Regulation of skeletal ryanodine receptors by dihydropyridine receptor II–III loop C-region peptides: relief of Mg^{2+} inhibition

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The aim of the present study was to explore interactions between surface-membrane DHPR (dihydropyridine receptor) Ca^{2+} channels and RyR (ryanodine receptor) Ca^{2+} channels in skeletal-muscle sarcoplasmic reticulum. The C region (^{725}Phe-Pro^{742}) of the linker between the 2nd and 3rd repeats (II–III loop) of the α_1 subunit of skeletal DHPRs is essential for skeletal excitation–contraction coupling, which requires a physical interaction between the DHPR and RyR and is independent of external Ca^{2+}. Little is known about the regulatory processes that might take place when the two Ca^{2+} channels interact. Indeed, interactions between C fragments of the DHPR (C peptides) and RyR have different reported effects on Ca^{2+} release from the sarcoplasmic reticulum and on RyR channels in lipid bilayers. To gain insight into functional interactions between the proteins and to explore different reported effects, we examined the actions of C peptides on RyR1 channels in lipid bilayers with three key RyR regulators, Ca^{2+}, Mg^{2+} and ATP. We identified four discrete actions: two novel, low-affinity ($> 10\ \mu$M), rapidly reversible effects (fast inhibition and decreased sensitivity to Mg^{2+} inhibition) and two slowly reversible effects (high-affinity activation and a slow-onset, low-affinity inhibition). Fast inhibition and high-affinity activation were decreased by ATP. Therefore peptide activation in the presence of ATP and Mg^{2+}, used with Ca^{2+} release assays, depends on a mechanism different from that seen when Ca^{2+} is the sole agonist. The relief of Mg^{2+} inhibition was particularly important since RyR activation during excitation–contraction coupling depends on a similar decrease in Mg^{2+} inhibition.

Key words: DHPR–RyR interaction, dihydropyridine receptor (DHPR) II–III loop, excitation–contraction coupling, magnesium inhibition, peptide C, ryanodine receptor (RyR).

INTRODUCTION

During EC (excitation–contraction) coupling, the action potential in the transverse tubule membrane of striated muscle fibres leads to a release of Ca^{2+} from the SR (sarcoplasmic reticulum). Two key components of this process are the DHPR (dihydropyridine receptor; an L-type Ca^{2+} channel), which is the voltage sensor in the transverse tubule membrane [1–4], and the RyR (ryanodine receptor), which is the Ca^{2+} release channel in the SR. Molecular interactions between these two Ca^{2+} channels play a key role in EC coupling and are fundamentally different in skeletal and cardiac muscles [5]. In the heart, transverse tubule depolarization triggers an influx of Ca^{2+} through the DHPR, which activates RyRs and calcium release [6–8]. In skeletal muscles, the influx of Ca^{2+} is not required [5] and RyR activation depends on a physical link between the DHPR and RyR [9] that is dependent on cytoplasmic levels of Ca^{2+}, ATP and Mg^{2+} [13–16].

A picture of how RyRs are regulated by the DHPR in skeletal muscles, in conjunction with intracellular Ca^{2+}, Mg^{2+} and ATP is emerging. Isolated RyRs are inhibited by cytoplasmic Mg^{2+} (the half-inhibitory concentration of Mg^{2+}, K_i, is $\sim$0.2 mM at physiological ionic strength [10]) and activated by Ca^{2+} ($\sim 1\ \mu$M [11]) as well as ATP ($\sim$0.3 mM, even in the absence of Ca^{2+}; [12]). During transverse tubule depolarization and activation of the RyR by the DHPR, (i) the sensitivity of RyRs to inhibition by Mg^{2+} is markedly reduced [13,14], (ii) ATP is a key activator of calcium release from the SR [15] and (iii) although Ca^{2+} is not the major RyR activator in EC coupling, it augments RyR activation [16,17]. These findings support Lamb and Stephenson's proposition [13] that, during EC coupling, DHPRs somehow relieve Mg^{2+} inhibition and thus permit RyR activation by ATP and Ca^{2+}. The regions of the DHPR that interact physically with the RyR have been examined in myocytes/myotubes as well as in cell-free systems. In the first case, cDNAs encoding chimaeric constructs of skeletal and cardiac DHPR α_1 subunits injected into *dysgenic* myotubes (i.e. myotubes lacking DHPRs [1,3,18]) revealed that the cytoplasmic loop between repeats II and III of the skeletal DHPR α_1 subunit (^{666}Glu-Leu^{791}) [2], and specifically residues ^{725}Phe-Pro^{742}, are the crucial determinants of skeletal EC coupling [19]. The recombinant II–III loop activates isolated skeletal but not cardiac RyRs [20]. Shorter synthetic sequences [21], A_S (^{671}Thr-Leu^{690}) and C_S (^{724}Glu-Pro^{760}), also interact with isolated RyRs [34–39]. A_S activates skeletal RyRs [21–25] or inhibits them by blocking the ion-conduction pathway [23]. In spite of the importance of residues ^{725}Phe-Pro^{742} for skeletal EC coupling [19], the regulation of RyRs by the corresponding synthetic peptide is not well understood. Ryanodine binding and Ca^{2+} release assays show that C_S is a much weaker activator of RyRs than A_S [21,22,26], whereas single-channel studies show that C_S strongly activates RyRs [25,27].

In the present study, we have further defined the interactions between C_S and skeletal RyRs (RyR1) in the presence of Ca^{2+}, Mg^{2+} and ATP, which are key physiological regulators of the RyR. Importantly, Mg^{2+} and ATP were present in previous experiments examining Ca^{2+} release from SR [21,22,26], but were not present in single-channel studies [25,27]. To characterize further the interaction with the RyR, we have compared the effects of C_S with those of related peptides, C_C (the cardiac C sequence), since

Abbreviations used: RyR, ryanodine receptor; DHPR, dihydropyridine receptor; DIDS, di-isothiocyanostilbene-2,2′-disulphonic acid; EC, excitation–contraction; SR, sarcoplasmic reticulum; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid.

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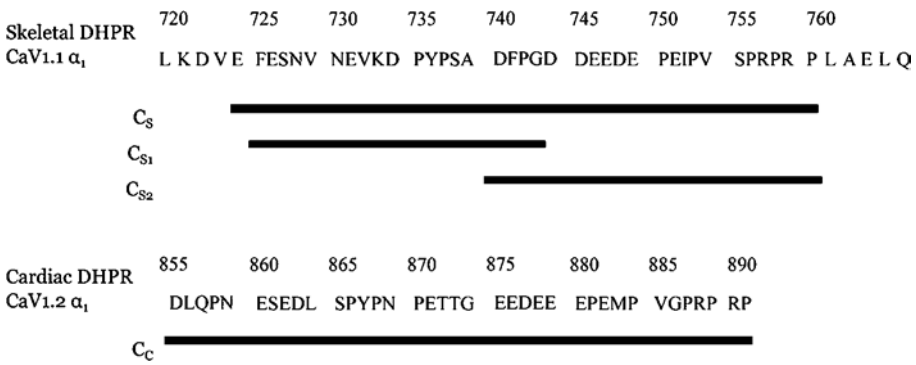

Figure 1 Sequences of the skeletal and cardiac DHPR C regions and the synthetic DHPR C-region peptides used in the present study

Note that several residues are given (double spaced) on either side of the skeletal C region used here to illustrate points made in the Discussion section.

replacement of the crucial region in the α_{1C} subunit with its skeletal counterpart restores skeletal EC coupling [19]. We also examined the two halves of C_S, the C_{S1} (^{725}Phe-Gly^{743}) and C_{S2} (^{740}Asp-Pro^{760}). C_{S2} is more potent ($K_i \sim 290\ \mu$M) compared with C_{S1} ($K_i \sim 3$ mM) in reversing the effects of A_S (30 μM) on Ca^{2+} release from SR vesicles, [^{3}H]ryanodine binding to RyRs and changes in MCA (4-methylcoumaryl-7-amide) fluorescence [22]. However, unlike C_{S2}, only the C_{S1} region is crucial for skeletal-type EC coupling in myocytes [19].

