DIRECT FLUORESCENCE MEASUREMENT OF DIFFUSIONAL WATER PERMEABILITY IN THE VASOPRESSIN-SENSITIVE KIDNEY COLLECTING TUBULE

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ABSTRACT A fluorescence method has been developed for accurate and instantaneous measurement of transepithelial diffusional water permeability (P_d) in perfused kidney tubules based on the sensitivity of the fluorophore aminonapthelane trisulfonic acid (ANTS) to solution H_2O/D_2O content. The fluorescence of ANTS was 3.2-fold lower in an H_2O buffer than in a D_2O buffer. The response of ANTS fluorescence to a change in solution H_2O/D_2O content occurred in <1 ms and was due to a collisional quenching mechanism. Isolated cortical (CCT) and outer medullary (OMCT) collecting tubules from rabbit were perfused with an isosmotic D_2O buffer at specified lumen flow rates (2–100 nl/min); tubules were bathed in isosmotic H_2O or D_2O buffers in which vasopressin (VP) could be added rapidly. Lumen fluorescence was monitored by quantitative epifluorescence microscopy at 380 ± 5 nm excitation and >530 emission wavelengths. P_d was determined from tubule geometry, lumen flow, ANTS fluorescence, and ANTS fluorescence vs. H_2O/D_2O calibration relation. The instrument response time for a change in bath H_2O/D_2O content was <4 s. At 37°C, P_d values (mean ± SE in cm/s × 10^4) were 6.4 ± 1.0 (−VP, n = 9) and 14.3 ± 1.1 (+250 μU/ml bath VP, n = 9) in the CCT, and 5.8 ± 1.0 (−VP, n = 6) and 15.3 ± 2.0 (+VP, n= 6) in the OMCT; at 23°C, P_d was 5.1 ± 0.6 (−VP, n = 4) and 7.8 ± 0.6 (+VP, n = 4) in the CCT. In response to rapid addition of 250 μU/ml vasopressin to the bath, CCT P_d remained unchanged for 71 ± 10 s (n = 9, 37°C) and 170 ± 45 s (n = 4, 23°C); this was followed by a slow increase in P_d ($T_{1/2}$ = 91 ± 17 s, 37°C; 119 ± 31 s, 23°C) to the new steady-state value. These results provide a new approach for study of transepithelial water transport in kidney tubules. Compared with ^3H_2O methods, the fluorescence method is superior in technical simplicity, time resolution, and accuracy. The improved time resolution is important for examination of the pre-steady-state kinetics of vasopressin-induced signalling events resulting in the hydroosmotic response.

INTRODUCTION

Measurement of transepithelial water permeability in perfused kidney tubules provides important information about the physiology of urinary concentration and dilution and about the regulation and biophysics of the membrane water pathway. The basal water permeability of the mammalian collecting tubule is very low. In response to binding of vasopressin (antidiuretic hormone; VP) to the basal membrane of tubule cells, there is a dramatic increase in the water permeability of the apical (luminal) membrane (Grantham and Burg, 1966; Schafer and Andreoli, 1972; Hall and Grantham, 1980; Hebert and Andreoli, 1980; Reif et al; 1984), thought to be due to the insertion of intracellular vesicles containing water channels into the apical surface (Harmanci et al., 1978; Brown and Orci, 1983).

Osmotic water permeability (P_f) is the transmembrane volume flow in response to an osmotic or hydrostatic gradient; diffusional water permeability (P_d) is the diffusional movement of water across a membrane in the absence of an osmotic driving force (Finkelstein, 1987). The ratio P_f/P_d is unity for water passage through membrane phospholipid, and >1 (usually >3) for water passage via channels or pores (Levine et al., 1984). Measurement of transepithelial P_d in perfused tubules has required perfusion of tubules with an isosmotic buffer containing ^3H_2O. P_d is determined from tubule geometry, lumen flow, and the ratio of ^3H_2O in the perfused buffer and the buffer collected over a timed period at the distal end of the tubule. The procedure is technically demanding and cannot be used to measure rapid transients in P_d in response to changes in physical or biochemical factors affecting tubule water permeability.

