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Ennis, Irene L., Carolina D. Garciarena, Néstor G. Pérez, Raúl A. Dulce, María C. Camilión de Hurtado, and Horacio E. Cingolani. Endothelin isoforms and the response to myocardial stretch. *Am J Physiol Heart Circ Physiol* 288: H2925–H2930, 2005. First published January 28, 2005; doi:10.1152/ajpheart.01202.2004.—Myocardial stretch elicits a biphasic increase in developed force with a first rapid force response and a second slow force response (SFR). The rapid phase is due to an increase in myofilament Ca^{2+} responsiveness; the SFR, analyzed here, is ascribed to a progressive increase in Ca^{2+} transients. Experiments were performed in cat papillary muscles to further elucidate the signaling pathway underlying the SFR. Although the SFR was diminished by BQ-123, a similar endothelin (ET)-1-induced increase in force was not affected: 23 ± 2 vs. 23 ± 3% (not significant). Instead, BQ-123 suppressed the contractile effects of ET-2 or ET-3 (21 ± 2 and 25 ± 3% vs. −1 ± 1 and −7 ± 3% respectively, $P < 0.05$), suggesting that ET-2 or ET-3, but not ET-1, was involved in the SFR. Each isoform activated the Na^+/H^+ exchanger (NHE-1), increasing intracellular Na^+ concentration by 2.0 ± 0.1, 2.3 ± 0.1, and 2.1 ± 0.4 mmol/l for ET-1, ET-2, and ET-3, respectively ($P < 0.05$). The NHE-1 inhibitor HOE-642 prevented the increases in force and intracellular Na^+ concentration induced by all the ET isoforms, but only ET-2 and ET-3 effects were sensitive to BQ-123. Real-time RT-PCR measurements of prepro-ET-1, -ET-2, and -ET-3 were performed before and 5, 15, and 30 min after stretch. No changes in ET-1 or ET-2, but an increase of ~60% in ET-3, mRNA after 15 min of stretch were detected. Stretch-induced ET-3 mRNA upregulation and its mechanical counterpart were suppressed by AT_1 receptor blockade with losartan. These data suggest a role for AT_1-mediated ET-3 released in the early activation of NHE-1 that follows myocardial stretch.

myocardial contractility; signal transduction; endothelin isoforms; sodium/hydrogen exchanger-1

IT WAS SHOWN BY Von Anrep (30) in 1912 that the dilatation of the heart induced by clamping the heart outflow was followed by a decline in heart volume toward the initial value. Later, Rosenblueth et al. (26) called attention to the fact that an increase in heart rate (Bowditch effect), as well as an increase in afterload (Anrep effect), triggered increases in contractility of the isolated canine right ventricle, which they named "the two-staircase phenomenon." Sarnoff et al. (28) in 1960 coined the term "homeometric autoregulation" to define the increase in contractility elicited by the Bowditch and Anrep phenomena. In 1973, Parmley and Chuck (23) were the first to reproduce the contractile effect of stretch in isolated strips of ventricular myocardium. They showed that when the muscle length was abruptly increased, there were corresponding rapid and slow increases in developed force. The rapid change in force is thought to be the basis of the Frank-Starling mechanism and is induced by an increase in myofilament Ca^{2+} responsiveness. The slow force response (SFR) develops in the next 10–15 min, and it is due to a progressive increase in the Ca^{2+} transient (1, 3, 17, 24). The source for this increase in Ca^{2+} remained unexplained until we proposed the activation of the Na^+/H^+ exchanger (NHE-1) as the cause for its production (3, 7, 8, 24). Studies in multicellular cardiac muscle preparations from cat, rabbit, rat, and failing human heart have provided evidence that the stretch induces a rise in intracellular Na^+ concentration ($[Na^+]_i$) through NHE-1 activation. The increase in $[Na^+]_i$ causes an increase in the Ca^{2+} transient through reverse-mode Na^+/Ca^{2+} exchange (NCX) (3, 24). Consistent with this proposed chain of events, the SFR was abolished by NHE-1 inhibition and reverse-mode NCX blockade (3, 24). Activation of NHE-1 was the result of an autocrine-paracrine loop involving the release of preformed angiotensin II (ANG II), which triggers the release/formation of endothelin (ET) (8). However, although a role of ET, but not ANG II, was confirmed in ferret papillary muscles by Calaghan and White (5), other investigators were not able to confirm this in rabbit (31) or failing human heart (32), challenging our proposed chain of events.

