Differential Expression of AE1 in Renal HCO₃⁻-secreting and -reabsorbing Intercalated Cells*

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The cortical collecting duct of the kidney contains two types of intercalated cells that transport HCO₃⁻ in opposite directions. HCO₃⁻ reabsorption takes place in the α-type intercalated cells, which express a CI/HCO₃⁻ exchanger on the basolateral membrane. This exchanger is the product of the anion exchanger 1 (AE1) or band 3 gene. HCO₃⁻ secretion occurs in the β-intercalated cells, which have a Cl/HCO₃⁻ exchanger on the apical membrane. Based on studies in an immortalized cell line, recently it was proposed that the apical anion exchanger of β-intercalated cells is also AE1 (van Adelsberg, J. S., Edwards, J. C., and Al-Awqati, Q. (1993) J. Biol. Chem. 268, 11293-11299). In the present study we reinvestigated this issue by determining the distribution of AE1 mRNA and protein in the two intercalated cell types using cells freshly isolated from the native epithelium.

Using quantitative reverse transcriptase polymerase chain reaction, we found that intercalated cells, isolated from rabbit kidney by fluorescence-activated cell sorting, have high levels of AE1 mRNA, whereas β-intercalated cells express very low levels. The ratio of AE1 mRNA levels in α- versus β-intercalated cells averaged 10.1 ± 2.6. In addition, metabolic acidosis increased the levels of AE1 mRNA by 3-5-fold in cortical collecting duct cells. This difference was confirmed by Northern blotting. Western blotting using an antibody against rabbit AE1 revealed a major immunoreactive product with a molecular weight of ~110 kDa in cortical collecting duct cells. Deglycosylation reduced the size of the immunoreactive product to ~90 kDa, which is compatible with the presence of a truncated form of AE1. Metabolic acidosis increased the intensity of the AE1 immunoreactive band. The level of AE1 immunoreactive protein was significantly higher in α-intercalated cells than in β-intercalated cells.

In aggregate, these data provide evidence for the differential expression of AE1 in HCO₃⁻-reabsorbing versus HCO₃⁻-secreting renal intercalated cells both at the mRNA and at the protein level. These results give no support to the concept that AE1 functions both as a basolateral and an apical anion exchanger in cortical collecting duct cells.

The fine regulation of acid/base balance by the kidney takes place in the collecting ducts. The cortical collecting duct (CCD)1 contains two types of intercalated cells (ICC), α- and β-type, which transport HCO₃⁻ in opposite directions (for review, see Ref. 1). HCO₃⁻ reabsorption occurs in α-ICCs, which are endowed with an apical H-ATPase and a basolateral Cl/HCO₃⁻ exchanger, which is the product of the AE1 (band 3) gene (2). The cell responsible for HCO₃⁻ secretion is the β-ICC, which functionally is a mirror image of the α-cell and is modeled with a basolateral H-pump and an apical Cl/HCO₃⁻ exchanger (1). Changes in acid/base balance are thought to result in an interconversion of the two ICC types by retargeting the transporters to opposite poles of the cells (3).

The opposing functional polarity of α- and β-ICC could be achieved either by expressing functionally similar but structurally different transporters for apical and basolateral acid or base extrusion or by targeting the same proteins to opposite poles in the two cells. There is immunohistochemical evidence that in the rat (but not in the rabbit) at least two subunits of the H-ATPase can occur with either apical or basolateral polarity in α- and β-ICCs, respectively (4). On the other hand, the question whether the same anion exchanger can occur in opposite membranes of α- and β-ICCs is still unresolved. The basolateral and apical anion exchangers differ in inhibitor sensitivity and kinetic properties (2), and, most importantly, antibodies directed against the basolateral exchanger fail to stain the apical membrane of β-ICCs (5, 6). Nevertheless, recently the possibility was raised that the apical exchanger of HCO₃⁻-secreting β-ICCs is the same protein as the basolateral exchanger of α-ICCs, i.e. AE1 (7). These authors reported that apical membrane preparations from cultured peanut lectin-positive cells (presumably β-ICC) contain a protein that reacts with an AE1 antibody on Western blots and has a size similar to that of AE1 (7).

