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Vasopressin Regulates Water Flow in a Rat Cortical Collecting Duct Cell Line Not Containing Known Aquaporins

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Abstract. Transepithelial water movements and arginine-vasopressin (AVP)-associated ones were studied in a renal cell line established from a rat cortical collecting duct ($RCCD_1$). Transepithelial net water fluxes (J_w) were recorded every minute in $RCCD_1$ monolayers cultured on permeable supports. Spontaneous net water secretion was observed, which was inhibited by serosal bumetanide (10^{-5} M), apical glibenclamide (10^{-4} M) and apical $BaCl_2$ (5×10^{-3} M). RT-PCR, RNAse protection and/or immunoblotting experiments demonstrated that known renal aquaporins (AQP1, AQP2, AQP3, AQP4, AQP6 and AQP7) were not expressed in $RCCD_1$ cells. AVP stimulates cAMP production and sodium reabsorption in $RCCD_1$ cells. We have now observed that AVP significantly reduces the spontaneous water secretory flux. The amiloride-sensitive AVP-induced increase in short-circuit current (I_{sc}) was paralleled by a simultaneous modification of the observed J_w: both responses had similar time courses and half-times (about 4 min). On the other hand, AVP did not modify the osmotically driven J_w induced by serosal hypertonicity. We can conclude that: (i) transepithelial J_w occurs in $RCCD_1$ cells in the absence of known renal aquaporins; (ii) the "water secretory component" observed could be linked to Cl^- and K^+ secretion; (iii) the natriferic response to AVP, preserved in $RCCD_1$ cells, was associated with a change in net water flux, which was even observed in absence of AQP2, AQP3 or AQP4 and (iv) the hydro-osmotic response to AVP was completely lost.

Key words: $RCCD_1$ cell line — Water-ion permeability — Aquaporins — AVP

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Introduction

The renal cortical collecting duct (CCD) plays an important role in the regulation of water and electrolyte balance. In renal tissue, arginine-vasopressin (AVP) acts through two different mechanisms: (i) the "natriferic response", corresponding to stimulation of sodium reabsorption via apical sodium channels and basolateral Na^+-K^+-ATPase [2, 17, 27]. Such Na^+ movement, coupled to the corresponding co-ions, will induce a transport-associated water movement, generally described as isotonic; (ii) the "hydro-osmotic response" mediated by an increase in water permeability through the insertion of aquaporin-2 (AQP2) in the apical membrane of CCD principal cells [1, 30]. In this case, water will move following the transepithelial osmotic gradient via apical AQP2 and basolateral resident AQP3 and AQP4.

AVP-regulated electrolyte transport has been previously reported in an epithelial cell line derived from the renal cortical collecting duct of the rat ($RCCD_1$), which maintains very high transepithelial resistance similar to that observed in the native tissue [2]. AVP increases the short-circuit current (through binding to V2 receptor) and cAMP production in $RCCD_1$ cells. AVP-induced current is inhibited by amiloride and it is also sensitive to chloride and potassium channel blockers [2, 6, 7], showing that $RCCD_1$ cells absorb Na^+ and secrete Cl^- upon AVP response. However, the characteristics of water movements (absorptive and/or secretory) and their regulation by AVP are unknown in $RCCD_1$ cells.

Even if the recent characterization and cloning of water channels has partially clarified their role in water transfers across epithelial barriers, functional studies should provide additional information on the water pathways in aquaporin-containing and in aquaporin-lacking epithelial cells. We have previously reported the characteristics of water permeability in a renal cell line

(LLC-PK1) transfected with AQP2 and grown in a permeable support [28]. Previous studies were performed in cells grown on glass coverslips, where transepithelial permeability could not be measured [13]. Now, we have studied the characteristics of water transport and its regulation by AVP in a tight epithelia formed by $RCCD_1$ cells on permeable supports. The minute-by-minute recordings of the transepithelial net water fluxes (J_w) were associated in different experimental conditions with the measurement of electrophysiological parameters (potential difference (ΔV), short-circuit current (I_{sc}) and transepithelial resistance (R_T)) and to mannitol permeability. Unexpectedly, we have observed a net water secretory flux in "basal" conditions. This spontaneous secretory J_w and the associated I_{sc} were inhibited by different pharmacological agents affecting ion transport across epithelia, which indicated coupling of water to specific ions. In addition, AVP modulated fluid movements simultaneously with the increase in I_{sc}. RT-PCR, RNAse protection and/or immunoblotting studies demonstrated the absence in $RCCD_1$ cells of the aquaporins normally expressed in the native tubules.

Materials and Methods

Cell Culture

Rat cortical collecting duct cells ($RCCD_1$) [2] were grown on Petri dishes and routinely passed once a week using trypsin. The defined culture medium (DM) was as follows: 1:1 Ham's F-12/DMEM; 14 mM $NaHCO_3$, 3.2 mM glutamine; 5×10^{-8} M dexamethasone; 3×10^{-8} M sodium selenite; 5 μg/ml insulin; 10 μg/ml epidermal growth factor; 5×10^{-8} M triodothyronine; 10 units/ml penicilin-streptomycin; 20 mM HEPES (pH 7.4) and 2% fetal bovine serum (Gibco BRL). For the experiments, cells between passages 30 and 45 were seeded on Transwell holders (3 μm pore Nucleopore filters; 4.5 cm^2 surface area; Costar) and cultured for 6–7 days.

