

**THE EFFECT OF OVARECTOMY ON INTRACELLULAR CALCIUM (Ca²⁺)
REGULATION IN GUINEA PIG CARDIOMYOCYTES**

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Running Head: Ovariectomy and Ca²⁺ handling

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Abstract

This study addressed the hypothesis that long-term deficiency of ovarian hormones, following ovariectomy, alters cellular calcium (Ca^{2+}) handling mechanisms in the heart resulting in the formation of a pro-arrhythmic substrate. It also tested if estrogen supplementation to ovariectomized animals reverses any alterations to cardiac Ca^{2+} handling and rescues pro-arrhythmic behaviour. Ovariectomy (OVx) or sham operations were performed on female guinea pigs using appropriate anaesthetic and analgesic regimes. Pellets containing 17β -estradiol (1mg, 60-day release), were placed subcutaneously in selected OVx animals (OVx+E). Cardiac myocytes were enzymatically isolated and electrophysiological measurements were conducted with a switch clamp system. In Fluo-4 loaded cells, Ca^{2+} transients were 20% larger and fractional sarcoplasmic reticulum (SR) Ca^{2+} release was 7% greater in the OVx group compared with the sham group. Peak L-type Ca^{2+} current was 16% larger in OVx myocytes with channel inactivation shifting to more positive membrane potentials creating a larger “window” current. SR Ca^{2+} stores were 22% greater in the OVx group and these cells showed a higher frequency of Ca^{2+} sparks and waves and shorter wave-free intervals. OVx myocytes showed higher frequencies of early afterdepolarizations (EADs) and a greater percentage of these cells showed delayed afterdepolarizations (DADs) following exposure to isoprenaline (ISO) compared with sham myocytes. The altered Ca^{2+} regulation occurring in the OVx group was not observed in the OVx+E group. These findings suggest that long-term deprivation of ovarian hormones in guinea pigs lead to changes in myocyte Ca^{2+} handling mechanisms that are considered pro-arrhythmogenic. 17β -estradiol replacement prevented these adverse effects.

Keywords

Ovariectomy, calcium regulation, arrhythmia, 17 β -estradiol

New & Noteworthy

OVx guinea pig cardiomyocytes have higher frequencies of Ca²⁺ waves and ISO-challenged cells display more EADs, DADs and extra beats compared with sham myocytes. These alterations to Ca²⁺-regulation are not observed in myocytes from OVx guinea pigs supplemented with 17 β -estradiol, suggesting that ovarian hormone deficiency modifies cardiac Ca²⁺-regulation potentially creating pro-arrhythmic substrates.

Introduction

Although the risk of cardiovascular disease (CVD) is lower in premenopausal women than in age-matched men with CVD developing about 10 years later in females compared with males (56), the risk increases noticeably in women after menopause (39, 45). Further, in animal models long-term deficiency of estrogen was associated with cardiac contractile dysfunction (36, 71), reduced myofilament force and increased Ca^{2+} sensitivity (37). Clinical observations show that while premenopausal women have lower incidence of sudden cardiac death compared with men – that could be related to reduced risk of malignant ventricular arrhythmias and delayed onset of coronary artery disease – postmenopausal women seem to lose this protection (19, 29, 40, 41, 46). Postmenopausal women are more vulnerable to arrhythmia-related sudden cardiac death compared with premenopausal women (54, 64). Notably, premenopausal women with ventricular premature beats that mostly originate from the right ventricle outflow tract show decreased premature beat frequency around ovulation when serum estradiol is at its peak (14).

While these clinical findings suggest ovarian hormones may provide some cardiovascular protection and reduce the incidence of pro-arrhythmic events (2, 32, 34, 65), large studies examining hormone replacement therapy (HRT) showed no cardiovascular benefit (47). These conflicting results led to the testing of a hypothesis which suggests that the timing of HRT is important and women receiving such therapy early after the menopause benefit from reduced risk of serious cardiovascular events (49). However, the association between serum estrogen levels and arrhythmias appears complex. For example, in acquired long-QT syndrome, drug-induced QT prolongation is more pronounced and the risk for polymorphic

ventricular tachycardia is greater when estradiol serum levels peak during the estrous cycle, suggesting a pro-arrhythmic role for estradiol (35). Therefore further investigation is required to clarify the effect of ovarian hormones on cardiac function and elucidate underlying mechanisms.

Although there have been some studies on EC coupling and Ca^{2+} regulation in cardiomyocytes following ovariectomy in rats and mice (8, 17, 26), the underlying mechanisms altered by estrogens remain ambiguous and, given the species used, may not be translatable to the human. More benefit may be achieved by developing and studying an animal whose cardiac EC coupling and Ca^{2+} regulation are more closely akin to the human.

Furthermore, ineffective Ca^{2+} regulation is potentially arrhythmogenic and so a deficiency of estrogen could provide a pro-arrhythmic substrate. Indeed, an increase in susceptibility to epinephrine-induced arrhythmias (for example, premature ventricular contractions) was noted in ovariectomized rats compared with gonad-intact animals (59). A clinical investigation also revealed that postmenopausal women who have idiopathic outflow tract ventricular arrhythmia had lower serum estradiol levels than control postmenopausal women, and the occurrence of ventricular arrhythmia events was lower in women with estrogen replacement (23).

The aims of the present study are to assess if long-term deprivation of ovarian estrogen in the guinea pig alters intracellular Ca^{2+} regulation in ventricular myocytes and leads to more arrhythmogenic events. We aim to identify which Ca^{2+} regulatory mechanisms are affected and if estrogen supplementation to ovariectomized guinea pigs reverses any alterations to cardiac Ca^{2+} handling and rescues pro-arrhythmic behaviour. Our rationale for using the guinea pig in these experiments is that in

many relevant physiological aspects it displays similar features to the human. The relative amounts of trans-sarcolemmal Ca^{2+} influx and SR Ca^{2+} release in their cardiac myocytes are almost identical to that of human in contrast to the corresponding fluxes in rat or mouse which are far removed from the human situation because of their very “high gain” EC coupling system (3). Most ionic currents shaping the ventricular action potential of the guinea pig are similar to human and very dissimilar to rat or mouse. In terms of sex hormone balance and steroidogenesis, again the guinea pig is similar to the human (33, 58).

Methods

All animal studies were carried out with the approval of the Home Office, United Kingdom and in accordance with the United Kingdom Home Office Guide on the Operation of the Animals (Scientific Procedures) Act 1986 under assurance number A5634-01.

Animal preparation

Female adult Dunkin-Hartley guinea pigs (400-500g) underwent either an ovariectomy (OVx) through a bilateral flank approach or a sham operation that left the gonads intact. Animals were anesthetized with 2-4 % isoflurane (Isoflo, Abbott)/ 95 % oxygen mix. Atropine sulphate (0.05 mg/kg s.c.; atrocare, Animalcare Limited) was administered to anaesthetized animals to reduce saliva secretions followed by prophylactic antibiotic (enrofloxacin, 5 mg/kg s.c.; Baytril, Bayer) and analgesic (carprofen, 4.4 mg/kg s.c; Rimadyl, Pfizer and buprenorphine, 0.05 mg/kg s.c; Vetergesic, Alstoe Ltd.). Local anaesthetic was injected (Bupivacaine, 2 mg/kg s.c;

Macaine Polyamp, AstraZeneca) around the incision site. Carprofen (Rimadyl, 15 mg in 300 mL water) was given orally for 2 days post-operatively.

To establish a long term absence of ovarian estrogen in OVx-operated animals, the three animal groups (Sham, OVx and OVx+E) were used 150 days post-surgery. Pellets (60-day release) containing 1mg 17 β -estradiol (E2) (Innovative Research of America) were implanted subcutaneously in the back region of selected OVx animals under isoflurane inhalation anaesthesia 90 days post-OVx surgery. Throughout the study, the animals were housed at 21 $\pm$ 1°C in a controlled lighting environment (12 hour light-dark cycles). After surgery, the guinea pigs were fed *ad libitum* with water and a casein-based, soy-free diet to minimize the uptake of phytoestrogens.

Echocardiography

M-mode echocardiography was performed by using a HP Sonos 5500 echo machine on unanaesthetized guinea pigs to avoid anaesthetic effects on heart function. The left ventricular internal diameters in diastole (LVIDd) and systole (LVIDs) were measured to calculate fractional shortening ($FS = (LVIDd - LVIDs) / LVIDd$).

Serum estradiol (E2)

Blood samples were collected in serum separator tubes (Becton Dickinson, UK) and centrifuged at 3000 rpm for 15 min at 4°C. The serum supernatant was transferred to low-binding protein Eppendorf tubes and stored at -80°C until assayed. Levels of serum E2 were measured by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (MyBioSource, San Diego, USA).

