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## TRANSIENT LOSS OF VOLTAGE CONTROL OF $Ca^{2+}$ RELEASE IN THE PRESENCE OF MAUROCALCINE IN SKELETAL MUSCLE

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## ABSTRACT

In skeletal muscle, sarcoplasmic reticulum (SR) calcium release is controlled by the plasma membrane voltage through interactions between the voltage sensing dihydropyridine receptor (DHPr) and the ryanodine receptor (RYr) calcium release channel. Maurocalcine (MCA), a scorpion toxin peptide presenting some homology with a segment of a cytoplasmic loop of the DHPr has been previously shown to strongly affect the activity of the isolated RYr. We injected MCA into mouse skeletal muscle fibers and measured intracellular calcium under voltage-clamp conditions. Voltage activated calcium transients exhibited similar properties in control and in MCA-injected fibers during the depolarizing pulses and the voltage dependence of calcium release was similar under the two conditions. However, MCA was responsible for a pronounced sustained phase of  $\text{Ca}^{2+}$  elevation that proceeded for seconds following membrane repolarization, with no concurrent alteration of the membrane current. The magnitude of the underlying uncontrolled extra-phase of  $\text{Ca}^{2+}$  release correlated well with the peak calcium release during the pulse. Results suggest that MCA binds to RYr that open upon membrane depolarization and that this interaction specifically alters the process of repolarization-induced closure of the channels.

## INTRODUCTION

Optimal control of voluntary movement and force production relies ultimately on the efficacy of excitation-contraction (e-c) coupling of skeletal muscle. The contraction of a skeletal muscle fiber is rapidly initiated and stopped by changes in the voltage across the plasma membrane, physiologically an action potential or a train of action potentials. The coupling between membrane depolarization and the resulting transient rise in cytoplasmic  $[Ca^{2+}]$  that activates the contractile machinery is ensured by two types of ion channel proteins facing each other, across an ~15 nm wide gap at the triadic junction; these two are the DHPr in the plasma/transverse-tubule membrane, that senses the changes in voltage and the RYr calcium release channel in the SR membrane. The opening and closure of the RYr calcium channel is subordinated to voltage-dependent conformational changes of the DHPr but the details of the functional interactions between the two still remain obscure. Since the early nineties several lines of evidence have highlighted the importance of the II-III cytoplasmic loop of the  $\alpha 1$  subunit of the DHPr in these interactions (1-3). Within this loop, a 20 amino acid fragment named domain A has received particular attention because the corresponding isolated peptide is capable of modulating  $Ca^{2+}$  release from SR preparations as well as the activity of the isolated RYr channel (4-9). The situation is, however, not so clear since both activating and inhibiting effects have been reported. Furthermore the relevance and exact function of this segment during physiological e-c coupling has been seriously challenged by negative results in studies that used expression of chimeras of the  $\alpha 1$  subunit in dysgenic myotubes (10-12).

MCa is a 33 amino acid toxin isolated from the venom of the scorpion *Scorpio Maurus Palmatus* (13). MCa is very similar to Imperatoxin A (IpTxa), a venom toxin from another scorpion and both share some sequence homology and a striking similarity in the spatial orientation of positively charged residues with domain A of the II-III loop of the DHPr (see 14, 15). Interestingly both MCa and IpTxa can enhance  $[^3H]$  ryanodine binding to SR vesicles, increase ion flow through isolated ryanodine receptors, elicit  $Ca^{2+}$  release from SR vesicles and modulate the properties of elementary calcium release events detected in permeabilized muscle fibers (6, 13, 14, 16, 17, 18, 19). In the case of MCa, there is also clear evidence that it binds to the same site of the RYr as domain A (20). This potentially makes these two toxins highly interesting tools to characterize the still elusive role of domain A of the DHPr in the e-c coupling process. Here we injected MCa into adult skeletal muscle fibers from mouse and measured intracellular  $[Ca^{2+}]$  under voltage-clamp conditions. Results show that MCa dramatically affects the process of repolarization-induced closure of the RYrs during physiological e-c coupling.

## METHODS

### *Preparation of the muscle fibers*

Experiments were performed on single fibers isolated from the *flexor digitorum brevis (fdb)* muscles from 4-8 weeks-old Swiss OF1 mice. All experiments were performed in accordance with the guidelines of the French Ministry of Agriculture (87/848) and of the European Community (86/609/EEC). Procedures for enzymatic isolation of single muscle fibers, partial insulation of the fibers with silicone grease and intracellular micro-injection of the dye indo-1, were as described previously (21-24). In brief, the major part of a single fiber was electrically insulated with silicone grease so that whole-cell voltage clamp could be achieved on a short portion of the fiber extremity. Prior to voltage-clamp, indo-1 was introduced into the myoplasm through local pressure micro-injection. Intracellular loading of either MCa or of its mutated analogue  $[Ala^{24}]McA$  was achieved by co-injection with the dye; peptides were present in the micro-injected solution at a concentration of 100  $\mu M$ . Following diffusion and

equilibration within the cytoplasm, this was believed to achieve a final cytoplasmic concentration within the 10 micromolar range (for details concerning micro-injections see 25). It should be noted that the effects seen in the present study required two orders of magnitude more M<sub>Ca</sub> than in experiments in more isolated systems. One likely reason for this discrepancy is that most of the injected M<sub>Ca</sub> was bound to sites within the fiber so that the free M<sub>Ca</sub> concentration was very much lower than the above estimated level.

In a separate series of experiments we also tested the effect of the injection of a synthetic domain A peptide corresponding to residues Thr<sup>671</sup>-Leu<sup>690</sup> of the  $\alpha 1$  subunit of the DHP<sub>r</sub>; this peptide was used at a concentration of 2 mM in the injecting pipette. Control data were always obtained from fibers issued from the same muscles as the toxin- or peptide-injected fibers. The number of fibers tested under a given set of conditions was always obtained from muscles taken from at least 3 different animals.

All experiments were performed at room temperature (20-22 °C).

### ***Electrophysiology***

An RK-400 patch-clamp amplifier (Bio-Logic, Claix, France) was used in whole-cell configuration. Command voltage pulse generation and data acquisition were done using commercial software (Biopatch Acquire, Bio-Logic) driving an A/D, D/A converter (Lab Master DMA board, Scientific Solutions Inc., Mentor, OH, USA). Analog compensation was systematically used to decrease the effective series resistance. Voltage-clamp was performed with a microelectrode filled with the intracellular-like solution (see Solutions). The tip of the microelectrode was inserted through the silicone, within the insulated part of the fiber. Membrane depolarizations (or series of depolarizations) were applied every 30 s from a holding command potential of -80 mV.

### ***Fluorescence measurements***

The optical set-up and the procedures used for indo-1 fluorescence measurements were as described previously (21, 22, 23, 26). In brief, a Nikon Diaphot epifluorescence microscope was used in diafluorescence mode. The beam of light from a high-pressure mercury bulb set on the top of the microscope was passed through a 335 nm interference filter and focused onto the preparation. The emitted indo-1 fluorescence light was collected by a 40x objective and simultaneously detected at  $405 \pm 5$  nm ( $F_{405}$ ) and  $470 \pm 5$  nm ( $F_{470}$ ) by two photomultipliers. The fluorescence measurement field was 40  $\mu$ m in diameter and the silicone-free extremity of each tested fiber was placed in the middle of the field. Background fluorescence at both emission wavelengths was measured next to each tested fiber and was then subtracted from all measurements.

