Postulated role of interdomain interactions within the type 1 ryanodine receptor in the low gain of Ca\(^{2+}\)-induced Ca\(^{2+}\) release activity of mammalian skeletal muscle sarcomplasmic reticulum

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frog (<4% of β-RyR) than in RyR1 in mammals (<15% of RyR3). The selective stabilization of RyR1 was attributed to two independent mechanisms: FKBP12 and 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid (CHAPS)-sensitive mechanisms (19). The latter mechanism seemed to play a major role (>70%), but the precise mechanism is not known.

In this study, we hypothesized that the interdomain interaction may account for the selective stabilization of the RyR1 channel, especially for the CHAPS-sensitive process. To test this hypothesis, we examined the effect of DP4 on the activity of RyR1. The results suggest that DP4 and CHAPS share a common mechanism in enhancing the CICR activity of RyR1, supporting our hypothesis. This conclusion is further supported by the finding of the present study that dantrolene (or its analog, azumolene) inhibits both DP4- and CHAPS-induced channel activation in the identical manner (e.g., by showing essentially identical concentration dependence of inhibition).

We also found that DP4 markedly enhanced caffeine sensitivity in releasing Ca²⁺ from the SR vesicles. These results indicate that the state at a reduced gain of the CICR activity of RyR1 is important in normal Ca²⁺ handling in skeletal muscle, and perturbation of this state may cause the channel dysfunction observed in some muscle diseases such as MH.

MATERIALS AND METHODS

Materials. Peptides (DP4 and DP4-mut) were synthesized using a synthesizer (model 431A; Applied Biosystems, Foster City, CA) with N-(9-fluorenylmethoxycarbonyl as the α-amino-protecting group, and they were purified by performing reverse-phase high-pressure liquid chromatography (36). [³H]Ryanodine (56 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). Azumolene (Proctor & Gamble, Norwich, NY) was kindly provided by Dr. Jerome Parness (Dept. of Anesthesia, Robert Wood Johnson Medical School, New Brunswick, NJ). Dantrolene was obtained from Sigma. Soybean phosphatide extract (lecithin, 95%) was from Avanti Polar Lipids (Alabaster, AL). All other reagents were of analytical grade.

[³H]Ryanodine binding. [³H]Ryanodine binding was performed with SR vesicles from bovine diaphragm which expresses both RyR1 and RyR3 or bovine epicranial muscle that expresses a single isoform of RyR1 (19). Briefly, the SR vesicles (100 μg of protein) were incubated with 8.5 nM [³H]ryanodine for 5 h at 25°C (or for 2 h at 37°C in the experiments with dantrolene and azumolene) (see Fig. 4) in a 100-μl solution containing 0.17 M NaCl, 20 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPSO), pH 6.8, 2 mM dithiothreitol, various concentrations of Ca²⁺ buffered with 10 mM EGTA (calculated using the value of 8.79 × 10⁻⁵ M⁻¹ as the apparent binding constant for Ca²⁺ of EGTA; see Ref. 4), and 1 mM β,γ-methyleneadenosine triphosphate (AMPPCP) unless otherwise indicated. For diaphragm SR vesicles, the amount of [³H]ryanodine bound to RyR1 and RyR3 were determined on the basis of the protein-bound radioactivity in the supernatant and the precipitated beads, respectively, after immunoprecipitating RyR3 with the anti-RyR3 antibody agarose mixture of L-β-(decane-5:2 wt/wt) and 400 μl of 0.17 M KCl, 20 mM MOPS, pH 6.8, 5 mM potassium phosphate, 10 mM phosphocreatine, 2 U/ml creatine kinase, and 2 μM fura-2. Fluorescence was measured using a Hitachi F-4500 fluorescence spectrophotometer with wavelength settings of 340 and 380 nm for excitation (alternating) and 510 nm for emission. The contaminating Ca²⁺ in the solution provided a sufficient extent of Ca²⁺ loading for Ca²⁺ release, and therefore no Ca²⁺ was added. Active loading of the SR vesicles with Ca²⁺ was started by addition of 1 mM Mg-ATP, which would give rise to ~0.3 mM free Mg²⁺. Free Ca²⁺ concentration in the cuvette declined with time and reached the steady state within 5 min. At this point, test reagents (DP4 or caffeine) were added and the changes in Fura-2 fluorescence were recorded.