We report here a new fluorescence method to measure P_d instantaneously and accurately based on the sensitivity of an impermeant fluorophore (aminonapthalene trisulfonic acid, ANTS) to solvent H_2O/D_2O composition. ANTS was the best of a series of fluorophores screened for H_2O/D_2O sensitivity; replacement of H_2O by D_2O results

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in a 3.2-fold increase in the quantum yield of ANTS. P_d was determined in perfused collecting tubules from lumen ANTS fluorescence, where lumen/bath were perfused with isosmotic D_2O/H_2O buffers, respectively. The method is applied to the measurement of P_d in cortical and outer medullary collecting tubules and of the pre-steady-state kinetics of P_d increase after rapid addition of vasopressin to the solution bathing the tubule.

METHODS

Materials

ANTS was obtained from Molecular Probes Inc., Junction City, OR. ^{3}H-Inulin was purchased from New England Nuclear, Boston, MA. Perfusion pipettes were made from glass tubing (Drummond Scientific Co., Broomall, PA).

Cuvette and Stopped-Flow Fluorescence Measurements

Spectral and lifetime studies were performed using an SLM 48000 multifrequency phase-modulation fluorometer (SLM Instruments, Urbana, IL) interfaced to an IBM PC/AT computer. Corrected fluorescence spectra were obtained using a rhodamine B quantum counter (excitation spectra) and monochromator/photomultiplier correction curve generated with a calibration lamp (emission spectra) (Lakowicz, 1983). For lifetime measurements, fluorescence was excited at 380 ± 1 nm and detected through two KV508 cut-on filters (Schott Glass, Duryea, PA). Ground state heterogeneity analysis was performed using at least six modulation frequencies (2–150 MHz) referenced to a 1,4-bis(4-methyl-5-phenyloxaxzol-2-yl) benzene solution in ethanol (1.45 ns lifetime).

Stopped-flow measurements were performed using a stopped-flow apparatus (Hi-Tech, Wiltshire, UK) interfaced to a MINC 11/23 computer (Digital Equipment Corp., Maynard, MA). Fluorescence was excited at 380 ± 8 nm using a 100-W Hg lamp and a monochromator in series with a 380 ± 10 nm six-cavity interference filter (Omega Optical, Brattleboro, VT), and detected through KV508 cut-on filters. The instrument dead time was 1.6 ms with 99% mixing efficiency in <0.5 ms.

In Vitro Microperfusion System

Isolated segments of rabbit cortical collecting tubule (CCT) and outer medullary collecting tubule (OMCT) were dissected and perfused in vitro as described previously (Burg et al., 1966). Briefly, kidneys from New Zealand White rabbits were cut in coronal slices. Fragments of tubules were dissected in a cooled (4°C) bath solution and transferred to a bath of 200 μl volume. Tubules were mounted between perfusion and holding pipettes. Luminal perfusion rate was maintained at 2–100 nl/min with a nanoliter infusion pump (Harvard Apparatus, Natick, MA) driving a 10-μl Hamilton syringe which was connected to the perfusion pipette with polyethylene tubing. The actual perfusion rate was calibrated against the set pump rate by timed collections of ^{3}H-inulin. With pipettes and tubule in place, the calibrated actual/set pump rates ranged from 0.8 (2 nl/min) to 0.94 (120 nl/min) for the Hamilton syringe used for all studies.

Bath solution was preheated to 37°C for most experiments and was continuously exchanged at 5–20 ml/min. At 20 ml/min the bath exchange time was <1 s. The bath solution contained (in mM): 142 NaCl, 4 Na_2HPO_4, 5 KCl, 1 $CaCl_2$, and 5 glucose, pH 7.40. The perfusate contained the same solutes plus 5 mM ANTS dissolved in D_2O. The NaCl concentration was decreased to maintain constant osmolarity. Solution osmolarities were measured with a vapor pressure osmometer (Wescor, Logan, VT) and were adjusted to 290 mosmol/kg water by addition of NaCl or of H_2O or D_2O. Solution pH was adjusted to 7.40 by addition of HCl or NaOH. For vasopressin experiments, synthetic arginine vasopressin (Pitressin, Parke-Davis, Morris Plains, NJ) was added to the bath solution at a concentration of 250 μU/ml. The length and inner diameter of the tubule were measured by an eyepiece micrometer in each experiment.

