Anesthesiology
76:132–138, 1992

EU 4093 Decreases Intracellular [Ca^{2+}] in Skeletal Muscle Fibers from Control and Malignant Hyperthermia–Susceptible Swine

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The mechanisms causing the malignant hyperthermia (MH) syndrome are related to a malfunction of intracellular Ca^{2+} homeostasis and can be prevented or reversed by dantrolene. EU 4093 (Azumolene®, 1-[[[5-(4-bromophenyl)-2-oxyzolyl] methylene]amino]-2-4-imidazolidinedione) is a 30-fold more water-soluble analogue of dantrolene that is believed to have the same effects as dantrolene on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in skeletal muscle and that should have similar efficacy in treating and preventing the clinical manifestations of MH in response to a halothane/succinylcholine challenge. To test this hypothesis, experiments were carried out in four controls (Yorkshire) and eight MH-susceptible crossbreed swine (Poland China × Pietrain). The resting $[Ca^{2+}]_i$ in normal muscle fibers measured by Ca^{2+}-selective microelectrodes was 111 ± 12 nM (mean ± standard deviation, n = 30), whereas in the MH muscles the resting $[Ca^{2+}]_i$ was 395 ± 36 nM, (n = 28) (P = 0.0001). EU 4093 decreased $[Ca^{2+}]_i$ in MH-susceptible skeletal muscle in a dose-related fashion from 207 to 38 nM after 0.5 to 2.0 mg/kg, respectively, and had a similar effect in control skeletal muscle (58 to 30 nM) after the same doses. In MH-susceptible swine, a dose of 2.0 mg/kg was successful in preventing any clinical signs of the MH syndrome during a subsequent halothane/succinylcholine challenge. A dose of 0.5 mg/kg was able to attenuate but not reverse the clinical signs of the MH syndrome after a halothane challenge, whereas a dose of 1.0 mg/kg was completely successful in reversing this effect in all subjects. These results indicate that EU 4093 is able to decrease the resting $[Ca^{2+}]_i$ in normal and MH-susceptible skeletal muscle and prevent and treat the clinical MH syndrome. These data suggest that it has a mechanism of action similar to that of dantrolene. (Key words: Ions, calcium: intracellular. Malignant hyperthermia, treatment: dantrolene; EU 4093. Measurement technique: ion-selective microelectrode. Skeletal muscle.)

MALIGNANT HYPERTHERMIA (MH) is a genetically transmitted syndrome that is induced by potent volatile anesthetics and/or depolarizing muscle relaxants.[1] The primary genetic defect in MH is currently believed to be an alteration in the structure of the sarcoplasmic reticulum (SR) Ca^{2+}-release channel (ryanodine receptor).[2,3] The pathophysiology of MH syndrome is related to a malfunction of intracellular Ca^{2+} homeostasis,[1,4–6] and there are significant differences in both the dissociation constant (Km) and the Ca^{2+} sensitivity for ryanodine binding by heavy SR vesicles in MH-susceptible swine.[7] Our previous studies demonstrated an abnormally high resting myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in susceptible swine and humans.[4,5,8] We have also demonstrated a rapid increase in $[Ca^{2+}]_i$ that accompanied the clinical manifestations of the syndrome when it is induced by halothane and/or succinylcholine.[9]

Dantrolene, a hydantoin derivative, has been shown to be uniquely effective in treating and preventing the clinical signs and symptoms of MH.[1,9] Dantrolene acts as a muscle relaxant is probably through its ability to reduce $[Ca^{2+}]_i$ in skeletal muscle.[10,11] It does this acutely by preventing Ca^{2+} ion release from the SR by reducing the frequency of spontaneous opening of the SR Ca^{2+}-release channel/ryanodine receptor.[12] Dantrolene decreases the increased $[Ca^{2+}]_i$ seen in MH-susceptible pigs and humans, and it appears that its ability to prevent the induction of an MH crisis is by way of this mechanism.[9,11,13] Dantrolene has also been shown to decrease $[Ca^{2+}]_i$ during the MH episode and is associated with the termination of the clinical signs and symptoms of the syndrome.[9,11] EU 4093 (Azumolene®) is hydantoin derivative related to dantrolene with improved water solubility (fig. 1). This water solubility could be a very useful characteristic. A vial of dantrolene contains only 20 mg of the lyophilized drug with 3,000 mg mannitol, which facilitates its dissolution, in 60 ml water. In contrast, EU 4093 may be prepared in an isotonic solution in a concentration of 10 mg/ml.