Solutions

Cells were bathed on either side with minimum medium containing 1:1 Ham's F12-DMEM and 14 mM $NaHCO_3$, pH 7.4 when bubbled with 5% CO_2/95% O_2. In some experiments, a "standard saline solution" containing (in mM): 139 NaCl, 5 KCl, 10 $NaHCO_3$, 1 $CaCl_2$, 1 $MgCl_2$, 0.8 $MgSO_4$, 20 HEPES, 5 glucose, pH 7.4; was used. In bicarbonate-free experiments, $NaHCO_3$ was osmotically replaced by sucrose. Finally, in chloride-bicarbonate free experiments the following "HEPES-sulfate solution" was used (in mM): 69.5 $NaSO_4$, 2.5 K_2 SO_4, 1 $CaSO_4$, 1.8 $MgSO_4$, 20 HEPES, 10 Tris, 5 glucose, 74 sucrose, pH 7.4.

Measurement of Water Fluxes

The transwell filters were directly inserted between two barrel lucite hemichambers so as to define two independent compartments, as previously described [28]. One of them (basolateral) was open to the atmosphere, while the other (apical) was hermetically sealed. A positive hydrostatic pressure gradient (4.5 cm of H_2O) was continuously applied to the apical bath. The closed chamber was connected with a small diameter polyethylene tube to the net water measurement system where the net water flux (J_w) was recorded every minute, as described elsewhere [8]. Briefly, the position of a liquid meniscus inside a capillary tube, was photoelectrically detected. Displacements to the right or to the left were proportional to the amount of water moving across the tissue layer. The data were computed in units of $\mu l.min^{-1}.cm^2$. The sensitivity of the system was 50 nl. The serosal bath was continuously bubbled with the appropriate CO_2/O_2 mixture to maintain the pH of the medium at 7.4 ± 0.1 (37°C).

In pharmacological studies, $RCCD_1$ cells were cultured in 6 holder clusters. To minimize random fluctuations, 3 holders were routinely taken as controls whereas the rest were tested with the employed agents (Student *t* test for nonpaired values was applied). Because of the use of this protocol, control data were different in different experimental series.

Electrophysiological Studies

Transepithelial voltage (V_T) and short-circuit current (I_{sc}) were continuously recorded employing an automatic voltage-clamp system (Physiological Instruments) and Navycite (ME2AG4) electrodes. Transepithelial resistance (R_T) was estimated every 90 sec from current deflections in response to a 1–2 mV/sec pulse.

Unidirectional ^{14}C-mannitol Fluxes

Transepithelial ^{14}C-mannitol fluxes were measured as previously reported [28]. ^{14}C-mannitol was added to the mucosal bath (1 μCi/ml) before water flux measurements. Samples (1 ml) were taken from the basolateral bath every 5 min. Mannitol permeability (P_s) was calculated taking into account corrections for sampling dilution and back-fluxes.

RT-PCR Studies

Total RNA from kidney (positive control) or $RCCD_1$ cells was isolated using SV total RNA Isolation System (Promega). Reverse transcription was performed on 2 μg of total RNA using the SuperScript Preamplification System for First Strand cDNA Synthesis (Gibco BRL). RNAs were placed in 50 μl of "RT reaction buffer" containing 1× PCR buffer, 0.5 μg oligo-dt primer, 0.1 mg/ml BSA, 10 mM DTT, 2.5 mM $MgCl_2$ and 10 U/μl RNAsin. The reaction was heated for 3 min at 80°C and cooled at 45°C. PCR buffer (25 μl) containing 1× PCR buffer, 0.1 mg/ml BSA, 10 mM DTT, 2.5 mM $MgCl_2$, 400 μM dNTPs and 100 units SuperScript II RT was added to half of the reaction. Control experiments in absence of the enzyme SuperScript II RT were performed on the other 25 μl. RT reaction was carried out for 1 hr at 45°C and stopped by heating for 2 min at 95°C.

PCR experiments (30 sec at 95°C, 30 sec at 56°C, 45 sec for 72°C for 30–35 cycles) were performed on 5 μl of the RT reaction using 10 pmol of specific primers for rat AQP1, AQP2, AQP3, AQP4, AQP6 and AQP7 (*see* Table 1). Internal positive control was included in each experiment using β-actin specific primers (sense: 5′ CGG AAC CGC TCA TTG CC 3′; antisense: 5′ ACC CAC ACT GTG CCC ATC TA 3′).

