Coupions in rat atria form distinct subgroups defined by their molecular partners

Meredith N. Schulson, David R. L. Scriven, Patrick Fletcher and Edwin D. W. Moore*

Department of Cellular and Physiological Sciences, University of British Columbia, Life Sciences Institute, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada

*Author for correspondence (edmoore@interchange.ubc.ca)

Accepted 5 November 2010

Journal of Cell Science 124, 1167-1174

© 2011. Published by The Company of Biologists Ltd

doi:10.1242/jcs.080929

Summary
Standard local control theory, which describes Ca\(^{2+}\) release during excitation–contraction coupling (ECC), assumes that all ryanodine receptor 2 (RyR2) complexes are equivalent. Findings from our laboratory have called this assumption into question. Specifically, we have shown that the RyR2 complexes in ventricular myocytes are different, depending on their location within the cell. This has led us to hypothesize that similar differences occur within the rat atrial cell. To test this hypothesis, we have triple-labelled enzymatically isolated fixed myocytes to examine the distribution and colocalization of RyR2, calsequestrin (Casq), voltage-gated Ca\(^{2+}\) channels (Ca\(v\)1.2), the sodium–calcium exchanger (Ncx) and caveolin-3 (Cav3). A number of different surface RyR2 populations were identified, and one of these groups, in which RyR2, Ca\(v\)1.2 and Ncx colocalized, might provide the structural basis for ‘eager’ sites of Ca\(^{2+}\) release in atria. A small percentage of the dyads containing RyR2 and Ca\(v\)1.2 were colocalized with Cav3, and therefore could be influenced by the signalling molecules it anchors. The majority of the RyR2 clusters were tightly linked to Ca\(v\)1.2, and, whereas some were coupled to both Ca\(v\)1.2 and Ncx, none were with Ncx alone. This suggests that Ca\(v\)1.2-mediated Ca\(^{2+}\)-induced Ca\(^{2+}\) release is the primary method of ECC. The two molecules studied that were found in the interior of atrial cells, RyR2 and Casq, showed significantly less colocalization and a reduced nearest-neighbour distance in the interior, compared with the surface of the cell. These differences might result in a higher excitability for RyR2 in the interior of the cells, facilitating the spread of excitation from the periphery to the centre. We also present morphometric data for all of the molecules studied, as well as for those colocalizations found to be significant.

Key words: Atrial myocyte, Excitation–contraction coupling, Ryanodine receptor, Ca\(v\)1.2, Sodium–Calcium exchanger, Caveolin-3, Calsequestrin

Introduction
Excitation–contraction coupling (ECC) in both atrial and ventricular myocytes depends on Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). During CICR, a small influx of Ca\(^{2+}\) through voltage-gated calcium channels (Ca\(v\)1.2) initiates a much larger release of Ca\(^{2+}\) from type 2 ryanodine receptors (RyR2) located in the adjacent junctional sarcoplasmic reticulum (SR) (Fabioti, 1983). In ventricular cells, a well-developed transverse and axial tubular system (TATS) spreads depolarization into the myocyte, resulting in a nearly synchronous SR Ca\(^{2+}\) release throughout the entire cell (Wier et al., 1995). Most atrial cells, however, lack such tubules and show a unique spatiotemporal pattern of Ca\(^{2+}\) release during ECC, characterized by a subsarcolemmal ring of elevated Ca\(^{2+}\) concentration, which variably propagates into the central bulk of the myocyte in direct proportion to cellular Ca\(^{2+}\) load and Ca\(^{2+}\) influx (Berlin, 1995; Huser et al., 1996; Mackenzie et al., 2001). Mackenzie and colleagues (Mackenzie et al., 2001) also showed that there are so-called ‘eager sites’ (those sites that respond very quickly to rises in Ca\(^{2+}\) concentration) along the periphery of atrial myocytes; these are separated by one or more ‘failure sites’, which exhibit weak regenerative Ca\(^{2+}\) responses with approximately one-quarter the magnitude of the ‘eager’ sites. Ca\(v\)1.2 channels are primarily distributed along the periphery of atrial myocytes and are highly colocalized with RyR2 (Carl et al., 1995). However, although this colocalization explains the responsiveness of peripheral sites to electrical depolarization, it does not explain why the so-called eager and failure sites, both classified as junctional RyR2, respond so differently to electrical stimulation.

Caveolin-3 (Cav3), the dominant isoform of caveolin in cardiomyocytes, is responsible for the formation of lipid rafts and caveolae in the cell membrane, but it is also found outside of these two structures (Patel et al., 2008). Rafts and/or caveolae have been postulated to play an important role in ECC; in the adult rat ventricular myocyte, removing cholesterol and disrupting the caveolae and/or rafts using methyl-\(\beta\)-cyclodextrin (MBC) reduced both the amplitude of the [Ca\(^{2+}\)] transient and the contraction (Calaghan and White, 2006). The use of MBC in rat arterial smooth muscle and neonatal cardiomyocytes, which lack a defined TATS, much like atrial myocytes, resulted in a reduction in the frequency, width and amplitude of Ca\(^{2+}\) sparks, without modulation of the current through the Ca\(v\)1.2 channels (Lohn et al., 2000). Their data suggest that caveolin might have a role in regulating local SR Ca\(^{2+}\) release, and might indicate the presence of Ca\(v\)1.2 channels near Cav3.
The type 1 sodium–calcium exchanger (Ncx) is known to be the sole member of the Ncx family of proteins present in the cardiomyocyte (Quednau et al., 1997) and has long been implicated in the regulation of ECC. First viewed as a possible alternative mechanism by which to activate CICR, it is now largely agreed that Ncx acts as a modulator of the process in cardiac myocytes (Bers, 2008). Structural data (Scriven et al., 2005) demonstrate that, whereas both couplons (Cav1.2 and RyR2) and Ncx molecules are closely associated with Cav3 on the surface of rat ventricular myocytes, they are only sparsely colocalized with one another within the cell interior. However, other studies have shown that Ncx is separate from Cav3, both in developing (Lin et al., 2009) and adult (Cavalli et al., 2007) ventricular cardiomyocytes. Owing to a lack of a well-developed TATS, Ncx is largely distributed along the cell periphery in atria (Bootman et al., 2006) but might colocalize with proteins such as RyR2 and Cav3 channels there. If so, it could act as an important modulator of ECC.

