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Functional and Molecular Adaptation of Cl^-/HCO_3^- Exchanger to Chronic Alkaline Media in Renal Cells

Valeria Rivarola, Paula Ford, Osvaldo Chara, Mario Parisi and Claudia Capurro

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

Key Words

AE isoforms • Intracellular pH • Acute and chronic alkalosis

Abstract

The Cl^-/HCO_3^- exchanger (AE) is one of the mechanisms that cells have developed to adjust pH_i. Despite its importance, the role of AE isoforms in controlling steady-state pH_i during alkalosis has not been widely investigated. In the present study, we have evaluated whether conditions simulating acute and chronic metabolic alkalosis affected the transport activity and protein levels of Cl^-/HCO_3^- exchangers in a rat cortical collecting duct cell line ($RCCD_1$). pH_i was monitored using the fluorescent dye BCECF in monolayers grown on permeable supports. Anion exchanger function was assessed by the response of pH_i to acute chloride removal. RT-PCR and immunoblot assays were also performed. Our results showed that $RCCD_1$ cells express two members of the anion exchanger gene family: AE2 and AE4. Functional studies demonstrated that while in acute alkalosis pH_i became alkaline and was not regulated, after 48 h adaptation; steady-state pH_i reached a value similar to the physiological one. Chronic treated cells also resulted in a 3-fold rise in Cl^-/HCO_3^- exchange activity together with a 2.2-fold increase in AE2, but not AE4, protein abundance. We conclude that $RCCD_1$ cells can adapt to chronic extracellular alkalosis reestablishing its steady-state pH_i and that AE2 would play a key role in cell homeostasis.

Introduction

The mammalian collecting duct (CD) is critical for the final regulation of acid-base balance. During disorders leading to metabolic alkalosis CD cells must not only regulate transepithelial HCO_3^- transport but also maintain intracellular homeostasis [1]. One of the mechanisms that cells have developed to adjust intracellular pH (pH_i) is Cl^-/HCO_3^- exchange [2, 3]. In the kidney, Cl^-/HCO_3^- exchange activity is nearly ubiquitous and the expression of four different isoforms (AE1-4) has already been described [3]. While it is widely accepted that AE1 plays a key role in the reabsorption of HCO_3^- in the collecting duct, the physiological role of the other AE isoforms

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

remains under study. It was suggested that AE2 would contribute to the regulation of pH_i (by exporting HCO_3^- in response to alkali load) and/or the regulation of cell volume [2, 3].

Despite its importance, the role of AE isoforms in controlling steady-state pH_i during acute and chronic alkalosis has not been widely investigated. Molecular studies showed that AE1 is down-regulated in acute metabolic alkalosis [4]. However, in the case of AE2 it was described that chronic metabolic alkalosis, generated by $NaHCO_3$ loads, induces an increase of AE2 mRNA in the rabbit cortical collecting duct (CCD) [5] but no changes at the protein level were observed in rat kidney cortex [6]. The aim of the present work was to study whether conditions simulating acute or chronic metabolic alkalosis affected the transport activity and protein levels of Cl^-/HCO_3^- exchanger. For this purpose we have used a rat cortical collecting duct cell line ($RCCD_1$), which exhibits features of both principal and intercalated cells, previously shown to be a good model of collecting duct cell function [7-11]. pH_i was monitored using the fluorescent dye 2',7'bis(2-carboxyethyl)-5-(and–6) carboxyfluorescein (BCECF) in monolayers grown on permeable supports, allowing independent and free access to the apical or the basolateral compartments. The results of the present study confirm that $RCCD_1$ cells express two members of the anion exchanger gene family, AE2 and AE4. From our functional studies we propose that AE2 and AE4 are both localized at the basolateral membrane. We demonstrated that only after 48 h adaptation to alkalosis, cells were able to regulate its steady-state pH_i. This regulation took place in parallel with an increase in AE2 protein abundance. We conclude that during chronic metabolic alkalosis $RCCD_1$ cells can reestablish its steady-state pH_i and that AE2 would play a key role in this process.

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; 5 X 10^{-8} M dexamethasone; 3 X 10^{-8} M sodium selenite; 5 µg ml^{-1} insulin; 5 µg ml^{-1} transferrin; 10 µg ml^{-1} epidermal growth factor; 5 X 10^{-8} M triodothyronine; 10 units ml^{-1} penicilin-streptomycin; 20 mM Hepes; pH 7.4) and 2% fetal bovine serum (FBS) (Invitrogen, San Diego, CA, USA) [7]. All experiments were performed on confluent cells, between the 20^{th} and 40^{th} passages, grown on permeable filters during six or seven days (Transwell, 3 µm pore size, 4.5 cm^2 growth area, Corning Costar, Corning, NY, USA).

Intracellular pH Studies

$RCCD_1$ monolayers, grown on permeable filters, were inserted between two lucite frames, separating two fluid compartments when diagonally placed in a quartz cuvette as previously reported [11]. Free access both to the apical and basolateral baths was possible. Measurements were made with a computerized and thermoregulated (37°C) spectrofluorometer (Jasco 770, Easton, MD, USA). For measurements, the cell monolayer was placed forming a 45° angle with 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 60 min 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 "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. Then pH-bathing solution was stepped between 6.6 and 8.5. The 510 / 439 ratio was linear over this pH range ($r = 0.99$, $n = 6$).