Fluorescence Measurement System

Luminal ANTS fluorescence was measured using an inverted epifluorescence microscope (Nikon Diaphot, Japan). To minimize instrument autofluorescence, a 25× long working distance fluorotar objective was used (Leitz Wetzlar, FRG). ANTS fluorescence was excited using a 100-W tungsten lamp powered by a stabilized DC supply (Oriel, Stratford, CT) in series with a KGl red/infrared blocking filter (Schott Glass) and a 380 ± 5 nm six-cavity interference filter blocked to 1,200 nm. Under these conditions fluorescence was barely visible to the unaided, dark-accomodated eye; there was no photobleaching or photodynamic injury to the tubule under these conditions. Emitted light was filtered through a 400-nm dichroic mirror and a GG530 sharp cut-on filter (Schott Glass). Fluorescence was detected by an R928S photomultiplier (Hamamatsu Corp., Middlesex, NJ) contained in a cooled housing (FACT 50, Thorn EMI Gencom, Inc., South Natick, MA). The signal was amplified by a DC power supply and amplifier (Ealing Corp., South Natick, MA) and interfaced to an IBM PC/AT computer via a ADALAB-PC analogue-to-digital interface board (Interactive Microware, Inc., State College, PA). The signal was filtered electronically using a single pole RC filter with a 1 s time constant; data was acquired at 30 points/s and averaged over 1-s intervals.

Experimental Protocol and Data Analysis

To eliminate effects of endogenous vasopressin, tubules were perfused for 60–120 min with vasopressin-free buffer at 37°C (Hall and Grantham, 1980; Hebert and Andreoli, 1980; Reif et al., 1984) at a lumen flow rate of 10 nl/min and bath exchange rate of 5 ml/min. Fluorescence was subsequently monitored over a 50–100 μm length of tubule near the holding pipette. The excitation and emission path contained iris diaphrams so that only the specified length of tubule was illuminated and measured. Under these conditions, background fluorescence (measured in every experiment) was <15% of total signal.

P_d was calculated from the relation (Grantham and Burg, 1966)

$$P_d = (V/A) \ln (C_{in}/C_{out}), \quad (1)$$

where V is lumen perfusion rate (nl/min), A is the inner tubule surface area (cm^2), and C_{in} and C_{out} represent the concentration of 'labeled' perfused water at the perfusion (C_{in}) and collection (C_{out}) sites. For ^3H_2O perfusion studies, C_{in} and C_{out} represent the radioactivity of perfused and collected fluids. For ANTS fluorescence studies, C_{in} and C_{out} represent the fraction of D_2O in the perfused fluid (C_{in} = 1.0) and in the fluid whose fluorescence is being measured (C_{out}). C_{out} was determined from the measured ANTS fluorescence and the ANTS fluorescence vs. fraction D_2O calibration (Fig. 1) as discussed below.

RESULTS

The approach used to measure diffusional water permeability in perfused tubules is based on the exchange of D_2O, perfused in the tubule lumen, with H_2O bathing the basal surface of the tubule. The H_2O/D_2O content of a point along the tubule lumen is determined by the tubule diameter, the length of tubule between the perfusion pipette and measuring point, the lumen flow rate, and P_d. The lumen H_2O/D_2O content is determined instantaneously from the fluorescence of a compound sensitive to H_2O/D_2O content.

