Vasopressin Stimulates Cl^- Transport in Ascending Thin Limb of Henle's Loop in Hamster

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Abstract

The effect of arginine vasopressin (AVP) on NaCl transport was investigated in the isolated microperfused hamster ascending thin limb of Henle's loop by measuring transepithelial voltage (Vt) and transmural $^{22}Na^+$ and $^{36}Cl^-$ fluxes. In the presence of a transmural NaCl concentration gradient (100 mM higher in the lumen), Vt was 8.4±0.4 mV. Addition of 1 nM AVP to the basolateral solution increased Vt to 9.6±0.4 mV, which corresponds to an increase in the Cl^- to Na^+ permselectivity ratio (P_{Cl}/P_{Na}) from 2.8±0.2 to 3.4±0.2. AVP at physiological concentrations increased Vt in a dose-dependent manner with an ED_{50} of 5 pM. AVP increased the Cl^- efflux coefficient from 99.6±6.3 to 131.4±10.6 × 10^{-7} cm^2/s without affecting the Na^+ efflux coefficient. 5-Nitro-2-(3-phenyl-propylamino)-benzoate (0.2 mM), a Cl^- channel inhibitor, in the perfusate decreased the basal Cl^- efflux coefficient and inhibited the AVP-induced increase in this parameter. The AVP-induced increase in Vt was not affected by [$d(CH_2)_5^1$,*O*-Me-Tyr^2, Arg^8] vasopressin, a V1 receptor antagonist, but was abolished by [$d(CH_2)_5$,D-Ile^2,Ile^4,Arg^8]vasopressin, a V2 receptor antagonist. The selective V2 agonist dDAVP in 1 nM also increased Vt from 8.6±0.7 to 9.5±0.6 mV. Dibutyryl cAMP and forskolin both increased Vt, whereas H89, an inhibitor of cAMP-dependent protein kinase, abolished the AVP-induced increase in Vt. These results demonstrate that AVP stimulates Cl^- transport in the ascending thin limb of Henle's loop by activating Cl^- channels via a signal transduction cascade comprising V2 receptors, adenylate cyclase, and cAMP-dependent protein kinase. The ascending thin limb of Henle's loop thus participates in the formation of concentrated urine as one of the target renal tubular segments of AVP. (*J. Clin. Invest.* 1995. 95:1623–1627.) Key words: renal tubule • microperfusion • V2 receptor • Cl^- channel • protein kinase A

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1. *Abbreviations used in this paper:* ATL, ascending thin limb of Henle's loop; AVP, arginine vasopressin; dDAVP, [deamino-Cys^1, D-Arg^8]-vasopressin; H89, *N*-[2-(*p*-bromocinnamylamino)-benzoate; NPPB, 5-nitro-2-(3-phenyl-propylamino)-benzoate; P_{Cl}/P_{Na}, Cl^- to Na^+ permselectivity ratio; Vt, transepithelial voltage.

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Introduction

Intense research activity has been directed towards determining the mechanism of action of arginine vasopressin (AVP)[1] in many target organs. The antidiuretic effect of AVP is mediated by its renal type (V2) receptors linked to adenylate cyclase (1). AVP stimulates transepithelial transport of water, urea, and NaCl in most of the renal tubular segments expressing V2 receptors, such as the collecting duct and the thick ascending limb of Henle's loop (1). However, in the ascending thin limb of Henle's loop (ATL), where V2 receptors are expressed and AVP stimulates cAMP formation, no action of AVP on transepithelial electrolyte transport has been positively demonstrated (2). This observation led us to disregard the ATL as a target tubular segment of AVP. It has remained unexplained for more than a decade why V2 receptors are expressed in the ATL.

