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Arginine-Vasopressin Modulates Intracellular pH via V1 and V2 Receptors in Renal Collecting Duct Cells

Valeria Rivarola, Paula Ford, María del Pilar Flamenco, Luciano Galizia and Claudia Capurro

Laboratorio de Biomembranas, Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Buenos Aires

Key Words

Na^+/H^+ and Cl^-/HCO_3^- exchangers • CFTR • $RCCD_1$ cell line

Abstract

Arginine-vasopressin (AVP) has been proposed to be involved in the modulation of acid-base transporters; however, the nature of the mechanisms underlying AVP direct action on intracellular pH (pH_i) in the cortical collecting duct (CCD) is not yet clearly defined. The aim of the present study was to elucidate which are the proteins implicated in AVP modulation of pH_i, as well as the receptors involved in these responses using a CCD cell line ($RCCD_1$); pH_i was monitored with the fluorescent dye BCECF in basal conditions and after stimulation with basolateral 10^{-8} M AVP. Specific V1- or V2-receptor antagonists were also used. RT-PCR studies demonstrated that $RCCD_1$ cells express V1a and V2 receptors. Functional studies showed that while V2-receptor activation induced a biphasic response (alkalinization-acidification), V1-receptor activation resulted in an intracellular acidification. The V2-mediated alkalinization phase involves the activation of basolateral NHE-1 isoform of the Na^+/H^+ exchanger while in the acidification phase CFTR is probably implicated. On the other hand, V1-mediated acidification was due to activation of a Cl^-/HCO_3^- exchanger. We conclude that in $RCCD_1$ cells AVP selectively activates, via a complex of V1 and V2 receptor-mediated actions, different ion transporters linked to pH_i regulation which might have physiological implications.

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Introduction

In the mammalian collecting duct (CD) arginine vasopressin (AVP) plays a critical role in adjusting urine volume and composition. Although the role of AVP in regulating water permeability is well known, its effects on electrolyte transport are more complex and not fully understood [1-3]. AVP has been proposed to be involved in the modulation of many electrolyte channels or transporters, including the epithelial Na^+ channel (ENaC), the Na^+/H^+ exchanger (NHE), the H^+-ATPase, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and the Cl^-/HCO_3^- exchanger (AE) [4-7]. Although some of these proteins are acid-base transporters, the nature of the mechanism underlying AVP

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Prof. Claudia Capurro, Ph.D.
Laboratorio Biomembranas, Dpto. Fisiología y Biofisica
Facultad de Medicina,Universidad de Buenos Aires
Paraguay 2155, Piso 7, 1121 Buenos Aires (Argentina)
Tel/Fax +54-11-4964-0503 (ext. 23), E-Mail capurro@mail.retina.ar

action on intracellular pH (pH_i) has not been yet clearly defined. For instance, Na^+/H^+ exchanger (NHE) activity was shown to be either inhibited by AVP in some nephron segments or stimulated in other segments as well as in several renal cell lines [8-10]. In addition, some authors proposed that AVP modulates in a different way Na^+/H^+ exchangers present in the basolateral (via activation) and luminal membrane (via inhibition) [8, 11]. Ganz et al. proposed that AVP also stimulates Na^+-dependent and independent Cl^-/HCO_3^- exchangers in mesangial cells [6]. Even more, in inner medullary collecting duct cells it was suggested that AVP stimulation of Cl^-/HCO_3^- exchangers would be involved in Cl^- secretion, although its activity was not evaluated [7]. In conclusion, AVP modulation of AE transporters in the CD cells is still unclear. AVP has also been postulated to modulate Cl^- transport via the CFTR, a channel that directly or indirectly also provides a bicarbonate conductance [12, 13]. However its involvement in pH_i response to AVP has not yet been investigated.

All these AVP actions may be regulated through two distinct types of receptors (V1 and V2) [14]. Most studies have proposed that AVP modulation of water and solute transport, when applied to the basolateral surface of the collecting duct, is mediated by V2 receptors [15]. On the other hand, V1 receptor has been implicated in distal tubule basolateral AVP stimulation of bicarbonate reabsorption [16] and in AVP luminal responses [4]. However, to date, the role of V1/V2 receptors in AVP action on pH_i in the CD remains obscure.

Therefore, the aim of the present study was to elucidate the mechanisms involved in AVP modulation of pH_i, as well as the receptors implicated in these responses using a rat cortical collecting duct cell line ($RCCD_1$). This cell line has previously proved to be a very useful model to achieve molecular and functional insights on "short-term" and "long-term" regulation by AVP of ion channels such as ENaC, K^+ channels and CFTR [5, 12, 17-20]. More recently, using this cell model, we have contributed to the understanding of the specific roles of different isoforms of the acid-base transporters NHE and AE (19, 21). To our knowledge $RCCD_1$ represents an attractive model to study the effects of AVP on acid-base transporters due to its capacity to maintain both the sensitivity to AVP and the expression of H^+/HCO_3^- extruders.

In the present work, pH_i response to basolateral AVP was monitored using the fluorescent dye 2',7'bis (2-carboxyethyl)-5-(and–6) carboxyfluorescein (BCECF-AM) in cell monolayers grown on permeable filters, a support that provides independent and free access to both the apical and the basolateral compartments. Our studies demonstrated, for the first time, that in $RCCD_1$ cells AVP is able to modulate, simultaneously, different proteins involved in pH_i regulation. We showed that the hormone stimulates NHE-1 and possibly CFTR via V2 receptors, as well as Cl^-/HCO_3^- exchange activity through V1 receptors.

