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Very High Water Permeability in Vasopressin-induced Endocytic Vesicles from Toad Urinary Bladder

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ABSTRACT The regulation of transepithelial water permeability in toad urinary bladder is believed to involve a cycling of endocytic vesicles containing water transporters between an intracellular compartment and the cell luminal membrane. Endocytic vesicles arising from luminal membrane were labeled selectively in the intact toad bladder with the impermeant fluid-phase markers 6-carboxyfluorescein (6CF) or fluorescein-dextran. A microsomal preparation containing labeled endocytic vesicles was prepared by cell scraping, homogenization, and differential centrifugation. Osmotic water permeability was measured by a stopped-flow fluorescence technique in which microsomes containing 50 mM mannitol, 5 mM K phosphate, pH 8.5 were subject to a 60-mM inwardly directed gradient of sucrose; the time course of endosome volume, representing osmotic water transport, was inferred from the time course of fluorescence self-quenching. Endocytic vesicles were prepared from toad bladders with hypoosmotic lumen solution treated with (group A) or without (group B) serosal vasopressin at 23°C, and bladders in which endocytosis was inhibited by treatment with vasopressin at 0–2°C (group C), or with vasopressin plus sodium azide at 23°C (group D). Stopped-flow results in all four groups showed a slow rate of 6CF fluorescence decrease (time constants 1.0–1.7 s for exponential fit) indicating a component of nonendocytic 6CF entrapment into sealed vesicles. However, in vesicles from group A only, there was a very rapid 6CF fluorescence decrease (time constant 9.6 ± 0.2 ms, SEM, 18 separate preparations) with an osmotic water permeability coefficient (P_f) of >0.1 cm/s (18°C) and activation energy of 3.9 ± 0.8 kcal/mol (16 kJ/mol). P_f was inhibited reversibly by >60% by 1 mM $HgCl_2$. The rapid fluorescence decrease was absent in vesicles in groups B, C, and D. These results demonstrate the presence of functional water transporters in vasopressin-induced endocytic vesicles from toad bladder, supporting the hypothesis that water channels are cycled to and from the luminal membrane and providing a functional marker for the vasopressin-sensitive water channel. The calculated P_f in the vasopressin-induced endocytic vesicles is the highest P_f reported for any biological or artificial membrane.

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INTRODUCTION

Transepithelial water permeability in the toad urinary bladder is regulated by the concentration of vasopressin at the serosal surface. There is a large body of evidence showing that the increase in luminal membrane water permeability in response to serosal vasopressin addition parallels the fusion of intracellular membrane vesicles (aggrephores) with the cell luminal membrane and the insertion of particle aggregates into the luminal membrane (Bourguet et al., 1976; Muller et al., 1980; Wade et al., 1981; Brown et al., 1983). The decrease in apical membrane water permeability following vasopressin removal, or continued vasopressin stimulation in the presence of a serosal-to-mucosal osmotic gradient, is thought to result from an endocytic process (Masur et al., 1984; Muller and Kachadorian, 1984; Harris et al., 1986). Water-permeable patches of apical membrane may be retrieved selectively for return to an intracellular compartment for later recycling to the luminal membrane.

The purpose of this study was to determine whether vasopressin-induced endocytic vesicles from toad urinary bladder contained functional water transporters. It is first demonstrated that vasopressin induces the appearance of a population of vesicles, labeled with either 6CF or fluorescein-dextran, having ~100-fold faster water transport than vesicles from toad bladders that have not been treated with vasopressin. A series of important control studies are then presented to establish the endosomal origin of the fluorescence signal arising from vesicles that have rapid water transport. Finally, the physical properties of the vasopressin-sensitive water channel are examined, including activation energy, inhibitory properties, and osmotic gradient dependence. These results provide evidence that the endocytic vesicles contain functional water transporters, and establish a method to examine the characteristics of the vasopressin-sensitive water transporter in isolated membrane vesicles. Development of a functional assay for water transport in endocytic vesicles provides an important approach to examine the cell biology of the exocytic/endocytic pathway for water channels and to assay the enrichment of vesicles containing water channels during purification procedures.

This work was presented in abstract form at the 33rd annual meeting of the Biophysical Society in Cincinnati, OH in February, 1989.

METHODS

Isolation of Endocytic Vesicles

Mexican toads (*Bufo marinus*) were maintained on wet peat moss. Toads were killed by decapitation and excised urinary hemibladders were mounted on PE-190 polyethylene tubing (Becton Dickinson & Co., Parsippany, NJ), serosa side out, using 3-0 silk suture. Bladders were rinsed with a 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 $CaCl_2$, 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 at 23°C. In some experiments, 50 mU/ml vasopressin (Pitressin; Parke-Davis, Morris Plains, NJ) was added to the serosal solution. After the initial incubation, the mucosal solution was replaced with 5 ml of 15 mM 6-carboxyfluorescein (6CF) or 50 mg/ml fluorescein-dextran (9,000 Da; Sigma Chemical Co., St. Louis, MO), in 5 mM K phosphate

titrated to pH 7.8 with KOH. The fluorescein-dextran was dialyzed to remove unconjugated fluorescein; thin layer chromatography confirmed that free fluorescein was absent in the mucosal solution and in the final endosome preparation. Hemibladders were incubated for 15 min with these solutions, followed by an additional 15 min with zero vasopressin in the serosal buffer A. In some experiments, the incubations with 6CF were performed at 0–2°C or in the presence of Na azide (see below).

