Critical Amino Acid Residues Determine the Binding Affinity and the Ca$^{2+}$ Release Efficacy of Maurocalcine in Skeletal Muscle Cells*

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Eric Estève‡‡§§, Sophia Smida-Rezgui‡, Sandor Sarkozy**, Csaba Szegedi**, Imed Regaya§, Lili Chen‡‡, Xavier Altafaj‡, Hervé Rochat§, Paul Allen§§, Isaac N. Pessah‡‡***, Isabelle Marty‡, Jean-Marc Sabatier§, Istvan De Waard‡, and Michel Ronjat‡‡

From the INSERM U 9931, CEA, CIS, 17 Rue des Martyrs, 38054 Grenoble Cedex 09, France, ‡‡Department of Physiology, University Medical School of Debrecen, H-4012, Debrecen, Hungary, §§CNRS UMR 6560, Faculté de Médecine Nord, Boulevard Pierre Dramard, 13916 Marseille Cedex 20, France, ††Department of Molecular Biosciences and Graduate Program in Neurosciences, University of California, Davis, California 95616, and §§Department of Anesthesia, Brigham and Women’s Hospital, Boston, Massachusetts 02115

Maurocalcine (MCa) is a 33 amino acid residue peptide toxin isolated from the scorpion Scorpio maurus palmatum. MCa and mutated analogues were chemically synthesized, and their interaction with the skeletal muscle ryanodine receptor (RyR1) was studied on purified RyR1, sarcoplasmic reticulum (SR) vesicles, and cultured myotubes. MCa strongly potentiates $[^3H]$ryanodine binding on SR vesicles (7-fold at $pCa$ 5) with an apparent EC$_{50}$ of 12 nM. MCa decreases the sensitivity of $[^3H]$ryanodine binding to inhibitory high Ca$^{2+}$ concentrations and increases it to the stimulatory low Ca$^{2+}$ concentrations. In the presence of MCa, purified RyR1 channels show long-lasting openings characterized by a conductance equivalent to 60% of the full conductance. This effect correlates with a global increase in Ca$^{2+}$ efflux as demonstrated by MCa effects on Ca$^{2+}$ release from SR vesicles. In addition, we show for the first time that external application of MCa to cultured myotubes produces a cytosolic Ca$^{2+}$ increase due to Ca$^{2+}$ release from 4-chloro-$m$-cresol-sensitive intracellular stores. Using various MCa mutants, we identified a critical role of Arg$^{24}$ for MCa binding onto RyR1. All of the other MCa mutants are still able to modify $[^3H]$ryanodine binding although with a decreased EC$_{50}$ and a lower stimulation efficacy. All of the active mutants produce both the appearance of a subconductance state and Ca$^{2+}$ release from SR vesicles. Overall, these data identify some amino acid residues of MCa that support the effect of this toxin on ryanodine binding, RyR1 biophysical properties, and Ca$^{2+}$ release from SR.

In skeletal muscles, contraction is triggered by the massive release of Ca$^{2+}$ from sarcoplasmic reticulum (SR).† The channel responsible for this release is the type-I ryanodine receptor (RyR1). RyR1 has been intensively studied because of its unique structural and functional organization. It forms part of a calcium mobilization complex in which RyR1 is apposed to a calcium mobilization complex in which RyR1 is apposed to the L-type voltage-dependent calcium channel (dihydropyridine receptor, DHPR) along many other structural and regulatory components (1). The activation of RyR1 requires a chain of events that starts with plasma membrane depolarization inducing a change in the conformation of DHPR itself transmitted to RyR1. The entire set of events is called excitation-contraction coupling (EC coupling).