RNAse Protection Assays

Experiments were performed on $RCCD_1$ cells grown on transwell filters (Costar) and lysed with a guanidium thiocyanate solution, as previously described [7]. Antisense cRNA probes for AQP3 (AQP_s) were synthesized using [^{32}P]-UTP (15 TBq/mmol; Amersham) with a Promega riboprobe kit. AQP3 probe yields a protected fragment of 274 bp (nucleotide 327–601). The glyceraldehyde-3-phosphate dehydroge-

Table 1. Primers used for aquaporins expression

	Primers	cDNA sequence location (nt)	Amplified fragment size (bp)	Data bank accession number
AQP1	up: 5′-GTC CCA CAT GGT CTA GCC TTG TCT G-3′	983–1008	362	107268
	dw: 5′-GGG AAG GGT CCT GGA GGT AAG TCA-3′	1321–1345		
AQP2	up: 5′-GCC CCT TGC AGG AAC CAG ACA-3′	1079–1100	277	D13906
	dw: 5′-GCC AAA GCG GGA ATG ACA GTC-3′	1335–1362		
AQP3	up: 5′-GGC TAA AAA CGC TCC CTG TAT CCA-3′	974–998	645	d17695
	dw: 5′-GGA GTT TCC CAC CCC TAT TCC TAA A-3′	1594–1619		
AQP4	up: 5′-TGC CAC CCA TTA AGG AAA CAG ATT-3′	1028–1052	540	u14007
	dw: 5′-GAT GCT GAG GGG GAA GAA GGA TTA T-3′	1543–1568		
AQP6	up: 5′-CTG CTT GTA TGG TGT CCC TGG TGT-3′	1003–1026	262	af083879
	dw: 5′-GGC CTT GGA AAA CTA ACT GGA TGG-3′	1241–1264		
AQP7	up: 5′-GCT GGC TGG GGC AAG AAA GTG-3′	931–952	323	ab000507
	dw: 5′-TTT ATT GCA GAA GGG TTG TGG TCA-3′	1230–1254		

nase (nt 707–871, protected fragment: 164 bp) and β-actin (nt 696–833, protected fragment: 137 bp) signals were used as internal standards. The effects of 24 hr dDAVP (10^{-8} M) and dibutyryl cAMP (10^{-3} M) were also tested.

Immunoblotting for AQP3

Confluent $RCCD_1$ cells were washed three times in cold PBS, and were incubated for 30 min in a cold lysis buffer containing (in mM): 150 NaCl, 20 Tris, 5 EDTA, 1% Triton 100×, 1 mM phenyl-methylsulfonyl fluoride, 10 μg/ml antipaine, 10 μg/ml leupeptine and 10 μg/ml pepstatine. The obtained lysate was clarified by centrifugation at 12,000 × *g* for 10 min. The homogenates were subject to 12% SDS-polyacriylamide minigels electrophoresis and transferred to nitrocellulose sheets (Bio-Rad Mini Protean II). Blots were blocked with 5% milk in PBS-T (in mM: 80 Na_2HPO_4, 20 NaH_2PO_4, 100 NaCl, and 0.1% Tween 20, pH 7.5) for 1 hr and incubated with purified anti-AQP3 (diluted 1:2,000; generously supplied by Dr. Tachnet from Saclay, France). The blots were then washed and incubated with horseradish peroxidase-conjugated goat antirabbit inmunoglobulin and bands were detected by the enhanced chemioluminiscense detection system (BCL, Amersham). Autoradiographs were obtained by exposing nitrocellulose to Kodak XAR film.

Results

Spontaneous Net Water Transport in $RCCD_1$ Cells

$RCCD_1$ cells were cultured on permeable filters for 6 or 7 days before they were mounted in the flux measurement chamber. Figure 1*A* shows that a significant and sustained spontaneous net secretory J_w (from the basolateral to the apical side, at 37°C) occurred in absence of any osmotic or chemical gradient and even against the hydrostatic gradient (4.5 cm of water) present in our measurement system. In this figure and all along the paper, negative values indicate secretory fluxes whereas positive values represent absorptive fluxes. It can be observed that this secretion was clearly inhibited at a lower temperature (27°C) and even reversed to an absorptive flux. This result suggests that the secretory J_w was associated with an active transport process. As expected, Fig. 1*B* shows that I_{sc} was also temperature-dependent.