Urine concentration is achieved by water reabsorption in the collecting duct according to the osmolar gradient across the tubule (3). In this process, the ATL plays an important role in the inner medulla by diluting urine and by maintaining the interstitium hypertonic through passive reabsorption of NaCl (3). The ATL is highly permeable to NaCl (4, 5) and impermeable to water (6). Moreover, Na^+ is reabsorbed through the paracellular shunt pathway (7, 8, 9) and Cl^- is reabsorbed through the cells via Cl^- channels in both the luminal and basolateral membranes (10–15). As for Cl^- transport, it has been suggested that there is a specific facilitated Cl^- transport system in the ATL (10, 16). This transport system is unique in that its permeability to Cl^- is > 10–20 times that apparent in the ascending thick limb of Henle's loop (6). Because of the electrogenicity and symmetry of the transepithelial NaCl diffusion potential, this transport system was initially considered to be mediated by Cl^- channels located in the tight junctions of the ATL (6). Subsequently, Kondo et al. (13) and Yoshitomi et al. (11) provided indirect and direct evidence for the presence of stilbene-sensitive Cl^- channels in both the luminal and basolateral membranes of the ATL. Several studies have supported the view that transcellular Cl^- transport is regulated by factors such as intracellular pH (12) and Ca^{2+} activity (15), as well as the water balance of the body (17). Kondo et al. demonstrated that intracellular acidification of ATL cells with *o*-nitrophenylacetate (12) or chelation of intracellular Ca^{2+} with Quin-II (15) inhibited Cl^- permeability. The effects of acidification and Ca^{2+} occurred in a noncompetitive manner, suggesting that both protons and Ca^{2+} are important regulators of Cl^- conductivity in the ATL (15). Recent studies in our laboratory have shown that the intracellular pH of ATL cells is maintained mainly by amiloride-sensitive Na^+/H^+ antiport in the basolateral membrane (18). These results suggest that ambient Na^+ concentration is one of the factors that regulate Cl^- permeability in the ATL. We have also shown that intracellular Ca^{2+} of ATL cells is kept low mainly by a calmodulin-sensitive Ca^{2+} ATPase and a dihydropyridine-sensitive Ca^{2+} channel in the basolateral membrane (19).

Recently, a molecular biological study demonstrated the presence of a specific 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS) sensitive Cl^- channel exclusively in this nephron segment (17), which is consistent with the idea that Cl^- is reabsorbed across ATL cells via DIDS-sensitive Cl^- channels in both the luminal and basolateral membranes. The amount of the mRNA encoding this Cl^- channel in the ATL increased when the animals were deprived of water, suggesting that this Cl^- channel plays an important role in concentrating urine (17).

We have now investigated the effect of AVP on Cl^- transport in the isolated microperfused hamster ATL and demonstrated that, at physiological concentrations, AVP stimulates Cl^- permeability in the ATL via V2 receptors, indicating that the ATL participates in the formation of concentrated urine as one of the target renal tubular segments of AVP.

Methods

In vitro microperfusion of isolated tubules. The ATL was microdissected and microperfused in vitro on an inverted microscope as previously described (7). Male golden hamsters weighing 50–80 g were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium and the left kidneys were removed. Briefly, a fragment of the ATL was microdissected under a stereoscopic microscope with fine forceps in a chilled dish filled with control Hepes-buffered solution containing (mM) 200 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 2 KH_2PO_4, 100 urea, 10 Hepes, 5.5 glucose, 5 L-alanine, 1 Na acetate (pH 7.4, bubbled with 100% O_2). The ATL was then transferred to a perfusion chamber mounted on the stage of an inverted microscope (IMT-2; Olympus, Tokyo, Japan). The distal end of the ATL with a fragment of the medullary thick ascending limb was sucked into a glass pipette, and the lumen of the ATL was cannulated. During the preincubation period, both sides of the ATL were microperfused with control Hepes-buffered solution. Viability of the tubules was ensured by isolating ATLs within 1 h after removal of the kidney.