Anion exchange activity in $RCCD_1$ cells was evaluated immediately after transition from Cl^- containing to Cl^--free solution. Upon Cl^- removal the initial 3 minutes of each experimental curve were fit, using GraphPad Prism (San Diego, CA. USA), to a simple exponential given by:

$$\Delta pH_i\ (t) = A.(1-e^{-k.t}) \qquad \text{Eq. 1}$$

where ΔpH_i is the difference, at time *t*; between initial pH_i and pH_i after the medium change, A is the maximal ΔpH_i and k is the rate coefficient. The rate of anion exchange activity (dpH_i/dt) was expressed as the first derivative of Eq. 1 at t = 0 (Eq. 2).

$$\frac{\delta pH_i}{\delta t}\ (t=0)=A.k \qquad \text{Eq.2}$$

RT-PCR Studies

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

PCR experiments were performed with 5 µl of the RT reaction using 25 pmol of specific primers for rat AE1, AE2, AE3 and AE4 (table 1). An internal positive control was included in each experiment using β-actin specific primers (sense: 5' CGG

Table 1. Specific rat primers used to detect the expression of AE 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.

	Primers	Amplified fragment size (bp)	cDNA Sequence Location (nt)	Data Bank accession number
AE1	5' CTC TGC TAC TCA AAC GCA GCC A 3	365	272-293	G203092
	5 TGT AGT CGA TGT GCG GAG CCT CA 3		615-637	
AE2	5' ACT GAG AGT GAT CCT CAC GT 3	537	1566-1585	G8394309
	5 CTC GCT TCT TTA GCA TCT GG 3		2084-2103	
AE3	5' GAT GAC AAG GAC AGT GGC TT 3'	725	1319-1338	G8394312
	5' TCT TCA GAG GTT GCC TCG GA 3'		2027-2046	
AE4	5' AGG CTT CTC GTG ATG AGG 3'	609	547-564	G22531598
	5' AAT CGC TGG GGT ACC AGC 3'		1138-1155	

AAC CGC TCA TTG CC 3'; antisense: 5' ACC CAC ACT GTG CCC ATC TA 3').

Cycling parameters were for AE1: 2 min at 94°C, 2 min at 55°C, 2 min at 72°C for one cycle and 1 min at 94°C, 1 min at 55°C, 1 min at 72°C for 34 cycles; for AE2: 2 min at 94°C, 2 min at 60°C, 2 min at 72°C for one cycle and 1 min at 94°C, 1 min at 60°C, 1 min at 72°C for 34 cycles; for AE3: 2 min at 94°C, 2 min at 55°C, 2 min at 72°C for one cycle and 30" at 94°C, 1 min at 55°C, 1 min at 72°C for 30 cycles and for AE4: 2 min at 94°C, 2 min at 60°C, 2 min at 72°C for one cycle and 1 min at 94°C, 1 min at 60°C, 2 min at 72°C for 40 cycles.

Immunoblotting Studies

Confluent $RCCD_1$ cells were washed three times in cold PBS, and were incubated for 30 min at 4°C in a cold lysis buffer containing: 150 mM NaCl, 20 mM Tris, 5 mM EDTA, 1% Triton 100 X, 1 mM phenyl-methylsulfonyl fluoride, 5 mg ml^{-1} aprotinin, 10 mg ml^{-1} antipaine, 10 mg ml^{-1} leupeptine and 10 mg ml^{-1} pepstatine. Cells were then collected with a rubber scrapper and homogenized using a 21 gauge syringe needle. The homogenates were subject to 7.5 % SDS-polyacriylamide minigels electrophoresis using the Tris-Tricine buffer system [12] and transferred to nitrocellulose sheets (Mini Protean II, Bio-Rad, Hercules, CA, USA). Blots were blocked with 5% milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0,1 % Tween 20, pH 7.5) for 1 h and incubated with purified rabbit anti-rat AE2, anti-rat AE3 or anti-human AE4 (2-5 mg ml^{-1}; Alpha Diagnostics International, San Antonio, TX, USA) overnight at 4°C. In some experiments excess of the specific peptide antigens were used (25 mg ml^{-1}) (Alpha Diagnostics International). The blots were then washed and incubated with horseradish peroxidase-conjugated goat antirabbit inmunoglobulin (1/10000, Sigma-Aldrich, St. Louis, MO, USA) and bands were detected by the enhanced chemiluminescence detection system (NEL, PerkinElmer Life Sciences, Boston, MA, USA). The autoradiographs were obtained by exposing nitrocellulose to ECL Hyperfilms (Amersham, Braunshweig, Germany) and digitized by laser scanner (ScanJet 6200C; Hewlett-Packard, Palo Alto, CA, USA). Bands were quantified using Scion Image software (Scion Corporation, Frederick, MD, USA). Densitometric values were normalized to the mean for the control group that was defined as 100% and results were expressed as mean ± SEM. The equity in protein loading in all blots was first verified by Ponceau S staining (Sigma-Aldrich).