Materials and Methods

Cell Culture

$RCCD_1$ cells were grown in DM medium (Dulbecco´s modified Eagle´s medium / Ham´s F-12, 1:1 v /v; 14 mM $NaHCO_3$, 3.2 mM glutamine; 0.5 µM dexamethasone; 0.3 µM sodium selenite; 5 µg ml^{-1} insulin; 5 µg ml^{-1} transferrin; 10 µg ml^{-1} epidermal growth factor; 0.5 µM triodothyronine; 100 units ml^{-1} penicilin-streptomycin; 20 mM Hepes; pH 7.4) and 2% fetal bovine serum (FBS) (Invitrogen, San Diego, CA, USA) [17]. All experiments were performed on confluent cells, between the 20th and 40th passages, grown on permeable filters (Transwell, 3 µm pore size, 4.5 cm^2 growth area, Corning Costar, Corning, NY, USA) during six or seven days.

Intracellular pH Studies

As previously reported, filters with $RCCD_1$ monolayers were inserted between two lucite frames and diagonally placed in a quartz cuvette, providing a separation of two fluid compartments [19]. Measurements were made with a computerized and thermoregulated (37°C) spectrofluorometer (Jasco 770, Easton, MD, USA). For the measurements, the cell monolayer was placed forming a 45° angle to the exploring beam. Fluorescence emission was monitored at 535 nm, with excitation wavelengths of 439 and 510 nm. For pH_i measurements, cells were loaded with 8 µM of 2',7'bis (2-carboxyethyl)-5-(and–6) carboxyfluorescein acetoxymethyl ester (BCECF/AM, Molecular Probes, Eugene, OR, USA) during 60min at 37°C, both from the apical and basolateral baths. The ratio of the BCECF fluorescence emitted from dye-loaded cells was calibrated in terms of pH, by incubating the cells in a "high K^+ solution" (140 mM KCl, 4.6 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 5 mM glucose) and then by permeabilizing the cells with 5 µM nigericin to balance extracellular pH (pH_o) with pH_i. The pH-bathing solution was stepped between 6.6 and 8.5. The 510 / 439 ratio was linear over this pH range ($r = 0.99$, $n = 6$).

pH_i was evaluated in cells bathed on each side with a control solution and after the addition of 10^{-8} M AVP or vehicle (water) to the basolateral bath. In all pharmacological studies, the respective vehicles were tested.

Anion exchange activity in $RCCD_1$ cells was evaluated as previously reported [21], immediately after transition from a Cl^- containing to a Cl^--free solution. Upon Cl^- removal, the initial 3 minutes of each experimental curve were fitted to a simple exponential curve by nonlinear regression (GraphPad Prism,

	Primers	Amplified fragment size (bp)	cDNA Sequence Location (nt)	Data Bank accession number
V1a	5' ATG CTG GTG GTG ATG ACA GCC GAC CGC TAC 3' 5' CAT CTG GAC AAT GAA GAA AGG CGC CCA GCA 3'	533	670-699 1173-1203	Z11690
V1b	5' GGA TGA GAA TGC CCC CAA TGA AGA 3' 5' GAG AGA GAG TGG CCC ATA CCT ACA 3'	532	1450-1473 1959-1988	U27322
V2	5' ATG GTG GGC ATG TAT GCC TCC TCC TAC ATG 3' 5' AGT GTC ATC CTC ACG GTC TTG GCC A 3'	460	399-427 835-859	Z11932

Table 1. Specific rat primers used to detect the expression of AVP receptor isoforms: RT-PCR experiments were performed using specific rat primers, both in rat kidney and in $RCCD_1$ cells mRNAs. Cycling parameters are detailed under Material and Methods section.

San Diego, CA. USA) and *d*pHi/*d*t was the first derivate at t = 0.

RT-PCR Assays

Total RNA from rat kidney (positive control) or $RCCD_1$ cells was isolated using SV total RNA Isolation System (Promega, Madison, WI, USA). Reverse transcription was performed on 2 µg of total RNA using M-MLV reverse transcriptase (Promega). RNAs were placed in 50 µl of "RT reaction buffer" containing: 1X M-MLV reverse transcriptase buffer (Promega), 0.5 µg oligo-dt primer and 10 U μl^{-1} RNAsin. The reaction was heated 3min at 80°C and cooled to 45°C. PCR buffer (25 µl) containing: 1X M-MLV reverse transcriptase buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs and 400 units M-MLV, was added to half of the reaction. Control experiments, in absence of the enzyme M-MLV, were performed on the remaining 25 µl. RT reaction was carried out for 1h at 45°C and stopped by heating 2min at 95°C.

PCR experiments were performed with 5 µl of the RT reaction using 25 pmol of specific primers for rat V1a, V1b and V2 (Table 1). An 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'). Cycling parameters were for V1a and V2: 2min at 94°C, 2min at 55°C, 2min at 72°C for one cycle and 1min at 94°C, 1min at 55°C, 1min at 72°C for 34 cycles; for V1b: 2min at 94°C, 2min at 63°C, 2min at 72°C for one cycle and 1min at 94°C, 1min at 63°C, 1min at 72°C for 34 cycles. To amplify V1b receptor products, a second PCR was performed using 5µl of the first reaction.

Solutions and chemicals

Control saline solution (CSS) contained (in mM): 139 NaCl, 30 Hepes, 10 $NaHCO_3$, 3 KCl, 1.8 $CaCl_2$, 1 $MgSO_4$, 1 KH_2PO_4, 1 K_2SO_4, 5.6 glucose. Bicarbonate-free solutions contained (in mM): 145 NaCl, 30 Hepes, 3 KCl, 1.8 $CaCl_2$, 1 $MgSO_4$, 1 KH_2PO_4, 1 K_2SO_4, 5.6 glucose. Cl^--free solutions were achieved by equimolar replacement of chloride with gluconate in CSS. All pH solutions were adjusted to 7.4 (at 37°C) using Tris-[hidroxymethyl] aminomethane (Sigma-Aldrich) and osmolality (320 ± 4 mOsm) was routinely measured in a pressure vapor osmometer (Vapro; Wescor, Logan, Utah, USA). Bicarbonate-containing-solutions were continuously bubbled with 5% CO_2 - 95 % O_2. When specified, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), ethylisopropylamiloride (EIPA), glibenclamide (Sigma-Aldrich) and HOE-694 (kindly provided by Dr. Juergen Puenter, Aventis Pharma Deuchland GmbH, Frankfurt, Germany) were used. AVP, V1 receptor antagonist: [deamino-Pen1, O-Me-Tyr2, Arg8]-Vasopressin, V2 receptor antagonist: [Adamantaneacetyl1, O-Et-D-Tyr2, Val 4, Aminobutyryl 6, Arg 8,9]-Vasopressin and V2 receptor agonist [dDAVP] were purchased from Sigma-Aldrich.