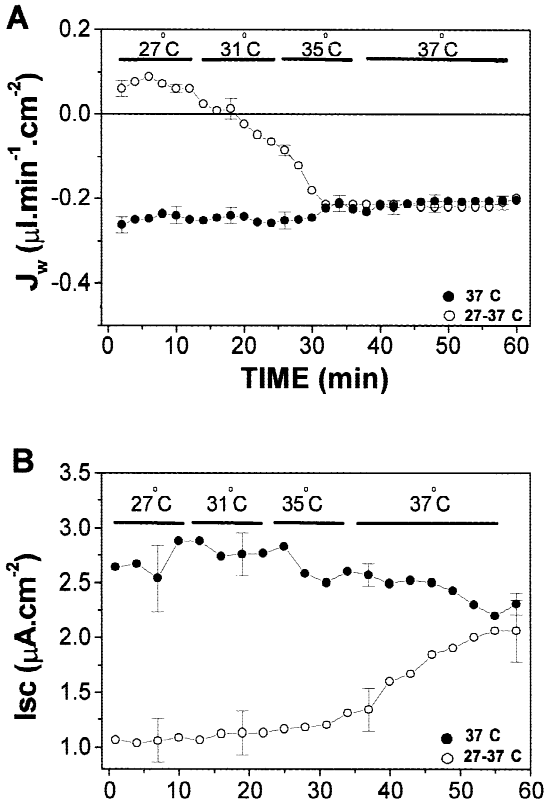

Fig. 1. Time course of water fluxes (J_w) and short-circuit current (I_{sc}) in $RCCD_1$ cells: effect of temperature. (*A*) Spontaneous secretory J_w observed in absence of any osmotic or chemical gradient (n = 13). Negative values indicate secretory fluxes and positive values absorptive fluxes. (*B*) I_{sc} evolution at different temperatures. In both figures, temperature varies between 27 and 37°C in experimental cells (open circles) or it is maintained at 37°C in controls (close circles).

No correlation was detected between the number of days cells were in culture and water secretion or trans-