The requirements for a fluorophore to be used as a

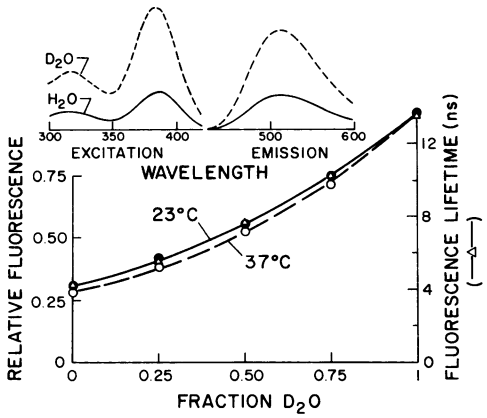

FIGURE 1 Fluorescence properties of ANTS. The relative fluorescence intensity of 5 mM ANTS in the perfusion buffer containing different H_2O/D_2O mixtures was measured near the tip of a perfusion pipette in the epifluorescence microscope. The pipette was immersed in a constant temperature bath. Bath flow was 20 ml/min and pipette flow was 50 nl/min. Relative fluorescence (F) vs. fraction D_2O (f_D) data were fitted to the quadratic relation: $F = 0.466 f_D^2 + 0.242 f_D + 0.284$ (37°C), and $0.387 f_D^2 + 0.300 f_D + 0.307$ (23°C). Fluorescence lifetimes were measured in a cuvette at an ANTS concentration of 0.1 mM. Multifrequency phase modulation data showed a single ANTS lifetime at all H_2O/D_2O ratios. (*Inset*) Fluorescence spectra of 0.1 mM ANTS in buffer containing 100% H_2O or 100% D_2O. Emission was 510 nm for excitation spectrum; excitation was 385 for emission spectrum.

luminal marker for diffusional water exchange are: (*a*) high sensitivity to H_2O/D_2O composition, (*b*) membrane impermeability, (*c*) bright fluorescence in the visible spectrum, (*d*) rapid response to changes in H_2O/D_2O composition, and (*e*) lack of toxicity to tubules. A series of fluorophores, including fluoresceins, rhodamines, coumarins, dansyl compounds, napthalenes, quinolines, indoles, and others, were screened for these characteristics. One fluorophore, ANTS, had the required properties. ANTS has been used as a fluid phase marker for monitoring vesicle fusion in liposome experiments (Smolarsky et al., 1977; Ellens et al., 1984).

The physical properties of ANTS are given in Fig. 1. ANTS has excitation peaks at 320 and 385 nm, with a single emission peak having a marked Stokes' shift at 510 nm. The quantum yield of ANTS is 0.73 in D_2O. The fluorescence of ANTS increases 3.2-fold as the fraction D_2O increases from 0 to 1, due to a change in quantum yield. Fluorescence lifetime studies show a parallel decrease in fluorescence intensity and lifetime, indicating a collisional mechanism for ANTS quenching by H_2O. The fluorescence of ANTS is insensitive to solvent polarity and O_2 tension. In stopped-flow experiments, mixture of ANTS in D_2O with H_2O buffer gives the entire change in fluorescence within the instrument dead time, indicating that ANTS responds in $\ll 1$ ms to changes in solution H_2O/D_2O content, as predicted for a diffusion-limited collisional mechanism.

The three negative charges on ANTS at neutral pH and the data on the entrapment of ANTS in liposomes (Smolarsky et al., 1977; Ellens et al., 1984) suggest that the membrane permeability of ANTS is extremely low. To confirm that ANTS was confined to the tubule lumen space, CCT were perfused at 37°C with 5 mM ANTS in an isosmotic H_2O buffer at a 10 nl/min perfusion rate. The bath was perfused with an isosmotic H_2O buffer containing vasopressin. Lumen fluid was collected using an 80-nl constant volume pipette. Under these conditions, there was no net transepithelial volume flow as measured by lumen ^{3}H-inulin concentration (0.03 ± 0.04 nl/min/mm, $n = 8$). The fluorescence of collected fluid (75.6 ± 0.4 arbitrary fluorescence units, mean ± SE, six tubules) did not differ from that of the perfused fluid (76.0 ± 0.2), indicating confinement of ANTS to the lumen space. After a 1-h lumen perfusion with ANTS followed by perfusion with ANTS-free solution, the increase in fluorescence signal above background was under 1% of that measured during the ANTS perfusion. Therefore cellular uptake of ANTS by diffusion or endocytosis does not contribute measurably to the fluorescence signal.