Statistics

Results are expressed as mean ± SEM. Student's t Test for paired or unpaired data was used according to the protocol; $p < 0.05$ was considered a significant difference.

Results

AVP modulates steady-state pH_i in $RCCD_1$ cells

The first set of experiments examined the effect of AVP on baseline pH_i in $RCCD_1$ cells grown on permeable supports. Figure 1A shows that the addition of 10^{-8} M basolateral AVP elicited a biphasic response in pH_i: an initial transient alkalinization, followed by an acidification restoring the steady-state pH_i. These effects were not observed in the presence of vehicle. AVP maximal alkalinization, calculated as the difference between baseline pH_i and that obtained at the first minute (ΔpH_i^{alk}), gave a value of 0.106 ± 0.016 pH units (n = 32). The acidification (ΔpH_i^{acid}) was estimated as the difference between pH_i values obtained at the first minute and the pH_i achieved 10 min after AVP stimulation. The mean acidification was -0.097 ± 0.008 pH units (n= 32).

The putative acid-base transporters involved in these responses were then investigated. Since we have previously reported that $RCCD_1$ cells express two isoforms (NHE-1 and NHE-2) of the Na^+/H^+ exchanger

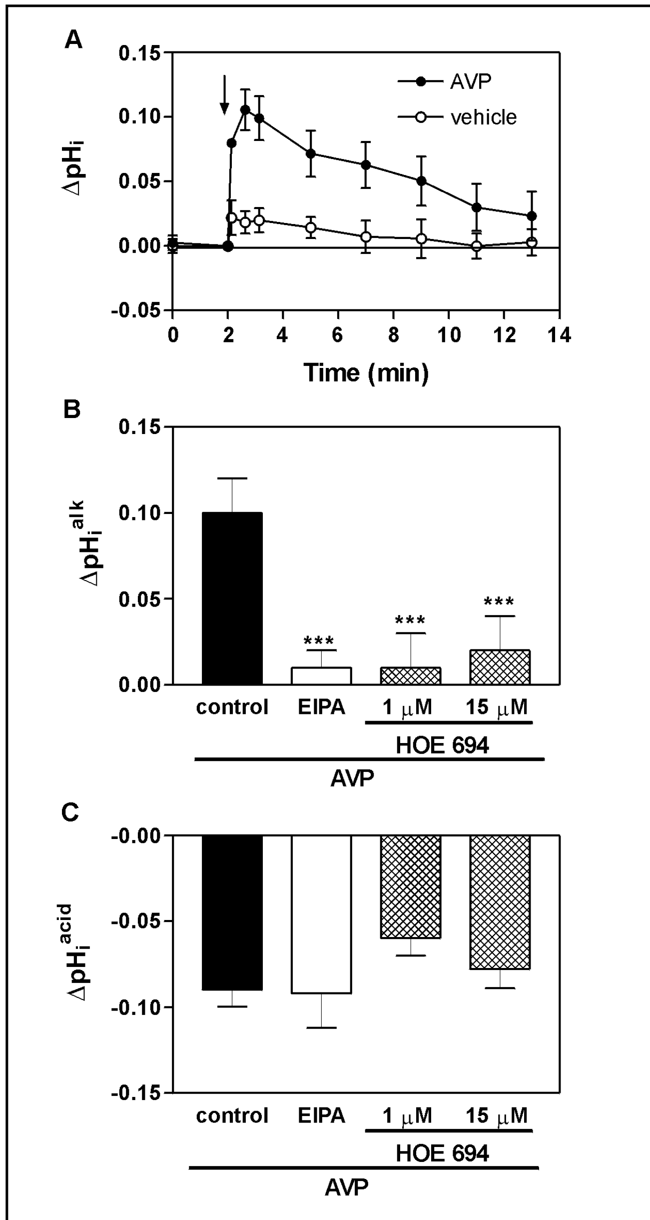

Fig. 1. Stimulation of Na^+/H^+ exchanger by AVP in $RCCD_1$ cells. A- Time course of pH_i after basolateral addition (at arrow) of 10^{-8} M AVP or of vehicle B- Effect of Na^+/H^+ exchanger inhibitors on AVP maximal alkalinization (ΔpH_i^{alk}). C- Effect of Na^+/H^+ exchanger inhibitors on AVP acidification (ΔpH_i^{acid}). In B and C values are mean ± SEM as difference respect to basal pH_i of 4 to 6 experiments. *** $p < 0.001$ compared to control.

