All three ryanodine receptor isoforms generate rapid cooling responses in muscle cells

Feliciano Protasi, Alexander Shtifman, Fred J. Julian, and Paul D. Allen. All three ryanodine receptor isoforms generate rapid cooling responses in muscle cells. Am J Physiol Cell Physiol 286: C662–C670, 2004. First published October 30, 2003; 10.1152/ajpcell.00081.2003.—The rapid cooling (RC) response in muscle is an increase in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) that is probably caused by Ca\(^{2+}\) release from the sarcoendoplasmic reticulum (SR). However, the molecular bases of this response have not been completely elucidated. Three different isoforms of the SR Ca\(^{2+}\) release channels, or ryanodine receptors (RyRs), have been isolated (RyR1, RyR2, and RyR3). In the current investigation, the RC response was studied in RyR-null muscle cells (1B5) before and after transduction with HSV-1 virions containing the cDNAs encoding for RyR1, RyR2, or RyR3. Cells were loaded with fluo 4-AM to monitor changes in [Ca\(^{2+}\)]\(_{c}\), and perfused with either cold (–0°C), room temperature (RT), or RT buffer containing 40 mM caffeine. Control cells showed no significant response to cold or caffeine, whereas robust Ca\(^{2+}\) transients were recorded in response to both RC and caffeine in transduced cells expressing any one of the three RyR isoforms. Our data demonstrate directly that RyRs are responsible for the RC response and that all three isoforms respond in a similar manner. Ca\(^{2+}\) release from RyRs is likely caused by a RC-induced conformational change of the channel from the closed to the open state.

THE MECHANISM THAT LINKS the depolarization of transverse tubules to the mechanical response in muscle fibers, i.e., excitation-contraction (E-C) coupling, has been extensively studied (35, 37). The sarcoendoplasmic reticulum (SR) Ca\(^{2+}\) release channels, or ryanodine receptors (RyRs), allow Ca\(^{2+}\) release from the SR lumen in response to depolarization of the plasma membrane and its invaginations, the transverse tubules (T tubules). RyRs have been isolated from a large variety of tissues where they play an important role in many cellular processes controlled by Ca\(^{2+}\) (for reviews see Refs. 8, 14, 25, 43). Three different isoforms have been identified: RyR1, the skeletal isoform (44, 49); RyR2, the cardiac isoform (27, 30); and RyR3, the brain isoform (7, 17). Interestingly, RyR3 is also found in skeletal muscle cells, smooth muscle cells, and many nonexcitable tissues.

The mechanism that controls muscle activation has been studied by using different agents such as caffeine, ryanodine, etc., and these compounds have been useful tools with which to study Ca\(^{2+}\) release from the SR (9, 15, 19). Ca\(^{2+}\) release from the SR can also be triggered and evaluated by quick drops in temperature to near 0°C, a procedure known as rapid cooling (RC). The development of such cooling responses seems to be due to enhanced Ca\(^{2+}\) release from the SR. RC-induced contractures, where force is used as the means of measuring changes in internal [Ca\(^{2+}\)], are completely reversible by simple return to the initial temperature.

The RC response has been observed in a variety of different muscle types (6, 20, 21, 36). Because the magnitude of the RC response is not influenced by changes in either the membrane potential or external [Ca\(^{2+}\)], the most likely source for the [Ca\(^{2+}\)] increase in the myoplasm is the Ca\(^{2+}\) release from the SR. However, the means by which the release is achieved are not yet completely understood. In rabbit cardiac muscle, depletion of the SR Ca\(^{2+}\) stores by pretreatment with caffeine has been shown to abolish the RC response (6). In addition, Bridge (6) showed that microscopic tension fluctuation detected by Fourier analysis was also abolished by cooling contractures, providing clear evidence for the involvement of the SR in the mechanism.

