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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 ± 0.1 vs. 1.7 ± 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

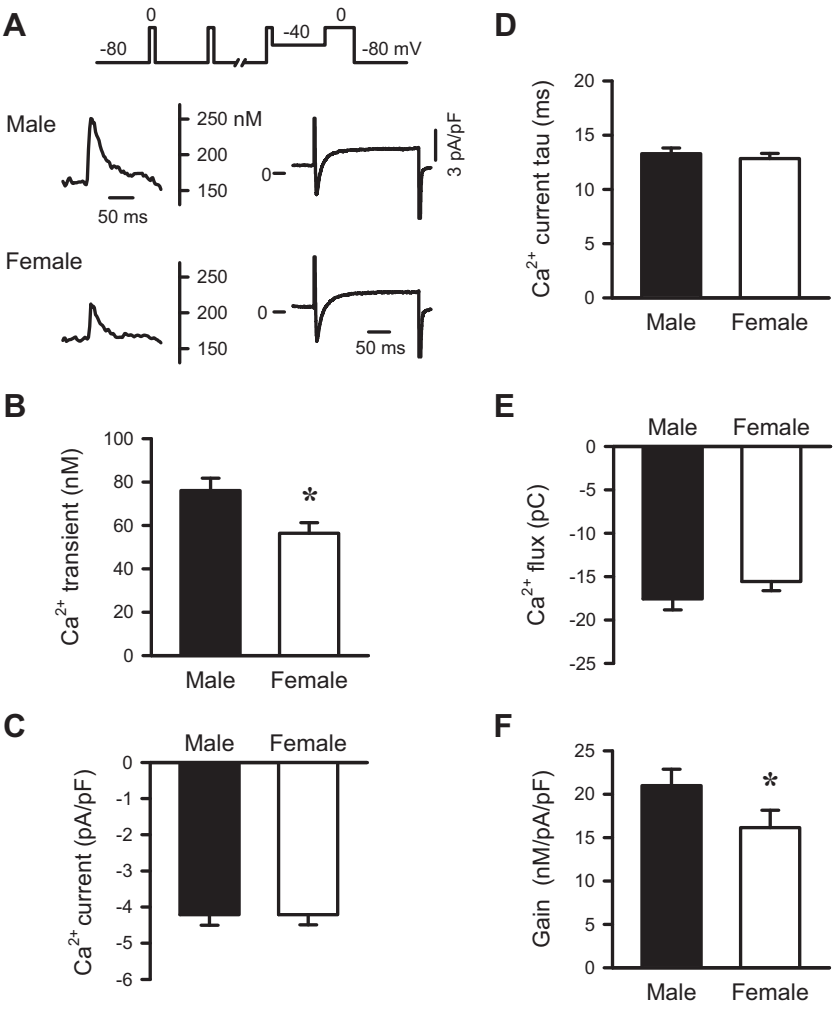

Fig. 1. Ca^{2+} transients and the EC coupling gain are smaller in myocytes from females in comparison to males, despite similar L-type Ca^{2+} current. A. The top panel shows the voltage clamp protocol, which consisted of conditioning pulses prior to a test step from −40 to 0 mV. Ca^{2+} transient (left panel) and Ca^{2+} current (right panel) recordings from male and female myocytes. B. Ca^{2+} transients were significantly smaller in myocytes from females. C. Peak Ca^{2+} current did not differ between males and females. D. Ca^{2+} current decay, tau, was similar between the sexes. E. Total Ca^{2+} flux was similar between the sexes. F. EC coupling gain was lower in females than males. (n = 43 male, 42 female cells; 23 male, 22 female animals; * denotes $P < 0.05$).

not due to a difference in peak Ca^{2+} current, which was similar in both sexes (Fig. 1C). Nor was this a result of differences in the decay or total flux of the current, as the time constant of inactivation (tau; Fig. 1D) and the total Ca^{2+} flux (Fig. 1E) were also similar between males and females. Typically, the size of the Ca^{2+} transient is proportional to the amount of Ca^{2+} entering the cell upon depolarization [11]. To quantify the amplification of Ca^{2+} signalling and compare between the sexes, EC coupling gain was calculated as a ratio of SR Ca^{2+} release to peak Ca^{2+} current. Results showed that gain was lower in females in comparison to males (Fig. 1F). These findings demonstrate that, although males and females exhibited similar Ca^{2+} currents, females had smaller Ca^{2+} transients, and thus lower gain.

Ca^{2+} transients and Ca^{2+} currents illustrated in Fig. 1 were recorded during a single voltage clamp step to 0 mV. Experiments were designed to determine if similar sex differences were seen over a range of membrane voltages. Fig. 2A shows Ca^{2+} transients measured during test steps to voltages between −40 and +80 mV. Peak Ca^{2+} transients were smaller in female myocytes than males (Fig. 2A), as determined by two-way repeated measures ANOVA. Underlying Ca^{2+} currents simultaneously measured in these myocytes did not differ between the sexes (Fig. 2B). To determine whether there were male–female differences in the voltage-dependence of activation of the Ca^{2+} current, steady-state activation curves were constructed. Fig. 2C shows that the steady-state activation was similar in males and females, as was the voltage of half-maximal activation (inset). The slope factor for steady-state activation was also similar in the two groups (4.8 ± 0.2 vs. 5.0 ± 0.2; $P = 0.408$). These results suggest that male and female myocytes have similar Ca^{2+} influx upon depolarization, but females exhibit smaller Ca^{2+} transients at physiologically relevant membrane voltages. EC coupling gain was also lower in females in comparison to males, and this difference was significant over a physiologically relevant range of membrane voltages (Fig. 2D). Thus, sex differences in Ca^{2+} transients and gain are present across a wide range of physiologically relevant voltages.

3.2. SR Ca^{2+} sparks are smaller in females, though SR Ca^{2+} content does not differ

To determine whether sex differences in Ca^{2+} transients were attributable to smaller SR Ca^{2+} release units, the properties of Ca^{2+} sparks were compared in quiescent myocytes from males and females. Fig. 3A shows representative Ca^{2+} sparks from male and female myocytes. As shown in Fig. 3B, spark frequency did not differ between the sexes. However, Ca^{2+} sparks in females were significantly smaller in amplitude in comparison to males (Fig. 3C). The width and duration of

