In vivo regulation of Na/Ca exchanger expression by adrenergic effectors

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Golden, Kish L., Jun Ren, Jessica O’Connor, Andrea Dean, Stephen E. DiCarlo, and James D. Marsh. In vivo regulation of Na/Ca exchanger expression by adrenergic effectors. Am J Physiol Heart Circ Physiol 280: H1376–H1382, 2001.—The Na/Ca exchanger encoded by the NCX1 gene plays an important role in calcium homeostasis in cardiac muscle. We previously identified three in vitro signaling pathways that are of major importance in the regulation of Na/Ca exchanger gene expression in neonatal cardiac myocytes, the protein kinase A (PKA) and protein kinase C (PKC) pathways, and intracellular Ca2+. To determine whether these pathways are important in vivo, we stimulated the PKA and PKC pathways and examined functional expression of the Na/Ca exchanger in adult rat heart. After a 3- and 7-day treatment, norepinephrine (200 μg·kg−1·h−1), isoproterenol (150 μg·kg−1·h−1), and phenylephrine (200 μg·kg−1·h−1) each stimulated a significant increase in NCX1 mRNA levels (35–85%, P < 0.05). Norepinephrine also stimulated a 35% increase in protein abundance (P < 0.05), a 20% decrease in relaxation duration (P < 0.05), and a 25% reduction in the fluorescence decay constant (P < 0.05) after a 7-day treatment. We conclude that a 7-day treatment of α- and β-adrenergic agonists increases the expression of functional Na/Ca exchangers in adult rat heart.

β-adrenergic receptors coordinately regulates the expression of the α1c-subunit of the L-type calcium channel [dihydropyridine (DHP) receptor] and the Na/Ca exchanger by activating protein kinase A (PKA) and protein kinase C (PKC) pathways. Specifically, β-adrenergic receptor activation in vitro increases expression of the DHP and NCX1 genes, whereas α-adrenergic signaling reduces their mRNA levels. The changes in the level of expression of these genes in vitro resulted in functional alterations as well. Intracellular calcium measurements reveal that when cardiac myocytes are pretreated with isoproterenol (Iso), there is an increase in the calcium transient amplitude, and a faster decay rate in the calcium transient, consistent with not only increased transcript abundance, but increased exchanger function (9).

In congestive heart failure in humans and animal models, circulating norepinephrine (NE) levels are markedly elevated and myocytes are under intense sympathetic stimulation, which is associated with abnormal calcium homeostasis (10, 24). We hypothesize that NE may influence the in vivo expression of the Na/Ca exchanger and hence calcium homeostasis by signaling through PKA and PKC pathways. To test this hypothesis, we treated rats with α- and β-adrenergic agonists over several days and subsequently analyzed expression of the Na/Ca exchanger transcript, protein, and we examined exchanger function.

METHODS

Adrenergic infusions. Adult male Sprague-Dawley rats were anesthetized with a ketamine-xylazine mixture (5:3, 1.32 mg/kg ip). A small lateral incision was made on the back of the neck. The skin was bluntly dissected to form a pocket, under which an osmotic minipump was implanted (Alza Scientific Products; Palo Alto, CA). The pumps were filled with either NE, phenylephrine (PE), or Iso dissolved in 0.001 N HCl or vehicle. In our study, the minipumps delivered 200 μg·kg−1·h−1 of NE and PE and 150 μg·kg−1·h−1 of Iso. After termination of the experiments, the rats were anesthetized, and the hearts were then removed and processed for Na/Ca

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exchanger message and protein levels. Other rats were treated similarly but cardiac myocytes were dissociated for studies on exchanger function.