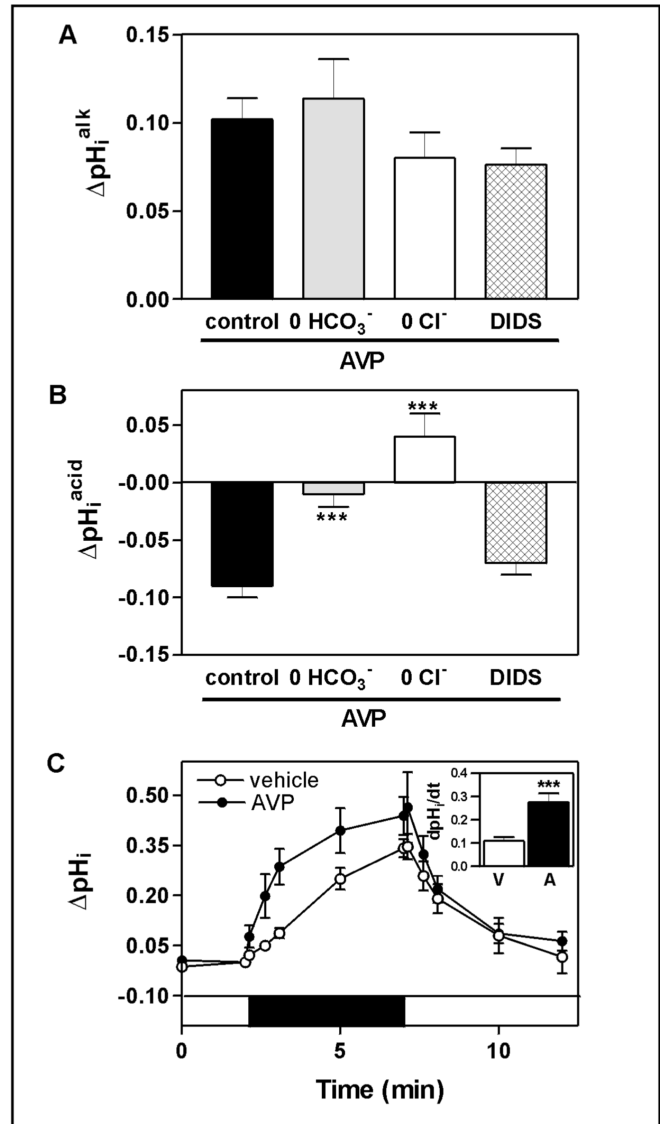

Fig. 2. Effect of AVP on Cl^-/HCO_3^- exchanger activity in $RCCD_1$ cells. A- Effect of HCO_3^- removal, Cl^- removal or 10^{-4} M DIDS on pH_i alkalinization induced by AVP (ΔpH_i^{alk}). B- Effect of HCO_3^- removal, Cl^- removal or 10^{-4} M DIDS on pH_i acidification induced by AVP (ΔpH_i^{acid}) *** $p < 0.01$, $n = 6$ compared to control. C- pH_i recorded in the presence of external Cl^- (open bar) and after basolateral Cl^- removal (solid bar) in cells pre-incubated for 10min with AVP (A) or vehicle (V). Inset: Rate of intracellular alkalinization (dpH_i/dt, pH units.min^{-1}) after basolateral Cl^- removal calculated in experiments in A. Values are mean ± SEM of 4 to 7 experiments. *** $p < 0.001$ compared to vehicle.

at the basolateral membrane [19], we examined whether the initial alkalinization was linked to this transporter activation. Therefore, experiments were first performed in cells that were previously incubated during 10 min with 10^{-4} M EIPA (an inhibitor of Na^+/H^+ exchanger) or with the vehicle (DMSO), and then stimulated with 10^{-8} M AVP. Figure 1B shows that after AVP stimulation the initial alkalinization was completely abolished in the presence of EIPA, indicating the participation of a Na^+/H^+ exchanger in this response. To determine which NHE

isoform might mediate the above-described alkalinization, we used the highly selective inhibitor HOE-694 which at low doses (1 μM) inhibits NHE-1 but not NHE-2, while at higher doses (15 μM) blocks both isoforms [22, 23]. As shown in Figure 1B, ΔpH_i^{alk} was completely blunted by 1 μM HOE-694 to the same extent as EIPA. A higher dose of HOE-694 did not elicit further inhibition. On the other hand, the acidification phase (ΔpH_i^{acid}) was not affected by any of these inhibitors (Figure 1C). Altogether, these results clearly suggest that the NHE-1 isoform of the Na^+/H^+ exchanger is activated by basolateral AVP.

As described, AVP elicited a biphasic response including an acidification phase. Our previous studies demonstrated that $RCCD_1$ cells express, at the basolateral membrane, two isoforms of the Cl^-/HCO_3^- exchanger: AE2 (DIDS-insensitive) and AE4 (DIDS-sensitive) [21]. Therefore, we then investigated whether these exchangers could be involved in the AVP-induced acidification. To test this hypothesis, cells were incubated either in HCO_3^- - free media, Cl^- - free media or with 10^{-4} M DIDS. Figure 2A shows that the initial alkalinization induced by AVP (ΔpH_i^{alk}) was not affected by any of these treatments. In contrast, the AVP-related acidification (ΔpH_i^{acid}) was abolished by chloride or bicarbonate removal but not affected by the addition of DIDS (Figure 2B). To further investigate the putative participation of a Cl^-/HCO_3^- exchanger in the acidification phase induced by AVP, Cl^-/HCO_3^- exchange activity was evaluated using the classically described technique of extracellular Cl^- removal [24]. These experiments were performed either in the presence or in the absence of 10^{-8} M AVP. Figure 2C shows that, upon AVP stimulation, the rate of anion exchange activity was significantly increased respect to the vehicle (inset). Altogether, these results demonstrated that a Cl^-/HCO_3^- exchanger is also activated by AVP.

V1a and V2 receptors are expressed in $RCCD_1$ cells

Although functional studies using the specific agonist dDAVP have demonstrated that $RCCD_1$ cells express the V2 receptor [5, 12, 17]; V1 receptor expression has not been previously tested. Then, before determining which AVP-receptors might mediate the above-described responses, the V1/V2 receptors expression in $RCCD_1$ cells was evaluated by RT-PCR. Experiments were carried out with primers for the different rat AVP-receptors previously reported in the literature (V1a, V1b and V2, Table I) [25, 26]. Assays were undertaken using total RNA extracted from $RCCD_1$ cells and rat kidneys (as positive controls). Figure 3 shows the results of representative RT-PCR experiments. As expected, RT-PCR of rat kidney RNA produced fragments of the proper size for all the tested receptors. On the other hand, only mRNAs for V1a (~533 bp) and V2 (~460 bp) are expressed in $RCCD_1$ cells. β-actin was used as an internal control for kidneys and $RCCD_1$ cells in all experiments (Figure 3B). These results confirm the expression of V1a and V2 receptors in $RCCD_1$ cells.