Initially it was suggested that the primary mechanism for Ca\(^{2+}\) release in response to RC was a rapid inhibition of the SR Ca\(^{2+}\) pump (SERCA), thus allowing for a large unopposed SR Ca\(^{2+}\) leak that would serve as the Ca\(^{2+}\) source for the RC response. Bers et al. (5) demonstrated the effect of ryanodine on RC contractures, showing that in the presence of the alkaloid, the RC contracture immediately after a contraction was greater than that seen under control conditions. After a shorter period of time, ryanodine abolished cooling contractures, again implicating the SR in the phenomenon (5). However, the unidirectional diastolic Ca\(^{2+}\) efflux rate, or leak rate, from rabbit ventricular myocytes is very low, suggesting that perhaps another mechanism is at work (2). Recent work (45) on skinned rat skeletal muscle has suggested that the Ca\(^{2+}\) released during RC contractures originates from the RyRs with a fraction of this release possibly coming through the inositol 1,4,5-trisphosphate (IP\(_{3}\)) receptor as well.

The goal of the present study was to obtain more conclusive evidence concerning the molecular basis for the release of SR Ca\(^{2+}\) by RC. Because both RyR2 and RyR3 rely on a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism for their activation, whereas RyR1 is activated through a direct mechanical coupling with the dihydropyridine receptor (DHPR), we thought it would be interesting to determine whether all three RyR isoforms respond similarly to RC and whether their responses were also independent of their activation mechanisms during

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the E-C coupling. The availability of a mouse cell line (1B5 cells) that does not express any type of RyR (26) but does express IP3 receptors, SERCA1, and all other proteins involved in E-C coupling provided us with the opportunity to make such an inquiry. In the present work, we have expressed all three RyR isofoms (types 1, 2, and 3) independently in 1B5 cells by using HSV-1 amplicon vectors (13, 46). We have previously shown that RyR proteins expressed with the use of this technique form functional Ca2+ release channels that are correctly targeted to junctions between the SR and exterior membranes (12, 26, 32, 33, 46–48). Results from the present work show that control 1B5 cells (RyR null) do not release an appreciable amount of Ca2+ in response to RC. However, a typical RC response was observed when any one of the three RyRs was expressed in these cells. The contribution of SERCA1 and IP3 receptors to RC, if any, seems to be secondary to the RyR-mediated response. This result establishes a direct relationship between the presence of RyRs and the RC response.

MATERIALS AND METHODS

Cell culture. The methods used to create the 1B5 cell line are described in detail elsewhere (26). 1B5 cells were expanded at 37°C in low-glucose DME medium containing 20% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and additional 2 mM L-glutamine (growth medium). After ~48 h the cells were replated in either 1) 35-mm dishes containing Thermoxan coverslips (Nunc, Naperville, IL) for immunocytochemistry or 2) 96-well plates with ultra-thin clear bottoms for Ca2+ imaging experiments (Corning, Costar, NY), coated with Matrigel (Collaborative Biomedical Products, Bedford, MA). When cells reached ~70% confluence, the growth medium was replaced with differentiation medium (containing 5% heat-inactivated horse serum instead of the 20% fetal bovine serum found in growth medium) to induce differentiation. The medium was changed every day, and the cells were either fixed for immunocytochemistry or imaged 5–6 days later.

cDNA packaging in HSV-1 virions and cell transfection. cDNAs encoding for RyR1, RyR2, and RyR3 were packaged into HSV-1 amplicon virions with the use of the helper virus-free packaging system. The methods used for preparation of HSV-1 virions are described in detail elsewhere (13, 46). Four to five days after the beginning of differentiation, the cells were infected with a differentiation medium containing HSV-1 virions at 3 × 10^5 infectious units/ml (a moiety of infection of ~3). This mixture was removed ~2 h later and replaced with differentiation medium. The cells were either fixed or imaged ~24–36 h after infection.

Immunocytochemistry. The cells were fixed in methanol at −20°C for a minimum of 20 min. After 5 min of rehydration in PBS, cells were blocked in PBS containing 1% BSA and 10% goat serum for 1 h. Cells were then first incubated at room temperature (RT) with primary antibodies for 2 h. This was followed by a 1-h incubation at RT with secondary antibodies. Code, specificity, working dilution, and sources of primary antibodies are as follows: 34C, recognizes RyR1 and RyR3, 1:20, Developmental Studies Hybridoma Bank, The University of Iowa (1); C3-33, recognizes RyR2, 1:20, gift of G. Meissner (18). The secondary antibody used was cyanine 3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA). An inverted fluorescence microscope (Olympus IX70) was used to view the specimens.

