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Endothelin-1 Regulates Cardiac L-Type Calcium Channels via NAD(P)H Oxidase-Derived Superoxide

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Abstract

It has been shown that reactive oxygen species (ROS) are involved in the intracellular signaling response to G-protein coupled receptor stimuli in vascular smooth muscle cells and in neurons. In the present study, we tested the hypothesis that NAD(P)H oxidase-derived ROS are involved endothelin-1 (ET-1)-induced L-type calcium channel activation in isolated cardiac myocytes. ET-1 (10 nM) induced a 2-fold increase in L-type calcium channel open-state probability (NPo). This effect of ET-1 was abolished by the ET_A receptor antagonist cyclo(D-Trp-D-Asp-Pro-D-Val-Leu) [BQ-123 (1 μM)] but was not altered in the presence of an ET_B receptor antagonist *N-cis*-2,6-dimethylpiperidinocarbonyl-*b*-tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH [BQ-788 (1 μM)]. Pre-treatment of cells with the ROS scavenger tempol (100 μM), polyethylene glycol-superoxide dismutase (SOD, 25 U/ml), or the NAD(P)H-oxidase inhibitor gp91ds-tat ([H]RKRRRQRRR-CSTRIRRL[NH₃]) (5 μM) significantly attenuated ET-1-induced increases in calcium channel NPo. Tempol, SOD, and gp91ds-tat alone had no effect on basal calcium channel activity. In addition, ET-1 significantly increased NAD(P)H oxidase activity and elevated intracellular superoxide levels in cultured cardiac myocytes. The superoxide generator, xanthine-xanthine oxidase (10 mM, 20 mU/ml), also increased calcium channel NPo in cardiac myocytes, mimicking the effect of ET-1. These observations provide the first evidence that ET-1 induces the activation of L-type Ca²⁺ channels via stimulation of NAD(P)H-derived superoxide production in cardiac myocytes.

Endothelin-1 (ET-1) was first characterized as a potent endothelium-derived vasoconstrictor peptide (Yanagisawa et al., 1988). It is also expressed in the heart and is released by endothelial, vascular smooth muscle, and myocardial cells, especially in cardiovascular disorders, such as congestive heart failure, cardiac hypertrophy, and ischemic heart disease (Moe et al., 2003; Angerio, 2005; Suzuki et al., 2005; Wainwright et al., 2005). Thus, it has been proposed that ET-1 plays a potential paracrine and autocrine role in cardiac functional and genetic regulation under pathophysiological conditions (Ito et al., 1991, 1993; Sugden,

2003). As such, it is not surprising that tremendous investigative efforts have been put forward to define the pathological and physiological circuits involved in ET-1 actions.

In cardiac myocytes, the L-type Ca^{2+} current (I_{CaL}) is the main depolarizing current contributing to the plateau-shape of the action potential and regulation of Ca^{2+} release from the sarcoplasmic reticulum (Cleemann and Morad, 1991). This channel is regulated by several second messengers, which explains why it mediates the action of numerous hormones and neuromediators, including ET-1. However, the effect of ET-1 on I_{CaL} still remains unclear. For example, some studies of ET-1 have shown clear increases in I_{CaL} (Lauer et al., 1992; Boixel et al., 2001), whereas others have shown a decrease in I_{CaL} (Cheng et al., 1995; Ono et al., 1995) or no effect (Tohse et al., 1990). These contradictory data may be due to the wide differences in experimental conditions, including different species, tissues, and preparations. In addition, the method employed to measure I_{CaL} could be the most important reason. Most of the initial studies were performed using the ruptured whole-cell patch clamp technique. In contrast, cell-attached single channel recording allows characterization of I_{CaL} without the alteration of intracellular components that occurs with whole-cell patch clamp technique. Therefore, it is essential to examine the effect of ET-1 on I_{CaL} and the underlying mechanism in intact cardiac myocytes.

