# Influence of $Na^+$-Independent $Cl^-$-$HCO_3^-$ Exchange on the Slow Force Response to Myocardial Stretch

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***Abstract***—Previous work demonstrated that the slow force response (SFR) to stretch is due to the increase in calcium transients ($Ca^{2+}$T) produced by an autocrine-paracrine mechanism of locally produced angiotensin II/endothelin activating $Na^+$-$H^+$ exchange. Although a rise in $pH_i$ is presumed to follow stretch, it was observed only in the absence of extracellular bicarbonate, suggesting $pH_i$ compensation through the $Na^+$-independent $Cl^-$-$HCO_3^-$ exchange (AE) mechanism. Because available AE inhibitors do not distinguish between different bicarbonate-dependent mechanisms or even between AE isoforms, we developed a functional inhibitory antibody against both the AE3c and AE3fl isoforms (anti-AE3Loop III) that was used to explore if $pH_i$ would rise in stretched cat papillary muscles superfused with bicarbonate after AE3 inhibition. In addition, the influence of this potential increase in $pH_i$ on the SFR was analyzed. In this study, we present evidence that cancellation of AE3 isoforms activity (either by superfusion with bicarbonate-free buffer or with anti-AE3Loop III) results in $pH_i$ increase after stretch and the magnitude of the SFR was larger than when AE was operative, despite of similar increases in $[Na^+]_i$ and $Ca^{2+}$T under both conditions. Inhibition of reverse mode $Na^+$-$Ca^{2+}$ exchange reduced the SFR to the half when the AE was inactive and totally suppressed it when AE3 was active. The difference in the SFR magnitude and response to inhibition of reverse mode $Na^+$-$Ca^{2+}$ exchange can be ascribed to a $pH_i$-induced increase in myofilament $Ca^{2+}$ responsiveness. **(*Circ Res*. 2003;93:1082-1088.)**

**Key Words:** myocardial $pH_i$ ■ $Na^+$-$H^+$ exchanger ■ AE3 ■ $Na^+$-$Ca^{2+}$ exchange ■ myocardial stretch



Since the experiments of Parmley and Chuck,[1] it is known that the stretch of cardiac muscle causes an increase in developed force (DF) in two well-characterized phases: an initial rapid rise followed by a slow rise that takes 15 to 20 minutes to complete development. The initial increase in force has been attributed to an increase in myofilament $Ca^{2+}$ responsiveness,[2] whereas the slower component, named the slow force response (SFR), is due to an increase in the $Ca^{2+}$ transient ($Ca^{2+}$T).[2,3] Recently, experiments in papillary muscles or trabecular preparations from rat,[3] cat,[4,5] and rabbit[6] provided evidence that the increase in $Ca^{2+}$T responsible for the SFR results from activation of the $Na^+$-$H^+$ exchanger (NHE) that increases intracellular $Na^+$ concentration ($[Na^+]_i$) driving the $Na^+$-$Ca^{2+}$ exchange (NCX) in reverse mode.

The increase in proton extrusion carried by the activated NHE might, by increasing $pH_i$, cause an increase in myofilament $Ca^{2+}$ responsiveness[7–9] that would also contribute to the increase in force during the SFR. However, although the rise in $pH_i$ is easily detected in the absence of bicarbonate, it is minimized or even canceled when there is $CO_2$ bicarbonate buffer in the medium.[3] This observation seems to result from a simultaneous activation of a bicarbonate-dependent acid loading mechanism after the release of endogenous angiotensin II/endothelin (Ang II/ET) by stretch which counteracts the effect of NHE stimulation.[3–5,10] The extrusion of intracellular $HCO_3^-$ ions, necessary to compensate for the increase in $pH_i$ that will otherwise occur because of NHE hyperactivity, seems to be carried by the $Na^+$-independent $Cl^-$-$HCO_3^-$ exchanger (AE). There are three different isoforms of the AE encoded by three different genes, AE1, AE2, and AE3.[11] In heart, all three AE gene products have been detected at the level of mRNA,[12–17] but the function of individual isoforms is under current investigation. Although AE3 is the most abundantly expressed isoform in cardiac tissue, there is one report that suggests that an AE1 variant is involved in the extrusion of $HCO_3^-$ in rat ventricular myocytes and that the other isoforms may have non–pH-regulatory functions.[18] The AE3 products found in cardiac tissue are the AE3c and the AE3fl isoforms.[15,17] Because at present the available pharmacological inhibitors of AE activity such as stilbene derivatives, do not distinguish between different AE isoforms and also have inhibitory effects on other anion exchangers and channels,[19,20] we developed an antibody common to both the AE3c and AE3fl isoforms to study the role of the AE3 in the compensatory mechanism that follows NHE-1 activation after stretch.

