Ca\(^{2+}\) entry-independent effects of L-type Ca\(^{2+}\) channel modulators on Ca\(^{2+}\) sparks in ventricular myocytes

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The aim of this study was to confirm and extend the original observations that DHPRs can modulate RyR2-mediated Ca\(^{2+}\) sparks in cat ventricular myocytes via Ca\(^{2+}\) entry-independent interactions. Experiments were carried out with cardiac myocytes permeabilized with saponin and with intact cells internally perfused via a patch pipette. Bath and patch pipette solutions were identical and contained 100 nM Ca\(^{2+}\). This prevented Ca\(^{2+}\) entry and changes in membrane voltage. Permeabilization also allowed us to control the sarcolemmal milieu surrounding RyR2 channels. Under these conditions, we determined that various L-type Ca\(^{2+}\) channel agonists and blockers (DHPR modulators) modified the frequency of Ca\(^{2+}\) sparks but had no direct effects on RyR2 channels or the sarco(endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump. A preliminary communication of these findings has appeared in abstract form (14).

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METHODS

Cardiac muscle preparations. The procedure for cell isolation was approved by the Institutional Animal Care and Use Committee of Loyola University Chicago Stritch School of Medicine. Adult mongrel cats of either sex were anesthetized with thiopental sodium (30 mg/kg ip). Following a thoracotomy, hearts were quickly excised, mounted on a Langendorff apparatus, and retrogradely perfused with collagenase-containing solution at 37°C according to methods described previously (39). All experiments were carried out at room temperature (20–22°C).

Heavy SR membrane fractions (SR microsomes) were used to study the activity of SERCA and of RyR2 channels reconstituted into planar bilayers. SR microsomes were obtained from the rat ventricle using cellular subfractionation methods (6). All preparations were kept in liquid nitrogen. Aliquots (15 μl each) of membranes were stored at −80°C. For experiments, aliquots were quickly thawed in water, kept on ice, and used within 5 h.

Measurements of Ca²⁺ sparks in permeabilized and intact myocytes. The sarcolemma of cat ventricular myocytes was permeabilized with saponin (0.005% for 30 s) (60). After permeabilization, cells were placed in an experimental solution containing (in mM) 100 K⁺-aspartate, 15 KCl, 5 KH₂PO₄, 5 MgATP, 0.4 EGTA, 0.12 CaCl₂, 0.75 MgCl₂, 10 phosphocreatine, 10 HEPES, and 0.04 fluo-3 pentapotassium salt. The solution also contained creatine phosphokinase (5 U/ml) and dextran (molecular weight: 40,000; 8%) and was titrated to pH 7.2 (KOH). The free [Ca²⁺]₀ and [Mg²⁺]₀ of this solution were 100 nM and 1 mM, respectively (calculated using WinMAXC 2.05, Stanford University).

Intact ventricular myocytes were voltage clamped (Axopatch 200B patch-clamp amplifier, Axon Instruments, Foster City, CA) using the whole cell ruptured-patch configuration. Cells were internally perfused with the same solution used for permeabilized cell experiments. The external solution had an identical ionic composition except for ATP, phosphocreatine, and creatine phosphokinase, which were excluded. Myocytes were voltage clamped at a holding potential of 0 mV −80°C. This allowed the detection of sparks with an amplitude of background noise of the fluorescence image. The detection threshold was set to 3.5 SD. This allowed the detection of sparks with an amplitude of ΔF/ΔF₀ ≈ 0.3 or larger. Ca²⁺ spark frequencies are expressed as numbers of observed sparks per second per 100 μm of scanned distance (sparks s⁻¹ 100 μm⁻¹). No corrections were made for missed events.

Measurements of Ca²⁺ uptake by SR microsomes. Ca²⁺ uptake by SR microsomes was measured with a spectrophotometer (Cory 50, Varian, Walnut Creek, CA) using the Ca²⁺-sensitive dye aequorin (APHH). SR membrane vesicles (50 μg/ml) were added to 1 ml phosphate buffer containing (in mM) 100 KH₂PO₄, 4 MgCl₂, 2 ATP, 0.01 ruthenium red, and 0.2 APIII; pH 7.0. Ca²⁺ uptake was initiated by the addition of 30 μM Ca²⁺ to the medium and measured as changes in absorbance of APIII (Δabs) between 710 and 790 nm. Ruthenium red (10 μM) was used to block Ca²⁺ leaks from the SR.

