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A spliced variant of AE1 gene encodes a truncated form of Band 3 in heart:

the predominant anion exchanger in ventricular myocytes

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SUMMARY

The anion exchangers (AE) are encoded by a multigenic family that comprises at least three genes, AE1, AE2 and AE3, and numerous splicoforms. Besides regulating intracellular pH (pH_i) via the Cl^-/HCO_3^- exchange, the AEs exert various cellular functions including generation of a senescent antigen, anchorage of the cytoskeleton to the membrane and regulation of metabolism. Most cells express several AE isoforms. Despite the key role of this family of proteins, little is known about the function of specific AE isoforms in any tissue, including the heart. We therefore chose isolated cardiac cells, in which a tight control of pH_i is mandatory for the excitation-contraction coupling process, to thoroughly investigate the expression of the AE genes at both the mRNA and protein levels. RT-PCR revealed the presence of AE1, AE2 and AE3 mRNAs in both neonatal and adult rat cardiomyocytes. AE1 is expressed both as the erythroid form (Band 3 or eAE1) and a novel alternate transcript (nAE1), which was more specifically characterized using a PCR mapping strategy.

Two variants of AE2 (AE2a and AE2c) were found at the mRNA level. Cardiac as well as brain AE3 mRNAs were expressed in both neonatal and adult rat cardiomyocytes. Several AE protein isoforms were found, including a truncated form of AE1 and two AE3s, but there was no evidence of AE2 protein in adult rat cardiomyocytes. In cardiomyocytes transfected with an AE3 oligodeoxynucleotide antisense, AE3 immunoreactivity was dramatically decreased but the activity of the Cl^-/HCO_3^- exchange was unchanged. In contrast, intracellular microinjection of blocking anti-AE1 antibodies inhibited the AE activity. Altogether, our findings suggest that a specific and novel AE1 splicoform (nAE1) mediates the cardiac Cl^-/HCO_3^- exchange. The multiple gene and protein expression within the same cell type suggest numerous functions for this protein family.

Key words: Anion exchanger, Band 3, pH, Cardiomyocyte

INTRODUCTION

Intracellular pH (pH_i) is a major regulator of diverse cellular processes including metabolic pathways, Ca^{2+} homeostasis, cell contractility, cell excitability (Busa and Nuccitelli, 1984), gene expression (Isfort et al., 1993) and cell death (McConkey and Orrenius, 1996; Gottlieb et al., 1996). A tight control of pH_i is essential to maintain the function of a cell that has permanently to face metabolic perturbations or plasmalemmal ion fluxes. Besides proton channels and pumps, three plasma membrane proteins regulate pH_i directly by the transport of either protons or bicarbonate ions. These include the alkalinizing Na^+/H^+ (NHE) and Na^+-dependent Cl^-/HCO_3^- (NBC) exchangers, and the acidifying, Na^+-independent Cl^-/HCO_3^- anion exchanger (AE) (Puceat et al., 1995).

The AEs are ubiquitously distributed in vertebrate tissues. The AE family is encoded by three genes, AE1, AE2 and AE3 (Alper, 1991), located on separate chromosomes (17, 7 and 2, respectively) (Yannoukakos et al., 1994) and encoding three different proteins. AE1 is expressed in spleen and encodes the erythroid Band 3 protein, a major membrane protein that has been studied extensively (Wang, 1994). AE2 is widely expressed in most tissues, at least as detected at the level of mRNA (Wang et al., 1996), while AE3 is expressed strongly in brain (Kopito et al., 1989) and in heart (Linn et al., 1992; Yannoukakos et al., 1994; Puceat et al., 1995). The three isoforms share 65% amino acid sequence homology in the membrane-associated transport domains, diverging to a greater extent in the N-terminal, cytoplasmic portion (Alper, 1991).

Further sequence diversity in the cytoplasmic domain results from alternative splicing of each gene product. Two isoforms of AE1, eAE1 and kAE1, have been cloned, and are the major protein isoforms present in erythrocytes and kidney, respectively. The kAE1 isoform is a truncation of the eAE1 protein starting at Met-79 of eAE1 (Brosius et al., 1989; Kudrycki and Shull, 1993). AE2 transcription can be initiated at three different points, generating alternatively spliced

mRNAs encoding three proteins N-terminally truncated at different points within the first 200 amino acids (Wang et al., 1996). AE3 transcripts comprise two isoforms present at highest concentrations in brain (bAE3) (Kopito et al., 1989) or in cardiac tissue (cAE3), with the 269 N-terminal amino acids of bAE3 being replaced by 73 alternate residues in cAE3.

The most striking characteristic of the AE family is the diversity of functions fulfilled by its members. Indeed, besides pH$_i$ regulation, the AEs modulate chloride and bicarbonate homeostasis in tissues such as stomach and kidney (Alper, 1991). AE1 also contributes to the flexibility of the erythrocyte cytoskeleton and, in turn, to cell shape (Jay, 1996). AE1 regulates glycolysis in erythrocytes by binding and inhibiting aldolase, glyceraldehyde 3-phosphate dehydrogenase and phosphofructokinase (Harrison et al., 1991). Moreover, trout AE1 expressed in *Xenopus* oocyte elicits transport of organic solute, stimulated by cell swelling (Fievet et al., 1998), and AE2 confers to the oocyte an NHE1-mediated cell volume regulation (Jiang et al., 1997). AE1 also generates a senescent antigen, a marker for cell removal by the immune system (Kay, 1981).

The association of some of the AE functions with particular domains in the protein emphasizes the power of alternative splicing in increasing the diversity of AE functions. For example, AE2 and AE3 contain an insertion in the 'Z-loop' (the extracellular peptide loop between membrane spans 5 and 6), which confers activity of a Cl$^-$ current to the AE protein and that is absent from AE1 (Motais et al., 1997). Only the eAE1 protein, however, contains one of the regions in its cytoplasmic N-terminal portion that is necessary for binding ankyrin or aldolase, and this is absent from the kAE1 isoform (Ding et al., 1994; Michaely and Bennett, 1995; Wang et al., 1995). Alternatively, kanadaptin, a recently identified protein, binds to kAE1 but not to eAE1 (Chen et al., 1998). Similarly, it has been hypothesized that the addition of 17 N-terminal residues in AE2a is responsible for AE2 cell sorting in polarized cells (Wang et al., 1996). The expression of a particular isoform would thus confer specific AE properties to the cell that could be altered by switching isoform expression.

In heart and other tissues all three AE gene products have been detected at the mRNA level (Kudrycki et al., 1990). However, detection of mRNA does not necessarily indicate protein translation, and furthermore mRNA may have been derived from more than one cell type. We previously reported the expression of AE1 and cAE3 proteins in cardiac myocytes isolated from adult and neonatal hearts (Korichneva et al., 1995a; Puceat et al., 1995). We also found that purinergic stimulation of cardiomyocytes activates a tyrosine kinase signaling pathway that leads to an acceleration of an AE1-dependent Cl$^-$/HCO$_3^-$ exchange (Puceat et al., 1998). A recent study shows the expression of both mRNA and proteins of two AE2 and two AE3 isoforms in smooth muscle cells (Brosius et al., 1997). Furthermore, changes in the expression of AE isoforms at different stages of cell development (Baltz, 1993) suggest a regulation of AE expression that may result in a substantially altered pH$_i$ behavior at the whole cell level. In light of the current knowledge about the dependence of AE cell function upon the expression and structure of a particular protein isoform, the characterization of the AE members expressed in a single cell type and their functional roles are of major physiological relevance.

