Endothelin-1 Stimulates the Na^+/Ca^{2+} Exchanger Reverse Mode Through Intracellular Na^+ (Na^+_i)–Dependent and Na^+_i-Independent Pathways

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Abstract—This study aimed to explore the signaling pathways involved in the positive inotropic effect (PIE) of low doses of endothelin-1 (ET-1). Cat papillary muscles were used for force and intracellular Na^+ concentration (Na^+_i) measurements, and isolated cat ventricular myocytes for patch-clamp experiments. ET-1 (5 nmol/L) induced a PIE and an associated increase in Na^+_i that were abolished by Na^+/H^+ exchanger (NHE) inhibition with HOE642. Reverse-mode Na^+/Ca^{2+} exchanger (NCX) blockade with KB-R7943 reversed the ET-1–induced PIE. These results suggest that the ET-1–induced PIE is totally attributable to the NHE-mediated Na^+_i increase. However, an additional direct stimulating effect of ET-1 on NCX after the necessary increase in Na^+_i could occur. Thus, the ET-1–induced increase in Na^+_i and contractility was compared with that induced by partial inhibition of the Na^+/K^+ ATPase by lowering extracellular K^+ (K^+_o). For a given Na^+_i, ET-1 induced a greater PIE than low K^+_o. In the presence of HOE642 and after increasing contractility and Na^+_i by low K^+_o, ET-1 induced an additional PIE that was reversed by KB-R7943 or the protein kinase C (PKC) inhibitor chelerythrine. ET-1 increased the NCX current and negatively shifted the NCX reversal potential (E_{NCX}). HOE642 attenuated the increase in NCX outward current and abolished the E_{NCX} shift. These results indicate that whereas the NHE-mediated ET-1–induced increase in Na^+_i seems to be mandatory to drive NCX in reverse and enhance contractility, Na^+_i-independent and PKC-dependent NCX stimulation appears to additionally contribute to the PIE. However, it is important to stress that the latter can only occur after the primary participation of the former. **(*Hypertension*. 2005;45:288-293.)**

Key Words: contraction ■ ion channels ■ endothelin

Endothelin-1 (ET-1) is a powerful inotropic agent that induces this effect acting through different intracellular signaling pathways.[1–4] However, at doses that increase contractility by ≈20%, the positive inotropic effect (PIE) is entirely attributable to activation of the reverse mode of the Na^+/Ca^{2+} exchanger (NCX).[5] This increase in contractility is similar in magnitude to that detected during the slow force response of myocardial stretch.[6–8] A sustained increase in intracellular Ca^{2+} through the NCX reverse mode induced by an increase in intracellular Na^+ (Na^+_i) produced by the activation of the Na^+/H^+ exchanger (NHE) may represent the signaling link between this transporter and cardiac hypertrophy.[9–11] In fact, we demonstrated recently that this mechanism is responsible for the increase in contractility induced by ET-1.[5] We proposed that ET-1 activates the NHE, which increases Na^+_i and shifts the NCX reversal potential (E_{NCX}) to a more negative voltage. These changes give more time for NCX to operate in reverse mode during the action potential and promote Ca^{2+} influx to the cell, determining the increase in force.[5]

It is well known that activation of protein kinase C (PKC) is a downstream pathway of ET-1 receptor binding.[4] Consistently, it has been shown that PKC phosphorylates the NCX protein after ET-1 stimulation.[12] Moreover, a direct PKC-dependent increase in the NCX current (I_{NCX}) induced by ET-1 in guinea pig myocytes was reported previously.[13] Thus, the possibility exists that a direct effect of ET-1 on NCX could also contribute to the PIE of this peptide after NCX is driven to the reverse mode by the mandatory increase in Na^+_i produced by activation of the NHE. In other words, the increase in Na^+_i could be a necessary but not exclusive condition to mediate the ET-1–induced Ca^{2+} influx through the NCX reverse mode. The investigation of this hypothesis constitutes the main purpose of the present study.

The overall results obtained herein allow us to suggest that the cardiac NCX reverse mode is modulated by ET-1 through 2 different pathways: (1) an Na^+_i-dependent one, consistent with a negative shift of E_{NCX} after a rise in Na^+_i attributable to the NHE activation; and (2) an Na^+_i-independent and PKC-dependent NCX stimulation. Both pathways appear to con-

Received September 17, 2004; first decision October 4, 2004; revision accepted November 29, 2004.
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***Hypertension* is available at http://www.hypertensionaha.org** **DOI: 10.1161/01.HYP.0000152700.58940.b2**

tribute to the ET-1–induced PIE. However, the latter seems to contribute to this effect only after primary participation of the former, which appears to be a mandatory step.