RyR channel recordings and analysis. Planar lipid bilayers were formed on 80- to 150-μm-diameter circular holes in Teflon septa, separating two 1.3-ml compartments, as previously described (11). Briefly, the trans compartment was filled with HEPES-Ca²⁺ solution [250 mM HEPES and 53 mM Ca(OH)₂; pH 7.4] and subsequently clamped at 0 mV by an Axopatch 200B patch-clamp amplifier (Axon Instruments). The cis compartment (ground) was filled with HEPES-Tris solution (250 mM HEPES and 120 mM Tris; pH 7.4). Bilayers of a 5:4:1 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (45–50 mg/ml in decane) were painted onto the holes of bilayer septa from the cis side. Subsequently, 500–1,000 mM CsCl and 1 mM CaCl₂ were added to the cis solution to promote vesicle fusion. Cardiac SR microsomes (5–15 μg) were then added to the cis solution. After fusion, ion currents >100 pA were observed at 0 mV (mediated by RyRs as well as by other cationic and anionic channels that permeate Cs⁺ or Cl⁻). Subsequently, the CsCl was removed by perfusing the cis chamber with HEPES-Tris solution (5 min at 4 ml/min). As shown previously (11), a mixture of BAPTA and dibromo-BAPTA was used to buffer free [Ca²⁺]₀ on the cis side of the surface of the channel ([Ca²⁺]₀, cis).

RyR2 recordings were filtered through a low-pass Bessel filter at 1–10 kHz and digitized at 20–100 kHz with a Digitida 1320 digital converter (Axon Instruments). Analysis was done using pCLAMP6 or pCLAMP9 software (Axon Instruments). Open times, closed times, and open probability (Pₒ) were determined by half-amplitude threshold analysis of single-channel recordings (11). For simplicity of the analysis, exponential fitting of dwell-time histograms (see Figs. 7 and 8) was performed assuming that all open and closed time distributions included only two components, which is an approximation (17). The simpler parameter of mean open time was used in Fig. 9 because of the limited number of events recorded, which did not allow accurate fitting of complex dwell-time distributions to the data.

Drugs and chemicals. The CaCl₂ standard for calibration was from World Precision Instruments (Sarasota, FL). Calciseptine and FS-2 were from Alomone Laboratories (Jerusalem, Israel). Nifedipine, nimodipine, FPL-64176 (FPL) and BayK were from Alomone Laboratories or Sigma Chemical (St. Louis, MO). Phospholipids were obtained from Avanti (Alabaster, AL). Fluo-3 was from Invitrogen/Molecular Probes (Carlsbad, CA). All other drugs and chemicals used in this work were analysis grade.

Statistical analysis. Data are presented as means ± SE of n measurements. Statistical comparisons between groups were performed with Student’s t-test of paired differences. Differences were considered statistically significant at P < 0.05.

RESULTS

DHPR channel blockers decreased Ca²⁺ spark frequency in permeabilized myocytes. It is known that DHPRs activate RyR2-mediated global Ca²⁺ transients (4, 56) and local Ca²⁺ sparks (42, 48, 53) through Ca²⁺ entry. Here, we tested the idea of possible Ca²⁺-independent modulation of SR Ca²⁺ release by DHPRs. First, we studied the effects of various antagonists of DHPRs on spontaneous Ca²⁺ sparks in saponin-permeabilized ventricular myocytes. Free [Ca²⁺]₀ in extracellular and intracellular solutions was buffered to ~100 nM, which renders the DHPR ineffective as a Ca²⁺ channel.

In Fig. 1A, we show the effect of nifedipine, an antagonist of the DHPR, on Ca²⁺ sparks. Nifedipine binds to specific sites on the DHPR, known as “dihydropyridine sites” (50, 51). Representative confocal linescan images of Ca²⁺ sparks and plots of F/F₀ from selected subcellular regions are shown in Fig. 1A,a under control conditions and 5 min after the addition of nifedipine (5 μM). Nifedipine significantly decreased the frequency of Ca²⁺ sparks by 55% (from 4.7 ± 0.5 to 2.1 ± 0.2 sparks s⁻¹ 100 μm⁻¹) from control values (n = 9 cells, P < 0.01; numbers of sparks analyzed under control conditions and
Fig. 1. Nifedipine (Nif) and nimodipine (Nimod) decreased Ca\textsuperscript{2+} spark frequency in saponin-permeabilized cat ventricular myocytes. 

**A:** confocal linescan images of Ca\textsuperscript{2+} sparks under control (Ctrl) conditions and 5 min after the addition of nifedipine (5 \mu M). Bottom traces are local $\Delta F/F_0$ profiles (where $\Delta F$ is the change in fluorescence and $F_0$ is initial fluorescence) of Ca\textsuperscript{2+}-release events. These $\Delta F/F_0$ plots were obtained by averaging fluo-3 fluorescence from the 1-\mu m-wide regions marked by boxes on the left of the linescan images. 

