Calmodulin sensitivity of the sarcoplasmic reticulum ryanodine receptor from normal and malignant-hyperthermia-susceptible muscle

Sean O'DRISCOLL*†, Tommie V. McCARTHY†, Hans M. EICHINGER‡, Wolf ERHARDT§, Frank LEHMANN-HORN* and Annegret HERRMANN-FRANK*‖

*Department of Applied Physiology, University of Ulm, D-89069 Ulm, Germany, †Department of Biochemistry, University College Cork, Ireland, ‡Experimental Station Thalhausen, Technical University of Munich, D-85402 Kranzberg, Germany, and §Institute of Experimental Surgery, Technical University of Munich, D-81675 Munich, Germany

Ca^{2+} release from sarcoplasmic reticulum (SR) of malignant-hyperthermia-susceptible (MHS) muscle is hypersensitive to Ca^{2+} and caffeine. To determine if an abnormal calmodulin (CaM) regulation of the SR Ca^{2+}-release-channel–ryanodine-receptor complex (RYR1) contributes to this hypersensitivity, we investigated the effect of CaM on high-affinity [^{3}H]ryanodine binding to isolated SR vesicles from normal and MHS pig skeletal muscle. CaM modulated [^{3}H]ryanodine binding in a Ca^{2+}-dependent manner. In the presence of maximally activating Ca^{2+} concentrations, CaM inhibited [^{3}H]ryanodine binding with no differences between normal and MHS vesicles. In the absence of Ca^{2+}, however, CaM activated [^{3}H]ryanodine binding with a 2-fold-higher potency in MHS vesicles. Significant differences between normal and MHS tissue were observed for CaM concentrations between 50 nM and 10 μM. A polyclonal antibody raised against the central region of RYR1 specifically inhibited this activating effect of CaM without affecting the inhibition by CaM. This indicates that the central region of RYR1 is a potential binding domain for CaM in the absence of Ca^{2+}. It is suggested that *in vivo* an enhanced CaM sensitivity of RYR1 might contribute to the abnormal high release of Ca^{2+} from the SR of MHS muscle.

INTRODUCTION

Activation of skeletal muscle is initiated by the release of Ca^{2+} ions from the intracellular Ca^{2+} store, sarcoplasmic reticulum (SR). Ca^{2+} release is mediated by the high-molecular-mass ligand-gated ryanodine-receptor–Ca^{2+}-release-channel complex (RYR1) (for recent reviews see [1,2]). The protein complex is composed of four identical subunits of about 5000 amino acids each. From the primary sequence, several regulatory binding sites have been deduced, including those for the physiologically occurring ligands Ca^{2+}, calmodulin (CaM) and ATP [3,4]. In various functional assays, Ca^{2+} in micromolar concentrations and ATP have been shown to activate the release channel, whereas Ca^{2+} in higher concentrations and Mg^{2+} were found to be inhibitory [1,2]. Similarly to Ca^{2+}, CaM also plays a dual role in the regulation of RYR1. CaM inhibits SR Ca^{2+} release when the release channel has been previously activated by Ca^{2+} [5–8]. Single-channel measurements revealed that CaM exerts this inhibitory effect by reducing the channel open time without changing the single-channel conductance [9]. Recently, it has been shown that CaM is also capable of activating SR Ca^{2+} release if the intracellular Ca^{2+} concentration is low [8,10]. While for the inhibitory effect micromolar CaM concentrations are necessary, CaM-dependent activation of RYR1 occurs in the nanomolar range. These data are corroborated by studies investigating the interaction of CaM with RYR1 on the structural level. Up to nine CaM-binding sites per RYR1 subunit have been suggested that bind CaM in the presence or absence of Ca^{2+} [11–13]. However, the activating and inhibitory effects of CaM have not yet been correlated with the suggested CaM-binding domains of RYR1.

In the present study, we investigated the effects of CaM on SR Ca^{2+} release of skeletal muscle of pigs susceptible or non-susceptible to pig stress syndrome, an equivalent to human malignant hyperthermia (MH). MH is an inherited muscle disorder in which predisposed individuals respond to volatile anaesthetics or depolarizing muscle relaxants with an accelerated cell metabolism, skeletal-muscle rigidity and a rapid rise in body temperature (for a review see [14]). An $Arg^{615} \rightarrow Cys$ mutation in the RYR1 gene is responsible for porcine stress syndrome [15], whereas in humans mutations in the RYR1 gene are thought to be causative for up to 50 % of all cases [15a]. *In vitro*, MH-susceptible (MHS) muscle is characterized by its enhanced Ca^{2+} and caffeine sensitivity, probably owing to an altered function of the mutant RYR1. In the experiments presented here, we compared the modulatory action of the ubiquitous endogenous messenger CaM in normal and MHS pig skeletal muscle and investigated its interaction with caffeine. Choosing binding of the high-affinity ligand ryanodine to isolated SR vesicles as the experimental approach, we were able to measure indirectly the functional state of the Ca^{2+}-release channel [16–18]. By the use of a site-directed polyclonal antibody, we identified a CaM-binding domain in the central region of the amino acid sequence of RYR1 that binds CaM in the absence of Ca^{2+}.