In vitro, the activity of RyR1 can be modulated by a number of different effectors such as Ca$^{2+}$, ryanodine, ATP, caffeine, and 4-chloro-$m$-cresol (CMC) (2, 3). Among these effectors, only few present high selectivity and affinity for RyR1. More specific pharmacological agents for RyR1 have been discovered in scorpion venoms (4, 5). One such peptide has been isolated from the venom of the chactid scorpion Scorpio maurus palmatus and has been termed maurocalcine (MCa). It is a 33-mer basic peptide reticulated by three disulfide bridges. MCa can be chemically synthesized without any loss of activity (5). It is potently active on RyR1 as it alters channel properties in the low nanomolar range (5, 6). MCa presents a strong sequence homology (82% amino acid sequence identity) with imperatoxin A (IpTxA), a toxin active on RyR1 and isolated from another scorpion venom (7–10). Besides the fact that MCa and IpTxA represent two of the most high affinity effectors of RyR1, they also share some amino acid sequence homology with a specific domain (domain A) of the II–III loop of Ca$_{v1.1}$, the subunit that carries the voltage sensor of DHPR (Fig. 1). Although the exact role of the domain A in the EC-coupling process is highly debated (11–13), we have recently shown by using plasmon resonance measurements that it is the single II–III loop sequence interacting with RyR1 (14). Nevertheless, this exceptional form of homology between a channel sequence and a toxin could be indicative that domain A possesses some kind of yet unresolved function in RyR1 regulation. Therefore, studying the MCa effects on RyR1 may produce several interesting hints on how to proceed further on investigating the role of domain A in EC coupling.

In this work, we synthesized several MCa analogues in which amino acid residues, also present in homologous position in domain A, were substituted by alanine residues. We then analyzed the effect of MCa and the analogues on $[^3H]$ryanodine

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¶¶Supported by a grant from the European Commission (RTN2-2001-00337) and AFM. To whom correspondence should be addressed. Tel.: 33-4-38-78-46-69; Fax: 33-4-38-78-50-41; E-mail: mronjat@cea.fr.
†The abbreviations used are: SR, sarcoplasmic reticulum; RyR1, ryanodine receptor; DHPR, dihydropyridine receptor; IpTxA, imperatoxin A; Fmoc, N-$\text{N}$-$\text{N}$-$\text{N}$-fluorenylmethoxycarbonyl; MOPS, 3-$(\text{N}$-$\text{N}$-$\text{N}$-$\text{N}$-morpholino)propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; LSSS, long-lasting subconductance state; EC, excitation-contraction; CMC, 4-chloro-$m$-cresol; MCa, maurocalcine.

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binding, Ca°-dependent activation of RyR1, channel activity, and Ca° release from SR vesicles. We identified an amino acid residue critical for the interaction of MCa with RyR1 and for the induction of a long-lasting subconductance state. The data also demonstrate a clear relationship in the ability of MCa to potentiate [3H]ryanodine binding, induce a subconductance state, and produce Ca° release from heavy SR vesicles. To probe the physiologic relevance of RyR1 as the main target of MCa, we tested the effect of the peptide on internal Ca° release in intact myotubes. We show that the addition of 100 nM MCa in Ca°-free extracellular medium induces an increase in cytosolic Ca° concentration and a complete inhibition of CMC-induced Ca° release.

EXPERIMENTAL PROCEDURES

Chemical Synthesis—N-$\text{Fmoc}$-t-amino acids, 4-hydroxymethyl-phenyloxyl 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 (15) 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 maurocalcine native-like) and named Ala/Lys°20, Ala/Lys°22, Ala/Lys°23, Ala/Lys°24, and Ala/Thr°26. Peptide chains were assembled stepwise on 0.25 meq of hydroxymethylphenyloxyl resin (1% cross-linked; 0.89 meq of amino group/g) using 1 mmol of Fmoc-amino acid derivatives as their hydroxybenzotriazole active esters in N,N,N,N-tetramethylpyrrolidone for 3 and 8 min, respectively. The $\text{N}_{\text{Fmoc}}$ amino acid derivatives. The side chain-protecting groups were as follows: ttrityl for Cys and Asn; tert-butyl for Ser, Thr, Glu, and Asp; pentamethylethoxy carbonyl for Arg; and tert-butylxycarbonyl for Lys. $\text{N}_{\text{Fmoc}}$-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 meq/g) was treated between 2 and 3 h at room temperature in constant shaking with a mixture of trifluoroacetic acid/H2O/thioanisol/ethanedithiol (88:6:3:v/v) and filtered, and the filtrate was precipitated by adding a 200- or 250-$\mu$m diameter aperture of a Delrin cap using a symmetrical buffer solution (250 mM KCl, 100 $\mu$M EGTA, 150 $\mu$M CaCL, 20 mM PIPES, pH 7.2). The chamber into the small aliquot of purified RyR1 selected at an appropriate concentration and defined by the cis (cytoplasmic) side, whereas the other channel labelled as the trans (luminal) side was kept free of gramicidin. To ensure the orientation of the incorporated RyR1, we tested the effect of free Ca° concentration 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 Axoscope 200 and pCLAMP 6.03 (Axon Instruments, Union City, CA).

