

Ca\(^{2+}\) Sparks and Waves in Canine Purkinje Cells

A Triple Layered System of Ca\(^{2+}\) Activation

Bruno D. Stuyvers, Wen Dun, Scot Matkovich, Vincenzo Sorrentino, Penelope A. Boyden, Henk E.D.J. ter Keurs

Abstract—We have investigated the subcellular spontaneous Ca\(^{2+}\) events in canine Purkinje cells using laser scanning confocal microscopy. Three types of Ca\(^{2+}\) transient were found: (1) nonpropagating Ca\(^{2+}\) transients that originate directly under the sarcolemma and lead to (2) small Ca\(^{2+}\) waves in a region limited to \(\sim 6-\mu m\) depth under the sarcolemma causing (3) large Ca\(^{2+}\) waves that travel throughout the cell (CWWs). Immunocytochemical studies revealed 3 layers of Ca\(^{2+}\) channels: (1) channels associated with type 1 IP\(_3\) receptors (IP\(_3\)R\(_1\)) and type 3 ryanodine receptors (RyR\(_3\)) are prominent directly under the SL; (2) type 2 ryanodine receptors (RyR\(_2\))s are present throughout the cell but virtually absent in a layer between 2 and 4 \(\mu m\) below the sarcolemma; (3) type 3 ryanodine receptors (RyR\(_3\)) is the dominant Ca\(^{2+}\) release channel in the Sub-SL. Simulations of both nonpropagating and propagating transients show that the generators of Ca\(^{2+}\) wavelets differ from those of the CWWs with the threshold of the former being less than that of the latter. Thus, Purkinje cells contain a functional and structural Ca\(^{2+}\) system responsible for the mechanism that translates Ca\(^{2+}\) release occurring directly under the sarcolemma into rapid Ca\(^{2+}\) release in the Sub-SL, which then initiates large-amplitude long lasting Ca\(^{2+}\) releases underlying CWWs. The sequence of spontaneous diastolic Ca\(^{2+}\) transients that starts directly under the sarcolemma and leads to Ca\(^{2+}\) wavelets and CWWs is important because CWWs have been shown to cause nondriven electrical activity. (Circ Res. 2005;97:0-0.)

Key Words: Purkinje \(\bullet\) Ca\(^{2+}\) sparks \(\bullet\) Ca\(^{2+}\) waves \(\bullet\) Ca transients \(\bullet\) automaticity

In cells devoid of t tubules such as atrial and Purkinje cells (Pcells), excitation–contraction coupling (ECC) involves Ca\(^{2+}\) release from stores located near the sarcolemma and subsequent Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) along a lattice of sarcoplasmic reticulum (SR) enveloping the sarcomeres, which then activate myofibrils throughout the cell.\(^1-5\) Drugs which affect SR function, such as thapsigargin and ryanodine, inhibit Ca\(^{2+}\) activation of Pcells.\(^6,7\) Conversely, spontaneous nonpropagating Ca\(^{2+}\) release and Ca\(^{2+}\) waves cause sarcolemma depolarization in both pacemaker cells and Pcells, which can lead to nondriven electrical activity even at normal [Ca\(^{2+}\)]\(_{c}\).\(^7-9\) Abnormal Ca\(^{2+}\) release in the network of Pcells may also be involved in lethal arrhythmias after myocardial infarction.\(^7,10\) Previous observations suggested that micro Ca\(^{2+}\) transients, spanning only a few micrometers and traveling over short distances, initiate cell-wide Ca\(^{2+}\) waves (CWWs) which in turn induce nondriven electrical activity in a Pcell aggregate.\(^7\) Here, we determined the mechanistic relationships between the different subcellular spontaneous Ca\(^{2+}\) events in canine Pcells using confocal microscopy.

Materials and Methods

Eighteen aggregates of 2 to 6 cells were enzymatically dispersed from the Purkinje network of canine left ventricle (n=9)\(^7\) and placed in a chamber on the stage of an inverted laser scanning confocal microscope (LSCM). Fluorescence was measured only in rod-shaped Pcells with typical junctional ends, clear striations, and membranes free of blebs.\(^2\)

Measurement and Analysis of Ca\(^{2+}\) Transients

Confocal line-scans were first positioned parallel to the long axis in the cell-center (longitudinal scans) and then moved to the lateral edge or to the top/bottom edge. The transition of fluorescence between the cytosol and extracellular fluid was used to localize the sarcolemma in transverse scans and scans through cell borders.

Local variations of [Ca\(^{2+}\)]\(_{c}\) along scan-lines were estimated from the pixel-to-pixel ratio F/F\(_0\) (F: instantaneous fluorescence; F\(_0\): reference fluorescence) and analyzed using custom programs in IDL (IDL 5.4, Research Systems).

To mechanistically understand the processes that contribute to both propagating and nonpropagating Ca\(^{2+}\) transients, we constructed a mathematical model of release, diffusion, binding, and uptake of Ca\(^{2+}\) in an array of (50) nodes. Ca\(^{2+}\) changes in scan line images were simulated by numerical integration of the differential equations\(^11\) for all Ca\(^{2+}\) fluxes.
Ca\textsuperscript{2+} transient; image on the right shows a simulation of the transient (see text and online supplement); the curvilinear displacement of the front of the Ca\textsuperscript{2+} transient (see upper inset; red lines) suggests that Ca\textsuperscript{2+} moves by diffusion from the original site of release under the sarcolemma (see text) and disappears by active removal.

**Immunolabeling**

The immunocytochemical protocol used was similar to that previously described.\textsuperscript{12} Primary antibodies used were anti-IP\textsubscript{3}R1 (1:1000), an antibody that recognizes all 3 RyR isoforms, anti-RyR\textsubscript{3} (IgG; clone C3–33 and clone 34C, respectively; Affinity Bioreagents Inc; 1:500), and anti-RyR\textsubscript{1} produced and verified as described previously.\textsuperscript{13,14} For each aggregate, serial slices (2-μm intervals) through the z-axis of the entire aggregate were imaged. Antibody label density across cells was obtained from a pixel-to-pixel average of fluorescence of 30 line arrays across the cells after correction for nonspecific fluorescence (see online supplement at http://circres.ahajournals.org).