Part of this work has been published in abstract form [19].

MATERIALS AND METHODS

Materials

Taq polymerase, restriction endonucleases and isopropyl-β-D-thiogalactopyranoside were purchased from Promega (Madison,

Abbreviations used: CaM, calmodulin; SR, sarcoplasmic reticulum; HSR, heavy SR; MH, malignant hyperthermia; MHS, MH-susceptible; RYR1, skeletal-muscle ryanodine receptor.

‖ To whom correspondence should be addressed.

WI, U.S.A.). An expression and purification kit (pTrcHis Xpress) was obtained from Invitrogen (San Diego, CA, U.S.A.). CaM and protease inhibitors were from Boehringer (Mannheim, Germany). Ryanodine was obtained from Calbiochem (Bad Soden, Germany), and [9,21-^{3}H(N)]ryanodine was from Du Pont NEN (Bad Homburg, Germany). A monoclonal antibody against CaM (C3545) and alkaline phosphatase-conjugated anti-mouse IgG were from Sigma-Aldrich Chemie (Deisenhofen, Germany). Alkaline phosphatase-conjugated protein P- and protein A-based IgG purification kits were from Pierce (BA Oud-Beijerland, The Netherlands). Other reagents for SDS/polyacrylamide gel electrophoresis, immunoblot analysis and nitrocellulose membranes were purchased from Bio-Rad (München, Germany). S&S glass-fibre filters for [^{3}H]ryanodine binding were obtained from Schleicher & Schüll (Dassel, Germany).

Isolation of 'heavy' SR vesicles

'Heavy' SR (HSR) vesicles were isolated from the Mm. longissimi dorsi of Pietrain pigs homozygous for the gene causing MH susceptibility and of pure-bred normal German landrace pigs as described previously [20] with slight modifications. Briefly, KCl-extracted crude SR membranes were layered on the top of discontinuous sucrose gradients (22/34/45 %, by weight) containing 0.6 M KCl, 10 mM K-Pipes, pH 7.0, and were centrifuged at 87000 ***g*** for 16 h (4 °C). HSR vesicles were recovered from the 34/45 % interface, pelleted at 124000 ***g*** for 60 min and resuspended in 0.3 M sucrose/0.1 M KCl/10 mM K-Pipes (pH 7.0). Resuspended membranes were rapidly frozen in liquid nitrogen and stored at −70 °C. To prevent proteolysis, the following protease inhibitors were included in several purification steps: 200 μM Pefabloc [4-(2-amino-ethyl)benzolsulphonylfluoride], 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A and 1 mM benzamidine.

Cloning, overexpression and purification of fusion protein RYR1-FP(2849–3183)

Total RNA was extracted from a human skeletal-muscle biopsy sample by guanidinium thiocyanate extraction as described previously [21]. First-strand cDNA synthesis was carried out in a volume of 20 μl, using 200 ng of random hexamer primers, 1 μg of total RNA and the Promega Reverse Transcription System. Amplification of nucleotide region 8525–9980 of RYR1 was performed, using primers designed from the published sequence [4], 5′-CACAAAGTGCCCAGACCTATG-3′ and 5′-CCCAGG-TTGTTGACGATGATTCT-3′. These primers flank the endogenous restriction sites for *Xho*I and *Hin*dIII to be used in the subsequent cloning procedure. The reaction was carried out in a total volume of 25 μl, with 0.5 μl of the cDNA mixture, 50 ng of each primer, 0.2 mM of each dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl, 0.1 % Triton X-100, pH 9.0, and 0.5 units of *Taq* polymerase. Amplification conditions involved denaturations at 94 °C for 5 min, followed by addition of *Taq* polymerase. Cycles (30) were then performed as follows: 94 °C for 1 min, 58 °C for 1 min, 78 °C for 1 min and finally a 10 min extension at 72 °C.

The PCR product was treated with the restriction endonucleases *Xho*I and *Hin*dIII, and the resulting fragment encoding amino acids 2849–3183 was directionally cloned in-frame into the polyhistidine-containing fusion-protein-expression plasmid pTrcHis B. *Escherichia coli* DH5α cells were transformed with the recombinant plasmid, and overexpression of the fusion protein was induced at A_{600} of 0.3 by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The cells were allowed to grow for a further 5 h before being harvested. The insoluble fusion protein [RYR1-FP(2849–3183)] was purified under denaturing conditions by immobilized-metal affinity chromatography according to the manufacturer's instructions (Invitrogen).

Production of antiserum and purification of polyclonal antibodies

Polyclonal antibodies against RYR1-FP(2849–3183) were prepared by injection of rabbits with fragmented 10 % SDS/polyacrylamide gel slices (see below) containing the purified protein. Booster injections were given 3 and 6 weeks later. Antiserum was collected 1 week after the second booster injection and was stored at −20 °C. Preimmune serum and antiserum were purified, using a protein A affinity column. Fractions containing IgG were concentrated by ultracentrifugation (Centricon-3; Amicon, Witten, Germany).