One intriguing finding that invited us to consider that ET-1 was probably not the isoform involved in the chain of events that follow myocardial stretch was the resistance of the positive inotropic effect of ET-1 to the ET_A-specific blocker BQ-123 demonstrated in the studies of Kasai et al. (15).

Three ET isopeptides (ET-1, ET-2, and ET-3) encoded by independent genes have been identified. Most of the cardiac effects of ET seem to be produced by ET-1, although the other two isoforms are also expressed in the heart. Endoh and colleagues (12) reported that the three isoforms elicit a positive inotropic effect, essentially with identical efficacies and potencies in the myocardium. However, although the effect of ET-1 was resistant to BQ-123, the effect of ET-3 was not (12, 15). Because the SFR was affected by BQ-123 (3, 5 ,24), we wonder whether ET-3, and not ET-1, was the isoform released by stretch. Experiments were undertaken to examine the role of ET isoforms in the SFR that follows myocardial stretch.

METHODS

This investigation conforms to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). All experiments were conducted in accordance with institutional guidelines, and the Institutional Animal Care and Use Committee at Universidad Nacional de La Plata approved the experimental protocol. The experiments were performed in cat papillary muscles from the right ventricle mounted and superfused as previously described (24). After they were mounted, the muscles were progressively stretched to determine the length at which they devel-

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oped the maximal twitch force (L_{max}). For the experiments in which the inotropic effects of ET-1, ET-2, or ET-3 were studied, the muscles were maintained at ~98% of L_{max} (L_{98}) throughout the protocol (30 min of incubation with each peptide in the absence and presence of the ET_A receptor blocker BQ-123 at 300 nmol/l). Changes in $[Na^+]_i$ were monitored as previously described (3, 24). For the stretch protocols, the muscles were set at 92% of L_{max} (L_{92}) and randomly assigned to one of the following protocols: *1*) 30 min at L_{92}, i.e., nonstretched muscles, *2*) 30 min at L_{92} and 5, 15, or 30 min at L_{98}, *3*) 10 min at L_{92} plus 20 min at L_{92} in the presence of 1 μmol/l losartan (an AT_1 receptor blocker) followed by 15 min of stretch to L_{98}, *4*) 30 min at L_{92}, 15 min of stretch to L_{98}, and 10 min of shortening to L_{92} for 10 min followed by incubation with BQ-123 for 20 more min and then 15 min of stretch to L_{98}. At the end of *protocols 1–3*, the superfusion solution was quickly replaced by RNA stabilization reagent (RNAlater, Qiagen) to freeze mRNA content for later determination. Total RNA was isolated from papillary muscles using the RNeasy kit (Qiagen). RNA (0.8 μg) was reverse transcribed using the Omniscript RT kit (Qiagen). A dilution of the resulting cDNA was used to quantify the relative content of mRNA by real-time PCR (iCycler iQ real-time PCR detection system, Bio-Rad) using appropriate primers and SYBR Green as the fluorescent probe. The following primers, designed using Primer3 software, were used: 5′-GGGTGTGAACCACGAGAAAT-3′ (forward) and 5′-CCACAGTCTTCTGAGTGGCA-3′ (reverse) for GAPDH, 5′-CAGACAAAGAACTCCGAGCC-3′ (forward) and 5′-GGTCTTGATGCTGTTGCTGA-3′ (reverse) for prepro-ET-1, 5′-CTCTCCTGGGACGCTAACTG-3′ (forward) and 5′-GGATGGCCTCTCTTGTCAAC-3′ (reverse) for prepro-ET-2, and 5′-TCTCCACAGACACGCTTACG-3′ (forward) and 5′-TGACTTCAGCCTTTGACGTG-3′ (reverse) for prepro-ET-3. PCR were performed with Platinum *Taq* DNA polymerase (Invitrogen). Fluorescence data were acquired at the end of extension. A melt analysis was run for all products to determine the specificity of the amplification. The cycle threshold values for each gene were measured and calculated by computer software (iCycler IQ OSS, version 3.0a, Bio-Rad).