Since the maximal rate of HCO₃⁻ secretion in the CCD of alkalotic rabbits is comparable to the rate of HCO₃⁻ reabsorption in the CCD of acidic animals (8) (if indeed the same protein functions both as the basolateral Cl/HCO₃⁻ exchanger in α-ICCs and the apical exchanger in β-ICCs), one can assume that AE1 mRNA and protein are expressed at comparable levels in α- and β-type ICCs. Also, if the apical and basolateral Cl/HCO₃⁻ exchangers are products of the same gene, one would expect that acidosis increases AE1 expression in α-ICCs to achieve maximal HCO₃⁻ reabsorption, whereas alkalosis would increase AE1 expression in β-ICC concurrent with enhanced HCO₃⁻ secretion. The findings of this study, however, do not conform with these predictions. Here we report that AE1 mRNA and protein are predominantly expressed in α-type ICCs, and there is a significant increase in the levels of both the mRNA and the protein in acidic versus alkalotic animals.

EXPERIMENTAL PROCEDURES

Animals—Male New Zealand white rabbits, weighing 1.5–2.0 kg, were used. The animals were kept on standard diet and had access to intercalated cells; AE1, anion exchanger 1; PCR, polymerase chain reaction; bp, base pair(s).

1 The abbreviations used are: CCD, cortical collecting duct; ICC, intercalated cells; AE1, anion exchanger 1; PCR, polymerase chain reaction; bp, base pair(s).
water ad libitum. Metabolic alkalosis was induced by intravenous infusion of 15 mmol/kg NaHCO3, 16–20 h before sacrifice. Metabolic alkalosis was achieved by an intragastric load of 15 mmol/kg of NH4Cl. To keep the amount of Na+ load constant, this latter group also received 15 mmol/kg NaCl intravenously. For the last 12 h before the experiments, the rabbits were on restricted food intake (3 oz). Urine samples were taken from the bladder immediately before sacrifice for the determination of urinary pH.

**Cell Isolation**—CCD cells were isolated from the renal cortex by solid phase immunoadsorption, using a monoclonal antibody against an ectoantigen on these cells (DT.17; Ref 11). To aid in the discrimination between live and dead cells, CCD cell preparations were also stained with DAPI (0.1 pg/ml), which is excluded from viable cells. The purity of the sorted cells was determined by immunocytochemistry using other cell-specific markers as described (11, 12), whereas α-ICCs were operationally defined as the DT.17- and peanut lectin agglutinin-negative population.

To isolate the α-intercalated cell types (i.e. α- and β-ICCs and principal cells) by fluorescence-activated cell sorting using cell-specific markers as described (11, 12), whereas α-ICCs were operationally defined as the DT.17- and peanut lectin agglutinin-negative population.

**RNA Isolation and cDNA Synthesis**—Total RNA was isolated using TRI Reagent™ (Molecular Research Center, Inc.) and poly(A)+ RNA was isolated by SDS lysis and oligo(dT) selection (13). RNA concentrations were calculated from the ODs at 260 nm. cDNA was synthesized using 0.5–2 μg of total RNA and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.).

**Reverse Transcription PCR**—Reverse transcription PCR was performed using primers 5 and 2 with 0.1, 0.3, and 1 ng of cDNA derived from CCD cells from acidotic (lanes 1–3, respectively) and alkalotic (lanes 4–6) rabbits. The expected size of the PCR product using these primers is 581 bp. The position of molecular weight standards is shown on the left.

**Western Blotting**—Immunoselected CCD cells were lysed in 1% SDS, which was followed by centrifugation at 12,000 x g for 10 min. From sorted α- and β-intercalated cells, protein factions were isolated using TRI Reagent™. Proteins were solubilized in 1% SDS, and protein concentrations were determined using the BCA protein assay reagents (Pierce). Proteins were separated by 7.5% polyacrylamide gel electrophoresis. The positions of the molecular weight standards were marked on the membrane. After 60 min of blocking with 2.5% Carnation nonfat dry milk and 50% fetal calf serum in phosphate-buffered saline at room temperature, the blots were probed with the anti-rabbit AE1 monoclonal antibody B4A2A (kindly provided by Drs. L. S. Ostedgaard and V. L. Schuster) diluted to 5 μg/ml in 10 mM Tris-HCl, 150 mM NaCl, 0.5% Tween 20 (TBST), for 60 min at room temperature. The blots were then washed 4 times with TBST and incubated with a horseradish peroxidase-coupled secondary antibody (anti-mouse IgG2a; Zymed) diluted to 0.5 μg/ml in phosphate-buffered saline containing 50% Stabilizyme HRP (conjugate stabilizer from BSI Corp.) for 60 min at room temperature and then washed 4 times with TBST. The reaction products were visualized using the enhanced chemiluminescence detection system (Amer sham Corp.).

