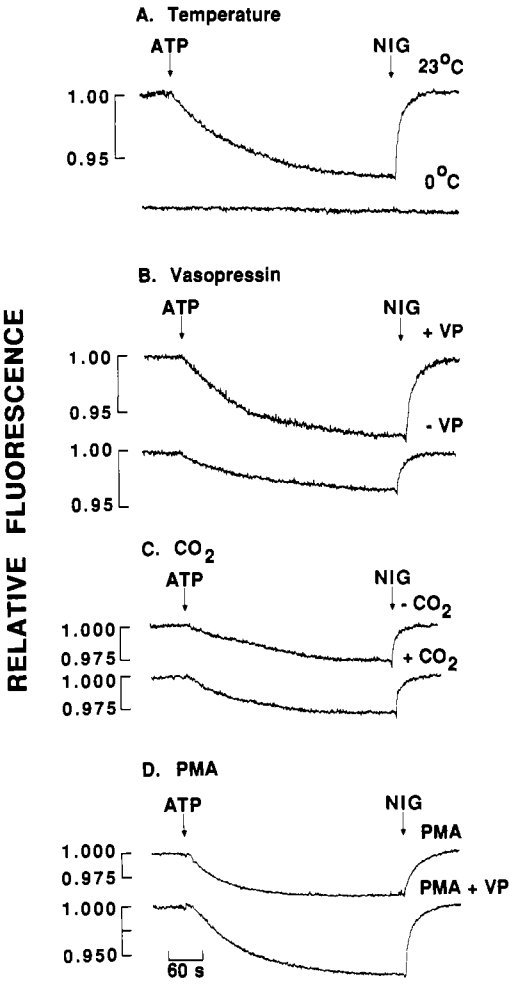

FIGURE 2: Cellular origin of ATP-dependent acidification in microsomal vesicles prepared from bladders labeled with mucosal 6CF for 10 min. Each set of data is from paired hemibladders; results of a series of paired studies are summarized in Table I. (A) Bladders were incubated with serosal vasopressin (50 milliunits/mL) for 30 min at 23 °C and labeled with 6CF for 10 min at 23 or 0 °C. (B) Bladders were incubated with 0 (–VP) or 50 milliunits/mL (+VP) vasopressin for 30 min prior to and during mucosal labeling. (C) Bladders were incubated with buffer A in which 40 mM NaCl was replaced by $NaHCO_3$ and bubbled with 5% CO_2 (pH 7.8) for 30 min. The mucosa was labeled with 6CF for 10 min after removal of CO_2/HCO_3. (D) Bladders were incubated for 30 min before and during mucosal labeling with mucosal PMA (1 μM) with or without serosal vasopressin (50 milliunits/mL).

microsomal pellet (Figure 2). Acidification was absent when endocytosis was inhibited (Shi & Verkman, 1989; Harris et al., 1986) by labeling the bladder at 0 °C instead of at 23 °C. The toad bladder contains both granular and mitochondrial-rich (MR) cells. Granular cells make up >85% of the apical membrane surface in the bladders of Dominican toads and are responsible for the vasopressin hydroosmotic response, whereas MR cells cause urinary acidification and are vasopressin insensitive (Anderson et al., 1985).

Figure 2B shows that acidification was remarkably increased when bladders were treated with serosal vasopressin before and at the time of mucosal labeling. However, acidification was not increased by a CO_2 withdrawal maneuver reported to increase endocytosis in MR cells from turtle bladder (Gluck, 1982). Endosomes were labeled in bladders in which CO_2 was first present and then withdrawn rapidly (Figure 2C, "+CO_2"). The lack of increased acidification suggests that few labeled endosomes originate from MR cells. Lastly, activation of granular cell protein kinase C by mucosal PMA is known to increase bladder water permeability, stimulate granule and aggrephore exocytosis, and increase endocytic retrieval of water channels (Masur et al., 1985; Shi et al., 1990b). PMA alone increased acidification, whereas the PMA and vasopressin effects were not additive (Figure 2D). Taken together, these data indicate that the observed ATP-dependent acidification was in a population of apical endocytic vesicles derived from toad bladder granular cells.