Materials and Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cat papillary muscles were used for registering developed force, intracellular pH (pH_i), and Na^+_i measurements, and isolated cat ventricular myocytes were used for patch-clamp experiments. Na^+_i and pH_i were determined by epifluorescence. A detailed description of these techniques is presented in an expanded Materials and Methods section, available online at http://www.hypertensionaha.org.

Experimental Protocols

To analyze the above-mentioned hypothesis, 3 different experimental approaches were assayed in the present work. (1) We compared the increase in Na^+_i and force promoted by ET-1 to those induced by partial inhibition of the Na^+/K^+ATPase; (2) We tested the ability of ET-1 to induce a PIE after driving the NCX in reverse mode by increasing Na^+_i through the partial inhibition of the Na^+/K^+ ATPase in the presence of NHE blockade; and (3) We evaluated the effects of ET-1 on outward I_{NCX} in the presence and absence of NHE inhibition.

Pharmacological Interventions

HOE642 (1 μmol/L) was used to inhibit the NHE, KB-R7943 (5 μmol/L), to inhibit the NCX reverse mode, and chelerythrine (1 μmol/L), to block PKC activation.

Statistics

Data are expressed as mean±SEM. To detect significant differences, paired or unpaired *t* test or repeated-measures 1-way ANOVA followed by Student Newman–Keuls as post hoc test were used as appropriate. A $P<0.05$ was considered to indicate significant differences.

Results

Figure 1 shows the effects of ET-1 (5 nmol/L) on contractile behavior (Figure 1A and 1B), on Na^+_i (Figure 1C), and on pH_i (Figure 1D) of isolated cat papillary muscles. Control values for force and Na^+_i across groups are shown in the table. The results indicate that the PIE of ET-1: (1) is abolished by both, NHE-1 blockade with HOE642 (1 μmol/L) and by the inhibitor of the NCX reverse mode, KB-R7943 (5 μmol/L); and (2) is accompanied by an increase in Na^+_i without changes in pH_i. The lack of changes in pH_i after NHE activation when bicarbonate is present in the medium has been suggested previously.[5,6,14] Note that a further increase in Na^+_i was detected after application of KB-R7943, suggesting that this compound blocked the exchange of Na^+_i for extracellular Ca^{2+}.

Although these experiments are suggesting that the PIE of this ET-1 dose is entirely attributable to stimulation of the NCX reverse mode, the possibility exists that the compound used for blocking this NCX operation mode would be acting on other mechanisms. However, as shown in Figure 2, this was not the case. We were unable to show any effect of KB-R7943 (5 μmol/L) on contractility under our control conditions (Figure 2A). These results also indicate that the NCX reverse mode does not contribute to basal contractility. KB-R7943 even failed to affect contractility after increasing it by enhancing extracellular Ca^{2+} concentration in a magnitude enough to increase developed force by ≈20% (Figure 2B), which is the effect produced by the dose of ET-1 used in the present work.