In this report, we thoroughly investigated expression of the AE isoforms and splicoforms in ventricular cardiomyocytes isolated from both neonatal and adult rat hearts. Using RT-PCR, we found that eAE1 but not kAE1, AE2 (AE2a, AE2c, but not AE2b) and AE3 (bAE3 and cAE3) are all expressed in cardiac cells at the mRNA level. Western analysis revealed the presence of a truncated form of AE1, and of both bAE3 and cAE3 proteins, while low levels of the AE2 protein were detected only in neonatal myocytes. We used an 'antisense strategy' and a microinjection approach to discriminate between the role of AE3 and AE1; we found evidence that AE1 but not cAE3 is the major Cl$^-$/HCO$_3^-$ exchange in ventricular myocytes, and that the majority of this protein in heart is encoded by a further, novel AE1 mRNA splice variant (nAE1). Our findings led us to question the respective roles of multiple AE isoforms in a single cell type.

MATERIALS AND METHODS

Animals

Wistar rats (200-250 g, 6-7 weeks old) were fed ad libitum and maintained on a 12h:12h light:dark cycle. The animals were maintained and treated according to the European Code of Practice.

Isolation and culture of neonatal cardiac myocytes

Cardiomyocytes were isolated from 2-3 day old Wistar rats as described elsewhere (Iwaki et al., 1990), with modifications. Hearts were removed, dissected free of atria and large vessels and minced in a nominally Ca^{2+}-free, Hepes-buffered balanced salt solution (in mM: NaCl 116, Hepes 20, KCl 5, NaH$_2$PO$_4$ 1, MgSO$_4$ 0.8, glucose 5.6, pH 7.35). The minced tissue was dissociated using collagenase CLS 2 (0.5 mg or 160 U/ml, Worthington, Freehold, NJ, USA) and pancreatin (0.15 mg/ml). Before plating, the cells were layered onto a Percoll density gradient system consisting of two layers of densities 1.056 and 1.08 g/ml. Fibroblasts were carefully removed and cardiomyocytes retrieved from the interface between the two layers. After dilution in Hepes buffer and centrifugation to remove Percoll, the cells were resuspended in plating medium (4:1 DMEM: medium 199, supplemented with 10% horse serum, 5% foetal calf serum, 50 i.u./ml penicillin and 50 mg/ml streptomycin) and cultured for 4 days. A sample of freshly isolated neonatal cardiac myocytes was recovered by centrifugation and RNA or protein extracted immediately.

Adult cardiac myocyte isolation

Cardiomyocytes were isolated from hearts of 200-250 g male Wistar rats as previously described (Puceat et al., 1995). This method routinely yielded 6-10×10^6 rod-shaped cells per heart. Briefly, the hearts were first perfused for 5 minutes at 35°C with a nominally Ca^{2+}-free Hepes-buffered solution containing (in mM): NaCl 117, KCl 5.7, NaHCO$_3$ 4.4, KH$_2$PO$_4$ 1.5, MgCl$_2$ 1.7, Hepes 21, glucose 11, taurine 20, and then for 55 minutes with the same solution containing 1.2 mg/ml collagenase (CLS4, Worthington) and 20 μM CaCl$_2$. The heart was removed from the perfusion apparatus and gently dissociated through a nylon gauze (mesh size 250 μm) and allowed to settle before being washed twice with collagenase-free Hepes buffer. The cells were then incubated for 15 minutes at 37°C. The Ca^{2+} concentration was then increased gradually in steps up to 0.3 mM. The cells were then washed with and resuspended in Hepes solution containing 1 mM Ca^{2+} and 0.25% bovine serum albumin. Dissociations resulting in lower yields were discarded. Approximately 2×10^6 cells were further purified on a Percoll gradient consisting of two layers of density 1.06 and 1.08 g/ml and centrifuged for 10 minutes at 400 ***g*** to remove the remaining contaminating non-myocytes. The resulting myocyte layer was recovered with a Pasteur pipette and diluted in Hepes solution to

allow settling of the myocytes. These were then centrifuged at 400 *g* for 10 seconds to remove extracellular fluid and immediately extracted with 900 µl of guanidinium thiocyanate solution (Chomczynski and Sacchi, 1987).

Other tissue samples (brain, kidney) were taken from pentobarbital-anesthetized rats, rinsed briefly in ice-cooled PBS and freeze-clamped with the blood remaining in situ. Mabin-Darby canine kidney (MDCK) cells were cultured to confluency in DMEM supplemented with 10% horse serum and extracted as for neonatal cardiomyocytes.

Reverse transcription and polymerase chain reaction

Total RNA was extracted and amplified by reverse transcription-polymerase chain reaction (RT-PCR). All PCR primer pairs were chosen to span intron-exon boundaries, to distinguish products amplified from contaminating genomic DNA. The small sizes of many introns in the AE genes resulted in efficient amplification of higher molecular mass cDNAs derived from DNA contaminating the RNA samples (not shown). Therefore all RNA samples were treated with DNAse prior to RT-PCR. The sizes of genomic DNA-derived fragments corresponded in all cases to predicted values from published intron sizes for mouse AE1 or rat AE2 and AE3 genes (Kopito et al., 1987; Linn et al., 1992; Wang et al., 1996). First strand cDNA was synthesized in an Omnigene thermal cycler using one cycle of 10 minutes at 25°C, 15 minutes at 42°C and 5 minutes at 94°C before cooling. The reaction was initiated using 300 ng total RNA and primed using oligodeoxythymidine (dT$_{16}$). This mixture was then used for PCR amplification in a total volume of 25 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 200 mM each of dATP, dCTP, dGTP and dTTP, 0.8 mM sense and antisense oligonucleotide primers, a 1:500 dilution of anti-Taq monoclonal antibody (Clontech, Palo Alto, CA, USA) and 0.625 U AmpliTaq polymerase. Samples were incubated for 29-36 cycles at 94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes, followed by 8 minutes at 72°C before cooling to 25°C. Samples (10 ml) were size-fractionated on 2% agarose gels, containing 0.2 mg/l ethidium bromide, by comparison with a 100 bp DNA ladder (Gibco, Life Technologies).

The amount of PCR product was quantified by scanning photographs of ethidium bromide fluorescent cDNA bands using a CCD camera interpreted by ScionImage 1.7.5 and intensity data obtained from the digitized image. The reliable range of DNA concentrations was monitored by including a range of concentrations of DNA ladder on each gel (with the marker dye omitted).