Fluorescence measurements. Twenty-four to thirty-six hours after transduction, the cells were loaded with a Ca2+ fluorescent dye, fluo 4-AM (Molecular Probes, Eugene, OR), to monitor the changes in the intracellular [Ca2+]. The loading procedure was performed as follows: 1) differentiation medium was removed, and the cells were washed twice with imaging buffer (IB) containing 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 6 mM glucose, 25 mM HEPES, 0.05% BSA, and 2 mM CaCl2; 2) cells were incubated for 30 min in 100 µl of IB containing 5 µM fluo 4-AM; and 3) the cells were washed again with IB.

The 96-well plates, containing the monolayer of differentiated 1B5 myotubes (either control or transduced) were placed on the stage of a Nikon Diaphot 300 inverted microscope equipped with an Olympus Uapo/340 ×40 oil-immersion objective (NA 1.35). The microscope was modified to incorporate a pair of separate three-dimensional micromanipulators on either side of the vertical post holding the condenser. Each well in the 96-well plate could be perfused in a controllable, accurate way by using an AutoMate eight-channel air pressure-controlled system incorporating multiple 50-ml reservoirs (Automate Scientific, Berkley, CA). Each reservoir could be rapidly switched into or out of the perfusion pathway with an ~50-µl dead volume. The perfusion inlet was positioned about 1 mm above the

Fig. 1. HSV-1 virions give excellent transfection efficiency. A and B: lack of labeling in control 1B5 cells confirms that ryanodine receptors (RyRs) were not expressed in 1B5 myotubes. C–E: immunostaining of transduced 1B5 cells shows that 60–80% of myotubes reacted positively to anti-RyR antibodies. Bar, 200 µm.
imaged myotubes to allow a very efficient and rapid stimulus of the imaged area.

In each experiment, a region of interest (ROI) with a size of approximately half the cell diameter was selected. The selected regions were from well-differentiated (large) myotubes and were carefully drawn to exclude all nuclei. The cells were imaged by using a PTI delta-RAM as the light source, and images were recorded at 30 fps with a Stanford Photonics 12-bit digital intensified charge-coupled device. The images were displayed and analyzed by using QED software (v1.3 QED, Pittsburgh PA). The ROIs were simultaneously recorded by using the strip chart utility in the QED software to monitor image intensity. Experiments were conducted in either Ca$^{2+}$-containing IB (see above) or Ca$^{2+}$-free IB. Ca$^{2+}$-free IB did not contain any added CaCl$_2$ and was supplemented in some experiments with either 2 mM EGTA or CdCl$_2$/LaCl$_3$ (0.5 mM/0.1 mM). All experiments were conducted at RT (21°C) except for the steps specifically dealing with the RC response. RC was achieved by precooling normal bathing solution in an ice bucket to −6°C. The cooling solution (~10–15 volumes of the media contained in the well) was applied to the well for 7−10 s at a rate of 1.5 volumes/s with the use of a prechilled glass pipette. At this time the perfusion system, which had been stopped just before the RC step, was restarted to perfuse the cells with RT IB, terminating the RC pulse. The traces in Figs. 3–6 are plotted as the change in fluorescence over baseline values, where the prestimulus background of each cell is subtracted from all signals.

Preparation of images. Pictures and negatives were scanned with a color flatbed scanner (UMAX Power Look II) at 300 dpi. Final images were created, mounted, and labeled using Microsoft Excel and PowerPoint 98, Adobe Photoshop v5.5, and Canvas v3.5.4 (Deneba Software).