Polyacrylamide gel electrophoresis and immunoblotting

Protein samples (5–30 μg) were denatured in Laemmli sample buffer at 95 °C for 3 min and separated in 10 % or 3.5–15 % SDS/polyacrylamide minigels. After electrophoresis, proteins were transferred to nitrocellulose membranes (0.20 or 0.45 μm pore size) at 400 mA for 1 h (transfer of fusion protein) or 20 h (transfer of RYR1) as described previously [22]. Membranes were blocked for 2 h in a solution containing 0.6 M NaCl, 40 mM Tris/HCl, pH 8.0, 0.2 % Tween-20 and 5 % skim milk powder, washed 3 times and then incubated for 1 h with primary antibody (1:1000 dilution) in blocking solution diluted 1:4. After three washes, membranes were incubated with alkaline phosphatase-conjugated protein P for 60 min and developed using 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue Tetrazolium.

CaM overlay

Proteins were transferred from 10 % SDS/polyacrylamide minigels to nitrocellulose of 0.2 μm pore size as described above. Membranes were washed 3 times with 0.1 M KCl/20 mM K-Pipes (pH 6.8) containing either 100 μM EGTA or 500 μM $CaCl_2$. The blots were then incubated in wash solution containing 500 nM CaM for 60 min. After two washes, a monoclonal anti-CaM antibody was added to the wash solution for 60 min. Blots were washed twice and subsequently were incubated for 60 min with secondary anti-mouse IgG antibodies linked to alkaline phosphatase. Finally, blots were developed, using 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue Tetrazolium as substrates.

[^{3}H]Ryanodine binding

HSR vesicles (at a protein concentration of about 600 μg/ml) were incubated with 10–15 nM [^{3}H]ryanodine in a medium containing 0.1 M KCl, 100 or 500 μM EGTA, 5 mM AMP, 10 mM K-Pipes, 200 μM Pefabloc, pH 6.8, and varying concentrations of Ca^{2+} for 2 h at 37 °C. Indicated concentrations of CaM, caffeine or IgG were added to the incubation medium. When the effect of IgG was investigated, vesicles were in addition preincubated with IgG for 30 min in the absence of [^{3}H]-ryanodine and CaM. The unbound ryanodine was separated from the protein-bound ryanodine by filtration of protein aliquots through S&S GF51 filters followed by washing three times with ice-cold buffer solution. Radioactivity remaining with the filters was measured by liquid scintillation counting. Non-specific

binding was determined by a 1000-fold excess of unlabelled ryanodine and subtracted from total binding. Each experiment was carried out in duplicate.

Miscellaneous methods

Protein concentration was determined by the method of [23], using BSA as a standard. Free concentrations of Ca^{2+} were calculated by a computer program, using binding constants described previously [24].

RESULTS

Modulation of high-affinity [^{3}H]ryanodine binding by CaM

[^{3}H]Ryanodine binding to isolated HSR vesicles was performed in the presence of 100 mM KCl, pH 6.8, i.e. conditions at which differences in the modulation of SR Ca^{2+} release from normal and MHS vesicles become most profound [25]. In the presence of maximally activating Ca^{2+} concentrations, MHS vesicles exerted an approx. 3-fold higher affinity for ryanodine (MHS: $K_d = 10.8 \pm 0.6$ nM; normal: $K_d = 29.7 \pm 2.2$ nM), whereas maximal activation was not significantly different (MHS: 17.3 ± 1.9 pmol/mg of protein; normal: 14.0 ± 0.6 pmol/mg of protein). The further experiments were carried out at [^{3}H]ryanodine concentrations slightly above the K_d value of MHS vesicles (10–15 nM).

Figure 1 shows the inhibitory effect of CaM on [^{3}H]ryanodine binding. Since CaM-mediated inhibition of SR Ca^{2+} release has been observed in the presence of activating Ca^{2+} concentrations [5–9], [^{3}H]ryanodine binding was initially activated by 50 μM Ca^{2+} (pCa = 4.3). CaM inhibited binding with an IC_{50} concentration of about 50 nM in both normal and MHS vesicles. Slight differences between normal and MHS tissue were only observed at higher, almost maximally inhibiting concentrations. The dose–response curve furthermore shows that CaM is not capable of inhibiting [^{3}H]ryanodine binding completely. The highest inhibition was about 40 % of control.

