Calcium and Cyclic Adenosine Monophosphate as Second Messengers for Vasopressin in the Rat Inner Medullary Collecting Duct

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Abstract

Vasopressin increases both the urea permeability and osmotic water permeability in the terminal part of the renal inner medullary collecting duct (terminal IMCD). To identify the second messengers that mediate these responses, we measured urea permeability, osmotic water permeability, intracellular calcium concentration, and cyclic AMP accumulation in isolated terminal IMCDs. After addition of vasopressin, a transient rise in intracellular calcium occurred that was coincident with increases in cyclic AMP accumulation and urea permeability. Half-maximal increases in urea permeability and osmotic water permeability occurred with 0.01 nM vasopressin. The threshold concentration for a measurable increase in cyclic AMP accumulation was ~ 0.01 nM, while measurable increases in intracellular calcium required much higher vasopressin concentrations (> 0.1 nM). Exogenous cyclic AMP (1 mM 8-Br-cAMP) mimicked the effect of vasopressin on urea permeability but did not produce a measurable change in intracellular calcium concentration. Conclusions: (*a*) Cyclic AMP is the second messenger that mediates the urea permeability response to vasopressin in the rat terminal IMCD. (*b*) Vasopressin increases the intracellular calcium concentration in the rat terminal IMCD, but the physiological role of this response is not yet known.

Introduction

Vasopressin increases both the urea permeability and the osmotic water permeability in the terminal part of the rat inner medullary collecting duct (terminal IMCD)[1] (1–3). A urea permeability response to vasopressin does not occur in earlier portions of the collecting duct system including the cortical collecting duct (4) and initial part of the inner medullary collecting duct (3). Thus, the action of vasopressin to increase urea permeability appears to be uniquely associated with the terminal IMCD. Urea reabsorption from the terminal collecting duct supplies most of the urea that accumulates in the inner medulla (5, 6). Consequently, urea transport across the terminal collecting duct epithelium and its regulation by vasopressin are important components of the urinary concentrating mechanism.

It is generally accepted that the water permeability response in collecting ducts results from occupation of specific vasopressin receptors coupled to the activation of adenylate cyclase and generation of cyclic adenosine monophosphate (cyclic AMP) in the cells. In contrast, the second messenger or second messengers involved in the urea permeability response of the terminal collecting duct have not been determined. It is reasonable to propose, based on prior studies of vasopressin-mediated stimulation of water permeability, that cyclic AMP mediates the vasopressin-induced increase in urea permeability in the terminal collecting duct. However, recent studies have demonstrated that another second messenger system, the phosphoinositide-calcium pathway, mediates vasopressin effects in several cell types including hepatocytes (7, 8), vascular smooth muscle cells (9), and renal glomerular mesangial cells (10). In this pathway, as discussed in detail in several recent reviews (8, 10–13), calcium acts as an intracellular messenger in concert with two organic second messengers, inositol trisphosphate and diacylglycerol.

The chief objective of the present investigation was to determine whether either cyclic AMP or intracellular calcium act as second messengers in the vasopressin-dependent stimulation of urea permeability in the terminal IMCD. Toward this end, we have carried out measurements of urea permeability, osmotic water permeability, intracellular calcium concentration, and cyclic AMP accumulation in isolated terminal collecting ducts from rats.

Methods

Solutions. The compositions of solutions used are shown in Table I. Solutions containing bicarbonate were equilibrated continuously with 94% O_2 and 6% CO_2. Hepes-buffered solutions were gassed with 100% O_2.

Chemicals were obtained from the following sources: synthetic atrial natriuretic factor (rat ANF 1-28; Peninsula Laboratories, Belmont, MA); BSA (Armour Pharmaceutical Co., Kankakee, IL); 1-deamino-8-D-arginine vasopressin (dDAVP; gift of Dr. Lewis Kinter, Smith Kline & French Laboratories, Philadelphia, PA); ionomycin (Calbiochem-Behring, La Jolla, CA); INDO-1 and INDO-1AM (Molecular Probes, Junction City, OR). Arginine vasopressin (AVP), bradykinin, collagenase (type 1), dimethylsulphoxide (DMSO), EGTA, HEPES, 3-isobutyl-1-methylxanthine (IBMX), 3-[*N*-Morpholino]-propanesulfonic acid (MOPS), TCA were obtained from Sigma (St. Louis, MO).

Tubule microperfusion studies. IMCD from the kidneys of pathogen-free Sprague-Dawley rats (Small Animal Breeding Facility, National Institutes of Health) were isolated and perfused in vitro using previously-described procedures (6). The tubule segments were dissected from the middle third of the inner medulla. When perfused, these tubules had a morphologic appearance consistent with that previously observed in the urea-permeable part of the inner medullary

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Received for publication 18 September 1987 and in revised form 28 December 1987.

1. *Abbreviations used in this paper:* ANF, atrial natriuretic factor; AVP, arginine vasopressin; DAG, diacylglycerol; dDAVP, 1-deamino-8-D-arginine vasopressin; DMSO, dimethylsulphoxide; IBMX, 3-isobutyl-1-methylxanthine; IMCD, inner medullary collecting duct; ITP, inositol tris-phosphate; MOPS, 3-[*N*-morpholino]propanesulfonic acid.

The Journal of Clinical Investigation, Inc.
Volume 81, June 1988, 1879–1888

collecting duct (terminal IMCD) (6). The tubule dissections were carried out at 15°C in solution 1, 3, or 5 (Table I) chosen to match the initial perfusate solution. The dissected tubules were mounted on concentric pipets for in vitro microperfusion at 37°C using method of Burg (14). The length and diameter of the perfused tubules were measured with an eyepiece micrometer.

Urea permeability measurements. The urea permeability of inner medullary collecting ducts was determined by measuring the flux of urea resulting from an imposed 5 mM bath-to-lumen urea gradient. Tubules were dissected in solution 1 (Table I), perfused with solution 1, and peritubular bath contained solution 2. The urea concentrations in collected fluid, perfusate and bath solutions were measured fluorometrically using a continuous flow ultramicrofluorometer (6). The method is linear within the range of sample sizes used in these experiments (0–100 pmol). In this range of sample sizes, the method can resolve differences in urea content of 4% or greater.