Myocyte isolation

166 Left ventricular myocytes were isolated from Sham, OVx or OVx+E guinea pig hearts
167 as previously described (31), but using the Liberase TL enzyme (Liberase, Roche).
168 Hearts were retrogradely perfused at 37°C with KH solution for 1-2 min. The
169 perfusate was switched to low Ca^{2+} solution for 4-5 min before adding Liberase TL
170 enzyme (5 mg in 50 ml enzyme solution) for 6-10 min. The hearts were then
171 removed from the cannula that supplied the retrograde perfusion, and the left
172 ventricles were chopped into 2-4 mm size tissues. The tissues were transferred to a
173 small jar containing the same enzyme solution, shaken and oxygenated. The
174 supernatant was filtered every 3 min until the digestion was complete. The flow-
175 through was centrifuged at low speed (400 rpm) for 1 min. The resulting cell pellet
176 was re-suspended in fresh low Ca^{2+} solution, in the absence of Liberase TL enzyme.
177 Finally, myocytes were stored in Dulbecco's modified Eagle's medium solution
178 (Gibco BRL, Life Technologies Ltd) at 20-22°C and were used within 6 to 8 h.

179 *Composition of solutions (in mM unless stated otherwise)*

180 Tyrode's solution (NT): NaCl 137, KCl 5.4, glucose 10, N-2-hydroxyethylpiperazine-
181 N'-2-ethanesulphonic acid (HEPES) 10, MgCl_2 1, CaCl_2 2; pH adjusted to 7.4 with 1M
182 NaOH.

183 Krebs-Henseleit (KH) solution: NaCl 119, KCl 4.7, MgSO_4 0.94, CaCl_2 1, KH_2PO_4 1.2,
184 NaHCO_3 25, glucose 11.5; pH adjusted to 7.4 with 1M HCl.

185 Low Ca^{2+} solution: NaCl 120, KCl 5, MgSO_4 5, Na pyruvate 5, glucose 20, taurine 20,
186 HEPES 10, NTA 5, CaCl_2 35 μM (estimated final free $[\text{Ca}^{2+}]$: 12-15 μM); pH adjusted
187 to 6.96 with 1M NaOH.

188 Enzyme solution: NaCl 120, KCl 5, MgSO₄ 5, Na pyruvate 5, glucose 20, taurine 20,
189 HEPES 10, CaCl₂ 200 μM; pH adjusted to 7.4 with 1M NaOH.

190 0Na⁺ 0Ca²⁺ solution: LiCl 140, KOH 6, glucose 10, HEPES 10, MgCl₂ 1, EGTA 1; pH
191 adjusted to 7.4 with 1M LiOH.

192 *Intracellular Ca²⁺ monitoring*

193 Ca²⁺ dye loading: Cells were loaded with the Ca²⁺-sensitive fluorescent dye Fluo-4
194 AM (Invitrogen, Life Technologies Ltd). Cells were incubated with 10 μM of the Fluo-
195 4 AM at RT on a rocking platform for 25 min. Thereafter the cell suspension was
196 centrifuged at low speed (400 rpm) for 1 min replacing the supernatant with fresh
197 Dulbecco's Modified Eagle's Medium. The cells were further incubated at RT for at
198 least 30 min to allow for de-esterification of the intracellular Ca²⁺ indicator.

199 Ca²⁺ transient: Cells were superfused at 37°C with NT solution. Ca²⁺ transients were
200 assessed during steady-state external field stimulation at 0.5 Hz as previously
201 described (52, 55). For Ca²⁺ transient decay rate determination, a standard single
202 exponential equation was fitted to the decay trace of a steady-state Ca²⁺ transient to
203 obtain a time constant from which a rate constant for the Ca²⁺ transient decay was
204 calculated (13). Following a SR Ca²⁺ content loading protocol (1 Hz stimulation for
205 20s), cells were rapidly superfused with 10 mM caffeine in NT or in 0Na⁺ - 0Ca²⁺
206 solution to induce a transient. The decay phase of the caffeine-induced Ca²⁺
207 transients were used to determine the rate constants of SERCA and NCX as
208 previously described (6). Fractional release (x/y) was calculated using the steady-
209 state Ca²⁺ transient amplitude (x) and the size of the caffeine evoked Ca²⁺ transient
210 in 0Na⁺ - 0Ca²⁺ solution (y) (1). One caveat of these approaches is that they are
211 oversimplified. When one extrusion system is inhibited the relative roles of the other

systems may change so more accurate estimations of their relative roles require consideration of cytosolic Ca^{2+} buffering which has not been measured here.

Ca^{2+} sparks and waves: Briefly, Ca^{2+} sparks and waves were acquired using a line-scanning confocal microscope, consisting of an inverted microscope with a Bio-Rad Radiance 2000 confocal attachment. Ca^{2+} sparks and waves were recorded during a 30 s quiescence period following a SR Ca^{2+} loading protocol (2 Hz stimulation for 20 s). When isoprenaline (ISO) was added to the superfusate in these types of experiment, it was used at a concentration of 0.5 μM . The SparkMaster plug-in for ImageJ (NIH, USA) was used to analyse Ca^{2+} sparks from the line scan images (42). The detection criterion for Ca^{2+} sparks was set at 4.2 times the standard deviation above the mean background value, instead of 3.8, to reduce false identification of Ca^{2+} sparks resulting from background noise. Spark mass was calculated using the following formula: Spark mass = amplitude $\times$ 1.206 $\times$ full width at half maximal amplitude (FWHM)³ (22). Ca^{2+} waves were analysed by using an ImageJ macro (52). Wave-free survival analysis was performed by using Kaplan-Meier Estimates.

Electrophysiological measurements

Action potential duration (APD), $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) inward current and L-type Ca^{2+} channel current ($I_{\text{Ca,L}}$) were recorded using a switch-clamping system (Axoclamp 2B amplifier, Molecular Devices, Sunnyvale, CA, USA) with high resistance microelectrodes to minimise cell dialysis and preserve the intracellular milieu. Sharp microelectrodes with resistances of 20-40 $\text{M}\Omega$ were filled with a solution containing 2M KCl, 5 mM HEPES, 100 μM EGTA, pH adjusted to 7.20 with KOH. In current clamp mode, action potentials were elicited with 3 ms current pulses (typically 1.5-2.5 nA) applied at a rate of 0.5 Hz. APDs were expressed as the time to

236 10% and 90% repolarisation (APD_{10} and APD_{90} , respectively). $I_{Ca,L}$ was recorded in
237 discontinuous single electrode voltage clamp mode (gain 0.5-1.5 nA/mV, and
238 switching rate 4-6 kHz) in the presence and absence of 200 μ M cadmium. $I_{Ca,L}$ was
239 evoked from a holding potential of -40 mV by 200 ms steps from -45 to +55 mV in 5
240 mV increments. Steady-state inactivation of $I_{Ca,L}$ was assessed with double pulse
241 protocols in which conditioning pulses of 200 ms duration and ranging from -45 to
242 +55 mV were followed by a 5 ms step to -40 and a 200 ms step to +5 mV. $I_{Ca,L}$ was
243 determined by subtracting the trace in the presence of cadmium from the original
244 trace at each voltage. Sarcoplasmic reticulum (SR) Ca^{2+} content was assessed by
245 integrating the inward NCX current induced by the rapid application of 10 mM
246 caffeine when cells were voltage-clamped at -80 mV, as previously described (15).

247 To elicit early and delayed afterdepolarizations (EADs and DADs, respectively), cells
248 were exposed to 50 nM isoprenaline (ISO) and paced at various frequencies (0.5, 1
249 and 2 Hz) for 30 s. The presence of EADs, appearing as transient repression of the
250 repolarisation process and/or reversal of the membrane potential during
251 repolarisation, was confirmed by the reversal of the negative first derivative (57) of
252 the membrane potential towards less negative and/or positive values, respectively.
253 DADs were counted if their amplitudes were ≥ 5 mV.

254 *mRNA expression of $Ca_v1.2$ and $Ca_v\beta 2$*

255 Total RNA from cells was extracted by using the RNeasy Mini Kit (Qiagen, UK)
256 according to the manufacturer's instructions. Primers were obtained from Invitrogen,
257 UK and made using a desalted purification method. Real-time RT-PCR was
258 performed using the one-step QuantiTect SYBR Green PCR kit (Qiagen, UK).
259 Primers were designed by using NCBI/Primer-Blast

260 (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Target gene sequences were
261 sourced from NCBI/Gene (<https://www.ncbi.nlm.nih.gov/gene/>). The primer
262 sequences were as follows:

263 $\text{Ca}_v1.2$ (encoded by CACNA1C): sense 5'-ACACTGCTGTGGACCTTCATC-3',
264 antisense 5'-TGCATACCAATCACGGCATA-3'.

265 $\text{Ca}_v\beta 2$ (encoded by CACNB2): sense 5'-CCAAGCCCAGTCAAACCTAGAAAAC-3',
266 antisense 5'-CAAACCTGGACGATGAATTCCTCC-3'.

