Stretch-Induced Alkalinization of Feline Papillary Muscle
An Autocrine-Paracrine System

H.E. Cingolani, B.V. Alvarez, I.L. Ennis, M.C. Camilión de Hurtado

Abstract—Myocardial stretch is a well-known stimulus that leads to hypertrophy. Little is known, however, about the intracellular pathways involved in the transmission of myocardial stretch to the cytoplasm and nucleus. Studies in neonatal cardiomyocytes demonstrated stretch-induced release of angiotensin II (Ang II). Because intracellular alkalinization is a signal to cell growth and Ang II stimulates the Na^+/H^+ exchanger (NHE), we studied the relationship between myocardial stretch and intracellular pH (pH_i). Experiments were performed in cat papillary muscles fixed by the ventricular end to a force transducer. Muscles were paced at 0.2 Hz and superfused with HEPES-buffered solution. pH_i was measured by epifluorescence with the acetoxymethyl ester form of the pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM). Each muscle was progressively stretched to reach maximal developed force (L_{max}) and maintained in a length that was ≈92% L_{max} (L_i). During the "stretch protocol," muscles were quickly stretched to L_{max} for 10 minutes and then released to L_i; pH_i significantly increased during stretch and came back to the previous value when the muscle was released to L_i. The increase in pH_i was eliminated by (1) specific inhibition of the NHE (EIPA, 5 μmol/L), (2) AT_1-receptor blockade (losartan, 10 μmol/L), (3) inhibition of protein kinase C (PKC) (chelerythrine, 5 μmol/L), (4) blockade of endothelin (ET) receptors with a nonselective (PD 142,893, 50 nmol/L) or a selective ET_A antagonist (BQ-123, 300 nmol/L). The increase in pH_i by exogenous Ang II (500 nmol/L) was also reduced by both ET-receptor antagonists. Our results indicate that after myocardial stretch, pH_i increases because of stimulation of NHE activity. This involves an autocrine-paracrine mechanism in which protein kinase C, Ang II, and ET play crucial roles. **(*Circ Res*. 1998;83:775-780.)**

Key Words: stretch, myocardial ■ pH, intracellular ■ Na^+/H^+ exchange ■ angiotensin ■ endothelin

Although it is well known that mechanical stimuli cause a variety of effects on the structure and function of the myocardial cells, little is known about how cells sense the mechanical stimuli, transmit the information to messenger systems, and finally regulate function and growth.[1,2] Highly regarded experiments demonstrate that the release of angiotensin II (Ang II) contributes to stretch-induced hypertrophy in cultured neonatal cardiac myocytes,[2–5] and that the effect was suppressed by the AT_1-receptor antagonist TCV-116.[4] The release of Ang II may involve an autocrine or paracrine mechanism because stretch-conditioned media mimicked the effect of stretch when transferred to nonstretched neonatal cardiomyocytes.[2] An increase in PKC activity is an effect detected after stretching cultured neonatal cardiomyocytes[2] and the adult heart.[6] Because Ang II, by mechanisms still unresolved but probably linked to PKC, activates the Na^+/H^+ exchanger (NHE),[7–8] the logical expectation is a rise in intracellular pH (pH_i) after myocardial stretch. Although we are unaware of measurements of myocardial pH_i before and during stretch, pressure overload increased NHE-1 mRNA levels in hearts.[9] Furthermore, the activity of mitogen-activated protein kinase (MAP kinase) was increased by stretch in cultured cardiomyocytes, and this increase was partially eliminated by NHE inhibition.[9] Nevertheless, an unknown is whether stretch alters NHE activity in multicellular preparations from adult hearts. This point is critical, because Ang II released after stretch might increase NHE activity[10–14] and promote the expression of endothelin (ET) as well as the upregulation of ET receptors.[15] ET can also increase myocardial pH_i through the activation of NHE.[16,17]

Experiments in feline papillary muscles were designed to monitor myocardial pH_i before and after stretch. Our results indicate that after myocardial stretch, the increase in myocardial pH_i is an early intracellular signal involving an autocrine-paracrine system.