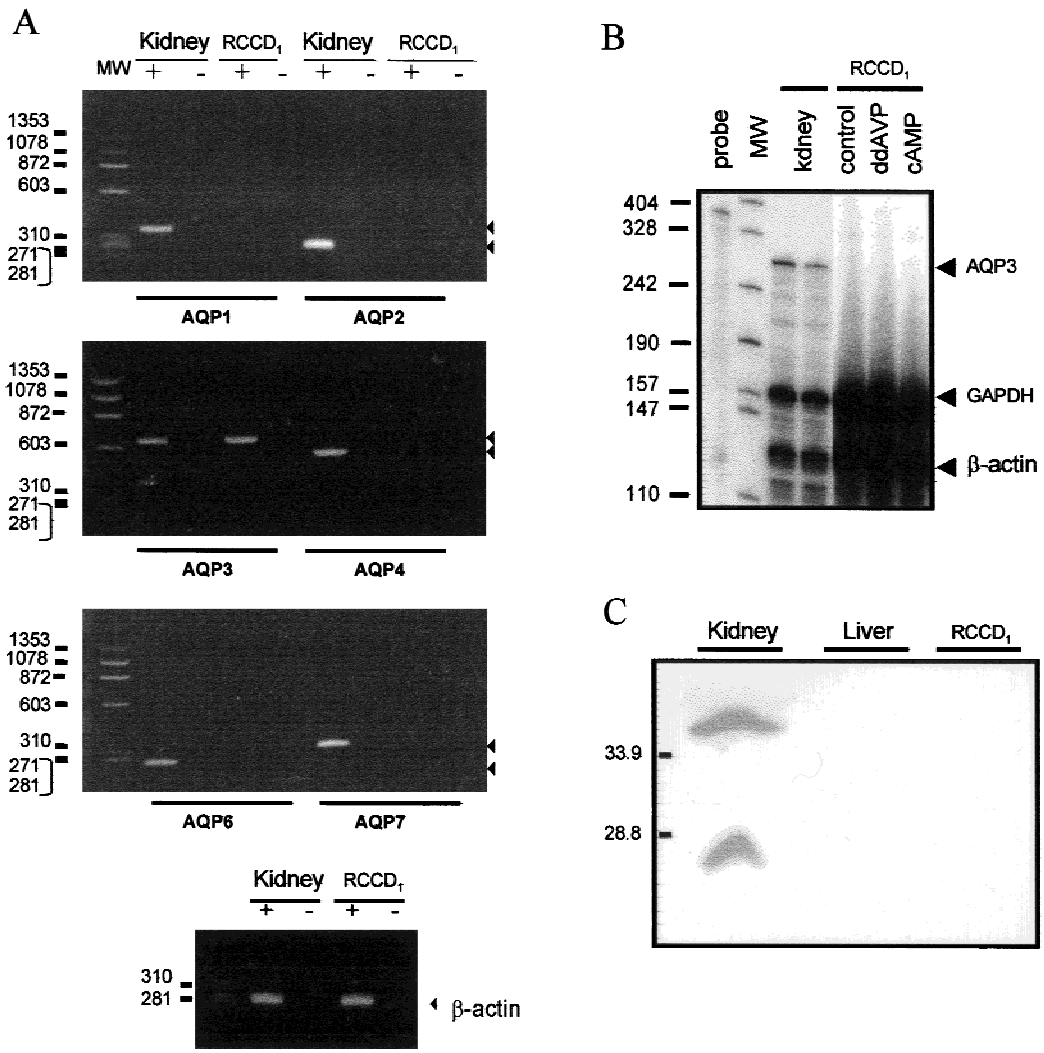

Fig. 2. AQPs expression in $RCCD_1$ cells. (*A*) RT-PCR experiments performed using specific primers for rat AQP1, AQP2, AQP3, AQP4, AQP6 and AQP7 in mRNAs from kidney (as positive control) and $RCCD_1$ cells. β-actin was used as an internal control. Assays were carried out in presence (+) or absence (−) of RT enzyme. An aliquot (10 μl) of each reaction was electrophoresed through a 2% agarose gel stained with ethidium bromide. Left lane, molecular weight marker (MW, ϕ X174 *Hae III* digested). Specific bands can be observed in all the positive controls but transcript was only observed for AQP3 in $RCCD_1$ cells. (*B*) RNAse Protection assay using specific probes for rat AQP3, GAPDH (164 nt) and β-actin (137 nt) as internal controls. Total RNAs from kidney or lysates of $RCCD_1$ were tested. $RCCD_1$ cells RNAs were hybridized in control conditions or after incubation with dDAVP (24 hr, 10^{-8} M) or dibutyryl cAMP (24 hr, 10^{-3} M). Protected fragments are indicated by arrows. First lane shows probe encoding for AQP3 (274 nt). MW molecular weight markers. (*C*) Immunoblot studies performed in homogenates of total kidney, liver and $RCCD_1$ cells. Samples were reacted with purified anti-AQP3 and revealed 27 kDa and 35 kDa AQP3 bands in positive control (kidney) but no bands were observed in liver (negative control) or $RCCD_1$ cells.

epithelial resistance [J_w ($\mu l.min^{-1}.cm^{-2}$) and R_T ($\Omega.cm^{-2}$) values: -0.152 ± 0.009 and 3221 ± 165 (day 6, $n = 15$); -0.165 ± 0.012 and 3361 ± 191 (day 7, $n = 8$)].

Known Renal Aquaporins are Not Expressed in $RCCD_1$ Cells

So as to determine whether aquaporins were expressed in $RCCD_1$ cells, RT-PCR experiments were performed using specific primers for AQP1, 2, 3, 4, 6 and 7, which are normally expressed in kidney [1, 18, 30, 31]. Results showed that while all mRNAs were present in the rat kidney (positive control) AQP3 was the only amplified transcript in $RCCD_1$ cells (Fig. 2*A*). To further investigate this last result, RNAse protection assay (a quantitative method to measure mRNA levels) was used. AQP3 mRNA was detected in rat kidney (positive control) but not in $RCCD_1$ cells. Furthermore, exposure of cells to dDAVP (10^{-8} M) or dibutyryl cAMP (10^{-3} M) for 24 hr did not induce AQP3 expression (Fig. 2*B*). In addition, immunoblot experiments showed the presence of AQP3

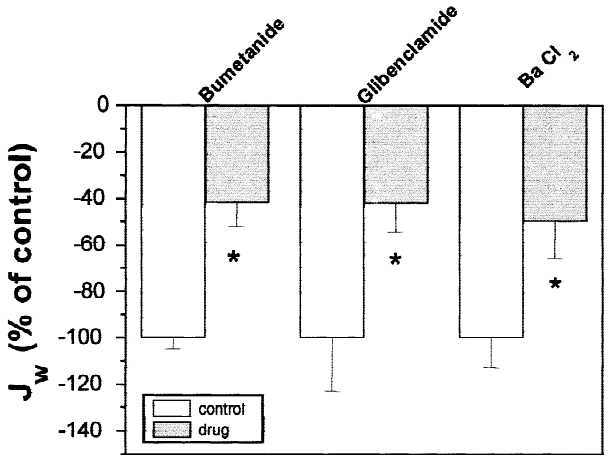

Fig. 3. Effects of ion transfer inhibitors on water secretion in $RCCD_1$ cells. 10^{-5} M Bumetanide was added to the serosal bath ($n = 6$), 10^{-4} M Glibenclamide and 5×10^{-3} M $BaCl_2$ were added to the mucosal bath ($n = 7$). Control experiments were performed in presence of the diluent alone. Experimental values are means ± SE as percentage of control values. * $P < 0.05$, ** $P < 0.01$ Student t test for unpaired data.

in the rat kidney (positive control) but not in the rat liver (negative control) or in $RCCD_1$ cells (Fig. 2*C*).

Effects of Ion Transport Inhibitors on Water Movements in $RCCD_1$ Cells

It has been proposed that chloride secretion in renal tissues and in $RCCD_1$ cells may involve chloride entry at the basolateral membrane through a Na^+-K^+-$2Cl^-$ cotransporter, and apical chloride exit through a glibenclamide-sensitive chloride conductance (presumably the cystic fibrosis transmembrane regulator; CFTR) [7, 14, 19, 23, 26]. Figure 3 shows that serosal addition of bumetanide (10^{-5} M), a specific blocker of the Na^+-K^+-$2Cl^-$ cotransporter, significantly decreased water secretion. On the other hand, apical glibenclamide addition resulted in a reduction of the net secretory J_w. These results suggest that water secretion may be coupled to chloride secretion, a mechanism recently proposed in $RCCD_1$ cells [7]. Since glibenclamide can also inhibit K^+ channels [16], we have evaluated the effect of the apical addition of 5×10^{-3} M barium chloride ($BaCl_2$), a potassium channel inhibitor. Figure 3 shows that $BaCl_2$ significantly decreased water secretion.