Statistics. The data are expressed as means ± SE of n repeated experiments. Student’s unpaired t-test was used to determine the significance of the differences between mean values.

RESULTS

CHAPS-induced RyR1 channel destabilization (activation) in the mammalian skeletal muscle shares a common mechanism with channel activation by DP4. Figure 1A demonstrates differential effects of DP4 on the [³H]ryanodine binding to RyR1 and RyR3 in bovine diaphragm muscle at 30 μM Ca²⁺. DP4 (100 μM) increased the [³H]ryanodine binding to RyR1 more than fivefold compared with the control level, whereas DP4-mut almost abolished the activating effect. In contrast, DP4 and DP4-mut produced virtually no effect on RyR3. Addition of CHAPS (as a 2:1 CHAPS-soybean lecithin wt/wt mixture) also increased the [³H]ryanodine binding to RyR1 but not to RyR3 under the same conditions (Fig. 1B), consistent with our previous results (19). These findings indicate that RyR1 is preferentially activated by DP4 or by CHAPS.

To test the hypothesis that DP4 and CHAPS act through a common mechanism, we performed quantitative analysis of the effects of DP4 and CHAPS on [³H]ryanodine binding to RyR1 sites for the ligand (Bmax); thus B/Bmax reflects apparent averaged activity of individual Ca²⁺ release channels. The Bmax value was calculated from the Scatchard plot of the amounts of bound [³H]ryanodine at various (1.8–36 nM) concentrations of [³H]ryanodine in the similar solution containing 1 M NaCl instead of 0.17 M. The Bmax values for RyR1 and RyR3 were 7.6–8.5 and 0.23–0.37 pmol/mg of protein, respectively.

Single-channel recordings. Single-channel recordings were obtained as described previously (22, 23). Lipid bilayers consisting of a mixture of l-α-phosphatidylethanolamine, l-α-phosphatidyl-l-serine, and l-α-phosphatidylcholine (5:3:2 wt/wt) in n-decane (40 mg/ml) were formed across a hole (~250 μm in diameter in a polystyrene partition separating cis- and trans-chambers. Native SR vesicles and the purified RyR1 were prepared from rabbit back muscle, which expresses only RyR1 (17). Channel currents were recorded in symmetrical solutions of 250 mM Cs-methanesulfonate (for the native SR vesicles) or KCl (for the purified RyR1) buffered at pH 7.4 with 20 mM HEPES-Tris at the holding potential of ~40 mV (cis). Experiments were performed at 18–22°C. Bilayers containing only a single channel were used for all experiments. Single channel current recording was performed using an Axopatch 1D patch-clamp amplifier (Axon Instruments, Union City, CA) were filtered at 1 kHz with an eight-pole, low-pass Bessel filter and collected at 5 kHz for analysis. Mean open probability (Pₒ) was calculated from recordings of duration >2 min using 50% threshold analysis performed with pClamp software version 6.04 (Axon Instruments).

Ca²⁺ release measurements. Ca²⁺ release from the isolated SR vesicles was fluorometrically measured by monitoring free Ca²⁺ concentration in the solution. Bovine epicranial SR vesicles (80 μg) were incubated at 25°C in a fluorometer cuvette containing 400 μl of 0.17 M KCl, 20 mM MOPS, pH 6.8, 5 mM potassium phosphate, 10 mM phosphocreatine, 2 U/ml creatine kinase, and 2 μM fura-2. Fluorescence was measured using a Hitachi F-4500 fluorescence spectrophotometer with wavelength settings of 340 and 380 nm for excitation (alternating) and 510 nm for emission. The contaminating Ca²⁺ in the solution provided a sufficient extent of Ca²⁺ loading for Ca²⁺ release, and therefore no Ca²⁺ was added. Active loading of the SR vesicles with Ca²⁺ was started by addition of 1 mM Mg-ATP, which would give rise to ~0.3 mM free Mg²⁺. Free Ca²⁺ concentration in the cuvette declined with time and reached the steady state within 5 min. At this point, test reagents (DP4 or caffeine) were added and the changes in fura-2 fluorescence were recorded.