Reactive oxygen species (ROS) are well recognized as important mediators of cardiovascular pathology, including hypertrophy and heart failure (Dhalla et al., 2000; Byrne et al., 2003b). Both mitochondria and NAD(P)H oxidase are capable of participating directly in the reduction of oxygen and producing superoxide. In vascular tissue the NAD(P)H oxidase is recognized as the predominant source of superoxide (Griendling et al., 2000). Recent studies are providing evidence for a role for NAD(P)H oxidase-derived ROS in cardiac pathology. Mice lacking the pg91phox subunit do not develop angiotensin II-induced cardiac hypertrophy (Byrne et al., 2003a). With the exception of the importance of ROS in cardiac pathophysiology, it is still not clear whether NAD(P)H oxidase-derived ROS play a role in the rapid response to ET-1 in cardiac myocytes. Identifying the mechanisms responsible for the action of ET-1 could help identify a new therapeutic target for ET-1-associated cardiac disorders.

In this study, we examined whether ET-1 increases intracellular ROS production via increased NAD(P)H-oxidase activity and whether the elevated ROS subsequently modulates the function of L-type calcium channels in cardiac myocytes.

Materials and Methods

Animals and Drugs

Adult male Sprague-Dawley rats (200–250 g) were obtained from Charles River Farms (Wilmington, MA). Rats were housed individually and kept on a 12/12-h light/dark cycle in a climate-controlled room. Rat chow and water were provided ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee of Northeast Normal University and by the North Dakota State University Institutional Animal Care and Use Committee.

Dihydroethidium (DHE) was purchased from Molecular Probes (Carlsbad, CA). Dulbecco's modification of Eagle's medium was obtained from GIBCO (Carlsbad, CA). Bay K 8644 was purchased from Calbiochem (San Diego, CA). The selective NAD(P)H oxidase inhibitor gp91ds-tat ([H]RKKRRQRRR-CSTRIRRL[NH₃]) and its control, scrambled gp91ds-tat ([H]RKKRRQRRR-CLRITRQSR[NH₃]), were synthesized by Tufts University Core Facility (Medford, MA). BQ-123, BQ-788, ET-1, ATP, GTP, HEPES, and others were purchased from Sigma-Aldrich (St. Louis, MO).

Ventricular Myocytes Isolation

Cardiac myocytes were enzymatically isolated from hearts obtained from rats anesthetized with sodium pentobarbitone (30 mg/kg i.p. injection). Each heart was rapidly excised and cooled in ice-cold Tyrode's solution containing 135 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1.0 mM MgCl₂, 5.5 mM glucose, 1.8 mM CaCl₂, and 10 mM HEPES, pH 7.4 (NaOH). After cannulation of the aorta, retrograde perfusion was started with Tyrode's solution via a recirculating system for 2 min. The heart was then perfused with Ca²⁺-free normal Tyrode's solution for 5 min, followed by perfusion with Ca²⁺-free normal Tyrode's solution containing collagenase (0.15 mg/ml) for 50 min. The ventricle was excised, cut into small pieces, and incubated at 37°C for 10 min in a storage solution containing 70 mM KOH, 50 mM glutamic acid, 40 mM KCl, 20 mM KH₂PO₄, 20 mM taurine, 3 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 0.5 mM EGTA, pH 7.4 (KOH). The separated ventricular myocytes were stored at 4°C in a refrigerator until they were used for electrophysiological recording.

Single Calcium Channel Recording

Single L-type calcium channel activity was measured by the patch-clamp technique in cell-attached mode. Patch pipettes were pulled with a three-step puller (Zeitz, Augsburg, Germany) and fire-polished to a final resistance of 2 to 5 MΩ. Cells were bathed in a bath solution containing 140mMpotassium aspartate, 10mMEGTA, and 10mMHEPES, pH 7.4 (KOH). The patch pipettes were filled with an internal pipette solution containing 110 mM BaCl₂, and 10 mM HEPES, pH 7.4 [Ba(OH)₂]. The potential of the electrode was adjusted to zero between the pipette solution and the bath solution immediately before seal formation. After a giga-seal between the pipette and myocyte had formed, the activity of L-type calcium channels was induced by stepping from a holding potential of -60 to +10 mV for 256-ms duration at a rate of 0.5 Hz. Single Ca²⁺ channel activity was recorded by L/M-PC patch-clamp amplifier (List, Darmstadt, Germany). Analysis of the unitary currents was performed with pClamp 8.0 software (Axon Instruments, Downingtown, PA), and the results were expressed as open-state probability (NPo).

