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Wide Long lasting Perinuclear Ca^{2+} Release Events Generated by an Interaction between Ryanodine and IP_3 Receptors in Canine Purkinje Cells

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Abstract

Purpose—to determine whether IP_3Rs contribute to the generation of wide long lasting perinuclear Ca^{2+} release events in canine Purkinje cells.

Methods—Spontaneous Ca^{2+} release events (elevations of basal [Ca^{2+}] equivalent to F/F_0 3.4SD over F_0) were imaged using Fluo-4AM and 2D confocal microscope. Only cells free of Ca^{2+} waves were analyzed. Subsarcolemmal region (SSL) was defined as 5μm from cell edges. Core was the remaining cell.

Results—The majority of events (94%, 0.0035 ± 0.0007 events(ev)/μm^2/sec, n=34 cells) were detected within a single frame (typical events, TE). However, a subpopulation (6.0%, 0.00022 ±0.00005 ev/μm^2/sec, n=41 cells: wide long lasting events, WLE) lasted for several frames, showed a greater spatial extent (51.0±3.9 vs. TE 9.0±0.3 μm^2, P<0.01) and higher amplitude (F/F_0 1.38±0.02 vs. TE 1.20±0.003, P<0.01). WLE event rate was increased by phenylephrine (10μM, P<0.01), inhibited by 2APB and U73122 (P<0.05), and abolished by tetracaine (1mM) and ryanodine (100μM). While SSL WLEs were scattered randomly, Core WLEs (n=69 events) were predominantly distributed longitudinally 18.2±1.6 ìm from the center of nuclei. Immunocytochemistry showed that IP_3R1s were located not only at SSL region but also near both ends of nucleus overlapping with RyRs.

Conclusion—In Purkinje cells, wide long lasting Ca^{2+} release events occur in SSL and in specific perinuclear regions. They are likely due to RyRs and IP_3R1s evoked Ca^{2+} release and may play a role in Ca^{2+} dependent nuclear processes.

Keywords

Purkinje cells; nucleus; Ca^{2+} transients; Phenylephrine

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INTRODUCTION

In the heart, the ryanodine receptor (RyR) is a major element in the control of the intracellular Ca^{2+} concentration, whereas the role of the inositol 1,4,5-trisphosphate receptor (IP_3R) remains unclear. It has been recently reported in rabbit ventricular myocytes that IP_3R is expressed predominantly in the nuclear envelope and as such, may play a role in the gene transcription through Ca^{2+}-dependent signaling pathways (excitation-transcription coupling) [1–3] and/ or response to endothelin [4]. In permeabilized atrial cells, the nucleus appears to be surrounded by its own Ca^{2+} store and IP_3 can induce nuclear Ca^{2+} release events [5].

Compared to ventricular myocytes, Purkinje cells exhibit a tenfold larger density of IP_3Rs [6,7]. A reduction of spontaneous activity in presence of 2APB [8] as well as a good correlation between Ca^{2+} transients and the cellular distribution of IP_3R1 specific antibody [9] support the idea that IP_3Rs are involved in Ca^{2+} activation of Purkinje fibers during both action potential evoked and spontaneous Ca^{2+} transients. IP_3Rs and related local Ca^{2+} releases have been localized near the sarcolemmal membrane and might constitute the primary event of the sequence leading to large Ca^{2+} transients and subsequent cell wide Ca^{2+} waves in Purkinje cells [9]. Cell wide Ca^{2+} waves in Purkinje cells can lead to non driven electrical activity [10]. However the existence of specific IP_3R related Ca^{2+} release activity in the nuclear region has not been considered. The aim of this study was to investigate the contribution of IP_3R to Ca^{2+} release events in the perinuclear region of canine Purkinje cells.

METHODS

All experiments were performed according to protocols approved by the Columbia University Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996).

Preparation

Purkinje cells were enzymatically dispersed from the Purkinje fibers of the canine heart (preparations=N=15) as previously described [11,12] and placed in a glass-bottomed perfused chamber on the stage of an inverted microscope and then loaded with 5μM Fluo-4 AM [9]. Fluorescence was measured only in rod-shaped Purkinje cells with typical junctional ends, clear striations and membranes free of blebs. All experiments were completed after cells had been superfused with Tyrodes solution (including 2mM Ca^{2+}, 4mM K^+, pH =7.4, 24°C) for at least 15 mins.

Cellular Ca^{2+} imaging

Ca^{2+}-related variations of Fluo-4 fluorescence were studied using a Yokogawa confocal scanning unit (CSU10, Yokogawa, Japan) attached to a Nikon TE200 microscope equipped with a Nikon x60 Fluorescence objective. The excitation light (wavelength: 488nm) source was a multiline 100 mW argon ion laser. Emitted fluorescence was filtered at 510 nm and sampled at a rate of 17 frames per sec by a CCD camera (ORCA-ER C4742–95, Hamamatsu Photonics KK, Japan). The Ca-related fluorescence was visualized through F/Fo ratio images as following: basal fluorescence (Fo) image was determined as the average of all frames; each fluorescence image was divided pixel-to-pixel by the corresponding Fo image.

Detection and analysis of Ca^{2+} events

Ca^{2+} release events were automatically detected through a series of consecutive F/Fo images using an IDL custom computer-based detection procedure. A Ca^{2+} event was defined as an elevation of basal [Ca^{2+}] equivalent to F/F_0 3.4 SD over F_0. The sensitivity of the detection

was 95 ± 2 % and the probability that the detection includes a false event was 6 ± 2 percent of total events (n=8, Figure S1 and S2 online supplement). Ca^{2+} event frequency and maximal event amplitude in different regions of the Purkinje cell were calculated as following: regions of interest (Subsarcolemmal (SSL) and Core ROIs) were first drawn on a cell image (ImageJ) and then superimposed on each of the binary images to determine the subcellular location (SSL or Core) of events (as in Figure S1). The amplitude, spatial extent, and location of events were determined using the custom-made IDL program (IDL6.0, Research Systems). In some cases (eg. Figure 1B) we have displayed Ca^{2+} events as pseudo linescans (Image J). For more details see Supplement.

