Water, Proton, and Urea Transport in Toad Bladder Endosomes That Contain the Vasopressin-sensitive Water Channel

LAN-BO SHI, D. BROWN, and A. S. VERKMAN

From the Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California 94143; and the Renal Unit, Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT   Vasopressin (VP) increases the water permeability of the toad urinary bladder epithelium by inducing the cycling of vesicles containing water channels to and from the apical membrane of granular cells. In this study, we have measured several functional characteristics of the endosomal vesicles that participate in this biological response to hormonal stimulation. The water, proton, and urea permeabilities of endosomes labeled in the intact bladder with fluorescent fluid-phase markers were measured. The diameter of isolated endosomes labeled with horseradish peroxidase was 90–120 nm. Osmotic water permeability (P_f) was measured by a stopped-flow fluorescence quenching assay (Shi, L.-B., and A. S. Verkman. 1989. *J. Gen. Physiol.* 94:1101–1115). The number of endosomes formed when bladders were labeled in the absence of a transepithelial osmotic gradient increased with serosal [VP] (0–50 mU/ml), and endosome P_f was very high and constant (0.08–0.10 cm/s, 18°C). When bladders were labeled in the presence of serosal-to-musosal osmotic gradient, the number of functional water channels per endosome decreased (at [VP] = 0.5 mU/ml, P_f = 0.09 cm/s, 0 osmotic gradient; P_f = 0.02 cm/s, 180 mosmol gradient). Passive proton premeability was measured from the rate of pH decrease in voltage-clamped endosomes in response to a 1 pH unit gradient (pH$_{in}$ = 7.5, pH$_{out}$ = 6.5). The proton permeability coefficient (P_H) was 0.051 cm/s at 18°C in endosomes containing the VP-sensitive water channel; P_H was not different from that measured in vesicles not containing water channels. Measurement of urea transport by the fluorescence quenching assay gave a urea reflection coefficient of 0.97 and a permeability coefficient of $<10^{-6}$ cm/s. These results demonstrate: (*a*) VP-induced endosomes from toad urinary bladder have extremely high P_f. (*b*) In states of submaximal bladder P_f, the density of functional water channels in endosomes is constant in the absence of an osmotic gradient, but decreases in the presence of a serosal-to-mucosal gradient, suggesting that the gradient has a direct effect on the efficiency of packaging of water channels into endosomes. (*c*) The VP-sensitive water channel does not have a high proton perme-

ability. (d) Endosomes that cycle the water channel do not contain urea transporters. These results establish a labeling procedure in which >85% of labeled vesicles from toad urinary bladder are endosomes that contain the VP-sensitive water channel in a functional form.

INTRODUCTION

The regulation of water permeability in amphibian urinary bladder is thought to involve an exocytic/endocytic cycling of vesicles containing water channels between an intracellular compartment and the cell apical plasma membrane (for recent reviews, see Handler, 1988; Harris and Handler, 1988; Brown, 1989; Verkman, 1989). Morphological studies have shown increased fusion of specialized membranes (aggrephores) with the apical membrane of toad bladder granular cells in response to vasopressin addition (Bourguet et al., 1976; Muller et al., 1980), and increased retrieval of endocytic markers in response to vasopressin removal and/or a serosal-to-mucosal osmotic gradient (Masur et al., 1984; Harris et al., 1986). Intramembrane particle aggregates, seen by freeze-fracture electron microscopy in aggrephores and in the luminal membrane upon vasopressin stimulation, have been proposed to be a morphological marker of water channels (Chevalier et al., 1974; Kachadorian et al., 1977; Brown et al., 1983).

We reported recently that vasopressin-dependent endosomes from toad bladder contained functional water channels and had the highest osmotic water permeability (P_f) of any artificial or biological membrane thus far examined; endosome P_f had low activation energy and was inhibited strongly and reversibly by mercurials (Shi and Verkman, 1989). Endosomes were labeled selectively in intact toad bladder by impermeant, fluid-phase fluorophores (6-carboxyfluorescein [6-CF] and fluorescein-dextran) that underwent rapid, concentration-dependent self-quenching when endosome volume decreased. P_f was measured in endosomes by a stopped-flow fluorescence technique in which the fluorescein-labeled endosomes were subjected to an inwardly directed osmotic gradient. It was found that endosomes with rapid water transport were present when bladders had been treated with vasopressin, but not in the absence of vasopressin or when endocytosis was turned off by low temperature or metabolic depletion with azide.

In this report, we make use of the sensitivity of the fluorescent endocytic markers to changes in volume and pH to address important questions about the transport characteristics of vasopressin-induced endosomes in toad bladder. (a) It is not known whether water channel retrieval by vasopressin-induced endosomes is an all-or-none phenomenon; in states of submaximal stimulation of water permeability in intact toad bladder, do individual endosomes contain fewer water channels or are there fewer endosomes that contain a fixed, large number of water channels? (b) Based on short-circuit current measurements in intact toad bladder, it has been proposed that the vasopressin-sensitive water channel serves as a pore for rapid proton movement (Gluck and Al-Awqati, 1980). If this is correct, then do endosomes containing the vasopressin-sensitive water channel have higher proton permeability than endosomes that do not contain water channels? (c) Vasopressin increases both water and urea permeability (Sands et al., 1987). However under some circumstances, high water permeability can be induced with vasopressin without elevation

in urea permeability in both toad bladder and collecting tubule (Carvounis et al., 1979; Knepper and Roch-Ramel, 1987). Do endosomes that cycle the water channel have urea tansporters? Fluorescence experiments were carried out to examine the osmotic water transport properties of endosomes formed in states of submaximal bladder water permeability, and to measure the passive proton and urea permeability of endosomes containing the vasopressin-sensitive water channel.

METHODS

Isolation of Endocytic Vesicles

Dominican toads (*Bufo marinus*, National Reagents, Bridgeport, CT) were maintained on wet peat moss. Toads were killed by decapitation and excised urinary hemibladders were mounted on polyethylene tubing, serosa side out, using 3–0 silk suture. Bladders were rinsed with toad Ringer solution (buffer A) containing 110 mM NaCl, 2.5 mM NaHCO$_3$, 3 mM KCl, 2 mM KH$_2$PO$_4$, 0.5 mM MgSO$_4$, 1 mM CaCl$_2$, 5 mM glucose, pH 7.8 at 23°C. Hemibladders were filled with 5 ml of buffer A and incubated for 30 min with 100 ml of aerated buffer A containing 0–50 mU/ml vasopressin (Pitressin, Parke-Davis, Morris Plains, NJ) in the serosal solution. The mucosal solution was then replaced with 5 ml of 15 mM 6CF, or 25 mg/ml fluorescein-dextran (9,000 D, Sigma Chemical Co., St. Louis, MO) in specified mixtures of buffer A and water, pH 7.8. The fluorescein-dextran was dialyzed to remove unconjugated fluorescein; thin layer chromatography confirmed that free fluorescein was absent from the mucosal solution and from the final endosome preparation. After specified incubation conditions (see Results), hemibladders were cooled to 0–2°C and rinsed extensively with 50 mM mannitol, 5 mM K phosphate, pH 8.5 (buffer B) for water and urea transport measurements, or with 100 mM KCl, 5 mM K phosphate, pH 7.5 (buffer C) for proton transport experiments. All subsequent procedures were carried out at 0–2°C.