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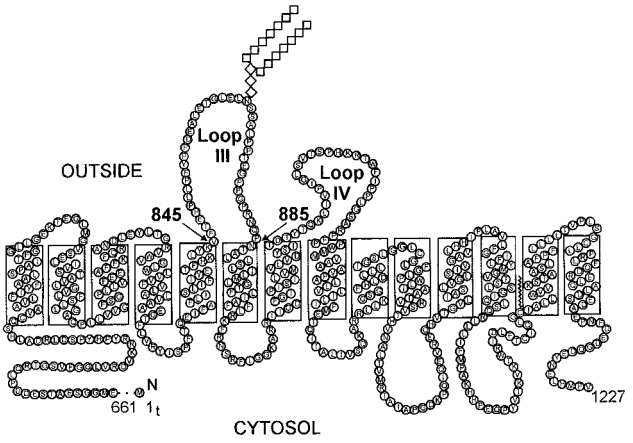


**Figure 1.** Transmembrane topology model for rat AE3. AE3 is the most abundant AE isoform in cardiac tissue and 2 different AE3 variants, AE3fl (1227 amino acids long) and AE3c (1034 amino acids), are coexpressed. The AE3c, the most abundant AE3 protein expressed in myocardium, contains a 73 amino acid unique N-terminal sequence that replaces the 270 amino acid unique N-terminal of the AE3fl. Isoforms show a high homology in the amino acid sequence of the transmembrane domains and extracellular loops. Topology model was developed by homology to AE1 and an existing AE1 topology model.[44] A polyclonal rabbit antibody raised against the third extracellular loop of AE3 (Loop III, between amino acids 845 to 885) was developed in our laboratory and assayed as an inhibitor of cardiac AE activity.

## Materials and Methods

Cat papillary muscles (n=34) from the right ventricle were mounted in a perfusion chamber placed on the stage of an inverted microscope for epifluorescence measurements and bathed with either a $CO_2$-$HCO_3^-$ or HEPES-buffered solution of a previously reported composition.[3,4] The muscles were paced at 0.2 Hz at a voltage 10% over threshold and isometric contractions were recorded. Cross sectional area (0.75 of the product of thickness by width) was $0.66 \pm 0.07$ mm$^2$, and it was used to normalize force records obtained with a silicon strain gauge. The stretch protocol, as previously described,[5] consisted in one abrupt stretch from 92% to 98% of $L_{max}$. All experiments were performed at 30°C. The effect of stretch on DF, intracellular $Na^+$, $Ca^{2+}$, and $H^+$ concentrations (measured with SBFI, Fura-2, and BECEF, respectively) was assessed in muscles in which AE was operative (bicarbonate-buffered medium) or inactivated by either removal of extracellular bicarbonate (HEPES-buffered medium) or the presence of the anti-AE3Loop III antibody (Figure 1). Detailed description of the development and characterization of the antibody and/or ionic determinations are in the expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org.

### Statistics

Data are expressed as mean±SEM. Student's *t* test or one-way ANOVA followed by Bonferroni's test were used as appropriate. A value of $P<0.05$ was considered to indicate significant differences.

## Results

### Characterization of the Rabbit Polyclonal Antibody Against AE3 Isoforms (anti-AE3Loop III)

The specificity of the rabbit polyclonal antibody (anti-AE3Loop III) for the AE3 isoforms was confirmed by immunoblot analysis with HEK293 cells transfected with AE3fl and AE3c isoforms, respectively (Figure 2A, lanes 1 and 2). This antibody also recognized both isoforms in rat and cat cardiac membranes (Figure 2A, lanes 3 and 4, respectively). Isoform specificity was also shown by the failure of the antibody to detect human AE1 on immunoblots (not shown). In addition to the specific recognition of the AE3 proteins in cardiac membranes, the anti-AE3Loop III showed an inhibitory property on bicarbonate-dependent acid loading mechanisms because it was able to decrease the rate of myocardial $pH_i$ recovery when exposed to acute intracellular alkaline loads (Figure 2B). The velocity of $pH_i$ recovery from intracellular alkalosis is a previously validated technique to analyze the acid-loading mechanisms.[21–23] To this purpose, intracellular $pH_i$ was suddenly increased by exposing the muscles during 10 minutes to trimethylamine chloride (TMA, inset of Figure 2B). The rate of $pH_i$ recovery was assessed by the slope of the $pH_i$ decay after peak intracellular alkalosis was attained. In order to induce different levels of intracellular alkalosis, three different TMA concentrations (20, 30, or