Total recording time in each experiment was 10–20 min for any 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 20–22 °C. The free Ca°+ concentration was calculated using the computer program and affinity constants published by Fabiato (21). Open probabilities were calculated using the common 50% criteria with a median dead zone of 5%. Amplitude and distribution were analyzed using Origin (Microcal Software, Northampton, MA). Values of the open probability are expressed as the means ± S.E.

Cell Culture—Primary cultures of rat satellite cells were obtained as described previously (16). Cells were seeded on laminin-coated plates, in a proliferation medium composed of Ham’s F-10 with glutamax-I (Invitrogen) supplemented with 20% fetal calf serum, 2.5 ng/ml basic fibroblast growth factor (Invitrogen), 100 U/ml penicillin-streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Gibco, Union City, CA). Protein concentration was measured by the Biuret method.

RESULTS

Synthesis of MCa and Analogues—Fig. 1 presents the primary structure of the different MCa analogues that we synthesized. The structural determination of MCa shows that the stretch of basic residues (from Lys°20 to Arg°27) forms a single basic-rich surface (22). We will define the residues forming this basic surface as residues belonging to the “basic class.” The opposite surface of the toxin contains four acidic residues (Asp°, Glu°, Glu°1, and Glu°2).

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Glu$^{12}$, Asp$^{15}$, and Glu$^{29}$) and one basic residue (Lys$^{6}$). We will define this acidic surface as the “acidic face.” This electrostatic charge distribution creates a marked anisotropy in which the role in MCa pharmacology is unknown. We choose to substitute charge distribution creates a marked anisotropy in which the role in MCa pharmacology is unknown. We choose to substitute

All of the MCa analogues also stimulate $[^3H]$ryanodine binding when the presence of varying external Ca$^{2+}$ concentrations (Fig. 2D). In the presence of MCa (saturating 100 nM), $[^3H]$ryanodine binding on SR vesicles still shows a classical bell-shaped dependence to external Ca$^{2+}$. Interestingly, we observed that the $[^3H]$ryanodine binding inhibition by high Ca$^{2+}$ concentration (pCa 3) was slightly decreased, whereas the $[^3H]$ryanodine binding stimulation by low Ca$^{2+}$ concentration (pCa 6 and 6.5) was increased. Indeed, while in the absence of MCa, ryanodine binding was only at 49% of its maximum in the presence of MCa, 39% of the maximum $[^3H]$ryanodine binding was still observed at pCa 3. Similarly, at pCa 6.5, ryanodine binding is at 70% of its maximum in the presence of MCa, whereas it is only at 4.8% of its maximum in the absence of MCa.

Characterization of the Effects of MCa Mutants on $[^3H]$Ryanodine Binding to RyR1—All of the MCa analogues also stimulate $[^3H]$ryanodine binding (Fig. 3A) with the exception of [Ala$^{24}$]MCa (up to 5 μM). For all of the functional analogues, the $EC_{50}$ of the stimulatory effect is reduced with values ranging between 49 ± 14 nM for [Ala$^{20}$]MCa and 432 ± 168 nM for [Ala$^{22}$]MCa, which would correspond to a reduction of affinity between 4- and 35-fold. [Ala$^{8}$]MCa also appeared less effective than MCa with an $EC_{50}$ of 199 ± 19 nM. These data show that the amino acid residues of MCa can be classified in three different groups: (i) residues belonging to the basic class that are important for interaction with RyR1 (Lys$^{20}$, Arg$^{23}$, and Arg$^{24}$); (ii) residues belonging or not to the basic class that appear less important for the interaction with RyR1 (Lys$^{19}$, Lys$^{16}$, and Thr$^{26}$); and (iii) a residue of the acidic face that appears to mildly affect the interaction with RyR1 (Lys$^{8}$).