Statistics. Values are means ± SE. Statistical analysis of results was performed with Student's *t*-test or ANOVA followed by the Student-Newman-Keuls post hoc test as appropriate. Significance level was set at $P < 0.05$.

RESULTS

Figure 1 shows the SFR to stretch and the effect of blocking the ET_A receptors with BQ-123 in cat papillary muscles. As in previous reports (3, 24, 31), after 10–15 min, the SFR reached a steady-state value that was ~ 20% higher than the initial rapid phase. It was previously demonstrated by us (3, 24) and others (2, 17) that the SFR is the result of a gradual increase in Ca^{2+} transients. The increase in Ca^{2+} transient amplitude is due to the NCX driven into its reverse mode by the increase in $[Na^+]_i$ produced by ET activation of the NHE-1 (3, 24). According to reports from others (5) and from our laboratory (3, 24), a significant diminution of the SFR was observed in the presence of the selective ET_A antagonist BQ-123 (Fig. 1).

Although ET-1 is the most studied ET isoform and its stimulation of ET_A receptors is widely accepted, it is known that the positive inotropic effect of this peptide is resistant to most of the available ET receptor blockers, either the nonselective receptor blockers that are supposed to block ET_A and ET_B receptors or the combination of selective ET_A and ET_B blockers (12, 15). However, this pharmacological feature of ET-1 was not considered by us when we described the effect of BQ-123 on the SFR. This apparently simple finding that the SFR was blunted by BQ-123, whereas the effect of ET-1

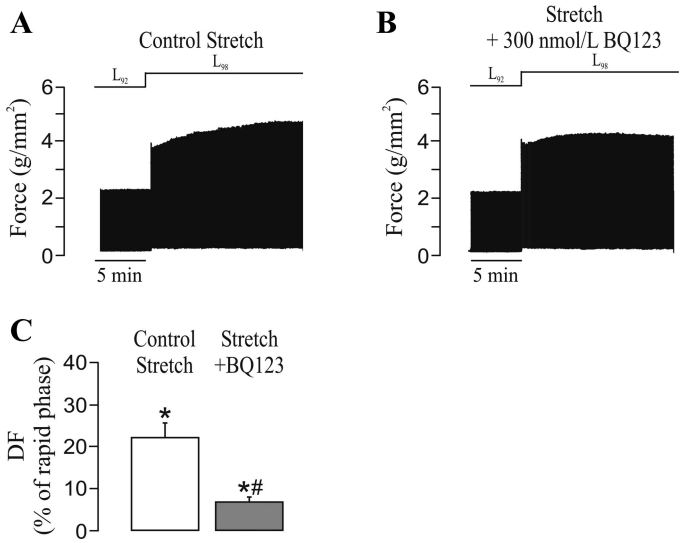

Fig. 1. *A*: stretch of cat papillary muscles from 92% of muscle length at maximum twitch force (L_{max}, L_{92}) to 98% of L_{max} (L_{98}) promotes characteristic biphasic response in force. Initial rapid phase was followed by the slow force response (SFR), which stabilized after 10–15 min at ~20% greater than the initial rapid phase. *B*: representative experiment in which stretching protocol was performed in the presence of BQ-123 (300 nmol/l). SFR was almost canceled by BQ-123, confirming participation of endogenous endothelin (ET) in development of SFR. *C*: averaged results obtained under experimental conditions described in *A* and *B*. SFR is expressed as percentage of initial rapid phase [developed force (DF)]. Values are means ± SE; $n = 4$. *$P < 0.05$ vs. initial rapid phase. #$P < 0.05$ vs. control.

was resistant to this compound, suggested to us that an ET isoform other than ET-1 might be responsible for NHE-1 activation.

As a first step to identify the isoform participating in development of the SFR, we carried out experiments in which an increase in force similar in magnitude to the SFR was mimicked by exogenous ET-1, ET-2, and ET-3, and their corresponding sensitivity to BQ-123 was explored. In pilot experiments, we found that the three isoforms were similar in efficacy and potency, in agreement with previous reports (12).