The 2D confocal approach taken here involves the use of a Nipkow spinning disk that reduces the level of light needed and thus increases the time one can sample the cell without obvious damage. In the case of these experiments, we chose 2 sec sampling of a confocal slice through the Purkinje cell at the level of the nucleus. While this allowed us to sample ALL subcellular sites in that plane at the same time (avg total 2D area of cells 4716±326 μm2, N=30), we may have not detected some events with our frame rate thus our frequency values may be an underestimation. All event rates were divided by area of cell viewed for analysis. Nevertheless what we did record and identify as an event was done with a program that showed a high degree of sensitivity and accuracy (see Figure S2). With most previous LSCM studies one only samples a very short line for brief periods of time. As such, WLEs may have occurred in atrial cells studied with LSCM, but they may have not been recorded since they were out of the view field in another subcellular region. Using our 2D system and overlaying the image of the Purkinje cell we were able to precisely locate WLEs.

In additional studies, we determined the characteristics of Ca^{2+} events in the absence and presence of pharmacological agents. For these studies, images were collected from groups of cells before (Control) and after 10min superfusion with an agent. We had previously determined that Ca^{2+} event characteristics in the absence of drug remained stable for more than 30mins (data not shown).

Immunocytochemistry

Freshly isolated Purkinje cells were prepared as in [9] and incubated overnight (at 4°C) with a rabbit polyclonal IP_3R1 antibody (PA3-901, Affinity Bioreagents Inc; 1:200) and a mouse monoclonal RyR2 antibody (MA3-916, Affinity Bioreagents Inc; 1:500). Specificity of IP_3R1 antibody was tested using the neutralizing peptide (PEP-019, Affinity Bioreagents Inc). The cells were then incubated for 1.5 hours with a mixture of Alexa Fluor 488-conjugated goat anti-rabbit IgG1, and Alexa Fluor 594-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 510 microscope set for dual excitation (100x, oil).

Statistics

Data are expressed as mean ± S.E. Comparisons were made using an unpaired Student *t*-test or ANOVA. Bonferroni was performed after ANOVA. N means number of cells, while n means number of events used in analysis. The difference was considered significant when $P < 0.05$.

RESULTS

Two types of non-propagating Ca^{2+} events

2D confocal imaging revealed two types of **spontaneous non-propagating Ca^{2+} release events** in canine Purkinje cells at physiologic $[Ca^{2+}]_o$ (2mM) (Figure 1): 1) Most Ca^{2+} events (94%) had characteristics similar to *Ca^{2+} sparks* previously observed in Purkinje cells by laser

scanning confocal microscopy [9]; named 'Typical Events' (TEs). In the present study, these Ca^{2+} release events were detected within a single frame (see arrowheads in Figure 1A) and showed amplitude and spatial extent of 1.20±0.003 F/Fo and 9.0±0.3 μm^2 respectively; 2) A small fraction (6%) of non-propagating events was composed of local Ca^{2+} elevations which lasted over several frames (120–360 ms), still remained localized, failing to propagate within confocal plane. These events had a 6 fold greater spatial extent (51.0±3.9 μm^2, $p<0.01$) and slightly larger amplitude (1.38±0.02 F/F_0, $p<0.01$) than TEs; named 'wide long lasting events' (WLEs). WLEs occurred at a ~16 fold reduced frequency than TEs (TEs: 0.0035 ±0.0007 ev/μm^2/sec, 1022 events, N=34 cells; WLEs: 0.00022±0.00005 ev/μm^2/sec, 150 events, N=41 cells, $P<0.01$; Figure 2). Both types of Ca^{2+} events were detected in the cell. However TEs occurred 6 times (0.0015±0.0004 vs 0.0089±0.00011 ev/μm^2/sec, $p<0.01$) and WLEs 4 times (0.00012±0.00004 vs 0.00052±0.00011 ev/μm^2/sec, $p<0.01$) more frequently in the subsarcolemma(SSL) than in the core (Figure 2). For the purposes of this report, we will focus on WLEs.

WLEs occur in the perinuclear region and occurrence is augmented with IP_3R activation

Imaging Ca^{2+} in 2D revealed that Core WLEs originated specifically near the nuclei (Figure 3). A specific procedure of image analysis was developed and applied to 39 cells to investigate the exact position of WLEs with respect to the nucleus. As shown in Figure 3, Core WLEs occurred predominantly along the longitudinal axis of the cell at both ends of the nucleus. Note none were found in the nucleus or in the nuclear envelope (Figure 3). The average nucleus area was 112um^2, the distance was 18.2μm, and the angle was 12.6 degrees.

WLEs were similar to compound sparks(Ca^{2+} events with multiple peaks (see LSCM data Figure 1B of [9]) described in rabbit portal vein myocytes [13], and canine Purkinje cell aggregates [9]. In these previous studies, arguments presented suggested that IP_3R Ca^{2+} events were initiators (modulators) of compound sparks. To examine whether IP_3R could be involved in the WLEs, Purkinje cells were exposed to phenylephrine, an alpha-adrenergic agonist which increases the production of InP_3 [14]. 10 min exposure to Phenylephrine(10μM) increased the WLE rate by 97% in the SSL (0.00130±0.00016 vs 0.00066±0.00016 (control) ev/sec/μm^2, N=29, $p<0.05$) and 400% in the Core (0.00045±0.00011 vs 0.00009±0.00004 (control) ev/sec/μm^2, N=29, $p<0.01$) while having no effect on the TE rate(Figure 4A), spatial extent and amplitude, or background fluorescence (Figure 4). WLE augmentations were abolished in presence of U-73122 (2μM), a PLC inhibitor, and 2APB (3μM). U73433 (2μM), an inactive analogue of U-73122, had no effect (Figure 4B). 2APB, in the absence of Phenylephrine, had no significant effect on WLE frequency(data not shown). It has been proposed that compound sparks could result from a sequence involving the initial activation of IP_3Rs by InP_3 and the subsequent trigger of Ca^{2+} release from adjacent RyRs [13]. Consistent with this hypothesis, we found that both ryanodine (100μM) and tetracaine (1mM) abolished WLEs in Purkinje cells (Figure 4B). Interestingly, on occasion a WLE gave rise to a Ca^{2+} wave (Figure 5) which propagated from the perinuclear region into the nucleus.

