Full Paper

Ca²⁺-Releasing Effect of Cerivastatin on the Sarcoplasmic Reticulum of Mouse and Rat Skeletal Muscle FibersRyotaku Inoue^{1,2}, Mitsuo Tanabe^{1,3,#}, Keita Kono^{1,3}, Kei Maruyama¹, Takaaki Ikemoto^{1,*}, and Makoto Endo¹¹Department of Pharmacology, Saitama Medical School, Moroyama-machi, Saitama 350-0495, Japan²Biological Research Laboratories, Sankyo Co., Ltd., Shinagawa-ku, Tokyo 140-8710, Japan³Pharmacology and Molecular Biology Research Laboratories, Sankyo Co., Ltd., Shinagawa-ku, Tokyo 140-8710, Japan

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Abstract. We analyzed the effect of HMG-CoA reductase inhibitors on Ca²⁺ release from the sarcoplasmic reticulum (SR) using chemically skinned skeletal muscle fibers from the mouse and the rat. Cerivastatin (>20 μM) released Ca²⁺ from the SR, while pravastatin showed only a little effect. The rates of Ca²⁺ release were increased by cerivastatin at all Ca²⁺ concentrations tested. Cerivastatin-induced Ca²⁺ release in the presence of Ca²⁺ was affected by adenosine monophosphate, Mg²⁺, and procaine in essentially the same way as for caffeine-induced Ca²⁺ release. The Ca²⁺-uptake capacity of the SR was reduced after co-treatment with ryanodine and cerivastatin at pCa 6.0 to a much greater extent than with ryanodine alone. Thus, cerivastatin-induced Ca²⁺ release in the presence of Ca²⁺ must be a result of the activation of the Ca²⁺-induced Ca²⁺ release (CICR) mechanism of the ryanodine receptor. However, even when CICR was maximally inhibited by Mg²⁺ and procaine, or in the practical absence of Ca²⁺ (pCa >8), cerivastatin still caused Ca²⁺ release. These results indicate that cerivastatin causes Ca²⁺ release also by activating some other mechanism(s) in addition to the activation of CICR. Either or both of these effects might be related to its adverse effect, rhabdomyolysis.

Keywords: calcium release, cerivastatin, ryanodine receptor, sarcoplasmic reticulum, skeletal muscle

Introduction

Ryanodine receptors (RyRs) play an important role as intracellular Ca²⁺-release channels in a wide variety of cells (1, 2). Recently, we reported that clofibrate acid (Clop), an antihyperlipidemic agent, causes Ca²⁺ release from the sarcoplasmic reticulum (SR) in a unique mode of action on RyR-Ca²⁺ release channels in skinned skeletal muscle fibers (3). It was previously reported that simvastatin, a different type of antihyperlipidemic agent, increased the cytoplasmic Ca²⁺ concentration via the release of stored Ca²⁺ in L6 rat myoblasts and resulted in cell injury, although the exact mechanism is still debated (4). These results raise the possibility

that both types of antihyperlipidemic agent may have a common ability to activate the RyR-Ca²⁺ release channel, which might have a causal relationship to their rare but serious adverse effect, rhabdomyolysis (5, 6).

Cerivastatin (Cer) is also classified as an HMG-CoA reductase inhibitor, as is simvastatin. These clinically used statins share an HMG-like moiety, which is essential for the inhibitory effect on the enzyme (7). In this study, we examined the effects of Cer on Ca²⁺ release in skinned skeletal muscle fibers mainly from the mouse, which we used in our previous experiments. In some experiments, however, we also used rat skinned muscle fibers because statin-induced myopathy was reported in the rat (8, 9). Our results demonstrated that Cer, a lipophilic type of statin, also had an ability to cause Ca²⁺ release from the SR. This effect of Cer, in the presence of Ca²⁺, was induced mainly by the activation of the Ca²⁺-induced Ca²⁺ release (CICR) mechanism of the RyR. However, Cer also caused Ca²⁺ release, even under

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conditions of maximal CICR inhibition. Thus, Cer may activate Ca^{2+} release in a complex manner.

