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Pre-Steady-State Analysis of the Turn-On and Turn-Off of Water Permeability in the Kidney Collecting Tubule

Michio Kuwahara and A.S. Verkman

Department of Medicine and Division of Nephrology, Cardiovascular Research Institute, University of California, San Francisco, California 94143

Summary. Water transport across the mammalian collecting tubule is regulated by vasopressin-dependent water channel insertion into and retrieval from the cell apical membrane. The time course of osmotic water permeability (P_f) following addition and removal of vasopressin (VP) and 8-Br-cAMP was measured continuously by quantitative fluorescence microscopy using an impermeant fluorophore perfused in the lumen. Cortical collecting tubules were subjected to a 120 mOsm bath-to-lumen osmotic gradient at 37°C with 10–15 nl/min lumen perfusion and 10–20 ml/min bath exchange rate. With addition of VP (250 μU/ml), there was a 23 ± 3 sec (SEM, n = 16) lag in which P_f did not change, followed by a rise in P_f (initial rate 1.4 ± 0.2 × 10^{-4} cm/sec^2) to a maximum of 265 ± 10 × 10^{-4} cm/sec. With addition of 8-Br-cAMP (0.01–1 mM) there was an 11 ± 2 sec lag. For [8-Br-cAMP] = 0.01, 0.1 and 1 mM, the initial rate of P_f increase following the lag was (units 10^{-4} cm/sec^2): 1.1 ± 0.1, 1.2 ± 0.1 and 1.7 ± 0.3. Maximum P_f was (units 10^{-4} cm/sec): 64 ± 4, 199 ± 9 and 285 ± 11. With removal of VP, P_f decreased to baseline (12 × 10^{-4} cm/sec) with a $T_{1/2}$ of 18 min; removal of 0.1 and 1 mM 8-Br-cAMP gave $T_{1/2}$ of 4 and 8.5 min. These results demonstrate (*i*) a brief lag in the P_f response, longer for stimulation by VP than by 8-Br-cAMP, representing the transient build-up of biochemical intermediates proximal to the water channel insertion step, (*ii*) similar initial dP_f/dt (water channel insertion) over a wide range of [8-Br-cAMP] and steady-state P_f values, and (*iii*) more rapid P_f decrease with removal of 8-Br-cAMP than with VP. These pre-steady-state results define the detailed kinetics of the turn-on and turn-off of tubule P_f and provide kinetic evidence that the rate-limiting step for turn-on of P_f is not the step at which VP regulates steady-state P_f. If water channel insertion is assumed to be the rate-limiting step in the turn-on of P_f, these results raise the possibility that water channels must be activated following insertion into the apical membrane.

Key Words fluorescence · water transport · vasopressin · kidney collecting tubule · kinetics

Introduction

Water permeability across the kidney collecting tubule is under direct hormonal control by the concentration of vasopressin circulating in the renal vasculature. In the absence of vasopressin, the water permeability of the apical membrane of collecting tubule cells is low, resulting in a low water permeability across the whole epithelium. Upon exposure of the cell basal membrane to vasopressin, there is a rapid and dramatic increase in the water permeability of the cell apical membrane, resulting in a >10-fold increase in transepithelial water permeability [1, 9, 15, 28]. The cellular mechanism by which vasopressin increases water permeability is the subject of great interest; it is thought that vasopressin activates adenylate cyclase, causing cAMP production and protein kinase activation, which ultimately induces cytoskeletal changes resulting in the insertion (exocytosis) of subcellular membranes containing functional water channels into the cell apical membrane [3, 11, 23, 24, 33]. It is thought that the regulation and turn-off of vasopressin-stimulated water permeability involves the retrieval of water permeable patches of apical membrane by endocytosis for subsequent storage in a subcellular compartment for later recycling to the apical membrane [5, 13, 31, 32].

The biochemical and physical factors regulating water channel exocytosis and endocytosis are poorly understood. The bulk of experimental evidence supporting current concepts of the vasopressin signaling mechanism is from two approaches: freeze-fracture electron microscopy, showing strong correlations between membrane morphology (apical membrane particle aggregates) and vasopressin presence [4, 12, 13], and steady-state measurements of osmotic water permeability across the intact collecting tubule in response to addition of specific activators and inhibitors of the hydrosmotic response. There is another important source of *functional* information, which has not been exploited because of technical difficulties in the measurement of osmotic water permeability in intact

kidney tubules—the pre-steady-state kinetics of the vasopressin hydrosmotic response. Based on the significant advances made in the understanding of enzymatic and cellular signaling mechanisms by analysis of pre-steady-state transients, we have examined the pre-steady-state kinetics of the vasopressin-induced hydrosmotic response in the intact kidney collecting tubule.