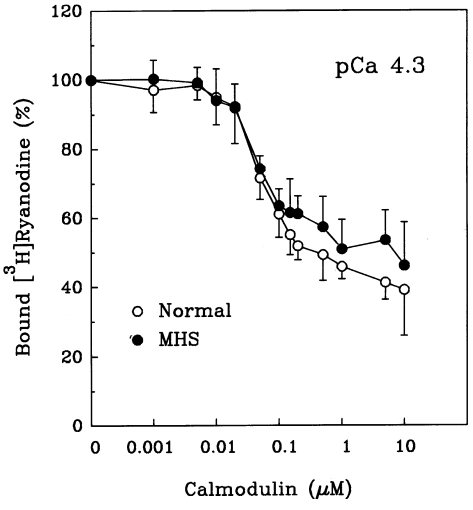

Figure 1 Inhibition of [^{3}H]ryanodine binding to normal and MHS SR vesicles by CaM

[^{3}H]Ryanodine binding was performed in the presence of 10 nM [^{3}H]ryanodine at a pCa of 4.3. Data were normalized to the amount of [^{3}H]ryanodine bound in the absence of CaM (normal: 100% = 2.26 pmol/mg of protein; MHS: 100% = 3.53 pmol/mg of protein). Error bars indicate S.D. ($n = 3$).

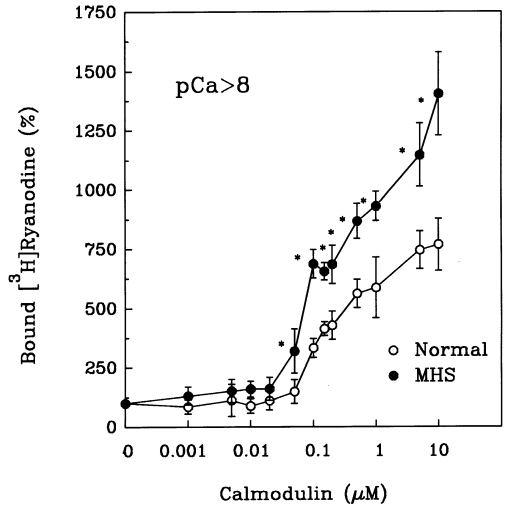

Figure 2 Activation of [^{3}H]ryanodine binding by CaM

[^{3}H]Ryanodine binding was carried out in the presence of 15 nM [^{3}H]ryanodine at 500 μM EGTA (pCa > 8). Data were normalized to the amount of [^{3}H]ryanodine bound in the absence of CaM (100% = 0.04 pmol/mg of protein). Error bars indicate S.D. ($n = 3$). Data points labelled by * are significantly different at $P < 0.05$ (Student's t test).

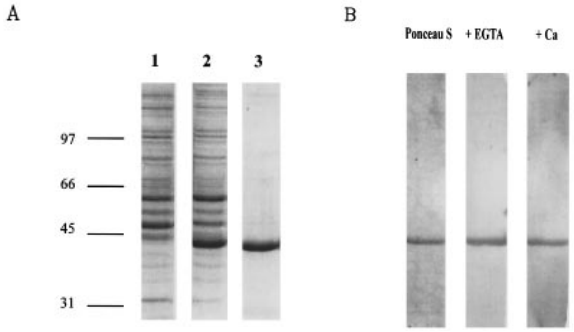

Figure 3 Expression and characterization of RYR1-FP(2849–3183)

(**A**) Cloning, overexpression, and purification of RYR1-FP(2849–3183) was carried out as described in the Materials and methods section. Samples (30 μg each) of total *E. coli* extracts from non-induced (lane 1) and induced cells (lane 2), and 4 μg of purified RYR1-FP(2849–3183) (lane 3), were loaded on an SDS/10% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Molecular-mass standards (migration indicated on the left-hand side; Bio-Rad) were phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa). (**B**) Ca^{2+} dependence of CaM binding to RYR1-FP(2849–3183). Purified RYR1-FP(2849–3183) was separated through a SDS/10% polyacrylamide gel and transferred to nitrocellulose. The left lane shows the Ponceau S staining of the Western blot. CaM overlay was carried out at 500 nM CaM in the absence of Ca^{2+} (100 μM EGTA) (middle lane) or in the presence of 500 μM Ca^{2+} (right lane).

Major differences between normal and MHS vesicles were, on the other hand, found for the activating effect of CaM. Activation of [^{3}H]ryanodine binding was tested in nominally Ca^{2+}-free solutions containing 500 μM EGTA, i.e. conditions permissive for CaM-mediated activation of SR Ca^{2+} release [8,10]. CaM potentiated binding in a concentration-dependent manner. The threshold for activation was 50 nM for MHS and 100 nM for normal vesicles (Figure 2). At the maximally applied concentration of CaM (10 μM), binding to MHS vesicles was 2-fold more enhanced compared with normal vesicles.

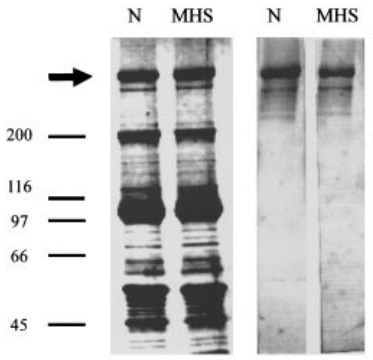

Figure 4 Interaction of polyclonal antiserum Ab(2849–3183) with RYR1

Samples (20 μg each) of normal (N) and MHS SR vesicles were separated on an SDS/3.5–15% polyacrylamide gel, stained with Coomassie Brilliant Blue (left two lanes) or transferred to nitrocellulose (right two lanes). The blot was then incubated with polyclonal antiserum Ab(2849–3183). Molecular-mass standards (migration indicated on the left-hand side; Bio-Rad) were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), BSA (66 kDa) and ovalbumin (45 kDa). The arrow indicates the position of RYR1.