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To test the hypothesis that DP4 and CHAPS act through a common mechanism, we performed quantitative analysis of the effects of DP4 and CHAPS on [³H]ryanodine binding to RyR1
using bovine epicranial muscle, in which a single isoform of RyR1 is expressed (19). The activity was expressed as $B/B_{\text{max}}$, in which $B$ values at a specified concentration of the ligand were normalized by $B_{\text{max}}$, obtained in separate experiments (see MATERIALS AND METHODS). This expression reflects apparent averaged activity of individual RyR channels (19). Figure 2A demonstrates concentration-dependent activating effects of CHAPS on $[^{3}\text{H}]$ryanodine binding to RyR1. Addition of CHAPS (as a 2:1 CHAPS-soybean lecithin w/w mixture) increased $[^{3}\text{H}]$ryanodine binding to RyR1 in a concentration-dependent manner (Fig. 2B, closed circles). The maximum enhancement of $B/B_{\text{max}}$ by CHAPS was greater than sixfold (from 0.026 ± 0.02 to 0.16 ± 0.1), and the concentration required for half-maximal activation was ~1%. The essentially identical CHAPS concentration dependence was obtained in the range of final concentration of 0.5–2% CHAPS with the SR vesicles that had been treated with 2% CHAPS and diluted to the CHAPS concentrations indicated (Fig. 2B, open circles). These results suggest that the effect of CHAPS is reversible and is determined by its final concentration.

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Figure 2B demonstrates concentration-dependent activating effects of CHAPS on $[^{3}\text{H}]$ryanodine binding to RyR1. Addition of CHAPS (as a 2:1 CHAPS-soybean lecithin w/w mixture) increased $[^{3}\text{H}]$ryanodine binding to RyR1 in a concentration-dependent manner (Fig. 2B, closed circles). The maximum enhancement of $B/B_{\text{max}}$ by CHAPS was greater than sixfold (from 0.026 ± 0.02 to 0.16 ± 0.1), and the concentration required for half-maximal activation was ~1%. The essentially identical CHAPS concentration dependence was obtained in the range of final concentration of 0.5–2% CHAPS with the SR vesicles that had been treated with 2% CHAPS and diluted to the CHAPS concentrations indicated (Fig. 2B, open circles). These results suggest that the effect of CHAPS is reversible and is determined by its final concentration.

We next examined whether the combined effects of these reagents were additive or nonadditive (Fig. 3). In the presence of 2% CHAPS in experiments in which the activation reached
the maximum (see Fig. 2B), the addition of DP4 did not cause a further increase in the activity up to 500 μM. With 1% CHAPS in experiments in which the activation was about half the maximum, DP4 showed a concentration-dependent activation but the maximal level attained was almost the same as the maximum attained in the presence of 2% CHAPS. Notably, the apparent EC_{50} value of DP4-induced activation was greater in the presence of 1% CHAPS than it was in its absence (from ~50 μM in the control to ~100 μM with 1% CHAPS), but again the maximum level remained the same. Thus the effects of DP4 and CHAPS are nonadditive. The highest attainable activation level was not due to the limitation of the assay system (e.g., saturation of [3H]ryanodine binding), because even much larger values were obtained with FK-506 (see Fig. 5) or at higher ionic strength (data not shown). These findings support the notion that DP4 and CHAPS act on RyR1 through a common activation mechanism.