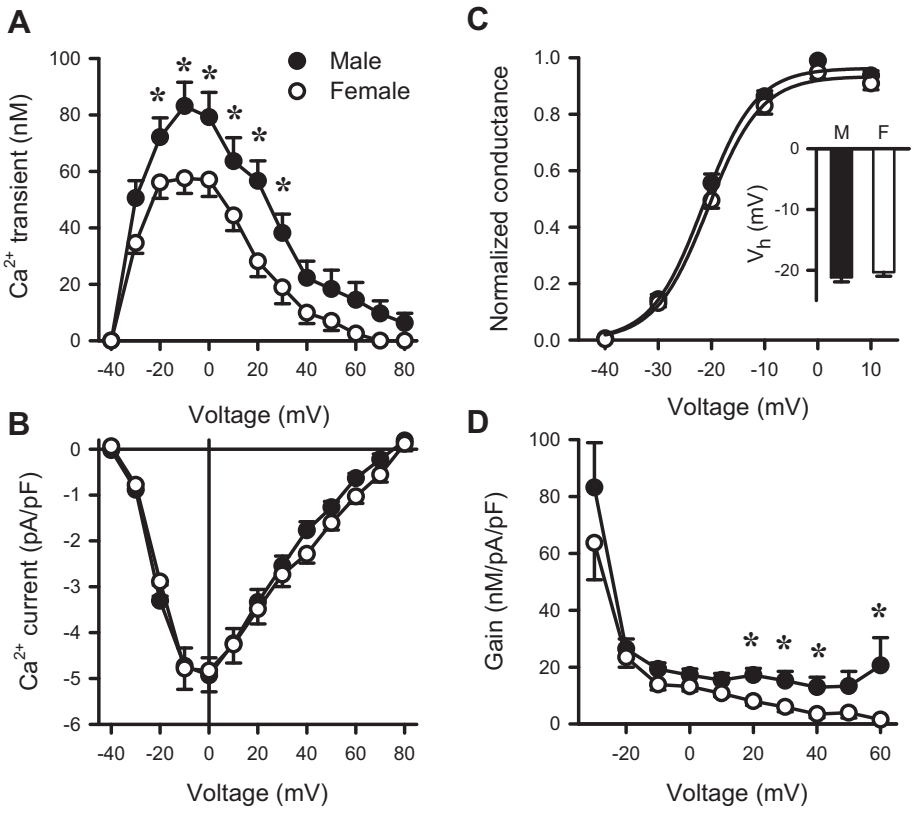

Fig. 2. Ca^{2+} current–voltage relationships and steady-state activation are similar between the sexes. A. Ca^{2+} transients were smaller in female myocytes in comparison to males as determined by two-way repeated measures ANOVA; voltages identified by a Holm-Sidak post-hoc analysis are indicated. B. Ca^{2+} current–voltage relationships were similar in myocytes from males and females. C. Steady-state activation curves were similar between the sexes. The half-maximal voltage of activation, V_h, did not differ between the sexes (inset). D. Overall, the EC coupling gain was lower in females than in males; specific voltages are indicated. (n = 22 male, 18 female cells; 13 male, 11 female animals; * denotes $P < 0.05$).

individual sparks did not differ, as the full width and full duration at half-maximum were similar between males and females (Fig. 3D and E). The time-to-peak of Ca^{2+} sparks was also similar between the sexes (Fig. 3F), though the tau of decay was faster in females than males (Fig. 3G). These results demonstrate that SR Ca^{2+} release units are smaller and decay faster in cells from females.

To elucidate whether smaller Ca^{2+} transients and sparks in female myocytes could be explained by lower SR Ca^{2+} stores, SR Ca^{2+} content was examined with rapid application of caffeine (Fig. 4A). Caffeine transient recordings from male and female myocytes are shown in Fig. 4A. Mean data revealed no sex difference in SR Ca^{2+} content, as shown in Fig. 4B. To quantify the amount of SR Ca^{2+} released as a percentage of the Ca^{2+} available in the SR, fractional release (Ca^{2+} transient/caffeine transient) was compared in the two groups. Fig. 4C shows that fractional release was lower in myocytes from females in comparison to males. Resting Ca^{2+} could also affect the gain of EC coupling, though diastolic Ca^{2+} levels did not differ between the sexes (Fig. 4D). These results indicate that smaller Ca^{2+} sparks and transients in females are not due to sex differences in SR Ca^{2+} content or diastolic Ca^{2+} concentration.

3.3. Intracellular cAMP levels are lower in females, and inhibition of PKA attenuates sex differences in SR Ca^{2+} release

Experiments were then designed to investigate contributions of the cAMP/PKA pathway in modulating SR Ca^{2+} release in males and females. Intracellular cAMP levels were measured in ventricular myocytes, and Fig. 5A shows that, under basal conditions, female cells had significantly lower levels of cAMP in comparison to males. Lower intracellular cAMP would cause less PKA activation and result in lower levels of phosphorylation of EC coupling targets in females than in males. To examine whether this contributed to sex differences in SR Ca^{2+} handling, experiments were performed with the selective PKA inhibitor H-89 (2 μM) [25], and results are presented as % of male control to facilitate comparisons between groups. Fig. 5B depicts representative Ca^{2+} transients and Ca^{2+} currents obtained in H-89 during a single 250 ms voltage clamp step from −40 to 0 mV. The inhibition of PKA reduced Ca^{2+} transients in both sexes, and importantly, eliminated the sex difference that was seen under basal conditions (Fig. 5C, $P = 0.646$). H-89 reduced Ca^{2+} current to a similar extent in myocytes from both sexes (Fig. 5C). Importantly, the male–female difference in basal gain was abolished (Fig. 5C, $P = 0.100$). These findings suggest that the cAMP/PKA pathway plays a key role in mediating sex differences in basal gain, as cAMP levels were lower in females and inhibition of PKA attenuated differences in Ca^{2+} transient amplitude and gain between males and females.

To determine whether H-89 would eliminate sex differences in subcellular Ca^{2+} release, individual SR Ca^{2+} sparks were measured in the absence and presence of 2 μM H-89. Fig. 5B shows examples of sparks with H-89 in both sexes. The inhibition of PKA eliminated the male–female difference in basal Ca^{2+} spark amplitude by reducing spark size to a similar extent in both sexes (Fig. 5D). In addition, H-89 attenuated the difference in Ca^{2+} spark decay between the sexes (Fig. 5D). The frequency of Ca^{2+} sparks was also dramatically reduced by H-89, but remained similar in males and females (Fig. 5D). These results demonstrate that male–female differences in individual spark amplitude and decay are no longer present when PKA is inhibited.

