Effects of a domain peptide of the ryanodine receptor on Ca\textsuperscript{2+} release in skinned skeletal muscle fibers

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Lamb, Graham D., Giuseppe S. Posterino, Takeshi Yamamoto, and Noriaiki Ikemoto. Effects of a domain peptide of the ryanodine receptor on Ca\textsuperscript{2+} release in skinned skeletal muscle fibers. Am J Physiol Cell Physiol 281: C207–C214, 2001.—Mutations in the central domain of the skeletal muscle ryanodine receptor (RyR) cause malignant hyperthermia (MH). A synthetic peptide (DP4) in this domain (Leu-2442–Pro-2477) produces enhanced ryanodine binding and sensitized Ca\textsuperscript{2+} release in isolated sarcoplasmic reticulum, similar to the properties in MH, possibly because the peptide disrupts the normal interdomain interactions that stabilize the closed state of the RyR (Yamamoto T, El-Hayek R, and Ikemoto N. J Biol Chem 275: 11618–11625, 2000). Here, DP4 was applied to mechanically skinned fibers of rat muscle that had the normal excitation-contraction coupling mechanism still functional to determine whether muscle fiber responsiveness was enhanced. DP4 (100 μM) substantially potentiated the Ca\textsuperscript{2+} release and force response to caffeine (8 mM) and to low [Mg\textsuperscript{2+}] (0.2 mM) in every fiber examined, with no significant effect on the properties of the contractile apparatus. DP4 also potentiated the response to submaximal depolarization of the transverse tubular system by ionic substitution. Importantly, DP4 did not significantly alter the size of the twitch response elicited by action potential stimulation. These results support the proposal that DP4 causes an MH-like aberration in RyR function and are consistent with the voltage sensor triggering Ca\textsuperscript{2+} release by destabilizing the closed state of the RyRs.

excitation-contraction coupling; voltage sensor; action potential; malignant hyperthermia; caffeine

Excitation-contraction (E-C) coupling in vertebrate skeletal muscle is the sequence of events by which an action potential on the surface membrane triggers Ca\textsuperscript{2+} release and contraction. The action potential propagates into the transverse tubular (T) system, and the dihydropyridine receptors (DHPRs) in the T system membrane act as voltage sensors, detecting the depolarization and opening adjacent ryanodine receptor (RyR)/Ca\textsuperscript{2+}-release channels in the apposing sarcoplasmic reticulum (SR) membrane by some protein-protein interaction, thereby releasing Ca\textsuperscript{2+} into the cytoplasm and activating the contractile apparatus (25, 39). It is known that the influx of extracellular Ca\textsuperscript{2+} through the DHPR is not necessary for initiating Ca\textsuperscript{2+} release, but it is unclear to what degree intracellular Ca\textsuperscript{2+} triggers or reinforces further Ca\textsuperscript{2+} release (so-called Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release) (6, 16, 29, 34, 43), particularly in adult mammalian skeletal muscle (12, 35). Malignant hyperthermia (MH) is an inherited disorder of skeletal muscle in humans and pigs that is triggered by volatile anesthetics, such as halothane, and by other factors, such as stress (11, 26). The syndrome is characterized by profound muscle rigidity and rapidly rising body temperature and acidosis, and is potentially fatal. The primary deficit producing MH in pigs and in many of the human cases involves abnormal regulation of the skeletal muscle RyR/Ca\textsuperscript{2+}-release channel (11, 26), caused by one of a number of different single-point mutations in either the NH\textsubscript{2}-terminal domain or central domain of the RyR (21, 40). The abnormality in RyR function can involve increased sensitivity to activation by cytoplasmic Ca\textsuperscript{2+} (41) and/or decreased inhibition of activity by high concentrations of Ca\textsuperscript{2+} (7, 26) and Mg\textsuperscript{2+} (20, 28), the latter being important because there is normally ~1 mM free Mg\textsuperscript{2+} in the cytoplasm, which exerts an important inhibitory effect on the RyRs at rest (see Refs. 12, 20, and 28). The precise functional abnormality of the RyR may well depend on the particular mutation involved (40), but interestingly, one study that directly compared the changes in RyR properties caused by a mutation in the NH\textsubscript{2}-terminal domain of the porcine RyR (Arg-615-Cys) with those for a mutation in the central domain of the human RyR (Gly-2434-Arg) found similar relative changes in both cases (33). In any case, most, if not all, of the mutations associated with MH cause an increase in the caffeine sensitivity of the Ca\textsuperscript{2+}-release process (40), and one of the key indicators used to diagnose an individual’s susceptibility to MH is a heightened responsiveness of muscle fibers in vitro to caffeine (11, 26).