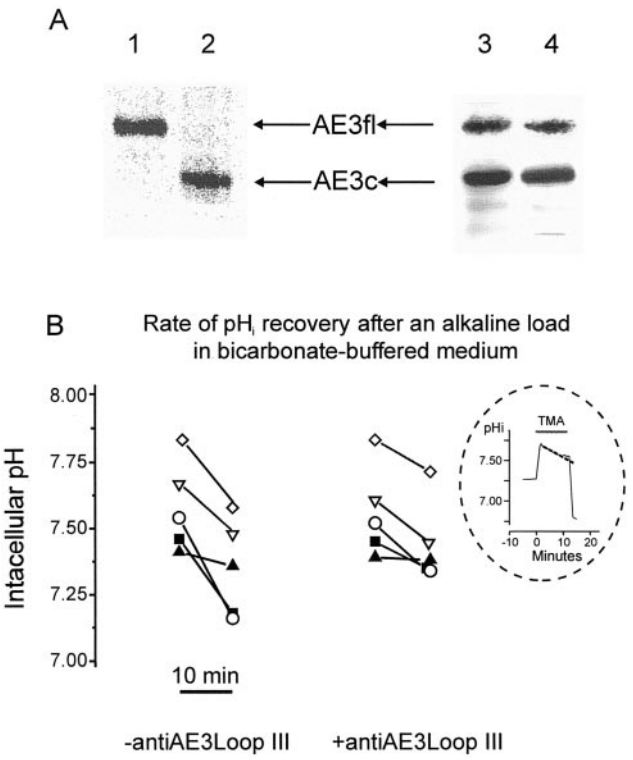


**Figure 2.** Characterization of the rabbit polyclonal anti-AE3Loop III. A, Immunoblot analysis with HEK293 cells transfected with rat AE3fl (lane 1) or AE3c cDNAs (lane 2) and with rat (lane 3) and cat (lane 4) cardiac membranes. Because Loop III is common to AE3 isoforms, the antibody specifically recognized both isoforms expressed in HEK293 cells and cardiac membranes. B, Effect of anti-AE3Loop III on the rate of $pH_i$ recovery from intracellular alkaline loads in papillary muscles bathed with $CO_2$/bicarbonate-buffered medium. Inset, Representative experiment showing the increase in $pH_i$ induced by sudden exposure to trimethylamine chloride (TMA) and the regression line (broken tracing) fitted to estimate the rate of $pH_i$ recovery. The TMA-induced alkaline load was performed in the absence (−antiAE3Loop III) and presence (+antiAE3Loop III) of antibody. Each symbol indicates a different preparation, and the connecting lines represent the rate of $pH_i$ recovery (pH unit/min). Muscles were exposed to 20, 30, or 40 mmol/L TMA in order to reach different peak $pH_i$ values. Note that the recovery rate was decreased by the antibody at any $pH_i$ value. Antibody does not affect myocardial buffer capacity because similar alkaline loads were obtained in its presence.

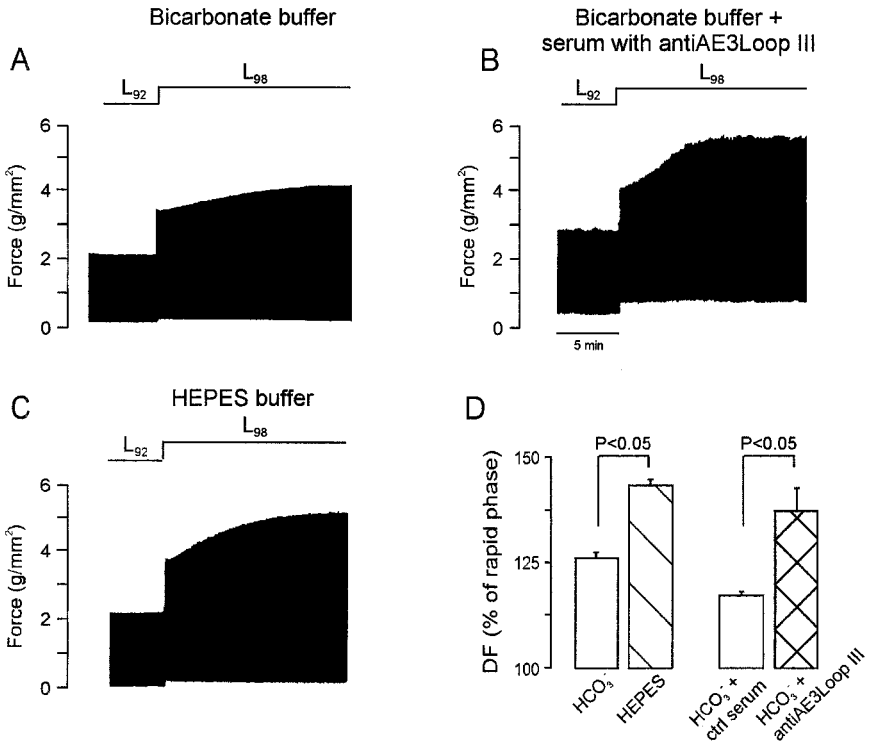


**Figure 3.** Force response to stretch in isolated cat papillary muscle. Typical slow records of the change in force after stretching cat papillary muscles under conditions that either allowed (A) or prevented AE activity by anti-AE3Loop III (B), or by removal of extracellular bicarbonate (C). Notice that when AE activity was absent the SFR was larger. D, Bars show the SFR magnitude 15 minutes after stretch, as percentage of the initial rapid phase.