Besides various effects on $EC_{50}$, all of the active analogues also present a significant decrease on the stimulation efficacy on $[^3H]$ryanodine binding. The stimulation efficacies of the analogues are on average 1.9-fold less than MCa itself and ranged between 3.2 ± 0.4-fold for [Ala$^{25}$]MCa and 4.7 ± 0.1-fold for [Ala$^{8}$]MCa. [Ala$^{23}$]MCa combines two negative changes com-
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Fig. 2. MCa affects [3H]ryanodine binding on heavy SR vesicles. A, MCa concentration dependence of [3H]ryanodine binding stimulation. [3H]Ryanodine binding was measured at pCa 5 in the presence of 5 nM [3H]ryanodine for 2.5 h at 37 °C. Nonspecific binding remained constant at all of the MCa concentrations and represented <8% of total binding. Data were fitted with a sigmoid function \( y = y_c + (a \times x^b/a + x^b) \), where \( y_c = 1.0 \pm 0.1 \) at MCa = 0 nM, \( a = 6.7 \pm 0.2 \) is the maximum stimulation factor over basal value, \( b = 1.3 \pm 0.1 \) the slope coefficient, and \( c = 12.5 \pm 0.8 \) nM is the EC50 for MCa effect. B, kinetics of [3H]ryanodine binding in the absence (filled circles) and presence of 100 nM MCa (open circles). Data were fitted with an exponential curve \( y = a \times (1 - \exp(-k_b)) \) with \( a \) being the maximal achievable normalized binding and \( k_b \) being the pseudo first order rate constant (\( k_{obs} \)) value for [3H]ryanodine binding. \( a = 0.99 \pm 0.01 \) and \( k_{obs} = 1.08 \times 10^{-2} \pm 0.22 \times 10^{-2} \text{ min}^{-1} \) (without MCa), and \( a = 0.94 \pm 0.01 \) and \( k_{obs} = 1.81 \times 10^{-2} \pm 0.23 \times 10^{-2} \text{ min}^{-1} \) (with 100 nM MCa). C, Scatchard representation of the concentration-dependent [3H]ryanodine binding in the absence (filled circles) and presence of 100 nM MCa (open circles). Experimental conditions in B and C were as described in A. D, extravesicular Ca\(^{2+}\) concentration dependence of [3H]ryanodine binding in the absence (filled circles) and presence of 100 nM MCa (open circles). The experiments were performed with 5 nM [3H]ryanodine for 2.5 h at 37 °C.

Characterization of the Effects of MCa and MCa Mutants on Ca\(^{2+}\) Release from SR Vesicles—To investigate the effect of MCa on Ca\(^{2+}\) release from heavy SR vesicles, we first actively loaded the vesicles by two consecutive additions of Ca\(^{2+}\) (50 \(\mu\)M and 20 \(\mu\)M) in the presence of ATP-Mg\(^{2+}\), pyrophosphate, and ATP-regenerating system. After Ca\(^{2+}\) loading reaches equilibrium, the addition of 1 \(\mu\)M MCa to the external medium induces Ca\(^{2+}\) release as shown in Fig. 4A. Similar results were obtained with 100 nM MCa (data not shown), but 1 \(\mu\)M MCa was chosen to allow a comparison with the effects of the lower affinity MCa analogues. External Ca\(^{2+}\) level reaches a plateau that corresponds to a new equilibrium between Ca\(^{2+}\) release and Ca\(^{2+}\) uptake rates as evidenced by the additional change produced by 4 \(\mu\)M A23187 calcium ionophore. The release of Ca\(^{2+}\) induced by 1 \(\mu\)M MCa was prevented by preincubating the SR vesicles with 10 \(\mu\)M ruthenium red, demonstrating that MCa-induced calcium release occurs through RyR (Fig. 4B). Similarly, the application of 1 \(\mu\)M MCa prevents the release of Ca\(^{2+}\) induced by the addition of 500 \(\mu\)M CMC, and conversely, the application of 500 \(\mu\)M CMC prevents an additional release of Ca\(^{2+}\) by 1 \(\mu\)M MCa (Fig. 4C). These data again demonstrate that MCa re-