Measurement of transepithelial voltage. Transepithelial voltage (Vt) was measured as previously described (20). The bath solution was connected to a heat-pulled glass capillary that allowed the continuous outflow of 3 M KCl solution, so that there was no change in the liquid junctional potential during solution exchange in the chamber. The KCl solution for the flowing boundary was also connected to the calomel half-cell, which was directly grounded. The perfusion pipette used to monitor Vt was filled with the solution connected to a 1–M KCl agar bridge in a thin polyethylene tube and then to an Ag-AgCl wire connected directly to one probe of a high-input impedance electrometer (FD-223; WPI, New Haven, CT). To avoid interference of the dilution potential of NaCl with Vt, we perfused the lumen of the ATL at a rate of > 20 nl/min. Bathing fluid was continuously exchanged at a rate of 5 ml/min. Vt was zero when both luminal and basolateral sides of the ATL were microperfused with control Hepes-buffered solution (37°C). Experiments were started after incubation of the tubules for 30 min in the presence of a transmural NaCl concentration gradient, which was imposed by perfusing the tubular lumen with control Hepes-buffered solution and bathing the tubule in a solution identical to the control solution but containing 100 mM NaCl and 280 mM urea.

Cl^- to Na^+ permselectivity ratios (P_{Cl}/P_{Na}) were determined from the measurement of the diffusion potential at zero volume flow produced by transepithelial ion activity gradients according to the Goldman equation:

$$Vt = 2.3 \cdot R \cdot T/F \cdot \log [\{a^b_{Na} + (P_{Cl}/P_{Na})a^l_{Cl}\}/ \{a^l_{Na} + (P_{Cl}/P_{Na})a^b_{Cl}\}] \quad (1)$$

where P_{Cl} and P_{Na} are the permeabilities of the epithelium to Cl^- and Na^+, respectively; a^b and a^l are the ion activities in the bath and luminal fluid, respectively; and R, T, and F have their usual meanings. When the diffusion potential for NaCl was measured, the concentration of NaCl in the bath was reduced by 100 mM by replacing NaCl with equiosmolal urea.

Measurement of lumen to bath Na^+ Cl^- flux. The lumen to bath flux of Na^+ or Cl^- was measured from the disappearance of $^{22}Na^+$ or $^{36}Cl^-$ from the perfusate as previously described (12–15) according to the equations:

$$Ke = (V/L) \ln (C_i/C_o) \quad (2)$$

$$J = [x]_p Ke \quad (3)$$

where Ke is the lumen to bath efflux coefficient; V is the fluid collection rate; L is the length of tubules; C_i and C_o are concentrations of isotope in the perfusate and collected fluid, respectively; J is the lumen to bath flux; and $[x]_p$ is the concentration of Na^+ or Cl^- in the perfusate. These parameters were always measured under the zero voltage condition, in which the compositions of the luminal perfusate and bathing fluid were identical control Hepes-buffered solution. Both $^{22}Na^+$ and $^{36}Cl^-$ were obtained as NaCl solution. The radioactivities of $^{22}Na^+$ and $^{36}Cl^-$ were measured with a gamma counter (ARC-500; Aloka, Tokyo, Japan) and a beta counter (LS6500; Beckman Instruments, Inc., Fullerton, CA), respectively. At least three samples were collected during each experimental condition, and the data were represented by the means of these samples. Collection of perfusate during each period was performed after a 30-min incubation at 37°C.

Statistical analysis. Results are expressed as means±SEM. Comparisons within a group with one treatment used the paired Student's *t* test. Comparisons between two groups employed the unpaired Student's *t* test. Multiple groups were analyzed using analysis of variance (ANOVA) and Bonferonni's modification of the *t* test. A value of $P < 0.05$ was accepted as statistically significant.

Chemicals. [Arg^8]-vasopressin (AVP), [deamino-Cys^1, D-Arg^8]-vasopressin (dDAVP), N^6, 2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cAMP), and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO); [$d(CH_2)_5{}^1$,*O*-Me-Tyr^2, Arg^8] vasopressin and [$d(CH_2)_5$,D-Ile^2,Ile^4,Arg^8]vasopressing from Peninsula Laboratories Inc. (Belmont, CA); *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) from Seikagaku (Tokyo, Japan); and 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB) from Research Biochemicals Inc. (Natick, MA). All other chemicals were from Wako (Osaka, Japan). $^{22}Na^+$ and $^{36}Cl^-$ were from Amersham International (Little Chalfont, United Kingdom).