Results are expressed as mean±SEM. Comparisons were performed on groups of data by ANOVA. The difference was significant when \( P<0.05 \) after Bonferroni adjustment.

**Results**

**Nonpropagating Ca\textsuperscript{2+} Transients**

Nonpropagating Ca\textsuperscript{2+} transients were ubiquitous throughout Pcells. Most (82%) of these events (amplitude=1.85±0.02 F/Fo; duration at half maximal amplitude \( T_{0.5} \)=41±1 ms; full width at half maximal amplitude \( \text{FWHM} \)=3.2±0.1 μm; rate=0.6±0.2 events per s per 100 μm; \( n=524 \)) were similar to Ca\textsuperscript{2+} sparks reported for rabbit Pcells.\textsuperscript{6,15} The remaining nonpropagating Ca\textsuperscript{2+} transients had amplitude above 3 F/Fo (amplitude=4.9±0.2 F/Fo; \( T_{0.5} \)=43±2 ms; \( \text{FWHM} \)=2.4±0.2 μm; rate=0.13±0.04 events per s per 100 μm scan; \( n=115 \); Figure 1A). Clusters of consecutive and/or simultaneous Ca\textsuperscript{2+} sparks (Figure 1B) were also detected predominantly in regions below the sarcolemma and were similar to compound sparks described previously.\textsuperscript{16} Early sparks in clusters were often followed by a progressive increase in amplitude of later sparks (Figure 1B), suggesting an avalanche of multiple Ca\textsuperscript{2+} releases from a single or several adjacent sites with summation of Ca\textsuperscript{2+}. However, amplitude of \(<5\% \) of this compound sparks exceeded 3 F/Fo.

Transversal scans revealed the presence of spontaneous nonpropagating Ca\textsuperscript{2+} transients with various amplitudes directly under the sarcolemma (Figure 1C and supplemental Figure Is). The asymmetrical spread of these events (Figure 1D) confirmed that releases occurred directly under and against the membrane. Multiple consecutive releases could occur from the same site under the sarcolemma and produce large rises of Ca\textsuperscript{2+}. The majority of the large (amplitude \( >3 \) F/Fo) nonpropagating events detected in our study actually occurred in this region (supplemental Figure Is).

**Propagating Ca\textsuperscript{2+} Transients**

Ca\textsuperscript{2+} transients also propagated as waves with linear fronts that extended from several micrometers to the full length of the scan-line (Figure 2). Two types of Ca\textsuperscript{2+} waves were identified: small waves or wavelets (amplitude \(<3 \) F/Fo) and large waves (amplitude \( \geq 3 \) F/Fo) or CWWs.\textsuperscript{1}

Wavelets had a short rise time, decayed exponentially after the peak, and lasted \( \approx 150 \) ms (Figures 2 and 6B and Table). Frequently wavelets started under the sarcolemma after nonpropagating large waves (amplitude \( \geq 3 \) F/Fo) or CWWs (Figure 2B and 2D) and in areas...
with prominent spark activity (Figure 2A). They propagated over a limited depth from the sarcolemma into the cell: 6.2±0.2 μm; n=224; 17 cells. We denote this 6-μm layer under the sarcolemma as the Sub-SL region. The waves traveled in the Sub-SL region over <10 μm (Table) and never triggered large nonpropagating Ca\(^{2+}\) transients. Interestingly, we found a significant reduction of the frequency of wavelets with 2APB (3 μmol/L), a modulator of IP\(_3\)-sensitive Ca\(^{2+}\) release channels \(^{17,18}\); 0.86±0.26 (control, n=31) versus 0.39±0.18 event/μm/100 μm (2APB, n=41); \(P<0.001\). Same 2APB effect was observed previously on nonpropagating Ca\(^{2+}\) transients near the sarcolemma.\(^{19}\)

CWWs typically extended from sarcolemma to sarcolemma (Figures 2C and 3), occurred at ≈5-fold lower frequency, and propagated along the aggregate at 2-fold lower velocity than wavelets (Table). CWWs lasted considerably longer than wavelets. They consistently exhibited “pseudo-plateaus” (Figures 2C, 3, and 6A) at a wide range of peak F/Fo (from 3 to 11 F/Fo; 21 waves) thus ruling out an artifact attributable to saturation of fluorescence at elevated [Ca\(^{2+}\)].

Transverse scans showed that small [Ca\(^{2+}\)]\(_i\) increases triggered wavelets (threshold: Thr <10 nmol/L; n=6) and wavelets triggered CWWs (Figures 3 and 4) at an ≈10-fold higher threshold (Thr≈70 to 120 nmol/L; n=5; Figure 3C).

Scans through an end-to-end boundary between cells confirmed the initiation sequence of Ca\(^{2+}\) waves and that the phenomenon perpetuates from cell to cell in the aggregate (Figure 3E).

**Figure 2.** Propagating Ca\(^{2+}\) transients. A, Wavelets sampled by 25-μm line-scans of the lateral (a: frames 1,2,3) and top (b: frames 2,3) Sub-SL. On line-scan images, wavelets often exhibited V-shapes (eg, frames a1, b3) suggesting that Ca\(^{2+}\) events started from one site in the Sub-SL and, from there, propagated at nearly constant velocity in all directions; note the presence of Ca\(^{2+}\) sparks (yellow arrows). B, 25-μm transverse scan-line through the cell-center shows that wavelets propagate in a layer of ~6-μm depth which may however extend occasionally deeper in the cell (frame 4); the width of the aggregate was 25 μm with 0 corresponding to the position of the sarcolemma. C, Longitudinal line-scans (25 μm) in the Pcell center reveal both typical Ca\(^{2+}\) sparks (F/Fo max=1.9, FWHM=1 to 2 μm) and CWWs (amplitude=8.3 F/Fo, velocity=147.2 μm/s); bottom figure (0.8 s by 25 μm) shows a representative example of diffusional flags (see text); note that speed is similar to that of Sub-SL wavelets (here 110 μm/s) and amplitude decreases without true front wave as shown by a-b profiles (see adjacent panel). D, Three-dimensional representation of frame 4 of Panel B illustrates large Ca\(^{2+}\) increases which are occasionally detected at the origin (white arrow) of wavelet under the membrane.

