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Intact calcium signaling in adrenergic-deficient embryonic mouse hearts

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ABSTRACT

Mouse embryos that lack the ability to produce the adrenergic hormones, norepinephrine (NE) and epinephrine (EPI), due to disruption of the dopamine beta-hydroxylase (*Dbh*$^{-/-}$) gene inevitably perish from heart failure during mid-gestation. Since adrenergic stimulation is well-known to enhance calcium signaling in developing as well as adult myocardium, and impairments in calcium signaling are typically associated with heart failure, we hypothesized that adrenergic-deficient embryonic hearts would display deficiencies in cardiac calcium signaling relative to adrenergic-competent controls at a developmental stage immediately preceding the onset of heart failure, which first appears beginning or shortly after mouse embryonic day 10.5 (E10.5). To test this hypothesis, we used ratiometric fluorescent calcium imaging techniques to measure cytosolic calcium transients, $[Ca^{2+}]_i$ in isolated E10.5 mouse hearts. Our results show that spontaneous $[Ca^{2+}]_i$ oscillations were intact and robustly responded to a variety of stimuli including extracellular calcium (5 mM), caffeine (5 mM), and NE (100 nM) in a manner that was indistinguishable from controls. Further, we show similar patterns of distribution (via immunofluorescent histochemical staining) and activity (via patch-clamp recording techniques) for the major voltage-gated plasma membrane calcium channel responsible for the L-type calcium current, $I_{Ca,L}$, in adrenergic-deficient and control embryonic cardiac cells. These results demonstrate that despite the absence of vital adrenergic hormones that consistently leads to embryonic lethality *in vivo*, intracellular and extracellular calcium signaling remain essentially intact and functional in embryonic mouse hearts through E10.5. These findings suggest that adrenergic stimulation is not required for the development of intracellular calcium oscillations or extracellular calcium signaling through $I_{Ca,L}$ and that aberrant calcium signaling does not likely contribute to the onset of heart failure in this model.

1. Introduction

The adrenergic hormones norepinephrine (NE) and epinephrine (EPI) provide critical stimulation of cardiac function during the transition from embryonic to fetal stages of development *in utero* [1–3]. Mice unable to produce these hormones due to disruption of the gene encoding for the adrenergic biosynthetic enzyme, dopamine β-hydroxylase (Dbh), begin to show signs of heart failure as early as embryonic day 10.5 (E10.5) and typically die shortly thereafter with the vast majority of deaths occurring during the period from E11-13 [1]. Adrenergic hormones have widespread actions on a number of systems, and it is not entirely clear which systems are those critically affected by their absence in the developing heart.

Prior studies have shown that adrenergic cells are present in the heart at early stages [4–6], where they were found to be associated with cardiac pacemaking and conduction tissue [7]. A subsequent study showed that pacemaking appeared to develop normally in the absence of NE and EPI, but there was delayed atrial-ventricular (A-V) conduction and increased propensity to develop arrhythmias in embryos that were NE&EPI-deficient [8]. Further, systems-based studies later showed significant changes in cardiac gene expression

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Abbreviations

AT	Atrium
$CaDV_{Max}$	Maximum Systolic Ca^{2+} transient Decline Velocity (Maximum velocity of cytosolic Ca^{2+} removal; Original value-AU/sec)
DHPR	Sarcolemmal L-type voltage-gated Ca^{2+} channel
Dbh	Dopamine β-hydroxylase
EPI	Epinephrine
NE	Norepinephrine
OT	Outflow Tract
RT_{90}	Return Time 90% (Time from Ca^{2+} peak to 10% peak during diastole)
RyR	Sarcoplasmic reticular Ca^{2+} release channel
SERCA2a	Cardiac isoform of the Sarcoplasmic Reticular Ca^{2+}-ATPase
VE	Ventricle

relating to metabolism that resulted in impaired energy metabolism [9,10]. The metabolic and A-V conduction defects likely contribute to the reduced viability of $Dbh^{-/-}$ embryos, but there could be other contributing causes as well.

In the present study, we specifically examined calcium signaling as a potentially important early target of adrenergic stimulation in early heart development. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations are crucial for excitation-contraction coupling, and they are well-known targets of adrenergic regulation in the heart [11–13]. Adrenergic hormones have been shown to regulate a number of specific targets involved in calcium signaling/handling in myocardial cells, and aberrant calcium signaling has been associated with heart failure phenotypes in adult models as well as in the clinic [14–19]. We also previously showed that expression from a number of genes involved in signal transduction, including one encoding for a voltage-dependent Ca^{2+} channel subunit, were significantly lower in NE&EPI-deficient embryos [9]. We thus set forth to test the hypothesis that Ca^{2+} signaling/handling is impaired in NE&EPI-deficient embryonic myocardium, as described in this paper.