Bladder epithelial cells were scraped with a glass slide and homogenized by 15 strokes of a Dounce homogenizer and 4 strokes of a Potter-Elvehjem homogenizer operating at low speed. The membrane suspension was centrifuged for 10 min at 2,000 *g*. The supernatant was centrifuged for 60 min at 100,000 *g*. The pellet was homogenized by 5–10 passages through a 23 gauge steel needle, resuspended in 10 ml of buffer B or C, and centrifuged for 60 min at 100,000 *g* to obtain the microsomal pellet containing fluorescently-labeled endosomes. Before experiments, the pellet was homogenized by three to five passages through 23 and 27 gauge steel needles. All experiments were performed within 1 h after microsome preparation. Protein concentration was determined by the method of Ohnishi and Barr (1978). From one hemibladder, the membrane yield was 0.4–0.6 mg protein in the microsomal pellet.

Morphological Studies

Endocytic vesicle morphology was studied by preparing the microsomal pellet using horseradish peroxidase as an endocytic marker (HRP, 10 mg/ml, type II, Sigma Chemical Co.) in place of 6CF or fluorescein-dextran. Vesicles were isolated as described above and fixed in 2.5% glutaraldehyde for 60 min at 2°C. Fixed vesicles were washed in buffer B, and then in 100 mM Tris, pH 8.2, pelleted, and reacted with the Tris buffer containing 1 mg/ml diaminobenzidine and 0.015% H$_2$O$_2$ for 20 min at 23°C to form an electron-dense deposit (Verkman et al., 1988). Vesicles were fixed again in 2.5% glutaraldehyde overnight, washed with 0.05 M glycine in phosphate-buffered saline, and collected in 2.5% agar for osmification, dehydration, and embedding in LX-112 (Ladd Industries, Burlington, VT). Thin sections of vesicles were examined using a Philips CM10 electron microscope.

The location of endosomes labeled with fluorescein-dextran in tissue sections was examined by a new method which preserved the location of fluorescein-dextran (Lencer et al., 1990b). After labeling the endosomes with mucosal fluorescein-dextran, bladders were washed extensively and fixed in 2% paraformaldehyde, 75 mM lysine and 10 mM periodate overnight at 4°C. Tissues were frozen by immersion in liquid N_2 and 1 μm semithin sections were cut on a Reichert FC4D ultracryomicrotome. Sections were examined on an Olympus BHS epifluorescence microscope (excitation 470 ± 10 nm, 510 nm dichroic mirror, 520 nm barrier filter) using a 60× or 100× oil immersion objective (NA 1.30).

Osmotic Water Permeability in Intact Toad Bladder

Transepithelial osmotic water permeability was measured in intact toad bladder sacs by the classical gravimetric method of Bentley (1958). Hemibladders were filled with 10 ml of buffer A and bathed in buffer A containing varying concentrations of vasopressin for 30 min at 23°C. All solutions were aerated vigorously throughout the experiment. The bladder weight was measured every min for 20 min after changing the mucosal solution to a fivefold dilution of buffer A with distilled water. In some experiments, serosal vasopressin was removed at the time the mucosal solution was made hypoosmotic.

Osmotic Water Permeability in Endosomes

Osmotic water permeability was measured by a stopped-flow fluorescence quenching technique (Chen et al., 1988; Verkman et al., 1988; Ye et al., 1989; Shi and Verkman, 1989). Suspensions of the microsomal pellet (~0.5 mg protein/ml) were subject to a 60 mM inwardly directed osmotic gradient in a Hi-Tech SF51 stopped-flow apparatus (Wiltshire, England) interfaced to a MINC/23 computer (Digital Equipment Corp., Maynard, MA). The measured instrument dead time was 1.7 ms. The sample was excited at 465 ± 5 nm using a single grating monochromator in series with a six-cavity broad band (420–490 nm) interference filter. Fluorescence was detected through an OG515 cut-on filter (Schott Glass, Duryea, PA). The light source consisted of a 100-W tungsten-halogen lamp powered by a stabilized DC supply. Sample temperature was controlled by a circulating water bath and monitored by an indwelling thermistor. Data were acquired at a maximal rate of 0.2 ms/point. 10–15 consecutive measurements of water permeability were performed on every sample for signal averaging.

Fluorescence kinetic data (512 points/experiment) were fitted to single or double exponential functions by a nonlinear regression procedure. The amplitude of the exponential curve was corrected for the instrument dead time by extrapolation of the fitted exponential curve to zero time. Osmotic water permeability coefficients (P_f) were calculated from time constants of the exponential function and endocytic vesicle geometry by standard equations as described previously (Chen et al., 1988). An endocytic vesicle surface-to-volume ratio of 6×10^5 cm^{-1} was used.

Passive Proton Permeability

Passive proton permeability was measured in fluorescein-dextran–labeled endosomes from the time course of fluorescence in response to a 1-pH-unit inward proton gradient. Strategies for measurement of proton permeability in biological membrane vesicles were reviewed recently (Verkman, 1987). Microsomes containing buffer C at pH 7.5 and the potassium ionophore valinomycin (4 μg/mg membrane protein) were mixed in the stopped-flow apparatus with an equal volume of buffer C titrated with HCl (pH ~2.8) so that when mixed, the final external pH was 6.5. Fluorescence was excited at 480 ± 5 nm and measured at >520 nm.

The initial proton flux (J_H; meq H$^+$/L/s, L is liter of cell water) was determined from the

initial rate of fluorescence decrease ($dF(0)/dt$; fluorescence units/s), the intravesicular buffer capacity ($B(pH)$; meq $OH^-/L/pH$ unit), the normalized pH vs. fluorescein-dextran fluorescence (f) calibration relation ($pH = 7.22 f^3 - 13.81 f^2 + 10.27 f + 3.82$; Ye et al., 1989), and the fluorescence intensities at pH 7.5 and 6.5 ($F_{7.5}$ and $F_{6.5}$) by the relation (Verkman and Ives, 1986),

$$J_H = \frac{dF(0)}{dt} B(pH) \frac{d(pH)}{dF} \left(\frac{f_{7.5} - f_{6.5}}{F_{7.5} - F_{6.5}} \right) \tag{1}$$