267 β -actin (encoded by ACTB): sense 5'-ACTTTGCTGCGTTACACCCT-3', antisense
268 5'-AATCAAAGTCCTCGGCCACA-3'.

269 Cycle threshold (C_t) values for target messenger RNA (mRNA) were normalized to
270 the housekeeping gene coding β -actin (ACTB) and the relative gene expression was
271 calculated using the $2^{-\Delta\Delta C_T}$ method (27).

272 *Acquisition systems and statistical analysis*

273 The electrophysiological and fluorescence data were recorded and analysed using
274 Clampex (ver. 10) acquisition and analysis software (Molecular Devices, Sunnyvale,
275 CA, USA). The Ca^{2+} spark morphological parameters of amplitude and mass were
276 log-transformed, resulting in normally distributed populations for statistical analysis.
277 Statistical differences between means were calculated using Student's t -test, two-
278 sample proportion test and a one-way ANOVA with a Fisher post-hoc test. Values
279 were expressed as mean $\pm$ S.D, and where appropriate, n =number of cells from a
280 total number of hearts (n =cells/hearts). Statistical significance is denoted as $*P < 0.05$,
281 $**P < 0.01$ and $***P < 0.001$.

282

Results

Physical characteristics of experimental animals

The serum estrogen level decreased significantly in OVx guinea pigs and increased when estrogen was replaced (the OVx+E treatment group) (Table 1). Average body weight (BW) significantly increased by 10 % in the OVx group but the ratio of heart weight (HW) to body weight remained unchanged compared with Sham (Table 1). Both uterine and heart weights normalized to body weight increased significantly in the OVx+E group (Table 1).

In vivo M-mode echocardiography

The fractional shortening (FS), assessed by measuring LVIDd and LVIDs, decreased in the OVx group but remained unchanged in the OVx+E group compared with Sham (Fig. 1). The OVx group showed larger LVIDs and LVIDd than those in the Sham group (Fig. 1).

Ca²⁺ transient amplitudes and SR Ca²⁺ fractional release

Cells were field-stimulated at 0.5 Hz to achieve steady states. Ca²⁺ transient amplitudes, measured as F/F_0 , were 20 % larger in the OVx group than in Sham (Fig. 2. A & B). The OVx group had greater SR Ca²⁺ fractional release compared with Sham (Fig. 2. C & D). Myocytes isolated from the OVx+E group had similar Ca²⁺ transient amplitudes and SR Ca²⁺ fractional release compared with myocytes isolated from the Sham group. The time-to-peak (TTP) of the Ca²⁺ transients remained unchanged between Sham, OVx and OVx+E groups (Fig. 3. A). Ca²⁺ transients measured from the OVx group had shorter decay times (R90) and faster decay rate constants compared with Sham and OVx+E, respectively (Fig. 3. C).

Cells from Sham and OVx+E had similar decay rate constants (Fig. 3. C). The rate constant representative of SERCA function was not different between Sham, OVx and OVx+E cells (Fig. 3. D). The rate constant indicative of NCX function was 20 % and 27 % faster in OVx myocytes compared with Sham and OVx+E, respectively (Fig. 3. E).

SR Ca²⁺ content

The SR Ca²⁺ content, obtained by integrating the caffeine-evoked inward NCX current (Fig. 4. A), was on average 20 % greater in the OVx group compared with the Sham group (Fig. 4. B). SR Ca²⁺ content was unaltered between cells isolated from Sham and OVx+E groups.

Ca²⁺ sparks and waves

Since SR Ca²⁺ content was greater in the OVx group we examined Ca²⁺ spark frequencies to assess if there were any changes in spontaneous Ca²⁺ release. In addition, we assessed if these cells had an increased tendency to produce Ca²⁺ waves when provoked by an ISO challenge. Spark frequencies were higher in the OVx group compared with Sham (Fig. 5. B). In the presence of 0.5 μ M ISO, myocytes showed more sparks, and the frequencies at which they occurred remained higher in cells isolated from the OVx group (Fig. 5. B). Spark amplitude and mass were also larger in OVx myocytes with or without ISO (Fig. 6). In conjunction with Ca²⁺ sparks, wave frequencies were higher in the OVx group compared with Sham (Fig. 7. B). In the presence of 0.5 μ M ISO, myocytes showed more waves and the frequencies at which they occurred remained higher in the OVx group (Fig. 7. B). Spark and wave frequencies significantly decreased in myocytes from animals supplemented with estrogen compared with the OVx group, and wave-

free survival times were longer in Sham and OVx+E in the absence or presence of ISO compared with OVx (Fig. 7. C & D).

L-type Ca^{2+} current amplitude and gating

This assessment of Ca^{2+} regulation indicated that there were changes in intracellular Ca^{2+} handling following long-term absence of estrogen. It was therefore necessary to measure L-type Ca^{2+} current to assess if changes in Ca^{2+} influx occurred following ovariectomy. The peak $I_{\text{Ca,L}}$ was 16 % larger in OVx than Sham and OVx+E (Fig. 8. B), and the amount of Ca^{2+} influx calculated during the 200 ms activating clamp step was 33 % larger in OVx myocytes compared with Sham and OVx+E myocytes (Fig. 8. D).

Voltage-dependent activation and steady-state inactivation curves were fitted with a Boltzmann equation ($y = 1/(1 + \exp[(x - V_{0.5})/K])$) (Fig. 9). The steady state inactivation of the L-type Ca^{2+} current shifted to more positive potentials in OVx myocytes ($V_{1/2, \text{inactivation}}$ shift: 3.0 mV, $P < 0.001$). Channel gating, determined by the L-type Ca^{2+} current inactivation shift, was unaltered in OVx+E compared with Sham myocytes (Fig. 9. A & B).

The probability of Ca^{2+} channels being open within the “window” range of voltages was calculated using the equation: $(1/\{1 + \exp[(V_{0.5, \text{activation}} - V)/K_{\text{activation}}]\}) \times 1/\{1 + \exp[(V - V_{0.5, \text{inactivation}})/K_{\text{inactivation}}]\})$ described previously (24). In Sham myocytes, the probability of Ca^{2+} channels being open was 0.07 at -15 mV while in OVx cells, this reached 0.15 at -10 mV, and subsequently decreased in the OVx+E group to 0.06 at -10 mV (Fig. 9.C).

L-type Ca^{2+} channel $\alpha 1c$ and $\beta 2$ subunit relative mRNA expression

A qRT-PCR was performed to measure relative mRNA expression levels to determine whether augmented OVx $I_{Ca,L}$ was a consequence of altered gene expression of $\alpha 1c$ or $\beta 2$ subunits. The relative mRNA expression of these subunits was unaltered between Sham, OVx and OVx+E groups (Fig. 10).

EADs and DADs induced by ISO

The right-shift in Ca^{2+} current inactivation, increasing the fraction of Ca^{2+} current that fails to inactivate within the “window” voltage range, may favour the generation of arrhythmic events following OVx. To assess pro-arrhythmic susceptibility following long-term deprivation of estrogen, the frequency of EADs or DADs after an ISO challenge was determined. Firstly, we measured action potentials in cells isolated from each group (Fig. 11. A). APD_{90} remained unchanged between Sham, OVx and OVx+E groups (Fig. 11. B), however APD_{10} was 1.3 times longer in cells isolated from the OVx group compared with Sham and OVx+E groups (Fig. 11. C). This may indicate more inward current flow early in the action potential supporting the Ca^{2+} current measurements.

EADs or DADs were elicited by pacing cells at 0.5, 1 or 2 Hz for 30 s in the presence of 50 nM ISO (Fig. 12). Myocytes from the OVx group showed higher frequency of EADs compared with the Sham group (Fig. 13. A) and, in addition, a higher percentage of OVx cells showed DADs when stimulated at 0.5 and 1 Hz compared with Sham and OVx+E (Fig. 13. B & C). OVx myocytes also had higher percentage of cells showing DAD-induced triggered extra beats during 0.5 Hz and 1 Hz stimulation compared with Sham and OVx+E cells (Fig. 13. D & E)

Discussion

Observations

This study addressed the hypothesis that long-term deficiency of ovarian hormones alters intracellular Ca^{2+} regulation in a way that may predispose cardiac myocytes to more potentially arrhythmogenic events. Alongside testing this central hypothesis, we assessed whether 17β -estradiol supplementation reversed altered intracellular Ca^{2+} handling, thus preventing pro-arrhythmic behaviour. Following ISO challenge, OVx myocytes had higher frequencies of EADs and a greater percentage of them showed DADs and DAD-induced triggered extra beats. Significantly fewer pro-arrhythmic events were observed in myocytes isolated from OVx guinea pigs with 17β -estradiol supplementation. These observations suggest that long-term deprivation of ovarian estrogen can modify Ca^{2+} regulation in ventricular myocytes in ways that may promote pro-arrhythmic behaviour.

