Signal Transduction and Ca\textsuperscript{2+} Signaling in Contractile Regulation Induced by Crosstalk Between Endothelin-1 and Norepinephrine in Dog Ventricular Myocardium

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Abstract—In certain cardiovascular disorders, such as congestive heart failure and ischemic heart disease, several endogenous regulators, including norepinephrine (NE) and endothelin-1 (ET-1), are released from various types of cell. Because plasma levels of these regulators are elevated, it seems likely that cardiac contraction might be regulated by crosstalk among these endogenous regulators. We studied the regulation of cardiac contractile function by crosstalk between ET-1 and NE and its relationship to Ca\textsuperscript{2+} signaling in canine ventricular myocardium. ET-1 alone did not affect the contractile function. However, in the presence of NE at subthreshold concentrations (0.1 to 1 nmol/L), ET-1 had a positive inotropic effect (PIE). In the presence of NE at higher concentrations (100 to 1000 nmol/L), ET-1 had a negative inotropic effect. ET-1 had a biphasic inotropic effect in the presence of NE at an intermediate concentration (10 nmol/L). The PIE of ET-1 was associated with an increase in myofilament sensitivity to Ca\textsuperscript{2+} ions and a small increase in Ca\textsuperscript{2+} transients, which required the simultaneous activation of protein kinase A (PKA) and PKC. ET-1 elicited translocation of PKC and from cytosolic to membranous fraction, which was inhibited by the PKC inhibitor GF 109203X. Whereas the Na\textsuperscript{+}/H\textsuperscript{+} exchange inhibitor Hoe 642 suppressed partially the PIE of ET-1, detectable alteration of pH did not occur during application of ET-1 and NE. The negative inotropic effect of ET-1 was associated with a pronounced decrease in Ca\textsuperscript{2+} transients, which was mediated by pertussis toxin–sensitive G proteins, activation of protein kinase G, and phosphatases. When the inhibitory pathway was suppressed, ET-1 had a PIE even in the absence of NE. Our results indicate that the myocardial contractility is regulated either positively or negatively by crosstalk between ET-1 and NE through different signaling pathways whose activation depends on the concentration of NE in the dog. (Circ Res. 2003; 92:1024-1032.)

Key Words: endothelin-1 ■ norepinephrine ■ myocardial contractility ■ Ca\textsuperscript{2+} transients ■ protein kinase C

During the course of cardiovascular disorders, such as congestive heart failure and ischemic heart disease, plasma levels of both endothelin-1 (ET-1) and norepinephrine (NE) tend to increase.\textsuperscript{1-4} The signal transduction processes that are triggered by the activation of receptors for these endogenous agonists are different, and, thus, it seems likely that crosstalk between ET-1 and NE might play a critical role in the regulation of cardiac function, determining hemodynamic responses to antagonists of \(\beta\)-adrenoceptors or endothelin receptors under various pathophysiological conditions. The available evidence implies that these endogenous regulators are engaged in crosstalk at different levels of their respective signaling pathways. For example, the positive feedback mechanism seems to exist at the level of the synthesis of NE by which ET-1 increases the plasma concentration of NE,\textsuperscript{5} whereas NE facilitates the expression of mRNA that encodes the prepro-ET-1\textsuperscript{6} and the production of ET-1.\textsuperscript{7}

ET-1 has a positive inotropic effect (PIE) in ventricular myocardium of most mammals, but it has no inotropic effect on canine ventricular myocardium.\textsuperscript{8,9} By contrast, ET-1 has a negative inotropic effect (NIE) in the presence of catecholamines and antagonizes the \(\beta\)-adrenoeceptor–mediated facilitatory regulation of contractile function in several mammalian species, including the dog.\textsuperscript{10-14} Regulation of myocardial contractility induced by crosstalk between ET-1 and NE has not been studied in detail. It has been reported that the acceleration of the hydrolysis of phosphoinositide and the subsequent generation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol might be responsible for the PIE of ET-1 in certain species,\textsuperscript{8} but the subcellular mechanism involved in the NIE of ET-1 has not been fully elucidated. We designed
the present study in an attempt to characterize the contractile regulation induced by crosstalk between ET-1 and NE over a wide range of concentration and its relationship to the regulation of Ca^{2+} signaling in canine ventricular myocardium. We examined subcellular mechanisms responsible for such regulation using selective inhibitors of protein kinases and other types of enzymes. ET-1 had a PIE and NIE, depending on the concentration of NE present before administration of ET-1. The effects were mediated by an increase in the myofilament sensitivity to Ca^{2+} or a decrease in Ca^{2+} transients, which were induced by activation of different signaling pathways in dog ventricular myocardium. Preliminary accounts of this study have been published elsewhere.13,15–18

Materials and Methods
All manipulations of animals were performed in accordance with the Guide for Animal Experimentation, Yamagata University School of Medicine, and Japanese Governmental Law (No. 105). Approval for all experiments with animals was obtained from the Committee for Animal Experimentation, Yamagata University School of Medicine, before the experiments, and the study was also carried out in accordance with the Helsinki Declaration. Mongrel dogs (7 to 10 kg) of both sexes were used in these experiments, which were performed as described previously.11,14,19

Isolation and Treatment of Canine Ventricular Trabeculae
The heart was excised, beating was ceased in cold Tyrode solution (~7°C) bubbled with 95% CO₂ and 5% O₂, and 2 to 4 thin trabeculae carneae of the right ventricular wall (<1 mm in diameter) were isolated and mounted in 20-mL organ baths that contained Krebs-Henseleit solution.8,11 The ventricular trabeculae were stimulated electrically with square-wave pulses of 5-ms duration and a voltage that was 20% above the threshold (~0.4 V) at a frequency of 0.5 Hz. The average length of muscle preparations was 7.39 ± 0.52 mm, and the average cross-sectional area was 1.43 ± 0.19 mm² (n = 165, from a total of 73 dogs).