Perinuclear localization of IP_3R1 and RyR2

The observation that WLEs occur in the SSL and near the nucleus, and the fact that the frequency of WLEs is sensitive to modulators of IP_3R strongly suggests the presence of IP_3Rs in the Purkinje perinuclear region. We sought to determine whether IP_3R1s are localized in the Core region by using a specific IP_3R1 antibody. The origin of this antibody differs from that used in our previous work [9]. Here we found that IP_3R1 was expressed in SSL as before [9], but also in the nuclear envelope and at the ends of the nucleus (Figure 6A). In the perinuclear region, the localization of IP_3R1s matched remarkably the distribution of WLEs (see Figure 3B). Interestingly, IP_3R1s and RyR2s localize at both ends of nucleus (Figure 6C). In fact the well known grid of RyRs overlays the puntate IP_3R1 staining at the nuclear poles. Blocking

peptides and secondary antibody alone images confirmed the existence of IP_3Rs in this region (Figure 6C,D).

Perinuclear Ca^{2+} storage

The characteristics of WLEs differ by region (Figure 2). Core WLEs were less frequent, had lower amplitude but comparable spatial extent compared to SSL WLEs. Thus a number of additional Purkinje cells were loaded with Fluo-5N (10μM), which enabled us to monitor Ca^{2+} in the sarcoplasmic reticulum in specific cell regions. As expected, the Fluo-5N fluorescence was distributed following a ~ 2μm striation pattern which was consistent with the distribution of RyR2s and revealed the 'sarcomeric' arrangement of the SR in canine Purkinje cells. Rapid caffeine (10mM) exposure induced a uniform decrease in both the 2μm-periodic signal and the perinuclear signal confirming the presence of large releasable Ca^{2+} stores in the perinuclear region of the Purkinje cell (data not shown).

DISCUSSION

In canine Purkinje cells, previous investigation of Ca^{2+} release activity by laser scanning confocal microscopy revealed the presence of various types of spontaneous Ca^{2+} release events [9]. In addition to Ca^{2+} sparks, large non-propagating events with very complex spatial and time courses were detected in a restricted region (SSL) extending 5 μm under the sarcolemma. In the present study, the use of a confocal spinning disk technique revealed the presence of similar wide large Ca^{2+} release events (WLEs) in the perinuclear region.

Our previous computational approach showed that the various spatiotemporal shapes of local Ca^{2+} events evidenced in Purkinje cells were primarily due to different forms of Ca^{2+} release channels in the SR [9]. Since large non-propagating Ca^{2+} events were detected in the SSL where IP_3R1s were detected by immunofluorescence, it was proposed that IP_3R activation was involved in the production of these large local Ca^{2+} elevations. Consistent with these data, we report here that production of IP_3 due to alpha-adrenergic stimulation notably increased the occurrence of WLEs in both the SSL and perinuclear Core regions with no effect on TEs. In addition, in the perinuclear region, the arrangement of IP_3R1 at each end of the nucleus matched remarkably the subcellular sites of the occurrence of Core WLEs suggesting an involvement of IP_3Rs in WLE generation. Alternative mechanisms for the alpha adrenergic increase in frequency of WLEs are unlikely. For instance, a phenylephrine IP_3 evoked increase in Ca^{2+} influx (L type Ca current) into these Purkinje cells could increase SR content which in turn could alter WLE frequency. However this scenario is unlikely since the effects on WLEs studied here occurred after 10mins superfusion. If an increase in SR content had occurred, it would have been only transient until a new equilibrium was reached. Second since Phenylephrine has been shown to have both IP_3 dependent and independent effects, it may be that IP_3R independent effects may relate to the increase in frequency of WLEs. We think this is unlikely since WLE frequency increase induced by Phenylephrine was sensitive to both the PLC inhibitor U73122 and the putative IP_3R blocker 2APB(Figure 4). By itself, the IP_3R-evoked Ca^{2+} release is very small in the cardiac myocyte, however, the small Ca^{2+} release from IP_3Rs could then be amplified by surrounding RyRs via CICR. These data differ from the findings in ventricular cells where IP_3R2 isoform was found to be confined to the nuclear envelope [15] or even facing the *inside* of the nucleus [1,16].

Like Purkinje cells, atrial cells also express a high level of IP_3Rs and IP_3R-dependent Ca^{2+} release. Endothelin has been shown to enhance typical Ca^{2+} spark frequency [7,17,18] as well as action potential dependent Ca^{2+} transients [17,19] via an IP_3R dependent mechanism. Recent work using saponin treated atrial cells and their isolated nuclei suggests that IP_3 can directly cause a slow rise(mins) in *nuclear* Ca^{2+} [5]. In these latter studies, there was no mention of an enhancement of wide long lasting perinuclear events (cytosolic, not nuclear) as

we describe here for Purkinje cells. Furthermore, there has not yet been a description as to the precise subcellular location of IP_3R dependent Ca^{2+} releases in Purkinje cells.

The functional nature of WLEs identified here in intact cells could be similar to that of prolonged Ca^{2+} releases described by Yang and Steel in rat cells [20]. However, there are key differences. First, Purkinje cell WLEs are long lasting yet an event lasting several seconds was never observed while rat prolonged events were sometimes >2 secs in duration. Second, Yang and Steele [20] reported that the rat prolonged Ca^{2+} release events were inhibited by ryanodine yet *not* modified by IP_3. In Purkinje cells, wide long lasting events are affected by ryanodine as well as by agents known to augment/antagonize IP_3 (Figure 4). Finally, subcellular location of IP_3R1 receptors in the perinuclear area of WLEs further suggests a role for IP_3R in their generation.