Materials and Methods

Preparation of skinned muscle fibers

Male mice (ICR, 5–9 weeks of age; Charles River Japan, Atsugi) and rats (Fisher, 5–6 weeks of age; Charles River Japan) were anesthetized with 50–80 $\text{mg} \cdot \text{kg}^{-1}$ sodium pentobarbital (i.p.) and killed by decapitation. Skinned fibers were prepared from extensor digitorum longus muscle bundles (EDL, width 2–3 mm) that were treated with saponin (50 $\mu\text{g} \cdot \text{mL}^{-1}$) for 45 min in a relaxing solution (4.76 mM ATP, 5.54 mM Mg methanesulfonate [MgMs_2], 108.6 mM potassium methanesulfonate [KMs], 20 mM NaN_3 , 20 mM piperazine- N,N' -bis(2-ethanesulfonic acid) [PIPES], and 1 mM EGTA; pH 7.0) to permeabilize the surface membrane (10). After washout of saponin, thin bundles (3–4 skinned fibers) were carefully dissected in relaxing solution, attached to a metal wire (with a silk filament), and inserted into a microcuvette (3, 11).

Measurement of Ca^{2+} release in skinned fibers

The Ca^{2+} released from the SR of the skinned fibers was quantified using Fura-2 (12). Details of the method are described elsewhere (11, 13). Briefly, thin bundles fixed in a capillary cuvette were mounted on the stage of a microscope (BHS-RFK; Olympus, Tokyo or TMD-300; Nikon, Tokyo) equipped with a fluorometer (CAM-200 or CAM-230; Nihon Bunko Kogyo, Tokyo) for measurement of the fluorescence intensity of Fura-2 at 510 nm, with alternating 340- and 380-nm excitations.

For the direct measurement of Ca^{2+} release in response to statins or caffeine, as shown in Fig. 1, the fluorescence intensity of 35 μM Fura-2 after Ca^{2+} loading in a fixed condition (pCa 6.7 and 4 mM MgATP for 60 s) was continuously monitored in the absence of ATP and EGTA and in the presence or absence of Ca^{2+} -releasing stimuli with or without 0.5 mM Mg^{2+} (3, 13).

The rate of Ca^{2+} -release was determined following the protocol diagrammatically represented in Fig. 2a, which consisted of Step 1) loading the SR with Ca^{2+} (pCa 6.7 and 4 mM MgATP for 60 s), Step 2) test (Ca^{2+} -releasing stimuli given for 4–600 s) after washout of loading solution, and Step 3) determination of the Ca^{2+} remaining in the SR by completely releasing it by applying a high concentration of caffeine and AMP. During the test period (Step 2), various concentrations of Ca^{2+} buffered with 10 mM EGTA in the presence or absence of statins or CICR activators (caffeine or AMP), sometimes together with various concentrations of Mg^{2+}

Fig. 1. Cer- and caffeine-induced Ca^{2+} release from the SR of mouse skeletal muscle fibers. Representative traces showing the time course of Ca^{2+} release from the SR in response to agents were obtained by measuring the change in Fura-2 intensity in the absence of ATP and EGTA and in the presence or absence of 0.5 mM Mg^{2+} . At the time indicated by the arrowhead, 20–500 μM Cer (a), 50 mM caffeine or 500 μM Cer (b), 500 μM Cer or 2 mM Pra (c) was applied. SR Ca^{2+} loading was carried out before each trial. The data in each panel were obtained from one and the same preparation, but those in different panels were from different preparations.