Materials and Methods

The experiments were performed in cat papillary muscles (mean cross-sectional area, 0.38±0.03 mm^2) dissected from the right ventricle. The animals were euthanized under anesthesia (pentobarbital sodium 35 mg/kg IP), and the hearts were quickly removed. The muscles were mounted in an experimental chamber placed on the stage of a CK2 Olympus inverted microscope. The general procedures for dissecting and mounting the muscles have been described by Camilión de Hurtado et al.[18] Briefly, each muscle was fixed to a

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hook by a silk thread tied at the chorda-muscle junction. The hook was attached to a micrometer allowing careful controlled stretching of the preparation. The ventricular end of the muscle was rigidly fixed to a force transducer to monitor developed tension. Muscles were paced with square wave pulses of 10 ms-duration and a voltage intensity 10% above threshold applied via 2 platinum wires running parallel to the preparations. Experiments were performed at 30°C, and the muscles were superfused (constant flow rate, 4.0 mL/min) with a HEPES-buffered solution to avoid the participation of bicarbonate-dependent mechanisms in the regulation of myocardial pH_i. The composition of the superfusing solution was (in mmol/L): NaCl 146.2, KCl 4.5, $CaCl_2$ 1.35, $MgSO_4$ 1.05, glucose 11.0, and HEPES 5.0. The pH of the solution was adjusted to 7.4 with 3 mol/L NaOH (total Na^+ concentration, 148.5 mmol/L) and gassed with 100% O_2.

Measurements of pH_i in the isolated muscles were made after loading the muscles with the acetoxymethyl ester form of the pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM, Molecular Probes), as previously described.[18] Washout of the extracellular space with dye-free solution was continued until stable fluorescence signals were obtained (usually within 90 minutes). BCECF fluorescence was excited at 450 and 495 nm, and the fluorescence emission monitored after passage through a 535±5 nm filter. To limit photobleaching, a neutral-density filter (1% transmittance) was placed in the excitation light path, and a manual shutter was used to select sampling intervals (for 3 seconds every 15 seconds) during the protocol. When the experiments began, fluorescence at H^+-insensitive wavelength was usually 5× greater than the original autofluorescence. At the end of each experiment, fluorescence emission was calibrated by the high K^+–nigericin method.[19] The calibration solution contained (in mmol/L): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 5.0, nigericin 0.01, sodium cyanide 4.0, and 2,3-butanedionemonoxime 20.0.[18] Buffer pH was adjusted with KOH to 4 different values ranging from 7.5 to 6.5. This calibration gave a linear relation (r=0.95±0.03, n=32) between buffer pH values and the fluorescence ratio (F_{495}/F_{450}). The fluorescence ratio was calculated as follows: ratio=$\text{fluorescence}_{495}-\text{autofluorescence}_{495}/\text{fluorescence}_{450}-\text{autofluorescence}_{450}$. When muscles are stretched, their volume that is visible to the photomultiplier is reduced, and consequently, the autofluorescence and pH-related signals may decrease. We found that in unloaded muscles the autofluoresece at 450 and 495 nm changed by 1.45±1.58% and 0.93±0.98%, respectively, which was not significantly different from zero (P>0.05, t test). Moreover, since BCECF is a ratio indicator, the importance of the change is greatly reduced because both recorded signals are almost equally affected, and therefore the change is largely eliminated by the use of ratios.

The experimental protocol designed for our study was as follows: after mounting, the values of slack length (L_s) and width of each muscle were determined with a reticle in the eyepiece of a SZ30 Olympus Zoom stereomicroscope set at a total magnification of ×30. Because the geometry of each long, slender papillary muscle approximates a cylinder, muscle cross-sectional area was calculated with the use of width/2 as radius value. Each muscle was progressively stretched to reach the length at which the force developed during the twitch was maximal (L_{max}). After L_{max} was determined, muscle length was reduced to ≈92% of L_{max}, and this length was maintained for 2 hours. Afterward, the muscle was stretched by manual adjustment of the micrometer to the previously determined value of L_{max}, and then released to the previous length. In the majority of the experiments, the stretch lasted 10 minutes; however, in a group of 4 muscles, the stretch was maintained for a longer period to assure pH_i had reached a steady value. Stretching of the muscles caused resting and developed tension to increase by 128±40% and 167±3%, respectively.

pH_i was measured before, during, and after the stretch protocol under control conditions and after treatment with 1 of the following compounds: 5 μmol/L 5-(N-ethyl-N-isopropyl)amiloride (EIPA), 5 μmol/L chelerythrine chloride, 10 μmol/L losartan, 50 nmol/L PD 142,893, and 300 nmol/L BQ-123. All compounds were purchased from Biochemical Research International, with the exception of losartan, which was a kind gift from Dupont Merck (West Point, Pa). The pH_i response to exogenous Ang II and ET-1 (Sigma-Aldrich) was assessed in the absence or presence of AT_1- or ET-receptor antagonists.