Effects of dantrolene and its analog on DP4-activated or CHAPS-activated [3H]ryanodine binding to RyR1. Dantrolene is an inhibitor of RyR1 used for the treatment of MH. It is widely accepted that its inhibitory effect on RyR1 is dependent on temperature and Ca^{2+} concentration, although many diverse and conflicting results have been reported (2, 9, 26). According to the more recent reports, dantrolene binds to the Leu^{590}, Cys^{609} region of RyR1 (28) and stabilizes interdomain interactions to reverse DP4-induced channel activation (7). Therefore, if DP4- and CHAPS-induced activation of RyR1 share a common mechanism mediated by destabilization of interdomain interactions, we expect that dantrolene will inhibit both DP4- and CHAPS-induced activation effects and show the identical pattern of inhibition. The ryanodine binding experiments shown in Fig. 4 were performed to test this hypothesis. Experiments were performed at 37°C instead of 25°C because dantrolene is more effective at a high temperature (2, 26). As shown in Fig. 4A, dantrolene inhibited [3H]ryanodine binding to RyR1 in the presence of 100 μM DP4 or 1% CHAPS and showed the identical concentration dependence of inhibition, as expected. The extent of inhibition was greater at 1 μM Ca^{2+} than at 30 μM Ca^{2+}, consistent with a previous report (2).

However, the extent of dantrolene inhibition of ryanodine binding was identical, regardless of the method of activation (i.e., DP4 or CHAPS). Azumolene at 10 μM (an equipotent, more water-soluble analog of dantrolene) inhibited [3H]ryanodine binding to RyR1 (2, 27); again, the extent of inhibition was identical, regardless of whether DP4 or CHAPS was used to activate ryanodine binding (Fig. 4B).

Dissociation of FKBP12 further increased [3H]ryanodine binding independently of DP4- and CHAPS-induced activation. FKBP12 is known to bind to RyR1 and RyR2 and to stabilize the channel in the closed state (11). A recent site-directed mutagenesis study demonstrated that Val^{2461} is the critical residue required for FKBP12 binding to RyR1 (3). Because Val^{2461} is located within the DP4 sequence, added...
containng 250 mM KCl (or Cs-methanesulfonate) at a holding potential of -40 mV (see MATERIALS AND METHODS). We used both the native SR vesicles and purified RyR1. Although RyR1 has bound calmodulin and FKBP12 in the native SR (15, 24), the purified RyR1 is free of such associated proteins (17). With the native SR vesicles, 10 μM DP4 increased mean Pₒ of the channel (Fig. 6A, left). A further increase in Pₒ was observed with 30 μM DP4. With the purified RyR1, DP4 also activated the channel in a concentration-dependent manner (Fig. 6A, right). No significant change in the current amplitude was observed in either specimen. There was no difference between

DP4 might have competed with the corresponding domain of RyR1 to dissociate FKBP12 from RyR1, resulting in activation of the channel. To test this possibility, we examined the effect of DP4 on the amount of the RyR1-bound FKBP12. Incubation of the SR vesicles with 100 μM DP4 did not affect the amount of FKBP12 bound to the SR vesicles, whereas 10 μM FK-506 completely dissociated FKBP12 from the vesicles (Fig. 5A). No effect was observed with 100 μM DP4-mut either. [³H]Ryanodine binding to RyR1 in the presence of the maximally activating concentration of DP4 (300 μM) was further increased by 10 μM FK-506, the extent of which was similar to that of the control (Fig. 5B). This was also true with RyR1 treated with 2% CHAPS (Fig. 5B), the effect of which was independent of FKBP12 (19). Taken together, these results suggest that DP4 as well as CHAPS activates RyR1 through the mechanism independent of FKBP12-RyR1 interaction.

Effect of DP4 on single Ca²⁺ release channel activity in native SR and purified RyR1. Single-channel currents through the RyR1 channel were recorded in symmetrical solutions containing 250 mM KCl (or Cs-methanesulfonate) at a holding potential of -40 mV (see MATERIALS AND METHODS). We used both the native SR vesicles and purified RyR1. Although RyR1 has bound calmodulin and FKBP12 in the native SR (15, 24), the purified RyR1 is free of such associated proteins (17). With the native SR vesicles, 10 μM DP4 increased mean Pₒ of the channel (Fig. 6A, left). A further increase in Pₒ was observed with 30 μM DP4. With the purified RyR1, DP4 also activated the channel in a concentration-dependent manner (Fig. 6A, right). No significant change in the current amplitude was observed in either specimen. There was no difference between
the purified RyR1 and the native SR vesicles with regard to the magnitude of activation by DP4 (Fig. 6B). Although the purified RyR1 contained 1% CHAPS, the detergent concentration had to be reduced to a negligible level when RyR1 was incorporated into the lipid bilayer. On the basis of the results shown in Fig. 2B, the effect of CHAPS is expected to be reversible. These findings suggest that DP4 directly activates RyR1 without any requirement of accessory modulators.