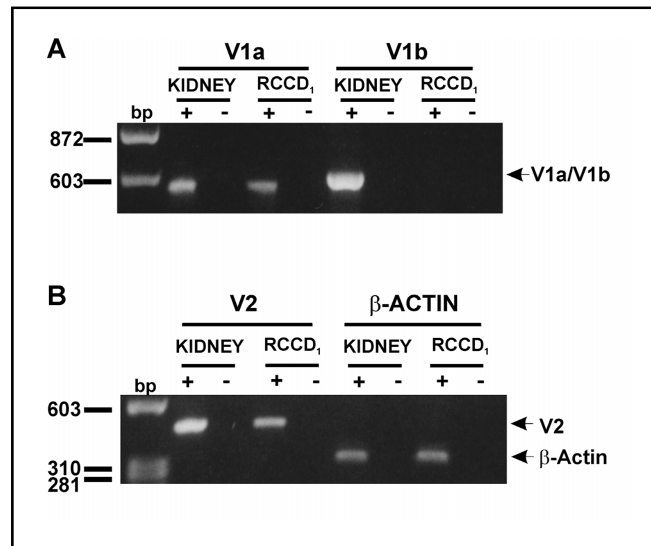

Fig. 3. AVP receptors expression in $RCCD_1$ cells. Representative RT-PCR experiments performed using specific primers for rat V1a or V1b (A) and V2 receptors (B) in mRNAs from rat kidney (positive control) and $RCCD_1$ cells. Assays were carried out in the presence (+) or absence (-) of RT enzyme. Left line, ΦX174 *Hae III* digested marker. β-actin was used as an internal control.

V1 and V2 receptors participate in AVP pH_i modulation

To detect which of the above mentioned receptors expressed in $RCCD_1$ cells could be involved in the AVP pH_i modulation response; we evaluated the effect of specific V1- or V2-receptor antagonists. In all experiments, cells were first incubated for 10 min with the antagonist and then stimulated by 10^{-8} M AVP. It is important to point out that the presence of antagonists did not affect steady-state pH_i, indicating no intrinsic effects (control: 7.47 ± 0.04, n=13; Anti V1: 7.45 ± 0.05, n=9; Anti V2: 7.44 ± 0.07, n=5). Figure 4A illustrates pH_i time-course in the presence of AVP plus either vehicle, 10^{-6} M V1-antagonist, 10^{-6} M V2-antagonist or both. It can be noted that in the presence of V1-antagonist AVP induced an alkalinization followed by a slightly lower pH_i recovery. In contrast, AVP incubation with V2-antagonist

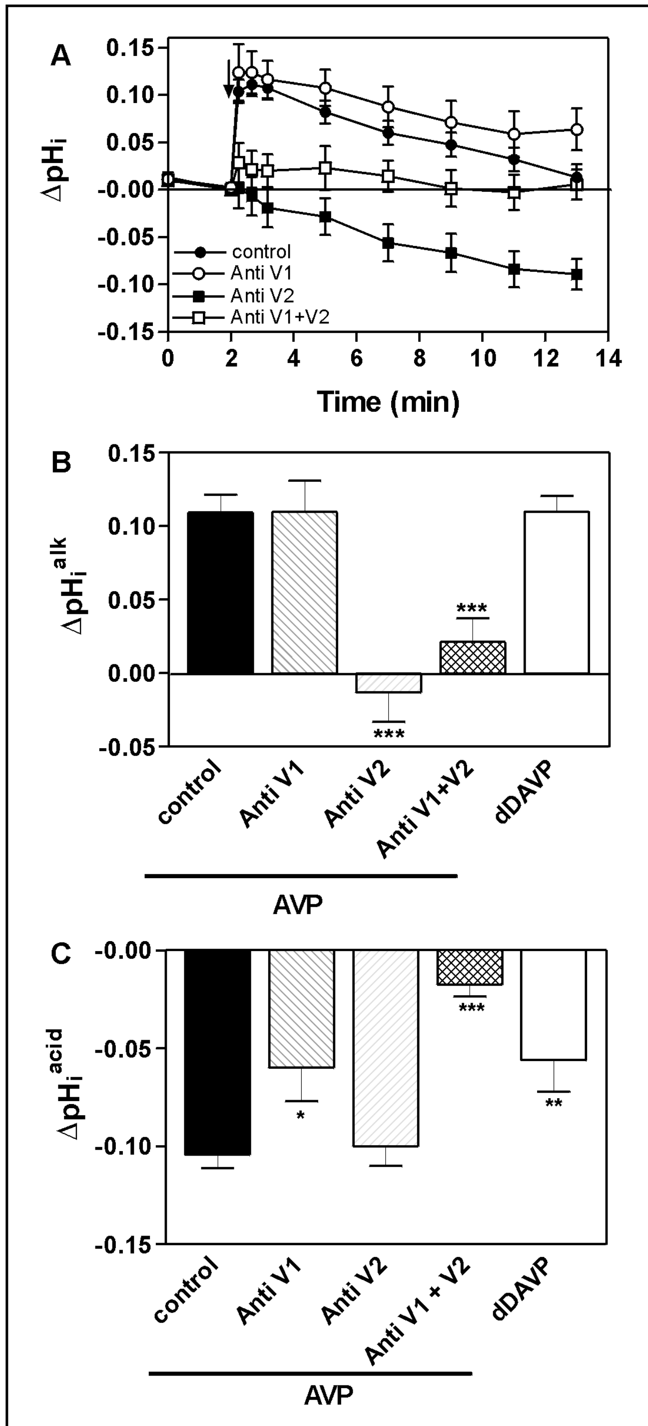

Fig. 4. V1 and V2 receptors are involved in the AVP-mediated pH_i modulation A- Time course of pH_i evolution after basolateral addition (at arrow) of 10^{-8} M AVP in cells pretreated with: 10^{-6} M V1-antagonist (Anti V1), 10^{-6} M V2-antagonist (Anti V2), both receptor antagonists or vehicle (control). B- Effects of Anti V1, Anti V2 or both on the AVP induced ΔpH_i^{alk} and effect of 10^{-8} M dDAVP. C- Effect of Anti V1, Anti V2 or both on the AVP induced ΔpH_i^{acid} and effect of 10^{-8} M dDAVP. Values are mean ± SEM of 4 to 8 experiments. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