SR Ca^{2+} content is an important determinant of the amount of Ca^{2+} released via ryanodine receptors (RyRs), and higher SR stores increases the frequency of Ca^{2+} sparks [26]. As such, we sought to determine whether inhibition of PKA affected SR Ca^{2+} release by altering SR Ca^{2+} content. However, SR content was unaffected by H-89, as shown by mean caffeine transient amplitude (Fig. 5E). Interestingly, H-89 did

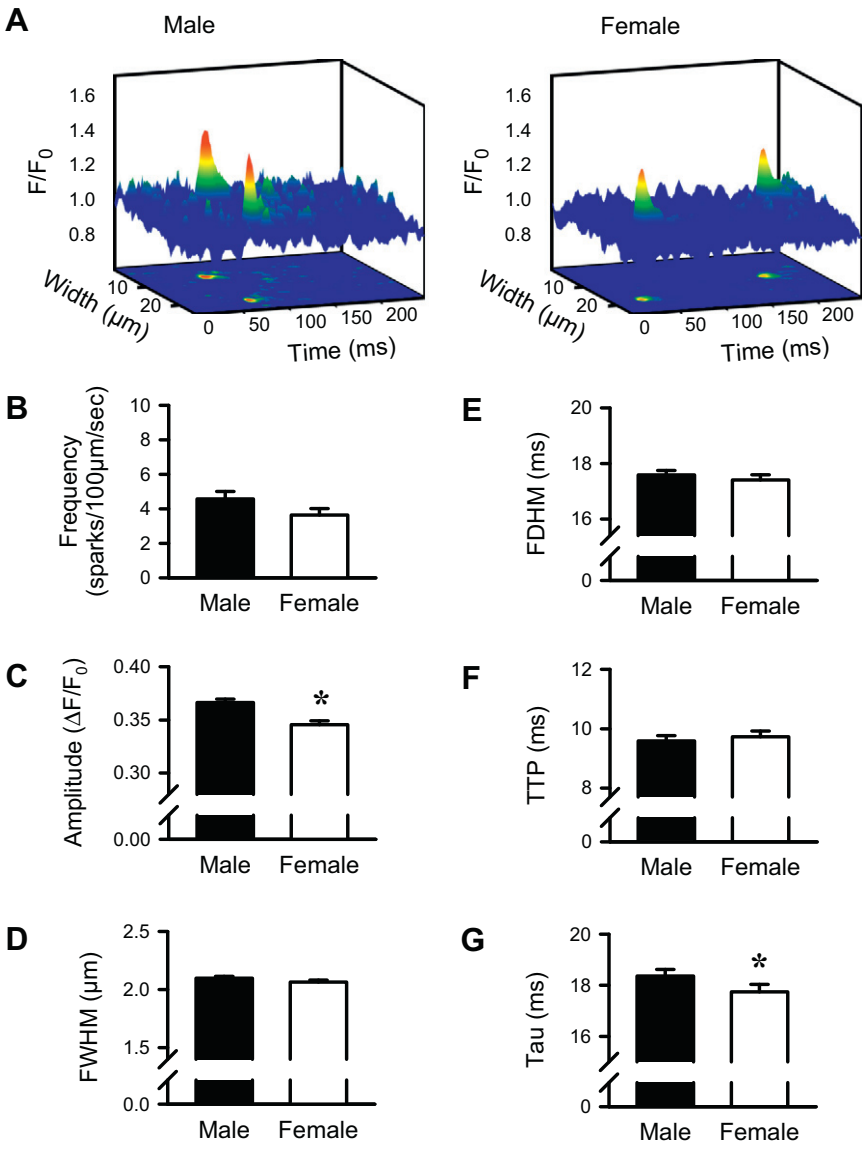

Fig. 3. Spontaneous SR Ca^{2+} sparks are smaller in myocytes from females in comparison to males, though spark frequency does not differ. A. Three dimensional representative recordings of Ca^{2+} sparks in quiescent myocytes from a male and a female. B. Spark frequency did not differ between the sexes. C. Mean spark amplitude was smaller in females than in males. D. Full width at half-maximum (FWHM) amplitude was similar for sparks in males and females. E. Spark duration, measured as full duration at half-maximum (FDHM) amplitude, did not differ between the sexes. F. Average time-to-peak (TTP) of Ca^{2+} sparks was similar in males and females. G. The decay of Ca^{2+} sparks, tau, was faster in females than males. (For spark frequency, n = 220 male, 217 female cells; for other parameters, n = 2623 male, 2147 female sparks; 12 male, 14 female animals; * denotes $P < 0.05$).

reduce fractional release in male, but not female myocytes and, in fact, abrogated the basal sex difference (Fig. 5E). Furthermore, while H-89 had no effect on diastolic Ca^{2+} levels in males, it reduced diastolic Ca^{2+} in female cells (Fig. 5E). These findings show that pharmacological inhibition of the cAMP/PKA pathway, which blocks sex differences in intracellular cAMP, eliminated differences in SR Ca^{2+} release, fractional Ca^{2+} release and EC coupling gain between males and females.

It is possible that inhibition of PKA decreases the amplitude of Ca^{2+} sparks by decreasing the phosphorylation of RyR2 by PKA. To determine if PKA inhibition attenuated male–female differences in Ca^{2+} transients and SR Ca^{2+} sparks by altering phosphorylation of RyR2, immunoblotting for phospho RyR2-S2808 was performed. Total RyR2 (Fig. 6A) and phospho RyR2-S2808 (Fig. 6B) did not differ between males and females under basal conditions, and H-89 did not significantly affect levels of either protein in males or females. As shown in Fig. 6C, the ratio of phospho RyR2-S2808 to total RyR2 was also similar between males and females under basal conditions. However, the inhibition of PKA with H-89 resulted in a significant reduction in the ratio of RyR2-S2808 to total in males, but had no effect in females.

3.4. Stimulation of adenylyl cyclase removes sex differences in EC coupling gain

To determine if responses to stimulation of cAMP production differed between the sexes, myocytes were exposed to a maximal concentration of the adenylyl cyclase activator, forskolin (10 μM) [22,27,28]. Forskolin increased intracellular cAMP to a similar level in both sexes (Fig. 7A). Experiments were then performed to determine if forskolin would also attenuate sex differences in EC coupling. Fig. 7B depicts representative Ca^{2+} transients and Ca^{2+} currents measured in the presence of forskolin during a 250 ms voltage clamp step from −40 to 0 mV. Ca^{2+} transient amplitude was increased by forskolin in both sexes, and was no longer smaller in females (Fig. 7C, $P = 0.229$). Ca^{2+} current was also increased by forskolin and remained similar between the sexes (Fig. 7C). As forskolin