Hemibladders were then rinsed extensively with 50 mM mannitol, 5 mM K phosphate, pH 8.5 (buffer B) at 2°C. All subsequent procedures were carried out using buffer B at 0–2°C so that final endosomes were loaded with buffer B. Bladder epithelial cells were scraped with a glass slide and homogenized by 15 strokes of a Dounce homogenizer and four strokes of a Potter-Elvehjem homogenizer operating at low speed. The membrane suspension was centrifuged for 10 min at 2,500 *g* to obtain a crude membrane pellet. 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 and centrifuged for 60 min at 100,000 *g* to obtain the microsomal pellet. For stopped-flow measurements, the pellet was homogenized by 3–5 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 average membrane yield was 0.5 mg protein in the microsomal pellet and 0.8 mg protein in the crude pellet.

Morphological Studies

Endocytic vesicle morphology was studied by preparing the microsomal pellet using horseradish peroxidase (10 mg/ml, type II; Sigma Chemical Co.) in place of 6CF or fluorescein-dextran. Vesicles were fixed in 2.5% gluteraldehyde and reacted with diaminobenzidine and H_2O_2 to form an electron-dense deposit (Verkman et al., 1988). Electron-dense vesicles isolated from toad bladders treated with vasopressin (group A protocol, see below) constituted ~5% of the total vesicles, and had a unimodal size distribution with a diameter of 100 ± 30 nm (mean ± SD, 30 electron-dense vesicles counted), which is similar to the size reported by Harris et al. (1986) for endosomes in thin sections of intact toad bladder. In vesicles prepared from bladders not treated with vasopressin (group B), there were 5–10-fold fewer vesicles containing the electron-dense deposit.

To visualize fluorescein-labeled endosomes in the intact toad bladder, bladder fragments were examined by epifluorescence microscopy immediately after labeling and rinsing with buffer A titrated to pH 8.5 with KOH. Bladder fragments were placed mucosa-side down on a thin glass slide (~0.12 mm thickness) and kept moist with buffer A at pH 8.5. The bladder was viewed with an inverted epifluorescence microscope (Nikon, Diaphot-TMD-EF; Nippon Kogaku, Tokyo, Japan) using a 100× fluorotar objective (Nikon, N.A. 1.30, oil immersion, working distance 0.17 mm). Excitation was 460 ± 5 nm with 510 dichroic mirror and 515 nm cut-on filter. Fluorescence was imaged with a SIT camera operating at fixed gain. The output was digitized by a frame grabber (DT2861; Data Translation, Marlboro, MA) in an 80286 computer with 80287 coprocessor and DT2858 auxillary processing board. Images were stored on a dedicated 100 MB hard disk drive.

Osmotic Water Permeability Measurements

Osmotic water transport was measured by a stopped-flow fluorescence quenching technique (Chen et al., 1988; Verkman et al., 1988; Ye et al., 1989). Suspensions of the crude pellet (~0.5 mg protein/ml) or the microsomal pellet (~0.5 mg protein/ml for 6CF studies, ~0.75 mg protein/ml for fluorescein-dextran studies) in buffer B were subject to a 60-mM inwardly directed osmotic gradient by mixture with an equal volume of buffer B containing 120 mM

sucrose. Experiments were carried out on a Hi-Tech SF51 stopped-flow apparatus (Wiltshire, England) interfaced to a Digital Equipment Corp. MINC/23 computer (Maynard, MA). 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 Technologies, Inc., 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. Each measurement required 0.075 ml of membrane suspension. All measurements were performed 10–15 times for signal averaging. The dead time of the stopped-flow apparatus was 1.7 ms as determined by reaction of dichloroindophenol with ascorbic acid. The electronic response time of the instrument was 0.5 ms.

Fluorescence kinetic data (512 points/experiment) were fitted to single 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 exponential functions and endocytic vesicle geometry by standard equations as described previously (Illsley and Verkman, 1986; Chen et al., 1988). An endocytic vesicle surface-to-volume ratio of 6×10^5 cm^{-1} was used based on the mean vesicle diameter of 100 nm.

Experimental Protocols

Endocytic vesicles were isolated using 6CF as the fluorescent marker by four major protocols. Group A: vasopressin was present during a 30-min incubation without 6CF and a first 15-min incubation with 6CF. Vasopressin was removed for a second 15-min incubation with 6CF; all incubations performed at 23°C. Group B: the same series of incubations at 23°C except that no vasopressin was present. Group C: the same incubations as in group A except that the two 15-min incubations with 6CF were performed at 0–2°C. Group D: the same incubations as in group A except that 0.1% Na azide was present in the serosal solution for the two 15-min incubations. Experiments were performed using two sets of paired bladders, comparing vesicles from groups A vs. B, A vs. C, and A vs. D. To measure the effects of temperature, mercurials, and osmotic gradient size on P_f, endocytic vesicles using the group A protocol were isolated from three toads.

