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cAMP-independent Activation of Protein Kinase A by the Peroxynitrite Generator SIN-1 Elicits Positive Inotropic Effects in Cardiomyocytes

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Abstract

The phosphatase vs. kinase equilibrium plays a critical role in the regulation of myocardial contractility. Previous studies have demonstrated that peroxynitrite exerts a biphasic effect on cardiomyocyte contraction, such that high peroxynitrite reduced β -adrenergic-stimulated myocyte contraction by inducing the dephosphorylation of phospholamban (PLB) via phosphatase activation. Conversely, low peroxynitrite increased basal and β -adrenergic-stimulated contraction also through a PLB-dependent mechanism. However, previous studies have not elucidated the mechanism underlying the positive effects of low peroxynitrite on myocyte contraction. In the current study, we examined the phosphatase vs. kinase equilibrium as a potential mechanism underlying the positive effects of peroxynitrite. SIN-1 (peroxynitrite donor, 10 μ mol/L) increased myocyte Ca^{2+} transient and shortening amplitude, accelerated myocyte relaxation, and enhanced PLB phosphorylation. Specific inhibition of PP1/PP2a with okadaic acid failed to inhibit this positive effect. However, inhibition of PKA with KT5720 completely abolished the effects of SIN-1 on myocyte contraction. Additionally, SIN-1 induced a significant increase in PKA activity in cardiac homogenates, which was inhibited with FeTPPS (peroxynitrite decomposition catalyst). Surprisingly, SIN-1 also increased activity in purified preparations (i.e., in the absence of cAMP) of PKA. Therefore, our data suggest that peroxynitrite directly activates PKA (independent from cAMP), resulting in the enhancement of myocyte contraction and relaxation through the phosphorylation of PLB.

Keywords

PKA; NOS1; Phospholamban; Phosphatase

INTRODUCTION

Activation of protein kinase A (PKA), which traditionally occurs with β -adrenergic receptor (β -AR) stimulation, is known to enhance cardiomyocyte contraction [1]. These effects occur primarily through the phosphorylation of phospholamban (PLB) by PKA at Serine16 [2], and

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can be reversed by phosphatases, namely protein phosphatase 1 (PP1) and 2a (PP2a) [3]. Consequently, the phosphatase vs. kinase equilibrium plays a critical role in regulating cardiomyocyte contractility.

Peroxynitrite (ONOO^-), the reaction product of nitric oxide and superoxide, exerts biphasic effects on cardiomyocyte function, such that low concentrations increase [4,5] and high concentrations decrease [4,6,7] contraction. We demonstrated that high peroxynitrite reduced β -AR-stimulated cardiomyocyte contraction by inducing the dephosphorylation of $\text{PLB}_{\text{Ser16}}$ via PP2a activation [7,8]. Conversely, low peroxynitrite increased basal and β -AR-stimulated cardiomyocyte contraction also through a PLB-dependent mechanism [4]. However, previous studies have not elucidated the mechanism underlying the positive inotropic effects of low peroxynitrite on myocyte contraction. Since these effects are dependent upon the phosphoprotein PLB, a shift in the phosphatase vs. kinase equilibrium is likely responsible. We previously showed that low peroxynitrite was without effect on protein phosphatase activity in cardiac homogenates [8]. Further, PKA is an enzyme which exhibits particular sensitivity to redox modification [9]. Here we investigate the phosphatase vs. kinase equilibrium by examining PP1/PP2a and PKA as potential signaling targets for low peroxynitrite.

MATERIALS AND METHODS

Cardiomyocyte isolation (male, C57BL/6), Ca^{2+} transient measurements, cell shortening measurements, and western blot analysis were performed as previously described [4,7,8]. PKA activity was measured using the PKA Kinase Activity Assay (Assay Designs, Ann Arbor, MI). Detailed methods are provided in the online supplement at <http://www.sciencedirect.com>.