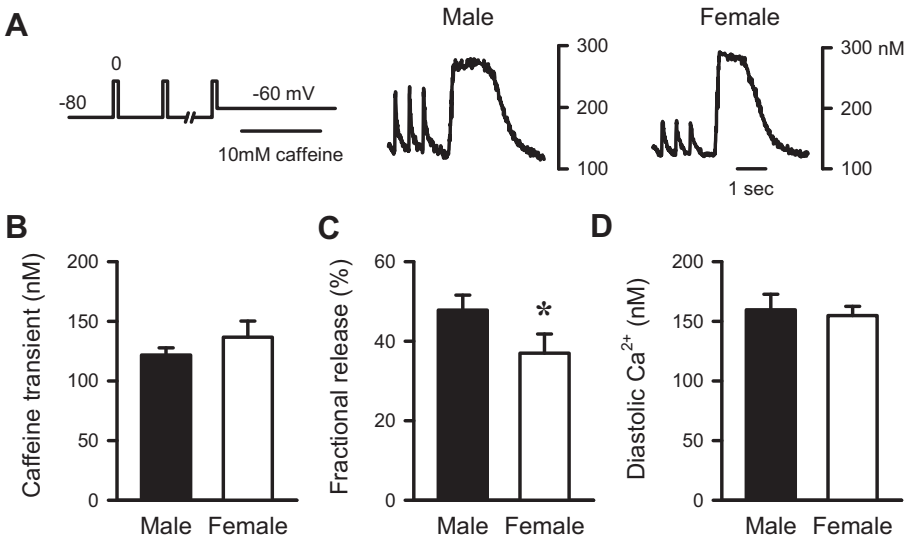

Fig. 4. SR Ca^{2+} stores and diastolic Ca^{2+} are similar between the sexes, but fractional release is lower in females. A. To measure SR Ca^{2+} content, the voltage clamp protocol consisted of a test step to −60 mV, during which 10 mM caffeine was applied to the cell for 1 s and caffeine transients were recorded, such as those shown from male and female myocytes. B. Mean caffeine transients did not differ between the sexes. C. Fractional release was lower in cells from females than males. D. Diastolic Ca^{2+} levels did not differ between the sexes. (n = 17 male, 13 female cells; 9 male, 8 female mice; * denotes P < 0.05).

increased both Ca^{2+} currents and Ca^{2+} transients, it had no significant effect on EC coupling gain, although it abolished the male–female difference that was present under basal conditions (Fig. 7C, P = 0.389).

To elucidate whether forskolin affected subcellular Ca^{2+} release units, Ca^{2+} sparks were compared in male and female myocytes in the absence and presence of 10 μM forskolin. Examples of sparks measured in forskolin are shown in Fig. 7B. Forskolin increased the amplitude of Ca^{2+} sparks in both groups and reversed the sex difference observed under basal conditions (Fig. 7D). Ca^{2+} spark decay was not affected by forskolin, but the sex difference observed under basal conditions was no longer present (Fig. 7D, P = 0.093). The frequency of Ca^{2+} sparks was unaffected by activation of adenylyl cyclase in males or females (Fig. 7D). These results suggest that, when intracellular cAMP is increased to similar levels in males and females, differences in Ca^{2+} transients, EC coupling gain, and individual SR Ca^{2+} sparks are eliminated.

3.5. PDE4B expression is increased in females, and PDE4 inhibition abolishes sex differences in EC coupling gain

To examine the mechanisms underlying sex differences in intracellular cAMP levels, experiments were performed to determine if degradation of cAMP differed between the sexes. Quantitative PCR was performed to measure mRNA levels of PDE, the enzyme responsible for breaking down cAMP. Specifically, PDE3 and PDE4 families were examined, as these are the major isoforms expressed in the ventricles [16,29]. Fig. 8A shows that ventricles from male and female mice had a similar pattern of expression of PDE3A and PDE3B, as well as PDE4A and PDE4D. However, PDE4B expression was significantly higher in females in comparison to males (Fig. 8A). This could increase cAMP degradation in the female heart and lead to lower levels of cAMP in comparison to males.

To determine whether an increase in the expression of PDE4B in females could account for basal male–female differences in EC coupling, experiments were performed in the presence of the selective PDE4 inhibitor rolipram (10 μM). Voltage clamp experiments with a test step from −40 to 0 mV revealed that inhibition of PDE4 increased the amplitude of Ca^{2+} transients in female myocytes, but not in males, thus eliminating the basal sex difference (Fig. 8B, P = 0.403). While rolipram had no effect on L-type Ca^{2+} current in myocytes from either sex, it increased EC coupling gain in females (P = 0.05) and eliminated the male–female difference in gain under basal conditions (Fig. 8B, P = 0.540). These results demonstrate that females have increased expression of PDE4B, which may reduce intracellular levels of cAMP and contribute to smaller Ca^{2+} transients and reduced EC coupling gain in female myocytes.

Experiments were then performed to determine whether PDE4 inhibition would abolish sex differences in individual SR Ca^{2+} sparks. In the presence of 10 μM rolipram, Ca^{2+} spark amplitude was increased in both sexes, although sparks remained smaller in female myocytes in comparison to males (Fig. 8C). Rolipram reduced Ca^{2+} spark decay in males and increased decay rates in females, so the sex difference present under basal conditions was reversed and sparks were prolonged in females (Fig. 8C). Spark frequency was unaffected by rolipram in either males or females (Fig. 8C). These results show that inhibition of PDE4 increases the amplitude and duration of Ca^{2+} sparks in females, which may contribute to the increase in Ca^{2+} transient amplitude and gain caused by rolipram.

4. Discussion

The goal of this study was to examine sex differences in myocardial Ca^{2+} handling in a murine model and discern a role for the cAMP/PKA pathway in mediating differences in SR Ca^{2+} release between males and females. Results indicate that myocytes from female mice had smaller Ca^{2+} transients, as well as smaller subcellular SR Ca^{2+} sparks in comparison to males. The reduction in SR Ca^{2+} release in cells from females occurred despite similar Ca^{2+} current, SR Ca^{2+} content and diastolic Ca^{2+} levels between the sexes. As such, females had lower EC coupling gain than males. We also found that basal cAMP levels were lower in females, which corresponded to an increase in the expression of PDE4B in comparison to males. Interestingly, both adenylyl cyclase activation and PDE4 inhibition eliminated differences in Ca^{2+} transient amplitude and EC coupling gain between the sexes. Importantly, inhibition of PKA decreased the ratio of phosphorylated to total RyR2 in males and had no effect in females. PKA inhibition also abolished male–female differences in Ca^{2+} transients, EC coupling gain, Ca^{2+} sparks, and fractional SR Ca^{2+} release. Overall, these observations suggest that the lower SR Ca^{2+} release and EC coupling gain characteristic of female cardiomyocytes is due to increased cAMP hydrolysis in females, which would likely result in less phosphorylation of SR targets, particularly RyR2, by PKA and thus alter SR Ca^{2+} handling.

