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15 **Altered Na/Ca exchange distribution in ventricular myocytes from failing hearts**

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49   **Abstract**

50   In mammalian cardiac ventricular myocytes, Ca efflux via Na/Ca exchange (NCX) occurs predominantly at t-

51   tubules. Heart failure is associated with disrupted t-tubule structure, but its effect on t-tubule function is

52   less clear. We therefore investigated t-tubular NCX activity in ventricular myocytes isolated from rat hearts

53   ~18 weeks after coronary artery ligation (CAL) or corresponding Sham operation. NCX current (I_{NCX}) and L-

54   type Ca current (I_{Ca}) were recorded using the whole-cell voltage-clamp technique in intact and detubulated

55   (DT) myocytes; intracellular ($[Ca]_i$) was monitored simultaneously using fluo-4. I_{NCX} was activated and

56   measured during application of caffeine to release Ca from sarcoplasmic reticulum (SR). Whole-cell I_{NCX} was

57   not significantly different in Sham and CAL myocytes, and occurred predominantly in the t-tubules in Sham

58   myocytes. CAL was associated with redistribution of I_{NCX} and I_{Ca} away from the t-tubules to the cell surface,

59   and an increase in t-tubular I_{NCX}/I_{Ca} density from 0.12 in Sham to 0.30 in CAL myocytes. The decrease in t-

60   tubular I_{NCX} in CAL myocytes was accompanied by an increase in the fraction of Ca sequestered by SR.

61   However, SR Ca content was not significantly different in Sham, Sham DT and CAL myocytes, but was

62   significantly increased by DT of CAL myocytes. In Sham myocytes there was hysteresis between I_{NCX} and

63    $[Ca]_i$, which was absent in DT Sham, but present in CAL and DT CAL, myocytes. These data suggest altered

64   distribution of NCX in CAL myocytes.

65

66   **New and Noteworthy**

67   NCX is normally located predominantly in the t-tubules of cardiac ventricular myocytes. However

68   redistribution of NCX occurs in myocytes from failing hearts, resulting in more uniform distribution

69   between t-tubule and surface sarcolemma; this alters access of NCX to Ca released from SR, and thus

70   cellular Ca handling.

71

72

1. Introduction

Contraction of cardiac ventricular myocytes is initiated by Ca influx across the cell membrane via the L-type Ca current (I_{Ca}), which activates ryanodine receptors (RyRs) in adjacent sarcoplasmic reticulum (SR) membrane, triggering Ca-induced Ca release (CICR) from the SR. Relaxation occurs as a result of Ca removal from the cytoplasm into the SR, via a Ca ATPase (SERCA), and across the cell membrane, predominantly via Na/Ca exchange (NCX; 2).

In mammalian ventricular myocytes, I_{Ca}, CICR and Ca efflux via NCX occur predominantly at invaginations of the surface membrane, called t-tubules (5). Presumably because of this colocation, Ca close to the site of CICR appears to be more effective than bulk cytoplasmic Ca at stimulating Ca efflux via NCX, and thus – since NCX carries 3 Na for each Ca – the associated inward (depolarizing) current (I_{NCX}; 35). This is important not only for normal Ca efflux but also because under conditions of Ca overload, spontaneous SR Ca release occurs, activating inward I_{NCX} and causing delayed after-depolarisations (DADs), which can trigger action potentials and arrhythmias (18). Because of the proximity of RyRs and NCX, it seems likely that such activity will arise predominantly at t-tubules.

Heart failure (HF) is associated with disruption of the t-tubule network (21, 22), and redistribution of I_{Ca} from the t-tubules to the surface membrane, although RyR distribution appears unaltered (7). However the effect of HF on the distribution of I_{NCX} is unknown, but is important because changes may alter the proximity of NCX to the site of CICR, and thus I_{NCX} and Ca efflux. In the present study we therefore investigated the distribution of I_{NCX} between the t-tubule and surface membranes, and its functional consequences, in myocytes from normal and HF rats.