### ***Calibration of the indo-1 response and $[Ca^{2+}]_i$ calculation***

The standard ratio method was used with the parameters:  $R = F_{405}/F_{470}$ , and  $R_{min}$ ,  $R_{max}$ ,  $K_D$ ,  $\beta$  having their usual definition. Results were either expressed in terms of indo-1 percent saturation or in actual free calcium concentration (for details of calculation, see 21, 25). *In vivo* values for  $R_{min}$ ,  $R_{max}$  and  $\beta$  were measured using procedures described previously (22, 23).

### ***Calculation of the rate of calcium release***

The method used to estimate the SR calcium release flux from the indo-1 transients was previously described (24): the time derivative of the total myoplasmic  $Ca^{2+}$  was calculated from the occupancy of the main intracellular calcium binding sites (27). The model included troponin C binding sites with a total sites concentration  $TN_{total}$  of 250  $\mu$ M, an “on” rate constant  $k_{on, CaTN}$  of  $0.0575 \mu M^{-1}.ms^{-1}$  and an “off” rate constant  $k_{off, CaTN}$  of  $0.115 ms^{-1}$ ; Ca-Mg binding sites on parvalbumin with a total sites concentration  $PV_{total} = 700 \mu$ M, “on” rate constant for  $Ca^{2+}$   $k_{on, CaPV} = 0.125 \mu M^{-1}.ms^{-1}$ , “off” rate constant for  $Ca^{2+}$   $k_{off, CaPV} = 5.10^{-4} ms^{-1}$ .

<sup>1</sup>, “on” rate constant for  $\text{Mg}^{2+}$   $k_{on, MgPV} = 3.3 \cdot 10^{-5} \mu\text{M}^{-1} \cdot \text{ms}^{-1}$ , “off” rate constant for  $\text{Mg}^{2+}$   $k_{off, MgPV} = 3 \cdot 10^{-3} \text{ms}^{-1}$ . The rate of calcium transport across the SR membrane was assumed to be proportional to the fractional occupancy of the SR pump sites with a dissociation constant  $Kd_{Ca_{pump}}$  of  $2 \mu\text{M}$  and a maximum pump rate of  $3 \mu\text{M} \cdot \text{ms}^{-1}$ . Resting  $[\text{Mg}^{2+}]$  was assumed to be  $1.5 \text{mM}$ . Indo-1 was assumed to be present at  $100 \mu\text{M}$  with values for the “on” and “off” rate constants of the Ca-indo-1 binding reaction of  $1 \cdot 10^8 \text{M}^{-1} \cdot \text{s}^{-1}$  and  $30 \text{s}^{-1}$ , respectively.

### ***Simulation of indo-1 transients***

Simulations of [Ca-indo-1] transients elicited by membrane depolarizations were performed according to the procedure of Timmer *et al.* (28), as described in Collet *et al.* (23). In brief, a synthetic  $\text{Ca}^{2+}$  release rate waveform was fed into a calcium distribution model that reproduced the main features of  $\text{Ca}^{2+}$  regulation in a muscle fiber. The model included the intracellular  $\text{Ca}^{2+}$  binding sites and uptake mechanism described in the previous paragraph. Resting  $[\text{Ca}^{2+}]$  was assumed to be  $0.1 \mu\text{M}$ .

### ***Solutions***

The intracellular-like solution contained (in mM) 120 K-glutamate, 5  $\text{Na}_2\text{-ATP}$ , 5  $\text{Na}_2\text{-phosphocreatine}$ , 5.5  $\text{MgCl}_2$ , 5 glucose, 5 HEPES adjusted to pH 7.20 with K-OH. The standard extracellular solution contained (in mM) 140 TEA-methanesulfonate, 2.5  $\text{CaCl}_2$ , 2  $\text{MgCl}_2$ , 10 TEA-HEPES and 0.002 tetrodotoxin, pH 7.20.

### ***Statistics***

Least-squares fits were performed using a Marquardt-Levenberg algorithm routine included in Microcal Origin (Originlab, Northampton, MA, USA). Data values are presented as means  $\pm$  S.E.M. for  $n$  fibers, where  $n$  is specified in Results. Statistical significance was determined using a Student’s t-test assuming significance for  $P < 0.05$ .

## **RESULTS**

The effect of MCa and of its mutated inactive analog  $[\text{Ala}^{24}]\text{MCa}$  (14) were tested on membrane current and intracellular calcium measured from isolated mouse skeletal muscle fibers. The mean initial resting  $[\text{Ca}^{2+}]$  level did not significantly differ between control fibers ( $58 \pm 12 \text{nM}$ ,  $n=16$ ), MCa-injected fibers ( $49 \pm 6 \text{nM}$ ,  $n=22$ ) and  $[\text{Ala}^{24}]\text{MCa}$ -injected fibers ( $62 \pm 11 \text{nM}$ ,  $n=7$ ), indicating that the peptides did not chronically alter the intracellular resting  $\text{Ca}^{2+}$  homeostasis. However, the presence of MCa produced a very remarkable change in the properties of the voltage activated  $\text{Ca}^{2+}$  transients. This is illustrated in Fig. 1A which shows superimposed indo-1 calcium signals that were elicited by successive step membrane depolarizations of 10, 30 and 50 ms duration to +10 mV in a control fiber, an MCa-injected fiber and an  $[\text{Ala}^{24}]\text{MCa}$ -injected fiber. In all fibers, and as classically described, voltage activated SR  $\text{Ca}^{2+}$  release produced a rapid rise in the calcium indicator saturation level and the signal remained elevated during the pulse. In the control fiber and in the  $[\text{Ala}^{24}]\text{MCa}$ -injected fiber, membrane repolarization turned off  $\text{Ca}^{2+}$  release and as a consequence the indo-1 signal returned towards its resting level with a time constant in the hundred of milliseconds range, as typically observed under normal conditions (see for instance 21, 22, 26). In the MCa-injected fiber, there was also a repolarization-induced sharp decay of the indo-1 signal, but surprisingly the decline was only partial and the signal was still very much elevated at the end of the records. Pulses of 10 and 30 ms duration to + 10 mV were applied in several fibers issued from the same muscles upon these three conditions. The graphs in Fig. 1 give mean values for the corresponding baseline  $[\text{Ca}^{2+}]$  level (Fig. 1B), the peak  $\Delta[\text{Ca}^{2+}]$  during the pulse (Fig. 1C), the  $\Delta[\text{Ca}^{2+}]$  measured at the time of the end of the records shown in Fig. 1A (Fig. 1D, that is 0.92 s after the onset of the pulses, which we will refer to as 1 s

after the pulses for simplicity) and the ratio of  $\Delta[\text{Ca}^{2+}]$  measured 1 s after the pulse ( $\Delta[\text{Ca}^{2+}]_{1s}$ ) to the preceding peak  $\Delta[\text{Ca}^{2+}]$  (Fig. 1E).  $\text{Ca}^{2+}$  values were obtained without correction for the kinetics of indo-1 Ca-binding. Fibers were all challenged successively with 10 and 30 ms-long pulses, with records taken either as shown in Fig. 1A or with longer records taken at a slower sampling rate (as shown in Fig. 2). There was no significant difference in the baseline  $[\text{Ca}^{2+}]$  (Fig. 1B) and peak  $\Delta[\text{Ca}^{2+}]$  level (Fig. 1C) between the three groups of fibers. Conversely, the mean absolute  $\Delta[\text{Ca}^{2+}]$  measured 1 s after the pulse was significantly larger, for both pulse duration, in the MCa-injected fibers than in either the control fibers or the  $[\text{Ala}^{24}]$ MCa-injected fibers (Fig. 1D). In average the  $\Delta[\text{Ca}^{2+}]_{1s}$  value was ~30 % of the preceding peak  $\Delta[\text{Ca}^{2+}]$  in the MCa-injected fibers, and ~5 % in the control and in the  $[\text{Ala}^{24}]$ MCa-injected fibers (Fig. 1E). The fact that this ratio exhibited a similar value for the two pulses duration in the presence of MCa indicates that the amplitude of the phenomenon correlated well with the preceding extent of  $\text{Ca}^{2+}$  release activation during the pulse.