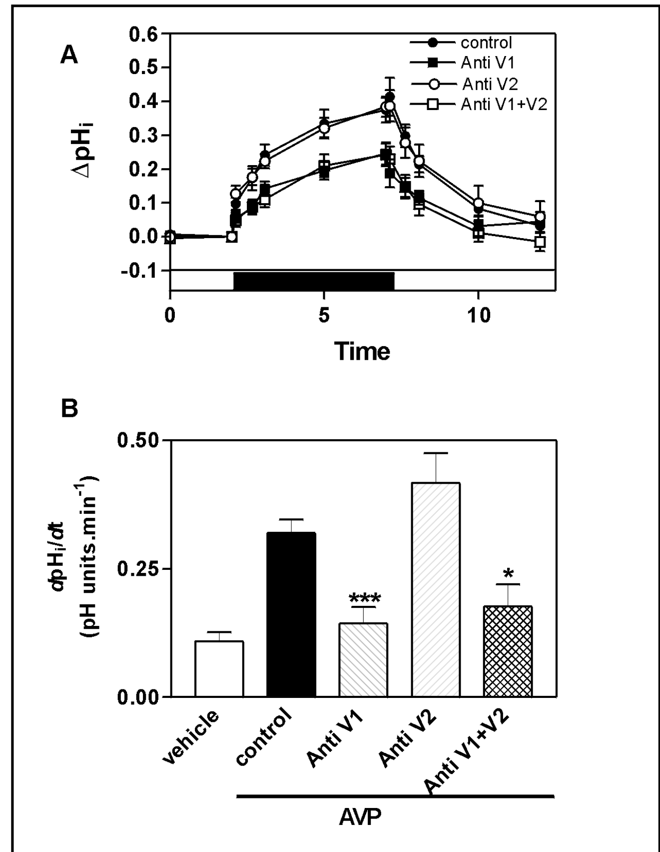

Fig. 5. Effect of V1 and V2 receptor antagonist on AVP-mediated Cl^-/HCO_3^- activation A- pH_i was recorded in the presence of external Cl^- (open bar) and after basolateral Cl^- removal (solid bar) in cells pre-incubated with AVP 10^{-8} M in the presence of 10^{-6} M V1 receptor antagonist (Anti V1), 10^{-6} M V2 receptor antagonist (Anti V2), both antagonists or vehicle (control). B- Rate of anion exchange activity (dpH_i/dt) after basolateral Cl^- removal in experiments without AVP (vehicle) and in those described in A. Values are the mean ± SEM of 6 to 9 experiments. *$p < 0.05$, *** $p < 0.001$ compared to control.

only elicited an acidification. Figure 4B resumes that the addition of V1-receptor antagonist did not affect the initial alkalinization. On the other hand, in the presence of a V2-receptor antagonist, the initial alkalinization was completely blunted. No additional effects were observed in the presence of both, V1 plus V2, antagonists. Moreover, the initial alkalinization was also obtained in the presence of 10^{-8} M dDAVP, a specific V2-receptor agonist. Experiments using the agonist dDAVP plus HOE-693 confirmed that this effect was NHE-1 dependent (ΔpH_i^{alk} pH units: dDAVP: 0.110 ± 0.021 vs. dDAVP + HOE: - 0.002 ± 0.018, n=6, $p < 0.001$). Together, these results confirm that the NHE1-mediated alkalinization