We recently reported new fluorescence techniques for the real-time measurement of osmotic and diffusional water permeability in kidney tubules based on the measurement of the fluorescence of impermeant fluorophores perfused through the tubule lumen [18, 19]. We report here the application of these methods to study the kinetics of turn-on and turn-off of osmotic water permeability in the rabbit cortical collecting tubule in response to rapid addition to and removal from the bath of vasopressin and the permeable cAMP analogue 8-bromo-cAMP. The kinetic results are interpreted in terms of a model for the regulated exocytic/endocytic cycling of water permeable membranes. The data support the utility of a kinetic approach for further examination of the complex biochemical regulation of collecting tubule water permeability.

Materials and Methods

Materials

Fluorescein sulfonate (FS) was obtained from Molecular Probes (Junction City, OR). Synthetic arginine vasopressin (Pitressin) was purchased from Parke-Davis (Morris Plains, NJ). 8-bromoadenosine-3′,5′-cyclic monophosphoric acid (8-Br-cAMP) was purchased from Sigma (St. Louis, MO).

In Vitro Microperfusion System

Isolated segments of rabbit cortical collecting tubule (CCT) were dissected and perfused in vitro as described previously [6, 19]. Briefly, kidneys from New Zealand white rabbits (1.5–2.5 kg) were cut in coronal slices. Tubules were dissected in a cooled (4°C) bath solution and transferred to a bath of 200 μl volume. Tubules were mounted between holding pipettes and the lumen was cannulated for perfusion. Luminal perfusion rate was maintained at 10–15 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. Luminal perfusion rate was calibrated against set pump rates as described previously [19].

The bath solution was preheated to 37°C and was exchanged continuously at 5–20 ml/min. Bath fluid composition was changed by adjusting a 5-way valve near the bath. At a bath exchange rate of 10 ml/min, the new fluid replaced the old fluid by >90% in <2 sec. The control bath solution contained (in mM): 115 NaCl, 5 KCl, 25 $NaHCO_3$, 10 Na acetate, 5 glucose, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$. Solution pH was adjusted to 7.4 by addition of HCl or NaOH after the solution was bubbled for 1 hr with 95% O_2/5% CO_2. The bath solution was bubbled continuously during the experiments. The perfusate contained (in mM): 150 NaCl, 2.5 K_2HPO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 1 FS, pH 7.4. Solution osmolalities were measured with a vapor pressure osmometer (Wescor, Logan, VT) and were adjusted to 290 mosmol/kg H_2O by addition of NaCl or H_2O. 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) was added to the bath solution at a concentration of 250 μU/ml. For cyclic AMP experiments, 8-Br-cAMP (0.01–10 mM) was added to the bath solution. These solutions were prepared just before the start of the experiment. The length and inner diameter of the tubule were measured by an eyepiece micrometer.

Fluorescence Measurement System

Luminal FS fluorescence was measured using an inverted epifluorescence microscope (Nikon Diaphot, Japan). Tubules were viewed through a 25× long working distance objective (LWD fluortar, N.A. 0.4, Leitz Wetzlar, Germany). Fluorescence was excited using a 100 W tungsten lamp powered by a stabilized DC supply (Oriel, Stratford, CT) in series with a 1.0 neutral density filter and a KG-3 infrared blocking filter (Schott Glass, Duryea, PA).

For measurement of luminal FS fluorescence, 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) contained in a cooled housing (FACT 50, Thorn EMI Gencom). The signal was amplified by an Ealing DC power supply and amplifier (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-sec time constant; data was acquired at a rate of 30 points/sec and averaged over 1-sec intervals.

Experimental Protocol and Data Analysis

To eliminate effects of endogenous vasopressin, tubules were perfused for 90 min with vasopressin-free buffer at 37°C [10, 15] at a lumen flow rate of 10 nl/min and bath exchange rate of 5 ml/min. For measurement of P_f, FS fluorescence was monitored at the tip of the holding pipette (inner diameter ~40 μm) just distal to the end of the tubule segment. As described previously [18], the advantages of this method are lack of effect on fluorescence signal of tubule motion and changes in tubule inner diameter, and elimination of photodynamic cell injury. The excitation and emission path contained iris diaphrams so that only the specified area was illuminated and measured. Under these conditions, background fluorescence was <0.5% of total signal.