When the increase in force was promoted by ET-1 (5.0 nmol/l), the positive inotropic effect was not affected by 300 nmol/l BQ-123 (Fig. 2). In contrast, when the SFR was mimicked by ET-2 or ET-3 (5.0 nmol/l each), the increase in force was abolished by BQ-123 (Figs. 3 and 4, respectively). Interestingly, the time course of the contractile response to myocardial stretch (Fig. 1) was better represented by exogenous ET-3 (Fig. 4) than ET-2 (Fig. 3) or ET-1 (Fig. 2).

Given the fact that the increase in $[Na^+]_i$ mediated by ET-induced NHE-1 stimulation is mandatory for the contractile response to stretch by favoring the NCX reverse mode of operation (3, 24) and that there was no evidence of the possible effects of ET-2 and ET-3 on NHE-1 activity, experiments were performed to explore whether these peptides were also able to activate the NHE-1, increasing $[Na^+]_i$ and, consequently, producing the positive inotropic effect. Figure 5 shows that similar increases in $[Na^+]_i$ and force were induced by ET-1, ET-2, and ET-3, demonstrating that the three isoforms were also equipotent in terms of NHE-1 activation. Figure 5 also shows that NHE-1 inhibition with 1 μmol/l HOE-642 canceled the in-

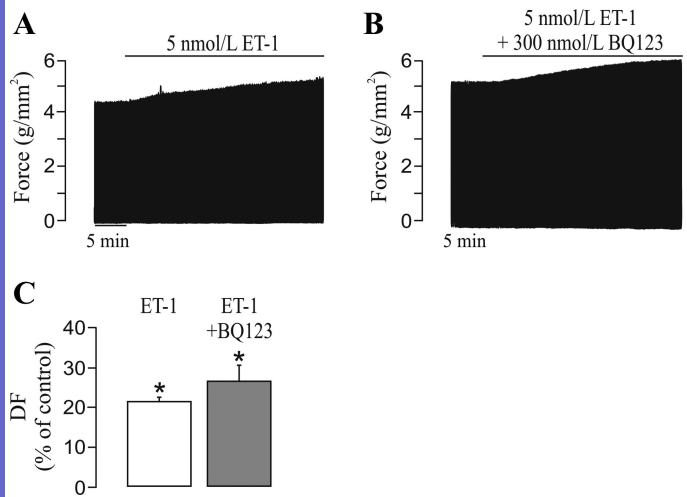

Fig. 2. *A*: after 30 min, addition of ET-1 (5 nmol/l) to a papillary muscle promoted an increase in DF similar in magnitude to that of SFR. *B*: experiment similar to *A*, but in the presence of 300 nmol/l BQ-123, a maneuver that did not prevent the increase in DF promoted by ET-1. *C*: averaged results of DF (expressed as percentage of pre-ET-1 value) after 30 min of incubation under experimental conditions described in *A* and *B* (without and with BQ-123). Values are means ± SE; $n = 7$ for ET-1 and $n = 5$ for ET-1 + BQ-123. *$P <$ 0.05 vs. before ET-1.

creases in $[Na^+]_i$ and force, indicating that, at this dose, the positive inotropic effect of each ET isoform is entirely mediated by NHE-1 activation and the subsequent rise in $[Na^+]_i$. Interestingly, the blockade of ET_A receptors with BQ-123 did not cancel the ET-1 effect on NHE-1 but completely suppressed the effect of ET-2 and ET-3 (Fig. 5). The lack of prevention of NHE-1 activation by this concentration of BQ-123 after this concentration of ET-1 is in agreement with the lack of blockade by this compound of the contractile response to ET-1 and further supports the notion that ET-1 was not the isoform involved in generation of the SFR.

We hypothesized that an indirect way to distinguish which isoform could be involved in the SFR would be to examine, at