Recently, a peptide called DP4, corresponding to a region in the central domain of the RyR (Leu-2442–Pro-2477) where MH mutations occur, has been used...
as a probe of RyR function (42). Interestingly, this peptide enhanced ryanodine binding and sensitized Ca2+ release in isolated SR, producing changes similar to those occurring with MH. It was proposed that the exogenous peptide has these effects because it competes with the corresponding region on the RyR for a binding site on another region of the RyR, thereby disrupting the normal interdomain interactions that stabilize the closed state of the RyR (42). In strong support of this, it was found that when DP4 had a single residue changed to match a MH mutation of the RyR (Arg-2458-Cys), the resulting peptide lacked any stimulatory effect, consistent with the ideas that the mutated sequence is unable to bind to a particular domain on the RyR as the normal sequence does and that this same disturbance in interdomain interactions is the cause of the heightened responsiveness of RyRs with MH mutations in vivo (42).

In the present study, we examine whether DP4 produces MH-like abnormalities in functioning muscle fibers, where the RyRs have normal associations with adjacent RyRs and other key molecules, and, in particular, where RyRs are under the control of the DHPR/voltage sensors in the T system. By using mechanically skinned muscle fibers in which the normal E-C coupling mechanism is retained and entirely functional (8, 16–18, 32, 37), we were able to examine whether DP4 causes enhanced caffeine sensitivity (11, 26) and increased responsiveness to low cytoplasmic [Mg2+] (28) and submaximal depolarization of the T system (5, 9), as occurs in MH-susceptible muscle fibers. In this way, we were able to show that DP4 indeed causes an MH-like abnormality in Ca2+ release in muscle fibers, giving strong support to the proposal that an aberration in interdomain interactions within the RyR is the basis of at least some forms of MH. Finally, we show that in contrast to its other effects, DP4 does not increase action potential-mediated Ca2+ release, which gives important insight into the relative roles of the voltage sensors and Ca2+-induced Ca2+ release in normal E-C coupling.

METHODS

Skinned fibers. Mechanically skinned fibers were obtained and used as described previously (16, 18). Briefly, Long-Evans hooded rats were anesthetized with halothane (2% vol/vol) in a bell jar and killed by asphyxiation, and then both extensor digitorum longus (EDL) muscles were removed. The muscles were placed in paraffin oil, except for the experiments on submaximal depolarization, in which they were first bathed in a NaCl solution with 10 μM tetrodotoxin (TTX) (in mM: 150 NaCl, 3 KCl, 2.5 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.3) for 30 min before being transferred to paraffin oil. Single muscle fibers were mechanically skinned under paraffin oil, and a segment was attached to a force transducer (AME875; Horten, Norway) at 120% of resting length. The skinned fiber was then placed in a 2-ml Perseps bath containing a potassium hexamethylene-diamine-tetraacetate (K-HDTA) solution (see below) for 2 min to allow the sealed T system to become normally polarized. Fibers were stimulated by rapid substitution of another appropriate solution or by applying electric field stimulation (40–50 V/cm, 2 ms) via two parallel platinum wires (32). In the field stimulation and depolarization (by ion substitution) experiments, fibers were used with their endogenous level of SR Ca2+ without any additional loading. In the caffeine and low-[Mg2+] experiments, the SR was repeatedly emptied and reloaded with Ca2+ as described below. All experiments were performed at room temperature (24 ± 2°C).

Solutions. All chemicals were obtained from Sigma, unless specified otherwise. The K+-HDTA solution contained (in mM), 126 K+, 37 Na+, 50 HDTA2− (Fluka, Buchs, Switzerland), 8 total ATP, 8.6 total magnesium, 10 creatine phosphate, 0.05 total EGTA, 90 HEPES, and 1 N5+, pH 7.10 ± 0.01 and pCa (= −log10 of [Ca2+]i) 7.0. The Na+-HDTA solution was identical except that K+ was replaced with Na+. The choline chloride (ChCl) solution was similar except all K+-HDTA was replaced with 100 mM ChCl, and the total HEPES was decreased to 50 mM and the total magnesium to 8.3 mM. The above solutions all contained 1 mM free Mg2+ and had an osmolality of 295 ± 10 mosmol/kg−1. The solution with a free [Mg2+] of 0.2 mM was similar to the standard K+-HDTA solution except that it contained only 5.3 mM total magnesium. Maximum Ca2+-activated force was determined using a solution (“max”) similar to standard K+-HDTA solution, but with 50 mM Ca2+-EGTA (20 μM free Ca2+) replacing all HDTA and the total magnesium reduced to 8.12 mM to keep the free [Mg2+] at 1 mM (36). Free [Ca2+]i of solutions was measured with a Ca2+-sensitive electrode (Orion Research, Boston, MA).

Contractile apparatus measurements. The Ca2+ sensitivity of the contractile apparatus was determined by exposing the skinned fiber to a sequence of solutions of progressively higher [Ca2+]i (with or without DP4) made by an appropriate mixture of the 50 mM Ca2+-EGTA solution and a similar solution with 50 mM free EGTA (36). Force measurements in the presence of DP4 were bracketed by similar sequences in the absence of DP4. The force produced by the fiber at each pCa under a given condition was expressed as a percentage of the corresponding maximum Ca2+-activated force and plotted against pCa. The scientific analysis program GraphPad Prism (GraphPad Software, San Diego, CA) was used to fit Hill curves to the force-pCa data obtained under each condition to obtain the pCa giving 50% of maximum force (pCa50) and the Hill coefficient.