The urea permeability (P_u) was calculated as: $P_u = (C_0V_0 - C_LV_L)/(\pi D\Delta C)$, where C_0 is the urea concentration in the perfusate, C_L is the urea concentration in the collected fluid, V_0 is the perfusion rate per unit tubule length, V_L is the collection rate per unit tubule length, ΔC is the mean urea concentration difference along the tubule, and D is the diameter of the perfused tubule. The perfusate urea concentration was zero for all experiments, thus obviating any need to measure V_0. The collection rate was determined by measuring the length of time needed to fill a calibrated volumetric pipette. In all experiments, the collection rate was increased to a level high enough (usually > 50 $nl \cdot min^{-1} \cdot mm^{-1}$) to avoid dissipation of > 50% of the imposed urea concentration gradient. For each experimental condition, a mean value for tubule flow rate, collected urea concentration, net flux, and permeability was calculated from the results of two or three collections.

In the absence of vasopressin, the urea permeability of the terminal IMCD falls to a stable level within 40–50 min after the temperature is raised to 37°C and this level is maintained for up to 120 min in the absence of added hormones (3). Consequently, in this study we chose to make all measurements of urea permeability between 45 and 170 min after warming the tubules. When tubules were exposed to two different concentrations of AVP or dDAVP, an additional equilibration period of 30–40 min was allowed before measurements at the second concentration.

In experiments in which the temporal response to AVP was measured, the bath exchange to the vasopressin-containing solution was 90% complete within 15 s. Bath solutions were preheated to 37°C to avoid changes in temperature or pH in the bath chamber during the exchange.

Water permeability measurements. The osmotic water permeability of inner medullary collecting ducts was determined by measuring the rate of water absorption resulting from an imposed osmotic gradient (100 mM bath-to-lumen NaCl gradient). Tubules were dissected in solution 3 (Table I), perfused with solution 3, and the peritubular bath contained solution 4. The osmolalities of the perfusate and bath were measured by vapor pressure osmometry (Wescor, Logan, UT) before the start of each experiment.

Creatinine was used as a volume marker. Previous studies have demonstrated that creatinine is a valid volume marker in the rat IMCD (3, 6). The creatinine concentrations in collected fluid, perfusate, and bath solutions were measured using a continuous flow colorimeter using the alkaline picrate method (15). The method is linear in the range of sample sizes used in these experiments (0–150 pmol) and can resolve differences between samples of 3% or greater. The NaCl permeability of this segment is low and is not affected by vasopressin (3). Consequently, dissipation of the transepithelial osmolality gradient by passive NaCl entry is negligible.

The perfusion rate V_0 was calculated using the equation $V_0 = V_L(X_L/X_0)$ where V_L is the collection rate, X_L is the collected creatinine concentration and X_0 is the perfusate creatinine concentration. The osmotic water permeability (P_f) was calculated using the equation of Al-Zahid et al. (16):

$$P_f = -\frac{V_0C_0}{AV_w}\left[\frac{C_0 - C_L}{C_0C_bC_L} + \frac{1}{(C_b)^2}\ln\frac{(C_L - C_b)C_0}{(C_0 - C_b)C_L}\right]$$

where C_0 and C_b are the osmolalities of the perfusate and bath, A is the luminal surface area, C_L is the collected fluid osmolality (calculated as $C_L = C_0 \cdot X_0/X_L$), and V_w is the partial molal volume of water. The luminal surface area was calculated as $A = \pi DL$ from the measured length (L) and diameter (D) of the perfused tubule.

In the absence of vasopressin, the osmotic water permeability of the terminal IMCD falls to a stable level within 40–50 min after the temperature is raised to 37°C and this level is maintained for up to 120 min in the absence of added hormones (3). Consequently, in this study we chose to make all measurements of osmotic water permeability between 45 and 170 min after warming the tubules.

Intracellular calcium measurements. The intracellular calcium concentration was measured in isolated perfused terminal inner medullary collecting ducts with the trapped intracellular calcium probe INDO-1 (17). Tubules were dissected at a higher temperature (21°C) than in other experiments to facilitate loading of the dye. The dissection solution was the same as the solution used to perfuse the tubule (solution 1, 3, or 5; Table I), except that the pH was reduced to 7.0. After several 0.3 to 0.5 mm tubules were dissected, tubules were loaded with INDO-1AM while in the dissection dish. INDO-1AM, a membrane permeant derivative of INDO-1, enters cells and is cleaved to the relatively impermeant compound INDO-1 by esterases normally present in the cells (17). Initial attempts to load INDO-1AM were unsuccessful because INDO-1AM forms dye micelles in aqueous solutions and the resulting dye concentration in free solution is probably very low. Dye loading was enhanced by first injecting 10 μl of 20 mM INDO-1AM (dissolved in DMSO) into 360 μl of a concentrated aqueous solution of bovine serum albumin (30 mg/ml dissolved with minimal shaking in 150 mM NaCl). Then, 15 μl of INDO-1AM/albumin solution was carefully injected into the dissection dish directly above the tubules (final concentration of INDO-1AM 0.8 μM; final dilution of DMSO 1:25,000 [vol/vol]). The tubules were exposed to INDO-1AM for 30 to 150 min at pH 7.0 and 21°C. Adequate loading was generally achieved within 45 to 60 min. INDO-1AM micelles adherent to the tubule were washed off before the tubules were transferred to the microscope spectrofluorometer.

The fluorescence emission spectra of intracellular INDO-1 were measured at 37°C in isolated perfused tubules with a specially designed epifluorescence microscope spectrofluorometer (18, 19), permitting simultaneous tubule perfusion and fluorescence spectroscopy. Light from a mercury arc lamp (Nikon, Inc.) was passed through a 366-nm interference filter (Corion, Holliston, MA), was reflected by a custom dichroic mirror (transmission > 400 nm; Andover Corp., Andover, MA), and then was focused to a 100–150 μm spot by a 63× objective (N.A. 1.25; Zeiss, Thornwood, NJ). The emitted light passed through the dichroic mirror, and was spread out onto the face of a silicon intensified vidicon camera by a monochromator (Instruments SA Inc., Metuchen, NJ). Since the excitation light was not detected by the camera, a barrier filter was not required. Emission spectra were obtained by averaging 5–10 readings over 0.5 to 1 s. Background autofluorescence of the tubule was < 5% of the INDO-1 fluorescence throughout the measured spectrum from 400 to 550 nm, precluding the need to subtract the autofluorescence spectrum from the spectrum of INDO-1. Furthermore, 10^{-8} M AVP added to the bath solution did not change background autofluorescence. A continuous flow bath exchange system prevented dye that may have leaked out of the cell from contributing to the measured fluorescence.