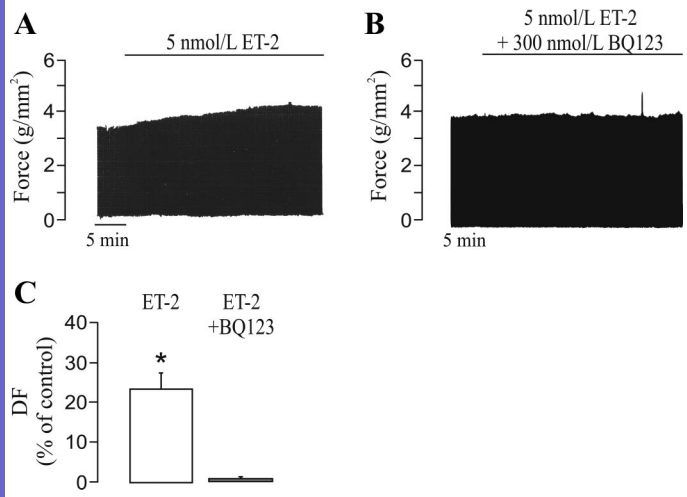

Fig. 3. *A* and *B*: representative experiments after addition of 5 nmol/l ET-2. Protocol is the same as in Fig. 2, except BQ-123 canceled the increase in DF. *C*: averaged results of DF after 30 min of incubation under experimental conditions described in *A* and *B* (without and with BQ-123). Values are means ± SE; $n = 4$. *$P <$ 0.05 vs. before ET-2.

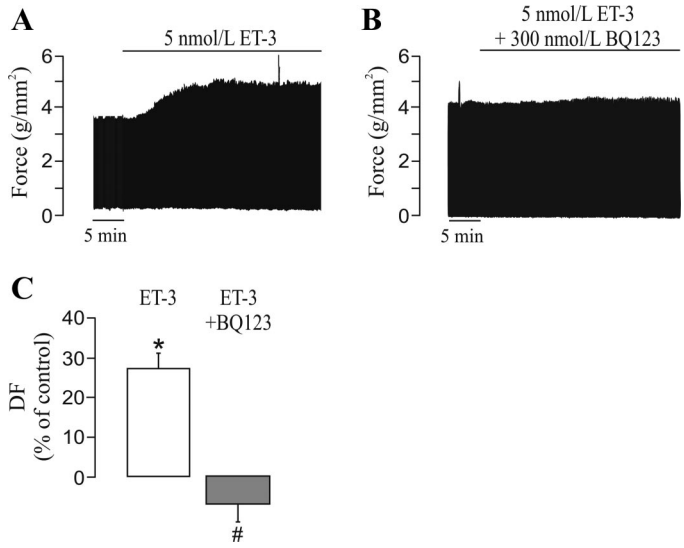

Fig. 4. *A*: the increase in DF after addition of 5 nmol/l ET-3 to a papillary muscle was of a similar magnitude to that obtained after addition of the same concentration of ET-1 and ET-2; thus similar to the SFR. *B*: similar to ET-2, BQ-123 canceled the increase in DF. *C*: averaged results of DF after 30 min of incubation under experimental conditions described in *A* and *B* (without and with BQ-123). Values are means ± SE; $n = 5$. *$P <$ 0.05 vs. pre-ET-3 control. #$P <$ 0.05 vs. ET-3.

different times after stretch, the relative abundance of the mRNA of the three isoforms. The rationale for this hypothesis is based on the assumption that the release/formation of a specific ET isoform immediately after stretch may be followed by an increase in its mRNA abundance to restore the intracellular pool. Therefore, a new set of experiments was carried out in which prepro-ET-1, -ET-2, and -ET-3 mRNAs were assessed by real-time RT-PCR before stretch (nonstretched muscles) and 5, 15, and 30 min after stretch. RT-PCR was used to corroborate expression of all three ET isoforms in cat papillary muscles. In accordance with a previous report (14), more amplification cycles were necessary to detect ET-2 than ET-1 and ET-3 in our preparation. Figure 6*A* shows the average values of the prepro-ET mRNA measurements. Prepro-ET-3 mRNA increased ~60% after 15 min of stretch and returned toward prestretch values after 30 min. No change in the amount of the other two prepro-ET isoform mRNAs was detected at 5, 15, or 30 min. Our previous experiments in cat papillary muscles indicated that, in the chain of events leading to the SFR, activation of the AT_1 receptors is upstream of the release/formation of ET. Along with our proposal that AT_1 receptors would be upstream of ET upregulation, another set of experiments exploring the effect of AT_1 receptor blockade on the prepro-ET mRNA profile was performed. The specific blockade of AT_1 receptors prevents the increase in prepro-ET-3 mRNA without affecting prepro-ET-1 and -ET-2 mRNA levels (Fig. 6*B*). Consistent with these results, the SFR was completely abolished in this group of papillary muscles: −1 ± 1% ($n = 4$) and 24 ± 1% ($n = 8$) in the presence and absence of losartan, respectively ($P < 0.05$). These data, therefore, confirm our proposal that the cross talk between the AT_1 receptors and ET-3 is a necessary step in induction of the SFR in this preparation.

