Charged surface area of Maurocalcine determines its interaction with the skeletal muscle calcium release channel

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Abstract

The 33 amino acid scorpion toxin Maurocalcine (MCa) has been shown to modify the gating of the skeletal-type calcium release channel (ryanodine receptor, RyR1) of the sarcoplasmic reticulum (SR). In this study, we explored the effects of MCa and its mutants ([Ala^{8}]MCa, [Ala^{19}]MCa, [Ala^{20}]MCa, [Ala^{22}]MCa, [Ala^{23}]MCa and [Ala^{24}]MCa) on single RyR1 channels incorporated into artificial lipid bilayers and on elementary calcium release events in rat and frog skeletal muscle fibers. MCa and its mutants induced long-lasting sub-conductance states (LLSS) on RyR1 that last for several seconds, however, their average length and frequency was altered, paralleling the spatial distance of the mutated amino acid from the critical residue, ^{24}Arg. If the mutation was placed further away from this critical residue the length and the frequency of LLSS decreased. While the effect of the toxin and its mutants was independent of the calcium concentration, it was strongly dependent on the direction of the current through the channel. If the direction was the same as in case of calcium release, the toxins were about 8-10 fold less effective as compared to the opposite current direction. In muscle fibers long-lasting calcium release events were observed after the addition of the toxins. The averaged length of these events correlated well with the duration of LLSS. In particular, the [Ala^{8}]MCa and [Ala^{19}]MCa mutants had similar effects as the wild type MCa and produced long, while the [Ala^{22}]MCa mutant induced shorter events. These data suggest that the effect of the toxin is governed by the large charged surface formed by residues ^{20}Lys, ^{22}Lys, ^{23}Arg, ^{24}Arg and ^{8}Lys. Our observations also indicate that the results from artificial lipid bilayer experiments mimic the *in situ* effects of MCa on RyR1.

Introduction

Excitation–contraction (EC) coupling in skeletal muscle fibers requires the functional interaction of two calcium channels, the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR1), residing in the transverse tubular membrane and in the membrane of the terminal cisternae of the sarcoplasmic reticulum (SR), respectively. In amphibian skeletal muscle cells, Ca^{2+} release from SR has been proposed to result from the summation of calcium release events, termed sparks, corresponding to the opening of a discrete number of ryanodine receptors (Tsugorka *et al.* 1995; Klein *et al.* 1996; Gonzalez *et al.* 2000*b*). In contrast, intact adult mammalian skeletal muscle fibers do not give rise to spontaneous Ca^{2+} release events (Shirokova *et al.* 1998). However, as it has recently been shown, a mild treatment of adult mammalian skeletal fibers with Saponin provokes the appearance of Ca^{2+} release events with various morphology (Kirsch *et al.* 2001; Zhou *et al.* 2003; Szentesi *et al.* 2004), long events with constant amplitude called embers (Gonzalez *et al.* 2000*a*) and sparks similar to those observed in frog skeletal muscle cells. It is still to be demonstrated, however, that these events are indeed the elementary building blocks of Ca^{2+} release in mammalian muscle and embers represent the opening of a single, or a few but coupled (Marx *et al* 1998) RyR1.

Two scorpion toxins, maurocalcine (MCa) and imperatoxin A (IpTxA), have been shown to modify RyR1 Ca^{2+} channel properties *in vitro* (El-Hayek *et al* 1995*b*; Fajloun *et al.* 2000) and proved to be useful tools for studying EC coupling and elementary events of Ca^{2+} release. It was also demonstrated that MCa induces Ca^{2+} release from SR vesicles, strongly potentiates [3H]ryanodine binding to SR vesicles, and increases the open probability of the purified RyR1 (Chen *et al.* 2003; Estève *et al.* 2003). In cultured rat myotubes, MCa induces Ca^{2+} release via the ryanodine receptor but has no effect on the depolarization-evoked Ca^{2+} transient. In contrast,

MCa does not induce any Ca^{2+} release or Ca^{2+} release events in intact adult mammalian fibers. On the other hand, in saponin-treated adult mammalian cells, MCa had a biphasic effect, it first induced a strong increase in calcium release event frequency and then it promoted the appearance of long-lasting embers (Szappanos *et al.* 2005). In addition, under voltage clamp conditions the toxin seemed to interfere with the repolarization-induced closure of RyRs raising the possibility that the peptide binds, preferentially to the open channel (Pouvreau *et al.* 2006).

Recent results have also drawn the attention to the charged amino acid residues around position 24 in MCa as the possible interaction site for the toxin binding to RyR1 (Estève *et al.* 2003; Szappanos *et al.* 2005). It is not clear, however, whether alterations in the cluster of negative charges around residue 24 would simply lower the affinity of toxin binding to RyR1 or also affect the quality of the interaction. It also awaits experimental confirmation that alterations seen on isolated channels incorporated into lipid bilayers would also appear under *in situ* conditions.