### Characteristics of Propagating Ca\(^{2+}\) Transients in Canine Purkinje Cells.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Velocity, μm/s</th>
<th>Amplitude, F/Fo</th>
<th>Duration, ms</th>
<th>Distance, μm</th>
<th>Rate, Event per s per 100 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Center (13 Cells)</td>
<td>F/Fo ≤3</td>
<td>91</td>
<td>180.5±14.3</td>
<td>1.80±0.04</td>
<td>73±12</td>
<td>5.6±0.4</td>
</tr>
<tr>
<td></td>
<td>F/Fo &gt;3</td>
<td>21</td>
<td>60.0±11.1</td>
<td>6.4±0.8</td>
<td>574±90</td>
<td>20.1±1.0</td>
</tr>
<tr>
<td>Sub-SL (12 Cells)</td>
<td>F/Fo ≤3</td>
<td>107</td>
<td>111.5±9.6</td>
<td>1.68±0.04</td>
<td>160±19</td>
<td>7.7±0.5</td>
</tr>
<tr>
<td></td>
<td>F/Fo &gt;3</td>
<td>22</td>
<td>49.3±6.8</td>
<td>4.8±0.4</td>
<td>471±80</td>
<td>16.2±1.4</td>
</tr>
</tbody>
</table>

Propagating Ca\(^{2+}\) transients detected by longitudinal scans of the cell-center and the Sub-SL were classified into events with amplitude ≤3 F/Fo and amplitude >3 F/Fo. Duration and travel distance were determined, respectively, from the time and spatial coordinates of the event in line-scan images. Rate is No. of events per second extrapolated to 100-μm scan; Velocity, averaged motion speed of event; velocity of a wave is calculated from the slope of the front on single-line-scan images; velocity of ‘wave-flag’ sampled in the cell-center (Figure 2C) is calculated by linear regression through Ca\(^{2+}\) maxima in the line-scan image. Note that diffusional flags cross the longitudinal scan-lines with angle, which leads to overestimate their velocity. Results are expressed as mean±SEM.
The suggestion that Ca\textsuperscript{2+} transients start near the sarcolemma, and from these sites, propagate through the Sub-SL and/or into the cell-center, was further corroborated by x/y scanning (supplemental Figures IVA, IVB, and IVC). The 2-dimensional confocal images showed that spontaneous nonpropagating Ca\textsuperscript{2+} transients (Figure 4C; see online video clips) were common in the Sub-SL and revealed the presence of small Ca\textsuperscript{2+} transients propagating parallel to the sarcolemma within a layer of 2-6 \textmu m thickness (Figure 4A, 2 through 4). Amplitude and propagation of these transients were similar to those of wavelets revealed by single line-scan technique (Figure 4D). Two-dimensional confocal images showed also that wavelets were frequently accompanied by diffuse increases of [Ca\textsuperscript{2+}] ("wave flag") below the Sub-SL (see online supplement). These “diffusional flags” were detected by single line-scans positioned further in the cell center and appeared as small events moving with same velocity than SubSL wavelets but with decreasing amplitude (see Figure 2C). On occasion, 2D animations clearly showed a wavelet initiating a CWW while traveling longitudinally in the SubSL (Figure 4B, 4 through 6; Figure 4D).

Our linescan observations were corroborated by fast confocal video imaging (Figure 4E), which indicates that Ca\textsuperscript{2+} wave propagation occurred on at least 2 functional subcellular levels, suggesting that elements required for propagation differ in 2 different regions.

### Immunolabeling of Ryanodine and IP\textsubscript{3} Receptors

We determined the nature and distribution of SR Ca\textsuperscript{2+} release channels in these Pcells. First, we found no labeling for IP\textsubscript{R2} and RyR\textsubscript{1} (results not shown). However, colabeling with IP\textsubscript{R1} and RyR\textsubscript{2} antibodies was positive and revealed 2 distinct regions: a layer of 2-6 \textmu m thick with IP\textsubscript{R1} label (Figure 5A.b and 5C; green) existed directly under the sarcolemma while intense RyR\textsubscript{2} labeling dominated most of the cell including the lateral IP\textsubscript{R1}-positive layer (Figure 5A.b and 5C; red). Analysis of IP\textsubscript{R1} and RyR\textsubscript{2}-positive regions showed that only 5% of the corresponding pixels overlapped. Furthermore, Figure 5A.b and 5C illustrate a novel and typical feature of antibody staining of Pcells, in that label for both RyR\textsubscript{2} and IP\textsubscript{R1} was extremely sparse in a continuous...
layer of 2 μm thick below the IP$_3$R$_1$-positive layer (see arrows). This apparent void of Ca$^{2+}$ channels was observed with RyR$_2$ antibody but not with a RyR antibody that recognized all isoforms of the channel (Figure 5A). We found that this void was filled by a specific RyR$_3$ antibody which actually labeled a layer of 7-μm thickness below the sarcolemma (Figure 5C) and was absent in the cell-center (Figure 5A.c, 5B.c, and 5C).

Properties of the Generators of Ca$^{2+}$ Transients

Using model simulations, we studied whether the large Ca$^{2+}$ waves are indeed generated by nonpropagating Ca$^{2+}$ releases near the sarcolemma, such as has been shown in rabbit Pcells, or are in fact caused by propagating Ca$^{2+}$ releases. We also used the simulations to identify the factors that determine the properties of wavelets and CWWs.

Sparks near the sarcolemma, in the Sub-SL and cell-center could be simulated accurately using similar parameters of the release (release time $\approx$ 40 ms) and uptake functions (see online supplement for details). The correlation between simulated and experimental data were robust for all events ($R^2$>0.97; Figure 1D and supplemental Figure IIIs). The Ca$^{2+}$ diffusion coefficient ($D_{\text{eff}}$) required to fit such sparks was $12.1 \pm 0.3 \ \mu$m$^2$/s in both the Sub-SL and cell-center; we used this value to simulate Ca$^{2+}$ wavelets and CWWs (Figure 6).