P_f was calculated from the relation [1],

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

where V_o is the initial lumen perfusion rate (cm³/min), A is the inner tubule surface area (cm²), V_w is the partial molar volume of water (18 cm³/mole), C_o and C_L are the initial and collected osmolalities, respectively, and C_b is the bath osmolality. Because the fluorescence intensity of FS is linearly proportional to its concentration at [FS] < 3 [17], C_L was determined from the

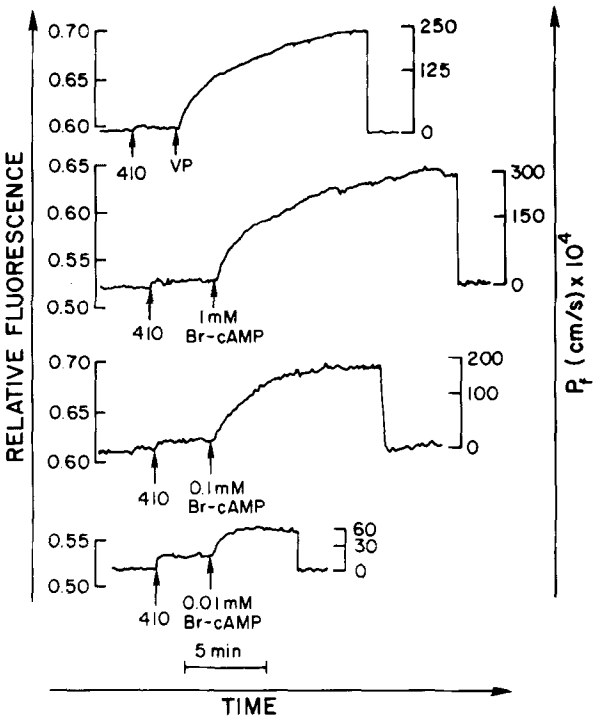

Fig. 1. Time course of P_f increase following addition of vasopressin or 8-Br-cAMP. CCT were perfused with 290 mOsm buffer containing 1 mM FS at a lumen flow rate of 10–15 nl/min at 37°C. Where indicated, bath fluid was changed from control (290 mOsm), to hyperosmotic (410 mOsm) buffer, to hyperosmotic buffer containing 250 μU/ml vasopressin or 0.01–1 mM 8-Br-cAMP. The rapid drop in fluorescence near the end of the experiment corresponds to a change in bath solution to control (290 mOsm) buffer. Relative fluorescence measured at the distal end of the tubule is given in arbitrary computer units; fluorescence was not measured at the proximal end of the tubule. P_f values (right-hand ordinate) were calculated from tubule geometry, lumen flow and relative fluorescence as described in Materials and Methods

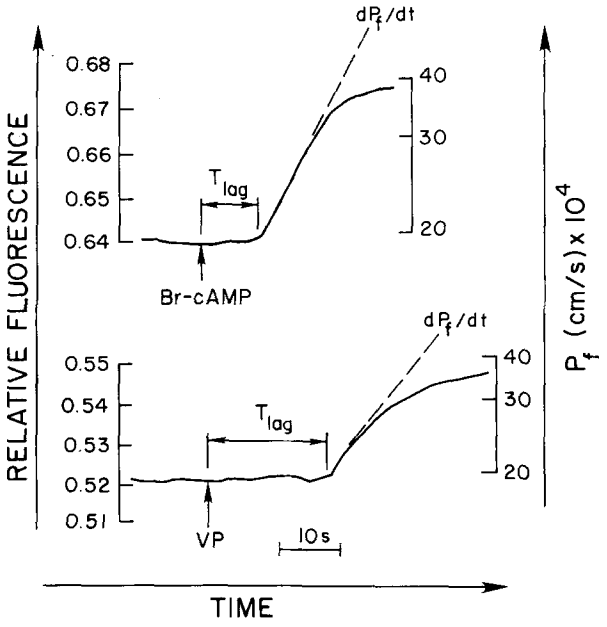

Fig. 2. Initial P_f response to 8-Br-cAMP and vasopressin. The perfusion protocol was the same as that used in Fig. 1. At the time of ligand addition, tubules were perfused with 290 mOsm buffer and bathed in 410 mOsm buffer. Concentrations were 1 mM (8-Br-cAMP) and 250 μU/ml (vasopressin). T_{lag} is the time between activator addition and the initial increase in P_f. dP_f/dt (dashed line) is the maximal slope of the P_f *vs.* time curve, which was identical to the initial slope after the lag phase was completed. dP_f/dt was generally constant over a >5-sec time interval

product of C_o and the ratio fluorescence intensities measured when bath osmolarity was 410 and 290 mOsm. In these studies fluorescence was measured at the distal end of the tubule; the fluorescence corresponding to C_o was inferred from the fluorescence in the absence of an osmotic gradient, which would equal the fluorescence at the proximal end of the tubule. It was reported previously that FS remained in the tubule lumen space and did not enter cells measurably under the conditions of the experiments [18].

