RAPID DEVELOPMENT OF VASOPRESSIN-INDUCED HYDROOSMOSIS IN KIDNEY COLLECTING TUBULES MEASURED BY A NEW FLUORESCENCE TECHNIQUE

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ABSTRACT The pre-steady-state kinetics of the vasopressin-induced increase in collecting tubule osmotic water permeability (P_f) has been measured by a new fluorescence technique. Isolated cortical collecting tubules (CCT) from rabbit kidney were perfused with physiological buffers containing the impermeant fluorophores fluorescein sulfonate (FS) and pyrenetetrasulfonic acid (PTSA). Tubules were subject to a 120 mOsm bath-to-lumen osmotic gradient in the presence and absence of 250 μU/ml vasopressin. The magnitude of transepithelial volume flow was determined from the self-quenching of FS, or from the ratio of PTSA/FS fluorescence, measured at 380 nm excitation and 420 ± 10 nm (PTSA) and >530 nm (FS) emission wavelengths. P_f was calculated from the magnitude of transepithelial volume flow, lumen and bath osmolarities, lumen perfusion rate, and tubule geometry. The instrument response time for a change in bath osmolality was <3 s. At 37°C, CCT P_f was (in units of cm/s × 10^4) 13 ± 2 (mean ± SE, 16 tubules) before, and 227 ± 10 after addition of vasopressin to the bath. CCT P_f began to increase in 23 ± 3 s after vasopressin addition and was half-maximal after 186 ± 20 s. At 23°C, P_f was 9 ± 1 (seven tubules) before, and 189 ± 12 after vasopressin addition. P_f began to increase in 40 ± 4 s and was half-maximal after 195 ± 35 s. After vasopressin removal from the bath, P_f decreased to its baseline value with a half-time of 14 min. These results establish a direct fluorescence method to monitor instantaneous transepithelial P_f in perfused tubules and show a very fast stimulation of CCT P_f in response to vasopressin.

INTRODUCTION

The stimulation of transepithelial water permeability by vasopressin in the mammalian kidney collecting tubule is an essential component of the urinary concentrating mechanism and of whole organism salt and water balance. The cellular mechanism by which vasopressin stimulates water permeability has been the subject of great interest; it is thought that vasopressin binding to V_2 receptors on the basal membrane of collecting tubule cells results in cAMP production, protein kinase activation, and ultimately, the insertion of intracellular vesicles containing functional water channels into the apical membrane (Harmanci et al., 1978; Wade et al., 1981; Brown and Orci, 1983; Verkman et al., 1988). The detailed sequence, mechanism, and pre-steady-state kinetics of the steps between cAMP production and increased water permeability are not known.

Of central importance for the examination of water permeation mechanisms in the intact kidney tubule is the accurate, time-resolved measurement of transepithelial osmotic water transport. The classical approach has been the timed collection of fluid perfused through the lumen of an isolated tubule segment subjected to a transepithelial osmotic gradient. The osmotic water permeability coefficient (P_f) is determined from lumen flow, tubule geometry, and the magnitude of transepithelial volume movement as assessed from the concentration of an impermeant luminal marker (e.g., ^{14}C-inulin) and/or the measured osmolality of collected fluid (Grantham and Burg, 1966; Schafer and Andreoli, 1972; Berry and Verkman, 1988). The procedure is technically demanding and cannot be used to examine very rapid changes in tubule P_f in response to activators and inhibitors of water transport.

We describe here a simple method for the accurate, instantaneous determination of transepithelial P_f in perfused tubules based on the measurement of fluorescence intensity of impermeant fluorophore(s) perfused through the tubule lumen. A measurement of fluorescence intensity or of a ratio of fluorescence intensities at two wavelengths provides a direct measure of the total magnitude of transepithelial volume movement and thus of tubule P_f. The method is applied to study the magnitude of P_f in cortical collecting tubules isolated from rabbit kidney, and the kinetics of P_f turn-on and turn-off in response to vasopressin addition and removal.

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METHODS

Materials

Fluorescein sulfonate (FS) and pyrenetetrasulfonic acid (PTSA) were obtained from Molecular Probes, Inc., Junction City, OR. Perfusion pipettes were made from glass tubing (Drummond Scientific Co., Broomall, PA).

In Vitro Microperfusion System

Isolated segments of rabbit cortical collecting tubule (CCT) were dissected and perfused in vitro as described previously (Burg et al., 1966; Kuwahara and Verkman, 1988). Briefly, kidneys from New Zealand White rabbits (1.5–2.5 kg) 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. Luminal perfusion rate was maintained at 2–80 nl/min with a nanoliter infusion pump (Harvard Apparatus Co., Inc., Natick, MA) driving a 10-μl Hamilton syringe which was connected to the perfusion pipette with polyethylene tubing. Luminal perfusion rate was calibrated against set pump rates as described previously (Kuwahara and Verkman, 1988).

The bath solution was maintained at 23 or 37°C and was exchanged continuously at 5–20 ml/min. Bath fluid composition was changed by adjusting a four-way valve near the bath. At a 20 ml/min bath exchange rate, the new fluid enters the bath 0.8 s after value adjustment and replaces the old fluid by >95% within an additional 1 s. The control bath solution contained (in mM): 142 NaCl, 4 Na_2HPO_4, 5 KCl, 1 $CaCl_2$, 5 glucose, pH 7.4. The perfusate contained the same solutes plus 10 mM FS or 2.5 mM FS and 5 mM PTSA. The NaCl concentration was decreased to maintain constant osmolality. Perfusate osmolalities were measured with a vapor pressure osmometer (Wescor Inc., Logan, UT) and were adjusted to 290 mosmol/kg H_2O by addition of NaCl or H_2O. Solution pH was adjusted to 7.4 by addition of HCl or NaOH. To determine transepithelial P_f, a bath-to-lumen osmotic gradient of 120 mOsm was imposed by adding sucrose to the control bath solution. 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.