**Deglycosylation**—In several experiments, the SDS-lysat was treated with 40,000 units/ml N-glycosidase (PNGase F; from New England Biolabs) at 37°C for 60 min, in the presence of 1% Nonidet P-40, before electrophoresis.

**RESULTS**

**Expression of AE1 mRNA in CCD Cells**—Using oligonucleotide primers 5 and 2 and cDNA derived from immunoselected rabbit CCD cells as template, a ~580-bp PCR product was amplified (Fig. 1). This PCR product is of the predicted size based on the published sequence of the mouse AE1 (18). The identity of this PCR product was verified by two independent methods. First, nested PCR was performed using sense and antisense primers with start positions corresponding to nucleotide sequences of other primers used for nested PCR are: primer 3 (upper), 5'-TGG ATG ATG GCC TCC GTT CTG C-3'; primer 4 (lower), 5'-TGG ATC GGC TTC TGG CTC ATC CT-3'; primer 2 (lower), 5'-CGT GGT GAT CTG AGA CTC AAG GAA-3'. These primer anneal to nucleotides 1531–1554 (primer 5) and 2089–2112 (primer 2) on the mouse AE1 and bracket a 581-bp sequence, which shows significant sequence divergence between AE1 and other members of the AE family, due to the presence of a cytoplasmic loop. The sequences of other primers used for nested PCR are: primer 3 (upper), 5'-TGG ATG ATG TTT GCC TCT GTT CTG C-3'; primer 4 (lower), 5'-GGA GAT GTA TTG GAC GAG GAA GCT-3'. These primer anneal to nucleotides 2038–2062 (primer 3) and 1585–1608 (primer 4) on the mouse AE1. The sequencing methods. First, nested PCR was performed using sense and antisense primers with start positions corresponding to nucleotide sequences of other primers used for nested PCR are: primer 3 (upper), 5'-TGG ATG ATG GCC TCC GTT CTG C-3'; primer 4 (lower), 5'-GGA GAT GTA TTG GAC GAG GAA GCT-3'. These primer anneal to nucleotides 2038–2062 (primer 3) and 1585–1608 (primer 4) on the mouse AE1.

**PCR Amplification**—Reactions were performed in a 20-μl total volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 75 μM dNTP, 200 ng of each primer, 0.1 units of Taq polymerase (Perkin-Elmer Corp.), and 1 μCi of [32P]dCTP (DuPont NEN, 3000 Ci/mmole) with varying amounts (0.1–3 ng) of template cDNA. Each sample was overlaid with 20 μl of Chill-Out™ (MJ Research, Inc.) to prevent evaporation. After an initial 2-min denaturation at 96°C, PCR was carried out for 30 cycles with denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and primer extension at 72°C for 1 min. The reaction mixtures were incubated for a final extension at 72°C for 8 min. The relative abundance of β-actin mRNA in each CCD cell sample was determined using primers and conditions as described (16). cDNA samples derived from pairs of rabbits (one acidic, the other alkalotic) were always amplified simultaneously in the same PCR.

After amplification, 4 μl of tracking dye was added to each sample, and 20 μl was run on a 6% polyacrylamide gel. Gels were dried, and the autoradiography in the PCR product was determined using a model 425 PhosphorImager™ (Molecular Dynamics).

**DNA Sequencing**—The 580-bp PCR AE1 product generated with rabbit CCD cDNA template was extracted by phenol/chloroform, and unincorporated primers and nucleotides were removed by centrifugal dialfiltration using a Microcon-100 ultrafiltration unit (Amicon). Direct sequencing was performed using primer 5 or primer 2 by the dye deoxy terminator chemistry on an ABI 373A automated sequencer.