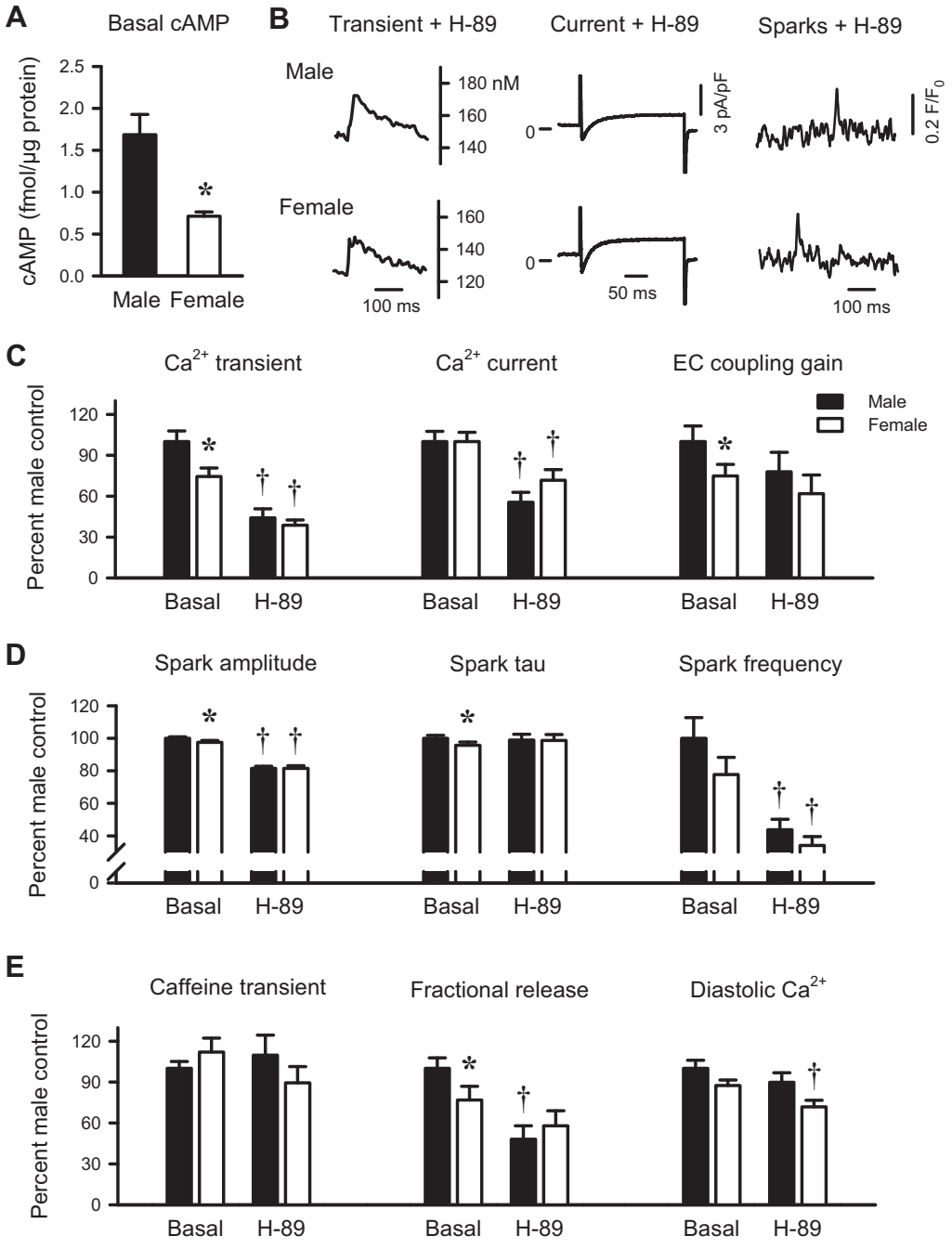

Fig. 5. Intracellular cAMP levels are lower in myocytes from females than males, and PKA inhibition with H-89 eliminates differences in SR Ca^{2+} release in male and female myocytes. A. Basal cAMP levels were lower in females. (n = 3 male, 3 female hearts in triplicate). B. Representative Ca^{2+} transients, Ca^{2+} current and sparks from male (top) and female myocytes (bottom) in the presence of 2 μM H-89. C. Ca^{2+} transients were decreased by H-89, and the basal sex difference was no longer present. Ca^{2+} current was also decreased and remained similar in myocytes from males and females. H-89 did not significantly affect EC coupling gain in cells from males or females, but abolished the basal sex difference. (n = 38 male, 46 female control cells; 14 male, 19 female H-89 cells; 14 male, 18 female animals). D. PKA inhibition decreased Ca^{2+} spark amplitude, and removed the basal difference between males and females. H-89 removed the male–female difference in spark decay, tau. Spark frequency was decreased by H-89 and remained similar between males and females. (For control, n = 137 male, 135 female cells; 1717 male, 1345 female sparks; 7 male, 7 female animals. For H-89, n = 101 male, 100 female cells; n = 517 male, 401 female sparks; 5 male, 5 female animals). E. Inhibition of PKA had no effect on caffeine transients in either sex. Fractional release was decreased in males by H-89, and the basal male–female difference was eliminated. H-89 decreased diastolic Ca^{2+} levels in female myocytes, but levels remained similar between the sexes. (n = 22 male, 14 control female cells; 9 male, 9 female H-89 cells; * denotes $P < 0.05$ compared to male, † denotes $P < 0.05$ compared to same-sex control).

Our previous study in rats showed that ventricular myocytes from females have smaller Ca^{2+} transients and Ca^{2+} sparks in comparison to males, as well as lower EC coupling gain [8]. However, whether similar sex differences are seen in cardiomyocytes from mice is controversial [30,31]. This study addressed the issue of whether similar sex differences are observed in a murine model, as these findings could enable future work with genetically-modified models. A major observation made in the present study is that basal Ca^{2+} transients are also smaller in ventricular myocytes from female C57BL/6 mice in comparison to males, while simultaneously measured L-type Ca^{2+} currents did not differ, which resulted in lower gain in females. Previous studies that have measured Ca^{2+} transients or Ca^{2+} currents independently have reported similar