**Procedures for catheter insertion.** Rats were instrumented with a Renathane catheter inserted into the descending aorta via the left common carotid artery for measurements of arterial blood pressure and heart rate as previously described (6). All of the rats were studied for 3 days after the surgical procedures. On 2 consecutive days, arterial pressure and heart rate were recorded for 2 h to obtain control (pre-drug) values. After we obtained control day values, osmotic minipumps were implanted. The rats were allowed 2 days to recover from the implant procedures. Subsequently, the rats were studied for 5 consecutive days to determine the effects of chronic infusion of NE or PE on arterial pressure and heart rate. An additional group of rats \((n = 3)\) followed an identical time line. However, the pumps were not implanted. These rats served as “time controls.”

**Preparation of RNA and Northern analysis.** Isolation of RNA and Northern blot analysis was performed as previously described (9). A 600-bp rat brain NCX1 cDNA probe, corresponding to nucleotides 1–600, was a gift of Dr. Kenneth Philipson. To normalize for possible differences in amount of RNA loaded on gels and/or transfer of total RNA from the gel, the filters were subsequently hybridized to a \((^{32}P\)dATP oligonucleotide complementary to 18S ribosomal RNA and analyzed by phosphorimager analysis (Molecular Dynamics; Sunnyvale, CA).

**Immunoblot analysis.** Total protein from whole hearts was isolated (9). Tissues were homogenized in buffer containing SDS with 1% 2-mercaptoethanol. After immunoblotting with an anti-Na/Ca exchanger polyclonal antibody (1:1,000) (Swant; Bellinzona, Switzerland) was completed, immunoreactive bands were detected by using chemiluminescence (Amersham) according to manufacturer instructions. The bands were quantified with the use of a laser densitometer (Molecular Dynamics).

**Cell shortening/relengthening and intracellular fluorescence measurements.** Single ventricular myocytes were enzymatically isolated (25). Mechanical properties of ventricular myocytes were assessed with the use of a SoftEdge videobased edge-detection system (Ionoptix; Milton, MA) (25). Cells were placed on a chamber mounted on the stage of an inverted microscope (model X-70, Olympus) and superfused (~2 ml/min at 37°C) with a buffer containing (in mM) 131 NaCl, 4 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, 10 HEPES (pH 7.4). Myocytes were field stimulated with the use of a pair of platinum wires at a frequency of 0.5 Hz.

A separate cohort of myocytes was loaded with fura 2-acetoxyethyl ester (AM) (0.5 \(\mu\)M) for 10 min, and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix) as previously described (26). While the myocytes were being stimulated to contract at 0.5 Hz, fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating cells at 360 nm for 0.5 s then at 380 nm for the duration of the recording protocol (333-Hz sampling rate). The 360-nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were inferred from the ratio of the fluorescence intensity at the two wavelengths.

**Statistical analysis.** Data were analyzed by using GB-STAT software (Dynamic Microsystems; Silver Spring, MD). Differences between variables were analyzed by the nonparametric Kruskal-Wallis one-way ANOVA. Data are shown as means ± SE.

Hemodynamic data are expressed as means ± SE. Two separate two-way ANOVA with repeated measures were used to compare mean arterial pressure or heart rate over time under the three experimental conditions of time control, NE infusion, and PE infusion. An \(\alpha\)-level of 0.05 was used to determine statistical significance.

**RESULTS**

**Effects of adrenergic agonists on hemodynamic variables.** Chronic infusion of PE at the dose studied did not significantly alter arterial pressure or heart rate (Fig. 1). In contrast, infusion of NE significantly increased arterial pressure by ~15 mmHg without any change in heart rate (Fig. 1). Additionally, time alone did not alter arterial pressure or heart rate when no drug was infused (Fig. 1). Because previous hemodynamic studies (1, 18, 27) on rats treated with pure \(\beta\)-adrenergic agonists at the same dose we used demonstrated either no change or a slight decline in mean arterial pressure, we did not perform hemodynamic measurements on animals treated with Iso.