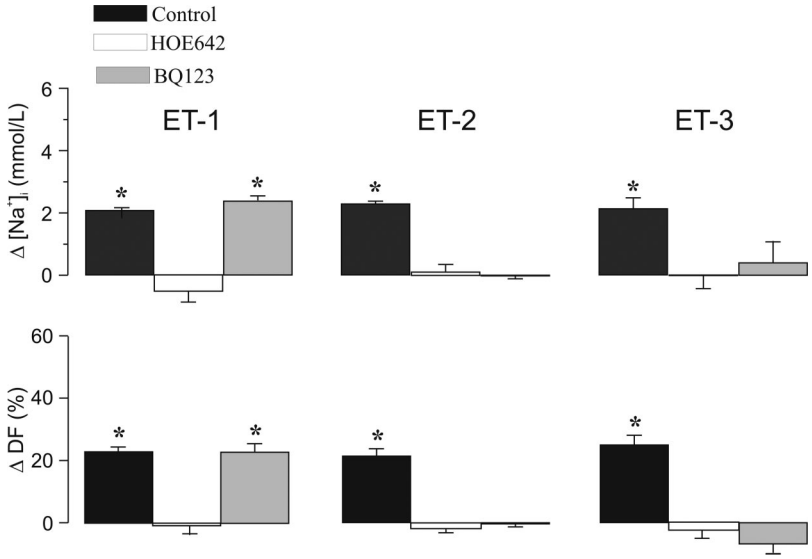

Fig. 5. ET-1, ET-2, and ET-3 induced similar increases in intracellular Na^+ concentration ($[Na^+]_i$) and force (n = 5, 3, and 4, respectively), demonstrating that ET isoforms are equipotent. Increases in $[Na^+]_i$ and force were canceled by blockade of Na^+/H^+ exchanger (NHE-1) with HOE-642 (n = 3 for ET-1 and ET-2 and n = 4 for ET-3), indicating that, at this dose, positive inotropic effect of each ET isoform is entirely due to NHE-1 activation. Blockade of ET_A receptors with BQ-123 did not cancel ET-1 effect on NHE-1 (n = 3) but completely suppressed effects of ET-2 and ET-3 (n = 3 and 4, respectively). *$P < 0.05$ vs. pre-ET stimulated values.

DISCUSSION

The experiments presented here represent the first evidence of a role of ET-3 in the myocardial response to stretch. In our multicellular preparation, myocardial stretch activates the tissue renin-angiotensin-endothelin system by releasing ANG II or by directly activating AT_1 receptors, leading to the release/formation of ET-3, which subsequently activates the NHE-1 and increases $[Na^+]_i$. Figure 7 depicts our hypothetical autocrine-paracrine chain of events that follows myocardial stretch.

Because exogenously applied ET-1, in concentrations that mimic the inotropic effect of the SFR, was not affected by BQ-123, it was difficult to accept that ET-1 could be involved in this chain of events. Although we do not have direct measurements of the ET isoform released by stretch, the transient increase in prepro-ET-3 mRNA detected 15 min after stretch, without significant changes in the other two isoforms, suggested a role for ET-3 in development of the SFR. It can result in a surprising rapid increase in the level of mRNA and its return to basal values in 30 min. However, it has been reported that the half-life of prepro-ET-1 mRNA is ~10 min (11), and a similar pattern of transient elevation of prepro-ET-1 mRNA has been described for the hypertrophic effect of exogenous ET-3 in neonatal rat myocytes (29). The measurement at the protein level itself would be stronger evidence of the isoform involved if the ET isoform is "de novo" synthesized before being released. However, the time required for the formation of ET is probably beyond the time scale of our experiments (~30 min). If we consider that the levels of ET may represent the spillover from the surrounding tissue with a short half-life, mRNA measurements are probably a reliable tool. This indirect approach allowed us to detect the mRNA upregulation of only the ET-3 isoform. We would like to emphasize that we measured ET mRNA abundance as an index of the restoration of the intracellular pool from which ET is released after myocardial stretch. This pool needs to be replenished after the preformed ET is released, inducing an increase in its mRNA. It can be argued that ET-1 or ET-2 mRNA could increase at a time different from the times examined by us; however, the fact that the mechanical effect was sensitive to BQ-123 and that BQ-123 blunted ET-3-induced, but not ET-1-induced, NHE-1 activation strongly supports the idea that ET-3 is the specific isoform involved in development of the SFR. More direct evidence of the isoform involved would probably require that the experiments be performed under specific inhibition of each isoform or that each isoform be genetically antagonized. Unfortunately, no compound is capable of specifically antagonizing each ET isoform, and tech-