Changes in I_{sc} were simultaneously evaluated in these experiments, the results of which are given in Table 2. Reduction of I_{sc} was observed in presence of apical chloride and potassium channel inhibitors (glibenclamide and $BaCl_2$) or in the presence of the basolateral $Na^+/K^+/2Cl^-$ inhibitor (bumetanide).

Additional experiments were performed in a medium devoid of bicarbonate to block the Cl^-/HCO_3^- exchanger activity. In bicarbonate-free solutions, no significant change in J_w was observed ($\mu l.min^{-1}.cm^{-2}$: control: -0.152 ± 0.009 *vs.* 0 HCO_3^-: -0.115 ± 0.033, NS, $n = 4$). Nevertheless, water secretion was significantly reduced in absence of both chloride and bicarbonate ($\mu l.min^{-1}.cm^{-2}$: control: -0.152 ± 0.009 *vs.* 0 Cl^- − 0 HCO_3^-: -0.051 ± 0.013, $P < 0.001$, $n = 4$).

Table 2. Effects of bumetanide, glibenclamide, $BaCl_2$ and amiloride on I_{sc} in $RCCD_1$ cells

	Control	Drug
Bumetanide (10^{-5} M)	1.87 ± 0.11 ($n = 6$)	1.38 ± 0.18 ($n = 6$)*
Glibenclamide (10^{-4} M)	2.04 ± 0.19 ($n = 9$)	1.60 ± 0.18 ($n = 8$)*
$BaCl_2$ (5×10^{-3} M)	1.78 ± 0.09 ($n = 7$)	1.32 ± 0.16 ($n = 7$)*
Amiloride (10^{-5} M)	3.20 ± 0.06 ($n = 3$)	2.24 ± 0.11 ($n = 3$)**

I_{sc} experiments were performed either in control conditions (in the presence of diluent) or in presence of serosal bumetanide (10^{-5} M), mucosal glibenclamide (10^{-4} M), $BaCl_2$ (5×10^{-3} M) or amiloride (10^{-5} M).
* $P < 0.05$, ** $P < 0.01$; Student t test for unpaired data.

Effects of AVP in Presence or in Absence of a Transepithelial Osmotic Gradient

It has been previously reported that AVP stimulates cAMP production and sodium transport in $RCCD_1$ cells [2]. We have now investigated whether AVP regulates the J_w reported in this paper. Figure 4*A* shows the effect of 10^{-8} M AVP, added to the basolateral bath. The basal J_w secretion (observed in absence of an osmotic gradient) was reduced by the hormone ($\Delta J_{w\ AVP\text{-}basal}$, $\mu l.min^{-1}.cm^{-2}$: 0.16 ± 0.02, $P < 0.01$; $n = 6$). Once the response to AVP was developed, mannitol addition to the basolateral bath (12.5 mOsM) resulted in an immediate and reversible reduction in the secretory J_w that became absorptive. In a different set of experiments a basolateral osmotic gradient was applied before AVP addition (Fig. 4*B*). An additional increase in J_w, after the initial response to the osmotic gradient was observed. The AVP-related flux response reverted upon AVP withdrawal. The osmotic water permeability coefficient (P_{osm}) was estimated from the J_w values associated to the applied osmotic gradient as previously reported [3, 20, 21]. No effect of AVP on P_{osm} was observed ($P_{osm} \times 10^{-3}$ $cm.sec^{-1}$, control: 6.27 ± 0.99 *vs.* AVP: 6.80 ± 0.30, NS, $n = 6$).

Together with the reduction of net secretory J_w, AVP significantly increased in absence of an osmotic gradient, I_{sc} and V_T with no changes in R_T ($\Delta I_{sc\ AVP\text{-}basal}$, $\mu A.cm^{-2}$: 2.24 ± 0.44, $P < 0.01$; $\Delta V_{T\ AVP\text{-}basal}$, mV: 0.62 ± 0.08, $P < 0.01$; $\Delta R_{T\ AVP\text{-}basal}$, $\Omega.cm^2$, −46 ± 20, NS; $n = 5$). Table 3 shows that the response to AVP was also observed (either in J_w or I_{sc}) at lower concentrations of the hormone (10^{-9} M) or in the presence of 10^{-3} M 8-Br-cAMP, a second messenger of AVP, which indicated the specificity of the response.