Fluorescence Measurement System

Fluorescence measurements were performed using an inverted epifluorescence microscope (Nikon Diaphot, Japan). To minimize autofluorescence, a 25× long working distance objective was used (LWD fluorotar, N.A. 0.4, Leitz Wetzlar, FRG). Fluorescence was excited using a 100-W tungsten lamp powered by a stabilized DC supply (Oriel Corp., Stratford, CT) in series with a 2.0 neutral density filter and a KG-3 infrared blocking filter (Schott Glass Technologies Inc., Duryea, PA).

For measurement of luminal FS fluorescence when FS was the only fluorophore present, excitation was at 480 ± 10 nm, with 510 nm dichroic mirror and >530 nm emission cut-on filter. Fluorescence was detected by an R928S photomultiplier (Hamamatsu, Middlesex, NJ) containing in a cooled housing (FACT 50, Thorn EMI Gencom, Inc., 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 an ADALAB-PC analogue-to-digital interface board (Interactive Microware, State College, PA). The signal was filtered electronically using a single pole RC filter with 0.3-s time constant; data was acquired at 30 points/s and averaged over 1-s intervals.

For measurement of P_f using the fluorophore pair FS and PTSA, fluorescence was excited using a six-cavity 380 ± 10 nm interference filter and a 400-nm dichroic mirror (Omega Optical, Inc., Brattleboro, VT). In some experiments, emitted light was focused onto a fused silica circular-to-rectangular fiberoptic cable and detected by an photodiode array (EG&G, Salem, MA) and model 1461 detector interface (Princeton Applied Research, Princeton, NJ). In some experiments, emitted light was split by a 50/50 dichroic beamsplitter (Omega Optical, Inc.) and detected using two Hamamatsu 9798Q photomultipliers, one with a 530-nm cut-on filter for detection of FS fluorescence and the other with a 400-nm cut-on filter in series with a 420 ± 10 nm interference filter for detection of PTSA fluorescence. As given in the Results, there was no overlap in emission spectra for these fluorophores.

Experimental Protocol and Data Analysis

To eliminate effects of endogenous vasopressin, tubules were perfused for 90–120 min with vasopressin-free buffer at 37°C (Hall and Grantham, 1980; Hebert and Andreoli, 1980) at a lumen flow rate of 10 nl/min and bath exchange rate of 5 ml/min. For measurement of P_f at 23°C, tubules were perfused for an additional 30 min at 23°C. Fluorescence was monitored over a 50–80-μm length of tubule near the holding pipette or at the tip of the holding pipette (diameter, ~40 μm) just distal to the end of the tubule segment. The advantages of the latter method are lack of effect on fluorescence signal of tubule motion and changes in tubule inner diameter, and elimination of photodynamic tubule injury. 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 and subtracted in every experiment) was <0.5% of total signal for FS and <25% of total signal for PTSA.

P_f was calculated from the relation (Al-Zahid et al., 1977),

$$P_f = -\frac{V_o C_o}{A v_w}\left[\frac{C_o - C_L}{C_o C_b C_L} + \frac{1}{(C_b)^2}\ln\frac{(C_L - C_b)C_o}{(C_o - C_b)C_L}\right], \quad (1)$$

where V_o is the initial lumen perfusion rate (nanoliters per minute), A is the inner tubule surface area (cm^2), v_w is the partial molar volume of water (18 cm^3/mol), C_o and C_L are the initial and collected osmolalities, respectively, and C_b is the bath osmolality. In isotopic studies C_L is determined by the radioactivity of the collected fluid. For fluorescence studies, C_L is determined by the fluorescence intensity (or intensity ratio) of the luminal fluid in the monitoring area (see below). For experiments in which the dependence of FS fluorescence (F) on lumen perfusion rate (V_o) was examined, P_f is fitted by a three-parameter nonlinear regression in which C_L/C_o is related to F by the calibration equation (see legend to Fig. 1)

$$F = a + b[0.0027(10C_L/C_o)^2 - 0.153(10C_L/C_o)], \quad (2)$$

where a and b are arbitrary scale factors related to instrument offset and gain.

Fluorescence Lifetime Measurements

The nanosecond fluorescence lifetime of PTSA in the presence of varying [FS] was determined to prove that FS quenches PTSA fluorescence by a collisional mechanism. Experiments were carried out on an SLM48000 multifrequency phase-modulation fluorimeter. Samples in 25-μl capillary tubes (to minimize inner filter effects) were excited at 380 ± 2 nm; fluorescence was detected through a 420 ± 10 cut-on filter. There was no signal from FS alone under these conditions. Phase and modulation lifetimes were determined at 10, 30, and 50 MHz referenced to a 1,4-bis(4-methyl-5-phenyloxazol-2-yl) benzene solution in ethanol (1.45 ns lifetime; Lakowicz, 1983).

RESULTS

Two methods were used to measure transepithelial P_f: a single-fluorophore and a double-fluorophore technique. In the single-fluorophore technique, tubules were perfused with the impermeant fluorophore FS which undergoes concentration-dependent self quenching. The measured FS signal was used to determine the FS concentration and thus the amount of FS-free water that has moved across

the collecting tubule epithelium in response to an osmotic gradient. In the double-label technique, tubules were perfused with the impermeant fluorophores FS and PTSA. The ratio of PTSA/FS fluorescence intensities measured simultaneously were used similarly to determine transepithelial volume flow. The double-label technique has the advantage that the fluorescence ratio is insensitive to changes in excitation light intensity, tubule motion, and changes in tubule lumen diameter. In addition, the double-label technique has application to measurement of tubule P_f by imaging methods (see Discussion).