Results of a series of paired experiments are summarized in Table I. The majority of membrane protein and entrapped fluorescent label was found in the low-speed and microsomal pellets. Acidification in these fractions was dependent upon vasopressin and PMA, but not on CO_2. As given below, rapid water transport was present in the microsomal pellet. It is important to note that the fractional decrease in fluorescence in response to ATP addition represents an average pH which is dependent upon the pH of individual vesicles and on the number of vesicles which acidify.

To examine ATP-dependent acidification in individual endosomes, we used a quantitative imaging technique developed recently (Shi et al., 1991). Microsomal vesicles were immobilized on a poly(L-lysine)-coated coverslip, mounted in a

Table I[a]

(A) ATP-Dependent Acidification and Water Transport in Vesicle Fractions

fraction	total protein/ bladder (mg)	rel entrapped fluores-cence	av pH after ATP	rel signal from vesicles with water channels
P_1 (low-speed)	0.8 ± 0.1	0.6	6.8	<0.1
P_2 (microsomal)	1.4 ± 0.1	1.0	7.2	1.0
P_3 (high-speed)	0.15 ± 0.06	0.12	7.5	0.2
S_3 (supernatant)	1.3 ± 0.1	<0.02		<0.1

(B) Influence of Bladder Pretreatment on ATP-Dependent Acidification

	microsomal fraction		low-speed fraction	
paired comparison	rel signal[b]	av pH	rel signal	av pH
vasopressin				
+VP	1	7.2	1	6.8
-VP	0.26 ± 0.02	7.4	0.36 ± 0.04	7.2
CO_2 withdrawal				
$+CO_2$	1	7.4	1	7.3
$-CO_2$	1.03 ± 0.03	7.4	0.96 ± 0.06	7.3
PMA effect (-VP)				
+PMA	1	7.3	1	7.1
-PMA	0.46 ± 0.08	7.4	0.44 ± 0.06	7.2
VP effect (+PMA)				
-VP	1	7.3	1	7.1
+VP	0.55 ± 0.1	7.1	0.45 ± 0.05	6.9

[a] Data are mean ± SEM from three to five separate sets of experiments. Average pH following ATP addition was determined from the fluorescence intensity and the 6CF fluorescence vs pH calibration relation. Data given in the lower half of the table are from paired hemibladder studies. [b] Relative signal is defined as the ATP-dependent drop in fluorescence relative to a control condition.

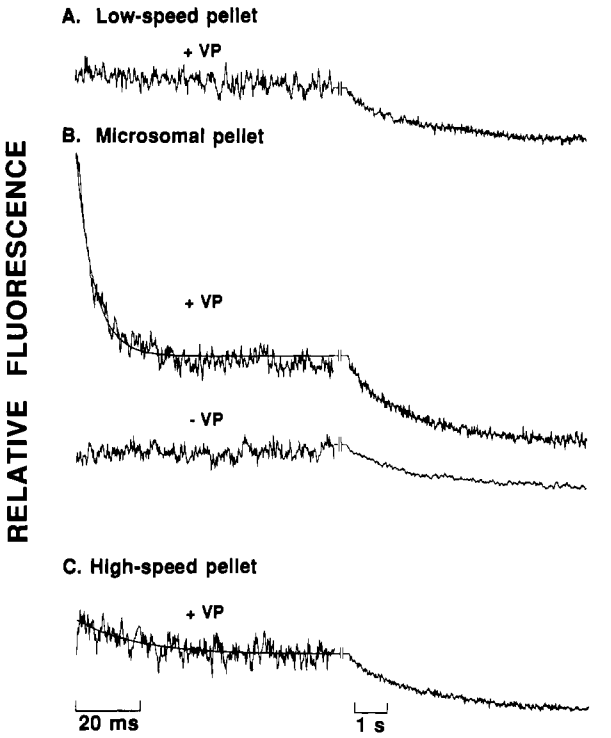

FIGURE 3: Osmotic water transport in endocytic vesicles fractions. Bladders treated or not treated with serosal vasopressin (50 milliunits/mL) were labeled with mucosal 6CF for 10 min. Vesicle fractions in buffer D were subjected to a 100 mM inwardly directed sucrose gradient in the stopped-flow apparatus. The fluorescence signal in the first 50 ms was expanded to show rapid water transport in a subpopulation of vesicles. Each curve is the average of 5–10 serial measurements. Single-exponential fitted curves are shown. Results from a series of measurements are summarized in Table I.