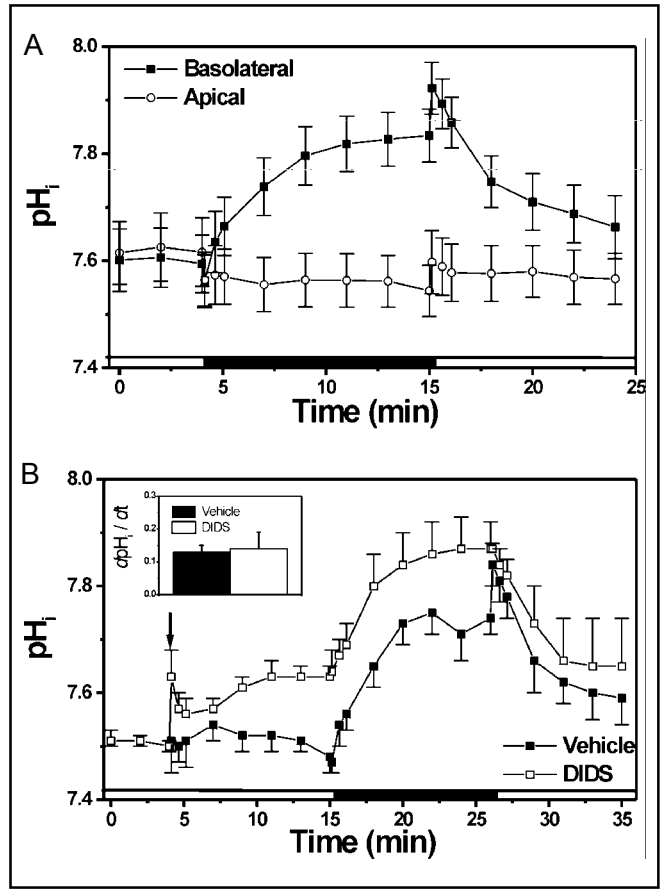

Fig. 1. Effect of Cl^- removal on pH_i in $RCCD_1$ cells. A- pH_i was recorded in the presence of external Cl^- (open bar) and after basolateral or apical Cl^- removal (solid bar). B- Evolution of pH_i in cells exposed, at the arrow, to 100 µM DIDS or vehicle (DMSO) in the presence of external Cl^- (open bar) and after basolateral Cl^- removal (solid bar). *Insert:* Rate of intracellular alkalinization (dpH_i/dt, pH units.min^{-1}) after basolateral Cl^- removal either in the presence of DIDS or vehicle. All experiments were performed at pH_o = 7.4.