In this study, we investigated the effects of different mutations of MCa on the Ca^{2+} release channel of SR and on the spontaneous Ca^{2+} release events in permeabilized adult skeletal muscle fibers from both amphibians and mammals. We show that MCa and its mutants induce – in a concentration-dependent manner – subconductance states in skeletal RyR when applied on the cytosolic side. Analysis of the concentration dependence of different mutants of the toxin suggests that the induction of these long-lasting subconductance states (LLSS) corresponds to a reversible, voltage-dependent binding and unbinding of MCa at a site accessible from the cytosolic side. These results strongly support the hypothesis that the effects of MCa depend on the charged surface region of the toxin and provide evidence for the binding of MCa to a channel site different from that of ryanodine.

Part of this work has been presented to the European Muscle Society (Szentesi *et al.*, 2006).

Material and methods

Chemical synthesis

N-α-Fmoc-L-amino acids, 4-hydroxymethylphenyloxy resin, and reagents used for peptide synthesis were obtained from PerkinElmer Life Sciences. The MCa and analogues were obtained by the solid-phase peptide synthesis (Merrifield, 1986) using an automated peptide synthesizer (Model 433A, Applied Biosystems Inc.). Analogues were obtained by point mutation (Ala instead of one amino acid in the sequence of native-like Maurocalcine) and named [Ala^{8}]MCa, [Ala^{19}]MCa, [Ala^{20}]MCa, [Ala^{22}]MCa, [Ala^{23}]MCa and [Ala^{24}]MCa. Peptide chains were assembled stepwise on 0.25 meq of hydroxymethylphenyloxy resin (1% cross-linked; 0.89 meq of amino group/g) using 1 mmol of *N*-α-Fmoc amino acid derivatives. The side chain-protecting groups were as follows: trityl for Cys and Asn; *tert*-butyl for Ser, Thr, Glu, and Asp; pentamethylchroman for Arg; and *tert*-butyloxycarbonyl for Lys. *N*-α-Amino groups were deprotected by treatment with 18 and 20% (v/v) piperidine/*N*-methylpyrrolidone for 3 and 8 min, respectively. The Fmoc-amino acid derivatives were coupled (20 min) as their hydroxybenzotriazole active esters in *N*-methylpyrrolidone (4-fold excess). After peptide chain assembly, the peptide resin (approximately 1.8 g) was treated between 2 and 3 h at room temperature in constant shaking with a mixture of trifluoroacetic acid/H_2O/thioanisol/ethanedithiol (88: 5/5/2, v/v) in the presence of crystalline phenol (2.25 g). The peptide mixture was then filtered, and the filtrate was precipitated by adding cold *t*-butylmethyl ether. The crude peptide was pelleted by centrifugation (3.000 x *g* for 10 min), and the supernatant was discarded. The reduced peptide was then dissolved in 200 mM Tris-HCl buffer, pH 8.3, at a final concentration of 2.5 mM and stirred under air to allow oxidation/folding (between 50 and 72 h, room temperature). The target products, MCa and analogues, were purified

to homogeneity, first by reversed-phase high pressure liquid chromatography (PerkinElmer Life Sciences, C18 Aquapore ODS, 20 µm, 250 x 10 mm) by means of a 60-min linear gradient of 0.08% (v/v) trifluoroacetic acid = 0–30% acetonitrile in 0.1% (v/v) trifluoroacetic acid/H_2O at a flow rate of 6 ml/min (λ = 230 nm). A second step of purification of MCa and analogues was achieved by ion-exchange chromatography on a carboxymethyl cellulose matrix using 10 mM (buffer A) and 500 mM (buffer B) sodium phosphate buffers, pH=9.0 (60-min linear gradient from 0 to 60% buffer B at a flow rate of 1 ml/min). The homogeneity and identity of MCa or analogues were assessed by the following: (i) analytical C18 reversed-phase high pressure liquid chromatography (Merck, C_{18} Li-Chrospher, 5 µm, 4 x 200 mm) using a 60-min linear gradient of 0.08% (v/v) trifluoroacetic acid/0–60% acetonitrile in 0.1% (v/v) trifluoroacetic acid/H_2O at a flow rate of 1 ml/min; (ii) amino acid analysis after acidolysis (6 N HCl/2% (w/v) phenol, 20 h, 118 °C, N_2 atmosphere); and (iii) mass determination by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

Preparation of heavy SR vesicles

Heavy SR (HSR) vesicles – containing vesicles formed from membrane fragments of the terminal cisternae of the SR – were isolated from rat skeletal muscle according to Lai and Meissner (1990) with slight modifications (Sarkozi et al., 2005). Following a homogenization in 100 mM NaCl, 20 mM EGTA, 20 mM Na-HEPES, pH=7.5, first crude microsomes were collected by centrifugation at 40,000 *g*. After removing the actomyosin contamination, by solubilization in 600 mM KCl, the microsome fraction was collected at 109,000 *g*. The pellet was resuspended and loaded onto a 20–45% linear sucrose gradient. HSR vesicles were extracted from the 36–38% region of the continuous sucrose gradient, collected by centrifugation and resuspended in 0.4 M sucrose, 10 mM K-PIPES, pH=7.0. Protein concentration was measured by the Biuret method.