perfusion chamber, and imaged at high magnification. Endosome pH was determined from fluorescein fluorescence measured before and after perfusion with buffer C containing 2 mM ATP, and after perfusion with buffer C containing 5 μM nigericin. In 160 labeled vesicles examined, 82% were insensitive to ATP, and 12% responded to ATP by a >25% decrease in fluorescence, corresponding to a >0.5 pH unit acidification. These results indicate that the averaged ATP-dependent acidification measured by cuvette fluorometry was due to strong acidification in a subpopulation of vesicles. Experiments were carried out below to measure osmotic water permeability in this subpopulation.

A potential concern in the use of 6CF as a fluid-phase marker of endocytosis is 6CF leakage from vesicles in intact bladder cells prior to cell scraping and homogenization at pH 8.5. Control studies were performed to determine whether significant leakage occurred. Direct examination of cells in intact bladders by confocal microscopy after 15 min of labeling showed well-demarcated endocytic vesicles without background haze. Increasing haze was observed after 30–60 min due to 6CF leakage and dispersal in the cell cytoplasm. In addition, there appeared after 15–30 min larger labeled vesicles probably corresponding to multivesicular bodies. To demonstrate that 6CF colocalized to endocytic vesicles containing entrapped dextran, bladders were colabeled with 6CF and rhodamine–dextran as described under Materials and Methods. Endocytic vesicles in the microsomal pellet were immobilized on a coated coverslip and imaged by using FITC and rhodamine filter sets as described by Lencer et al. (1990b). In 100 labeled vesicles examined, every vesicle labeled with rhodamine–dextran contained 6CF; there were no vesicles that contained 6CF alone. It was shown in separate experiments that 6CF (or rhodamine)-labeled vesicles were not visible using the rhodamine (or FITC) filter sets. Taken together with previous results (Shi & Verkman, 1989; Shi et al., 1990a), these data support the conclusion that 6CF is a valid marker of endocytosis as used in the present studies.

Do Functional Proton Pumps and Water Channels Colocalize? Figure 3 shows the time course of 6CF fluorescence in response to a 100 mM inwardly directed sucrose gradient. The osmotic gradient induced water efflux and a decrease in vesicle volume resulting in fluorescence self-quenching due to an increased concentration of entrapped fluorophore. In the low-speed pellet (Figure 3A), there was a slow decrease in fluorescence corresponding to water efflux in vesicles with low osmotic water permeability (P_f < 0.002 cm/s). In the microsomal pellet from vasopressin-treated bladders (Figure 3B), there was a very fast decrease in fluorescence with $P_f \sim 0.1$ cm/s; rapid water permeability was not observed in the absence of vasopressin, or as shown previously (Shi & Verkman, 1989; Shi et al., 1990a), when endocytosis was turned off by low temperature, azide addition, and gluteraldehyde fixation of the mucosal membrane. The rapid water permeability has been shown to arise from endocytic retrieval of vasopressin-sensitive water channels from the apical membrane of bladder granular cells. The microsomal fraction also contained a population of endocytic vesicles with low water permeability. A very small signal with high water permeability was measurable in the high-speed fraction (Figure 3C). The results of a series of measurements on vesicle fractions are summarized in Table I. Importantly, the microsomal fraction contained some labeled vesicles with proton pumps and some (possibly the same vesicles) with water channels.