ET-1 was administered at a single concentration to each muscle preparation. Selective inhibitors were administered 20 to 30 minutes before the addition of ET-1 and were present in the organ bath throughout respective experiments. Pertussis toxin (PTX) at 0.5 μg/mL was allowed to act for 10 hours before experiments were started.

Preparation and Analysis of Canine Ventricular Myocytes
A portion of the free wall of the left ventricle that is supplied via a branch of the left anterior descending artery was excised. The artery was cannulated and perfused with Tyrode’s solution that contained 1.0 mg/mL collagenase and 0.1 mg/mL protease via a recirculating system for 15 to 25 minutes at room temperature (24°C). Then the muscle was perfused with Tyrode’s solution that contained 0.2 mmol/L CaCl₂ and cut into small pieces ~3×3 mm² with a...
scalpel. The resultant cells in suspension were rinsed several times with Tyrode’s solution that contained gradually increasing concentrations of Ca²⁺ up to 1.8 mmol/L.

Procedures used for loading of indo-1, superfusion of myocytes, measurements of fluorescence, and cell length are presented in detail in online data supplement, available at http://www.circresaha.org.

Subcellular Localization of Protein Kinase C Isoforms
The subcellular fractionation procedures, antibodies, and Western blotting techniques are described in the online data supplement.

Statistical Analysis
Experimental values are presented as mean±SE. Significant differences between mean values were estimated by a repeated-measures ANOVA or by Student’s t test with analytic software STATVIEW J-4.5 (Abacus Concepts). P<0.05 was judged to indicate a significant difference.

Results
Influence of NE on the Inotropic Effects of ET-1
Endothelin-1 at 1 to 100 nmol/L did not, by itself, affect the peak twitch force. However, in the presence of NE at the subthreshold concentration of 1 nmol/L, ET-1 at 10 and 100 nmol/L had a definite PIE in association with negative lusitropic and clinotropic (the effect on time to peak tension) effects (data not shown), and these effects of ET-1 were concentration-dependent (Figures 1A and 1B). The EC₉₀ value for ET-1 in the presence of NE at 1 nmol/L was 34.0±5.90 nmol/L (determined in 37 preparations from 14 dogs, including data presented in Reference 15).

NE had a concentration-dependent PIE at 10, 100, and 1000 nmol/L equivalent to 11.5±4.1% (n=5), 80.4±8.4% (n=5), and 248.3±27.4% (n=8) of the basal force, respectively, and the threshold concentration and EC₅₀ value were 3 nmol/L and 0.87±0.09 μmol/L, respectively. When the NE concentration before the administration of ET-1 was increased, the PIE of ET-1 was converted to a NIE, depending on the concentration of NE. In the presence of 10 nmol/L NE, 10 nmol/L ET-1 induced a biphasic inotropic response (ie, a transient NIE followed by a long-lasting PIE); in the presence of NE at higher concentrations (≥100 nmol/L), ET-1 had a definite NIE (Figure 1C). In the presence of NE at 1000 nmol/L, the NIE of ET-1 was markedly reduced, and ET-1 did not have any inotropic effect in the presence of 10 μmol/L NE (Figure 1D).

Regulation of Ca²⁺ Signaling by Crosstalk Between ET-1 and NE
In canine ventricular myocytes, neither ET-1 (10 nmol/L) nor NE (0.1 and 1 nmol/L) by itself affected the cell shortening and Ca²⁺ transients (Figure 2A). When 10 nmol/L ET-1 was administered in the presence of 0.1 nmol/L NE, ET-1 induced an increase in cell shortening (Figures 2A and 2B, bottom) in association with a small increase in Ca²⁺ transients (Figure 2B, top).

The increase in cell shortening induced by 10 nmol/L ET-1 was equivalent to that produced by an increase in extracellular...
lar Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{o}$) to 3.6 mmol/L. However, the increase in Ca$^{2+}$ transients induced by ET-1 was significantly ($P<0.05$) smaller than that induced by [Ca$^{2+}$]$_{o}$ of 3.6 mmol/L (Figure 2C), an indication that the increase in cell shortening induced by ET-1 was attributable, at least in part, to an increase in the myofilament sensitivity to Ca$^{2+}$.

NE at 100 nmol/L induced a pronounced increase in the maximum cell shortening together with a remarkable increase in Ca$^{2+}$ transients. ET-1 markedly decreased the NE-induced increase in cell shortening and Ca$^{2+}$ transients (Figures 3A and 3B). The NE-induced increases in cell shortening and Ca$^{2+}$ transients were inhibited by ET-1 to an essentially similar extent (Figure 3C), and the relationship between the amplitudes of cell shortening and Ca$^{2+}$ transients that was observed with NE alone was unaffected by ET-1 (data not shown).