40 mmol/L) were used, only one of them on each muscle, both in the absence (1:100 control serum) and in the presence of the antibody (1:100 serum containing anti-AE3Loop III). Figure 2B shows that the slope of the $pH_i$ recovery phase (denoted by the lines connecting each pair of symbols) was reduced in the presence of the antibody. On average, the rate of $pH_i$ recovery was reduced by 55±14% in the presence of anti-AE3Loop III. When only the last 5 minutes of the recovery phase were computed, in order to ensure complete equilibration of the $CO_2$-$HCO_3^-$ buffer system,[23] the reduction of the velocity of $pH_i$ recovery by the antibody was even greater (68±15%). However, the inhibition of $pH_i$ recovery was not complete. Although unlikely, (see later sections) the possibility exists that the amount of antibody used was not enough to block all the AE3 units. Alternatively, we should also consider that in addition to AE3 other isoforms, including the $Cl^-$-$OH^-$ (CHE),[23] contribute to the recovery of $pH_i$ during alkalosis. Nevertheless, these results support that extrusion of $HCO_3^-$ from myocardial cells after an alkaline load is influenced by the AE3 isoforms.

## Influence of AE Activity on the SFR to Stretch

Figures 3A through 3C shows typical records of the changes in DF elicited when papillary muscles were stretched under experimental conditions that would allow ($CO_2$ bicarbonate-buffered medium) or prevent AE activity (HEPES or AE inhibition by anti-AE3Loop III). The initial rapid phase of the increase in force was of similar magnitude under all experimental conditions. Immediately after the stretch DF increased, on average, from 2.56±0.13 g/mm$^2$ to 3.76±0.18 (n=8) in HEPES, from 2.18±0.13 to 3.42±0.17 (n=8) in bicarbonate, from 2.67±0.29 to 3.71±0.49 (n=3) in bicarbonate plus control serum, and from 2.60±0.31 to 3.88±0.46 (n=6) in bicarbonate with serum+anti-AE3Loop III. However, the increase in DF during the SFR was larger in those circumstances in which AE activity was prevented, ie, in HEPES buffer and after AE inhibition with the anti-AE3Loop III. Figure 3D shows the overall results of this series of experiments. When AE activity was prevented (HEPES buffer or bicarbonate buffer+anti-AE3Loop III), the magnitude of the SFR was about 2-fold larger than when AE was contributing to $pH_i$ regulation (bicarbonate or bicarbonate plus control serum).

One possibility to explain the larger SFR in HEPES-buffered medium and after inhibition of AE activity by anti-AE3Loop III is that under these experimental conditions (AE3 inactive), stretch-induced NHE activation causes an increase in $pH_i$. A rise in $pH_i$ may increase contractility by increasing myofilament $Ca^{2+}$ responsiveness.[7–9]

Figure 4A shows that stretch caused a significant elevation of $pH_i$ in the absence of bicarbonate, where AE is not operative in accordance with our previous reports.[3,4] In the presence of bicarbonate but with AE3 isoforms inactivated by the antibody, a rise in $pH_i$ was also detected (Figure 4B). Under this condition, however, the rise in $pH_i$ was of lesser magnitude. The difference can be explained by the fact that in the presence of bicarbonate cell buffering power increases by 2.3 times,[24] thereby attenuating the change in $pH_i$ produced by a given $H^+$ efflux or influx ($J_{H+}$). When calculated, the $J_{H+}$ values with and without bicarbonate were 1.37±0.21 (n=5) versus 1.42±0.32 (n=6) mmol/L per min, respectively (NS). In other words, although the increase in $pH_i$ when the antibody inhibits AE3 activity was lesser in bicarbonate than in the absence of bicarbonate, the magnitude of $J_{H+}$ was similar. These results, therefore, provide experimental evidence to support our previously proposed hypothesis[3] that the

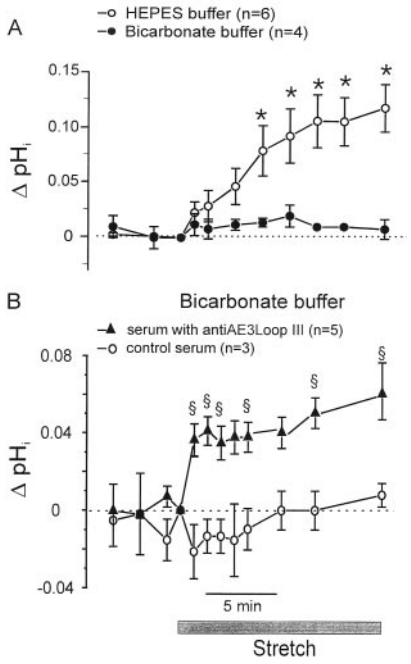


**Figure 4.** Effect of stretch on myocardial $pH_i$. A, Stretch induced a rise of $pH_i$ in muscles bathed with HEPES-buffered medium that was absent in those bathed with bicarbonate buffer. *$P<0.05$ vs bicarbonate. B, When AE activity was inhibited by anti-AE3Loop III, $pH_i$ rose after stretch despite the presence of extracellular bicarbonate. Note the different scale in A and B. §$P<0.05$ vs control serum.