Alpha adrenergic stimulation in Purkinje cells; functional effect?

It has been known for a long time that alpha agonist stimulation of normal canine Purkinje fibers can cause an increase in automaticity [21]. Further PLC alone when superfused over normal Purkinje fibers also induces an increase in automaticity. Since both ryanodine and verapamil were found to decrease this PLC-induced automaticity some have suggested a role for an increase in intracellular Ca^{2+} [22]. In our studies on intact Purkinje cells on rare occasion we observed a WLE giving rise to a cell wide Ca^{2+} wave(see Figure 5). This cell wide wave could under appropriate circumstances lead to nondriven electrical activity[10]. These results would be consistent with enhanced automaticity in the presence of phenylephrine.

Alpha adrenergic stimulation has been reported to affect L type Ca^{2+} currents but increases, decreases and no changes have been reported (eg.[23]). This suggests that alpha adrenergic stimulation of Ca^{2+} influx will depend on the dominance of the DAG/PKC pathway [24] and the one initiated by IP_3 activation. Such could then contribute to systolic Ca^{2+} increases. Here we report that under the conditions of our study, phenylephrine causes no change in typical Ca^{2+} spark events (TEs) in the normal Purkinje cell.

It is more likely that the specific WLE augmentation in perinuclear areas is related to intracellular Ca^{2+} signaling that is dissociated from normal Purkinje cell EC coupling. Thus we speculate that perinuclear IP_3Rs may have a different role from SSL IP_3Rs. For some cardiac cells, IP_3R is expressed predominantly in the nuclear envelope and may play a role in the gene transcription through Ca^{2+}-dependent signaling pathways (excitation-transcription coupling) [1 3 25 26]. Perinuclear IP_3Rs in Purkinje cells may also be related to gene transcription and cellular processes. The locations of perinuclear WLEs and IP_3R are close to the nucleus (Figure 3). Thus, it is likely that perinuclear IP_3R can mobilize large Ca^{2+} stores under neurohumoral stimuli in Purkinje cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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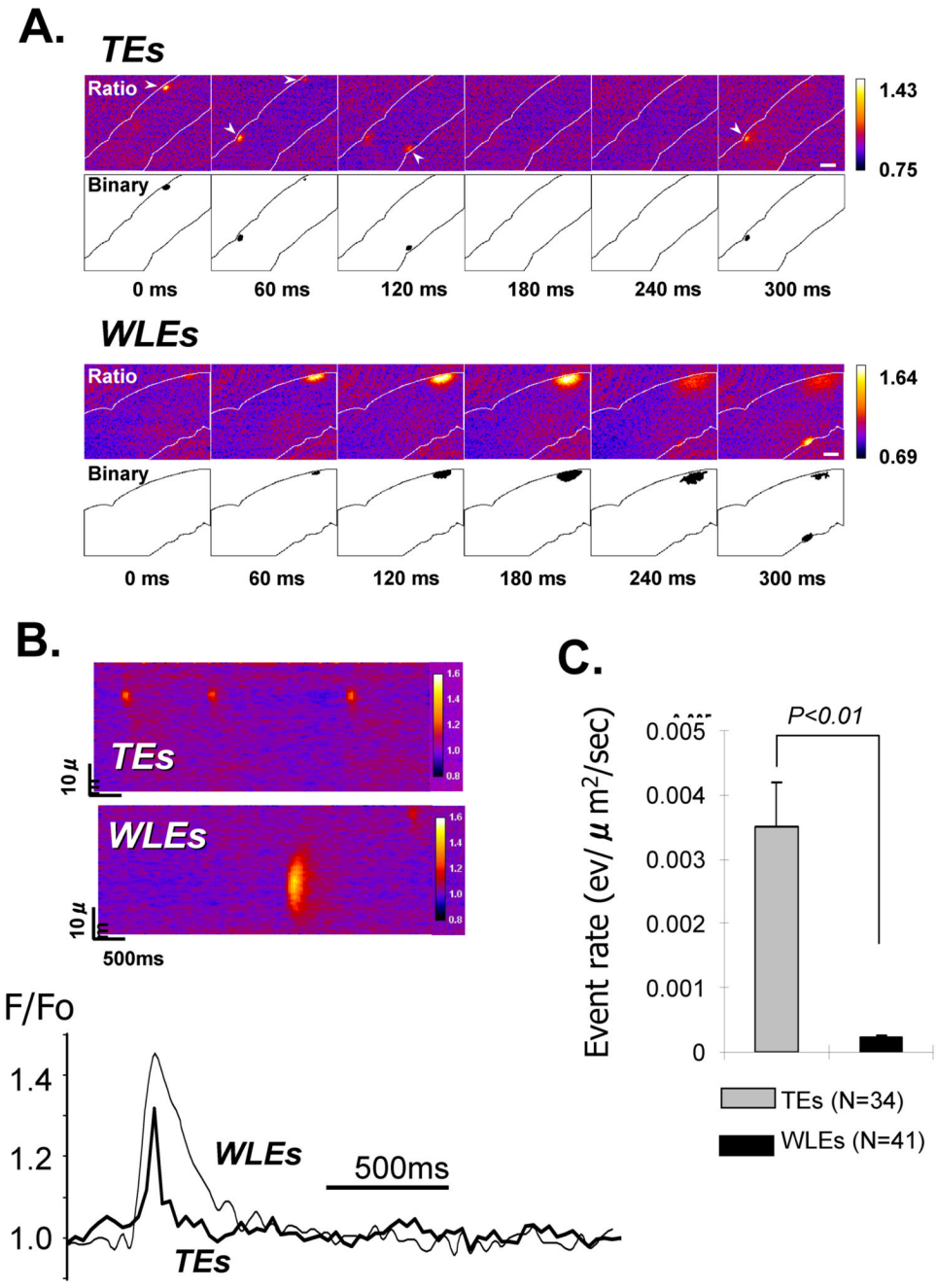