In the presence of NE at an intermediate concentration of 10 nmol/L, ET-1 had a biphasic effect, inducing a transient decrease in cell shortening that was associated with a decrease in Ca$^{2+}$ transients, followed by a long-lasting increase in cell shortening that was associated with a statistically insignificant alteration of Ca$^{2+}$ transients (Figures 4A and 4B). Summary of these data are presented in Figure 4C. Our findings indicate that, in the presence of 10 nmol/L NE, the inotropic response to ET-1 involves a combination of facilitatory and inhibitory effects.

Similar results were obtained in aequorin-loaded canine right ventricular trabeculae (see the online data supplement).

**Signal Transduction Pathway for the ET-1–Induced PIE**

Figure 5 shows the effects of selective inhibitors of cAMP-mediated and protein kinase C–mediated (PKC-mediated) pathways on the PIE of ET-1 in the presence of a subthreshold concentration of 1 nmol/L NE. The PIE of ET-1 was abolished by treatment of trabeculae with timolol (1 μmol/L), which blocks β-adrenoceptors, and with H-89 (1 μmol/L), an inhibitor of protein kinase A (PKA: Figure 5A). These inhibitors, at the concentrations used, did not affect the PIE of NE that was induced by activation of β-adrenoceptors (data not shown). Carbachol (0.1 μmol/L), which selectively inhibits the cAMP-mediated PIE, also reversed the PIE of ET-1 (Figure 5B). These results indicate that the PIE of ET-1 in the presence of NE requires the simultaneous activation of PKA and PKC signaling pathways.
Signal Transduction Pathway for the ET-1-Induced NIE

At concentrations that completely inhibited the PIE of 10 nmol/L ET-1, the inhibitors of PKC and PLC did not suppress but, in fact, enhanced the NIE of ET-1 that was induced in the presence of 100 nmol/L NE (Figure 6A).

Prior treatment with PTX (0.5 μg/mL) and the treatment with LY83583 (10 μmol/L), an inhibitor of guanylyl cyclase (GC), with KT5823 (0.3 μmol/L), an inhibitor of cGMP-dependent protein kinase (PKG), or with cantharidin (10 μmol/L), an inhibitor of protein phosphatase (PP), almost completely suppressed the NIE of ET-1 in the presence of 100 nmol/L NE (Figure 6B). These selective inhibitors had no effects on the basal force and on the PIE of NE mediated by β-adrenoceptors (data not shown).

Unmasking of the PIE of ET-1 by Suppression of Inhibitory Pathways

After the pretreatment of trabeculae with PTX (Figure 7A) and the treatment with KT5823 (Figure 7B) or with cantharidin (Figure 7C), ET-1 (10 nmol/L) had a PIE even in the absence of NE.

Influence of GF 109203X and Hoe 642 on PKCα Translocation

We had examined the subcellular distribution of 4 major PKC isoforms (α, β, δ, and ε) by immunoblotting with the use of isoform-specific antibodies. We found that the dog right ventricle expressed α and ε isoforms, whereas no significant immunoreactivity was detected for β and δ. Because the subcellular localization of the PKCα isoform did not change
in response to pharmacological stimuli, we reported data for the PKCζ isoform in the present study.

Representative immunoblots of the PKCζ isoform are shown in Figure 8A. The membrane-associated immunoreactivity was markedly increased in response to phorbol dibutyrate and ET-1/NE. The translocation of PKCζ to the membranous fraction by ET-1/NE was completely blocked by GF 109203X (a selective PKC inhibitor, 1 μmol/L) but not by Hoe 642 (a Na+/H+ inhibitor, 1 μmol/L) (Figure 8B). Under the same experimental condition, Hoe 642 suppressed significantly (P<0.05) the PIE induced by combination of ET-1 with NE by ∼40% (control, 151±5.6%; Hoe 642, 132±4.8%; n=4 each).

**Discussion**

In the present study, we demonstrated that the extent and quality of the inotropic effects of ET-1 in canine ventricular myocardium are determined by the concentration of simultaneously applied NE, with the functional outcome of coupling subsequent to stimulation of ET receptors being dependent, apparently, on the extent of β-stimulation by NE. The regulation of contraction induced by ET-1 involves activation of PKC, of a G protein–coupled cAMP/PKA pathway, and of a PTX-sensitive G protein–coupled cGMP/PKG/PP pathway.

By itself, ET-1 did not induce any inotropic response, but it had a definite PIE in the presence of NE at 0.1 to 1 nmol/L, which did not significantly affect the basal force. The PIE of ET-1 was converted to a NIE when the concentration of NE was increased. ET-1 had a prominent NIE in the presence of NE at 100 nmol/L and higher, and these observations are essentially consistent with previous reports that ET-1 attenuated the PIE and positive chronotropic effect of β-stimulation in the dog,\(^{11}\) rat,\(^{21}\) and guinea pig.\(^{22}\) Our results indicate that in the presence of NE, positive and negative inotropic responses compete with one another and that, in the presence of NE at high concentrations, the PIE of ET-1 is completely replaced by a NIE. This crosstalk between ET-1 and NE might contribute significantly to the variable inotropic responses to ET-1; in previous studies, ET-1 had no effect,\(^{8,9,23}\) a PIE,\(^{4,8}\) and a NIE,\(^{24-26}\) including a time-dependent component to these phenomena.\(^{27}\)