Effect of DP4 on the responses of RyR1 to various modulators of CICR. We have shown that the stabilization of the RyR1 channel may be attributed primarily to a reduction in the gain of CICR activity, but not to an alteration in its response to the CICR modulators (19). To understand the molecular and functional mechanisms of the stabilization, we examined the effect of DP4 on the response of RyR1 to endogenous CICR ligands (Ca$^{2+}$, Mg$^{2+}$, and adenine nucleotides) and to caffeine, a representative CICR modulatory drug.

DP4 (100 \( \mu \)M) increased [\( ^{3} \)H]ryanodine binding sixfold without substantial changes in Ca$^{2+}$ dependence (Fig. 7A). The EC$_{50}$ value for Ca$^{2+}$ activation was slightly but significantly (\( P < 0.05 \)) smaller in the presence of 100 \( \mu \)M DP4 (3.2 \( \pm \) 0.5 \( \mu \)M) than that in control (5.1 \( \pm \) 0.9 \( \mu \)M), whereas the IC$_{50}$ value for Ca$^{2+}$ inactivation was unchanged (0.32 \( \pm \) 0.02 mM in control and 0.36 \( \pm \) 0.03 mM with 100 \( \mu \)M DP4). The concentration dependence of Mg$^{2+}$ inhibition is shown in Fig. 7B. Mg$^{2+}$ decreased the [\( ^{3} \)H]ryanodine binding with or without 100 \( \mu \)M DP4, showing similar concentration dependence: the IC$_{50}$ values for Mg$^{2+}$ were 0.21 \( \pm \) 0.06 mM (without DP4) and 0.13 \( \pm \) 0.02 mM (with 100 \( \mu \)M DP4) (Fig. 7B).

AMPPCP, a nonhydrolyzable ATP analog, increased the [\( ^{3} \)H]ryanodine binding to SR vesicles at 30 \( \mu \)M Ca$^{2+}$ (Fig. 8A, open circles). The ryanodine binding was increased seven- to eightfold by 100 \( \mu \)M DP4 at all AMPPCP concentrations (Fig. 8A, closed circles). Normalized values (Fig. 8A, inset) revealed that there was no significant difference in the activation pattern associated with AMPPCP; the EC$_{50}$ value, 0.3 mM, was about the same under both conditions. This suggests that DP4 does not affect the sensitivity of RyR1 to adenine nucleotide. The concentration dependence of caffeine activation is shown in Fig. 8B. Caffeine increased the binding at 1 \( \mu \)M Ca$^{2+}$ in a concentration-dependent manner (Fig. 8B, open circles). The ryanodine binding was further enhanced by 100 \( \mu \)M DP4 at all caffeine concentrations used (Fig. 8B, closed circles). In addition, DP4 slightly sensitized RyR1 to caffeine; the EC$_{50}$ value for caffeine with 100 \( \mu \)M DP4 (~1.2 mM) was about half that of the control (~2.4 mM) (Fig. 8B, inset).

Effect of DP4 on Ca$^{2+}$ release from isolated SR vesicles. Ca$^{2+}$ release from the isolated SR vesicles was fluorometrically determined with the bovine epicranial muscle SR (see MATERIALS AND METHODS). DP4 induced transient Ca$^{2+}$ release from the Ca$^{2+}$-loaded SR, and this effect was concentration dependent, although the releasing action was weak (Fig. 9A). Caffeine also induced concentration-dependent Ca$^{2+}$ release: Ca$^{2+}$ release was marginal at 2 mM caffeine and massive at 10 mM caffeine (Fig. 9B, left). Pretreatment with 30 \( \mu \)M DP4 markedly enhanced Ca$^{2+}$ release by 2 mM caffeine, but not with 10 mM caffeine (Fig. 9B, right). This potentiating effect of 30 \( \mu \)M DP4 was examined in the presence of various concentrations of caffeine (Fig. 9C). The threshold concentration of caffeine was decreased from ~1 mM in the absence of DP4 to ~0.2 mM in the presence of 30 \( \mu \)M DP4. The potentiating effect of DP4, however, appeared to decrease with an increase in the caffeine concentration. This is probably due to the limitation of the amount of releasable Ca$^{2+}$ stored in the SR, because the amount of Ca$^{2+}$ released by 10 mM caffeine alone (open circle) was similar to that obtained with simultaneous addition of caffeine and DP4 (reversed triangle). Taken together, these findings suggest that DP4 sensitizes the RyR1 channel to caffeine in releasing Ca$^{2+}$ from the SR.