In Fig. 1A, it is noticeable that within the 30 s period of time between two consecutive pulses,  $[\text{Ca}^{2+}]$  had eventually returned to its initial resting level as witnessed by the absence of change in the baseline indo-1 saturation level. The MCa-induced sustained post-pulse  $[\text{Ca}^{2+}]$  level was thus a transient phenomenon. Examples of its overall time course are illustrated in Fig. 2 which shows 15 s-long indo-1 saturation records in a control fiber and in two MCa-injected fibers. Fibers were depolarized by short pulses to +10 mV of various duration. Although somewhat variable in pattern and amplitude, the post-pulse  $[\text{Ca}^{2+}]$  elevation observed in the presence of MCa always lasted for seconds and vanished with an extremely slow time course.

This long-lasting elevated intracellular  $[\text{Ca}^{2+}]$  level had to result from either an additional influx of  $\text{Ca}^{2+}$  into- or a reduced  $\text{Ca}^{2+}$  efflux from- the myoplasm. The possibility that MCa produced an extra-  $\text{Ca}^{2+}$  influx across the plasma membrane following a step membrane depolarization is unlikely because there was no specific change in the membrane current during and after the pulses in the MCa-injected fibers, as compared to the control ones. This is illustrated in Fig. 3 which shows indo-1 calcium transients and corresponding membrane current traces displayed at a high y scale magnification, elicited by three consecutive 5 ms-long pulses to +10 mV applied to a control fiber (Fig. 3A) and to an MCa-injected fiber (Fig. 3B). Whereas the presence of MCa induced a very reproducible long-lasting  $\text{Ca}^{2+}$  elevation after each of the pulses, the membrane current traces were very similar to those from the control fiber. Using data from the fibers used in Fig. 1, the mean change in membrane current  $\Delta I$  (from the pre-pulse holding level) measured at the end of the 10 ms-long pulses was  $-0.21 \pm 0.3$  (n=3),  $0.72 \pm 0.41$  (n=5) and  $1.46 \pm 0.8$  (n=5) A/F in control fibers, MCa-injected fibers and  $[\text{Ala}^{24}]$ MCa-injected fibers, respectively. When measured 1 s after the pulse,  $\Delta I$  was  $-0.024 \pm 0.006$  (n=5),  $-0.021 \pm 0.004$  (n=9) and  $-0.01 \pm 0.004$  A/F (n=5), in the same groups, respectively. For the two sets of measurements there was no significant difference between the three groups.

Furthermore, in 3 fibers injected with MCa we took measurements in the presence and in the absence of extracellular calcium. For this each tested cell was superfused continuously with the extracellular solution using a polyethylene capillary perfusion system operated by gravity. Figure 4 shows membrane currents and indo-1 calcium signals measured from one of these fibers in response to a 20 ms long voltage step to +10 mV in the control condition (left), in the absence of extracellular calcium (replaced by magnesium, middle) and after returning to the control calcium containing solution (wash, right). The membrane current records are presented on an expanded x scale for clarity. They were corrected for the passive linear components by subtracting the adequately scaled current measured in response to a 20 mV hyperpolarization. The membrane current traces in the presence of the control external solution clearly exhibit a negative inward phase due to the activation of the slow inward calcium current (see for instance 30). As expected this current was lost in the absence of extracellular calcium but there was no simultaneous effect on the amplitude of the post-pulse

sustained phase of  $[Ca^{2+}]$  elevation that resulted from the presence of MCa. In the 3 fibers tested the peak change in  $Ca^{2+}$  measured 1 s after the end of the pulse in the absence of extracellular calcium corresponded to  $86 \pm 9$  % of the value measured before in the presence of extracellular calcium. We believe that this definitely excludes the possibility of a significant contribution of external calcium to the effect of MCa.

The MCa-induced post-pulse elevated  $Ca^{2+}$  level was thus of intracellular origin. Since the phenomenon was only triggered upon voltage activation of the fibers and because the initial fast decline of the calcium transients appeared unaffected, there is little doubt that the underlying mechanism was a transient dysfunction of SR  $Ca^{2+}$  release. This of course is also in agreement with the results from previous measurements on SR vesicles and single ryanodine receptor channels that showed a selective effect of MCa on the calcium release channel activity with no concurrent alteration of the SERCA-mediated  $Ca^{2+}$  uptake (14, 29). The features of the MCa-induced post pulse  $Ca^{2+}$  elevation are consistent with a slowly decaying extra- phase of  $Ca^{2+}$  release proceeding for seconds following membrane repolarization. This is simulated in Fig. 5 using a model of calcium distribution (see Methods). Figure 5A shows synthetic  $Ca^{2+}$  release flux traces in control conditions (thin trace) and in the presence of MCa (thick trace). The inset in Fig. 5A shows an enlarged view of the  $Ca^{2+}$  release flux traces assumed to be activated by the depolarizing voltage step (V). The  $Ca^{2+}$  flux waveform was made to have an early transient component that spontaneously decayed during the pulse towards a steady level. Upon repolarization, the flux was made to turn off with a time constant of 3 ms. Figure 5B shows the same traces on a longer time scale. The control and the MCa flux traces were made to be identical until the end of the pulse; thereafter the difference between the two was the presence of an additional small slowly decaying extra-phase of  $Ca^{2+}$  release in the MCa trace. This slowly decaying component was simulated as having a single exponential time course with a time constant of 8 s and an amplitude of 0.2  $\mu$ M/ms. Figure 5C and Fig. 5D show the indo-1 saturation traces calculated from the above fluxes on a short and a long time scale, respectively. Results clearly show that a slowly decaying post-pulse extra- $Ca^{2+}$  flux can well account for the results obtained in the presence of MCa. We also took advantage of the simulation to quantitatively estimate the amplitude of the transmembrane calcium current that would have been necessary to account for the observed effect of MCa (if the effect were to be solely due to  $Ca^{2+}$  entry across the plasma membrane). Using a value of 40  $\mu$ m for the diameter of the voltage-clamped end portion of the fiber and assuming a ratio of capacitance to apparent membrane surface of 5  $\mu$ F/cm<sup>2</sup> (21, 30) and a fraction of total fiber volume accessible to  $Ca^{2+}$  of 0.7, the MCa-induced extra- $Ca^{2+}$  input flux of 0.2  $\mu$ M/ms peak amplitude in Fig. 5 would have corresponded to a peak transmembrane calcium current of  $\sim 5$  A/F amplitude. Since such a current was clearly absent from the records obtained in the presence of MCa (Fig. 3) this possibility was definitely discarded, in accordance with the results from Fig. 4.