**Differential Regulation of Ca\(^{2+}\) Signaling by ET-1**

The PIE of ET-1 in the presence of a threshold concentration of NE was associated with a small increase in Ca\(^{2+}\) transients. Figure 5. Effects of inhibitors of PKA-mediated and PKC-mediated pathways on the PIE of ET-1 in isolated canine ventricular myocardium. A, Effects of treatment with timolol (1 μmol/L), H-89 (1 μmol/L), staurosporine (STS; 10 nmol/L), H-7 (10 μmol/L), and neomycin (NEO; 10 μmol/L) individually on the PIE of ET-1 in the presence of 1 nmol/L NE. ***P<0.001 vs 1 nmol/L NE alone. B, Effects of carbachol (CCh; 0.1 μmol/L) on the PIE of ET-1 in the presence of 1 nmol/L NE. Numbers in parentheses indicate numbers of preparations examined.

***P<0.001 vs 1 nmol/L NE plus 10 nmol/L ET-1.

Figure 6. Effects of inhibitors of PKC and Gi pathways on the NIE of ET-1 in isolated canine ventricular myocardium. A, Effects of treatment with staurosporine (STS; 10 nmol/L), H-7 (10 μmol/L), and neomycin (NEO; 10 μmol/L) individually on the NIE of ET-1 in the presence of 100 nmol/L NE. B, Effects of prior treatment with PTX (0.5 μg/mL), LY29383 (LY; 10 μmol/L), KT5823 (KT; 0.3 μmol/L), and cantharidin (Cant; 10 μmol/L) individually on the NIE of ET-1. The force of contraction before the addition of ET-1 was taken as 100% for each preparation, and changes in the force recorded 20 minutes after the application of ET-1 are expressed as a percentage relative to the maximum force. Numbers in columns indicate numbers of preparations examined. ***P<0.001 vs 100 nmol/L NE alone.
This increase was significantly smaller than that induced by an increase in $[\text{Ca}^{2+}]$, that elicited a PIE equivalent to that of ET-1 (Figure 2). These observations imply that the PIE of ET-1 is associated definitively with an increase in the myofilament sensitivity to $[\text{Ca}^{2+}]$, being consistent with previous findings with ET-1 in other species. \textsuperscript{19,28–30} Because ET-1 did not have a PIE in the presence of inotropic interventions, such as dihydroouabain or an increase in $[\text{Ca}^{2+}]$, ET-1 might require a weak $[\text{Ca}^{2+}]$-stimulation for induction of its PIE. This synergistic action of ET-1 and NE indicates that there is a critical difference in the regulation between the dog and other mammals. \textsuperscript{8,19,29–31} In mice, $[\text{Ca}^{2+}]$-stimulation and ET-1 regulate cardiac contractility in opposite directions in part through phosphorylation of troponin I on distinct sites, \textsuperscript{32} indicating that the phosphorylation of contractile proteins plays a crucial role in the regulation, the role of which could not be determined in the present study.

In contrast to the PIE, the NIE of ET-1 was accompanied by a pronounced decrease in $[\text{Ca}^{2+}]$ transients (Figure 3), which may play a key role in the NIE of ET-1. We investigated in dog ventricular myocytes that ET-1 inhibited significantly the increase in L-type $[\text{Ca}^{2+}]$ current ($I_{\text{Ca}}$) induced by isoproterenol. \textsuperscript{14} Because the inhibitory action of ET-1 on the isoproterenol-induced increase in $I_{\text{Ca}}$ was suppressed by the treatment with PTX in rabbit myocytes, the inhibition of the cAMP-mediated increase in $I_{\text{Ca}}$ via the PTX-sensitive inhibitory pathway activated by ET-1 might contribute, to some extent, to the ET-1-induced decrease in $[\text{Ca}^{2+}]$ transients. Involvement of effects on other processes, such as PKA and SR $[\text{Ca}^{2+}]$ release, however, is not excluded.

Present observations with indo-1-loaded myocytes are consistent with findings obtained with aequorin-loaded dog ventricular trabeculae (online data supplement).\textsuperscript{18}

Subcellular Mechanisms for the PIE of ET-1
Neomycin, staurosporine, and H-7, at a concentration that did not affect the PIE of NE mediated by $\beta$-adrenoceptors, abolished the PIE of ET-1. Furthermore, timolol and H-89 completely suppressed the PIE of ET-1 (Figure 5A), whereas carbachol reversed the PIE of ET-1 (Figure 5B). These observations together imply that the ET-1–induced PIE and increase in the myofilament sensitivity to $[\text{Ca}^{2+}]$ require the simultaneous activation of PKC and PKA.

Although the $[\text{Na}^{+}]$–$[\text{H}^{+}]$ exchange inhibitor Hoe 642 inhibited partially the PIE of ET-1, we could not detect an appreciable alteration of pH in single myocytes loaded with the fluorescent pH probe SNARF-1 (online data supplement).

We found recently that the $[\text{Ca}^{2+}]$-sensitizing actions of OR-1896 and levosimendan were abolished by carbachol,\textsuperscript{33–35} an indication that a $[\text{Ca}^{2+}]$-sensitizing mechanism might exist that requires accumulation of cAMP. Although myosin-binding protein C, which is phosphorylated via a cAMP/PKA signaling pathway, might be a candidate for the source of increased sensitivity to $[\text{Ca}^{2+}]$, the target proteins...
responsibility for such a cAMP-mediated increase in Ca\(^{2+}\)-sensitivity remain to be identified.