FIGURE 1. Typical (TEs) and Wide long lasting (WLEs) Ca^{2+} events in intact canine Purkinje cells

A: Two sequences of consecutive serial frames showing respectively TEs (**top**) and WLEs (**bottom**); each series of F/Fo images (upper part) is accompanied by the corresponding binary representations (lower part); the computational procedure for the detection of non-propagating events was based on the selection of a F/Fo cutoff value; the equivalent 'binary' images were constructed by giving the values of '0' or '1' to pixels depending whether the intensity was below or above the cutoff. Note that the time scale is relative to t=0 of the first frame of the sequence with 60ms per frame; **B**: Quantitative comparison between TEs and WLEs using pseudo-line scan images: the reconstruction of line scans from 2D images of panel A enables

comparisons between local *F*/*Fo* profiles of TEs and WLEs such as those shown in the lower panel. **C**: Compared to TEs, WLEs are less frequent in intact Purkinje cells. N is number of cells.

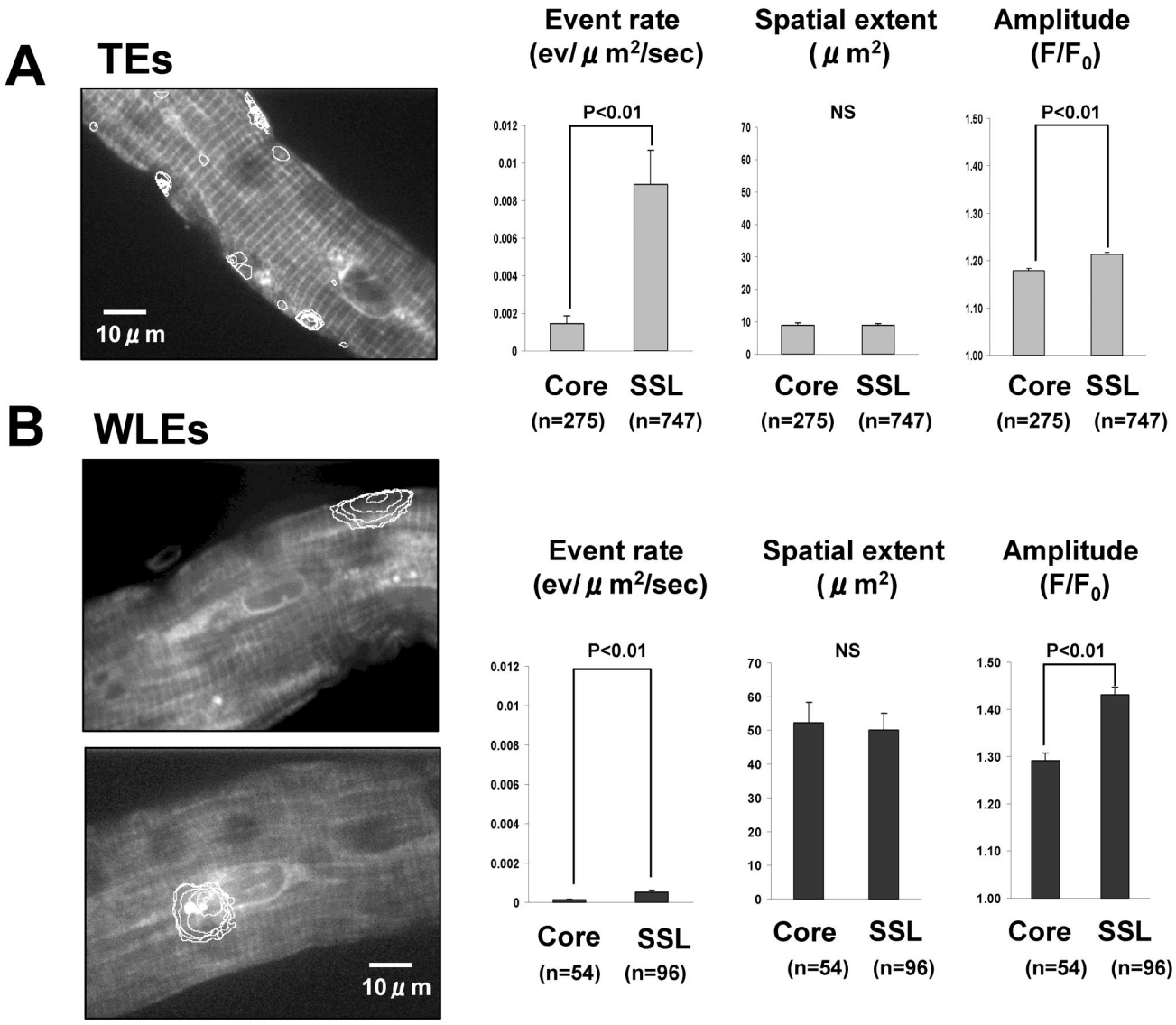

FIGURE 2. Characteristics in Typical events(TE) (gray bars) and Wide long lasting events(WLE) (black bars) by subcellular regions (SSL (<5μm from sarcolemma) and Core)

A. Stacked Cell Images with representative Typical Ca^{2+} events in Core and SSL. Summary bar graphs(right) show the differences between Core and SSL in Event rate (ev/μm^2/sec, left), Spatial extent (μm^2, middle) and Amplitude(*F/Fo*, right) for Typical events. n = number of events. TEs: 34 cells; WLEs: 41 cells.

B. Stacked Cell Images with representative Wide long lasting Ca^{2+} events in Core(lower) and SSL(upper). Summary bar graphs(right) show the differences between Core and SSL in Event rate (ev/μm^2/sec, **left**), Spatial extent(μm^2, **middle**) and Amplitude (F/Fo, **right**) of WLE events.