**Results**

**Controls**

Before beginning the triple-labelling experiments, a series of controls was performed in order to ensure the validity of our protocols. First, rat atrial myocytes were labelled with antibodies against RyR2, Cav1.2, Casq, Ncx or Cav3 individually. This was done in order to examine not only the distribution of each protein, but also to ensure that the labelling characteristics did not change when the cells were incubated with multiple antibodies simultaneously. Deconvolved images from the surface and interior (left- and right-hand panels respectively, for each pair of images) of the labelled cells are shown in Fig. 1. At the surface (Fig. 1C, left-hand panel), clusters of Cav1.2 were spread evenly across the membrane. Ncx labelling on the surface (Fig. 1D, left-hand panel), by contrast, was dense but somewhat uneven, and appeared as even larger aggregates than those of Cav1.2. Note that the very bright staining at one side of the membrane is an artefact created by imaging the sarcolemma in parallel with the optical axis of the microscope (edge effect). An antibody directed against the Cav3 protein also collected in large clusters on the sarcolemmal membrane (Fig. 1E, left-hand panel) that appeared to be oriented in longitudinal striations. Cav1.2, Ncx and Cav3 proteins were only expressed to a very small degree in the interior of atrial myocytes (Fig. 1C–E, right-hand panels) and the antibodies against these three proteins label only faintly in this region. This labelling probably represents either synthesis and/or degradation of the proteins or the presence of rudimentary t-tubules in some atrial cells.

The ability of the Zenon kits to eliminate cross-reaction between the secondary antibodies against two monoclonal primary antibodies was tested using primary antibodies against proteins that are known to localize to distinct regions of the cell (RyR2 and the nuclear pore complex). We labelled myocytes simultaneously with these two antibodies using the manufacturer’s protocol and were able to confirm that there was no cross-talk between the two labels (supplementary material Fig. S1). The results of these control experiments confirmed that we could label with two monoclonal antibodies simultaneously without fear of cross-reaction.

The final step in confirming the validity of our triple-labelling experiments was to compare the colocalization values between pairs of proteins that occurred in more than one experiment, to see

**Fig. 1. Single-labelling of atrial myocytes.** An atrial myocyte segment labelled with antibodies against RyR2 (A), Casq (B), Cav1.2 (C), Ncx (D) or Cav3 (E). For each antibody, the image shown is either a 1.5-μm-thick section taken from the cell surface (left panels), or a single 250-nm plane taken from approximately the middle of the cell (right panels). For the interior segments of RyR2 (A) and Casq (B), there is a discrete subsarcolemmal ‘gap’ with no labelling of either protein (upper inset, arrows), whereas the lower inset shows that some longitudinal elements of labelling are present (arrowheads). Scale bar: 5 μm.
whether they were the same as one another. We found no significant differences (see supplementary material Table S1 for a detailed analysis), and, for this reason, pooled the results for the dual colocalizations that were common to more than one experiment (Table 1). With the completion of these control experiments and calculations, we were confident that the labelling characteristics of each protein involved in our triple-labelling experiments are independent of one another and that our results are a true indication of the structural organization of atrial myocytes and the association of these proteins within the cells.

**Triple-labelling experiments**

The first of three triple-labelling experiments reported here examines the potential association between RyR2, Ncx and Ca\(\text{\textsubscript{1.2}}\) in rat atria. Fig. 2 shows a section from a representative atrial cell, with RyR2 in blue, Ncx in green and Ca\(\text{\textsubscript{1.2}}\) in red. Each image (A–C) is the same 1-\(\mu\)m-thick layer from the surface of the myocyte. Fig. 2A shows the entire field of view; this exhibited a significant edge effect because of the abundance of Ncx labelling. In this region, the cell curvature caused an increase in apparent object colocalization owing to the reduced resolution along the P-axis.

In this region, the cell curvature caused an increase in apparent colocalization owing to the reduced resolution along the Z-axis. To prevent the edge effect from causing errors in our calculations, we used the cell segment shown in Fig. 2B (outlined segment in Fig. 2A) to measure colocalization. Fig. 2C shows the colocalization between the three molecules. The primary association appears to be between RyR2 and Ca\(\text{\textsubscript{1.2}}\) (magenta; Table 1). There were also a significant number of RyR2–Ca\(\text{\textsubscript{1.2}}\)–Ncx triplets (white; Table 2). Neither the Ncx–RyR2 (cyan) nor the Ca\(\text{\textsubscript{1.2}}\)–Ncx (yellow) represent significant colocalizations (Table 1). Ncx seems to be mainly organized into large clusters that are either completely separate from the RyR2–Ca\(\text{\textsubscript{1.2}}\) unit, and do not associate with either protein (e.g. Fig. 2B), or that are integral to the RyR2–Ca\(\text{\textsubscript{1.2}}\) unit.

