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## Hyperactivity and altered mRNA isoform expression of the $\text{Cl}^-/\text{HCO}_3^-$ anion-exchanger in the hypertrophied myocardium

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### Abstract

**Objective:** The aim was to examine the regulation of the cardiac  $\text{Na}^+$ -independent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (AE) mRNA isoform expression in association to the enhanced AE activity in the hypertrophied myocardium of spontaneously hypertensive rats (SHR). **Methods:** AE activity was determined by the initial rates of the  $\text{pH}_i$  recovery from imposed intracellular alkalinization (forward mode of exchange) and the  $\text{pH}_i$  rise induced by  $\text{Cl}^-$  removal (reverse mode). Net  $\text{HCO}_3^-$  ( $J_{\text{HCO}_3^-}$ ) efflux and influx were respectively determined. AE mRNA isoforms were analyzed by Northern blot with specific probes to detect AE1, AE2 and AE3 mRNAs. **Results:** Initial  $J_{\text{HCO}_3^-}$  efflux after imposed alkaline load ( $\text{pH}_i \cong 7.5$ ) was higher in SHR than in normotensive WKY rats ( $3.01 \pm 0.33$ ,  $n=7$ , vs.  $0.64 \pm 0.29$  mM/min,  $n=5$ ,  $P<0.05$ ).  $J_{\text{HCO}_3^-}$  influx induced by  $\text{Cl}^-$  deprivation was also increased in SHR,  $4.24 \pm 0.56$  mM/min ( $n=10$ ) versus  $2.31 \pm 0.26$  ( $n=10$ ,  $P<0.05$ ) in WKY. In arbitrary units, the 4.1-kb AE1 mRNA decreased in SHR ( $0.15 \pm 0.01$ ,  $n=7$ ) compared to WKY ( $0.29 \pm 0.06$ ,  $n=7$ ,  $P<0.05$ ), whereas the 3.6-kb mRNA did not change. AE2 mRNAs were similarly expressed in WKY and SHR. Cardiac specific AE3 (cAE3) mRNA decreased in SHR,  $1.10 \pm 0.16$  arbitrary units ( $n=8$ ) versus  $1.79 \pm 0.24$ , ( $n=8$ ,  $P<0.05$ ) in WKY. Full length AE3 (flAE3) mRNA increased from  $0.69 \pm 0.06$  (WKY,  $n=8$ ) to  $1.25 \pm 0.19$  arbitrary units in SHR ( $n=8$ ,  $P<0.05$ ). **Conclusions:** The increase in flAE3 mRNA expression in cardiac tissue from the SHR is an adaptive change of the hypertrophied myocardium that might be in connection with the increased activity of the AE. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Gene expression; Hypertension; Hypertrophy; Ion exchangers; Membrane transport

### 1. Introduction

Intracellular pH ( $\text{pH}_i$ ) plays a central role in the regulation of different cellular events including  $\text{Ca}^{2+}$  homeostasis [1,2], metabolism [3] ionic conductance [4,5], myofilament sensitivity to  $\text{Ca}^{2+}$  [6], gene expression [7] and cell death [8,9]. Therefore, the control of  $\text{pH}_i$  is essential to maintain the normal function of the cell, which is permanently exposed to metabolic changes or ion fluxes across the plasmalemma. There are at least three plasma membrane proteins that control  $\text{pH}_i$  by either proton or bicarbonate transport. These are the alkalinizing  $\text{Na}^+/\text{H}^+$

exchanger (NHE) [10] and  $\text{Na}^+/\text{HCO}_3^-$  cotransport (NBC) [11,12], and the acidifying  $\text{Na}^+$ -independent  $\text{Cl}^-/\text{HCO}_3^-$  anion-exchanger (AE) [13]. A novel chloride-dependent acid loader ( $\text{Cl}^-/\text{OH}^-$  exchanger) has also been described in the guinea-pig ventricular myocyte [14].

We have previously reported enhanced AE and NHE activities in the hypertrophied myocardium of spontaneously hypertensive rats (SHR) [15]. Moreover, regression of cardiac hypertrophy after pretreatment with enalapril normalized the activities of both ion-exchangers [16]. The hyperactivity of the AE has been also shown in erythrocytes from hypertensive patients [17]. The AE family comprised of three genes, AE1, AE2 and AE3 [3], is ubiquitously distributed in vertebrate tissues. The AE proteins share about 65% amino acid sequence homology

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in the membrane-associated transport domains but are more diverging in the N-terminal cytoplasmic domain [13]. Further sequence diversity in the cytoplasmic domain results from alternative splicing of each gene product [18–20]. Two isoforms of AE1, eAE1 and kAE1, have been cloned, and are the major protein isoforms present in erythrocytes and kidney, respectively [18,19]. The AE2 gene contains three distinct promoters, which leads to the production of three N-terminal variants of AE2 that exhibit tissue-specific pattern expression [20]. In heart all three AE gene products have been detected at the level of mRNA [20–24] although function of individual protein products is still under current investigation. The AE3 is the most abundant isoform in cardiac tissue. Two different AE3 variants are co-expressed in cardiac tissue: full-length AE3 (fAE3), which was found as the major anion-exchanger in brain and retina, and cardiac AE3 (cAE3) that encodes a shorter AE3 protein including a unique 73 amino acid domain at the N-terminus [22–25]. The purpose of the present study is to examine the regulation of the cardiac AE mRNA isoform expression in association to the enhanced AE activity in the hypertrophied myocardium of SHR.

## 2. Methods

### 2.1. Animals

Experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (US Department of Health and Human Services) with age-matched spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats, which were originally derived from Charles River Breeding Farms, Wilmington, MA. All animals were identically housed under controlled lighting and temperature with free access to standard rat chow and tap water. Beginning at 12 weeks of age, systolic blood pressure (SBP) was measured weekly by the standard tail-cuff method [26]. On the day of the experiments, the animals were deeply anesthetized with ether and their hearts were removed. Atria and all adjacent connective tissue were removed and the remaining tissue blotted and weighed to determine heart weight (HW). The ratio between HW and body weight (BW) was used to determine the degree of hypertrophy.

### 2.2. AE activity assay

From each heart, a papillary muscle was dissected free and mounted, as previously described [15], in an organ bath on the stage of an inverted microscope (Olympus CK2). Muscles were superfused with the following  $\text{HCO}_3^-/\text{CO}_2$ -buffered solution (in mM): 128.3 NaCl; 4.5 KCl; 1.35  $\text{CaCl}_2$ ; 20.23  $\text{NaHCO}_3$ ; 0.35  $\text{NaH}_2\text{PO}_4$ ; 1.05  $\text{MgSO}_4$ ; 11 glucose. The solution was equilibrated with

$\text{CO}_2/\text{O}_2$  gas mixture to assure a  $\text{pCO}_2$  value of 35 mmHg at the chamber level, giving a  $\text{pH}_o$  of  $\text{CO}_2/\text{HCO}_3^-$ -buffered solutions  $7.37 \pm 0.01$  at  $30^\circ\text{C}$ .