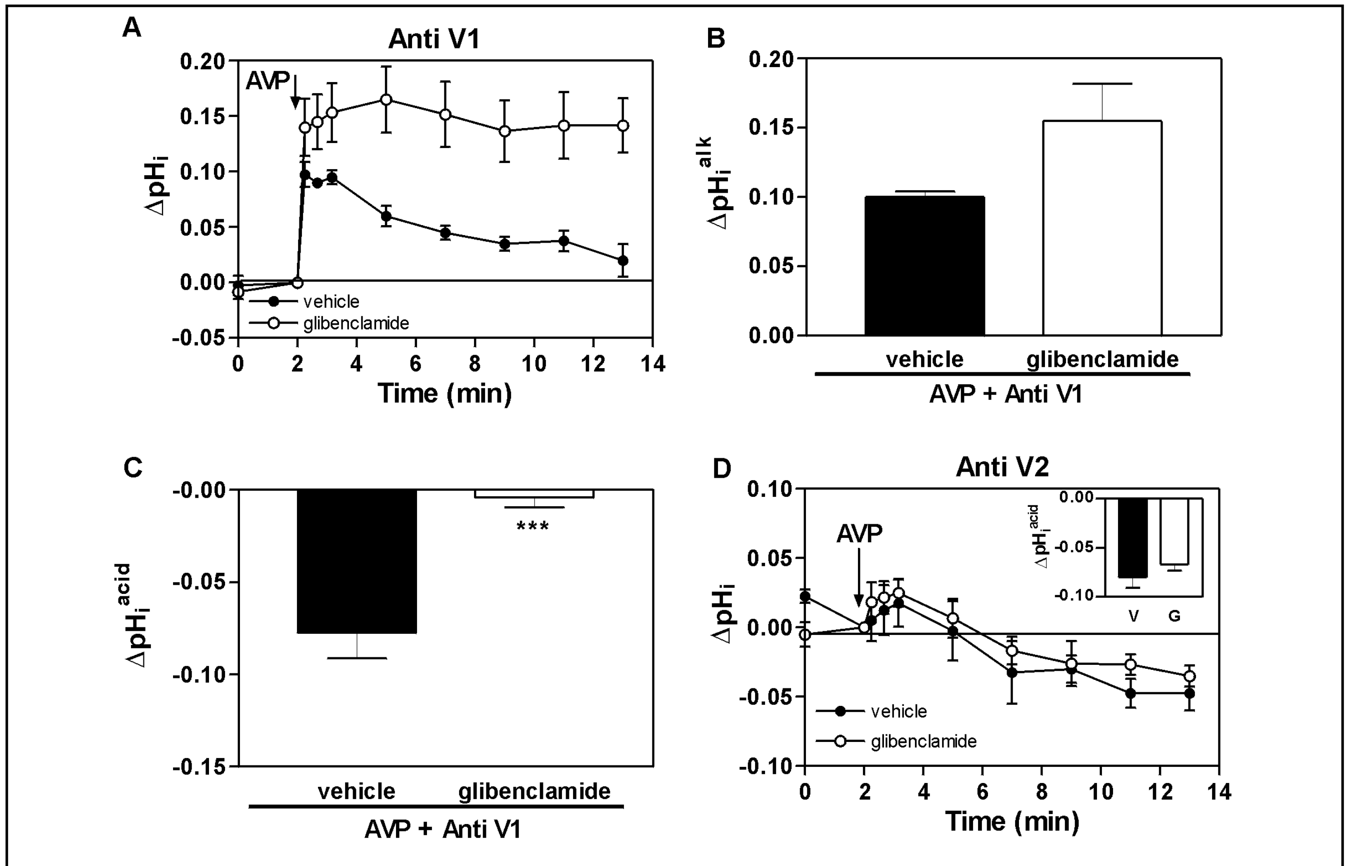

Fig. 6. Effect of glibenclamide on AVP response via V2 receptor. A- Time course of pH_i after basolateral addition (at arrow) of 10^{-8} M AVP in the presence of 10^{-6} M V1 receptor antagonist plus glibenclamide or vehicle. B and C- ΔpH_i^{alk} and ΔpH_i^{acid}, respectively, in conditions described in A. D- Time course of pH_i after basolateral addition (at arrow) of 10^{-8} M AVP in the presence of 10^{-6} M V2 receptor antagonist plus glibenclamide or vehicle. Insert: ΔpH_i^{acid} in both conditions (glibenclamide or vehicle). Values are mean ± SEM as difference respect to basal pH_i of 4 to 6 experiments, *** $p < 0.001$ compared to vehicle.

phase above described takes place via a V2-receptor pathway.

Concerning the AVP-related acidification Figure 4C shows that, in the presence of V1-antagonist, ΔpH_i^{acid} was slightly lower than that observed in cells treated with AVP plus vehicle. A similar response was obtained activating V2-receptor with the specific agonist dDAVP; in contrast, the V2-antagonist did not affect the acidification phase. However, ΔpH_i^{acid} was completely blunted in the presence of both antagonists, which suggests that V2 receptors may also be involved in the AVP-induced acidification.

These stated results describe that a Cl^-/HCO_3^- exchanger contributes to the AVP-induced acidification. In order to evaluate which receptor would be involved in this response, Cl^- removal experiments were performed in the presence of the specific antagonists (Figure 5A). It can be observed that the AVP-induced stimulation of Cl^-/HCO_3^- anion exchanger activity was significantly blocked in the presence of V1-receptor antagonist (Figure 5B). In contrast, this response was not affected by V2-receptor antagonist. Moreover, V1 plus V2 antagonists elicit the same response than that observed with V1 antagonist. According to these results, it can be proposed that in $RCCD_1$ cells AVP stimulates a Cl^-/HCO_3^- exchanger via the V1-, but not V2-, receptor pathway. However, V2-receptor could also be involved in the acidification response if another mechanism is implicated. To further investigate this hypothesis we first evaluated if CFTR is involved in the AVP induced acidification.

Previous studies (including ours in $RCCD_1$ cells) have shown that dDAVP stimulates chloride secretion, via CFTR, in mammalian CCD and IMCD cells [12, 27]. Even more, several authors have proposed that, besides

chloride transport, CFTR also provides HCO_3^- conductance [28] and this was confirmed in $RCCD_1$ cells [18]. To test if CFTR channels are involved in the AVP-induced acidification, cells were pretreated for 10 min with 10^{-4} M glibenclamide (an inhibitor of CFTR) or with the vehicle and then stimulated with AVP. Results showed that while glibenclamide did not affect the initial alkalinization phase induced by AVP (ΔpH_i^{alk} pH units; vehicle: 0.08 ± 0.006 vs. glibenclamide: 0.06 ± 0.008, n=8, N.S.) the acidification phase was significantly reduced (ΔpH_i^{acid} pH units; vehicle: -0.091 ± 0.008 vs. glibenclamide: -0.023 ± 0.006, n=8, $p < 0.001$). These results suggest that the acidification phase induced by AVP probably involves, besides a Cl^-/HCO_3^- exchanger, the CFTR channel. To investigate which receptor would be involved in this response experiments were performed in cells pretreated for 10 min with glibenclamide or vehicle, then incubated 10 min with the respective antagonist (V1 or V2) and finally stimulated by 10^{-8} M AVP. Figure 6 A-C shows that in the presence of the V1-receptor antagonist and glibenclamide while the initial alkalinization takes place, pH_i was not able to recover. In contrast, using the V2-receptor antagonist, the AVP induced acidification was not affected by glibenclamide (Figure 6D). These results suggest that CFTR channels may participate in the AVP-induced acidification via the V2-receptor pathway.