Large Ca$^{2+}$ sparks were reproduced accurately by assuming a larger Ca$^{2+}$-release flux and a longer Ca$^{2+}$-release time (up to 100 ms), without a change in other parameters.

Large Ca$^{2+}$ waves are expected to travel faster than small waves; actually, we found the opposite when we compared CWWs with wavelets: wavelets propagated on average twice as fast as CWWs (Table). The simulations ruled out that this observation was caused by differences in $D_{\text{eff}}$ (see above) or extrusion kinetics (online supplement). The spacing$^{20,21}$ of Ca$^{2+}$ channels (Figure 5A.a) appeared to be similar in the Sub-SL and cell-center and cannot, therefore, explain our observation either. Finally, we tested whether the observed difference in velocity between wavelets and CWWs was
caused by different Ca\textsuperscript{2+} thresholds of the generators. The velocity appeared to be inversely proportional to Thr (see supplemental Figure III), and CWW propagation required a Ca\textsuperscript{2+} threshold up to 20-fold higher than the one for wavelet propagation consistent with the experimentally determined values (Figure 3). In addition, the simulations suggested 2 other aspects of the unique and distinct nature of CWWs as compared with wavelets (Figure 6): a 10-fold longer lasting Ca\textsuperscript{2+} release occurred during a CWW (200 to 500 ms) compared with that of either wavelets or sparks (20 to 45 ms), whereas the calculated total Ca\textsuperscript{2+} release in CWWs was 40-fold larger (Cf. insets Figure 6).

**Discussion**

**Ca\textsuperscript{2+} Sparks**

We show here that, at normal [Ca\textsuperscript{2+}], Ca\textsuperscript{2+} sparks occur ubiquitously throughout canine Pcells with characteristics similar to those of ventricular myocytes,\textsuperscript{22,23} including the presence of a subpopulation of large sparks. Large sparks in this study appear, however, wider and larger than those observed in rat cardiac myocytes.\textsuperscript{24–26} The presence of compound sparks\textsuperscript{16} with, on average, amplitude <3 and large width in the population of nonpropagating Ca\textsuperscript{2+} transients may explain why, in Pcells, large nonpropagating events were slightly narrower than small events (see FWHM above).

Accuracy of the correlation between amplitude and localization of Ca\textsuperscript{2+} sparks is limited because local Ca\textsuperscript{2+} releases can occur outside the confocal plane. Nevertheless, although large single sparks were seen occasionally in the cell-center, the majority was found directly under the sarcolemma and in the SubSL region. Our observations differ from those in rabbit Pcells, where sparks are exclusively found directly under the sarcolemma.\textsuperscript{6,15} The large sparks of canine Pcells are similar to the large 2APB-sensitive Ca\textsuperscript{2+} events reported in rabbit portal vein myocytes.\textsuperscript{18} The compound sparks in the Sub-SL and repetitive Ca\textsuperscript{2+} spark generation from single sites directly under the SL (see online supplement) suggest that both near-synchronous activation of multiple Ca\textsuperscript{2+} release units\textsuperscript{20} and rapidly repetitive activation in one site may occur in these regions.

This is consistent with the hypothesis that activation of IP\textsubscript{3}R may recruit adjacent Ca\textsuperscript{2+} channels including IP\textsubscript{3}Rs and RyRs (or vice versa);\textsuperscript{18,27} our findings that 2 types of SR-Ca\textsuperscript{2+} channels coexist (Figure 5) and that Ca\textsuperscript{2+} events are
sensitive to 2APB \textsuperscript{17,19} make it probable that this IP\textsubscript{3}/RyR interaction indeed occurs in canine Pcells.

**Ca\textsuperscript{2+} Transients in the Cell-Center: Diffusion or Propagation?**

Cordeiro et al observed no spontaneous Ca\textsuperscript{2+} release centrally in rabbit Pcells although RyRs were present,\textsuperscript{6,15} and thus concluded that central RyRs were “silent.” Our observations do not support the same conclusion, as we found that spontaneous Ca\textsuperscript{2+} sparks were common in the cell-center (eg, Figure 2), thus showing that RyRs are active in canine Pcells. This species difference in spontaneous Ca\textsuperscript{2+} release may also explain why canine Pcell aggregates and fibers exhibit nondriven electrical activity\textsuperscript{2,7} whereas rabbit Purkinje fibers do not.\textsuperscript{28}

Cordeiro et al proposed that the large central Ca\textsuperscript{2+} elevation evoked by the AP in rabbit Pcells resulted only from diffusion of Ca\textsuperscript{2+} released from peripheral sarcolemmal sites.\textsuperscript{6,15} Our simulations of Ca\textsuperscript{2+} release directly under the sarcolemma (Figure 1C) are consistent with this conclusion with respect to large sparks.\textsuperscript{6,15} In contrast, the spatiotemporal Ca\textsuperscript{2+} distributions of both wavelets and CWWs in canine Pcells (Figure 6) could be reproduced only by incorporating the propagation of Ca\textsuperscript{2+} release from node to node in the model, confirming that propagated CICR is responsible for these Ca\textsuperscript{2+} waves. Furthermore, our simulations show that the lower velocity of CWWs requires that Thr of Ca\textsuperscript{2+} release elements involved in the propagation be an order of magnitude higher than that of wavelets; this was consistent with our experimentally determined values (Figure 3). Finally, the typical plateau of CWW as well as the simulated Ca\textsuperscript{2+} release function are novel findings for a cardiac cell and suggest a distinct Ca\textsuperscript{2+} release mechanism. During CWWs, one or both of the following may occur: the release channels open completely, but the flux declines because of a decreased gradient across the channel.\textsuperscript{29} Alternatively, irreversible channel opening could be induced by an interaction between the permeant ion and the channel, similar to the mechanism that has been proposed for skeletal muscle.\textsuperscript{30} The determination of such mechanism is beyond the scope of this study. However, once we understand the mechanism of this persistent Ca\textsuperscript{2+} release, we should be able to reduce it in the intact Pcell. Such a reduction in the amplitude of CWWs and thus the depolarization that accompany them would be antiarrhythmic.