The papillary muscles were loaded with the acetoxymethyl ester form of 2'-7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM, Molecular Probes, Eugene, OR) to determine  $\text{pH}_i$  as previously described [15,16]. Briefly, BCECF fluorescence was excited at 450 and 495 nm and the fluorescence emission monitored after passage through a  $535 \pm 5$  nm filter. The  $\text{pH}_i$  was calculated from the ratio of fluorescence intensities at 495 and 450 nm after the subtraction of background fluorescence from each reading. A calibration curve was constructed at the end of each experiment with the high  $\text{K}^+$ -nigericin method [27]. The  $\text{pH}_i$  calibration solution contained (mM) 140.0 KCl; 1.0  $\text{MgCl}_2$ ; 1.0  $\text{CaCl}_2$ ; 5.0 HEPES; 0.01 nigericin; 4.0 sodium cyanide and 20.0 2,3-butanedionemonoxime; with pH value adjusted to four different values ranging from 7.5 to 6.5.

AE activity was examined operating as an alkali extruder (forward mode) and also during the reverse mode of operation. In the forward mode, the general principle of the technique is to induce an intracellular alkaline load with a weak base like trimethylamine (TMA) which results in a rapid increase in  $\text{pH}_i$  due to the influx of uncharged TMA, and the subsequent combination of most of these molecules with intracellular  $\text{H}^+$ . It has been previously demonstrated that while there is a spontaneous return of  $\text{pH}_i$  towards the baseline value in the presence of  $\text{HCO}_3^-$ -buffered medium, no significant  $\text{pH}_i$  recovery from TMA-induced alkalosis is detected in  $\text{HCO}_3^-$ -free solutions [28,29] making the technique a valid tool to study the AE activity. Thus, the  $\text{pH}_i$  values recorded during the first minute after the peak intracellular alkalosis of the TMA-pulses were fitted to a straight line to determine the initial rate ( $\text{dpH}_i/\text{dt}$ ) of  $\text{pH}_i$  changes [28,30]. In order to induce different degrees of intracellular alkalosis different TMA concentrations (10, 20 and 30 mM trimethylamine hydrochloride, Sigma Chemical Co.) were used. One, two or three TMA concentrations were tested on each muscle with a total of 11 alkali-loads performed on five separate muscles. To induce the reversal of the AE activity, the superfusing solution was switched to a  $\text{Cl}^-$  free solution with Na gluconate used as a substitute [31]. The removal of extracellular  $\text{Cl}^-$  induces the efflux of intracellular  $\text{Cl}^-$  coupled to an inward movement of  $\text{HCO}_3^-$  (reverse mode) that causes a rise in  $\text{pH}_i$ . The  $\text{pH}_i$  values recorded during the first minute after  $\text{Cl}^-$  removal were fitted to a straight line to estimate the initial rate of alkalization induced by reversal of AE activity [31,32]. Net  $\text{HCO}_3^-$  fluxes ( $J_{\text{HCO}_3^-}$ ) were determined as the product of  $\text{dpH}_i/\text{dt}$  times the total intracellular buffering power ( $\beta_t$ ) which was computed from the initial change in  $\text{pH}_i$  after the alkaline and acid load of the TMA pulses as  $\beta_t = [\text{TMA}]_i / \Delta\text{pH}_i$  [29,32,33]. The concentration of internal TMA ( $[\text{TMA}]_i$ ) was calculated from the concentration of external TMA and its  $\text{pK}_a$

using the Henderson–Hasselbach equation. A  $pK_a$  value of 9.80 was used for the calculations. The values of  $\beta_i$  were  $77.0 \pm 11.62$  mM/pH unit in SHR vs.  $66.75 \pm 10.31$  in WKY (NS) at a mean  $pH_i$  of  $7.32 \pm 0.02$  whereas they were  $52.8 \pm 8.2$  vs.  $46.1 \pm 5.8$  mM/pH unit (NS) in SHR and WKY, respectively, at  $pH_i$  of  $7.09 \pm 0.02$ . Although these values of  $\beta_i$  are lower (~50%) than those used to compute previously reported  $J_{HCO_3^-}$  [15], the results are in agreement with those from other and our laboratories that showed no difference between WKY and SHR when measuring intrinsic buffer capacity [15,16,34].

### 2.3. RNA isolation

Heart tissue was rapidly homogenized with TRIZOL™ reagent (Gibco BRL) and total RNA isolation was performed following the instructions of the manufacturer. Total RNA from each heart was approximately 1.2 mg. Poly(A<sup>+</sup>) RNA was isolated from 1-mg total RNA (Poly-ATrac, Promega) with a yield of approximately 10–12 µg/mg of total RNA. RNA was quantified by UV spectrophotometry.

### 2.4. Northern blots

Poly(A<sup>+</sup>) RNA (4–6 µg) was denatured with formaldehyde and formamide and resolved in 1% agarose gels, described [35] with the exception that the formaldehyde concentration was reduced to 0.22 M in the gel and buffer. RNAs (0.28–6.58 kb from Promega) were used as standards. mRNAs were transferred to nylon membranes (Inmobilon S from Millipore) and cross-linked to the membrane with ultraviolet light (320 nm for 2 min). Blots were prehybridized for 4 h at 42°C in 50% formamide, 5×SSC, 1×Denhardt's, 1% sodium dodecyl sulfate (SDS), 50 µg/ml salmon sperm DNA, and 20 mM sodium phosphate at pH 6.6. Hybridization was performed for 16–18 h at 42°C in 10 ml 50% formamide, 5×SSC, 1×Denhardt's, 1% SDS, 50 µg/ml salmon sperm DNA,

10% dextran sulfate and 20 mM sodium phosphate at pH 6.6 containing  $4 \times 10^6$  cpm/ml of radiolabeled probe. Blots were washed  $2 \times 15$  min at room temperature in  $2 \times$ SSC, 0.2% SDS followed by two washes for 30 min at 65°C in  $0.2 \times$ SSC, 0.2% SDS. The membranes were exposed to Kodak Biomax™ MS film with an enhancing screen for 4 to 6 days at  $-70^\circ\text{C}$ . The autoradiographs were quantified with an UltraScan XL densitometer (Pharmacia). The results were normalized with those obtained by hybridization with a radiolabeled probe to detect GAPDH mRNA.