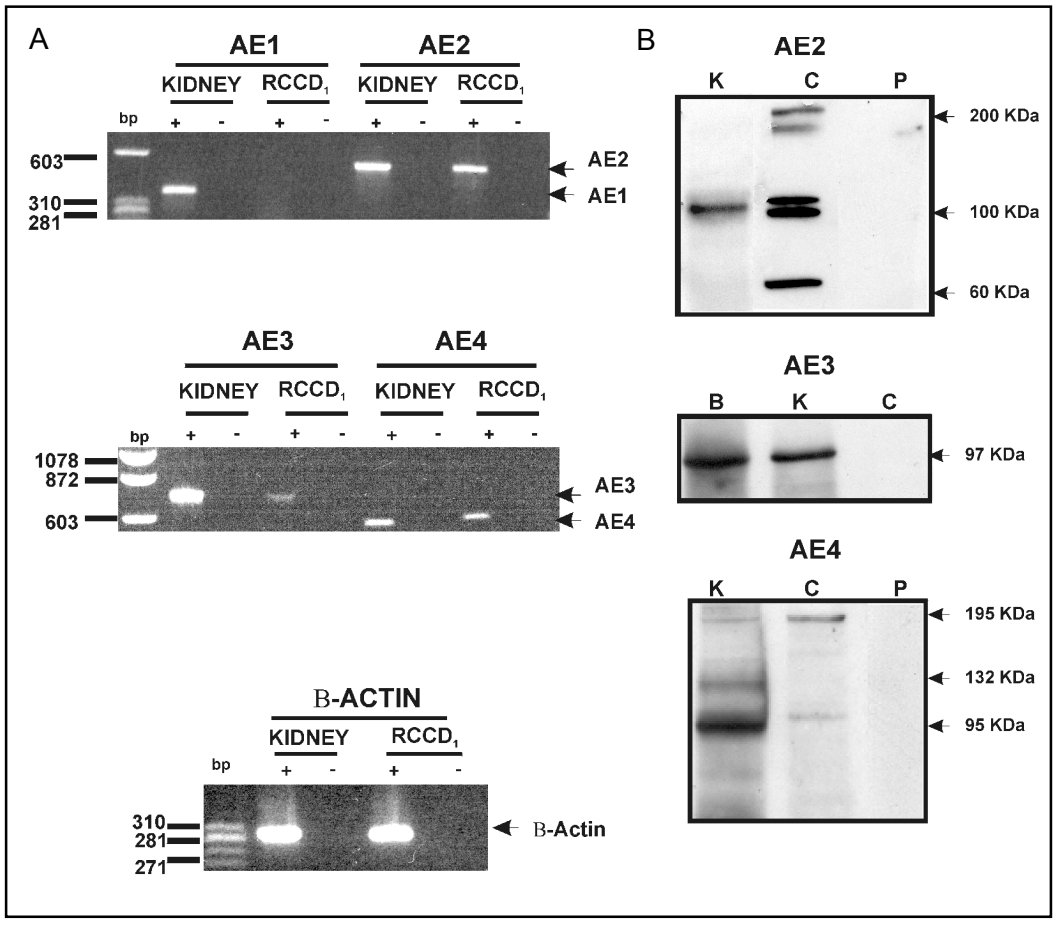

Fig. 2. AE-isoform expression in $RCCD_1$ cells. A- Representative RT-PCR experiments performed using specific primers for rat AE isoforms in mRNA from rat kidney (positive control) and $RCCD_1$ cells. Assays were carried out in the presence (+) or absence (-) of RT enzyme. Left line, ΦX174 *Hae III* digested marker. RT-PCR experiments using specific primers for rat AE1 and AE2 (*upper panel*), for rat AE3 and AE4 (*middle panel*) and for rat β-actin used as internal control (*lower panel*). B-Immunoblotting studies using specific antibodies for AE2, AE3 and AE4 isoforms. All lines were loaded with 100 μg protein either of rat brain (B), $RCCD_1$ cells (C) or rat kidney (K). Specific peptide antigen was assayed in $RCCD_1$ cells (P). *Upper panel*: AE2 antibody, *Middle panel*: AE3 antibody, *Lower panel*: AE4 antibody. Experiments are representative of at least four assays.

Solutions and chemicals

In functional studies, cells were bathed on each side with a control saline solution containing (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 glucose. Cl^--free solutions were achieved by equimolar replacement of chloride with gluconate. Solution´s pH was adjusted to 7.4 or 7.8 (at 37°C) using Tris-[hidroxymethyl] aminomethane (Sigma-Aldrich) and osmolalities were routinely measured in a pressure vapor osmometer (Vapro; Wescor, Logan, Utah, USA). Solutions were continuously bubbled with 5% CO_2 - 95 % O_2.

To evaluate cell adaptation to alkaline environment, media were generated by the addition of $NaHCO_3$ up to pH 7.8 ($NaHCO_3$ media). Cells were then maintained in these media for 1 h or 48 h before functional or molecular studies were performed. In some experiments appropriate amounts of NaCl were added to the control media to account for the differences in osmolality (~60 mOsM, NaCl media).

When specified the inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) was used (Sigma-Aldrich).

Statistics

Results are expressed as mean ± SEM. Student's *t* test for unpaired data was used, $p < 0.05$ was considered significant.

Results

$RCCD_1$ cells express Cl^-/HCO_3^- exchange activity

At resting pH_i a Cl^-/HCO_3^- exchanger would be expected to move HCO_3^- out of the cell, Cl^- removal should reverse the exchanger and cause cell alkalinization. Therefore, the first set of experiments examined Cl^-/HCO_3^- exchange activity in $RCCD_1$ cells by monitoring the rate of intracellular alkalinization in response to removal of extracellular Cl^-. Fig. 1A shows that removal of Cl^- from basolateral bath resulted in an intracellular alkalinization reaching a maximal ΔpH of 0.25 ± 0.02 pH units (n = 14). The mean rate of this intracellular

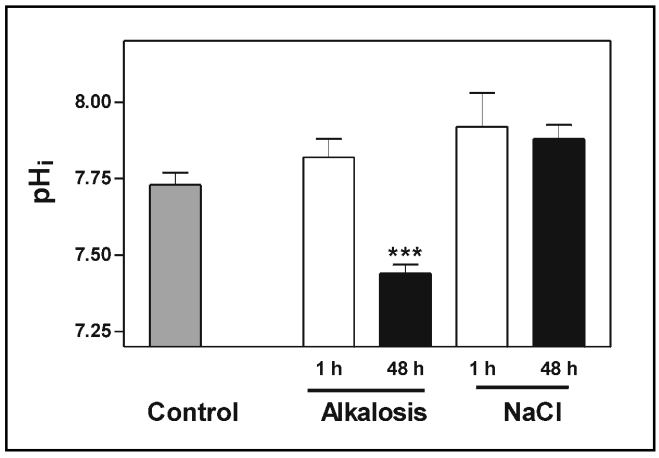

Fig. 3. Effect of alkaline media adaptation on steady-sate pH_i in $RCCD_1$ cells. Cells were incubated in control media or adapted for 1 h or 48 h to a $NaHCO_3$ media. Some experiments were performed adapting cells to a NaCl media for 1 h or 48 h. pH_i was measured at external pH = 7.8. Each bar is mean ± SEM for 5-7 separate experiments. *** $p < 0.001$ compared with control cells.