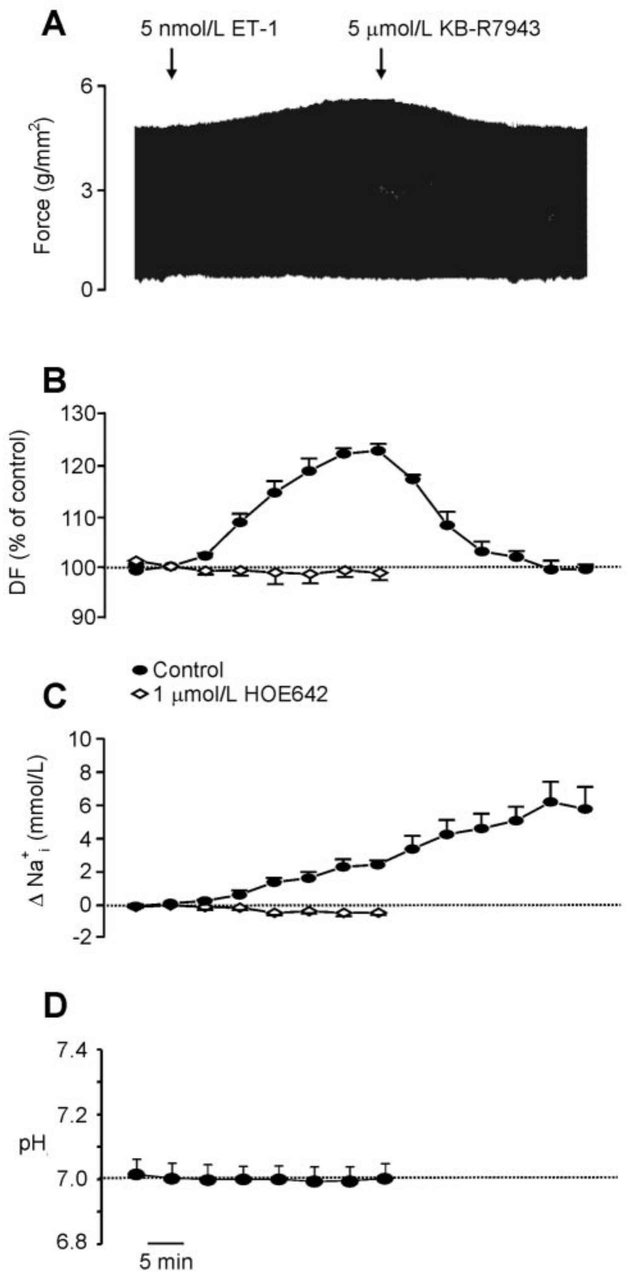

Figure 1. Effect of ET-1 on contractility and Na^+_i in cat papillary muscles. A, Typical force record of an isometrically contracting cat papillary muscle showing the PIE promoted by 5 nmol/L ET-1 and its suppression after inhibition of the NCX reverse mode with 5 μmol/L KB-R7943. B, Averaged results of 5 experiments like those shown in A (filled symbols) and cancellation of the ET-1–induced PIE after inhibition of NHE with 1 μmol/L HOE642 (open symbols; n=4). C, Increase in force observed with ET-1 was accompanied by a rise in Na^+_i that continued after NCX inhibition (filled symbols). Preincubation with HOE642 prevented the increase in developed force (DF) and Na^+_i, supporting the notion that NHE activation, and thus Na^+_i increase, is a mandatory step to increase force (open symbols). D, Addition of ET-1 did not produce any change in papillary muscle pH_i (n=4).

To test the possibility that in addition to the Na^+_i-dependent mechanism, there is also a direct stimulatory effect of ET-1 on NCX, pilot experiments in which an increase in Na^+_i promoted by partial inhibition of the Na^+/K^+ ATPase with ouabain were performed. Although no systematic studies were done to estab-

Control Values of Developed Force (DF) and Na^+_i of the Different Experimental Groups

Controls	DF, g/mm^2	Na^+_i, mmol/L	n
Before ET-1	3.77±0.39	5.18±0.29	5
Before low K^+_o, 1.8 mmol/L	4.33±0.37	5.25±1.03	4
Before low K^+_o, 0.9 mmol/L	4.44±0.38	5.71±0.80	3
HOE642 before ET-1	3.53±0.50	4.65±1.01	4
HOE642 before low K^+_o (Figure 4)	3.49±1.01		3
HOE642 before low K^+_o (Figure 5)	3.50±0.40	5.40±0.28	3

lish the relationship between the increase in Na^+_i and developed force in our preparations, no consistent relationship between both parameters was found after 1, 2, or 5 μmol/L ouabain (data not shown). These results might be explained by nonspecific effects of this compound[15,16] or failure to select the right dose. Then we decided to increase Na^+_i by partial inhibition of the Na^+/K^+ ATPase by lowering extracellular K^+ (K^+_o). Figure 3 shows that when Na^+_i was increased by this maneuver, the increase in developed force showed a linear relationship with Na^+_i. This increase in developed force was blunted by KB-R7943 (5 μmol/L; Figure 3, inset), indicating that it was attributable to activation of the NCX reverse mode. However, when the Na^+_i levels were augmented by ET-1–induced activation of NHE, the increase in force lies above this relationship. Therefore, for a given Na^+_i, the increase in developed force was