This study was performed to test the effects of EU 4093

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Received from the Department of Anesthesia, Brigham and Women's Hospital, Boston, Massacusetts; Centro de Biofisica y Bioquimica, Instituto Venezolano Investigaciones Cientificas, Caracas, Venezuela; the Department of Anesthesia, Massachusetts General Hospital, Boston, Massachusetts; and the Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massacusetts. Accepted for publication September 20, 1991. Supported by grants from the Muscular Dystrophy Association, CONICIT of Venezuela grant S1-1277, and National Institutes of Health Center Grant GM 15904-19. EU 4093 (Azumolene®) was donated by Norwich Eaton Pharmaceuticals.

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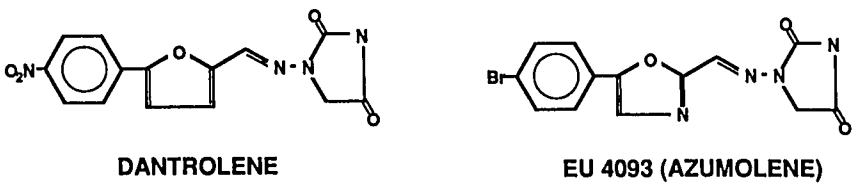

FIG. 1. Chemical structures of dantrolene and EU 4093 (Azumolene®).

on resting $[Ca^{2+}]_i$ in control and MH-susceptible swine and to ascertain whether it was effective in preventing and/or treating the MH syndrome, because its superior water solubility characteristics would significantly facilitate clinical preparation and administration of the drug during a clinical MH crisis.

Materials and Methods

The experiments were carried out at the Boston Biomedical Research Institute on each of four control Yorkshire swine and eight MH-susceptible crossbred swine (Poland China × Pietrain) on two occasions 3 weeks apart in random order according to a protocol approved by the institutional animal care committee of the Boston Biomedical Research Institute. Susceptibility to MH was confirmed by a qualitative standard "barnyard" challenge with halothane, as previously described.[14] For this challenge, limb rigidity, tachycardia (20 beats/min above baseline), and increase in the body temperature of more than 1° C in 3 min were taken as indications of susceptibility to MH. EU 4093 (Azumolene®) was provided by Norwich Eaton Pharmaceuticals (Norwich, NY). An isotonic solution of EU 4093 was prepared using guidelines provided by the manufacturer and contained 10 mg/ml EU 4093, 2.2% (vol/vol) glycerol, and potassium hydroxide to adjust the *p*H to 10.2.

Anesthesia was induced with intravenous (iv) sodium thiopental (10–15 mg/kg) and maintained with fentanyl iv (0.025 mg/kg) and a mixture of N_2O and O_2 (66:34%). Spontaneous movements were prevented with pancuronium iv (0.15 mg/kg). Additional iv doses of 0.025 mg/kg of fentanyl and 0.06 mg/kg of pancuronium were given every 15–30 min as needed. Animals were monitored for heart rate, hemoglobin O_2 saturation, end-tidal P_{CO_2}, and rectal temperature. Fluids and all drugs used were administered *via* an ear vein catheter. The two experimental protocols followed in this study are shown in figure 2 (in which letters indicate the stages of the experiments during which the *in vivo* measurements of $[Ca^{2+}]_i$ were carried out).

Dose Response/Prevention

In these experiments (fig. 2A), all of the MH-susceptible and all of the control swine were given cumulative 0.5-mg/kg iv doses of EU 4093 at 15-min intervals to a final dose of 2 mg/kg. $[Ca^{2+}]_i$ measurements were made before the first dose and after each subsequent dose of EU 4093. After the 2-mg/kg measurements were made, paralysis was reversed with neostigmine, and the animals were exposed to halothane (2%) for 30 min and another measurement made. The pigs were then given succinylcholine (2 mg/kg) in two doses 15 min apart before a final measurement 5 min later and the discontinuance of anesthesia.