pared with wild-type MCa, a decrease in the apparent affinity and the lowest [3H]ryanodine binding stimulation efficacy. Two explanations can be put forward to explain why [Ala24]MCa is ineffective on [3H]ryanodine binding. Firstly, it may not bind to RyR1, which is the simplest hypothesis. Secondly, it may bind onto RyR1 but cannot produce allosteric changes required to enhance [3H]ryanodine binding. In this latest case, [Ala24]MCa would compete with MCa for binding on RyR1. To test this hypothesis, we measured the effect of MCa (20 and 100 nM) on [3H]ryanodine binding in the presence of increasing concentrations of [Ala24]MCa. Fig. 3B shows that the effect of MCa is not altered by the presence of up to 1 \(\mu\)M [Ala24]MCa, demonstrating that this analogue is not able to bind onto RyR1. We also checked whether or not the absence of the effect of [Ala24]MCa on [3H]ryanodine binding is related to experimental pCa conditions. Indeed, no [3H]ryanodine binding stimulation was observed at pCa values ranging between 2 and 7 (Fig. 3C).
leases Ca\(^{2+}\) from SR vesicles by acting on RyR. Fig. 4D shows that the application of 1 \(\mu\)M of any one of the mutated MCa analogues induces Ca\(^{2+}\) release from SR vesicles with the exception of [Ala\(^{23}\)]MCa and [Ala\(^{24}\)]MCa. However, these two last MCa analogues are not equivalent since application of 10 \(\mu\)M [Ala\(^{23}\)]MCa resulted in Ca\(^{2+}\) release, whereas 10 \(\mu\)M [Ala\(^{24}\)]MCa remained inactive (Fig. 4E). The present observation made for [Ala\(^{24}\)]MCa is in complete agreement with its lack of effect on \(^{3}H\)ryanodine binding (Fig. 3B). The results obtained with [Ala\(^{23}\)]MCa are also coherent with the data on \(^{3}H\)ryanodine binding (lower affinity and lower stimulation efficacy), although a discrepancy exists between the [Ala\(^{23}\)]MCa concentration needed for Ca\(^{2+}\) release and those inducing an increase in \(^{3}H\)ryanodine binding.

**Modification of RyR1 Calcium Channel Conductance by MCa and Analogues**—Application of 200 nM MCa to the cis face of the bilayer-recording chamber (cytoplasmic side of RyR1) induces a characteristic long-lasting subconductance state (LLSS) (Fig. 5A). This subconductance state represents 60% of the full conductance state, and the channel spends 54.8 \pm 6.2% of its time in this subconductance level. A much less frequent smaller LLSS (48% of the full conductance) was described in earlier reports (5, 6), but the experimental conditions were slightly different (purified RyR1 versus junctional SR vesicles and K\(^{+}\) current versus Cs\(^{+}\) current in previous reports (5, 6)).