Neonatal Rat Cardiomyocyte Culture

Primary cultured cardiomyocytes were prepared from neonatal rats according to previously published procedures with minor modulation (Gruh et al., 2006). In brief, ventricles from 1-day-old Sprague-Dawley rats were digested at 37°C in Hanks' solution containing collagenase (class II; Worthington Biochemical, Lakewood, NJ). Isolated cells were suspended in culture medium composed of 8% fetal bovine serum, Dulbecco's modification of Eagle's medium, and 0.2% penicillin-streptomycin solution. The cell suspension was

placed in 35-mm diameter tissue culture dishes at a cell density of 4×10^6 /dish and incubated at 37°C in a humidified atmosphere with 95% O₂ + 5% CO₂.

Measurement of Intracellular ROS Level

ROS generation was determined using the oxidant-sensitive fluorogenic probe DHE (excitation wavelength, 488 nm; emission wavelength, 610 nm) essentially as described elsewhere (Hool et al., 2005). In brief, primary cultured cardiomyocytes were loaded with 100 nM DHE for 30 min at 37°C. The cells were then incubated with PBS for 5 min, followed by 10 nM ET-1 for 5 min. In another set of cardiomyocytes, cells were pretreated with BQ-123 (1 μM) or vehicle control for 10 min before ET-1 treatment. Ethidium fluorescence within cardiomyocytes was detected by a fluorescent microscope (Nikon, Melville, NY), and its intensity in individual cells was analyzed using Quantity One Software (Bio-Rad, Hercules, CA). Each treatment condition was run in triplicate within experiments, and each set of experiments was performed using three separate culture dishes.

Measurement of NAD(P)H Oxidase Activity

The lucigenin-derived chemiluminescence method was used to measure ET-1-induced NAD(P)H oxidase activity in primary cultured cardiomyocytes. Cardiomyocytes were treated with ET-1 (10 nM) or PBS for 5 min and washed in ice-cold PBS, and cells were scraped and then sonicated for 1 s. Ten minutes before recording luminescence, NAD(P)H (100 μM) and lucigenin (5 μM) were added and light emission was recorded during the next 10 s by a Wallac 1450 Micro-Beta JET Luminometer (PerkinElmer Life and Analytical Sciences, Waltham, MA). Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin standards. Data were presented as counts per minute per milligram of protein.

Data Analysis

All data are expressed as mean ± S.E. Comparisons between experimental groups were performed using analysis of variance followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

Results

ET-1 Stimulates L-type Calcium Channel

ET-1 has positive inotropic and chronotropic actions in a variety of cardiac preparations (Kramer et al., 1991). To investigate the role of I_{CaL} in these responses to ET-1, we used cell-attached patch-clamp single channel recording to assess the effect of ET-1 on I_{CaL} in isolated rat cardiac myocytes without disruption of intracellular environment. Application of ET-1 (10 nM) evoked a significant increase in the calcium channel NPo (Fig. 1, A and B). The channel open time was increased from 2.95 ± 0.33 to 4.74 ± 0.42 ms ($n = 11$, $P < 0.05$), and the channel close time was decreased from 88.40 ± 14.73 to 35.31 ± 12.41 ms ($n = 11$, $P < 0.05$) by application of ET-1 (10 nM). However, the perfusion of cardiomyocytes with ET-1 (10 nM) did not significantly alter the unitary current amplitude, as shown in Fig. 1A (1.42 ± 0.11 and 1.40 ± 0.18 pA in cells before and after treatment with ET-1, respectively, $n = 11$, $P > 0.05$). The increases in NPo of I_{CaL} evoked by ET-1 were partially reversed after

10-min washout with fresh bath solution (from 0.021 ± 0.002 to 0.017 ± 0.002 , $n = 4$, $P < 0.05$; the baseline NPo was 0.014 ± 0.03). In another series of experiments, the addition of a specific I_{CaL} activator Bay K 8644 (100 nM) significantly increased NPo of I_{CaL} without changing the unitary current amplitude, mimicking the effect of ET-1 on this channel in cardiac myocytes (Fig. 1, C and D). The Bay K 8644-induced increases in NPo of I_{CaL} were completely reversed after 10-min washout with fresh bath solution (from 0.026 ± 0.03 to 0.017 ± 0.01 , $n = 4$, $P < 0.05$; the baseline NPo was 0.015 ± 0.02).