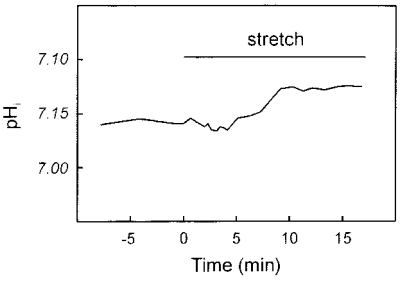

Figure 1. Time course of stretch-induced increase in pH_i. This figure shows an experiment in which the stretch protocol lasted for more than 10 minutes.

Statistics

Data were expressed as mean±SEM, and analyzed by the Student t test, repeated-measures ANOVA, or 1-way ANOVA, as appropriate. Values of P<0.05 were considered significant.

Results

In pilot experiments, we observed that stretch induced an increase in pH_i that reached a steady value in about 10 minutes. Figure 1 shows the results of 1 of these experiments. For this reason, the stretch protocol for the subsequent experiments was restricted to 10 minutes. Figure 2A shows the changes in pH_i in a representative experiment in which the muscle was stretched during 10 minutes and then released. Myocardial pH_i returned to baseline value after the release of the muscle. The pH_i values before and after stretch, as well as the maximal attained during the 10-minute stretch protocol, are shown in Figure 2B (n=11). The increase in pH_i in the overall experiments reached a maximum of 0.09±0.01 (P<0.05) in 8.31±0.71 minutes (n=15).

Because the only pH_i regulatory mechanism in the absence of bicarbonate is the NHE, an activation of this exchanger probably mediates the decrease in intracellular proton concentration. Similar experiments to those shown in Figure 1 were repeated in the presence of 5 μmol/L EIPA (Figure 3).

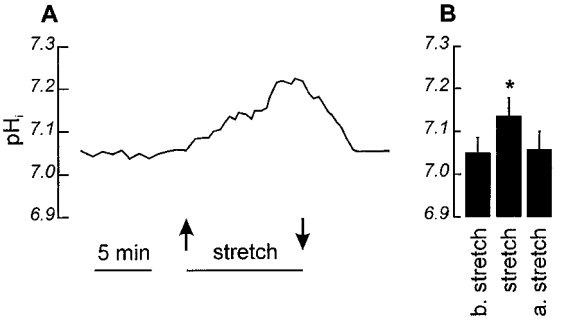

Figure 2. Stretch-induced intracellular alkalinization. A, Results of a representative experiment in a papillary muscle superfused with a bicarbonate-free (eg, HEPES-buffered) solution. Muscle length was kept at 92% of L_{max}, suddenly stretched to L_{max} for 10 minutes, and then released to the initial length. Arrows indicate the beginning and the end of stretching. B, Mean values of pH_i before (b), during, and after (a) the stretch protocol (n=11). *Indicates significant difference.

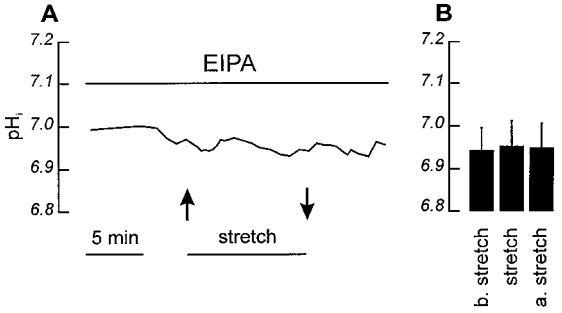

Figure 3. Inhibition of NHE activity abolished stretch-induced intracellular alkalinization. A, Results of a representative experiment in which a papillary muscle was stretched in the presence of 5 μmol/L EIPA to inhibit NHE activity. Arrows indicate the beginning and the end of stretching. Inhibition of NHE activity decreased myocardial pH_i value and suppressed the stretch-induced intracellular alkalinization. B, Mean values of pH_i before (b), during, and after (a) the stretch protocol (n=6).