When the tubule is perfused with ANTS in an isosmotic D_2O buffer and bathed in an isosmotic H_2O buffer in the presence of vasopressin, the lumen fluorescence increases with increased lumen perfusion rate (Fig. 2). At low lumen flow, there is nearly complete exchange of lumen D_2O for bath H_2O at the region of the tubule being illuminated and

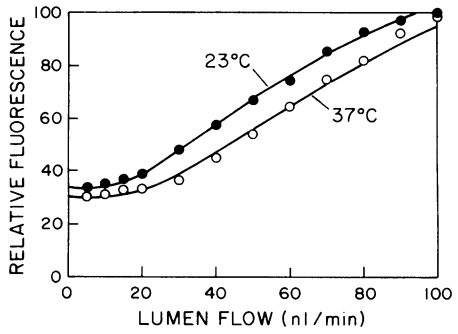

FIGURE 2 P_d determination by flow dependence of ANTS fluorescence. CCT were perfused with isosmotic D_2O perfusion buffer containing 5 mM ANTS at specified lumen flow rates. Fluorescence intensity and tubule diameter were measured at each lumen flow rate after the fluorescence signal stabilized. The distances between the perfusion pipette and the measuring area were 1.2 mm (23°C) and 1.0 mm (37°C). The average lumen diameter increased from 21 to 29 μm with increasing lumen flow from 5 to 100 nl/min. Plotted relative fluorescence represents the fluorescence intensity corrected to the tubule diameter measured at a 20 nl/min flow rate. Correction factors were generated from the square of the ratio of tubule diameter at a lumen flow of 20 nl/min to the tubule diameter at a given lumen flow. Correction factors were determined independently by measurement of ANTS fluorescence in an equivalent experiment in which lumen was perfused with ANTS in H_2O buffer; under these conditions changes in fluorescence were due only to changes in lumen diameter. Both methods gave equivalent correction factors. Relative fluorescence (F_r) vs. lumen flow (V) data were fitted to the relation $F_r = A + B \cdot F[\exp(P_d A/V)]$, where $F[x]$ is the function which converts fraction D_2O to fluorescence units (see legend to Fig. 1) and A and B are arbitary parameters related to amplifier gain and offset. Fitted P_d values were 20.4 ± 1.5 cm/s (37°C) and 10.2 ± 0.7 cm/s (23°C).

measured (typically 0.7–1.0 mm from perfusion pipette). As flow approaches zero, measured fluorescence asymptotically approaches the constant value predicted for ANTS in an H_2O buffer. At high lumen flow, there is less transit time for volume movement through the tubule resulting in less exchange of lumen D_2O for bath H_2O, giving a higher fluorescence signal. As P_d increases (23 to 37°C), the curve is shifted to the right; a higher lumen flow is required to effect the same amount of D_2O/H_2O exchange. Data were fitted to Eq. 1 as described in the legend to Fig. 2. P_d values for the CCT were $(20.4 \pm 1.5) \times 10^{-4}$ cm/s (37°C, +VP) and $(10.2 \pm 0.7) \times 10^{-4}$ cm/s (23°C, +VP). In the absence of VP, P_d obtained by the flow dependence protocol was $(6.2 \pm 0.5) \times 10^{-4}$ cm/s (37°C) (data not shown).

The kinetics of the response of lumen fluorescence to a change in the bath H_2O/D_2O composition is given in Fig. 3. CCT were perfused with an isosmotic D_2O buffer containing ANTS. The bath was switched between isosmotic H_2O and D_2O buffers. The curve shows a rapid signal response (half time <4 s) without lag. Because of the small lumen volume (~0.2 nl for a 1-mm tubule length), there is rapid replacement of the lumen space with new perfused fluid (~1 s at 10 nl/min lumen flow), predicting a very brief transient response. Therefore rapid physiological changes in P_d are measurable with this perfusion system.

The pre-steady-state kinetics of P_d increase after vasopressin addition to the bath are shown in Fig. 4. Interestingly, there is a lag phase in which P_d does not change, followed by a slow increase in P_d (decrease in fluorescence). Results for a series of tubules are summarized in Table I. P_d increased significantly in all groups. The lag phase in P_d for the CCT at 37°C was similar to that for the OMCT at 37°C, but significantly shorter than the lag phase at 23°C ($P < 0.025$). These results are quite reproducible in different tubules, and give important information about the intracellular vasopressin signalling events (see Discussion).