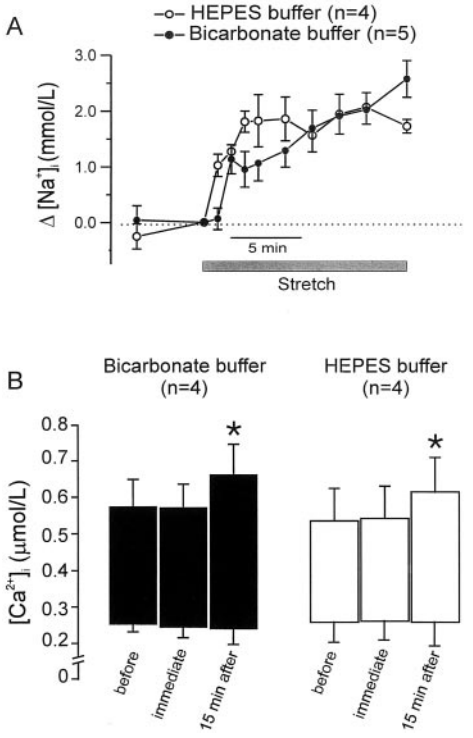


**Figure 5.** Changes in $[Na^+]_i$ and $Ca^{2+}$ transients after myocardial stretch in the absence and in the presence of bicarbonate. Myocardial stretch caused a similar rise in $[Na^+]_i$ in the presence and in the absence of extracellular bicarbonate (A). B, Shows changes in intracellular $Ca^{2+}$ after stretch. Bar bottom limits indicate diastolic $Ca^{2+}$ levels, whereas bar upper limits indicate peak $Ca^{2+}$ values. Before, immediate, and after 15 minutes refers to before stretch, immediately after stretch, and after 15 minutes of stretch values, respectively. Note that peak systolic $Ca^{2+}$ did not change immediately after stretch, but was larger after 15 minutes. Increase in peak systolic $Ca^{2+}$ was of similar magnitude in HEPES buffer compared bicarbonate, (n=4 each). Diastolic $Ca^{2+}$ did not change significantly at any time after stretch.

lack of $pH_i$ changes after myocardial stretch in bicarbonate-buffered medium could be due to compensation through AE activity of the stretch-induced NHE-1 stimulation. At the same time, the data indicate that the AE3 isoforms are the ones involved in the phenomenon because the acid-loading blockade with the antibody causes an effect on $pH_i$ of the same magnitude as the removal of bicarbonate. In addition, further support of stretch-induced simultaneous stimulation of NHE-1 and AE activity is given by the fact that myocardial stretch in the presence of bicarbonate causes a fall in $pH_i$ when NHE-1 activity is inhibited with 1.0 $\mu$mol/L HOE 642. Under this condition, we found a decrease in $pH_i$ by $0.03\pm0.01$ pH units (n=4, $P<0.05$) that peaked in 3 to 4 minutes and gradually faded away. The mechanism(s) underlying the fading is currently under study but we should keep in mind that the $Na^+$-$HCO_3^-$ cotransport (NBC) may be blunting the decrease in $pH_i$. The activation of NBC by Ang II was reported.[25,26]

Because myofilament responsiveness is a function of $pH_i$ and not of $J_{H+}$, it is reasonable that the mechanical response to stretch will be lesser in a more buffered tissue, being other factors constant, than in bicarbonate-free buffer ($133.2\pm3.84\%$, n=6, and $142.8\pm1.2\%$, n=8, respectively; $P<0.05$) as shown in Figure 3D. It is also reasonable to expect that the difference in the magnitude of SFR in HEPES compared with bicarbonate alone were even larger ($142.8\pm1.2\%$ versus $125.7\pm1.3\%$, n=8; $P<0.05$) because the $pH_i$ difference was also larger ($\approx$0.10 pH units). Of note, a 20% increase in contractility was reported to occur in response to a rise in $pH_i$ of $\approx$0.10 pH units due to respiratory alkalosis in ferret perfused whole hearts.[27]

## Stretch-Induced Increase of $[Na^+]_i$ and $Ca^{2+}$T in the Absence and Presence of Extracellular Bicarbonate

The increase in $[Na^+]_i$ mediated by stretch-induced stimulation of NHE activity in the absence and presence of bicarbonate is shown in Figure 5A. Without bicarbonate, $[Na^+]_i$ rose from a baseline value of $4.2\pm1.8$ to $6.0\pm1.8$ mmol/L (n=4), whereas in its presence, $[Na^+]_i$ increased from $5.4\pm1.3$ to $7.8\pm1.3$ mmol/L (n=5, NS) after the stretch.