### 2.5. Hybridization probes

Probes to detect the different AE mRNA isoforms were prepared by PCR from plasmid cDNA cloned into the expression vector pRBG4. All probe sequences were selected to be free of repetitive DNA elements using the RepeatMasker Software<sup>1</sup> and were in unique sequence regions on the basis of alignments of the AE1, AE2 and AE3 cDNAs. For AE3 mRNA detection, probes were generated using pJRC31, containing rat cAE3 cDNA fragment and pJRC32, containing rat flAE3 cDNA fragment [36]. The probes were: (1) AE3com, 222 bp of the gene that codes for amino acids 428–501, common to rat cAE3 and flAE3, generated by PCR using pJRC32 as template, forward primer 5'-AACGATGACAAG-GACAGTG and reverse primer 5'-GCTTTTCCCCCG-GTGACCG. (2) cAE3, 317 bp that codes for amino acids 1–73 unique to rat cAE3 generated by PCR using pJRC31 as template, forward primer 5'-GAAACCTTAC-CACGTCCAGC and reverse primer 5'-CAAGCAG-CTACCCCTGCCAGG. (3) flAE3, 349 bp that codes for amino acids 154–269 of rat flAE3, generated by PCR using pJRC32 as template, forward primer 5'-GGAGCCTCCACCCCGAGGGC and reverse primer 5'-CTTCATGTCGTCCAGATCGGC (see Fig. 1).

For AE1 mRNA detection, a 306 bp of mouse AE1 cDNA, corresponding to amino acids 285–384 of AE1,

<sup>1</sup><http://ftp.genome.washington.edu/cgi-bin/RepeatMasker software>.

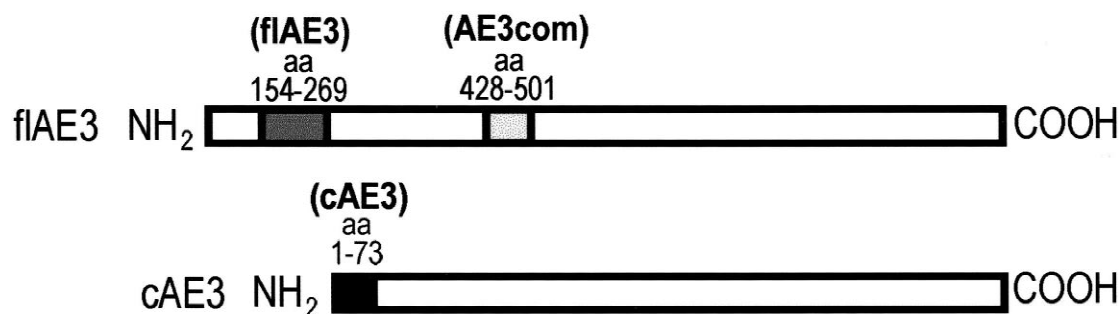


Fig. 1. Schematic representation of the flAE3 and cAE3 isoforms of rat AE3. AE3 common (AE3com) is a 222 bp probe of the rat gene that codes for amino acids 428–501 of rat AE3 gene. This probe detects both cAE3 and flAE3 mRNAs. cAE3 is a 317 bp probe of the rat gene that codes for amino acids 1–73 specific for the cardiac AE3 isoform. flAE3 is a 349 bp fragment of the rat gene that codes for amino acids 154–269 of rat AE3 to detect specifically the full-length AE3 isoform.

was generated by PCR using pRG106 [37] as template, forward primer 5'-GAGGCTCCGCATGTGCGAC and reverse primer 5'-GTCACCAGGGCCCCCTTTTC.

For AE2 mRNA detection, a 411 bp fragment of mouse AE2 cDNA, corresponding to amino acids 92–228 of AE2, was generated by PCR using pBSL103 [38] as template, forward primer 5'-GATGCCCGACGTCGCAAG and reverse primer 5'-TCGATGTCCAGGCTGGGC. Probes to detect rat AE1 and AE2 mRNAs were prepared from mouse cDNA based on sequence homology between mouse and rat genes.

For GAPDH mRNA, a 749 bp probe of the gene that codes for amino acids 11–248 of the rat GAPDH was used [39]. The expression level of GAPDH mRNA was reported unaltered in cardiac myocytes from SHR [40].

To verify all probes, DNA sequencing with an Applied Biosystems 373 A DNA sequencer, was performed by the Core Facility in the Department of Biochemistry, University of Alberta.

The probes were radiolabeled by random hexamer labeling (Gibco, BRL) using  $\alpha$ - $^{32}$ P-dCTP (New England Nuclear, Life Sciences) to a specific activity of  $2 \times 10^9$  cpm/ $\mu$ g.

## 2.6. Statistical analysis

The results are expressed as the mean  $\pm$  S.E.M. of the indicated number of independent experiments. Statistical significance was assessed by the Student's *t*-test. ANCOVA test was used to compare the slopes of the regression lines relating  $J_{\text{HCO}_3^-}$  to  $\text{pH}_i$  in both rat strains.

## 3. Results

### 3.1. Animals characteristics

The characteristics of the animals used in this study are shown in Table 1. SBP, HW and cardiac hypertrophy (HW/BW) were statistically higher in the SHR ( $n=30$ ) compared to WKY rats ( $n=30$ ) ( $P<0.05$ ).

Table 1  
General characteristics of WKY and SHR rats

Animals	SBP (mmHg)	BW (g)	HW (mg)	HW/BW (mg/g)
WKY ( $n=30$ )	124.9 $\pm$ 1.1	328.1 $\pm$ 10.9	0.863 $\pm$ 0.026	2.66 $\pm$ 0.04
SHR ( $n=30$ )	179.3 $\pm$ 2.2*	303.6 $\pm$ 7.7	1.010 $\pm$ 0.024*	3.33 $\pm$ 0.05*

SBP, systolic blood pressure; BW, body weight; HW, heart weight.  
\* $P<0.05$ .