RyR1 Ca^{2+} channel reconstitution and single-channel recording analysis

Measurements of channel activity were carried out using purified RyR1 incorporated into planar lipid bilayers. RyR1 was purified from rat SR vesicles as previously described (Lai et al., 1988). The bilayers were formed using phosphatidylethanolamine, phosphatidylserine, and L-phosphatidylcholine in a ratio of 5:4:1 dissolved in *n*-decane up to the final lipid concentration of 20 mg/ml (Herrmann-Frank et al., 1996). Bilayers were formed across a 200 or 250 μm diameter aperture of a Delrin cap using a symmetrical buffer solution (in mM: 250 KCl, 0.1 EGTA, 0.15 $CaCl_2$, 20 PIPES, pH 7.2). Small aliquot of purified RyR1 was added into the chamber which defined as the *cis* (cytoplasmic) side, whereas the other chamber labeled as *trans* (luminal) side, and was kept on ground potential. To ensure the orientation of the incorporated RyR1, we tested the effect of Ca^{2+} on both side. After successful incorporation of the RyR1 channel, free calcium concentration in the *cis* chamber was adjusted to 238 nM by the addition of EGTA. Electrical signals were filtered at 1 kHz through an 8-pol low-pass Bessel filter and digitized at 3 kHz using Axopatch 200 and pCLAMP 6.03 (Axon Instruments, Union City, CA, USA). Total recording time in each experiment was 10–20 min for each experimental condition tested. After changing conditions, at least 5 min were allowed for equilibration, which appeared to be enough to reach the new equilibrium of the parameters. Single channel measurements were carried out at 22 ± 1 °C. The free Ca^{2+} concentration was calculated using the computer program and affinity constants published by Fabiato (1988). Open probabilities were calculated using the common 50% criteria with a medial dead zone of 5%. Current amplitude distribution was analyzed using Origin (Microcal Software, Northampton, MA, USA). The LLSS ratio was calculated as the fraction of time the channel spent in the LLSS from a given (usually several minutes long) total recording time.

Isolation of single skeletal muscle fibers

Single skeletal muscle fibers were isolated manually from the *m. semitendinosus* of frogs (*Rana esculenta*) as described previously (Szentesi *et al.* 1997). Briefly, the frogs were killed by rapid decapitation, followed by pithing. The mechanical separation of fibers was carried out in Ringer's solution (in mM, 115 NaCl, 2.5 KCI, 1.8 $CaCl_2$ and 5 HEPES) then transferred to the recording chamber. The fiber was permeabilized by applying the following solution containing 0.004% Saponin for 3 minutes (in mM, 100 K-glutamate, 10 HEPES, 1 EGTA, 5 $MgCl_2$, 5 Na_2-ATP, 10 sodium phosphocreatine, 10 glucose, 0.13 $CaCl_2$ and 8% dextran, 0.05 Fluo-3). After permeabilization Saponin was removed and 100 μM Fluo-4 was applied. The pH of the solutions was set to 7.0.

Single skeletal muscle fibers from the *extensor digitorum communis* muscles of rats were isolated enzymatically as described previously (Szentesi *et al.* 1997). Briefly, rats of either sex were anaesthetized and killed by cervical dislocation. After removal the muscles were treated with collagenase 1.5 mg/ml (Sigma, Type I) for 1–1.5 h at 37°C. Fibers were allowed to rest for at least 20 min after dissociation. The selected fiber was transferred to the recording chamber filled with relaxing solution (in mM: 150 K-glutamate, 10 Hepes, 1 EGTA, 2 $MgCl_2$). The surface membrane was permeabilized using a relaxing solution (in mM: 125 potassium glutamate, 10 Hepes, 1 EGTA, 5 $MgCl_2$, 5 Na2-ATP, 10 sodium phosphocreatine, 10 glucose, 0.13 $CaCl_2$ and 8% dextran, 0.05 mM Fluo-3) containing 0.002% saponin. This solution was then changed to a K_2SO_4-based internal solution (in mM: 95 K_2SO_4, 10 Hepes, 1 EGTA, 6 $MgCl_2$, 5 Na_2-ATP, 10 sodium phosphocreatine, 10 glucose, 0.26 $CaCl_2$, 0.1 Fluo-4 and 8% dextran). The pH of the solutions was set to 7.2. The MCa and its mutants were added from 1 mM stock solution. The killing of animals was in accordance with the guidelines of the European Community (86/609/EEC) following a protocol approved by the institutional animal care committee.