Results

Effects of AVP on Vt. In the absence of a transmural NaCl concentration gradient, Vt was zero, as Imai and Kokko described (10). Reduction of NaCl in the basolateral solution from 200 to 100 mM by isotonic replacement with urea deflected Vt in a lumen-positive direction, because the transepithelial permeability of Cl^- is greater than that of Na^+ (10). In the presence of a transmural NaCl concentration gradient, Vt was 8.4±0.4 mV ($n = 10$). Addition of 1 nM AVP to the basolateral solution increased Vt to 9.6±0.4 mV in 10 min ($n = 10$, $P < 0.0001$) (Fig. 1). After removal of AVP, Vt did not decrease for more than 30 min. AVP (1 nM) increased P_{Cl}/P_{Na} from 2.8±0.2 to 3.4±0.2 ($n = 10$, $P < 0.0001$), which corresponds to an increase of 20.7±2.9%. In the absence of AVP, Vt did not change during the entire experimental period. AVP at physiological concentrations increased Vt in a dose-dependent manner, with an ED_{50} of 5 pM (Fig. 2).

Effects of AVP on Na^+ and Cl^- permeability. To determine whether the increase in Vt was attributable to the increase in Cl^- permeability, we examined the changes in the lumen to bath efflux coefficient of $^{36}Cl^-$. AVP (1 nM) increased the Cl^- efflux coefficient from 99.6±6.3 to 131.4±10.6 × 10^{-7} cm^2/s

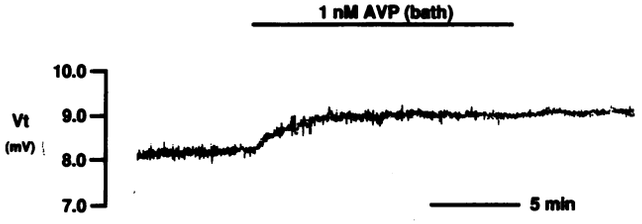

Figure 1. Representative tracing of the effects of AVP in the bath solution on Vt of an isolated microperfused ATL. Vt was measured in the presence of a transmural NaCl concentration gradient (100 mM higher in the lumen). Addition of 1 nM AVP to the basolateral solution increased Vt. Vt did not reduce for more than 30 min after removal of AVP.

($n = 8$, $P < 0.005$) (Table I), which corresponds to an increase of 31.3±6.3%. However, AVP did not affect the Na^+ efflux coefficient (Table I). In the absence of AVP, the Cl^- efflux coefficient did not change during the entire experimental period. The extent of AVP-induced increase in P_{Cl}/P_{Na} did not differ significantly from that in the Cl^- efflux coefficient. These results indicate that the increase in Vt upon addition of AVP to the basolateral solution was attributable to the increase in Cl^- permeability. To confirm that the AVP-induced increase in Cl^- permeability resulted from activation of the Cl^- channel, we examined the effect of NPPB, a Cl^- channel inhibitor. Addition of 0.2 mM NPPB to the perfusate decreased the basal Cl^- efflux coefficient and inhibited the AVP-induced increase in this parameter (Table I), indicating that AVP stimulates Cl^- permeability by activating the Cl^- channel.