**Mechanism of Initiation of Wavelets and CWWs**

Spontaneously occurring Wavelets start commonly after sparks directly under the sarcolemma or in the Sub-SL (Figure 2), and, in turn, initiate CWWs (Figures 3 and 4). The effect of 2APB observed in this study, and in the previous study of Boyden et al,\textsuperscript{19} suggests that IP\textsubscript{3}/R-mediated Ca\textsuperscript{2+} release is instrumental in initiating/modulating Ca\textsuperscript{2+} wavelets. We never observed wavelets, which arrived at the sarcolemma and subsequently induced Ca\textsuperscript{2+} release directly under the sarcolemma. This directional asymmetry suggests that elements propagating wavelets and those causing Ca\textsuperscript{2+} sparks directly under the sarcolemma are functionally distinct. Furthermore, for adequate simulation of sparks near the sarcolemma, a 2-fold longer Ca\textsuperscript{2+} release pulse (supplemental Figure IIc) was required compared with that needed for wavelets (Figure 6), suggesting that Ca\textsuperscript{2+} transients directly under the sarcolemma and in the Sub-SL result from different Ca\textsuperscript{2+} release mechanisms.

Large CWWs span the entire cell suggesting that their underlying SR-Ca\textsuperscript{2+} release elements are ubiquitous. The amplitude duration and speed of the CWWs requires that the SR-Ca\textsuperscript{2+} release elements that mediate CWWs have a high CICR threshold and release a large Ca\textsuperscript{2+} flux for hundreds of ms. Larger and smaller low-threshold wavelets were found in the Sub-SL, suggesting that the SR-Ca\textsuperscript{2+} release elements that reside in this region have a low CICR threshold and release a small Ca\textsuperscript{2+} flux for tens of ms. These functionally distinct Ca\textsuperscript{2+} release elements must overlap in the Sub-SL, where both CWWs and wavelets can exist. This arrangement predicts that the probability for wavelets to trigger CWWs is small, as reported previously\textsuperscript{2} (Table). On the other hand, when the high CICR threshold of the CWW generators has been surpassed, Ca\textsuperscript{2+} release elements in the SR network will propagate the wave as far as this network reaches.
In summary, we show here that canine Pcells contain 3 functionally distinct Ca\(^{2+}\) release systems: system (1) is restricted to a thin layer (2 \(\mu\)m) directly under the sarcolemma and opposed to system (2) in the Sub-SL; system (2) partially overlaps with system (3) that drives Ca\(^{2+}\) release from sarcolemma to sarclemma.

**Ca\(^{2+}\) Release Channel Elements**

We demonstrate here that a sophisticated triple-layered system of SR-Ca\(^{2+}\) release channel architecture underlies the above-proposed hierarchy of Ca\(^{2+}\) wave generation. Like in atrial cells, \(^5\) we observed IP\(_3\)-RyR under the sarcolemma. However, different from atrial cells, the IP\(_3\)R1 isoform was detected in Pcells. RyR\(_3\) formed a clear striated pattern in the cell, similar to the pattern shown in rabbit Pcells\(^6\), as well as directly under the membrane near IP\(_3\)R\(_3\)s, but were virtually absent in a 2-\(\mu\)m layer (“void”) below the sarclemma, in the subSL region. This void was specific for RyR\(_3\) because an antibody that recognized all three RyR isoforms showed no void. In fact, RyR\(_3\) labeling was found in high density filling the void between RyR\(_2\) and IP\(_3\)R\(_1\) (Figure 5).

**Functional Implications**

Although a detailed pharmacological analysis of Ca\(^{2+}\) transient generators in canine Pcells is beyond the scope of this study, our findings reveal that Ca\(^{2+}\) activation in spontaneous Pcells differs substantially from that of ventricular myocytes. It can be explained in the following way (Figure 7):

First, a layer (2 \(\mu\)m) directly under the sarcolemma contains both IP\(_3\)R\(_3\)s and RyR\(_3\)_s, which are either separately or in combination responsible\(^18\) for the spontaneous large sparks directly under the sarcolemma. Second, the Sub-SL (6 \(\mu\)m thick from the sarcolemma) is a layer of RyR\(_3\)_s, which partially overlaps the previous IP\(_3\)R/RyR layer; RyR\(_3\)_s will generate sparks in the unstimulated Pcell. Because of their low threshold, RyR\(_3\)_s will readily respond to Ca\(^{2+}\) release directly under the sarclemma by the generation of Sub-SL wavelets. RyR\(_3\)_s indeed show spontaneous Ca\(^{2+}\) activity at normal diastolic [Ca\(^{2+}\)]\(_c\), when expressed in HEK cells,\(^31\) and pure RyR\(_3\) release is more sensitive to caffeine than other RyR isoforms.\(^31\) Their distribution would make them a source of sparks and wavelets in the Sub-SL if they also exhibit a low threshold for CICR in canine Pcells.

Third, RyR\(_3\)_s overlap with widely distributed RyR\(_3\)_s, thus allowing RyR\(_3\)_s to respond to Ca\(^{2+}\) release from the RyR\(_3\)_s Sub-SL network and generate CWWs. RyR\(_3\)_s channels release a large amount of Ca\(^{2+}\), thereby recruiting all available Ca\(^{2+}\) release channels in the generation of CWWs. This model could serve as a safe unidirectional system that ensures organized activation of Pcells in response to an action potential.

In conclusion this study provides novel functional and structural evidence for a triple layered system of Ca\(^{2+}\) activation in canine Pcells involving IP\(_3\)R\(_3\), RyR\(_3\), and RyR\(_3\) explaining a well recognized feature of the Pcell aggregate: the nondriven electrical activity.\(^7\)

**Acknowledgments**

This work was supported by grants from the National Institutes of Health (HL-58860) and the Alberta Heritage Foundation for Medical Health (HL-58860) and the Alberta Heritage Foundation for Medical Health (HL-58860) and the Alberta Heritage Foundation for Medical Health (HL-58860) and the Alberta Heritage Foundation for Medical Health (HL-58860) and the Alberta Heritage Foundation for Medical
Research (AHFMR); H.E.D.J.K. is an AHFMR Medical Scientist. We thank Dr W.G. Wier for his help during the construction of the laser scanning confocal microscope and G. Groves for his logistic support.