94 **2. Materials and Methods**

95 ***2.1 Surgical model of heart failure***

96 Ligation of the left anterior descending coronary artery of adult male Wistar rats (CAL) was performed as

97 described previously (7). The corresponding sham operation (Sham) was identical except that no tie was

98 placed around the coronary artery. All procedures were performed in accordance with UK legislation and

99 approved by the University of Bristol Ethics Committee. As reported in another study using cells from these

100 animals, CAL had no significant effect on body weight or tibia length, but significantly increased heart and

101 lung weights relative to body weight and tibia length, decreased ejection fraction and increased left

102 ventricular diastolic and systolic volumes, indicative of early stage heart failure (7).

103

104 ***2.2 Myocyte isolation and detubulation***

105 Myocytes were isolated from the left ventricular free wall and septum of Sham and CAL animals 18.6 ± 0.3

106 and 18.5 ± 0.3 weeks after surgery, respectively, as described previously (7), and stored for 2-8 hours

107 before use on the day of isolation. Myocyte detubulation (DT) - physical and functional uncoupling of the t-

108 tubules from the surface membrane - was achieved using formamide-induced osmotic shock, as described

109 previously (4, 6, 7).

110

111 ***2.3 Solutions***

112 All reagents were obtained from Sigma-Aldrich (Poole, UK) unless otherwise specified. Cells were

113 superfused with solution containing (in mmol/L): 133 NaCl, 1 $MgSO_4$, 1 $CaCl_2$, 1 Na_2HPO_4, 10 D-glucose, 10

114 HEPES, pH 7.4 (NaOH); 5 CsCl was added to inhibit K currents. The pipette solution contained (in mmol/L):

115 110 CsCl, 20 TEACl, 0.5 $MgCl_2$, 5 MgATP, 10 HEPES, 0.4 GTP-Tris, pH 7.2 (CsOH), and 0.1 pentapotassium salt

116 of the fluorescent Ca indicator fluo-4 (Life Technologies Ltd, Paisley, UK).

117

118 ***2.4 Measurement of I_{Ca} and I_{NCX}***

119 Myocytes were placed in a chamber mounted on a Diaphot inverted microscope (Nikon UK Ltd, Kingston-

120 upon-Thames, UK). Membrane currents and cell capacitance were recorded with the whole-cell patch-

121 clamp technique, using an Axopatch 200B patch clamp amplifier, a Digidata 1440A A/D converter and

122 pClamp 10 software (Molecular Devices (UK) Ltd., Reading, UK), which was used for data acquisition (at 2

123 kHz) and analysis. Pipette tip resistances were typically 1.5-3.0 MΩ when filled with pipette solution.

124   Holding potential was -80 mV; a 300 ms ramp to -40 mV was used to inactivate I_{Na}, followed by step

125 depolarization to 0 mV for 300 ms to activate I_{Ca}, at a frequency of 1 Hz. I_{Ca} was measured as the difference

126 between peak inward current and current at the end of the pulse to 0 mV. Once steady-state was achieved,

127 stimulation was stopped and, after 10 s quiescence, caffeine (10 mmol/L) was rapidly applied to the cell to

128 cause spatially and temporally uniform release of SR Ca (3); the resulting inward current due to Ca

129 extrusion via NCX was recorded at -80 mV. Following wash-off of caffeine, stimulation was restarted and

130 continued until a steady-state was reached. The protocol was then repeated, but $NiCl_2$ (10 mmol/L) was

131 applied 10 s before application of caffeine in the continued presence of Ni, to inhibit NCX.

132   Membrane currents were normalized to membrane capacitance (a function of membrane area) to

133 give current density. The distribution of membrane current between the surface and t-tubular membranes

134 was calculated from the currents measured in intact (whole cell) and DT (surface membrane only)

135 myocytes, as described previously (7).