If the increase in $Ca^{2+}$T occurring during the SFR were produced by a reverse mode of the NCX promoted by the rise in $[Na^+]_i$, it should also be of similar magnitude in the presence and absence of bicarbonate, and this was the case. Figure 5B summarizes the data of intracellular $Ca^{2+}$ levels measured before, immediately after and 15 minutes after stretch. As previously reported,[2,3] the peak systolic $Ca^{2+}$ did not change immediately after stretch, but increased significantly during the development of the SFR. Fifteen minutes after the stretch, values of peak systolic $Ca^{2+}$ were $15.4\pm5.4\%$ and. $12.3\pm3.9\%$ over

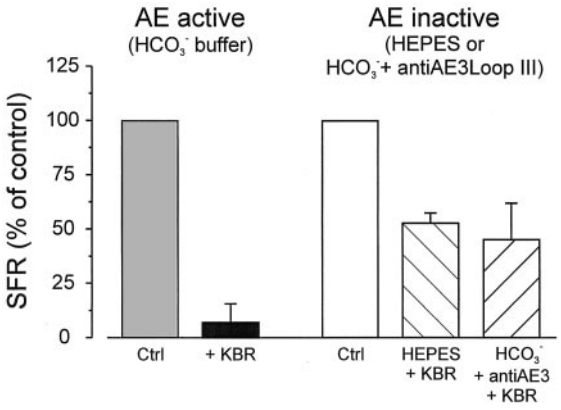


**Figure 6.** Effect of inhibition of reverse mode of NCX with KB-R7943 on the SFR. KB-R7943 completely suppressed the SFR when there was AE activity (bicarbonate buffer) but only reduced it by ≈50% when AE activity was absent (HEPES buffer or anti-AE3Loop III). Results are expressed as percentage of control (C) in each series of experiments.

baseline, in the presence and absence of bicarbonate respectively (n=4 each, NS). These data together with the lack of changes in diastolic $Ca^{2+}$ at any time during the SFR are in agreement with previous reports.[2,3]

### Effect of KB-R7943 on the SFR in the Presence and Absence of AE Activity

To further investigate the differences in the SFR magnitude and the mechanisms involved in its development, we explored the effect of reverse mode of NCX inhibition with KB-R7943 in the presence and absence of AE activity. To do this, the effect of KB-R7943 on the SFR in HEPES buffer or in bicarbonate plus the anti-AE3Loop III was compared with that observed when AE was operative and able to compensate stretch-induced changes in $pH_i$ (bicarbonate-bathed muscles). Figure 6 shows that KB-R7943 almost completely suppressed the SFR when AE was active, whereas it only decreased the SFR by ≈50% when $pH_i$ was not compensated (inactive AE). The KB-R7943 insensitive fraction of the SFR likely results from an increase in myofilament responsiveness due to $pH_i$ increase,[7–9] because a larger SFR was elicited with similar increments in $Ca^{2+}$T. This contention is further supported by the comparison of the twitches and $Ca^{2+}$T shown in Figure 7 that were obtained in muscles with active (A) or inactive (B) AE at the time of complete SFR development. Although the amplitude of the $Ca^{2+}$T is of about similar magnitude under both conditions, DF is larger (5.4±0.2 versus 4.3±0.2 g/mm$^2$, n=8 each; $P<0.05$) and the maximal velocity of force development faster (22.3±1.3 versus 16.4±1.3 g/mm$^2$ per second; $P<0.05$) when the AE was rendered inactive by the removal of bicarbonate from the medium (Figure 7B). In addition, when AE was inactivated, twitch relaxation was slower as assessed by $t_{50}$, $t_{90}$, and $\tau$ values (Table). An increase in myofilament responsiveness should be accompanied by a decrease in magnitude and shortening of the $Ca^{2+}$T. The increase in peak systolic $Ca^{2+}$ was slightly lesser in the absence of bicarbonate although the difference did not reach statistical significance. The prolongation of the $Ca^{2+}$T may suggest changes in intracellular $Ca^{2+}$ handling, although