References

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Circ Res. published online June 9, 2005;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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http://circres.ahajournals.org/content/early/2005/06/09/01.RES.0000173375.26489.fe.citation

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/06/11/01.RES.0000173375.26489.fe.DC1
**Ca^{2+} sparks and waves in Canine Purkinje Cells;**
A Triple Layered System of Ca^{2+} Activation.

Bruno D. Stuyvers #, Wen Dun *, Scot Matkovich **, Vincenzo Sorrentino §,
Penelope A. Boyden *, Henk E.D.J. ter Keurs *#

# Department of Medicine, Physiology & Biophysics
University of Calgary
Calgary, Alberta, Canada

* Department of Pharmacology

**Department of molecular Cardiology
Center for Molecular Therapeutics
Columbia University
New York, NY, USA

§ Department of Neuroscience
University of Siena, Italy

This work was supported by grants HL-58860 from NHLBI, Bethesda, MD, USA and by CIHR and AHFMR, Canada

**Short title:** Ca^{2+} Sparks and Ca^{2+} Waves in Purkinje Cells

**Subject Codes:** 132, 136

**Key words:** Purkinje, Ca^{2+} sparks, Ca^{2+} waves, Ca-transients, automaticity

**Address for Correspondence:**
Bruno D. Stuyvers
Cardiovascular Research Group
Department of Medicine, Physiology & Biophysics
University of Calgary
Health Science Center/R1665
3330 Hospital Dr. N.W.
Calgary, Alberta
T2N 4N1 Canada
Tel: (403) 220-3374
Fax: (403) 270-0313
Email: stuyvers@ucalgary.ca
Supplemental Methods.

Fluo-4 Fluorescence Measurements: Ca\(^{2+}\) data acquisition

We used a custom Laser Scanning Confocal Microscope (LSCM) designed after \(^1\): The excitation source was the 488 nm line of a 100 mW Argon laser (MELLES GRIOT, CA, USA). Fluo-4 fluorescence (F) was sampled (at 510 nm) using an avalanche photodiode module (EG&G Optoelectronics, Montreal, Canada), which outputs TTL pulses at a rate determined by the light intensity. The pulses are counted and sent as 8-bit digital data (Electronics provided by Michael J., UMB, Baltimore, MD, USA) to a variable-scan digital video capture board (NI PCI-1424, National Inst.). Line scan images (pixels of 0.1\(\mu\)m x 10 \(\mu\)s) were constructed from 500 consecutive 25 \(\mu\)m lines obtained at 333 Hz. X/Y confocal scans were generated by a dual mirror set; the latter required precise centering of the incident laser beam on both mirrors and horizontal scan lines less than 50 \(\mu\)m to avoid loss of focus during scanning. The dual mirror set was used for 2D scans of small regions of an aggregate; 10\(\mu\)m x 10\(\mu\)m frames were sampled at a rate of 8 per second (112 lines/frame, 1.2 ms/line). The point-spread function in x-z plane of the LSCM (equipped with a 60x, 1.2 N.A. water immersion objective) was determined using latex fluorescent microbeads (Molecular Probe) with a sub-resolution diameter of 0.1 \(\mu\)m. The resolution of the LSCM was 550 and 310 nm along the axial and lateral axes respectively.

Scanning, light signal processing and digital video reconstruction were controlled by a microcomputer equipped respectively with a D/A board, a digital input/output board and the digital video capture board, driven by a custom Labview program (Labview 6.1 and IMAQ, National Instruments; OS: Win2K) modified from an original version generously provided by Dr. W.G.Wier (UMB, Baltimore, MD, USA). \(^2\).
Analysis of Fluo-4 Fluorescence: Expression of Ca\textsuperscript{2+} data

Local variations of [Ca\textsuperscript{2+}], along a scan line were estimated from the pixel-to-pixel ratio F/Fo, where Fo is the basal fluorescence intensity of Fluo-4 along the line. For 1D-scan line images, a Fo-line was obtained by averaging the 30 lines of a sub-array selected such that it showed stable background fluorescence and no evidence of photo-damage to either the dye or the cell. For x/y scans, F/Fo images were obtained by dividing each pixel of 2D F-Frames by the corresponding pixel of a Fo-frame. Fo-frame was selected among the serial images such that it displayed only background fluorescence. To resolve the local variations in [Ca\textsuperscript{2+}], and minimize the effects of motion, the Fo-frame was selected as close as possible to the frame(s) containing the Ca\textsuperscript{2+}-variation of interest. Most images included a cell edge, which made identification of motion artifacts straightforward as mismatch between F and Fo frames; this allowed us to take motion into consideration in the interpretation of F/Fo images or to chose a more appropriate Fo-image.

We used a combination of transverse and longitudinal scans to characterize the propagation of Ca\textsuperscript{2+} transients in space. In both transversal and longitudinal directions, line-scanning is suitable for quantification of wave velocity provided that the measured distance and duration reflect the true propagation of the event. Thus, we designed a wave analysis program (IDL) to verify first that wave data complied with the conditions that 1) the wave propagates at constant velocity and 2) the linescan nearly matches the propagation trajectory of the event. Whether the velocity was constant was easily verified from the linearity of the front of the linescan image of the wave. Then, we estimated the degree of overlap between propagation trajectory and scan line by measuring the variation in time of the maximal fluorescence intensity; if a continuous variation of more than 6 % was detected, we assumed that the wave trajectory diverged too much from the line and we discarded the data from
velocity calculation. Nevertheless, a possible misalignment of the direction of propagation of small Ca\textsuperscript{2+} transients could not be completely ruled out, which may have caused an overestimate of the propagation velocity of some wavelets.