Fig. 2. Enhancing effect of Cer on Ca^{2+} release from the SR of mouse skeletal muscle fibers. a) Experimental protocols for measurement of CICR rate. Rounded boxes represent the SR. Step 1: in situ Ca^{2+} pump ATPase was used to load the SR in the presence of 4 mM MgATP at pCa 6.7 for 90 s. WASH: MgATP and Ca^{2+} were washed out in the presence of 1 mM EGTA. Step 2: Test solution (various Ca^{2+} concentrations, buffered with 10 mM EGTA and no ATP) was applied to induce CICR in the presence or absence of statins. This procedure was omitted in protocol 1. Step 3: After washout of the test solution, the total release of Ca^{2+} remaining in the SR was induced by addition of the assay solution containing a high concentration of caffeine (50 mM) and AMP (25 mM) in the presence of Fura-2 (35 μM). The difference in the amounts of Ca^{2+} assayed between the two types of protocol was taken to represent the amount of Ca^{2+} released during the test procedure in protocol 2. b) The SR was loaded with a fixed amount of Ca^{2+} , which was then released by treating the fiber with or without 500 μM Cer in the absence of Mg^{2+} at pCa 6.0 for the period of time shown on the abscissa. Remaining Ca^{2+} in the SR after the treatment was plotted on the ordinate (mean $\pm$ S.E.M., $n = 3$ or 4). An exponential curve was fitted by a nonlinear least square method. Error bars smaller than the symbols are not shown. c) The rates of Ca^{2+} release induced by 20–500 μM were measured at pCa 6.0 in the absence of Mg^{2+} (mean $\pm$ S.E.M., $n = 7$ –16). ** $P < 0.01$ (vs without Cer).

or procaine, were given. At Step 3), the Ca^{2+} remaining in the SR after treatment with a test solution was determined from the change in fluorescence intensity of Fura-2. The amount of Ca^{2+} released during Step 2 was estimated by comparing the results of assays with or

without Step 2 (protocol 2 vs protocol 1 in Fig. 2a). The time course of the amount of Ca^{2+} remaining in the SR was adequately described by a single exponential fall with increasing duration of application of a test solution with or without statins, as shown in Fig. 2b.

Therefore, the activity of Ca^{2+} release was expressed in terms of decay rate constants, based on the amount of Ca^{2+} remaining in the SR, as was the case in previous reports (3, 11). The effects of Cer or pravastatin (Pra) could be tested repetitively in the same preparation, since the increased Ca^{2+} -release rates returned to the control value after washout of these statins.

For measurement of the Ca^{2+} -uptake capacity of the SR in Fig. 7, the Ca^{2+} content of the SR was also estimated by the magnitude of the response to caffeine in the presence of Fura-2 after a standard loading procedure but without the test procedure mentioned above (protocol 1 in Fig. 2a) (3, 13). The Ca^{2+} contents of the SR after treatment with ryanodine were compared with that before the treatment.

All experiments were carried out at room temperature (21–23°C). Statistical significance was tested by Dunnett's test for multiple comparisons (Fig. 7) or by the paired *t*-test for comparisons between data with and without an agent.

Experimental solutions

The basic composition of all experimental solutions used in this report, including the test solutions and other solutions, was as previously described (11). Test solutions of various pCa's (<8) were obtained by mixing a 10 mM EGTA-containing solution and a 10 mM CaEGTA-containing solution. Stock solutions of statins were made by dissolving Cer and Pra in dimethyl sulfoxide (DMSO) and in ultrafiltered water (Mill-Q; Millipore, Billerica, MA, USA), respectively, to obtain concentrations of 10–250 mM (Cer) or 1 M (Pra) and added in the test solution. All test solutions with or without these agents were adjusted to contain 0.2% DMSO. The pH of all solutions was adjusted to 7.0 with KOH. Although rather high concentrations of Cer and Pra were used, they did not appreciably bind divalent cations, as evidenced by the fact that the presence of these agents, up to 500 μM and 2 mM, respectively, altered neither the free Mg^{2+} nor the free Ca^{2+} concentration measured by the fluorescence change of Mag-Fura-2 (14) and Fura-2, respectively.

Materials

Cerivastatin sodium and pravastatin sodium were synthesized by Sankyo Co., Ltd. (Tokyo). Na_2ATP was obtained from Boehringer Mannheim (Mannheim, FRG), saponin from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, USA), Fura-2 and Mag-Fura-2 from Molecular Probes, Inc. (Eugene, OR, USA), and EGTA from Dojindo Laboratories (Kumamoto). AMP and *p*-aminobenzoic acid diethylaminoethyl ester (procaine) were purchased from Sigma Chemical Co. (St. Louis,

MO, USA). All other chemicals were of the highest reagent grade available.