In some experiments, vesicles from groups A vs. B were studied with 6CF replaced by fluorescein-dextran using four sets of paired bladders. In some experiments the group A protocol was used except that 6CF was present in the serosal solution instead of in the mucosal solution. In some experiments the two 15-min incubations with 6CF were replaced with a single 10-min incubation with zero serosal vasopressin and a hypoosmotic mucosal solution containing 6CF (see Results).

RESULTS

Rapid Osmotic Water Transport in Vasopressin-induced Endosomes

Fig. 1 shows the time course of 6CF and fluorescein-dextran fluorescence in endocytic vesicles from toad bladder in response to a 60-mOsmol inwardly directed osmotic gradient, which caused a 50% reduction in vesicle volume. The osmotic gradient caused water efflux, a decrease in vesicle volume, and 6CF or fluorescein-dextran fluorescence self-quenching, resulting in a time course of decreasing fluorescence. Data in Fig. 1 *A* are shown for one set of experiments, typical of four, in which endocytic vesicles labeled with 6CF were prepared from bladders that had

been exposed (group A protocol, see Methods) or not exposed (group B) to vasopressin. The signal amplitudes were normalized for the protein concentration in the final microsomal pellet. For both groups of endocytic vesicles, there was a slow, multiexponential time course of decreasing fluorescence over ~5 s. The curve shapes for the slow fluorescence decrease were similar. For toad bladders that were treated with vasopressin, there was in addition a very fast decrease in fluorescence with a time constant of ~10 ms at 18°C. In 18 separate samples prepared by the group A protocol and used for various comparisons in this study, the very fast fluorescence decrease was present in every preparation and had a mean time constant of

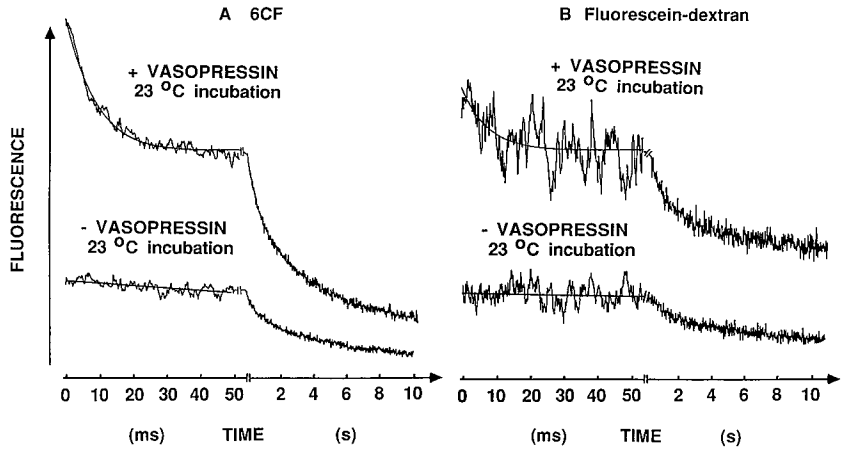

FIGURE 1. Vasopressin effect on osmotic water transport in endocytic vesicles from toad bladder. Endocytic vesicles were prepared from toad bladders as described in Methods. Paired hemibladders were incubated in the presence (group A protocol) or absence (group B) of serosal vasopressin prior to cell scraping and homogenization. Stopped-flow measurements of water permeability were carried out at 18°C using a 60-mOsmol inwardly directed osmotic gradient as described in Methods. Each curve shown with two different time scales is the average of 10 repeated measurements. Signal amplitudes were normalized to the protein concentration in the vesicle suspension to allow a direct comparison of curves. A single exponential fit through the first 50 ms of the + vasopressin data is given. (*A*) 6CF was present in the mucosal solution. (*B*) Fluorescein-dextran was present in the mucosal solution. Results for a series of measurements performed on separate bladder preparations using 6CF are given in Table I.

9.6 ± 0.2 ms (SEM) at 18°C. There was no change in fluorescence when vesicles were mixed with an isosmotic buffer, indicating the absence of mixing artifacts in these experiments.

As explained in the Methods section, a signal average of 10–15 experiments was required to obtain the signal quality of the curves in Fig. 1 *A*; this required most of the microsomal pellet prepared from two hemibladders. Fluorescein-dextran has also been shown to be an endocytic marker that undergoes fluorescence self-quenching (Chen et al., 1988; Ye et al., 1989), however, the signal-to-noise ratio for studies performed with fluorescein-dextran is >10-fold poorer than that obtained

for equivalent studies performed with 6CF. Nonetheless, we believed it was important to show that results similar to those for 6CF could be obtained with fluorescein-dextran, a well-established impermeant endocytic marker. For these studies, a high concentration of fluorescein-dextran was used (50 mg/ml) and the complete microsomal pellet from four hemibladders was required to obtain an acceptable signal. In one set of experiments typical of three shown in Fig. 1 *B*, vasopressin induced the appearance of a population of vesicles with rapid water transport, similar to, but of lower signal quality than, results obtained using 6CF. In three sets of studies with fluorescein-dextran in vesicles prepared by the group A protocol, the time constant for the rapid fluorescence decrease was 8 ± 1 ms (18°C).