The selectivity of staurosporine and H-7 was checked by comparing the concentration-dependent effect of these agents on the \(\alpha\)- and \(\beta\)-mediated PIE,\(^{37,38}\) which is discussed in detail in online data supplement.

Treatment with cantharidin and with KT5823 and pretreatment with PTX unmasked the PIE of ET-1 even in the absence of NE. These findings indicate that the signaling process that leads to activation of G-proteins, PKG, and phosphatases might be highly effective even in the baseline state, counteracting the G\(_{q}\)/G\(_{s}\)-mediated PIE via suppression of cAMP-mediated signaling and leading to the absence of a PIE of ET-1; ie, ET-1 stimulates both G\(_{q}\) and G\(_{i}\) proteins, accounting for the activation of facilitative and inhibitory (Figure 7).

**Subcellular Mechanisms for the NIE of ET-1**

The selective inhibitors that abolished the PIE enhanced the NIE of ET-1 (Figure 6), an indication that the NIE of ET-1 is mediated by signaling processes that are different from those involved in the induction of the PIE of ET-1. Because the NIE of ET-1 was almost completely inhibited by pretreatment with PTX, the accumulation of cAMP induced by \(\beta\)-stimulation might be suppressed by the G\(_{i}\)-mediated deactivation of adenyl cyclase. However, this scenario is unlikely, because the NIE of ET-1 is not accompanied by a significant reduction in the accumulation of cAMP that is mediated by \(\beta\)-stimulation.\(^{10,11,39}\) Although \(\beta\)-adrenoceptors that are coupled to G\(_{i}\) proteins are activated by NE\(^{40}\) and could come into play in the NIE of ET-1 in the presence of high concentrations of NE, the findings with ET-1 in the presence of various \(\beta\)-adrenoceptor agonists, including the \(\beta\)-selective agonist zinterol, exclude the essential role of \(\beta\)-subtype in the NIE of ET-1\(^{41}\) (online data supplement).

Both LY83583, an inhibitor of GC, and KT5823, an inhibitor of PKG, suppressed the NIE of ET-1, an indication that G\(_{i}\)-coupled cGMP/PKG signaling might be responsible for the inhibitory action of ET-1, whereas nitric oxide is not involved in such regulation.\(^{10,11}\) It has been reported that G\(_{i}\)-coupled receptors, such as muscarinic M\(_{3}\) and adenosine A\(_{1}\) receptors,\(^{42}\) counteract the effect of activation of PKA, in part via the activation of PP: the muscarinic agonists acetylcholine and carbachol inhibit protein phosphatase inhibitor-1 (PPI-1) through G\(_{i}\)-mediated stimulation of the PP activity with resultant dephosphorylation of a variety of functional proteins that are phosphorylated by PKA.\(^{43–47}\) The muscarinic inhibition could occur without a concomitant decrease in the cAMP content or in the PKA activity.\(^{45,46}\) Our observation that an inhibitor of PP, cantharidin, abolished the NIE of ET-1 is consistent with the results of previous investigations described above. In this context, it is noteworthy that the NIE of ET-1 is more susceptible to cantharidin than the NIE of carbachol and the PIE of NE mediated by \(\beta\)-adrenoceptors,\(^{48}\) an indication that the differential effect of cantharidin on various signaling processes is exerted in dog ventricular myocardium.

In summary, ET-1 has a positive effect, biphasic effect, or NIE, depending on the extent of \(\beta\)-stimulation in canine ventricular myocardium. The PIE of ET-1 might be attributable to activation of the PLC/PKC pathway, requiring simultaneous activation of the process that is mediated by cAMP. By contrast, the NIE of ET-1 can be ascribed to activation of phosphatases via G\(_{i}\)-coupled cGMP/PKG signaling. The PIE was associated with an increase in the myofilament sensitivity to Ca\(^{2+}\), whereas the NIE was attributable to a decrease in Ca\(^{2+}\) transients. The crosstalk between ET-1 and NE might play a crucial role in the regulation of myocardial contractility under pathophysiological conditions that are associated with elevated plasma levels of endogenous regulators.

**Acknowledgments**

This work was supported in part by Grants-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by a Research Grant for Cardiovascular Disease (11-1) from the Ministry of Health and Welfare, Japan.

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_Circ Res._ 2003;92:1024-1032; originally published online April 10, 2003;
doi: 10.1161/01.RES.0000070595.10196.CF

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/92/9/1024

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Materials and Methods

Sources of chemicals
Endothelin-1 (Peptide Institute, Osaka, Japan); staurosporine (Boehringer Mannheim GmbH, Mannheim, Germany); (-)-norepinephrine bitartrate, timolol maleate, H-7, neomycin sulfate, prazosin hydrochloride, carbamylcholine chloride (carbachol), H-89 (Sigma Chemical, St. Louis, MO); GF 109203X (); Hoe 642 (); pertussis toxin (Funakoshi, Tokyo, Japan); LY83583, KT5823 (Wako Pure Chemical Ltd, Osaka, Japan).