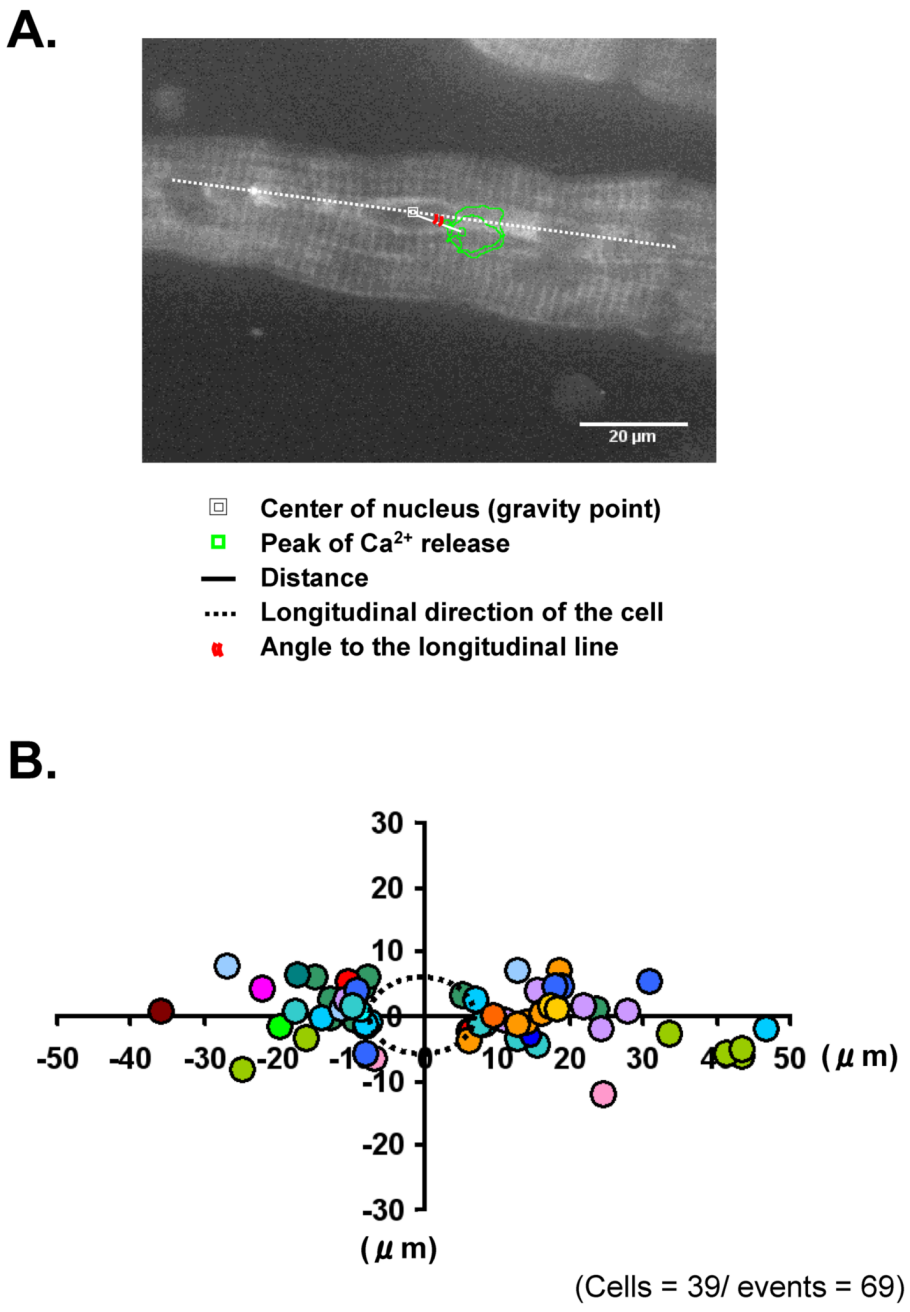

FIGURE 3. Location of Wide long lasting Ca^{2+} events in Core plane of Purkinje cells

A; Typical 2D confocal scan of Purkinje cell through nuclear level. Shown are various measures made to determine subcellular locale of WLEs. **B**; A plot illustrating the location of Core WLEs in reference to nucleus. Each dot represents a specific data point. Note that WLEs occur along the longitudinal axis of cell and not in core area transverse to nucleus. Total number of normal Purkinje cells/events as indicated.