Control Studies

It is important to prove that the rapid fluorescence decrease observed in the presence of vasopressin in Fig. 1*A* represents the self-quenching of 6CF that has entered vesicles by an endocytic route. It is possible to be seriously misled in these kinds of studies. For example, lucifer yellow, another endocytic probe, has been shown in activated macrophages to enter endocytic structures by diffusion through the plasma membrane and into intracellular vesicles (Steinberg et al., 1988), giving an apparent endocytic pattern. It has been shown that vasopressin increases the paracellular permeability in toad bladder to impermeant solutes such as sucrose (Parisi et al., 1981), raising the possibility that 6CF might gain access to the basal cell membrane and become entrapped in water-permeable basal membrane endosomes. In addition, if apical membrane endosomes containing 6CF fused with a lysosomal compartment containing functional water channels, then a rapid fluorescence decrease might be observed that did not represent endocytic retrieval of water channels. Functional and morphological studies were carried out to rule out these possibilities.

Two strategies were used to turn off endocytosis. In the first, bladders were exposed to vasopressin at 23°C and cooled to 0–2°C during incubation with 6CF (group C). Functional and morphological studies have established that this temperature effectively turns off toad bladder endocytosis (Harris et al., 1986). In one set of experiments, typical of four, the very fast fluorescence decrease was absent when bladders were cooled during the incubation with mucosal 6CF (Fig. 2 *A*). However, the slower exponential decrease remained. Similar experiments were performed in the presence of sodium azide to turn off endocytosis by metabolic depletion (Fig. 2 *B*). Again, in one set of experiments, typical of three, the slower fluorescence decrease was present, however the 10-ms process was absent. These data indicate that the 10-ms process is the result of vasopressin-dependent endocytosis while the slower fluorescence decrease arises in part from a nonspecific, nonendocytic population of membranes with entrapped 6CF. The nonspecific entrapment of mucosal labels is possible because of binding of the label compound to the mucosal glycocalyx with subsequent entrapment during vesiculation caused by the homogenization procedure.

Results from a series of experiments performed using paired hemibladders are summarized in Table I. The fast fluorescence decrease was measured only in the presence of vasopressin and when bladders were incubated with the endocytic

marker 6CF at 23°C. The fast fluorescence decrease was absent in the absence of vasopressin treatment or when endocytosis was turned off by low temperature or Na azide. Similar stopped-flow measurements were performed using the low speed crude pellet (see Methods) to assess the enrichment of endosomes containing water channels. The population of endocytic vesicles containing water transporters prepared by the group A protocol was greatly enriched in the final microsomal pellet compared with the crude pellet; the fast fluorescence decrease was not measurable in the crude pellet. The slower fluorescence decrease was present in the crude pellet

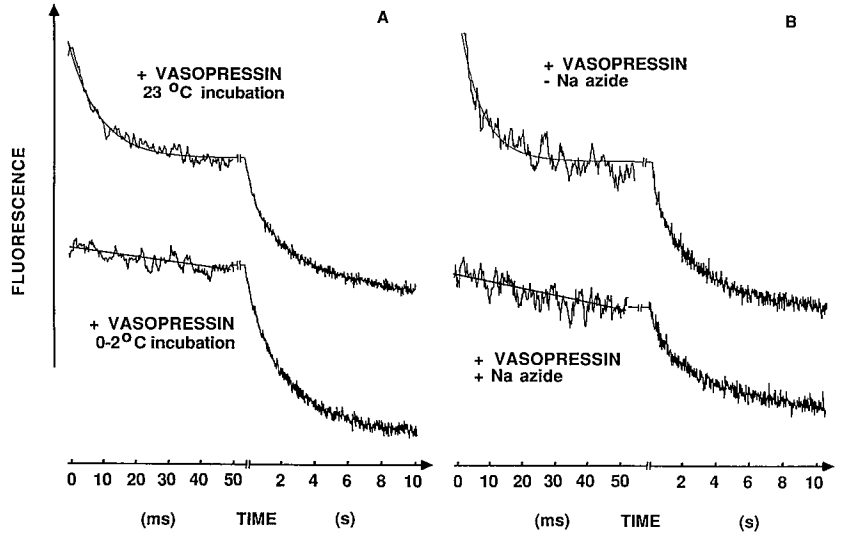

FIGURE 2. Effect of incubation temperature and Na azide on osmotic water transport in endocytic vesicles from toad bladder. Endocytic vesicles were prepared from toad bladders as described in Methods. (*A*) Paired hemibladders were incubated in the presence of vasopressin for 30 min at 23°C, followed by 15 min in the presence of vasopressin at 23°C (group A protocol) or 0–2°C (group C), followed by 15 min in the absence of vasopressin at 23°C (group A) or 0–2°C (group C). (*B*) Paired hemibladders were incubated according to the group A protocol or to the same protocol with Na azide present in the serosal solution (group D). Curves are the average of 10 repeated experiments and have been normalized for protein concentration as in Fig. 1. A single exponential fit through the first 50 ms of the 23°C incubation data is given. Results for a series of measurements performed on separate bladder preparations are given in Table I.