As already pointed out in Fig. 1 and also observable in Fig. 2 a specific feature of the MCa effect was that the amplitude of the post-pulse  $[Ca^{2+}]$  elevation was larger as the pulse duration was increased, indicating that the size of the post-pulse uncontrolled  $Ca^{2+}$  flux depended upon the previous extent of voltage-dependent  $Ca^{2+}$  release activation. This was also true when, for a same duration, the pulse amplitude was increased within the voltage range of activation of  $Ca^{2+}$  release (see Fig. 6A). This suggests that the more release channels are activated by voltage, the more will potentially be affected by MCa and remain open after membrane repolarization. Despite its impressive effect on the post-pulse  $[Ca^{2+}]$  level, MCa seemed to only marginally, if at all, affect  $Ca^{2+}$  release during the depolarizing pulses. As an example, Fig. 6 shows the results from experiments aimed at comparing the voltage dependence of  $Ca^{2+}$  release between control and MCa-injected fibers. Indo-1  $Ca^{2+}$  signals were recorded in response to the pulse protocol shown at the top of Fig. 6A. It consisted of a

sequence of 3 consecutive step depolarizations of 30 ms duration to the indicated values; each pulse was then incremented by 5 mV and the sequence was repeated. Figure 6A shows an example of corresponding indo-1 saturation signals in a control fiber and in an MCa injected fiber while Fig. 6B shows the  $\text{Ca}^{2+}$  release traces ( $d[\text{Ca}]_T/dt$ , see Methods) calculated from the above transients. For clarity only the release flux trace calculated from the last sequence of indo-1 transients (in response to the pulses to  $-30$ ,  $-10$  and  $+10$  mV) is shown. Although during a single sequence the sustained post-pulse  $[\text{Ca}^{2+}]$  level in the presence of MCa may have affected the properties of  $\text{Ca}^{2+}$  release during the next pulse, the voltage level for both threshold and maximal activation of  $\text{Ca}^{2+}$  release were similar in the two fibers. The mean voltage dependence of the peak  $\text{Ca}^{2+}$  release flux from identical experiments in 3 control fibers and 4 MCa-injected fibers is shown in Fig. 6C. Superimposed curves were calculated with the mean parameters obtained from fitting a Boltzmann function to the individual series of data points in each fiber. Mean corresponding values for the peak release flux, midpoint voltage and steepness factor were  $7.1 \pm 0.6 \mu\text{M}/\text{ms}$ ,  $-22 \pm 2$  mV and  $4.6 \pm 0.3$  mV in control fibers and  $5.4 \pm 0.5 \mu\text{M}/\text{ms}$ ,  $-17.3 \pm 3$  mV,  $3.8 \pm 0.1$  mV in MCa-injected fibers, respectively. Mean values for the peak release flux and midpoint voltage did not significantly differ while the steepness factor was slightly but significantly reduced in the presence of MCa ( $P=0.05$ ). Figure 6D presents the voltage dependence of the mean values for the post-pulse release flux in the control and in the MCa-injected fibers; values were measured 500 ms following the onset of each pulse (time indicated by the open arrows in panel B). In the MCa-injected fibers the amplitude of the post-pulse flux clearly had a voltage dependence similar to that of the peak release flux, whereas the control fibers exhibited no residual post-pulse flux. The strong dependence of the amplitude of the post-pulse release flux upon the amplitude of the peak flux is further illustrated in Fig. 6E where corresponding individual values for these two parameters were plotted versus each other. Fitting a straight line through the individual series of MCa data points in each fiber indicated that the post-pulse release flux corresponded to  $4.3 \pm 0.005$  % of the peak flux activated during the pulse.

Since MCa presents some homology with domain A of the II-III loop of the  $\alpha 1$  subunit of the DHPr, it was of particular interest to directly test the effect of domain A under the present conditions. A series of measurements was thus performed with fibers micro-injected with a synthetic domain A peptide (see Methods). Figure 7 illustrates a series of results from these experiments; this figure is organized in the same format as Figure 1 in order to ease the comparison. Figure 7A shows superimposed indo-1 calcium signals elicited by step depolarizations of 10, 20 and 50 ms duration to  $+10$  mV in a control fiber and in a fiber injected with the domain A peptide. The graphs give mean values for the corresponding baseline  $[\text{Ca}^{2+}]$  level (Fig. 7B), the peak  $\Delta[\text{Ca}^{2+}]$  during the pulse (Fig. 7C), the  $\Delta[\text{Ca}^{2+}]$  measured 0.92 s (referred to as 1 s) after the onset of the pulses (Fig. 7D) and the ratio of  $\Delta[\text{Ca}^{2+}]$  measured 1 s after the pulse ( $\Delta[\text{Ca}^{2+}]_{1s}$ ) to the preceding peak  $\Delta[\text{Ca}^{2+}]$  (Fig. 7E). The y scale in panels B, D and E is the same as in the corresponding panels of Figure 1. Data are from 4 control fibers and 4 fibers injected with peptide A. Obviously, the presence of peptide A in the intracellular medium did not produce any reproducible alteration in the properties of the  $\text{Ca}^{2+}$  transients that would be consistent with the effect of MCa.

## DISCUSSION

The present results show that during physiological excitation-contraction coupling, the step of repolarization-induced closure of the  $\text{Ca}^{2+}$  release channels is transiently disrupted by MCa, a toxin peptide that exhibits some homology with the domain A of the II-III loop of the DHPr in terms of both amino acid sequence (13) and three-dimensional structure (31). MCa was previously shown to have potentiating or activating effects on [ $^3\text{H}$ ]ryanodine binding to- and  $\text{Ca}^{2+}$  release from- SR vesicles, on isolated RYr channel activity, on  $\text{Ca}^{2+}$  release from

intact myotubes and on spontaneous  $\text{Ca}^{2+}$  release events in permeabilized muscle fibers (13, 14, 19, 29). More recently MCA and domain A were shown to share common binding sites on RYr1 (20) which, added to the fact that MCA effect on RYr1 is clearly inhibited by domain A (29), would provide convincing support for the possibility that the two act on the same target through an analogous mechanism. The fact that the inhibition did not correspond to a simple competitive process (29) is likely to be due to the complexity of the overall mechanism because of the presumable 4:1 stoichiometry of the MCA (or domain A) -RYr1 interaction.

Here we show that, within the physiological operating mode of e-c coupling of an intact mammalian skeletal muscle fiber, MCA forces  $\text{Ca}^{2+}$  release channels to remain open following a transient membrane depolarization. The effect of MCA was specific because a single amino-acid substitution (Arg<sup>24</sup> by an alanine), previously shown to prevent MCA alteration of RYr1 activity (14), induced the loss of its effectiveness. The simplest conclusion from our results is that repolarization-induced closure of MCA-modified RYr channels is temporarily impaired. At this point two alternatives may be considered.

The first possibility would be that the effects of MCA arise from its homology with domain A and tell us something about the dynamic physiological interactions that take place between the DHPr and the RYr during e-c coupling. In this framework however, one may have expected an excess of free peptide A in the myoplasm to produce a similar effect to that of MCA. In contrast, fibers injected with a synthetic domain A peptide did not exhibit a sustained  $\text{Ca}^{2+}$  elevation following a step depolarization. This makes it hard to further argue in favor of this first hypothesis although it remains possible that the synthetic domain A peptide does not yield the exact native functional configuration of the corresponding endogenous segment within the II-III loop or that the exogenous domain A is functional but acts in an undistinguishable manner from the endogenous one. Alternatively, the difference observed here between peptide A and MCA in terms of functional effect may be related to the biochemical and single channel data indicating that peptide A does not compete with MCA (29) or IpTx A (32) for certain effects on the ryanodine receptor. Finally, although here we did not detect any obvious effect of peptide A, it should be mentioned that in mechanically skinned rat skeletal muscle fibers with functional excitation-contraction coupling, peptide A was reported to elicit small spontaneous force responses in some fibers and to potentiate responses to depolarization in all fibers (33).