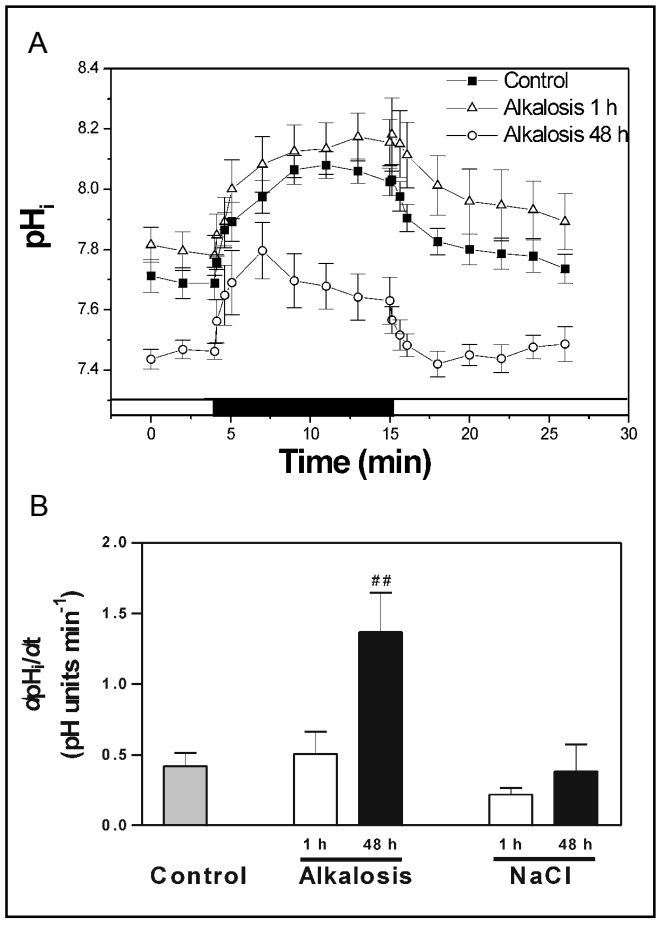

Fig. 4. Effect of alkaline media adaptation on Cl^-/HCO_3^- exchange activity in $RCCD_1$ cells. A- pH_i was recorded in the presence of external Cl^- (open bar) and after basolateral Cl^- removal (solid bar) in cells preincubated in control media or adapted for 1 h or 48 h to alkaline media. B- Rate of intracellular alkalinization (dpH_i/dt) after basolateral Cl^- removal in experiments in A and in cells adapted to NaCl media. All experiments were performed at external pH = 7.8. ## $p < 0.01$ compare to control cells.

alkalinization (dpH_i/dt) was 0.14 ± 0.04 pH units.min^{-1} (n = 14). Readdition of basolateral Cl^- caused the pH_i to return to the baseline. In contrast, the removal of Cl^- from apical solution caused no changes in pH_i. These results evidence the presence of Cl^-/HCO_3^- exchange activity at the basolateral membrane of $RCCD_1$ cells.

To further characterize Cl^-/HCO_3^- exchange activity $RCCD_1$ cells were incubated with basolateral 100 μM DIDS for ten minutes before Cl^--removal experiments. Figure 1B shows that the addition of the inhibitor, in the presence of external chloride, caused pH_i to increase by 0.11 ± 0.03 pH units (n = 5). However, the rate of basolateral Cl^-/HCO_3^- exchange activity was not inhibited by DIDS (dpH_i/dt, pH units.min^{-1}: vehicle: 0.13 ± 0.02 vs. DIDS: 0.14 ± 0.05, n = 5). These results suggest that more than one isoform of Cl^-/HCO_3^- exchanger could be present at the basolateral membrane of $RCCD_1$ cells, one sensitive and the other insensitive or partially sensitive to DIDS.

Cl^-/HCO_3^- Exchanger Isoforms Expression in $RCCD_1$ Cells

To determine which AE gene products might mediate the above-described Cl^-/HCO_3^- exchange activity, RT-PCR assays were performed in $RCCD_1$ cells. Experiments were carried out using specific primers for the four rat Cl^-/HCO_3^- exchanger isoforms at present described (AE1-4, Table 1). Assays were undertaken using total RNA extracted from $RCCD_1$ cells and rat kidneys (positive controls). Figure 2A shows the results of representative RT-PCR experiments. As expected, RT-PCR of rat kidney RNA produced fragments of the proper size for all isoforms tested. On the other hand, only mRNAs for AE2 (~537 bp), AE3 (~725 bp) and AE4 (~609 bp) were expressed in $RCCD_1$ cells (three to four different experiments). β-actin was used as an internal control for kidneys and $RCCD_1$ cells in all experiments (Fig. 2A).

Immunoblotting studies were performed using specific antibodies against AE2, AE3 and AE4 isoforms