The intensity of a fluorophore measured in a segment of tubule lumen is proportional to the number of fluorophore molecules within the tubule segment and to the brightness (quantum yield and molar absorptivity) of the fluorophore. Therefore any impermeant fluorophore with good brightness that undergoes a concentration-dependent change in fluorescence intensity within the tubule lumen is usable as a single label marker of transepithelial volume flow occurring between the perfusion pipette and the measuring area. For the first set of studies, the fluorescein derivative FS was used in its self-quenching concentration range. As reported previously (Chen et al., 1988), FS has peak excitation and emission wavelengths of 480 and 530 nm, respectively, with quantum yield near unity and molar absorptivity of 93,000 $M^{-1}cm^{-1}$. FS does not alter rates of water transport. Fig. 1 shows the relative fluorescence of 10–20 mM FS measured near the tip of a perfusion pipette immersed in a constant temperature bath. FS fluorescence decreased with concentration due to a collisional quenching mechanism. The FS fluorescence vs. [FS] relation was slightly sensitive to temperature, but insensitive to buffer composition at physiological ionic strengths.

Fig. 2 shows an isolated CCT perfused with 10 mM FS.

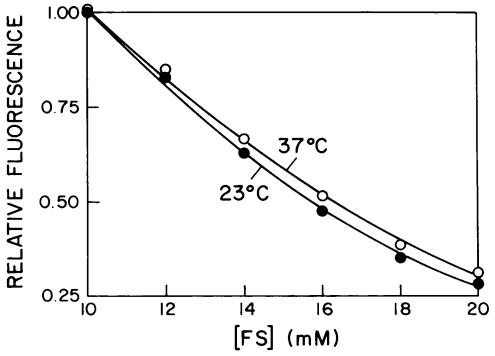

FIGURE 1 Self quenching of fluorescein sulfonate. Relative fluorescence intensity of FS was measured in the control buffer containing 10 mM FS; concentrations of all components of this buffer were increased proportionately to prepare higher [FS] buffer to parallel experimental conditions in which solute-free water is transported from lumen to bath. Fluorescence (F) was measured near the tip of a perfusion pipette (~25 μm diameter) at 480 nm excitation and >530 nm emission wavelengths. Data were fitted empirically to a quadratic relation with: $F = 0.0027[FS]^2 - 0.153[FS] + 2.27$ (37°C) and $F = 0.0036[FS]^2 - 0.183[FS] + 2.48$ (23°C).

Tubule length was 1.6 mm. In the top photo, the full tubule length between perfusion and collection pipettes was illuminated to demarcate the tubule lumen. In the bottom photo, the tubule segment in the region of the collecting pipette is shown at higher magnification; under experimental conditions a small area (50–80 μm) of tubule lumen near the collection pipette was illuminated as shown in the figure. For some experiments a small area of collection pipette just beyond the end of the tubule was illuminated to minimize tubule motion artifacts and effects of changes in tubule lumen diameter on the fluorescence signal.

Experiments were carried out to prove that FS is impermeant under the conditions of the experiment. CCT were perfused at 37°C with 10 mM FS at 10 nl/min in the absence of an osmotic gradient. Lumen fluid was collected using an 80-nl constant volume pipette. The fluorescence of collected fluid (69.3 ± 0.6 fluorescence units, mean ±SE, $n = 6$) did not differ from that of the perfused fluid (70.2 ± 0.5, $n = 4$). Because there was no net transepithelial volume flow under these conditions as shown previously (Kuwahara and Verkman, 1988), these results indicate that FS is confined to the lumen space. After a 90-min lumen perfusion with 10 mM FS followed by perfusion with FS-free solution, the increase in signal above background was <1% of that measured during the FS perfusion (0.7 ± 0.1%, $n = 3$). Therefore cellular uptake of FS does not contribute measurably to the fluorescence signal.

To determine the effect of lumen perfusion rate on lumen FS fluorescence, CCT were perfused with 290 mOsm solution containing 10 mM FS and bathed in a 410 mOsm solution. Lumen flow was varied between 2 and 80 nl/min with a bath exchange rate of 10 ml/min (Fig. 3). Data in the absence (−VP) and presence (+VP) of vasopressin were obtained for the same tubule. As flow approaches zero, measured fluorescence approaches the constant value predicted from the fluorescence calibration curve for complete osmotic equilibration. At high lumen flow, there is less transit time for osmotic equilibration resulting in a lower lumen FS concentration giving a higher fluorescence signal. At a higher P_f in the presence of vasopressin, the data were shifted to the right. The curves fitted through the data were based on the theoretical dependence of FS fluorescence on lumen flow (Eqs. 1 and 2) and give P_f values of $(12 \pm 2) \times 10^{-4}$ cm/s (−VP) and $(286 \pm 14) \times 10^{-4}$ cm/s (+VP).

An important application of the real-time method to measure P_f was the examination of the rapid kinetics of P_f stimulation after vasopressin addition to the bath. To determine the response characteristics of the combined perfusion, optical, and electronic systems, CCT were perfused with a 290 mOsm solution containing 10 mM FS at a lumen flow rate of 10 nl/min (Fig. 4). Bath solution was changed between 290 and 410 mOsm at a flow rate of 10 ml/min. The curve shows a rapid signal response (mean half-time of 2.9 s) without lag. The finite response time is quite consistent with a bath exchange time of ~1.2 s (200