EXPERIMENTAL

Peptides

Peptides with the sequences shown in Figure 1 were synthesized as in [23].

Isolation of SR vesicles, lipid bilayers, solutions, single-channel recording and analysis

These techniques have been described previously in [28,29]. The *cis* (cytoplasmic) bath contained either 230 mM $CsCH_3O_3S$ + 20 mM CsCl + 10 mM TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid; pH 7.4 adjusted with CsOH) (250 mM Cs^+ solution) or 80 mM $CsCH_3O_3S$ and 20 mM CsCl, 10 mM TES (100 mM Cs^+ solution). The *trans* (luminal) solution contained either 30 mM $CsCH_3O_3S$ + 20 mM CsCl + 10 mM TES (50 mM Cs^+ solution) or 250 mM Cs^+ solution + 0.1–1.0 mM $CaCl_2$. In experiments where vesicle fusion was performed with *cis* and *trans* baths containing 250 mM Cs^+ solution, 500 mM mannitol was added to the *cis* bath to produce the osmotic gradient necessary for vesicle fusion. [Ca^{2+}] was determined using a Ca^{2+} electrode (Fluka, Rolconkoma, NY, U.S.A.). [Mg^{2+}] was estimated from the total amount of $MgCl_2$ using published association constants [30] and the program 'Bound and Determined' [31]. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.), ATP, $CsCH_3O_3S$, CsCl and $MgCl_2$ from Sigma, $CaCl_2$ from BDH Chemicals (Poole, Dorset, U.K.) and TES, CsOH and DIDS (di-isothiocyanostilbene-2,2′-disulphonic acid) from MP Biomedicals (Seven Hills, NSW, Australia).

Positive current is defined as flow of positive charge from cytoplasm to lumen (*cis* to *trans*). Electrical potential is expressed as $V_{cytoplasm} - V_{lumen}$ (i.e. $V_{cis} - V_{trans}$). Channel activity was recorded at +40 mV. Channels were exposed to different conditions by flowing solutions over the bilayer using the local perfusion system with an exchange time of 1–2 s, depending on the flow rate [32].

DIDS modification

In one experiment, RyRs were modified by DIDS to slow the gating kinetics of the channels. The RyRs were almost fully activated by 30–90 s exposure to 500 μM DIDS at 1 mM cytoplasmic Ca^{2+} ($P_0 \pm$ S.E.M. $= 0.94 \pm 0.01$, $n = 11$). Removal of DIDS decreased P_0 to approx. 0.8 because the reversible component of DIDS activation was removed [32].

Statistics

The means were weighted with the number of channels in the bilayer. The S.E.M. was determined as:

$$\sqrt{\left[\sum_i n_i(x_i - X)^2\right] \Big/ \left[N(N-1)\sum_i n_i\right]}$$

where N is the number of experiments, n_i the number of RyRs in the bilayer in the ith experiment, x_i the test value in the ith experiment and X the weighted mean of the test values x_i, where

$$X = \left[\sum_i (n_i x_i) \Big/ \sum_i (n_i)\right]$$

RESULTS

Activation of RyRs by 100 nM–1 μM DHPR C-region peptides

In the present study, RyRs were activated under control and test conditions either by cytoplasmic Ca^{2+} alone (100 μM) or by cytoplasmic ATP (2 mM) in the presence of free [Ca^{2+}] between 20 and 200 μM. It has previously been shown that C_S ($\leqslant 10\ \mu$M) activates RyRs at cytoplasmic [Ca^{2+}] over the range 1 nM–100 μM [27]. We show here that activation after adding peptide C_S to the cytoplasmic side of the RyR depended on the presence of ATP (Figure 2). In the absence of ATP, the addition of 1 μM C_S to the cytoplasmic side of the channels substantially increased RyR activity, whereas removal of peptide did not return P_0 to control levels within the time frame of the experiments (Figure 2A). In contrast, in the presence of ATP, RyRs had a $P_0 \sim 0.8$ and were not stimulated further by the addition of 1 μM C_S (Figure 2B).

RyR activation by the four peptides (C_S, C_{S1}, C_{S2} and C_C) was measured during rapid application of 100 nM peptide to bilayers by local perfusion for 30–90 s. These exposures were brief enough and at sufficiently low concentrations that the inhibitory effects of the peptides (see below) were minor. Peptides were removed by flowing control solution over the bilayer for 30–120 s. Under control conditions, in the presence of 100 μM cytoplasmic Ca^{2+},

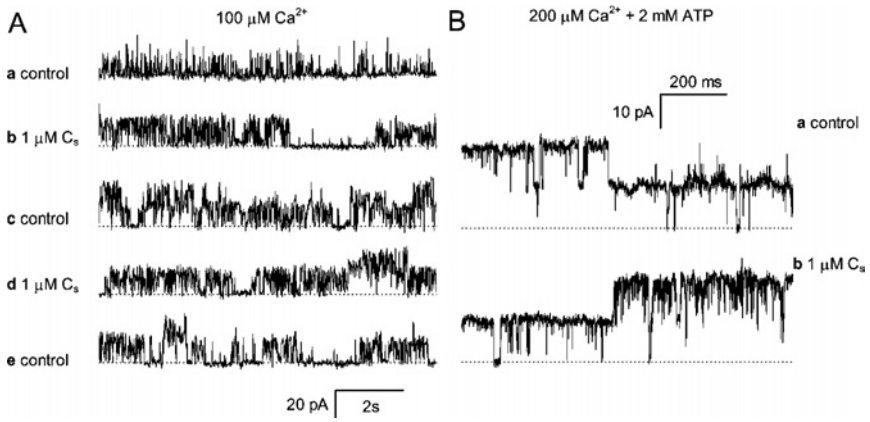

Figure 2 RyRs are activated by the DHPR C-region peptide C_S in the cytoplasmic bath

(**A**) Current traces in a single experiment on two RyRs. The current baselines are denoted by the broken lines and channel openings are seen as upward current transitions. Channels were recorded (**a**) in *cis*/*trans* 250 mM/50 mM Cs^+, 0.1 mM/1 mM Ca^{2+}, (**b**) during exposure to 1 μM C_S, (**c**) 15 s after removal of C_S from the solution, (**d**) during a second exposure to 1 μM C_S and (**e**) after removal again of the peptide. Reapplication of C_S produced no additional increase in channel activity. (**B**) Two RyRs are activated by 2 mM ATP + 0.5 mM Ca^{2+} (free $[Ca^{2+}] = 200$ μM) in the cytoplasmic bath (**a**) under control conditions and (**b**) during exposure to 1 μM C_S.

the RyRs had P_0 values of approx. 0.1. Application of each of the peptides increased channel activity with efficacies in the order $C_S > C_{S1} \sim C_{S2} > C_C$ (Figure 3A, black bars). Curiously, current levels marginally increased after the removal of peptides (Figure 3A, open bars).