$dF(0)/dt$ was determined from the initial slope of an exponential fitted to the first 2 s of the fluorescence time course. $B(pH)$ was determined by two independent methods: (a) from $dF(0)/dt$ measured at two different exogenous buffer strengths (see Results), and (b) from the magnitude of the pH decrease in response to a 15-mM inward gradient of formic acid, a weak acid in which the protonated moiety permeates rapidly and undergoes almost complete dissociation within the endosome. $d(pH)/dF$ was determined from the calibration relation to be 4.3 pH units at pH 7.5. The two calibration points, $F_{7.5}$ and $F_{6.5}$, were determined by subjecting microsomes to a 1-pH-unit inward proton gradient in the presence of the potassium/proton exchanger nigericin (5 μM). In the presence of nigericin, intravesicular pH equilibrates with a half-time of 1-2 s. The fluorescence at the start of the data trace was $F_{7.5}$; the stable fluorescence intensity after pH equilibration was $F_{6.5}$. The two calibration points were required to provide an absolute pH scale for fluorescence intensities measured in the stopped-flow apparatus. $F_{7.5}$ and $F_{6.5}$ were determined in every experiment. The factor $f_{7.5}-f_{6.5}$ was determined from the calibration relation to be 0.48. The proton permeability coefficient (P_H in centimeters per second) was calculated from the ratio $J_H/([\Delta H^+]S/V)$, where S/V is endosome surface-to-volume ratio. It is recognized that P_H, or any other parameter describing proton permeability, changes in a complex manner with pH, pH gradient, and membrane potential (Verkman and Ives, 1986; Nagel, 1987). Therefore P_H values in different membranes must be compared with identical driving forces.

Urea Permeability Measurements

The urea permeability of endosomes was measured by a stopped-flow fluorescence quenching technique in which vesicles in buffer B were subject to a 60-mM inwardly directed urea gradient. Urea permeability was measured from the time course of endosome volume, inferred from the fluorescence intensity of entrapped 6CF. In response to the urea gradient, there was rapid water efflux from endosomes resulting in 6CF self-quenching, followed by urea uptake and water influx resulting in an increase in 6CF fluorescence intensity to its pre-gradient level (Chen et al., 1988). Because of the functional heterogeneity in the water permeability characteristics of the microsomal pellet (see below), determination of the urea permeability coefficient (P_{urea}) required the comparison of experimental curves with curves calculated from a simulation routine. Theoretical curves were calculated by numerical integration of the Kedem-Katchalsky equations for coupled hydraulic water and neutral solute flow (Kedem and Ketchalsky, 1958) in which two noninteracting vesicle populations with different P_f and P_{urea} values were assumed to exist (see Results).

RESULTS

Water Transport Measurements in Intact Toad Bladder

Measurements of transepithelial water transport in intact toad bladder were carried out to establish the best conditions for subsequent endosome experiments. It has been reported that both a serosal-to-mucosal osmotic gradient and vasopressin

removal from the serosal solution enhance the endocytic retrieval of fluid-phase markers (Masur et al., 1984; Harris et al., 1986). In Fig. 1 A bladders were incubated in the absence of an osmotic gradient for 30 min with vasopressin to load the apical membrane of bladder granular cells with functional water channels. At time zero, the mucosal solution was diluted to initiate mucosal-to-serosal osmotic water flow. In the continued presence of serosal vasopressin (+ VP, upper curve), mucosal-to-serosal volume flow was high and decreased slowly due to one or more physiological down-regulatory processes such as vasopressin receptor or water channel internalization. When vasopressin was removed at time zero (− VP, lower curve), there was a rapid decrease in volume flow with a half-time of <5 min; baseline water permeability was attained after 10 min. Fig. 1 B shows a dose-response relationship for vasopressin-stimulated osmotic water flow in intact toad bladder. Bladders were
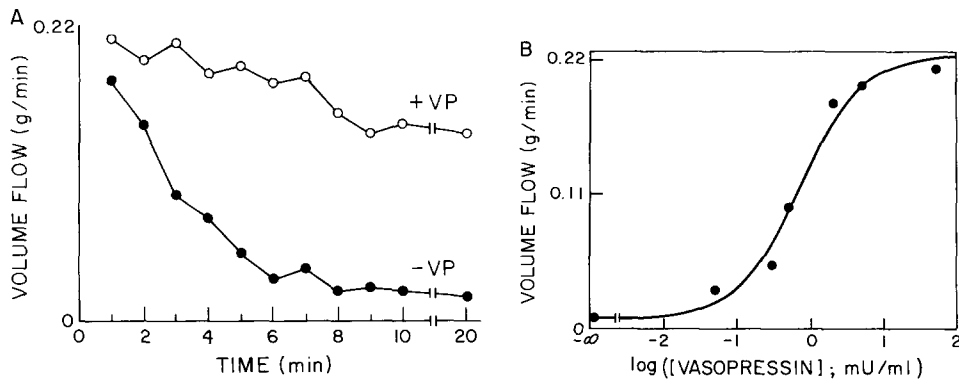

FIGURE 1. Vasopressin-induced water flow in intact toad urinary bladder. Experiments were carried out at 23°C. (A) Bladders were incubated for 30 min in the presence of 50 mU/ml serosal vasopressin in the absence of an osmotic gradient. At time zero, the mucosal solution was replaced by a fivefold dilution of buffer A with distilled water in the presence (+ VP) or absence (− VP) of serosal vasopressin. Volume flow was measured gravimetrically at 1-min intervals. (B) Dose-response relation for serosal vasopressin. Experiments were carried out as above for varying concentrations of vasopressin in the serosal solution throughout the experiment. Data were fitted to a single-site saturable binding model with $K_{1/2} = 0.6 \pm 0.2$ mU/ml vasopressin.

incubated with varying concentrations of serosal vasopressin for 30 min and then subjected to a serosal-to-mucosal osmotic gradient in the continued presence of vasopressin. Water flow was determined from the volume flow over the first 3 min where little physiological down-regulation occurred. A single-site saturable binding model was fitted to the data with a $K_{1/2}$ of 0.6 mU/ml vasopressin. These results were used as described below to prepare toad bladders in states of known submaximal water permeability.

Morphological Studies of Endosomes

The cellular location of fluorescein-dextran–labeled endosomes was examined by fluorescence microscopy of semithin tissue sections. Bladders were incubated with

no osmotic gradient in the presence or absence of vasopressin for 30 min, followed by 10 min in the presence of mucosal fluorescein-dextran (see Methods). Fluorescent endosomes were apically located and much more dense when bladders were treated with vasopressin. These findings are very similar to results reported for the kidney collecting tubule studied by the same morphological technique (Lencer et al., 1990*b*). The permeability properties of these labeled endosomes present in a bladder cell microsomal fraction are studied below.

The size of labeled endosomes was determined by electron microscopy. Endosomes containing HRP were isolated from bladders that were treated and not treated with vasopressin as described in Methods. HRP-labeled endosomes were visualized by formation of an electron-dense deposit by reaction with diaminobenzidine and H_2O_2. Fig. 2 shows an electron micrograph of HRP-labeled endosomes from a vasopressin-treated bladder. Examination of a series of electron micrographs showed that labeled endosomes were about five times more numerous in bladders that had been treated with vasopressin compared with control bladders. The average diameter of endosomes from bladders treated with vasopressin was 103 ± 25 nm (mean ± SE, $n = 85$); of those not treated with vasopressin the diameter was 120 ± 30 nm ($n = 32$). Knowledge of endosome size was required for calculation of absolute permeability coefficients.