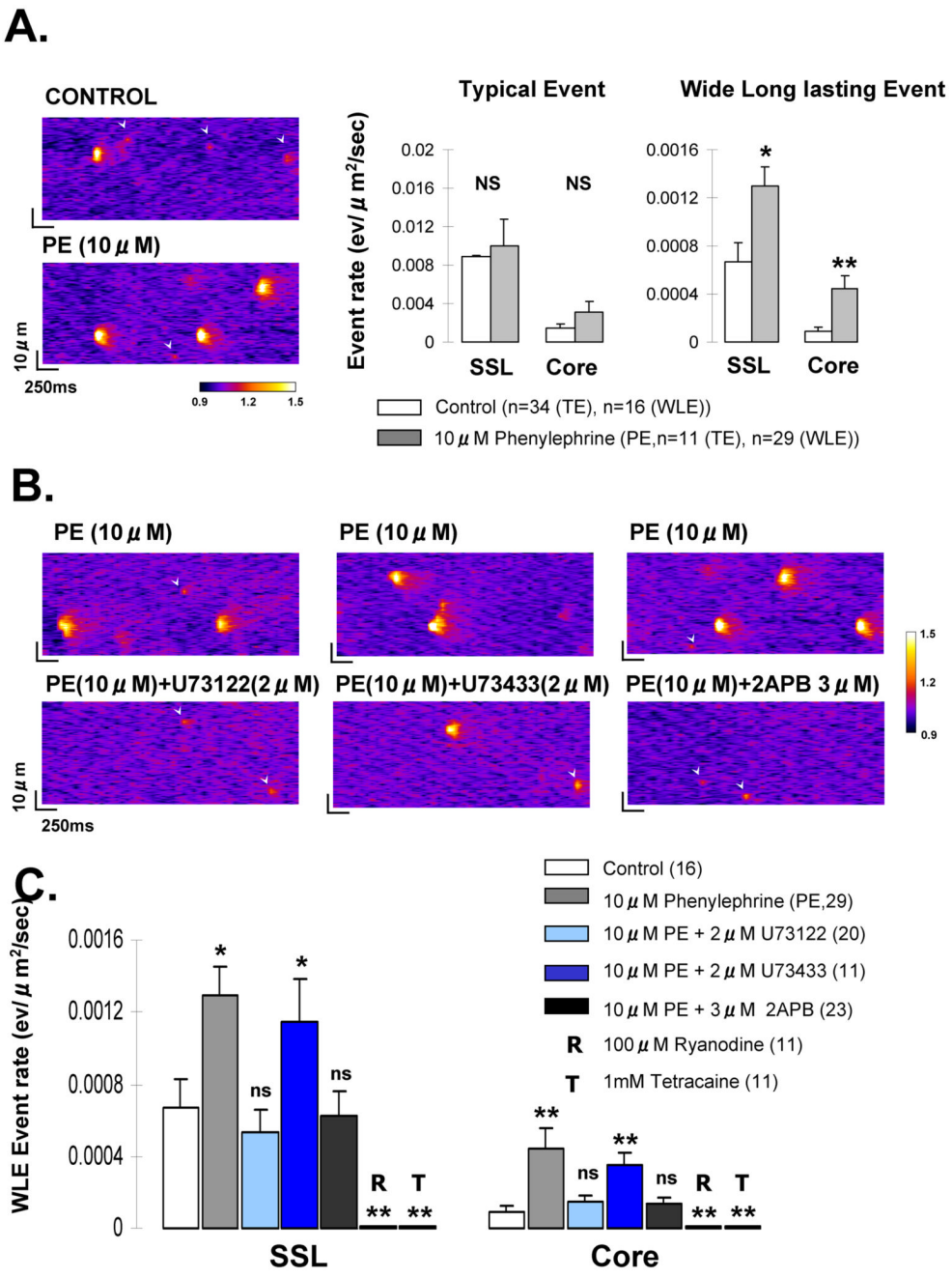

FIGURE 4. Effects of Pharmacological Agents on WLEs

Pseudo-linescan images of Ca^{2+} events in control(absence of any drug), in presence of Phenylephrine (PE) alone(**Panels A,B**) PE plus U73122, PE plus U73433, PE plus 2APB. **Panel A** right illustrates the effects of PE alone on Typical event and Wide long lasting event rate in SSL and Core of normal Purkinje cells. Calibration F/Fo Bar below. **Panel B** Calibration F/Fo Bar to the right. Arrowheads indicate the occurrence of TEs. **C**. Summary bar graph showing the effects of various agents on the spontaneous WLE Ca^{2+} event rate (ev/μm^2/sec) in Core (right) and SSL (left) of Purkinje cells. The height of each bar indicates average event rate value (+SEM). ( )=the number of cells. *P<0.05 vs Control, **P<0.01 vs Control. ns = no significant difference between Control and agent-treated cells.