The calcium dependent shift in the emission spectrum of INDO-1 was quantitated using an intensity ratio method (17). The two wavelengths used for emission intensity measurements were 409 nm and 480 nm. These wavelengths were chosen to maximize the sensitivity of the measurements. These wavelengths were identified by subtracting the emission spectrum of INDO-1 in the presence of calcium from the emission spectrum in the absence of calcium and identifying the mini-

Table I. Composition of Solutions

	Solution No.							
	1	2	3	4	5	6	7	8
NaCl	118	118	118	237	133	117	135	133
$NaHCO_3$	25	25	25	25		25		
K_2HPO_4	2.5	2.5	2.5	2.5	2			2
KCl							5	
NaH_2PO_4						1	1	
NaHepes					10		10	10
HCl					6		6	6
$CaCl_2$	1	1	1	1	1	1	1	
EGTA								2
Creatinine			4	4				
Raffinose	5							
Urea		5	5	5				

Solutions also contained (in mM) glucose (5.5) and $MgSO_4$ (1.2). Solutions containing bicarbonate were bubbled with 6% CO_2/94% O_2. Bicarbonate-free solutions were bubbled with 100% O_2.

mum and maximum (20). The $[Ca^{2+}]$ was calculated from the ratio (R) of the intensities measured simultaneously at the two wavelengths using (17):

$$[Ca^{2+}] = K_d \cdot (s_{f2}/s_{b2}) \cdot (R_{max} - R)/(R - R_{min}) \quad (1)$$

where K_d is the dissociation constant of INDO-1 (250 nM [17]), R_{max} and R_{min} are the emission intensity ratios obtained in the presence and absence of calcium; the remaining two terms are the emission intensities of INDO-1 at 480 nm in the absence (s_{f2}) and presence (s_{b2}) of calcium.

Calibration to determine R_{min}, R_{max}, and s_{f2}/s_{b2} in the cells was done in isolated perfused inner medullary collecting ducts by altering extracellular calcium in the presence of the calcium ionophore ionomycin. (Calibration was done in the cells because preliminary experiments showed that the intracellular environment alters the spectral properties of INDO-1 as is true for other trapped intracellular probes.) Tubules were dissected in Ca-free EGTA solution (solution 8, Table I). They were loaded with INDO-1, and perfused using solution 8 in both the peritubular bath and luminal perfusate. The bath solution contained 5–10 μM ionomycin. Emission spectra were collected every 10–20 min until the intensity ratio reached a stable value. The bath solution was exchanged to solution 5 containing calcium and 5–10 μM ionomycin. Emission spectra were taken frequently until the intensity ratio reached a stable value. Fig. 1 shows the emission spectra of intracellular INDO-1 at low and high bath calcium concentrations. R_{min} was 0.372, R_{max} was 1.047, and s_{f2}/s_{b2} was 1.624.

The course of a typical experiment in which intracellular calcium was measured is as follows. After mounting the tubule on the perfusion pipets and warming it to 37°C, a continuous bath exchange was established at 7–10 cm^3/min fed from a heated bath exchange reservoir mounted above the perfusion apparatus. After a 30–40 min equilibration period, the basal intracellular calcium concentration was determined as the mean of two or more measurements. Then, agent or vehicle was added to the bath exchange reservior with vigorous mixing. Because of the dead space between the bath exchange reservoir and the perfusion chamber, there is a 25-s lag between addition of agent to bath exchange reservoir and complete exchange of the peritubular bath. (This method of changing the peritubular bathing solution is utilized to avoid motion of the tubule or changes in bath temperature which may influence the intracellular calcium measurement.) Timed measurements of intracellular calcium were made for 10 min after each change of the peritubular bath solution. At the end of each experiment, sufficient AVP or dDAVP to form a 10^{-8} M solution was added to the bath exchange reservoir and additional measurements were made to provide a positive control for each tubule studied. The peak intracellular calcium value is defined as the maximum reading in the first 5 min following addition of agent or vehicle. These values were corrected for variability in the measurement method by subtracting the maximum deviation from the mean during the pre- and postpeak periods. For the 57 observations described in Table II, the average maximal deviation was 11.0 nM. This value approximates the minimum peak height detectable with the method. The *steady state* intracellular calcium value was defined as the average value in the period between 5 and 10 min after addition of agent or vehicle. Since bleaching of the dye limited the number of measurements, the results of preliminary studies were used to guide selection of measurement times.

For intracellular calcium measurements, the tubules were generally

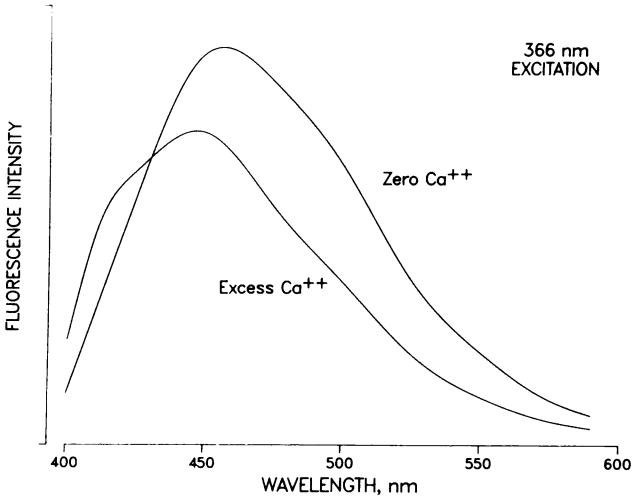

Figure 1. Emission spectra of intracellular INDO-1. Spectra were determined in the presence of the calcium ionophore ionomycin (5–10 μM) in the nominal absence of extracellular calcium (Zero Ca^{2+}; solution 8 [Table I]) and in the presence of 1 mM extracellular calcium (Excess Ca^{2+}; solution 5 [Table I]).

Table II. Measurements of Intracellular Calcium Concentration

Agent (No. of tubules)	Concentration	Intracellular calcium concentration		
		Baseline	Peak	Steady state
	M	*nM*	*nM*	*nM*
Vehicle (11)		137±9	137±10	127±8
AVP (20)	10^{-8}	131±10	263±14*	140±13
AVP (7)	10^{-10}	116±16	131±21	134±20
dDAVP (7)	10^{-8}	130±9	221±17*	138±9
8-Br-cAMP (4)	10^{-3}	77±20	82±22	80±21
ANF (4)	5×10^{-8}, 10^{-7}	141±11	142±10	133±11
Bradykinin (4)	10^{-6}	130±22	130±20	132±13

* $P < 0.05$ vs. baseline value.