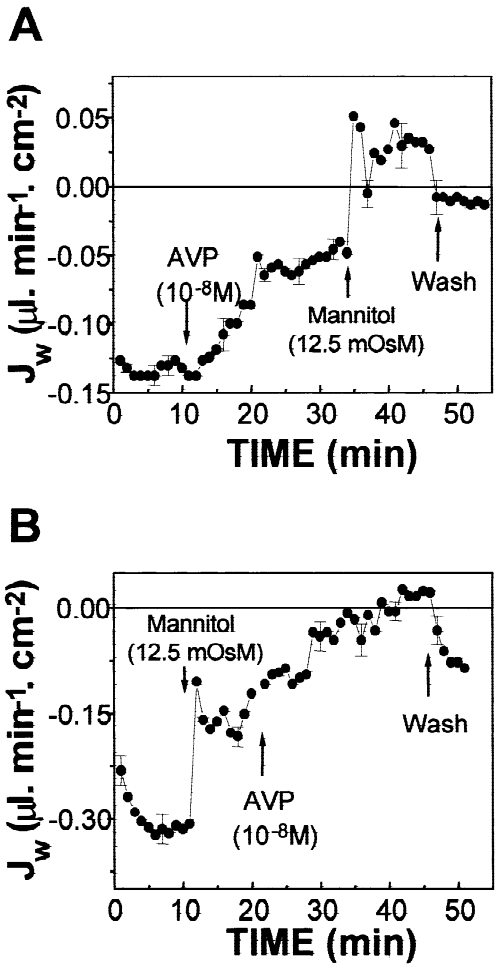

Fig. 4. Time course of water fluxes (J_w) in $RCCD_1$ cell line: Effects of AVP and of an osmotic gradient. (*A*) Effects of AVP (10^{-8} M) and the subsequent osmotic challenge (serosal mannitol, 12.5 mOsM) on water fluxes ($n = 3$). (*B*) J_w evolution in the presence of a serosal osmotic gradient (mannitol, 12.5 mOsM) and the subsequent effect of AVP (10^{-8} M, $n = 3$).

Table 3. Effects of AVP and 8 Br-cAMP on J_w and I_{sc} in $RCCD_1$ cells

	ΔJ_w (µl.min^{-1}.cm^{-2})	ΔI_{sc} (µA.cm^2)	*n*
AVP (10^{-8} M)	0.160 ± 0.020	2.24 ± 0.44	6
AVP (10^{-9} M)	0.040 ± 0.006**	1.68 ± 0.57	3
8-Br-cAMP (10^{-5} M)	0.085 ± 0.009*	1.60 ± 0.21	3

Net water fluxes and short-circuit current were simultaneously measured at different concentrations of AVP or in presence of cAMP. Results are expressed as delta between AVP effect minus basal effect. * $P < 0.05$, ** $P < 0.01$; Student *t* test for unpaired data.

To evaluate whether AVP action could be mediated by paracellular water movements, unidirectional ^{14}C-mannitol fluxes (a marker of the paracellular pathway) were measured simultaneously with J_w (Fig. 5). While

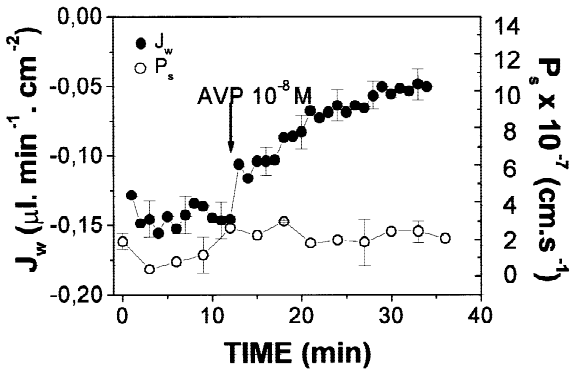

Fig. 5. Simultaneous measurements of J_w and ^{14}C-mannitol permeability (P_s) in $RCCD_1$ cell line. AVP (10^{-8} M) was added (arrow) to the serosal bath. J_w is expressed in µl.min^{-1}.cm^{-2} and P_s in cm.sec^{-1} ($n = 4$). Filled circles (●) correspond to J_w and open circles (○) to ^{14}C-mannitol permeability (P_s).

AVP clearly decreased water secretion, no changes were detected in mannitol permeability (P_s).

The AVP-induced water flux in $RCCD_1$ observed in the absence of external osmotic gradients is likely to be dependent on ion transport rates. Figure 6 shows the simultaneously recorded time course of the relative changes in J_w and I_{sc} induced by AVP. A close parallelism was observed between these two parameters. Moreover, AVP action was abolished after the apical addition of 10^{-5} M amiloride, an inhibitor of the sodium ENaC channel, either on water fluxes [AVP (J_w % of control value): −100 ± 10 *vs.* AVP + amiloride: −45 ± 11) or in I_{sc} (Table 2).

Discussion

This study focuses on water movements and their AVP regulation in an immortalized rat cortical collecting duct cell line ($RCCD_1$). When grown on a permeable support, these cells exhibit high transepithelial resistance associated with a significant potential difference and short-circuit current [2]. These observations, together with ultrastructural features and expression of cell-specific markers, support the notion that the $RCCD_1$ cell line expresses characteristics of at least two different cell types that may correspond to the principal (absorptive) and intercalated (secretory) cells, present in the native cortical collecting ducts [2].

The results presented here show that absorptive and secretory water fluxes can be dissociated in $RCCD_1$ cells, with a predominant net secretory J_w in basal conditions. Although the ability to reverse the direction of net fluid transport is typical of other absorptive epithelia, as the intestine, the capacity of the renal epithelia to secrete fluid has not been widely recognized [25].