Measurement of elementary calcium release events

Fibers were imaged with an LSM 510 META laser scanning confocal microscope (Zeiss, Oberkochen, Germany) as reported previously (Szentesi *et al.* 2004). Line-scan images of fluorescence ($F(x,t)$) were taken at 1.54 ms line^{-1} and 512 pixels line^{-1} (pixel size 0.142 µm) parallel to the fiber axis. Fluo-4 was excited at 488 nm and emitted light was collected through a band-pass filter and digitized at 12 bit. Elementary calcium release events were captured using an automatic computer detection method (e.g. Cheng *et al.* 1999) as described in our previous report (Szentesi *et al.* 2004). The eventless portion of the image was used to calculate baseline fluorescence ($F_0(x)$). The program also determined the amplitude and as $\Delta F/F_0$, spatial half-width measured at the time of the peak (full width half-maximum; FWHM) and duration for sparks and average amplitude, duration and FWHM for embers. FWHM was calculated by fitting a Gaussian function to the spatial distribution obtained by averaging 3 lines at the peak for sparks or by all lines except the first and last 10 ms of the event for embers.

Chemicals

Protease inhibitors were obtained from Boeringer (Mannheim, Germany), Fluo-3, Fluo-4, were purchased from Molecular Probes Inc. (Eugene, OR, USA), while all other chemicals, unless otherwise specified, were from Sigma (St Louis, MO, USA).

Results

Effect of the wild type MCa on isolated RyR1

The effect of the MCa-RyR interaction on the channel function was tested using RyR1 incorporated into planar lipid bilayer (Fig. 1). Applying 50 nM MCa on the cytoplasmic (*cis*) side of the channel induced long-lasting subconductance states, which corresponded to about 60% of the full conductance (Fig. 1B). In case of the wild type MCa, the subconductance state displayed an average LLSS time which was several thousand times longer than that of the mean open time of the unmodified channel (Fig. 1A and B). Between LLSS – in the presence of the toxin – the channel showed normal gating with increased activity. This manifested as an approximately 10-fold increase in the open probability during the inter-LLSS periods in the presence of 200 nM MCa *cis* ($P_{o,\,control}$ = 0.02 ± 0.007; $P_{o,\,MCa}$ = 0.21 ± 0.016). Interestingly, the MCa modified channel retained its ryanodine sensitivity since MCa also induced a subconductance state of the ryanodine-modified RyR1 (Fig. 1C). These results provide evidence for the binding of MCa to a channel site different from that of ryanodine. It should be stressed that in the parallel presence of ryanodine (1 μM) and MCa (50 nM) the effect of ryanodine is the determinant as the channel spends most of its time in the "half conductance state" characteristic for ryanodine, which then alternates with the LLSS induced by the scorpion toxin.

Effects of mutated analogues of MCa on the isolated RyR1

If, by analogy with other peptide toxin-ion channel associations (Hidalgo et al., 1995; Dudley et al., 1995; Gurrola et al., 1999), the high-affinity MCa-RyR1 interaction is mediated by electrostatic forces, mutations in the binding domain of MCa should alter its electrostatic potential and/or the pattern of the subconductance states. Fig. 2 demonstrates that synthetic MCa derivatives activate the isolated RyR and induce subconductance states, in a similar fashion – but

to different extents – as the wt MCa. Mutations within the cluster of basic amino acids of residues 20-24 elicited dramatic effects. Replacing ^{24}Arg with Ala ([Ala^{24}]MCa mutant) in the wt MCa sequence essentially abolished the MCa induced subconductance states (Fig. 2F). Mutants [Ala^{20}]MCa, [Ala^{22}]MCa and [Ala^{23}]MCa exhibited subsequently shorter and shorter transitions to the subconductance state, paralleling the spatial position of their mutation with respect to residue 24 (Fig. 2C-E). In contrast, replacing Lys8 or Lys19 with Ala yielded almost the same LLSS as did the wt MCa (Fig. 2A and B).

Polarity dependence of the MCa effects

The parameters of the LLSS events induced by MCa and its mutants depended on the polarity of the membrane potential, that is, on the direction of the current. When the charge carrier (K^+ or Ca^{2+}) moves from the *trans* to the *cis* side the current direction is identical to that when calcium is released from the SR ("physiological direction"). Using membrane potentials to drive the ions in the physiological direction, the LLSS ratios for each and every mutant were found to be lower as compared to the opposite polarity – "reverse direction" – (Table 2). The effect of the toxins seemed independent of the actual value of the holding potential in the voltage range of 20-120 mV, only the polarity (current direction) had appreciable effect. The MCa-modified channel remained ohmic since the channel conductance was independent of the voltage and of the polarity. It is also interesting, that the MCa effect is identical at 50 µM and at 240 nM free calcium concentration in case of the wild type (Fig. 1A and B) and the [Ala^8]MCa mutant, while the LLSS ratio is slightly lower at low calcium. The length of LLSS is also calcium independent in both current directions (data not shown).