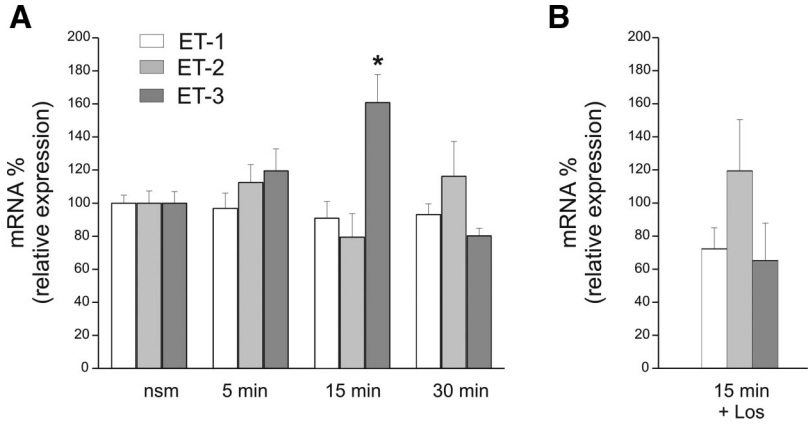

Fig. 6. *A*: significant increase in expression of prepro-ET-3 mRNA (~ 60%) after 15 min of stretch (n = 6) but no change in expression of prepro ET-1 and -ET-2 mRNAs at any time (n = 7 and 4, respectively) compared with nonstretched muscles (nsm, n = 4). *B*: ANG II AT_1 receptor blockade with losartan (1 μmol/l, Los) completely abolished increase in prepro-ET-3 mRNA induced by myocardial stretch without affecting prepro-ET-1 and -ET-2 mRNAs (n = 4). *$P < 0.05$ vs. nsm.

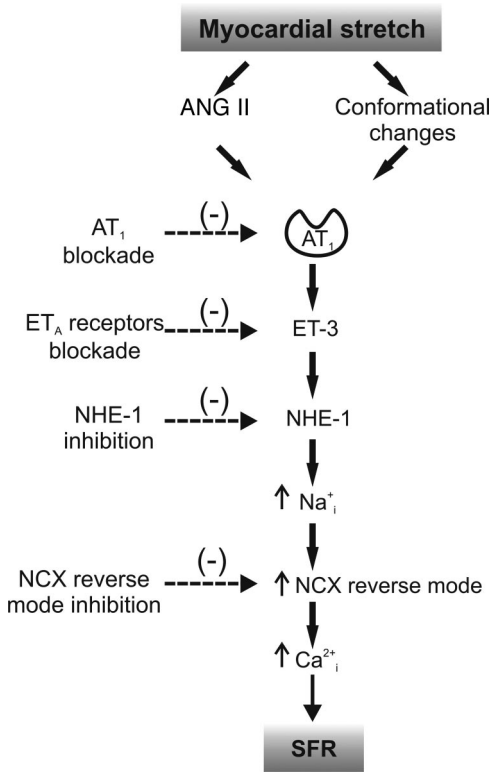

Fig. 7. Schematic representation of proposed autocrine-paracrine cascade of events after myocardial stretch. Myocardial stretch activates AT_1 receptors by release of endogenous ANG II or by direct induction of a conformational change of the receptors. Stimulation of AT_1 receptors induces release/formation of ET-3, which, through BQ-123-sensitive receptors, will activate NHE-1, increasing $[Na^+]_i$. Rise in $[Na^+]_i$ will drive $Na+/Ca^{2+}$ exchange (NCX) in reverse mode, determining the increase in Ca^{2+} transient and, consequently, development of SFR. Several pharmacological interventions corroborating this sequence of events are depicted at *left*. Cells other than myocytes (perhaps endothelial cells) could be contributing to the cascade, making the myocardial response to stretch a paracrine mechanism.

niques to genetically manipulate ET expression in the hearts from which papillary muscles are being isolated are not available to us.