**A,a:** average linescan images of sparks ($n$ = 18 events each) observed under control conditions and with nifedipine (5 \mu M). 

**A,c-f:** numerical data of spark characteristics [frequency (c), amplitude (d), duration (e), and width (f)] under control conditions and in the presence of nifedipine. 

**B:** confocal linescan images of Ca\textsuperscript{2+} sparks under control conditions and in the presence of nimodipine (1 \mu M). Bottom traces are local $\Delta F/F_0$ profiles of Ca\textsuperscript{2+}-release events in the regions marked by boxes. 

**B,b-e:** average data of Ca\textsuperscript{2+}-release events [frequency (b), amplitude (c), duration (d), and width (e)] under control conditions and in the presence of nimodipine. *Significant nifedipine or nimodipine effects on Ca\textsuperscript{2+} spark frequency compared with control ($P < 0.01$).
in the presence of nifedipine were 583 and 461, respectively; Fig. 1A,c). Nifedipine did not significantly change activation and decay kinetics (Fig. 1A,b) or the average amplitude, duration, or width of Ca<sup>2+</sup> sparks (Fig. 1A,d-f). Increasing the nifedipine concentration up to 20 μM did not produce any further inhibition of Ca<sup>2+</sup> sparks. The effect of nifedipine was almost irreversible upon washout.

In Fig. 1B, we show experiments with nimodipine, another blocker that binds to the dihydropyridine site with 5–10 times higher affinity than nifedipine. After 5 min of exposure, nimodipine (1 μM) greatly decreased spark frequency by 60% (4.5 ± 0.6 to 1.8 ± 0.6 sparks·s<sup>-1</sup>·100 μm<sup>-1</sup>) from control values (n = 5 cells, P < 0.01; numbers of sparks analyzed under control conditions and in the presence of nimodipine were 273 and 198, respectively; Fig. 1B,a and b). Nimodipine (as found for nifedipine) did not affect the amplitude, duration, and width of Ca<sup>2+</sup> sparks (Fig. 1B,c-e). Thus, both antagonists of DHPR nifedipine and nimodipine caused a similar degree (55–60%) of decrease in Ca<sup>2+</sup> spark frequency without altering the spatiotemporal characteristics of sparks.

Figure 2 shows the effects of calciseptine (A) and FS-2 (B). These are two peptides known to block DHPR channels and to interact with the dihydropyridine binding site (57). These two peptides also decreased Ca<sup>2+</sup> spark frequency of permeabilized myocytes. As found for nifedipine and nimodipine (Fig. 1), calciseptine and FS-2 were also without effect on the amplitude, duration, or width of sparks (not shown). In contrast to dihydropyridines, the effects of these water-soluble peptides were reversed by superfusion (not shown).

Not all DHPR channel blockers inhibited Ca<sup>2+</sup> sparks in permeabilized myocytes. Verapamil, which binds to phenylalkylamine sites (50), had no effect on Ca<sup>2+</sup> sparks (Fig. 3A). The presence of verapamil did not prevent the action of nifedipine, which remained effective at decreasing the spark frequency (Fig. 3B).

DHPR channel agonists increased the frequency of Ca<sup>2+</sup> sparks in permeabilized myocytes. The effects of two DHPR channel agonists, BayK and FPL, are shown in Fig. 4. BayK (1 μM) increased spark frequency by 53% (from 3.2 ± 0.6 to 4.9 ± 0.8 sparks·s<sup>-1</sup>·100 μm<sup>-1</sup>, n = 5 cells, P < 0.01; numbers of sparks analyzed under control conditions and in the presence of BayK were 242 and 308, respectively; Fig. 4A,a and b). BayK had little or no effect on spark amplitude, duration, and width (Fig. 4A,c-e). Figure 4B,a and b, shows that FPL (5 μM) also increased the frequency of Ca<sup>2+</sup> sparks by 69% (from 4.2 ± 0.9 to 7.1 ± 1.3 sparks·s<sup>-1</sup>·100 μm<sup>-1</sup>, n = 4 cells, P < 0.05). As with the other tested compounds, the amplitude, duration, and width of sparks did not change significantly (Fig. 4B,c-e).

In summary, our results suggest that L-type Ca<sup>2+</sup> channel antagonists and agonists produce opposite effects on Ca<sup>2+</sup> spark frequency in permeabilized cells that were independent of Ca<sup>2+</sup> entry but did not affect the spatiotemporal properties of individual Ca<sup>2+</sup> sparks.