RESULTS

Immunocytochemistry. Control 1B5 cells were differentiated under the same conditions as cells that were transduced with HSV-1 virions. Control cells do not react with either of the anti-RyR antibodies used to detect RyR expression in transduced myotubes (Figs. 1 and 2, A and B). 34C recognizes both RyR1 and RyR3, whereas C3-33 is specific for RyR2. Lack of staining in nontransduced myotubes confirms that these cells do not express detectable levels of any RyR. After infection with HSV-1 amplicon virions, 1B5 myotubes reacted positively with anti-RyR antibodies (Figs. 1 and 2, C–E). The number of myotubes transduced by HSV-1 amplicon virions varied slightly from culture to culture (see Tables 1 and 2). Infection with HSV-1 amplicon virions does not appear to cause cell death or obvious changes in cell size and shape and does not affect the level of differentiation. Figure 1, C–E, shows low-magnification images of myotubes 24−36 h after infection, immunostained with anti-RyR antibodies. The number of cells stained with anti-RyR antibodies agrees with functional results showing that ~60−80% of the myotubes respond to caffeine in each sample group (see Ca$^{2+}$ imaging and Tables 1 and 2 for more detail). High-magnification immunofluorescence images reveal that all of the RyR constructs were targeted to their expected location in SR-exterior

Table 1. Summary of experiments in 2 mM Ca$^{2+}$

<table>
<thead>
<tr>
<th></th>
<th>Total No. of Cells Imaged</th>
<th>No. of Cells Responding to Caffeine</th>
<th>No. of Cells Responding to RC</th>
<th>%Transduced Cells Responding to RC</th>
<th>No. of Cells Responding to Br-A23187</th>
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<tbody>
<tr>
<td>Control</td>
<td>128</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24 of 28</td>
</tr>
<tr>
<td>RyR1</td>
<td>115</td>
<td>67 (58%)</td>
<td>66</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>RyR2</td>
<td>126</td>
<td>76 (60%)</td>
<td>73</td>
<td>96%</td>
<td></td>
</tr>
<tr>
<td>RyR3</td>
<td>134</td>
<td>97 (72%)</td>
<td>96</td>
<td>99%</td>
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Quantitative results from functional experiments in 2 mM Ca$^{2+}$. Control 1B5 cells did not respond to rapid cooling (RC) as transduced cells did. The slowly developing, relatively small responses shown in Fig. 3 were not counted for inclusion in these data. Exposure to Br-A23187 was performed only in control cells to determine whether sarcoplasmic reticulum (SR) internal stores were loaded with Ca$^{2+}$. By far, the majority of transduced 1B5 cells did respond to RC. RyR, ryanodine receptor.

AJP-Cell Physiol • VOL 286 • MARCH 2004 • www.ajpcell.org
Table 2. Summary of experiments in Ca$^{2+}$-free buffer

<table>
<thead>
<tr>
<th></th>
<th>No. of Cells Responding to Caffeine</th>
<th>No. of Cells Responding to RC</th>
<th>%Transduced Cells Responding to RC</th>
<th>No. of Cells Responding to Br-A23187</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>20 of 20</td>
</tr>
<tr>
<td>RyR1</td>
<td>68</td>
<td>55 (81%)</td>
<td>51</td>
<td>93%</td>
</tr>
<tr>
<td>RyR2</td>
<td>92</td>
<td>77 (84%)</td>
<td>75</td>
<td>97%</td>
</tr>
<tr>
<td>RyR3</td>
<td>64</td>
<td>57 (61%)</td>
<td>57</td>
<td>100%</td>
</tr>
</tbody>
</table>

Quantitative results from functional experiments in Ca$^{2+}$-free buffer. Control 1B5 cells did not respond to RC as transduced cells did. As in Table 1, the slowly developing, relatively small responses shown in Fig. 3 were not counted for inclusion in these data. Exposure to Br-A23187 was performed only in control cells to determine whether SR internal stores were loaded with Ca$^{2+}$. By far, the majority of transduced 1B5 cells did respond to RC. The absence of external Ca$^{2+}$, or blocked surface membrane L-type Ca$^{2+}$ channels, did not influence the ability of transduced 1B5 cells to respond to either RC or caffeine.