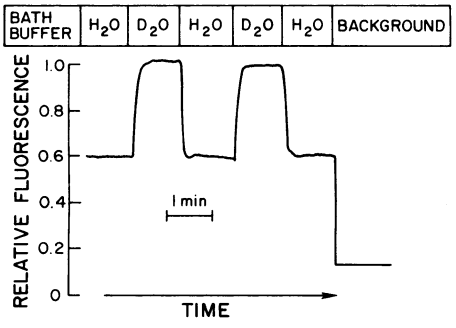

FIGURE 3 Response of lumen fluorescence to bath composition. CCT (length, 0.9 mm) was perfused with D_2O buffer containing 5 mM ANTS at 37°C at a lumen flow rate of 20 nl/min. Bath buffer was switched between isosmotic buffers containing 100% H_2O and 100% D_2O at a flow rate of 10 ml/min. The average fitted exponential time constant for the signal response was 3.2 s. The background signal was measured by moving the perfused tubule out of the measuring area.

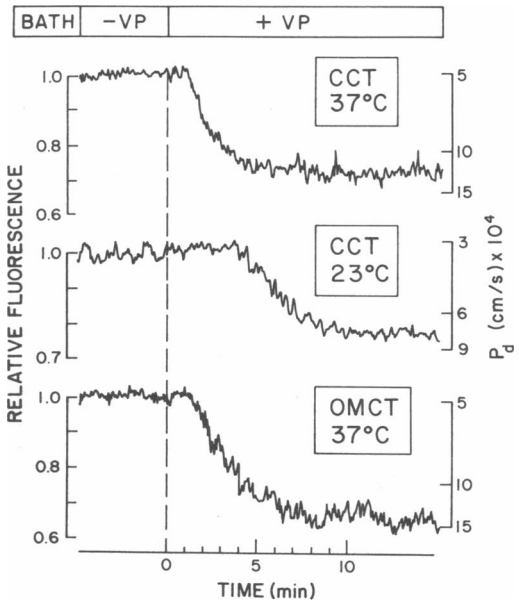

FIGURE 4 Pre-steady-state kinetics of vasopressin effect on P_d. Tubules were perfused with isosmotic D_2O buffer containing 5 mM ANTS at a lumen flow rate of 20–30 nl/min. Tubule lengths and inner diameters were 0.68 mm and 24.2 μm, respectively (CCT, 37°C)), 0.71 mm and 23.1 μm (CCT, 23°C), and 0.62 mm and 23.1 μm (OMCT, 37°C). Tubules were perfused at least 60 min at 37°C in the absence of vasopressin before the start of the experiment. Tubules were bathed in an isosmotic buffer containing H_2O. Where indicated, bath fluid was changed to an isosmotic H_2O buffer containing 250 μU/ml vasopressin at a rate of 10 ml/min. In response to vasopressin, fluorescence signal decreased gradually after a time lag. After the vasopressin response reached a stable value, tubules were perfused at a lumen flow of 2 nl/min and the fluorescence signal and inner diameter were measured. Under these conditions, it is assumed that all luminal fluid is H_2O (see Fig. 2). Inner diameter of tubules did not change in response to vasopressin addition. P_d values (right ordinate) were calculated from tubule geometry, lumen flow, relative fluorescence, the ANTS fluorescence vs. fraction D_2O calibration curve, and the fluorescence signal measured at a lumen flow rate of 2 nl/min.

DISCUSSION

A method has been described for measurement of transepithelial diffusional water permeability in perfused tubules based on the sensitivity of an impermeant fluorophore (ANTS) to solution H_2O/D_2O content. In previous reports of P_d measurement in renal proximal and collecting tubules, transepithelial P_d was measured by perfusion and timed collection of ^3H_2O. There are two important advantages of a fluorescence method over the ^3H_2O perfusion method: (*a*) Improved time resolution. The instantaneous values of P_d can be measured in real time with 1 s resolution. (*b*) Technical simplicity. Measurement of P_d by fluorescence does not require collection of perfused fluid, the major source of difficulty and error in the isolated perfused tubule technique. In addition, the fluorescence method does not require the use of ^3H_2O with high specific activity. Because the tubule is mounted for fluorescence studies, parallel determination of the time courses of P_d

