Published in final edited form as:

Biochem Biophys Res Commun. 2012 July 6; 423(3): 600–605. doi:10.1016/j.bbrc.2012.06.024.

PKC- ϵ mediates multiple endothelin-1 actions on systolic Ca^{2+} and contractility in ventricular myocytes

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Abstract

Endothelin-1 (ET-1) induces positive inotropy (enhanced contractility) in cardiac muscle, but establishing underlying cellular mechanisms has been controversial in part because of a growing number of signaling pathways and end effectors targeted by ET-1. Here we present evidence that ET-1 induces positive inotropism in ventricular tissue by increasing both systolic Ca^{2+} and myofilament Ca^{2+} sensitivity. To examine the roles of PKC- δ and PKC- ϵ in these acute responses to ET-1, kinase inactive dominant negative PKC (dn-PKC) constructs were expressed in adult rat ventricular myocytes. Yellow fluorescent protein (YFP) was fused to dn-PKC constructs to visualize expression and localization of dn-PKC in living myocytes. Due to an alanine to glutamate mutation in the pseudosubstrate site, dn-PKCs constitutively translocated to anchoring sites and were unaffected by agonist or phorbol ester treatment. Dn-PKC- δ -YFP mainly distributed at Z-lines and at intercalated disks in adult myocytes, whereas dn-PKC- ϵ -YFP stained the surface sarcolemma, T-tubules/Z-lines and perinuclear region. Myocytes expressing dn-PKC- δ -YFP showed normal systolic Ca^{2+} and contractile responses to ET-1. In contrast, the entire ensemble of ET-1 responses was blocked in myocytes expressing dn-PKC- ϵ -YFP including increased Ca^{2+} transients, enhanced myofilament Ca^{2+} sensitivity, and positive inotropy. This report provides direct evidence that PKC- ϵ is activated early and robustly following ET-1 stimulation and thus mediates multiple intracellular changes underlying the acute actions of ET-1 on myocardium.

Keywords

PKC- ϵ ; endothelin-1; systolic Ca^{2+} ; positive inotropy

INTRODUCTION

The 21 amino acid peptide hormone endothelin-1 (ET-1) is a strong vasoconstrictor in vascular tissues, but also regulates a variety of biological processes in non-vascular tissues.

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In myocardium, ET-1 regulates the function of ion channels, ion exchangers and contractile proteins, which in turn affect cardiac muscle performance in many ways [1]. Acute exposure to ET-1, for example, exerts a positive inotropic effect in cardiac tissues of many mammalian species [2,3,4,5,6]. Thus, ET-1 may have beneficial effects in the short term to preserve heart function under conditions of mechanical stress, hypoxia and neurohumoral stimulation. In the long term (days to years), chronic ET-1 exposure may be detrimental, leading to hypertrophy and heart failure. Increased levels of circulating ET-1 and upregulation of ET receptors have been documented in failing hearts [7,8,9,10,11]. Identifying basic cellular mechanisms underlying the effects of ET-1 on Ca^{2+} handling and contractility would provide a foundation on which to understand its acute physiological and chronic pathological roles in the heart.

In many tissues including myocardium, ET-1 binds to G-protein coupled ET_A receptors and activates phosphoinositide-specific phospholipase C (PLC), which in turn produces diacylglycerol (DAG) and activates PKC [12,13,14]. Experiments using transgenic overexpression or pharmacological inhibition have implicated PKC signaling pathways in regulation of contractility and Ca^{2+} handling in the heart. Of the three major PKC isoforms (e.g. PKC- α , PKC- δ and PKC- ϵ) expressed in the healthy mammalian heart, PKC- α has been shown by gain- and loss-of-function strategies to be a negative regulator of cardiac contractility and Ca^{2+} handling [15]. In our previous study, phorbol ester initiated positive inotropy in PKC- δ and PKC- ϵ overexpressing cultured adult rat ventricular myocytes [16]. Later, we identified PKC- ϵ as a major PKC involved in ET-1 induced positive inotropy in adult rat ventricular myocytes [17]. However, the precise mechanism of PKC- ϵ in ET-1 induced positive inotropy is lacking.