Expression and characterization of a fusion protein covering a predicted CaM-binding domain

From the primary sequence of RYR1, three CaM-binding sites in the central amino acid region have been deduced for the human receptor [4] and one for the rabbit receptor [3]. To investigate whether this region is essential for the binding of CaM, we expressed as a fusion protein the region encoding the human amino acid sequence 2849–3183, which contains two of the predicted binding sites. Comparing the sequences of the human and pig RYR1 in this region revealed sequence identity of $> 98\,\%$. The overexpressed protein was purified, as verified by SDS/polyacrylamide gel analysis (Figure 3A), and analysed for CaM binding. CaM-overlay studies showed that the purified fusion protein RYR1-FP(2849–3183) bound CaM in the absence and presence of Ca^{2+} (Figure 3B), indicating that this amino acid region might be involved in the regulation of the Ca^{2+}-release channel by CaM. The purified RYR1-FP(2849–3183) was used for the production of polyclonal antibodies, and the specificity of the polyclonal antiserum [Ab(2849–3183)] was characterized by immunoblotting. Figure 4 (left panel) shows a SDS/polyacrylamide gel that was loaded with aliquots of SR vesicles of normal and MHS muscle. The corresponding Western blot (Figure 4, right panel) revealed that in both vesicle types Ab(2849–3183) specifically reacted with RYR1.

Interaction of IgG(2849–3183) with CaM regulation of RYR1

Since the expressed fusion protein has been shown to bind CaM, we investigated whether the polyclonal antibodies directed against this region could have an effect on the CaM regulation of the Ca^{2+}-release channel. IgG was purified from Ab(2849–3183), and its effects on [^{3}H]ryanodine binding were examined under conditions permissive for activation and inhibition by CaM. When [^{3}H]ryanodine binding to MHS SR was maximally activated by Ca^{2+}, IgG(2849–3183) applied at a 4:1 ratio of IgG:SR protein inhibited binding to about 80 % of control (Table 1). Application of higher concentrations of IgG did not result in a stronger inhibition. In the presence of 200 nM CaM, the percentage of CaM-mediated inhibition was the same as in the absence of CaM. This result was confirmed for normal SR vesicles (results not shown).

Table 1 Effects of IgG(2849–3183) on the inhibition of [^{3}H]ryanodine binding to MHS SR vesicles by CaM

[^{3}H]Ryanodine binding was performed at a pCa of 4.3 in the absence and presence of 200 nM CaM. The [^{3}H]ryanodine concentration was 10 nM. The labels 0, 1 and 4 in the left column refer to the applied ratio of [IgG]:[SR protein]. Data represent the means $\pm$ S.D. of three different experiments.

	Bound [^{3}H]ryanodine (pmol/mg of protein)	
[IgG]:[SR protein]	0 nM CaM	200 nM CaM
0	4.04 $\pm$ 1.14	2.20 $\pm$ 0.59
1	3.80 $\pm$ 1.04	2.11 $\pm$ 0.72
4	3.30 $\pm$ 0.89	1.74 $\pm$ 0.40

Table 2 Effects of IgG(2849–3183) on the activation of [^{3}H]ryanodine binding to MHS SR vesicles by CaM

[^{3}H]Ryanodine binding was performed in the presence of 15 nM [^{3}H]ryanodine at 500 μM EGTA (pCa $>$ 8) and the indicated concentrations of CaM. The labels 0, 1 and 4 refer to the ratio of [IgG]:[SR protein] present. Data represent the means $\pm$ S. D. of three different experiments.

	Bound [^{3}H]ryanodine (pmol/mg of protein)		
[IgG]:[SR protein]	0 nM CaM	150 nM CaM	1 μM CaM
0	0.022 $\pm$ 0.011	0.163 $\pm$ 0.026	0.268 $\pm$ 0.020
1	0.029 $\pm$ 0.004	0.144 $\pm$ 0.044	0.214 $\pm$ 0.034
4	0.021 $\pm$ 0.006	0.077 $\pm$ 0.027	0.133 $\pm$ 0.040

Table 2 describes the inhibitory effect of IgG(2849–3183) on high-affinity [^{3}H]ryanodine binding to MHS SR vesicles when binding was activated by CaM. Distinct inhibition was already observed for low concentrations of IgG. At a ratio of IgG to SR protein of 1:4, CaM-activated binding was reduced to 50 % of control. To prove the specificity of the inhibitory effect of IgG(2849–3183), we also investigated the effect of preimmune IgG under the same experimental conditions. Preimmune IgG, however, did not interfere with [^{3}H]ryanodine binding. At the maximally applied ratio of preimmune IgG:SR protein of 4:1, binding was slightly increased to 103 % of control in the presence of 1 μM CaM.