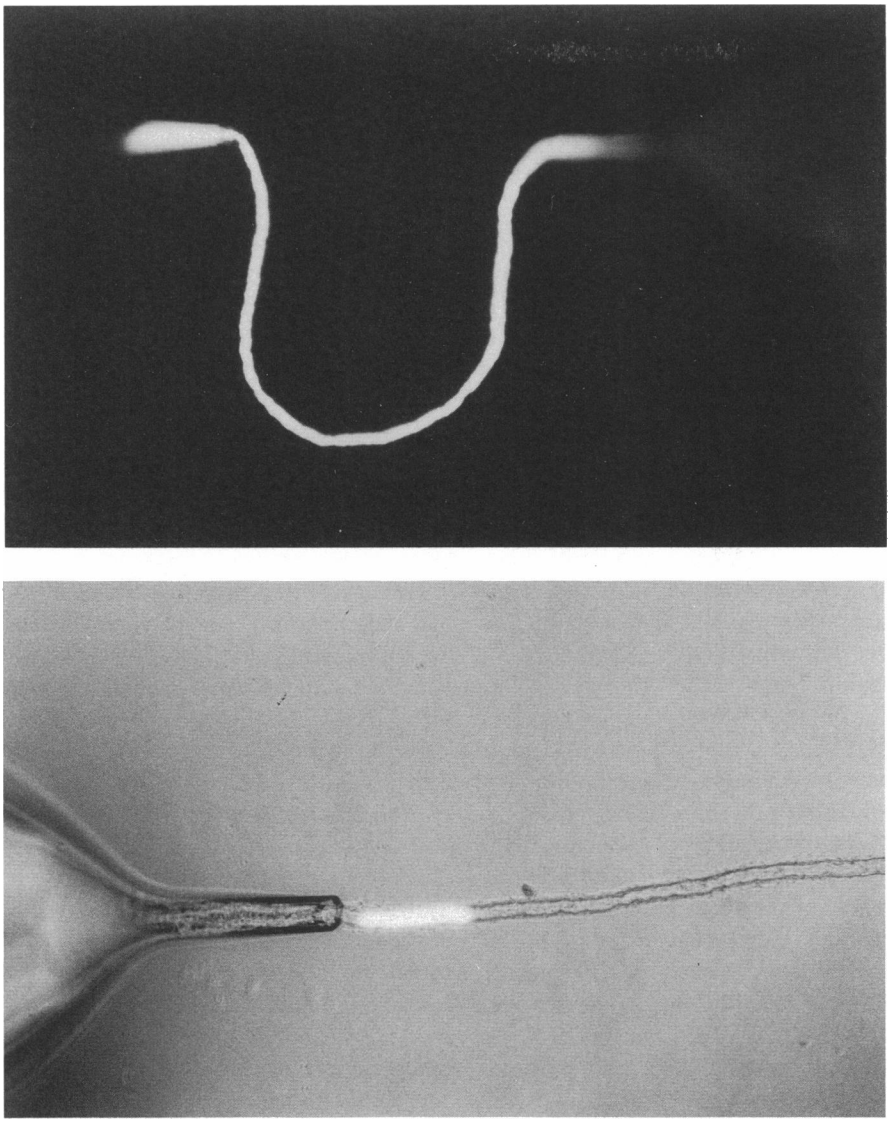

FIGURE 2 Measurement of CCT P_f by luminal FS fluorescence. Rabbit CCT was dissected and mounted between perfusion and collection pipettes as described in Methods. (*Top*) The tubule length was 1.6 mm with diameter 26 μm; for calibration, the distance between the tips of the pipettes was 0.65 mm. The complete tubule was illuminated by epifluorescence (480 nm excitation, >530 nm emission). (*Bottom*) Tubule and collection pipette at higher magnification. Only a small segment of tubule is illuminated by epifluorescence, similar to the experimental protocol.

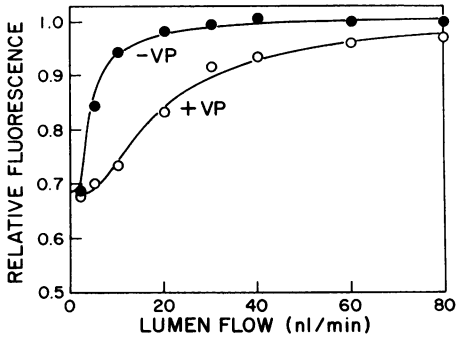

FIGURE 3 P_f determination by dependence of FS fluorescence on lumen flow. CCT were perfused with 290 mOsm solution containing 10 mM FS at specified lumen flow rates (2–80 nl/min). Bath osmolality was 410 mOsm with an exchange rate of 10 ml/min. Relative fluorescence at each lumen flow was recorded just beyond the end of the tubule in the collection pipette after the fluorescence signal stabilized (60–120 s). Tubule length was 1.80 mm with a diameter of 24.2 μm. Relative fluorescence vs. lumen flow data were fitted to Eq. 1 as described in Methods with P_f (in units of cm/s $\times 10^4$) = 12 ± 2 (−vasopressin) and 286 ± 14 (+250 μU/ml vasopressin).

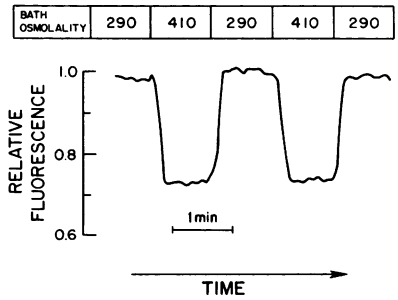

FIGURE 4 Response of lumen fluorescence to bath osmolality in the presence of vasopressin. CCT was perfused with a 290 mOsm buffer containing 10 mM FS at 10 ml/min. Bath osmolality was switched between 290 and 410 mOsm at an exchange rate of 10 ml/min. Both bath solutions contained 250 μU/ml vasopressin. The average half-time for the signal response was 2.9 s.