Water Permeability Measurement in Endosomes

In our previous study, it was established that vasopressin-induced endosomes from toad bladder had very high P_f (0.06–0.1 cm/s) and low activation energy (4 kcal/mol), and that P_f was inhibited >70% by $HgCl_2$ (Shi and Verkman, 1989). A single labeling protocol was used. Bladders were labeled with a mucosal fluorescent marker for 15 min in the presence of vasopressin and a maximal 180 mosmol serosal-to-mucosal osmotic gradient, and then for an additional 15 min in the absence of vasopressin, with continued presence of the gradient. This protocol was chosen to maximize endocytosis based on reports that both osmotic gradients and vasopressin withdrawal enhanced the uptake of fluid-phase endocytic markers (Masur et al., 1984; Harris et al., 1986). The absolute amount of endocytosis could not be quantitated in these previous morphological studies, and importantly, it was not possible to determine whether the fluid-phase labels were incorporated into endosomes containing water channels.

The influence of osmotic gradients and serosal vasopressin concentration on the number and water permeability of endosomes was examined to (*a*) determine optimal labeling conditions which maximized the relative and absolute number of endosomes containing functional water channels, and (*b*) evaluate the efficiency of water channel packaging into endosomes in states of submaximal bladder water permeability. The effects of (*a*) osmotic gradient size, (*b*) serosal vasopressin concentration, and (*c*) vasopressin removal were examined independently.

The influence of osmotic gradient size on water permeability in endosomes was examined first. Bladders were exposed to maximal vasopressin (50 mU/ml) for 30 min in the absence of an osmotic gradient. The mucosal solution was then replaced with a solution of specified osmolality containing 15 mM 6CF; vasopressin was left in the serosal solution. Labeling was terminated after 15 min. The time course of
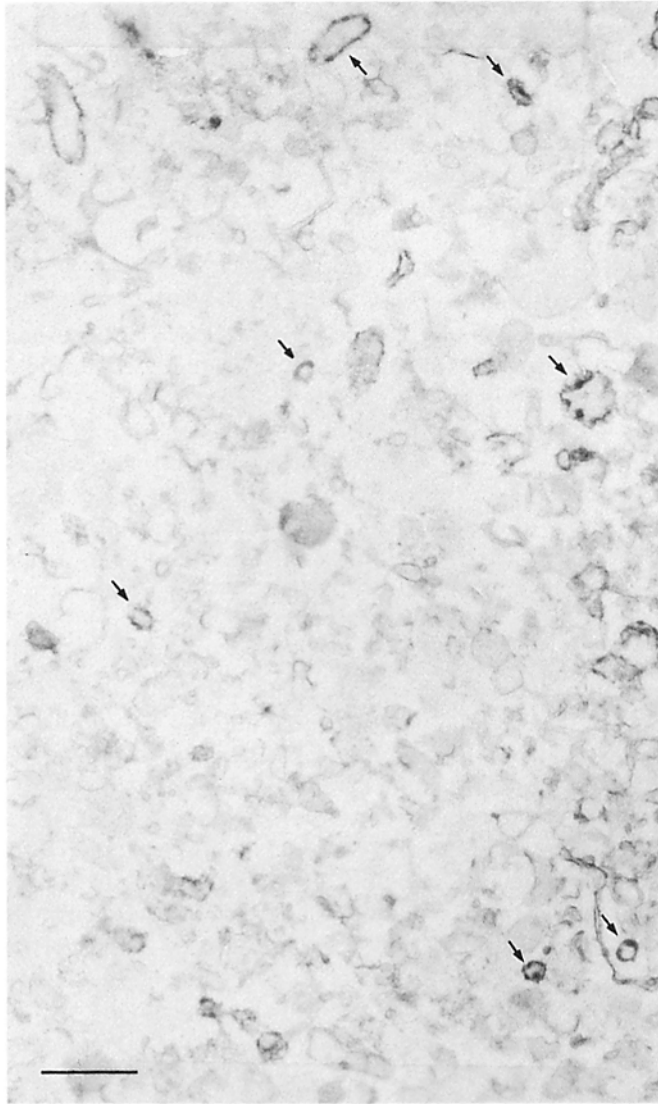
FIGURE 2.   Morphology of toad bladder endosomes. Endosomes were labeled with the electron-dense marker HRP in vasopressin-treated bladders and isolated as described in Methods. Thin sections were examined by electron microscopy. Bar, 1 μm. Arrows indicate HRP-labeled vesicles.

endosome fluorescence in response to a 60-mM inwardly directed osmotic gradient is shown in Fig. 3. The osmotic gradient caused outward osmotic water flow, decreased endosome volume, and 6CF fluorescence self-quenching, resulting in a time-dependent decrease in the fluorescence signal. The total kinetic signal contained two distinct components: a rapid fluorescence decrease with a time constant for an exponential function of 5–8 ms, followed by a slower fluorescence decrease

with half-time of 0.5–2 s. The data are summarized in Table I, *top*. An increased serosal-to-mucosal osmotic gradient resulted in an increase in the total signal amplitude, defined as the composite amplitude for the fast and slow kinetic processes. The amplitude of the fast process, which is proportional to the number of endosomes containing functional water channels, decreased with increasing gradient size. These results indicate that measurement of the total uptake of a fluid-phase endocytic marker is not a valid measure of the total amount of water channel retrieval. Table I, *top* also shows that endosome P_f decreased with increasing osmotic gradient size. This surprising finding is evaluated further below.

In our previous study in which endosomes were labeled by two 15-min incubations with mucosal 6CF in the presence of a maximal osmotic gradient, it was shown that the fast process arose from true endocytic 6CF uptake while the slow process
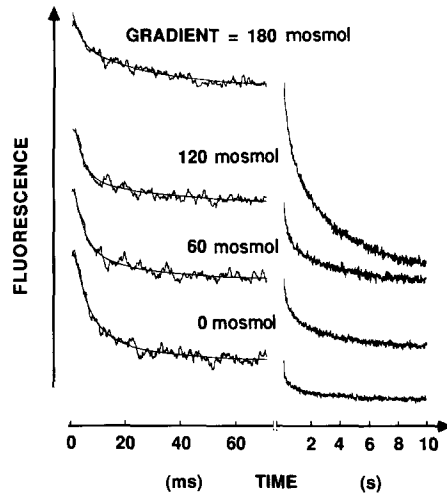

FIGURE 3. Influence of transepithelial osmotic gradients on water transport in labeled vesicles from toad bladder. Bladders were incubated for 30 min at 23°C with 50 mU/ml serosal vasopressin. The mucosal solution was then changed to a hypoosmotic or isosmotic buffer containing 6CF with continued presence of serosal vasopressin. Endosomes were isolated as described in Methods and subjected to a 60 mosmol inwardly directed osmotic gradient at 18°C. Each curve is the average of 10–15 experiments. Signals in this and subsequent data were normalized for total microsomal protein so that amplitudes could be compared directly. A biexponential function fitted to the first 80 ms of each curve is given.

was in part due to nonendocytic 6CF uptake. Similar and additional control studies were performed in the absence of an osmotic gradient (Table I, *bottom*). Endocytosis was inhibited by incubation with 6CF at 0–2°C, by the addition of Na azide to the bath, or by mucosal glutaraldehyde fixation. The rapid process was absent with each of these maneuvers, while the slow process was reduced partially. These studies demonstrate that the fast kinetic process arises from endocytic vesicles containing water channels.