It has been shown that both ET_A and ET_B receptors are expressed in cardiomyocytes (Wainwright et al., 2005). Thus, we examined the endothelin-receptor subtype(s) involved in the L-type calcium current response to ET-1 by pretreatment of the cells with specific receptor antagonists. Data are shown in Fig. 2A, indicating that the ET_A receptor antagonist BQ-123 (1 μ M) completely blocked the ET-1-induced increase in calcium channel NPo. By contrast, the ET_B receptor antagonist BQ-788 (1 μ M) did not alter the calcium channel responsiveness to ET-1 under the same treatment conditions. In these experiments, neither BQ-123 (1 μ M) nor BQ-788 (1 μ M) alone significantly changed the basal calcium channel activity.

NAD(P)H Oxidase-Derived ROS Are Involved in I_{CaL} Response to ET-1

Initially, we used tempol, a superoxide scavenger, to determine the role of ROS in ET-1-induced increases in L-type calcium channel NPo in freshly isolated cardiac myocytes. Figure 2B shows that, under control conditions, ET-1 (10 nM) caused a 2-fold increase in NPo of I_{CaL} and that treatment with tempol (100 μ M) resulted in an 80% attenuation of the ET-1-induced increase in NPo of I_{CaL} ($P < 0.05$ compared with ET-1 alone). However, in the presence of tempol (100 μ M), Bay K 8644 (100 nM) increased the NPo of I_{CaL} from 0.014 ± 0.002 to 0.026 ± 0.004 ($n = 6$, $P < 0.01$), indicating that tempol has no effect on Bay K 8644-induced I_{CaL} . Tempol (100 μ M) alone did not significantly change the basal calcium channel activity in isolated cardiac myocytes (Fig. 2B). Next, we used gp91ds-tat to confirm the involvement of ROS and further to identify the source of ROS in the ET-1 action. Pretreatment of cardiomyocytes with gp91ds-tat (5 μ M) significantly attenuated ET-1-induced increases in NPo of I_{CaL} ($P < 0.05$, compared to ET-1 alone; Fig. 2B). In the presence of gp91ds-tat (5 μ M), Bay K 8644 (100 nM) increased the NPo of I_{CaL} from 0.015 ± 0.01 to 0.025 ± 0.03 ($n = 6$, $P < 0.01$). In contrast, scrambled gp91ds-tat (5 μ M) did not alter the ET-1-induced activation of I_{CaL} under the same treatment conditions (data not shown). Neither gp91ds-tat nor scrambled gp91ds-tat alone had any significant effect on the basal I_{CaL} activity in cardiac myocytes.

ET-1 Increases ROS Production in Cardiomyocytes

The fluorogenic probe, DHE, was used to assess the effect of ET-1 on ROS production in primary cultured cardiomyocytes. Under control conditions, ethidium fluorescence was low in PBS-treated cardiomyocytes (Fig. 3B); however, treatment of these same cells with 10 nM ET-1 resulted in a significant increase in the density of ethidium fluorescence within cardiomyocytes (Fig. 3, C and G). The ET-1-induced increase in fluorescence was completely inhibited by the ET_A endothelin receptor antagonist BQ-123 (1 μ M; Fig. 3, F and G). BQ-123 (1 μ M) alone did not alter the basal ethidium fluorescence (Fig. 3, E and G). In

contrast, the ET_B endothelin receptor antagonist BQ-788 (1 μ M) had no significant effect on this action of ET-1 (Fig. 3G). In addition, the ET-1-induced increase in ethidium fluorescence was attenuated by gp91ds-tat (5 μ M) (845 ± 61 and 926 ± 77 ethidium fluorescence intensity of cells treated with gp91ds-tat and gp91ds-tat + ET-1, respectively, $n = 13$, $P > 0.05$). However, the scrambled control gp91ds-tat did not alter the stimulatory action of ET-1 on ROS production (751 ± 79 and 1522 ± 98 ethidium fluorescence intensity of cells treated with scrambled gp91ds-tat and scrambled gp91ds-tat + ET-1, respectively, $n = 11$, $P < 0.01$). These results indicate that, via activation of ET_A receptors, ET-1 increases superoxide production, which is blocked by inhibition of NAD(P)H oxidase.