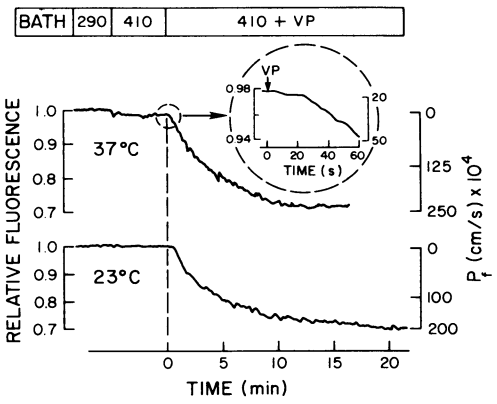

FIGURE 5 Pre-steady-state kinetics of vasopressin effect on P_f. CCT were perfused with 290 mOsm buffer containing 10 mM FS at a lumen flow rate of 10 nl/min at 37°C or 23°C. Where indicated, bath fluid was changed from control (290 mOsm) to hyperosmotic solution (410 mOsm), to a hyperosmotic solution containing 250 μU/ml vasopressin. P_f values (right ordinate) were calculated from tubule geometry, lumen flow, relative fluorescence, and FS fluorescence vs. concentration calibration curve in Fig 1. (*Inset*) Initial P_f response is shown on an expanded scale.

μl bath at a 10 ml/min exchange rate), a half-time for lumen exchange of ~2.5 s (1.77 mm tubule length, 25.4 μm tubule diameter, and 10 nl/min lumen flow), and an electronic response time of ~0.5 s (RC time constant = 0.3 s). Therefore rapid physiological changes in P_f are measurable with this perfusion system.

The pre-steady-state kinetics of P_f response after bath osmolality change and vasopressin addition are shown in Fig. 5. Upon increase in bath osmolality from 290 to 410 mOsm in the absence of vasopressin, there is a slight decrease in fluorescence signal due to the nonzero basal P_f. In response to addition of 250 μU/ml vasopressin to the bath, the fluorescence signal decreased further after a lag period. As shown in the circle with an expanded scale, the lag phase lasted 22 s after vasopressin addition, followed by a gradual increase in P_f (decrease in fluorescence). P_f reached a stable value of 232 × 10^{-4} cm/s in 12 min. A similar experiment was performed at 23°C in the lower part of the figure.

The results of a series of similar measurements are summarized in Table I. The mean tubule length was 1.60 ± 0.06 mm (n = 23) with a range from 1.10 to 2.12 mm. For most measurements the lumen perfusion rate was 10 nl/min (range 8–15 nl/min). The inner tubule diameter was 24.3 ± 0.8 μm in the 290 mOsm bath solution, 26.0 ± 0.8 μm in the 410 μm bath solution, and 24.2 ± 0.9 μm in the 410 mOsm bath solution containing vasopressin. In response to vasopressin, there was a 17-fold increase in P_f at 37°C and a 21-fold increase in P_f at 23°C. P_f values

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

Temperature	n	P_f −VP	P_f +VP	T_{lag}	$T_{1/2}$
°C		cm/s × 10^4		s	s
37	16	13 ± 2	227 ± 10	23 ± 3	186 ± 20
23	7	9 ± 1	189 ± 12*	40 ± 4‡	195 ± 35

The perfusion protocol given in Fig. 5 was used. Values are mean ± SE for experiments performed using n separate tubules. *$P < 0.05$ and ‡$P < 0.01$ compared with corresponding data at 37°C. T_{lag} was defined at the difference in time between the addition of bath vasopressin and the start of the vasopressin response. $T_{1/2}$ was defined as the time between the start of the fluorescence decrease and the time at which fluorescence decreased to 50% of the difference between initial and final fluorescence values. For tubules studied at 37°C, three out of 19 tubules were excluded from the analysis because of drift in fluorescence signal with apparent $T_{1/2}$ values exceeding 400 s; one out of seven tubules was excluded at 23°C for the same reason with $T_{1/2}$ exceeding 500 s.

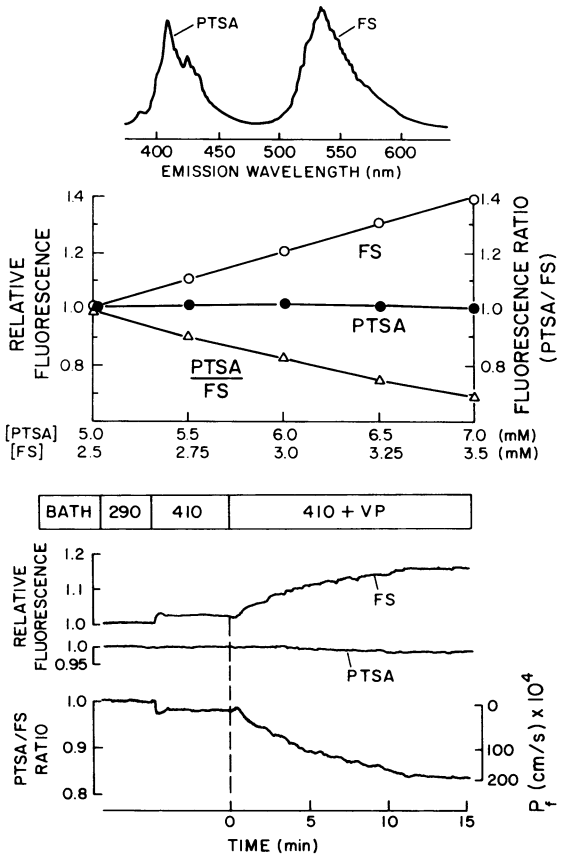

FIGURE 6 (*Top*) Calibration of FS and PTSA fluorescence in the doubly labeled lumen. The fluorescence emission spectrum of a solution of 5 mM PTSA and 2.5 mM FS in control buffer was obtained using a photodiode array as described in Methods. Fluorescence was excited at 380 nm. The lower calibration curves were measured using a beamsplitter and two photomultipliers as described in Methods. Relative fluorescence was normalized to unity for the 5 mM PTSA, 2.5 mM FS solution. (*Bottom*) Time course of P_f measured by dual emission detection. CCT was perfused with 290 mOsm buffer containing 5 mM PTSA and 2.5 mM FS at 37°C. The perfusion protocol was the same as in Fig. 5. Fluorescence was excited at 380 nm with dual detection of PTSA and FS fluorescence. P_f values were calculated using the PTSA/FS fluorescence ratio vs. concentration curve given at the top of the figure. P_f (in cm/s × 10^4) was 15 (−vasopressin) and 190 (+250 μU/ml vasopressin).

were significantly higher at 37°C than at 23°C. The lag time (T_{lag}) in which no measureable change in P_f occurred was significantly longer at 23°C (40 ± 4 s) than at 37°C (22 ± 3 s), however the half-time for increase in P_f ($T_{1/2}$) was not significantly different.