Pre-steady-state kinetic data for the response of P_f to vasopressin or 8-Br-cAMP were analyzed as follows (*see* Figs. 1 and 2). The lag time (T_{lag}) was determined from the time between the addition of activating ligand to the bath (start of bath fluid change) and the start of the increase in P_f. T_{lag} was corrected for the time elapsed between adjustment of the 5-way valve and 50% fluid exchange (~1.5 sec). $T_{1/2}$ was defined as the time between the start of P_f response to vasopressin (or 8-Br-cAMP) and the time at which P_f reached 50% of the maximal response. The initial rate of P_f response (dP_f/dt, cm/sec^2 · 10^4) was determined from the initial increase in fluorescence following the lag period, which was linear for 5–10 sec. Data are expressed as means ± SE. Statistical comparisons were made using paired or non-paired Student's *t* test.

Results

Turn-On Kinetics of the Vasopressin Hydrosmotic Response

Figure 1 (top curve) shows the increase in transepithelial osmotic water permeability (P_f) following addition of vasopressin to the bath compartment (bathing the cell basal membrane). After dissection, tubules were perfused and bathed in an isosmotic buffer (290 mOsm) for 90 min at 37°C to eliminate effects of endogenous vasopressin and to allow tubule cell metabolism to stabilize. Upon increase in bath osmolality to 410 mOsm in the absence of vasopressin, there was a rapid (<2 sec), small increase in fluorescence at the distal end of the tubule corresponding to lumen-to-bath osmotic water movement and an increase in concentration of the fluorescent marker at the distal end of the tubule. Upon addition of vasopressin to the bath, there was a brief period of time in which P_f did not increase measurably (~20 sec, *see* Fig. 2, lower curve), followed by a slow increase in P_f over ~10 min. Upon return of bath osmolality to 290 mOsm, there was a rapid (<2 sec) return of fluorescence to its baseline value, indicating that the fluorescence signal was

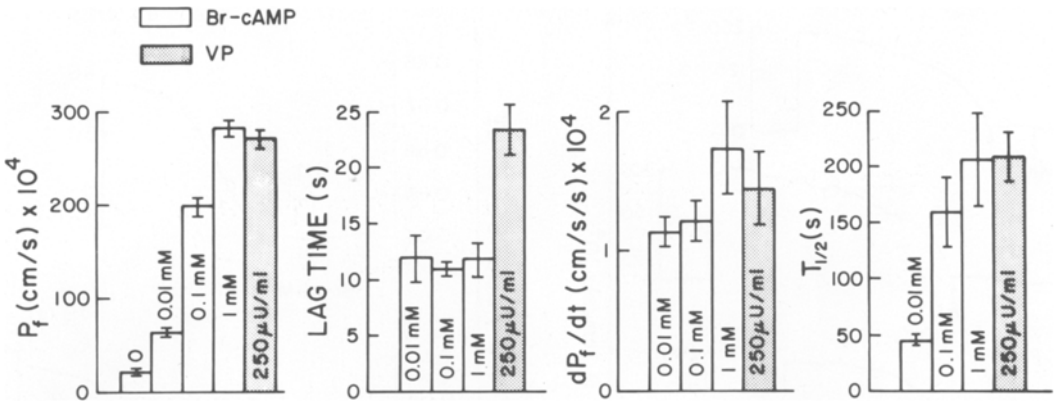

Fig. 3. Pre-steady-state kinetics for the activation of P_f by 8-Br-cAMP and vasopressin. The perfusion protocol was the same as that used in Fig. 1. Definitions for the lag time (T_{lag}) and dP_f/dt are given in Fig. 2. $T_{1/2}$ is the time, after the initial lag phase, in which the P_f response is half maximal. Data represent the mean ± SE for measurements performed on a series of n separate tubules (n = 7, 0.01 mM 8-Br-cAMP; n = 17, 0.1 mM 8-Br-cAMP; n = 14, 1 mM 8-Br-cAMP; n = 16, vasopressin)

without drift. These results are in agreement with data reported previously obtained using a fluorescence self-quenching and a double fluorophore label technique [18].

It is postulated that the lag period gives information about the pre-steady-state kinetics of the biochemical processes preceeding the insertion of water channels into the apical membrane, whereas the time course of increasing P_f gives information about the rate-limiting process of water channel activation, possibly the exocytosis of vesicles containing water channels into the cell apical membrane. It was reported previously that the lag time and the increase in P_f for the first few minutes after the lag period was independent of the presence and direction of a transepithelial osmotic gradient [20]. Therefore, the early response of P_f to stimulation by vasopressin and by varying concentrations of the cAMP analogue 8-Br-cAMP was determined to examine the kinetics of the turn-on of water permeability.