136

137 ***2.5 Measurement of intracellular Ca***

138   Fluo-4 fluorescence was excited at 450-488 nm and emitted fluorescence collected at wavelengths

139 > 560 nm. Normalised Fluo-4 fluorescence (F/F_0) was converted to intracellular free Ca concentration ($[Ca]_i$)

140 as follows:

141   Equation 1: $$[Ca^{2+}]_i = \frac{K_d \, F/F_0}{\frac{K_d}{[Ca^{2+}]_{rest}} - F/F_0 + 1}$$

142 where resting Ca ($[Ca]_{rest}$) was assumed to be 0.1 µmol/L and the dissociation constant (K_d) for fluo-4 *in situ*

143 was 1.1 µmol/L (8, 20).

144   The rate of decay of Ca transients was obtained by fitting single exponential functions to the

145 declining phase of the I_{Ca}- and caffeine-induced Ca transients. The fitted rate constants (*k'*) were multiplied

146 by the appropriate (Sham or CAL) buffering power (calculated by plotting $[Ca]_i$ against $[Ca]_{total}$, as described

5

147   previously; 12, 33), to correct for the effect of buffering power and thus allow comparison of the rate of Ca

148   extrusion in Sham and CAL myocytes. This corrected rate constant (k) during application of caffeine (k_{Caff})

149   was used as an index of the rate of total sarcolemmal Ca efflux, and that in the presence of caffeine plus Ni

150   (k_{Ni}) as the rate of the slow (non-SR, non-NCX) Ca extrusion pathways. The rate of Ca removal via NCX (k_{NCX})

151   was calculated as k_{Caff}-k_{Ni}, and the rate of Ca uptake by SR (k_{SR}) was calculated as the difference between

152   the rate of decline of the I_{Ca}-induced Ca transient and k_{Caff}. These rate constants were also used to calculate

153   the percentage contribution of these pathways to Ca removal from the cell cytoplasm, as described

154   previously (25, 36).

155

156   ***2.6 Statistical analysis***

157   Data are expressed as mean ± SEM. The errors of derived variables, and the subsequent statistical analysis,

158   were calculated using propagation of errors from the constituent measurements. Student's t-tests and 2-

159   way ANOVA with the Bonferroni *post hoc* test were used as appropriate. Statistical significance was taken

160   as $p<0.05$. All statistical tests were performed on the number of cells. Sample sizes ('n-numbers') are given

161   as c/h, where c is the number of cells used from h hearts.

162

163

164 **3. Results**

165 ***3.1 The effect of CAL on NCX distribution***

166   Cell capacitance (a function of membrane area) was significantly larger in CAL myocytes

167 (240.2±20.8 pF (Sham) vs 375.0±63.0 pF (CAL); n=12/6 and 8/4, respectively; p=0.004); this was

168 accompanied by a non-significant increase in cell volume (33.5±5.1 pl (Sham) vs. 49.0+6.1 pl (CAL)) resulting

169 in no significant difference in cell surface area:volume ratio, as reported previously during cellular

170 hypertrophy (15, 29).

171   Figure 1A shows representative caffeine-induced Ca transients (top) and accompanying membrane

172 currents (bottom) recorded from Sham and CAL myocytes. Caffeine-induced Ca transient amplitude was

173 significantly smaller in CAL compared with Sham myocytes (2.42±0.63 µM (Sham) vs. 0.78±0.44 µM (CAL);

174 P<0.05) and buffering power, assessed as described in the Methods, was significantly larger in CAL

175 myocytes (134±66 (Sham) vs. 294±46 (CAL); p<0.01). Representative caffeine-induced Ca transients and I_{NCX}

176 in DT Sham and CAL cells are shown in Figure 1B: DT did not significantly alter peak $[Ca]_i$ in Sham cells

177 (2.06±0.69 µM), but increased peak $[Ca]_i$ in CAL cells (to 2.30±1.09 µM).

178   In Sham myocytes, the decline of the caffeine-induced Ca transient, corrected for buffering power

179 (see Methods), was significantly slowed by DT (k_{Caff}: 106.8±9.5 (Sham) vs 46.3±5.0 s^{-1} (Sham DT); p<0.001;

180 Figure 1C), consistent with loss of t-tubular Ca efflux pathways. In CAL myocytes, the decline of the

181 caffeine-induced Ca transient was not significantly different from Sham myocytes, and not significantly

182 altered by DT (k_{Caff}: 120.4±15.2 (CAL) vs 141.8±7.7 s^{-1} (CAL DT); Figure 1C), consistent with reduced t-tubular

183 sarcolemmal Ca efflux in CAL myocytes.