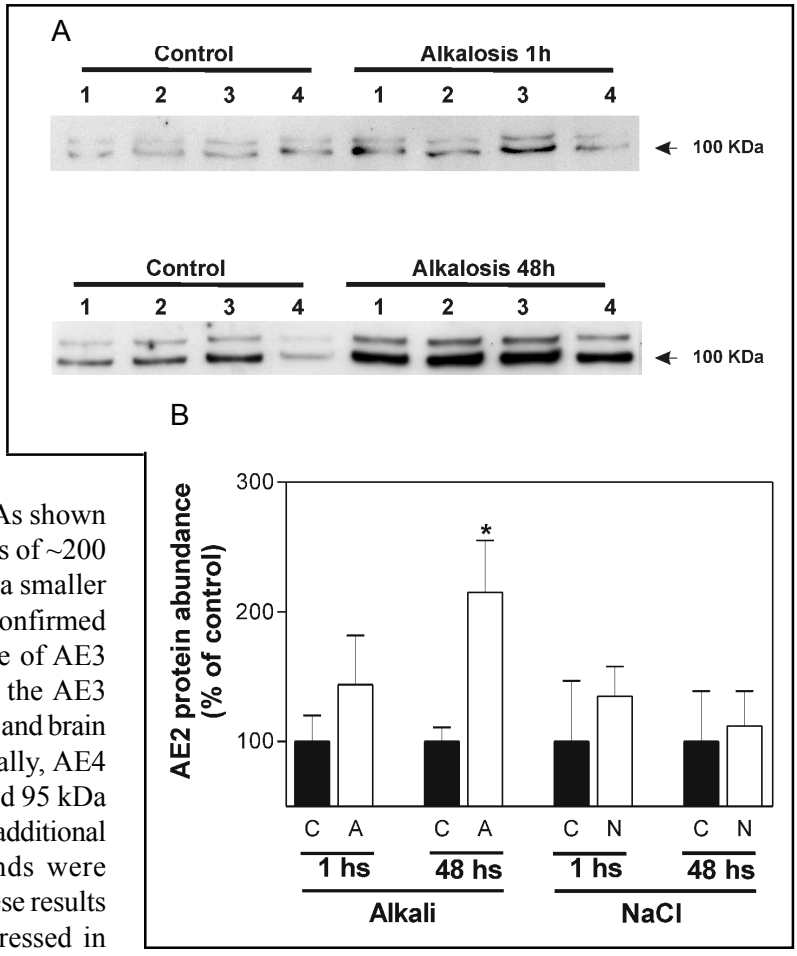

Fig. 5. Effects of alkaline media adaptation on AE2 protein abundance in $RCCD_1$ cells. A- Representative immunoblots of cells incubated either in control or alkaline media during 1 h or 48 h. Numbers represent four independent experiments. Each line was loaded with 40 µg protein. B- Densitometric analysis of experiments in A and in cells adapted to NaCl media. C: Control, A: Alkaline media, N: NaCl media. * $p < 0.05$ compare to control cells.

(positive mRNAs expressed in $RCCD_1$ cells). As shown in Fig. 2B the AE2 antibody detected two bands of ~200 and 100 kDa (both migrated as a doublet) plus a smaller band of ~60 kDa. The specificity of AE2 was confirmed with the peptide antigen. Despite the presence of AE3 mRNA no positive signal was apparent with the AE3 antibody in $RCCD_1$ cells. However, in rat kidney and brain a positive band of ~97 kDa was detected. Finally, AE4 antibody recognized positive bands of ~195 and 95 kDa both in kidney and in $RCCD_1$ cells. In kidney, an additional band of ~132 kDa was also observed. Bands were specifically blocked by the peptide antigen. These results confirm that isoforms AE2 and AE4 are expressed in $RCCD_1$ cells.

Effects of alkaline media on Cl^-/HCO_3^- exchanger activity in *$RCCD_1$ cells*

Cell adaptation to acute and chronic alkalosis was assayed in $RCCD_1$ cells preincubated in $NaHCO_3$ media (pH 7.8) for 1 h and 48 h or in control media (pH 7.4). In order to evaluate the effects of alkalosis on Cl^-/HCO_3^- exchanger activity all functional experiments were performed using an external alkaline pH (7.8). Fig. 3 shows baseline pH_i measurements in each of these situations. It can be observed that in both, $RCCD_1$ cells with no alkaline pretreatment (control) and $RCCD_1$ cells pretreated during 1 h with $NaHCO_3$ media, resting pH_i was alkaline (7.73 ± 0.04, n = 10 and 7.82 ± 0.06, n = 11, respectively). In contrast, when cells were incubated during 48 h in $NaHCO_3$ media, pH_i decreased (7.44 ± 0.03, n = 6) to values not statistically different from $RCCD_1$ physiological pH_i [11]. As $NaHCO_3$ media have an osmolality higher than control ones, we also tested in parallel baseline pH_i of cells adapted to a medium of equivalent osmolalities (NaCl media, pH = 7.4). Results showed that after 1 h or 48 h treatment with NaCl media pH_i, measured at external pH of 7.8, was alkaline (NaCl, 1 h; 7.92 ± 0.11, n = 7 vs. 48 h: 7.88 ± 0.05, n = 5, NS). These results indicate that restoration of baseline pH_i, after 48 h alkalosis, is due to cell adaptation to chronic alkaline media and not to an increase in external osmolality.

To evaluate Cl^-/HCO_3^- exchange activity in each of these conditions, Cl^- removal experiments were performed (Fig. 4A). The mean rates of intracellular alkalinization after chloride removal (dpH_i/dt) in each of these conditions are summarized in Fig. 4B. It can be observed that there are no differences between non-treated cells (control) or 1 h alkalosis-treated cells. In contrast, chronic alkalosis-treated cells resulted in a significant increase in the rate of basolateral Cl^-/HCO_3^- activity. Control experiments incubating cells in NaCl media, during 1 h or 48 h, showed no statistical differences, demonstrating no effect of osmolality on this response (Fig 4B). Moreover, none of these pretreatments were able to elicit apical Cl^-