In response to additions of 0.01, 0.1 and 1 mM 8-Br-cAMP (Fig. 1, lower three curves), there were brief lag periods followed by time courses of increasing P_f. Steady-state P_f was dose dependent. As seen using an expanded time scale in Fig. 2 (top curve), the lag time was notably shorter when tubules were stimulated with 8-Br-cAMP than when stimulated by a maximal concentration of vasopressin. The initial rate of increase in P_f after the lag period, dP_f/dt (in units of cm/sec^2), was determined from the slope of the fluorescence increase within the first 5–10 sec after P_f began to increase. In all experiments, this slope was equal to the maximal value of dP_f/dt after activator addition. It was shown previously that in the sustained presence of maximal vasopressin, the transient response of transepithelial volume flux in going from zero to its steady-state value occurred within 3 sec [18]. This response time was much faster than the maximal value of dP_f/dt in response to activator addition.

Results for steady-state P_f, lag time, dP_f/dt and $T_{1/2}$ for a series of tubules are summarized in Fig. 3. P_f increased with increasing 8-Br-cAMP concentrations with a calculated K_d of 0.04 mM; there was no further increase in P_f using 10 mM 8-Br-cAMP (*not shown*). These steady-state results are in agreement with P_f values reported previously [8]. P_f measured at 1 or 10 mM 8-Br-cAMP was not significantly different from that measured at a saturating vasopressin concentration. The lag time was independent of the 8-Br-cAMP concentration, but significantly longer than the lag time for stimulation of P_f by vasopressin. Interestingly, although the vasopressin-stimulated steady-state P_f increased 6.2-fold over the 8-Br-cAMP concentration range tested, there was little increase in dP_f/dt. In addition, dP_f/dt for stimulation of P_f by vasopressin was not significantly different than that for stimulation by 8-Br-cAMP. $T_{1/2}$, the time at which the half-maximal P_f response was attained, increased significantly with increasing concentrations of 8-Br-cAMP. Possible mechanisms for these findings are discussed below.

Turn-Off Kinetics of the Vasopressin Hydrosmotic Response

Figure 4 shows the turn-off phase of P_f following removal of 8-Br-cAMP and vasopressin. P_f was fully stimulated by addition of activating ligand to the bath solution for ~10 min. With continued stim-

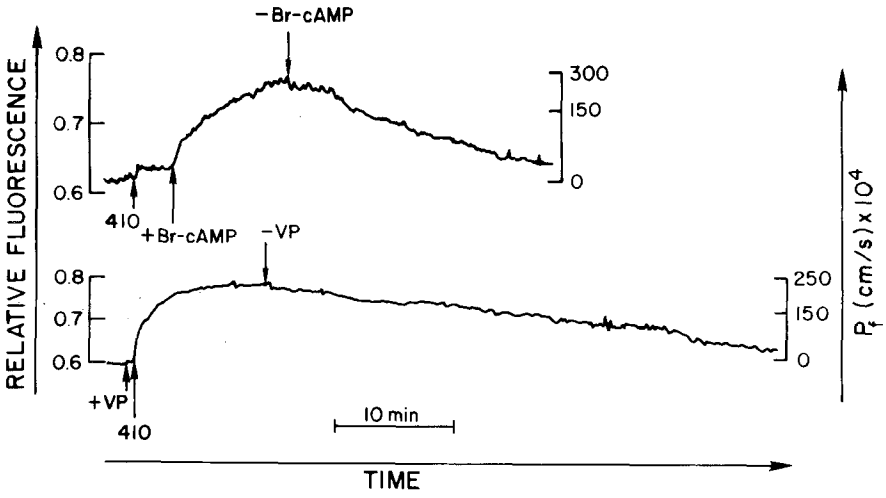

Fig. 4. Time course of P_f following removal of 8-Br-cAMP and vasopressin. CCT were perfused at 37°C with 290 mOsm buffer containing 1 mM FS. After maximal P_f response was obtained (~10 min), 1 mM 8-Br-cAMP (upper panel) or 250 μU/ml vasopressin (lower panel) was removed from a hyperosmotic bath solution (410 mOsm)

ulation of P_f by vasopressin or 8-Br-cAMP, there was <5% change in P_f over 30 min (*data not shown*), indicating little physiological down-regulation of P_f in the CCT under the conditions of the experiment. Upon removal of activating ligand, P_f declined to its baseline value measured before ligand addition. The rate of P_f decrease was greater for ligand removal after stimulation of P_f by 8-Br-cAMP than by vasopressin.