The kinetics of RyR activation by 100 nM of each of the peptides was measured by compiling ensemble current traces from all experiments (Figure 3B). The exponential time constants for activation (τ_a) and their associated rate constants are given in Table 1. The activation time constant for C_{S2} was similar to the time of solution exchange and, thus, was a lower estimate of the actual activation rates. Activation by C_S, C_{S1} and C_C was significantly slower.

In the presence of ATP, C_S did not increase RyR activation (Figures 3A and 4). The results presented in Figure 4 show the concentration dependence of RyR activation by ATP in the presence and absence of C_S. In the absence of C_S (Figure 4, open circles), ATP (with 100 μM Ca^{2+}) increased P_0 from 0.19 ± 0.07 to 0.63 ± 0.14, whereas in the presence of C_S (Figure 4, filled circles), RyRs had P_0 of 0.4 ± 0.1 and ATP could increase this to 0.75 ± 0.11. Hill fits to the data showed that K_a for ATP activation was 400 μM both in the presence and absence of C_S.

Fast inhibition of RyRs is most apparent with $\geqslant$ 10 μM DHPR C-region peptides

Fast inhibition of RyRs described in this section has not been reported previously. The existence of fast inhibition was indicated by several previous observations. First, we noticed that application of $\geqslant 10$ μM C_S to RyRs caused less initial activation when compared with that induced by 100 nM peptide (e.g. Figure 3). Secondly, RyR activity increased when 100 nM or 1 μM of the peptides was removed (e.g. Figures 2A and 3A), indicating that activity was decreased in the presence of the peptide. In seven other experiments, application of 10 μM C_S increased P_0 from 0.03 ± 0.01 to 0.07 ± 0.02 and then P_0 increased further to 0.12 ± 0.03 on washout of C_S. These observations showed that RyRs were reversibly inhibited by C_S and that the inhibition masked peptide activation. To measure the concentration dependence of inhibition, RyRs were permanently (within the time frame of the experiment) stimulated at the beginning of each experiment by a brief (30 s) application of 10 μM C_S in the presence of 100 μM Ca^{2+}. When C_S was re-applied, P_0 decreased

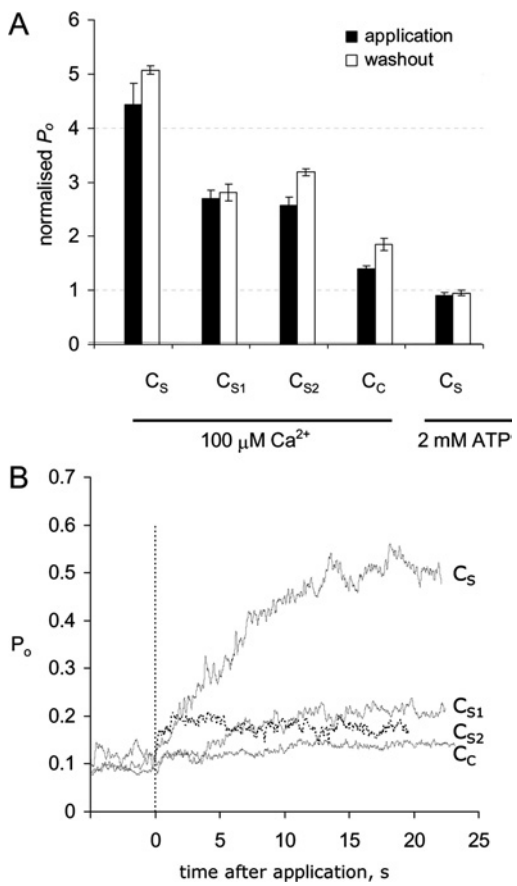

Figure 3 RyR activation by the DHPR C-region peptides C_S, C_{S1}, C_{S2} and C_C

(**A**) Channel activity is expressed as P_0. The degree of activation in response to peptide application (black bars) and peptide washout (open bars), normalized to control values before the addition of peptide. The four data groups on the left show the effect of 100 nM peptide in the presence of 100 μM Ca^{2+}. The data on the right show the effect of 1 μM C_S in the presence of 2 mM ATP + 0.5 mM Ca^{2+} (free $[Ca^{2+}] = 200$ μM). Control values of P_0 and the number of experiments are given in Table 1. (**B**) Time course of RyR activation. Ensemble traces were compiled from the RyR current summed from all experiments. Initially, RyRs were partially activated in the presence of 100 μM cytoplasmic Ca^{2+} with P_0 values of approx. 0.1. The peptide was applied at zero time for 20–30 s. The exponential time constants, activation rates and number of experiments are given in Table 1.

Table 1 Activation time constants (τ_a) for the DHPR C-region peptides

Values of τ_a were obtained from exponential fits to the current-activation time courses shown in Figure 3. P_{min} is the mean P_0 before peptide application. N is the total number of bilayer experiments and n the total number of channels summed for each current time course.

	P_{min}	N	n	τ_a (s)	k_{on} ($M^{-1} \cdot s^{-1}$)
C_S	0.12	4	88	7.63 ± 0.25	$(13.1 \pm 0.4) \times 10^5$
C_C	0.09	9	375	4.7 ± 0.07	$(21 \pm 0.3) \times 10^5$
C_{S1}	0.08	10	216	3.80 ± 0.15	$(26 \pm 1) \times 10^5$
C_{S2}	0.07	6	80	0.92 ± 0.05	$(10.9 \pm 0.6) \times 10^6$

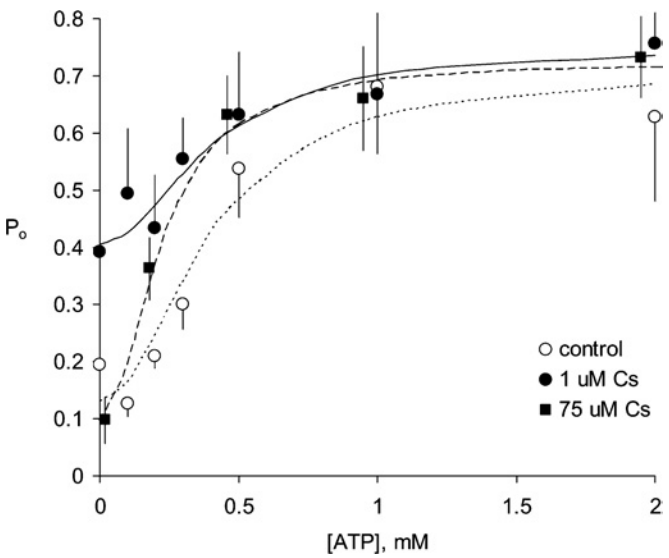

Figure 4 Activation of RyRs in the presence and absence of C_S

ATP (2 mM) alone (○, $N = 6$), ATP + 1 μM C_S (●, $N = 4$), ATP + 75 μM C_S (■, $N = 4$). The curves show Hill fits (Hill coefficient = 2) to each data set. The fitted Hill parameters are: (· · · · · ·), $K_a = 0.4$ mM, $P_{max} = 0.71$, $P_{min} = 0.13$; (——), $K_a = 0.4$ mM, $P_{max} = 0.75$ and $P_{min} = 0.41$; and (- - - -), $K_a = 0.23$ mM, $P_{max} = 0.72$ and $P_{min} = 0.1$. The baths contained (*cis*/*trans*) 250 mM/50 mM Cs^+ and 0.1 mM/1 mM Ca^{2+} (free).