Nontransduced myotubes do not respond to RC. Typical results from control 1B5 myotubes are shown and summarized in Fig. 3 and Tables 1 and 2. Cells were challenged in sequence with RT buffer, cold buffer to near 0°C (RC), 40 mM caffeine, and a Ca$^{2+}$ ionophore (Br-A23187). Figure 3 shows experiments in which the IB contained 2 mM Ca$^{2+}$ (A) or experiments performed in Ca$^{2+}$-free buffer (nominally Ca$^{2+}$-free buffer supplemented with 2 mM EGTA) (B). In Fig. 3, A and B, the three traces represent responses of three different cells in the same field of view. In the RT buffer measurement, as in the RC measurement, the buffer was applied by using manual pipetting to rule out possible mechanical artifacts during the RC procedure. As shown in Fig. 3 and summarized in Tables 1 and 2, cells did not respond to application of RT buffer, although occasionally a very small change in fluorescence was observed, indicating that there is a slight motion artifact (visible in some of the traces in Fig. 3B, arrow). In response to sudden lowering of temperature (RC), nontransduced 1B5 myotubes exhibited a small, slow increase in fluo 4 fluorescence. These responses do not resemble those observed in myotubes transduced with the RyRs. In fact, the size of these transients is much smaller, and the onset time is much slower than those seen in transduced cells (compare Fig. 3 to Figs. 4 and 5). External Ca$^{2+}$ entry through surface membrane channels does not seem to be responsible for the small transients observed in response to RC. In fact, as demonstrated by Fig. 3B (Ca$^{2+}$-free external solution), the RC-elicited Ca$^{2+}$ transients appear to be identical to those triggered in the presence of extracellular Ca$^{2+}$ (Fig. 3A). To test for the possible role of the RyRs in this response, we conducted the classic caffeine challenge (10). Caffeine application did not elicit Ca$^{2+}$ release in control 1B5 myotubes, confirming the complete absence of RyRs in these cells (see Tables 1 and 2). To demonstrate that internal SR stores were loaded with Ca$^{2+}$ in the RyR-null myotubes, we perfused nontransduced 1B5 cells with a buffer containing a Ca$^{2+}$ ionophore (Br-A23187, 10 μM). Exposure of the cells to this compound produced a strong increase in fluo 4 fluorescence, indicating that internal stores contained a large amount of Ca$^{2+}$ (Tables 1 and 2). As shown in Fig. 3A, a small fraction of the cells did not appear to respond to the ionophore treatment (light gray trace), indicating that SR internal stores are not well developed in some myotubes (see also Refs. 32 and 33 for more detail on differentiating 1B5 cells).

RC responses in RyR-transduced myotubes. Transduced cells expressing any one of the three RyR isoforms differed significantly from the nontransduced cells in their responses to RC or caffeine (Fig. 4 and Table 1). A typical response of 1B5

membrane junctions, or calcium release units (CRUs) (see Ref. 31 for review), as indicated by the fluorescence being clustered into multiple intense foci (Fig. 2, C–E). When the focus level was adjusted above and below the sharpest point, it was clearly evident that the majority of these immunopositive foci are located at, or very near, the surface membrane, on both the ventral and dorsal sides of the myotubes, in agreement with previous work (see Refs. 32 and 33 for more detail).

Fig. 3. Control 1B5 cells do not respond to either rapid cooling (RC) or caffeine. Experiments were performed with the use of either imaging buffer (IB) containing 2 mM Ca$^{2+}$ (A) or nominally Ca$^{2+}$-free buffer containing 2 mM EGTA, i.e., a Ca$^{2+}$-free buffer (B). The 3 traces in both A and B show the response of 3 different cells in the same field of view: room temperature (RT) IB invariably produced no change or, at most, a small change indicative of a motion artifact (B, arrow); RC induced a small increase in fluorescence (ΔF) with a slow upstroke phase; and 40 mM caffeine applied at RT produced no response. Application of 10 μM Br-A23187, a Ca$^{2+}$ ionophore, at RT usually produced a strong increase in fluorescence, indicating that internal stores (sarcoplasmic reticulum) did contain Ca$^{2+}$. Caff, caffeine; a.u., arbitrary units.