Primer compositions

Two series of primers were designed. The first series was designed to amplify the described splice variants of AE1, AE2 and AE3. The rat eAE1 sequence was deduced by combining the 5′ end of rat eAE1 sequence up to the beginning of exon 4 (GenBank sequence L02943; Kudrycki and Shull, 1993) with the full-length rat kAE1 sequence from exon 4 onwards (GenBank sequence J04793; Kudrycki and Shull, 1989) and used for PCR primer selection. For eAE1, a sense primer, complementary to nucleotides 8-31 of eAE1, 5′ ACA TGC AGG ACC ACG AGA AAG TGC, and an antisense primer complementary to nucleotides 601-622, 5′ ATG CT GGT GAG GAA GAA GAG G, were designed to amplify a region of 615 bp in length (also called AE1-V). For kAE1, sense −441 to −418, 5′ TGG GGA GAA GAG AGG AGA TAC AGC and antisense primers 270-289, 5′ TGC GTT TGA GTA GCA GAG CC, could generate two possible products, k1 = 420 bp, or k2 = 730 bp. The other isoform-specific primers were: for AE2a, sense 33-52, 5′ CGC AGA TTC TTT GCA CAC GC, antisense 233-254, 5′ AGT GGA TGA TGG ATA TGG TGG G (GenBank sequence J05166; Kudrycki et al., 1990); AE2b, sense −13 to +8, 5′ TGC ACT CCC GCA GGA TGA CTC A, antisense 538-559, 5′ CCA TCT CCT CCA CCA GAG TTC C; AE2c, sense −429 to −408, 5′ GCA GAG AAT AGG AGT TCG CTG G, antisense −172 to −151, 5′ AGC CTC ACT CTC ATC AGT AGG G (GenBank sequences U45886, U45887;

Wang et al., 1996); bAE3, sense 334-357, 5′ AAA GAG AAA ACC TCT GCT CCC CCC, antisense 963-986, 5′ AAC ATC AGT TCA TTC AGC TCC ACG, product 653 bp (GenBank sequence J05167; Kudrycki et al., 1990); and for cAE3, sense −101 to −80, 5′ AGG AAA CCT TAC CAC GTC CAG C, antisense 606-629, 5′ TGG TCA GAT ACA ATC ATG GTC TCC, product 730 bp (GenBank sequence M87060; Linn et al., 1992). The specificity of the PCR primer sets for each AE mRNA isoform was ensured by choosing primers from the unique 5′ ends of the mRNA. The second series of primers was designed for mapping of the AE1 mRNA. These were chosen from the deduced rat eAE1 sequence corresponding to the following nucleotides: AE1-IV, sense 149-172, 5′ AGC AAA CAG CCA CAG ACT ACA TCC, antisense 537-560, 5′ ACT CCC TCC AGA TCC TTT AGG TCC, product 412 bp; AE1-III, sense 530-553, 5′ ACG CTG AGG ACC TAA AGG ATC TGG, antisense 1874-1897, 5′ TTA TCA GGA TGG AGA TGG GGA CCC, product 1368 bp; AE1-II, sense 970-993, 5′ CTG GAT AGC TTT CTG GAC TGT AGC, antisense 1874-1897, 5′ TTA TCA GGA TGG AGA TGG GGA CCC, product 928 bp; AE1-I, sense 1688-1711, ACT ACC CGC TAC AAG AGA GCT ATG, antisense 2160-2183, ATG CCA ACA ACC AGC AGC AAG TCC, product 496 bp; 3′ end AE1 (common to all splice variants) sense 2675-2698, 5′ TCA TCT TCA GAG AGC TAG AAC TCC, representing nucleotides, antisense 3295-3318, 5′ AAG ACA TTA GGG GTA TTT CTG GGC, product 644 bp.

Estimation of mRNAs by RT-PCR

Semiquantitative measurements of mRNA expression for the AE1 mapping experiment were performed in the linear range of PCR amplification in order to maintain differences in cellular mRNA levels throughout the experiment. Initially PCR amplifications were performed for different numbers of cycles, at 2-step intervals for each PCR primer pair, in order to determine the cycle number range over which the percentage of amplification was constant. Next a range of concentrations of total RNA was amplified to determine the point of saturation of the reverse transcription step. In our experiments, RT-PCR product yields were kept under 60-70 ng DNA, to stay within the linear range of measurement (determined using different amounts of fX174 *Hae*III digest, Promega, Madison, WI, USA).

Transfection of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes maintained in culture for 2-3 days were transfected with 250 nM AE3 oligodeoxynucleotide sense (AGG GGA ATG ACA AG) or antisense (CTT GTC ATT CCC CT) corresponding to nucleotides −6 to +7 of rat cAE3 cDNA (Linn et al., 1992) using 3 µg/ml DOSPER (1,3-di-oleoxyloxy-2-(6-carboxy-spermyl)-propylamid, Boehringer, Meylan, France) for 6 hours in serum- and antibiotic-free medium. This protocol allows for 95% of transfection efficiency, as assessed by FACS analysis of cells transfected with fluorescein-conjugated ODN. The high yield of transfected cells favoured the use of neonatal rat cardiac cells in this set of experiments. After washing, the cells were kept in medium added with 5% heat-inactivated fetal calf serum for 36 hours and pH$_i$ was monitored as described above.

Cell fractionation

Neonatal and adult cardiac myocytes were washed in ice-cooled PBS for centrifugation at 1000 *g* for 4 minutes at 4°C (neonatal) or 30 seconds (adult). The pellet was thoroughly resuspended in hypotonic lysis buffer (containing in mM: Tris 10, Na$_4$P$_2$O$_7$ 10, EDTA, 1, MgCl$_2$, 1, NaF, 10, pH 8.0, supplemented with 0.1 mM PMSF). After centifugation, the pellet was resuspended with NET buffer (containing in mM: NaCl 150, EDTA 5, Tris-HCl 50, pH 8.0) supplemented with 1% Nonidet-P40, 50 mM NaF, 0.1 mM PMSF and 10 µg/ml leupeptin, and kept on ice for 15 minutes. Myofilaments were removed by centrifugation at 12000 *g* for 20 minutes at 4°C and the resulting supernatant ('crude membrane fraction') mixed with 4× Laemmli buffer and boiled for 2 minutes prior to western analysis.

Monitoring of pH$_i$ in cardiac cells

Isolated ventricular cells were loaded for 20 minutes at 37°C with 5 μM Snarf-1/AM (Molecular probes, Eugene, OR). The cells were transferred to the stage of an epifluorescence microscope and superfused with a medium containing 117 mM NaCl, 5.7 mM KCl, 1.2 NaH$_2$PO$_4$, 24 mM NaHCO$_3$, 1.7 mM MgCl$_2$, 1.0 mM CaCO$_3$ bubbled with O$_2$/CO$_2$, 95%/5%. In Cl$^-$-free solution containing 19 mM NaHCO$_3$, sodium gluconate, KHCO$_3$ and MgSO$_4$ replaced NaCl, KCl and MgCl$_2$, respectively. The field was illuminated at 514 nm with a Xenon lamp. The images were recorded at 580 and 640 nm using a W-viewer and a CCD camera (Hamamatsu). The ratio of fluorescence intensity of images acquired at each wavelength was calculated on-line by a computer (Argus software, Hamamatsu). An external calibration using nigericin allowed for the calculation of pH$_i$ as described previously (Puceat et al., 1991a). The experiments were performed at 35±1°C. The Cl$^-$/HCO$_3^-$ activity was estimated from the slope of alkalinization calculated within the first 2 minutes following the removal of Cl$^-$ ions from the cell superfusing buffer. The equivalent H$^+$ flux (dpH/dt) was calculated assuming a cell buffering capacity of 35 and 40 mM in neonatal (Korichneva et al., 1995b) and adult (Puceat et al., 1995) rat cardiomyocytes, respectively.

pH$_i$ measurements in transfected HEK 293 cells

HEK 293 cells were cultured in DMEM supplemented with 10% fetal calf serum. Cells plated on laminin-coated coverslips in 35 mm dishes were transfected with 10 μg/ml of DOTAP and 1 μg of plasmids encoding human cAE3 subcloned in pMEP (a pMT3SV2 modified vector, a gift from S. Alper) together with 1 μg GFP plasmid (Green Lantern, Gibco). After 24-48 hours, cells were serum starved for at least 5 hours before experiments. HEK 293 cells were loaded with 5 μM Snarf-1 AM for 20 minutes at 37°C. Transfected cells were selected from the green fluorescence when excited at 488 nm. The AE activity was estimated from the slope of alkalinization that developed when Cl$^-$ ions were removed from the superfusing buffer as described for cardiomyocytes. Intrinsic buffering capacity of transfected cells was estimated as previously described (Puceat et al., 1991b). This capacity was 15.2±0.5 mM/pH unit ($n=7$) and was linearly dependent upon pH$_i$ with a maximal value of 44 mM/pH unit at pH$_i$ of 7.2 and a minimal value of 11 mM/pH unit at pH$_i$ of 7.6 ($n=15$).