Treatment

In the treatment experiments (fig. 2B), control values of clinical observations and resting $[Ca^{2+}]_i$ were made in all of the MH-susceptible and control animals. After reversing paralysis with neostigmine, the animals were exposed to halothane (2%). This triggered the clinical syndrome in all of the MH-susceptible animals within 2 min, at which time a second set of $[Ca^{2+}]_i$ measurements and clinical observations were made. The halothane was discontinued after this measurement (approximately 5–10 min after the commencement of halothane), and 0.5 mg/kg of EU 4093 was administered. This was followed by a third set of measurements of $[Ca^{2+}]_i$ and clinical observations 5–10 min later. After these measurements, a second dose of 0.5 mg/kg of EU 4093 (cumulative dose 1

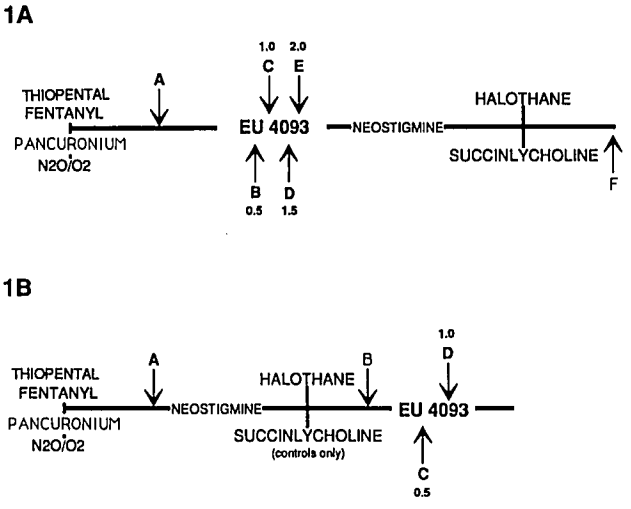

FIG. 2. The experimental protocol followed for the dose–response and prevention study (*A*) and MH treatment study (*B*). The letters (A, B, C, D, E, F and A, B, C, D) indicate the times when measurements of $[Ca^{2+}]_i$ and resting membrane potential were carried out in control and MH-susceptible muscle fibers *in vivo*. The doses of EU 4093, indicated by numbers above the letters (where appropriate), indicate the cumulative dose of EU 4093 (milligrams per kilogram) administered.

mg/kg) was administered, and the final measurements of $[Ca^{2+}]_i$ and clinical observations were made 5–10 min afterward. In the control animals, halothane was continued for 1 h. After 30 min, two 1-mg/kg doses of succinylcholine were administered at 15-min intervals to confirm the lack of susceptibility, and a second set of measurements was made. EU 4093 was not required and not administered to these animals at this time.

Ca^{2+}-Selective Microelectrodes

Glass microelectrodes with a tip outer diameter of 0.5 μm or less were pulled from glass capillaries with filaments (WPI-1B150F). They were prepared and calibrated as described previously.[11] The microelectrode tips were first back-filled with the neutral ligand ETH 1011, and the remaining part was filled with *p*Ca 7 solution. The calibration was carried out before and after every five measurements, and data were discarded if the calibration curves before and after each recalibration differed by more than 4 mV from *p*Ca 6–7 (physiologic range). We confirmed that the calibration and response time of these ion-selective microelectrodes was not influenced by changes in *p*H from 6.1 to 7.9, by any of the drugs used in this study, or by changes in free Mg^{2+} (0–4 mM) or monovalent cations (Na^+ [20-150 mM] and K^+ [2–120 mM]) (data not shown).

Recording Procedure

After induction of anesthesia, a 5-cm incision was made over the peroneus longus muscle of the right hind leg. After the muscle was identified, its surface was freed of connective tissue, and then the superficial muscle fibers were exposed and kept covered with warm swine physiologic solution (37° C). This muscle was chosen because it was not susceptible to motion artifacts induced by artificial ventilation and arterial pulsations. Motion artifacts were further reduced by means of flexible connections between the electrode capillaries and the connections to the voltmeters. Resting membrane potential (Vm) measured in one cell and Ca^{2+}-specific potential measured in a second cell were determined as described previously.[11] Signals were displayed on digital voltmeters (Simpson M-465) and recorded on a two-channel recorder (Linear M 005). Each measurement of $[Ca^{2+}]_i$ was made after a separate impalement of the muscle cells with the microelectrodes and was done by subtracting the average Vm for a condition from the individual Ca^{2+}-specific potential.

Criteria for Collecting Experimental $[Ca^{2+}]_i$ Data

Data that conformed to the following criteria were accepted.