Table 1. Dual colocalizations

<table>
<thead>
<tr>
<th>Molecule pairs</th>
<th>Colocalization (%)</th>
<th>Reverse pairs</th>
<th>Colocalization (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RyR2–Cav3</td>
<td>8.36±2.40</td>
<td>Cav3–RyR2</td>
<td>7.77±2.60</td>
<td>5</td>
</tr>
<tr>
<td>RyR2–Ncx</td>
<td>10.02±1.29</td>
<td>Ncx–RyR2</td>
<td>8.17±1.19</td>
<td>17</td>
</tr>
<tr>
<td>RyR2–Caspq</td>
<td>61.31±3.08</td>
<td>Caspq–RyR2</td>
<td>54.47±2.39</td>
<td>9</td>
</tr>
<tr>
<td>RyR2–Ca(\text{\textsubscript{1.2}})</td>
<td>52.90±7.77</td>
<td>Ca(\text{\textsubscript{1.2}})–RyR2</td>
<td>59.25±7.52</td>
<td>13</td>
</tr>
<tr>
<td>Cav3–Ca(\text{\textsubscript{1.2}})</td>
<td>4.22±0.78</td>
<td>Ca(\text{\textsubscript{1.2}})–Cav3</td>
<td>3.70±0.81</td>
<td>5</td>
</tr>
<tr>
<td>Ncx–Caspq</td>
<td>7.28±2.07</td>
<td>Caspq–Ncx</td>
<td>8.96±1.94</td>
<td>9</td>
</tr>
<tr>
<td>Ncx–Ca(\text{\textsubscript{1.2}})</td>
<td>7.19±1.01</td>
<td>Ca(\text{\textsubscript{1.2}})–Ncx</td>
<td>9.89±1.60</td>
<td>8</td>
</tr>
</tbody>
</table>

Colocalizations that occur at a significantly greater level than that expected by chance (\(P<0.01\)) are shown in bold. Values in italics represent colocalizations that occur at a significantly lesser level than that expected by chance (\(P>0.975\)), suggesting that they are in separate domains. Values are number of voxels in the colocalizing objects divided by the total number of voxels for that molecule.

Colocalizations that occur at a significantly greater level than that expected by chance (\(P<0.01\)) are shown in bold. Values in italics represent colocalizations that occur at a significantly lesser level than that expected by chance (\(P>0.975\)), suggesting that they are in separate domains. Values are number of voxels in the colocalizing objects divided by the total number of voxels for that molecule.

**Fig. 2. Triple-labelling of RyR2, Ncx and Ca\(\text{\textsubscript{1.2}}\).** An atrial myocyte labelled with antibodies against RyR2 (blue), Ncx (green) and Ca\(\text{\textsubscript{1.2}}\) (red). All images are 1-\(\mu\)m-thick slices from the cell surface. (A) View of the entire cell, showing the surface distribution and colocalization of RyR2, Ncx and Ca\(\text{\textsubscript{1.2}}\). (B) Segment of the cell surface from the area shown in A. (C) The same segment, showing only the object colocalization of RyR2–Ca\(\text{\textsubscript{1.2}}\)–Ncx (white), RyR2–Ca\(\text{\textsubscript{1.2}}\) (magenta), Ncx–RyR2 (cyan) and Ncx–Ca\(\text{\textsubscript{1.2}}\) (yellow). Scale bars: 5 \(\mu\)m.

Fig. 3 shows triple labelling of RyR2 (blue), Casq (red) and Ncx (green) in a 1-\(\mu\)m-thick segment from the cell surface of a representative atrial myocyte. The full cell is shown in Fig. 3A, the outlined segment shows the area that excluded the edges and was used for the analysis. Fig. 3B is an expanded version of the boxed region in Fig. 3A, whereas Fig. 3C shows the object colocalization between the three molecules. The most dominant form of association between these proteins was RyR2 with Casq alone (magenta), with about 50% of the voxels colocalized (Table 1). There were a significant number of RyR2–Casq–Ncx triplets (white; Table 2), as well as a few dual colocalizations [Ncx–RyR2 (cyan) and Ncx–Casq (yellow)] neither of which is significant (Table 1). It is worth noting that there was a large amount of Ncx that was not colocalized with either RyR2 or Casq (Fig. 3B).

RyR2 and Casq are the only molecules studied that have a substantial presence in the interior of the atrial cell. A comparison of the surface and interior colocalization of RyR2 and Casq (Table 3), showed that there was significantly less colocalization in the interior than at the surface, irrespective of whether one compares object colocalization or the number of voxels in the colocalized objects. In addition, the size of both the RyR2 and Casq objects differs significantly between the interior and surface, with Casq smaller in the interior, whereas the RyR2 was larger (Table 4). Furthermore, the median nearest-neighbour distances for both RyR and Casq were significantly less (\(P<0.05\)) in the interior of the cell than they were on the surface (Table 5), although neither the cluster diameter nor the inter-cluster distance differed between the interior and surface (Table 6).