Microinjection of neonatal rat cardiomyocytes

Microinjection of neonatal rat cardiac cells was performed according to Shubeita et al. (1992). The pipette concentration of antibodies was 900 μg/ml in KCl 150 mM, EGTA 0.025 mM, Pipes 1 mM, pH 7.2. Two anti-AE1 antibodies raised against residues 605-616 or 831-842 of the rat eAE1 protein (Popov et al., 1997) (RKFKNSTYFPGK or FKPPKYHPDVPF, respectively) were affinity-purified and microinjected together. pH$_i$ was recorded 1 hour after injection.

Western blotting

Electrophoresis and western blotting analysis were performed as described previously (Puceat et al., 1995). The polyclonal (anti-AE1 and anti-AE3) and monoclonal (anti-AE2) antibodies used in the current study were raised against peptide sequences common to all splice variants of each AE isoform, corresponding to: (1) residues 605-616 of the rat eAE1 protein (RKFKNSTYFPGK) (Popov et al., 1997); (2) the 14 C-terminal amino acids of the rat AE2 protein (CEGVDEYNEMPMP) (Zolotarev et al., 1996) and (3) residues 999-1010 of cAE3 (QDRELQALDSED) common to both cAE3 and bAE3.

Materials

The GeneAmp RNA PCR Kit containing MuLV Reverse Transcriptase, AmpliTaq DNA Polymerase and other reagents was purchased from Perkin Elmer. Oligonucleotide primers were purchased from Eurogentec (Belgium) and Life Technologies (France).

RESULTS

Before experiments designed to identify and characterize the protein mediating the Cl$^-$/HCO$_3^-$ exchange in cardiac cells, we used RT-PCR and western blotting to screen the expression of both AE mRNA and proteins in this cell type.

Multiple AE isoforms and spliced products mRNAs and proteins are expressed in isolated rat cardiomyocytes

We used RT-PCR to identify all mRNA isoforms of the AE family that are expressed in cardiomyocytes free of cardiac non-myocytes. To detect alternatively spliced AE mRNAs, specific sense primers were chosen from the unique 5′ region of each isoform, while the antisense primers were located in a
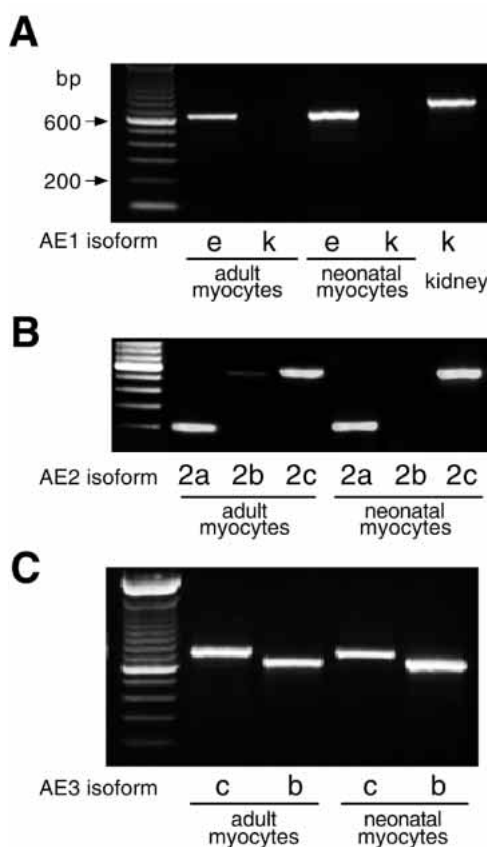

Fig. 1. RT-PCR products of splice isoforms of AE1, AE2 and AE3 in isolated adult and neonatal rat cardiac myocytes. Total RNA (300 ng) from freshly isolated adult or neonatal rat cardiomyocytes was reverse transcribed and subjected to 36 cycles of amplification. (A) Expression of eAE1 and kAE1 in cardiac myocytes and in kidney. Kidney total RNA (300 ng) was also subjected to RT-PCR analysis with a primer set specific for kAE1 for comparison with cardiac myocyte RNA. (B) Expression of AE2a, b and c in adult and neonatal rat cardiomyocytes. Total RNA was reverse transcribed and subjected to PCR with primer sets specific for AE2a, b or c. (C) Expression of cAE3 and bAE3 spliced mRNAs in adult and neonatal cardiomyocytes. Primers were designed to amplify cDNAs from the 5′ regions specific to each mRNA splicoform. The results are representative of those obtained from three tissue samples. The identity of the amplified PCR fragments was checked by digesting the cDNA products with restriction enzymes.

sequence common to all AE1, AE2 or AE3. PCR amplification for a maximum number of cycles revealed that both adult and neonatal rat ventricular myocytes expressed eAE1 but not kAE1. The shorter transcript of kAE1 (420 bp) did not amplify from either kidney or cardiac RNA after 36 cycles (Fig. 1A). mRNA of the three N-terminal AE2 variants recently described by Wang et al. (1996) were detected in adult rat cardiomyocytes with AE2a and AE2c, however, yielding much more RT-PCR product than AE2b. AE2b mRNA could not be amplified from total RNA of neonatal rat cardiomyocytes (Fig. 1B).

RT-PCR products for both bAE3 and cAE3 were generated from the total RNA of cardiomyocytes isolated from both neonatal and adult rat ventricles (Fig. 1C). A lower number of RT-PCR cycles was required to amplify cAE3 relative to bAE3. This was indicative of the relative mRNA levels for each, as the amplification efficiency of the two primer sets was similar. A greater amount of bAE3 product was generated within 30 cycles in neonatal rat cardiomyocytes than in adult myocytes (Fig. 1C).

To determine whether mRNA was effectively translated into the corresponding protein, we analyzed, by western blotting, proteins in membrane prepared from freshly isolated adult and cultured neonatal rat cardiac myocytes. An antibody directed against a peptide corresponding to the conserved amino acids 605-616 of the rat eAE1 protein specifically recognized a protein migrating with an apparent molecular mass of 80 kDa in membrane fractions prepared from both freshly isolated rat adult and cultured neonatal cardiac myocytes (Fig. 2A), but failed to detect immunoreactivity at 90 kDa (kAE1) or 100 kDa (eAE1) detectable in kidney cells (Fig. 1A) or erythrocyte membrane fractions, respectively.