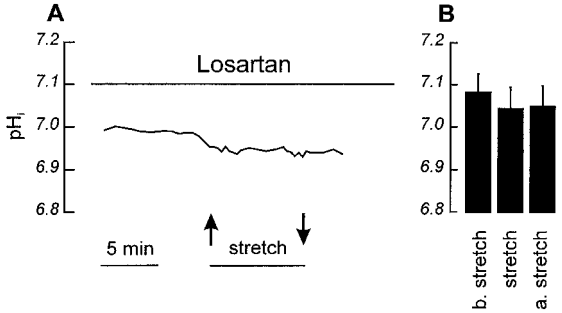

Figure 5. Blockade of AT_1 receptors abolished stretch-induced intracellular alkalinization. A, Results of a representative experiment in which a papillary muscle was stretched in the presence of the specific blocker of AT_1-subtype receptors, losartan (10 μmol/L). Arrows indicate the beginning and the end of stretching. B, Mean values of pH_i before (b), during, and after (a) the stretch protocol (n=4). No significant change in pH_i was detected when the muscles were stretched after blockade of the AT_1 receptors.

Under this condition, we were unable to detect any significant change in pH_i with stretch, which strongly suggests that the activation of NHE was underlying the increase of pH_i, as illustrated in Figure 1. Although the possibility of nonspecific actions of EIPA on other processes cannot be completely excluded,[20] this amiloride analogue seems to be specific for NHE at the dosage used here.[14,21] Previous results from our laboratory, which used the same experimental preparation (ie, isolated cat papillary muscle), showed that 5 μmol/L EIPA inhibited by ≈70% the recovery from hypercapnic acidosis.[22]

The activation of NHE may be the result of PKC stimulation induced either by the release of Ang II and/or ET, by the increase in tensile stress, or through other mechanisms. Figure 4 shows that in the presence of a PKC inhibitor, such as chelerythrine, the increase in pH_i mediated by stretch was eliminated, which suggests the involvement of a PKC-dependent pathway in stretch-induced activation of NHE. Both a tendency of baseline pH_i to decrease and a small, statistically insignificant decrease in pH_i after stretch were observed in the presence of chelerythrine. The decline in baseline pH_i probably results from either a decrease in basal

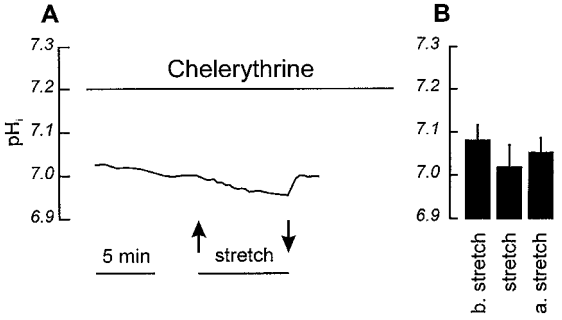

Figure 4. Inhibition of PKC activity abolished stretch-induced intracellular alkalinization. A, Results of a representative experiment in which a papillary muscle was stretched in the presence of 5 μmol/L chelerythrine. Arrows indicate the beginning and the end of stretching. A slight decrease in pH_i that did not reach statistical significance was observed after the addition of chelerythrine. B, Mean values of pH_i before (b), during, and after (a) the stretch protocol (n=4). No significant change in pH_i was detected after stretching the papillary muscles in the presence of chelerythrine.

phosphorylation(s) or from nonspecific inhibitory actions of chelerythrine, as recently reported in cardiac muscle.[23] The small decrease in pH_i after stretch observed in the presence of chelerythrine may be a consequence of the known stretch-induced increase in intracellular calcium concentration.[24,25] An increase in intracellular calcium can displace protons from binding sites and decrease pH_i.[26]

Figure 5 shows the increase in pH_i after stretch was also eliminated by blockade of AT_1 receptors with 10 μmol/L losartan. These results suggest that the increase in pH_i after myocardial stretch came from the release of Ang II, which in turn increased PKC activity leading to NHE hyperactivity. Similarly to chelerythrine, losartan induced a slight (not statistically significant) decrease of baseline pH_i. We speculate that both pharmacological interventions decrease NHE activity probably decreasing basal phosphorylation. Implicit in our speculation is the assumption of some basal production of Ang II and activation of PKC.