184   To investigate the role of NCX in these changes, exposure to caffeine was repeated in the presence

185 of Ni, to inhibit NCX. Figure 1D shows the rate of Ca extrusion via NCX (k_{NCX}); DT significantly decreased k_{NCX}

186 in Sham cells (86.8±7.8 (Sham) vs 34.3±4.8 s^{-1} (Sham DT); p<0.0001), compatible with loss of t-tubular NCX.

187 In CAL myocytes, k_{NCX} was not significantly different from Sham myocytes, and not significantly altered by

188 DT (102.5±114.5 (CAL) vs 102.2±8.0 s^{-1} (CAL DT)), implying that although the rate of Ca extrusion via NCX is

189 similar in Sham and CAL myocytes, there is little Ca extrusion via t-tubular NCX in these cells.

190   We used I_{NCX} in intact and DT myocytes to quantify its distribution between the surface and t-tubule

191   membranes. Since NCX activity depends on Ca adjacent to the exchanger, we measured I_{NCX} at a $[Ca]_i$ of 400

192   nmol/L during the descending phase of the caffeine transient, when Ca has been shown to be uniformly

193   distributed throughout the cytoplasm in both intact and DT cells (3). Figure 1E shows that whole cell I_{NCX}

194   density determined in this way was not significantly different in Sham and CAL myocytes, and that in Sham

195   myocytes, I_{NCX} density is significantly greater in the t-tubule membrane than at the cell surface, as reported

196   previously (14), resulting in a t-tubule:surface sarcolemma I_{NCX} ratio of 25:1. However, the distribution of

197   I_{NCX} is different in CAL myocytes, decreasing at the t-tubules by ~50%, and increasing at the surface

198   membrane by ~300%, resulting in no significant difference in I_{NCX} density between the two membranes in

199   these cells, and an I_{NCX} t-tubule: surface sarcolemma ratio of 3:1. This suggests that the slower Ca extrusion

200   via NCX following DT of Sham cells is due to loss of t-tubular NCX and that the lack of effect of DT on the

201   rate of Ca extrusion via NCX in CAL cells is due to its relocation away from t-tubules.

202

203   ***3.2 The effect of CAL on cellular Ca handling***

204   The preceding data show redistribution of I_{NCX} in CAL myocytes. NCX is one of the major Ca efflux pathways

205   which compete for cytoplasmic Ca (1, 25), so that a decrease of NCX activity at the t-tubules (the site of

206   CICR and SERCA; 24, 32), might alter the balance of Ca removal via NCX and SR (30), thereby altering SR Ca

207   content, and thus Ca release and I_{NCX}. We therefore determined the effect of CAL on the contribution of

208   different pathways to Ca removal.

209   Figure 2A shows the percentage contribution of different pathways to Ca removal from the

210   cytoplasm. There was no significant difference in the contribution to Ca removal by the "slow" Ca extrusion

211   pathways (sarcolemmal Ca ATPase and mitochondria) between the 4 groups. In Sham myocytes, DT caused

212   a small decrease in the fraction of Ca removed via NCX and increase in Ca removal by the SR, consistent

213   with loss of NCX following DT. In CAL myocytes, the fraction of Ca removed via NCX was smaller than in

214   Sham cells, despite no significant change in whole-cell I_{NCX} density at a given free $[Ca]_i$ (Figure 1E); this was

215   accompanied by a significant increase in the fraction of Ca removed by SR. DT of CAL myocytes had no

216   significant effect on the fraction of Ca removed via NCX or SR, consistent with less t-tubular NCX activity in

8

217   these cells. These data suggest decreased access of NCX to Ca ions, and thus Ca efflux, in CAL compared to