(relative normalized amplitude 0.6), supporting the interpretation that it represents nonspecific, probably large membrane fragments with entrapped 6CF.

To show that the fast fluorescence decrease does not arise from basal membrane endocytosis, the group A protocol was repeated with 15 mM 6CF in the serosal solution, instead of in the mucosal solution. The microsomal pellet was prepared in the usual manner and vesicles were subject to a 60-mM inwardly directed osmotic gradient in the stopped-flow apparatus. In two sets of experiments, there was no rapid fluorescence decrease; there was a low amplitude, slow fluorescence decrease with a time constant of ~2 s and an amplitude of ~10% of that measured in vesicles

prepared by the group A protocol with 6CF in the mucosal solution. Therefore, basal membrane endocytosis does not contribute importantly to the measured fluorescence signals.

The possibility that vasopressin-induced endosomes fuse with intracellular membranes having different water permeability properties was next examined. The two 15-min incubations with mucosal 6CF were replaced by a single 10-min incubation with mucosal 6CF after the removal of vasopressin from the serosal solution. If significant changes in water permeability properties of endosomes occurred by fusion events, then the shorter incubation might result in a change in the time constant for the fast fluorescence decrease or a marked decrease in its signal amplitude. This was not the case. In three separate preparations, the time constant (9 ± 1 ms, SEM,

TABLE I

Osmotic Water Transport in Endocytic Vesicles from Toad Bladder

	Fast process		Slow process	
	Time constant	Relative amplitude	Time constant	Relative amplitude
	ms		*s*	
Vasopressin effect				
+VP (group A)	11 ± 1	1.0	1.6 ± 0.2	1.5 ± 0.3
−VP (group B)	—	0.0	1.7 ± 0.2	0.3 ± 0.1
Effect of incubation temperature				
23°C (group A)	10 ± 2	1.0	1.5 ± 0.3	1.3 ± 0.1
0–2°C (group C)	—	0.0	1.0 ± 0.4	2.6 ± 0.4
Effect of Na azide addition				
−Azide (group A)	9 ± 2	1.0	1.4 ± 0.2	1.3 ± 0.2
+Azide (group D)	—	0.0	1.6 ± 0.2	0.4 ± 0.1

Experiments were carried out using paired hemibladders. Osmotic water permeability was compared in endocytic vesicles prepared by group A vs. B, A vs. C, and A vs. D protocols as described in Methods. Vesicles were subject to a 60 mOsmol inwardly directed osmotic gradient at 18°C. Data are the mean ± SD for experiments performed on three to four separate vesicle preparations. Data for the first 50 ms ("fast process") and for the 0.1–5-s time interval ("slow process") were fitted to single exponential functions. The relative amplitude of the fast exponential process for the group A vesicles was set to unity. Relative amplitudes were normalized for total protein in the vesicle suspension used for stopped-flow measurements.

$n = 3$) did not differ significantly from that measured in the group A protocol (9.6 ± 0.2 ms, SEM, $n = 18$). The signal amplitude of the fast component, normalized for total protein, was ~40% of that measured in microsomes prepared by the group A protocol. It is therefore unlikely that the rapid transport in endocytic vesicles is an artifact of membrane fusion.

Toad bladders labeled with 6CF by group A and B protocols were viewed by epifluorescence microscopy (Fig. 3). The image of the vasopressin-treated group A bladder showed the presence of widely distributed, punctate areas of fluorescence in many cells. This pattern is characteristic of endocytic fluorophore uptake (Lencer et al., 1990) and is not visually different from the pattern observed when bladders

were labeled with fluorescein-dextran using the group A protocol (not shown), or that reported by Harris et al. (1986) for bladder labeling by rhodamine-dextran. In contrast, there was very little labeling of bladders not treated with vasopressin (group B protocol). Similar images showing very little 6CF uptake were obtained when endocytosis was inhibited in the group C and D protocols (not shown).