**Regulation of Na/Ca exchanger mRNA abundance by adrenergic agonists.** To examine the in vivo effects of chronic infusion of \(\alpha\)- and \(\beta\)-adrenergic agonists on the expression of NCX1, we treated animals for 3 and 7 days with NE, Iso, or PE. After a 3-day treatment, NE produced significant cardiac hypertrophy as determined by an increase in the heart weight-to-body weight ratios compared with sham-operated control rats.
animals (Fig. 2); Iso also produced cardiac hypertrophy. Heart weight-to-body weight ratios were 5.27 ± 0.30 mg/g (n = 5; P < 0.01) and 6.69 ± 0.70 mg/g (n = 5; P < 0.01) for NE and Iso, respectively, compared with 4.43 ± 0.29 mg/g (n = 5) for control animals. On the other hand, PE infusion for 3 days did not produce a significant increase in heart weight-to-body weight ratio (4.59 ± 0.50 vs. 4.43 ± 0.29) (Fig. 2). When Na/Ca exchanger message levels were assessed after a 3-day infusion, Na/Ca exchanger expression relative to that of 18S ribosomal RNA was increased ~37% in hearts from animals treated with either NE or Iso (n = 5; P < 0.05) (Fig. 3). PE infusion for the same amount of time produced an 87% increase in Na/Ca exchanger mRNA abundance (n = 5; P < 0.05).

After a 7-day infusion, PE induced a significant increase in heart weight-to-body weight ratios but with a less robust hypertrophic effect than that produced with either NE or Iso (Fig. 4). The degree of hypertrophy induced by NE after a 3-day infusion is further augmented when the infusion is continued for 7 days; the hypertrophic response of Iso infusion after 3 days is unaltered after a 7-day infusion (Fig. 4). When mRNA abundance for the Na/Ca exchanger was determined, NE, Iso, and PE all produced significant increases in Na/Ca exchangerexpression after infusion for 7 days (Fig. 5).

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To determine whether the NE-induced increase in Na/Ca exchanger gene expression resulted in changes in protein levels, we performed immunoblot analysis employing a polyclonal antibody raised against the purified dog cardiac sarcolemmal Na/Ca exchanger. Figure 6 shows an immunoblot of proteins obtained from adult rat hearts after NE treatment. After a 7-day exposure, NE stimulated a 35% increase in Na/Ca exchanger protein, compared with untreated myocytes (n = 6, P < 0.05). PE or Iso infusion produced comparable increases in Na/Ca exchanger protein levels (data not shown).

**Effect of NE on myocyte shortening and relengthening.** To determine the consequences of 7-day NE treatment on cardiac myocyte shortening and relaxation properties, we employed a video-based edge-detection system to assess myocyte mechanical properties. The cells were washed extensively and measurements were made in the absence of catecholamines. A representative trace is shown in Fig. 7A. The average resting cell length of ventricular myocytes from control animals was 149.19 ± 6.04 μm, and peak shortening (normalized to cell length) in response to electrical stimulation was 5.30 ± 0.30% (Fig. 7). NE infusion for 7 days did not affect resting myocyte cell length. However, peak twitch amplitude was 143% of controls (5.30 ± 0.30 vs. 7.60 ± 0.60%, n = 40, P < 0.01). NE also decreased the time-to-peak shortening (Fig. 7C). Furthermore, isolated cardiac myocytes from NE-treated rats displayed an accelerated rate of relaxation, or time to 90% relaxation (215 ± 16 vs. 176 ± 13 ms; n = 38, P < 0.01). See Fig. 7D for an example.

**Fig. 6.** Immunoblot analysis of Na/Ca exchanger protein abundance after a 7-day treatment. **Top:** immunoblots from a 6% polyacrylamide gel were probed with a polyclonal antibody raised against the purified dog cardiac sarcolemmal Na/Ca exchanger. **Bottom:** NE treatment produced approximately a 35% increase in Na/Ca exchanger protein levels, compared with controls. n = 8 rats; *P < 0.05