TABLE I
PRE-STEADY-STATE KINETICS OF VASOPRESSIN EFFECT ON P_d

Tubule segment	n	Temp	P_d		T_{lag}	$T_{1/2}$
			−VP	+VP		
		°C	$cm/s \times 10^4$		s	s
CCT	9	37	6.4 ± 1.0	14.3 ± 1.1	71 ± 10	91 ± 17
CCT	4	23	5.1 ± 0.6	7.8 ± 0.6	170 ± 45	119 ± 31
OMCT	6	37	5.8 ± 1.0	15.3 ± 2.0	87 ± 14	110 ± 13

The perfusion protocol given in the legend to Fig. 4 was used. Values are mean ± SE for experiments performed using n separate tubules. Data from every perfused tubule was included in the analysis. The mean tubule length between perfusion pipette and measuring area was 0.75 ± 0.03 mm (n = 19) with a range from 0.62 to 1.06. For most measurements the lumen perfusion rate was 30 nl/min (range 20–40 nl/min). T_{lag} was defined as the time between addition of bath vasopressin and the start of the vasopressin response. $T_{1/2}$ was defined as the difference in time between the start of the vasopressin response and the time at which fluorescence decreased to 50% of the difference between initial and final fluorescence values. Addition of 250 μU/ml vasopressin increased P_d significantly in all groups ($P < 0.001$ in CCT at 37°C; $P < 0.025$ in CCT at 23°C; $P < 0.005$ in OMCT at 37°C).

and other parameters that are measurable by fluorescence, such as P_f, calcium, or pH, is possible.

The use of a fluorescent marker to discriminate between solution H_2O and D_2O content was reported by Lawaczeck (1984). They observed that indol compounds underwent a ~30% increase in fluorescence when H_2O was replaced by D_2O. However, low sensitivity of indols to H_2O/D_2O composition, low quantum yield, low excitation (280 nm) and emission (320 nm) wavelengths, and lack of membrane impermeability make these compounds unsuitable for studies in perfused tubules. Fortunately, it was possible to identify one compound, ANTS, which was impermeant (three negative charges at neutral pH) and strongly sensitive to H_2O/D_2O composition (3.2-fold increase in quantum yield in D_2O) with excellent optical characteristics for quantitation by epifluorescence microscopy. Fluorescence lifetime and stopped-flow studies showed that ANTS fluorescence was quenched by H_2O by a collisional mechanism and that ANTS fluorescence responded in <1 ms to changes in solution H_2O/D_2O content (Ye and Verkman, 1988). Perfusion studies showed that ANTS was not taken up measurably into tubule cells and that ANTS was confined to the luminal space. Tubules were perfused for more than 2 h with 5 mM ANTS in D_2O buffer and illuminated at 380 nm without morphological evidence of toxicity and without impairment of the vasopressin-induced hydroosmotic response.

A potential difficulty in the determination of P_d by isotopic labeling of water protons is a possible underestimate in P_d resulting from decreased diffusion of ^2H_2O (D_2O) or ^3H_2O compared with ^1H_2O. It is hard to assess this concern theoretically. In the red cell, where P_d has been measured for ^1H_2O (nuclear magnetic resonance; Fabry and Eisenstadt, 1975), D_2O (light scattering; Lawaczeck, 1984) and ^3H_2O (rapid mixing flow tube; Brahm, 1982), there is good agreement in P_d values obtained by the three techniques. The P_d value in the CCT reported here (14×10^{-4} cm/s, 37°C, +VP) is in good agreement with those reported in ^3H_2O perfusion studies ($11–31 \times 10^{-4}$ cm/s, 37°C, +VP; Hall and Grantham, 1980; Hebert and Andreoli, 1980). At present it is not possible to apply proton nuclear magnetic resonance methods to measure transepithelial P_d in perfused tubules because of inadequate sensitivity and spatial resolution of imaging methods. In one report, proton nuclear magnetic resonance was used to determine basolateral P_d in a suspension of proximal tubules from rabbit kidney (Verkman and Wong, 1987); basolateral membrane P_d (2×10^{-3} cm/s, 37°C) was similar to reported transepithelial P_d (5×10^{-3} cm/s; Berry, 1985) in the rabbit proximal tubule. It is not possible to obtain purified suspensions of kidney collecting tubules for similar studies.