To determine whether endocytic vesicles containing the vasopressin-sensitive water channel acidified in response to ATP, the pH of endosomes with high P_f was measured by a

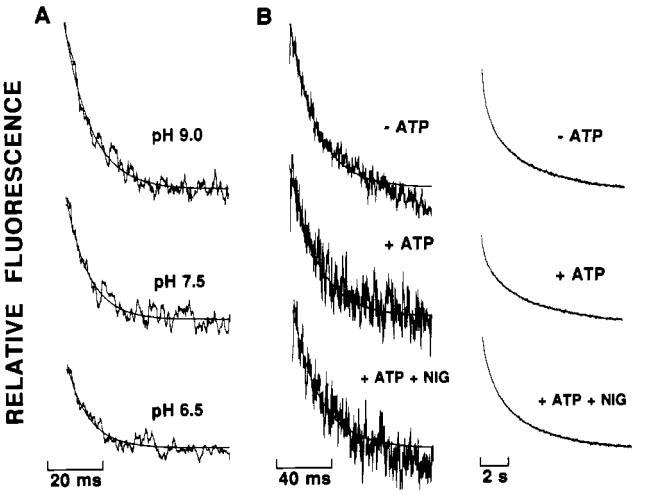

FIGURE 4: Measurement of ATP-dependent acidification in endocytic vesicles with high and low osmotic water permeability. Vesicles from the microsomal fraction were labeled with 6CF in vasopressin-treated bladders. (A) Vesicles in buffer C at pH values indicated were subjected to a 100 mM inwardly directed sucrose gradient. (B) Vesicles in buffer D containing valinomycin at pH 7.5 were subjected to a 100 mM sucrose gradient prior to ATP addition (−ATP), 3 min after addition of 2 mM ATP (+ATP), and immediately after addition of 5 μM nigericin to the suspension containing vesicles and ATP (+ATP + NIG). The y scale is the same for all curves to permit direct comparison. In three sets of experiments, relative signal amplitudes (relative preexponential factor for exponential data fits, 0–50 ms for the fast component and 0.2–5 s for the slow component) were 1.0 (−ATP), 0.98 ± 0.04 (+ATP), and 0.98 ± 0.03 (+ATP + NIG) (fast component, left) and 1.0 (−ATP), 0.78 ± 0.06 (+ATP), and 1.03 ± 0.05 (+ATP + NIG) (slow component, right).

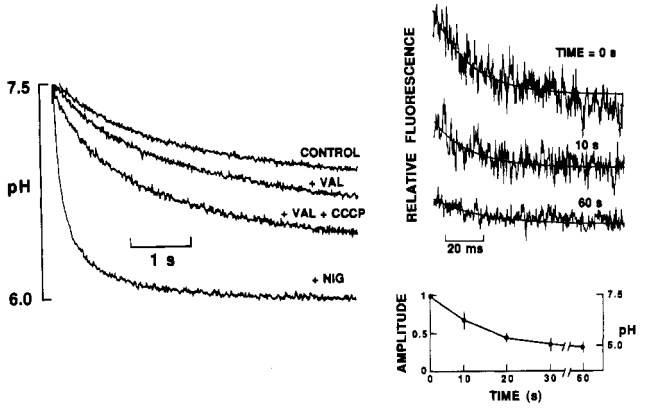

FIGURE 5: Passive proton permeability in 6CF-labeled endocytic vesicles containing the vasopressin-sensitive water channel. (Left) Microsomal vesicles in buffer C at pH 7.5 were subjected to a 1.5 pH unit inwardly directed proton gradient in the stopped-flow apparatus. Where indicated, valinomycin (10 μg/mg of protein), CCCP (10 μM), and nigericin (5 μM) were added. (Right, top). Vesicles were subjected to a 1.5 pH unit inwardly directed proton gradient at the different indicated times before osmotic water efflux was induced by a 100 mM sucrose gradient. The decrease in fluorescence was due to osmotic water efflux; curve amplitudes provide a direct measure of internal pH at the time of water efflux (see text). (Right, bottom). Curve amplitudes (mean ± SE) from five sets of measurements as a function of time between exposure to the pH gradient and the osmotic gradient.