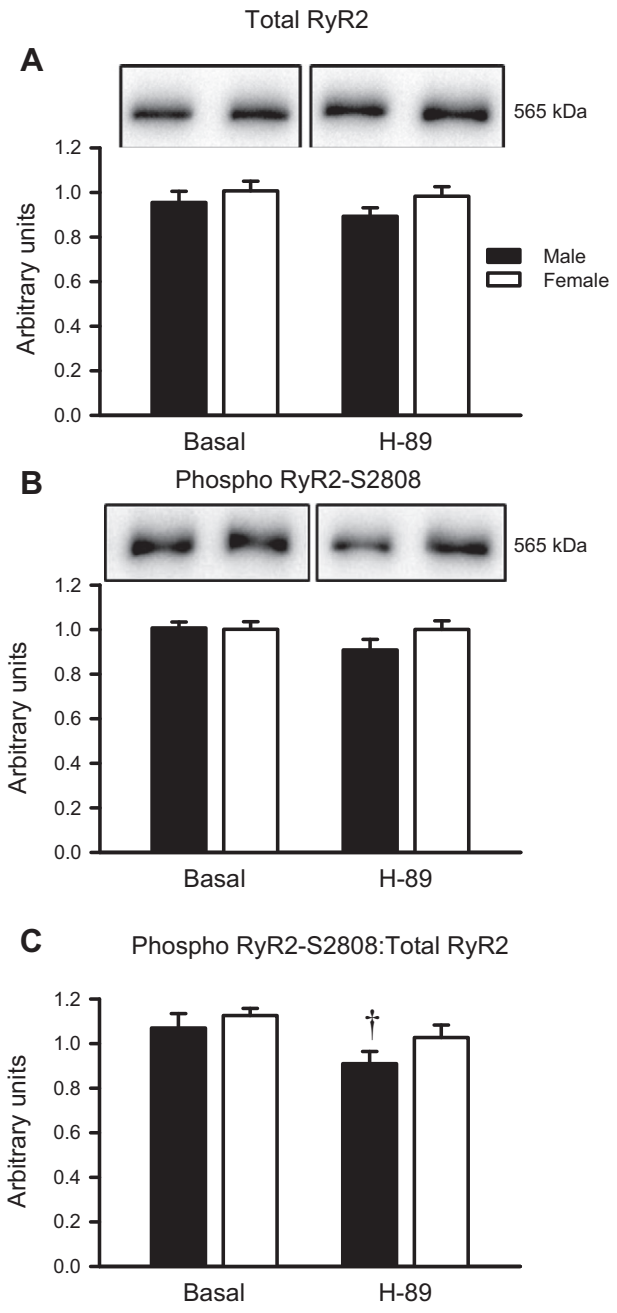

Fig. 6. Inhibition of PKA decreased the ratio of phospho RyR2-S2808 to total RyR2 in males but not in females. A. Total RyR2 protein did not differ between males and females, and H-89 did not alter RyR2 levels in either sex. B. Phospho RyR2-S2808 protein levels were similar in males and females under control conditions, and H-89 had no significant effect in males or females. C. The ratio of RyR2-S2808 to total RyR2 is similar in males and females under basal conditions. Inhibition of PKA with H-89 reduced the ratio in males, but had no effect in females. n = 6 male, 6 female hearts (†denotes $P < 0.05$ compared to same-sex control).

findings in myocytes from rats [5,8,10,32,33]. Together, these results show that male–female differences in Ca^{2+} current are unlikely to be responsible for less SR Ca^{2+} release in females in comparison to males.

Our results indicate that male–female differences in SR Ca^{2+} release are not due to differing SR Ca^{2+} content or cytosolic Ca^{2+} concentration, as both parameters were similar between the sexes. These findings are in agreement with a number of studies in rats and other rodents that have reported similar diastolic and SR Ca^{2+} between males and females [8,10,30,34]. The present study also found that Ca^{2+} sparks were smaller in amplitude and decayed more quickly in myocytes from female mice in comparison to males, as observed previously in rats [8]. These smaller and faster subcellular Ca^{2+} release units in myocytes from females may sum to form smaller Ca^{2+} transients than in males. As these measurements were obtained from quiescent myocytes and are independent of Ca^{2+} current activation, the amplitude of Ca^{2+} sparks is indicative of the intrinsic gating of ryanodine receptors [35]. Our results, taken together with the report by Farrell et al. [8], suggest that smaller Ca^{2+} sparks and lower EC coupling gain are fundamental properties of female cardiomyocytes.

A key finding in our study is that, under basal conditions, intracellular cAMP levels are smaller in myocytes from females in comparison to males. Lower cAMP levels in female myocytes would be expected to cause less activation of PKA, which would result in less phosphorylation of EC coupling components in comparison to males. The present study examined the functional consequences of PKA inhibition on Ca^{2+} handling and made the novel observation that H-89 abolished sex differences in both subcellular Ca^{2+} sparks and Ca^{2+} transients, resulting in similar EC coupling gain between males and females. This was not due to an effect of PKA inhibition on SR Ca^{2+} stores, and therefore, fractional release was similar between the sexes with H-89. Furthermore, even though Ca^{2+} channels are phosphorylated under the basal state, sex differences in Ca^{2+} current are not involved as inhibition of PKA reduced current in males and females to a similar extent. Our group has previously identified a role for PKA in maintaining SR Ca^{2+} release in female myocytes in the absence of β-adrenergic stimulation [22]. The present study suggests that a similar or even larger role exists for PKA in regulating basal SR Ca^{2+} release in male myocytes. Together, these results suggest that lower cAMP levels in females cause less basal activation of PKA, which in turn attenuates Ca^{2+} sparks, and thus Ca^{2+} transient amplitude and EC coupling gain.

Our results show that basal RyR2 protein levels do not differ in ventricles from male and female mice. Interestingly, we also found that there was no sex difference in RyR2 phosphorylation at S2808 under basal conditions, which was unexpected given the difference in SR Ca^{2+} release. The basis for this is unclear, but could be related to male–female differences in PKA-mediated phosphorylation at another site, such as S2030 [36]. Nonetheless, our data clearly show that inhibition of PKA caused a marked reduction in the ratio of phospho RyR2-S2808 to total RyR2 in males, but had no effect in females. This male-selective effect of PKA inhibition on RyR2 phosphorylation could explain why H-89 abolishes sex differences in Ca^{2+} transients, Ca^{2+} sparks and the gain of SR Ca^{2+} release. It is possible that there are higher local levels of cAMP around RyR2 in males than in females and that this compartmentalization of cAMP contributes to sex differences in SR Ca^{2+} release.

Sex differences in response to stimulation of the cAMP/PKA pathway were examined with a maximal concentration of forskolin, which resulted in similar intracellular cAMP between male and female cardiomyocytes. Previous studies with β-adrenergic receptor agonists have found no male–female difference in cAMP levels [12,37], though males may have a minor increase in adenylyl cyclase activity [38]. Importantly, the present study found that, although forskolin increased Ca^{2+} currents in both males and females, it abolished differences in Ca^{2+} transients, Ca^{2+} sparks, and EC coupling gain between males and females. Nichols et al. [39] have shown that forskolin (0.1 μM) increases RyR2 phosphorylation at S2808. Though controversial, previous work has suggested that PKA phosphorylation of RyR2 increases open probability and thus SR Ca^{2+} release [40–43]. Our results suggest that exposing male and female myocytes to similar intracellular cAMP attenuates sex differences in SR Ca^{2+} release, which could be due to similar levels of PKA-mediated phosphorylation of RyR2.