Results from these experiments are summarized in Tables 1 and 2.
myotubes expressing RyR1, RyR2, or RyR3 is demonstrated in Fig. 4. Note that in Fig. 4, each panel shows three traces from three different cells in the same field of view. The cells were initially challenged with cold buffer to near 0°C (RC), then with 40 mM caffeine (RT), and later with IB buffer equilibrated to RT. The first two steps were then repeated to establish the reproducibility of the technique. In Fig. 4C, all three traces show a response to RC and caffeine, indicating that all three cells were successfully transduced by the RyR3 virus. In contrast, only two of the three cells responded to RC and caffeine in Fig. 4, A and B. The third cell in each of these two experiments was not transduced by the virus, thus making it possible to compare the RC response in control and transduced cells side by side (compare light gray traces in Fig. 4, A and B, with other traces in the same panels and with Fig. 3). As was the case in control cultures, the response to RC in nontransduced 1B5 myotubes (Fig. 3 and light gray trace in Fig. 4, A and B) is much slower and smaller than the RC response from transduced cells (Fig. 4).

In cells expressing RyR, the magnitude of the response to RC was strictly correlated with the presence and magnitude of the caffeine response: in fact, RC- and caffeine-elicted transients in the same cell were always comparable in size (see Fig. 4). Note that almost all of the cells that responded to caffeine also responded to RC (see Tables 1 and 2 and Fig. 4). The cell-to-cell variation in the absolute size of both the RC and caffeine and the correlation in size between the two responses in the same myotube are likely to be directly related to the amount of RyR expressed and fluo 4 loading. Note that in RyR2- and RyR3-transduced cells, spontaneous oscillations of intracel-
lular $[\text{Ca}^{2+}]$ were often observed (see Ref. 40 for more detail).

To address the possible contribution of the $\text{Ca}^{2+}$ influx in the RC response, we conducted experiments in which the extracellular $\text{Ca}^{2+}$ either was removed or its influx was blocked by DHP channel agonists (see Fig. 5 and Table 2). In Fig. 5, the RC response was elicited in RyR-transduced myotubes bathed in nominally $\text{Ca}^{2+}$-free IB supplemented with either 0.5 mM CdCl$_2$ and 0.1 mM LaCl$_3$ (left) or with 2 mM EGTA (right). As in Figs. 3 and 4, each panel shows results from three separate IB5 cells transduced with RyR1, RyR2, or RyR3. The cells were challenged in sequence with RT buffer, followed by RC and, finally, 40 mM caffeine. RT buffer did not elicit any significant responses, although occasionally there was a small motion artifact similar to that indicated by the arrow in Fig. 5C. Neither removal of $\text{Ca}^{2+}$ from the extracellular bathing medium (IB) nor blocking of $\text{Ca}^{2+}$ entry affected the responses to RC and caffeine. In addition, the order of magnitude and morphological characteristics of both the RC and caffeine responses were seemingly unchanged from those elicited in $\text{Ca}^{2+}$-containing or $\text{Ca}^{2+}$-replete buffer (compare Figs. 4 and 5). As in Fig. 4, the magnitude of the caffeine and RC responses in the same cell appear to be proportional, regardless of the type of RyR isoform expressed. Furthermore, the percentage of transduced cells responding to RC is unchanged (compare Tables 1 and 2). The absence of external $\text{Ca}^{2+}$ seems to affect only the frequency of spontaneous $\text{Ca}^{2+}$ oscillations, suggesting a possible role of $\text{Ca}^{2+}$ entry in triggering those events (see Ref. 40 for more detail).

Ryanodine blocks both RC and caffeine responses. To strengthen the correlation between RyR expression and RC response, we pretreated cells for 45 min with 500 $\mu$M ryanodine (37°C) to specifically block the ryanodine receptors (Fig. 6). Adjacent, similarly transduced wells were tested in parallel with caffeine to confirm the RyR expression. As shown in Fig. 6, cells were challenged in sequence with RC, followed by 40 mM caffeine. Figure 6 demonstrates that pretreatment with 0.5 mM ryanodine completely blocked the characteristic RC as well as the caffeine response, showing that the transients presented in Figs. 4 and 5 are predominantly a result of $\text{Ca}^{2+}$ release through the RyRs. Note that the slow-onset, small-amplitude increase in fluorescence in response to RC, described in control cells (Fig. 3), was not affected by exposure to ryanodine, indi-