RESULTS

SIN-1 (10 $\mu\text{mol/L}$, peroxynitrite donor) significantly increased basal Ca^{2+} transient (CaT) and shortening amplitudes (CaT: 1.0 ± 0.2 vs. 1.3 ± 0.2 $\Delta\text{F}/\text{F}_0$, Shortening: 4.6 ± 0.8 vs. 5.6 ± 1.0 % RCL), and accelerated the time to 50% relaxation (RT_{50}) (CaT: 282 ± 15 vs. 264 ± 14 ms, Shortening: 350 ± 25 vs. 312 ± 21 ms) in cardiomyocytes (Fig. 1A–C). These effects were inhibited by FeTPPS (peroxynitrite decomposition catalyst) in a previous study [7]. Consistent with the enhanced contraction and accelerated relaxation, SIN-1 induced an increase in $\text{PLB}_{\text{Ser16}}$ phosphorylation (0.65 ± 0.06 vs. 0.88 ± 0.06 A.U., $p < 0.05$; Fig. 1D). The positive effect of SIN-1 on CaT amplitude (0.9 ± 0.1 vs. 1.2 ± 0.1 $\Delta\text{F}/\text{F}_0$, $P < 0.05$) and decline (299 ± 10 vs. 275 ± 13 ms, $p < 0.05$) remained despite specific inhibition of PP1/PP2a with okadaic acid (OA, 1 $\mu\text{mol/L}$) (supplemental Fig. 1). This same concentration of OA inhibited the negative inotropic effect of high peroxynitrite in a previous study [7].

We next examined PKA using the specific inhibitor KT5720 (1 $\mu\text{mol/L}$). PKA inhibition alleviated the positive effect of SIN-1 on basal contraction (CaT amplitude: 1.0 ± 0.2 vs. 1.1 ± 0.2 $\Delta\text{F}/\text{F}_0$, Shortening: 4.8 ± 0.7 vs. 4.9 ± 0.8 % RCL) and RT_{50} (CaT: 295 ± 18 vs. 306 ± 17 ms, Shortening: 275 ± 34 vs. 261 ± 30 ms; Fig. 2A–D). PKA inhibition also blunted the effect of the β -adrenergic agonist isoproterenol (0.01 $\mu\text{mol/L}$) on CaT amplitude (3.2 ± 0.4 vs. 2.0 ± 0.3 $\Delta\text{F}/\text{F}_0$, $P < 0.05$) in control experiments (supplemental Fig. 2A–C).

In experiments examining PKA activity, Fig. 2E shows that SIN-1 induced a 22% increase in PKA activity in cardiac homogenates (8.2 ± 0.5 vs. 10.1 ± 0.3 $\text{Abs}_{450}/\text{mg}$ protein; $P < 0.05$, Fig. 2D). FeTPPS (10 $\mu\text{mol/L}$) abolished this effect. Surprisingly, SIN-1 also significantly increased PKA activity in purified preparations containing the regulatory and catalytic subunits of PKA (0.01 ± 0.01 vs. 0.06 ± 0.01 Abs_{450} ; Fig. 2F). cAMP was not added in these experiments. We also tested the effects of NO (spermine NONOate, 300 $\mu\text{mol/L}$) and hydrogen peroxide

(H₂O₂, 100 μmol/L) on PKA activity. NO induced a slight increase in PKA activity (but nominal compared to SIN-1), while H₂O₂ had no effect (data not shown). Furthermore, KT5720 had no effect on unstimulated PKA activity (0.00±0.01 Abs₄₅₀) but abolished the effect of SIN-1 on PKA activity (0.00±0.005 Abs₄₅₀). As a positive control, cAMP (10 μmol/L) significantly increased PKA activity by 218% in cardiac homogenates (26.0±0.5 Abs₄₅₀/mg) and in purified preparations of PKA (0.08±0.2 Abs₄₅₀) (supplemental Fig. 2D–E).

DISCUSSION

In our current study, we reveal a novel mechanism whereby low peroxynitrite induces a direct, cAMP-independent activation of PKA, which leads to the phosphorylation of PLB_{Ser16}, and the enhancement of myocyte contraction and relaxation (Fig. 1). The positive effects of peroxynitrite, produced via SIN-1, observed in the current study are consistent with previous reports [4,5]. Inhibition of PP1/PP2a did not alter the effects of SIN-1 (Supp. Fig. 1). This contrasts directly with the effect of high peroxynitrite [7,8], but is consistent with the negligible effect of low peroxynitrite on phosphatase activity [8]. Therefore, peroxynitrite does not appear to exert differential effects on cardiomyocyte contraction through a biphasic regulation of phosphatase activity. However, since OA is more selective for PP2a than for PP1, we cannot completely rule out PP1 as a potential target for the effects of peroxynitrite.