**DHPR channel modulators did not change SR Ca<sup>2+</sup> load and did not affect SERCA pump activity.** It is known that Ca<sup>2+</sup> spark frequency depends on the SR Ca<sup>2+</sup> load (7, 28, 43). Therefore, the effect of DHPR channel modulators on the amplitude of the [Ca<sup>2+</sup>]<sup>i</sup> transient induced by the addition of 20 mM caffeine was examined. The magnitude of the transient was used as an index of SR Ca<sup>2+</sup> load. Figure 5 shows confocal linescan images and ΔF/F<sub>0</sub> plots of Ca<sup>2+</sup> release induced by the application of 20 mM caffeine under both control conditions (left) and in the presence of nifedipine (5 μM; right). Nifedipine did not cause any changes in SR Ca<sup>2+</sup> load during the first minute. Incubation with this modulator for >5 min produced a small increase of 12 ± 6% in the amplitude of the caffeine-induced [Ca<sup>2+</sup>]<sup>i</sup> transient (n = 4, not significantly different from control).

In parallel experiments, we tested the direct effect of DHPR modulators on Ca<sup>2+</sup> uptake by isolated SR microsomes. The aim was to determine the possible effects of DHPR modulators on the kinetics of SERCA-mediated Ca<sup>2+</sup> uptake, which could affect the rate of refilling of the SR and consequently the frequency of sparks (7, 28, 43). Figure 6A shows the time course of Ca<sup>2+</sup> uptake into SR microsomes in response to a Ca<sup>2+</sup> spike. Recordings are shown for control conditions as well as for SR microsomes incubated with nifedipine or BayK.

In all cases, after the addition of Ca<sup>2+</sup> there was rapid uptake. The net Ca<sup>2+</sup> uptake equals SR Ca<sup>2+</sup> influx (mediated by
SERCA) minus SR Ca2+ leak (via RyRs). Because the experiments were conducted in the presence of ruthenium red (10 μM), the RyR-mediated Ca2+ leak was fully inhibited. Time courses of Ca2+ uptake after the spike were similar under all conditions. This suggests that nifedipine and BayK did not affect the rate of Ca2+ uptake into SR microsomes. Figure 6B shows time constants of SR Ca2+ uptake (expressed as percentages of control values) for all DHPR modulators used in our study. None of the compounds tested had any effect on SERCA-mediated SR Ca2+ uptake kinetics at the doses used to modify Ca2+ sparks. Some compounds (FPL, nifedipine, and BayK) had minor effects on SERCA-mediated loading (~10–20% inhibition) when tested at very high levels (20–50 times higher concentrations than those used in Ca2+ spark experiments). Thus, the effects of DHPR modulators on Ca2+ sparks could not be explained by changes of SR Ca2+ load or SERCA pump activity.

**DHPR channel modulators did not affect the behavior of RyR2 channels reconstituted into planar lipid bilayers.** RyR2 channels from rat ventricular myocytes were reconstituted into planar lipid bilayers following methods previously described (11). Examples of single RyR2 recordings under control conditions and in the presence of nifedipine are shown in Fig. 7A. Experiments were conducted in the presence of Mg2+ and ATP at cytoplasmic levels. Channels were partially activated with 5 μM cytosolic Ca2+. The addition of nifedipine did not change single-channel current amplitude (Fig. 7A). Nifedipine was also without effect on RyR2 P0 values (Fig. 7B) and on the dwell time distribution of channel events (Fig. 7, C and D). Similar results (i.e., no significant effect on RyR2 channel properties) were found with the DHPR blockers FS-2 (n = 9) and calcisentinel (n = 6).

The effects of DHPR agonists on RyR2 channels were also tested. As shown in Fig. 8, BayK did not significantly change current amplitude, P0, or dwell-time distributions of RyR2 channels in 5 μM Ca2+. Similarly, FPL (5 or 10 μM) was without effect on RyR2 current amplitude, P0, or dwell-time distribution (n = 8; results not shown). This lack of effect on the kinetics of Ca2+-activated RyR2 is in agreement with our Ca2+ spark experiments, where the magnitude and spatiotemporal characteristics of sparks were not affected (see above).

In cells, Ca2+ sparks originate from channels that are exposed to lower Ca2+ levels ([Ca2+]cyt = 100 nM). Consequently, kinetic data from RyR2 channels activated by micromolar levels of Ca2+, similar to previously reported tests (17, 41), do not adequately answer the key question of whether DHPR modulators affect spontaneous RyR2 gating at resting [Ca2+]cyt. Therefore, we studied RyR2 gating using cytosolic bathing solutions containing Ca2+, Mg2+, and ATP, as in the Ca2+ spark experiments. Since, at 100 nM, Ca2+-activated RyR2 channels are quite inactive (P0 < 0.01), testing these conditions required long recordings (8- to 12-min duration).