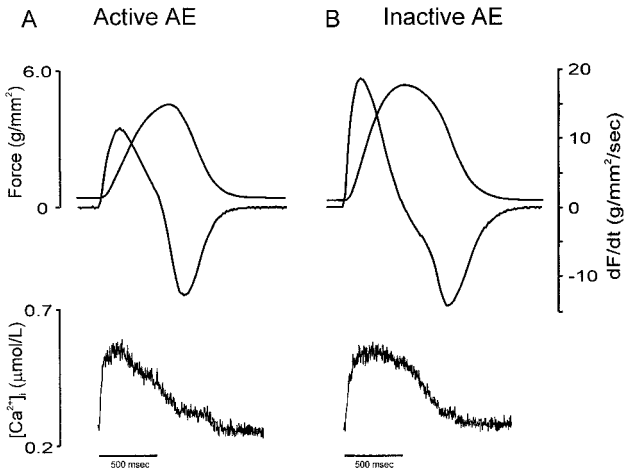


**Figure 7.** Comparison of the twitches and $Ca^{2+}$T after 15 minutes of stretch in muscles with active and inactive AE. Representative records of twitch force, its first derivative and the corresponding $Ca^{2+}$T are shown from top to bottom. Note that the amplitude of the $Ca^{2+}$T is similar under both conditions, but DF is larger, the maximal velocity of force development is faster, whereas relaxation of twitch force and $Ca^{2+}$T are slower in the presence of inactive AE.

modifications in the rate of crossbridge attachment and detachment[28] cannot be ruled out. About $Ca^{2+}$ handling, two main mechanisms determine the rate of $Ca^{2+}$T decay: the SR $Ca^{2+}$ uptake and the NCX-mediated $Ca^{2+}$ efflux. Alkalosis was shown to increase the NCX-mediated $Ca^{2+}$ efflux,[29] which would accelerate the decay of the $Ca^{2+}$T. Instead, the activity of type 1 phosphatase, which controls the level of phospholamban phosphorylation, is activated by intracellular alkalosis[30] and this may consequently be reducing the rate of $Ca^{2+}$ uptake (see also 'Limitations of This Study).

## Discussion

The results presented herein show that the autocrine/paracrine (Ang II/ET) loop triggered by stretch[4,5,8] simultaneously activates the NHE and AE activities and that the interplay between these two mechanisms determines the effect of stretch on myocardial $pH_i$ and the contractile response. When the increase in $H^+$ extrusion through activated NHE is compensated by $HCO_3^-$ efflux through the AE, there is no change in $pH_i$ and the SFR is totally due to the increase in $Ca^{2+}$T through the reverse mode of NCX promoted by the rise in $Na^+_i$. When AE activity is prevented, the $Ca^{2+}$T increases similarly but myocardial $pH_i$ rises after stretch and the SFR is larger probably because a $pH_i$-dependent increase in myofilament $Ca^{2+}$ responsiveness contributes to force development in addition to the increase in $Ca^{2+}$T.

The fact that the stretch of isolated cardiomyocytes is followed by release of preformed Ang II, acting in an

**Twitch Relaxation Indexes**

| Condition | $t_{50}$, ms | $t_{90}$, ms | $\tau$, ms | n |
|---|---|---|---|---|
| Active AE (bicarbonate) | 277.6±17.9 | 470.5±24.4 | 327.6±18.1 | 8 |
| Inactive AE (HEPES) | 373.8±23.4 | 583.8±30.9 | 427.5±25.6 | 8 |
| *P* | <0.05 | <0.05 | <0.05 | |

autocrine/paracrine fashion, has been known since the work of Sadoshima and Izumo.[31] In the same preparation, Ito et al[32] demonstrated that Ang II induced the release of ET-1 and upregulation of preproET-1 mRNA levels whereas Yamazaki et al[33] showed that stretched cardiomyocytes released ET-1 to the surrounding culture medium. The chain of events linking the autocrine-paracrine loop triggered by the stretch and the SFR has been proposed by us since 1999,[3] when we provided pharmacological evidence that Ang II and ET in a PKC-dependent pathway activate the NHE-1 leading to the increase in $Na^+_i$ and reverse mode of NCX. Interestingly, Bluhm et al[34] analyzing the potential contribution of sarcolemmal ion fluxes to the SFR development in an ionic model of cardiac myocytes, found that the SFR could be mimicked by an increase in $[Na^+]_i$ that concurred with an increase in $Ca^{2+}$ entry through the NCX.

Stretch-induced NHE-1 hyperactivity will be accompanied by an increase in $pH_i$ and in $Na^+_i$ in the absence of bicarbonate and also when the AE3 isoforms are inactivated. In either of these conditions, the increase in contractility will be a function of both alterations: the rise in $pH_i$ and in $Ca^{2+}$T. If AE3 is operative, $pH_i$ will not rise and the increase in $Na^+_i$ by increasing the $Ca^{2+}$T, will be the cause of the SFR.