218   Sham myocytes, presumably as a result of its change in location away from the site of SR Ca release at the

219   t-tubules, possibly exacerbated by decreased Ca release at the t-tubules as a result of redistribution of I_{Ca}

220   and altered t-tubule morphology (7, 21). They also suggest that this redistribution leads to an increase in

221   fractional SR Ca uptake, which would be expected to increase SR Ca release, given its steep dependence on

222   SR Ca content (26, 34).

223   To determine whether the changes in NCX distribution were quantitatively different from the

224   redistribution of I_{Ca} reported previously (7), which might alter Ca balance across the t-tubule membrane,

225   and thus the effect of loss of t-tubules during HF on cellular Ca balance, we calculated I_{Ca} distribution in

226   Sham and CAL myocytes. We have previously reported changes in I_{Ca} distribution in CAL myocytes (7) when

227   Ca was buffered using BAPTA in the patch pipette. It was therefore possible that the redistribution of I_{Ca}

228   might differ under the present conditions, in which no exogenous Ca buffer, apart from fluo-4, was present.

229   However, in agreement with previous work, DT of Sham myocytes decreased I_{Ca} density, from -3.71±0.25 to

230   -2.70±0.25 pA/pF, while DT of CAL myocytes had no significant effect on I_{Ca} density (-2.71±0.19 (CAL) vs -

231   2.93±0.21 pA/pF (CAL DT)). These data were used to calculate the distribution of I_{Ca} between the t-tubule

232   and surface membranes, as described previously (7). Figure 2B shows that, consistent with previous work

233   (7), I_{Ca} density was significantly higher in the t-tubules of Sham myocytes than in their surface membrane,

234   and that in CAL myocytes, t-tubular I_{Ca} density was significantly smaller than in Sham myocytes (t-tubular

235   I_{Ca}: -9.39±3.70 (Sham) vs -1.80±0.99 pA/pF (CAL); n=12/6 and 8/4, respectively; P<0.05), and not

236   significantly different from I_{Ca} density in the surface membrane. Since CAL has little effect of the rate of

237   inactivation of I_{Ca} at the t-tubule or surface membranes (7), Ca influx via I_{Ca} will reflect these changes in

238   current density.

239   The ratio of t-tubular I_{NCX}/I_{Ca} density obtained from these data is 0.12 in Sham myocytes and 0.30 in

240   CAL myocytes. Thus t-tubular density of I_{NCX} relative to I_{Ca} is greater in CAL than in Sham myocytes; this will

241   result in DT causing greater loss of NCX (and thus Ca efflux) relative to I_{Ca} (and thus Ca influx) in CAL cells,

242   which would be expected to increase cellular Ca loading. This is consistent with the observation that SR Ca

243   content was not significantly affected by DT in Sham myocytes, but was significantly increased (P<0.05) by

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244 DT in CAL myocytes (SR Ca content: 78.8±8.2 μM (Sham); 63.3±9.8 μM (Sham DT); 58.7±5.8 μM (CAL);

245 105.4±17.4 μM (CAL DT)).

246

247 **3.3 The effect of CAL on the relationship between $[Ca]_i$ and I_{NCX}**

248 The data above suggest that decreased t-tubular NCX in CAL myocytes decreases access of NCX to Ca

249 released from the SR, thereby increasing fractional SR Ca uptake; they also suggest that SR Ca load might be

250 increased following loss of t-tubules, as a result of the relative changes in Ca influx and efflux. The

251 consequent increase in SR Ca content will, by altering release, alter NCX activity. However, I_{NCX} activity may

252 also be altered directly as a result of the change in the colocation of NCX and RyRs.

253 Previous work has shown hysteresis in the relationship between bulk cytoplasmic Ca and I_{NCX} during

254 spontaneous and caffeine-induced Ca release, with a larger current for a given Ca when Ca is increasing

255 than when it is decreasing (35). We have proposed that this is because I_{NCX} occurs predominantly at t-

256 tubules, where NCX will be exposed to a higher Ca than that in the bulk cytoplasm during Ca release (28). If

257 so, DT would be expected to reduce the hysteresis, and CAL to change it because of altered NCX

258 distribution.