### 3.2. AE activity in 'forward mode'

It has been shown that after an imposed intracellular alkaline load,  $\text{pH}_i$  gradually returns towards the baseline value and this recovery has been ascribed to the activity of the AE. Fig. 2A shows the time-course of the changes in  $\text{pH}_i$  that follows the application and washout of TMA in a representative experiment. A rapid rise of  $\text{pH}_i$  from a baseline value of  $\text{pH}_i$  of  $\sim 7.17$  to  $\sim 7.55$  was obtained in this experiment. On average the value of  $\text{pH}_i$  before the TMA-pulse was  $7.16 \pm 0.02$  in SHR and  $7.14 \pm 0.03$  in WKY (NS). The intracellular alkalinization was followed by a recovery due to the activity of the AE. The  $\text{pH}_i$  recovery from alkalosis was interrupted by the washout of TMA, which caused an intracellular acidification. The rate of the  $\text{pH}_i$  recovery from TMA-induced alkalosis in WKY and SHR rats was determined by the slope of the initial decay in  $\text{pH}_i$  values as illustrated by the broken line in Fig. 2A. The overall velocity of  $\text{pH}_i$  recovery from the alkaline load was faster in SHR than in WKY ( $0.039 \pm 0.007$ ,  $n=11$  vs.  $0.010 \pm 0.003$  pH unit/min,  $n=11$ ). From the data of the rate of  $\text{pH}_i$  recovery from the alkaline load, the relationship between net  $\text{HCO}_3^-$  efflux ( $J_{\text{HCO}_3^-}$ ) and  $\text{pH}_i$  was determined and the results obtained are plotted in Fig. 2B. The lines relating  $\text{HCO}_3^-$  efflux to  $\text{pH}_i$  show the known activation of the AE by the increase in  $\text{pH}_i$ . Two lines with different slopes for WKY and SHR rats (ANCOVA,  $P<0.0001$ ) were obtained. For the sake of comparison, those experiments in which  $\text{pH}_i$  attained similar peak values during the TMA pulses were selected to compare the  $J_{\text{HCO}_3^-}$  during the  $\text{pH}_i$  recovery phase in the SHR and WKY rats. At a common  $\text{pH}_i$  value the  $J_{\text{HCO}_3^-}$  in the SHR was higher, as shown in Fig. 2C. These results confirm previous data from our laboratory [15,16] showing that the hyperactivity of the AE is not secondary to a more alkaline  $\text{pH}_i$ . In our previous report [15], the two lines relating  $J_{\text{HCO}_3^-}$  and  $\text{pH}_i$  were parallel suggesting that the difference in AE activity between normotensive and hypertensive myocardium was independent of  $\text{pH}_i$ . In the results described herein non-parallel lines were obtained, which suggests that the difference in activity of the AE between both preparations increases with the increase in  $\text{pH}_i$ . The difference in  $\beta_i$  values in both studies (different techniques used) can explain a difference in the absolute values of  $J_{\text{HCO}_3^-}$  but not the different slopes detected. In any case, it should be more reasonable to speculate that if the AE was activated with the increase in  $\text{pH}_i$ , the difference between normotensive and hypertensive hearts would become more evident at the higher values of  $\text{pH}_i$  and be absent at  $\text{pH}_i$  values at which the AE activity is negligible.

### 3.3. AE activity after $\text{Cl}^-$ deprivation

Fig. 3A shows the time-course of the  $\text{pH}_i$  changes

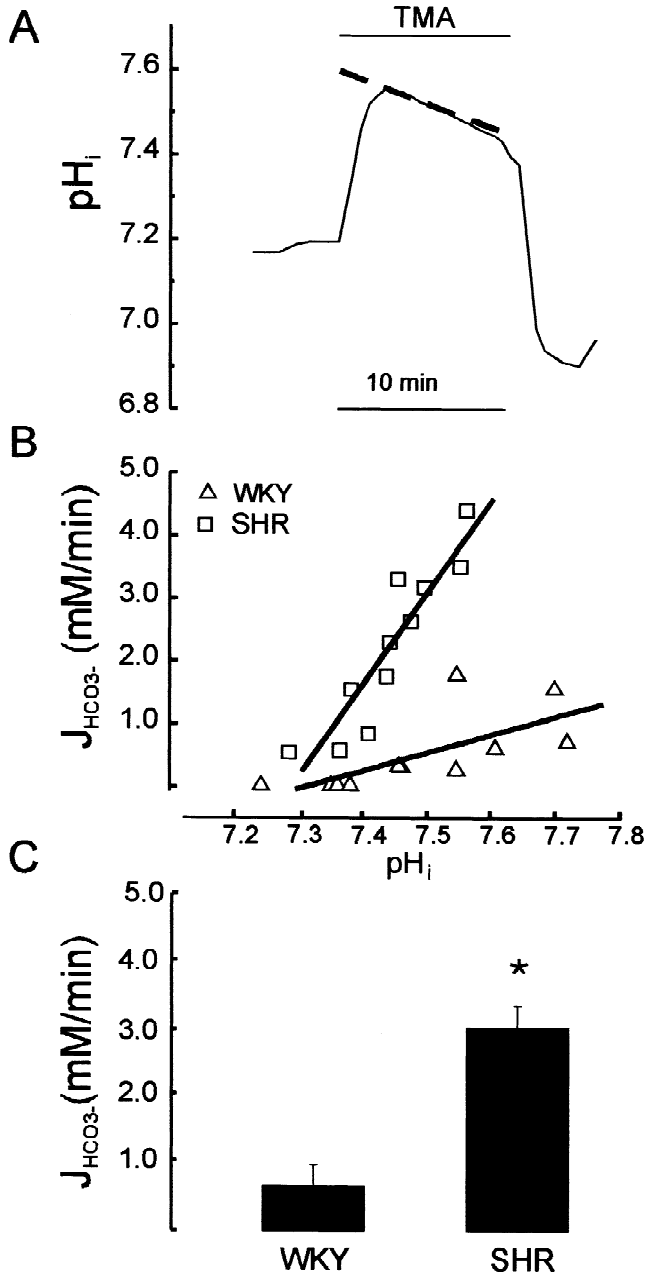


Fig. 2. Recovery from imposed intracellular alkalinization in the myocardium of WKY and SHR rats. (A) Representative experiment showing the time-course of the changes in  $pH_i$  caused by the application and washout of TMA in a papillary muscle loaded with BCECF. The straight line fitted by the least square method and used to estimate the initial rate of  $pH_i$  recovery is shown (broken line). (B) Net  $HCO_3^-$  efflux ( $J_{HCO_3^-}$ , see Methods) in papillary muscles from WKY and SHR rats was plotted as a function of  $pH_i$ . Each point represents the values determined during different TMA pulses (10, 20 or 30 mM final concentration). Total number of muscles used from each rat strain was 5. (C) Mean values ( $\pm$ S.E.M.) of estimated  $J_{HCO_3^-}$  during the recovery of  $pH_i$  during TMA-induced alkaline loads in WKY and SHR rats. Experiments in which  $pH_i$  attained similar peak values during the TMA pulses were selected for the comparison (mean peak  $pH_i$  values were  $7.49 \pm 0.02$ ,  $n=7$ , in SHR and  $7.52 \pm 0.03$ ,  $n=5$ , in WKY, NS). \* $P < 0.05$  vs. WKY.

