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Original article

# Sex differences in SR $Ca^{2+}$ release in murine ventricular myocytes are regulated by the cAMP/PKA pathway

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ABSTRACT

Previous studies have shown that ventricular myocytes from female rats have smaller contractions and $Ca^{2+}$ transients than males. As cardiac contraction is regulated by the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, we hypothesized that sex differences in cAMP contribute to differences in $Ca^{2+}$ handling. $Ca^{2+}$ transients (fura-2) and ionic currents were measured simultaneously (37 °C, 2 Hz) in ventricular myocytes from adult male and female C57BL/6 mice. Under basal conditions, diastolic $Ca^{2+}$, sarcoplasmic reticulum (SR) $Ca^{2+}$ stores, and L-type $Ca^{2+}$ current did not differ between the sexes. However, female myocytes had smaller $Ca^{2+}$ transients (26% smaller), $Ca^{2+}$ sparks (6% smaller), and excitation–contraction coupling gain in comparison to males (23% smaller). Interestingly, basal levels of intracellular cAMP were lower in female myocytes ($0.7 \pm 0.1$ vs. $1.7 \pm 0.2$ fmol/μg protein; $p < 0.001$). Importantly, PKA inhibition (2 μM H-89) eliminated male–female differences in $Ca^{2+}$ transients and gain, as well as $Ca^{2+}$ spark amplitude. Western blots showed that PKA inhibition also reduced the ratio of phospho:total RyR2 in male hearts, but not in female hearts. Stimulation of cAMP production with 10 μM forskolin abolished sex differences in cAMP levels, as well as differences in $Ca^{2+}$ transients, sparks, and gain. To determine if the breakdown of cAMP differed between the sexes, phosphodiesterase (PDE) mRNA levels were measured. PDE3 expression was similar in males and females, but PDE4B expression was higher in female ventricles. The inhibition of cAMP breakdown by PDE4 (10 μM rolipram) abolished differences in $Ca^{2+}$ transients and gain. These findings suggest that female myocytes have lower levels of basal cAMP due, in part, to higher expression of PDE4B. Lower cAMP levels in females may attenuate PKA phosphorylation of $Ca^{2+}$ handling proteins in females, and may limit positive inotropic responses to stimulation of the cAMP/PKA pathway in female hearts.



## 1. Introduction

Studies in humans have identified important differences in normal cardiac contractile function between the sexes. For example, at rest, women have a higher ejection fraction in comparison to men [1,2]. However, in response to exercise, men are able to increase their ejection fraction more than women [1,3]. These findings suggest that women are less able to augment contractile function in response to increasing demand than men. The majority of studies with animal models concur with these observations. Specifically, smaller contractions have been reported in both working heart models and in cardiac muscles from female animals in comparison to males, especially in conditions of high demand such as increased pacing frequencies [4–7].

Previous studies in the rat model have found that these results translate to isolated myocytes, in that cells from females exhibit smaller contractions and $Ca^{2+}$ transients in comparison to males, especially with faster, more physiological stimulation frequencies (e.g. 1 to 4 Hz) [8–10]. As sarcoplasmic reticulum (SR) $Ca^{2+}$ release is lower in females, but $Ca^{2+}$ current is similar in both sexes, excitation–contraction (EC) coupling gain is lower in myocytes from female rat hearts [8]. These observations suggest that differences in $Ca^{2+}$ regulation in individual cardiomyocytes contribute to sex differences in cardiac contractile function. However, whether these can be generalized to other species is

*Abbreviations:* cAMP, cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; EC, excitation–contraction; Fura-2 AM, fura-2 acetoxymethyl; FR, fractional release; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IV, current–voltage; PCR, polymerase chain reaction; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase type A; RyR2, ryanodine receptor type 2; SERCA2, sarcoplasmic reticulum calcium ATPase type 2; SR, sarcoplasmic reticulum.

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unclear, as few studies have investigated male–female differences in EC coupling at the cellular level in other animal models.

Interestingly, although information is limited, some studies suggest that there are also differences in responses to β-adrenergic receptor stimulation in male and female cardiomyocytes. Activation of β-adrenergic receptors is known to regulate EC coupling by increasing production of cyclic adenosine monophosphate (cAMP), thus activating protein kinase A (PKA) [11]. PKA phosphorylates various components of the EC coupling pathway to increase inotropy and lusitropy [11]. Few studies have examined sex differences in response to stimulation of the cAMP/PKA pathway, and these studies have found that the β-adrenergic agonist isoproterenol elicits smaller increases in $Ca^{2+}$ currents, $Ca^{2+}$ transients and contractions in myocytes from females in comparison to males [10,12]. This is accompanied by a smaller increase in isoproterenol-stimulated cAMP levels in female myocytes [12]. However, whether there are male–female differences in basal cAMP levels has not been investigated. If basal cAMP is lower in females, this would be expected to cause less PKA activation, and could explain lower SR $Ca^{2+}$ release and EC coupling gain in female myocytes in comparison to males. Levels of cAMP are critically regulated by phosphodiesterase (PDE) enzymes, which are responsible for hydrolysis and breakdown of cyclic nucleotides [13,14]. In hearts from male animals, PDE3 and PDE4 have been largely implicated in modulating $Ca^{2+}$ handling and EC coupling [15–17], though PDE1 and PDE2 have also been suggested to have minor contributions [18,19]. Whether expression of major PDE isoforms is similar in females has not yet been investigated.