Ca^{2+} transient and SR Ca^{2+} load

OVx myocytes had greater SR Ca^{2+} content and larger Ca^{2+} transient amplitudes supporting a previous report (17). We also found OVx myocytes had larger peak $I_{\text{Ca,L}}$ and corresponding Ca^{2+} influx. We suggest the greater SR Ca^{2+} content may result from the loading effect of the larger Ca^{2+} influx. Not only is the size of SR Ca^{2+} release proportional to the size of the Ca^{2+} current (4), but also increased Ca^{2+} influx loads the SR with more Ca^{2+} (16) that augments fractional SR Ca^{2+} release (50). The Ca^{2+} transient decay rate constant was significantly greater in OVx compared with Sham and OVx+E. While the rate constant of SERCA function

remained unchanged between Sham, OVx and OVx+E, the rate constant of NCX function significantly increased in OVx hearts compared with Sham and OVx+E, leading to a faster Ca^{2+} transient decay in the OVx group. The enhanced NCX forward-mode may help to facilitate Ca^{2+} efflux from the cell in response to increased Ca^{2+} influx following ovariectomy and could potentially prolong the action potential and increase the likelihood of EAD formation (57). Upregulated NCX function was also reported in an ovariectomized rat model and was suggested to be associated with NCX phosphorylation by upregulation of protein kinase A (PKA) (26).

Ca^{2+} sparks and waves

Our data show significant increases in the frequency of Ca^{2+} sparks and waves following ovariectomy. Spark amplitudes and mass were also larger in OVx myocytes. All these indices are indicative of an enhanced SR Ca^{2+} load (48) which is proposed to alter ryanodine receptor Ca^{2+} sensitivity at luminal regulatory sites (21) or directly at the store-sensing gate in the receptor (10), and increase single channel current amplitude that governs cluster behaviour (20). These changes increase the probability of Ca^{2+} wave formation and propagation that are considered to be pro-arrhythmic (62). These will induce sufficient Ca^{2+} -sensitive inward current, particularly forward-mode NCX current, to cause depolarization of the cardiac cell membrane producing spontaneously triggered beats under certain conditions (51). Therefore OVx myocytes may have increased tendency to develop arrhythmias, which could explain the finding that ovariectomy increased the

susceptibility to ischemia-induced arrhythmias (29) and the increased incidence of arrhythmias in post-menopausal women (40).

In addition, we also investigated the cells responses to β -adrenergic stimulation (ISO challenge). Cells from Sham, OVx and OVx+E all produced significantly more sparks and waves when treated with ISO. OVx myocytes again showed higher frequencies of sparks and waves compared with Sham. Contrastingly, OVx+E myocytes had comparable responses to ISO challenge as Sham myocytes. Therefore, OVx myocytes were more sensitive to adrenergic stimulation which elicited significantly more SR Ca^{2+} release events. Estradiol supplementation suppressed these potentially adverse effects.

Ca^{2+} current

L-type Ca^{2+} current was measured to determine if it could be responsible for the increases in cellular Ca^{2+} content. We found the peak $I_{\text{Ca,L}}$ and the corresponding Ca^{2+} influx were larger in OVx myocytes. Furthermore, we observed that current inactivation shifted to more positive voltages and the probability of Ca^{2+} channels being open within the “window” voltage range was greater in OVx myocytes. Both parameters may favour the generation of pro-arrhythmic events (5, 70). Evidence suggests that the activity of L-type Ca^{2+} channels increased following ovariectomy and these changes are reversed by estrogen supplementation (25, 38, 60). The changes appear to take place without alterations to the relative amounts of gene expression for L-type Ca^{2+} channel subunits $\alpha 1c$ and $\beta 2$. This was a similar finding to the levels of mRNA coding for L-type Ca^{2+} channel α -subunits in smooth muscle cells of coronary arteries following estrogen withdrawal (60). It is tempting to suggest that the increased $I_{\text{Ca,L}}$ may result from a change in phosphorylation status of the

channel. Enhanced basal activity of PKA and increased basal activity of L-type Ca^{2+} channel has been shown in rat OVx myocytes, and the increased basal activity of L-type Ca^{2+} channel was reversed by a PKA inhibitor (25). In addition, CaMKII δ and phosphorylated CaMKII were both upregulated in cardiomyocytes from an ovariectomized rat model, and were restored to normal levels following estrogen replacement (30).

EADs and DADs

A key finding in our study was the observation that OVx myocytes had higher frequencies of EADs and a greater percentage of these cells showed DADs and DAD-induced triggered extra beats during ISO challenge. Significantly fewer pro-arrhythmic events were observed in myocytes isolated from OVx guinea pigs with 17β -estradiol supplementation. To the best of our knowledge, this is the first ISO challenge experiment to induce EADs/DADs in cardiomyocytes from ovariectomized animals. EADs are likely to occur when a proportion of Ca^{2+} channels reactivate or fail to inactivate, especially in the case of increased window current (68). Late phase EADs may share the same underlying mechanisms as DADs which are SR Ca^{2+} overload and spontaneous Ca^{2+} release (63). Adrenergic stimulation-induced EADs are regarded as the underlying mechanism of torsade de pointes ventricular tachycardia and sudden death in patients with idiopathic long QT syndrome (LQTS) (43). Our observations may help explain an *in vivo* whole heart study showing increase in susceptibility to adrenergic stimulation-induced arrhythmias in ovariectomized rats compared with gonad-intact animals (59).

Comparing Ca^{2+} handling in ovariectomized animal models

Table 2 summarises the changes in cardiac myocyte Ca^{2+} handling in ovariectomized animal models. We have reported an increased peak $I_{\text{Ca,L}}$ in OVx that is in good agreement with effects seen in rat and mouse OVx models (18, 25). The amount of mRNA coding for the L-type Ca^{2+} channel was unaltered following OVx; a finding similar to Fares *et al* in the rat (18) but it is uncertain if L-type Ca^{2+} channel protein expression changes (11, 18). We suggest the increased $I_{\text{Ca,L}}$ may result from the change in phosphorylation status of the channel. Further research using PKA/CaMKII inhibitors is required to assess the role of L-type Ca^{2+} channel phosphorylation in estrogen-mediated responses.

Most studies of the effects of OVx report increased Ca^{2+} transient amplitudes (12, 17, 18, 25, 26) although discrepancies exist (9, 61). OVx in the guinea pig speeds the decay of the Ca^{2+} transient and our results suggest that this is due to faster Ca^{2+} removal via NCX. While mRNA and protein expression of NCX are unaltered in most studies (18, 26), there is one report of increased activity of NCX resulting from PKA phosphorylation (26). The rate constant representing SERCA function remained unaltered following OVx implying unchanged Ca^{2+} uptake into the SR but the expression and regulation of SERCA seems to vary among studies (7, 8, 11, 18, 26, 61).

We report an increased spark frequency, amplitude and mass in the OVx group, which supports similar findings in the mouse, an animal whose cardiac EC coupling is more reliant on SR Ca^{2+} release than the guinea pig (17, 18).

All these effects following OVx could be reversed with estradiol supplementation confirming that it is the withdrawal of estrogen that mediates the changes in Ca^{2+} regulation.

In this study we have used an ISO challenge to assess if OVx creates a pro-arrhythmic substrate. OVx myocytes had more sparks and waves following the ISO challenges compared with Sham and OVx+E cells. Furthermore, these cells had a higher frequency of EADs and a higher percentage of DADs and DAD-induced triggered extra beats. To further explore the pro-arrhythmic mechanism, the gating of L-type Ca^{2+} channel was investigated and was found to shift towards a more positive E_m with larger window current allowing a higher probability of reactivation of L-type Ca^{2+} channels. These novel findings may provide new insights for the mechanisms underlying postmenopausal arrhythmia.

Myofilament Ca^{2+} sensitivity

Our data also showed a decrease in *in vivo* fractional shortening and increases in LVIDd and LVIDs in the OVx group compared with the ovary-intact sham group, observations that are difficult to reconcile with increased Ca^{2+} current and SR Ca^{2+} content. A possible explanation is that there are changes to myofilament Ca^{2+} sensitivity in OVx hearts. Ca^{2+} desensitization and/or decreased ATPase activity has been observed in *in vitro* studies of TnT mutant proteins (28, 44, 53, 69) and accompanies many dilated cardiomyopathy phenotypes (69) that show decreases in *in vivo* fractional shortening yet increases to the Ca^{2+} transient as found here. A deficiency of female sex hormones alters the cardiac contractile machinery (36, 46, 66) but the few studies that have been done in rats present an ambiguous picture of the effects of ovariectomy (37, 67).