As expected when the AE was inactive and $pH_i$ rose, the blockade of the reverse mode of operation of NCX only suppressed part of the mechanical change because the contractile effect of the increase in $pH_i$ remained present (Figure 6). However, under more physiological conditions where no change in $pH_i$ took place, the SFR was totally due to the increase in $Ca^{2+}$T and could be abolished by inhibiting the NCX in reverse mode or preventing the increase in $Na^+_i$.[3,5]

Boron's group reported in 1989[35] that the resulting $pH_i$ after growth factors stimulation of renal mesangial cells in the presence of bicarbonate, will depend on the interplay between the activity of the acid extruders and the alkaline extruder, the AE. The latter mechanism, first described in myocardium by Vaughan-Jones,[36] is known to be regulated by increases in $pH_i$, ATP, $\beta$-adrenergic agonists, and aldosterone, as well as by growth factors.[21,36–40]

The fact that Ang II and ET-1 stimulate NHE and AE activities[21,40] in a PKC-dependent manner makes it difficult to dissect if a common intracellular pathway or different PKC isoforms or downstream effectors are involved in the activation of both exchangers. On one hand, it has been shown by some authors of the present study that exogenous applied Ang II induces AE3 activation by PKC-$\epsilon$–dependent phosphorylation of the AE3fl isoform on Ser67.[41] Myocardial stretch probably shares the same pathway. On the other hand, Moor and Fliegel[42] demonstrated that ET induces NHE-1 stimulation through the 42/44 mitogen-activated protein kinase and p90, which are both known as downstream effectors from PKC.

Because the antibody against the third extracellular loop binds to both AE3c and AE3fl, we were unable to dissect if one or both of these isoforms is/are responsible for the extrusion of $HCO_3^-$ from the cell counteracting the increase in proton efflux through NHE-1. Nevertheless, our data are in agreement with those of Yannoukakos et al[43] who demonstrated that both AE3 isoforms have the ability to transport Cl in exchange for $HCO_3^-$, but not with those reported by Richards et al.[18] After myocardial stretch (when enhanced AE activity was probably the result of Ang II/ET action), the antibody was able to fully block the acid loading process to the same extent as when external bicarbonate was removed to render inactive the AE. It can be therefore speculated that different AE isoforms may be the target for different interventions. Perhaps the AE3 isoforms, and particularly the AE3fl, are the ones linked to PKC-dependent activation. In addition to be PKC-$\epsilon$–dependent phosphorylated by Ang II,[41] the AE3fl isoform is upregulated in the hypertrophied myocardium of the SHR where $pH_i$ is compensated in spite of the enhanced NHE-1 activity.[17]

## Limitations of the Study

There are several limitations of our study that deserve to be examined.

(1) KB-R7943, in addition to inhibiting the reverse mode of NCX, can also inhibit $Na^+$, L-type $Ca^{2+}$, and $K^+$ currents.[44,45] The possibility therefore exists, that the compound was interfering with some other mechanism(s) in addition to inhibit NCX in reverse. However, in our hands and at the concentration used (5.0 $\mu$mol/L), we were unable to detect any significant effect of KB-R7943 on basal contractility of cat papillary muscles or on the increase in contractility (20%) caused by increasing extracellular $[Ca^{2+}]$ (unpublished results, 2003). This lack of KB-R7943 effect contrasts with the ability of the compound to cancel the increase in contractility after the rise in $[Na^+]_i$ due to stretch-induced NHE-1 activation and suggests that in our experimental model and at the concentration used, it was primarily blocking the NCX in reverse.

(2) The effects of stretch on $[Na^+]_i$ were compared in the absence and presence of bicarbonate. However, in the presence of bicarbonate, the activity of the NBC might play a role in determining $[Na^+]_i$. In spite that we did not detect a statistically significant difference between the stretch-induced rise of $[Na^+]_i$ with or without bicarbonate, the increase in $[Na^+]_i$ showed a tendency to be larger in bicarbonate (see Figure 4), an effect that can be due to the activation of NBC. The activation of this exchanger by Ang II has been previously reported.[25,26]

(3) A careful analysis of the kinetics of the changes in $pH_i$, $[Na^+]_i$, $[Ca^{2+}]_i$, and DF after stretch in the presence and absence of bicarbonate was not performed. When bicarbonate was absent, the rise in $pH_i$ was gradual, but when the AE3 isoforms were inactivated by the antibody the change in $pH_i$ was more abrupt. The reason for this difference is not apparent to us. However, we should keep in mind that in the latter condition, other bicarbonate-dependent mechanisms like the above mentioned NBC might be playing a role.

(4) A prolongation of the $Ca^{2+}$T was detected in the absence of bicarbonate, whereas an increase in $pH_i$ took place. The mechanism for this prolongation of the $Ca^{2+}$T is not known at present. One potential explanation could be a $pH_i$ dependency of the Fura-2 fluorescence ratio, which was shown to increase with increasing $pH_i$.[46]

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