Both increases in intracellular Ca^{2+} and increases in myofilament Ca^{2+} sensitivity have been shown to contribute to the positive inotropic effects of ET-1 under various conditions [5,18]. A variety of mechanisms have been proposed including (among others) myosin light chain phosphorylation by MLCK, troponin I phosphorylation by p21 activated kinase or PKC, phosphorylation of Na^+/H^+ exchanger by MAP kinases, and phosphorylation of K^+ channels or L-type Ca^{2+} channels by PKC or tyrosine kinases [19,20,21,22,23,24]. Whether one or a few mechanisms dominate *in vivo* or whether many mechanisms operate together in a coordinated fashion is unknown.

The purpose of this study was to identify cellular mechanisms of ET-1 mediated positive inotropy in adult rat ventricular myocytes and examine the role of novel PKC isoforms in regulating these processes. Electrically-paced twitches, systolic Ca^{2+} transients, and myofilament Ca^{2+} sensitivity were measured after ET-1 stimulation. Adenovirus gene delivery of dominant negative PKC constructs was used to inhibit endogenous PKC function in an isoform selective manner.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. X-rhod-1 was from Molecular Probes (Eugene, OR). Go6976 was from EMD Biosciences (San Diego, CA).

Dominant negative PKC constructs

Dn-PKC was generated through a double mutation by converting alanine (Ala) to glutamate (Glu) (amino acid 147 for PKC- δ and amino acid 159 for PKC- ϵ) and lysine (Lys) to arginine (Arg) (amino acid 376 for PKC- δ and amino acid 436 for PKC- ϵ). This double mutation permanently impairs the ATP-binding site of the enzyme but still allows the

enzyme to compete for substrates and anchoring sites, thereby effectively attenuating the activity of each PKC isoform [25]. These constructs are referred to as dn-PKC- δ -YFP and dn-PKC- ϵ -YFP.

Adenovirus construction

Generation of recombinant adenoviruses was accomplished using AdEasy adenoviral vector system (Stratagene) according to the manufactures' instructions as described previously [16].

Cardiac myocyte adenoviral infection

All manipulations of animals have been reviewed by and received approval from the Animal Care Committee of the University of Wisconsin. Ventricular myocytes were isolated from 3 month old male Sprague-Dawley rats with enzymatic digestion, then plated onto laminin coated coverslips and infected with adenoviruses as described previously [16]. After 2 days after infection, transfection efficiency reached to 100%, but we limited all the measurements within 40 hrs to minimize myocyte degeneration during culture period and to test cells at different expression levels.

Western blotting and *in vitro* kinase assay

Adenovirus infected myocytes were incubated in lysis buffer (in mmol/L: 50 Tris-Cl, pH 7.4, 250 NaCl, 3 EGTA, 3 EDTA, 1 DTT, 0.3 sodium orthovanadate, 10 sodium fluoride, 0.5 PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 1% Triton X-100). After centrifugation, 10 μ g total protein was subjected to 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Western blot analysis of adenovirus infected myocytes was performed with polyclonal anti-GFP, PKC- α and PKC- δ antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and polyclonal anti-PKC- ϵ antibody from Calbiochem (San Diego, CA). To examine kinase activities of GFP or YFP fused PKCs including wild-type and dominant negative constructs, recombinant PKC proteins were immunoprecipitated with polyclonal anti-GFP antibody and were incubated with 1 μ Ci of [γ - 32 P]ATP and 1 μ g histone H1 in kinase buffer (in mmol/L: 20 HEPES, pH 7.7, 2 MgCl₂, 2 MnCl₂, 0.01 ATP, 1 DTT, 0.3 sodium orthovanadate, 10 sodium fluoride, 0.5 PMSF, 1 μ g/ml leupeptin and 1 μ g/ml aprotinin) at 22°C for 30 min. Kinase reaction samples were then subjected to 10% SDS-PAGE. The phosphorylated gel was washed with 1x PBS buffer, dried and autoradiographed.