Data expressed as mean±SE. The *peak* value was taken as the maximum reading in the first 5 min following addition of agent or vehicle corrected for variability in the measurement method as indicated in the text (see Methods). The steady state value was taken as the average value in the period between 5 and 10 min after addition of agent or vehicle. Experiments in Hepes-buffered and bicarbonate-buffered solutions were pooled. The numbers of tubules studied in bicarbonate buffered solutions were: AVP 10^{-10} M, 4; AVP 10^{-8} M, 7; 8-Br-cAMP, 2; bradykinin, 1. The responses were virtually identical with the two buffers.

perfused and bathed with a HEPES-buffered solution (solution 5; Table I). Additional intracellular calcium measurements were made in tubules perfused using the same bicarbonate-buffered solutions as the urea permeability protocol (solutions 1 and 2, Table I).

Cyclic AMP accumulation studies. Cyclic AMP accumulation in microdissected nonperfused segments of rat inner medullary collecting duct was determined as described previously (21). A brief summary of the experimental approach follows. Pathogen-free Sprague-Dawley rats (85–150 g body wt) were decapitated and the left kidney was perfused with 10 ml of an ice-cold solution (solution 6, Table I) containing 1 mg/ml collagenase. The middle third of the inner medulla was isolated, dissected into four strips, and incubated in solution 6 containing collagenase for 30 min at 37°C. Segments of inner medullary collecting ducts were microdissected at 17°C in solution 7 containing 0.05% BSA. After measuring the tubule lengths with an ocular micrometer, tubule segments were transferred in 2 μl of dissection solution to 18 μl of solution 7 containing 0.56 mM IBMX at 4°C. The microdissected IMCDs were then equilibrated for 10 min at 37°C in a shaking water bath. 20 μl of prewarmed solution 7 containing AVP or vehicle and 0.5 mM IBMX was then added, and the incubation was continued at 37°C. The incubation time after addition of AVP or vehicle was 2 min in the dose-response experiments and was varied between 0 and 10 min in the time course experiments. The reaction was stopped by adding 50 μl of 10% TCA followed by immediate vortexing. After centrifugation, the supernatant was frozen at −20°C overnight. In each experiment, blank samples (containing medium and TCA without tubules) were processed.

The TCA was removed by extraction with 0.75 ml water-saturated ether. The aqueous phase was dried, then 100 μl of 50 mM sodium acetate buffer (pH 6.2) was added to each sample. The cAMP content was measured by radioimmunoassay (New England Nuclear, Boston, MA) as described previously (21). Appropriate cAMP standards were carried through the entire assay procedure for determination of standard curves. 50% of the tracer displacement occurred at 99.8±4.8 fmol ($n = 2$).

To measure the time course of the response to vasopressin, IMCDs with a total length of ~ 1 mm were used for each determination of cAMP accumulation. For the dose-response experiments, the tubule lengths used for each determination were 1.5 mm (control and 10^{-12} M AVP), 1.0 mm (10^{-11} M AVP), 0.5 mm (10^{-10} M AVP), and 0.25 mm (10^{-9} AVP and greater).

Statistics. All results are reported as mean±1 SE. Statistical significance was determined using t tests (paired and unpaired). $P < 0.05$ indicated statistical significance.

Results

Time-course of response to AVP

Urea permeability. Fig. 2 shows the time-course of urea permeability changes in response to 10^{-11} M and 10^{-8} M of AVP. The responses were normalized to express the percent maximal change, facilitating comparisons among tubules. An increase in the urea permeability was detected within 2 min. By 5 min, the urea permeability increased to ~ 60% of the full response. After the initial rapid rise in permeability which occurred within 5–10 min, there was a slower increase that was complete within 30 min after addition of AVP. The mean unstimulated urea permeability in these four tubules was 25±9 × 10^{-5} cm/s. The urea permeability rose to an average of 79±17 × 10^{-5} cm/s within 40 min of AVP addition in the four tubules.

cAMP accumulation. Fig. 3 shows the time course of cAMP accumulation in microdissected nonperfused segments of the rat IMCD after addition of AVP (5×10^{-10} M) and in tubules not exposed to AVP. 0.5 mM IBMX was present in both groups. In the presence of AVP, an increase in cAMP accumulation was detected at 1 min. cAMP accumulation increased steadily over 10 min in both control and AVP-stimulated tubules.

Intracellular calcium. Fig. 4 shows the time course, in one tubule, of intracellular calcium concentration following addition of vehicle, then 10^{-8} M AVP. The results from all tubules are shown in Table II. The mean intracellular calcium concentration did not change significantly when vehicle (either water or 160 mM NaCl) was added to the bath (Table II).

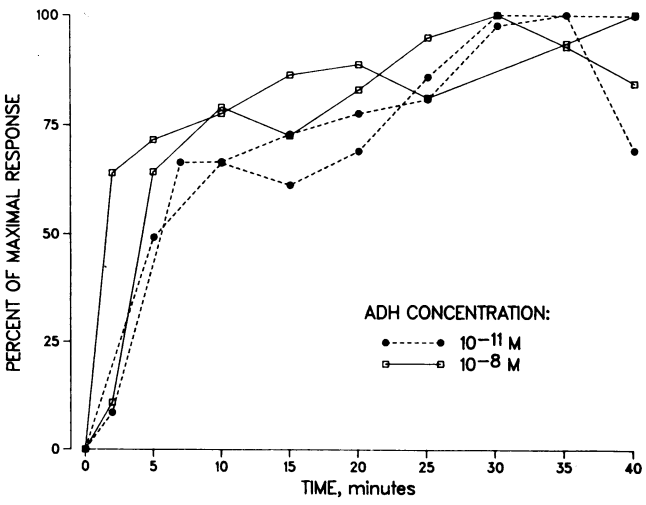

Figure 2. Time course of vasopressin-stimulated urea permeability response in isolated perfused inner medullary collecting ducts from rats. Vasopressin was added at zero time. ADH, antidiuretic hormone (arginine vasopressin).

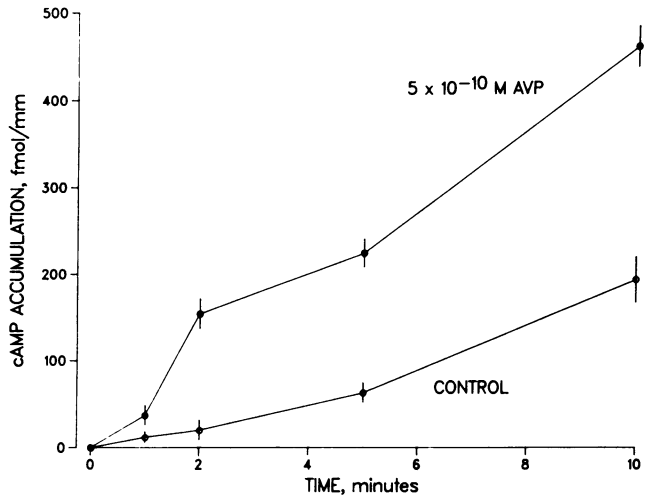

Figure 3. Time course of vasopressin-stimulated increase in cAMP accumulation by isolated nonperfused rat inner medullary collecting ducts.