Receptor and signal transduction mechanism that mediate the effect of AVP. To determine whether the AVP-induced increase in Cl^- permeability is mediated by V1 or V2 receptors, we examined the effects of receptor-specific antagonists on the AVP-induced increase in Vt. Addition of [$d(CH_2)_5{}^1$,*O*-Me-Tyr^2, Arg^8] vasopressin, a V1 receptor antagonist Vt, or [$d(CH_2)_5$,D-Ile^2,Ile^4,Arg^8]vasopressin, a V2 receptor antagonist, did not affect Vt (Fig. 3 *A*). The AVP-induced increase in Vt in the presence of the V1 receptor antagonist corresponds to an increase in P_{Cl}/P_{Na} by 16.0±2.4%, which was not significantly different from that without antagonists. In the presence of the V2 receptor antagonist, the AVP-induced increase in Vt corresponds to an increase in P_{Cl}/P_{Na} by 2.2±1.5%, which was significantly different from that without antagonists. Furthermore, dDAVP, a selective V2 agonist, in 1 nM also increased Vt from 8.6±0.7 to 9.5±0.6 mV ($n = 7$; $P < 0.05$) (Fig. 3 *B*).

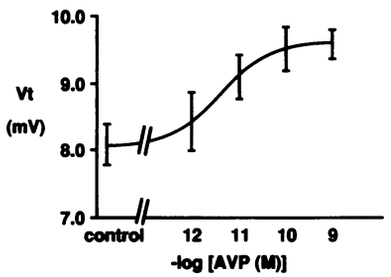

Figure 2. Dose-response relation between the concentration of AVP in the bath solution and Vt. Vt was measured in the presence of a transmural NaCl concentration gradient (100 mM higher in the lumen). Various concentrations of AVP were added to the basolateral solution. The steady state Vt values are expressed as means±SEM ($n = 4$).

Table I. Effects of AVP on Na^+ and Cl^- Efflux Coefficients

		L	Condition	V	Ke	Δ Ke
	n	*mm*		*nl/min*	*10^{-7} cm²/s*	*10^{-7} cm²/s*
Cl^-	8	0.25±0.04	Control	34.5±2.6	99.6±6.3	32.0±6.7
			AVP	35.0±2.7	131.4±10.6*	
Na^+	6	0.26±0.04	Control	34.8±4.0	37.8±3.3	0.7±1.2
			AVP	34.2±3.8	38.5±4.2	
Cl^-	6	0.27±0.04	NPPB	32.1±2.3	41.2±8.1	5.3±1.2‡
			NPPB + AVP	30.7±2.1	46.4±9.2§	

L, tubular length; V, perfusion rate; Ke, efflux coefficient; ΔKe, difference in efflux coefficients between the paired samples. The lumen to bath fluxes of Na^+ or Cl^- were measured from the disappearance of $^{22}Na^+$ or $^{36}Cl^-$ from the perfusate. The compositions of the luminal perfusate and bathing fluid were identical control Hepes-buffered solution. For the AVP condition, 1 nM AVP was added to the bathing solution. NPPB (0.2 mM) was added to the luminal solution as indicated. Results are expressed as means±SEM. * $P < 0.005$ compared with the value without AVP (paired *t* test); ‡ $P < 0.01$ compared with the value without NPPB (unpaired *t* test). § $P < 0.05$.

We then examined the signal transduction system associated with this effect of AVP. Addition of dibutyryl cAMP (0.5 mM), and forskolin (25 μM) to the basolateral solution increased Vt (Fig. 4 *A*). Furthermore, addition of H89, a highly specific inhibitor of cAMP-dependent protein kinase, abolished the AVP-induced increase in Vt (Fig. 4 *B*).

Discussion

The present study demonstrates that AVP stimulates Cl^- transport in the ATL via V2 receptors, adenylate cyclase, and cAMP-dependent protein kinase. Thus, AVP stimulates Cl^- reabsorption and decreases tubular Cl^- concentration in the ATL. Cl^- is the key electrolyte in the control of extracellular fluid volume homeostasis (21). Tubuloglomerular feedback is sensitive to Cl^- concentration in the luminal solution in the thick ascending limb of Henle's loop, because the proximal tubular pressure oscillates in association with oscillations in tubular flow and the early distal tubular Cl^- activity (21). Cl^- transport through macula densa cells is important in the tubuloglomerular feedback mechanism and in macula densa–mediated renin release (21). It is therefore consistent that AVP regulates Cl^- transport rather than Na^+ transport in the ATL.