**Fig. 7.** Effect of chronic (7 day) β-adrenergic stimulation (NE) on cardiac contraction in adult rat ventricular myocytes. **A:** representative traces showing myocyte shortening and relengthening from a control and a NE-treated myocyte. **B–D:** bar graphs exhibiting the effect of chronic NE treatment on cell peak shortening (PS), time-to-peak shortening (TPS), and time-to-90% relaxation (TR_{90}). n = 81–87 myocytes per group; *P < 0.05 vs. control.
Effect of NE on intracellular Ca\(^{2+}\) transients. To determine whether augmented NCX1 expression alters the Ca\(^{2+}\) transient functionally, adult myocytes were isolated from control and 7-day NE-treated animals, and loaded with fura 2. The Ca\(^{2+}\) transient amplitude and rate of decay constant (\(\tau\)) were recorded. During the recordings, both groups were perfused with HEPES buffer (see METHODS) in the absence of any catecholamines. Representative traces are shown in Fig. 8A. Myocytes isolated from NE-treated animals displayed an increased amplitude of the Ca\(^{2+}\) transient (Fig. 8C) and a slight but significant increase in diastolic Ca\(^{2+}\) levels (Fig. 8D). In the presence of 5 \(\mu\)M thapsigargin, to block sarcoplasmic reticulum (SR) Ca\(^{2+}\) uptake, the \(\tau\) of the Ca\(^{2+}\) transient was markedly faster (smaller \(\tau\)) in myocytes isolated from NE-treated animals, suggesting an enhanced rate of intracellular Ca\(^{2+}\) removal via the Na/Ca exchanger and not augmented sequestration (Fig. 8B) [297 ± 19 ms (control) vs. 214 ± 10 ms; \(P < 0.01\)]. The increase in the calcium transient decay rate may also be at least partially influenced by the increased calcium transient amplitude (4). In cardiac myocytes, the plasma membrane Ca\(^{2+}\)-ATPase contributes only minimally (<5%) to relaxation of the calcium transient (2, 3).

DISCUSSION

NE exerts its actions on cardiac myocytes by signaling through \(\beta\)- and \(\alpha\)-adrenergic receptors, and thus by regulating \([Ca^{2+}]_i\), can alter contractile performance. Sustained adrenergic signaling can produce \(\beta\)-adrenergic receptor downregulation and blunted transmembrane signaling. In patients with advanced congestive heart failure, a 3-day infusion of a \(\beta\)-adrenergic agonist (dobutamine) is sometimes used to improve cardiac function; the beneficial effects on cardiac output and symptoms are often sustained long after conclusion of the infusion (13). The mechanism of this effect is unclear. The current study of a similar treatment regimen in an animal model provides new mechanistic insights and provides some understanding of the molecular remodeling of the calcium regulating processes that occurs.

Until recently, very little was known about the molecular signals that regulate expression of the Na/Ca exchanger. In vitro studies (9) from our laboratory have demonstrated that adrenergic signaling can modulate the expression of the Na/Ca exchanger. Activation of PKA was demonstrated to augment the mRNA levels for the Na/Ca exchanger. To determine whether the in vivo activation of PKA can regulate Na/Ca exchanger expression, we treated animals with NE and Iso and measured functional expression of the Na/Ca exchanger. After a 3- and 7-day treatment with either NE or Iso, there was a slight but significant hypertrophic response. Our findings are consistent with the results of Boluyt et al. (5), where Iso infusion for 2 and 4 days increased ventricular weight-to-body weight...
ratios by 27 and by 47%, respectively (5). Although NE treatment also produced a hypertensive response, and thus might induce hypertrophy, the hypertrophic effects of Iso stimulation in vivo do not appear to be mediated by changes in hemodynamic variables. When blood pressure measurements were performed on animals after chronic exposure to Iso at the dose we studied, there was minimal change in arterial pressure (1, 18, 27). Additionally, other reports (19) demonstrate that Iso directly produces hypertrophy. The hypertrophic response to NE may be a consequence of afterload changes and/or direct transcriptional effects mediated via both α- and β-adrenergic receptors (28). Zimmer et al. (28) demonstrated that treatment with α- and β-adrenergic antagonists reverses the NE-induced increases in RNA-to-DNA ratios and heart weight-to-body weight ratios. The hypertrophic response to a pure β-adrenergic agonist (Iso) and a relatively pure α-adrenergic agonist (PE) are not due to hemodynamic changes and are likely direct transcriptional effects.