MCa mutants alter the channel gating in two different ways

The effect of the toxin, in the presence of 240 nM ionized calcium, on the LLSS ratio and on the length of these events is demonstrated in Fig. 3. As the site of the mutation was closer and closer to the critical residue 24, the LLSS ratio became smaller and smaller (Fig. 3A). Furthermore, the place of the mutation affects the average LLSS event length more substantially than that of the LLSS ratio (Fig. 3B). Note also that the LLSS ratio as well as the frequency of the events increased with the increase in the toxin concentration. The average length of the LLSS events, on the other hand, seemed to be independent of the toxin concentration (Fig. 3B). These results highlight the existence of a localized binding-site of MCa on RyRs and suggest that the MCa - RyR interaction can be modified with the place of the mutation. In order to further investigate the effect of the different mutations of MCa at the level of EC coupling, elementary calcium release events (ECRE) were studied on adult skeletal muscle fibers.

Effects of MCa analogues on event properties of permeabilized mammalian fibers

We have previously shown that MCa can initiate, or modify the properties of spontaneous calcium release events in intact adult mammalian striated muscle fibers (Szappanos *et al.* 2005). We thus tested whether the different mutants of MCa would alter calcium release in a similar way as they do for the isolated channel. To this end, cells from rat skeletal muscle were permeabilized, placed in a sulfate based internal solution containing Fluo-4 and imaged with a confocal scanner. Fibers showed spontaneous calcium release events (Fig. 4A) in accordance with previous observations (Kirsch *et al.* 2001; Zhou *et al.* 2003; Szappanos *et al.* 2005). Shortly after the addition of 50 nM MCa (2–10 min) the frequency at which ECRE occurred increased considerably (Fig. 4B). In addition to sparks long openings with or without sparks were also observed (Fig. 4B inset). For testing the specificity of the mutants of MCa, we added the [Ala^{19}]MCa analogue of MCa which was found to have similar effects on the occurrence

(Fig. 4C) and duration (187±4 vs. 185±3 ms; n = 767 and 1109; for MCa and the mutant, respectively) of embers as the wild type toxin. It should be noted that the above duration of the embers is a lower estimate since in some cases the embers were longer than the time window of the measurements thus their beginning, end or both were missing from the image and, in addition, the average may contain events that originate from non-modified channels. It should also be stressed here that we have demonstrated earlier that the [Ala^{24}]MCa analogue has no effects on the occurrence of ECRE and, furthermore, the events in its presence are similar to those under control conditions (Szappanos et al. 2005).

To demonstrate that events having length of several seconds do occur in the presence of MCa longer imaging times were also used. This, however, was not done routinely to avoid unnecessary photo damaging the cells. Nevertheless, as presented in Fig. 5A for the Ala^{19} mutant, events of long durations were indeed captured. Note that even with this increased recording time the beginning or end of some events were still missed. It is also intriguing that, unlike in frogs (see below), where a spark preceded the long ember, the events in rats always started as a sudden step to an increased fluorescence level which was then maintained during the entire event.

Occasionally (22 events out of the several hundred), however, stepwise changes in fluorescence were detectable as presented in Fig. 5B. The amplitudes of these steps were slightly greater than the original, 158±18% for the second step as normalized to the first, indicating the opening of an additional, but independently gated channel. In addition to simple stepwise changes in fluorescence, events with complex kinetics were also captured (Fig. 5C). These showed long openings, lasting for several hundreds of milliseconds, which alternated with a burst-like pattern clearly resembling that seen for isolated channels (Fig. 2).

Effects of MCa analogues on amphibian fibers

While mammalian fibers readily produce calcium release events of long duration and small amplitude under control conditions, amphibian fibers are normally devoid of such events. To further explore the effects of the mutations of MCa and to derive quantitative measures for their duration we used frog skeletal muscle fibers where a long event with small amplitude would clearly mark a toxin modified channel. Figure 6 thus presents line-scan images recorded under control conditions (Fig. 6A) and in the presence of wt MCa, [Ala^{8}]MCa, [Ala^{19}]MCa and [Ala^{22}]MCa (Fig. 6B-E, respectively). The wt toxin and the two mutants decreased event frequency but produced long lasting calcium release events which were never observed under control conditions in frogs but resembled those reported for IpTxA (Shtifman et al. 2000). In line with data from single channels, the [Ala^{22}]MCa mutant caused shorter calcium release events then either of the other two mutants (see also Fig. 7A).