The slower fluorescence decrease probably represents a combination of nonendosomal, and vasopressin-dependent and -independent (constitutive) endocytic uptake of the fluorescent marker. Several maneuvers to decrease nonspecific binding of the fluorescent marker to mucosal membranes, including extensive washing with albumin, rinsing with acetyl-cystine (mucomyst), and hypotonic cell swelling

before cell scraping, did not result in a decrease in the amplitude of the slower fluorescence decrease. Therefore, the interpretation of subsequent experiments on vasopressin-induced endosomes must take into account the apparently unavoidable presence of a >10% contamination by a population of vesicles with slow water transport that have taken up the fluorescent marker.

Experiments were carried out at submaximal concentrations of vasopressin to examine whether the number of functional water channels per endosome retrieved by vasopressin-induced endocytosis was dependent upon the density of water channels on the toad bladder apical membrane surface. If the number of water channels per endosome were constant, then with decreasing vasopressin, the water permeability of individual endosomes would not change, however the number of endo-

TABLE I

Influence of Transepithelial Osmotic Gradients on Endosome P_f

Gradient	Relative total amplitude	Fast process			Slow process
		Amplitude	τ	P_f	τ
mosmol			*ms*	*cm/s*	*s*
Effect of serosal-to-mucosal osmotic gradients on water transport					
180	1.50	0.32	7.4	0.079	1.1
120	1.35	0.45	6.6	0.088	0.9
60	1.15	0.62	6.5	0.090	0.9
0	1.00	0.82	5.6	0.102	1.0
Control studies at 0 gradient					
0 VP	0.45	0			1.6
50 mU/ml VP, incubation conditions:					
0–2°C	0.41	0			1.4
+NaN₃	0.29	0			1.3
+glut	0.15	0			2.5

(*Top*) Bladders were treated with 50 mU/ml vasopressin for 30 min with 0 osmotic gradient and then labeled with 6CF with specified serosal-to-mucosal gradients (see Methods). Endosomes were subjected to a 60-mosmol inwardly directed osmotic gradient as in Fig. 3. Mean values are given for experiments performed on three separate bladder preparations; individual measurements were repeated 10–15 times. The relative total amplitude is the amplitude of the total fluorescence signal, normalized for microsome protein, and set equal to unity for the 0 gradient experiment. Amplitudes and time constants for a biexponential fit are given. P_f values were calculated as described in Methods. (*Bottom*) Bladders were labeled with 6CF in the absence of vasopressin (0 VP), and with vasopressin when endocytosis was turned off by incubation with 6CF at 0–2°C, addition of 1% NaN₃ to the bath, and mucosal fixation with 0.5% gluteraldehyde.

somes with rapid water transport would decrease. Alternatively, the water permeability of vasopressin-induced endosomes might decrease in states of submaximal bladder water permeability. Fig. 4 *A* shows water transport in endosomes prepared from bladders treated with five different concentrations of vasopressin (see Fig. 1 for dose-response relation in intact toad bladder). There was no transepithelial osmotic gradient in these experiments. Results are summarized in Table II. With increasing serosal [vasopressin] there was an increasing number of endosomes containing functional water channels. However, P_f in these endosomes changed rela-

tively little, showing constant and efficient packaging of water channels in states of submaximal bladder water permeability. There was little effect of [vasopressin] on the amplitude and time course for the slow process.

Fig. 4 *B* shows a similar experiment performed in the presence of a 180 mosmol serosal-to-mucosal osmotic gradient. Data are summarized in Table II. With increasing serosal [vasopressin] endosome P_f increased significantly, and the amplitude of the slow process increased slightly. These results raise the possibility that packaging of water channels into endosomes formed in the presence of a serosal-to-mucosal osmotic gradient may be inefficient (see Discussion).
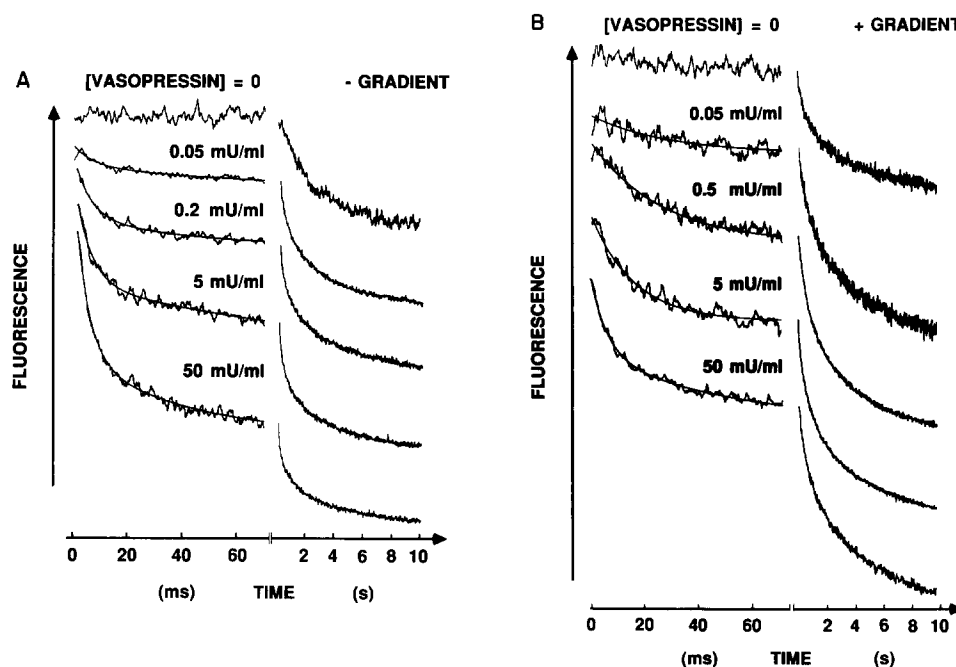

FIGURE 4. Vasopressin dose-response relation for water permeability of endosomes formed in the absence (*left*) and presence (*right*) of a 180 mosmol serosal-to-mucosal osmotic gradient. Endosomes were prepared in buffer B from toad bladders treated with the indicated concentration of serosal vasopressin. Endosomes were subjected to a 60 mosmol inwardly directed osmotic gradient at 18°C. Each curve is the average of 15 experiments; fitted biexponential functions are shown. Fitted results are summarized in Table II.