In conclusion, ovariectomy had detrimental effects on Ca^{2+} regulation in guinea pig cardiac myocytes. The dysregulation of Ca^{2+} handling contributes to a higher frequency of spontaneous or triggered SR Ca^{2+} release events. With β -adrenergic

stimulation, the probability of formation of EAD and DAD, and DAD-induced triggered extrasystoles was higher in the ovariectomy group. These findings suggest long-term absence of ovarian hormones lead to adverse changes in cardiac Ca^{2+} handling mechanisms that may cause the formation of a more pro-arrhythmic substrate. 17β -estradiol replacement prevented these changes.

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Table 1. Physical characteristics of experimental animals

	n	BW (g)	UW (g)	HW (g)	UW/BW (%)	HW/BW (%)	Serum E2 (pg/mL)
Sham	9	784 ± 85	1.52 ± 0.47	2.38 ± 0.08	0.19 ± 0.05	0.31 ± 0.04	7.80 ± 4.37
OVx	10	870 ± 67*	0.37 ± 0.08***	2.66 ± 0.23***	0.04 ± 0.01***	0.31 ± 0.02	4.19 ± 1.93*
OVx+E	10	711 ± 57*	3.89 ± 0.60***	2.48 ± 0.13	0.55 ± 0.10***	0.35 ± 0.04*	24.22 ± 3.83***

Values expressed as means ± S.D.

n=number of animals; BW=Body weight, HW=Heart weight, UW=Uterine weight

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ represent significant difference compared with Sham group.

One-way ANOVA, Fisher's test.

756 **Table 2. Changes in cardiomyocyte Ca²⁺ handling in ovariectomized animal models**

OVx animal	Guinea pig (this study)	Rat	Mouse
Peak I _{Ca,L}	↑	A↑ ⁽²⁵⁾	↑ ⁽¹⁸⁾ , ↔ ⁽¹⁷⁾
L-type Ca ²⁺ channel	mRNA↔	WB↑ ⁽¹¹⁾ mRNA↔ ⁽¹¹⁾	WB↓ ⁽¹⁸⁾
PKA		E↑ ⁽²⁶⁾ A↑ ⁽²⁵⁾	
CaMKIIδ and phosphorylated CaMKII		WB↑ ⁽³⁰⁾	
SR Ca ²⁺ content	↑		↑ ^(17, 18)
Ca ²⁺ transient amplitude	↑	↑ ^(12, 25, 26) , ↓ ⁽⁹⁾	↑ ^(17, 18) , ↓ ⁽⁶¹⁾
Ca ²⁺ transient TTP	↔		↓ ⁽¹⁸⁾
Ca ²⁺ transient rate constant	↑	↑ ^(12, 26)	↑ ^(17, 18) , ↓ ⁽⁶¹⁾
Fractional SR Ca ²⁺ release	↑		↔ ^(17,18)
SERCA	R↔	A↔ ⁽²⁶⁾ , ↓ ⁽⁸⁾ WB↔ ^(11, 26) , ↓ ^(7, 8) mRNA↔ ⁽¹¹⁾ , ↓ ⁽⁸⁾ PLB↔ ^(7, 8, 11)	WB↔ ⁽¹⁸⁾ ↓ ⁽⁶¹⁾
NCX	R↑	A↑ ⁽²⁶⁾ RC↑ ⁽²⁶⁾ WB↔ ⁽²⁶⁾ , ↓ ⁽¹¹⁾ mRNA↔ ⁽¹¹⁾	WB↔ ⁽¹⁸⁾
RyR2		A↑ ⁽²⁶⁾ WB↔ ⁽¹¹⁾	
Spark frequency	↑		↑ ^(17, 18)
Spark amplitude	↑		↑ ⁽¹⁷⁾
Spark mass	↑		↑ ⁽¹⁸⁾ , ↔ ⁽¹⁷⁾
Wave frequency	↑		
ISO induced EADs & DADs	↑		

757 A = Activity, E = Expression of fluorescence intensity, PLB = Phospholamban, WB = Western blot,

758 R = Rate constant, RC = Relaxation contribution

759 ↑: increase, ↔: no difference, ↓: decrease all compared with Sham group

760

Fig. 1. Representative *in vivo* M-mode echocardiograms measuring LVIDd and LVIDs from A: Sham, B: OVx and C: OVx+E-operated guinea pigs. D, E, F: Mean data of LVIDd, LVIDs and FS respectively (* P <0.05, ** P < 0.01, *** P <0.001 Sham, n=9; OVx, n=10, OVx+E, n=10).

Fig. 2. The effect of long-term absence of estrogen on Ca^{2+} handling. A: Representative traces comparing Ca^{2+} transients between Sham, OVx and OVx+E Fluo-4 loaded-myocytes. B: Myocytes from OVx animals elicited a larger Ca^{2+} transient compared with Sham and OVx+E (** P <0.01; Sham, n=44/4; OVx, n=55/4; OVx+E, n=40/3). C: Experimental protocol used to measure fractional SR Ca^{2+} release in myocytes (see Methods). D: Fractional SR Ca^{2+} release was larger in OVx myocytes compared with Sham myocytes (** P < 0.01; Sham, n=34/3, OVx, n=48/4; OVx+E, n= n=40/3).

Fig. 3. Ca^{2+} transient parameters. Mean time-to-peak differences are shown in panel A and time-to-90% decay variation in panel B for Sham, OVx and OVx+E groups. Panels C shows the mean differences in decay rate constants for the transients and Panels D and E the variation in rate constants for SERCA and NCX respectively (* P <0.05, ** P <0.01, *** P <0.001; Sham, n=56/3; OVx, n=38/3; OVx+E, n=33/3).

Fig. 4. A: Typical traces of caffeine-induced NCX inward current (upper) and the integrated current used to measure SR Ca^{2+} content (lower). B: SR Ca^{2+} content was significantly greater in OVx myocytes compared with Sham and OVx+E myocytes (Sham= 51 ± 10 $\mu\text{M}/\text{l}$ accessible cell volume, OVx= 62 ± 13 $\mu\text{M}/\text{l}$ accessible cell volume, OVx+E= 50 ± 11 $\mu\text{M}/\text{l}$ accessible cell volume, *** P <0.001; Sham, n=33/3; OVx, n=34/3; OVx+E, n=33/5).

Fig. 5. A: The detection of Ca^{2+} sparks in Sham, OVx and OVx+E Fluo-4 loaded-myocytes using line-scanning confocal microscopy. B: Mean spark frequency was higher in OVx myocytes compared with Sham and OVx+E myocytes in the presence and absence of ISO (** P <0.01, *** P <0.001; Sham, n=55/5; OVx, n=36/3, OVx+E, n=40/4).

Fig. 6. The effect of long-term absence of estrogen on (A) Ca^{2+} spark amplitude and (C) mass. Spark amplitudes were larger in OVx than in Sham and OVx+E in the absence (A) and presence (B) of ISO. OVx myocytes had larger spark masses

compared with Sham and OVx+E in the absence (C) and presence (D) of ISO
(* P <0.05, ** P <0.01, *** P <0.001; Sham, n=55/5; OVx, n=36/3, OVx+E, n=40/4).

Fig. 7. Measuring Ca^{2+} wave frequency and wave-free survival in Sham, OVx and OVx+E Fluo-4 loaded-myocytes. A: Typical traces of Ca^{2+} waves recorded in Sham, OVx and OVx+E myocytes. B: Wave frequency was higher in OVx compared with Sham and OVx+E both in the absence (NT) and in the presence of ISO (ISO 0.5 μM) (** P <0.01, *** P <0.001; Sham, n=61/5; OVx=38/3; OVx+E=40/4). C&D: Wave-free survival times in the absence (C) and presence of ISO (D). Wave-free survival time was longer in Sham compared with OVx myocytes in NT and following ISO challenge (* P <0.05 and *** P <0.001 respectively, log rank test). The wave-free survival was significantly longer in the OVx+E group compared with OVx group only in the presence of ISO (** P <0.01, log rank test).

Fig. 8. A: The voltage clamp protocol and the corresponding traces of $I_{\text{Ca,L}}$ (cadmium-sensitive currents) elicited by voltage steps. B: The current-voltage (I-V) relationships determined by the typical protocol shown in panel A for Sham, OVx and OVx+E myocytes. Peak $I_{\text{Ca,L}}$ was larger in OVx myocytes compared with Sham and OVx+E (***) P <0.001; Sham, n=40/5; OVx, n=44/5; OVx+E, n=37/7). C: Representative traces of peak $I_{\text{Ca,L}}$ (upper) and calculated Ca^{2+} influx (lower) in Sham, OVx and OVx+E myocytes. D: Mean data from experiments shown in panel C. Ca^{2+} influx was larger in OVx myocytes compared with Sham and OVx+E myocytes (***) P <0.001).