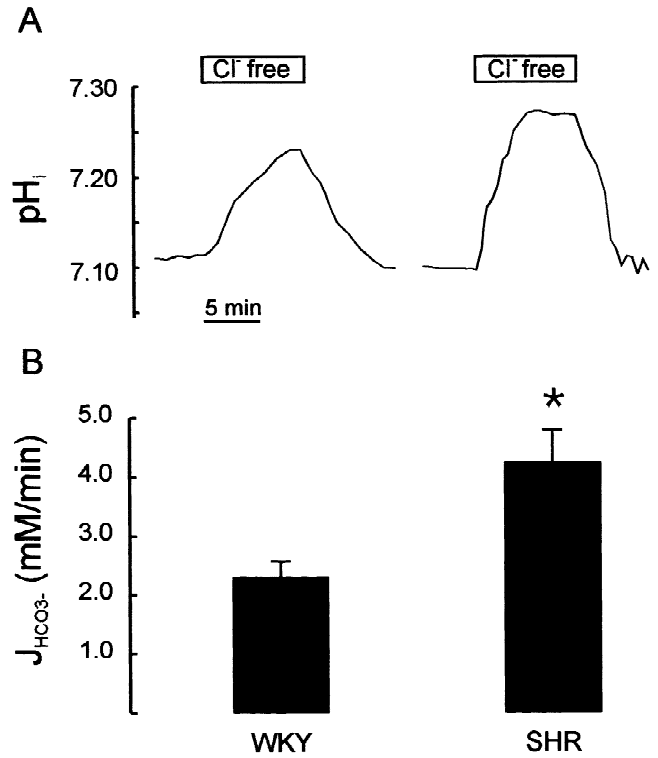


Fig. 3. Effect of the withdrawal and re-addition of external  $Cl^-$  on the  $pH_i$  of papillary muscles from WKY and SHR rats. (A) Typical experiments showing the time-course of  $pH_i$  changes caused by the removal and re-addition of external  $Cl^-$  in WKY (left) and SHR (right). (B) Mean values ( $\pm$ S.E.M.) of net  $HCO_3^-$  influx ( $J_{HCO_3^-}$ ) in WKY ( $n=10$ ) and SHR ( $n=10$ ) induced by the removal of  $Cl^-$ , as estimated by the product of  $dpH_i/dt$  times buffer capacity. \* $P < 0.05$  vs. WKY.

induced by the withdrawal and re-addition of  $Cl^-$  in experiments with papillary muscles from WKY and SHR rats. The removal of extracellular  $Cl^-$  causes the reversal of the AE and the influx of  $HCO_3^-$  induces, consequently, a rise of  $pH_i$ . After the re-addition of  $Cl^-$  there is a gradual recovery towards  $pH_i$  baseline. Steady  $pH_i$  values before the removal of  $Cl^-$  was similar in WKY ( $7.12 \pm 0.04$ ,  $n=10$ ) and SHR ( $7.14 \pm 0.01$ ,  $n=10$ ). The rate of rise of  $pH_i$  in SHR was  $\sim 70\%$  faster than in WKY, as measured 1 min after  $Cl^-$  washout. Thus, mean values of the initial rate of  $pH_i$  rise were  $0.074 \pm 0.009$  pH unit/min in SHR ( $n=10$ ) vs.  $0.048 \pm 0.006$  in WKY ( $n=10$ ,  $P < 0.05$ ). From these data, the net initial influx of  $HCO_3^-$  elicited by the withdrawal of  $Cl^-$  was estimated and the bars in Fig. 3B summarize the results obtained. There was an increase in  $J_{HCO_3^-}$  in the SHR compared to WKY.

The increased activity of the AE in the hypertrophied myocardium of SHR was detected therefore, in the forward and reverse modes of exchange. Although the enhanced activity of the AE in the myocardium of hypertensive rats was detected by measuring the bicarbonate fluxes carried by the exchanger operating in either forward or reverse mode, the magnitude of the fluxes were different. The

different values obtained may be explained by the difference in the driving forces after an alkaline load or  $\text{Cl}^-$  deprivation.

### 3.4. AE3 mRNA expression

Northern blots of poly( $\text{A}^+$ ) RNA (3–4  $\mu\text{g}$ ) isolated from rat cardiac tissue were probed with three different probes (Fig. 1). The common AE3 (AE3com) probe hybridized with two transcripts of 4.4 kb and 3.8 kb in length in normotensive rat cardiac tissue (Fig. 4A-1). The expression of the 3.8-kb mRNA was higher than that of 4.4-kb mRNA. This is consistent with previously reported results [23]. To determine the relative mRNA expression level of both isoforms in cardiac tissue from normotensive and SHR, Northern blots of cardiac mRNA were hybridized separately with specific probes to detect cAE3 and fAE3 (Fig. 4A2–3). Compared to cardiac tissue from normotensive rats, in the hypertrophied myocardium of SHR a decrease of cAE3 mRNA expression was observed (Fig. 4A-2). Conversely, an increased expression of fAE3 mRNA was obtained. The overall results obtained of cAE3 and fAE3 mRNA expression in cardiac tissue from both groups of rats are shown in Fig. 4B, where it shows ~40%

decrease in cAE3 and ~80% increase in fAE3 in the SHR compared to WKY.

### 3.5. AE1 and AE2 mRNA expression

Northern blot hybridization of 5–6  $\mu\text{g}$  poly( $\text{A}^+$ ) RNA with a probe that codes for amino acids 285–384 of rat AE1 showed two bands of 3.8 and 4.1-kb (Fig. 5A). The overall data in arbitrary units of AE1 mRNA from normotensive and SHR rat cardiac tissue are shown in Fig. 5B. Compared to WKY rats a decrease in the 4.1-kb mRNA expression in SHR was observed. The 3.8-kb transcript was similarly expressed in cardiac tissue from both WKY and SHR rats. Fig. 5C shows the expression level of AE2 mRNA in cardiac tissue from WKY and SHR rats. The 411 bp that codes for amino acids 92–228 of rat AE2 showed a single band of 4.4-kb mRNA. The overall results are shown in Fig. 5D. There was no difference in the AE2 mRNA expression level between WKY and SHR cardiac tissue.

Relatively to AE3 variants, longer autoradiographic exposures of the blot were necessary to detect AE1 and AE2 mRNAs.