Note also that these long events seem to always start with a "classical" spark. From the several thousand long events measured in the presence of MCa or its mutants only a handful had no leading spark, as the one presented in the inset of Fig. 6B for the wt MCa.

The mutation of MCa alters the morphology of ECRE

We found similar effects of the different mutants of MCa on the length of embers in permeabilized frog skeletal muscle fibers as was shown above on LLSS length. As shown in Figure 7A, the mutants [Ala^{8}]MCa and [Ala^{19}]MCa were almost as effective as the wt MCa both at 50 and at 100 nM. In contrast, the [Ala^{22}]MCa mutant induced approximately 60 and 50% shorter events than did the native toxin at the two concentrations, respectively. We also tested [Ala^{24}]MCa but in the presence of this analogue we did not observe embers as in toxin free control solution (data not shown).

As mentioned above, the toxin modified events, in most cases, display a starting spark followed by a prolonged ember. Figure 7B plots the amplitude and FWHM of these components for the wt toxin. The events that were assumed to be the closest to the confocal plane, as judged by their large amplitude, were selected from those included into panel A. As shown in Fig. 7B the starting sparks had 5-6 times greater amplitudes and slightly larger FWHM than the trailing embers.

Discussion

In this work we evaluated how the replacement of different amino acids in the sequence of the wt MCa affects its ability to modify the gating of the skeletal type RyR. We show that mutations of MCa within the cluster of negatively charged residues surrounding ^{24}Arg decrease the appearance of long-lasting subconductance states on isolated channels reconstituted into planar lipid bilayers and long-lasting embers in saponin-treated adult striated muscle fibers. The extent of this effect depended on the direction of the current in lipid bilayer experiments indicating a voltage dependent binding of the toxin to the channel.

Effect of mutations in MCa on the lengths of LLSS and ECRE

In previous studies we have demonstrated that MCa increases the frequency of ECRE together with a decrease in the amplitude of the individual events in saponin-treated adult mammalian striated muscle fibers (Szappanos et al., 2005). In addition, it induces long-lasting embers with durations usually exceeding 200 ms, occasionally lasting longer than 1.5 s. On purified RyR1 reconstituted into planar lipid bilayers, the toxin induced both an increase in the open probability of the channel and the appearance of long-lasting open events characterized by a reduced conductance (Chen *et al.* 2003; Estève *et al.* 2003). As a consequence of these effects, MCa caused a dramatic increase in the [^{3}H]ryanodine binding to RyR1 as well as in Ca^{2+} release from SR vesicles. All these effects were completely abolished by the point mutation of the Arg residue in position 24 of MCa (Estève *et al.* 2003; Szappanos et al., 2005) supporting the hypothesis that the basic amino acid-rich region is important for the functional effect of MCa on RyR1.

Here we show that the effects of MCa described for LLSS and for ECRE depend on the position of mutation of the toxin and correlate with the distance of the mutation from the charged surface of MCa. Indeed, the effects of [Ala^8]MCa and [Ala^{19}]MCa mutants on ECRE were almost

identical to the wt toxin, in the presence of [Ala^{22}]MCa the length of embers decreased, and finally the [Ala^{24}]MCa analogue did not show any effect on ECRE. These results are in direct agreement with the observations originating from lipid bilayer experiments with these analogues illustrating that the capability for inducing the long-lasting sub-conductance state of RyR1 depends on the place of mutation.

Changing amino acids at different positions – by substituting a charged amino acid with a neutral one – thus reveals that the dramatic effect of the mutations within the cluster of basic amino acids cannot be solely attributed to the change of the net electrical charge of the peptide since mutations distant to the cluster but producing the same net electrical charge had relatively minor effects. Since structural changes within MCa – due to its triple disulphide bonds – are unlikely to accompany the mutations, this cluster must represent the interacting site with the RyR.

The properties of the MCa binding site on RyR

Recent evidence has positioned the MCa binding site to residues 3351–3507 on RyR1 (Altafaj *et al.* 2005). The results presented here suggest that the toxin binding site is distant from the high affinity calcium binding (regulatory) site because the effects of the toxins were calcium independent. In addition, it seems also to be distinct from the ryanodine binding site (see below). Finally, it should be noted that the calcium release channel was still ohmic after binding the toxin or its mutants indicating an allosteric modification of the RyR conformation by the toxins.

The fact that the LLSS length was found to be independent of the concentration of MCa – or of the concentration of its analogues – suggests that one MCa binds to a RyR monomer. On the other hand, since the inter LLSS open probability is higher (by approximately 10 fold) than under control conditions indicates that the binding of 1, 2, or 3 MCa to the tetrameric channel preconditions its gating.