Repeated Ca2+ load-release cycles for low [Mg2+] and caffeine-induced release. Because fibers were skinned under paraffin oil, the SR initially contained its normal endogenous level of Ca2+. Where required, the SR was fully and rapidly depleted of Ca2+ by exposing the fiber for 1 min to a low-[Mg2+] K+-HDTA solution (0.015 mM Mg2+) with 30 mM caffeine and 0.5 mM free EGTA (pCa 8) present to chelate the released Ca2+, having first preequilibrated the fiber for 10 s in the standard K+-HDTA solution with 0.5 mM EGTA to ensure that EGTA was present in the fiber (see Ref. 13). After depletion, the fiber was washed for 1 min in the standard K+-HDTA solution with 0.5 mM EGTA (pCa 8). The SR was then reloaded with Ca2+ (to approximately the original endogenous level) by exposure to a loading solution (with 1 mM total EGTA at pCa 6.7) for a set time (in the 15- to 20-s range). The relative Ca2+ content of the SR could be estimated from the time integral of the force response when inducing full release of SR Ca2+ by exposure to the caffeine-low [Mg2+] solution (e.g., see Fig. 2); this response was highly reproducible for any given loading time (see Ref. 13).

To examine the ability of the DP4 peptide to trigger Ca2+ release at 0.2 mM Mg2+ (e.g., see Fig. 2), each EDL fiber was fully depleted of Ca2+ and reloaded as above (with the loading terminated by a 2-s exposure to a K+-HDTA solution with
0.5 mM free EGTA) and then 2) equilibrated for 20 s in the standard K+–HDTA solution (1 mM Mg2+ and 50 μM EGTA, pCa 7.0) with or without peptide, 2) exposed for 15 s to the 0.2 mM Mg2+ solution (50 μM EGTA, pCa 7.0) with or without peptide, and 3) once again fully depleted of Ca2+ with the 30 mM caffeine-low [Mg2+] solution (full release: "F. R." in Fig. 2). When examining the effect of DP4 on caffeine-induced Ca2+ release, the sequence was the same as above except that the fiber was exposed to a solution with 8 mM caffeine (at 1 mM Mg2+) instead of the 0.2 mM Mg2+ solution.

DP4 and DP3 peptides. DP4 peptide (2442LIQAGKGEAL-RIRAILRLSVPLDDLGVISLQIP2477) and DP3 peptide (324DTAPKRDVEGMGPPEIKYESLCFVQHY3351) were synthesized on an Applied Biosystems (model 431A) synthesizer employing N-(9-fluorenyl)methoxycarbonyl as the α-amino protecting group and were cleaved and deprotected with 95% trifluoroacetic acid. Purification was carried out by reverse-phase high-pressure liquid chromatography using a Rainin Instruments preparative C8 column. The peptides were dissolved in double-distilled water at 30 mM and mixed with the appropriate solutions to give a final concentration of 20 or 100 μM, with the same amount of distilled water being added to the matching control solutions.

Force traces and analysis. In force traces, unless otherwise indicated, the skinned muscle fiber was bathed in the standard K+–HDTA solution (1 mM Mg2+ and 50 μM total EGTA, pCa 7.0). In the text, mean values are ± SE of the mean. Statistical probability (P) was determined with Student’s paired t-test, with P < 0.05 considered significant.