Results

Effects of cerivastatin on Ca^{2+} release

In skinned skeletal muscle fibers, without addition of any activating agents, Ca^{2+} only slowly leaked out from the SR after Ca^{2+} loading in medium containing 0.5 mM Mg^{2+} (but neither ATP nor EGTA) (Fig. 1). Under this condition, Cer caused a dose-dependent Ca^{2+} release from the SR (>20 μM , Fig. 1a), and the amount of Ca^{2+} released in 90 s by Cer (500 μM) was much the same as that released by a high concentration of caffeine (50 mM, Fig. 1b). After Ca^{2+} release by Cer (500 μM) for 90 s, 50 mM caffeine did not induce further Ca^{2+} release (data not shown). These results indicate that, under this condition, Cer is able to induce Ca^{2+} release from the same Ca^{2+} store, the SR, which is influenced by caffeine. On the other hand, Pra, which is a hydrophilic type of statin, showed only a small effect, even at a high concentration of 2 mM (Fig. 1c).

For quantitative measurement of Ca^{2+} release, however, it is inappropriate to monitor the Ca^{2+} concentration outside the SR directly as in Fig. 1 because the CICR channels would be further activated by the released Ca^{2+} under such weakly buffered conditions (15). Therefore, we examined the effect of Cer on the rate of Ca^{2+} release in the presence of a high concentration of EGTA (10 mM)- Ca^{2+} buffer, as described in the Materials and Methods. Figure 2b shows the time course of Ca^{2+} release at pCa 6.0. The amount of Ca^{2+} in the SR declined with increasing duration of the test procedure (Fig. 2a) in the absence and presence of Cer (500 μM). The rate of Ca^{2+} release was significantly and dose-dependently increased by Cer at pCa 6.0 (Fig. 2: b and c). A similar effect was observed at both pCa 7.0 and 5.0 (data not shown).

Cer-induced Ca^{2+} release in the presence of various concentrations of Ca^{2+}

We examined the effect of Cer on the rates of Ca^{2+} release under various concentrations of Ca^{2+} in the absence of Mg^{2+} . Cer (500 μM) significantly increased the Ca^{2+} -release rate not only at pCa 7.0–4.5, but also at pCa >8 (10 mM EGTA and no added Ca^{2+}) (Fig. 3a, filled circles vs open circles). We also carried out similar experiments using rat skeletal muscle fibers. The features with which Cer augmented Ca^{2+} release rates at all Ca^{2+} concentrations studied (pCa >8 and pCa 7.0–4.5) were similar to those of the mouse (Fig. 3b).

Fig. 3. Effect of Cer on the rate of CICR. The rate of Ca^{2+} release from the SR of mouse (a) or rat (b) skeletal muscle fibers was measured with or without 500 μM Cer at a range of pCa >8–4.5 in the absence of Mg^{2+} (mean $\pm$ S.E.M., $n = 3–9$ for mouse, $n = 3$ or 4 for rat). * $P < 0.05$, ** $P < 0.01$ (vs without Cer). The paired data with or without Cer were obtained from the same preparation at each Ca^{2+} concentration. Error bars smaller than the symbols are not shown.

Effect of CICR modulators on Cer-induced Ca^{2+} release

In the previous sections, we demonstrated that Cer has an ability to cause Ca^{2+} release from the SR (Fig. 1), and its effect is dependent on the Ca^{2+} concentration (Fig. 3). Apart from the enhancing effect at pCa >8, the results of Fig. 3 suggest that Cer activates the CICR mechanism of the RyR. Therefore, we examined the effects of CICR modulators on Cer-induced Ca^{2+} release.

First, we tested the effect of AMP, which is a well-known potentiator of CICR. At pCa 6.0, AMP significantly increased the rates of Ca^{2+} release both in the presence and absence of caffeine as expected (Fig. 4,

Fig. 4. Dependence on AMP concentration of the rate of Cer- or caffeine-induced Ca^{2+} release in mouse skeletal muscle fibers. The rate of Ca^{2+} release with 500 μM Cer or 20 mM caffeine was measured under various AMP concentrations (0–10 mM) at pCa 6.0 in the presence of 0.5 mM Mg^{2+} (mean $\pm$ S.E.M., $n = 5$ for Cer, $n = 3$ for caffeine and AMP alone). The data (0–10 mM AMP) in each line were obtained from the same preparation. * $P < 0.05$, ** $P < 0.01$ (0 vs 2 mM or 2 vs 5 mM AMP in each line). N.S., not significant. Error bars smaller than the symbols are not shown.

open circles and filled triangles). While Cer-induced Ca^{2+} release was also significantly augmented, this increasing effect of AMP was saturated at 2 mM (Fig. 4, filled circles). Similar effects of AMP on both Cer- and caffeine-induced Ca^{2+} release were observed at pCa >8, 7.0, and 5.0 (data not shown).