In Fig. 4, we show an atrial myocyte labelled with antibodies against RyR2 (blue), Ca\(\text{\textsubscript{1.2}}\) (red) and Cav3 (green). Fig. 4A shows a 1-\(\mu\)m-thick layer, from the bottom cell surface, with a marked edge effect. In Fig. 4B, we display the segment outlined in Fig. 4A that excludes the edge regions. Object colocalization is shown in
Table 2. Triple colocalizations

<table>
<thead>
<tr>
<th>Molecule triplets</th>
<th>Colocalization (%)</th>
<th>Molecule triplets</th>
<th>Colocalization (%)</th>
<th>Molecule triplets</th>
<th>Colocalization (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RyR2–Casq–Cav1.2</td>
<td>5.81±0.95</td>
<td>Cav3–Ca,1.2–RyR2</td>
<td>3.54±1.24</td>
<td>Ca,1.2–RyR2–Cav3</td>
<td>5.58±1.56</td>
<td>5</td>
</tr>
<tr>
<td>RyR2–Casq–Cav1.2</td>
<td>5.81±0.95</td>
<td>NCX–Ca,1.2–RyR2</td>
<td>11.92±2.07</td>
<td>Ca,1.2–RyR2–Ncx</td>
<td>15.26±2.19</td>
<td>8</td>
</tr>
<tr>
<td>RyR2–Casq–Cav1.2</td>
<td>5.81±0.95</td>
<td>Ncx–Casq–RyR2</td>
<td>4.73±1.09</td>
<td>Casq–RyR2–Ncx</td>
<td>5.85±0.96</td>
<td>9</td>
</tr>
</tbody>
</table>

Colocalizations that occur at a significantly greater level than that expected by chance (P<0.01) are shown in bold. Each colocalization is calculated with respect to the first molecule listed. Values are number of voxels in the colocalizing objects divided by the total number of voxels for that molecule.

Fig. 4C, with the triple colocalization RyR2–Ca,1.2–Cav3 (white; Table 2) and the colocalization between RyR2 and Ca,1.2 (magenta; Table 1) being highly significant. Colocalization of RyR2 with Cav3 (cyan) was not significant, whereas the observed values for the Cav3–Ca,1.2 pair (yellow) were much less than those expected by chance (Table 1), indicating that these two molecules are probably in separate domains. The large colocalization between RyR2 and Ca,1.2 was reflected in the large number of tightly coupled magenta clusters with a few triplets (white) interspersed.

Our analysis technique (Fletcher et al., 2010) allowed us to derive three useful metrics for describing the positioning of the molecules within the atrial cell; these are the median nearest-neighbour distance of the individual molecules (Table 5); the cluster diameter, a measure of how closely associated the colocalized molecules are (Table 6); and the inter-cluster distance, a measure of how separated the colocalizations are. The values in Table 6 include only those colocalizations that were statistically significant (see Tables 1 and 2).

Discussion

A number of our results correspond well with earlier findings in rat ventricular myocytes (Scriven et al., 2000). The association of RyR2 with Ncx in atrial cells was not significantly different from that expected by chance, and atrial RyR2 had large and significant colocalizations with both Ca,1.2 and Casq. The nearest-neighbour distances for the surface RyR2 and Ca,1.2 (Table 5), as well as the inter-cluster distance for the RyR2–Ca,1.2 (Table 6), were remarkably similar to those found in the ventricle (Scriven et al., 2010), with differences of 20 nm or less in their values. In addition, the nearest-neighbour distance for Cav3 indicated that its density was higher than that for either RyR2 or Ca,1.2, which agrees with our earlier finding from the ventricle surface (Scriven et al., 2005). Ncx had a nearest-neighbour value (and thus density) similar to that of Cav3, although we do not have values from the ventricle surface that we can compare it with. Results that differ from those in the ventricle involve Cav3: RyR2 and Cav3 alone were not significantly colocalized, unlike ventricular myocytes, where surface Cav3 seems to associate equally with RyR2 and Ca,1.2, presumably at the dyads (Scriven et al., 2005). In atrial cells, there was a small but significant population of RyR2, Ca,1.2 and Cav3 triplets (Table 2).

The role of Ncx in ECC has been subject of an extended debate. Numerous models with various assumptions have produced conflicting results (Sher et al., 2008), and, although it is well established that Ncx plays a major role in Ca\(^{2+}\) extrusion in the cardiomyocyte, its contribution to the Ca\(^{2+}\) influx, which could trigger CICR, is uncertain. Evidence from rabbit neonatal ventricular cells, which do not have a well-developed TATS, as for atrial myocytes, shows that they have a heightened level of RyR2–Ncx colocalization (Dan et al., 2007) and that they rely on Ncx-mediated CICR (Huang et al., 2008) during their early development. Our results indicate that this mechanism is absent in the mature atria; as there was no significant colocalization between RyR2 and Ncx, couplings consisting solely of these two molecules (and excluding Ca,1.2) are unlikely to exist and cannot contribute to CICR. As ~50% of the RyR2 colocalized only with Ca,1.2 channels, it is probable that Ca,1.2-mediated CICR is the primary method of initiating Ca\(^{2+}\) release in the atrial myocyte.

We found a statistically significant number (~13%) of RyR2–Ca,1.2–Ncx triplets on the atrial surface, with a spacing of approximately twice that of the simple RyR2–Ca,1.2 couplon (Table 6). This result raises questions as to the role of Ncx in these triplets and why only a limited number of dyads are involved. Bers (Bers, 2008) argues that, if the Ncx faces the restricted space of the dyadic cleft, the high Ca\(^{2+}\) concentrations that exist during the action potential would limit the role of Ncx to Ca\(^{2+}\) extrusion. Numerous models with various assumptions have produced conflicting results (Sher et al., 2008), and, although it is well established that Ncx plays a major role in Ca\(^{2+}\) extrusion in the cardiomyocyte, its contribution to the Ca\(^{2+}\) influx, which could trigger CICR, is uncertain. Evidence from rabbit neonatal ventricular cells, which do not have a well-developed TATS, as for atrial myocytes, shows that they have a heightened level of RyR2–Ncx colocalization (Dan et al., 2007) and that they rely on Ncx-mediated CICR (Huang et al., 2008) during their early development. Our results indicate that this mechanism is absent in the mature atria; as there was no significant colocalization between RyR2 and Ncx, couplings consisting solely of these two molecules (and excluding Ca,1.2) are unlikely to exist and cannot contribute to CICR. As ~50% of the RyR2 colocalized only with Ca,1.2 channels, it is probable that Ca,1.2-mediated CICR is the primary method of initiating Ca\(^{2+}\) release in the atrial myocyte.