Does CaM interfere with the caffeine activation of RYR1?

Caffeine is diagnostically used to determine the susceptibility of patients to MH. The contracture force of skeletal-muscle fibre bundles dissected from a biopsy sample is measured in the presence of increasing concentrations of caffeine. According to the European test protocol, a threshold contracture of 200 mg at 2 mM caffeine or less is considered to indicate MHS if the sensitivity to halothane is also enhanced. To examine whether CaM contributes to this enhanced caffeine sensitivity of MHS muscle, we measured the activation of [^{3}H]ryanodine binding in the presence of increasing caffeine and CaM concentrations. Caffeine by itself activated [^{3}H]ryanodine binding in a concentration-dependent manner (Figure 5). This activation was further potentiated by CaM. However, the potentiating effect of CaM was not different in normal and MHS vesicles. In the

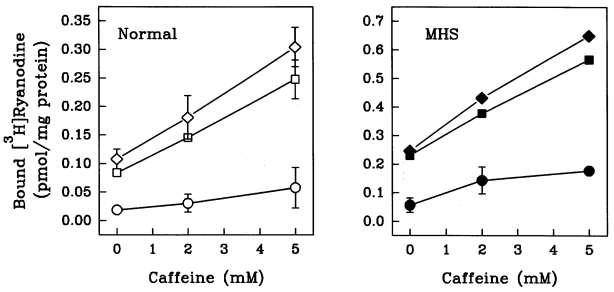

Figure 5 Effects of CaM and caffeine on [^{3}H]ryanodine binding

[^{3}H]Ryanodine binding was performed in the presence of 15 nM [^{3}H]ryanodine at a pCa of 7 and the indicated concentrations of CaM [0 nM (○, ●); 200 nM (□, ■); 1 μM (◇, ◆)] and caffeine. Error bars indicate S.D. ($n = 2$). If no error bars are shown, they are encompassed within the symbol.

presence of 2 mM caffeine, binding was further enhanced about 1.5–2-fold by 200 nM CaM and 2.5–3-fold by 1 μM CaM for both vesicle types (Figure 5).

DISCUSSION

MHS muscle is characterized by its abnormal Ca^{2+} metabolism, which is probably due to an altered function of the mutant RYR1/Ca^{2+}-release channel. The increased Ca^{2+} metabolism is accompanied by an enhanced sensitivity to Ca^{2+}-releasing drugs such as caffeine, which has become a valuable diagnostic tool to determine the susceptibility of patients to MH. The effect of caffeine and other SR Ca^{2+}-releasing compounds has been extensively studied in isolated SR membrane fractions and, in a more direct approach, on the gating properties of the reconstituted Ca^{2+}-release channel [20,25–29]. However, less information is available about the regulation of the mutant receptor by the known endogenous ligands of RYR1, Ca^{2+}, Mg^{2+}, CaM and ATP. Among these, Ca^{2+} is the best characterized modulator. A decreased Ca^{2+} sensitivity has been described for inhibiting Ca^{2+} concentrations and, depending on the experimental approach, an enhanced sensitivity for micromolar activating concentrations [26,27,29–32]. CaM is an ubiquitous intracellular messenger that activates enzymes and ion channels in numerous cell types (for reviews see [33,34]). Depending on the cytoplasmic Ca^{2+} concentration, CaM activates or inactivates SR Ca^{2+} release [5–10]. Although CaM-dependent phosphorylation has been suggested to regulate skeletal-muscle SR Ca^{2+} release [35–37], both the activating and inhibiting effects of CaM do not require ATP, indicating a direct interaction of CaM with the RYR1 channel complex.

In order to compare the CaM modulation of SR Ca^{2+} release in normal and MHS muscle, high-affinity [^{3}H]ryanodine binding to isolated SR vesicles was used as the experimental approach. Binding of [^{3}H]ryanodine to the RYR1/Ca^{2+}-release-channel complex is increased by ligands that activate the SR Ca^{2+}-release channel and is reduced by other regulators that inhibit SR Ca^{2+} release [16–18]. Because of the similar pharmacological dependence of binding and SR Ca^{2+} release, [^{3}H]ryanodine binding to RYR1 thus reflects the functional state of the Ca^{2+}-release channel. CaM-induced inhibition of Ca^{2+}-stimulated [^{3}H]ryanodine binding was not different in normal and MHS SR vesicles. A half-maximal inhibition was achieved at a CaM concentration of approximately 50 nM, which confirms previously reported values [5–8]. These data indicate that the closing of the Ca^{2+}-release channel by the Ca^{2+}-bound form of CaM is not altered in MHS muscle. CaM has also been found to bind with high affinity to RYR1 in the absence of Ca^{2+} [8,11,13]. Functionally, binding of this Ca^{2+}-free form of CaM is correlated with an activation of SR Ca^{2+} release [8,10]. In Ca^{2+}-free solutions (500 μM EGTA), CaM activated [^{3}H]ryanodine binding to both normal and MHS vesicles but with a higher potency in the latter (Figure 2). Significant differences started at low concentrations of 50 nM. At the maximally applied concentration of 10 μM CaM, binding was 2-fold more increased in MHS vesicles compared with normal. At this level, saturation of binding was almost achieved for normal SR but not for MHS vesicles. Since the total concentration of CaM of skeletal muscle, however, has been determined as 2 μM [38], we did not apply distinctly higher concentrations that exceed this physiological range.