823

824 Fig. 9. Activation and inactivation plots of the L-type Ca^{2+} channel from Sham, OVx
825 and OVx+E myocytes. The activation curve was plotted using the test voltage and
826 the corresponding normalized conductance (G/G_{max}). Conductance (G) was
827 calculated using the equation: $G = I / (V - V_{\text{rev}})$, where V was the test voltage, I was
828 the peak current at the test voltage and V_{rev} was the reversal potential obtained by
829 extrapolating the tail part of the I-V curve to its intersection with the voltage axis.
830 Steady-state inactivation was obtained by normalizing the individual currents to the
831 maximal current (I/I_{max}) and plotting against the test voltage. A&B: L-type Ca^{2+}
832 channel gating shifted to more positive E_m in OVx myocytes compared with Sham
833 ($V_{1/2, \text{inactivation}}$ shift: 3.0 mV, $P < 0.001$). Channel gating was unaltered in OVx+E
834 compared with Sham myocytes (Sham, $n=40/5$; OVx, $n=46/6$, OVx+E, $n=37/7$). C:
835 The probability of having the channel opening within the “window” range of voltages
836 in Sham myocytes peaked at -15 mV with a probability value of 0.07. This value
837 increased to 0.15 at -10 mV in OVx cells. In OVx+E cells the probability value was
838 0.06 at -10 mV.

839

840 Fig. 10. The relative amounts of $\text{Ca}_v1.2$ and $\text{Ca}_v\beta 2$ mRNA following ovariectomy. A:
841 Relative $\text{Ca}_v1.2$ mRNA expression (normalized to β -actin mRNA) was unaltered
842 between Sham, OVx and OVx+E myocytes (Sham, $n=7$; OVx, $n=7$; OVx+E, $n=6$). B:
843 The relative $\text{Ca}_v\beta 2$ mRNA expression (normalized to β -actin mRNA) did not change
844 following OVx and OVx+E compared with Sham (Sham, $n=7$; OVx, $n=7$; OVx+E, $n=6$).

845

846

Fig. 11. Assessing the long-term absence of estrogen on action potential duration (APD). A: Representative recordings of action potentials from ventricular myocytes. B: Mean data show APD₉₀ remained unchanged between Sham, OVx and OVx+E. C: Mean data indicate APD₁₀ was longer in OVx compared with Sham and OVx+E, but no differences in APD₁₀ were observed between Sham and OVx+E (***P*<0.001; Sham, n=7; OVx, n=7; OVx+E, n=6).

Fig. 12. Identifying early and delayed afterdepolarizations following ISO challenges. Panels A1-3 show typical traces of EAD formation in action potentials with short (A1), normal (A2) and long duration (A3) and the corresponding first derivative (dV/dt) traces from OVx myocytes. The action potentials were recorded during 2 Hz stimulation in 50 nM ISO. Panel B shows typical DADs that form in one OVx myocyte during 0.5 Hz stimulation in 50 nM ISO. One recording illustrates a DAD-induced (triggered) extrasystole.

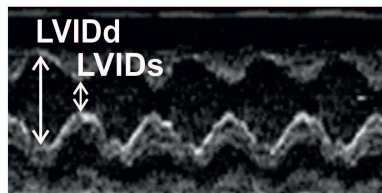
Fig. 13. Quantifying early and delayed afterdepolarizations following ISO challenges. Panel A: OVx myocytes had a higher mean frequency of EADs per 10 beats compared with Sham and OVx+E myocytes following 50 nM ISO challenges at 2 Hz stimulation (Sham=2.8 ± 2.4, OVx=4.7 ± 2.5, OVx+E=3.4 ± 2.5, **P*<0.05; Sham, n=39/5; OVx, n=30/4; OVx+E=30/5). Panels B and C show the percentage of cells with DADs in the three groups Sham, OVx and OVx+E following treatment with 50 nM ISO, during 0.5 Hz (**P*<0.05, ****P*<0.001; Sham, n=37/5; OVx, n=45/4; OVx+E=33/5) and 1 Hz (**P*<0.05; Sham, n=42/5; OVx, n=40/4; OVx+E=32/5) stimulation respectively. Panels D and E show the percentage of cells in each group

871 that exhibit DAD-induced extrasystoles during 0.5 and 1 Hz stimulation respectively
872 (* $P < 0.05$).

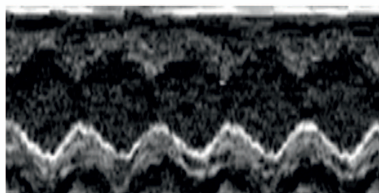
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Fig. 1

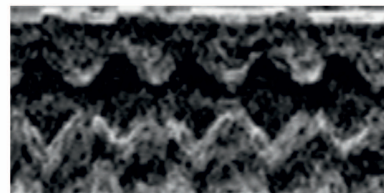
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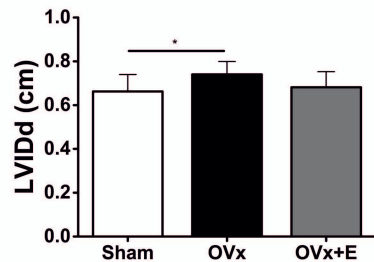
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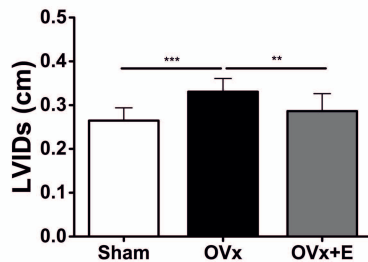
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D



E



F

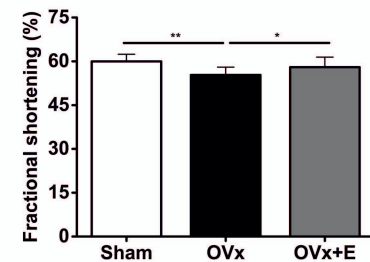


Fig. 2

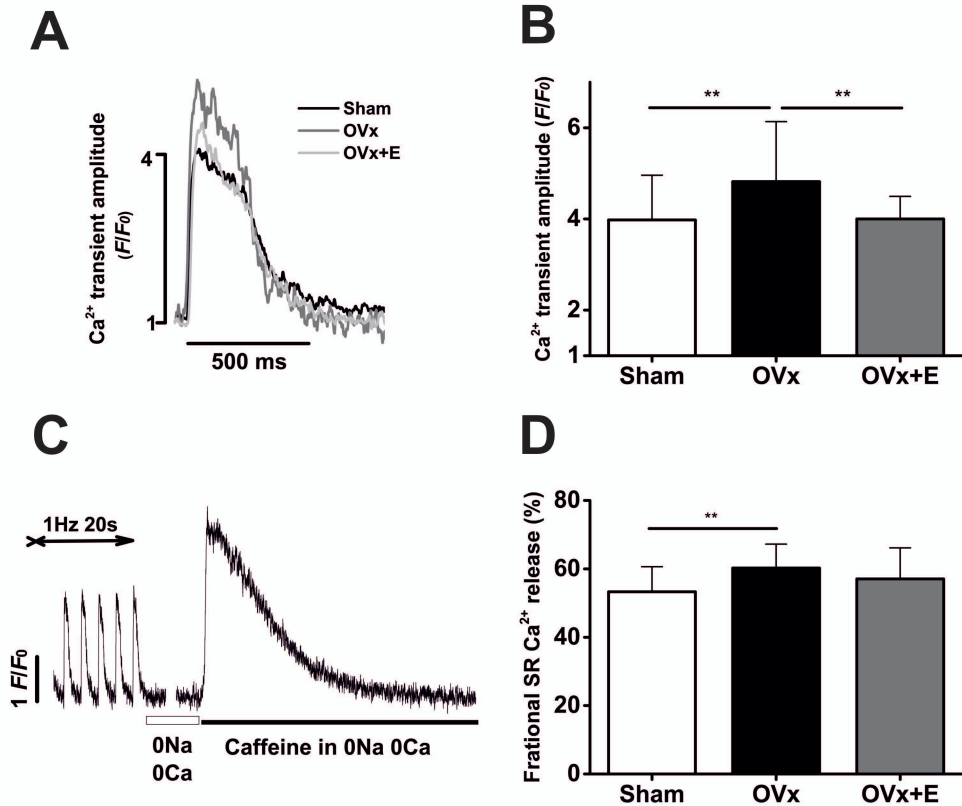
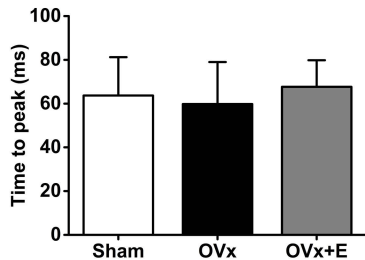
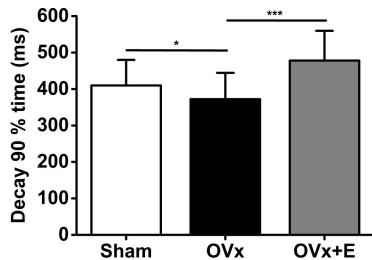