2. Materials and methods

2.1. Animals

$Dbh^{-/-}$ mice were produced and embryos collected at E10.5, as previously described by breeding heterozygous ($Dbh^{+/-}$) mating pairs [9]. As no significant differences have been observed between wild-type ($Dbh^{+/+}$) and heterozygous ($Dbh^{+/-}$) hearts at E10.5 in any examined parameter, here or in earlier studies [1,8], these two genotypes were combined into a single group referred to as "NE&EPI-competent." Homozygous knockout ($Dbh^{-/-}$) mice were designated as "NE&EPI-deficient" due to their inability to produce NE or EPI.

The head was rapidly removed for DNA extraction and genotyping by PCR methods and primers as previously described [9]. All animal procedures were performed in accordance with protocols approved by the Georgetown University and University of Central Florida Animal Care and Use Committees and conform with NIH guidelines.

2.2. Immunofluorescent histochemistry

Immunofluorescent histochemical staining of hearts was performed essentially as described previously [7], using a mouse anti-Dihydropyridine Receptor (DHPR) α2 subunit antibody (D-219; Sigma) and mouse anti-Sodium channel (pan) antibody (S-8809, Sigma). Secondary donkey anti-mouse (FITC and Texas Red) antibodies were obtained from Jackson ImmunoResearch.

2.3. Whole-cell patch-clamp recordings

Cardiomyocytes were isolated from individual hearts and seeded onto laminin-coated 12 mm glass coverslips [20]. L-type Ca^{2+} current ($I_{Ca,L}$) was measured by the whole-cell patch-clamp technique as previously described [21].

2.4. Ex vivo intracellular Ca^{2+} measurements

Hearts were isolated under aseptic conditions and placed dorsal-side up on the gelatin-coated coverslip bottom of cell culture chambers (Cat. #CSTPKG, Cell MicroControls, Norfolk, VA), secured in place by inserting a stainless-steel minutiens pin through the outflow tract into a ridge of silicone rubber adhered to the coverslips, and cultured 20–48 h in supplemented Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone Labs, Logan, UT) that had been charcoal stripped to remove catecholamine and steroid hormones, as previously described [8]. Hearts that did not recover spontaneous beating during culture were excluded (regardless of genotype).

Cell chambers were placed onto an inverted microscope attached to a Myocyte Calcium Recording System (IonOptix, Milton, MA) and superfused with unmodified DMEM at 37 °C, pH = 7.4. The ratiometric cytoplasmic Ca^{2+} dye Indo-1 AM (TEFLabs, Austin, TX) was loaded by incubation in Hepes-buffered modified Tyrode's solution (in mM: 134 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 D-glucose; pH balanced with NaOH) containing 10 μM Indo-1 AM ester with 0.003% pluronic F-127 and 25 μM probenecid at room temperature for 60–90 min at room temperature, followed by 10 min re-equilibration in DMEM at 37 °C. During the re-equilibration period, hearts were momentarily (10–20 s) and reversibly exposed to DMEM with 5 mM Ca^{2+} to re-synchronize contractions.

Stimulation treatments were applied in sequence shown in Fig. 1. Ca^{2+} transients were recorded for 10–20 s (10–20 heartbeats) from the common ventricle immediately before application of each respective drug ("baseline") and every 30–60 s during drug exposure until transient height either stabilized or began to decline. High extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) and caffeine were quickly (≤5 min) and completely reversible upon washout (replacement of DMEM containing stimulant with DMEM alone).

Ca^{2+} ratio transients were recorded in IonWizard v4.4 (IonOptix) at 1000 Hz. Background fluorescence measurements (405/485 nm) at 340 nm excitation were taken immediately prior to Indo-1 loading and subtracted from subsequent analysis. Ca^{2+} transients were smoothed by lowpass Butterworth filter, then averaged to create a mean transient, which was analyzed in IonWizard v5. As all examined drugs increase intracellular Ca^{2+} concentration, Ca^{2+} transient values were determined from interval with highest Ca^{2+} transient height (5–10 min) and are reported as % baseline.

2.5. Statistical analysis

Data are expressed as mean ± SEM. Student *t*-tests were performed and graphs made in Origin 6.0, with $p < .05$ required to reject a null hypothesis. Values of *n* indicate the number of independent animals or cells studied.