Twitch measurements

Cell twitches were initiated by electric field stimulation with a SD9 stimulator (Grass Instrument, Quincy, MA) in a modified PH1 chamber (Warner Instrument, Hamden, CT) mounted on a Zeiss inverted microscope. The stimulation protocol was 0.4 Hz, 10-ms duration, and 60 V at 22°C. Individual myocytes were monitored with a model VED 104 video edge detector (Crescent Electronics, Sandy, UT) and cell shortening was recorded using Felix software (Photon Technology International, West Sussex, UK).

Confocal imaging and Ca²⁺ transient measurements

Confocal images were acquired with a Bio-Rad Radiance 2100 laser scanning confocal microscope. Ca²⁺ indicator, X-rhod-1 was loaded to cultured myocytes and Ca²⁺ signals were recorded with the line scan oriented along the long axis of the myocyte at a speed of 2 ms/line, using the Lasersharp 2000 software. X-rhod-1 was excited at 543 nm with a Green HeNe laser, and emitted fluorescence was collected at 600 nm. Ca²⁺ transients were measured with a computer program running IDL software.

RESULTS

Construction and characterization of dn-PKC mutants

PKC enzymes have been proposed to be central downstream players in ET-1 signaling. We focused on Ca^{2+} -independent PKC isoforms because while the general PKC inhibitor, chelerythrine, consistently and fully blocked ET-1 mediated positive inotropy, the Ca^{2+} -dependent PKC inhibitor, Go6976, did not (data not shown). The efficacy of Go6976 was tested in previous study shown by blocking phorbol ester induced negative inotropic response [16]. Therefore, we explored strategies for selective inhibition of PKC- ϵ and PKC- δ , the two Ca^{2+} -independent PKC isoforms highly expressed in mammalian hearts.

In our hands, peptide-based PKC isoform inhibitors had limitations with regard to consistency and efficacy of action in ventricular myocytes [26]. An alternative isoform-specific inhibitor strategies (i.e. use of dominant negatives) was explored here. Dn-PKC constructs were expressed in adult rat ventricular myocytes by use of adenoviruses. To reveal expression levels and subcellular localization of dn-PKC constructs, each was fused with YFP at their N-terminus.

Dn-PKC-YFP constructs expressed in myocytes were characterized by Western blots, immunoprecipitation and *in vitro* kinase assays. Adenoviruses expressing GFP alone or wild-type PKC-GFP constructs were used as controls. After standard adenovirus infection with ~50 MOI, cultured myocytes were harvested in the lysis buffer. Western blot analysis of lysed samples was performed using primary antibodies to GFP, and bands were detected at the expected molecular weights for the fusion proteins (Figure 1A). Expressions of endogenous PKC isoforms including PKC- α , - δ , and - ϵ were not changed by introducing either wild-type or dominant-negative PKC- δ and PKC- ϵ (Figure 1B). Lysed samples also were immunoprecipitated with a GFP antibody to isolate fluorescent PKCs from the endogenous PKC isoforms expressed in myocytes. Immunoprecipitated GFP variants were then subjected to *in vitro* kinase reactions with histone H1 as a substrate. Quantified kinase activities of wt-PKC-GFPs and dn-PKC-YFPs are shown in Figure 1C. Dn-PKC-YFP constructs showed only basal kinase activity at a similar level to GFP alone (no kinase fusion) indicating complete loss of kinase activity, as expected.

Localization of full length dn-PKC-YFP constructs

Adult ventricular myocytes expressing dn-PKC- δ -YFP and dn-PKC- ϵ -YFP showed regular striated patterns especially near the nucleus (Figure 1D and 1E). To test whether this regularity occurred at T-tubules, Z-lines, or some other structures, we stained dn-PKC-YFP expressing myocytes with a Z-line marker (α -actinin), or a T-tubular marker (membrane binding dye di-8-Anepps). Both structures show striations at about 2 μm intervals when visualized with their own markers, but Z-lines are continuous and T-tubules are punctate (Figure 1F and 1G).

Dn-PKC- δ -YFP mainly distributed at intercalated discs and in a striation-like pattern particularly around the nucleus of cardiac myocytes (Figure 1D). The abundant Z-line protein α -actinin showed a regular striation staining pattern that matched dn-PKC- δ -YFP localization patterns (Figure 1F). Dn-PKC- δ -YFP also showed some level of co-localization with di-8-Anepps staining patterns, but the discrete and continuous striated staining of dn-PKC- δ -YFP was more consistent with cytoskeletal rather than membrane anchoring (Figure 1G).