Table III shows the influence of vasopressin removal on endosome water permeability. Bladders were first incubated with 50 mU/ml serosal vasopressin for 30 min in the absence of an osmotic gradient to cause maximal water channel exocytosis. Bladders were then labeled with the fluorescent marker for 15 min in the presence or absence of an osmotic gradient and in the presence or absence of serosal vasopressin. The data show that vasopressin removal causes the formation of endosomes containing water channels, however the number of such endosomes is less than that formed in the presence of vasopressin. These results are consistent with the hypoth-

TABLE II

Influence of [Vasopressin] and Osmotic Gradients on Endosome P_f

[VP]	Relative total amplitude	Fast process			Slow process
		Amplitude	τ	P_f	τ
mU/ml			*ms*	*cm/s*	*s*
0 gradient					
0	0.36	0			1.9
0.05	0.53	0.18	7.1	0.081	1.1
0.5	0.60	0.34	6.7	0.085	1.1
5	0.85	0.53	6.9	0.082	1.0
50	1.00	0.82	5.6	0.102	1.1
180 mosmol gradient					
0	0.79	0			1.7
0.05	1.04	0.12	37	0.015	1.6
0.5	1.35	0.21	22	0.024	1.6
5	1.48	0.29	10	0.060	1.9
50	1.62	0.45	7.6	0.077	1.7

Experiments were carried out on three to four separate endosome preparations as described in the legend to Fig. 4. Values were calculated as described in the legend to Table I.

esis that the exocytic-endocytic cycling is continuous in the presence of vasopressin. However, the appearance of a significant population of endosomes with high P_f over the brief time period in which P_f was above baseline is consistent with the conclusion that vasopressin withdrawal induces a transient "wave" of endocytosis (Masur et al., 1984; Harris et al., 1986). Interestingly, endosome P_f decreased when vasopressin was removed. This apparent P_f may represent an average value for P_f of endosomes formed at different times after vasopressin removal.

The data in Tables I–III indicate that the greatest relative and absolute number of

TABLE III

Influence of Vasopressin Removal on Endosome P_f

	Relative total amplitude	Fast process			Slow process
		Amplitude	τ	P_f	τ
			ms	*cm/s*	*s*
0 gradient					
VP present	1.00	0.81	5.8	0.100	1.3
VP removed	0.68	0.40	8.3	0.071	1.1
180 mosmol gradient					
VP present	1.58	0.40	7.9	0.074	1.6
VP removed	0.38	0.14	18	0.033	1.4

Experiments were carried out on three separate endosome preparations. Bladders were incubated for 30 min in the presence of 50 mU/ml serosal vasopressin with no osmotic gradient. The mucosal solution was then replaced with an isosmotic (0 gradient) or hypoosmotic (180 mosmol serosal-to-mucosal gradient) solution containing 6CF. Vasopressin was left in (VP present) or removed (VP absent) from the bath. Endosomes were isolated after a 15-min incubation period with the mucosal 6CF.

endosomes containing functional water channels were formed in the absence of a transepithelial osmotic gradient and with continuous presence of 50 mU/ml serosal vasopressin. This protocol will be used to prepare endosomes containing the vasopressin-sensitive water channel for subsequent proton and nonelectrolyte permeability studies.

Passive Proton Permeability in Endosomes

The passive proton permeability in endosomes was measured to determine whether the vasopressin-sensitive water channel served as a rapid conduit for proton movement, in a manner similar to the membrane pores induced by amphotericin or gramicidin A. Endosomes containing fluorescein-dextran were voltage-clamped with K/valinomycin and subjected to a 1-pH-unit inward proton gradient in the stopped-flow apparatus (Fig. 5). The time course of proton entry was determined from the
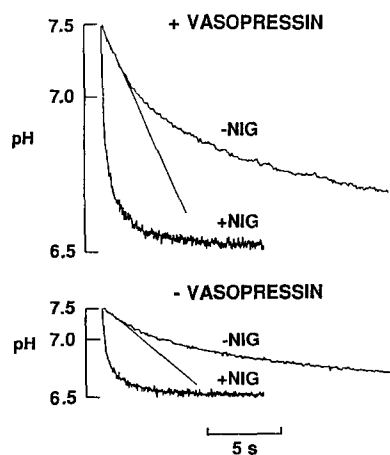

FIGURE 5. Passive proton permeability in endosomes. Fluorescein-dextran–labeled endosomes were isolated from toad bladder treated and not treated with 50 mU/ml vasopressin in the absence of an osmotic gradient. Endosomes in buffer C were voltage-clamped with K/valinomycin and subjected to a 1-pH-unit inward proton gradient in the presence and absence of 5 μM nigericin at 18°C. Each curve is the average of four experiments. Lines drawn are tangent to the initial part of the curves. See text for fitted flux rates and permeability values.

endosome fluorescence. A two-point calibration procedure was used in every experiment to convert fluorescence to pH as described in Methods.

The top curve in Fig. 5 shows proton movement in endosomes prepared from vasopressin-treated bladders. Addition of nigericin to the endosomes resulted in rapid pH equilibration. In four sets of measurements on separate endosome preparations, the initial rate of pH decline in the absence of nigericin was 0.24 ± 0.02 pH units/s (SD). The same initial rate of pH decline was measured when the time base was 0.5 s instead of 10 s, showing that faster components of pH equilibration were not present. The rate of pH decline was not influenced by doubling the valinomycin concentration, indicating the adequacy of the voltage clamp. In endosomes from bladders not treated with vasopressin (*lower curves*), the total fluorescence signal was smaller because fewer labeled vesicles were present, however, the rate of fluorescence decrease (0.23 ± 0.03 pH units/s, $n = 4$) was not different from that of endosomes from vasopressin-treated bladder.

To determine absolute proton permeability coefficients (P_H), knowledge of vesicle

buffer capacity and surface-to-volume ratio was required. From the magnitude of the fall in pH after a 15-mM inward formate gradient, buffer capacities of 34 and 38 mM OH^-/pH unit were calculated in vesicles from bladders treated and not treated with vasopressin, respectively. These results are in agreement with buffer capacities of 36 and 39 mM OH^-/pH unit calculated from the decrease in the initial rate of pH decline when buffer C was replaced with a buffer having a 10-fold higher buffer capacity (100 mM KCl, 50 mM K phosphate, pH 7.5; measured buffer capacity 32 mM OH^-/pH unit at pH 7.5). From the buffer capacities, and an endosome surface-to-volume ratio of 6×10^5 cm^{-1}, P_H values of 0.051 and 0.047 cm/s were calculated for endosomes from bladders treated and not treated with vasopressin, respectively. The magnitude of and similarities between these values suggest that the vasopressin-sensitive water channel does not contribute significantly to endosome passive proton permeability. It should be noted that these P_H values should be considered to be upper limits to the passive proton permeability; proton movement coupled to movement of Cl or K was not examined in these studies.