As shown in Fig. 9, spontaneous single opening events were very infrequent (ranging from a few dozen to a few hundred for the whole 8–12 min recordings). Experiments with cells showed that DHPR modulators induce changes in spark frequency from −60 to +70%. Thus, a similar change in the frequency of spontaneous channel openings should be observed if there was a direct effect of DHPR modulators on RyR2. We found, however, that FPL had no significant effect on the frequency and mean duration of spontaneous channel openings under resting [Ca2+]cyt conditions (Fig. 9). Similar results (no effect) were obtained when comparing the frequency and duration of spontaneous events under control conditions and in the presence of other DHPR modulators (20 μM nifedipine, n = 4; and 5 and 10 μM Bay-K8644, n = 5). These results strongly support the notion that RyR2 is not directly affected by DHPR modulators.
Effects of DHPR modulators on Ca\textsuperscript{2+} sparks in myocytes internally perfused with a patch pipette. The results shown above imply that DHPR modulators affect Ca\textsuperscript{2+} sparks in the absence of Ca\textsuperscript{2+} entry and that their effect does not appear to involve RyR2 or SERCA modulation. These results would suggest that Ca\textsuperscript{2+} entry-independent DHPR-RyR2 interactions modulate sparks in permeabilized myocytes. The presence of those interactions in intact myocytes was also tested (i.e., whether they were not an artifact induced by the process of saponin permeabilization). For this purpose, intact myocytes were internally perfused through a patch pipette. In these experiments, the pipette and bathing solutions were identical to the ones used for permeabilized cell experiments. Thus, any ion gradients (particularly Ca\textsuperscript{2+}) across the sarcolemma were eliminated. As it is well established for low Ca\textsuperscript{2+} solutions, the electrical seal between the pipette and cell membrane was quite leaky. This precluded experiments with modifications of the holding potential. As shown in Fig. 10, nifedipine also decreased spark frequency in internally perfused myocytes. On average, nifedipine (5 \mu M) decreased the frequency of Ca\textsuperscript{2+} sparks from 4.0 \pm 0.6 to 2.5 \pm 0.3 sparks/s/(100 \mu m)^2, or by 37\% from control values (n = 4 cells, P < 0.01). Similar to
saponin-permeabilized cells, nifedipine did not significantly change the spatiotemporal characteristics of Ca\(^{2+}\) sparks.

**DISCUSSION**

We found that Ca\(^{2+}\) spark frequency in permeabilized ventricular myocytes is affected by various L-type Ca\(^{2+}\) channel agonists and blockers (DHPR modulators). In permeabilized cells, the membrane potential was collapsed, and our bathing solutions (both in the cytosol and extracellular space) contained low-Ca\(^{2+}\) (100 nM, corresponding to typical resting [Ca\(^{2+}\)].\(_{cyt}\) in cardiac myocytes) and identical Ca\(^{2+}\) levels. The elimination of the transsarcolemmal Ca\(^{2+}\) gradient renders DHPRs ineffective as Ca\(^{2+}\) channels. Thus, the effects of DHPR modulators on Ca\(^{2+}\) spark frequency were independent of DHPR-mediated Ca\(^{2+}\) entry.

In the absence of DHPR-mediated Ca\(^{2+}\) entry, agonists/blockers of DHPR remain effective as Ca\(^{2+}\) spark modulators. However, the DHPR modulators had no effect on the spontaneous activity of RyR2 channels under resting conditions or on the rate of SR Ca\(^{2+}\) loading by the SERCA pump. Therefore, the observed effects of DHPR modulators on RyR2-mediated Ca\(^{2+}\) sparks of permeabilized myocytes suggest the existence of a physical/functional, Ca\(^{2+}\) entry-independent link between cardiac DHPRs in T tubules and RyR2 in the SR.

**DHPR modulators affect Ca\(^{2+}\) sparks in the absence of Ca\(^{2+}\) entry.** We found that various DHPR blockers decreased the frequency of Ca\(^{2+}\) sparks. All the effective DHPR blockers (nifedipine, nimodipine, calciseptine, and FS-2) are known to competitively interact with the dihydropyridine binding site (31, 50). Contrarily, verapamil, which binds to the site of phenylalkilamines and does not affect the DHPR inactivation process, was without effect (31). An increase in spark frequency was found with a DHPR agonist (BayK) that interacts with the dihydropyridine site (50, 51) and also with FPL, which appears to have a different mechanism of action and involves binding to a novel benzolpyrrole site (50). We did not test all other available classes of DHPR modulators. Consequently, the picture of the pattern of specificity of binding versus efficiency for spark modulation in the absence of Ca\(^{2+}\) entry is still incomplete. Nonetheless, the dihydropyridine site appears to play a significant role in Ca\(^{2+}\) spark modulation.