Then, we investigated the effects of CICR inhibitors on Cer-induced Ca^{2+} release. Mg^{2+} is a well-known inhibitor of CICR. At pCa 6.0, the enhancing effect of Cer was reduced by the presence of Mg^{2+} in a dose-dependent manner, as is the case with caffeine, although the extent of the inhibition by high concentrations of Mg^{2+} (>1 mM) on Cer-induced Ca^{2+} release was much smaller than that on caffeine-induced Ca^{2+} release (Fig. 5a). Under Ca^{2+} -free conditions (pCa >8), Cer-induced Ca^{2+} release was also inhibited by Mg^{2+} , although the magnitude of inhibition was rather small altogether (Fig. 5b).

Procaine also inhibits CICR, and therefore, caffeine-induced Ca^{2+} release was strongly inhibited with or without Ca^{2+} in our preparations (3). We examined the effect of procaine on Cer-induced Ca^{2+} release. Procaine strongly inhibited Cer-induced Ca^{2+} release at pCa 6.0 (Fig. 6a) and 7.0 (data not shown). On the other hand, at pCa >8, the rate of Ca^{2+} release augmented by the action of Cer could not be reduced by adding procaine

Fig. 5. Inhibition by Mg^{2+} of the rate of Cer- or caffeine-induced Ca^{2+} release in mouse skeletal muscle fibers. The rate of Ca^{2+} release with 500 μM Cer or 20 mM caffeine was measured under various Mg^{2+} concentrations (0–10 mM) at pCa 6.0 (a) (mean $\pm$ S.E.M., $n = 3–10$ for Cer, $n = 4$ for caffeine) or at pCa >8 (b) (mean $\pm$ S.E.M., $n = 4$ for Cer, $n = 3$ for caffeine). $P < 0.05$ (1 or 1.5 mM vs 10 mM Mg^{2+}). Error bars smaller than the symbols are not shown.

at all (Fig. 6b). Tetracaine has been shown to inhibit CICR with a higher potency than procaine (16). It was also reported that while procaine could not inhibit physiological Ca^{2+} release, tetracaine could (17). In contrast to procaine, tetracaine significantly reduced the rate of Cer-induced Ca^{2+} release not only at pCa 6.0 and 7.0, but also at pCa >8 (Fig. 6c).

These results suggest that Cer can enhance CICR. However, apart from this action, it might also enhance a different mode of Ca^{2+} release, somewhat similar to physiological Ca^{2+} release, as indicated by its effect at pCa >8 , which was inhibited by tetracaine but not by

Fig. 6. Effect of procaine or tetracaine on Cer-induced Ca^{2+} release in mouse skeletal muscle fibers. The rate of Ca^{2+} release with 50–500 μM Cer at pCa 6.0 (a) or 200–500 μM Cer at pCa >8 (b) was measured in the presence or absence of 10 mM procaine under Mg^{2+} -free conditions (mean $\pm$ S.E.M., $n = 3$ or 4 for pCa 6.0, $n = 3–6$ for pCa >8). The paired data with or without procaine were obtained from the same preparation at each Cer concentration. $*P < 0.05$, $**P < 0.01$ (vs Cer alone). N.S., not significant. c: The rate of Ca^{2+} release with 500 μM Cer at pCa $>8–6.0$ was measured in the presence or absence of 500 μM tetracaine under Mg^{2+} -free conditions (mean $\pm$ S.E.M., $n = 3$). The paired data with or without tetracaine were obtained from the same preparation at each Ca^{2+} concentration. $*P < 0.05$, $**P < 0.01$ (vs Cer alone).

procaine.