stopped-flow fluorescence technique (Ye et al., 1989). Figure 4A validates the principle of the method. Endocytic vesicles at different internal pH were subjected to a 100 mM inwardly directed sucrose gradient. There was a rapid decrease in the fluorescence signal with similar time course; however, the amplitude of the signal paralleled the 6CF fluorescence vs pH titration curve. At pH 6.5, the signal amplitude was 53 ± 3% of that at pH 9.0, in agreement with the measured pK_a of 6.4 for 6CF. Therefore, the measurement of signal amplitude provides an approach to resolve the internal pH of populations of endocytic vesicles with different water permeabilities. For each component of water permeability, the ratio of single amplitudes before and after ATP addition (or the amplitude after nigericin addition compared with that before nigericin addition) provides a quantitative measure of vesicle pH. If there are vesicles with both high and low water permeabilities present in the sample, the ATP-dependent pH response of each type of vesicle can thus be measured separately.

Figure 4B shows the fast and slow time course of 6CF fluorescence in microsomal vesicles prepared from vasopressin-treated bladders. ATP addition did not alter the amplitude of the fast decrease in fluorescence corresponding to labeled endocytic vesicles containing the vasopressin-sensitive water channel. ATP addition decreased remarkably the amplitude of the slow decrease in fluorescence corresponding to labeled vesicles with low water permeability; the decrease in signal amplitude was reversed by nigericin. In three separate sets of measurements performed at an initial pH of 7.5, the pH following ATP addition was 7.5 ± 0.1 (SE) in vesicles with high P_f and 6.8 ± 0.2 in vesicles with low P_f. These results indicate that endocytic vesicles which contain water channels do not acidify in response to ATP.

If endocytic vesicles with high water permeability do not contain an extraordinarily high proton leak, the lack of ATP-dependent acidification indicates the lack of a functional proton pump. It was reported that vesicles containing the vasopressin-sensitive water channel had a passive proton permeability (P_H) of 0.05 cm/s that was not different from the P_H measured in endocytic vesicles with low water permeability (Shi et al., 1990a). Additional control experiments were performed in 6CF-labeled endocytic vesicles with buffers used in the present study to validate this important conclusion. Figure 5 (left) shows the time course of vesicle acidification following a 1.5 pH unit inwardly directed proton gradient. In endocytic vesicles prepared from vasopressin-treated bladders, P_H in the absence of valinomycin (0.047 cm/s) increased to 0.056 cm/s in the presence of a saturating concentration of valinomycin (10 μg/mg of vesicle protein). The absolute pH scale was determined by addition of nigericin to collapse the pH gradient rapidly as reported previously (Shi et al., 1990a). To demonstrate the adequacy of the voltage clamp, addition of the electrogenic protonophore CCCP resulted in a 2.1-fold increase in the rate at which the pH gradient collapsed. These results indicate that the methodology was capable of detecting a population of vesicles with very high P_H.

Additional experiments were carried out to measure passive proton permeability directly in the population of endocytic vesicles with high P_f. Vesicles initially at pH 7.5 were exposed to a 1.5 pH unit inwardly directed H gradient, allowed to equilibrate partially, and then exposed to a 100 mM inwardly directed sucrose gradient (Figure 5, right). The signal amplitude decreased with increasing time after exposure to the the pH gradient. By the same principle applied in Figure 4, the amplitude of the stopped-flow signal provided a measure of the pH in labeled vesicles with high and low P_f. Averaged signal amplitudes from five sets of measurements are shown in Figure 5 (right, bottom). The signal amplitude decreased by 50% after a ~10-s equilibration time, in agreement with the data in Figure 5, left. Therefore, the passive proton permeability of endocytic vesicles containing the vasopressin-sensitive water channel was not higher than that reported by Shi et al. (1990a) in vesicles with low P_f that demonstrate

ATP-dependent acidification. Taken together, these results show that a high passive proton permeability does not account for the lack of ATP-dependent acidification in endocytic vesicles with high P_f.