However, 10^{-8} M AVP significantly increased the intracellular calcium concentration. The peak response occurred 87±10 seconds (n = 20) after AVP was delivered to the peritubular bath chamber. In general, the response lasted ~ 2–3 min, with return to near baseline values by 4–5 min. A rise in intracellular calcium concentration was seen in response to 10^{-8} AVP in solutions buffered by either Hepes (solution 5) or bicarbonate (solutions 1 and 2).

AVP dose-response relationships

Urea permeability. Both 10^{-11} M (series A, Table III) and 10^{-10} M (series B, Table III) AVP increased the urea permeability of the rat IMCD. The increase with 10^{-11} M was 55% of the maximal response (defined as the response to 10^{-8} M AVP). The increase with 10^{-10} M AVP was 79% of maximal. Thus, the concentration of AVP that caused a half maximal increase in urea permeability was about 10^{-11} M. The mean results are plotted on Fig. 5, expressed as percent maximal change. In additional experiments (Table III), 10^{-8} M dDAVP, a selective V_2 receptor agonist, significantly increased the urea permeability by about twofold.

Osmotic water permeability. 10^{-11} M AVP increased the osmotic water permeability of the rat IMCD (Table IV). This increase was 44% of the maximal response (defined as the response to 10^{-8} M AVP). 10^{-10} M had about the same effect as 10^{-8} M AVP in two tubules (data not shown). Based on these limited data, the dose-response relationship for osmotic water permeability appears to be similar to that for urea permeability (Fig. 5).

cAMP accumulation. The dose-response relationship for cAMP accumulation, measured in microdissected nonperfused segments of rat IMCD, is shown in Fig. 6. There was a significant increase in cAMP accumulation at 10^{-11} M AVP. The concentration of AVP that caused a half maximal increase in cAMP accumulation was about 5×10^{-10} M.

Intracellular calcium. Fig. 7 shows the time course of intracellular calcium concentration in response to 10^{-10} and 10^{-8} AVP. The results for all experiments are summarized in Table II. At 10^{-10} M, AVP did not significantly alter the intracellular calcium concentration, despite the fact that this concentration caused nearly maximal increases in urea permeability and osmotic water permeability.

Response to exogenous cAMP

The cyclic AMP analogue 8-Br-cAMP (1 mM added to the peritubular bath) reversibly increased the urea permeability of rat inner medullary collecting ducts (Table III). However, 1 mM 8-Br-cAMP did not significantly alter the apparent intracellular calcium concentration (Fig. 8; Table II).

Effect of INDO-1 on urea permeability response

It might be argued that 10^{-10} M AVP normally increases intracellular calcium, but did not do so in these experiments because intracellular INDO-1 acted as a calcium buffer. If this were true and if calcium normally mediates the urea perme-

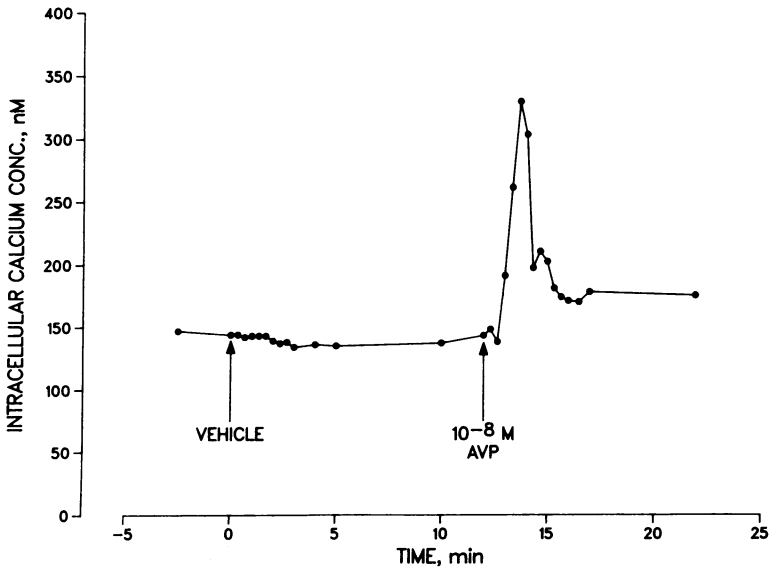

Figure 4. Effects of vehicle and 10^{-8} M AVP on intracellular calcium concentration in an isolated perfused rat IMCD.

Table III. Urea permeability response to AVP, dDAVP, and 8Br-cAMP

	Collection rate	Collected concentration	Urea permeability	Percent of maximal response
	nl/min/mm	*mM*	*$\times 10^{-5}$ cm/s*	
A AVP dose-response series A (n = 5)				
Control	43±4	0.70±0.22	11±4	
10^{-11} AVP	55±11	1.22±0.19	29±8*	55±14‡
10^{-8} AVP	71±11	1.39±0.34	46±18*	
B AVP dose-response series B (n = 5)				
Control	87±17	1.24±0.30	49±12	
10^{-10} AVP	110±26	1.94±0.40	107±24*	79±4‡
10^{-8} AVP	130±29	1.82±0.24	121±26*	
C dDAVP (n = 4)				
Control	86±17	0.67±0.08	21±6	
10^{-8} dDAVP	112±6	1.00±0.10	43±6*	
D 8 Bromo-cAMP (n = 4)				
Control	51±6	0.83±0.09	19±4	
1 mM 8Br-cAMP	87±11	1.35±0.14	50±6*	
Recovery	100±13	0.86±0.08	35±4§	

* Indicates urea permeability with agent (AVP, dDAVP, or 8Br-cAMP) was significantly different from control values ($P < 0.05$).
§ indicates significant reversal of 8 bromo-cAMP effect ($P < 0.05$).
‡ indicates percent maximal change was significantly different from zero ($P < 0.05$).
Measured urea concentrations in bath and perfusate were 5.0 mM±0.0 and 0.0±0.0, respectively. Perfusion rates were increased in the presence of vasopressin to maintain collected urea concentration at < 2.5 mM, i.e., to prevent dissipation of the bath-to-lumen urea gradient. Mean tubule diameter was 27.9±0.5 μm and did not differ among groups. Mean tubule length was 0.45 mm.