RESULTS

Response to caffeine. We first sought to examine whether DP4 enhances the response of a muscle fiber to caffeine. Intact muscle fibers from MH-susceptible humans and pigs display increased responsiveness to caffeine; this is one of the major diagnostic features of MH (11, 26). Caffeine triggers Ca2+ release and contraction in skinned muscle fibers, similar to its action in intact fiber. Because the responsiveness to caffeine increases when the SR is loaded more heavily with Ca2+ (14), the effect of DP4 was examined at a constant SR Ca2+ load level, close to the level present endogenously in each fiber. In this set of experiments, each mechanically skinned rat EDL muscle fiber was first fully depleted of all its SR Ca2+ by exposure to a 30 mM caffeine-low [Mg2+] solution (see METHODS), with the relative area of the resulting force response being indicative of the amount of Ca2+ that had been present endogenously (see Ref. 13). The SR was then reloaded to approximately the original level, and the fiber equilibrated for 20 s under standard conditions (pCa 7.0 weakly buffered with 50 μM EGTA and 1 mM free Mg2+) before being exposed to a similar solution with 8 mM caffeine for 15 s. This concentration of caffeine was chosen because it typically produces a detectable, but relatively small, response at such a level of SR Ca2+ loading (e.g., Fig. 1), which thus allows identification of any excitatory or inhibitory effect of DP4. The response to 8 mM caffeine was potentiated in the presence of 100 μM DP4 in every fiber examined (e.g., Fig. 1). On average, the peak of the force response to caffeine was increased more than threefold, with the mean peak response, with and without DP4, being 12 and 39%, respectively, of the maximum Ca2+-activated force in those fibers (Table 1). Paired comparison of the response before and after addition of 100 μM DP4 in each fiber indicated a significant increase in peak force, equivalent to 27 ± 5% of maximum Ca2+-activated force, in the 20 fibers examined. This increase in force was due to increased Ca2+ release, because DP4 had no significant effect on the properties of the contractile apparatus as determined by exposing skinned fibers to heavily Ca2+-buffered solutions (see METHODS). (In the presence of DP4, the maximum Ca2+-activated force was 97 ± 2% of that in the absence of DP4, and the mean changes in the pCa50 and Hill coefficient were 0.00 ± 0.02 and −0.2 ± 0.1 in the three fibers examined, with the control values being 5.80 ± 0.02 and 4.6 ± 0.5, respectively.) DP4 produced less potentiation of caffeine-induced responses at 20 μM than at 100 μM, as shown by the mean force increases in Table 1, with the difference also being apparent when both concentrations were tested in the same fiber (not shown). The potentiating effect of DP4 on caffeine-induced Ca2+ release was largely reversed by washout of the peptide in <2 min (Fig. 1 and Table 1).

DP3, a peptide corresponding to a different region of the RyR (Asp-324–Val-351), had no effect at a concentration of 100 μM on caffeine-induced responses in any of the three fibers examined; the mean change in the force response to 8 mM caffeine upon adding DP3 was 1 ± 2% of maximum Ca2+-activated force in that fiber (paired comparison, n = 3), with the mean response of the fibers to caffeine being 14 ± 2% of maximum force before exposure to DP3, 15 ± 1% in the presence of DP3, and 16 ± 1% after washout of DP3. This lack of effect of DP3 on the response to caffeine is in accord with the observation that other similar-sized peptides such as DHPR peptides C8 and A8(20) have no effect on the Ca2+-activated force in that fiber (paired comparison, n = 3), with the mean response of the fibers to caffeine being 14 ± 2% of maximum force before exposure to DP3, 15 ± 1% in the presence of DP3, and 16 ± 1% after washout of DP3. This lack of effect of DP4 on caffeine-induced Ca2+ release was largely reversed by washout of the peptide in <2 min (Fig. 1 and Table 1).

Fig. 1. DP4 potentiates the force response to 8 mM caffeine. A skinned extensor digitorum longus (EDL) fiber of the rat was subjected to repeated load-release cycles in which the sarcoplastic reticulum (SR) was loaded with Ca2+ (20 s at pCa 6.7, 1 mM total EGTA) to a set level (close to the endogenous level) and the fiber was exposed to a solution with 8 mM caffeine with or without 100 μM DP4 present, before the SR was again fully depleted of Ca2+ (latter not shown). DP4 was added as appropriate during the 20-s equilibration period preceding caffeine exposure (see METHODS). Maximum Ca2+-activated force was ascertained with a heavily buffered Ca2+ solution with 20 μM free Ca2+ (max). Time scale: 2 s during caffeine responses and 30 s during maximum activation.
caffeine-induced force response in this skinned fiber preparation (15), shows that the potentiating effect of DP4 is not due to some nonspecific effect of high peptide concentrations.

**Response to low [Mg^{2+}].** Skinned fibers from MH-susceptible pigs (with the endogenous level of SR Ca^{2+} release Ca^{2+} and produce a substantial force response when the free [Mg^{2+}] in the bathing solution is decreased from the normal physiological concentration (1 mM) to 0.2 mM, whereas fibers from normal pigs do so in only a small proportion of cases under the same conditions (28). Similarly, skinned fibers from rat EDL muscle with SR Ca^{2+} loading at close to the endogenous level never produced any force response when lowering the free [Mg^{2+}] from 1 mM to 0.2 mM in the absence of DP4 (e.g., Fig. 2 and Table 1), as found previously (15, 18). (Each fiber was subjected to repeated depletion-load cycles similar to when testing caffeine responsiveness in Fig. 1; see METHODS). However, when 100 μM DP4 was present, the same stimulus produced Ca^{2+} release and a substantial force response in every fiber (e.g., Fig. 2), with the peak of the force on average reaching 24% of the maximum Ca^{2+}-activated force in the same fibers (Table 1). When the SR was fully depleted of its remaining Ca^{2+} immediately after such a response, the relatively small size of the resulting force response (e.g., Fig. 2) further confirmed that a substantial amount of Ca^{2+} had been released from the SR during exposure to 0.2 mM Mg^{2+} in the presence of DP4. Thus the presence of DP4 made the rat fibers hyperresponsive to low intracellular [Mg^{2+}], much like what happens in MH. This effect of DP4 was completely reversed by washout of the peptide (Table 1).