An antibody raised against the 14 C-terminal amino acids of the rat AE2 protein revealed a broad band centered around 170 kDa in MDCK (kidney cell line) whole-cell lysate (Fig. 2B, lane 3) but failed to show immunoreactivity with 200 µg protein from adult rat myocyte membrane (Fig. 2B, lane 1). A low level of immunoreactivity was, however, detectable in cultured neonatal rat cardiac myocytes at the same apparent molecular mass. Two further bands were detected in MDCK cells, a broad one around 120 kDa and a more intense band at

115 kDa. A band migrating at 115 kDa was also present in neonatal myocytes. This latter requires further examination but may represent the product of AE2c variant detected by PCR. The antibody also showed a minor cross-reactivity with a Band 3-related protein (kAE1) in MDCK cells (at 80-90 kDa), as might be expected from the 71% amino acid homology of the AE2 immunogenic peptide with the AE1 C terminus. While the RT-PCR products became visible in fewer than 30 amplification cycles, the AE2 proteins were of low abundance in neonatal rat myocytes and not detected at all in adult rat cardiac cells by western blot analysis.

An antibody raised against a peptide sequence from the C-terminal domain common to both AE3 splice isoforms (residues 999-1010 of cAE3) recognized a 120 kDa protein in both adult and neonatal rat myocytes, as detected previously (Puceat et al., 1995; Yannoukakos et al., 1994); it was accompanied by immunoreactivity at 150-160 kDa in neonatal rat cardiomyocytes corresponding to bAE3 as compared with the 160 kDa protein detected in brain sample (Fig. 2C).

Cardiac myocytes express a novel isoform of AE1

In a former study, we reported that antibodies reacting with the N-terminal, 42 kDa cytosolic domain of eAE1 from human erythrocytes failed to react with the 80 kDa AE1 protein (Puceat et al., 1995). This suggested that in heart, the AE1 protein migrating with an apparent molecular mass of 80 kDa (Fig. 2A) might differ in its N-terminal sequence. To determine the splicing region in the eAE1 gene from which a new splicoform (nAE1) could be generated, we designed PCR primers chosen from the eAE1 sequence to cover regions just 3′ to possible splicing sites, assumed to be the exon-exon boundaries around the larger introns (indicated by arrowheads in Fig. 3A). Exon-exon boundary sequences were taken from those of the mouse AE1 gene, which are nearly identical to those of the human sequence (Sahr et al., 1994). AE1-V, -IV, -III, -II, -I and 3′-end AE1 primer sets included nucleotides 8-622, 149-560, 530-1897, 970-1897, 1688-2183 and 2675-3318, respectively, of the eAE1 coding sequence (Kudrycki and Shull, 1989, 1993).To allow for differences in the total amount of AE1 mRNA (i.e. the sum of all AE1 transcripts) between different tissues, RNAs were diluted prior to RT to

Fig. 2. Expression of AE1, AE2 and AE3 proteins in isolated adult and cultured neonatal rat cardiac myocytes. (A) Anti-AE1 western blot analysis of 200 µg of membrane protein from freshly isolated adult (lane 2), neonatal rat cardiomyocytes cultured for 4 days (lane 3) or freshly isolated adult cells blotted with the anti-AE1 antibody in the presence of 10 µg/ml of the immunizing peptide (lane 1) or from MDCK (right panel). (B) Anti-AE2 western blot analysis of 200 µg membrane protein prepared from whole hearts of adult rats (lane 1), neonatal rat cardiomyocytes cultured for 4 days (lane 2), freshly isolated adult cardiomyocytes (lane 3) or 100 µg protein from MDCK cells (lane 4). (C) Anti-AE3 western blot analysis of 200 µg membrane protein from freshly isolated rat adult cardiomyocytes (lane 1), from neonatal rat cardiomyocytes cultured for 4 days (lane 2), or from brain (right panel). Immunoreactivity was revealed using an ECL detection. No immunoreactivity was observed when using the preimmune sera. Molecular masses were estimated from the positions of prestained standards. Each blot is representative of at least three experiments.
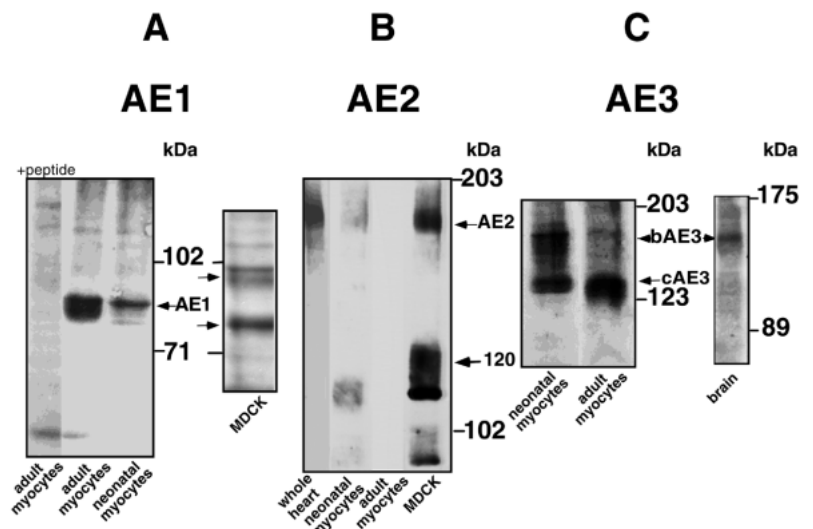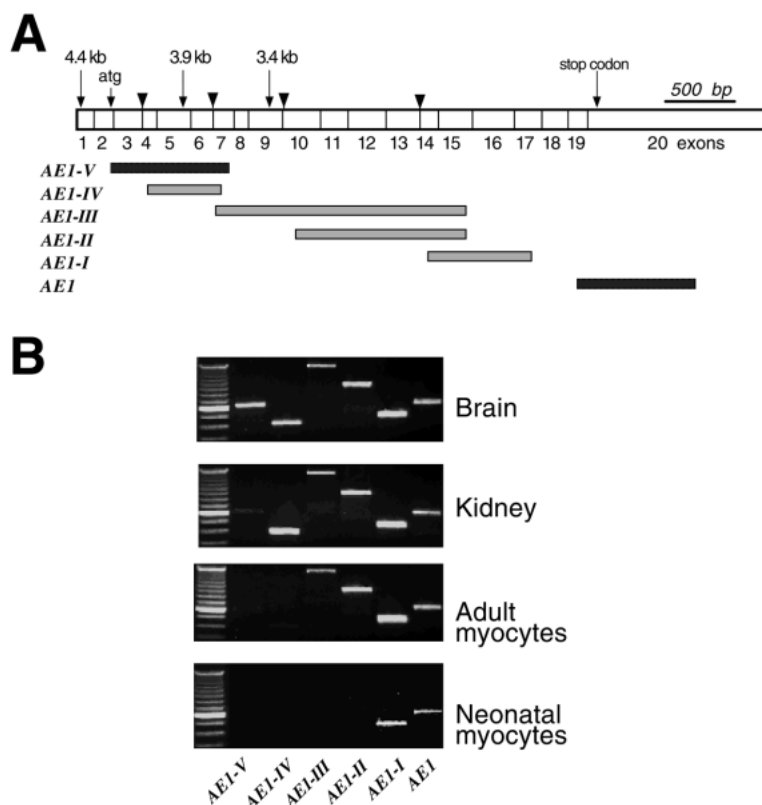
Fig. 3. Mapping of cardiac AE1 mRNA by RT-PCR.
(A) Cartoon depicting the positions of AE1 mapping primers relative to exon-exon boundaries in eAE1 mRNA sequence (conserved between species; Sahr et al., 1994). Arrowheads show the positions of some of the larger introns (introns 3, 6, 9 and 13). Sizes of the RT-PCR products are drawn to scale. Numbers (in kb) above the sequence are aligned with the assumed start positions of their respective mRNAs.
(B) Relative levels of AE1-V, -IV, -III, -II, -I and 3′-end AE1 RT-PCR products in brain, kidney, adult rat and 4 day-cultured neonatal rat cardiomyocyte RNA. Total RNA from brain (30 ng), kidney (6 ng), freshly isolated adult rat cardiac myocytes (300 ng) and neonatal rat cardiomyocytes cultured for 4 days (300 ng) was reverse transcribed and amplified for 32, 30, 33, 33, 32 or 32 cycles with AE1-V, -IV, -III, -II, -I or 3′-end AE1 primers, respectively. The cDNA products were size-fractionated by agarose electrophoresis and the band intensities recorded with a CCD camera interpreted by Scion Image 1.7.5. Note that the use of 32 amplification cycles was responsible for the lower product yield here of eAE1 (primer set AE1V) in cardiac cells in this figure compared to Fig. 1 (36 cycles). The gels are representative of 3 similar experiments for each primer set.