We found a statistically significant number (~13%) of RyR2–Ca,1.2–Ncx triplets on the atrial surface, with a spacing of approximately twice that of the simple RyR2–Ca,1.2 couplon (Table 6). This result raises questions as to the role of Ncx in these triplets and why only a limited number of dyads are involved. Bers (Bers, 2008) argues that, if the Ncx faces the restricted space of the dyadic cleft, the high Ca\(^{2+}\) concentrations that exist during the action potential would limit the role of Ncx to Ca\(^{2+}\) extrusion. Numerous models with various assumptions have produced conflicting results (Sher et al., 2008), and, although it is well established that Ncx plays a major role in Ca\(^{2+}\) extrusion in the cardiomyocyte, its contribution to the Ca\(^{2+}\) influx, which could trigger CICR, is uncertain. Evidence from rabbit neonatal ventricular cells, which do not have a well-developed TATS, as for atrial myocytes, shows that they have a heightened level of RyR2–Ncx colocalization (Dan et al., 2007) and that they rely on Ncx-mediated CICR (Huang et al., 2008) during their early development. Our results indicate that this mechanism is absent in the mature atria; as there was no significant colocalization between RyR2 and Ncx, couplings consisting solely of these two molecules (and excluding Ca,1.2) are unlikely to exist and cannot contribute to CICR. As ~50% of the RyR2 colocalized only with Ca,1.2 channels, it is probable that Ca,1.2-mediated CICR is the primary method of initiating Ca\(^{2+}\) release in the atrial myocyte.

We found a statistically significant number (~13%) of RyR2–Ca,1.2–Ncx triplets on the atrial surface, with a spacing of approximately twice that of the simple RyR2–Ca,1.2 couplon (Table 6). This result raises questions as to the role of Ncx in these triplets and why only a limited number of dyads are involved. Bers (Bers, 2008) argues that, if the Ncx faces the restricted space of the dyadic cleft, the high Ca\(^{2+}\) concentrations that exist during the action potential would limit the role of Ncx to Ca\(^{2+}\) extrusion. Numerous models with various assumptions have produced conflicting results (Sher et al., 2008), and, although it is well established that Ncx plays a major role in Ca\(^{2+}\) extrusion in the cardiomyocyte, its contribution to the Ca\(^{2+}\) influx, which could trigger CICR, is uncertain. Evidence from rabbit neonatal ventricular cells, which do not have a well-developed TATS, as for atrial myocytes, shows that they have a heightened level of RyR2–Ncx colocalization (Dan et al., 2007) and that they rely on Ncx-mediated CICR (Huang et al., 2008) during their early development. Our results indicate that this mechanism is absent in the mature atria; as there was no significant colocalization between RyR2 and Ncx, couplings consisting solely of these two molecules (and excluding Ca,1.2) are unlikely to exist and cannot contribute to CICR. As ~50% of the RyR2 colocalized only with Ca,1.2 channels, it is probable that Ca,1.2-mediated CICR is the primary method of initiating Ca\(^{2+}\) release in the atrial myocyte.
increased excitability. We hypothesize that these dyads form the structural basis for the ‘eager sites’ of Ca\(^{2+}\) release (Mackenzie et al., 2001) at the surface of atrial myocytes.

Another small (~3%) but significant group of triplets is RyR2–Ncx–Casq, whose sparsity is reflected in their large intercluster distances (Table 6). Although it is possible that these represent RyR2–Ncx dyads modulated by Casq, this seems unlikely given the absence of this type of dyad without Casq. We believe that this is a subgroup of the RyR2–Cav1.2–Ncx conglomerate, in which the Ncx is close enough (probably in the dyad) to colocalize with Casq. As Casq does not always colocalize with RyR2, we can split the RyR2–Casq group into two – one in which the RyR2 excitability is modulated by Casq, and one in which it is not. Confirmation of this hypothesis would require quadruple labelling so that we can look at all four proteins simultaneously.

If we assume that Casq acts as both a luminal Ca\(^{2+}\) sensor for, and a P\(_i\) inhibitor of, RyR2 channels when it is bound (Gyorke et al., 2004; Terentyev et al., 2007), the lower colocalization of RyR2 and Casq in the interior of atrial myocytes means it is probable that they are more excitable than their surface counterparts. This would facilitate the diffusion of Ca\(^{2+}\) waves from the periphery to the centre, which is necessary in cells lacking a t-tubular structure. In addition, the nearest-neighbour distances for both RyR2 and Casq are significantly less in the interior than on the surface of the cell (Table 5), which would increase the likelihood of inward diffusion of the Ca\(^{2+}\) waves. Woo and colleagues (Woo et al., 2003a) noted a larger number of combined sparks (i.e. sparks that consisted of two to five single sparks) in the centre of atrial cells compared with that evident in their periphery, which could support a role for Casq as a modulator of excitability. They also reported that spontaneous Ca\(^{2+}\) sparks are more frequent in the cell periphery than in the interior, but attributed this to an interaction between the \(\alpha\)-C-terminal tail of Casq, Ca\(1.2\) and RyR2 (Woo et al., 2003b), which occurs only in the cell periphery, rather than to any effect of Casq. A second significant finding is that the sizes of the Casq clusters (both colocalized and not) are significantly smaller in the interior of the cell compared with at the periphery (Table 4). Although it is difficult to make an exact correspondence between voxel numbers and molecule density, it seems probable that there is much less Casq within the cell than along its edge, which might represent smaller stores of Ca\(^{2+}\). By contrast, RyR2 clusters are larger in the interior than in the exterior. It is not clear whether this is due to the limited space available at the surface or to some functional difference.