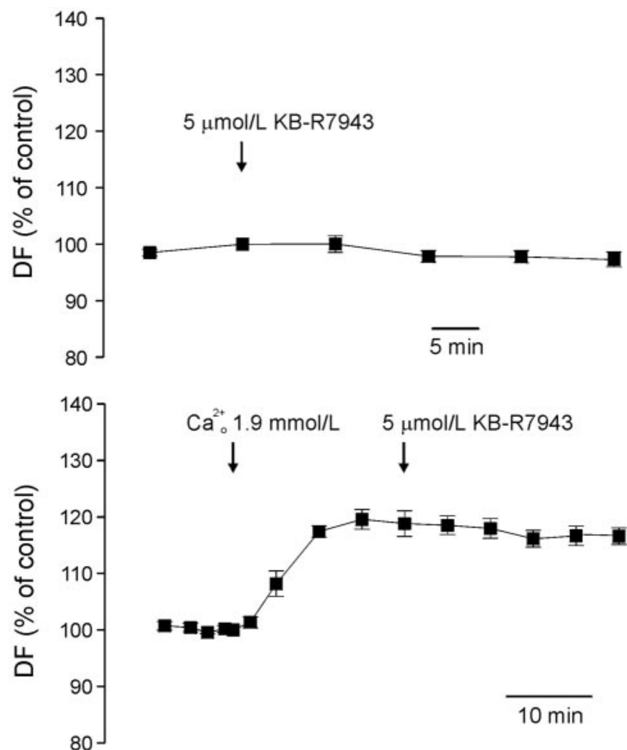

Figure 2. Effect of KB-R7943 on basal contractility and after increasing force by increasing extracellular Ca^{2+} concentration. Inhibition of NCX reverse mode by 5 μmol/L KB-R7943 did not affect basal contractility (A) or an increase in force of ≈20% promoted by augmentation of extracellular Ca^{2+} to 1.9 mmol/L (B), supporting the notion that at this concentration, KB-R7943 does not elicit nonspecific effects. DF indicates developed force.

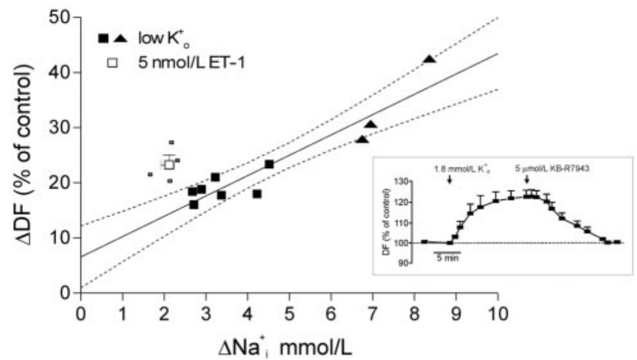

Figure 3. Effect of increasing Na^+_i by partial inhibition of the Na^+/K^+ ATPase on cardiac contractility. When Na^+_i was increased by partial inhibition of Na^+/K^+ ATPase after lowering K^+_o to 2 different concentrations (1.8 mmol/L, solid squares; 0.9 mmol/L, solid triangles), the increase in developed force (DF) showed a linear relationship with Na^+_i ($y=6.54+3.69x$). This increase in force may conceivably be assigned to activation of the NCX reverse mode because it was reverted by KB-R7943 (5 μmol/L; inset). However, when Na^+_i levels were augmented by ET-1–induced NHE activation, the results lie above the relationship. In other words, for a similar increase in Na^+_i, the increase in force was significantly greater in the presence of ET-1, suggesting that this peptide is driving NCX in reverse mode not only through an NHE-mediated increase in Na^+_i but also by an additional effect that favors more Ca^{2+} influx.

greater when the increase in Na^+_i was attributable to ET-1 than when it was induced by low K^+_o. This finding suggests that ET-1 is driving the NCX in reverse mode by increasing Na^+_i by NHE activation, but it also has additional effects favoring the Ca^{2+} influx through NCX.

If ET-1 is directly stimulating NCX through a PKC pathway,[12,13] and this mechanism is inducing additional effects to NHE activation, we should detect a further increase in developed force at higher-than-normal Na^+_i and under NHE blockade. Figure 4 shows that this was the case. After the low K^+_o-induced PIE and despite the NHE inhibition, an additional increase in force was now evident when ET-1 was added to the extracellular solution (Figure 4A). KB-R7943 reversed both PIEs, that of low K^+_o and that of ET-1, indicating that they were attributable to activation of the NCX reverse mode. On average, ET-1 induced an increase of developed force of 10.8±0.4% with respect to the steady-state value obtained with low K^+_o (Figure 4B). To confirm that this ET-1–induced PIE is Na^+_i independent, we performed additional experiments in which we simultaneously recorded force and Na^+_i (Figure 5). A similar pharmacological protocol of Figure 4 was followed, but in this case, instead of KB-R7943, the PKC inhibitor chelerythrine (1 μmol/L) was added after the ET-1–induced PIE reached a steady-state level. Figure 5 clearly shows that under these experimental conditions, ET-1 produces a PIE in the absence of changes in Na^+_i and that this increase in contractility is completely reversed by PKC inhibition. These overall results indicate that ET-1 is able to induce a PKC-dependent PIE in the presence of NHE inhibition only after the increase in Na^+_i produced by low K^+_o, which drives the NCX to the reverse mode. These experiments also indicate that part of the PIE of ET-1 is Na^+_i independent and thus unrelated to the change in E_{NCX} produced by the increase in Na^+_i.