Results for a series of tubules are summarized in Fig. 5. The time at which P_f decreased to 50% of its maximal value was 15 min for vasopressin, 8.5 min for 1 mM 8-Br-cAMP, and 4 min for 0.1 mM 8-Br-cAMP. P_f returned to its baseline value (~15 × 10^{-4} cm/sec) after 60 min (vasopressin), 30 min (1 mM 8-Br-cAMP) and 10 min (0.1 mM 8-Br-cAMP).

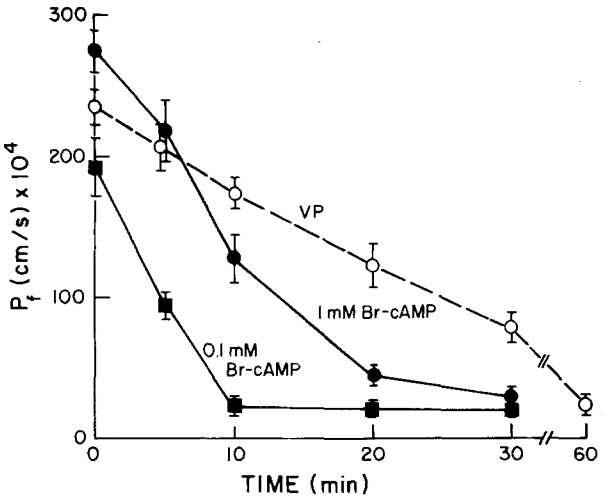

Fig. 5. Effect of removal of 8-Br-cAMP and vasopressin on P_f. The perfusion protocol was similar to that in Fig. 4. 8-Br-cAMP (0.1 or 1 mM) or vasopressin (250 μU/ml) was added to a hyperosmotic bath solution (410 mOsm). After ~10 min, 8-Br-cAMP or vasopressin was removed from the bath solution, as represented by zero time. Data points are the mean ± SE for measurements performed using a series of n separate tubules (n = 5, 0.1 mM 8-Br-cAMP; n = 6, 1 mM 8-Br-cAMP; n = 6, vasopressin)

Discussion

The majority of physiological data on water transport in vasopressin-sensitive epithelia has been obtained from steady-state measurements of water permeability in the presence and absence of activators and inhibitors of the hydrosmotic response. In the kidney collecting tubule, serial measurements of transepithelial osmotic water permeability have required multiple timed collections of perfused fluid, each over several minutes, limiting the detection of rapid transients in P_f following ligand addition [10, 16, 26, 28]. In toad urinary bladder, specialized measurement devices have been built to measure water transport with 1 min time resolution [25], however the presence of significant unstirred layers on the bladder serosal surface makes difficult the analysis of rapid transients in water permeability in response to added ligands. We recently reported a simple fluorescence method for the real-time measurement of osmotic and diffusional water permeability in perfused kidney tubules based on the detection of fluorescence of impermeant markers perfused through the tubule lumen [18–20]. The method did not interfere with normal tubule function and allowed measurement of transepithelial water permeability with time resolution of under 2 sec.

The analysis of pre-steady-state data provides information not obtainable from steady-state measurements of transepithelial P_f. Pre-steady-state

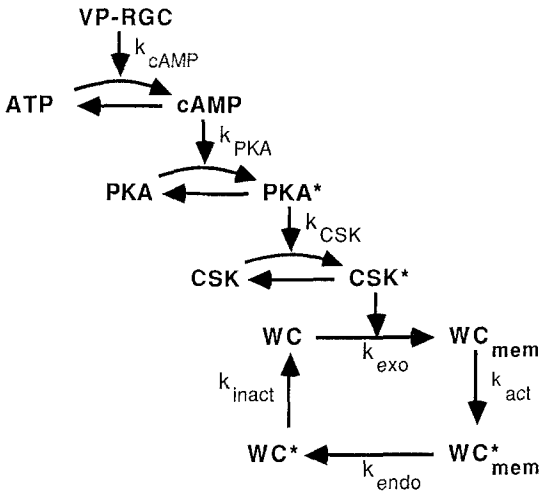

Fig. 6. Working kinetic model for vasopressin-dependent hydrosmosis in the kidney collecting tubule. Abbreviations: VP, vasopressin; RGC, receptor-guanine nucleotide-catalytic subunit complex of adenylate cyclase; PKA, protein kinase A; CSK, cytoskeleton; WC, water channel in endosome; WC_{mem}, water channel in the cell apical membrane; *k*'s, rate constants for individual reaction steps. Asterisks refer to activated states