Physical Properties of the Water Channel

The apparent activation energy (E_a) for P_f was measured from the temperature dependence of the fluorescence time course (Fig. 4, *top two curves,* and Table II). The time constants for osmotic equilibration decreased from 9.8 to 7.5 ms with a temperature increase from 18 to 30°C, giving an E_a of 3.9 kcal/mol (16 kJ/mol). E_a values of <6 kcal/mol have been associated with water passage through specific pores and channels (see Finkelstein, 1987). Estimated E_a for the slower fluorescence

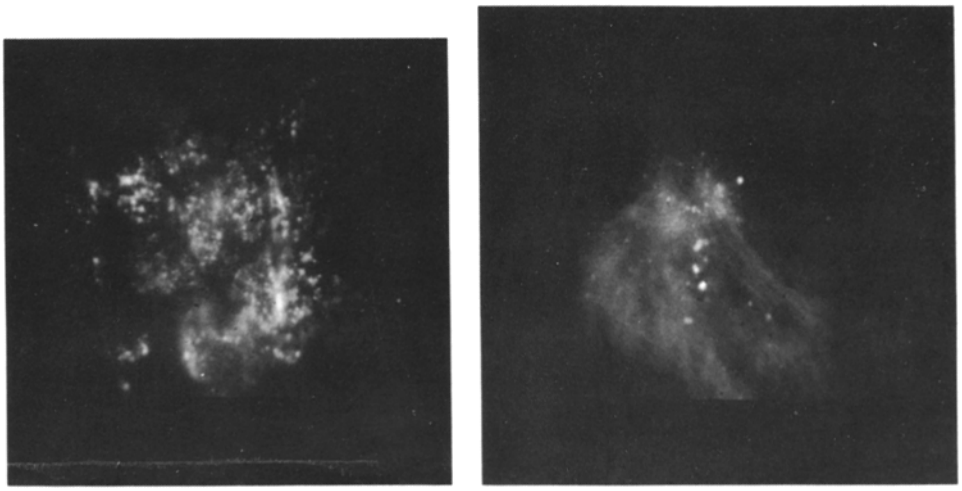

FIGURE 3. Epifluorescence micrographs of toad bladder endosomes labeled with 6CF. Bladders prepared by group A (*left*) and B (*right*) protocols were imaged as described in Methods. The same signal amplification and offset were used for both images. The total width of each photo corresponds to 80 μm.

decrease was 9 kcal/mol, suggesting the absence of a facilitated pathway for osmosis in the nonendocytic membranes containing 6CF.

Effects of $HgCl_2$, an inhibitor of osmotic water transport in red cells, the renal proximal tubule and the intact toad urinary bladder, were examined. In three sets of experiments, 0.5 mM $HgCl_2$ inhibited osmotic water permeability in the vasopressin-induced endocytic vesicles by 22 ± 5% (SEM, $n = 3$). 1 mM $HgCl_2$ inhibited osmotic water permeability by 66 ± 8% (SEM, $n = 3$); inhibition was reversed by >80% with the addition of 5 mM mercaptoethanol after the incubation with 1 mM $HgCl_2$. In three sets of experiments, osmotic water permeability in endocytic vesicles was not inhibited significantly by 0.05, 0.1, and 0.2 mM $HgCl_2$.

To determine the absolute osmotic water permeability coefficient (P_f) for the vasopressin-dependent endocytic vesicles, the dependence of fluorescence on vesi-

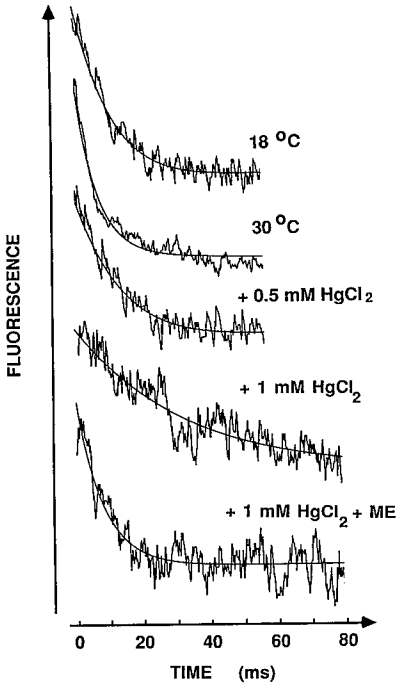

FIGURE 4. Effect of temperature and $HgCl_2$ on osmotic water transport in endocytic vesicles from toad bladder. Endocytic vesicles were prepared from toad bladders incubated with vasopressin at 23°C (group A protocol) as described in Methods. Osmotic water transport was measured in response to a 60-mOsmol inwardly directed osmotic gradient at 18 and 30°C, and at 18°C after incubation for 5 min with 0.5 mM and 1 mM $HgCl_2$. In the curve labeled "1 mM $HgCl_2$ + ME," 5 mM mercaptoethanol was added to the vesicles incubated with 1 mM $HgCl_2$ just before the stopped-flow experiment. Curves are the average of 10 repeated experiments. Fitted single exponential time constants are summarized in Table II and in the text.

cle volume was established and used in the numerical analysis as described previously (Chen et al., 1988). In one set of experiments, typical of two, the relative signal amplitudes, determined by single exponential fits to the first 50 ms of the fluorescence time course, were 0 (0 mOsmol gradient), 0.38 (30 mOsmol gradient), 0.52 (45 mOsmol gradient), 0.73 (60 mOsmol gradient), and 1.0 (120 mOsmol gradient). Based on these results and the surface-to-volume ratio for the endocytic vesicles of 6×10^5 cm^{-1}, P_f was 0.058 ± 0.002 cm/s (18°C) for a 60-mOsmol inward gradient (18 separate preparations). If a significant nonmembrane second barrier to osmosis was present or if vesicle surface area decreased with vesicle shrinkage, apparent P_f might decrease with increasing osmotic gradient size (Illsley and Verkman, 1986). P_f calculated in response to inwardly directed osmotic gradients of 30