In summary, in addition to the enhanced activity of the

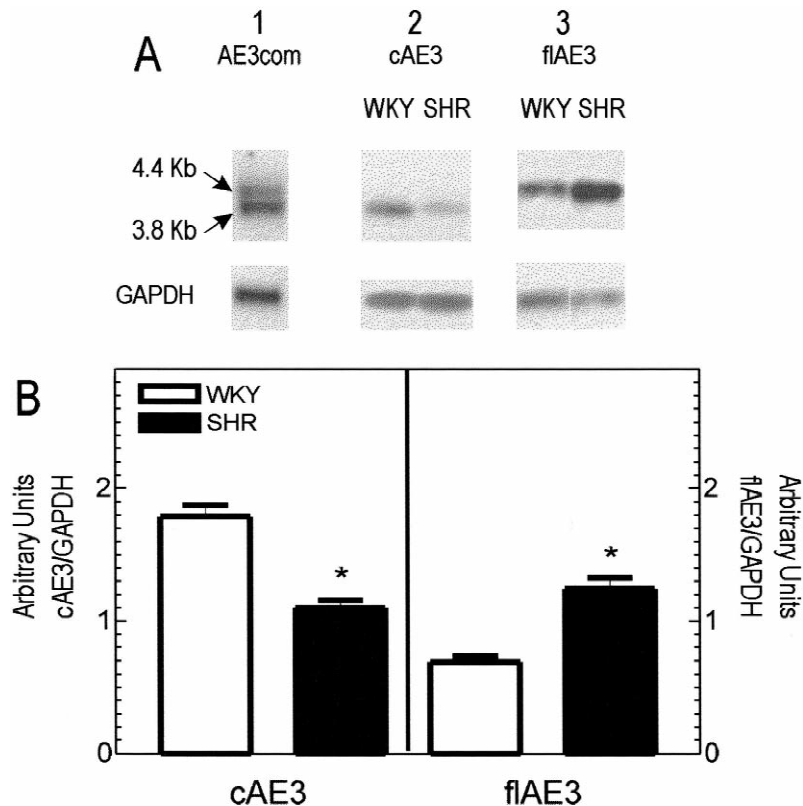


Fig. 4. Expression of AE3 mRNAs in cardiac tissue from WKY and SHR rats. (A) Northern blots with 3–4  $\mu\text{g}$  poly( $\text{A}^+$ ) RNA from the indicated rat tissues were analyzed as described in Methods with the following probes. (1) AE3com: 222 bp probe, to detect both rat fAE3 and cAE3 mRNAs; (2) cAE3: 317 bp probe for cAE3 mRNA; (3) fAE3: 349 bp probe for fAE3 mRNA. Autoradiographic exposure times were 4 days (AE3 mRNAs) and 14–20 h (GAPDH). (B) In arbitrary units (see Methods) the graph shows the expression level of cAE3 and fAE3 mRNAs in cardiac tissue from WKY ( $n=8$ ) and SHR ( $n=8$ ). Bars are the means  $\pm$  S.E.M. \* $P<0.05$  vs. WKY.

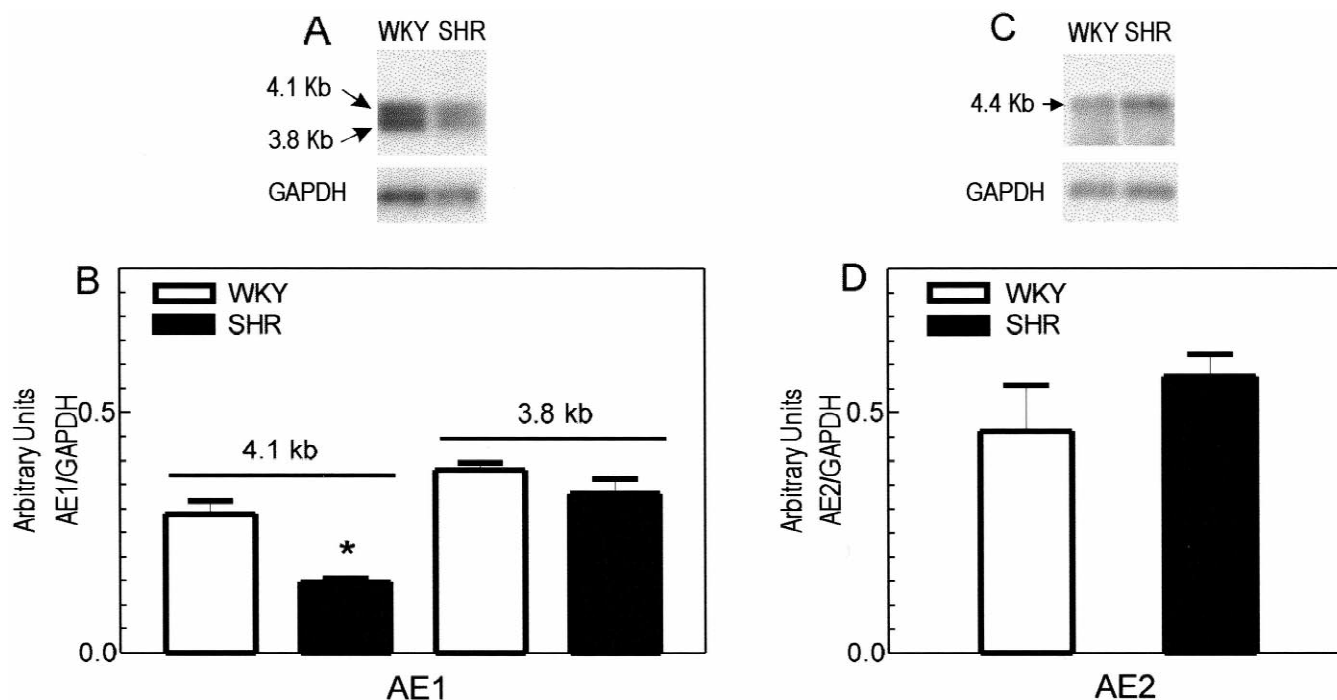


Fig. 5. Expression of AE1 and AE2 mRNAs in cardiac tissue from WKY and SHR rats. Northern blots with 5–6  $\mu\text{g}$  poly(A<sup>+</sup>) RNA from the indicated rat tissues was analyzed as described in Methods. (A) 306 bp probe, to detect rat AE1 mRNAs. (B) In arbitrary units (see Methods) the graph shows the expression level of AE1 mRNAs in cardiac tissue from WKY ( $n=7$ ) and SHR ( $n=7$ ). Bars are the means  $\pm$  S.E.M. Compared to WKY, a decrease in the 4.1 kb mRNA expression in the SHR was observed. ( $P<0.05$ ). The 3.8 kb mRNA was similarly expressed in WKY and SHR. (C) 411 bp probe, which specifically detects rat AE2 mRNA. (D) In arbitrary units the graph shows the expression level of AE2 mRNA in cardiac tissue from WKY ( $n=6$ ) and SHR ( $n=6$ ). Bars are the means  $\pm$  S.E.M. The 4.4 kb AE2 mRNA was equally expressed in both groups of animals. Autoradiographic exposure times were 5 to 6 days (AE1 and AE2 mRNAs) and 14–20 h (GAPDH).

AE in the SHR; the results presented herein are showing no changes in the mRNA expression of the AE2 and 3.8-kb AE1, a decrease of the 4.1-kb AE1 and cAE3, and an increase of fAE3.

#### 4. Discussion

The enhanced activity of the AE in the hypertrophied myocardium was previously reported by this laboratory [15]. We are now showing that the hyperactivity of the AE can be detected either in the forward or in the reverse mode of exchange. The fact that the increased activity of the AE was also detected after Cl<sup>-</sup> deprivation, in addition to reinforce the data, eliminates the possibility of out of equilibrium conditions induced by the alkaline load.