**Northern Blot Analysis**—Northern blotting was carried out using standard protocols (17). In brief, 2.5 μg of poly(A)+ RNA originating from immunoselected CCD cells from acidotic and control rabbits was fractionated on a 1.2% agarose gel containing 1% formaldehyde. RNA was transferred to a nylon membrane (0.45 μm; MSI Inc.) and probed with gel-purified PCR fragment generated with primers 5 and 2 and labeled with 32P during the PCR. Prehybridization was performed at 42°C for 10–12 h in 5× SSC, 5× Denhardt’s solution, 50% formamide, 100 μg/ml salmon sperm DNA, and 0.5% SDS. Hybridization was done using the same conditions as for prehybridization for 12 h. Two washes were carried out at room temperature for 15 min each, with 1× SSC, 0.1% SDS, followed by two washes with 0.25× SSC, 0.1% SDS. After a final wash at 45°C for 15 min with 0.1× SSC, 0.1% SDS, the blot was exposed to x-ray film.
otides 2038 and 2112 (primers 3 and 2) and to nucleotides 1551 and 1608 (primers 5 and 4 on the mouse AE1). The nested primer pairs yielded PCR products with the expected sizes (74 and 77 bp, respectively), indicating that the 580-bp product is indeed amplified from AE1 cDNA. Second, the identity of the primer pairs yielded PCR products with the expected sizes (74 and 2038 and 2112 (primers 3 and 2) and respectively (14, 19). On the other hand, the AE1 PCR product shows only 62% homology to the corresponding region of the rabbit ileal AE2 (20), 61% to the mouse AE2 (21), and 56% to the mouse AE3 (22).

The level of β-actin mRNA was determined with a similar PCR technique in each CCD cDNA sample. When the relative level of β-actin mRNA in samples containing increasing amounts of cDNA was compared with that of AE1 mRNA, we found that AE1 mRNA is abundant in CCD cells, as the slope of AE1 mRNA versus increasing amounts of starting cDNA was only about an order of magnitude lower than that of β-actin.

Effect of Metabolic Acidosis and Alkalosis on the Expression of AE1 mRNA in CCD Cells—To examine whether changes in acid/base balance result in an altered expression of AE1 mRNA, we induced metabolic acidosis or alkalosis in pairs of rabbits. Urinary pH averaged 5.4 ± 0.4 in acidic versus 8.3 ± 0.2 in alkalotic rabbits (p < 0.001). AE1 mRNA levels were determined in cDNAs derived from isolated CCD cells, and the results are summarized in Fig. 2. The relative abundance of AE1 mRNA, calculated from the ratio of [32P]dCTP incorporated into the 580-bp PCR product and into the 350-bp β-actin PCR product, was significantly higher in acidic than in alkalotic animals. The average increase in AE1 mRNA levels in acidosis versus alkalosis was 4.53-fold. This difference was confirmed by Northern blotting of CCD mRNAs obtained from pooled samples from three acidic and three control rabbits using the PCR product as probe (Fig. 3). In both cases a single hybridizing mRNA species was detected with a size of approximately 4.5 kilobases, and its intensity was significantly higher in mRNA obtained from acidic than from control animals (Fig. 3).

Distribution of AE1 mRNA in Different CCD Cell Types—Fig. 4A summarizes the steady-state AE1 mRNA levels in the different cell types of the collecting duct, isolated by fluorescence-activated cell sorting. It is obvious, that α-ICCs have abundant levels of AE1 mRNA, whereas β-ICCs and principal cells expressed very low levels. When we compared the levels of AE1 mRNA in a given cell type originating from acidic versus alkalotic rabbits, we found that both α-ICCs and β-ICCs expressed significantly higher levels in acidic rabbits (average increase following acidosis was 13.8- and 6.5-fold in α-ICC and β-ICC, respectively; Fig. 4B). At the same time, the relative abundance of AE1 mRNA in α- versus β-cells were unaltered by changes in acid/base balance; AE1 mRNA in α-ICC versus AE1 mRNA in β-ICC was 10.69 ± 2.5 in acidic and 10.77 ± 5.3 in alkalotic rabbits (n = 5 for each group), suggesting that acidosis and alkalosis are not accompanied by opposite changes in AE1 mRNA expression in HCO3-secretion versus HCO3-reabsorbing intercalated cells.
Effect of Metabolic Acidosis and Alkalosis on the Level of AE1 Protein in CCD Cells—To determine whether the acidosis-induced increase in AE1 mRNA levels in CCD cells is accompanied by similar changes at the protein level, we performed Western blot analysis using a monoclonal antibody against rabbit AE1 (VB4A4, a generous gift of Drs. L. S. Ostedgaard and V. L. Schuster). Western analysis revealed the presence of a major immunoreactive band in both rabbit red blood cells (Fig. 5A, lanes 1 and 2) and CCD cell lysates obtained from normal (N) and acidotic (A) rabbits. Further analysis has shown that the band with molecular weight ~110 kDa corresponds to AE1, and the bands with lower molecular weights probably correspond to degradation products generated during the protease treatment used to obtain single cell preparations for cell sorting.