For measurement of tubule P_f by a ratiometric method using a double-fluorophore label, a series of fluorophore combinations were screened for impermeability, acceptable brightness at low illumination intensities, and lack of spectral overlap when excited at a single wavelength. The fluorophore pair PTSA and FS were chosen. Fig. 6 (*top*) shows the emission spectrum of a perfusion solution containing 5 mM PTSA and 2.5 mM FS obtained at the tip of a perfusion pipette using a photodiode array spectrometer. At an excitation wavelength of 380 nm, the two emission peaks are separated clearly. To calibrate the FS and PTSA concentration dependences of this perfusion solution, emitted fluorescence was divided by a dichroic beamsplitter and measured simultaneously by two photomultipliers. Using appropriate emission filters (420 ± 10 nm, PTSA; >530 nm, FS), fluorescence from the two probes could be measured without cross interference.

With proportionately increasing FS and PTSA concentrations, FS fluorescence increased while the PTSA fluorescence changed little, resulting in a decrease in the PTSA/FS fluorescence ratio. The FS fluorescence intensity increases in direct proportion to its concentration because little self quenching occurs below 5 mM FS (Kim et al., 1988). In the absence of FS, PTSA fluorescence is almost linear with [PTSA] (0–8 mM), indicating absence of PTSA self quenching (not shown). The PTSA lifetime was 10.6 ns in the absence of FS; PTSA lifetime decreased monotonically from 7.5 to 5.9 ns with increasing [FS] (2.5–3.5 mM). Therefore, the lack of increase in PTSA fluorescence occurs because of quenching of PTSA emission by FS. The PTSA/FS ratio thus provides an intensity and geometry independent measure of transepithelial volume flow.

Fig. 6 (*bottom*) shows the time course of P_f measured by the double-label technique. CCT was perfused at 37°C with a 290 mOsm solution containing 5 mM PTSA and 2.5 mM FS. As transepithelial volume flow increased, FS fluorescence increased and PTSA fluorescence decreased slightly, resulting in a time course of decreasing PTSA/FS fluorescence ratio. The P_f values and time course are similar to those measured by the single-label technique.

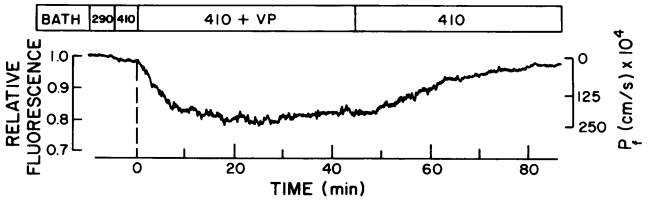

FIGURE 7 Time course of P_f after vasopressin addition and removal. The perfusion protocol was the same as in Fig. 5. CCT were perfused at 37°C with 290 mOsm solution containing 10 mM FS. In response to addition of 250 μU vasopressin to the bath, P_f increased from 12 × 10^{-4} cm/s to a maximal value of 219 × 10^{-4} cm/s in 20 min. Upon vasopressin removal, P_f decreased to a baseline value of 18 × 10^{-4} cm/s with a half-time of 14 min.

Finally, the effect of vasopressin addition and removal on P_f were determiend (Fig. 7). Addition of 250 μU/ml vasopressin increased P_f from 12 × 10^{-4} cm/s to 219 × 10^{-4} cm/s over 20 min. With continued vasopressin stimulation, P_f decreased slightly over the next 30 min. When vasopressin was removed from the bath, P_f decreased slowly to its baseline value with a half-time of ~14 min. Thus the P_f response was reversible in the fluorescence assay.

DISCUSSION

We report here a simple fluorescence method for the real-time measurement of transepithelial osmotic water permeability in perfused kidney tubules. The measurement of fluorescence of impermeant luminal fluorophores replaces the classical method of obtaining timed collections of perfused fluid for assay of labeled inulin concentration and/or solution osmolality. Two fluorescence assays were developed. In the first assay, the self quenching of fluorescein sulfonate (FS) was used as a sensitive indicator of FS concentration at the distal end of the tubule, and thus of the magnitude of transepithelial volume flow. The FS fluorescence signal from a segment of tubule lumen is sensitive to FS concentration, lamp intensity, tubule lumen diameter, and tubule motion artifacts. The latter two sources of errors were minimized by measuring the fluorescence signal just beyond the tip of the tubule in the collection pipette.

To eliminate the latter three sources of error rigorously, we developed a double-label technique. A series of fluorophores were screened for the characteristics of (*a*) membrane impermeability, (*b*) brightness when excited at a single, common wavelength, (*c*) separability of emission spectra, and (*d*) different dependences of emission intensities on concentration when used together. The fluorophore combination PTSA and FS fulfilled these criteria. Simultaneous measurement of PTSA and FS emission intensities at low excitation intensity was accomplished by constructing a 50/50 beamsplitter at the output of an epifluorescence microscope. Two photomultipliers fitted with appropriate filters were used to detect emission intensities. A double-label technique is essential for measurement of tubule P_f by imaging methods, where intensities must be normalized for tubule geometry and optical focus at all points along the tubule. By use of imaging, P_f can be measured from the dependence of fluorescence ratio on length along the tubule, providing an additional dimension of information to strengthen the regression procedure.