Upon further examination of the phosphatase vs. kinase equilibrium, we demonstrated that PKA inhibition with KT5720 completely abolished the contractile effects of SIN-1 (Fig. 2). Further, SIN-1 induced an increase in PKA activity in cardiac homogenates, which was inhibited by FeTPPS, thus implicating peroxynitrite as the causal species (Fig. 2). Surprisingly, SIN-1 also increased activity in purified preparations of PKA (Fig. 2). These experiments were performed in the complete absence of cAMP, as confirmed by the low basal PKA activity, suggesting that peroxynitrite directly activates PKA. Since SIN-1 is not a direct peroxynitrite donor, and produces other reactive species, we also tested the effects of NO and H₂O₂ on PKA activity. We observed that NO had nominal effects compared to SIN-1 and H₂O₂ had no effect on PKA activity. Therefore, our results indicate that peroxynitrite leads to positive inotropy via direct PKA activation. PKA is a redox sensitive enzyme that can be regulated by various oxidants (i.e., H₂O₂) [9]. However, our current results contrast with the H₂O₂-mediated activation of PKA, as this is the first report to demonstrate the direct activation of purified PKA, independent from cAMP. Future studies will need to confirm this novel effect within cardiac myocytes. Compared with cAMP alone, the activating effect of peroxynitrite on PKA was not as robust, but this is consistent with the contractile effects of low peroxynitrite (Fig. 1) compared to β-AR stimulation (Supp. Fig. 2).

The peroxynitrite-mediated increase in PKA activity may relate to the physiological regulation of cardiomyocyte function by neuronal nitric oxide synthase (NOS1), an enzyme that is expressed constitutively within cardiomyocytes and colocalizes with xanthine oxidoreductase [10]. We demonstrated that NOS1 regulates cardiomyocyte contraction by modulating PLB_{Ser16} phosphorylation and signals through the production of peroxynitrite [11]. Therefore, NOS1 may regulate cardiomyocyte contraction by inducing PKA activation via peroxynitrite production.

In conclusion, the positive effect of peroxynitrite is produced from the direct, cAMP-independent activation of PKA. This activation shifts the phosphatase vs. kinase equilibrium, which leads to the phosphorylation of PLB_{Ser16} and the enhancement of myocyte contraction. Although other targets for peroxynitrite exist within the cardiomyocyte (e.g., L-type Ca²⁺ channel [12]), PKA remains a major target for the effects of low peroxynitrite. The results of this study may also have important ramifications with regard to redox regulation and traditional PKA signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A–C) Individual, steady-state CaT (top) and shortening (bottom) traces, and pooled data (mean $\pm$ S.E.M.) demonstrating the effect of control and SIN-1 on (B) amplitude and (C) time to 50% relaxation ($n = 9$ –12 myocytes/2–4 hearts). D) Representative western blot for PLB_{Ser16} phosphorylation and PLB_{Total}, and pooled data (mean $\pm$ S.E.M.) for control and SIN-1 ($n = 3$ hearts/group). * $p < 0.05$ vs. Control.

Figure 2.

A–D) Individual, steady-state CaT (top) and shortening (bottom) traces, and pooled data (mean $\pm$ S.E.M.) demonstrating the effect of control and SIN-1 on (B) amplitude, (C) % Δ from control, and (D) time to 50% relaxation $\pm$ KT5720 ($n = 6$ –11 myocytes/2–4 hearts). E) Pooled data (mean $\pm$ S.E.M.) for PKA activity with control and SIN-1 treated cardiac homogenates $\pm$ FeTPPS ($n = 5$ hearts/group). F) Pooled data (mean $\pm$ S.E.M.) for purified PKA activity for control and SIN-1 $\pm$ KT5720 ($n = 6$ /group). * $p < 0.05$ vs. Control, ** $p < 0.05$ vs. SIN-1 alone.