obtain equivalent amounts of AE1 mRNA, as indicated by the yield of the most 3′ RT-PCR product. To maintain differences in the amounts of mRNA sequence between tissues the number of PCR amplification cycles was limited, to ensure that amplification was in the log-linear range (32, 30, 33, 33, 32 and 32 cycles, for AE1-V to 3′ end sets, respectively). Brain was assumed to contain a majority of the eAE1 spliced variant, as in northern blots only a single mRNA band binds a cDNA probe from the conserved 3′ region (Kudrycki et al., 1990).

Fig. 3B illustrates the amounts of RT-PCR product from each primer set, from the 5′-end primer set AE1-V to the common 3′-end primer set AE1, in control (brain) and tested tissues (kidney, adult myocytes, neonatal myocytes). In kidney RNA, the ratios of the amounts of product from AE1-IV, -III, -II and -I primers to 3′-end AE1 were very similar to those obtained from brain RNA, while the ratio of AE1-V to AE1 was considerably lower ($10\pm2\%$, $n=3$). In adult myocytes, the ratios of AE1-III, -II and -I to AE1 were similar to those from brain RNA, while both AE1-V and AE1-IV product ratios were $3\pm1\%$ ($n=3$) of those obtained from brain RNA. Furthermore, only AE1-I and 3′-end AE1 products could be amplified from cultured rat neonatal myocyte RNA (Fig. 3B).

AE1 mediates the Cl⁻/HCO₃⁻ exchange activity in cardiomyocytes

Two strategies were designed to identify the AE isoform that mediates the Cl^-/HCO_3^- exchanger in cardiac cells (Pucéat et al., 1998). First, neonatal rat cardiomyocytes were transfected with an antisense oligodeoxynucleotide (ODNAS) to prevent AE3 expression. Observation in confocal microscopy of cells transfected with a fluorescein-conjugated AE3-ODNAS 12 hours after transfection revealed a patchy cytosolic

fluorescence likely to be endosomal vesicles, a strong perinuclear staining but a weak nuclear distribution of the ODN (Fig. 4A). 36 hours after transfection, Fig. 4B shows that most if not all cells took up the ODN and that the latter was homogeneously distributed within the cells. Fig. 4C illustrates the results obtained in cells transfected with the AE3-ODNAS compared to those obtained in AE3-ODNS transfected cells. Neither the ODNS nor the ODNAS affected the Cl^-/HCO_3^- exchange activity while the ODNAS specifically decreased the level of the 120 kDa cardiac AE3 protein by $70\pm9\%$ ($n=4$). AE1 expression was not affected. It could be argued that both the cAE3 and bAE3 proteins still expressed in ODNAS-transfected cells are sufficient to exchange Cl^- for HCO_3^- ions. Thus, an alternative approach targeted at the AE1 protein was used. The truncated cardiac AE1 is not cloned. The translation initiation site of this gene is thus not known and the antisense knock out of AE1 expression is thus rather difficult to design. We decided instead to microinject neonatal rat cardiomyocytes with two specific anti-AE1 antibodies, raised against the third and sixth cytoplasmic loops (Popov et al., 1997; Tang et al., 1998) of rodent eAE1 (Pucéat et al., 1998), regions close to domains presumed to be the pore of the transporter (Muller-Berger et al., 1995; Popov et al., 1997; Tang, 1998). Microinjection into neonatal rat cardiomyocytes of a mixture of the two purified antibodies that specifically recognize the 80 kDa cardiac AE1 (Fig. 2A) dramatically decreased the rate of anion exchange by $79\pm9\%$ ($n=40$, Fig. 4D). The AE activity was unaffected in cells injected with purified rabbit IgG (5.85 ± 0.12 H⁺ equivalents/minute, $n=14$) or the same antibody mixture preincubated with the immunizing peptides ($n=19$, Fig. 4D).

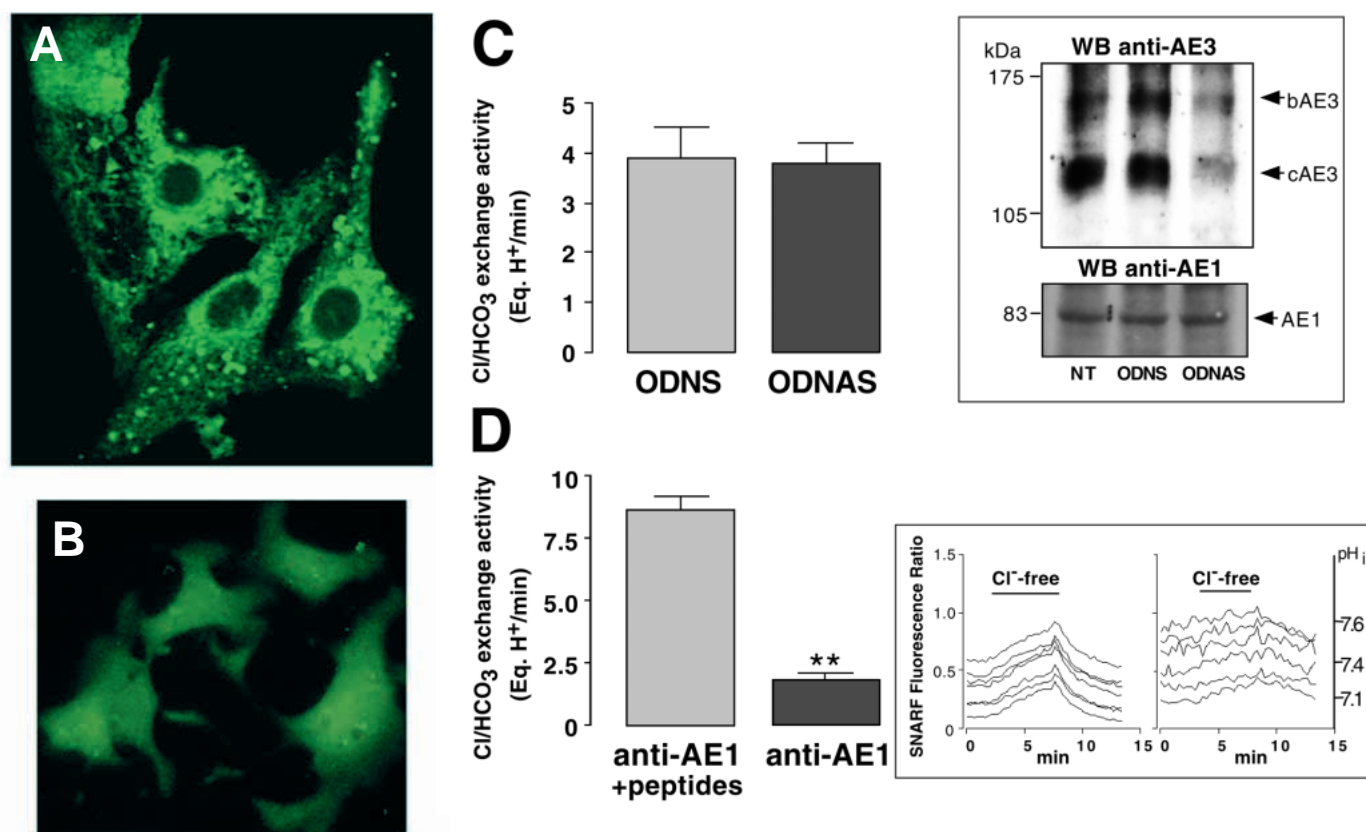HEK 293 cells had been shown to lack AE activity. We