ET-1 Stimulates NAD(P)H Oxidase in Cardiomyocytes

Treatment of primary cultured cardiomyocytes with ET-1 (10 nM) resulted in a 2-fold increase in NAD(P)H oxidase activity (Fig. 4A). This effect of ET-1 was completely blocked by preincubation of cardiomyocytes with the ET_A endothelin receptor antagonist BQ-123 (1 μ M). However, the ET_B endothelin receptor antagonist, BQ-788 (1 μ M), has no effect on ET-1-induced increases in NAD(P)H oxidase activity (Fig. 4A). In addition, preincubation of cardiomyocytes with gp91ds-tat (5 μ M), but not with scrambled gp91ds-tat (5 μ M), significantly attenuated the ET-1-induced activation of NAD(P)H oxidase (Fig. 4B).

Superoxide Contributes to ET-1-Induced I_{CaL} Activation

To identify whether superoxide contributes to ET-1-induced I_{CaL} activation, we recorded I_{CaL} activity in the presence or absence of a cell-permeable superoxide scavenger, PEG-SOD (25 U/ml), in isolated cardiac myocytes. The results are shown in Fig. 5A, indicating that pretreatment of cardiomyocytes with PEG-SOD (25 U/ml) significantly attenuated ET-1-induced I_{CaL} activation and that PEG-SOD alone had no effect on the basal I_{CaL} activity. In addition, in the presence of PEG alone, ET-1 (10 nM) still increased I_{CaL} NPo from 0.012 ± 0.002 to 0.019 ± 0.003 ($n = 5$, $P < 0.01$), indicating that PEG itself has no effect on ET-1-induced I_{CaL} activation. Moreover, superfusion of cardiomyocytes with a superoxide generator, xanthine-xanthine oxidase (X-XO: X = 10 mM; OX = 20 mU/ml) increased I_{CaL} activity, mimicking the effects of ET-1 (Fig. 5A). These results suggest that superoxide is involved in the ET-1-induced I_{CaL} activation in cardiomyocytes. Next, we examined the role of H₂O₂ in the action of ET-1 on I_{CaL} in cardiac myocytes. Pretreatment of cells with PEG-catalase (250 U/ml) did not significantly alter ET-1-induced I_{CaL} activation. Superfusion of cardiomyocytes with H₂O₂ (1 μ M) had no effect on I_{CaL}. Taken together, these data suggest that the stimulatory effect of ET-1 on I_{CaL} is mediated by intracellular superoxide production in cardiac myocytes.

Discussion

The present studies provides the first evidence that ET-1-induced activation of L-type calcium channels (I_{CaL}) is mediated by NAD(P)H oxidase-derived superoxide production in cardiac myocytes. This conclusion is supported by the following observations that 1) ET-1 increased the open-state probability of I_{CaL} recorded in cell-attached patches of cardiac myocytes and that this effect of ET-1 was diminished by a specific ET_A receptor antagonist; 2) the ET-1-induced increase in I_{CaL} was attenuated by the superoxide scavengers, tempol

and PEG-SOD; 3) superfusion of cardiac myocytes with a superoxide generator, xanthine-xanthine oxidase, also increased I_{CaL} activity, mimicking the action of ET-1; 4) incubation of cultured cardiomyocytes with ET-1 increased intracellular superoxide levels and NAD(P)H oxidase activity; and 5) pretreatment of cardiac myocytes with the NAD(P)H oxidase inhibitor, gp91ds-tat, attenuated ET-1-induced superoxide production and increases in I_{CaL} open-state probability.