The P_f values obtained here by the fluorescence method in the presence of vasopressin (189 × 10^{-4} cm/s at 23°C, 227 × 10^{-4} cm/s at 37°C) were similar to those reported

previously by the fluid collection method at 23–25°C ($\sim 169 \times 10^{-4}$ cm/s, Grantham and Burg, 1966; 186×10^{-4} cm/s, Schafer and Andreoli, 1972; 227×10^{-4} cm/s, Al-Zahid et al., 1977; 252×10^{-4} cm/s, Hebert and Andreoli, 1980; 170×10^{-4} cm/s, Hall and Grantham, 1980), and at 37–38°C (446×10^{-4} cm/s, Al-Zahid et al., 1977; 232×10^{-4} cm/s, Fine et al., 1978; 216×10^{-4} cm/s, Hall and Grantham, 1980; 234×10^{-4} cm/s, Ando et al., 1986; 409×10^{-4} cm/s, Jones et al., 1988).

Upon addition of vasopressin to the bath solution, P_f gradually increased after a brief lag period, reaching a stable value in ~20 min at 23°C and in ~10 min at 37°C (Fig. 5). This time course is in general agreement with that reported previously at 23°C (50 min in Fig. 3 of Schafer and Andreoli, 1972; 20 min in Fig. 1 of Al-Zahid et al., 1977; 25 min in Fig. 1 of Hall and Granthan, 1980) and at 37°C (10 min in Fig. 1 of Fine et al., 1978; 15 min in Fig. 2 of Hall and Grantham, 1980; 30 min in Fig. 3 of Reif et al., 1984; 20 min in Fig. 3 of Jones et al., 1988). However, in these previous reports it was not possible to analyze the P_f response with time resolution better than a few minutes (typically 5–10 min) because of the time required for collections of perfused fluid. The analysis of the time course for P_f increase after vasopressin exposure in the present study shows that $T_{1/2}$ is 186 s at 23°C and 195 s at 37°C (Table I). The lag time (T_{lag}), in which no measurable change in P_f occurs in response to vasopressin, was determined for the first time in the present study to be 23 s at 37°C and 40 s at 23°C.

The very brief lag time for initiation of the vasopressin-induced hydroosmotic response is quite remarkable. According to the present working model for the vasopressin effect, vasopressin binding to basal membrane V_2 receptors activates adenylate cyclase to produce cAMP which in turn activates a protein kinase. Then, presumably in response to one or more phosphorylation events, there are major cytoskeletal alterations resulting in insertion of intracellular membranes containing water channels into the apical surface. Therefore, within 23 s, vasopressin (*a*) diffuses to its receptor, (*b*) binds to the receptor, (*c*) activates the catalytic subunit of adenylate cyclase via the G_s regulatory protein to produce cAMP, (*d*) resulting in activation of protein kinases, (*e*) transduction of the biochemical signal to cytoskeletal elements, (*f*) physical movement of intracellular vesicles containing water channels to the apical surface, and (*g*) fusion of vesicles followed by unfolding of the new membrane to transport water. There are in addition modulatory inputs from calcium and protein kinase C pathways. At present, the exact sequence and rate-limiting steps of the activation cascade are not known.

The kinetics of turn-on of P_f given here differ significantly from the kinetics of turn-on of diffusional water permeability (P_d) reported in the accompanying paper (Kuwahara and Verkman, 1988). P_d did not change significantly (T_{lag}) for 71 s at 37°C and 170 s at 23°C after vasopressin addition to the bath. The corresponding lag times for the P_f response were 23 s at 37°C and 40 s at 23°C. After vasopressin addition, P_d was half-stimulated in ~160 s at 37°C and in ~290 s at 23°C. Corresponding times for approximate half-stimulation of P_f after vasopressin addition were 210 s at 37°C and 235 s at 23°C.

Although the detailed methodology for measurement of P_f and P_d were different, it is interesting to discuss possible reasons for the different turn-on kinetics. Although lumen perfusion rate was generally higher for measurement of P_d, there was little effect of lumen flow over the range 10–30 nl/min on tubule diameter and on P_f and P_d. A serial barrier to diffusional water flow (e.g., cytoplasmic or external unstirred layer) could not account for the prolonged lag in the P_d response and the similar times between vasopressin addition and half-stimulation of P_f and P_d. A serial barrier for P_d should have little effect on the lag time and shorten slightly the time between vasopressin addition and half-stimulation of P_d. There are, however, two notable differences in the protocol for measurement of P_f and P_d. P_f was measured in the presence of a bath-to-lumen osmotic gradient whereas P_d was measured in the absence of an osmotic gradient. In recent work, we provided evidence that the magnitude and direction of water flow through collecting tubule cells appears to modulate directly rates of water channel insertion and retrieval (Kuwahara and Verkman, 1989). In addition, although D_2O probably does not alter the magnitude of steady-state diffusional water flow, there are multiple biochemical or cytoskeletal processes which might be modified by D_2O, causing a delay in pre-steady-state activation processes for water channel insertion. Further experiments are required to establish with certainty the mechanisms for the apparent differences in the turn-on kinetics of osmotic and diffusional water transport.