259 To test this idea we plotted free $[Ca]_i$ against current density during application of caffeine for each

260 of the 4 groups of cells. Figure 3 shows the average hysteresis loops for each group of cells; each loop

261 consists of the data from all the cells in that group. The left panels show the relationship in intact (top) and

262 DT (bottom) Sham myocytes. Intact cells show a hysteresis, as described previously (35), with more current

263 at a given $[Ca]_i$ during the rising phase. However this hysteresis is reduced in DT myocytes, consistent with

264 idea that the hysteresis arises at the t-tubules, due to proximity of NCX to SR Ca release via RyRs.

265 The right panels show similar traces obtained from CAL myocytes. The loop obtained in CAL

266 myocytes was smaller than in Sham cells, presumably because the mean rise of free $[Ca]_i$ was smaller in

267 these cells (Figure 1A), although the hysteresis was maintained (see inset). Interestingly, the hysteresis is

268 still apparent in DT CAL myocytes, suggesting that the hysteresis arises at the surface membrane, in

269 contrast to its t-tubular location in Sham myocytes, and consistent with redistribution of NCX to the surface

270 membrane in these cells.

10

271

272 **4. Discussion**

273 The present study shows that in CAL myocytes NCX activity decreases at the t-tubules and increases at the

274 surface membrane. This is similar to the redistribution of I_{Ca} that we have reported previously (7), and is

275 associated with changes in the fraction of Ca removed by NCX and SR, the balance of Ca influx vs efflux

276 across the t-tubule membrane, and the relationship between $[Ca]_i$ and I_{NCX}. This is important in

277 understanding the role of the t-tubules, and their loss, in Ca handling and the generation of DADs in HF.

278

279 **4.1 Redistribution of NCX in CAL myocytes**

280 I_{NCX} decreases at the t-tubules and increases at the cell surface of CAL myocytes, with no change in whole-

281 cell current. Although I_{NCX} depends on Ca at the cytoplasmic face of the exchanger, this redistribution is

282 unlikely to be due to local differences in Ca, because caffeine was used to release total SR Ca content and

283 I_{NCX} distribution calculated at a fixed Ca, late during the descending phase of the caffeine transient, when

284 Ca, and thus stimulation of NCX, is likely to be uniform throughout the cell. The redistribution is also

285 unlikely to be due to changes in t-tubule density, which is unaltered in this model of heart failure (7), nor is

286 it likely that a change in surface area: volume ratio, which was not significantly different in Sham and CAL

287 myocytes, contributes to the observed changes.

288 The mechanism of redistribution is unclear, although reminiscent of the redistribution of beta-2

289 adrenoceptors and I_{Ca} from their normal t-tubular location to a more uniform distribution in cells from

290 failing hearts (6, 7, 27). It has been suggested that localisation of NCX activity at the t-tubules is due, in part,

291 to local protein kinase A (PKA) activity (10); however PKA activity appears to increase at the t-tubules in CAL

292 myocytes (7) making it unlikely that this can explain the decrease in t-tubular I_{NCX} observed in these cells.

293 These changes may reflect reversion in HF towards a more neonatal phenotype, in which cell activation is

294 dependent upon Ca influx and efflux across the surface, rather than t-tubule, membrane (11, 31), and a

295 general loss of t-tubular protein localisation.

296

297 **4.2 Functional consequences of redistribution**

11

298 Computer modelling suggests that the relative location of NCX, SERCA and sarcolemmal Ca ATPase alters

299 their ability to compete for cytoplasmic Ca, and thus the amount of Ca removed by each pathway (30). The

300 present work shows that DT of Sham myocytes decreases Ca efflux via NCX, as a result of loss of t-tubular

301 NCX, and increases SR Ca uptake. A similar decrease in Ca efflux via NCX, and increased Ca uptake via

302 SERCA, occurred in CAL myocytes, compared to Sham cells, even though total NCX density at a given $[Ca]_i$ is

303 the same in CAL and Sham myocytes. This can be explained by redistribution of NCX away from the t-