We next examined whether the LLSS induced by MCa could be correlated to Ca\(^{2+}\) release from heavy SR vesicles and stimulation of \(^{3}H\)ryanodine binding. Therefore, we tested the effect of [Ala\(^{24}\)]MCa on the conductance level of RyR1 in lipid bilayers (Fig. 5B). Indeed, this mutant peptide has no effect on \(^{3}H\)ryanodine binding (Fig. 3A) nor on the Ca\(^{2+}\) release from SR vesicles (Fig. 4D). As expected, we show that the application of 200 nM [Ala\(^{24}\)]MCa does not induce any LLSS of RyR1. Applications of higher concentrations (up to 750 nM) also were without any effect (data not shown). Conversely, the application of 200 nM of an active MCa analogue, [Ala\(^{20}\)]MCa, that conserves MCa-like properties (for \(^{3}H\)ryanodine binding and Ca\(^{2+}\) release from SR vesicles) produces the appearance of the charac-
FIG. 4. Ca$^{2+}$ release from heavy SR vesicles induced by MCa and MCa analogues. A, heavy SR vesicles were actively loaded with Ca$^{2+}$ by sequential addition of 50 and 20 μM CaCl$_2$ in the monitoring chamber. After each increment, the absorbance was monitored until the added Ca$^{2+}$ was taken up by SR and the trace relaxed close to its original baseline (CaCl$_2$ additions constituting ~70–80% of their loading capacity). In these conditions, the addition of 1 μM MCa produces Ca$^{2+}$ release. The addition of 4 μM A23187 empties the SR vesicles (open arrow). Calibration of Ca$^{2+}$ release is performed by two consecutive additions of 20 μM Ca$^{2+}$ (data not shown). B, similar experiment as in A but with a preliminary application of 10 μM ruthenium red (RR). RR produces a deflection of absorbance due to its optical properties. C, traces illustrating the lack of effect of 500 μM CMC after MCa application (left) and, conversely, the lack of effect of 1 μM MCa after application of 500 μM CMC (right). D, similar experiments as in A but with 1 μM of various MCa analogues. Two consecutive applications were performed for [Ala$^{23}$]MCa. E, traces illustrating the maintenance of effect of MCa on Ca$^{2+}$ release despite the prior application of [Ala$^{23}$]MCa (left) or [Ala$^{24}$]MCa (right).
FIG. 5. MCa but not [Ala24]MCa induces long-lasting openings of RyR1. Single channel recordings were taken as described under “Experimental Procedures.” The holding potential was −80 mV, the conductance of the RyR was approximately 500 pS. The time duration of each record is 20 s. Channel openings are downward deflections, and C and O designate the closed and opened state of the channel, respectively. Each toxin was applied at a concentration of 200 nM in the cis chamber. Free Ca²⁺ concentration was 238 nM in the cis chamber. A, control and MCa-induced RyR1 channel activity. Left panel, representative current traces. Right panel, current amplitude histograms. B, as in A but for [Ala24]MCa. C, as in A but for [Ala20]MCa.
**Fig. 6. MCa induces a cytosolic Ca\(^{2+}\) transient in cultured myotubes.** A, confocal images of myotubes after 3 days in culture. Left, transmitted light image (differential interference contrast). Right and bottom, Fluo-4 fluorescence images acquired before (control) and at indicated times after application of 100 nM MCa. B, MCa-induced fluorescence variations of Fluo-4-loaded myotubes measured in the absence (−) and presence (+) of extracellular Ca\(^{2+}\). C, mean peak value of Fluo-4 fluorescence variation induced by 100 nM MCa with or without extracellular Ca\(^{2+}\). D, fluorescence variation induced by 250 \(\mu\)M CMC without or with (black line) or without (gray line) a preapplication of 100 nM MCa. E, mean peak value of fluorescence variations induced by CMC without or with preapplication of 100 nM MCa. F, percent of myotubes as a function of the peak value of the fluorescence change induced by CMC without or with preapplication of MCa.

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Intracellular Ca\(^{2+}\) Release in Intact Myotubes Induced by MCa—We next analyzed the effect of MCa on cytosolic Ca\(^{2+}\) variations by changes in Fluo-4 fluorescence levels in cultured myotubes. Extracellular application of 100 nM MCa on a group of representative myotubes produces a fast increase in intracellular Ca\(^{2+}\) level (Fig. 6A). The time course of the change in fluorescence level (\(\Delta F/F\)) shows that Ca\(^{2+}\) increase occurs within 3 s followed by a rapid decrease back to the basal level (Fig. 6B). MCa produces a similar elevation in cytosolic Ca\(^{2+}\) when extracellular medium is supplemented with 50 \(\mu\)M La\(^{3+}\) and deprived of Ca\(^{2+}\) by the addition of 1 mM EGTA (Fig. 6B). This observation demonstrates that MCa-induced Ca\(^{2+}\) mobilization occurs from internal sources. An analysis of the average change in fluorescence level confirms that there is no significant difference in cytosolic Ca\(^{2+}\) elevation in the absence or presence of extracellular Ca\(^{2+}\) (Fig. 6C). In similar experimental conditions, extracellular application of 250 \(\mu\)M CMC also induces a rapid and transient Ca\(^{2+}\) elevation (Fig. 6D) (24). A prior incubation of the myotube with 100 nM MCa almost totally prevents CMC-induced peak Ca\(^{2+}\) elevation (Fig. 6D). This observation was confirmed by an average quantification of fluorescence variation (mean \(\Delta F/F = 79 \pm 67\) (n = 53) for CMC alone versus 4 \pm 7 (n = 91) for CMC after MCa) (Fig. 6E). We also analyzed the cell variability in response to CMC alone or CMC after MCa application (Fig. 6F). In the presence of CMC, most myotubes (85.6%) produce important changes in fluorescence (\(\Delta F/F > 50\%\)), whereas after incubation with MCa, an opposite profile of responses is observed with 47% of the cells showing <10% change in fluorescence (\(\Delta F/F = 0–10\%\)). These results demonstrate that the CMC-sensitive Ca\(^{2+}\) stores are also MCa-sensitive. Because CMC is known to act on intracellular Ca\(^{2+}\) stores by the bias of RyR (2), this is another indication that MCa acts on RyR-dependent Ca\(^{2+}\) stores. To confirm that the effect of MCa on Ca\(^{2+}\) elevation is the result of an interaction with RyR, we also tested the effect of the mutant [Ala\(^{24}\)]MCa. We show that it is unable (i) to produce a Ca\(^{2+}\) release from myotubes (Fig. 7A) and (ii) to modify CMC effect (Fig. 7B).