An indication of the position of Ncx in relation to the RyR2–Ca\(1.2\) is given by the cluster diameters of RyR2–Ncx–Ca\(1.2\) and RyR2–Ncx–Casq, which were approximately twice those of RyR2–Casq (Table 6). This suggests that although Ncx is close enough to the RyR2–Ca\(1.2\) couplon to influence [Ca\(^{2+}\)] within the dyadic space, it is further away from RyR than is Casq, and it is not in contact with these molecules. This means that it is unlikely that either fluorescence resonance energy transfer (FRET) or immunoprecipitation could be used to confirm this result. Furthermore, a recent paper (Nichols et al., 2010) has shown that, despite their well-known close association in both the ventricular and atrial cardiomyocyte, RyR2 and Ca\(1.2\) do not co-immunoprecipitate, making it unlikely that RyR2 and Ncx would do so.

The two significant dual colocalizations, RyR–Casq, 1.2 and RyR–Casq (Table 1), as measured in different experiments, have colocalization values that are not significantly different from each other and sum to greater than 100% of the total RyR. In addition, they have identical cluster diameters and similar intercluster distances (Table 6). In the ventricle, electron micrographs show that Casq is associated with the couplon (Franzini-Armstrong et al., 1987), so it is probable that there is significant overlap between the two groups, and that they form the triplet RyR–Casq, 1.2–Casq. If there is complete overlap between the two groups, and all of the

---

**Table 3. RyR–Casq colocalization**

<table>
<thead>
<tr>
<th>Molecular pair</th>
<th>Interior colocalization (%)</th>
<th>Surface colocalization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Object</td>
<td>Voxels in objects</td>
</tr>
<tr>
<td>Casq with RyR2</td>
<td>16.35±1.82</td>
<td>37.56±2.41</td>
</tr>
<tr>
<td>RyR2 with Casq</td>
<td>28.07±4.03</td>
<td>47.28±5.18</td>
</tr>
</tbody>
</table>

\(n=9\). All values listed for interior colocalization are significantly less \((P<0.05)\) than their corresponding values at the surface. Values from the surface include RyR–Casq that is colocalized with Ncx.

---

**Table 4. RyR and Casq object sizes**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Mean object size (voxels)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Interior</td>
</tr>
<tr>
<td>Casq</td>
<td>8.08±1.02</td>
</tr>
<tr>
<td>RyR2</td>
<td>25.19±2.19</td>
</tr>
</tbody>
</table>

\(n=9\) cells. All objects, whether colocalized or not, are included in the calculations.

*\(P<0.05\) compared with that in the interior.

---

**Fig. 4. Triple-labelling of RyR2, Cav3 and Ca\(1.2\).** An atrial myocyte labelled with antibodies against RyR2 (blue), Cav3 (green) and Ca\(1.2\) (red). All images are 1-μm-thick slices from the cell surface. (A) View of the entire cell, showing surface distribution and colocalization of RyR2, Cav3 and Ca\(1.2\). (B) Segment of the cell surface from the area shown in A. (C) The same segment, showing only the object colocalization of RyR2–Cav3–Ca\(1.2\) (white), RyR2–Ca\(1.2\) (magenta), Cav3–Ca\(1.2\) (yellow) and RyR2–Cav3 (cyan). Scale bars: 5 μm.
Cav3 alone is not statistically significant, which suggests that Cav3 molecules. The proportion of total RyR2 colocalized with Cav3, which we suspect is due to dyads being close to a group of between Cav3 with either RyR2 or Cav1.2: the only significant ‘naked’ RyR is definitely present in the interior of the atrial cell, as is not far-fetched to suggest its existence on the atrial surface. A surprising finding was that there was no significant pairing between Cav3 with either RyR2 or Ca1,1.2: the only significant association of Cav3 that we found was in the triplet RyR2–Ca1,1.2–Cav3, which we suspect is due to dyads being close to a group of Cav3 molecules. The proportion of total RyR2 colocalized with Cav3 alone is not statistically significant, which suggests that Ca1,1.2 channels in the RyR2–Ca1,1.2–Cav3 group are immediate neighbours of Cav3, rather than occupying lipid rafts or caveolae at these sites. Thus, a small proportion of the dyads are close enough to be affected by signalling molecules associated with Cav3, but the cluster diameters (Table 6) indicate that, although Cav3 is close to the couplon, it is probably not in physical contact with the sarcolemmal Ca1,1.2. The functional consequences of this association are difficult to estimate; there are multiple signalling molecules (e.g. β-adrenergic and A1 receptors) associated with Cav3. Cholesterol removal, which disrupts caveolae and lipid rafts, has been shown to cause a reduction in the frequency, width and amplitude of Ca2+ sparks (Lohn et al., 2000), although it is not clear whether this effect is due to a disruption of signalling between Cav3-associated molecules and the dyad or to the deleterious effects of cholesterol removal from the membrane. If it is the former, molecules associated with Cav3 might alter the open probability of RyR2, resulting in a change in the spark characteristics. Because they were derived from different experiments, one could theorise that the two triplets RyR2–Ca1,1.2–Ncx and RyR2–Ca1,1.2–Cav3 actually represent the quadruplet RyR2–Ca1,1.2–Cav3–Ncx. However, as the number of RyR2–Ca1,1.2–Cav3 triplets is far fewer than those of RyR2–Ca1,1.2–Ncx (Table 2), and their intercluster distances are very different (Table 6), it is unlikely that the groups match up. Even if significant numbers of the quadruplet did exist, there would still be a large amount of unmatched RyR2–Ca1,1.2–Ncx. Finally, isolated Ca1,1.2 was significantly less colocalized with Cav3 than one would expect by chance, implying that these two molecules are specifically directed to separate cellular domains. This differs from ventricular tissue, where Cav3 and Ca1,1.2 are colocalized on the cell surface (Scriven et al., 2005; Balijepalli et al., 2006).