1. The microelectrode calibration curve performed before and after each five impalements differed by no more than 4 mV between (*p*Ca 6–7).
2. The membrane potential of the muscle fibers had to be more negative than −79 mV.
3. Neither the membrane potential nor the ion specific potential could increase by more than 4 mV during the Ca^{2+} measurements.

Results are presented as mean ± standard deviation. A Mann Whitney U test was done to compare the resting $[Ca^{2+}]_i$ levels and a Kruskal-Wallace to compare variance between and within groups in the treatment and dose response/prevention groups. Significance was accepted at the $P < 0.05$ level. Nonparametric multiple comparisons were performed using the nonparametric comparisons portion of Statview 512+™ (Abacus Inc).

Results

While there was no significant difference in the resting membrane potential between these control and MH-susceptible muscle fibers, the resting $[Ca^{2+}]_i$ was 3.6 times greater in MH-susceptible muscle fibers than in controls. The mean $[Ca^{2+}]_i$ in controls was 110 ± 10 nM, n = 30 (range 90–140 nM), whereas it was 393 ± 31 nM, n = 28 (range 330–470 nM) in the MH-susceptible swine ($P = 0.0001$).

EU 4093 0.5–2.0 mg/kg decreased resting $[Ca^{2+}]_i$ in a dose-dependent fashion with no effect on Vm at any dose in both MH-susceptible and control animals. Figure 3 shows a typical dose–response relationship for a susceptible muscle fiber, when the animal received (iv) 0.5 (fig. 3B), 1.0 (fig. 3C), 1.5 (fig. 3D), and 2.0 mg/kg (fig. 3E) of EU 4093. The recording of $[Ca^{2+}]_i$ after each dose of EU 4093 shows the reduction of myoplasmic $[Ca^{2+}]$ as a function of drug dose. Figure 4 summarizes the results of these experiments. In MH-susceptible animals EU 4093 induced a reduction in resting $[Ca^{2+}]_i$ from 398 ± 26 nM to 212 ± 27, 145 ± 24, 78 ± 10, and 38 ± 6 nM for a dose of 0, 0.5, 1.0, 1.5, and 2.0 mg/kg respectively. ($P < 0.05$ for all comparisons.) For the same doses in normal swine, resting $[Ca^{2+}]_i$ decreased from 104 ± 8 nM to 58 ± 10, 40 ± 8, 32 ± 4, and 31 ± 4 nM. After each 0.5-mg/kg dose of EU 4093, both groups of animals had a transient increase in heart rate of 35 ± 4 beats/min that lasted 2–3 min and then returned to normal. A cumulative dose of 2 mg/kg of EU 4093 prevented any change in end-tidal O_2 concentration ($P_{ET}CO_2$), hemoglobin O_2 saturation, heart rate, respiratory rate, or body temperature as well as the increment in $[Ca^{2+}]_i$ following a halothane and succinylcholine challenge when compared to control values ($P > 0.10$).

It is interesting to note that after the administration of 2 mg/kg EU 4093, all pigs had apparent muscle weakness,