304 tubules in CAL cells, so that it no longer has "privileged" access to Ca released from the SR as a result of its

305 proximity to RyRs; this will reduce Ca extrusion via NCX, and allow a greater fraction of the cytoplasmic Ca

306 to be removed by SERCA, much of which also appears to be located at t-tubules (24), where Ca release

307 occurs. Although a large fractional decrease in Ca extrusion via NCX results in a relatively small fractional

308 increase in SR Ca uptake, this reflects the relatively small fraction of Ca removed by NCX compared to the

309 SR. Altered NCX location in CAL myocytes, which was measured when cytoplasmic Ca was relatively

310 uniformly distributed, is likely to be important during the systolic Ca transient, since NCX activity close to

311 the site of CICR at the t-tubules, where the majority of Ca efflux normally occurs (Figure 1D), will be

312 reduced, although this may be offset by reduced Ca release at the t-tubules due to decreased t-tubular I_{Ca}.

313   It is notable that although fractional SR Ca uptake was greater in CAL than in Sham myocytes

314 (Figure 2A), SR Ca content was not significantly different, and the caffeine-induced rise of cytoplasmic Ca

315 was smaller (Figure 1A). However, calculated peak $[Ca]_i$ depends on $[Ca]_{rest}$, which was taken as 0.1 μmol/L

316 in the present study; increasing $[Ca]_{rest}$ would increase peak $[Ca]_i$, but previous reports of $[Ca]_{rest}$ in CAL have

317 been inconsistent, showing an increase, decrease, or no change (9, 17, 19, 37). Nevertheless the present

318 observations may be reconciled by increased *fractional* SR Ca uptake being offset by decreased local I_{Ca},

319 which would tend to decrease SR Ca content, and the increased Ca buffering observed in CAL myocytes,

320 which would decrease free Ca for a given release. This may also account for the greater slope of the

321 relationship between Ca and I_{NCX} in CAL myocytes (Figure 3), since a given Ca extrusion would result in a

322 smaller change in free Ca. However DT increased SR Ca content sufficiently to cause a larger caffeine-

323 induced rise of cytoplasmic Ca with hysteresis evident in I_{NCX} between the rising and falling phases of Ca

324 release. An alternative explanation for the increased SR Ca uptake is increased SERCA activity in CAL

12

325 myocytes, although this seems unlikely, since previous work has shown decreased SERCA activity in HF (16)

326 and this alone would not explain the lack of effect of DT on the contribution of different efflux pathways to

327 Ca removal.

328 It is also notable that despite the decreased percentage contribution of NCX to Ca removal in CAL

329 myocytes, k_{NCX} was not significantly different from that in Sham cells. Thus it appears that NCX can rapidly

330 remove Ca from the cytoplasm in the absence of a functional SR (k_{NCX}) but its fractional contribution is

331 decreased, presumably because its ability to compete with SERCA is decreased as a result of its relocation.

332 This redistribution of I_{NCX} is also likely to be important because HF is associated with disorganisation

333 and loss of t-tubules (13, 21–23), and redistribution of I_{Ca} (and thus Ca release) from the t-tubules to the

334 surface membrane. The present work shows that t-tubular I_{NCX}/I_{Ca} density is higher in CAL myocytes than in

335 Sham, suggesting that loss of t-tubules will lead to greater loss of Ca efflux, compared to influx, in CAL

336 myocytes, and thus greater Ca accumulation, consistent with the observed effect of DT on SR Ca content in

337 these cells. Thus loss of t-tubules in HF may result in increased SR Ca content, which will increase both

338 systolic Ca release and the probability of spontaneous SR Ca release, and thus of DADs.