Despite the sequence homology between domain A of the DHPr and MCA and their common binding site on the RYr, it is also possible that MCA acts through a mechanism that is independent from the function of domain A. Since the toxin had no detectable effect on resting  $[\text{Ca}^{2+}]$  in the absence of depolarization, MCA is likely to have poor access to its binding site under resting conditions. Depolarization-induced opening of the RYr channels most likely then reveals the MCA binding site, allowing interaction with the toxin.  $\text{Ca}^{2+}$  transients exhibited no major alteration during the depolarizing pulses in the presence of MCA: the absolute peak amplitude of the transients and the voltage dependence of  $\text{Ca}^{2+}$  release were similar to those observed under control conditions. MCA thus does not appear to strongly impair the functional operating mode of the overall population of RYr channels once the membrane is depolarized. This may indicate that MCA-bound channels behave normally during the depolarization though one also cannot exclude the possibility that the fraction of MCA-bound channels was low enough that even if operating in an altered mode their contribution would be too little to seriously affect the overall release flux during the pulse. Conversely, membrane repolarization clearly reveals the activity of the MCA-bound channels. Once bound, MCA thus likely prevents the conformational change required for channel closure upon membrane repolarization. In that sense MCA could be pictured to act in an analogous way as ryanodine which, as we have previously showed, produces a use-dependent increase in SR  $\text{Ca}^{2+}$  leak due to RYr channels remaining locked open after a transient membrane depolarization (23). A noticeable difference is, however, that MCA-modified

channels eventually return to a normal gating mode, presumably due to the dissociation of M<sub>Ca</sub>, as indicated by the return of intracellular [Ca<sup>2+</sup>] to its resting level.

The present effect of M<sub>Ca</sub> has a striking similarity with a previously described alteration of e-c coupling in frog skeletal muscle where low myoplasmic [Mg<sup>2+</sup>] levels could also induce a long-lasting elevation of myoplasmic [Ca<sup>2+</sup>] following a transient membrane depolarization (34). The similarity with the present effect of M<sub>Ca</sub> may be indicative that the putative site for Mg<sup>2+</sup> inhibition of Ca<sup>2+</sup> release is somehow linked to the M<sub>Ca</sub> binding site.

In conclusion, this work demonstrates that the scorpion venom toxin M<sub>Ca</sub> specifically alters the physiological process of repolarization-induced closure of the RYr channels. Further investigation of the properties of this effect will certainly prove helpful to unravel the molecular steps involved in the control of the RYr channels during excitation-contraction coupling.

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## FIGURE LEGENDS

**Figure 1. Incomplete decay of step depolarization-activated  $\text{Ca}^{2+}$  transients in MCa-injected fibers.** *A*, indo-1 saturation transients elicited by consecutive depolarizing steps of 10, 30 and 50 ms duration to +10 mV in a control fiber (top) in an MCa-injected fiber (middle) and in a fiber injected with the mutated analog [Ala<sup>24</sup>]MCa. *B*, mean values for the baseline  $[\text{Ca}^{2+}]$  level in control (n=5), MCa-injected (n=9) and [Ala<sup>24</sup>]MCa-injected fibers (n=5) challenged by a 10 and a 30 ms-long pulse to +10 mV. *C* and *D*, corresponding values for the peak  $\Delta[\text{Ca}^{2+}]$  and the  $\Delta[\text{Ca}^{2+}]$  measured ~1 s after the onset of the pulse, respectively. *E*, corresponding mean values for the ratio of the  $\Delta[\text{Ca}^{2+}]$  measured ~1 s after the onset of the pulse (mean shown in *C*) to the peak  $\Delta[\text{Ca}^{2+}]$  during the pulse (mean shown in *D*). See text for details.

**Figure 2. The long lasting  $[\text{Ca}^{2+}]$  elevation following the end of a step depolarization in MCa-injected fibers.** Traces correspond to indo-1 saturation records of 15 s duration in a control fiber and in two MCa-injected fibers depolarized by short pulses to +10

mV of various duration. Pulses of 5 and 10 ms duration were applied in the control fiber (left). Pulses of 5, 10 and 30 ms duration were applied in the two MCa-injected fibers.

**Figure 3. The MCa-induced long-lasting post-pulse  $[Ca^{2+}]$  elevation is not associated with a change in the holding membrane current.** In *A* and *B*, the top series of traces correspond to successive (from left to right) indo-1 saturation transients elicited by a 5 ms long pulse to +10 mV in a control fiber (*A*) and in an MCa-injected fiber (*B*); the bottom series of traces show the corresponding membrane current records at a high gain.

**Figure 4. The MCa-induced long-lasting post-pulse  $[Ca^{2+}]$  elevation persists in the absence of extracellular calcium.** All records are from the same fiber injected with MCa. The top series of traces corresponds to the changes in membrane current elicited by a 20 ms long pulse to +10 mV. The indo-1 calcium signals measured simultaneously are shown underneath on a more compressed time scale. Records were taken in the presence of the control extracellular solution (left), in the absence of extracellular calcium (middle) and after returning to the calcium containing control solution (right). Membrane current records were corrected for the linear components.

**Figure 5. Simulation of the effect of MCa using a simple model of intracellular  $Ca^{2+}$  distribution.** *A* and *B*, synthetic  $Ca^{2+}$  release flux traces in control conditions (thin trace) and in the presence of MCa (thick trace). In *A* and *B* the same traces are shown on a short and long time scale, respectively. In *A*, the inset focuses on the peak phase of the release traces. In *B*, the inset shows the two traces on an expanded y scale. The only difference between the control and the MCa trace is the presence of a small slowly decaying extra- phase of  $Ca^{2+}$  release in the presence of MCa. This extra phase is best seen in the inset of panel *B*. *C* and *D*, corresponding calculated indo-1 saturation traces on a short and a long time scale, respectively.

**Figure 6. Voltage dependence of  $Ca^{2+}$  release in control fibers and in MCa-injected fibers.** *A*, indo-1 saturation transients measured in response to the pulse protocol illustrated on top, in a control fiber (left) and in an MCa-injected fiber (right). The protocol consisted of a sequence of 3 consecutive step depolarizations of 30 ms duration. During the first sequence the 3 successive steps were to -50, -30 and -10 mV, respectively. The amplitude of each of the 3 steps was then incremented by 5 mV and the sequence was repeated. The sequence was applied 5 times so that during the last run the 3 steps were to -30, -10 and +10 mV, respectively. Indo-1 transients obtained in response to the 5 sequences are shown superimposed. *B*,  $Ca^{2+}$  release flux calculated from the above indo-1 transients; only the release flux from the indo-1 trace obtained in response to the successive pulses to -30, -10 and +10 mV is shown. The y scale bar in each panel corresponds to 1  $\mu$ M/ms. The horizontal dotted line corresponds to the initial baseline level. Filled and open arrows point to the peak and post-pulse release flux levels that were measured, respectively; amplitudes were measured from the pre-pulse level. The post-pulse flux values were taken as the average from a 100 ms long portion of trace, 500 ms following the pulse onset. The mean from these measurements is reported in the following panels. *C*, mean voltage dependence of the peak  $Ca^{2+}$  release flux in control fibers (filled circles, n=3) and in MCa-injected fibers (open circles, n=4). *D*, corresponding mean voltage dependence of the post-pulse  $Ca^{2+}$  release flux. *E*, plot of the individual values of the post-pulse release flux versus the corresponding peak release flux in the control (filled symbols) and the MCa-injected fibers (open symbols). Different symbols correspond to the different fibers.