![Fig. 5. External $\text{Ca}^{2+}$ is not involved in the RC response. A–C: experiments shown were performed in nominally $\text{Ca}^{2+}$-free IB supplemented with either 0.5 mM CdCl$_2$ and 0.1 mM LaCl$_3$ (left) or 2 mM EGTA (right). Application of IB at RT invariably produced no change or, at most, a very small change in fluorescence indicative of motion artifact (C, arrow). RC resulted in a very rapid and large increase in fluorescence. Application of 40 mM caffeine at RT also produced a rapid and large increase in fluorescence. Note that IB5 cells that expressed RyR2 or RyR3 still show spontaneous activity, although less pronounced than that in IB containing $\text{Ca}^{2+}$ (see Fig. 4). Results from experiments performed in $\text{Ca}^{2+}$-free IB are summarized in Table 2.](http://ajpcell.physiology.org/Downloadedfromhttp://ajpcell.physiology.org/)}
Note that the slow increase in fluorescence caused by RC observed in control 1B5 (Fig. 3) was not blocked by ryanodine.

cating that this change in fluorescence is not mediated by release of Ca\(^{2+}\) from RyRs.

### DISCUSSION

In the present work we sought to investigate the role of the three RyR isoforms in response to RC. The motivation for such an inquiry was prompted by the fact that the three different RyR isoforms are activated via different mechanisms during muscle E-C coupling. Both RyR2 and RyR3 rely on a CICR mechanism for their activation, whereas RyR1 activation is independent from external Ca\(^{2+}\), relying instead on a direct mechanical coupling with the DHPR. We thought it would be interesting to determine whether all three RyR isoforms respond to RC similarly and whether the response is independent of the activation mechanism used during the E-C coupling.

With the aid of an RyR knockout cell line (1B5 cells), we were able to express the different isoforms independently and establish that all three RyR isoforms respond with a similar robust Ca\(^{2+}\) transient to RC (Figs. 3 and 4). We also showed compelling evidence that the Ca\(^{2+}\) transients generated by the RC are not dependent on the influx of Ca\(^{2+}\) through the DHPRs (Fig. 5). In addition, our data suggest that, within our system, the involvement of the SR Ca\(^{2+}\) pumps and IP\(_3\) receptors in the RC process, if any, is minimal because the responses in the absence of RyRs are extremely small compared with those seen as a result of RC (Fig. 3).

From examination of the traces in Figs. 4 and 5, it is clear that there is a strong correlation between the expression of RyRs, a rapid Ca\(^{2+}\) release in response to both caffeine and RC application, irrespective of the RyR isoform expressed in the myotubes. This is compelling evidence in favor of the hypothesis that release of Ca\(^{2+}\) from RyRs mediates the RC response.

Further support for this hypothesis is provided by the experiments shown in Fig. 6 in which the rapid, large-amplitude RC response was completely blocked by micromolar concentrations of ryanodine.

The RyRs are extremely large ion channels that have been resolved to 30-nm resolution by cryoelectron microscopy (34, 38). These studies most likely depict the receptor in a closed state, because it has a very low unstimulated channel open probability (\(P_o\)), and they reveal a fourfold symmetry axis consistent with a homotrameric composition. Using similar cryoelectron microscopic techniques, Orlova et al. (29) and Serysheva et al. (39) activated the RyR with Ca\(^{2+}\) and 3',5'-methyladenosine 5'-triphosphate and kept it in the open state with ryanodine, demonstrating changes in RyR structure caused by activation. Their results suggest that the RyR undergoes large rearrangements of structure in both the transmembrane and cytoplasmic segments as it transitions between the open and closed states. It is, therefore, tempting to speculate that similar changes occur in the RyR as it is exposed to RC, i.e., a rapid decrease in temperature induces conformational changes, which lead to the activation of, and consequent Ca\(^{2+}\) release from, the RyRs. If indeed RC provides a means for eliciting conformational changes within the RyR, this approach could serve as a useful tool in the study of the conformational states of the RyRs in the bilayers and during Ca\(^{2+}\) spark production (16, 41).