ET-1 has a positive inotropic effect in many isolated cardiac preparations (Kramer et al., 1991). It is well known that an increase in the intracellular calcium transient contributes to this positive inotropic effect and that the basis for the increased Ca^{2+} transient is stimulation of I_{CaL} . The results of the present study are consistent with this hypothesis, inasmuch as ET-1 caused an increase in the open-state probability of I_{CaL} in rat cardiac myocytes. However, previous investigations examining the effect of ET-1 on I_{CaL} have produced conflicting results, with decreases (Lauer et al., 1992; Boixel et al., 2001), increases (Cheng et al., 1995; Ono et al., 1995), and no effect (Tohse et al., 1990) on basal I_{CaL} reported. These discrepancies may be due to differences in experimental conditions, particularly differences in the electrophysiologic recording methods used (e.g., rupture of the cell membrane). One important reason for the discrepant findings regarding the effects of ET-1 on I_{CaL} in cardiac myocytes may be related to the expression of ET receptors. Cardiac myocytes express both ET_A - and ET_B endothelin receptors (Moe et al., 2003; Wainwright et al., 2005). In general, the ET_A receptor subtype is more abundant (90%) and is considered more important for the cardiac stimulatory effects of ET-1 on I_{CaL} as demonstrated in the present study. Although ET_B receptors may not contribute to the action of ET-1 on I_{CaL} , as shown in the present study in rat cardiac myocytes, they may contribute to inhibitory effects of ET-1 (Kedzierski and Yanagisawa, 2001). Thus, the ratio between ET_A and ET_B receptors in cardiac myocytes from different animal species could play a role in the observed action of ET-1 on I_{CaL} . Another important reason for these discrepant findings may be related to the interaction with other regulatory factors and intracellular second messengers, such as cyclic AMP, β -adrenoceptor agonists, protein kinase A, and protein kinase C (PKC). It has been shown that the effect of ET-1 on cardiac contractility and L-type calcium current varies depending on the presence of norepinephrine, β -adrenoceptor agonists, or cAMP analogs (Watanabe and Endoh, 1999; Chu et al., 2003). For example, in canine ventricular myocardium, ET-1 induces either a positive inotropic effect or a negative inotropic effect, which is determined by the presence of a low concentration or high concentration of norepinephrine, respectively. Moreover, in rabbit cardiomyocytes, ET-1 has a biphasic effect on calcium current, causing a transient decrease followed by a long-lasting increase in current (Watanabe and Endoh, 1999). The ET-1-induced inhibition of L-type calcium current was enhanced in the presence of an activated cAMP-dependent pathway. This interaction between ET-1 and norepinephrine in the regulation of cardiomyocyte contractility and calcium channel current could play an important role in the pathophysiology of heart failure, which is associated with increased sympathetic nerve activity and elevated plasma norepinephrine levels. Nevertheless, studying the effect of ET-1 on calcium channel current under different neurohormonal conditions will help improve our understanding of the cellular mechanisms underlying the pathogenesis of cardiac diseases, such as heart failure and cardiac remodeling.

The present observations demonstrate that ET-1 increases intracellular superoxide levels by stimulation of NAD(P)H oxidase and that increased superoxide production is involved in the stimulatory effect of ET-1 on I_{CaL} in cardiac myocytes. Although superoxide may be rapidly converted to hydrogen peroxide, it is unlikely that the stimulatory effect of ET-1 on I_{CaL} is mediated by hydrogen peroxide because the hydrogen peroxide scavenger, catalase, had no effect on the response to ET-1 and authentic hydrogen peroxide itself had no effect on I_{CaL} . These results are supported by data from others in vascular smooth muscle cells showing that ET-1 increases superoxide production via ET_A -receptor/NAD(P)H oxidase pathways (Callera et al., 2003; Li et al., 2003). Given that extracellular calcium influx plays essential roles in ET-1-induced vascular contraction and cell proliferation, it is not surprising that the ET-1-ROS signaling pathway is involved in the action of other cardiovascular regulatory factors, such as angiotensin II-induced positive inotropic effects, leptin-induced cardiac hypertrophy, and atrial natriuretic peptide-mediated antihypertrophic effects (Xu et al., 2004; Cingolani et al., 2006; Laskowski et al., 2006). However, the nature of the intracellular signaling mechanisms by which ROS increases I_{CaL} in cardiac myocytes remains to be clarified. One possibility may be a direct redox-dependent mechanism, because the pore-forming α_{1C} -subunit of the cardiac L-type Ca^{2+} channel contains more than 10 cysteine residues (Mikami et al., 1989) that can potentially undergo redox modification. Indeed, oxidization of Src homology groups causes stimulation of I_{CaL} , whereas GSH and dithiothreitol, which reduce disulfide bonds, inhibit this current in ferret ventricular myocytes (Campbell et al., 1996).