TABLE II

Temperature Dependence of Water Transport in Endocytic Vesicles

Temperature	Fast process	Slow process
°C	*ms*	*s*
18	9.8	1.8
30	7.5	1.0
E_a(kcal/mol)	3.9 ± 0.8	9 ± 2

Stopped-flow measurements were carried out at 18 and 30°C for three separate sets of endocytic vesicles prepared by the group A protocol. Data were analyzed as described in the legend to Table I. Activation energies (E_a) were calculated for each set of paired measurements from the slopes of Arrhenius plots. The mean ± SD for the three separate sets of E_a values are given.

and 45 mOsmol was 0.10–0.12 cm/s and 0.07–0.08 cm/s (range for two sets of experiments performed on separate preparations), respectively. These results suggest that the true membrane P_f is >0.1 cm/s (see Discussion).

DISCUSSION

On the basis of studies correlating morphology and water permeability in intact toad bladder, it is generally believed that water transport units, viewed by freeze-fracture electron microscopy as particle aggregates, are cycled to and from the cell mucosal surface in response to vasopressin action. This scheme is based on the unproven assumption that membrane fragments that are inserted into and retrieved from the cell mucosal membrane contain functional water transporters. The purpose of the present study was to determine by a direct functional assay whether vasopressin-induced endocytic vesicles from the luminal membrane of toad urinary bladder contained water transporters. Endocytic vesicles were labeled in intact toad bladder by a membrane-impermeable fluorophore that underwent concentration-dependent self-quenching in response to osmotically induced endosome shrinkage. Osmotic water transport in isolated endocytic vesicles was measured by a stopped-flow fluorescence technique having millisecond time resolution. It was found that endocytic vesicles from mucosal membrane, formed in response to vasopressin addition and removal from the serosal surface in the presence of a serosal-to-mucosal osmotic gradient, had very high water permeability, and as described below, the highest water permeability reported in any biological or artificial membrane. Endocytic vesicles with high water permeabilities were not present in control toad bladders not exposed to vasopressin, and bladders in which endocytosis was turned off by low temperature or by energy depletion with the addition of Na azide. These findings provide evidence that functional water transporters are retrieved from the bladder mucosal membrane by vasopressin-dependent endocytosis, which supports the membrane cycling hypothesis for control of transepithelial water permeability by vasopressin. In addition, the ability to assay water permeability in endocytic vesicles provides a functional marker to characterize the vasopressin-sensitive water transporter and to follow endosome purity in fractionation procedures.

Because of the very high water permeability in the endocytic vesicles and the small quantities of membrane material available from toad bladder cells, there were a number of technical refinements in the experimental design necessary to measure water permeability. The fluorescence labeling procedure chosen for these studies was based on reported data from several laboratories showing stimulation of toad bladder endocytosis by a serosal-to-mucosal osmotic gradient in the presence of serosal vasopressin and in response to removal of serosal vasopressin (Masur et al., 1984; Harris et al., 1986; Harris and Handler, 1988). Bladders were first maximally stimulated by vasopressin for 30 min in the absence of a transepithelial osmotic gradient to load water transporters onto the mucosal membrane. The fluid-phase marker 6CF was added to the mucosal surface at the same time that a maximal serosal-to-mucosal osmotic gradient was applied. Bladders were incubated for 15 min under these conditions followed by an additional 15 min after vasopressin removal. The total time of 30 min was chosen to obtain an acceptable yield of labeled endocytic vesicles with minimal incorporation of endocytic vesicles into

other subcellular compartments such as lysosomes and multivesicular bodies. The homogenization and differential centrifugation procedures were optimized empirically to minimize trauma to endocytic vesicles and to maximize the yield of endocytic vesicles in the final microsomal pellet. The influence of osmotic gradient size and vasopressin concentration on the formation of endocytic vesicles containing water channels was not examined in this study, but is reported in later studies (Shi et al., 1990).

A high concentration of 6CF was chosen to label endosomes. 6CF is a fluid-phase marker that has a high quantum yield and molar absorptivity, and is membrane impermeable at pH 8.5 (Chen et al., 1988). 6CF undergoes concentration-dependent self-quenching with a response time of much less than 1 ms. Although fluorescein- or rhodamine-labeled dextrans also undergo fluorescence self-quenching and have been used widely as fluid phase markers of endocytosis, the total fluorescence signal from endocytic vesicles labeled with fluorescein-dextran at high concentration was less than 10-fold of that obtained using 15 mM 6CF, making it difficult, but possible to measure rapid osmotic water transport. To measure the fluorescence time course of rapid osmotic water transport using 15 mM 6CF, the signal-to-noise ratio was ~15:1 for the average of 10–15 individual time courses, each consisting of 512 data points that were obtained using a single-pole 0.5 ms RC filter. The signal-to-noise ratio was 3:1 when 7.5 mM CF was used. The use of a low–ionic strength, low-osmolarity buffer for loading endosomes was important for the measurement of rapid osmosis. The apparent half-time for equilibration of endosome volume by osmotic water transport increases with increasing internal osmolarity and osmotic gradient. The half-time for osmotic water movement would have been ~2 ms if a 210-mOsmol internal buffer were used, a time comparable to the instrument dead time. In addition, the self-quenching efficiency of 6CF decreases markedly with increasing ionic strength. The rapid time course of osmotic water transport was not measurable when 25 mM KCl was used in place of mannitol.