Experimental procedures for indo-1-loaded canine ventricular myocytes
Myocytes were loaded with a fluorescent dye acetoxyethyl ester of indo-1 (indo-1/AM) by incubation in a 5 μmol/L solution indo-1 for about 3 min at room temperature (24°C). After loading, cells were pelleted by centrifugation at 200 rpm for 1 min. The cells in the pellet were resuspended in HEPES-Tyrode solution. The myocytes were then placed in a perfusion chamber and perfused with bicarbonate buffer for about 10 min.

The myocytes in the perfusion chamber were placed on the stage of an inverted microscope (Diaphot TMD 300; Nikon, Tokyo, Japan). Perfusion was initiated with bicarbonate buffer that contained 1.8 mmol/L CaCl₂ at a rate of 1 ml/min at room temperature and cells were stimulated electrically by square-wave pulses at a voltage that was about 30-40% above the threshold, at a frequency of 0.5 Hz.

For measurements of fluorescence, indo-1 was excited with light from a xenon lamp (150 W), at a wavelength of 355 nm, reflected by a 380 nm long-pass dichroic mirror, and fluorescence was detected with a fluorescence spectrophotometer (CAM-230; Japan Spectroscopic Co., Tokyo, Japan). Excitation light was applied to myocytes intermittently through a neutral density filter to minimize the photobleaching of indo-1. Emitted fluorescence was collected via an objective lens (CF Fluor DL40; Nikon) and
then separated by a 580 nm long-pass dichroic mirror to allow simultaneous measurements of emitted fluorescence at 405 nm and at 500 nm through band-pass filters. A bright-field image of individual cells was projected onto the photodiode array of an edge detector (C6294-01; Hamamatsu Photonics K.K., Hamamatsu, Japan) with temporal resolution of 5 ms and cell length was monitored at the same time as fluorescence from indo-1.

Myocytes were perfused with a solution that contained the agent of interest. Perfusion of prazosin (300 nmol/L) was initiated 10 min before the application of NE. When the response of myocytes to NE reached a steady state, fluorescence from indo-1 was measured and then the perfusate was switched to a solution that contained the agent of interest.

Measurements of cell length and fluorescence from indo-1 were stored and displayed by means of a computer (Power Macintosh 8100/100AV; Apple Computer Inc., Cupertino, CA, U.S.A.) equipped with an A/D converter (MP-100A; BIOPAC Systems Inc., Santa Barbara, CA, U.S.A.) at 200 Hz and analyzed after low-pass filtering (cutoff frequency of 20 Hz). The data used for statistical analysis were obtained by signal-averaging of five successive tracings of cell shortening and Ca\(^{2+}\) transients. In the analysis of data, diastolic cell length and the ratio of indo-1 fluorescence at 405 nm and 500 nm prior to the first application of an agent of interest in individual experiments were regarded as the basal values for each myocyte. They were assigned values of 100%, and all the data were expressed as percentages relative to these basal values.

Aequorin-loaded canine right ventricular trabeculae

Mongrel dogs of either sex (8-12 kg) were anesthetized by intravenous administration of pentobarbital sodium (30 mg/kg). Hearts were rapidly excised and free-running trabeculae (< 1 mm in diameter) were dissected from the free wall of the right ventricle.

For simultaneous detection of contractile force and intracellular Ca\(^{2+}\) transients, the Ca\(^{2+}\)-sensitive bioluminescent protein aequorin was loaded by the modified macro-injection technique, as described elsewhere in detail.\(^1\) The muscle was electrically stimulated by square-wave pulses of 5-ms duration at a voltage about 20%
above the threshold at 0.5 Hz in modified Krebs-Henseleit solution at 37 °C. The composition of the solution was as follows (in mM): NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 24.9, KH2PO4 1.2, and glucose 11.1 (with 0.057 mM ascorbic acid and 0.027 mM EDTA). The solution was bubbled with 95% O2-5% CO2 and maintained pH 7.4 in the control condition. Aequorin light signals were detected with a photomultiplier (9789A; Thorn EMI Electron Tubes, Ruislip, UK) and light signals were smoothed by a low-pass filter (cut-off frequency of 100 Hz; Multi-channel SR filter 3315; NF Electron Instruments). Signals were recorded on digital audiotape (PC-108M; Sony Magnescale, Tokyo, Japan) for subsequent analysis. The muscle preparation was equilibrated for about 120 min after aequorin-loading procedure, meanwhile the bioluminescence declined to a steady low level. During the equilibration period, the muscles were stretched initially at a resting tension of 5 mN and the length was later adjusted to give the developed tension of 90% of the Lmax. Fifty to hundred-fifty signals of Ca2+ transients and isometric contractions were averaged to improve the signal-to-noise ratio by means of data analysis software (Visual Designer: Intelligent Instrumentation, Tucson, AZ, USA) in IBM PC/AT personal computer (FMV-Deskpower S13; Fujitsu, Tokyo, Japan). The 2.5th root of the peak amplitude of aequorin signals was calculated as an indicator of the peak [Ca2+]i.4

In each preparation, the maximal response to isoproterenol (ISOmax) was determined at the end of experiments after washout of the drugs for 2 h and the increase in contractile force and the amplitude of Ca2+ transients induced by inotropic interventions were expressed as a percentage of ISOmax.