There is some evidence that the levels of $Ca_V1.2$ protein, which is a subunit of the L-type Ca^{2+} channel, are higher in females in comparison to males in rat and rabbit models [44,45]. However, as reviewed by Parks and Howlett [46], there is a general consensus in the literature that Ca^{2+} current does not differ between male and female rodents. The present study shows that Ca^{2+} current remains similar between

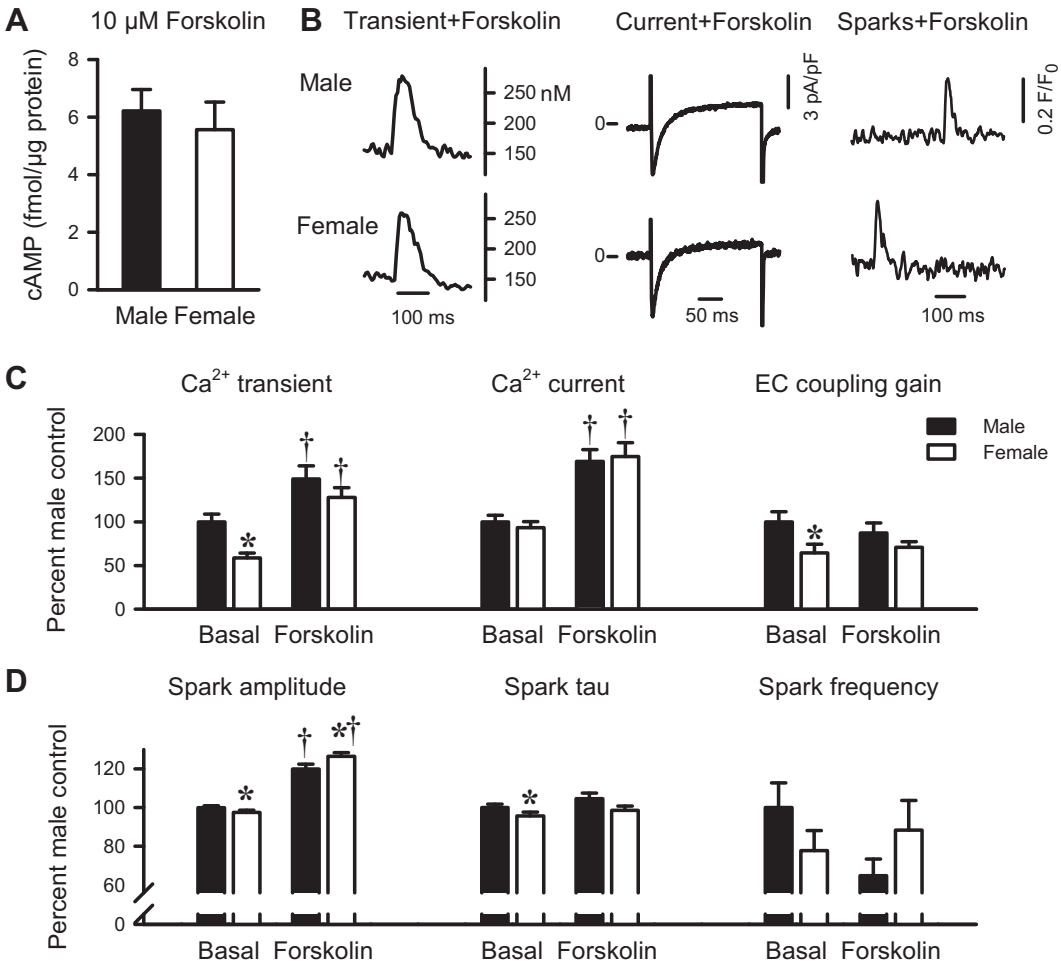

Fig. 7. Adenylyl cyclase activation attenuates male–female differences in cAMP levels, Ca^{2+} transients and sparks. A. In the presence of forskolin, intracellular cAMP did not differ between the sexes. (n = 3 male, 3 female hearts in triplicate). B. Representative Ca^{2+} transients, Ca^{2+} current and sparks from male (top) and female myocytes (bottom) in the presence of 10 μM forskolin. C. Forskolin increased Ca^{2+} transient amplitude in both males and females, and removed the basal sex difference. Ca^{2+} current was also increased by forskolin in males and females, and remained similar between the sexes. Forskolin eliminated sex differences in EC coupling gain. (n = 21 male, 24 female controls cells; 15 male, 18 female forskolin cells; 10 male, 11 female animals). D. Forskolin increased Ca^{2+} spark amplitude in both sexes, and reversed the male–female difference seen under control conditions. Forskolin did not alter Ca^{2+} spark decay, but removed the difference observed under basal conditions. Spark frequency was unaffected by forskolin in either sex. (For control, n = 137 male, 135 female cells; 1717 male, 1345 female sparks; 7 male, 7 female animals. For forskolin, n = 97 male, 99 female cells; n = 836 male, 1096 female sparks; 5 male, 5 female animals; * denotes $P < 0.05$ compared to male, † denotes $P < 0.05$ compared to same-sex control).

the sexes upon inhibition of PKA with H-89 or activation of adenylyl cyclase with forskolin. These findings suggest that L-type Ca^{2+} channels are exposed to similar cAMP levels under basal conditions and upon activation or inhibition of the cAMP/PKA pathway. It is possible that compartmentalization of functional cAMP pools around L-type Ca^{2+} channels is responsible for comparable Ca^{2+} currents between the sexes. Male–female differences may exist in other post-translational modifications of L-type Ca^{2+} channels, which could negate the increase in Ca^{2+} channel expression that has been shown in female rodent models.

Previous studies using only male rodents have implicated specific PDE isoforms in controlling functional compartments of cAMP within cardiomyocytes [47]. Importantly, ours is the first study to examine the expression pattern of PDE isoforms in ventricles from female rodents, and to compare with levels in males. PDE3 and PDE4 were examined, as these are the two main families implicated in the regulation of cardiac contractile function [16,29,47,48]. We made the novel observation that PDE4B mRNA expression was increased in female ventricles in comparison to males. Otherwise, the pattern of expression in females and males was similar to previous results obtained in isolated myocytes or whole ventricles of mice and rats [15,27]. Specifically, studies have suggested that PDE4B is complexed with L-type Ca^{2+} channels [49], while PDE4D and PDE3A have been shown to localize to RyR2 and SERCA2a [50–53]. It is possible that compartmentalization differs in females in comparison to males, and that an increase in the expression of PDE4B in females could increase breakdown of a specific functional pool of cAMP, perhaps around RyR2 as suggested by the results of the present study. Future studies to investigate PDE4B localization in female myocytes are warranted, as this could be involved in lower SR Ca^{2+} release in females.

The present study examined the effect of inhibiting all PDE4 isoforms, and determined that rolipram abolished differences in Ca^{2+} transients and EC coupling gain between myocytes from males and females. Many previous studies have reported no inotropic effect of rolipram on cardiomyocytes [24,50,54], however, all of these studies have examined myocytes from male animals only. Our results indicate that rolipram increases Ca^{2+} transient amplitude selectively in female myocytes to a level similar to males, thus resulting in comparable EC coupling gain between the sexes. Rolipram also increased the amplitude of Ca^{2+} sparks in both male and female cells, although sparks remained smaller in females. Interestingly, inhibition of PDE4 reduced the rate of decay of individual sparks in males, while prolonging decay in females. Therefore, it is possible that although sparks remain smaller in females, PDE4 inhibition prolongs spark duration in comparison to males, which would result