DISCUSSION

Our recent work showed that the CICR activity of RyR1 was considerably lower than that of RyR3 in native SR vesicles, whereas after CHAPS treatment, the two purified isoforms showed equal activity with unchanged Ca$^{2+}$ sensitivity (19).
Sensitivity to adenine nucleotide was unchanged. RyR1, but not RyR3, is therefore considered to be in the stabilized state in the SR, where the gain of CICR is reduced. Although removal of FKBP12 partly explained the stabilization, the remaining major part was reversed by CHAPS, indicating critical involvement of molecular interactions. Ikemoto and colleagues (10, 32, 36) showed that DP4, a synthetic peptide corresponding to the Leu2442-Pro2477 region, stimulated RyR1 activity, but that DP4-mut observed in MH did not. They proposed a hypothesis that an interdomain interaction between the NH2-terminal domain and the central domain containing the DP4 region regulates channel activity and that “unzipping” of the interacting domains destabilized the channel, resulting in enhanced Ca2+ release activity observed in MH (5, 6).

The main aim of the present study was to test whether the hypothesized interdomain interaction is involved in the CHAPS-induced activation and/or destabilization of the RyR1 channel. We examined the effects of CHAPS and DP4 on RyR1 of bovine skeletal muscle SR vesicles using a [3H]ryanodine binding assay. We have demonstrated that 1) DP4 and CHAPS activated RyR1 but not RyR3 (Fig. 1); 2) the two reagents showed the identical level of maximal activation (Fig. 2), and these activation effects were nonadditive (Fig. 3); 3) RyR1 activated by DP4 or CHAPS was inhibited by dantrolene and azumolene, showing an identical pattern of inhibition (Fig. 4); and 4) activation by DP4 or CHAPS was independent of FKBP12-dependent stabilization (Fig. 5). All of these findings suggest that DP4 and CHAPS share a common mechanism for the activation of RyR1.

An increasing body of evidence supports the hypothesis that the interactions between the two key domains harboring many
of the reported MH mutations (NH2-terminal and central domains) play an important role in the regulation of RyR1 Ca2+ channels. Thus domain peptides and antibodies that bind specifically to either of these domains produced MH-like hyperactivation and hypersensitization of RyR1 channels (8, 10, 32, 36). The channel activation by these agents is well correlated with increased accessibility of the fluorescent probe, attached to either of these domains, to a macromolecular fluorescence quencher, indicative of an increased gap between the interacting domains, namely, domain unzipping (8, 37). Furthermore, dantrolene binds to the Leu596-Cys609 region of the NH2-terminal domain of RyR1 (28) inhibited DP4- and anti-DP4 antibody-induced channel activation, accompanied by a decrease in the probe accessibility to the quencher (i.e., a decrease in the gap of the interacting domains, or domain zipping) (7). As described above, CHAPS- and DP4-induced channel activation share various common features in the process of not only channel activation but also channel inhibition by dantrolene. Thus it seems that the actual mechanism of CHAPS-induced activation and/or destabilization of the RyR1 channel is mediated by destabilization of the interacting domain pair consisting of the NH2-terminal and central domains. It is likely, then, that the hypothesized interdomain interaction is affected by CHAPS, resulting in enhanced activity of RyR1, the effect that is indistinguishable from the one caused by DP4. However, little is known about how the conformational signal elicited in the interacting domains can be transmitted to the channel, although there must be some mechanisms by which the channel is functionally coupled with the operation of these domains. There remains a strong possibility that DP4 and CHAPS change allosteric domain-domain and domain-channel interactions, reducing the free energy barrier to facilitate the change from the closed to the open state of the channel.