Another major question that arises from our studies concerns the mechanisms that are involved in the ET-1-stimulated NAD(P)H oxidase activation in cardiac myocytes. At present, little is known with regard to such mechanisms in cardiac myocytes. However, several clues are emerging from other research areas that may provide future direction for addressing this question. Activation of PKC is involved in ET-1-induced increases in cardiac I_{CaL} via an unknown mechanism (He et al., 2000), and PKC-dependent NAD(P)H oxidase activation is the main source of intracellular ROS production in response to angiotensin II in vascular smooth muscle cells (Touyz and Schiffrin, 2001; Yasunari et al., 2002; Ungvari et al., 2003) and in neurons (Wang et al., 2006). Therefore, future studies examining whether PKC-mediated phosphorylation of NAD(P)H oxidase contributes to the ET-1-induced ROS elevation in cardiac myocytes should provide important information regarding intracellular signaling.

In summary, ET-1 increases I_{CaL} activation via stimulation of ET_A receptors in cardiac myocytes. The ET-1-induced increase in I_{CaL} is mediated by NAD(P)H oxidase-derived superoxide production. Given that activation of L-type calcium channels is involved in ET-1-related cardiac pathophysiological conditions (Chu et al., 2003; Hirotsani et al., 2004; Angerio, 2005; Sugden and Clerk, 2006), ROS-dependent activation of I_{CaL} may contribute to ET-1-induced intracellular calcium mobilization, cardiac excitation-contraction, myocyte proliferation, and consequently disorders, such as heart failure and cardiac hypertrophy.

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ABBREVIATIONS

ET-1	endothelin-1
NAD(P)H	nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
NPo	channel open-state probability
DHE	dihydroethidium
I_{CaL}	L-type calcium channel current
SOD	superoxide dismutase
PEG	polyethylene glycol
X-XO	xanthine-xanthine oxidase
PKC	protein kinase C
BQ-123	cyclo(D-Trp-D-Asp-Pro-D-Val-Leu)
BQ-788	<i>N-cis</i> -2,6-dimethylpiperidinocarbonyl- <i>b</i> -tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH
gp91ds-tat	[H]RKKRRQRRR-CSTRIRRL[NH ₃]
PBS	phosphate-buffered saline
Bay K 8644	1,4-dihydro-2,6-dimethyl-5-nitro-4-[2'-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester

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Fig. 1.

Effects of ET-1 and Bay K 8644 on I_{CaL} recorded in cell-attached patches of isolated cardiomyocytes. A, representative tracings showing I_{CaL} activity recorded in a cardiomyocyte before and after application of ET-1 (10 nM). The bars show the baseline (I_{CaL} closing state). The patch membrane was depolarized to 10 mV from the holding potential of -60 mV for 256 ms. B, bar graphs are mean $\pm$ S.E. of I_{CaL} open probability in cardiomyocytes ($n = 11$) before and after application of ET-1 (10 nM). *, $P < 0.01$ compared with control. C, representative tracings showing I_{CaL} activity recorded in a cardiomyocyte under control condition and after superfusion with Bay K 8644 (Bay K, 100 nM). The bars indicate the channel closing state. D, bar graphs are mean $\pm$ S.E. of calcium channel open probability in cardiomyocytes ($n = 12$) before and after application of Bay K 8644 (Bay K, 100 nM). *, $P < 0.005$ compared with control.

Fig. 2.