Discussion

AVP stimulates Na^+/H^+ exchange and CFTR via V2 receptor in $RCCD_1$ cells

In this study, we demonstrated that AVP stimulation, via V2-receptor, induces a biphasic response on baseline pH_i (alkalinization-acidification). A number of evidences, here presented, strongly demonstrated that the alkalinization phase is a consequence of a direct activation of the isoform NHE-1 of the Na^+/H^+ exchanger, via V2 receptor. First, the alkalinization phase was completely blunted in the presence of 1 μM HOE, a dose necessary to block NHE-1 but not NHE-2. Second, this response was mimicked with the specific V2-receptor agonist dDAVP and it was completely blunted in the presence of dDAVP plus 1 μM HOE. Finally, the alkalinization phase was not observed in the presence of V2-receptor antagonist but it was achieved with the addition of V1-receptor antagonist.

The proposal that AVP stimulates NHE-1 is in agreement with previous reports in mesangial, MDCK and LLC-PK1 cells [6, 8, 9, 29]. However, *Schlatter et al.* reported, in clusters of principal cells of rat CCD, that NHE activity is inhibited by AVP [10]. However, in this work, because of CCD clusters conformation, apical membrane is probably mainly exposed to AVP stimulus. Conversely, we are here exclusively evaluating the effects of basolateral AVP. Exposition of $RCCD_1$ cells to 10^{-8} M apical AVP only induces a progressive acidification (ΔpH_i: -0.032 ± 0.016 pH units 10 min after AVP addition, n=5). Therefore, we suggest that AVP activates NHE-1 via V2-receptor and this effect is only observed when the hormone is added from the basolateral bath.

V2-receptors stimulation also induces a decrease in pH_i (acidification phase). This was demonstrated using the specific V2-receptor agonist dDAVP as well as specific antagonists. Even more, the herein observed acidification phase induced by AVP, via V2-receptors, was completely blocked by glibenclamide, probably indicating the participation of CFTR in this response. Previous studies (including ours in $RCCD_1$ cells) have shown that: 1- dDAVP and cAMP agonists stimulate CFTR and this effect is blocked by glibenclamide in mammalian CCD and IMCD cells [12, 27]; 2- in $RCCD_1$ cells AVP increases CFTR mRNA and protein levels [12]; and 3- CFTR directly or indirectly provides a bicarbonate conductance [13, 18, 28]. Nevertheless, glibenclamide, while inhibits CFTR also blocks ATP-sensitive K^+ channels or p-glycoproteins [30, 31]. However, although ATP-sensitive K^+ channels may be regulated by AVP, this effect does not involve alkali extrusion. In addition, it was described that p-glycoproteins block alkali efflux [32] and they are not regulated by AVP. Then, it is reasonable to postulate that activation of CFTR by AVP, via V2-receptors, may induce an intracellular acidification due to HCO_3^- efflux of the cell.

AVP stimulates Cl^-/HCO_3^- exchange via V1 receptor in $RCCD_1$ cells

V1 receptor expression has been reported in all CCD cells [33]. However to date, AVP actions mediated by V1-receptors in the CCD still remain obscure. It was previously proposed that, in rabbit CCD, a Na^+ conductance is stimulated after basolateral AVP addition through V1-receptors [34]. Furthermore, these receptors have been associated to AVP-related stimulation of HCO_3^- reabsorption in distal collecting ducts [16], but it has not been functionally determined whether AVP can modulate Cl^-/HCO_3^- transporters through V1a receptors. Our present results demonstrate that $RCCD_1$ cells expressed V1a receptors and that the AVP-induced acidification,

via this receptor, was dependent on the activation of a Cl^-/HCO_3^- exchanger. This is the first time that modulation of Cl^-/HCO_3^- exchanger by AVP in the kidney is proposed. In mesangial cells, Ganz et al. demonstrated that AVP was able to stimulate Na^+-dependent as well as Na^+-independent Cl^-/HCO_3^- exchangers [6]. In the collecting duct, Kizer et al. showed that electrogenic Cl^- secretion was stimulated by AVP in an IMCD cell line. This secretion was dependent on apical CFTR, basolateral Cl^-/HCO_3^- exchanger and $Na^+/2Cl^-/K^+$ cotransport [7]. We here demonstrated, for the first time, that AVP directly stimulates a Cl^-/HCO_3^- exchanger in the CCD. We have previously reported that $RCCD_1$ cells express, at the basolateral membrane, two isoforms of the Cl^-/HCO_3^- exchanger: AE2 and AE4. While AE2 is DIDS-insensitive, AE4 is sensitive to the inhibitor [21]. Our present studies show that AVP is able to stimulate a DIDS-insensitive Cl^-/HCO_3^- exchanger isoform. Therefore, we can speculate that this isoform would be AE2.

In summary, our present results demonstrate that in the $RCCD_1$ cells AVP activates, via a complex of V1a and V2 receptor-mediated actions, different proteins involved in ion transport and pH_i regulation. V2 receptor activation resulted in a biphasic response (alkalinization-acidification) due to the stimulation of NHE-1 isoform and probably CFTR. On the other hand, AVP actions mediated by V1a-receptor results in an acidification due to an increase in Cl^-/HCO_3^- exchange activity.

The enhanced ability of CCD cells to selectively regulate, in the presence of AVP, different ion transporters linked to pH_i may have several physiological implications. For instance, a key stimulus for AVP release is an acute increase in external osmolality. In this situation cell volume decreases (shrinkage) and "regulatory volume increase" (RVI) mechanisms are triggered in order to promote solute and water influxes into the cell. Then, it is reasonable to speculate that AVP may help the activation of transporters involved in RVI (i.e. Na^+/H^+ and Cl^-/HCO_3^- exchangers) which could be essential for intracellular homoeostasis maintenance. Although the potential application of these results to the physiological state is still speculative, the proposal that AVP selectively regulates different acid-base transporters may contribute to further understand the larger integrative transport functions of renal epithelia by means of studying single transport molecules.

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