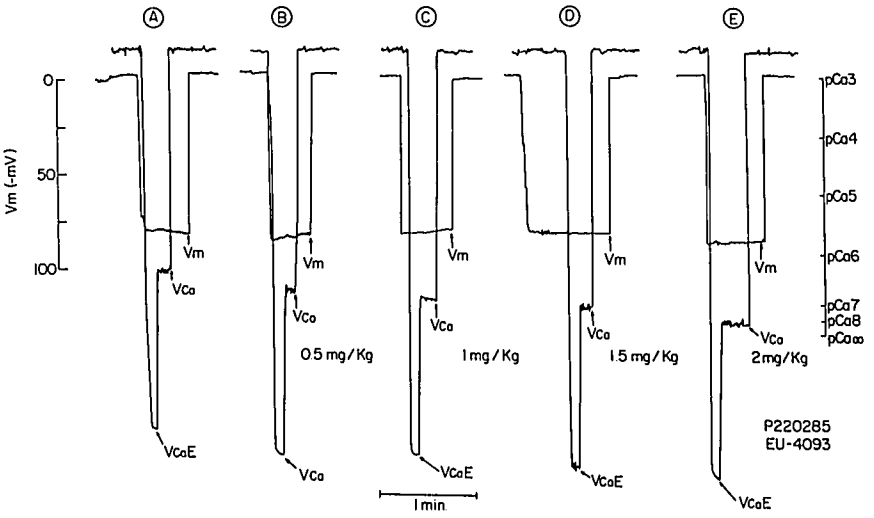

FIG. 3. Recording of the resting membrane potential (Vm) and intercellular free calcium (VCa) from a typical MH-susceptible skeletal muscle fibers *in vivo* before and after the susceptible animal received EU 4093 at different doses. *A*: The value for Vm and $[Ca^{2+}]_i$ (−78 mV, 550 nM) before the animal received a dose of EU 4093; *B*: after 0.5 mg/kg Vm and $[Ca^{2+}]_i$ were −81 mV and 330 nM; *C*: after 1.0 mg/kg (cumulative dose) Vm and $[Ca^{2+}]_i$ were −78 mV and 110 nM; *D*: after 1.5 mg/kg (cumulative dose) Vm and $[Ca^{2+}]_i$ were −78 mV and 88 nM; and *E*: after 2.0 mg/kg (cumulative dose) Vm and $[Ca^{2+}]_i$ were −81 mV and 40 nM. Calibration curves for Vm and *p*Ca are on the left and right axes, respectively.

demonstrated by difficulty in walking, for more than 2 h after the discontinuance of anesthesia.

In the absence of EU 4093 pretreatment, a halothane challenge (without succinylcholine) in all of the susceptible animals produced a large increase in resting $[Ca^{2+}]_i$ from a resting value 393 ± 10 nM, (mean ± standard deviation) (n = 28) to 5,530 ± 410 nM (n = 12) ($P < 0.001$) with no accompanying change in Vm ($P > 0.70$). This was accompanied by an increase in PET_{CO_2}, a reduction in hemoglobin O_2 saturation, a marked increase in heart rate, an increased respiratory rate, and an increase in rectal temperature (table 1). The administration of EU 4093, 0.5 mg/kg, reduced $[Ca^{2+}]_i$ to 674 ± 44 nM ($P < 0.001$), but although it lowered $[Ca^{2+}]_i$ sufficiently to reduce the limb rigidity that is characteristic of the syndrome in these swine, it did not reverse the physiologic symptoms of the syndrome (table 1). All of the symptoms of the syndrome were reversed by the second 0.5-mg/kg EU 4093 dose, (cumulative 1 mg/kg), and $[Ca^{2+}]_i$ decreased to 79 ± 6 nM ($P < 0.05$), which is slightly below the resting value seen in normal swine without EU 4093. In the typical experiment shown in figure 5, exposure to halothane triggered the MH episode, which was associated with a significant increase in the $[Ca^{2+}]_i$ from a resting value of 470 to 4,090 nM (fig. 5B). The administration of 0.5 mg/kg of EU 4093 was successful in partially reducing the increase in resting $[Ca^{2+}]_i$ (630 nM) (fig. 5C) that had followed the halothane challenge; the second dose of EU 4093 (cumulative 1 mg/kg) (fig. 5D) was accompanied as well by a reduction of $[Ca^{2+}]_i$ to 50 nM. The mean data for these experiments is summarized in figure 6.

Discussion

In the current study we have once again demonstrated that there is a significantly higher resting $[Ca^{2+}]_i$ in MH-susceptible swine than is found in normal swine. It is not possible from this study to determine with certainty that the observed increase in resting $[Ca^{2+}]_i$ in MH-susceptible swine is not a breed-specific observation because we studied two and only two different breeds of pigs. However, we have previously reported similar results in purebred Poland-China pigs[4] and have found similar results in purebred Pietrain MH-susceptible swine (unpublished data) and in MH-susceptible humans[5,13] of various ethnic