Fig. 4. AE1 mediates the Cl^-/HCO_3^- exchange in neonatal rat cardiomyocytes. (A) Confocal microscopy of cells transfected with a fluorescein-conjugated AE3 ODNAS and observed 12 hours after transfection. The picture shows an optical section (0.5 μm) selected at the middle of the cells. (B) Fluorescein-conjugated ODN transfected cells observed 36 hours after transfection. (C) Cells were then loaded with Snarf1 to measure pH$_i$. The Cl^-/HCO_3^- exchange activity was measured from the alkalinizing slope that developed when Cl^- ions were removed from the external solution. The results illustrated by the bar graph are expressed as the mean ±s.e.m. of ODNS (n=22) and ODNAS (n=35) transfected cells. Basal pHi was 7.29±0.03 in ODNS-transfected cells and 7.28±0.04 in ODNAS-transfected cells. The inset on the right show a typical anti-AE3 western blot; 200 μg of whole cell lysate protein prepared from non-transfected (NT), ODNS- or ODNAS-transfected cells were subjected to SDS-PAGE and analysed in western blots using an anti-AE3 antibody. (D) Neonatal rat cardiomyocytes were microinjected with anti-AE1 antibodies (n=40) and the Cl^-/HCO_3^- exchange activity was measured as described above. Antibodies preincubated with 10 μg of immunizing peptides served as controls (n=19).The inset on the right show typical recordings of anion exchange activity measured in cells microinjected with anti-AE1 antibodies preincubated in the presence of the immunizing peptides (left panel) or in the absence of the immunizing peptides (right panel). The recordings illustrate measurements performed on one microscope field. Three microscope fields were analysed in each experimental condition and the experiments were carried out in two separate cultures.

therefore chose this cell line to co-express cAE3 and GFP. In contrast to Lee et al (1991) and Ruetz et al. (1993) or Tang et al. (1998), however, we observed a large endogenous anion exchange activity in non-transfected and GFP-transfected cells (3.75±0.34 H$^+$ equivalents/minute; n=36) maintained in serum containing medium or serum starved for 5-24 hours before pH$_i$ measurements were taken. cAE3-transfected cells did not exhibit a significantly different anion exchanger activity (4.35±0.48 H$^+$ equivalents/minute; n=27).

DISCUSSION

In the present study, we first report that all AE genes (AE1, AE2 and AE3) are expressed and that at least three AE mRNAs are translated into proteins in a single cell type, namely cardiac myocytes isolated from neonatal and adult rat hearts (Table 1), devoid of non-muscular cells. This argues against the tissue specificity of AE isoforms, a hypothesis that has been so far raised in the light of mRNA expression detected by northern blotting. RT-PCR is a more sensitive approach than northern blot analysis. Although our findings concerning the expression of AE1 and AE3 are in agreement with those of Kudrycki et al. (1990) obtained by northern blotting of RNA extracted from whole heart, we have further shown that the isolated ventricular

Table 1. Expression of AE mRNA and proteins in isolated rat cardiomyocytes

Rat	Neonate		Adult	
AE isoforms	mRNA	protein	mRNA	protein
AE1	eAE1, nAE1	nAE1	eAE1, nAE1	nAE1
AE2	AE2a,c	AE2*	AE2a,b,c	Not detected
AE3	cAE3,bAE3	cAE3,bAE3	cAE3,bAE3	cAE3

*Low immunoreactivity.

myocyte expresses AE1, AE2 and AE3 genes, and have found that several spliced variants considered to be relatively tissue-specific are expressed in this cell type.

Kudrycki et al. (1990) observed that expression of specific AE mRNA was restricted to some tissues. Similarly from northern blot studies, bAE3 and cAE3 were considered as a neuronal and cardiac-specific isoform, respectively (Kopito et al., 1989; Yannoukakos et al., 1994). Our data lead us to clarify this point. We have detected an AE3 protein migrating with an apparent molecular mass of 160 kDa that does not react with an antibody directed against the N-terminal sequence of the rat AE3c protein (Puceat et al, 1995). This provides convincing evidence for the expression of the 'brain' isoform in the heart. Interestingly, we have also observed expression of the 'cardiac' protein isoform of AE3 in microsomes prepared from whole brain, as well as its RT-PCR product (results not shown). Our findings rule out the hypothesis as to the tissue-specific expression of these isoforms. The level of AE3b RT-PCR product in neonatal rat cardiac myocytes was higher than in adult cells relative to AE3c, as observed by Yannoukakos and colleagues (1994), suggesting that differential regulation of expression of the splice variants is possible. This is consistent with a specific role for AE3b in neonatal myocytes that may be altered or lost in adult heart.

Expression of AE2 mRNA was reported in whole heart by Wang et al. (1996), and the appearance of a single band by northern analysis in that report suggested that the predominant spliced isoform was AE2a. In the current study we have demonstrated that AE2 mRNA is expressed in pure cardiac myocytes. While the RT-PCR product became visible in less than 30 cycles, mRNA expression may not necessarily be accompanied by protein expression. Western analysis with a specific monoclonal anti-AE2 antibody was able to detect a protein band of 170 kDa and another at 115 kDa in MDCK (kidney) cells, in membrane prepared from whole rat heart, but at much lower levels in neonatal myocytes. The strong AE2 immunoreactivity observed in membrane prepared from whole heart contrasts with the lack of AE2 immunoreactivity in membrane prepared from isolated ventricular myocytes. This thus reveals that AE2 expression is restricted to non-cardiomyocytes. Altogether, our data indicate that, in the cardiomyocyte, AE2 is regulated at the translational level and during cell development. The widespread tissue distribution of AE2 mRNA (Wang et al., 1996) might not be accompanied by a similarly widespread translation into proteins.