Since the AE is an acidifying mechanism (extrudes HCO<sub>3</sub><sup>-</sup>), its enhanced activity would result in a lower pH<sub>i</sub> of the hypertrophied heart. However, due to the simultaneous hyperactivity of the NHE detected in the myocardium of the SHR, no significant changes in the steady state of pH<sub>i</sub> was detected in the hypertrophied myocardium in the presence of HCO<sub>3</sub><sup>-</sup> ([15] and present results). The cellular mechanisms by which the hypertensive rat (or the hypertrophied myocardium) is presenting an increased activity of the AE accompanying the increased activity of

the NHE are not known. The enhanced activity of the AE is not secondary to a more alkaline pH<sub>i</sub> due to the hyperactivity of the NHE1, since we are showing herein that in the hypertrophied heart AE activity is higher than the control, even when compared at the same pH<sub>i</sub>. The possibility that an enhanced activity of the exchanger induced by an initial phosphorylation could lead to an increased expression of the AE mRNA is an alternative to be analyzed. In connection with this, the expression of other exchanger mRNAs more widely analyzed, like the NHE1, has been shown to increase following an initial hyperactivity induced by post-translational mechanisms [41,42].

The AE is a family of three genes, AE1, AE2 and AE3 [3]. The AE proteins share most of the amino acids sequence homology in the membrane-associated transport domains but are more diverging in the cytoplasmic domain [13]. Alternative splicing of each gene product results in further sequence diversity in the cytoplasmic domain [13]. The most abundant AE isoforms in cardiac tissue are the cAE3 followed by fAE3 isoforms. This was reported previously by others [23] and confirmed in the present study in normotensive rat cardiac tissue. AE1 and AE2 variants were detected with longer autoradiographic exposures of the blot. It is not yet definitely known which AE isoform is responsible for the AE activity in cardiac tissue.

Because of the abundance of its mRNA, the cAE3 isoform becomes a strong candidate for the AE involved in the regulation of myocardial  $\text{pH}_i$ . Our results show no changes in the mRNA expression of the AE2 and 3.8-kb AE1, a decrease of the 4.1-kb AE1 and cAE3, and an increase of the fAE3.

We are not aware of previous studies analyzing the expression of the different AE isoforms in the hypertrophied myocardium. In the present study we report for the first time that the hypertrophied myocardium of the SHR presents a higher expression of the fAE3 mRNA isoform than their normotensive controls.

In a recent study, the level of fAE3 RT-PCR product relative to cAE3 product was higher in neonatal rat cardiac myocytes than in adult cells [43]. Furthermore, it has been reported that the cardiac-specific transcript (cAE3) in mouse heart increased substantially between the fetal and adult stages while fAE3 mRNA decreased [23]. In connection to this, it has been shown that AE exchanger activity is necessary for maintaining embryo intracellular  $\text{pH}_i$ , and in conditions where the external environment is even moderately alkaline, embryo development depends on functional AE [44]. Taken together, the changes in transcriptional regulation of specific genes during cardiac hypertrophy reflect a return to an earlier stage of cardiomyocyte differentiation.

On the basis of our results, the hypertrophied myocardium of SHR expresses fetal pattern of AE3 mRNA expression. We cannot conclude that the increased AE activity of the hypertrophic myocardium is the result of the over-expression of fAE3 mRNA isoform observed in this study. An increase in fAE3 mRNA expression does not necessarily mean an increase in protein translation and activity of the exchanger. However, a change in the expression level of AE3 mRNA isoforms in the hypertrophied myocardium was detected and this change is similar to the pattern found in cardiomyocytes at the early stage [23,43]. This allows us to suggest that the increase in fAE3 mRNA expression is an adaptive change of the hypertrophied myocardium that might be in connection with the increased activity of the AE. Future studies should be focussed to investigate whether or not the over-expression of fAE3 mRNA is responsible for the increased activity of the exchanger.

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### References

- [1] Orchard CH, Kentish JC. Effects of changes of pH on the contractile function of cardiac muscle. *Am J Physiol* 1990;258:C967–C981.
- [2] Kusuoka H, Backx PH, Camilión de Hurtado MC, Azan-Backx M, Marban E, Cingolani HE. Relative roles of intracellular  $\text{Ca}^{2+}$  and pH in shaping myocardial contractile response to acute respiratory alkalosis. *Am J Physiol* 1993;265:H1696–H1703.
- [3] Schaffer SW, Safer B, Ford C, Illingworth JR. Respiratory acidosis and its reversibility in perfused rat heart: regulation of citric acid cycle activity. *Am J Physiol* 1978;234:H40–H51.
- [4] Kohlardt M, Haap K, Figulla HR. Influence of low extracellular pH upon the Ca inward current and isometric contractile force in mammalian ventricular myocardium. *Pflügers Arch* 1976;366:31–48.
- [5] Fry CH, Poole-Wilson PA. Effect of acid–base changes on excitation–contraction coupling in guinea pig and rabbit cardiac ventricular muscle. *J Physiol (Lond)* 1981;313:141–160.
- [6] Fabiato A, Fabiato F. Effects of pH in the myofilaments and sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscle. *J Physiol (Lond)* 1978;276:233–255.
- [7] Isfort RJ, Cody DB, Asquith TN, Ridder GM, Stuard SB, LeBoeuf RA. Induction of protein phosphorylation, protein synthesis, immediate early-gene expression and cellular proliferation by intracellular pH modulation. Implications of the role of hydrogen ions in signal transduction. *Eur J Biochem* 1993;213:349–357.
- [8] McConkey DJ, Orrenius S. Signal transduction pathways in apoptosis. *Stem Cells* 1996;14:619–631.
- [9] Gottlieb RA, Gruol DL, Zhu JY, Engler RL. Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase and apoptosis. *J Clin Invest* 1996;97:2391–2398.
- [10] Aronson PS. Kinetics properties of the plasma membrane  $\text{Na}^+/\text{H}^+$  exchanger. *Annu Rev Physiol* 1985;47:545–560.
- [11] Roos A, Boron WF. Intracellular pH. *Physiol Rev* 1981;61:296–434.
- [12] Lagadic-Gossman D, Buckler KJ, Vaughan-Jones RD. Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J Physiol (Lond)* 1992;458:361–384.
- [13] Alper SL. The band 3-related anion-exchanger (AE) gene family. *Annu Rev Physiol* 1991;53:549–564.
- [14] Sun B, Leem CH, Vaughan-Jones RD. Novel chloride-dependent acid loader in the guinea-pig ventricular myocyte: part of dual acid-loading mechanisms. *J Physiol (Lond)* 1996;495:65–82.
- [15] Pérez NG, Alvarez BV, Camilión de Hurtado MC, Cingolani HE. Intracellular pH regulation in myocardium of the spontaneously hypertensive rat: compensated enhanced activity of the  $\text{Na}^+/\text{H}^+$  exchanger. *Circ Res* 1995;77:1192–1200.
- [16] Ennis IL, Alvarez BV, Camilión de Hurtado MC, Cingolani HE. Enalapril induces regression of cardiac hypertrophy and normalization of  $\text{pH}_i$  regulatory mechanisms. *Hypertension* 1998;31:961–967.
- [17] Alonso A, Arrázola A, Garciandía A, Esparza N, Gómez-Alamillo C, Díez J. Erythrocyte anion-exchanger activity and intracellular pH in essential hypertension. *Hypertension* 1993;22:348–356.
- [18] Brosius III FC, Alper SL, García AM, Lodish HF. The major kidney band 3 gene transcript predicts an amino-terminal truncated band 3 polypeptide. *J Biol Chem* 1989;264:7784–7787.
- [19] Kudrycki KE, Shull GE. Rat kidney band 3  $\text{Cl}^-/\text{HCO}_3^-$  exchanger mRNA is transcribed from an alternative promoter. *Am J Physiol* 1993;264:F540–F547.
- [20] Wang Z, Schultheis PJ, Shull GE. Three N-terminal variants of the AE2  $\text{Cl}^-/\text{HCO}_3^-$  exchanger are encoded by mRNAs transcribed from alternative promoters. *J Biol Chem* 1996;271:7838–7843.