Imai and Kusano observed that AVP induced an approximately twofold increase in cAMP concentration in the ATL (2). However, they failed to detect an effect of AVP on Cl^- permeability in the hamster ATL microperfused in vitro (2); it is possible that the tubules were too long (0.3–0.4 mm) or the perfusion rates too slow (12–16 nl/min) to detect such an effect. We observed that the changes in Cl^- permeability were not easy to detect with long tubules or slow perfusion rates, because basal Cl^- permeability in the ATL was high. The dose of vasopressin in the previous study was relatively high (1–10 mU/ml), which also might have been responsible for the negative results because such pharmacological doses of vasopressin have been shown to reduce adenylate cyclase responsiveness as well as the rate of cAMP production (22, 23).

Cl^- transport across the ATL is more sensitive to the baso-

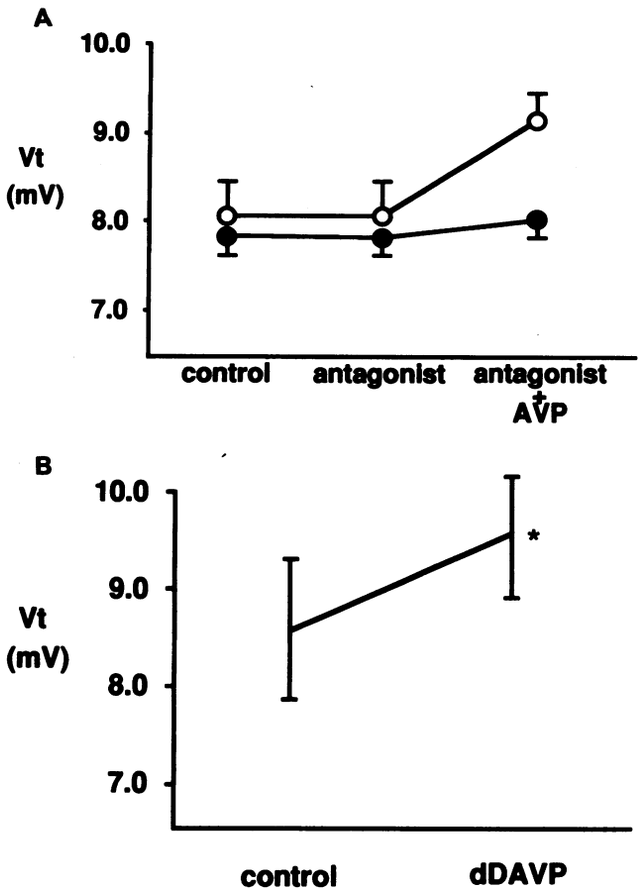

Figure 3. Receptor that mediates the effect of AVP. (*A*) Effect of a V1 receptor antagonist (*open circles*) and a V2 receptor antagonist (*closed circles*) on the AVP-induced increase in Vt. [d(CH_2)$_5$^{1},*O*-Me-Tyr^2, Arg^8] vasopressin (0.1 μM) and [d(CH_2)$_5$,D-Ile^2,Ile^4,Arg^8] vasopressin (1 μM) were used as V1 and V2 receptor antagonists, respectively. After addition of antagonist to the bath fluid for 15 min, AVP (1 nM) was added to the bath solution (n = 6). (*B*) Effect of 1 nM dDAVP in the bath fluid on Vt (n = 7). *P < 0.05.