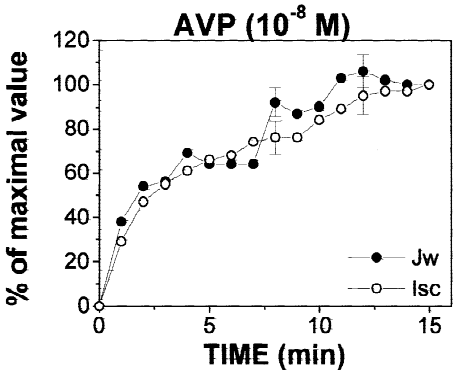

Fig. 6. Simultaneous recording of time-course changes in J_w and I_{sc} induced by AVP in $RCCD_1$ cells. Results are expressed as a percentage of the maximum value (AVP effect minus basal effect). A clear parallelism between both parameters was observed ($n = 5$).

Water Secretion in $RCCD_1$ Cells

Fluid secretion in $RCCD_1$ cells occurs in the absence of any chemical or osmotic gradient and even against a hydrostatic pressure difference, features suggesting energy-requiring mechanisms for the transport of fluid. Moreover, fluid secretion was strongly dependent on medium temperature. The observed inhibitory effects of chloride and potassium transport blockers on J_w and I_{sc}, are consistent with specific coupling of K^+, Cl^- and water in the reported secretion. These results are in agreement with the recent demonstration that $RCCD_1$ cells express CFTR and the Na^+-K^+-$2Cl^-$ cotransporter [7]. In these cells, net chloride secretion appears to involve Cl^- entry into the cell through a basal Na^+-K^+-$2Cl^-$ cotransporter and, likely, apical exit through the CFTR [7]. Previous studies have also shown the expression of CFTR in the collecting duct and of a secretory isoform of the Na^+-K^+-$2Cl^-$ cotransporter in rat renal intercalated cells and mIMCD-3 cells [4, 11].

Since it is well established that different types of aquaporins are present in the kidney [1, 18, 30, 31], they were the first candidates as water pathways in $RCCD_1$ cells. Our RT-PCR studies have only shown the presence of the messenger RNA for AQP3. However, RPA and immunoblotting studies failed to confirm this result, suggesting the very low abundance of transcripts and, consequently, the absence of AQP3 expression in $RCCD_1$ cells. We have previously reported, using the same experimental device, the characteristics of water movements in LLC-PK1 cells whether or not transfected with AQPs [28]. While the initially observed J_w was not different from zero in LLC-PK1 cells (either in wild-type or in AQP1- or AQP2-transfected cells) [28], $RCCD_1$ cells showed significant spontaneous secretory J_w in basal conditions. For this reason, $RCCD_1$ cells are a suitable model in which coupling between water and ion transfer in a "non-containing AQPs" epithelial barrier can be studied.

Effect of AVP on Water Movements in $RCCD_1$ Cells

The absence of AQP2 expression is crucial for the loss of the "hydro-osmotic response" to AVP since no change in osmotic permeability was elicited by AVP or cAMP in $RCCD_1$ cells. A similar situation was previously reported in LLC-PK1 cells, where the hydro-osmotic response to AVP was reconstituted only after transfection with AQP2 [13, 28].

In $RCCD_1$ cells, a significant decline in net secretory J_w was also observed after the addition of AVP, in the absence of any osmotic gradient. This observation could be interpreted as representing an increase in the "water absorptive component" coupled to an amiloride-sensitive Na^+ absorption and/or a decrease in the "water secretory component" coupled to Cl^- and K^+. Based on the reported observations, we can speculate that probably water transfer was not simply the result of an osmotic gradient built up by ions transport or a nonspecific effect on paracellular permeability. The simultaneous measurement of J_w and I_{sc} indicates that if an osmotic gradient was generated after AVP action, it would be established very rapidly, because no delay was observed between both responses (within the limits of our experimental setup). Moreover, it was previously reported that AVP by itself did not appear to exert a direct effect on paracellular permeability, as judged from its lack of effect on sucrose, insulin or mannitol fluxes in IMCD cells [10]. Our results in $RCCD_1$ cells are in line with these observations, since mannitol permeability was not affected by the presence of AVP.

Water and Ions Pathways in $RCCD_1$ Cells

It is generally accepted that water can cross cell membranes through two different pathways: the lipid bilayer or specific water channels (aquaporins) [1, 5, 9, 15, 18, 22]. These two alternatives imply, in the case of ion-coupled transfers, the development of a "local osmosis gradient" which, to our knowledge, was not experimentally demonstrated. A third possible mechanism (ion-water cotransport proteins) has been recently proposed to explain the water flux associated with the K^+-Cl^- or the Na^+-glucose cotransporters [32, 33]. Along this line, it has been reported that CFTR may also function as a water channel [12, 24]. Our results, together with the absence of known renal AQPs expression, led us to speculate that ionic transporters rather than the lipid bilayer could be the molecular pathways involved in the observed water movements. The evidence supporting the water-ions cotransport hypothesis is still limited and

sometimes indirect. Future experiments are needed to clarify this point.

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