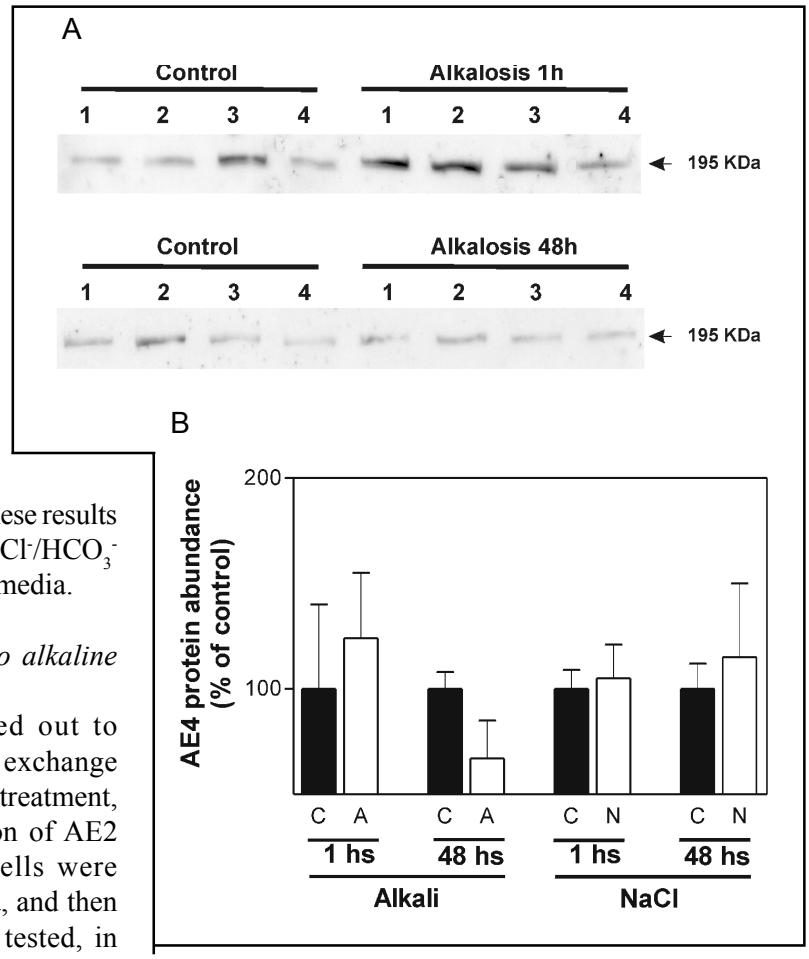

Fig. 6. Effects of alkaline media adaptation on AE4 protein abundance in $RCCD_1$ cells. A- Representative immunoblots of $RCCD_1$ cells incubated either in control or alkaline media during 1 h or 48 h. Numbers represent four independent experiments. Each line was loaded with 40 µg protein. B- Densitometric analysis of experiments in A and in cells adapted to NaCl media. C: Control, A: Alkaline media, N: NaCl media.

$/HCO_3^-$ activity (data not shown). Altogether, these results confirm a significant activation of basolateral Cl^-/HCO_3^- exchange after chronic incubation in alkaline media.

AE2 and AE4 expression in response to alkaline media in $RCCD_1$ cells

Immunoblotting studies were carried out to determine whether the observed Cl^-/HCO_3^- exchange activity increments, after alkaline media pretreatment, were associated to changes in the expression of AE2 and/or AE4 isoforms. For this, $RCCD_1$ cells were incubated during 1 or 48 h in $NaHCO_3$ media, and then total cell proteins were extracted. We also tested, in parallel, the effect of cells exposed to NaCl media on AE2 and AE4 abundance. Fig. 5A shows the results of immunoblots of cells exposed to alkaline media during 1 h or 48 h, which were probed with anti-AE2 antibody. AE2 expression was not affected after an acute adaptation to alkalosis, but it was 115 ± 40 % increased after 48 h incubation in alkaline media (Fig. 5B). This increase was not observed in NaCl control experiments.

On the other hand, regardless of the metabolic alkalosis state (acute or chronic) AE4 level expression was not changed (Fig 6 A and B). These results indicate that AE2, but not AE4, isoform would be involved in pH_i regulation after alkaline adaptation.

Discussion

Functional and molecular expression of Cl^-/HCO_3^- exchange in $RCCD_1$ cells

The first step of this work was to use the $RCCD_1$ cell model to investigate functional distribution and gene expression of Cl^-/HCO_3^- exchanger. Our molecular studies confirmed that AE2 and AE4, two members of the anion exchanger gene family, are expressed in $RCCD_1$ cells. These results are in agreement with previous reports proposing that AE2 is expressed along the entire collecting duct system and AE4 in the intercalated cells of collecting duct [5, 13-15]. In the case of AE3, although mRNAs were detected in $RCCD_1$ cells, immunoblotting studies failed to confirm polypeptide expression. This lack of AE3 expression suggests a very low abundance of transcripts. This result is consistent with a previous report, which indicates that AE3, in the kidney, is only observed in the afferent arteriole and large arteries [16].

Our functional studies demonstrate that there is only basolateral Cl^-/HCO_3^- exchange activity in $RCCD_1$ cells, suggesting that AE2 and AE4 would be localized at the basolateral membrane. Basolateral membrane localization of AE2 and AE4 in rat kidney collecting duct has been previously reported [13, 14]. Our results also show that baseline pH, in the presence of external Cl^-, was significantly increased by 100 µM DIDS suggesting a

contribution of a DIDS-sensitive Cl^-/HCO_3^- exchanger in the maintenance of the physiological steady-state pH_i in $RCCD_1$ cells. However, after external Cl^- removal, Cl^-/HCO_3^- exchange activity was not inhibited by DIDS. Therefore, it can be proposed that more than one Cl^-/HCO_3^- exchange isoform may be involved in pH_i homeostasis. The sensitivity of some AE isoforms to DIDS is controversial. For instance, while several authors have proposed a high sensitivity of AE2 in many systems [17-19], others have demonstrated low sensitivity in renal epithelial cells [20-22]. On the other hand, in HEK and LLC-PK_1 cells transfected with rat AE4 cDNA, very sensitive Cl^-/HCO_3^- activity to H_2DIDS was reported [14] whereas rabbit AE4 expressed in *Xenopus laevis* oocytes was shown to be insensitive to DIDS [15]. Considering that sensitivity to stilbenes, in renal cell lines is low for AE2 and high for rat AE4, we can speculate that in $RCCD_1$ cells, at 100 μM,DIDS-insensitive activity may be associated to AE2 while DIDS-sensitive activity to AE4.