**Immunostaining of SR Ca\textsuperscript{2+} release channels.**

Freshly isolated aggregates were attached to laminin-coated glass cover slips, washed in PBS, fixed for 30 min with 4% paraformaldehyde and washed in PBS again, after which they were permeabilized with 0.7 % Triton X-100 for 20 min, followed by another PBS wash. Cells were, then, incubated with 10% normal goat serum to block nonspecific binding. For the IP\textsubscript{3}R\textsubscript{1} isoform-specific antibody, a rabbit polyclonal antibody was produced using the previously described epitope sequence RIGLLGHPPHMNVNPQPA comprising the most distal C-terminal amino acids of IP\textsubscript{3}R\textsubscript{1}\textsuperscript{3} and was recently used in \textsuperscript{4}. The antiserum was produced using the PolyQuik regimen and purified via peptide-affinity purification (Zymed Laboratories, So. San Francisco). Specificity of IP\textsubscript{3}R\textsubscript{1} antibody was tested against recombinant IP\textsubscript{3}R\textsubscript{1}, IP\textsubscript{3}R\textsubscript{2} and IP\textsubscript{3}R\textsubscript{3} (data not shown).

To investigate the degree of colocalization of IP\textsubscript{3}R\textsubscript{1} and RyR\textsubscript{2}, primary antibodies were used simultaneously and incubated with cells overnight at 4°C. After washing, the cells were incubated for 1.5 hours with the mixture of secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG1 and Alexa Fluor 568-conjugated goat anti-mouse IgG1 (Molecular Probes)). Cells were re-suspended in Citiflour Mounting Medium (Agar Scientific), plated onto microscope slides and examined using a Zeiss LSM 410 microscope with dual excitation.
Mathematical simulation of Ca\textsuperscript{2+} transients.

Parameters, variables and units

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{Parameter} & \textbf{Description} \\
\hline
t & time (s) \\
\hline
t_s & start time (s) \\
\hline
\(a\) & amplitude of the Ca\textsuperscript{2+}-release (mol/L) \\
\hline
\(\tau_{\text{on}}\) & on-time constant (s) \\
\hline
\(\tau_{\text{off}}\) & off-time constant (s) \\
\hline
\([\text{Ca}]_{\text{Rest}}\) & Resting [Ca\textsuperscript{2+}] (nmol/L) \\
\hline
\([\text{Ca}]_t\) & instantaneous [Ca\textsuperscript{2+}] (nmol/L) \\
\hline
\([\text{Ca}]_N\) & [Ca\textsuperscript{2+}] in the node (nmol/L) \\
\hline
R & Ca\textsuperscript{2+} - release flux (number of Ca\textsuperscript{2+} ions/s) \\
\hline
U & Ca\textsuperscript{2+} - uptake flux (number of Ca\textsuperscript{2+} ions/s) \\
\hline
U_{\text{MAX}} & maximal Ca\textsuperscript{2+} - uptake flux (number of Ca\textsuperscript{2+} ions/s) \\
\hline
EC_{\text{U50}} & Ca\textsuperscript{2+} at half maximal uptake (nmol/L) \\
\hline
D & diffusion coefficient \\
\hline
\(k_+^{\text{ATP}}, k_+^{\text{TnC}}, k_+^{\text{Fluo4}}\) & Ca\textsuperscript{2+} on-rate constants for ATP, TnC and Fluo-4. \\
\hline
\(k_-^{\text{ATP}}, k_-^{\text{TnC}}, k_-^{\text{Fluo4}}\) & Ca\textsuperscript{2+} off-rate constants for ATP, TnC and Fluo-4. \\
\hline
[ATP], [TnC], [Fluo4] & ATP, TnC and Fluo-4 concentrations (nmol/L) \\
\hline
[Ca.ATP], [Ca.TnC], [Ca.Fluo4] & Ca\textsuperscript{2+}-ATP, Ca\textsuperscript{2+}-TnC, Ca\textsuperscript{2+}-Fluo4 Concentrations (nmol/L) \\
\hline
\end{tabular}
\end{table}

To examine Ca\textsuperscript{2+} movement within the Purkinje cell a mathematical model of Ca\textsuperscript{2+} release Ca\textsuperscript{2+} diffusion Ca\textsuperscript{2+} binding and Ca\textsuperscript{2+} re-uptake was constructed. The model was simplified after the model...
of Michailova et al.\textsuperscript{5} and assumes an array of elements which release Ca\textsuperscript{2+} as a pulse. We assumed a pulse shaped Ca\textsuperscript{2+} release flux ($R$) with exponential rise and fall:

$$R = a(1 - e^{-(t-t_\text{off})/\tau_\text{off}}) \cdot \frac{[Ca]_{\text{Rest}}}{[Ca]_i}$$

The release flux was assumed to be inversely proportional with [Ca\textsuperscript{2+}]\textsubscript{i}. Opening of the Ca\textsuperscript{2+} channels was either initiated (spontaneous) or was triggered by CICR if [Ca\textsuperscript{2+}] in a node exceeded a variable threshold (Thr). Ca\textsuperscript{2+} release through open channels was assumed to decay inversely proportional to [Ca\textsuperscript{2+}]\textsubscript{i} near the channel\textsuperscript{5,6}. The presence or absence of feedback of [Ca\textsuperscript{2+}]\textsubscript{i} on the simulated release flux did not affect \Delta t for sparks and required only adjustment of $R$, but the presence of feedback appeared essential for adequate simulation of Ca\textsuperscript{2+} transients that propagated as cell wide waves. Since it is likely that a rise in [Ca\textsuperscript{2+}]\textsubscript{i} always accompanies a reduction of the Ca\textsuperscript{2+} gradient across the release channel, we chose to enable this feedback for all transients. Released Ca\textsuperscript{2+} binds to ATP, Fluo-4 and C troponin (TnC) according to the reaction of the general form:

$$Ca^{2+} + \text{Ligand} \quad \Leftrightarrow \quad Ca-Ligand$$

with ligand concentrations (Ligand) and rate constants ($K^L_+$, $K^L_-$) as given by Csernoch et al.\textsuperscript{7} adjusted to 26\textdegree C assuming a Q\textsubscript{10} of 2. We assumed that [Mg\textsuperscript{2+}]\textsubscript{i} was constant (1 mM) and that ATP only binds Ca\textsuperscript{2+} and Mg\textsuperscript{2+}.