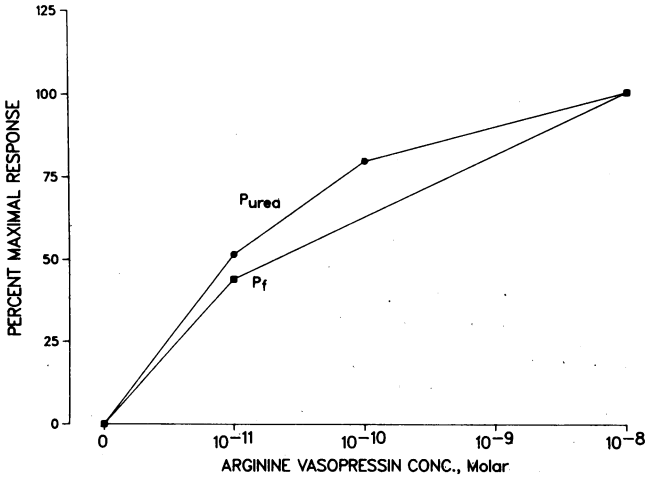

Figure 5. Comparison of dose-response relationships for effects of arginine vasopressin on urea permeability (P_{urea}) and osmotic water permeability (P_f) in rat inner medullary collecting ducts. See Tables III and IV for detailed descriptions of these experiments including standard error values. The concentration of vasopressin required for half-maximal P_{urea} and P_f responses was approximately 10^{-11} M.

Table IV. Osmotic Water Permeability Response to Arginine Vasopressin

	Collection rate	Collected/perfused creatinine	Osmotic water permeability
	nl/min/mm		*μm/s*
Control	9.4±0.8	1.11±0.02	63±3
10^{-11} AVP	9.4±1.3	1.26±0.04	171±38*
10^{-8} AVP	11.3±1.3	1.37±0.04	310±38*

Data are reported as mean±SE (n = 5). Tubule diameter was 29.8±0.6 μm; tubule length was 0.69±0.11 mm.
* $P < 0.05$ vs. control.

ability response to AVP, then INDO-1 loading should prevent the permeability response to 10^{-10} M AVP. However, in two INDO-1 loaded tubules (not shown), 10^{-10} M AVP caused a 2.3-fold increase in urea permeability, a response comparable to that seen in the absence of INDO-1 (Table III). Therefore, regardless of whether INDO-1 substantially buffers intracellular calcium, this result adds further support to the view that vasopressin can increase the urea permeability in the absence of a rise in intracellular free calcium concentration.

Effect of other hormones and analogues on intracellular calcium

dDAVP. dDAVP 10^{-8} M added to the peritubular bath significantly increased the calcium concentration (Table II). The peak intracellular calcium concentration was similar to that seen with 10^{-8} M AVP. As shown in Figs. 9 and 10, the time course of the increase in intracellular calcium concentration in response to 10^{-8} M dDAVP is similar to that seen with 10^{-8} M AVP.

Atrial natriuretic factor. Atrial natriuretic factor (rat ANF_{1-28}, 5×10^{-8} or 10^{-7} M added to the peritubular bath) did not have a significant effect on intracellular calcium concentration (Fig. 9; Table II). Furthermore, prior exposure to ANF

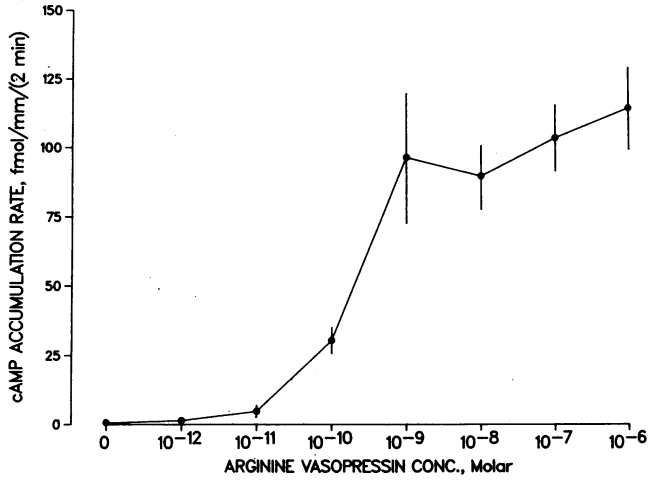

Figure 6. Dependence of cAMP accumulation on concentration of arginine vasopressin. Experiments were done in microdissected rat inner medullary collecting ducts. Effects at and above 10^{-11} M were statistically significant.

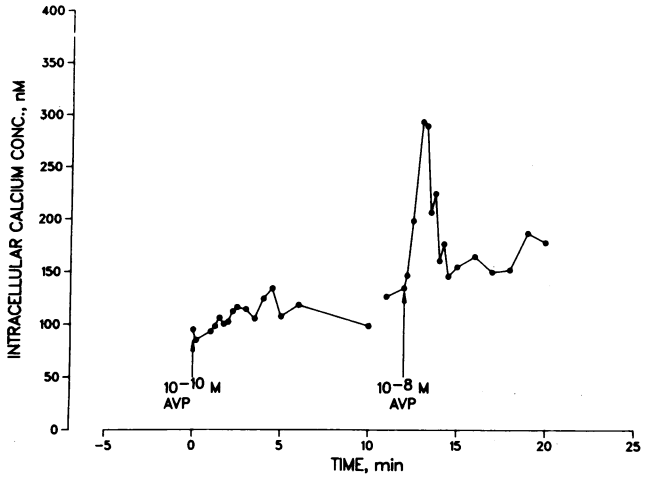

Figure 7. Effects of 10^{-10} M and 10^{-8} M arginine vasopressin (AVP) on intracellular calcium concentration in an isolated perfused rat inner medullary collecting duct. In a total of seven tubules, there was no significant effect of 10^{-10} M AVP on intracellular calcium (Table II).

in the peritubular bath did not prevent the rise in intracellular calcium concentration in response to 10^{-8} M dDAVP (Fig. 9).

Bradykinin. Bradykinin, 10^{-6} M added to the peritubular bath, did not have a significant effect on intracellular calcium concentration (Fig. 10; Table II). Furthermore, prior exposure of the tubules to bradykinin in the peritubular bath did not prevent the rise in intracellular calcium concentration in response to 10^{-8} M dDAVP (Fig. 10).