The exact mechanisms by which the opposing functional polarity of HCO₃⁻ secreting β-ICCs and HCO₃⁻ reabsorbing α-ICCs is achieved are unclear. Whereas there is evidence that the same H-ATPase subunits can occur with either basolateral or apical polarity (4, 23), the idea that the two membranes harbor the same anion exchanger remains controversial. It has been recently proposed that anion exchange in both α- and β-ICC is mediated by the same AE1 gene product (7).

The major findings of this study speak against this hypothesis. If the apical anion exchanger of β-ICCs were the product of the same gene as the basolateral exchanger of α-ICCs, the mRNA levels in the two cell types should be comparable. Our results demonstrate that the levels of AE1 mRNA are significantly higher in α-ICC than in β-ICC. In fact, mRNA levels of AE1 detected in β-ICCs are comparable with those seen in principal cells, a cell type that is probably not involved in HCO₃⁻ transport (cf. Ref. 24). In addition, the observed ratio of AE1 mRNA expression in α- versus β-ICCs is likely to be an underestimate for two reasons. First, for technical reasons, the α-ICC population isolated in our experiments is not 100% homogeneous, and therefore not all cells express AE1. Second, as we reported earlier (6, 11), the β-ICC population (isolated as peanut lectin positive cells) includes about 10% α/β hybrid cells. Thus, the expression of AE1 mRNA in “true” β-ICC is probably even lower than the above results would suggest.

The low levels of AE1 mRNA in β-ICC make it unlikely that the apical exchanger of β-ICC is encoded by this gene, unless there is a significant discrepancy between mRNA and protein levels in the two cell types. Such discrepancy might arise if the translation of AE1 mRNA is much more efficient in β-cells than in α-cells and/or the protein is significantly more stable in the apical than in the basolateral membrane. In this case, the low levels of AE1 mRNA found in β-cells might suffice to maintain AE1 protein levels comparable with those seen in α-cells. Our Western blot data, however, refute this possibility, as the pattern of expression of AE1 protein was similar to that observed at the mRNA levels, i.e. sorted α-cells contained significantly more AE1 protein than β-ICCs did.

Another line of indirect evidence arguing against AE1 being the apical exchanger of β-ICCs is our observation that AE1 mRNA and protein levels in CCD cells (containing both α- and β-ICCs) are increased by metabolic acidosis and/or decreased by metabolic alkalosis. If the apical and basolateral exchangers were the product of the same gene, one would not expect to see significant changes in the mRNA levels in acidic versus alkalotic animals, since in acidosis the basolateral exchanger of
α-ICC, which mediates HCO$_3^-$ reabsorption, should be expressed at increased levels, whereas in alkalosis, the apical exchanger of β-ICCs, which mediates the extrusion of HCO$_3^-$ from the cell into the urine, should increase. This, however, is not the case; both in acidosis and in alkalosis, α-ICCs expressed significantly higher (about 10-fold) levels of AE1 mRNA than did β-ICCs.

Our results agree well with those obtained by Da Silva et al. (25) demonstrating that in rats chronic respiratory acidosis results in an increase in the steady-state levels of AE1 mRNA and with preliminary histochemical observations indicating more intensive staining for AE1 in the collecting ducts of acidic than alkalotic rats (26). Our Western blot data demonstrate that the higher mRNA levels observed in acidosis are accompanied by higher AE1 protein levels, suggesting that the increased transcription is important for the physiological regulation occurring in acidosis or alkalosis.

In aggregate, these data are difficult to reconcile with the idea that AE1 functions as the apical anion exchanger of β-ICCs. The reason for the differences between our findings and that of Van Adelsberg et al. (7) is not clear. One likely possibility is that the two studies utilized different experimental systems: we have determined AE1 mRNA levels in freshly isolated ICCs, whereas their study examined AE1 protein in cultured ICCs and a transformed cell line. Since our earlier studies revealed a remarkable plasticity of β-ICCs in culture, which spontaneously differentiate into α-ICC and principal cells (27, 28), it is possible that the cultured cells in Van Adelsberg's study were also inhomogeneous, and contained both β- and α-ICCs, which might be responsible for the expression of AE1.

In summary, the results of this study suggest that HCO$_3^-$ secretion and reabsorption in the CCD are probably mediated by structurally different anion exchangers. Our recent observations that another member of the anion exchanger family, AE2 (2), is expressed at high levels in CCD cells and changes in acid/base balance regulate AE1 and AE2 expression in opposite directions (29) reinforce the notion that multiple anion exchangers participate in the regulation of HCO$_3^-$ transport in these cells.

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