339 The proximity of the majority of NCX adjacent to RyRs at the t-tubules may also be important in the

340 genesis of arrhythmias due to activation of NCX by spontaneous SR Ca release in conditions of Ca overload

341 (28). The hysteresis between bulk cytoplasmic Ca and I_{NCX} observed during application of caffeine or during

342 spontaneous Ca release (35; Figure 3) is consistent with Ca released from SR having privileged access to

343 NCX. The observation that DT of Sham myocytes decreased this hysteresis suggests that it arises at the t-

344 tubules as a result of the proximity of the majority of NCX to the site of Ca release in the t-tubules.

345 However this hysteresis was evident in CAL and DT CAL myocytes, so that it appears to be occurring at the

346 surface of these cells. The hysteresis in CAL cells cannot be explained by a change in $[Ca]_{rest}$ altering the

347 calibration of $[Ca]_i$, which would alter the x-axis gain of the hysteresis loops, but the hysteresis would

348 remain. It seems likely, therefore, that the hysteresis in CAL myocytes is due to the redistribution of I_{NCX} to

349 the surface membrane resulting in enhanced I_{NCX} in response to Ca at the cell surface, which itself may be

350 increased by the observed redistribution of I_{Ca}, even in the apparent absence of changes in RyR distribution

351 (7). Thus it appears that privileged access occurs at the cell surface in CAL myocytes, so that loss of t-

13

352   tubules in HF may not protect against DADs, which may be generated at the cell surface and exacerbated

353   by the increase in SR Ca content that accompanies loss of t-tubules in these cells.

354

355   **4.3 Conclusions**

356   These data suggest that the cellular distribution of NCX is altered in CAL myocytes, and that this will alter

357   NCX activity both directly, by altering the proximity of NCX to the site of SR Ca release, and indirectly, by

358   increasing SR Ca uptake, both in intact myocytes, by decreasing the ability of NCX to compete with SERCA,

359   and following loss of t-tubules, which will result in greater loss of NCX than I_{Ca}. These changes will alter I_{NCX},

360   and thus action potential configuration, Ca balance, and the probability, magnitude and site of DAD

361   generation in HF.

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369   **Disclosures**

370   None

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Figure legends

466

467   Figure 1. A. Representative records of $[Ca]_i$ and the associated inward current (I_{NCX}) during the application

468   of 10 mM caffeine (open bar) in Sham and CAL cells. Scale bars represent 2 s. B. Representative records of

469   $[Ca]_i$ and the associated inward current (I_{NCX}) during the application of 10 mM caffeine (open bar) in DT

470   Sham and CAL cells. Scale bars represent 2 s. C. Mean rate constants for the decline of the caffeine-induced

471   Ca transient (k_{Caff}); n=12/6, 11/5, 8/4, 7/4 for Sham, Sham DT, CAL and CAL DT respectively. D. Mean k_{NCX};

472   n= 12/6, 11/5, 8/4, 5/3 for Sham, Sham DT, CAL and CAL DT respectively. E. I_{NCX} density in the whole cell,

473   and at the surface and t-tubule membranes, determined at 400 nM $[Ca]_i$ during the declining phase of the

474   caffeine transient. *P<0.05, **P<0.01, ***P<0.001 and **** P<0.0001 with Bonferroni post-test.

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477   Figure 2. A. Mean percentage contribution of different pathways to Ca removal from the cytoplasm of

478   Sham, DT Sham, CAL and DT CAL cells; n=12/6, 11/5, 8/4, 5/3 respectively. B. Distribution of I_{Ca} density

479   between the surface and t-tubule membranes; n=12/6 and 8/4 for Sham and CAL respectively; only

480   statistical comparisons between t-tubule and surface membranes are shown. * P<0.05, ** P<0.01 and ***

481   P<0.001 with Bonferroni post-test.

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484   Figure 3. Averaged hysteresis loops for I_{NCX} density *vs* $[Ca]_i$ during application of caffeine in Sham, DT Sham,

485   CAL and DT CAL myocytes. n=11/5, 9/5, 7/4, 7/4 respectively. Inset for CAL shows loop on expanded scales:

486   X and Y-scale bars represent 0.1 µM and 0.2 pA/pF respectively.

487
488

A.

B.

SHAM

CAL

SHAM DT

CAL DT