**Twitch responses.** It was also possible to use skinned fibers to examine what, if any, effect DP4 had on the normal E-C coupling mechanism in skeletal muscle. Transverse electric field stimulation (2 ms, 50 V/cm) triggers an action potential in the (sealed) T system of a mechanically skinned muscle fiber, thereby eliciting a twitch response that is highly comparable to that in an intact fiber, with repeated stimulation (e.g., 50 Hz) eliciting a tetanic force response (32). In the rat EDL fibers examined here (n = 6), the twitch response

### Table 1. Effect of DP4 on force response to different stimuli

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>[DP4], μM</th>
<th>Before DP4</th>
<th>In DP4</th>
<th>After Washout</th>
<th>Change</th>
</tr>
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<tbody>
<tr>
<td>Caffeine</td>
<td>20</td>
<td>5 ± 3% (7)</td>
<td>24 ± 7% (7)</td>
<td>14 ± 3% (5)</td>
<td>19 ± 6% (7)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>100</td>
<td>12 ± 4% (20)</td>
<td>39 ± 6% (20)</td>
<td>24 ± 5% (15)</td>
<td>27 ± 5% (20)</td>
</tr>
<tr>
<td>Mg^{2+} (0.2 mM)</td>
<td>100</td>
<td>0 ± 6% (11)</td>
<td>24 ± 5% (11)</td>
<td>1 ± 1% (9)</td>
<td>24 ± 5% (11)</td>
</tr>
<tr>
<td>Submax. depol. 100</td>
<td>33 ± 10% (5)</td>
<td>53 ± 7% (5)</td>
<td>41 ± 5% (5)</td>
<td>20 ± 5% (5)</td>
<td></td>
</tr>
<tr>
<td>Action potential</td>
<td>100</td>
<td>46 ± 6% (6)</td>
<td>45 ± 6% (5)</td>
<td>47 ± 4% (4)</td>
<td>2 ± 2% (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE (no. of fibers in parentheses) of the peak force response to the indicated stimulus before addition of DP4, in the presence of DP4, and after washout of DP4 (not examined in every fiber). Each response was expressed as a percentage of the maximum Ca^{2+}-activated force. *Significant increase in the response in the presence of DP4 (paired t-test). Caffeine (8 mM) and low [Mg^{2+}] were applied as shown in Figs. 1 and 2. Submaximal depolarization (Submax. depol.) was performed by ionic substitution (Na\(^+\) release, when it was 30 s. Maximum Ca\(^{2+}\) loading; see METHODS), the fiber was equilibrated for 20 s in the standard solution with 1 mM free Mg\(^{2+}\) was subjected to repeated load-release cycles as in Fig. 1. After the SR was loaded to a particular level (20-s loading; see METHODS), the fiber was equilibrated for 20 s in the standard solution with 1 mM free Mg\(^{2+}\) and then exposed to a similar solution with 0.2 mM Mg\(^{2+}\) for 15 s before release of all Ca\(^{2+}\) remaining in the SR (full release, F. R.) was induced with a 30 mM caffeine-low [Mg^{2+}]-0.5 mM EGTA solution (see METHODS). There was never any force response at 0.2 mM Mg\(^{2+}\) in the absence of DP4 (e.g., 1st section), but a large response was induced in the presence of 100 μM DP4 (e.g., 2nd and 3rd sections) with a relatively small force response produced when the SR was subsequently emptied of its remaining Ca\(^{2+}\) (F. R.), confirming that a substantial amount of Ca\(^{2+}\) had been released during the exposure to low [Mg^{2+}] with DP4. The trace preceding max shows the response when full release of Sr Ca\(^{2+}\) was induced immediately after loading (i.e., without exposure to the 0.2 mM Mg\(^{2+}\) solution); its similarity to the preceding response indicates that little if any Ca\(^{2+}\) was lost from the SR in 0.2 mM Mg\(^{2+}\) in the absence of DP4. Time scale: 2 s throughout, except during max and wash period between 0.2 mM Mg\(^{2+}\) and full release, when it was 30 s. Maximum Ca\(^{2+}\)-activated force determined as in Fig. 1.
reached 46 ± 6% of maximum Ca\textsuperscript{2+}-activated force, with a mean 20–80% rise time of 17 ± 2 ms and a rate constant of relaxation of 43 ± 2 s\textsuperscript{-1} (exponential fit to final 50% of force decay), and 50 Hz of stimulation elicited a tetanic response that reached maximum force (e.g., Fig. 3). Interestingly, the presence of 100 μM DP4 had no significant effect on the peak size of the twitch response (e.g., Fig. 3; see mean data in Table 1) or on its rise time or decay rate (mean 103 ± 2% and 98 ± 3% of preceding control value in absence of DP4, n = 6). There was no noticeable difference in the twitch response 30 and 90 s after addition of DP4. After washout of DP4, the peak of the twitch response was not significantly different from that before DP4 exposure (Table 1), and, likewise, the rise time and decay rate were unchanged (101 ± 5% and 103 ± 9% of values for initial response, respectively, in the 4 fibers examined). The peak of the twitch response is submaximal, where force varies steeply with [Ca\textsuperscript{2+}], so it should be a sensitive indicator of the amount of Ca\textsuperscript{2+} released. Thus the lack of any change in the size (and rate of rise) of the twitch response indicates that DP4 had no significant effect on the amount of Ca\textsuperscript{2+} released by action potential stimulation. Furthermore, the fact that the decay rate of the twitch was also unchanged in DP4 indicates that the peptide had no significant effect on Ca\textsuperscript{2+} uptake by the SR, because inhibition of uptake causes very marked potentiation and prolongation of the twitch response in this skinned fiber preparation (Posterino and Lamb, unpublished observations).