However, we thought that Ang II could promote the synthesis and release of ET from cardiomyocytes, endothelial cells, and even from fibroblasts.[15,27–29] ET has been shown to increase PKC activity,[16] to activate the NHE,[12,14,16,17] and to act as a growth factor increasing c-*fos* expression as well as DNA and protein synthesis.[30] For these reasons, experiments were performed in which the papillary muscles were stretched after the blockade of ET receptors with a nonselective (PD 142,893) or a selective ET_A antagonist (BQ-123). Figure 6 shows both antagonists of ET receptors suppressed the increase in pH_i induced by stretch.

A reasonable interpretation of these data is that stretching the papillary muscle induces the release of Ang II from myocardial cells, which in turn causes synthesis and/or release of ET. This is in agreement with previous results in neonatal cardiomyocytes showing that Ang II induces (within 30 minutes) a PKC-dependent increase in prepro ET-1 mRNA levels and the release of ET-1.[15]

The well-known effect of Ang II to increase myocardial pH_i,[8,10–14] previously suggested to be the main mechanism for its positive inotropic effect,[10] may be the result of an autocrine-paracrine system mediated through ET receptors. If

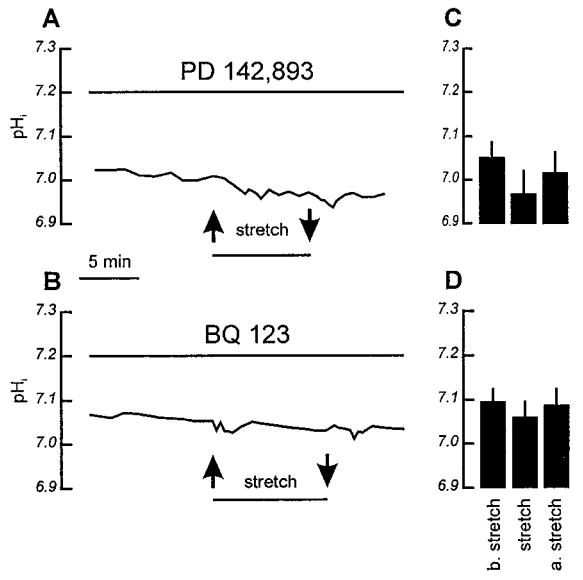

Figure 6. Blockade of ET receptors abolished stretch-induced intracellular alkalinization. A and B, Results of representative experiments in which papillary muscles were stretched in the presence of either a nonselective or a selective ET_A antagonist are shown. Arrows indicate the beginning and the end of stretching. C and D, Mean values of pH_i before (b), during, and after (a) the stretch in the presence of PD 142,893 and BQ-123 (n=4), respectively. No significant change in pH_i was detected when the muscles were stretched after the blockade of ET receptors.