Various groups have reported that Ca\(^{2+}\) entry is required for changes in spark frequency associated with voltage or with the use of BayK (1, 48). However, our present findings support the work of Bers and colleagues (23, 44), who also detected effects of BayK on sparks of intact myocytes (without permeabilization) in Ca\(^{2+}\)-free solutions. This suggested the existence of some kind of physical interaction between DHPR and RyR2. In the present study, permeabilized cells were utilized to gain better control of Ca\(^{2+}\) levels in the “cytosolic milieu.” This condition prevents any effect of DHPR modulators related to changes in cytosolic Ca\(^{2+}\) that concomitantly could affect SR load (28, 43).

In permeabilized cells, nifedipine was an effective inhibitor of sparks. In intact cells, direct effects of nifedipine on Ca\(^{2+}\) sparks were not found; however, nifedipine inhibited the action of BayK (23, 44). The differential effect of nifedipine may be related to the preferred binding of this compound to the open or inactivated conformations of the DHPR, which are favored when the membrane potential collapses (as in permeabilized...
cells) (22, 31, 50, 51). At -80 mV (as in intact cells), DHPR channels are mostly in the closed state, which has a much lower affinity for nifedipine. The addition of BayK stabilizes some channels in open and inactivated conformations, which bind nifedipine with much higher affinity (22, 31, 50, 51). Thus, studies with intact and permeabilized cells are complementary and suggest that sparks are favored by maneuvers (permeabilization) or compounds that stabilize the DHPR in the open state (BayK and FPL).

A possible limitation of our experiments is that they were all carried out under conditions of nonphysiologically low extracellular Ca$^{2+}$. However, Bers and coworkers (30) have shown that the effects of BayK are relatively independent of membrane potential, SR Ca$^{2+}$ load, or extracellular Ca$^{2+}$ levels, suggesting that the DHPR-RyR interaction may take place under more physiological conditions.

Another concern, from EM studies and from studies showing a low DHPR-to-RyR2 ratio (~0.15 to 0.3) in cardiac myocytes, is that a minority of RyR2 channels are directly linked to cardiac DHPRs (3, 5, 18, 19). This is in contrast to fast skeletal muscle, where the DHPR-to-RyR1 ratio can reach 2 and where RyR1s at the face membrane of the terminal cisternae are found to physically overlap with DHPR tetrads at the T tubule (5, 18, 19). However, in some slow twitch skeletal muscles, DHPR-to-RyR ratios are considerably lower than 2, indicating that many RyR1s would not directly interact with DHPR tetrads (33). This suggests that DHPR control of RyRs may operate with different DHPR-RyR geometries at the T tubule. Furthermore, it is becoming increasingly clear that groups of RyR1 or RyR2 channels can gate coupled (13, 29). This coupled gating provides a possible mechanism for control of most cardiac RyR2s even if only a few of them are directly linked to DHPRs. In support of this notion is the present finding that DHPR modulators affect Ca$^{2+}$ spark frequency but do not change spark properties such as amplitude, duration, and spatial width (i.e., the probability of triggering a spark is changed but not the normal dynamics of Ca$^{2+}$ release from a RyR2 cluster once it is activated).

DHPR modulators do not affect SERCA or RyR2 channels. In ventricular myocytes, it is thought that sparks arise from spontaneous activation of clusters of RyR2 channels. Therefore, the observed alteration of Ca$^{2+}$ spark frequency by DHPR modulators could be explained by direct or indirect effects of these agents on gating properties of the RyR. Direct effects would be expected to change the amplitude, width, and (possibly) duration of sparks; however, no such effects were observed. Indirect effects could be related to changes in SR Ca$^{2+}$ load as well as the kinetics of Ca$^{2+}$ repletion of the SR (21, 43) occurring through modulation of the SERCA pump. As shown in Figs. 5 and 6, none of the DHPR modulators