Discussion

The evidence acquired in the present study supports the view that the vasopressin-mediated increase in urea permeability in the terminal IMCD results from activation of adenylate cyclase and generation of cyclic AMP in the cells. The present studies have also demonstrated that vasopressin causes a transient rise in intracellular calcium concentration. However, vasopressin-mediated increases in urea permeability and osmotic water permeability are not dependent on a rise in intracellular calcium. The physiologic role of the vasopressin-stimulated increase in intracellular calcium has not yet been identified.

We review the basis for these conclusions and the relationship of the present results to prior observations.

Physiologic role of vasopressin in the IMCD. The IMCD consists of at least two morphologically and functionally distinct subsegments (3, 22). The initial part (initial IMCD), found in the outer third of the inner medulla, contains two cell types (intercalated and principal cells) and resembles the collecting duct from the inner stripe of the outer medulla (23). The terminal IMCD, the focus of the present study, contains only one cell type, usually termed a principal cell. The principal cell in the terminal IMCD differs from the principal cell of the initial IMCD. It contains a specialized urea transport pathway, probably a carrier, which mediates an extremely rapid rate of passive urea transport across the terminal collecting duct epithelium (3, 6). In the initial IMCD and more proximal collecting duct segments, this urea transport pathway is either absent or present in much lower activity (3). The urea transport pathway in the terminal IMCD is important for the urinary concentrating mechanism because it supplies nearly all of the urea that accumulates in the inner medullary interstitium (5, 6).

In this study, we have established that physiologic concentrations of vasopressin (i.e., 10^{-11} to 10^{-10} M) can cause large increases in the urea permeability of the rat terminal IMCD. This result is consistent with an important physiologic role for vasopressin in the regulation of urea transport across the terminal IMCD.

Cyclic AMP as a second messenger for vasopressin in the IMCD. The results of the present study are consistent with the view that the effect of vasopressin to increase urea permeability in the terminal IMCD is mediated by cyclic AMP. Three pieces of evidence, considered together, support this conclusion. First, vasopressin increases cyclic AMP accumulation in

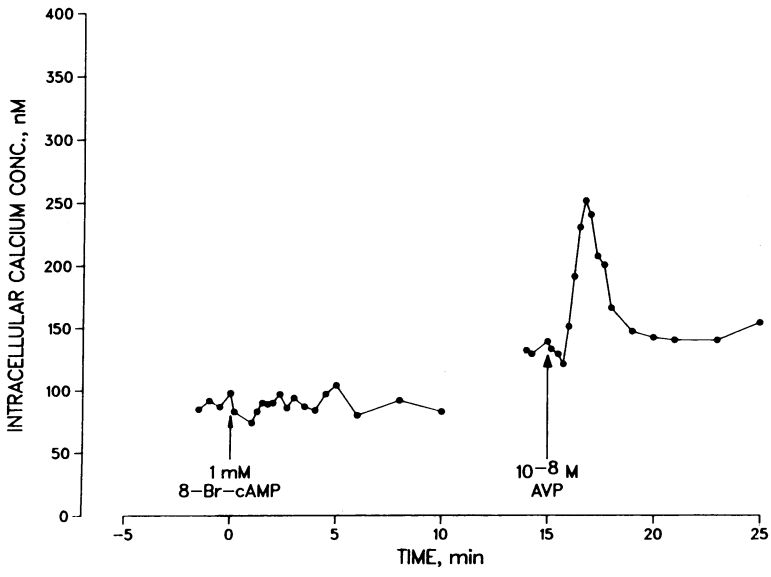

Figure 8. Effects of 1 mM 8-Br-cAMP and 10^{-8} M AVP on intracellular calcium concentration in an isolated perfused rat IMCD. In a total of four tubules, there was no significant effect of 1 mM 8-Br-cAMP on intracellular calcium (Table II).

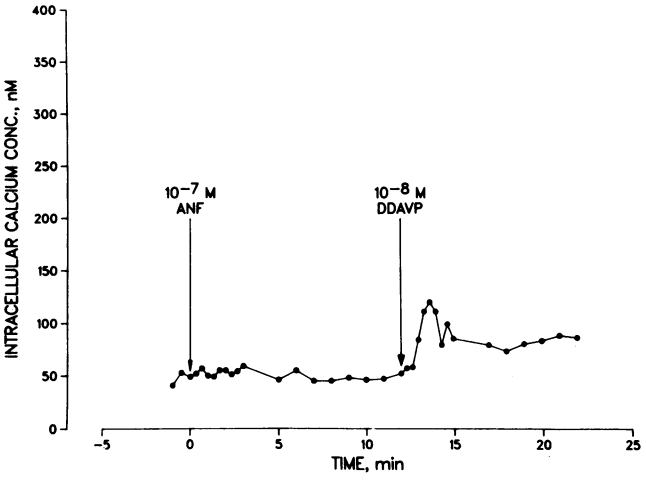

Figure 9. Effects of 10^{-7} M ANF and 10^{-8} M dDAVP on intracellular calcium concentration in an isolated perfused rat inner medullary collecting duct. In a total of four tubules, there was no significant effect of ANF on intracellular calcium (Table II).

the terminal IMCD, and the rise cyclic AMP precedes the rise in urea permeability (compare Figs. 2 and 3). Second, the dose-response relationship for stimulation of cyclic AMP accumulation by vasopressin shows a threshold in the physiologic range of vasopressin concentrations (Fig. 6). Third, a cyclic AMP analogue, 8-Br-cAMP, increased the urea permeability of isolated perfused IMCD when added to the peritubular bath (Table III).

Cyclic AMP accumulation increases with vasopressin concentration up to 10^{-9} M vasopressin (Fig. 6). In contrast, urea permeability increases to a maximal or nearly maximal level in response to only 10^{-10} M vasopressin. Thus, it appears that vasopressin can increase cyclic AMP accumulation to much higher levels than required to elicit a maximal urea permeability response. At present, it is not known what function can be served by the generation of excess cyclic AMP.