Submaximal depolarization. DP4 potentiated direct stimulation of the RyR/Ca\textsuperscript{2+}-release channel [by caffeine (Fig. 1) and by reduced cytoplasmic [Mg\textsuperscript{2+}] (Fig. 2)] but did not affect the amount of Ca\textsuperscript{2+} released by action potential stimulation (Fig. 3). This disparity might have resulted from differences in the relative potency of the stimuli (note that the action potential triggered much more rapid release of SR Ca\textsuperscript{2+} than the other stimuli, indicative of much greater activation of the RyR) or may have been due to some fundamental difference between activating the RyR directly or via the voltage sensors/DHPRs in the T system. This was investigated further by examining the effect of DP4 on Ca\textsuperscript{2+} release elicited by submaximal stimulation of the voltage sensors using the ionic substitution method (8, 16–18, 37) to partially depolarize the T system. Because depolarization of the T system in skinned fibers normally elicits an action potential that would cause maximal activation of the voltage sensors, it was necessary to abolish any action potentials by exposing the muscle fibers to 10 μM TTX before skinning (see METHODS and Ref. 32). In the five EDL fibers used under such circumstances in this study, rapid substitution of the K\textsuperscript{+}-based bathing solution with a Na\textsuperscript{+}-based (zero K\textsuperscript{+}) solution (see METHODS) elicited a reproducible, submaximal force response (e.g., Fig. 4), which on average, was only 33 ± 10% of maximum Ca\textsuperscript{2+}-activated force in those fibers. In the same fibers, depolarization of the T system by substitution of the K\textsuperscript{+}-based solution with a matching ChCl-based solution produced a much larger response (mean 82 ± 9% of maximum: 87–97% in the case of 4 of the 5 fibers), which indicates that the Na\textsuperscript{+} substitution was causing only partial depolarization of the T system. This is not unexpected, because when substituting Na\textsuperscript{+} for K\textsuperscript{+}, the T system is depolarized because of removal of the strong polarizing effect of K\textsuperscript{+}, and the total effect may be reduced by the polarizing effect of any other process (e.g., Na\textsuperscript{+}/K\textsuperscript{+} exchange). In contrast, ChCl substitution depolarizes the T system, not only by reducing cytoplasmic [K\textsuperscript{+}] but also by
increasing the cytoplasmic concentration of the relatively permeable anion Cl\(^-\) (2) and, consequently, is considerably more potent. [It is necessary, though, to keep the exposure to ChCl relatively brief to avoid direct effects on the SR that can elicit a further slow phase of Ca\(^{2+}\) release (18).]

When 100 μM DP4 was present, the response to Na\(^+\) substitution was substantially increased in every fiber examined (e.g., Fig. 4), with the mean of the peak response increasing by >50% in relative terms (see Table 1). When expressed as a percentage of the maximum Ca\(^{2+}\)-activated force, the mean increase in the size response to depolarization by Na\(^+\) substitution in the presence of DP4 (20%; i.e., from 33 to 53% of maximum force) was similar to the increase found when stimulating with caffeine or low [Mg\(^{2+}\)] (Table 1). The potentiating effect of DP4 was largely reversed within 1 min by washout of the peptide (e.g., Fig. 4 and Table 1).