Dn-PKC- ϵ -YFP stained the perinuclear region, the surface sarcolemma around the perimeter of the cell, and gave a striated pattern in the vicinity of the nucleus (Figure 1E). Co-staining with anti- α -actinin antibody or di-8-Anepps again revealed co-localization with the T-

tubules/Z-lines of myocytes (Figure 1F and 1G). In the case of dn-PKC- ϵ -YFP, the striated staining was more punctate suggesting membrane association. The perinuclear staining appeared to reflect association of dn-PKC- ϵ -YFP with the Golgi apparatus.

Effects of ET-1 on myocyte contractile function

Recently we reported that rat ventricular myocytes in short term culture (less than 40 hr) showed a normal rod-shaped morphology, intact transverse tubules (T-tubules) and basal twitch properties similar to freshly isolated myocytes [27]. In the present study, we tested the effect of ET-1 on myocyte contractile function in these cultured ventricular myocytes. ET-1 induced about a 60% increase in twitch amplitude (Figure 2A).

To determine the mechanism of ET-1 mediated positive inotropic responses, we examined changes in intracellular Ca^{2+} handling after ET-1 stimulation. The long-wavelength calcium indicator X-rhod-1 was used to permit accurate measurement of Ca^{2+} signals in cells expressing fluorescent protein constructs [28]. Intracellular Ca^{2+} measurements showed that ET-1 enhanced systolic Ca^{2+} transient amplitude by about 55 %, which was developed in parallel with the increase in twitch amplitude (Figure 2B and 2C).

Effects of Dn-PKC-YFP expression on ET-1 induced contractility change

Dn-PKC constructs characterized as shown in Figure 1 were used as isoform-selective inhibitors in an effort to block endogenous PKC function. The dn-PKC- ϵ and dn-PKC- δ isoforms did not appear to affect basal twitch amplitudes (data not shown). As previously reported, ET-1-induced positive inotropic effect was abolished by dn-PKC- ϵ -YFP expression but not by dn-PKC- δ -YFP expression (Figure 2A) [17]. This result indicates that ET-1 initiated positive inotropy was selectively mediated by the ϵ -isoform of PKC.

Experiments with ET-1 stimulation were also associated with an increase in the systolic Ca^{2+} transient. Myocytes expressing the dn-PKC- δ -YFP construct also showed about a 55% increase in Ca^{2+} transient amplitude with ET-1 stimulation (Figure 2C). In contrast, myocytes expressing dn-PKC- ϵ -YFP did not show changes in systolic Ca^{2+} after ET-1 stimulation (Figure 2C) indicating that PKC- ϵ is the major player in ET-1 induced intracellular Ca^{2+} increase.

ET-1 induced increase in Ca^{2+} sensitivity and involvement of PKC ϵ

Next, we determined the impact of ET-1 stimulation on myofilament Ca^{2+} sensitivity, which may also affect twitch amplitude and kinetics. To test the possibility that the sensitivity of the myofilament Ca^{2+} regulatory system is altered by ET-1, we examined the relationship between free Ca^{2+} and twitch shortening during postrest potentiation. Living rat myocytes showed a typical transient potentiation of the twitch and Ca^{2+} transient responses after a 2 minute rest period (related to the negative staircase force-frequency response in this species). ET-1 stimulation did not alter the maximum Ca^{2+} release or twitch response after the rest period, and the kinetics of the transient potentiation were maintained (not shown) [29]. A comparison between relative intracellular $[\text{Ca}^{2+}]$ and relative shortening during postrest potentiation showed a linear relationship (Figure 3). The slope of the linear relationship decreased in response to ET-1 such that more twitch shortening occurred at a given free $[\text{Ca}^{2+}]$. This observation indicates that myofilament Ca^{2+} sensitivity was enhanced by ET-1. Overall, the data reveal that ET-1 stimulation induced a positive inotropic response in this system by increasing both systolic Ca^{2+} and myofilament Ca^{2+} sensitivity.