In our cells, the presence and magnitude of the RC response was strongly correlated with the caffeine response, i.e., to the expression of RyRs in our system. Nevertheless, as shown in Fig. 3 (and in some of the traces in Figs. 4 and 5), control 1B5 myotubes that did not express any RyR did produce a very slow onset, small-amplitude fluorescence increase in response to RC that, nonetheless, was much smaller than that elicited in transduced myotubes. What could possibly cause these slow, small responses to RC in control 1B5 cells? There are at least three possible reasons for this behavior: 1) a temperature effect on the fluorescent dye fluo 4, because low temperatures have been shown to affect the sensitivity of single-wavelength dyes, such as fluo 4 (28); 2) the balance between a Ca\(^{2+}\) leak from the SR and its restitution by the Ca\(^{2+}\)-ATPase pump may be altered; in fact, if the leak rate were less sensitive to cooling than the pump rate, then a small, transient rise in cytoplasmic [Ca\(^{2+}\)] could occur; and finally, 3) a recent report suggests that a small component of RC-induced contracture may be due to Ca\(^{2+}\) release via an IP\(_3\)-sensitive store in addition to the main RyR component (45). In support of this hypothesis, slow and small depolarization-induced Ca\(^{2+}\) transients caused by Ca\(^{2+}\) release from IP\(_3\) receptors can be observed near the nuclei in control 1B5 myotubes (11). Although the contribution of other extracellular components cannot be excluded from our conclusions, one fact remains certain: in our system, the major contributor to the elevation in intracellular [Ca\(^{2+}\)] in response to RC was undoubtedly the RyR.

One issue that is difficult to resolve is the mechanism by which the transients decay during the RC response. This has been previously addressed by Bers et al. (3, 4). From their findings in cardiac cells, the slow decline in intracellular [Ca\(^{2+}\)] appears to be due to the reaccumulation of Ca\(^{2+}\) in the SR via the Ca\(^{2+}\)-ATPase. However, the authors did not exclude the possible contribution of the Na\(^{+}\)/Ca\(^{2+}\) exchanger, which may be responsible for the removal of as much as 50%
of myoplasmic Ca$^{2+}$ during the relaxation. The decay phase of the Ca$^{2+}$ transient is a complicated process involving many intracellular components. In fact, in addition to the clear role of the SR and plasma membrane Ca$^{2+}$ transporters, numerous intracellular proteins may also be involved in the termination of Ca$^{2+}$ release, i.e., buffering by calcium binding proteins. Furthermore, there is a distinct possibility that Mg$^{2+}$ and Ca$^{2+}$ may be intimately involved in the termination of the release during the RC (22–24).

In 1991, Sitsapesan et al. (42) presented work, quite relevant to the findings presented here, in which the effects of cooling on conductance and gating in adult sheep cardiac (RyR2) SR Ca$^{2+}$ release channels under voltage control were studied in lipid bilayers. Although in their experiments there was no real RC, since cooling from one temperature (higher) to another (lower) could take several minutes, there was a marked increase in $P_o$ as the channel cooled. Because $P_o$ was seen to be much more temperature sensitive than the conductance decrease, the net effect of cooling was a marked increase in channel current. This finding fits in nicely with our results and, as the authors pointed out, could help to explain the RC response in muscle. It would appear that the cooling technique they used influenced prolongation of the open state, rather than acting to open the channel. Taking into account the marked differences between their relatively slow cooling and our RC techniques, these results generally fit in with the view that cooling alone can drastically modify RyR channel function, possibly by inducing structural changes as speculated above.

The present study demonstrates directly that that all three RyR isoforms (RyR1, skeletal isoform; RyR2, cardiac isoform; RyR3, brain isoform) respond to RC in a similar manner. No significant differences were detected among different RyR isoforms. The RC response is most likely caused by a cooling-induced conformational change in the RyR, leading to an increase in $P_o$ of the channel. Whether the RC effect represents a direct effect of the temperature on the RyR and/or an indirect effect mediated by one (or more) of the several proteins that interact with it in muscle CRUs remains to be determined. With the ever-growing use of knockout cell lines and molecular manipulations of the RyRs, it is imperative to know whether transduction of the cells, as well as the expression of the functional molecule of interest, was successful. Although pharmacological means are the most common way of solving this problem, questions always remain regarding the second-order, not readily detectable effects of such agents. This technique provides a relatively easy, noninvasive, and nonpharmacological method for determining a successful transduction and expression of functional RyRs.

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