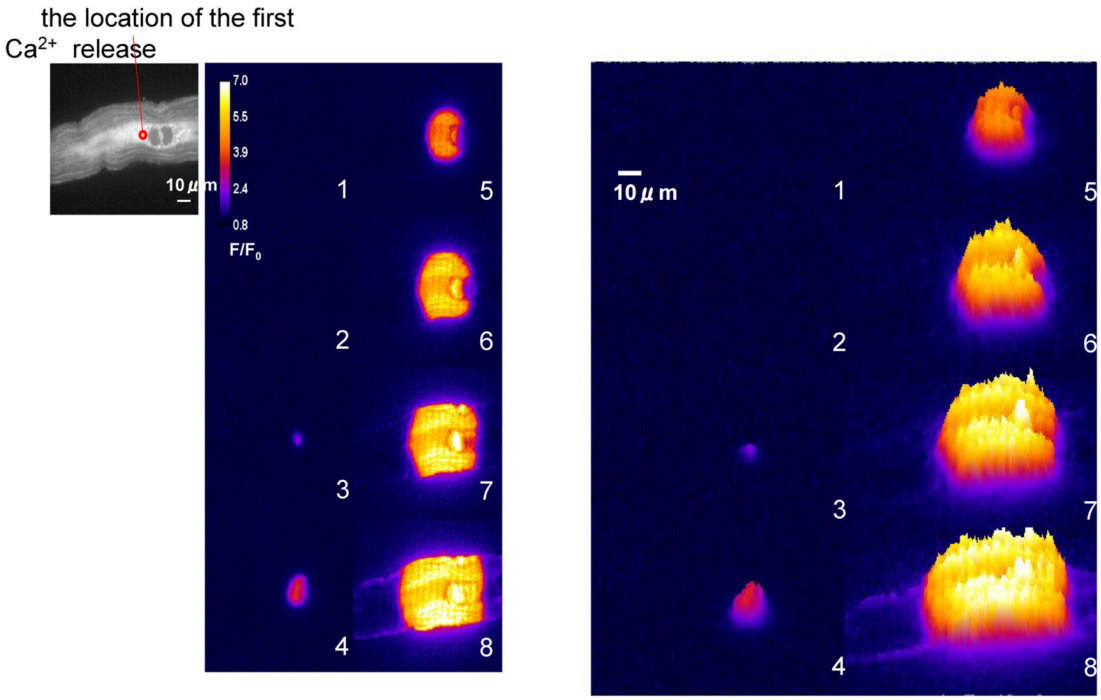

Figure 5B

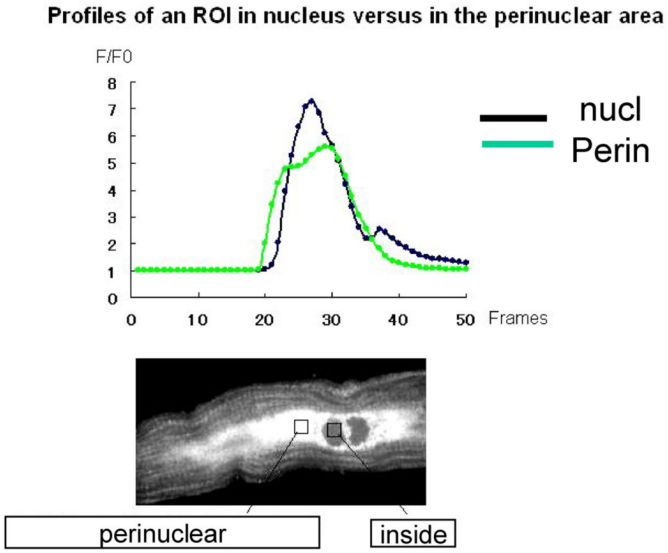

FIGURE 5. Perinuclear Ca^{2+} event initiating a Cell wide Ca^{2+} wave in a normal Purkinje Cell

A. Consecutive images showing the initiation of a Cell wide Ca^{2+} wave from a WLE in the perinuclear region (see upper inset) of an intact Purkinje cell. Middle panels show F/Fo images 1–8 in 2D representation. Right panel shows same images as pseudo 3D. Color bar in middle is calibration of F/Fo. Only on rare occasion did a WLE give rise to a Cell wide Ca^{2+} wave in normal canine Purkinje cells. **B**. Linear profiles from two ROIs during the propagation of Ca^{2+} from perinuclear area to inside nucleus.

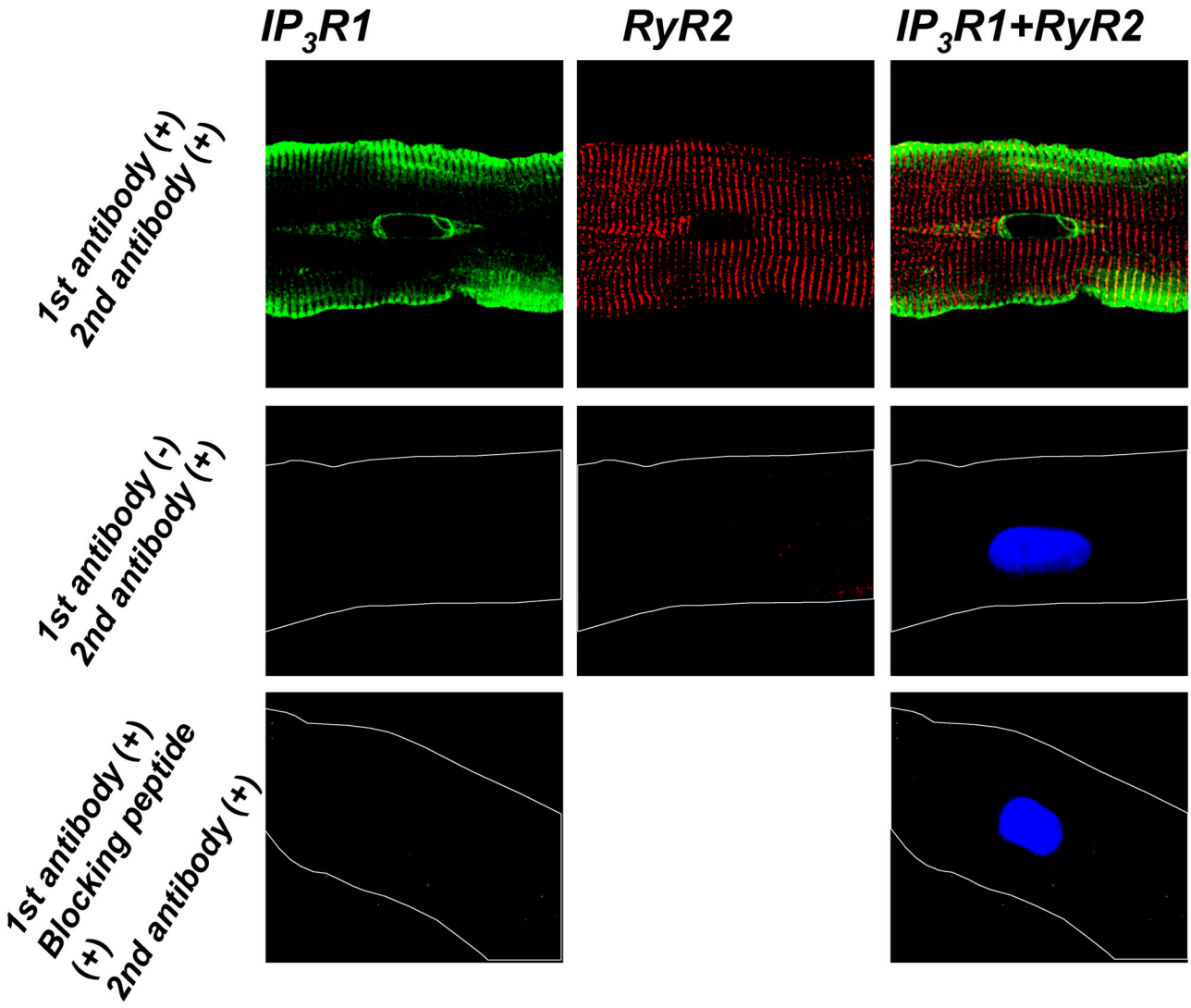

FIGURE 6. Co-localization of SR Ca^{2+} release channels, IP_3R1(left) and RyR2(middle), in a normal Purkinje cell

Overlap images are to the **right**. Costaining showing that IP_3R1 (green) is located in the sarcolemma and perinuclear regions while RyR2 (red) is throughout the cell. As shown secondary antibody alone showed no signal (middle images) and primary IP_3R1 antibody plus blocking peptide showed no staining (lower images). Lower images also stained for nucleus (blue,Dapi).