**DISCUSSION**

Comparison of DP4 effects and MH. Previous work has shown that DP4, a synthetic peptide corresponding to a region in the central domain of the skeletal muscle RyR, made RyRs in isolated SR vesicles hyperactive and also hypersensitive to agonists such as polylysine (42). Here it is shown that DP4 also has stimulatory and sensitizing effects in functioning muscle fibers, where the RyRs are assembled in their normal in situ array, with their normal physical associations with other molecules in the SR, and with the voltage sensors/DHPRs in the T system. Importantly, the change in properties occurring in the presence of DP4 makes the fibers resemble those from MH-susceptible muscle. Specifically, in the presence of DP4, 1) the responsiveness to caffeine is heightened (e.g., Fig. 1) like in MH (11, 26), 2) the release mechanism becomes hyperactive such that there is Ca\(^{2+}\) release and contraction when the free [Mg\(^{2+}\)] is lowered to 0.2 mM (Fig. 2), as seen with fibers from MH-susceptible pigs (28), and 3) the response to submaximal depolarization of the T system is potentiated, as found with K\(^+\) depolarization in intact fibers (9) and ionic substitution in SR vesicles (5) in MH. The fact that the twitch response was not potentiated in DP4 (Fig. 3) is also in accord with the properties of MH muscle, where the twitch response in muscle bundles is not significantly different from that in normal muscle when the fibers are strongly polarized (with 2 mM extracellular K\(^+\)) (9), as is likely to be the case with the T system in the skinned fibers here (31). The reason why the twitch response remains constant in DP4 is discussed later (see Relevance to E-C coupling mechanism).

**Likely basis of DP4 effects.** The molecular basis of the effects of DP4 was not directly studied in the fiber experiments here but can be inferred from the effects of DP4 in isolated SR (42), which were in close accord, including in terms of the concentration dependence of DP4 (e.g., stimulatory effect at 20 μM DP4 being ~50–80% of that at 100 μM; cf. Table 1). Yamamoto et al. (42) found that DP4 increased the sensitivity of ryanodine binding to activation by cytoplasmic Ca\(^{2+}\) ~2.5-fold and decreased the sensitivity to inhibition at high [Ca\(^{2+}\)] by ~2-fold. Significantly, these relative changes are very similar to the differences found between normal and MH SR vesicles for the same parameters, for both porcine and human MH mutations of the RyR (2.5- to 3-fold increase in affinity for Ca\(^{2+}\) activation and ~2-fold decrease in affinity for Ca\(^{2+}\) inhibition) (33). This close similarity further supports the proposal that DP4 induces an MH-like aberration in RyR function (42). Furthermore, the changes found in the inhibitory effect of Mg\(^{2+}\) are also in accord. Mg\(^{2+}\) acts with similar affinity as Ca\(^{2+}\) at the low-affinity “Ca\(^{2+}\) inhibition” site (which is better described as a “Ca\(^{2+}\)/Mg\(^{2+}\) inhibition” site or simply as a “Mg\(^{2+}\) inhibition” site, given that it would normally be occupied by Mg\(^{2+}\) rather than by Ca\(^{2+}\) under physiological conditions) (12, 19, 20, 22, 23). Yamamoto et al. (42) found similar inhibition of RyR activation with high concentrations of either Ca\(^{2+}\) or Mg\(^{2+}\), with DP4 reducing the affinity for Mg\(^{2+}\) by ~1.5-fold. This also fits with the 1.5- to 3-fold decrease in affinity for Mg\(^{2+}\) inhibition found in single RyRs from MH-susceptible pigs compared with those from normal pigs (20).

It is also important to consider the absolute concentrations involved in the Mg\(^{2+}\) inhibitory effect. The dissociation constant for the Ca\(^{2+}\)/Mg\(^{2+}\) inhibitory site (K\(_i\)) is ~0.05–0.1 mM in solutions with close to physiological ionic strength, such as in the fiber experiments here (and it is increased markedly at the higher ionic strength and/or [Cl\(^-\)] used in many other experiments) (4, 20, 23). This explains why lowering the [Mg\(^{2+}\)] from the physiological level of ~1 mM to 0.2 mM in the experiments here caused little if any Ca\(^{2+}\) release in the absence of DP4 (e.g., Fig. 2 and Table 1). Furthermore, if DP4 reduces the affinity of the Ca\(^{2+}\)/Mg\(^{2+}\) site two- or threefold (see above) (i.e., K\(_i\) raised to ~0.1–0.3 mM), lowering the [Mg\(^{2+}\)] to 0.2 mM could be expected to elicit some Ca\(^{2+}\) release, given the stimulatory effect of the ATP present in all the solutions (see Refs. 12 and 22). The released Ca\(^{2+}\) would then be able to trigger further Ca\(^{2+}\) release from the SR, particularly with the heightened Ca\(^{2+}\) activation occurring in DP4. Thus the changes in RyR function observed in the SR vesicles (42) readily account for the response to low [Mg\(^{2+}\)] found in the presence of DP4 in this study, with a similar explanation accounting for the responsiveness of MH muscle to low [Mg\(^{2+}\)] (28).

The ability of DP4 to potentiate the response to partial depolarization of the T system (e.g., Fig. 4 and Table 1) is also to be expected from the change in the properties of the RyR. It is not necessary to propose that DP4 has an effect on the voltage sensor/DHPR itself. Increasing the responsiveness of the RyR (e.g., by increasing Ca\(^{2+}\) activation and/or decreasing Mg\(^{2+}\) inhibition) would be expected to cause a larger response for a given level of submaximal stimulation of the RyRs by the voltage sensors (i.e., at some given submaximal level of T system depolarization). This has recently been formulated in an explicit model for the
case of voltage sensor activation of Ca\(^{2+}\) release in normal and MH muscle (3), which describes how altering the ligand sensitivity of RyR activation can shift the voltage dependence of Ca\(^{2+}\) release without there being any change in the activation characteristics of the voltage sensor.