**Figure 7. Voltage-activated  $Ca^{2+}$  transients in fibers injected with a synthetic peptide corresponding to domain A of the II-III loop of the DHP  $\alpha$ 1 subunit.** *A*, indo-1 saturation transients elicited by consecutive depolarizing steps of 10, 20 and 50 ms duration to

+10 mV in a control fiber (top) and in a peptide *A*-injected fiber (bottom). *B*, mean values for the baseline  $[Ca^{2+}]$  level in control (n=4) and peptide *A*-injected fibers (n=4) challenged by the pulse protocol shown in *A*. *C* and *D*, corresponding values for the peak  $\Delta[Ca^{2+}]$  and the  $\Delta[Ca^{2+}]$  measured ~1 s after the onset of the pulse, respectively. *E*, corresponding mean values for the ratio of the  $\Delta[Ca^{2+}]$  measured ~1 s after the onset of the pulse (mean shown in *C*) to the peak  $\Delta[Ca^{2+}]$  during the pulse (mean shown in *D*).

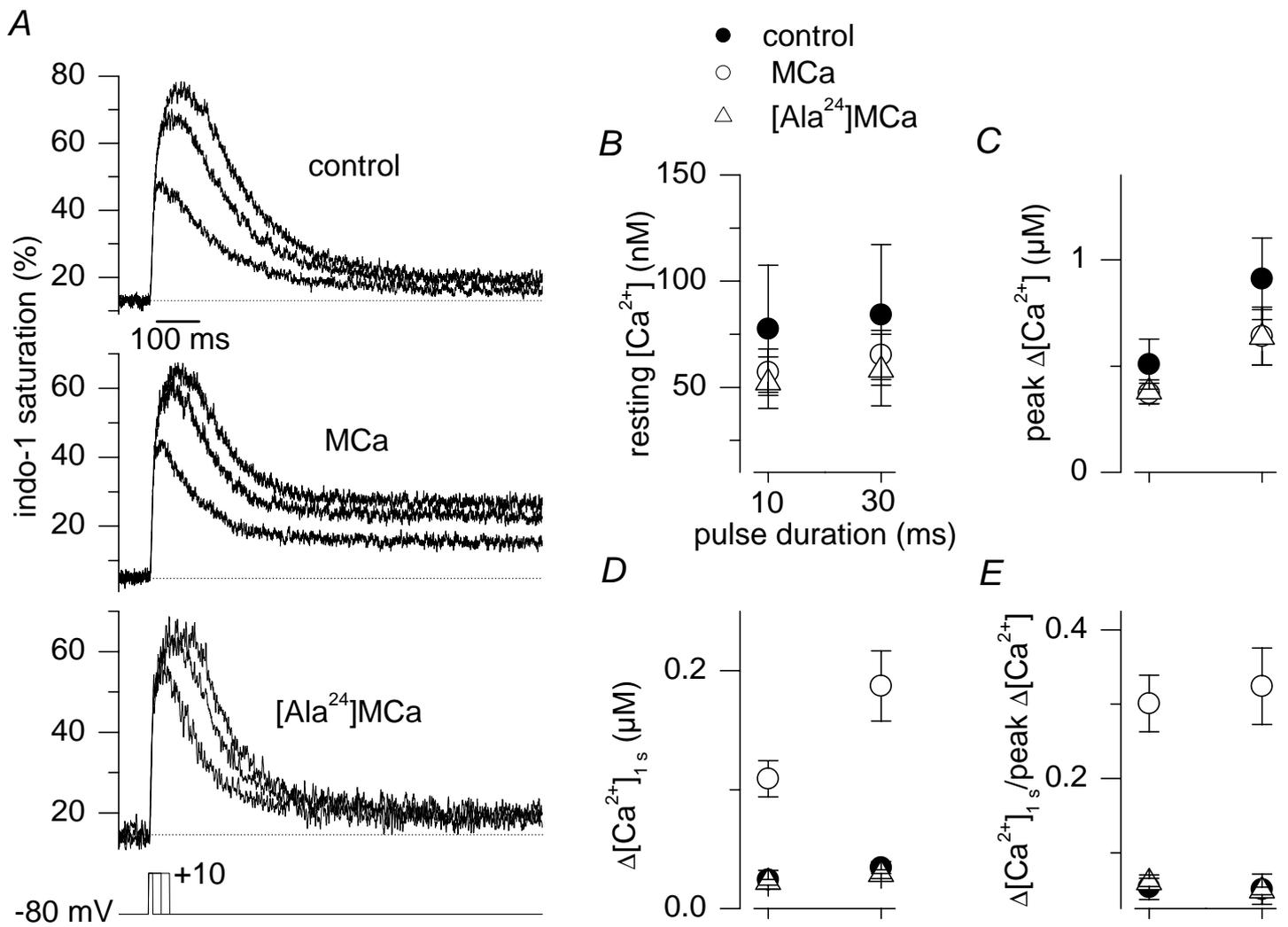


Figure 1

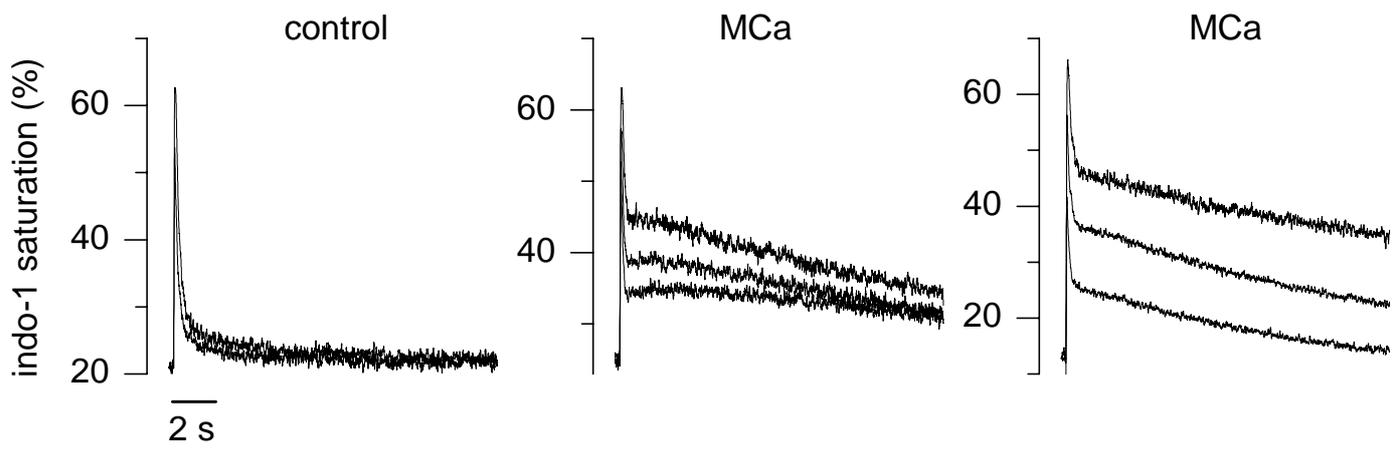


Figure 2

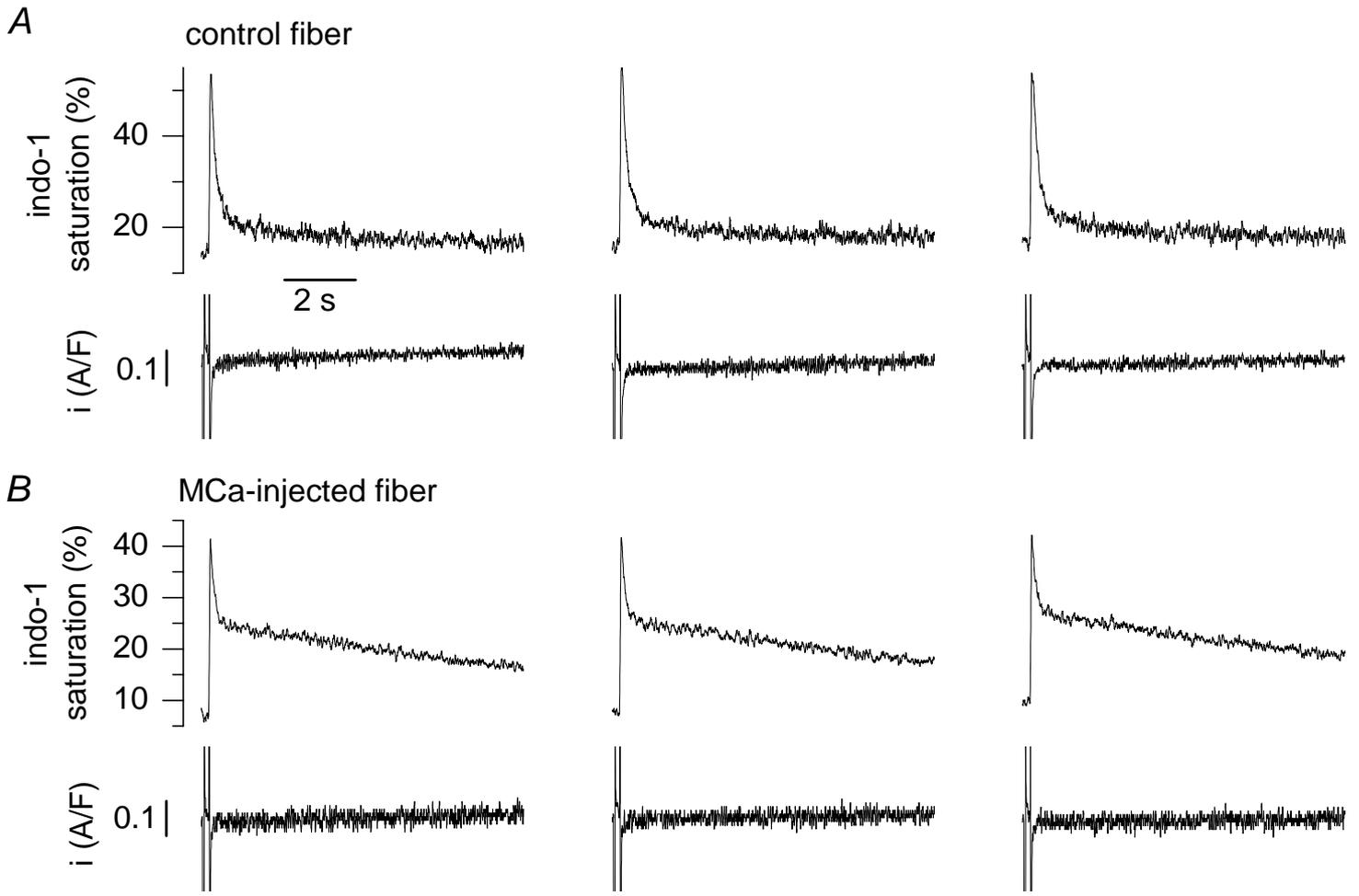


Figure 3

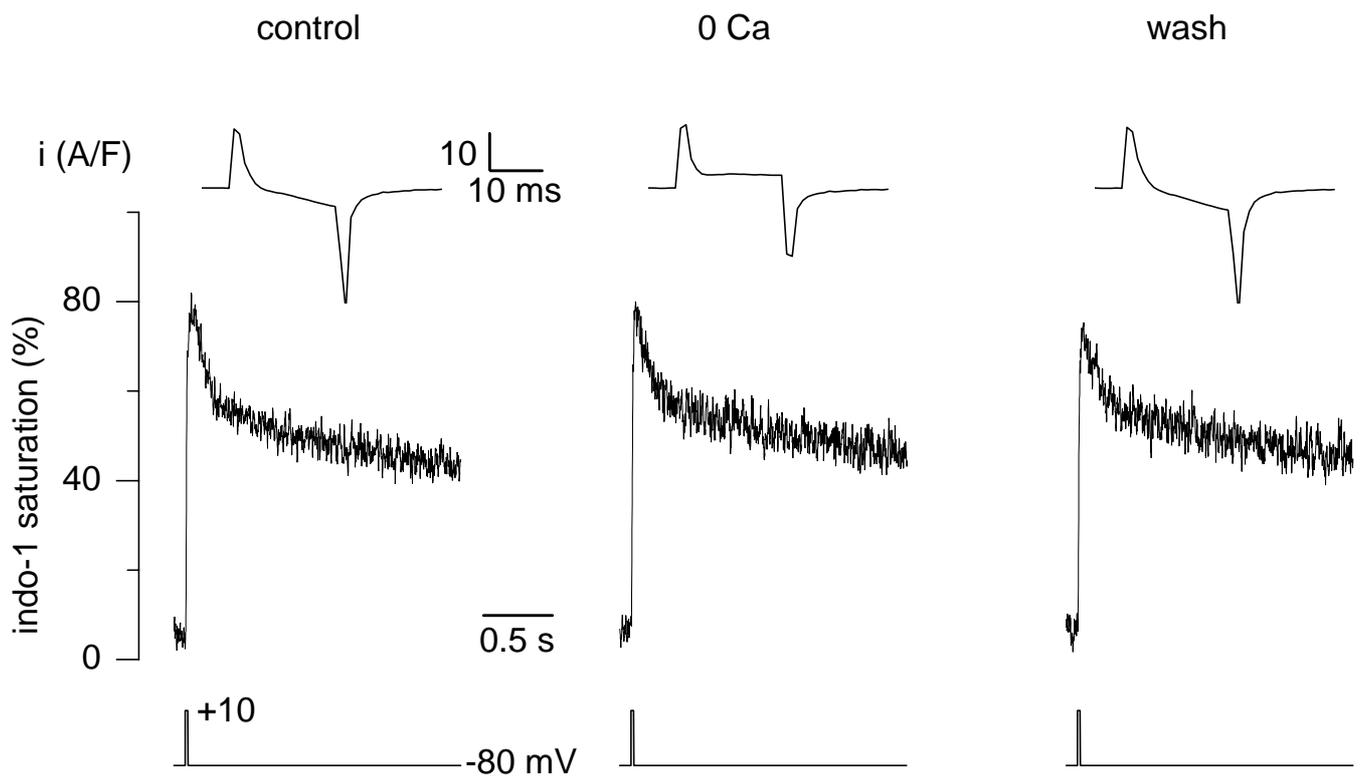


Figure 4

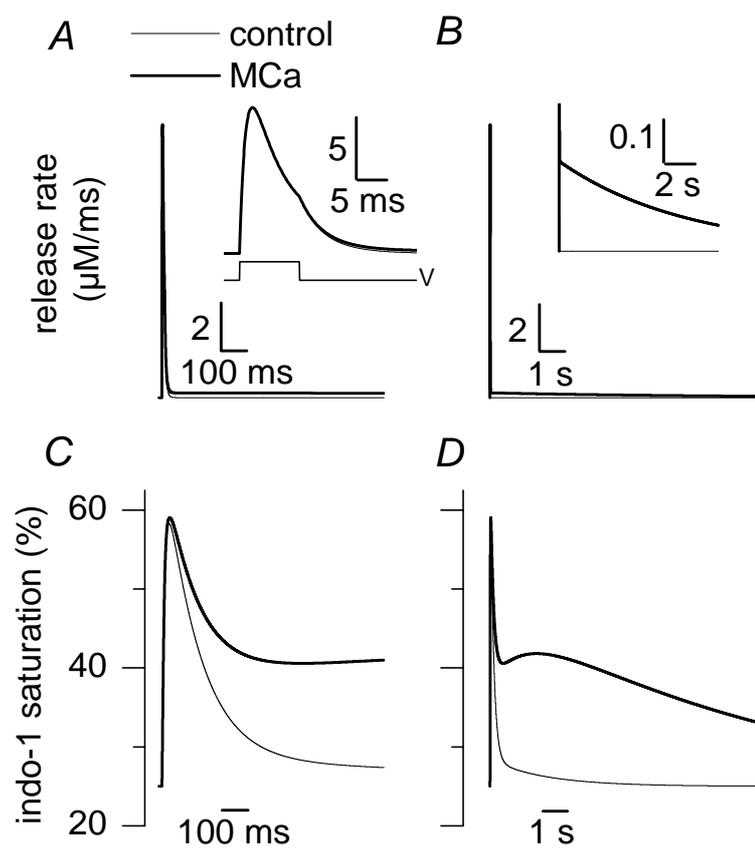
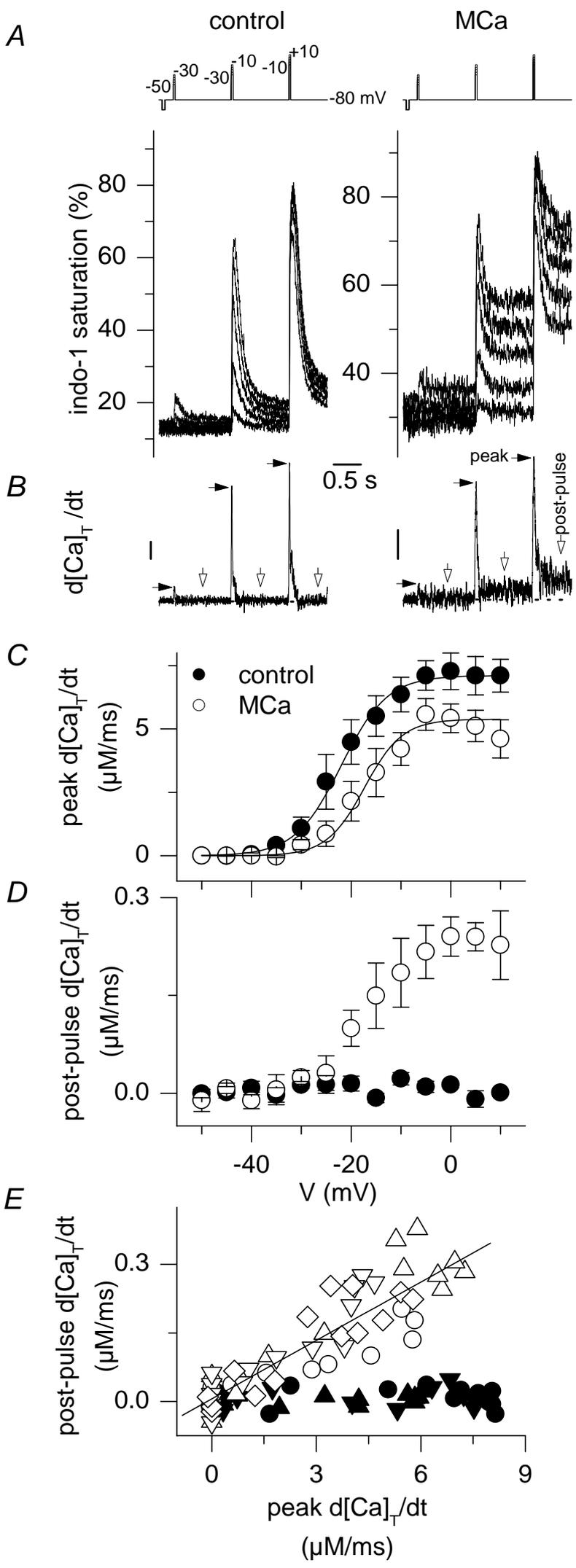


Figure 5

Figure 6



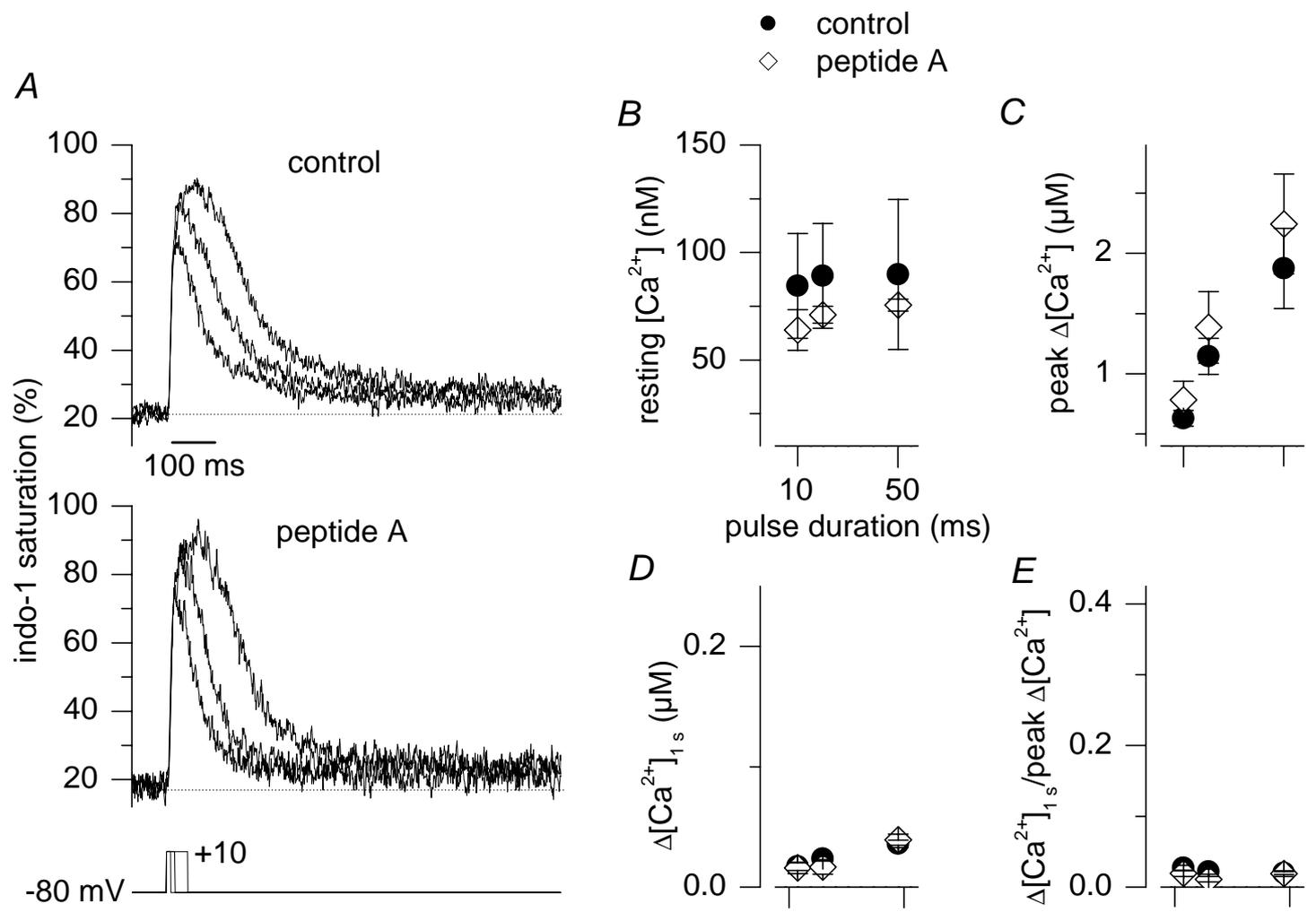


Figure 7