If there are changes induced by ET-1 occurring through the NCX in reverse independent of the alterations in the E_{NCX}, we

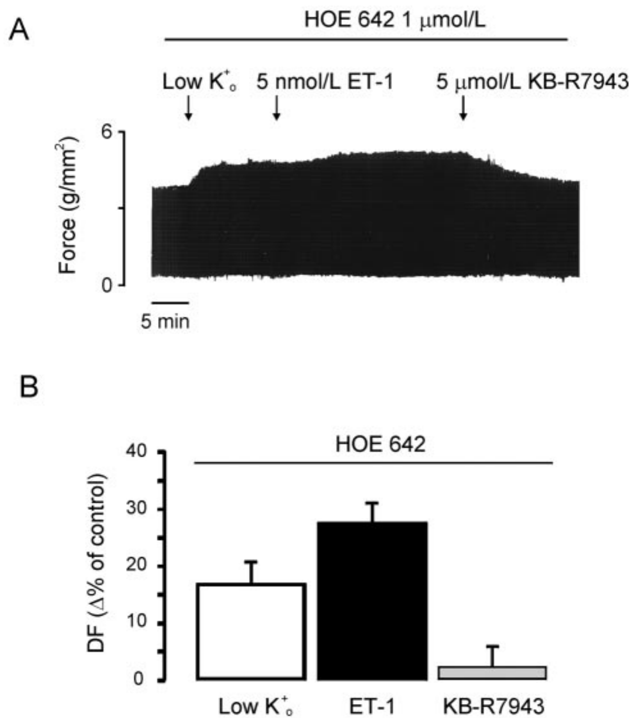

Figure 4. ET-1 is able to further increase force after an increase in contractility induced by low K^+_o in the presence of NHE inhibition. A, representative continuous recording of force of a papillary muscle pretreated with 1 μmol/L HOE642 and exposed successively to low K^+_o, 5 nmol/L ET-1, and 5 μmol/L KB-R7943. In the continuous presence of HOE642 in the extracellular medium, ET-1 applied after the increase in contractility produced by low K^+_o was able to induce an additional PIE that was completely reversed by KB-R7943. B, Averaged changes in developed force (DF) at steady state of 3 experiments like those shown in A.