With continued exposure to vasopressin at 37°C, a gradual decrease in P_f was seen after 30 min (Fig. 7). This effect has been termed "flux inhibition" and has been observed in rabbit CCT at 37°C (Hall and Grantham, 1980) and in the toad urinary bladder (Edelman et al., 1964; Schwartz and Walter, 1967; Eggena et al., 1968). The etiology of "flux inhibition" has not been resolved, however effects of vasopressin receptor down-regulation (Kirk, K., submitted for publication) and apical endocytosis of water channels have been postulated (Harris et al., 1986). With removal of vasopressin from the bath solution, P_f returned gradually to its baseline value in 35 min (Fig. 7). This result is consistent with a previous study in which P_f returned to baseline after 30 min of vasopressin removal (Reif et al., 1984). The long time required for the decrease in P_f probably results from several factors including the kinetics of vasopressin-receptor dissociation, cAMP degradation, protein dephosphorylation, and water channel endocytosis. Further experiments are required to define precisely the sequence and rate-limiting steps of the P_f turn-off response.

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REFERENCES

Ando, Y., H. R. Jacobson, and M. D. Breyer. 1987. Phorbol myristate acetate, dioctanoylglycerol and phosophatidic acid inhibit the hydroosmotic effect of vasopressin on rabbit cortical collecting tubule. J. Clin. Invest. 80:590–593.

Al-Zahid, G., J. A. Schafer, S. L. Troutman, and T. E. Andreoli. 1977. Effect of antidiuretic hormone on water and solute permeation, and the activation energies for these processes in mammalian cortical collecting tubules. *J. Membr. Biol.* 31:103–129.

Berry, C. A., and A. S. Verkman. 1988. Osmotic gradient dependence of osmotic water permeability in proximal convoluted tubule. *J. Membr. Biol.* In press.

Brown, D., and L. Orci. 1983. Vasopressin stimulates formation of coated pits in rat kidney collecting ducts. *Nature (Lond.).* 302:253–255.

Burg, M., J. Grantham, M. Abramow, and J. Orloff. 1966. Preparation and study of fragments of single rabbit nephrons. *Am. J. Physiol.* 210:1293–1298.

Chen, P. -Y., D. Pearce, and A. S. Verkman. 1988. Membrane water and solute permeability determined quantitatively by self-quenching of an entrapped fluorophore. *Biochemistry.* 27:5713–5718.

Edelman, I. S., M. J. Peterson, and P. F. Gulyassy. 1964. Kinetic analysis of the antidiuretic action of vasopressin and adenosine 3'5' monophosphate. *J. Clin. Invest.* 43:2185–2194.

Eggena P., R. Walter, and I. L. Schwartz. 1968. Relationship between hydroosmotic flow and the inhibited response of the toad bladder to vasopressin. *Life Sci.* 7:59–63.

Fine, L. G., D. Schlondorff, W. Trizna, R. M. Gilbert, and N. S. Bricker. 1978. Functional profile of the isolated uremic nephron. Impaired water permeability and adenylate cyclase responsiveness of the cortical collecting tubule to vasopressin. *J. Clin. Invest.* 61:1519–1527.

Grantham, J. J., and M. B. Burg. 1966. Effect of vasopressin and cyclic AMP on permeability of isolated collecting tubules. *Am. J. Physiol.* 211:255–259.

Hall, D. A., and J. J. Grantham. 1980. Temperature effect on ADH response of isolated perfused rabbit collecting tubules. *Am. J. Physiol.* 239:F595–F601.

Harmanci, M. C., W. A. Kachadorian, H. Valtin, and V. A. DiScala. 1978. Antidiuretic hormone-induced intramembraneous alterations in mammalian collecting ducts. *Am. J. Physiol.* 235:F440–F443.

Harris, H. W., J. B. Wade, and J. S. Handler. 1986. Transepithelial water flow regulates apical membrane retrieval in antidiuretic hormone-stimulated toad urinary bladder. *J. Clin. Invest.* 78:703–712.

Hebert, S. C., and T. E. Andreoli. 1980. Interactions of temperature and ADH on transport processes in cortical collecting tubules. *Am. J. Physiol.* 238:F470–F480.

Jones, S. M., G. Frindt, and E. E. Windhager. 1988. Effect of peritubular [Ca] or ionomycin on hydrosmotic response of CCTs to ADH or cAMP. *Am. J. Physiol.* 254:F240–F253.

Kim, Y., N. P. Illsley, and A. S. Verkman. 1988. Rapid fluorescence assay of glucose and neutral solute transport using an entrapped volume indicator. *Anal. Biochem.* 172:403–409.

Kuwahara, M., and A. S. Verkman. 1988. Direct fluorescence measurement of diffusion water permeability in the vasopressin-sensitive kidney collecting tubule. *Biophys. J.* 54:587–593.

Kuwahara, M., and A. S. Verkman. 1989. Transcellular water flow directly modulates water channel exocytosis and endocytosis in kidney collecting tubule. *Biophys. J.* 54:587–593.

Lakowicz, J. R. 1983. Principles of Fluorescence Spectroscopy. Plenum Publishing Corp., New York. 19–51.

Reif, M. C., S. L. Troutman, and J. A. Schafer. 1984. Sustained response to vasopressin in isolated rat cortical collecting tubule. *Kidney Int.* 26:725–732.

Schafer, J. A., and T. E. Andreoli. 1972. Cellular constraints to diffusion. The effect of antidiuretic hormone on water flows in isolated mammalian collecting tubules. *J. Clin. Invest.* 51:1264–1278.

Schwartz, I. L., and R. Walter. 1967. Factors influencing the reactivity of the toad bladder to the hydro-osmotic action of vasopressin. *Am. J. Med.* 42:769–776.

Verkman, A. S., W. Lencer, D. Brown, and D. A. Ausiello. 1988. Endosomes from kidney collecting tubule cells contain the vasopressin-sensitive water channel. *Nature (Lond.).* 333:268–269.

Wade, J. B., D. L. Stetson, and S. A. Lewis. 1981. ADH action: evidence for a membrane shuttle hypothesis. *Ann. NY Acad. Sci.* 372:106–117.