- [21] Kudrycki KE, Newman PR, Shull GE. cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. *J Biol Chem* 1990;265:462–471.
- [22] Linn SC, Kudrycki KE, Shull GE. The predicted translation product of a cardiac AE3 mRNA contains an N terminus distinct from that of the brain AE3  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. Cloning of a cardiac AE3 cDNA, organization of the AE gene and identification of an alternative initiation site. *J Biol Chem* 1992;267:7927–7935.
- [23] Linn SC, Askew GR, Menon AG, Shull GE. Conservation of an AE3  $\text{Cl}^-/\text{HCO}_3^-$  exchanger cardiac specific exon and promoter region and AE3 mRNA expression patterns in murine and human hearts. *Circ Res* 1995;76:584–591.
- [24] Kobayashi S, Morgans CW, Casey JR, Kopito RR. AE3 anion-exchanger isoforms in the vertebrate retina: development regulation and differential expression in neurons and glia. *J Neurosci* 1994;14:6266–6279.
- [25] Yannoukakos D, Stuart-Tilley A, Fernandez HA, Fey P, Duyk G, Alper SL. Molecular cloning, expression and chromosomal localization of two isoforms of the AE anion-exchanger from human heart. *Circ Res* 1994;75:603–614.
- [26] Buñag RD. Validation in awake rats of a tail-cuff method for measuring systolic pressure. *J Appl Physiol* 1973;34:279–282.
- [27] Thomas JA, Buchsbaum RN, Zimniak A, Racker E. Intracellular pH measurements in Erlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* 1979;18:2210–2218.
- [28] Xu P, Spitzer KW.  $\text{Na}^+$ -independent  $\text{Cl}^-/\text{HCO}_3^-$  exchange mediates recovery of  $\text{pH}_i$  from alkalosis in guinea pig ventricular myocytes. *Am J Physiol* 1994;267:H85–H91.
- [29] Wallert MA, Frolich O.  $\text{Na}^+/\text{H}^+$  exchange in isolated myocytes from adult rat heart. *Am J Physiol* 1989;257:C207–C213.
- [30] Pucéat M, Clément O, Vassort G. Extracellular  $\text{MgATP}$  activates the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in single rat cardiac cells. *J Physiol (Lond)* 1991;444:241–256.
- [31] Désilets M, Pucéat M, Vassort G. Chloride dependence of pH modulation by  $\beta$ -adrenergic agonist in rat cardiomyocytes. *Circ Res* 1994;75:862–869.
- [32] Jiang L, Stuart-Tilley A, Parkash J, Alper SL.  $\text{pH}_i$  and serum regulate AE2-mediated  $\text{Cl}^-/\text{HCO}_3^-$  exchange in CHOP cells defined transient transfection status. *Am J Physiol* 1994;267:C845–C856.
- [33] Camilión de Hurtado MC, Alvarez BV, Pérez NG, Ennis EL, Cingolani HE. Angiotensin II activates  $\text{Na}^+$ -independent  $\text{Cl}^-/\text{HCO}_3^-$  exchange in ventricular myocardium. *Circ Res* 1998;82:473–481.
- [34] Siczkowski MO, Davies JE, Ng LL. Sodium–hydrogen antiporter protein in normotensive Wistar Kyoto and spontaneously hypertensive rats. *J Hypertens* 1994;12:775–781.
- [35] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
- [36] Sterling D, Casey JR. Regulation by intracellular pH and transport activity of AE3  $\text{Cl}^-/\text{HCO}_3^-$  exchange proteins. *Biochem J* 1999;344:221–229.
- [37] Ruetz S, Lindsey AE, Ward C, Kopito RR. Functional activation of plasma membrane anion-exchangers occurs in a pre-Golgi compartment. *J Cell Biol* 1993;121:37–48.
- [38] Lee BS, Gunn RB, Kopito RR. Functional differences among nonerythroid anion-exchangers expressed in transfected human cell line. *J Biol Chem* 1991;266:11448–11454.
- [39] Tso JY, Sun XH, Kao TH, Reece KS, Wu R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity. *Nucleic Acid Res* 1985;13:2485–2502.
- [40] Bayraktutan U, Yang Z-K, Shah AM. Selective dysregulation of nitric oxide synthase type 3 in cardiac myocytes but not coronary microvascular endothelial cells of spontaneously hypertensive rats. *Cardiovasc Res* 1998;38:719–726.
- [41] Dyck JR, Maddaford TG, Pierce GN, Fliegel L. Induction of expression of the sodium–hydrogen exchanger in rat myocardium. *Cardiovasc Res* 1995;29:203–208.
- [42] Gan XT, Zhang Y, Karmazyn M. Induction of hypertrophy and NHE1 gene expression by acidosis in cultured neonatal rat ventricular myocytes (Abstract). *J Mol Cell Cardiol* 2000;32:143.
- [43] Richards SM, Jaconi ME, Vassort G, Pucéat M. A spliced variant of AE1 gene encodes a truncated form of Band 3 in heart: the predominant anion-exchanger in ventricular myocytes. *J Cell Sci* 1999;112:1519–1528.
- [44] Zhao Y, Chauvet PJ-P, Alper SL, Baltz JM. Expression and function of bicarbonate/chloride exchangers in the preimplantation mouse embryo. *J Biol Chem* 1995;270:24428–24434.