The osmotic water permeability coefficient (P_f) in the vasopressin-induced endocytic vesicles, for small osmotic gradients, was ~0.1 cm/s at 18°C. This value is much higher than the P_f of 0.0001–0.005 cm/s for lipid bilayers and biological membranes that do not contain water channels (Fettiplace and Haydon, 1980). This value is also notably higher than the P_f reported for membranes known to contain water channels including human erythrocytes (0.02 cm/s; Macey, 1984), proximal tubule apical and basolateral membranes (0.01–0.03 cm/s; Meyer and Verkman, 1987), and vasopressin-induced endosomes from rat kidney papilla (0.03 cm/s; Verkman et al., 1988). In toad bladder granules, which fuse, together with aggrephores, with the mucosal membrane in response to vasopressin stimulation (Masur et al., 1986), P_f was reported to be 0.0005 cm/s at 23°C (Verkman and Masur, 1988). The high P_f in toad bladder endocytic vesicles therefore constitutes strong evidence in itself for the presence of functional water channels. The measured P_f of ~0.1 cm/s should be regarded as a lower limit for the true P_f of the endocytic vesicle membrane because of possible second barriers to water diffusion, such as cytoskeletal constraints or unstirred layers that might limit the detection of faster rates of water permeability (Illsley and Verkman, 1986). The increase in apparent P_f with

decreasing osmotic gradient is consistent with but does not prove this possibility. If <5% of the toad bladder luminal membrane consists of inserted intracellular membrane fragments, then the true P_f of a fully functional endosome membrane might be >0.4 cm/s, 20 times the P_f of a vasopressin-stimulated bladder.

In addition to high P_f, other characteristics supporting the presence of water channels in a membrane are a low activation energy (generally under ~6 kcal/mol) a ratio of osmotic-to-diffusional water permeability coefficients (P_f/P_d) >1 and inhibition of P_f by mercurials (Finkelstein, 1987; Verkman, 1989). E_a for the vasopressin-dependent endocytic vesicles was ~4 kcal/mol, which is consistent with the presence of a functional water channel. P_f/P_d could not be determined because P_d would be immeasurably fast with current magnetic resonance or fluorescence techniques (Ye and Verkman, 1989); if $P_f/P_d = 10$, the exchange time for labeled water would be ~0.1 ms. P_f was inhibited reversibly by >60% by 1 mM $HgCl_2$ with a K_d of >0.5 mM. A similar inhibition of transepithelial P_f by $HgCl_2$ was reported recently in intact toad bladder where apical membrane water channels were fixed with gluteraldehyde (Hoch et al., 1989). In the red cell, P_f was inhibited 90% by $HgCl_2$ with a K_d of ~0.02 mM (Macey, 1984). In proximal tubule apical membrane vesicles and apical endosomes, P_f was inhibited ~70% with a K_d of ~0.3 mM (Meyer and Verkman, 1987; Ye et al., 1989). In proximal tubule basolateral membranes, P_f was inhibited ~75% with a K_d of 0.07 mM (Meyer and Verkman, 1987). The lower inhibitory potency of $HgCl_2$ in toad bladder endocytic vesicles compared with red cells suggests that the vasopressin-sensitive water channel is biochemically different from the water channel in red cells.

Although notable differences exist between toad urinary bladder and kidney collecting tubule in the morphology of presumed water channel–containing intracellular membranes, the regulation of apical membrane water permeability by vasopressin involves a cycle of exocytosis and endocytosis of membranes containing water channels. The present results and those reported recently for endosomes from papillary collecting tubule (Verkman et al., 1988) show that functional water channels are retrieved from the apical membrane surface in both types of epithelia by vasopressin-dependent endocytosis. For studies of the biophysical properties of the vasopressin-dependent water channel, the toad bladder endocytic preparation has the advantage in that it is possible to control directly the endocytic process in the in vitro toad bladder without the potentially confounding factors of the in vivo labeling procedure required in the mammalian kidney. For isolation of large quantities of endocytic material for biochemical characterization of water channels and for water channel isolation, the kidney preparation has the potential advantage that large quantities of membranes containing water channels can be isolated. The results reported here establish the use of vasopressin-induced endocytic vesicles as selective carriers of the vasopressin-dependent water channel, and thus provide a basis for further studies of the transport and biochemical characteristics of water channels.

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