Primary sequence predictions suggested the presence of up to three CaM-binding sites in the central region of the RYR1 sequence [3,4]. CaM overlay studies using RYR1 fusion proteins further indicated a CaM-binding region (amino acids 2937–3225) that binds CaM in the absence and presence of Ca^{2+} [13]. To investigate if the different functional effects of CaM are due to binding of CaM to this part of RYR1, the cDNA encoding amino acids 2849–3183 was overexpressed in bacteria, and the purified, expressed fusion protein was used for the production of polyclonal antibodies. The sequence of this fusion protein overlaps to a great extent with the suggested CaM-binding region, amino acids 2937–3225. CaM overlay studies revealed that RYR1-FP(2849–3183) also binds CaM in the presence and absence of Ca^{2+} (Figure 3). The functional effects of purified IgG directed against this fusion protein, however, were different when the Ca^{2+} concentration in the experimental assay was changed. IgG did not interfere with the inhibiting effect of CaM but decreased the amount of bound [^{3}H]ryanodine when binding was activated by CaM in the absence of Ca^{2+}. These data strongly suggest that the activating effect can be assigned to binding of the Ca^{2+}-free form of CaM to the central amino acid region of RYR1. Electron microscopy studies revealed that CaM binds near the periphery of the cytoplasmic portion of the RYR1 channel complex [39]. Since the central part of RYR1 is a surface-exposed area [40], binding to this region appears to be highly probable. As RYR1-FP(2849–3183) also bound CaM in the presence of Ca^{2+}, we cannot exclude that binding of the Ca^{2+}-bound form of CaM to this region is correlated with the inhibiting effect of CaM. It is suggested that the occupation of additional binding sites outside the central amino acid region of RYR1 is required for the CaM-induced inhibition of SR Ca^{2+} release. This assumption is supported by CaM overlay studies showing the existence of additional CaM-binding domains that bind CaM in the presence of Ca^{2+} outside this region [12,13].

The activation of [^{3}H]ryanodine binding by CaM is lower in normal tissue. In addition, the inhibition of CaM-activated binding by IgG was lower in normal vesicles compared with MHS vesicles (results not shown). These data suggest that the conformation of the central part of RYR1 may be altered in MHS tissue. A direct sterical interaction between this region and that containing the $Arg^{615} \rightarrow Cys$ mutation could explain the enhanced CaM sensitivity of MHS muscle. Alternatively, the $Arg^{615} \rightarrow Cys$ mutation might induce a conformational change in the suggested C-terminal pore-forming region of RYR1, transferring the ion channel into a preactivated state. In this case, activation of SR Ca^{2+} release by CaM would be facilitated in MHS muscle. This alternative would favour a scheme for the topology of RYR1 where the central region is in close contact with the transmembrane part of the channel complex.

Does CaM contribute to the increased caffeine sensitivity of MHS muscle? Our results show that CaM further stimulated

caffeine-activated [^{3}H]ryanodine binding, but the CaM-specific potentiation was not different in normal and MHS vesicles (Figure 5). Assuming that the free CaM concentration is not different in normal and MHS muscle, it is suggested that CaM does not further increase the caffeine sensitivity of MHS muscle but transfers the channel closer to the threshold for activation.

In summary, we have shown that the SR Ca^{2+}-release channel in MHS muscle is more sensitive to activating CaM concentrations. This activating effect could be functionally assigned to binding of CaM to the central part of the receptor. It is of interest that in this region a phosphorylation site has been identified that is activated by CaM-dependent kinases [41]. Further characterization of this region will be necessary to clarify a possible interaction between these regulatory sites.

We thank M. Richter for experimental assistance in the initial stage of the experiments. This work was partially supported by fellowships from EMBO and DAAD to S.O'D.