To prevent photodynamic tubule injury and to minimize signal background and fluctuation, several technical details of the measurement system are notable. A long working distance objective with minimal autofluorescence at 380 nm was selected from screening of a series of objectives. The glass lens in the turret of the Nikon microscope required for parfocality was removed because of excessive autofluorescence (>10× background signal). Coaxial excitation and emission iris diaphrams were set to illuminate and measure over a small tubule length to minimal off-axis scattering. The red end of the ANTS emission spectrum (>530 nm) was measured to minimize signal contribution from autofluorescence of tubule cells and of the dichroic mirror. A tungsten lamp powered by a stabilized DC supply gave superior optical stability compared with an Hg arc lamp, with adequate intensity at 380 nm for ANTS excitation without photodynamic tubule injury.

The pre-steady-state time course of P_d increase after addition of vasopressin to the bath provides a new dimension of information not obtainable from steady-state measurements. After vasopressin addition, there is a lag phase in which no measurable increase in P_d occurs, followed by a slow increase in P_d. The lag phase is prolonged at 23°C. The current hypothesis for the signalling events by which vasopressin binding to V_2 receptors on the basal membrane of collecting tubule cells leads to an increase in transepithelial water permeability involves first the activation of adenylate cyclase and production of cAMP (Grantham and Burg, 1966). Through a series of poorly understood events, the cAMP signal induces major cytoskeletal changes in the collecting tubule cell (Kirk et al., 1984) resulting in the insertion of new membrane from intracellular stores into the apical membrane (Wade et al., 1981; Harmanci et al., 1978). The cAMP signal also causes changes in apical membrane morphology and increased retrieval of apical membrane, possibly through a clathrin-

associated mechanism (Brown and Orci, 1983). Recent work from this laboratory showed that a vasopressin-inducible population of endosomes from mammalian collecting tubule contained the vasopressin-sensitive water channel (Verkman et al., 1988), providing strong evidence for the water channel recycling hypothesis.

Because of the ordered sequence of steps required for increase in apical membrane water permeability, it is likely that the lag phase in P_d after vasopressin addition consists of one or more rate-limiting steps proximal to the water channel insertion step. The subsequent slow increase in P_d probably represents the insertion of active water channels, or possibly the activation of water channels that have been inserted into the apical membrane. Although these possibilities are intriguing, it is not the intent of the present study to define the kinetics and ordering of the vasopressin-induced signalling events. Further studies involving parallel measurements of P_d, P_f, cell calcium, and other parameters in response to addition of vasopressin and other activators/inhibitors of the hydroosmotic response are required for definition of individual signalling events (see accompanying paper, Kuwahara et al., 1988).

Measurement of the luminal ANTS signal by epifluorescence microscopy has provided a method to determine transepithelial P_d in perfused tubules with time resolution of 1 s and measured accuracy of under 5% for detection of changes in P_d. However, because the ANTS fluorescence signal depends upon both buffer H_2O/D_2O composition and the total lumen volume which is illuminated and detected photometrically, tubule motion can potentially cause signal artifacts that are unrelated to changes in P_d. To correct for tubule motion, the tubule lumen can be perfused with a pair of fluorophores, one sensitive to H_2O/D_2O composition (ANTS, excitation 380 nm, emission >500 nm) and the other insensitive to H_2O/D_2O composition (pyrenetetrasulfonic acid [PTSA], excitation 380 nm, emission 420 nm). With excitation at 380 nm, the ratio of emission intensities at >500 nm and 420 nm, measured using a beamsplitter and two photomultipliers, depends only on solution H_2O/D_2O composition. We have applied this dual detection approach recently for measurement of P_f in perfused tubules using the pair of fluorophores PTSA and fluorescein sulfonate (Kuwahara et al., 1988). Another modification to the method reported here for improved accuracy of P_d measurements is to use spatially resolved detection (SIT or intensified CCD camera) to measure fluorescence along the full tubule length. The length dependence of the fluorescence intensity (Eq. 1) would provide a major constraint in the fitting procedure, likely to give a marked improvement in the accuracy of P_d determination and in the resolution of small changes in P_d.

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