The objectives of this study were: 1) to determine whether basal differences in cAMP levels contribute to sex differences in SR $Ca^{2+}$ release; and 2) to investigate the underlying cellular mechanisms responsible for male–female differences in SR $Ca^{2+}$ release. Experiments measured $Ca^{2+}$ currents, $Ca^{2+}$ transients, $Ca^{2+}$ sparks and intracellular cAMP levels in ventricular myocytes from male and female mice. The effects of pharmacologically activating or inhibiting the cAMP/PKA pathway on sex differences in $Ca^{2+}$ handling properties were examined, as was a role for PDE. Results indicate that the cAMP/PKA pathway plays a major role in attenuating SR $Ca^{2+}$ release in myocytes from females, and suggest that increased cAMP degradation by PDE4B may be responsible.

## 2. Materials and methods

An expanded Methods section is available on the online Data Supplement.

### 2.1. Isolation of ventricular myocytes

Experiments conformed to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals and were approved by the Dalhousie University Committee on Laboratory Animals. Adult C57BL/6 male and female mice (5–10 months) were obtained from Charles River Laboratories (St. Constant, QC). Ventricular myocytes were isolated by perfusion of enzymes through the aorta as previously described [20]. Quiescent rod-shaped myocytes with clear striations were used in experiments.

### 2.2. Myocyte $Ca^{2+}$ handling

Myocytes were incubated with fura-2 acetoxymethyl (AM) (5 μM; Invitrogen, Burlington, ON) for 20 min in darkness, and then superfused with buffer (pH 7.4; 37 °C).

Transient outward $K^+$ current was inhibited with 4-aminopyridine, while $Na^+$ current was inhibited with lidocaine and inactivated by a pre-pulse to −40 mV prior to test pulses. Discontinuous single electrode voltage clamp recordings (sample rate 5–6 kHz) were made with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA) and high resistance microelectrodes (18–28 MΩ) to avoid buffering internal $Ca^{2+}$ and to minimize intracellular dialysis. Clampex v8.2 software (Molecular Devices) was used to generate protocols. $Ca^{2+}$ transients were measured with a DeltaRam fluorescence system and Felix v1.4 software (Photon Technologies International (PTI), Birmingham, NJ). An in vitro calibration curve was used to calculate intracellular $Ca^{2+}$ concentrations, as previously described [20,21]. All voltage clamp protocols were preceded by five 50 ms conditioning pulses from −80 to 0 mV (2 Hz). $Ca^{2+}$ currents and transients were recorded simultaneously during 250 ms test pulses to varying potentials. A single 250 ms voltage clamp test step from −40 to 0 mV was used to activate $Ca^{2+}$ transients and $Ca^{2+}$ currents in experiments where myocytes were exposed to drugs. $Ca^{2+}$ current, measured as the difference between peak current and the end of the test pulse, was normalized to cell capacitance. $Ca^{2+}$ current decay (tau) was quantified by fitting traces with an exponential function and total $Ca^{2+}$ flux was measured as the integral of the $Ca^{2+}$ current. Steady-state activation of the L-type $Ca^{2+}$ current was obtained by calculating conductance as: $g = I_{Ca} / (V - E_{rev})$. Steady-state activation of each cell was fitted with the Boltzmann equation: $d = 1 / \{1 + \exp[-(V_C - V_h) / k]\}$. SR $Ca^{2+}$ content was measured by rapid application (1 s) of caffeine solution, which was nominally $Ca^{2+}$- and $Na^+$-free to inhibit extrusion of $Ca^{2+}$ by $Na^+$–$Ca^{2+}$ exchange.

$Ca^{2+}$ sparks were recorded in myocytes incubated with fluo-4 AM (20 μM) as previously described [22]. Cells were placed in a chamber on a laser scanning confocal microscope (Zeiss LSM 510-Meta, Carl Zeiss Canada, Toronto, ON) and superfused with buffer (37 °C). Solvent alone (0.02 and 0.04% DMSO) had no effect on $Ca^{2+}$ currents, $Ca^{2+}$ transients, or $Ca^{2+}$ sparks in males or females.

### 2.3. Enzyme immunoassay, immunoblotting, and quantitative PCR

Intracellular cAMP levels were determined in isolated ventricular myocytes treated with control, DMSO solvent control (0.1%), or forskolin (10 μM), as previously reported [22]. Total RyR2 and phospho RyR2-S2808 protein levels were determined by immunoblotting, as previously described [23]. Polyacrylamide gels (6%) were loaded with equal amounts of total sample protein (10 μg). Primary antibodies used were ryanodine receptor (RyR; AbCam, Cambridge, UK; 1:5000) and RyR2 phospho Serine-2808 (RyR2-S2808; Badrilla, Leeds, UK; 1:2500). Quantitative mRNA expression of PDE isoforms was measured in ventricles, as described previously [24], using intron spanning primers for PDE3A, PDE3B, PDE4A, PDE4B and PDE4D isoforms (Supplemental Table 1). GAPDH was used as a reference gene.

### 2.4. Statistical analyses

Sigmaplot (v11.0, Systat Software Inc.) was used for all statistical analyses and figures. Differences between means ± S.E.M. were significant for $P < 0.05$.

## 3. Results

### 3.1. $Ca^{2+}$ transients are smaller and EC coupling gain is lower in female myocytes in comparison to males

Experiments were designed to examine sex differences in $Ca^{2+}$ handling in myocytes from male and female C57BL/6 mice. Ventricular myocytes were voltage clamped and basal $Ca^{2+}$ handling properties were measured during a single 250 ms test step to 0 mV (Fig. 1A, top panel). Cell capacitance, a measure of membrane area, was similar in male and female myocytes (235.4 ± 12.2 and 215.3 ± 8.9 pF, $P = 0.377$). Fig. 1A depicts representative $Ca^{2+}$ transients (left panels) and L-type $Ca^{2+}$ currents (right panels) recorded simultaneously in myocytes from a male and a female mouse. Mean data revealed that $Ca^{2+}$ transient amplitude was significantly smaller in myocytes from females in comparison to males (Fig. 1B). This was