Reduction of the Ca^{2+} -uptake capacity of the SR after co-application of ryanodine and Cer

If Cer-induced Ca^{2+} release is indeed the result of enhancement of CICR, the RyR- Ca^{2+} release channel must be activated by Cer, because CICR is the result of an activation of the RyR- Ca^{2+} release channel. To test this, we used ryanodine, which preferentially binds to activated CICR channels and locks them in an open state. When RyR- Ca^{2+} release channels are locked open by ryanodine, the Ca^{2+} -uptake capacity of the SR irreversibly decreases (3, 13, 18). Therefore, if Cer indeed activates the RyR, simultaneous application of ryanodine and Cer should cause a reduction of the Ca^{2+} -uptake capacity of the SR. Thus, the effect of ryanodine treatment with or without Cer (200 μM) on the Ca^{2+} -uptake capacity of the SR of mouse and rat skeletal muscle fibers was examined.

First, the amount of Ca^{2+} accumulated in the SR with the constant loading procedure was measured by releasing it completely with a high concentration of caffeine (run 1, 100%). Before the loading procedure of the next runs, we applied ryanodine, Cer, or both for 90 s at pCa 6.0 in the absence of ATP. When Cer alone was applied, little decrease in the Ca^{2+} uptake capacity was observed in the subsequent runs (Fig. 7a, filled circles). After the treatment of ryanodine alone, the Ca^{2+} uptake capacities were decreased appreciably (Fig. 7a, open triangles, runs 2–4), as reported previously (3). After co-application of ryanodine and Cer, the Ca^{2+} uptake capacities of the SR were further decreased (Fig. 7a, filled triangles, runs 2–4). These results suggest that the channel activity under the condition of pCa 6.0 was sufficient for ryanodine to bind to the CICR channel proteins within 90 s to a detectable amount in mouse skeletal muscles, and Cer further elevated the CICR channel activity so that larger numbers of the channel were locked in an open state by ryanodine.

When we carried out similar experiments using rat skeletal muscle fibers, the reduction of Ca^{2+} uptake capacities after treatment with ryanodine alone were much smaller than those of mouse skeletal muscles (vs Fig. 7a) as expected from the results in Fig. 3 that the rate of Ca^{2+} release of rat skeletal muscles at pCa 6.0 was much lower than that of mouse. In contrast, after simultaneous treatment with ryanodine and Cer, the Ca^{2+} uptake capacity remarkably decreased (Fig. 7b, filled triangles). Thus, these results indicated that Cer activated the RyR at pCa 6.0 in both mouse and rat skeletal muscles, leading to Ca^{2+} release from the SR.

Fig. 7. Enhancement of reduction of the Ca^{2+} -uptake capacity of the SR of skeletal muscle fibers after co-application of ryanodine and Cer. Ca^{2+} -uptake capacity was determined after active Ca^{2+} loading to the SR, by measuring the amount of Ca^{2+} released by application of 50 mM caffeine. Four successive measurements were made from the same preparations of mouse (a) or rat (b). The data were normalized by the magnitude of the caffeine response in the first run. Between the runs, skinned fibers were treated for 90 s with a solution containing Ca^{2+} (pCa 6.0) in the absence of Mg^{2+} and adenine nucleotide, to which either Cer (200 μM) alone, ryanodine (30 μM) alone, or both ryanodine and Cer were added (mean $\pm$ S.E.M., $n = 5$ or 6 for mouse, $n = 4 - 6$ for rat). * $P < 0.05$ (vs ryanodine alone, Dunnett's test).

Enhancing effect of Cer on Ca^{2+} release independent of Ca^{2+} concentration

As described in the previous sections, Cer had an ability to activate the CICR mechanism in the presence of Ca^{2+} ; however, it also enhanced procaine-insensitive Ca^{2+} release in the absence of Ca^{2+} . The enhancing effect