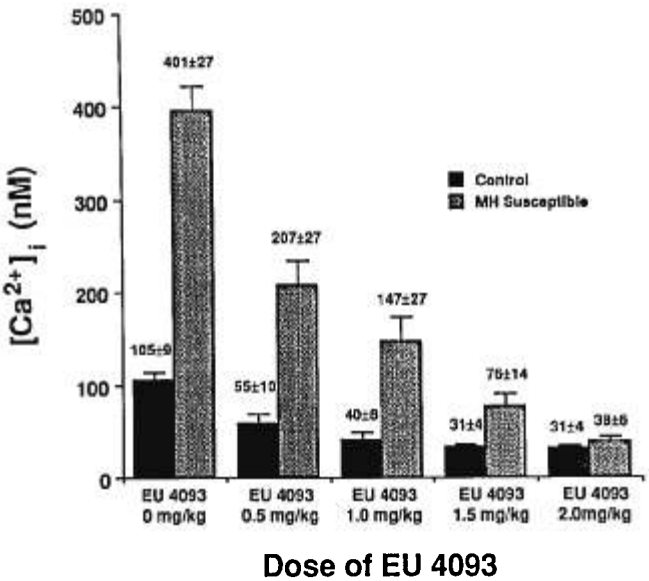

FIG. 4. Average values (mean ± SD) of resting $[Ca^{2+}]_i$ in control and MH-susceptible swine after a stepwise cumulative dose administration of EU 4093, which was 398 ± 26 to 212 ± 27 M,*† 145 ± 24 nM*† 78 ± 4 nM,*† and 38 ± 6 nM for doses of 0. 0.5, 1.0, 1.5, and 2.0 mg/kg respectively for MH-susceptible swine and 104 ± 8 to 58 ± 10 nM,* 40 ± 4 nM,* 31 ± 4 nM,* and 31 ± 4 nM* respectively. *$P < 0.05$ compared to 0 mg/kg of EU 4093. †$P < 0.05$ compared to control.

TABLE 1. Physiologic Parameters Associated with Control and MH-Susceptible Swine before and after Exposure to Halothane and after Treatment with EU 4093

	Treatment	PET_{CO_2} (mmHg)	Hb_{O_2} Saturation (%)	Heart Rate (beats/min)	Respiratory Rate (breaths/min)	Temperature (° C)
MH-susceptible swine	Control	38 ± 4	100 ± 1	120 ± 5	15†	38.1 ± .2
	After Halothane	97 ± 12*	84 ± 3*	215 ± 15*	24 ± 4	39.2 ± .3*
	After EU 4093 (0.5 mg/kg)	85 ± 14*	86 ± 4*	210 ± 12*	34 ± 5	40 ± .3*
	After EU 4093 (1.0 mg/kg)	49 ± 5	100 ± 2	100 ± 10	29 ± 4	39.1 ± .4
Control swine	Control	38 ± 4	100 ± 1	124 ± 6	15†	38.1 ± .2
	After halothane and succinylcholine	37 ± 3	100 ± 1	84 ± 4	21 ± 4	38 ± .3

* $P < 0.05$ compared to control.
† Controled ventilation.
Mean ± SEM.

PET_{CO_2} = end-tidal carbon dioxide concentration; Hb_{O_2} = hemoglobin oxygen.

origin. Likewise, we have found similar results to those found in our Yorkshire swine in Poland-China Yorkshire crossbred[4] MH-negative swine and in MH-negative humans.[5,13] These data lead us to believe that the increased resting $[Ca^{2+}]_i$ seen in MH-susceptible individuals is a characteristic of MH susceptibility and is not a breed-specific, or, for that matter, a species-specific, finding.

Unfortunately, it was technically impossible to measure $[Ca^{2+}]_i$ *in vivo* using the ideal circumstance, *i.e.*, with two electrodes in a single muscle cell. This two-cell technique may lead to a possible error in the measurements of $[Ca^{2+}]_i$ because if Vm in the cell in which either $[Ca^{2+}]_i$ or Vm is being measured was very different than its surrounding cells, this would affect the accuracy of the measurement of $[Ca^{2+}]_i$ and go undetected. We feel confident that our measurements of $[Ca^{2+}]_i$ accurately represent the true circumstance, for two reasons. First, *in vitro* studies in muscles from similar animals and humans, where both electrodes are in the same cell, yield identical results. Second, we have determined the variance of Vm under these same conditions[4,9] and found it to be insufficient to create an error larger than 5% in measurements of $[Ca^{2+}]_i$ with a single electrode. We have always used the second electrode in what is most likely to be a second cell for each measurement to confirm that a specific treatment does not change Vm; however, it would have been possible to use a constant value and not change the validity of the results observed.