Discussion

The granular cell of toad urinary bladder is the target for the hydroosmotic action of vasopressin. There is a considerable body of evidence that the cellular mechanism for this action is the exocytosis and endocytosis of membrane patches containing functional water channels (Brown, 1989; Handler, 1988; Verkman, 1989). The water permeability of individual water channels is probably not subject to physiological regulation (Shi et al., 1990b). The maximal water permeability of endocytic vesicles formed in response to vasopressin action is extremely high (>0.1 cm/s); it has been estimated that >5 % of the vesicle membrane surface is dedicated to water transport (Shi et al., 1990a). In recent studies, mRNA encoding water channels from toad bladder and kidney medulla expressed functional water channels in Xenopus oocytes (Zhang et al., 1990; Zhang & Verkman, 1991), suggesting that the vasopressin-sensitive water channel is a distinct protein. The purpose of the study reported here was to examine whether the large number of water channels retrieved from the apical membrane and packaged into endocytic vesicles enter an intracellular pathway of progressive acidification and processing.

In response to vasopressin, we found that a fraction of the endocytic marker was sorted into vesicles containing ATP-dependent proton pumps; however, vesicles with high water permeability did not acidify in response to ATP. It cannot be determined from the data presented whether endocytosed label in vesicles which acidify passed through vesicles containing water channels. In each subcellular fraction examined, no individual vesicles were found to contain both water channels and functional proton pumps. This finding suggests a conservative mechanism for the intracellular trafficking of the vasopressin-sensitive water channel. Water channels in endocytic vesicles may not enter the acidification pathway leading to lysosomal degradation. It is proposed that the water channels remain in vesicles that undergo little processing prior to reinsertion into the apical membrane in response to a subsequent stimulation by vasopressin. These results are in agreement with preliminary data in toad bladder of Franki et al. (1988), showing that the morphological marker of acidic vesicles, DAMP, was absent from vasopressin-induced endocytic vesicles labeled with horseradish peroxidase. A similar conclusion was reported in the kidney collecting tubule (Lencer et al., 1990b), where vasopressin-induced endosomes did not measurably transfer their fluorescent label to acidic vesicles, nor did vesicles labeled with FITC–dextran stain with the rhodamine-labeled antilysosomal antibody LGP-120.

We found that vasopressin-induced endocytic vesicles from toad bladder granular cells transferred their fluorescent label (6CF or FITC–dextran) to acidic intracellular vesicles which contain an NEM-inhibitable proton pump, in agreement with results in a number of other cell systems (Galloway et al., 1983; Okuma et al., 1982; Yamashiro et al., 1987; Ye et al., 1989). However, to determine whether vesicles containing water channels acidified, it was necessary to measure the pH, after ATP addition, of vesicles with high and low water permeability. This was accomplished by making use of the concentration and pH dependences of entrapped 6CF. Vesicles containing 6CF with very high water permeability did not acidify, whereas those with low water permeability acidified. The assay to resolve the pH in separate vesicle populations was developed for studies of water channel and proton pump localization in endosomes from mammalian proximal tubule (Ye et al., 1989). In proximal tubule, >95% of endosomes which contained water channels acidified in response to ATP, a conclusion opposite to that in the toad bladder endosomes. In proximal tubule endosomes, the kinetics of acidification were regulated in part by phosphorylation of a chloride channel which provided an electrical shunt to minimize the positive interior membrane potential due to inward proton pumping (Bae & Verkman, 1990). In the experiments reported here, the presence of potassium and valinomycin ensured that ATP-dependent acidification was not blocked by the rate-limiting movement of a counterion.