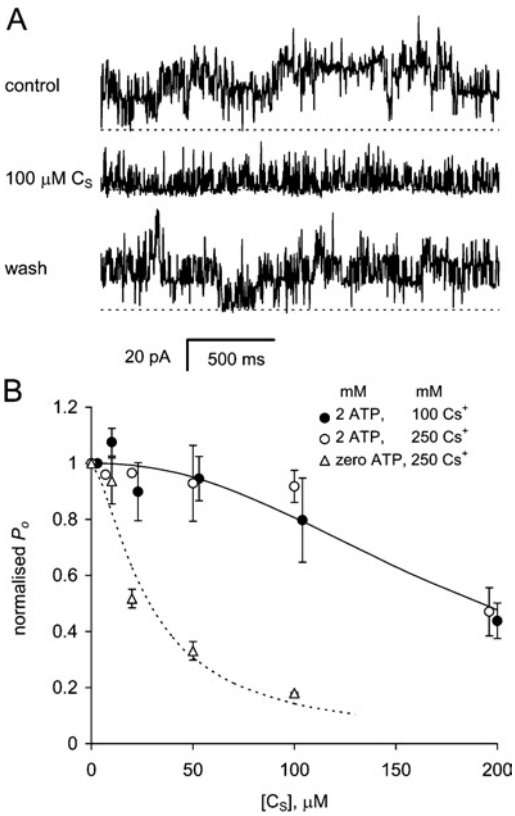

Figure 5 Fast inhibition of RyRs by the DHPR C-region peptide C_S

(**A**) The gating of four RyRs are recorded at + 40 mV. Channel activity during application and withdrawal of 100 μM C_S reveals reversible peptide-induced inhibition. Before the control recording, the channels were exposed to 10 μM C_S for 30 s so that the channels were activated by the peptide in the control and test situations. The broken line denotes the current baselines in each record. The baths contained (*cis*/*trans*) 250 mM/50 mM Cs^+ and 0.1 mM/0.1 mM Ca^{2+}. (**B**) Concentration dependences of RyR inhibition by C_S, normalized to control values under three experimental conditions. Cytoplasmic solutions contained 250 mM Cs^+ and 0.1 mM Ca^{2+} (△; P_0 for control = 0.24 ± 0.05, $N = 5$); 100 mM Cs^+ and 2 mM ATP, 0.1 mM Ca^{2+} (free $[Ca^{2+}] = 40$ μM) (○; P_0 for control = 0.74 ± 0.13, $N = 5$); or 250 mM Cs^+, 2 mM ATP, 0.1 mM Ca^{2+} (free $[Ca^{2+}] = 20$ μM) (●; P_0 for control = 0.42 ± 0.11, $N = 3$). Curves show Hill fits to the data with $K_i = 28$ mM and $n_i = 1.5$ (· · · · · ·) and $K_i = 190$ μM and $n_i = 2.2$ (——).

in a concentration-dependent manner (Figure 5B, triangles) with $K_i = 28$ mM and $n_i = 1.5$. When the same method was used in the presence of ATP, RyRs were inhibited by C_S with a lower affinity, $K_i = 190$ μM and $n_i = 2.2$ (Figure 5B, circles), in 100 or 250 mM Cs^+ solutions. Figure 4 shows that, in the absence of ATP, RyRs are inhibited by 75 μM C_S compared with 1 μM C_S and that this inhibition is markedly alleviated by [ATP] as low as 0.2 mM.

In an effort to define the kinetics of inhibition more clearly, we used channels whose gating kinetics were slowed by DIDS modification (see the Experimental section). DIDS-modified RyRs had a lower frequency of channel closures that made it easier to distinguish the closing events due to peptide inhibition (Figure 6A). The DIDS-modified RyRs were exposed to cycles of peptide application (10–60 s) and washout (20–90 s). Peptide inhibition occurred within 15–30 s of peptide application (Figures 6A and 6B). Peptide inhibition was fully reversed within 20–60 s of washout. Rates of RyR inhibition and recovery were determined from exponential fits to the current response. The apparent affinities for the DHPR C-region peptides, calculated from the on- and off-rates of inhibition, are given in Table 2. C_S and C_C (50 μM) each produced similar inhibition of DIDS-modified RyRs (Figure 6C). Peptides C_{S1} and C_{S2} at 50 μM were much weaker inhibitors than C_S or C_C. C_{S2} was a more potent inhibitor than C_{S1} at 150 μM.

Slow inhibition by 50–150 μM DHPR C-region peptides

The C peptides also induced a very slow low-affinity inhibition. It was shown previously that when C_S was applied to RyRs in bilayers at sufficiently high concentrations (50–150 μM) for several minutes, it produced channel closure that can take several minutes to reverse, if at all [27]. In the present study, we report that C_{S1}, C_{S2} and C_C at 50–150 μM induced a similar inhibition. C_{S1} inhibited 9/13 channels within 30–420 s, C_{S2} inhibited 3/9 channels within 60–260 s and C_C inhibited 2/7 channels within approx. 40 s.

Ca^{2+}/Mg^{2+} inhibition of RyRs is modified by 25–50 μM DHPR C-region peptides

Inhibition of RyRs by cytoplasmic Mg^{2+} is an important regulator of Ca^{2+} release in muscles. We have shown that Mg^{2+} inhibits RyRs by binding to either the Ca^{2+}-activation sites (*A*-sites) or to low-affinity sites (*I*-sites) where Ca^{2+}, Mg^{2+} and other bivalent cations can bind and inhibit the channel [33,34]. The effect of DHPR C-region peptides on Ca^{2+}/Mg^{2+} inhibition of RyRs was

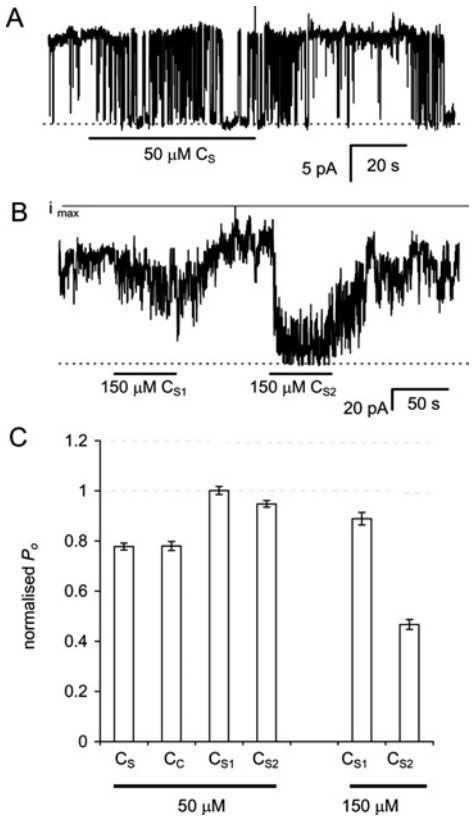

Figure 6 Fast inhibition of DIDS-modified RyRs by DHPR C-region peptides

(**A**) The gating of a single, DIDS-modified RyR during application and withdrawal of 50 μM C_S under conditions described in Figure 5(A). Baselines are shown by the broken line. (**B**) Inhibition of seven DIDS-modified RyRs in one bilayer subjected to application and removal of 150 μM peptides C_{S1} and C_{S2}. The solid line labelled 'i_{max}' denotes the current when all channels are open. (**C**) DIDS-modified RyRs inhibited by DHPR C-region peptides. Data are normalized to P_0 values in the absence of a peptide. The control values and number of experiments are given in Table 2.