Fig. 3

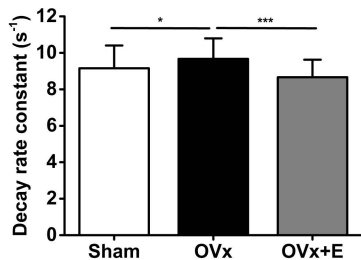
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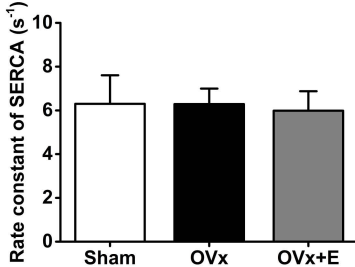
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C



D



E

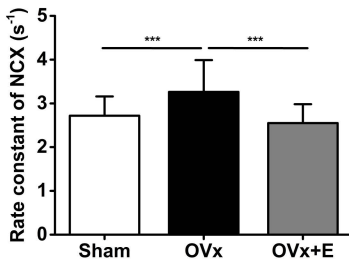
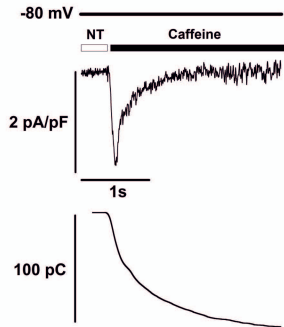


Fig. 4

A



B

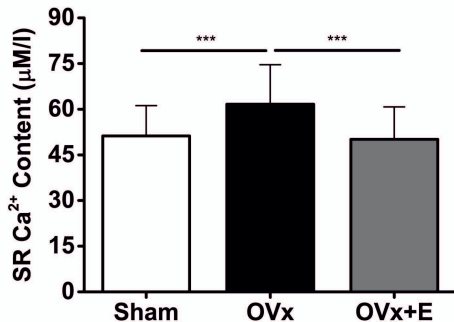
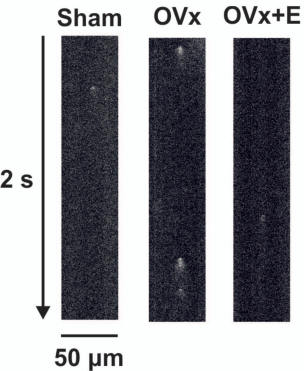


Fig. 5

A



B

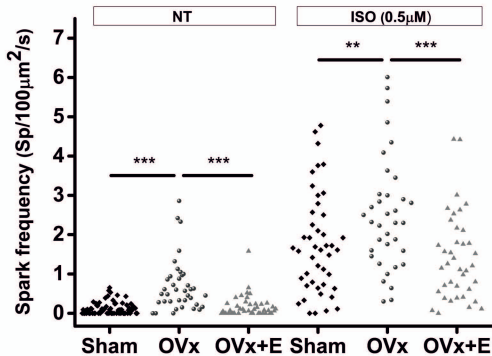
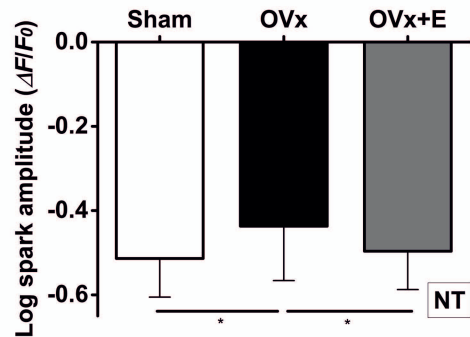
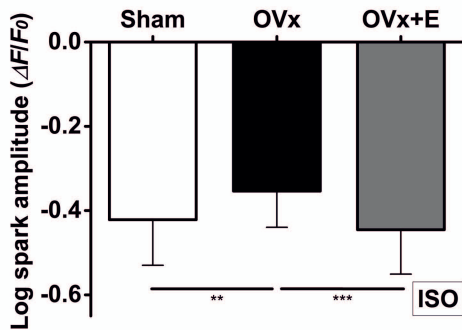


Fig. 6

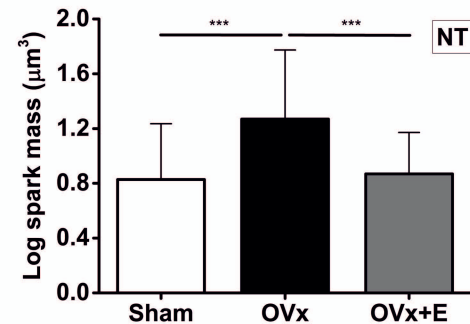
A



B



C



D

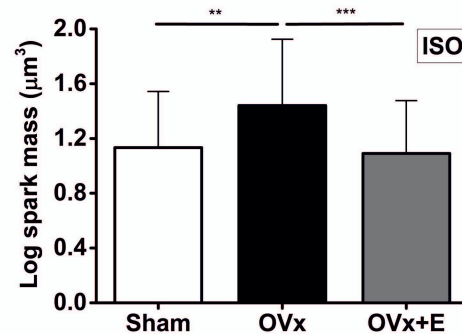
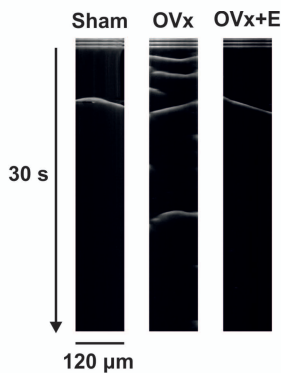
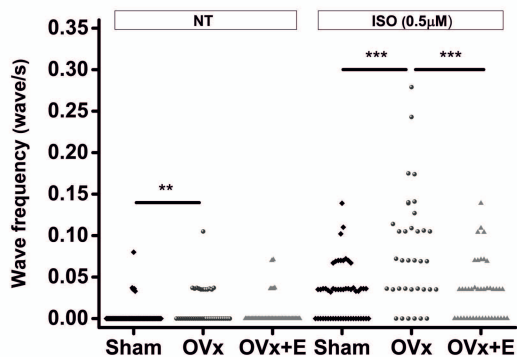


Fig. 7

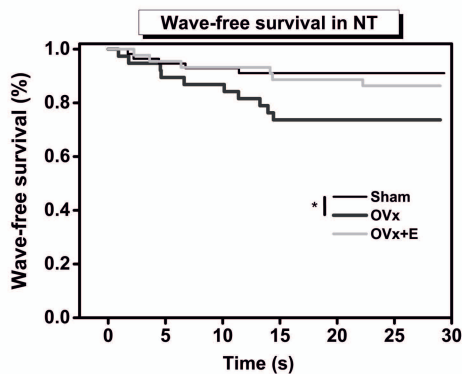
A



B



C



D

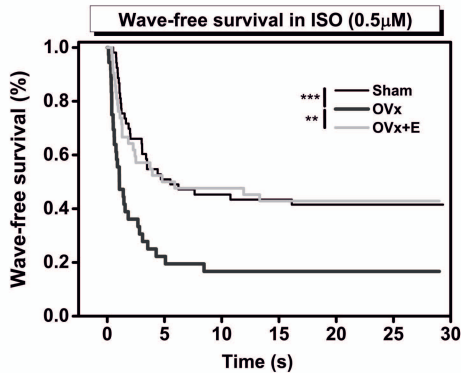
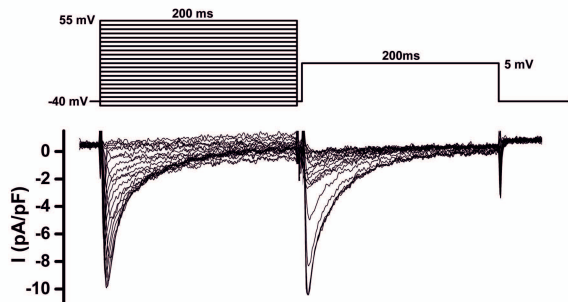
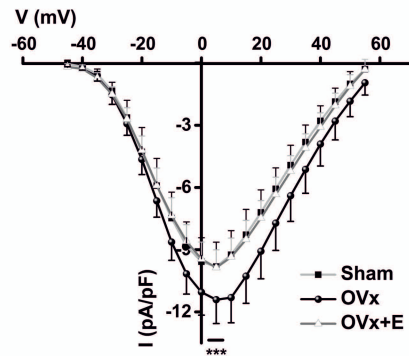