### Materials and Methods

All chemicals were purchased from Sigma–Aldrich unless otherwise stated. Animal handling was performed in accordance with the guidelines of the Canadian Council on Animal Care.

### Cell isolation and preparation

Atrial myocytes were isolated from freshly excised hearts using the method of Rodrigues and Severson (Rodrigues and Severson, 1997). Briefly, adult male Wistar rats weighing 200–250 g were given 200 units of heparin (Organon Canada, Toronto, ON) intraperitoneally at 15–20 minutes prior to killing with sodium pentobarbital (20 mg per 100 g of body weight; MTC Pharmaceuticals, Cambridge, ON). The heart was immediately excised and hung for retrograde Langendorff perfusion with warm (37°C) Joklik MEM (M0518) supplemented with 23 mM NaHCO3, 1.2 mM MgSO4, and 1 mM DL-carnitine (final concentrations), and equilibrated with 95% O2 and 5% CO2. Perfusion was adjusted to give a flow of 7 ml/minute and maintained for 5 minutes to drain the heart of blood. It was then switched to a Joklik solution containing 162 units/ml type II collagenase (Worthington Biochemical, Lakewood, NJ). Once the heart softened, the atria were separated from the ventricles, minced and filtered through a Nitex nylon mesh (200 μm). Preparations contained 30–50% rod-shaped quiescent cells, which were incubated for 20 minutes at 37°C in M-199 (M4530) supplemented with 25 mM HEPES (pH 7.4), 1 mM DL-carnitine, 0.1 mM insulin, 0.56 mM penicillin, 0.14 mM streptomycin sulphate, 2 mM EGTA and 0.01 g/ml fatty-acid-free BSA (final concentrations). Cells were then fixed for 10 minutes in freshly made 2% paraformaldehyde. Fixation was quenched with 100 mM glycine (pH 7.4) for 10 minutes, after which cells were washed three times for 10 minutes each in PBS (137 mM NaCl, 8 mM NaH2PO4, 2.7 mM KCl and 1.5 mM KH2PO4, pH 7.4). Cells were then permeabilized with 0.1% Triton X-100 for 10 minutes followed by three 10-minute washes in PBS.

### Immunolabelling

Primary antibodies were against: RyR2 (mouse monoclonal; MA3-916, Affinity BioReagents, Golden, CO); calsequestrin (rabbit polyclonal; PA1-913, Affinity BioReagents, Golden, CO).
Incubations involving one monoclonal and one polyclonal primary antibody were performed sequentially overnight at 4°C. All labelling experiments involving two monoclonal primary antibodies were performed using mouse IgG1 Zenon labelling kits (Invitrogen). Experiments involving one polyclonal and two monoclonal antibodies were performed in one of two ways. In the first method, cells were incubated with one polyclonal antibody overnight at 4°C, and then with the appropriate monoclonal antibody. The second monoclonal primary was conjugated to its fluorochrome using a Zenon labelling kit and then added to cells for 1 hour at room temperature. In this method, the polyclonal antibody overnight at 4°C and then with an antibody conjugated to its fluorochrome. This was followed by incubation with a secondary antibody that had been conjugated to its fluorochrome. The results were identical to those obtained using mouse IgG1. All labelling experiments involving two polyclonal antibodies were performed using mouse IgG1 Zenon labelling kits (Invitrogen).

We also performed this experiment by labelling RyR overnight at 4°C, with the appropriate secondary antibodies without cross-reaction. Igs secondary antibody with the Zenon labelling kit. The results were identical to those obtained using mouse IgG1. All labelling experiments involving two polyclonal antibodies were performed using mouse IgG1 Zenon labelling kits (Invitrogen).

Control experiments
We used the Zenon kit (Invitrogen) to label monoclonal antibodies against the RyR and the nuclear pore complex (NPC). These two molecules are known to be in separate domains. Supplementary material Fig. S1 shows the results from this experiment. RyR and its secondary antibody (Alexa-Fluor-594-conjugated; shown in red) and NPC (Alexa-Fluor-488-conjugated; shown in green) were detected in the respective regions of the cell – the Z lines (arrows) and nuclear pore region (arrowhead), respectively. There is negligible red labelling in the nuclear region and/or green labelling at the Z-lines, indicating no cross-reaction between the secondary antibodies. Colocalization analysis of these two proteins confirms what we see in Fig. S2 (0.32% RyR colocalizes with NPC, 6.88% NPC colocalizes with RyR, 2.21% RyR colocalizes with RyR). NPC with RyR is a result of a much lower density of NPC as compared with RyR, and also of colocalization along the z-axis where RyR lies on top of, and underneath, the nuclear membrane. We also performed this experiment by labelling RyR overnight at 4°C, then adding a goat Alexa-Fluor-594-conjugated anti-mouse-IgG secondary antibody for 1.5 hours at room temperature. This was followed by incubation with a primary antibody that had been conjugated to a goat Alexa-Fluor-488-conjugated anti-mouse-IgG secondary antibody with the Zenon labelling kit. The results were identical to those obtained using mouse IgG1. All labelling experiments involving two polyclonal antibodies without cross-reaction.