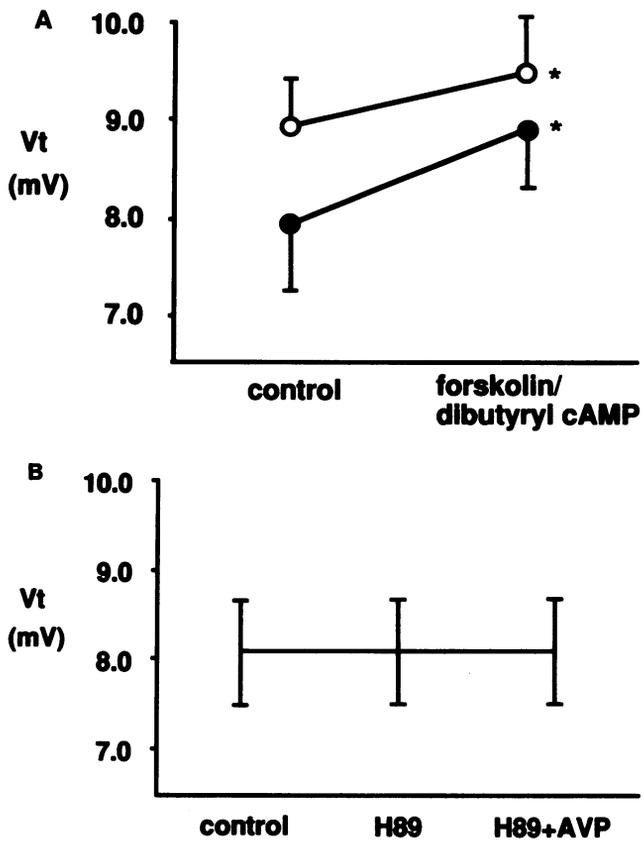

Figure 4. Signal transduction system that mediates the effect of AVP. (*A*) Effect of 0.5 mM dibutyryl cAMP (*closed circles*) and 25 μM forskolin (*open circles*) in the bath fluid on Vt (n = 5, respectively). *Significantly different from control value (P < 0.05; paired t test). (*B*) Effect of H89 on AVP-induced increase in Vt. After addition of 5 μM H89 to the bath fluid and perfusate for 15 min, 1 nM AVP was added to the bath solution (n = 6).

lateral pH than the luminal pH (12) and is regulated by Ca^{2+} preferentially in the bathing solution (15). Furosemide in the bathing solution inhibited Cl^- transport across the ATL, but the inhibitor had no effect on Cl^- transport when added to the luminal solution (13). We recently demonstrated with the use of a Na^+-sensitive fluorescent probe, SBFI, that the furosemide-sensitive Na^+ transporter is absent in the ATL (9). Therefore, we conclude that furosemide inhibited the Cl^- channel in the basolateral membrane but not in the luminal membrane. These observations indicate that Cl^- channels in the apical and basolateral membranes have different physiological characteristics. Thus, the hormonal regulation of the Cl^- channels in these two membranes also might be different. The present study indicates that Cl^- transport across the ATL is inhibited by NPPB in the luminal solution. However, this observation does not clarify whether AVP stimulates Cl^- channels in the apical or basolateral membrane, or both. Patch clamp experiments or measurements of intracellular Cl^- concentration are required to provide a definitive answer to this question. But ATL cells are too small and exhibit too much interdigitation to attach a patch pipette. Our preliminary attempts to measure intracellular Cl^- concentration with a chloride-sensitive fluorescent probe, MQAE, were not successful because the dye was not sufficiently loaded. Intracellular Cl^- concentration measurement with double-barreled, ion-selective microelectrodes, as described by Kondo et al. (24), is also impossible in ATL cells because they are too small to impale. Therefore we cannot clarify this point at this moment.

In the inner medulla of the kidney, where the final regulation of water and electrolyte excretion occurs, the inner medullary collecting duct was thought to be the only target of AVP to affect transepithelial transport. However, our results indicate that the ATL participates in the formation of concentrated urine as another target segment of AVP, where AVP stimulates NaCl reabsorption according to the transmural NaCl concentration gradient. The resulting enhancement of urine dilution and interstitial hypertonicity synergistically stimulate water reabsorption in the inner medullary collecting duct, which occurs together with the direct effect of AVP on the inner medullary collecting duct to increase water permeability. We conclude that AVP promotes concentrating urine not only by increasing water permeability in the collecting duct, but also by stimulating dilution of urine in the entire ascending limb.

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