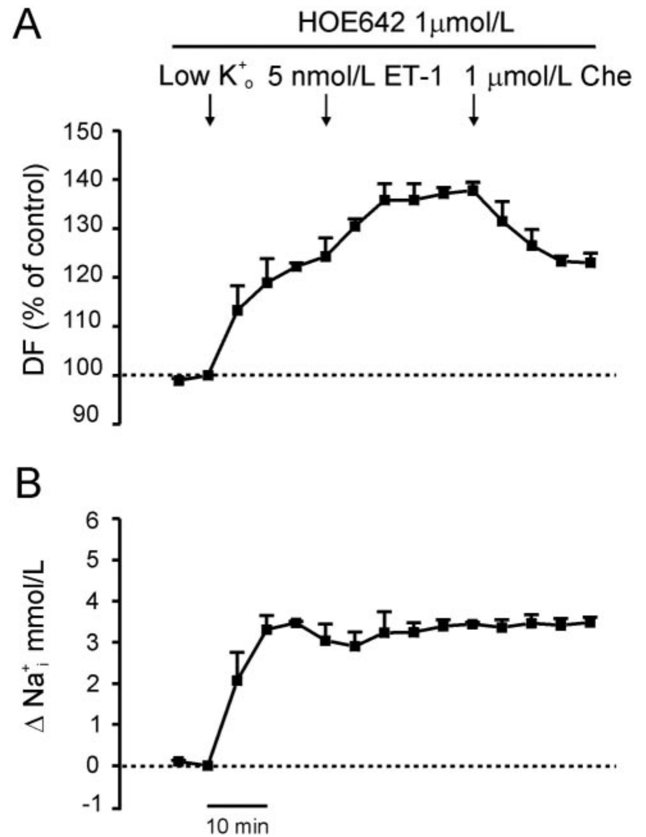

Figure 5. PKC mediates the additional increase in force promoted by ET-1 after the increase in contractility induced by low K^+_o in the presence of NHE inhibition. A, Average time course changes of developed force (DF) in 3 muscles subjected to a similar sequence of interventions to that shown in Figure 4A except for the addition of the PKC inhibitor chelerythrine (Che; 1 μmol/L) instead of KB-R7943. Once again, ET-1 applied after the increase in contractility produced by low K^+_o further increased force, but this time, only this additional effect was reversed by chelerythrine, suggesting a direct PKC-mediated phosphorylation effect of ET-1 on NCX. B, Interestingly, neither the additional PIE promoted by ET-1 nor its reversion by chelerythrine affected Na^+_i, giving strong support to the notion that ET-1 exerts Na^+-dependent (Figure 1) and -independent effects on force.

should detect changes in the I_{NCX} insensitive to NHE blockade. Thus, we next recorded whole-cell currents evoked by depolarizing steps between −80 and +80 mV in 10-mV increments from a holding potential of −40 mV. Figure 6A shows the average current-voltage (I-V) relationship for I_{NCX} before and after addition of ET-1 to the bath solution. A statistically significant ET-1–induced enhancement in outward I_{NCX} was observed at potentials higher than −10 mV with 1 nmol/L ET-1 and at potentials higher than −40 mV with 10 nmol/L ET-1. A significant increase in inward I_{NCX} was also observed at −70 and −80 mV with 10 nmol/L ET-1. A linear fitting of the individual data points of the I-V for each cell was performed to detect the 0 current level of I_{NCX}, representing E_{NCX}, in the absence and presence of ET-1. ET-1 dose-dependently produced a significant E_{NCX} negative shift (Figure 6B).

To evaluate whether the ET-1–induced E_{NCX} negative shift was attributable to an increase in Na^+_i secondary to the activation of the NHE, experiments in the presence of HOE642 were performed. Figure 6C shows the effect of HOE642 on the average I-V for I_{NCX} before and after the addition of ET-1 to the bath solution. A statistically significant ET-1–induced enhancement in outward I_{NCX} was observed at potentials >+40 mV with 1 nmol/L ET-1 and at potentials higher than −30 mV with 10 nmol/L ET-1. A significant increase in inward I_{NCX} was also observed at −70 and −80 mV with 10 nmol/L ET-1. HOE642 did not affect basal I_{NCX} (Figure 6A and 6C). No shift in E_{NCX} was observed in the presence of HOE642 (Figure 6D), indicating that the ET-1–induced negative shift observed in the absence of this inhibitor was attributable to the increase in Na^+_i generated by activation of NHE. No significant changes in basal E_{NCX} were observed with the NHE inhibitor (−39.2±5.4 and −49.5±5.1 in the absence [n=8] and presence of HOE642 [n=9], respectively).

To further analyze the net effect of ET-1 on NCX, we compared the relative increase in I_{NCX} induced by ET-1 in the absence and presence of the NHE inhibitor. ET-1 induced a greater augmentation of I_{NCX} in the absence of HOE642 than in the presence of this NHE inhibitor (at 0 mV with 10 nmol/L ET-1; 3.5±0.8- and 1.9±0.5-fold increase in the absence [n=8] and presence of HOE642 [n=9], respectively; $P<0.05$). The most likely interpretation of these results is that when the E_{NCX} negative shift was prevented by HOE642, stimulation of the NCX reverse mode was limited to the direct effect of ET-1 on NCX. On the other hand, when Na^+_i was allowed to rise in the absence of NHE inhibition, both effects of ET-1 on the NCX