Dn-PKC- δ -YFP expressing myocytes also showed an increase in myofilament responsiveness to Ca^{2+} after ET-1 as determined by the postrest potentiation protocol

(Figure 3). Thus, dn-PKC- δ -YFP myocytes were indistinguishable from control myocytes in their response to ET-1. In contrast, myocytes expressing dn-PKC- ϵ -YFP did not show changes in myofilament responsiveness to Ca^{2+} after ET-1 (Figure 3) indicating that PKC- ϵ is critically involved in ET-1 induced myofilament Ca^{2+} sensitivity.

DISCUSSION

In the present study, we demonstrate that underlying positive inotropic response to ET-1 was an increase of cytosolic Ca^{2+} and myofilament Ca^{2+} sensitivity. Previously, we identified PKC- ϵ as a major signaling molecule that is involved in ET-1 induced positive inotropic effect in adult rat ventricular myocytes [17]. Here, PKC- ϵ dominant negative construct also abolished ET-1 induced increase of cytosolic Ca^{2+} and myofilament Ca^{2+} sensitivity confirming that PKC- ϵ is the critical upstream molecule for ET-initiated contractility changes.

Recent studies have implicated other kinases or phosphatases such as MAP kinases, Akt, tyrosine kinases and calcineurin as possible mediators of ET-1 actions [30,31,32]. The results of the present study re-focus attention onto PKC- ϵ and its effects on excitation-contraction coupling and on myofilament properties to account for acute ET-1 actions on myocardium. Results from our own lab have provided evidence for similarities between ET-1 and PKC dependent stimulation of L-type Ca^{2+} current [33]. We have also shown that ET_A receptors, PLC- β 1 and activated PKC- ϵ are co-localized with L-type Ca^{2+} channels in cardiac T-tubules [12], where they mediate a positive inotropic response. These earlier observations are completely consistent with the 50% increase in systolic Ca^{2+} initiated by ET-1 in the present study. Importantly, the new results now link these changes in systolic Ca^{2+} with activation of a specific PKC isoform, namely PKC- ϵ .

What intracellular mechanisms might underlie altered myofilament Ca^{2+} sensitivity? A number of studies have concluded that altered phosphorylation of myofilament proteins underlie increases in myofilament Ca^{2+} sensitivity. Phosphorylation of myosin regulatory light chain has been a leading candidate to account for this [34], but PKC does not directly phosphorylate this myofilament protein. Recent investigations with transgenic mice provided evidence for PKC dependent enhancement of Ca^{2+} sensitivity via phosphorylation of troponin I [24]. Intracellular alkalinization has long been considered an important mechanism of enhancing myofilament Ca^{2+} sensitivity [35,36]. In our recent study, changes in intracellular pH after ET-1 stimulation were monitored using rat ventricular myocytes [17]. Surprisingly, no strong correlation between inotropic responses and intracellular alkalinization was observed in those experiments [17]. This evidence suggests that ET-1 increases cardiac myofilament Ca^{2+} sensitivity via mechanisms other than intracellular alkalinization. The precise mechanism of altered myofilament Ca^{2+} sensitivity needs to be further investigated.

The use of fluorescent fusion dn-PKCs in the present work significantly extends the range and impact of experiments by permitting PKC localization and contractile regulation to be examined in living myocytes in parallel. The YFP tags revealed that the dn-constructs displayed isoform specific distribution patterns which were remarkably similar to patterns of corresponding GFP tagged wild-type PKC isoforms after phorbol ester stimulation from our previous study [16]. In that study, active PKC- ϵ -GFP accumulated at the surface sarcolemma, Golgi apparatus and T-tubules, with the latter two locations correlating most closely with positive inotropy. In the present study, accumulation of dn-PKC- ϵ -YFP in these same subcellular locations blocked the positive inotropic response to ET-1.