Given the above constrains, and assuming that MCa and its mutants bind to RyR1 via a common structural motif, then corresponding mutations should evoke parallel changes in the affinity if the continuity of the basic surface is essential, probably because the electric field-induced orientation of the toxin plays an important role in its binding to the channel. Since the continuity of the charged surface cluster seems equally – if not more – important than the charge itself, we conclude that the orientation of the peptide is the most important factor in determining the binding of the toxin. This suggests that the MCa binding site is near to the opening of the channel pore – where the electric gradient is extremely large – and thus the electric momentum dominates the orientation of the MCa molecule. In the above framework, due to the necessity of a suitable orientation of the charged surface, the interaction of MCa with the RyR should be influenced by the polarity of the membrane potential. In line with the above reasoning we found that the dissociation constants of the peptides were polarity dependent.

We have recently suggested that in intact cells MCa binds preferably to the open conformation of the RyR (Pouvreau et al. 2006). It should be noted that this conclusion was derived from comparing the prolonged calcium release after repolarisation to that of maximal calcium release during the depolarizing pulse. This prolonged calcium release should correspond to the LLSS state seen in lipid bilayer experiments. This suggests that the binding site for the toxin which is responsible for the induction of LLSS is not, or is less accessible when the channel is closed. Clearly, a binding site within or in close association to the channel pore would fulfill this criteria. If the binding site is indeed found within the pore the movement of positively charge ions through the channel (the calcium release process) should hinder the binding of the positively charged toxin. A similar phenomenon is expected to take place in bilayer experiments if the holding potential is set to drive the charge carrying ions in the physiological direction. This is

indeed what was observed strengthening the idea that the binding site is somehow associated with the pore itself.

Combined effect ryanodine and MCa

Normally low concentrations of ryanodine induce a characteristic "half conductance" state of RyR from which the channel only occasionally closes, and only for a short time (for a few milliseconds), and never switches to the full open state unless the ryanodine is removed from the bathing solution (for review see Meissner and el-Hashem, 1992). In the case of the simultaneous presence of ryanodine and MCa the "half conductance state" characteristic to the alkaloid dominates channel behavior. Nevertheless, long closing periods – due to the presence of MCa – become apparent, during which the channel enters a "half of the half" conductance state clearly indicating that the binding of the toxin and the alkaloid are independent of one another. The free gating of the channel between these two states ensures that not only the binding but also the state into which MCa transforms the channel is independent of that induced by ryanodine.

The binding site for MCa and its effect on ECRE

MCa and its structural analogue Imperatoxin A have been shown to induce extremely long calcium release events in amphibian skeletal muscle fibers (Gonzalez *et al.* 2000b; Stifmann *et al.* 2000; Szappanos *et al.* 2005). These events are most likely the direct consequence of the calcium release channel entering into the LLSS following the binding of the toxin molecule. Several pieces of evidence support this conclusion. First, the length of these long events correlates well with the length of LLSS. Second, mutations in MCa cause parallel changes in the ability of the toxin to induce LLSS on isolated channels and to modify the kinetics of ECRE. Finally, neither the wild type MCa nor its mutated analogues have any effects on the SR calcium pump (data not shown). This, on the one hand, clearly confirms that these long events are due to

the calcium released through a single toxin-modified channel while calcium sparks are generated by the opening of a group of calcium release channels. On the other hand, it gives further insight into the interaction of MCa and the calcium release channel. As discussed above the toxin seems to prefer the open conformation when it binds to RyR. If MCa would only bind to the open channel the prediction would be that long events should tail calcium sparks. This is clearly the case for amphibians where long events are generally made up of a leading spark and a tailing ember. It should be noted, however, that a few events (2% of those where long embers were observed) are devoid of the leading ember. This indicates that there is a possibility, albeit much lower, for the toxin to bind to the closed channel. We, of course, cannot exclude the possibility that the binding actually occurred right after the opening of the channel and this then prevented neighboring channels to open and the spark from being formed. While the above scenario is a real, although unlikely possibility in amphibians, it clearly cannot account for the observation in mammals where all long events are without leading sparks. Taken together, our data are in line with the hypothesis that MCa and its mutants bind preferably, but not solely to the open channel.

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Table 1: Polarity and concentration dependence of LLSS ratio with different MCa mutants.

MCa analogue	[toxin] (nM)	Reverse direction	Physiological direction*
wt	50	88.1 ± 10.5	9.8 ± 1.1
	200	91.5 ± 11.2	43.8 ± 5.2[#]
[Ala[8]]MCa	50	52.5 ± 6.1	3.5 ± 0.74
	200	86.7 ± 9.1[#]	5.4 ± 0.60[#]
[Ala[20]]MCa	50	34.8 ± 6.4	3.1 ± 0.72
	200	68.4 ± 8.2[#]	3.9 ± 0.63
[Ala[24]]MCa	50	0.34 ± 0.18	not measured
	200	0.62 ± 0.21	not measured

[#] indicates that the difference is significant (p=0.01) between the LLSS ratios determined at 50 nM and 200 nM toxin concentration. *Physiological direction was defined as the direction in which cat ions move during the release of calcium from the SR. The LLSS ratio was determined as described in Materials and methods and expressed as percentage.