Ca\textsuperscript{2+} uptake flux ($U$) was assumed to follow Hill kinetics. We used the parameters for Ca\textsuperscript{2+} uptake by the SR measured for Cardiac myocytes in the laboratory\textsuperscript{8}:

$$U = \frac{U_{\text{MAX}} \cdot [Ca]_{\text{Hill}}}{EC_{U_{50}}^{\text{Hill}} + [Ca]_{\text{Hill}}}$$
The Hill coefficient \((Hill = 2.2)\) was assumed to be constant. \(EC_{U50}\) and \(U_{MAX}\) were fitted to the experimental data.

The one dimensional diffusion equation was solved using Crank’s finite difference method \(^9\) from:

\[
\frac{d[Ca]}{dt} = D \frac{1}{x} \left( \frac{dx}{dC} \right) \frac{1}{dx} + R - U - k_{ATP}^{ATP} [ATP] - [Ca.ATP] + k_{ATP}^{ATP} [Ca.ATP] - k_{+}^{ATP} [Ca.ATP] - k_{+}^{ATP} [Ca.ATP]
\]

\[
\]

\]

- *Initial conditions for [Ca\(^{2+}\)]\(_i\) and buffer concentrations-*

The cytosolic \([Ca^{2+}]_i\) was assumed to be 70 nM \(^{10}\) and the calculations were started with the buffers in equilibrium, using a physical diffusion coefficient \(D\) for \(Ca^{2+}\) (in water) of \(3.0 \cdot 10^{-6}\) cm\(^2\) s\(^{-1}\) \(^9\).

For simplification we have neither incorporated other ligands nor other buffering systems known to exist in Purkinje cells \(^{11;12}\). The calculations were performed with an integration interval of 10\(^{-7}\) s. Rise times of a spark and a wave were simulated by fitting the rate constants of \(Ca^{2+}\) channel opening and closing, their open time (\(\Delta t\)), and \(R\) to the experimentally observed rising phase of the \(Ca^{2+}\) transient. The decline of a \(Ca^{2+}\) transient was simulated by fitting \(U_{max}\) and \(EC_{U50}\) to the observed decline of the transients. The diffusion coefficient \(D\) was, then, obtained by fitting the simulated spatio temporal \(Ca^{2+}\) contour to the \(Ca^{2+}\) sparks experimentally observed in those regions (see Fig.2s).

Finally, propagation of a \(Ca^{2+}\) wave was simulated by fitting the threshold (Thr) for CICR in the nodes to the velocity of experimentally observed waves.

Accurate simulations were obtained when the amount of \(Ca^{2+}\) released from the SR was 10.2 \(\mu\)mol/L (\(Ca^{2+}\) pulse duration: 50ms) and 296.6 \(\mu\)mol/L (\(Ca^{2+}\) pulse duration: 450 ms) during sub-SL wavelets and CWW respectively (see Fig.6 in the main text); on average, the \(Ca^{2+}\) pulse duration for wavelets or sparks was 20-45 ms \textit{versus} 200-500 ms for CWWs.
Supplemental Results

Fig.1s: Spatiotemporal representation and analysis of Ca\(^{2+}\) transients near the sarcolemma.

Typical examples of Ca\(^{2+}\) sparks (A) and local Ca\(^{2+}\) transients occurring near the SL with various amplitudes (B-F). Frames C and D show clearly 2 consecutive transients and their partial summation, which leads occasionally to large elevation of [Ca\(^{2+}\)], such as those shown in frames E and F. The transients were sampled with 25 µm confocal scan lines positioned transversally across a Purkinje cell aggregate. Each raw frame (upper) is accompanied by the corresponding analysis frame (lower) that shows the line contours of [Ca\(^{2+}\)]. The initial contour is drawn for 1.2 F/Fo and the contour increment is 0.4 F/Fo.

Fig.2s: Simulation of non-propagating Ca\(^{2+}\) events in the cell-centre, in the Sub-SL and near the sarcolemma.

Frames to left of the figure show spatiotemporal representations (frame size: 25µm x 1.5s) of experimental (a) and simulated (b) non-propagating Ca\(^{2+}\) transients in the cell-centre (panel A: ‘core’), in the sub-SL (panel B) and near the sarcolemma (panel C). Traces to the right of each panel were obtained from profiles as indicated in a and b of panel A. For each panel, graphs c and e thus illustrate respectively the time course and spatial distribution (at the time of peak Ca\(^{2+}\)) of the measured (blue traces) and the simulated (red traces) non-propagating Ca\(^{2+}\)-transients. Insets (d) in each panel show the event-initiating Ca\(^{2+}\)-release flux (yellow line) superimposed on the corresponding simulated Ca\(^{2+}\) variation during a typical spark (red line). Ca\(^{2+}\) transients were reproduced by simulation of release, uptake, binding and diffusion of Ca\(^{2+}\) in the absence of regenerative Ca\(^{2+}\)-release. The estimate of the amount of Ca\(^{2+}\) released from the SR was 9.1 µmol/L (Ca\(^{2+}\) pulse duration: 25ms) during a spark in
the core and in the sub-SL and 17.7 µmol/L (Ca$^{2+}$ pulse duration: 60 ms) during a spark near the sarcolemma.

**Figure 3s: Influence of Ca$^{2+}$-threshold (Thr) on the propagation velocity of Ca$^{2+}$ waves.**

The propagation velocity (Vprop) of Cell Wide Waves (Left Panel) and Sub-SL wavelets (Right Panel) was calculated for different values of Thr by using the Ca$^{2+}$ activation model. On average, velocities of CWWs measured experimentally (see Table in the main text) were obtained for 10-50 times larger values of Thr than Vprop of wavelets.
**Video Clip 1:** 2D confocal imaging of propagating \( \text{Ca}^{2+} \) Transients in the SubSL region
See Figure 4 for details.

**Video Clip 2:** Trigger of \( \text{Ca}^{2+} \) waves by SubSL \( \text{Ca}^{2+} \) events
See Figure 4 for details.

**Video Clip 3:** High speed (Nipkow) confocal imaging of SubSL wavelets.

Frames (34 µm x25 µm) were captured at 27 fps from a high speed spinning disk confocal system. Confocal plan was positioned across the Purkinje cell-centre; 10 pixels/µm.
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