In summary, the acute effects of ET-1 on cardiac contractile function have been studied using dn-PKC constructs. Inhibition of PKC- ϵ function selectively blocked ET-1 mediated positive inotropy and Ca^{2+} signaling. On the basis of these data, we propose that ET-1 affects systolic Ca^{2+} , and myofilament Ca^{2+} sensitivity and that PKC- ϵ is the upstream kinase involved in all of these intracellular regulatory processes. The data provide strong overall support for the hypothesis that PKC- ϵ is a central kinase activated early and robustly by ET-1, and that it up-regulates contractility and Ca^{2+} handling in ventricular myocytes.

Acknowledgments

This work is supported by NIH grant R01 HL081386. Imaging was performed at the Keck Center for Biological Imaging at the University of Wisconsin with valuable technical assistance of Lance A. Rodenkirch. The authors give special thanks to Dr. Jeffery W. Walker who deceased during the preparation of this manuscript. Dr. Walker was authors' PhD advisor and guided for this study.

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Highlights

- ✓ Dn-PKC ϵ and dn-PKC δ showed distinct subcellular localization in adult myocytes.
- ✓ ET-1 induces positive inotropy, increase in $[Ca^{2+}]_i$ and increase in Ca sensitivity.
- ✓ The effects of ET-1 were abolished by dn-PKC ϵ overexpression but not by dn-PKC δ .
- ✓ The data suggest that PKC ϵ has a critical role in ET-1-induced changes.

Figure 1. Expression and characterization of dn-PKC-YFPs in myocytes

A. Western blotting of myocyte lysates using anti-GFP antibody. Each lane indicates myocyte lysates prepared 40 hours after adenovirus infection. Total 20 μ g lysates were loaded at each lane and the same blot was reprobed with each antibody after stripping for panel B. GFP alone at about 25 kDa clearly showed up in lane 1 and other GFP fused recombinant PKC proteins located at over 100 kDa. B. Western blotting using PKC isoform specific antibodies. C. *In vitro* kinase assay. GFP alone and fluorescent tagged PKCs were immunoprecipitated with anti-GFP antibody from myocyte lysates and tested for kinase activity using histone H1 as a substrate. Dn-PKC-YFPs show almost no kinase activity compared to wild-type PKC-GFPs. Data represent mean $\pm$ SD; *P<0.02 versus each isoform of wild-type PKC-GFP. D and E. Representative confocal images of dn-PKC- δ -YFP (D) and dn-PKC- ϵ -YFP (E) using the 514 nm laser. Arrows and arrowheads indicate the most prominent features of each localization pattern. F and G. Co-localization of dn-PKC-YFPs with Z-line/T-tubular markers in myocytes. F. Myocytes were decorated with mouse monoclonal anti- α -actinin antibody (and an Alexa 568-labeled secondary antibody) as a Z-line marker. Localization of dn-PKC-YFP constructs (Left, green), α -actinin (Middle, red), and merged image (Right) are shown. G. Myocytes were stained with di-8-Anepps, a marker of T-tubular membranes. Localization of dn-PKC-YFP constructs (Left, green), di-8-Anepps (Middle, blue), and merged image (Right) are shown. Bar = 10 μ m.

Figure 2. Effects of dn-PKC-YFPs expression on ET-1 induced positive inotropy and Ca^{2+} signaling

A. Twitch amplitude after 15 minutes treatment of 10 nmol/L ET-1 in control myocytes and myocytes expressing dn-PKC-YFPs. B. Confocal line scan images recorded from an X-rhod-1 loaded myocyte (2 ms/line). Representative images were captured before (left) and 15 min after addition of 10 nmol/L ET-1 (right) (n=10). The Ca^{2+} signal is shown as a fluorescence ratio (F/F_0) with the fluorescence signal (F) being normalized to the signal prior to contraction (F_0). C. Ca^{2+} transient amplitude after ET-1 activation in control myocytes and myocytes expressing dn-PKC-YFPs. Data represents mean $\pm$ SEM; *P<0.001 compared to control.

Figure 3. Relationships between twitch amplitudes and intracellular Ca^{2+} amplitudes

The relationship between free Ca^{2+} and twitch shortening during postrest potentiation was examined to determine myofilament Ca^{2+} sensitivity. Twitch and Ca^{2+} transient amplitudes were normalized to their respective maxima. The results are representative of data obtained in five experiments on separate culture preparations.