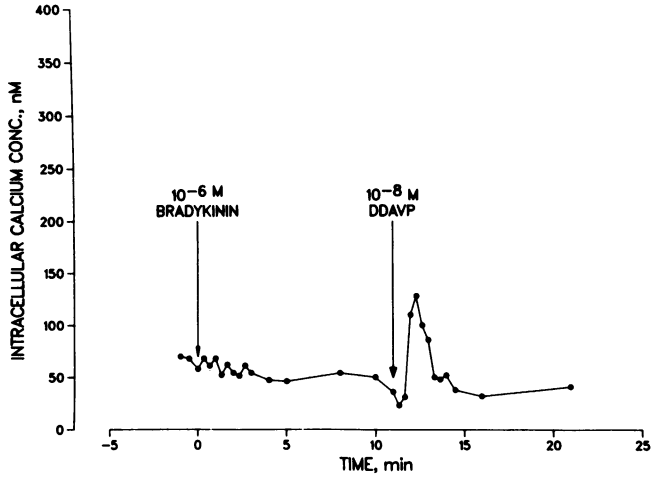

Figure 10. Effects of 10^{-6} M bradykinin and 10^{-8} M dDAVP on intracellular calcium concentration in an isolated perfused rat inner medullary collecting duct. In a total of four tubules, there was no significant effect of 10^{-6} M bradykinin on intracellular calcium (Table II).

It has been demonstrated in previous studies (1, 3) that vasopressin also increases osmotic water permeability in the terminal IMCD. The present results confirm this conclusion (Table IV). As in other parts of the collecting duct system, the water permeability response in the terminal IMCD is thought to be mediated by cyclic AMP. The dose-response relationship for stimulation of water permeability by vasopressin is consistent with this conclusion. That is, the concentration of vasopressin required to elicit a water permeability response is similar to that required to increase urea permeability and cyclic AMP accumulation.

Calcium as a putative second messenger for vasopressin in the IMCD. This paper contains the first reported evidence that vasopressin increases the intracellular calcium concentration in the terminal IMCD. The increase was a transient phenomenon with peak intracellular calcium concentrations occurring 60–90 s after addition of vasopressin to the peritubular bath. The intracellular calcium concentration then fell to near baseline levels within 3–5 min after vasopressin addition (Fig. 4). The time course is similar to vasopressin-mediated increases in intracellular calcium activity seen in hepatocytes (8), cultured renal mesangial cells (24, 25), cultured smooth muscle cells (9, 26, 27), and cultured pig kidney cells (LLC-PK_1) (28, 29). The calcium transients in these tissues are thought to result from activation of the phosphoinositide cascade by vasopressin. The rise in intracellular calcium may regulate several processes in the cell. One important role is the activation, in concert with diacylglycerol (DAG), of protein kinase C.

Because the intracellular calcium transients demonstrated in the terminal IMCD in the present study were similar to those that are linked to the phosphoinositide system in other tissues, it is reasonable to propose that the demonstrated rise in intracellular calcium in the IMCD is also due to activation of the phosphoinositide system, although direct proof of this possibility is lacking. Previous studies have demonstrated the presence of a functional phosphoinositide pathway in cultured rabbit IMCD cells (30, 31). Further studies are needed to determine what role the phosphoinositide pathway plays in regulation of IMCD function.

Different vasopressin receptor subtypes have been implicated in the activation of the phosphoinositide-calcium pathway (V_1 receptors) and activation of adenylate cyclase (V_2 receptors) (7). The present studies were not designed to resolve the receptor subtype linked to the demonstrated rise in intracellular calcium in the terminal IMCD. Nevertheless it is interesting that the V_2 selective vasopressin analogue dDAVP appeared to be nearly equipotent with arginine vasopressin in stimulating a rise in intracellular calcium (Table II). This raises the possibility that the receptor responsible for vasopressin-mediated increases in intracellular calcium in the IMCD may differ from its counterpart in other tissues. One possibility is that the rise in intracellular calcium results from the binding of vasopressin to oxytocin receptors. Oxytocin receptors have not been demonstrated in the IMCD, but are present in cultured renal tubule epithelia (LLC-PK_1) where they are linked to the phosphoinositide-calcium pathway (32).

Although vasopressin increases intracellular calcium in the rat terminal IMCD, it appears unlikely that calcium is a second messenger involved in the vasopressin-dependent increases in urea and water permeability. Two pieces of evidence support this conclusion. First, a nearly maximal increase in

urea permeability was observed in response to 10^{-10} M vasopressin, a concentration that produced no measurable change in intracellular calcium concentration.[2] The permeability response to 10^{-10} M vasopressin occurred regardless of whether or not the cells were loaded with INDO-1. Therefore, calcium buffering by INDO-1 (if present) did not prevent the permeability response. Second, a large increase in urea permeability was seen in response to 1 mM 8-Br-cAMP despite the absence of a measurable change in intracellular calcium activity. Thus, it appears that large increases in urea permeability can occur without a demonstrable change in intracellular calcium concentration.

If calcium is not a second messenger for stimulation of urea and water permeability by vasopressin, then what physiological role could a rise in intracellular calcium in response to vasopressin play? It could be argued that the concentration of vasopressin required to raise intracellular calcium is so high that under physiological conditions, circulating concentrations are unlikely to rise high enough to cause the response. Thus, calcium-mediated responses to vasopressin may be important only in pathological conditions, such as hemorrhage, in which circulating vasopressin levels reach very high values. However, we are reluctant to draw such a conclusion based on the data obtained in the present study. Our reluctance stems from the fact that the in vitro conditions for our studies were chosen on the basis of a need to assure viability of the epithelium (6) and are not representative of the environment normally experienced in the inner medulla during antidiuresis. It is possible that the dose-response relationship for mobilization of intracellular calcium by vasopressin is altered by factors such as osmolality, ionic strength, and urea concentration that may vary in the inner medulla. Thus, we consider it reasonable to assume that vasopressin-induced changes in intracellular calcium could play some (as yet undiscovered) physiological role in the regulation of IMCD function.

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2. The kinetics of vasopressin binding to its receptor(s) must be considered in the interpretation of experiments comparing the effects of a given concentration of vasopressin on intracellular calcium, cAMP accumulation and urea permeability. It could be argued that the intracellular calcium and cAMP responses (measured within 2 min of vasopressin addition) could have been limited if binding of vasopressin to its receptor(s) had not yet reached an equilibrium state. In contrast, the urea permeability measurements (carried out at least 40 min after vasopressin addition) would have been made well after binding had reached equilibrium. Two observations suggest, however, that differences in vasopressin concentration dependencies among physiological responses do not depend on differing receptor occupancies. First, a urea permeability increase was readily detectable after 2 min exposure to vasopressin even at 10^{-11} M vasopressin (Fig. 2), indicating that the increases in intracellular calcium and urea permeability have different concentration dependencies even when compared after the same duration of vasopressin exposure. (This conclusion does not depend on whether or not vasopressin binding to its receptor(s) reached equilibrium.) Second, the rate of cAMP accumulation was already maximal within 2 min of vasopressin exposure (Fig. 3) suggesting that binding of vasopressin to V_2 receptors had already reached an equilibrium state.

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