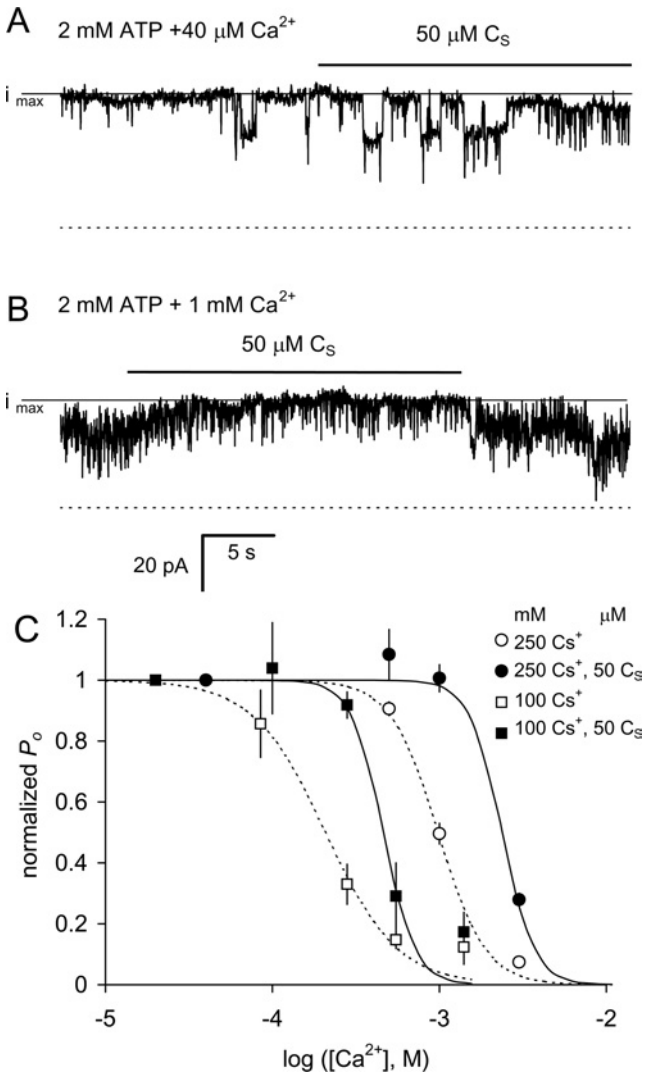

Figure 7 Alleviation of Ca^{2+} inhibition by C_S

(**A**, **B**) The gating of three RyRs recorded at + 40 mV. The cytoplasmic solution contained 250 mM Cs^+ and 2 mM ATP and the indicated free [Ca^{2+}]. In these experiments, luminal [Ca^{2+}] = 1 mM. (**A**) Channel activity in 40 μM Ca^{2+} is slightly inhibited by application of 50 μM C_S (note the increased frequency of short closures). (**B**) The presence of 1 mM Ca^{2+} has two effects on channel activity. It slightly decreased the channel conductance by partially blocking the Cs^+ flow and decreased the open probability of channel opening (the latter being Ca^{2+} inhibition). During application of 50 μM C_S in the presence of 1 mM Ca^{2+}, channel activity reversibly increased as a result of decreased Ca^{2+} inhibition. The broken lines denote the current baselines in each record and the solid lines labelled 'i_{max}' denote the current when all channels are open. (**C**) Ca^{2+} inhibition normalized to P_0 at 10–40 μM Ca^{2+} in the presence of 250 mM Cs^+ (○, ●) and 100 mM Cs^+ (□, ■) and in the absence (○, □) or presence of 50 μM C_S (●, ■). The data are the mean P_0. The Hill fit parameters for all the experiments, including normalizing values at low [Ca^{2+}] and [Mg^{2+}], are given in Table 3.

measured to determine whether the peptides could mimic the change in the bivalent ion affinity of *I*-sites, which is so integral to EC coupling (see the Introduction section). To avoid effects of high-affinity peptide activation, RyRs were subjected at the start of each experiment to two cycles of exposure to peptides and washout to ensure that the channels were permanently activated by the peptide. To measure inhibition at the *I*-sites, RyRs were locally perfused with solutions containing various [Ca^{2+}] or [Mg^{2+}] in the presence of 2 mM ATP. Ca^{2+} and Mg^{2+} concentration dependences were obtained from the peptide-modified RyRs, both in the presence and absence of a peptide.

Figure 7 shows that application of C_S rapidly and reversibly alleviates Ca^{2+} inhibition of RyRs in the presence of ATP. C_S

Table 2 Time constants for inhibition (τ_i) and recovery (τ_r) and the associated reaction rates k_{on} and k_{off} for the DHPR C-region peptides on DIDS-modified RyRs

P_{max} is the value of P_0 in the absence of peptide and *N* the total number of experiments and *n* the total number of channels over which the averages for τ were taken.

	P_{max}	*N*	*n*	τ_i (s)	k_{on} ($M^{-1} \cdot s^{-1}$)	*N*	*n*	τ_r (s)	k_{off} (s^{-1})	K_i (μM) (k_{off}/k_{on})
50 μM C_S	0.85 ± 0.01	14	923	3.3 ± 0.2	(6.1 ± 0.4) × 10^3	13	809	12.9 ± 0.7	(7.8 ± 0.1) × 10^{-2}	13
50 μM C_C	0.87 ± 0.02	8	488	2.4 ± 0.2	(8.3 ± 0.7) × 10^3	8	488	23.4 ± 2.2	(4.3 ± 0.4) × 10^{-2}	5.2
150 μM C_{S1}	0.81 ± 0.02	7	76	1.0 ± 0.1	(6.7 ± 0.7) × 10^3	4	48	3.6 ± 0.3	(2.8 ± 0.2) × 10^{-1}	42
150 μM C_{S2}	0.79 ± 0.02	5	67	3.5 ± 0.2	(1.9 ± 0.1) × 10^3	4	60	10.2 ± 0.7	(9.8 ± 0.7) × 10^{-2}	52

Table 3 Summary of the parameters of Hill fits to the [Ca^{2+}] and [Mg^{2+}] dependences of P_0 in the absence and presence of DHPR C-region peptides

P_{max} is the P_0 at low bivalent-ion concentration, K_i the half-inhibitory concentration of X^{2+} and n_i the Hill coefficient. N is the number of bilayers and n the number of channels measured.

DHPR C-region peptide	Experimental conditions	X^{2+}	P_{max}	K_i (μM)	n_i	N	n
–	250 mM Cs^+, 2 mM ATP	Ca^{2+}	0.86 ± 0.03	980 ± 055	3.0 ± 0.6	5	51
50 μM C_S	250 mM Cs^+, 2 mM ATP	Ca^{2+}	0.84 ± 0.06	2300 ± 400	2.6 ± 1.1	4	31
–	100 mM Cs^+, 2 mM ATP	Ca^{2+}	0.73 ± 0.06	210 ± 037	2.0 ± 0.5	3	40
50 μM C_S	100 mM Cs^+, 2 mM ATP	Ca^{2+}	0.49 ± 0.05	460 ± 071	4.5 ± 2.8	3	40
–	100 mM Cs^+, 2 mM ATP	Mg^{2+}	0.69 ± 0.07	250 ± 40	2.3 ± 0.7	5	70
50 μM C_S	100 mM Cs^+, 2 mM ATP	Mg^{2+}	0.46 ± 0.07	420 ± 110	2.7 ± 2.0	6	65
25 μM C_{S1}	100 mM Cs^+, 2 mM ATP	Mg^{2+}	0.58 ± 0.06	570 ± 120	1.7 ± 1.4	4	75
25 μM C_{S2}	100 mM Cs^+, 2 mM ATP	Mg^{2+}	0.62 ± 0.07	350 ± 41	3.2 ± 2.0	4	12
*Native	250 mM Cs^+	Ca^{2+}		710 ± 80		18	
*MHS	250 mM Cs^+	Ca^{2+}		1160 ± 220		13	
*Native	100 mM Cs^+	Ca^{2+}		204 ± 32		18	
*MHS	100 mM Cs^+	Ca^{2+}		957 ± 250		9	