The fact that myocardial stretch induces the release of ANG II and ET is well known since pioneering studies by Sadoshima et al. (27) and Yamazaki et al. (33), who showed that mechanical stretch of cultured neonatal rat cardiomyocytes caused the release of preformed ANG II to the surrounding medium and that the peptide acted as the initial stimulus of the stretch-induced hypertrophic response. The release of ANG II triggered by stretch was also demonstrated in adult cardiomyocytes by Leri et al. (19). Moreover, Liang and Gardner (20), studying the expression of the brain natriuretic peptide gene induced by mechanical stretch of cultured neonatal myocytes, found that it was completely blocked by AT_1 receptor blockade with losartan or by blockade of ET_A receptors, implying a role for ANG II and ET as autocrine/paracrine mediators. These authors also found that ANG II and ET were arrayed in series, because the effect of ANG II was cancelled by ET receptor blockade, whereas the effect of ET was unaffected by AT_1 blockade (20). Previous studies from our laboratory in feline papillary muscles also showed that the cross talk between ANG II and ET follows the same direction, because ANG II effects were suppressed by ET receptor blockade while effects of exogenously applied ET-1 were insensitive to blockade of AT_1 receptors with losartan (8, 25). Although a recent study challenged the release of ANG II after myocardial stretch in the necessary amount to stimulate the AT_1 receptors, conformational changes of these receptors induced by the stretch itself seem to be able to trigger intracellular pathways that are also prevented by AT_1 blockade (35).

Species-dependent variations in the positive inotropic effect of ET-3 that have been previously reported (34) could probably account for the fact that the role of ET in development of the SFR was confirmed in the rat (3), cat (24), and ferret (5), but not rabbit (31), myocardium. Nor was involvement of ET detected in the failing human myocardium (32); however, we are not aware of reports in normal human hearts. Although the source of the ET released after myocardial stretch remains controversial (5, 24), endothelial/endocardial cells could be the source of ET-3. If this were the case, the injury of these cells during some experimental protocols could be another explanation for the discrepancies. However, the presence of the SFR along with stretch has been reported in isolated cardiomyocytes by two different groups of investigators (4, 13).

Although the stimulation of myocardial NHE-1 by ET-1 is a well-known fact (6, 18, 22), the effect of ET-2 and ET-3 on the exchanger has been less explored (16). We demonstrated here that ET-1, ET-2, and ET-3 stimulate the NHE-1 and increase $[Na^+]_i$ in a similar magnitude. The rise in $[Na^+]_i$ entirely accounts for the positive inotropic effect of each isopeptide. However, only the rise in $[Na^+]_i$ induced by ET-2 and ET-3 was cancelled by the concentration of BQ-123 selected here, perhaps as a reflection of the participation of different subtypes of ET receptors, as previously suggested (12, 21).

Myocardial stretch has been widely analyzed as one of the main causes of cardiac hypertrophy, particularly in neonatal rat cardiomyocytes, and the role of the ANG II-ET-aldosterone system in this phenomenon was demonstrated. On the other hand, myocardial response to stretch in multicellular preparations has been also extensively studied in regard to the Frank-Starling mechanism and two phases of the contractile response. However, the possibility that both myocardial responses to stretch resulted from the same intracellular signaling pathway was not considered until the finding that the SFR, the in vitro mechanical counterpart of the Anrep effect, was the result of an autocrine-paracrine loop triggered by the sequential activation of AT_1 receptors, the release of ET, and the activation of NHE-1 (Fig. 7) (10). Moreover, the possible link between the SFR and myocardial hypertrophy is supported by the fact that the chronic and specific blockade of the NHE-1 was shown to effectively regress cardiac hypertrophy in several experimental models (see Ref. 9 for review).

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