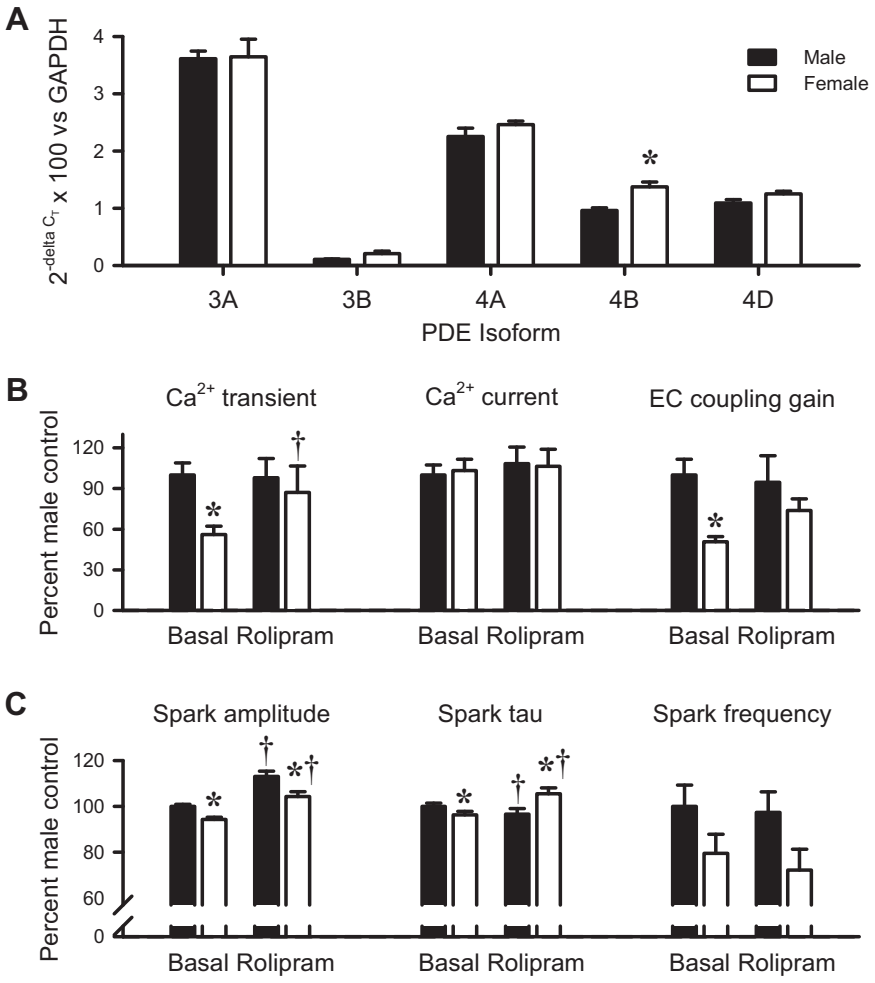

Fig. 8. Ventricles from female mice possess higher PDE4B mRNA levels, and inhibition of PDE4 eliminates male–female differences in Ca^{2+} transients and EC coupling gain. A. Quantitative mRNA expression of PDE3A, PDE3B, PDE4A, and PDE4D relative to GAPDH was similar between the sexes, while females exhibited an increase in PDE4B expression. (n = 3 male, 3 female hearts in triplicate). B. Rolipram only increased Ca^{2+} transient amplitude in females, and abolished the basal male–female difference. Ca^{2+} current, measured simultaneously, was unaffected by rolipram in either sex. Inhibition of PDE4 increased gain in females ($P = 0.05$) and removed the basal sex difference. (n = 21 male, 17 female control cells; 11 male, 10 female rolipram cells; 10 male, 9 female animals) C. Rolipram increased the amplitude of subcellular Ca^{2+} sparks in both sexes, and sparks remained larger in males. Ca^{2+} spark decay was shortened by rolipram in males, but was prolonged in females. Spark frequency was not altered by rolipram in either sex. (For control, n = 220 male, 217 female cells; 2624 male, 2147 female sparks; 12 male, 14 female animals. For rolipram, n = 87 male, 116 female cells; n = 982 male, 944 female sparks; 5 male, 7 female animals; * denotes $P < 0.05$ compared to male, † denotes $P < 0.05$ compared to same-sex control).

in similar total Ca^{2+} release. The amount of Ca^{2+} released during a spark has been shown to be regulated by the intrinsic gating of RyR [35], which may become similar in males and females upon inhibition of PDE4.

A limitation to our study is that experiments measured total cellular cAMP, and therefore did not take into account potential differences that may exist in compartmentalization of cAMP within male and female cardiomyocytes. However, our results do demonstrate an important difference that exists in total cAMP content, and thus overall PKA activity between males and females. It is also possible that other PDE isoforms contribute to sex differences in SR Ca^{2+} release. For example, recent findings have suggested that PDE2 may play a role in regulating basal EC coupling in the heart [19], although the impact on EC coupling was modest and was only examined in males. The present study used high resistance microelectrodes and discontinuous single electrode voltage clamp (switch clamp) to measure Ca^{2+} current, as in many previous studies by our group and others (e.g. [22,30,55–57]). This has the advantage of limiting dialysis of important intracellular components such as cAMP and avoids buffering intracellular Ca^{2+} levels. Although access resistance is higher with this approach than with patch pipettes, we continuously monitored the output of the switching circuit during switch clamp to ensure adequate settling time for accurate voltage measurement and optimal accuracy of voltage control. An alternative approach to minimize internal dialysis would be to use the perforated patch-clamp technique, although this technique also has higher access resistance than patch pipettes and so is often used in conjunction with switch clamp [58]. Another limitation to consider is that experiments were performed in C57BL/6 mice, and thus results cannot be directly applied to humans. Echocardiography and working heart studies in mice have reported either no sex difference or reduced ejection fraction in female in comparison to male mice [59,60]. However, studies in humans have identified higher ejection fraction at rest in women than men [1,2]. These differing results suggest that species differences may exist between mice and humans, which could be due to variations in autonomic tone.

In conclusion, these results suggest that the cAMP/PKA pathway plays a role in sex differences in SR Ca^{2+} release by attenuating the magnitude and duration of individual Ca^{2+} release units in female myocytes. This study suggests that increased degradation of cAMP by PDE4B in females may result in sex differences in the activity of PKA. Whether sex steroid hormones are involved in these male–female differences is not yet understood. However, testosterone has been shown to inhibit PDE activity in the ventricles of male rats [61] and this could explain the higher levels of cAMP observed in male cells. Interestingly, results from Kravtsov et al. [62] suggest that ovariectomy

increases PKA activity in female rats. Together with our study, this suggests that oestrogen may suppress SR Ca^{2+} release, which is at least partly due to decreased signalling via the cAMP/PKA pathway. Ultimately, these findings imply that female hearts may have limited positive inotropic responses to stimulation of the cAMP/PKA pathway, which could be due to lower basal activity in female ventricular myocytes. Less SR Ca^{2+} release in females would limit Ca^{2+} overload while simultaneously limiting inotropic responses in conditions of higher demand. This could be protective against cardiovascular disease resulting from high Ca^{2+} levels, however cardioprotection may occur at the expense of increased inotropy.

Disclosure statement

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.07.006.

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