The β-adrenergic-agonist-induced hypertrophy after a 3- and 7-day infusion was accompanied by changes in mRNA and protein abundance that paralleled alterations in gene expression observed in pressure overload-induced hypertrophy. Menick et al. (17) reported rapid upregulation of Na/Ca exchanger message and protein levels in an in vivo model of acute right ventricular pressure overload in felines. Normalization of transcript abundance in the setting of hypertrophy is complex. We normalized to the abundance of 18S ribosomal RNA; ribosomal RNA itself increases in cardiac hypertrophy (16). Thus the magnitude of increase in Na/Ca exchanger abundance we found probably is an underestimate.

In animal models of heart failure and cardiac hypertrophy there is impaired relaxation that is causally associated with prolongation of the calcium transient. An almost universal finding is downregulation of SR Ca\textsuperscript{2+}-ATPase function. It appears that increased expression of functional Na/Ca exchangers in response to β-adrenergic infusion could be compensatory for the downregulation of SR Ca\textsuperscript{2+}-ATPase function. Hasenfuss et al. (11) demonstrated that unaltered Na/Ca exchanger protein levels in the presence of decreased SR Ca\textsuperscript{2+}-ATPase protein abundance in some humans with end-stage heart failure is associated with combined systolic and diastolic dysfunction (11). On the other hand, upregulation of Na/Ca exchanger protein in the presence of reduced SR Ca\textsuperscript{2+}-ATPase protein levels in other humans is associated with only systolic dysfunction. In our functional studies, the time to relaxation of the calcium transient is reduced in myocytes isolated from animals treated with β-adrenergic agonists, consistent with enhanced exchanger function. Because the calcium transient τ is faster in the presence of a SR Ca\textsuperscript{2+}-ATPase inhibitor, we view it as very likely that the mechanism for the increase in relaxation rate is, at least in part, due to enhanced calcium efflux across the sarcolemma via the Na/Ca exchanger.

Contrary to our in vitro findings demonstrating downregulation of Na/Ca exchanger expression in response to α-adrenergic stimulation, the in vivo activation of α-adrenergic receptors by treating animals with PE augmented Na/Ca exchanger mRNA levels. Our in vitro experiments with PE were done in the presence of propranolol, and thus our in vivo results might be explained by a direct effect of PE on β-adrenergic receptors. Alternatively, it may be due to an indirect effect caused by PE-induced depletion of myocardial NE stores (8). We also cannot rule out the possibility of variant signaling pathways that exist between these two models. The hypertrophic response of PE infusion was not mediated by changes in hemodynamic variables. Thus it appears that increases in Na/Ca exchanger mRNA and protein abundance is the result of transmembrane signaling in the myocyte, and probably enhanced transcription. Of note, within the three tissue-specific alternative promoters of the Na/Ca exchanger gene there are multiple potential AP-1 binding regions as well as a pair of consensus binding sites for the cAMP response element binding protein that might mediate enhanced transcription in response to α- and β-adrenergic signaling (20). Our results are consistent with the findings of others that there is an increase in Na/Ca exchanger expression and function in hypertrophy (17, 22).

In summary, we have demonstrated that α- and β-adrenergic stimulation in vivo increases NCX1 gene expression and protein abundance. Our physiological data are consistent with enhanced Na/Ca exchanger function. These findings may reflect, in part, the molecular remodeling that occurs in human heart after intravenous catecholamine treatment. In the nonfailing rat heart, α- and β-adrenergic stimulation produces alterations in gene expression that may have a salutary effect on diastolic function.

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