* Native and MHS refer to normal and malignant hyperthermia-susceptible RyRs respectively from pig skeletal muscles [39].

caused a small decrease in activity of channels that were not Ca^{2+}-inhibited [i.e. when the cytoplasmic [Ca^{2+}] was 40 μM (Figure 7A)]. Increasing cytoplasmic [Ca^{2+}] to 1 mM resulted in a decrease in P_0 due to Ca^{2+} inhibition at the *I*-sites (Figure 7B). Addition of C_S to the inhibited channels caused a rapid increase in the current, which decreased as soon as the peptide was removed. The presence of C_S (50 μM) decreased the sensitivity of RyRs to inhibition by Ca^{2+} under high (250 mM) and low (100 mM) ionic strength conditions (Figure 7C). The half-inhibitory concentration K_i and Hill coefficient n_i for Ca^{2+} and Mg^{2+} are given in Table 3. The K_i for *I*-site inhibition is shifted to higher bivalent cation concentrations when the ionic strength is higher (Figure 7C and [35]). This ionic strength dependence was retained in the presence of C_S.

Similarly, DHPR C-region peptides decreased RyR sensitivity to Mg^{2+} inhibition. Mg^{2+} inhibition was measured with 10–40 μM free cytoplasmic Ca^{2+} to saturate the *A*-sites and ensure that inhibition was mainly due to Mg^{2+} binding to the low-affinity *I*-sites [33]. Figure 8 shows the effect of DHPR C-region peptides on Mg^{2+} inhibition at low ionic strength. C_S (50 μM) caused a decrease in RyR activity in the absence of Mg^{2+} (Figure 8A). In the presence of 0.3 mM Mg^{2+}, the peptide addition had a stimulating effect (Figure 8B). Comparison of these records indicates that C_S decreased the amount of Mg^{2+} inhibition in these channels (Figure 8C). Experiments using 25 μM C_{S1} and C_{S2} also showed that these peptides shifted Mg^{2+} inhibition to higher concentrations (Figure 8C and Table 3). After washout of these peptides, the K_i values for Ca^{2+} and Mg^{2+} inhibition at high and low ionic strengths were similar to those seen previously in native RyRs (Table 3), indicating that Ca^{2+}/Mg^{2+} inhibition was not altered during high-affinity activation by C_S.

DISCUSSION

In the present study, we find that regulation of RyRs by DHPR C-region peptides is strongly dependent on the cytoplasmic Ca^{2+}, Mg^{2+} and ATP. This dependence can explain previous differences between the effects of the peptides on Ca^{2+} release from SR and on single-channel activity. A significant finding was that the DHPR C-region peptides decreased the apparent affinity of the RyR for Mg^{2+} inhibition. This is a particularly important result since the C region of the DHPR II–III loop is the determinate of skeletal-type EC coupling [2], a process that is proposed to activate RyRs by relieving their Mg^{2+} inhibition [13]. The experiments revealed other novel effects of these peptides on RyR activity. In the present study, the inhibition of RyRs by C_S seen previously [27] is shown to be due to two mechanisms that are clearly distinguished by their kinetics. One mechanism produces inhibition with onset and recovery times of minutes after peptide application and removal. On the time scale of the bilayer experiments, this inhibition process is non-reversible. The other mechanism causes reversible inhibition with onset and recovery times of seconds. Fast inhibition occurs at the same time as, and partially masks, a slowly reversible activation process and both effects are substantially decreased in the presence of ATP.

A DHPR II–III loop peptide relieves Ca^{2+}/Mg^{2+} inhibition

The ability of the C-region peptide to decrease Mg^{2+} inhibition reveals a tantalizing parallel between the actions of the peptide and changes in RyR activity during EC coupling in skeletal muscles. It is surprising that the mere addition of the C_S section of the DHPR II–III loop to the RyR in bilayers can mimic an effect of the DHPR on RyRs during EC coupling. The 2-fold decrease in RyR affinity for Mg^{2+} caused by C_S is much smaller than the 10–100-fold decrease that occurs during EC coupling in skinned muscle fibres [36] and in triad preparations [37]. However, the effect of C_S indicates that, in this very different isolated system, there is a loose interaction between the DHPR C-region peptides and the RyR, which can affect Ca^{2+}/Mg^{2+} inhibition. Such an interaction is consistent with a recent finding by Gallant [38] that the $Arg^{615} \rightarrow Cys$ mutation in pig skeletal RyRs not only decreases the apparent affinity of the RyR for Ca^{2+} and Mg^{2+} inhibition [39] but also decreases the activating effect of C_S. Although the $Arg^{615} \rightarrow Cys$ mutation and C_S both relieve Ca^{2+}/Mg^{2+} inhibition, the peptide and the mutation differ in their ionic strength dependences. The $Arg^{615} \rightarrow Cys$ mutation has a larger effect on Ca^{2+}/Mg^{2+} inhibition at low ionic strength (Table 3), whereas C_S has the same effects both at high and low ionic strength.

Different peptide mechanisms are revealed by different experimental conditions

Effects of C-region peptides depend on the type of RyR agonists present. A slowly reversible activation by C_S is seen when RyRs are activated by Ca^{2+} alone (without ATP; e.g. Figure 3). In the presence of ATP, this type of activation is absent. However, if Mg^{2+} is also present, C_S can reversibly activate RyRs by decreasing their sensitivity to Mg^{2+} inhibition (e.g. Figure 8). These findings can reconcile disparate reports in the literature about the effect