Imaging, deconvolution and analysis
Images were captured using a Zeiss AxioObserver inverted microscope with a 63x/1.4 objective and were then passed through a narrow bandpass filter (Semrock, Rochester, NY) specific for the chosen fluorophore. Images were captured on a thermoelectrically cooled charged-couple device (CCD) camera with an 80% quantum efficiency (STi S1502AB chip). The z-position of the sample was controlled by a PZM2000 piezo stage (Applied Scientific Instrumentation, Eugene, OR). The pixel size of these images was 95 nm in x- and y-planes, and images were acquired at 250-mm intervals in the z-plane. Images were deconvolved using the algorithm developed by Carrington and colleagues (Carrington et al., 1995). All images were dark-current- and background-subtracted, and flat-field corrected, to allow for non-uniformity in illumination and camera gain across the field of view. After deconvolution, the images were aligned using fiducary markers that emitted in all wavelengths. Finally, images were peeled, one layer of voxels at a time, and numbered from 0 (surface) to 12 (centre) (Srivastava et al., 2005). Because of some uncertainty in the exact position of the surface, the ~1 layer (immediately outside the surface) was also included. Colocalization and labelling density (number of hit voxels over the total number of voxels) were measured as a function of the distance into the cell; ‘surface’ and ‘interior’ represented the values obtained from layers 1 ~10 and +10, respectively. Interior colocalization and density was only calculated for the RyR2-Ca2+ pair, as no other proteins had significant labelling in the interior. All values were obtained from a segment of focal plane chosen to eliminate possible edge effects (Srivastava et al., 2008). Voxels or blobs encompassing all three antibodies (e.g. A with B and C) were counted as triple colocalized and separate from the dual colocalizations (e.g. A with B, B with C, and A with C) given that the triple grouping was thought to be functionally different from the doublets (Fletcher et al., 2010).

We thank William Catterall for the gift of the anti-Ca2,1,2 antibody (National Institutes of Health grant R01 HL085372) and Qixia Yu for technical assistance. This work was supported by grants from the Canadian Institutes of Health Research (MOP12875), the Heart and Stroke Foundation of British Columbia and the Yukon, and the Natural Sciences and Engineering Research Council of Canada (to E.D.W.M.).

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/7/1167/DC1

References


Table S1. Group and global means for dual colocalizations

<table>
<thead>
<tr>
<th></th>
<th>RyR2–Ncx–Casq</th>
<th>RyR2–Ncx–Ca\textsubscript{1.2}</th>
<th>RyR2–Cav3–Ca\textsubscript{1.2}</th>
<th>Global</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (n=9)</td>
<td>Mean (n=8)</td>
<td>Mean (n=5)</td>
<td></td>
</tr>
<tr>
<td>RyR2 with Cav3</td>
<td></td>
<td></td>
<td>8.36</td>
<td>2.40</td>
</tr>
<tr>
<td>Cav3 with RyR2</td>
<td></td>
<td></td>
<td>7.77</td>
<td>2.60</td>
</tr>
<tr>
<td>RyR2 with Ca\textsubscript{1.2}</td>
<td>50.22</td>
<td>5.71</td>
<td>57.19</td>
<td>9.63</td>
</tr>
<tr>
<td>Ca\textsubscript{1.2} with RyR2</td>
<td>57.37</td>
<td>4.60</td>
<td>62.25</td>
<td>10.10</td>
</tr>
<tr>
<td>Cav3 with Ca\textsubscript{1.2}</td>
<td></td>
<td>4.22</td>
<td>0.78</td>
<td>4.22</td>
</tr>
<tr>
<td>Ca\textsubscript{1.2} with Cav3</td>
<td></td>
<td>3.70</td>
<td>0.81</td>
<td>3.70</td>
</tr>
<tr>
<td>Ncx with RyR2</td>
<td>7.72</td>
<td>1.27</td>
<td>8.73</td>
<td>1.09</td>
</tr>
<tr>
<td>RyR2 with Ncx</td>
<td>10.11</td>
<td>1.43</td>
<td>9.90</td>
<td>1.10</td>
</tr>
<tr>
<td>Ncx with Ca\textsubscript{1.2}</td>
<td>7.19</td>
<td>1.01</td>
<td>7.19</td>
<td>1.01</td>
</tr>
<tr>
<td>Ca\textsubscript{1.2} with Ncx</td>
<td>9.89</td>
<td>1.60</td>
<td>9.89</td>
<td>1.60</td>
</tr>
<tr>
<td>Casq with Ncx</td>
<td>8.96</td>
<td>1.94</td>
<td>8.96</td>
<td>1.94</td>
</tr>
<tr>
<td>Ncx with Casq</td>
<td>7.28</td>
<td>2.07</td>
<td>7.28</td>
<td>2.07</td>
</tr>
<tr>
<td>Casq with RyR2</td>
<td>54.47</td>
<td>2.39</td>
<td>54.47</td>
<td>2.39</td>
</tr>
<tr>
<td>RyR2 with Casq</td>
<td>61.31</td>
<td>3.08</td>
<td>61.31</td>
<td>3.08</td>
</tr>
</tbody>
</table>

The values represent the mean percentage colocalization of the voxels in colocalized objects.