Role of ET_A receptor, ET_B receptor, and superoxide in ET-1-induced increases in I_{CaL} in isolated cardiomyocytes. A, bar graphs showing the effect of an ET_A receptor antagonist, BQ-123 (BQ123, 1 μ M), and an ET_B receptor antagonist, BQ-788 (BQ788, 1 μ M), on ET-1-induced increases in I_{CaL} in cell-attached patches. Data are mean $\pm$ S.E. channel open probability in cardiomyocytes ($n = 7$ and 8) treated under the following conditions: Control, ET-1 (10 nM), BQ-123 or BQ-788, and BQ-123 + ET-1 or BQ-788 + ET-1. *, $P < 0.05$ compared with the respective control. B, bar graphs showing the effect of a superoxide scavenger, tempol (TP, 100 μ M), and a NAD(P)H oxidase inhibitor, gp91ds-tat (GP, 5 μ M), on ET-1-induced increases in I_{CaL} in cell-attached patches. Data are mean $\pm$ S.E. of channel open probability in cardiomyocytes ($n = 9$ and 7) treated under the following conditions: control, ET-1 (10 nM), TP or GP, and TP + ET-1 or GP + ET-1. *, $P < 0.05$ compared with control.

Fig. 3.

Effects of ET-1 on superoxide production within cardiomyocytes. Superoxide levels were detected using the fluorogenic probe DHE in primary cultured cardiomyocytes as detailed under *Materials and Methods*. A through C, cardiomyocytes that were treated under the following conditions: cardiomyocytes in normal optical phase (A); fluorescence micrograph of cardiomyocytes loaded with DHE and treated with PBS (B); and fluorescence micrograph of the same cardiomyocytes as shown in B (C), following treatment with ET-1 (10 nM). D through F, cardiomyocytes that were treated under the following conditions: cardiomyocytes in normal optical phase (D); fluorescence micrograph of cardiomyocytes loaded with DHE after treatment with BQ-123 (BQ123, 1 μ M) for 5 min (E); and fluorescence micrograph of the same cardiomyocytes as shown in E (F), following treatment with ET-1 (10 nM). Bar = 20 μ m. G, bar graphs summarizing ethidium fluorescence intensity before and after treatment with ET-1 in the presence of vehicle control (PBS), BQ-123 (BQ123, 1 μ M), or BQ-778 (BQ778, 1 μ M). In each treatment condition, 20 to 23 cardiomyocytes were used for quantification of fluorescence. They were derived from three experiments and at least three dishes in each experiment. Data are mean $\pm$ S.E. *, significant difference from the respective control ($P < 0.01$).

Fig. 4.

Effects of ET-1 and gp91ds-tat on NAD(P)H oxidase activity in cardiomyocytes. A, effect of ET-1 and receptor involvement. Primary cultured cardiomyocytes were pretreated under the following conditions: vehicle control, BQ-123 (BQ123 1 μ M), or BQ-788 (BQ788 1 μ M) for 10 min. This was followed by incubation with 10 nM ET-1 for an additional 5 min. Cells were collected, and NAD(P)H activity was measured and expressed as mean light emission (counts per milligram of protein per minute). Data are mean $\pm$ S.E. ($n = 7$). *, significantly different from respective control treatment ($P < 0.05$). B, effect of gp91ds-tat. Primary cultured cardiomyocytes were pretreated under the following conditions: vehicle control, gp91ds-tat (GP, 5 μ M), or scrambled gp91ds-tat control (ScrGP, 5 μ M) for 10 min before treatment with 10 nM ET-1 for 5 min as described in A. Data are mean $\pm$ S.E. ($n = 9$). *, significantly different from respective control ($P < 0.05$).

Fig. 5.

Identification of ROS involved in ET-1-induced increases in I_{CaL} activity. A, role of superoxide. I_{CaL} were recorded in cell-attached patches of isolated cardiomyocytes under the following conditions: control, superfusion of ET-1 (10 nM), superfusion of PEG-SOD (SOD, 25 U/ml), and PEG-SOD plus ET-1 or control, superfusion of xanthine-xanthine oxidase (X-XO: X, 10 mM; XO, 20 mU/ml). Data are mean $\pm$ S.E. of calcium channel open probability in cardiomyocytes ($n = 9-10$). *, $P < 0.05$ compared with respective control. B, role of hydrogen peroxide. I_{CaL} were recorded in cell-attached patches of cardiomyocytes under the following treatment conditions: control, superfusion of ET-1 (10 nM), superfusion with PEG-catalase (CAT, 250 U/ml), and PEG-Catalase plus ET-1 or control, superfusion of H_2O_2 (1 μ M). Data are mean $\pm$ S.E. of calcium channel open probability in cardiomyocytes ($n = 11$ and 9). *, $P < 0.05$ compared with respective control.