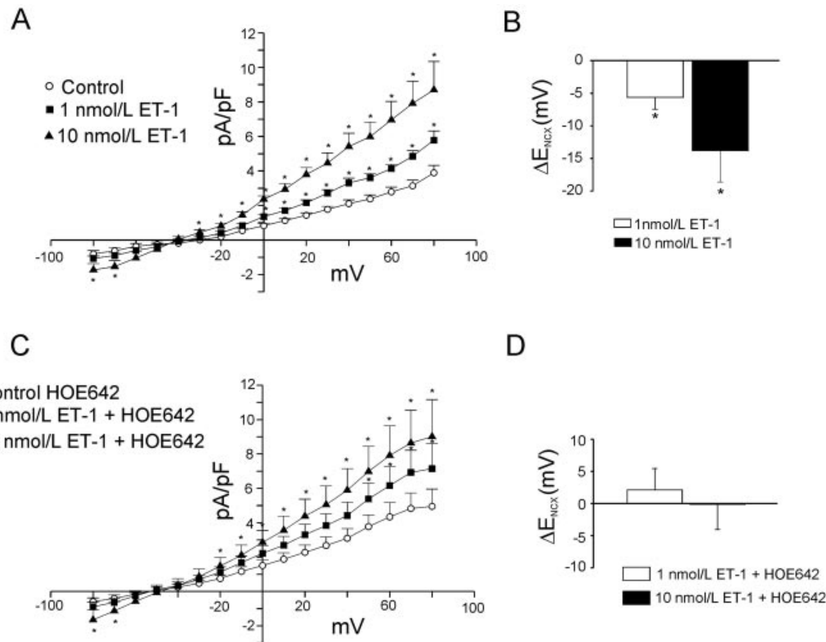

Figure 6. Effects of ET-1 on I_{NCX} in the absence and presence of NHE inhibition. A, ET-1 effects on average I-V relations for I_{NCX} in the absence of HOE642 (n=8). Whole-cell currents were evoked by depolarizing pulses from −80 to +80 mV in 10-mV increments from a holding potential of −40 mV. Currents were recorded before (control) and after successive addition of 1 nmol/L ET-1, 10 nmol/L ET-1, and 10 mmol/L $NiCl_2$ in the continuous presence of ET-1. I_{NCX} was obtained as Ni^{2+}-sensitive currents, after subtraction of the currents in the presence of Ni^{2+} to those in the absence and presence of ET-1. B, Negative shift of the E_{NCX} (n=8) obtained from I_{NCX} after successive addition of 1 and 10 nmol/L ET-1 and expressed as the difference from control (ΔE_{NCX}). C, ET-1 effects on average I-V relations for I_{NCX} in the presence of 1 μmol/L HOE642 (n=9). Currents were recorded in the continuous presence of HOE642 and before (control) and after successive addition of 1 nmol/L ET-1, 10 nmol/L ET-1, and 10 mmol/L $NiCl_2$ in the presence of ET-1. D, ΔE_{NCX} (n=9) obtained from Ni^{2+}-sensitive currents after successive addition of 1 and 10 nmol/L ET-1 in the presence of HOE642. Asterisk indicates statistically different from control.

were evident: the increase in the driving force for the reverse mode induced by the E_{NCX} negative shift and direct stimulation of NCX.

Discussion

We can conclude that a low dose of ET-1, equipotent to the slow force response to myocardial stretch[6–8] and to doses of angiotensin II that promote the release of ET-1,[5] increases myocardial force by increasing Na^+_i through the activation of NHE, which in turn stimulates the NCX reverse mode. In the present work, we attempted to answer the following question: Is this increase in Na^+_i the only mechanism responsible for these mechanical changes? A simplistic approach would be that if prevention of the increase in Na^+_i by blocking NHE prevented development of the mechanical counterpart, then the increase in Na^+_i is the determinant of the contractile effect. However, the possibility that the increase in Na^+_i could be a necessary but not an exclusive mechanism to induce the Ca^{2+} influx through the NCX in reverse should be considered. In this regard, we are presenting evidence that although blockade of the NCX reverse mode abolished the mechanical counterpart manifested after a rise in Na^+_i, induced either by ET-1 or by Na^+/K^+ ATPase inhibition, for a given increase in Na^+_i, the increase in developed force was greater when activation of the NCX reverse mode was induced by ET-1 than when the Na^+/K^+ ATPase was inhibited. Moreover, we also demonstrated that in the presence of NHE inhibition, ET-1 applied after the increase in Na^+_i produced by low K^+_o was able to induce an additional PIE that was completely reversed by inhibition of the NCX reverse mode.

These results strongly suggest that factors affecting the NCX reverse mode other than the increase in Na^+_i and the consequent E_{NCX} negative shift are participating when the increase in force is mediated by ET-1. In this regard, our electrophysiological experiments are indicating that ET-1 is also producing a direct stimulation of NCX. A phosphorylation of NCX by a PKC-dependent mechanism has been proposed[12,13] and might represent the subcellular pathway leading to direct stimulation of this transporter. Consistently, we showed in the present work that PKC mediates the Na^+_i-independent PIE detected in the experiments in which ET-1 was applied after low K^+_o in the presence of NHE inhibition. The possibility that ET-1–induced activation of different PKC isoforms may represent a common pathway for both, Na^+_i-dependent and -independent effects on NCX, constitutes an interesting hypothesis that deserves further research.