Adaptation of $RCCD_1$ cells to external alkaline pH

To study functional adaptation to changes in extracellular pH, we performed experiments with $RCCD_1$ cells that were previously grown in media that mimics metabolic alkalosis. Our studies showed that only when the cells were adapted chronically to alkalosis (48 h) steady-state pH_i reached physiological values. This normalization of pH_i during chronic treatment would be critical for cell homeostasis because changes in pH_i affect a wide array of biological processes. Moreover, after Cl^- removal, the rate of alkalinization increased 3- fold in chronic alkalosis. The observed restoration of the steady-state pH_i and the higher Cl^-/HCO_3^- exchange activity, in chronically treated cells, were associated to a 2.2-fold increase in AE2 abundance, without changes in AE4 expression.

Since we have found a correlation between activity and AE2 abundance, we are proposing that AE2 would have a specific role in normalization of pH_i. This proposal is also supported by the fact that in acute alkaline-treated cells pH_i was not reestablished after one hour of incubation and no change in AE2 abundance was observed.

The molecular results presented here are in line with a previous study, in rabbit CCD, reporting that AE2 mRNA was significantly increased following both *in vivo* and *in vitro* $NaHCO_3$ chronic alkali load [5]. On the other hand, there are no studies concerning AE4 pH modulation except that its localization was not influenced by the metabolic state of the rat (acidosis or alkalosis) [14]. We have demonstrated herein that AE4 expression was not affected by chronic alkalosis in $RCCD_1$ cells.

Even if there is no previous functional study reporting adaptation of Cl^-/HCO_3^- exchangers to alkalosis, pH-sensitivity of AE isoforms has been largely evaluated after immediate changes in pH [23]. In these situations it was previously reported that while AE1 exchange activity is stable across a wide range of pH, AE2 and AE3 are activated by alkaline pH_i [21, 24, 25]. Moreover, AE2 would also be highly sensitive to changes in extracellular pH (pH_o) [26]. At the molecular level, Stewart *et al.* have characterized the presence of a pH-sensitive surface on the AE2 N-terminal cytoplasmic domain that contributes to the pH_o- and pH_i-dependence of AE2-mediated anion transport [26, 27]. These concerns are consistent with our present results in non-adapted cells (control), which showed a rise in Cl^-/HCO_3^- exchange activity upon exposition to an external pH of 7.8 when compared with cells exposed to an external pH of 7.4 (dpH_i/dt, pH units.min^{-1}: 0.422 ± 0.093 and 0.144 ± 0.043 respectively, $n = 8$, $p < 0.02$). Even though, at a constant external pH of 7.8, non-adapted cells showed an increase in Cl^-/HCO_3^- exchange activity, their pH_i is alkaline. Therefore, this external pH modulation of the transporter was apparently not sufficient to reestablish pH_i in $RCCD_1$ cells. Considering these results and the fact that AE2/AE4 protein expression is not modified in non-adapted cells, we are suggesting that basal protein abundance may be insufficient to regulate pH_i under this condition.

Our present results also demonstrated that acute or chronic NaCl treated cells did not modified its baseline pH_i, Cl^-/HCO_3^- exchange activity or AE2 protein abundance. It has been previously shown that AE2 expressed in *Xenopus* oocytes is activated by hypertonicity secondary to an activation of Na^+/H^+ exchange [28]. We have previously demonstrated that, in $RCCD_1$ cells, the isoform NHE-2 of the Na^+/H^+ exchanger is mainly activated after hypertonic shocks [11]. Stimulation of NHE-2 by hypertonicity alkalinizes the cell and thereby, could secondary activate a Cl^-/HCO_3^- exchange. However, in $RCCD_1$ cells, pH_i remained alkaline during more than 15 min probably indicating no activation of a Cl^-/HCO_3^- exchange. Altogether these results let us to hypothesize that external increase in osmolality in $RCCD_1$ cells is not affecting Cl^-/HCO_3^- exchange activity at least under the conditions studied. This lack of Cl^-/HCO_3^- exchange activation by hyperosmolality may be due to the requirement of

additional factors (other than intracellular alkalinization). For instance, in mouse medullary thick ascending limb of Henle´s loop (TAL), it has been reported that Cl^-/HCO_3^- exchange is activated by hypertonicity only after vasopressin or cAMP stimulation [29, 30].

Regarding AE2 protein abundance Quentin *et al*. has recently proposed that chronic sodium loading, either as $NaHCO_3$ or NaCl, increased AE2 abundance to a similar extent in the outer medulla, whereas in the kidney cortex, only chronic NaCl loading led to an increase in AE2 protein expression [6]. However, this work mainly evaluated AE2 expression in both, cortical and medullary TAL, but not specifically in the collecting duct. Therefore, it is possible that AE2 modulation may vary between different nephron segments.

In summary, our present results demonstrate, for the first time, that cells expressing Cl^-/HCO_3^- exchange can adapt to chronic extracellular alkalosis regulating its steady-state pH_i and increasing AE2 abundance. We propose that during chronic alkalosis AE2 would play a key role in reestablishing cell homeostasis.

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