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**Fig. 7.** Nifedipine did not affect single ryanodine receptor (RyR) channel activity. A: representative RyR2 channel recordings under control conditions (left) and after the addition of 20 μM nifedipine (right). All recordings were made at a holding potential of 0 mV with luminal Ca$^{2+}$ as the current carrier. Cytosolic solutions contained near-physiological levels of total ATP (5 mM) and free [Mg$^{2+}$] (1 mM). Cytosolic [Ca$^{2+}$] was 5 μM, which partially activated the channels (for details, see METHODS). B: mean open probability ($P_o$) values of RyR2 channels under control conditions and with cumulative doses of nifedipine (1–40 μM) ($P_o$ values are mean values ± SE from $n = 6$ experiments). C and D: examples of dwell open (C) and closed (D) times under control conditions and in the presence of 20 μM nifedipine (representative of $n = 4$ experiments with single channels). RyR2 $\tau$ values were obtained by fitting the logarithmic dwell time distributions [open (o) or closed (c) event distributions] with two components. Openings of control RyR2 channels distributed with $\tau_o = 1.7 ± 0.2$ ms (68 ± 5% of the events) and $\tau_c = 6.9 ± 1.0$ ms (32 ± 5% of the events). Closures distributed with $\tau_c = 1.2 ± 0.2$ ms (31 ± 4% of the events) and $\tau_c = 8.7 ± 1.6$ ms (69 ± 4% of the events). For nifedipine, $\tau_o = 2.0 ± 0.4$ ms (23 ± 5% of the events), $\tau_o = 6.4 ± 0.9$ ms (67 ± 3% of the events).
tested had a significant effect on the SR load or on SR Ca\textsuperscript{2+} uptake kinetics. Inhibitory effects of the DHPR modulators were observed only when added at much higher (20–50 times higher) doses than those effective to modulate sparks (results not shown). These findings are consistent with previous reports in the literature. BayK was found to inhibit SERCA with an IC\textsubscript{50} of 30–50 M (61). Nifedipine and nimodipine were shown to have a dual effect (activation first and subsequent inhibition) on SERCA (10, 54). If the effects observed here reflect the action of these DHPR modulators on SERCA, we

Fig. 8. BayK did not affect the activity of RyR2 channels reconstituted into planar lipid bilayers. A: representative RyR2 channel recordings under control conditions (left) and in the presence of BayK (10 μM; right). Experimental conditions were as described in Fig. 7. B: lack of effect of BayK on RyR2 \( P_o \) values (means ± SE; \( n = 6 \) experiments). C and D: examples of representative dwell time [open (C) and closed (D) time] distributions of channel events. Control dwell times were \( \tau_1 = 1.0 ± 0.3 \text{ ms (40 ± 4% of the events)} \), \( \tau_2 = 5.5 ± 0.6 \text{ ms (60 ± 4% of the events)} \), \( \tau_3 = 0.9 ± 0.2 \text{ ms (49 ± 13% of the events)} \), and \( \tau_4 = 10.4 ± 0.8 \text{ ms (51 ± 13% of the events)} \). For BayK, \( \tau_1 = 1.5 ± 0.2 \text{ ms (38 ± 3% of the events)} \), \( \tau_2 = 6.3 ± 0.5 \text{ ms (62 ± 3% of the events)} \), \( \tau_3 = 0.8 ± 0.3 \text{ ms (47 ± 11% of the events)} \), and \( \tau_4 = 11.5 ± 1.7 \text{ ms (53 ± 11% of the events)} \).

Fig. 9. FPL did change the frequency and duration of RyR2 openings under resting Ca\textsuperscript{2+} conditions. Data are shown for 4-min recordings of RyR2 channel currents at a holding potential of 0 mV with luminal Ca\textsuperscript{2+} as the current carrier. As above (Figs. 7 and 8), cytosolic solutions contained physiological levels of total ATP and free [Mg\textsuperscript{2+}]. Cytosolic [Ca\textsuperscript{2+}] was 100 mM (the same as for permeabilized myocytes; for details, see METHODS). Under these conditions, RyR2 channels had a \( P_o \) of \( <0.01 \). All events with a duration of \( >0.05 \text{ ms} \) were selected and are shown on expanded time scales. A: control. B: FPL (5 μM). From \( n = 9 \) experiments under conditions as in the examples (8- to 12-min recordings each), we estimated that RyR opening events per channel per minute were \( 0.875 ± 0.132 \text{ vs. 0.708 ± 0.125} \) and mean open times were \( 5.1 ± 1.5 \text{ vs. 6.0 ± 2.3 ms (control vs. FPL, respectively).} \)
would expect a decrease in Ca\(^{2+}\) spark frequency with BayK because inhibition of SERCA decreases SR Ca\(^{2+}\) load. Furthermore, nifedipine would increase spark frequency since the low doses utilized here should slightly increase SERCA activity.