We confirmed the presence of an AE1-related protein in isolated adult and neonatal myocytes with an apparent molecular mass of 80 kDa, while AE1e (Band 3) migrates at 100 kDa (Puceat et al., 1995). We have previously reported that antibodies reacting with the N-terminal, 42 kDa cytosolic domain of the AEe (Band 3) protein from erythrocytes failed to react with the AE1 protein product (Puceat et al., 1995). This suggested that the AE1 peptide in heart is not the erythroid form and might be transcribed from one of the two smaller mRNA species (i.e. 4.1 and 3.6 kb) detected in heart (Kudrycki et al., 1990). The AE1 in heart (nAE1) has a molecular mass close to the one of the truncated kidney AE1 (Brosius et al., 1989). Using primers that amplified AE1k from kidney mRNA, however, we could not detect AE1k mRNA in cardiac cells. The forward primer specific for AE1k was chosen from the extreme 5′ end of the mRNA in order to amplify both the

AE1k1 and AE1k2 mRNAs (Kudrycki and Shull, 1993). Although we failed to amplify the shorter AE1k1 transcript in kidney because of its low abundance in rat (Kudrycki and Shull, 1993), it seems unlikely that AE1 protein in the heart was translated from AE1k mRNA.

To further characterize the AE1 splicoform(s) expressed in cardiac myocytes, we designed a PCR mapping strategy using primer sets that amplify different regions of the AE1e mRNA (Fig. 3A). Since all the alternate splicing events so far described for AE genes result in differences in the 5′ end of the mRNAs (Kudrycki and Shull, 1993; Linn et al., 1992; Wang et al., 1996), and since Kudrycki (1990) identified other AE1-related mRNAs by northern analysis using a 3′ probe, we assume that the 3′ end of all the AE1-derived products was conserved. The yields of AE1-I products were the same in all four tissues that we screened (Fig. 3B), indicating that the 3′ region coded by exons 13-20 of the AE1 gene are conserved in all the AE1 splice variants. In contrast, the amount of product from the AE1-V primer set was greatly reduced in kidney RNA compared to brain (assumed to be all eAE1), as expected from the absence of exons 1 and 2 from kAE1. The amount of AE1-IV product was lower in adult myocyte mRNA, indicating that the bulk of the AE1 mRNA lacked some part of the sequence encoded by exons 4-6. The bulk of the AE1 mRNA in cultured neonatal myocytes appears to comprise a mRNA differing further downstream, missing AE1III and -II regions (coded by exons 7-14 and 10-14, respectively). Similar results were obtained using mRNA instead of total RNA, or using random hexamer priming of the RT step (data not shown). Altogether, our findings suggest the existence in adult and neonatal myocytes of AE1 mRNAs that are spliced in one of introns 4-6 and 10-13, respectively, and not in intron 3 as for kAE1. This further supports the existence of new AE1 transcripts in cardiac cells. This finding is of great physiological significance because in one of our previous reports (Puceat et al., 1995), we suggested that an AE1-related protein may mediate the Cl$^-$/HCO$_3^-$ exchange in cardiac cells. In the current study, we used 'an antisense stategy' and a microinjection approach to discriminate between AE3s and AE1 as Cl$^-$/HCO$_3^-$ exchangers. While AE3 expression was significantly decreased in antisense transfected cells, we failed to detect any change in the rate of Cl$^-$/HCO$_3^-$ exchange. Imaging the cells transfected with a fluorescein-conjugated AE3 antisense revealed that most of cells were efficiently transfected, ruling out the possibility of recording pH$_i$ in a non-transfected cell. Although the antisense transfection was primarily designed to knock out cAE3 expression, the 160 kDa bAE3 protein was also significantly decreased (by 59±6%, n=4; Puceat et al., 1998). This latter effect is likely to be due to the binding of the ODNAS to the sequences nt 306-332 and nt 1320-1326 of bAE3 cDNA (Kudrycki et al., 1990), which share 66% homology with the initiation translation site of cAE3, and to the low level of bAE3 protein in isolated rat ventricular cardiomyocytes (Fig. 2C). Anyhow, AE1 expression was not affected and so this approach turned out to be successful for specifically looking at the participation of AE3s proteins in the Cl$^-$/HCO$_3^-$ exchange. In oocytes injected with cRNA encoding cAE3 or bAE3, the Cl$^-$/HCO$_3^-$ exchange activity was just twice as active as in water-injected oocytes (Yannoukakos et al., 1994), while under the same experimental conditions, a 10- to 20-fold increase in Cl$^-$/HCO$_3^-$ exchange

activity in oocytes expressing AE1 or AE2 was observed (Humphreys et al., 1994). Lee et al. (1991) also observed a much lower exchange activity in HEK 293 cells expressing bAE3 than in cells expressing AE2. Altogether, AE3 seems to be a poor candidate for performing, at least as a major function, the exchange of Cl$^-$ for HCO$_3^-$ ions in cardiac cells.

We did not find any AE2 protein in adult rat cardiomyocytes and only a very low expression of AE2s in neonatal rat cardiomyocytes (see Fig. 2). Furthermore, the poor DIDS sensitivity of AE2 protein (Lindsey et al., 1990) (EC$_{50}$, 150 μM; Lee et al., 1991; 19 μM; Humphreys et al., 1994) makes it an unlikely candidate for the cardiac Cl$^-$/HCO$_3^-$ exchange, which is fully inhibited by low concentrations of DIDS (EC$_{50}$: 3 μM; Puceat et al., 1995; Korichneva et al., 1995a).

Microinjection of anti-AE1 antibodies directed against domains of the protein close to the region presumed to be the ionic pore (Muller-Berger et al., 1995; Popov et al., 1997; Tang et al., 1998a) dramatically decreased the rate of transport of the Cl$^-$ and HCO$_3^-$ anions. This effect is specific since the same experiments carried out with rabbit IgG, or the same antibodies preincubated with the immunizing peptides, did not affect the anion exchange activity. The microinjection approach brings a strong argument in favour of the AE1 as a major Cl$^-$/HCO$_3^-$ exchanger, at least in neonatal rat cardiac cells. Despite its limited tissue distribution (i.e. erythrocytes, kidney cells and cardiac cells) it should be noted that AE1 turns out in each case to be the major Cl$^-$/HCO$_3^-$ exchanger in these cells.

We thus found three AE protein isoforms in adult (i.e. 'novel' nAE1, cAE3 and bAE3) and as many as five in neonatal cardiac cells (i.e. 'novel' nAE1, AE2a/b, cAE3 and bAE3). This multiple expression raises a question of basic cell physiology as to the physiological and functional significance of expression of numerous proteins belonging to the same family. So far, one can only speculate about these functions. It is likely that not all AE proteins exhibit an anion exchange activity. Using an approach of knock out by oligonucleotide antisense and of intracellular microinjection of blocking antibodies, we obtained evidence that 'cardiac' AE1 (nAE1) but not cAE3 performs the function of Cl$^-$/HCO$_3^-$ exchanger. It is reasonable to believe that some of these AEs could regulate the pH of intracellular compartments, as already suggested in some cells (Kopito, 1990). The availability of pH-sensitive green fluorescent protein targeted to the intracellular compartments (Llopis et al., 1998) should allow us to examine this possibility. The non-pH-regulatory functions of this protein family are undoubtedly as important as the anion exchange one and should thus be investigated in cardiac cells. Finally, a differential regulation of these genes during cell development or under neurohormonal control, as suggested from the presence of gene-specific promoter regulatory sites (e.g. AP1 and AP2) in AE1 (Kudrycki and Shull, 1989) but not in AE2 or AE3 (Wang et al., 1996; Linn et al., 1992), could also explain the presence of multiple isoforms in a same cell type. Together with other approaches such as a cDNA antisense approach to fully knock out AE expression, we are in the process of investigating such a differential gene regulation in order to better understand the roles of individual AE isoforms expressed in cardiac cells.

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