EU 4093 is a hydantoin derivative, structurally related to dantrolene sodium; however, its chemical structure makes the compound 50-fold more water-soluble than dantrolene sodium (10 *vs.* 0.2 mg/ml, respectively). It was presumed that because of its structural similarity this compound would have many of the pharmacologic characteristics of dantrolene sodium. The present study demonstrates that EU 4093 has similar effects to our previous study with dantrolene[9] on decreasing $[Ca^{2+}]_i$ in skeletal muscle, and, as is the case with dantrolene, has no effect

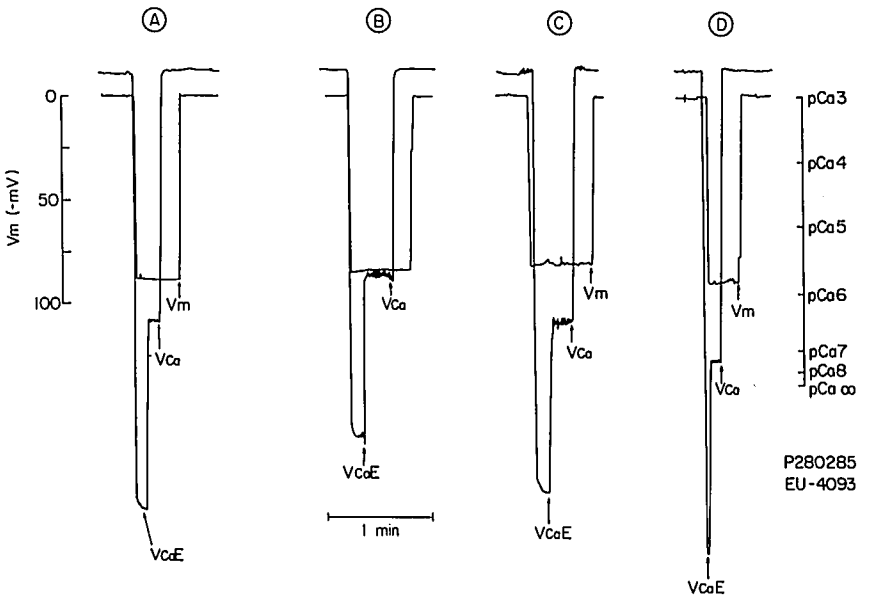

FIG. 5. Recording of the resting membrane potential (Vm) and intracellular free calcium potential (VCa) from a typical MH-susceptible skeletal muscle fibers *in vivo* before the MH syndrome was initiated by halothane 2% and after the susceptible animal received EU 4093 to reverse the syndrome. *A*: The control value for Vm and $[Ca^{2+}]_i$ (−82 mV, 470 nM) before the animal received halothane; *B*: after reversal of the pancuronium and administration of halothane 2% Vm and $[Ca^{2+}]_i$ were −78 mV and 4,090 nM; *C*: after administration of EU 4093 0.5 mg/kg Vm and $[Ca^{2+}]_i$ were 78 mV and 630 nM; and *D*: after 1.0 mg/kg (cumulative dose) Vm and $[Ca^{2+}]_i$ were −82 mV and 50 nM. Calibration curves for Vm and *p*Ca are on the left and right axes, respectively.

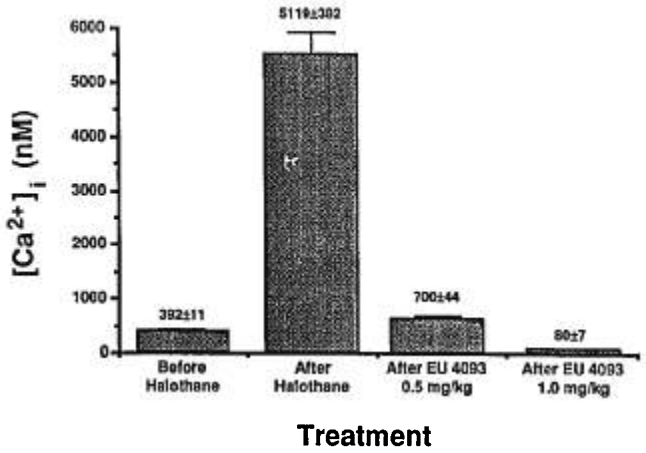

FIG. 6. Average values (mean ± SD) of resting $[Ca^{2+}]_i$ in MH-susceptible swine before and after exposure to halothane, which triggered the MH syndrome. This was followed by a cumulative dose administration of EU 4093 of 0.5 and 1.0 mg/kg respectively. These were 393 ± 10* to 5530 ± 410 nM,† 674 ± 44 nM,* and 79 ± 6 nM,*†‡ respectively. The latter dose was successful in reversing the MH symptoms. †$P < 0.05$ compared to prehalothane measurement. *$P < 0.01$ compared to the halothane measurement. ‡$P < 0.05$ compared to 0.5 mg/kg EU 4093 measurement.

on the resting membrane potential. By comparing the data from these two studies it can be seen that for a given dose, EU 4093 was 30–50% more potent than dantrolene in reducing $[Ca^{2+}]_i$ in skeletal muscle of normal and MH-susceptible swine when studied in similar animals and under similar conditions. It was also 30–50% more potent than dantrolene in treating the pathophysiologic aspects of the MH syndrome (ED_{100} 1 *vs.* 2 mg/kg).