The ability to detect a functional proton pump requires that the ATP-dependent pH gradient not be dissipated by a high passive proton permeability. Direct measurements of the passive proton permeability (P_H) of vasopressin-induced endocytic vesicles gave a relatively low P_H, similar to that measured in endosomes not containing water channels from toad bladder, and endosomes containing water channels and proton pumps from mammalian proximal tubule (Ye et al., 1989). To demonstrate slow proton permeation in labeled endocytic vesicles which contain the vasopressin-sensitive water channel, the stopped-flow assay was used to measure P_H in vesicles with high water permeability. It was shown that a pH gradient dissipated slowly in endocytic vesicles with high water permeability, supporting the conclusions that (a) the vasopressin-sensitive water channel is not a conduit for protons (Shi et al., 1990a) and (b) the lack of ATP-dependent acidification in endocytic vesicles containing the water channel was due to the absence of functional proton pumps rather than to a high passive proton permeability. It is unlikely, though possible, that nonfunctional proton pumps are present in the nonacidifying endosomes containing water channels.

In summary, our results show that the vasopressin-sensitive water channel is present in endocytic vesicles in toad bladder granular cells that do not acidify in response to ATP. Other vesicles with low water permeability from the same cells do acidify and receive a measurable fraction of the fluorescent endocytic marker. The specialized trafficking mechanism for the vasopressin-sensitive water channel may be important for the efficient recycling of water channels without extensive sorting and processing. Efficient recycling of water channels without degradation is particularly important because of the large number of water channels that are retrieved continuously, even during the sustained action of vasopressin. Further studies are required to define the exact step(s) between water channel endocytosis and reinsertion into the apical membrane.

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Registry No. ATP, 56-65-5; ATPase, 9000-83-3; H^+, 12408-02-5; pitressin, 113-79-1; water, 7732-18-5.

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Solubilization and Localization of Weakly Polar Lipids in Unsonicated Egg Phosphatidylcholine: A ^{13}C MAS NMR Study†

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ABSTRACT: The weakly polar lipids cholesteryl ester, triacylglycerol, and diacylglycerol incorporate to a limited extent into the lamellar structure of small unilamellar vesicles. The localization of the carbonyl group(s) at the aqueous interface was detected by [^{13}C]carbonyl chemical shift changes relative to the neat unhydrated lipid [Hamilton, J. A., & Small, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6878-6882; Hamilton, J. A., & Small, D. M. (1982) *J. Biol. Chem. 257*, 7318-7321; Hamilton, J. A., Bhamidipati, S. B., Kodali, D. R., & Small, D. M. (1991) *J. Biol. Chem. 266*, 1177-1186]. This study uses ^{13}C NMR to investigate the interactions of these lipids with unsonicated (multilamellar) phosphatidylcholine, a model system for cellular membranes and surfaces of emulsion particles with low curvature. Magic angle spinning reduced the broad lines of the unsonicated dispersions to narrow lines comparable to those from sonicated dispersions. [^{13}C]Carbonyl chemical shifts revealed incorporation of the three lipids into the lamellar structure of the unsonicated phospholipids and a partial hydration of the carbonyl groups similar to that observed in small vesicles. Other properties of interfacial weakly polar lipids in multilayers were similar to those in small unilamellar bilayers. There is thus a general tendency of weakly polar lipids to incorporate at least to a small extent into the lamellar structure of phospholipids and take on interfacial properties that are distinct from their bulk-phase properties. This pool of surface-located lipid is likely to be directly involved in enzymatic transformations and protein-mediated transport. The ^{13}C magic angle spinning NMR method may be generally useful for determining the orientation of molecules in model membranes.

Cholesteryl esters (CE),[1] diacylglycerols (DAG), and triacylglycerols (TAG) are weakly polar water-insoluble lipids that form stable monolayers at an air–water interface but do not swell in water to form lamellar structures (Small, 1986). The esterified form of cholesterol is a key chemical component involved in the transport and metabolism of cholesterol. A structural derivative that decreases the polarity of unesterified cholesterol, CE can be sequestered into the core of plasma

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[1] Abbreviations: CE, cholesteryl ester; DAG, diacylglycerol; TAG, triacylglycerol; MAS, magic angle spinning; PC, phosphatidylcholine; TO, triolein; CO, cholesteryl oleate; DPG, dipalmitoyl-*sn*-glycerol; NOE, nuclear Overhauser enhancement; T_1, spin-lattice relaxation time.

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