We previously proposed that the stabilization of RyR1 might cause reduction in the gain of CICR activity because the ligand sensitivity remains unchanged in the SR vesicles (19). In this study, 100 μM DP4 consistently increased the \( \frac{B}{B_{\text{max}}} \) value of \([^3\text{H}]\)ryanodine binding (6- to 7-fold), regardless of the presence and absence of the CICR modulators, including Ca2+, Mg2+, AMPPCP, and caffeine (Figs. 7 and 8). No obvious alteration by DP4 was observed in the sensitivity to inactivating Ca2+ (Fig. 7A), Mg2+ (Fig. 7B), or activating AMPPCP (Fig. 8A), whereas slight sensitization was observed with activating Ca2+ (~1.5-fold) (Fig. 7A) or caffeine (~2-fold) (Fig. 8B). The enhancing effect of DP4 with slight sensitization was also reported with rabbit skeletal muscle SR (36). These results suggest that the primary effect of DP4 is to increase the gain of CICR activity. This supports our hypothesis that the stabilization of RyR1 primarily causes the reduction in gain of CICR.

The importance of the CICR gain seems to have been overlooked often in previous investigations. It is difficult to compare CICR activity directly between different samples (e.g., SR vesicles from different tissues or cells expressing different RyR mutants) because of different RyR contents. Therefore, comparisons generally have been made after normalization of the activity with its peak activity (e.g., \([^3\text{H}]\)ryanodine binding at the optimum Ca2+ (see Fig. 7, A and B) to correct for the RyR contents. Such normalization is useful for determining the sensitivity to ligands (e.g., Ca2+, Mg2+, or caffeine) but may lead to misinterpretation of the CICR gain. We could overcome this problem by using the \( \frac{B}{B_{\text{max}}} \) expression, which reflects apparent average activity of individual channels and holds the information about CICR gain (19). Thus the \( \frac{B}{B_{\text{max}}} \) expression connoting the CICR gain is useful for the evaluation of CICR activity.

The concept of CICR gain may also explain some species differences with regard to caffeine sensitivity. For instance, it was found that caffeine easily causes contracture of frog skeletal muscle, but that it often is abortive in mammalian skeletal muscle (25, 35). In frog skeletal muscle, β-RyR may account for most of the CICR activity because it shows a \( \frac{B}{B_{\text{max}}} \) value (0.2–0.25) greater than that for α2-RyR (as low as 0.009) (20). RyR1 demonstrated higher Ca2+ sensitivity for activation (EC50 of ~5 μM in this study) than β2-RyR (EC50 of ~16 μM; see Ref. 20) but much lower \( \frac{B}{B_{\text{max}}} \) (~0.02) than β-RyR. Thus the lower sensitivity to caffeine in mammalian skeletal muscle could reasonably be explained by lower CICR gain of RyR1.

Lamb et al. (10) recently reported that DP4 potentiated caffeine-induced Ca2+ release from the SR of rat skinned skeletal muscle fibers. They also found that DP4 by itself induced Ca2+ release when the cytoplasmatic Mg2+ concentration ([Mg2+]i) was set at 0.2 mM, but not when it was set at 1 mM, suggesting that the Ca2+-releasing effect of DP4 by itself does not occur at physiological [Mg2+]i level, which was assumed to be ~1 mM. In the present study, DP4 induced small, transient Ca2+ release from the SR vesicles (Fig. 9A), in which the free [Mg2+]i was calculated to be ~0.3 mM. At 1 mM [Mg2+]i, no significant Ca2+ release by DP4 (up to 100 μM) was observed (data not shown). Thus activation by DP4 alone is not sufficient for Ca2+ release in the presence of 1 mM Mg2+. Thus the enhanced CICR activity induced by DP4 is likely to mimic the altered Ca2+ handling observed in the MH phenotype. The findings to date also imply that the low CICR gain of the RyR1 channel is critically important in normal Ca2+ handling in skeletal muscle.

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