REFERENCES

1 Coronado, R., Morrissette, J., Sukhareva, M. and Vaughan, D. M. (1994) Am. J. Physiol. **266**, C1485–C1504
2 Meissner, G. (1994) Annu. Rev. Physiol. **56**, 485–508
3 Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) Nature (London) **339**, 439–445
4 Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., Meissner, G. and MacLennan, D. H. (1990) J. Biol. Chem. **265**, 2244–2256
5 Meissner, G. (1986) Biochemistry **25**, 244–251
6 Plank, B., Wyskovsky, W., Hohenegger, M. and Suko, J. (1988) Biochim. Biophys. Acta **938**, 79–88
7 Fuentes, O., Valdivia, C., Vaughan, D., Coronado, C. and Valdivia, H. H. (1994) Cell Calcium **15**, 305–316
8 Tripathy, A., Xu, L., Mann, G. and Meissner, G. (1995) Biophys. J. **69**, 106–119
9 Smith, J. S., Rousseau, E. and Meissner, G. (1989) Circ. Res. **64**, 352–359
10 Buratti, R., Prestipino, G., Menegazzi, P., Treves, S. and Zorzato, F. (1995) Biochem. Biophys. Res. Comm. **213**, 1082–1090
11 Yang, H., Reedy, M. M., Burke, C. L. and Strasburg, G. M. (1994) Biochemistry **33**, 518–525
12 Chen, S. R. W. and MacLennan, D. H. (1994) J. Biol. Chem. **269**, 22698–22704
13 Menegazzi, P., Larini, F., Treves, S., Guerrini, R., Quadroni, M. and Zorzato, F. (1994) Biochemistry **33**, 9078–9084
14 MacLennan, D. H. and Phillips, M. S. (1992) Science **256**, 789–794
15 Fujii, J., Otsu, K., Zorzato, F., De Leon, S., Khanna, V. K., Weiler, J. E., O'Brien, P. J. and MacLennan, D. H. (1991) Science **253**, 448–451
15a MacLennan, D. H. (1995) Curr. Opin. Neurol. **8**, 397–401
16 Pessah, I. N., Stambuk, R. A. and Casida, J. E. (1987) Molec. Pharmacol. **31**, 232–238
17 Michalak, M., Dupraz, P. and Shoshan-Barmatz, V. (1988) Biochim. Biophys. Acta **939**, 587–594
18 Chu, A., Diaz-Munoz, M., Hawkes, M. J., Brush, K. and Hamilton, S. L. (1990) Molec. Pharmacol. **37**, 735–741
19 O'Driscoll, S., Herrmann-Frank, A., McCarthy, T. V. and Lehmann-Horn, F. (1996) Biophys. J. **70**, A164
20 Mickelson, J. R., Ross, J. A., Reed, B. K. and Louis, Ch.F. (1986) Biochim. Biophys. Acta **862**, 318–328
21 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochemistry **162**, 156–159
22 Towbin, H., Staehelin, T. and Gordon, (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
24 Fabiato, A. (1988) Methods Enzymol. **157**, 378–417
25 Shomer, N. H., Mickelson, J. R. and Louis, Ch.F. (1994) Am. J. Physiol. **36**, C1253–C1261
26 Mickelson, J. R., Litterer, L. A., Jacobson, B. A. and Louis, Ch.F. (1990) Arch. Biochem. Biophysics **278**, 251–257
27 Valdivia, H. H., Hogan, K. and Coronado, R. (1991) Am. J. Physiol. **261**, C237–C245
28 Fill, M., Stefani, E. and Nelson, T. (1991) Biophys. J. **59**, 1085–1090
29 Herrmann-Frank, A., Richter, M. and Lehmann-Horn, F. (1996) Biochem. Pharmacol. **52**, 149–155
30 Mickelson, J. R., Gallant, E. M., Litterer, L. A., Johnson, K. M., Rempel, W. E. and Louis, Ch.F. (1988) J. Biol. Chem. **263**, 9310–9315
31 Fill, M., Coronado, R., Mickelson, J. R., Vilven, J., Ma, J., Jacobson, B. A. and Louis, Ch.F. (1990) Biophys. J. **50**, 471–475
32 Shomer, N. H., Louis, C. F., Fill, M., Litterer, L. and Mickelson, J. R. (1993) Am. J. Physiol. **264**, C125–C135
33 Cohen, P. and Klee, C. B. (eds.) (1988) Calmodulin, Molecular Aspects of Cellular Regulation, Elsevier, Amsterdam
34 Saimi, Y. and Kung, C. (1994) FEBS Lett. **350**, 155–158
35 Wang, J. and Best, P. M. (1992) Nature (London) **359**, 739–741
36 Herrmann-Frank, A. and Varsányi, M. (1993) FEBS Lett. **332**, 237–242
37 Hain, J., Mayrleitner, M., Fleischer, S. and Schindler, H. (1994) Biophys. J. **67**, 1823–1833
38 Klee, C. B. and Vanaman, T. C. (1982) Adv. Protein Chem. **35**, 213–321
39 Wagenknecht, T., Berkowitz, J., Grassucci, R., Timerman, A. P. and Fleischer, S. (1994) Biophys. J. **67**, 2286–2295
40 Marks, A. R., Fleischer, S. and Tempst, P. (1990) J. Biol. Chem. **265**, 13143–13149
41 Suko, J., Maurer-Fogy, I., Plank, B., Bertel, O., Wyskovsky, W., Hohenegger, M. and Hellmann, G. (1993) Biochim. Biophys. Acta **1175**, 193–206

Received 15 March 1996/3 June 1996; accepted 13 June 1996