transients contain information about the sequence of reactions in a multistep activation cascade, as well as the rate constants for individual reaction steps. In principle, the site of action of stimulatory and inhibitory ligands can be resolved if the pre-steady-state data is sufficiently detailed. In the present study, we have obtained pre-steady-state data for the turn-on and turn-off of transepithelial water transport as a functional marker to examine the kinetics of water channel exocytosis/activation and endocytosis/inactivation. There is a large body of evidence in amphibian urinary bladder and in kidney collecting tubule correlating transepithelial water permeability with the presence, in electron micrographs, of specialized structures (particle aggregates) on the cell apical surface [4, 12]. Functional water transport measurements provide important complementary information to the morphological studies, because water channel function cannot be evaluated from electron micrographs, and because it has been very difficult to obtain reliable information about the kinetics of water channel cycling from a series of static micrographs taken from separate tissue samples.

A working hypothesis for the vasopressin signal transduction mechanism is given schematically in Fig. 6 to provide a framework for the interpretation of kinetic results. This is a simplified model of the cAMP pathway for the regulation of water permeability. This mechanism does not include explicitly the modulatory inputs from protein kinase C, calcium-calmodulin and prostaglandin pathways [2, 21, 29, 30], nor does it contain features of proposed vasopressin-dependent second barriers to water permeability [22] or physical changes in apical membrane fluidity and morphology [14]. It was not the intent of this initial study to examine the complex interrelations among multiple signaling mechanisms. It is clear, however, that a pre-steady-state approach can be applied in the presence of non-cAMP ligands to examine non-cAMP signaling processes.

In the simplified model in Fig. 6, vasopressin binding to a V_2 receptor (R) on the basal surface of collecting tubule cells results in the activation of adenylate cyclase (GC) and production of cAMP. The cAMP is then thought to activate a cAMP-dependent protein kinase (PKA), presumably by dissociation of the regulatory and catalytic subunits of PKA. The activated protein kinase (PKA*) then, through one or more unknown steps, which likely involve protein phosphorylation, induces the activation of important cytoskeletal elements (CSK) causing the insertion (exocytosis, rate constant k_{exo}) of water channels (WC) into the apical membrane. Because it is not known whether the water channels are functional immediately upon insertion into the apical membrane or whether additional biochemical or physical modifications of the channel occur, a separate activation step (rate constant k_{act}) is included to give functional water channels in the apical membrane (WC^*_{mem}). Based on the water channel shuttle hypothesis [33] and on recent data showing the presence of functional water channels in vasopressin-dependent endosomes from collecting tubule [32], a step for water channel retrieval (endocytosis, rate constant k_{endo}) is given, followed by water channel inactivation (rate constant k_{intact}) required to complete the cycle. It is assumed that measured transepithelial osmotic water permeability is a functional measure of WC^*_{mem}.

In response to addition of a maximal concentration of vasopressin to the solution bathing the tubule, there was a 23 sec lag time in which P_f did not change measurably. With addition of 0.01–1 mM 8-Br-cAMP, the lag time shortened to 11 sec and was independent of 8-Br-cAMP concentration. In terms of the working model for vasopressin signaling, the lag time represents the composite times for ligand diffusion to the cell basal surface, the pre-steady-state accumulation of biochemical intermediates proximal to the water channel insertion and activation steps, and the response time of the measurement system. It is unlikely that ligand diffusion to tubule cells is a rate-limiting process because: (*i*) the unstirred layer associated with the perfused tubule is extremely small under the conditions of the ex-

periment, (*ii*) the lag time was independent of the concentration of 8-Br-cAMP, and (*iii*) the lag time was longer for a maximal concentration of vasopressin than for 8-Br-cAMP. Similarly, a lag phase cannot be accounted for by the instrument response time; a change in bath osmolarity in a vasopressin-stimulated tubule resulted in a change in fluorescence signal at the distal end of the tubule without lag and with a half time of under 2 sec [18].

Therefore, we attribute the lag phase to the accumulation of biochemical intermediates proximal to the water channel activation step (*cAMP, PKA*, CSK** and WC_{mem} in Fig. 6). The decreased lag time for stimulation of P_f by 8-Br-cAMP than for stimulation by vasopressin likely results from the time required for vasopressin binding to its receptor and activation of adenylate cyclase. Studies of the kinetics of vasopressin binding to V_2 receptors in LLC-PK1 cells [27] and of the pre-steady-state activation of adenylate cyclase [7] are consistent with a 12-sec time (difference between 23 and 11 sec) for these processes. The remaining 11-sec lag time for stimulation of P_f by 8-Br-cAMP is then attributed to the pre-steady-state accumulation of PKA*, CSK* and WC_{mem}. On the basis of data presented, it is not possible to assign lag times for these individual processes; however, the rapid activation of PKA in other systems and the complex nature of cytoskeletal activation and insertion of endosomes into the apical membrane make it likely that the 11-sec lag time results from activation of cytoskeletal processes. The independence of the lag time on the concentration of 8-Br-cAMP indicates the presence of a rate-limiting unimolecular reaction with zero order kinetics (e.g., reaction steps described by rate constants k_{PKA}, k_{CSK}, k_{exo} or k_{act}).