Because of the strong and reversible inhibition of endosome P_f reported for $HgCl_2$ (Shi and Verkman, 1989), effects of $HgCl_2$ and the related mercurial p-chloromercuribenzene sulfonate (pCMBS) on P_H were studied. In two sets of experiments using 0.5 and 1 mM $HgCl_2$ (10-min incubation), concentrations at which P_f was inhibited 30–70%, P_H increased 1.4–2.5-fold. Similar studies with 2 mM pCMBS (30 min incubation) gave a 1.8-fold increase in P_H. These are probably nonspecific effects of the mercurials on the membrane protein and lipid structure, obscuring possible inhibitory effects of these compounds. We have observed similar mercurial-induced proton leaks in artificial liposomes and proximal tubule vesicles.

Urea Permeability in Endosomes

To determine whether endosomes containing water channels had rapid urea transport, 6CF-labeled endosomes in buffer B were subjected to a 60-mM inwardly directed urea gradient. Fig. 6 A shows the time course of endosome fluorescence in response to 60-mM gradients of several polar nonelectrolytes, and the impermeant solute, sucrose. The urea and sucrose curves were nearly identical over the first 50 ms. In three sets of studies on separate endosome preparations, the ratio of signal amplitudes of the urea-to-sucrose curves, giving the urea reflection coefficient (Chen et al., 1988), was 0.97 ± 0.04 (SD). A near unity reflection coefficient indicates separate pathways for water and urea movement across the endosomal membrane.

To estimate the urea permeabity coefficient (P_{urea}) for the water channel–containing endosomes, the time course of fluorescence was measured over a longer time period. Over 60 s (not shown), there was slow urea influx. The presence of functional heterogeneity in water transport presents a potential difficulty in the measurement of rapid urea transport in the population of endosomes containing water channels. Rapid urea transport in the endosomes containing water channels would be observed as an increase in fluorescence over 1 s. However, there was at the same time a decrease in fluorescence due to water transport in the population of labeled vesicles with slow water transport (see Fig. 3). A mathematical model was therefore

constructed to establish an upper limit to P_{urea} for the water channel–containing endosomes. Fig. 6 *B* shows the predicted time course of fluorescence for several values for P_{urea}. The experimental urea curve shows no increase in fluorescence over 1 s, indicating that P_{urea} is under 10^{-6} cm/s. This low value for P_{urea} suggests the absence of a functional urea transporter in endosomes containing the vasopressin-sensitive water channel (see Discussion). For the more permeable nonelectrolytes formamide and ethylene glycol, there is an increase in fluorescence over 1 s, demonstrating that a high P_{urea} is measurable with this methodology.
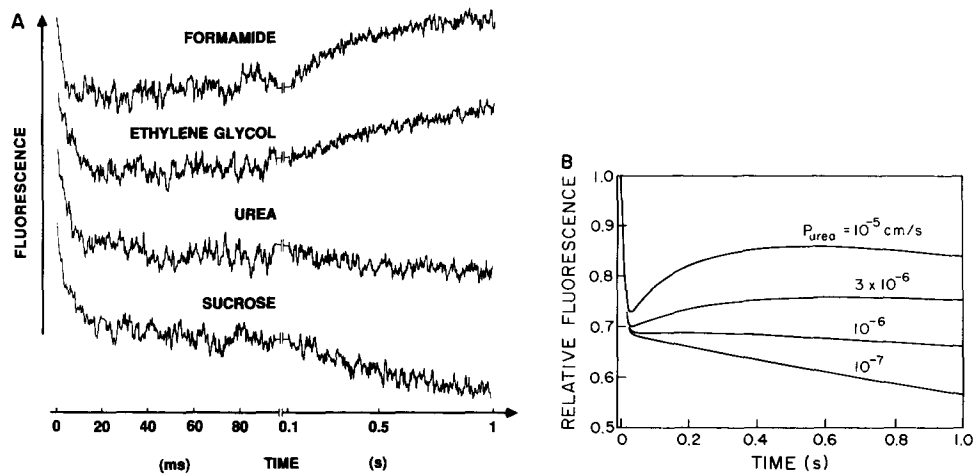

FIGURE 6.   Urea transport in vasopressin-induced endosomes. (*A*) Endosomes were prepared from vasopressin-treated bladders in the absence of a transepithelial osmotic gradient. Endosomes were isolated in buffer B and subjected to 60-mM inwardly directed gradients of the indicated solutes at 18°C. Each curve is the average of 10–15 experiments. (*B*) Theoretical curves showing the time course of relative fluorescence after exposure of endosomes to a 60-mM inward urea gradients. Curves were generated using the Kedem-Katchalsky equations (1958) for coupled osmotic water and urea flow for two noninteracting vesicle populations of equal number. This is a worst case analysis in which the influence of inward urea transport on the fluorescence over 1 s is slightly underestimated. Parameters: For the water channel–containing endosomes, $P_f = 0.06$ cm/s, P_{urea} was varied, and the urea reflection coefficient was 1. For the signal arising from vesicles with slow water transport, $P_f = 0.0003$ cm/s, $P_{urea} = 6 \times 10^{-8}$ cm/s, and the urea reflection coefficient was 1.

DISCUSSION

The purpose of these studies was to examine the water, proton and urea transport properties of endocytic vesicles that retrieve the vasopressin-sensitive water channel from the apical membrane of granular cells of toad urinary bladder. Fluid-phase fluorescent markers of endocytosis were used to label endosomes in intact toad bladder. Fluorescence microscopy of semithin fixed frozen sections of bladder showed that endosomes labeled with fluorescein-dextran were located near the apical membrane of granular cells and that the number of endosomes was increased greatly by treatment of bladders with vasopressin. To measure osmotic water, pro-

ton and urea transport in endosomes, a microsomal fraction was prepared from bladder cells by scraping, homogenization, and differential centrifugation. Only transport in the fluorescein-labeled vesicles was measured in the transport assays; the presence of nonfluorescent, unlabeled vesicles did not influence the assay. Electron micrographs of microsomal vesicles labeled with the electron-dense marker HRP showed that ~5% of microsomes prepared from vasopressin-treated bladder contained HRP, whereas <1% of microsomes from untreated bladder contained HRP. Control experiments showed that the rapid water transport in fluorescein-labeled vesicles from vasopressin-treated bladders arose from a vasopressin-dependent, apical endocytic process.

The current hypothesis is that apical membrane water permeability in toad bladder is regulated by the vasopressin-dependent cycling of vesicles containing water channels between an intracellular compartment and the apical plasma membrane. In response to vasopressin addition, there appears to be fusion of aggrephores, which on freeze-fraction electron microscopy contain particle aggregates, with the apical membrane. This process causes the apical insertion of the particle aggregates, assumed to represent water channels, resulting in increased apical membrane water permeability. In response to vasopressin removal, functional water channels are thought to be retrieved by endocytosis, however the intracellular fate of retrieved water channels is unknown. Whereas water channel endocytosis appears to occur via clathrin-coated pits in kidney collecting tubule (Brown and Orci, 1983; Brown et al., 1988; Strange et al., 1988), there is little information about the mechanism of endocytosis in amphibian urinary bladder. It has been shown recently that endosomes from kidney collecting tubule and toad bladder, which retrieve the vasopressin-sensitive water channel, do not contain an H^+ ATPase, suggesting that these endosomes may not enter the usual degradative pathway by lysosomal fusion (Lencer et al., 1990a). Our studies here demonstrate that functional water channels are retrieved into endosomes formed from a water-permeable apical membrane in toad bladder.