**Subcellular localization of PKC isoforms**

Membranous and cytosolic fractions of detergent-extracted PKC were prepared as previously reported.5,6 The subcellular localization of PKC isoforms was examined by quantitative immunoblotting. Equal amounts of cytosolic and membranous protein extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes.5,6 To ensure equivalent protein loading and equal transfer efficiency, the nitrocellulose membrane was stained with Ponceau S. After blocking, membranes
were incubated with PKC-isofrom specific primary antibodies (Transduction Laboratories). Then, blots were incubated with a secondary antibody and visualized by enhanced chemiluminescence (Amersham Life Science). The degree of labeling was quantified by a computer program (NIH) and expressed in relative scan units.

We had examined the subcellular distribution of 4 major PKC isoforms (α, β, δ, and ε) by immunoblotting with the use of isoforms-specific antibodies. We found that the left ventricle of dog expressed α and ε isoforms, whereas no significant immunoreactivity was detected for β and δ. Therefore, alterations in the PKC localization of α and ε isoforms were examined in the present study. Since actual decreases in absolute protein abundance of cytosolic PKC are not typically observed in the intact heart, we used the membrane-associated immunoreactivity of PKC isoforms as indices of translocation. Since the subcellular localization of the PKCα isoform did not change in response to pharmacological stimuli (data not shown), we reported data for the PKCε isoform in the present study.

**Measurements of intracellular pH**

Cytosolic pH was monitored with the fluorescent pH probe SNARF-1 according to Gambassi et al (1992). Isolated myocytes bathed in Krebs-Henseleit solution were loaded by a 10-minute exposure to 4 μmol/L SNARF-1 AM. The optical system used to monitor pH is a modification of that used for indo-1 fluorescence measurements. SNARF-1 fluorescence was excited at 530 nm. Emitted SNARF-1 fluorescence at 590 nm and 640 nm represents, respectively, the H⁺-bound and H⁺-free forms of the indicator, and their ratio is a measure of pH.

**Statistical analysis**

Data are expressed as means ± S.E.M. For analysis of multiple measurements obtained from a single preparation, we used one-way analysis of variance (ANOVA) for repeated measures with Bonferroni’s test. A P value smaller than 0.05 was considered to indicate statistically significant difference.
Results

Effects of ET-1 and NE on aequorin-loaded canine right ventricular trabeculae

In an aequorin-loaded canine right ventricular trabecula, neither ET-1 (100 nmol/L) nor NE (0.1 nmol/L) by itself affected the contractile force and the amplitude of Ca$^{2+}$ transients. When ET-1 (100 nmol/L) was administered in the presence of NE (0.1 nmol/L), ET-1 induced a positive inotropic effect (PIE) in association with a small increase in the amplitude of Ca$^{2+}$ transients (Online Figure 1). Online Figure 2 shows summarized data. ET-1 (100 nmol/L) elicited a significant PIE in the presence of NE (0.1 nmol/L), but Ca$^{2+}$ transients were not affected significantly. The PIE of 100 nmol/L ET-1 was equivalent to that produced by an increase in extracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]o) to 3.6 mmol/L. However, the increase in the amplitude of Ca$^{2+}$ transients induced by ET-1 was smaller than that induced by a [Ca$^{2+}$]o of 3.0 mmol/L (Online Figure 3), an indication that the PIE of ET-1 was due, at least in part, to an increase in the sensitivity of myofilaments to Ca$^{2+}$ ions.

Norepinephrine at a high concentration (1 μmol/L) induced a pronounced PIE together with a remarkable increase in the amplitude of Ca$^{2+}$ transients (Online Figure 4, middle panel). ET-1 markedly decreased the NE-induced PIE and increase in the amplitude of Ca$^{2+}$ transients (Online Figure 4, right panel). The NE-induced PIE and increase in Ca$^{2+}$ transients were inhibited by ET-1 to a similar extent (Online Figure 5) and the relationship between the PIE and amplitude of Ca$^{2+}$ transients that was observed with NE alone was essentially unaffected by ET-1 (Online Figure 6).

Effects of the combination of NE and ET-1 on intracellular pH

In canine ventricular myocytes loaded with the fluorescent pH$_i$ probe SNARF-1, cytosolic pH was not altered appreciably during increased cell shortening in response to NE (100 nmol/L) and ET-1 (10 nmol/L) (n = 5). Under the same experimental condition, pimobendan (10 μmol/L) increased pH$_i$, significantly approximately 0.1 pH units (n =
Discussion

Involvement of $\beta_2$-adrenoceptors and/or NO in the NIE of ET-1

It has been shown that $\beta_2$-adrenoceptors are coupled to $G_i$ proteins and contribution of this subtype becomes greater when the concentration of NE is increased.\textsuperscript{10} We carried out the experiments using various sympathomimetic amines, including a selective beta-2 adrenoceptor agonist zinterol, to elucidate whether the NIE of ET-1 is partly due to the subtype selective activation of adrenoceptors, namely $\beta_2$-adrenoceptors. It was found that the biphasic effect, i.e., the NIE followed by a long-lasting PIE, was exerted in response to ET-1 in the presence of zinterol, epinephrine, dobutamine, denopamine and isoproterenol in a similar manner as ET-1 does during the induction of a PIE of a similar extent by norepinephrine in the current study.\textsuperscript{11} Therefore, the activation of $\beta_2$-adrenoceptors may not play a crucial role in the NIE of ET-1 in canine ventricular myocardium. In addition, we have shown by means of pharmacological procedures that the production of NO does contribute to the NIE of ET-1 in the presence of $\beta$-adrenoceptor stimulation in the canine ventricular myocardium.\textsuperscript{12}