Thus the effects of DP4 observed in the SR vesicle experiments (42) can account for the observations in functioning fibers in this study and, by extension, also for the characteristics of MH-susceptible muscle fibers. The present experiments do not identify the precise action of DP4 on the RyR but are consistent with the proposal that DP4 disturbs the interdomain interactions that stabilize the closed state of the RyR (42) (see Introduction). The strongest support for this proposal was the fact that a variant of DP4, which had a single residue changed to give a RyR sequence associated with MH, lacked all ability to stimulate the RyR (i.e., to perturb the closed state) (42). The effect of this mutated peptide could not be tested in the present experiments because of solubility problems occurring with the skinned fiber solutions, where the major anion was HDTA\(^{2-}\) rather than Cl\(^{-}\). However, in view of the close similarity in the stimulatory effects of DP4 in the skinned fiber and SR vesicle experiments, and also the similar ineffectiveness of DP3 in both preparations (see Results), it would be surprising if the DP4-mutant peptide was not ineffective in the fibers as it is in the SR vesicles.

Because the DP4 peptide encompasses the region of the RyR thought to bind the important regulatory protein FKBP12 (Lys-2458–Val-2468) (1), it could be proposed that DP4 exerts its stimulatory action by removing FKBP12 from the RyR rather than by interfering with the normal interdomain interactions within the RyR. However, this seems unlikely because washout of DP4 resulted in reversal of its activatory effects (Table 1) even though there was no FKBP12 present in the solutions to replace any that had been removed from the RyRs. Furthermore, although Pro-2462 is thought to be critical to FKBP12 binding, changing the corresponding Pro to Glu in DP4 had no appreciable effect on the stimulatory action of the peptide in SR vesicles (Yamamoto and Ikemoto, unpublished observations).

**Relevance to E-C coupling mechanism.** Irrespective of whether or not DP4 produces an MH-like aberration in RyR function, an additional important finding was that an agent potentiated Ca\(^{2+}\) release when the RyRs were stimulated directly by caffeine or by low [Mg\(^{2+}\)], but not when they were stimulated by action potential-mediated activation of the voltage sensors/DHPRs. Because Ca\(^{2+}\) release elicited by submaximal depolarization of the T system was potentiated in DP4, it was apparent that the above disparity was not due to some fundamental difference between stimulating the RyR directly or via the voltage sensors. Instead, it seems that it was due to a difference in the efficacy of the stimuli, possibly because an action potential may fully activate the RyRs so that no further upmodulation is possible; indeed, to account for the extremely high Ca\(^{2+}\) efflux rate occurring during an action potential, it is likely that many, if not all, RyRs must open fully or to a high level (24). Interestingly, DP4 could not have caused any increase in Ca\(^{2+}\) release during or after the declining phase of the action potential even though RyRs would not then be still maximally activated, because any such additional Ca\(^{2+}\) efflux would have resulted in an increase in the twitch response. The lack of additional Ca\(^{2+}\) release was not due to depletion of SR Ca\(^{2+}\), because further Ca\(^{2+}\) release could be triggered <10 ms later by a subsequent action potential. It is possible that the total amount of Ca\(^{2+}\) released through an individual RyR during an action potential is fixed by some Ca\(^{2+}\)- or use-dependent inactivation process (10, 30, 38). Because DP4 increased the sensitivity to caffeine-induced Ca\(^{2+}\) release [and hence presumably to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (6)], the lack of any increase in Ca\(^{2+}\) release in the twitch response in DP4 implies either that all the RyRs in adult mammalian muscle are quite insensitive to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (12, 18, 35) or that every Ca\(^{2+}\)-sensitive RyR activates and then becomes completely unresponsive (inactivated) during the course of the release phase of the action potential. The former possibility is supported by a recent study showing that even high concentrations of caffeine are ineffective at inducing Ca\(^{2+}\) release in adult mammalian skinned fibers in the presence of physiological free [Mg\(^{2+}\)] (14). E-C coupling in mammalian muscle evidently does not involve initiation or reinforcement of Ca\(^{2+}\) release by an unconstrained Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism and instead can be explained by tight voltage sensor control of the RyRs, in which voltage sensor activation substantially reduces the inhibitory effect of Mg\(^{2+}\) on the release channels and possibly also increases the Ca\(^{2+}\) sensitivity of Ca\(^{2+}\) activation (e.g., Ref. 12). If this is correct, voltage sensor activation could be viewed simply as exerting a “destabilizing” effect on the RyR that is similar to, though stronger than, that occurring with DP4 application or naturally in MH mutations.

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