No published evidence was found on inhibitory FPL effects on SERCA pump as observed here for high doses (>20 \(\mu M\)). Similarly, we found no reports on SERCA modulation by FS-2 or calciseptine to compare with our results (no effect). In general, our results and those in the literature indicate that the concentrations of the DHPR modulators utilized here have no significant effects on SERCA.

In the present study, DHPR modulators had no significant effects on RyR2 channel properties, which is in agreement with previous reports (e.g., Refs. 36 and 44). Unlike previous tests (17, 55), the solutions utilized in our planar lipid bilayer experiments contained the endogenous RyR2 modulators ATP and Mg\(^{2+}\) (12), which allowed better comparison with cellular experiments. We tested channels exposed to resting Ca\(^{2+}\) levels (100 nM), which opened infrequently \((P_o < 0.01, 3–15\) events/min) as well as RyR2 partially activated with micromolar [Ca\(^{2+}\)] \((P_o \sim 0.1–0.2)\). The results indicate that DHPR modulators did not affect spontaneous openings of RyR2 at resting Ca\(^{2+}\) levels or \(P_o\) and open-closed time distributions of RyR2 activated by micromolar Ca\(^{2+}\).

It has been reported that high doses of BayK and FPL can activate purified RyR2s (41, 55), although no effects were found at concentrations comparable with those in our Ca\(^{2+}\) spark experiments. It is speculated that purified RyR2s are sensitive to BayK and FPL because CHAPs solubilization (used for the purification of RyRs) alters the pharmacological properties of RyRs (35, 41). CHAPs solubilization also interrupts interactions between channels and dissociates them from factors and regulatory ancillary proteins (e.g., calsequestrin, FK506 binding protein 12.6, calmodulin, and triadin). These isolated and stripped RyRs may allow BayK to access binding sites that are not accessible in native channels (29, 41).

In summary, present and previous evidence suggest that DHPR modulators do not affect SERCA or RyR2 channels when they are tested at the concentrations used in our cellular experiments. This would indicate that DHPR modulators targeted another receptor present in cells that affects spontaneous gating of local arrays of RyR2 (and consequently Ca\(^{2+}\) sparks). We propose that such a spark modulator is the DHPR channel. In our experimental conditions, the DHPR was ineffective as a Ca\(^{2+}\) channel (transmembrane voltage and the Ca\(^{2+}\) gradients between the extracellular milieu and the cytosol have been cancelled). Thus, the results here cannot be explained by CICR but likely reflect physical DHPR-RyR interactions.

**Physiological significance of DHPR-RyR interactions in the heart.** It is well accepted that RyR2-dependent Ca\(^{2+}\) release during E-C coupling in the heart is mainly mediated by Ca\(^{2+}\) entry through DHPR channels (i.e., by CICR). The coexistence of CICR with a voltage-sensitive release mechanism (VSRRM) was proposed by Ferrier and coworkers (for a review, see Ref. 22). However, the physiological relevance of a VSRRM component of E-C coupling is still a matter of debate, and followup studies have questioned the experimental evidence for VSRRM (for a review, see Ref. 2).

The observations reported by Bers and coworkers with BayK (23, 30, 43, 44) as well as our observations with BayK and other DHPR modulators (nifedipine, calciseptine, FS-2, and FPL) cannot be attributed to CICR or to direct effects of these agents on RyR2s or the SERCA pump. Under these experimental conditions, the DHPR does not communicate with the RyR2 via Ca\(^{2+}\) entry to modulate RyR2-mediated
sparks. This makes physical interaction a likely candidate for DHPR-RyR2 communication in the heart. Furthermore, DHPR-RyR interactions have also been described in non-muscle tissues such as neurons (15, 37). Therefore, physical DHPR-RyR interactions appear not to be an exclusive characteristic of skeletal muscle. Accumulating evidence suggests that this mechanism of regulation and/or modulation of intracellular Ca\(^{2+}\) release may emerge as a common feature of electrically excitable cells.

In skeletal muscle, it is evident that RyR1-DHPR interactions reduce the expression and propagation of sparks in resting fibers (47, 59). Accordingly, Ca\(^{2+}\)-release experiments (32) and bilayer experiments (12) found that there is a significant RyR1-mediated Ca\(^{2+}\) leak under ionic conditions that mimic those in resting skeletal cells. This suggests that negative control is essential to prevent SR Ca\(^{2+}\) depletion in resting fibers.

Available data have also suggested an inhibitory role of DHPRs for RyR2-mediated sparks in cardiac cells at rest. RyR2 activity in cells was much lower than expected from its expression (15). Functional and/or physical interactions. Further studies are required to fully understand the nature of these DHPR-RyR2 interactions as well as to determine their potential significance for the control of cardiac E-C coupling both in health and disease.

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