Selectivity of PKC inhibitors

The selectivity of PKC inhibitors, such as staurosporine and H-7, have been shown to be low in vitro. Therefore we checked the selectivity by comparing the concentration-dependent inhibitory action of these inhibitors on $\alpha$- and $\beta$-mediated PIE in ventricular myocardium.\textsuperscript{10-14} At the concentrations employed in the current study, both staurosporine and H-7 suppressed selectively the $\alpha$-adrenoceptor-mediated PIE with no suppressant action on the $\beta$-mediated PIE. It is surprising that H-7 inhibits PKA ($IC_{50} = 3$ $\mu$mol/L) and PKC ($IC_{50} = 6$ $\mu$mol/L) at approximately identical concentrations in vitro.\textsuperscript{13} but in intact ventricular muscle H-7 is able to inhibit selectively the $\alpha$-mediated
PIE that has been shown to be associated with acceleration of phosphoinositide hydrolysis and resultant activation of PKC. Staurosporine possesses likewise an identical inhibitory potencies on PKA (IC$_{50}$ = 8.2 nmol/L)$^{16}$ and PKC (IC$_{50}$ = 2.7 nmol/L).$^{17}$ and inhibits also the tyrosine kinase activity,$^{16}$ but it showed also a selective inhibitory action on α-mediated PIE.$^{13,14}$ Although these pieces of evidence are indirect, it is important to perform this type of experiments to examine the selective action of protein kinase inhibitors in intact tissue.

References


7. Paul K, Ball NA, Dorn GW 2nd, Walsh RA: Left ventricular stretch stimulates angiotensin II-mediated phosphatidylinositol hydrolysis and protein kinase C ε


**Legends**

Online Figure 1. The positive inotropic effect of ET-1 at 100 nmol/L in the presence of NE at 0.1 nmol/L in an aequorin-loaded canine right ventricular trabecula electrically stimulated at 0.5 Hz at 37°C.

Online Figure 2. Effects of ET-1 at 100 nmol/L on Ca\(^{2+}\) transients and force of contraction in the presence of NE at 0.1 nmol/L in aequorin-loaded canine right ventricular trabeculae electrically stimulated at 0.5 Hz at 37°C.

Online Figure 3. Relationship between (peak light)\(^{324}\) and force of contraction during induction of the PIE by ET-1 at 100 nmol/L in the presence of NE at 0.1 nmol/L, elevation of [Ca\(^{2+}\)]\(_{o}\) (3.0, 3.5, and 4.0 mmol/L), and isoproterenol (0, 10, 30, and 100 mmol/L) in aequorin-loaded canine right ventricular trabeculae electrically stimulated at 0.5 Hz at 37°C. Numbers in parentheses: numbers of experiments.

Online Figure 4. The ET-1 (100 nmol/L)-induced inhibition of the increase in Ca\(^{2+}\) transients and isometric contractions induced by NE at 1 μmol/L in an aequorin-loaded canine right ventricular trabecula electrically stimulated at 0.5 Hz at 37°C.

Online Figure 5. Effects of ET-1 at 100 nmol/L on the increase in Ca\(^{2+}\) transients and force of contraction induced by NE at 1 μmol/L in aequorin-loaded canine right ventricular trabeculae electrically stimulated at 0.5 Hz at 37°C.
Online Figure 6. Relationship between (peak light)$^{12,5}$ and force of contraction during induction of the inotropic effect by NE 1 μmol/L + ET-1 100 nmol/L, elevation of $[\text{Ca}^{2+}]_i$ (3.0, 3.5, and 4.0 mmol/L) and isoproterenol (0, 10, 30, and 100 mmol/L) in aequorin-loaded canine right ventricular trabeculae electrically stimulated at 0.5 Hz at 37°C. Numbers in parentheses: numbers of experiments.
Online figure 1
Contro
NE 0.1 nmol/L
ET-1 100 nmol/L

Force of contraction

% of ISOmax

Force of contraction (Peak light)\(^{1/2.5}\)

Online figure 2

\[\text{Force of contraction} \quad \ast : P < 0.01 \quad (n = 8)\]

\[\text{Force of contraction} \quad \bullet : (\text{Peak light})^{1/2.5}\]

Control \quad NE 0.1 nmol/L \quad NE 0.1 nmol/L + ET-1 100 nmol/L
(Peak light) \(^{1/2.5}\)

% of ISOmax

<table>
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<th>Force of contraction</th>
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- [Ca^{2+}]_o (8)
- NE 0.1 nmol/L + ET-1 100 nmol/L (8)
- ISO (8)

Online figure 3
Online figure 4
Online figure 5

![Graph showing force of contraction and percentage of ISOmax](image)

Legend:
- Open square: Force of contraction
- Solid square: (Peak light)^{1/2.5}
- Asterisk: P < 0.01 (n = 6)

- Control
- NE 1 μmol/L
- NE 1 μmol/L + ET-1 0.1nmol/L

Percentage of ISOmax:
- 0
- 10
- 20
- 30
- 40
Online figure 6