The initial rate of increase in P_f after the lag phase (dP_f/dt) can be interpreted as a functional marker for the rate-limiting step of water channel activation. It was surprising that dP_f/dt differed little for stimulation of P_f by 0.01, 0.1 and 1 mM 8-Br-cAMP, a concentration range over which the 8-Br-cAMP-dependent component of steady-state P_f increased by a factor of 6.2 (*see* Fig. 3). The constancy of dP_f/dt cannot be accounted for by a limiting instrument response time; maximal dP_f/dt was more than 10-fold slower than the instrument response capability for measurement of P_f. In addition, the increase in $T_{1/2}$ with increasing 8-Br-cAMP concentration provides independent evidence that the rate of water channel insertion decreases relative to the magnitude of steady-state P_f as [8-Br-cAMP] increases. If hormonal regulation of steady-state P_f occurred by control of the rate of exocytosis at a constant rate of endocytosis, then dP_f/dt should increase in proportion to steady-state P_f. Since the rate of water channel endocytosis increases with increasing vasopressin [13], water channel exocytosis must increase $\geqslant$6.2-fold over the 8-Br-cAMP concentration range 0.01 to 1 mM in order to implicate exocytosis as the key step for the hormonal regulation of water transport. Our results are not consistent with this simple interpretation.

From a kinetic viewpoint, the finding that dP_f/dt remains constant under conditions where steady-state P_f increases formally requires that: (*i*) there is a saturable, rate-limiting reaction in the activation cascade leading to the formation of WC^*_{mem}, and (*ii*) the rate-limiting step is not the step in which steady-state P_f is regulated by cAMP. It is not possible from the kinetic data available to determine which step in the activation cascade is rate-limiting. Because of the requirement for physical organization and movement, the cytoskeletal activation/water channel exocytosis step may be the rate-limiting process. If there were a limited number of "carriers" to shuttle water channels to the apical surface, then this insertion process would be saturable. If water channel insertion is the rate-limiting, saturable process, then the step controlling steady-state P_f must be different, likely at a point in the activation cascade distal to the saturable step. This analysis raises the interesting possibility that water channel activation may be required following water channel insertion. Further experimental work is required to validate these ideas.

In response to removal of the activating ligand from the bath solution, P_f returned to its unstimulated value with half times of 18 min for vasopressin, and 8.5 and 4 min for 8-Br-cAMP concentrations of 1 and 0.1 mM, respectively (*see* Fig. 5). These results suggest that the longer time required for turn-off of hydrosmosis for vasopressin removal resulted from the slow kinetics of vasopressin-receptor unbinding; an unbinding half time of 5 min was reported for unbinding of 10 nM vasopressin from receptors in intact cultured LLC-PK1 cells [27]. The slower decrease in P_f with vasopressin removal can also result from the kinetics of adenylate cyclase turn-off; however, direct kinetic measurements in adenylate cyclase from turkey erythrocytes suggest that this time is under 15 sec [7], much shorter than the time required for turn-off of P_f. The significantly shorter half time for turn-off of P_f at 0.1 mM 8-Br-cAMP than for that at 1 mM 8-Br-cAMP has several possible interpretations. If rates of water channel endocytosis are similar for 0.1 and 1 mM 8-Br-cAMP, then the lower steady-state P_f for 0.1 mM 8-Br-cAMP would result in a decreased half time for turn-off of P_f because fewer water channels must be retrieved. Alternately, because the intracellular concentration of 8-Br-cAMP drops faster for

removal of 0.1 mM than of 1 mM 8-Br-cAMP, subsequent reactions in the signaling cascade may be inactivated more rapidly. Direct measurements of endocytosis using fluorescent or other markers in real time are required to distinguish among these possibilities.

In summary, the methods and results reported here provide a kinetic approach to examine the mechanism by which vasopressin signals the hydrosmotic response. This new approach has yielded information about the pre-steady-state kinetics of the turn-on and turn-off of transepithelial osmotic water transport in response to addition and removal of vasopressin and 8-Br-cAMP in the perfused collecting tubule. A pre-steady-state approach should be of utility in defining the site of action of activators and inhibitors of the hydrosmotic response, and the role of intracellular calcium in the vasopressin signaling mechanism.

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