Taking into account the values of the ET-1–induced E_{NCX} negative shift detected in the present study and considering that Ca^{2+}_o, Ca^{2+}_i, and Na^+_o were 2 mmol/L, 200 nmol/L, and 140 mmol/L, respectively, the change in Na^+_i can be calculated with the equation: $E_{NCX}=3E_{Na}-2E_{Ca}$, where E_{Na} and E_{Ca} are the equilibrium potentials for Na^+ and Ca^{2+}, respectively. Thus, the values of the estimated increase in Na^+_i were 1.57 ± 0.51 and 5.22 ± 1.32 mmol/L for 1 and 10 nmol/L ET-1 (n=8), respectively. These values are of the same order of magnitude to those measured in the bulk of the cytosol by epifluorescence in papillary muscles after addition of 5 nmol/L ET-1 (Figure 1C). However, it is important to note that the increase in Na^+_i in the isolated myocytes might reflect changes of this ion in a space in which intracellular dialysis with the solution of the patch pipette cannot maintain the Na^+ concentration at a constant level.

Although it is difficult to imagine how the increase in Na^+_i can only enhance the NCX reverse mode without slowing the forward mode, we would like to call attention to the fact that the selective inhibitor of the NCX reverse mode blunted the contractile response. However, we cannot completely rule out a contribution of a reduced forward mode of the NCX to the increase in Ca^{2+}_i.

Together, the results presented herein indicate that the cardiac NCX reverse mode is modulated by ET-1 through 2 different pathways: (1) a negative shift of E_{NCX} after a rise in Na^+_i because

of NHE activation, and (2) an increase in the turnover rate of the NCX units induced by a PKC-dependent pathway. Both pathways appear to contribute to the ET-1–induced PIE. However, the increased turnover rate seems to contribute to this effect only after the change in E_{NCX} produced by NHE activation, which appears to be a necessary step. The fact that basal levels of Na^+_i are increased in some myocardial diseases such as hypertrophy[17,18] or heart failure[18,19] permit the speculation that it would not be mandatory that the aforementioned mechanisms should take place in series. Thus, the relative contribution of these 2 pathways to the ET-1–induced increase in contractility in pathological states is an interesting forthcoming perspective that deserves future attention.

Study Limitations

The main limitations of our study rely on the comparison of the contractile response with an increase in Na^+_i levels by 2 different interventions, namely ET-1 and low K^+_o. Although we demonstrated that both interventions increase contractility by NCX in reverse, and that for a given increase in Na^+_i, ET-1 induced a greater increase in contractility than inhibition of the Na^+/K^+ ATPase, the following possibilities should be analyzed: (1) Despite an equal Na^+_i in the bulk of the cytosol, the increase of Na^+_i induced by ET-1 would be higher in a fuzzy space close to NCX. However, a colocalization of NCX and NHE,[20,21] and also of NCX and the Na^+/K^+ ATPase,[22] have been reported; (2) ET-1 could induce a greater prolongation of the action potential duration (APD) than low K^+_o, then the influx of Ca^{2+} through the NCX reverse mode would be greater than that induced by Na^+/K^+ ATPase inhibition. Although the increase in APD by ET-1 has been reported,[23,24] low K^+_o can also induce a prolongation of APD,[25] and the differences between both interventions are difficult to evaluate; (3) The way selected by us to increase Na^+_i (low K^+_o), by hyperpolarizing the cell, may promote Ca^{2+} efflux through the forward mode of NCX, thus reducing PIE. However, no negative inotropic effect was observed after complete block with KB-R7943 of the low K^+_o-induced PIE (Figure 3, inset); and (4) The increase in Na^+_i produced by either ET-1 or inhibition of the Na^+/K^+ ATPase may affect regulation of ion channels (ie, Na^+-activated K^+ channels[26]) and alter membrane potential. However, in our experiments, these potential effects of increased Na^+_i unlikely influence the contractile behavior because the PIE induced by ET-1 or Na^+/K^+ ATPase inhibition was completely cancelled by selective inhibition of the NCX reverse mode.

Acknowledgments

This work was supported by grant (PEI) 6020 from the Consejo Nacional de Investigaciones Científicas (CONICET), Argentina (E.A.A.) and grants PICT 05-08512 from Agencia Nacional de Promoción Científica y Tecnológica, Argentina, and PIP 02255 from CONICET (H.E.C.).

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