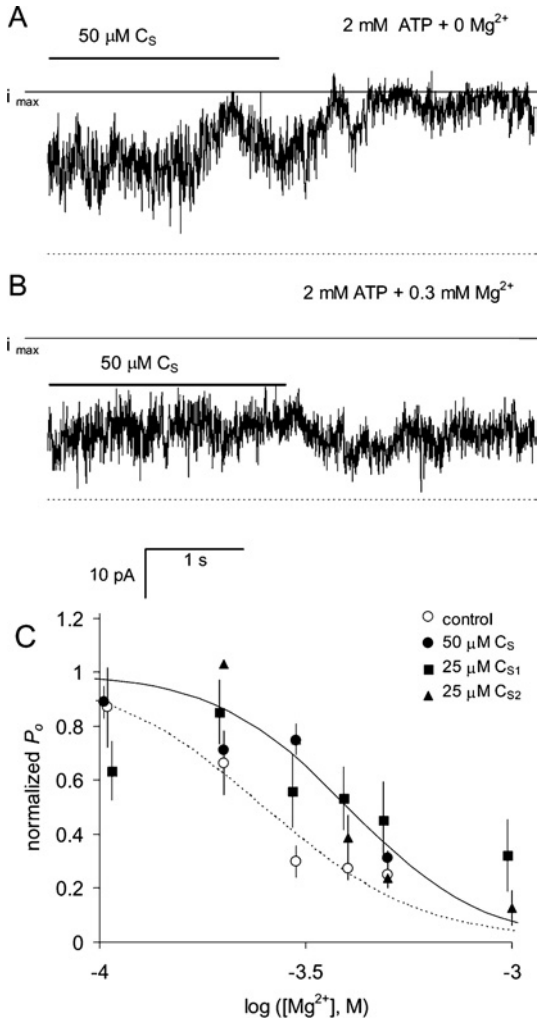

Figure 8 Alleviation of Mg^{2+} inhibition by DHPR C-region peptides

(**A**, **B**) The gating of five RyRs recorded at + 40 mV. The cytoplasmic solution contained 100 mM Cs^+ and 2 mM ATP and the indicated [Mg^{2+}] and luminal [Ca^{2+}] = 1 mM. (**A**) Channel activity in 10 μM Ca^{2+} is partially inhibited in the presence of 50 μM C_S. (**B**) In the presence of 0.3 mM Mg^{2+}, 50 μM C_S slightly increased channel activity. Comparison of the traces in (**A**, **B**) shows that Mg^{2+} produced less inhibition in the presence of C_S. The broken lines denote the current baselines in each record and the solid lines labelled 'i_{max}' denote the current when all channels are open. (**C**) Mg^{2+} inhibition in 100 mM Cs^+ in the presence and absence of peptides. Data are normalized to P_0 at 10 μM Ca^{2+} and zero Mg^{2+}. In the presence of peptides (filled symbols), RyRs are less sensitive to bivalent ion inhibition. The broken and solid curves are Hill fits to ○ and ● respectively. The Hill fit parameters for all the experiments, including normalizing values at low [Ca^{2+}] and [Mg^{2+}], are given in Table 3.

of these peptides on RyR1. This and other single-channel studies [25,27] show that RyR activation by peptide C_S is much stronger and occurs with higher affinity than that revealed by measurements of Ca^{2+} release from SR vesicles where $K_a > 10$ μM (C. S. Haarmann, A. F. Dulhunty and D. R. Laver, unpublished work; [21,22,26]). So far, single-channel studies have measured the effects of C_S under conditions where Ca^{2+} is the sole agonist, whereas Ca^{2+} release experiments were performed in the presence of ATP and Mg^{2+} since these ligands are required for vesicle loading. Therefore C_S-induced activation of SR Ca^{2+} release could be due to the relief of Mg^{2+} inhibition, which is a mechanism different from the high-affinity activation seen with Ca^{2+} alone. In the present study, we show that C_{S2} is a stronger and faster inhibitor of RyR than C_{S1}, which may also explain why C_{S2} is more potent than C_{S1} in reversing the activation by A_S [22].

C_S has no effect on Ca^{2+} release in skinned fibres but decreases depolarization-induced Ca^{2+} release, suggesting that C_S competes with the II–III loop for its activation site on the RyR [24]. It is unlikely that fast inhibition by C_S could occur in these skinned fibre experiments due to the presence of ATP in the cytoplasmic bathing solution. It is intriguing that reversible inhibition is regulated by the physiological agonist ATP. Physiologically, reversible inhibition in the absence of ATP and its abolition in the presence of ATP could contribute to the decrease in Ca^{2+} release seen during fatigue where ATP levels are decreased.

Activation by C_S (^{724}Glu-Pro^{760}) in the present study appears to differ from that seen with a similar peptide (^{720}Leu-Glu^{765}) [25]. C_S activates with $K_a < 100$ nM and is effectively non-reversible, whereas activation by residues ^{720}Leu-Glu^{765} had $K_a \sim 10$ μM and is readily reversible. The additional amino acids at each end of C_S (LKDV and PLAEL; Figure 1) appear to decrease significantly its binding affinity. This is not surprising given the frequency of charged amino acids near the ends of C_S and in additional amino acids in ^{720}Leu-Glu^{765}. In a parallel situation, a significant loss of activity is seen when the predominantly positively charged peptide A_S (^{671}Thr-Leu^{690}) is modified by the addition of residues 691–710, which contain several negative charges [40].

DHPR C region in EC coupling

Two requirements for changing cardiac to skeletal EC coupling in *dysgenic* myotubes expressing CaV1.2 are the localization of DHPRs opposite every other RyR and the activation of RyRs via an external Ca^{2+}-independent interaction with the DHPR. Both these requirements are fulfilled when the C-region sequence is skeletal, in an otherwise cardiac context [2,19,41]. Some effects of C-region peptides are consistent with interactions between DHPRs and RyRs that may contribute to skeletal EC coupling. First, the localizing interaction is expected to be of high affinity and specific for the skeletal C region. Indeed, RyR activation by C_S is greatest with the skeletal DHPR peptides that bind and activate with high affinity and have low off rates. Secondly, the activating interaction in EC coupling might be the desensitization of RyRs to Mg^{2+} inhibition [36]. Although the latter effect is not specific for the DHPR isoform, isoform specificity may depend on co-localization of DHPRs and RyRs (via the DHPR C region or by other regions that may, or may not, be conserved between CaV1.1 and CaV1.2). There is a further parallel between our results and EC coupling studies. We find that activation by C_S is stronger than that by either C_{S1} or C_{S2}. This is consistent with a stronger EC coupling if the whole C region is of skeletal sequence compared with the situation when C_{S1} is skeletal and C_{S2} is cardiac [19]. There appears to be a lack of C-region isoform specificity in fast inhibition, which indicates that both cardiac and skeletal DHPRs and RyRs could interact physically with each other if they were appropriately targeted. This appropriate targeting appears to be dependent on the C_{S1} region [19].

In conclusion, the action of these peptides critically depends on the presence of the key physiological regulators, Ca^{2+}, Mg^{2+} and ATP, so that peptide effects on isolated RyRs might not equate to their action in muscle but rather provide pointers to mechanisms underlying EC coupling. The manifold effects of the peptides do suggest that the C region of the DHPR is capable of a complex influence over RyR activity in resting muscles and during EC coupling.

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REFERENCES

1 Knudson, C. M., Chaudhari, N., Sharp, A. H., Powell, J. A., Beam, K. G. and Campbell, K. P. (1989) Specific absence of the alpha 1 subunit of the dihydropyridine receptor in mice with muscular dysgenesis. J. Biol. Chem. **264**, 1345–1348

2 Tanabe, T., Beam, K. G., Adams, B. A., Niidome, T. and Numa, S. (1990) Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. Nature (London) **346**, 567–569

3 Tanabe, T., Beam, K. G., Powell, J. A. and Numa, S. (1988) Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. Nature (London) **336**, 134–139

4 Adams, B. A. and Beam, K. G. (1990) Muscular dysgenesis in mice: a model system for studying excitation-contraction coupling. FASEB J. **4**, 2809–2816

5 Armstrong, C. M., Bezanilla, F. M. and Horowicz, P. (1972) Twitches in the presence of ethylene glycol bis(-aminoethyl ether)-N,N′-tetracetic acid. Biochim. Biophys. Acta **267**, 605–608

6 Fabiato, A. (1985) Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. J. Gen. Physiol. **85**, 291–320

7 Nabauer, M., Callewaert, G., Cleemann, L. and Morad, M. (1989) Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. Science **244**, 800–803

8 Beuckelmann, D. J. and Wier, W. G. (1988) Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. J. Physiol. (Cambridge, U.K.) **405**, 233–255

9 Schneider, M. F. and Chandler, W. K. (1973) Voltage dependent charge movement of skeletal muscle: a possible step in excitation-contraction coupling. Nature (London) **242**, 244–246

10 Lamb, G. D. (2002) Voltage-sensor control of Ca^{2+} release in skeletal muscle: insights from skinned fibers. Front. Biosci. **7**, d834–d842