of Cer did not result from the perforation of SR membrane or denature of proteins on the SR since Ca^{2+} release caused by Cer completely ceased after wash-out of the drug and Ca^{2+} uptake capacities were not essentially impaired by Cer as shown in Fig. 7. Thus, a specific mechanism different from CICR may be involved in the effect of Cer on Ca^{2+} release. In order to support this notion and also to examine whether the mechanism is Ca^{2+} -concentration-dependent, we examined the effect of various concentrations of Ca^{2+} on Cer-induced Ca^{2+} release under conditions where CICR was maximally inhibited (Fig. 8). For this purpose, we used 10 mM Mg^{2+} together with 10 mM procaine (Fig. 8) as in the case of our previous report (3). Under this condition, the Ca^{2+} -dependent increase in Ca^{2+} release rates was fully abolished, as reported previously (3), and caffeine (20 mM) also failed to increase Ca^{2+} release rates (data not shown). Thus, CICR activity was strongly inhibited under this condition. However, Cer still caused an appreciable increase in the rate of Ca^{2+} release (Fig. 8, filled circles) in the presence or absence of Ca^{2+} . Although it is difficult to exclude completely the possibility that some CICR activity still remained under this condition, these results strongly suggest that Cer might also exert a Ca^{2+} -releasing effect, regardless of Ca^{2+} concentrations, through a mechanism different from that of CICR.

Fig. 8. Enhancing effect of Cer on Ca^{2+} release in the presence of a high concentration of Mg^{2+} and procaine in mouse skeletal muscle fibers. The rate of Ca^{2+} release with or without 500 μM Cer was measured at a range of pCa >8–5.0 in the presence of 10 mM Mg^{2+} and 10 mM procaine (mean $\pm$ S.E.M., $n = 4-9$). The paired data with or without Cer were obtained from the same preparation at each Ca^{2+} concentration. ** $P < 0.01$ (vs without Cer). Error bars smaller than the symbols are not shown.

Discussion

Activation of RyR by Cer

In this paper, we described a strong enhancing effect of Cer on Ca^{2+} release in saponin-skinned skeletal muscle fibers. At pCa 7.0–4.5, Cer-induced Ca^{2+} release has some features in common with those of CICR: 1) the rate of Cer-induced Ca^{2+} release was much higher at higher Ca^{2+} concentrations than at lower Ca^{2+} concentrations (Fig. 3: a and b), and it was further enhanced by a CICR activator, AMP (Fig. 4); 2) procaine, a CICR inhibitor, reduced the rate of Cer-induced Ca^{2+} release in the presence of Ca^{2+} (Fig. 6a); and 3) Mg^{2+} suppressed Cer-induced Ca^{2+} release in a concentration-dependent manner (Fig. 5a). Although the Mg^{2+} -concentration-dependence of Cer-induced Ca^{2+} release and caffeine-induced Ca^{2+} release appear different, since Cer-induced Ca^{2+} release may include a component through a different mechanism, if we subtract the values for the rates of Cer-induced Ca^{2+} release in Fig. 5b from those in Fig. 5a, by assuming that the rates of Cer-induced Ca^{2+} release in the presence of Ca^{2+} in Fig. 5a are a sum of those through CICR and through such a different mechanism, we could probably obtain a similar Mg^{2+} concentration dependence of Cer-induced Ca^{2+} release as that of caffeine-induced Ca^{2+} release. These data indicate that Cer has an enhancing effect on CICR in the presence of Ca^{2+} . In agreement with these results, Cer was shown to activate the RyR, since ryanodine could lock the RyR in an open state in the presence of Cer, thereby causing a reduction of the Ca^{2+} -uptake capacity of the SR at pCa 6.0 (Fig. 7).

Although more than 20 μM of Cer was required to potentiate CICR (Figs. 1 and 2b), optimal Ca^{2+} -releasing concentrations of Clof or other types of CICR activators (caffeine or adenine nucleotides) are much higher (>several hundred μM). Thus, Cer might be classified as a type of activator that can cause Ca^{2+} release at relatively low concentrations, as is 4-chloro-*m*-cresol (19, 20) or doxorubicin (21, 22).

A feature of the enhancing effect of Cer on CICR is distinct from that of caffeine. Caffeine causes Ca^{2+} release, as shown in Fig. 1, since it strongly potentiates CICR by increasing its Ca^{2+} sensitivity (2, 23, 24). On the other hand, adenine nucleotides potentiate CICR without altering the Ca^{2+} -dependence of CICR (2, 23, 24). Similarly to AMP, as shown in Fig. 3, the Ca^{2+} sensitivity of Ca^{2+} release with Cer appeared to be unchanged in both mouse and rat. Therefore, it would not be surprising to observe that the magnitude of potentiation by AMP for Cer-induced Ca^{2+} release saturates at about 2 mM (Fig. 4).