Figure legends

Figure 1. *Effect of Maurocalcine and Ryanodine on the calcium channel.*

Solubilized Ryanodine receptor of the rat was incorporated into a lipid bilayer and the channel current was recorded under voltage clamp condition using 45 mV as the holding potential at two different *cis* [Ca^{2+}] (50 µM left; 240 nM right). The charge carrier was 250 mM KCl, channel openings are represented by downward deflections. The closed state of the channel is marked between the current traces using the symbol —. *A* and *B*, Single channel recordings obtained under control conditions and in the presence of 200 nM MCa demonstrating the long lasting subconductance state, lasting for several seconds, for the latter. *C*, Recording in the presence of 200 nM MCa and 1 µM Ryanodine. The Ryanodine induced specific "half" conductance state is labeled as Rya, while the MCa induced state in the presence of Ryanodine is labeled as MCa.

Figure 2. *Effect of MCa and its mutants on the RyR1 of the rat.*

Representative current traces in the presence of MCa and its mutants. Experimental conditions are identical to those for Fig. 1 left column. The traces in the right and left column show identical single channel recordings obtained using different time scales to better visualize the effects of different mutants. Between the two columns the closed state is marked as —. Note that, if the place of the mutation gets closer and closer to the critical residue 24, the length and the frequency of the LLSS decreases while the single channel conductance does not change.

Figure 3. *Parameters of the LLSS differ for different mutants.*

Single channel records were taken at 50 nM (●), at 200 nM (■) and at 500 nM (▼) toxin concentration and the LLSS ratio (A) and the average length of the LLSS events (B) were

determined. LLSS ratio was calculated as the ratio of the time spent in the sub-conductance state over the total time, and expressed as percentage. Each symbol represents the mean of at least 20 events taken from at least 8 bilayers. Note that if the site of the mutation was placed more and more distant from the critical residue 24, the channel spends less and less time in the LLSS state. Note also that while the concentration of the toxin did influence the LLSS ratio it had little effect, if any, on its average length.

Figure 4. *Effect of wt and mutant MCaon calcium release events in rat skeletal muscle fibers.* Line-scan images in control (A) and in the presence of wt MCa (B) and [Ala^8]MCa mutant (C) in Saponin skinned rat skeletal muscle fibers. Scanning was made parallel with the fiber axis. Images were corrected for background fluorescence (F/F_0) and pseudo-colored. Note the larger number of long lasting embers as compared to control in the presence of both types of the toxin. The inset shows that wt MCa causes similar long events as the [Ala^8]MCa mutant.

Figure 5. *Long lasting calcium events caused by [Ala^{19}]MCa mutant in rat skeletal muscle fibers.* (A) Pseudo-colored line-scan image taken in the presence of [Ala^{19}]MCa. Same scanning direction and image correction was applied as in Fig. 4 but the length of acquisition was eight times longer. Note the endless events on either side of the image. (B) A representative ember with a secondary opening taken from a long scan. (C) A representative event demonstrating the successive re-openings of the channel. The traces below the images show the average of 5 neighboring lines at the middle of the event while the traces next to the images represent the spatial spread of the event calculated by averaging the lines between the times marked by white arrows.

Figure 6. *Effect of wt and mutant MCa on calcium release events in frog skeletal muscle fibers.* Pseudo-colored line-scan images under control conditions (A) and in the presence of wt MCa (B), [Ala^{8}]MCa (C), [Ala^{19}]MCa (D) and [Ala^{22}]MCa mutant (E) in frog skeletal muscle fibers. Same scanning direction and image correction was applied as in Fig. 4. Note the decreased number of long lasting embers in the presence of [Ala^{22}]MCa as compared to those measured in the presence of wt MCa and the other mutants. The inset in panel B displays a rare event where the long opening was not preceded by a spark.

Figure 7. *Effect of MCa and its mutants on the parameters of calcium release events*

(A) The average length of the events measured in the presence of wt MCa and its different mutants calculated from images similar to those presented in Fig. 6. Empty bars represent averages measured in the presence of 50 nM, while filled bars give values in the presence of 100 nM of the toxins. The numbers above the bars give the number of events included into the average. Only those events were considered whose lengths could reliably be estimated. Note the fewer number of events for the [Ala^{22}]MCa mutant. (B) The parameters of the leading spark (hatched bars) and the trailing ember (cross-hatched bars) for selected large events (n = 66) measured in the presence of the wt toxin (50 nM). In case of the trailing ember the average amplitude is given.