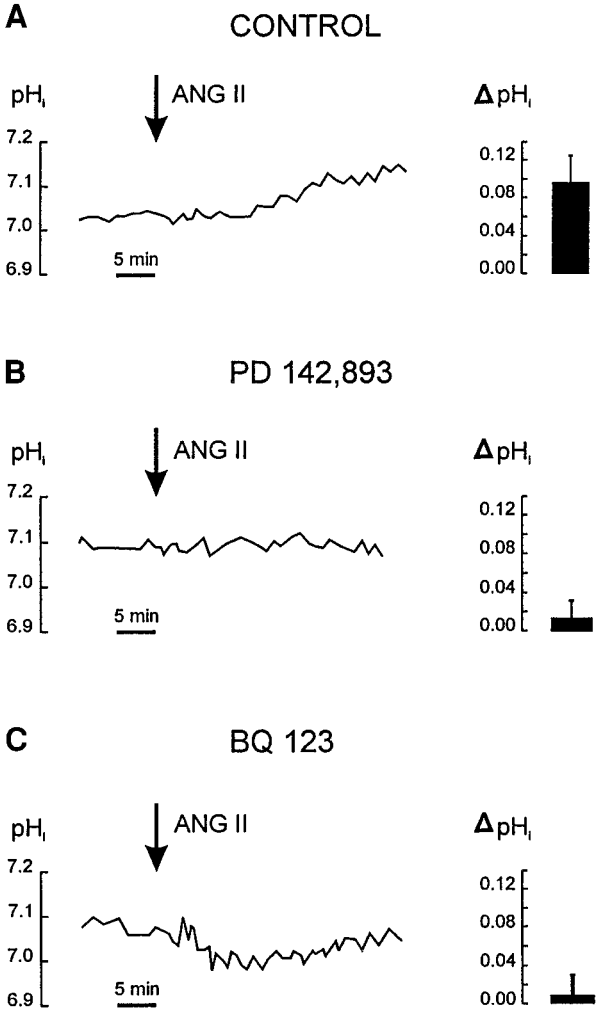

Figure 7. Ang II-induced intracellular alkalinization is mediated by ET receptors. A, Tracings from a representative experiment illustrate the increase in pH_i elicited by Ang II. The hormone was added to the superfusate (HEPES buffer) at 500 nmol/L final concentration at the time indicated by the arrow. Bar on the right side shows the mean (±SEM) increase in pH_i induced by Ang II in 5 experiments of the same type. B and C, Results from representative experiments in which 500 nmol/L Ang II was applied in the presence of either a nonspecific (PD 142,893 at 50 nmol/L) or a selective ET_A antagonist (BQ-123 at 300 nmol/L). Both the nonselective and the specific ET_A antagonist canceled the alkalinizing effect of Ang II. A slight decrease in pH_i was detected in some experiments (C) after the addition of Ang II in the presence of ET-receptor blockers. The acidifying effect of the hormone when its stimulatory effect on NHE activity is prevented has been previously reported. Bars on the right side indicate mean values (±SEM) of the overall experiments of each type (n=5 in B, n=4 in C).

this interpretation is correct, the effect of Ang II on pH_i should be reduced by ET receptor blockade. This possibility was explored in the experiments shown in Figure 7. The effect of ET receptors blockade on the pH_i response to Ang II was analyzed in these experiments. Figure 7A shows the increase in myocardial pH_i that resulted after the application of 500 nmol/L Ang II. Pilot experiments have shown that the increase in pH_i induced by exogenous Ang II was related to its concentration; for example, the rise in pH_i was maximal at 1 μmol/L, and 30% or 96% of the maximal effect was obtained with 10 and 500 nmol/L, respectively. Figure 7B and 7C shows the suppression of Ang II effect on myocardial pH_i by the nonselective endothelin receptor antagonist PD 142,893, as well as by the selective ET_A blocker, BQ-123. However, a potential explanation for our findings is that PD 142,893 and BQ-123 were nonspecifically inhibiting the binding of Ang II to the AT_1 receptors. This possibility seems improbable because the positive inotropic effect of Ang II was preserved in the presence of both ET receptor antagonists. Developed tension increased by 120±49% (n=4) after Ang II in the presence of PD 142,893 and by 143±25% (n=4) in the presence of BQ-123; whereas, under control conditions the same concentration of Ang II increased myocardial contractility in a similar extent (144±36%, n=5). These results rule out the possibility of nonspecific blockade of AT_1 receptors by ET antagonists. A previous report has suggested a causal link between the increase in pH_i and in contractility after Ang II.[10] However, our results demonstrate that intracellular alkalinization does not play a significant role in the positive inotropic effect of Ang II in myocardial tissue.

The dissociation between the positive inotropic effect of Ang II and the increase in pH_i has been previously reported from our laboratory.[31]

The pH_i response to exogenous ET-1 in our preparation of intact papillary muscle superfused with HEPES-buffered medium is shown in Figure 8. ET-1 induced a concentration-dependent increase in pH_i with a maximum within the nanomolar range. ET-1 at 10 pmol/L caused an elevation of pH_i similar to that seen during stretch (0.11±0.04). ET-1 concentrations around this order of magnitude have been

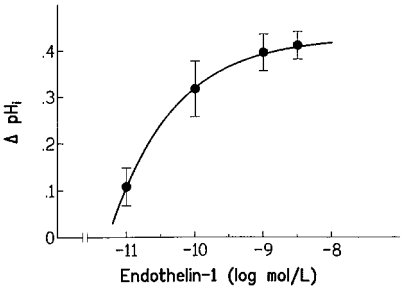

Figure 8. Concentration-pH_i response relationship to exogenous ET-1 in papillary muscles superfused with HEPES (n=4).

found in cultures of cardiomyocytes[3,32] and of endothelial cells.[27]

The pH_i response to ET-1 was not changed by the blockade of AT_1-subtype of receptors. When 10 pmol/L ET-1 was assayed in the presence of losartan, about the same increase in pH_i was detected (0.12 ± 0.02, n=4). These results indicate that the activation of NHE by ET is a consequence of the direct effect of the peptide through ET receptors and argue against the possibility that both Ang II and ET may be required for activation of NHE.