Opening mode of Cer-induced Ca^{2+} release in the absence of Ca^{2+}

Cer could significantly increase the rate of Ca^{2+} release, even in the absence of Ca^{2+} and AMP (Fig. 3, $\text{pCa} > 8$). Procaine failed to inhibit the effect of Cer on Ca^{2+} release in the absence of Ca^{2+} , unlike at $\text{pCa} 6.0$ (Fig. 6: a and b), whereas the Ca^{2+} release induced by caffeine was inhibited by procaine in our skinned fibers, even in the absence of Ca^{2+} (3). Moreover, under conditions where CICR was maximally inhibited, Cer still strongly potentiated Ca^{2+} release (Fig. 8). These results indicate that Cer activates a Ca^{2+} -release mechanism other than CICR. Although we cannot rule out the possibility that Cer in the absence of Ca^{2+} might cause Ca^{2+} release through an RyR-independent mechanism, it might activate RyR channels in a mode different from CICR, for Cer-induced Ca^{2+} release in the absence of Ca^{2+} is inhibited by tetracaine (Fig. 6c), which is an inhibitor of the RyR- Ca^{2+} release channels of skeletal muscle not only in the mode of CICR, but also in another, physiological mode. In this respect, the effect of Cer in the absence of Ca^{2+} is similar to that of Clof, which activates RyR in a mode similar to the physiological mode (3, 25). However, while adenine nucleotide (AMP) showed an inhibitory effect on Clof-induced Ca^{2+} release, it showed an enhancing effect on Cer-induced Ca^{2+} release, even at $\text{pCa} > 8$. Also, while the inhibitory effect of Mg^{2+} on Clof-induced Ca^{2+} release saturated at about 1 mM, that on Cer-induced Ca^{2+} release increased further in the range above 1 mM (Fig. 5), although the magnitude of inhibition was rather small throughout the range of Mg^{2+} concentrations. Therefore, the mode of action of Cer in the absence of Ca^{2+} is also likely to be different from that of Clof. Further elucidation of the detailed molecular mechanism of Cer-induced Ca^{2+} release, including the question as to whether Cer affects the RyR- Ca^{2+} release channel directly or indirectly through the RyR-associated protein, may provide a key for understanding the opening mechanism of the RyR- Ca^{2+} release channel.

Cer-induced Ca^{2+} release and rhabdomyolysis

It is reported that statins used clinically may cause myopathy, which in some cases progresses to rhabdomyolysis as a serious side effect (5, 6, 26). The exact pathogenetic mechanism of statin-induced myopathy remains unknown. In skeletal muscle, an excessive increase in the intracellular level of Ca^{2+} beyond a physiological range frequently results in muscle damage (27–31). Therefore, it is conceivable that Ca^{2+} release via the RyR activated by statins may cause muscle damage, which leads to myopathy. It should be noted that the Ca^{2+} -releasing effect of Cer was much stronger

than that of Pra (Fig. 1c), and Cer was associated with a tenfold higher incidence of myotoxicity than Pra or any other statin (32, 33). However, there is a large discrepancy between the effective concentration required for the activation of Ca^{2+} release and inhibition of HMG-CoA reductase ($>20 \mu\text{M}$ vs nM, respectively) (34, 35). A chronic influence of low concentrations of Cer on the RyR- Ca^{2+} release channel might produce Ca^{2+} release. Alternatively, a certain genotype of RyR- Ca^{2+} release channel might be very sensitive to Cer. Of course, at present, we do not know whether the activation of the RyR by Cer could induce myopathies leading to rhabdomyolysis since statins, including Cer, have pleiotropic effects (36, 37). Further investigation of the properties of Ca^{2+} release induced not only by Cer but also by other statins may contribute to the understanding of their serious adverse effect, rhabdomyolysis, as well as to the elucidation of the mechanisms of Ca^{2+} release via the RyR.

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