Fig. 8

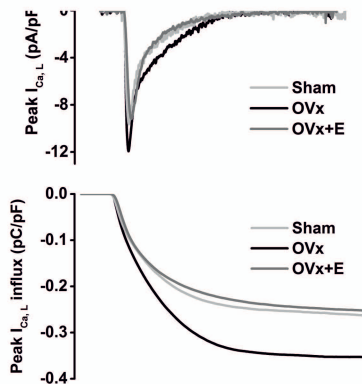
A



B



C



D

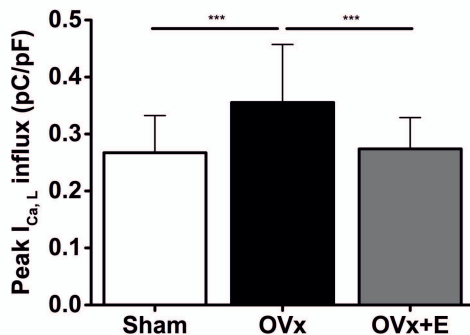
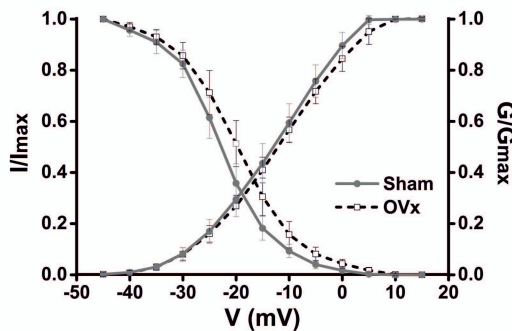
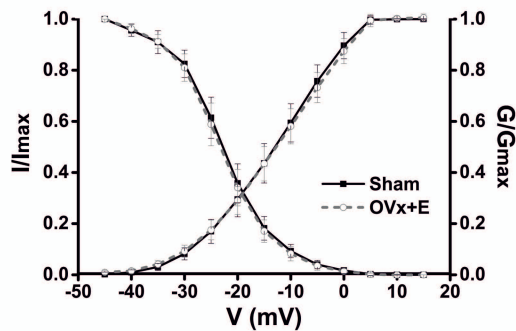


Fig. 9

A



B



C

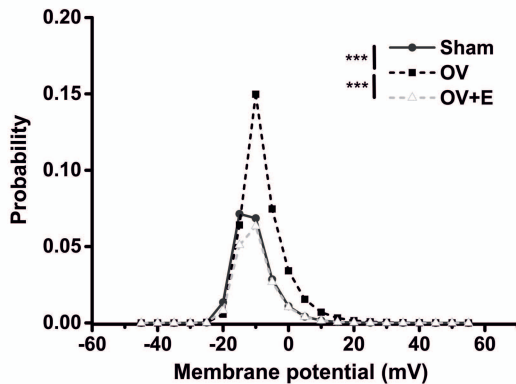
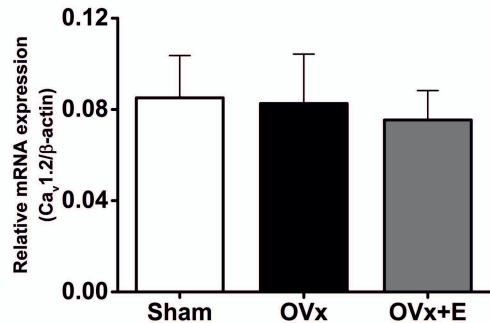


Fig. 10

A



B

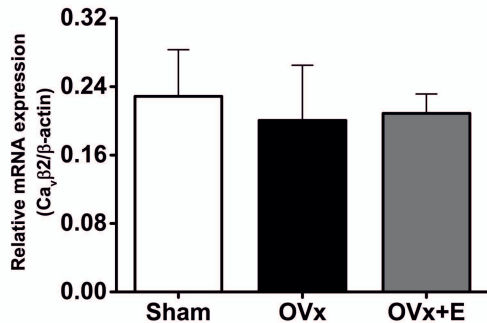


Fig. 11

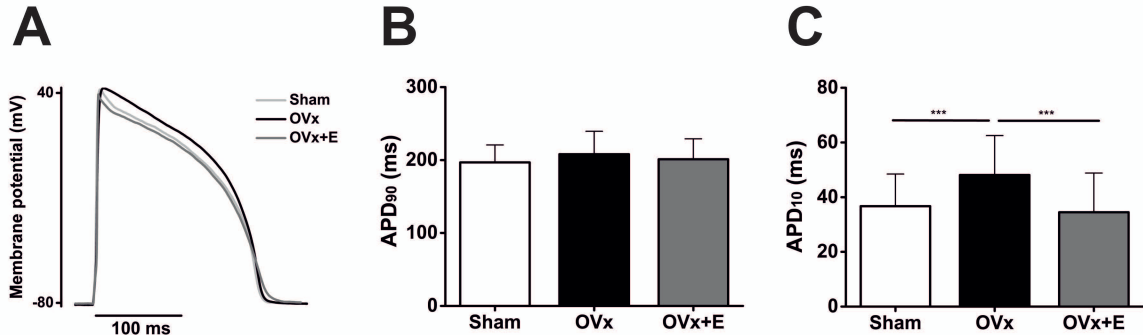


Fig. 12

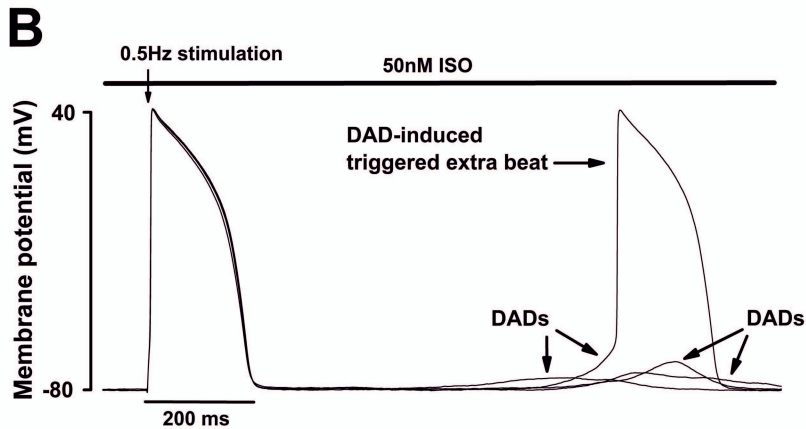
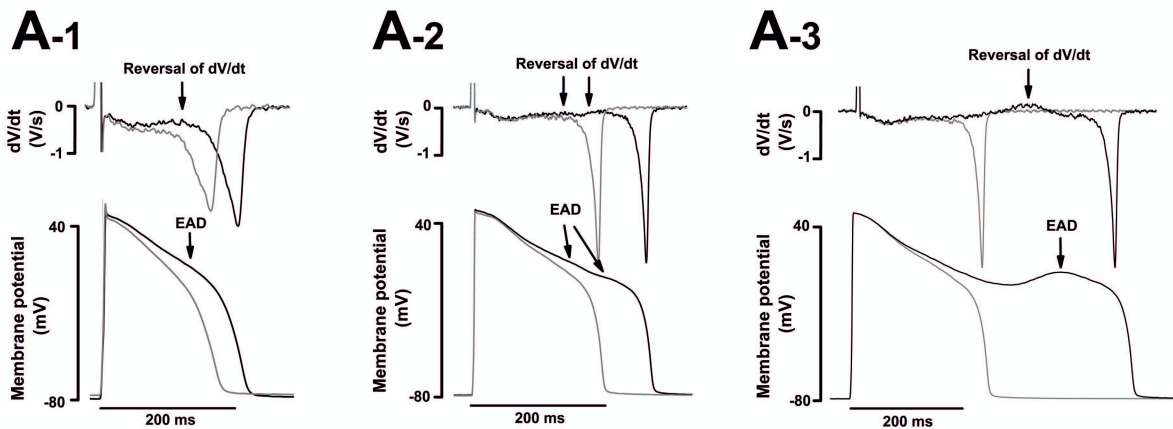
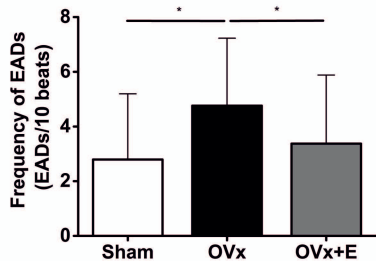
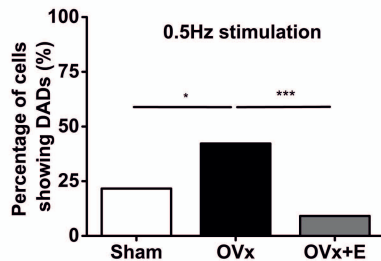


Fig. 13

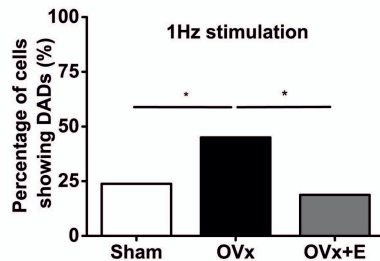
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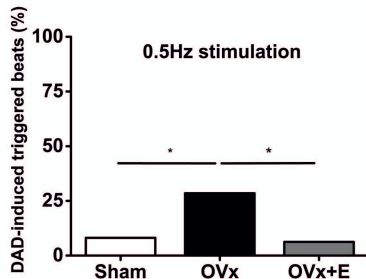
B



C



D



E