Discussion

In an adult feline papillary-muscle preparation, the increase in muscle length induces release of Ang II, which will secrete ET by an autocrine-paracrine system. The effect of ET after Ang II action seems to underlie the increase in myocardial pH_i. A similar hypothetical mode has been proposed by Schlüter et al[33] for the action of mechanical load on protein synthesis in myocytes. These authors proposed that Ang II was released into the medium after mechanical load. Activation of AT_1 receptors by locally released Ang II increases the number of ET receptors and initiates the release of ET. ET stimulates PKC and, subsequently, protein synthesis.[33] Our experiments were not designed to study the influence of mechanical load on protein synthesis. However, activation of MAP kinase after the stretching of neonatal myocytes and the partial blockade of MAP kinase by NHE inhibition has been reported.[9] The description in a recent article of how NHE inhibition attenuated stretch-induced protein synthesis in neonatal cardiomyocytes states that it would be interesting to know how mechanical stretch activates the antiporter.[34] Our data provide evidence about the mechanisms involved and also describe stretch-mediated alkalinization in adult myocardium.

The potential importance of the increase in myocardial pH_i after the increase in muscle length is not a previously recognized effect of stretch. However, we should be cautious to extrapolate these findings to the more physiological conditions in which the bicarbonate-dependent mechanisms are operative. An acid-loading bicarbonate-dependent mechanism, the sodium independent Cl^-/HCO_3^- exchanger, is also activated by Ang II.[35] Therefore, an argument can be made that if the alkalinizing effect of stretch can be detected only in the absence of bicarbonate, the finding is merely a biochemical curiosity. Even if this were true (ie, no significant changes in pH_i after stretch when the bicarbonate-dependent mechanisms are operative), our findings allow us to arrive at the following conclusions: (1) Mechanical stretch causes the release of Ang II from feline, adult papillary muscles. In agreement with a recent report,[6] these results provide direct evidence of the autocrine-paracrine mechanism in the adult heart. (2) An activation of the NHE is detected after mechanical stretch. This activation is eliminated not only by AT_1-receptor blockade but also by 2 different ET antagonists. (3) Under our experimental conditions, the increase in myocardial pH_i detected after Ang II was because of the autocrine-paracrine effect of ET. Both the selective ET_A and the nonselective ET-receptor antagonists suppressed the increase in pH_i mediated by Ang II. (4) Whether or not myocardial pH_i changes under physiological conditions (ie, in HCO_3-containing solutions), an increase in $[Na^+]_i$ caused by the hyperactivity of NHE in the presence of Ang II is expected. The increase in $[Na^+]_i$ may still be present even if the effect of the increased activity of NHE on pH_i were eliminated by the enhanced activity of the anion exchanger.[35] This $[Na^+]_i$ increase will lead to a secondary increase in $[Ca^{2+}]_i$, through the Na^+/Ca^{2+} exchanger and may contribute to the positive inotropic effect of Ang II. Although we cannot completely rule out the contribution of increases in pH_i to the increase in contractility induced by Ang II, our results indicate that, at least in feline myocardium, intracellular alkalinization does not play a significant role in its positive inotropic effect.

Our experiments were performed in a multicellular preparation. This preparation, although more representative of mechanisms operating in the whole heart, does not enable dissection of the particular cell type(s) involved in the chain of events triggered by stretch. These factors are particularly important because papillary muscles contain endothelial endocardial cells and fibroblasts in addition to myocytes. A recent suggestion is that fibroblasts are the source of ET released by Ang II and play a critical role in the hypertrophy of cardiomyocytes in culture.[29]

Nevertheless, we were able to demonstrate in an adult multicellular preparation that stretch of the muscle activates NHE through a mechanism involving PKC activation and an autocrine-paracrine system in which Ang II and ET are steps in the chain of events. The implications of this phenomenon on protein synthesis and myofilament responsiveness after stretch deserves further investigation. A recent report shows that ET_A-receptor antagonists diminished the hypertensive response to Ang II,[36] and hints at the possibility that some of the effects thought to be caused by Ang II are in fact mediated by ET.

Acknowledgments

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