The ED_{100} was chosen as an endpoint because of its clinical relevancy and because of the steepness of the dose–effect relationship. These data on the potency and ability of EU 4093 to reverse the clinical MH syndrome in susceptible swine have been confirmed by the recent report of Dershwitz and Sreter.[15] It is not possible to determine the potency of EU 4093 in reducing $[Ca^{2+}]_i$ as accurately in control swine as in MH-susceptible swine because the calibration curve for the intracellular Ca^{2+} electrodes is somewhat nonlinear between pCa 7 and 8. In our studies of the effects of dantrolene in the prevention of MH symptoms we found that there is a threshold $[Ca^{2+}]_i$ close to 200 nM below which MH was prevented in all cases and above which the syndrome could be triggered in all cases.[9] This suggests that a dose between 0.5 and 1.0 mg/kg of EU 4093 should be effective in the prevention of MH; however, this hypothesis was not tested in the present study. Both dantrolene and EU 4093 in equipotent doses (2 *vs.* 1 mg/kg) caused muscle weakness in the pigs, as demonstrated by difficulty in walking for more than 2 h after treatment, similar to the weakness reported in humans following dantrolene administration.[1]

Resting $[Ca^{2+}]_i$ is a balance between active Ca^{2+} uptake by the SR and passive release or "leak" from the Ca^{2+} release channel in the SR. As is demonstrated by the effects of EU 4093 on both normal and MH-susceptible muscle, this leak can be acutely altered and basal $[Ca^{2+}]_i$ reduced. It seems apparent from these and previous studies that either the probable increased passive "leak" from the SR in susceptible animals must be very large or somehow there is a change the set point of the SR Ca^{2+} ATPase to permit the increased resting $[Ca^{2+}]_i$. The presence of an increased passive leak has been demonstrated previously.[16] However, if it were high enough to "overwhelm" the Ca^{2+} ATPase, one would expect it to cause an increased basal metabolic rate, and this is not the case. The SR Ca^{2+} ATPase in susceptible animals has been shown to have normal activity and is sufficient to lower their $[Ca^{2+}]_i$ to resting levels after a contraction with a normal time course. To date, there have been no studies that have demonstrated a change in the Ca^{2+} sensitivity of the pump, but most of these studies have been done with micromolar Ca^{2+} in the assay solution and have not examined the Ca^{2+} sensitivity of the pump.

Previous investigation has shown that dantrolene's mechanism of action is an inhibition of SR Ca^{2+} release during activation and a reduction of passive Ca^{2+} efflux.[16–19] This effect could be mediated by changing the non–ionic charge movement[20] or by acting directly on the SR[10,16,21] or both. The mechanism for these changes is most probably due to the ability of dantrolene to reduce the burst frequency of the SR Ca^{2+} release channels.[12] It is possible, but not demonstrated, that EU 4093 may have similar effects. In isolated SR vesicles from control and MH swine, EU 4093 was able to significantly decrease SR Ca^{2+} release at a concentration of 10^{-7} M or greater, suggesting a mechanism of action similar to that of dantrolene on the Ca^{2+} release channel.[22] It has also been shown to block ryanodine binding to purified heavy SR vesicle membranes enriched for the Ca^{2+} release channel/ryanodine receptor.[21] Below 10^{-5} M, EU 4093 does not change the Ca^{2+} uptake by the SR, but at concentrations greater than 10^{-5} M, it has been shown to increase SR Ca^{2+} uptake by 15 and 75% in control and MH vesicles respectively.[23] These effects appear to be mediated by a change in the activity of the Ca^{2+}–Mg^{2+} ATPase by EU 4093.[24]

The authors thank Alyce Russo and Dhuwya Otero for their secretarial assistance and illustrations.

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