11 Smith, J. S., Coronado, R. and Meissner, G. (1986) Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+}. J. Gen. Physiol. **88**, 573–588

12 Laver, D. R., Lenz, G. K. and Lamb, G. D. (2001) Regulation of the calcium release channel from rabbit skeletal muscle by the nucleotides ATP, AMP, IMP and adenosine. J. Physiol. (Lond.) **537**, 763–778

13 Lamb, G. D. and Stephenson, D. G. (1992) Importance of Mg^{2+} in excitation-contraction coupling in skeletal muscle. News Physiol. Sci. **7**, 270–274

14 Lamb, G. D., Recupero, E. and Stephenson, D. G. (1992) Effect of myoplasmic pH on excitation-contraction coupling in skeletal muscle fibres of the toad. J. Physiol. (Lond.) **448**, 211–224

15 Blazev, R. and Lamb, G. D. (1999) Low [ATP] and elevated [Mg^{2+}] reduce depolarization-induced Ca^{2+} release in rat skinned skeletal muscle fibres. J. Physiol. (Lond.) **520**, 203–215

16 Anderson, K. and Meissner, G. (1995) T-tubule depolarization-induced SR Ca^{2+} release is controlled by dihydropyridine receptor- and Ca^{2+}-dependent mechanisms in cell homogenates from rabbit skeletal muscle. J. Gen. Physiol. **105**, 363–383

17 Lamb, G. D., Junankar, P. R. and Stephenson, D. G. (1995) Raised intracellular [Ca^{2+}] abolishes excitation-contraction coupling in skeletal muscle fibres of rat and toad. J. Physiol. (Lond.) **498**, 349–362

18 Chaudhari, N. (1992) A single nucleotide deletion in the skeletal muscle-specific calcium channel transcript of muscular dysgenesis (mdg) mice. J. Biol. Chem. **267**, 25636–25639

19 Nakai, J., Tanabe, T., Konno, T., Adams, B. and Beam, K. G. (1998) Localization in the II-III loop of the dihydropyridine receptor of a sequence critical for excitation-contraction coupling. J. Biol. Chem. **273**, 24983–24986

20 Lu, X., Xu, L. and Meissner, G. (1994) Activation of the skeletal muscle calcium release channel by a cytoplasmic loop of the dihydropyridine receptor. J. Biol. Chem. **269**, 6511–6516

21 El-Hayek, R., Antoniu, B., Wang, J., Hamilton, S. L. and Ikemoto, N. (1995) Identification of calcium release-triggering and blocking regions of the II-III loop of the skeletal muscle dihydropyridine receptor. J. Biol. Chem. **270**, 22116–22118

22 Saiki, Y., El-Hayek, R. and Ikemoto, N. (1999) Involvement of the Glu724-Pro760 region of the dihydropyridine receptor II-III loop in skeletal muscle-type excitation-contraction coupling. J. Biol. Chem. **274**, 7825–7832

23 Dulhunty, A. F., Laver, D. R., Gallant, E. M., Casarotto, M. G., Pace, S. M. and Curtis, S. (1999) Activation and inhibition of skeletal RyR channels by a part of the skeletal DHPR II-III loop: effects of DHPR Ser687 and FKBP12. Biophys. J. **77**, 189–203

24 Lamb, G. D., El-Hayek, R., Ikemoto, N. and Stephenson, D. G. (2000) Effects of dihydropyridine receptor II-III loop peptides on Ca^{2+} release in skinned skeletal muscle fibers. Am. J. Physiol. **279**, C891–C905

25 Stange, M., Tripathy, A. and Meissner, G. (2001) Two domains in dihydropyridine receptor activate the skeletal muscle Ca^{2+} release channel. Biophys. J. **81**, 1419–1429

26 Yamamoto, T., Rodriguez, J. and Ikemoto, N. (2002) Ca^{2+}-dependent dual functions of peptide C. The peptide corresponding to the Glu724-Pro760 region (the so-called determinant of excitation-contraction coupling) of the dihydropyridine receptor alpha 1 subunit II-III loop. J. Biol. Chem. **277**, 993–1001

27 Haarmann, C. S., Green, D., Casarotto, M. G., Laver, D. R. and Dulhunty, A. F. (2003) The random-coil 'C' fragment of the dihydropyridine receptor II-III loop can activate or inhibit native skeletal ryanodine receptors. Biochem. J. **372**, 305–316

28 Ahern, G. P., Junankar, P. R. and Dulhunty, A. F. (1994) Single channel activity of the ryanodine receptor calcium release channel is modulated by FK-506. FEBS Lett. **352**, 369–374

29 Laver, D. R., Roden, L. D., Ahern, G. P., Eager, K. R., Junankar, P. R. and Dulhunty, A. F. (1995) Cytoplasmic Ca^{2+} inhibits the ryanodine receptor from cardiac muscle. J. Membr. Biol. **147**, 7–22

30 Marks, P. W. and Maxfield, F. R. (1991) Preparation of solutions with free calcium concentration in the nanomolar range using 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid. Anal. Biochem. **193**, 61–71

31 Brooks, S. P. and Storey, K. B. (1992) Bound and determined: a computer program for making buffers of defined ion concentrations. Anal. Biochem. **201**, 119–126

32 O'Neill, E. R., Sakowska, M. M. and Laver, D. R. (2003) Regulation of the calcium release channel from skeletal muscle by Suramin and the disulfonated Stilbene derivatives DIDS, DBDS, and DNDS. Biophys. J. **84**, 1674–1689

33 Laver, D. R., Baynes, T. M. and Dulhunty, A. F. (1997) Magnesium inhibition of ryanodine-receptor calcium channels: evidence for two independent mechanisms. J. Membr. Biol. **156**, 213–229

34 Meissner, G., Darling, E. and Eveleth, J. (1986) Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. Biochemistry **25**, 236–244

35 Shomer, N. H., Louis, C. F., Fill, M., Litterer, L. A. and Mickelson, J. R. (1993) Reconstitution of abnormalities in the malignant hyperthermia-susceptible pig ryanodine receptor. Am. J. Physiol. **264**, C125–C135

36 Lamb, G. D. and Stephenson, D. G. (1991) Effect of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibres of the toad. J. Physiol. (Cambridge, U.K.) **434**, 507–528

37 Ritucci, N. A. and Corbett, A. M. (1995) Effect of Mg^{2+} and ATP on depolarization-induced Ca^{2+} release in isolated triads. Am. J. Physiol. **269**, C85–C95

38 Gallant, E. M., Hart, J., Eager, K., Curtis, S. and Dulhunty, A. F. (2004) Caffeine sensitivity of native RyR channels from normal and malignant hyperthermic pigs: effects of a DHPR II-III loop peptide. Am. J. Physiol. **286**, C821–C830

39 Laver, D. R., Owen, V. J., Junankar, P. R., Taske, N. L., Dulhunty, A. F. and Lamb, G. D. (1997) Reduced inhibitory effect of Mg^{2+} on ryanodine receptor-Ca^{2+} release channels in malignant hyperthermia. Biophys. J. **73**, 1913–1924

40 Casarotto, M. G., Green, D., Pace, S., Young, J. and Dulhunty, A. F. (2004) Activating the ryanodine receptor with dihydropyridine receptor II-III loop segments: size and charge do matter. Front. Biosci. **9**, 2860–2872

41 Grabner, M., Dirksen, R. T., Suda, N. and Beam, K. G. (1999) The II-III loop of the skeletal muscle dihydropyridine receptor is responsible for the bi-directional coupling with the ryanodine receptor. J. Biol. Chem. **274**, 21913–21919

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