The data in Table II show that endosomes formed in the absence of a transepithelial osmotic gradient have a high P_f that is nearly independent of the serosal vasopressin concentration. With increasing vasopressin, the number of endosomes containing water channels increased. This represents efficient packaging of water channels into endosomes; a maximal number of water channels are concentrated into a minimum number of endosomes. A very selective endocytic process must be responsible for "concentrating" water channels in specialized patches of apical membrane destined for endocytosis. This process is particularly impressive at low vasopressin, when the mean density of apical membrane water channels is quite low.

However, in the presence of a large serosal-to-mucosal osmotic gradient, the water permeability of endosomes decreased. Under these conditions the endocytic process "samples" a partially averaged density of water channels in a patch of apical membrane; water channels do not appear to be maximally concentrated into a minimum number of endosomes. While such a concentrating mechanism may be efficient for subsequent intracellular sorting, it may be energetically unfavorable or physically impossible to concentrate water channels in the presence of a large

osmotic gradient. These results are in general agreement with recent morphological measurements of Kachadorian et al. (1989) showing that individual cells have a graded, rather than an all-or-none, response to vasopressin. It is proposed that a large osmotic gradient may force an endocytic event to occur before the sorting of apical membrane components has been completed. It is important to emphasize that by functional measurements, it is not possible to determine whether inactive, or partially active water channels are present on endosomal membranes. It will be important to correlate our functional results with label freeze-fracture studies performed on endosomes formed in states of submaximal water permeability.

The physical nature of the vasopressin-sensitive water channel is unknown. If the water channel is a narrow channel like gramicidin A, which excludes small solutes such as urea, then it is interesting to estimate the number of water channels per endosome and the endosome passive proton permeability. From the osmotic water permeability of a single gramicidin A channel (9.6×10^{-15} cm^3/s; Rosenberg and Finkelstein, 1978), the endosome P_f (0.1 cm/s) and the endosome surface area (3×10^{-10} cm^2), it is calculated that each endosome contains ~3,300 gramicidin A–like water channels. Because each gramicidin A channel has a radius of 0.2 nm, it is calculated that ~1% of the endosome surface area is the aqueous area bounded by the pore walls. If the protein or specialized lipid area of the water channel were five times larger than the aqueous pore area, then ~5% of total endosome surface area would be devoted to water channels. This value is quite reasonable because it is likely that the principal and maybe sole function of vasopressin-induced endosomes is to cycle water channels.

The passive proton permeability of a gramicidin A–like channel is very high; lipid bilayer studies show that $P_H/P_f >> 10$ (Hladky and Haydon, 1972). In recent studies, the estimated single-channel gramicidin proton permeability is ~10^{-12} cm^3/s, giving P_H/P_f ~100 (Decker and Levitt, 1988). It has been proposed that protons leap rapidly from water molecule to molecule through such a pore (Grotthus mechanism). However, a high proton permeability is not a necessary property of a membrane with high water permeability; the human erythrocyte has a high P_f that is >90% inhibitable by mercurials, and a low passive proton permeability. Macey (1984) has observed that $P_H/P_f << 0.001$ in red cells, and concludes that protons are excluded from the red blood cell water channel. Our results show that the passive proton permeability of vesicles containing the vasopressin-sensitive water channel is not different from that of vesicles not containing water channels, with P_H/P_f ~0.5, much less than P_H/P_f ~100 for the gramicidin channel. We conclude that, like the red cell water channel, the vasopressin-sensitive water channel does not conduct protons. It was not possible, however, in these studies to detect the presence of an incremental proton permeability from the vasopressin-sensitive water channel that is much smaller than the endogenous passive proton permeability.

Our finding that the vasopressin-sensitive water channel does not have high passive proton conductance is different from the conclusions of Gluck and Al-Awqati (1980). From measurements of short-circuit current, cell pH, and ^3H_2O uptake in intact toad bladder, they concluded that vasopressin increased the apical membrane proton conductance in parallel to transepithelial diffusive water permeability. It is difficult to reconcile these findings with our conclusion that P_H in vasopressin-

induced endosomes is no higher than P_H in endosomes not containing water channels. In the intact toad bladder epithelium, it is difficult to exclude several possible reasons for an increase in apparent proton conductance with vasopressin, including changes in (a) apical membrane surface area of geometry, (b) apical membrane fluidity, (c) the apical or basolateral membrane ion conductances, and (d) paracellular transport properties.

There is evidence that the pathways for osmotic water and urea transport are distinct in vasopressin-sensitive epithelia. Water permeability can be increased in the toad bladder (Carvounis et al., 1979), and in several segments of the collecting tubule (Knepper and Roch-Ramel, 1987) without effect on urea permeability. In the rat inner medullary collecting tubule stimulated by vasopressin, Knepper et al. (1989) showed that the urea reflection coefficient was near unity, indicating separate physical pathways for water and urea transport. The studies here show that vasopressin-dependent endosomes that contain functional water channels have a urea reflection coefficient near unity and a urea permeability coefficient of $<10^{-6}$ cm/s. This permeability is much less than that of red cells that contain a specific urea transporter (10^{-3} cm/s, Macey, 1984), but similar to permeabilities for urea in simple lipid bilayers (10^{-7} to 10^{-6} cm/s, Galluci et al., 1971). These results support the existence of distinct regulatory pathways for water and urea transport in the toad bladder. It is not known whether the regulation of urea transport involves membrane cycling, as for water channels, or biochemical modification of in situ apical membrane urea transporters, as proposed for regulation of Na channel activity by vasopressin.

In summary, we have made use of the volume and pH-dependent properties of fluorescein endocytic markers to examine the transport characteristics of vasopressin-induced endosomes arising from the apical membrane of toad urinary bladder. A labeling procedure was developed in which >85% of labeled vesicles were endosomes that contained the vasopressin-sensitive water channel. Several unanticipated effects of osmotic gradients and vasopressin concentration on the packaging of apical membrane water channels into endosomes were found. In states of submaximal bladder water permeability in the absence of a transepithelial osmotic gradient, endosomes were packaged maximally with water channels. However, in the presence of an osmotic gradient inducing mucosal-to-serosal volume movement, individual endosomes contained a submaximal number of functional water channels. Endosomes containing water transporters did not have rapid proton transport indicating that the vasopressin-sensitive water channel, like the red cell water channel, is not a conduit for protons. Physiologically this is important to prevent vasopressin-induced intracellular acidosis because of the low pH of urine. Endosomes containing the water channel do not contain urea transporters, suggesting that regulation of urea transport occurs by an independent mechanism. Further biochemical and molecular biological studies of the vasopressin-sensitive water transporter are required to begin to define its structural and molecular properties.

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