**DISCUSSION**

In skeletal muscles, RyR1 appears to represent the main target of MCa. We have previously shown that this toxin stimulates \(^{3}H\)ryanodine binding, stabilizes RyR1 into a subconductance state, and produces Ca\(^{2+}\) release from heavy SR vesicles. In this work, we further investigated the biochemical and functional properties of MCa. Using synthetic mutated analogues of MCa, we defined some critical amino acid residues of MCa that are required for its effects on RyR1. We also show for the first time that MCa is able to induce intracellular Ca\(^{2+}\) release in intact myotubes. This effect is conserved in the absence of external Ca\(^{2+}\), demonstrating that the increase in...
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FIG. 7. [Ala^{24}]MCa does not induce Ca^{2+} release in intact myotubes. A, variation of the cytoplasmic Ca^{2+} concentration upon external application of 1 μM [Ala^{24}]MCa as measured in the experiments depicted in Fig. 6. B, mean peak value of fluorescence variations induced by 250 μM CMC without or with preapplication of 1 μM [Ala^{24}]MCa.

cytoplasmic Ca^{2+} concentration is due to the release of Ca^{2+} from internal stores. In these conditions, Ca^{2+} release induced by CMC, a RyR1 agonist, is completely inhibited by the preapplication of MCA. Conversely, the application of the inactive [Ala^{24}]MCa analogue does not induce any Ca^{2+} release from internal stores nor does it inhibit CMC-induced Ca^{2+} release. These data demonstrate that Ca^{2+} release induced by MCA occurs through RyR1 activation. The cellular effect of MCA is of the greatest importance because it demonstrates that the toxic effect of the toxin can be explained by its action on RyR1 despite the intracellular location of this target. This result also reveals that MCA has the ability to cross the plasma membrane of skeletal muscle cells, a property that is shared by some other basic peptides (25). Cell penetration is expected to occur rapidly as Ca^{2+} elevation can be observed a few seconds after external application of MCA.

We have identified Arg^{24} as a crucial amino acid residue for the effects of MCA. Indeed, the replacement of Arg^{24} by an alanine induces a complete loss of MCA effects on [^{3}H]ryanodine binding, on the appearance of subconductance events, on Ca^{2+} release from SR vesicles, and on intact myotubes. Conversely, mutated analogues that appeared less crucial for RyR1 function. Recently, it was proposed that the three-dimensional structural surface of MCA and IpTxA mimics that of domain A of the 1 subunit of DHPR that shares sequence similarities with both MCA and IpTxA and induces a subconductance state different from those observed with the toxin (65 and 86% of the full-conductance state for domain A) (6). Based on sequence identity, it is probable that MCA and IpTxA bind more than one common site(s) or regions on the RyR1 tetramer. Interestingly, the binding of two structurally related molecules on the same channel can produce highly different conductance states of RyR1. Therefore, it is tempting to postulate that once MCA or IpTxA is bound on RyR1, structural modification of these ligands could produce important changes in the conductance properties of RyR1. This concept of RyR1 conductance modulation could be applied to domain A to provide an explanation for its role in the regulation of RyR1 function. Recently, it was proposed that the three-dimensional structural surface of MCA and IpTxA mimics that of domain A (23). It would be interesting to investigate whether conformational changes in domain A during membrane depolarization are of nature to modify RyR1 channel conductance.

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