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Effects of Dantrolene on Myoplasmic Free [Ca^{2+}] Measured In Vivo *in Patients Susceptible to Malignant Hyperthermia*

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Malignant hyperthermia (MH) is a genetic disease characterized by hypermetabolism in skeletal muscle following a triggering stimulus and can be reversed or pretreated with dantrolene sodium. The myoplasmic free [Ca^{2+}] was measured, using Ca^{2+} selective microelectrodes *in vivo* in the superficial fibers of the sartorius muscle of eight MH-susceptible and eight control subjects. Both groups received continuous epidural anesthesia with chloroprocaine 3%. In both the control and MH muscle fibers, the myoplasmic free [Ca^{2+}] was measured before and after the intravenous administration of a cumulative dantrolene dose of 0.5, 1.5, and 2.5 mg/kg. The mean resting myoplasmic free [Ca^{2+}] was 0.112 ± 0.004 μM (mean ± SEM n = 32) in the control and 0.485 ± 0.022 μM (n = 33) in the MH subjects. In the MH subjects, dantrolene induced a dose-dependent reduction in myoplasmic free [Ca^{2+}]. The 0.5-mg/kg dose reduced it to 0.326 ± 0.017 μM (n = 22), the 1.5-mg/kg dose to 0.233 ± 0.015 μM (n = 25), and the 2.5-mg/kg dose to 0.092 ± 0.008 μM (n = 26). In controls, dantrolene also reduced resting myoplasmic free [Ca^{2+}] but to a lesser extent. The 0.5-mg/kg dose reduced it to 0.096 ± 0.004 μM (n = 22), the 1.5-mg/kg dose to 0.077 ± 0.003 μM (n = 23), and the 2.5-mg/kg dose to 0.068 ± 0.002 μM (n = 27). The results of the study extend our previous findings in humans and swine and demonstrate that it is possible to measure myoplasmic free [Ca^{2+}] *in vivo* in humans. They also demonstrate that the effect of administration dantrolene on human skeletal muscle *in vivo* is associated with a dose-dependent reduction in the myoplasmic free [Ca^{2+}]. (Key words: Malignant hyperthermia, ions: calcium. Measurement techniques, calcium: microelectrodes. Neuromuscular relaxants: dantrolene.)

MALIGNANT HYPERTHERMIA (MH) is a potentially fatal genetic myopathy that occurs if susceptible individuals are exposed to volatile anesthetic agents and or depolarizing muscle relaxants.[1,2] It is now well established that the pathophysiology of MH syndrome is related to a malfunction of the intracellular Ca^{2+} homeostasis in skeletal muscle.[3–6] The ryanodine receptor, which is proposed to be the Ca^{2+}-release channel of the sarcoplasmic reticulum, has been shown to be abnormal and have abnormal function in MH-susceptible pigs.[7–9] A defect in the function of this protein would be likely to produce an alteration of Ca^{2+} homeostasis. An abnormally high resting myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) has been found in MH-susceptible swine[4] and patients.[3,5,6] In addition, a marked increase in $[Ca^{2+}]_i$ has been shown to occur during an experimentally induced MH episode.[4] The hydantoin derivative, dantrolene sodium, is the only drug proven effective for the prophylaxis and therapeutic management of MH.[10–12] Dantrolene depresses excitation–contraction coupling without interfering with either the electrical properties (resting and action potential) of skeletal muscle or neuromuscular transmission.[13] Furthermore dantrolene does not act on the contractile proteins or specifically change nonionic charge movement during excitation–contraction coupling.[14] It has been shown to block Ca^{2+} release from the sarcoplasmic reticulum but to have no effect on Ca^{2+} uptake by this organelle. We have shown previously *in vitro* that dantrolene decreases resting $[Ca^{2+}]_i$ in muscle fibers from susceptible patients and swine.[6,15] In the present investigation we studied the effect of dantrolene sodium on $[Ca^{2+}]_i$ *in vivo* in control subjects and MH-susceptible subjects who received increasing doses of dantrolene sodium.

Materials and Methods

After obtaining informed consent as approved by the committee for the protection of patients for research, eight MH-susceptible volunteers and eight control volunteers without any antecedent history of neuromuscular disease received continuous epidural anesthesia with chloroprocaine 3%. MH susceptibility in the susceptible subjects was confirmed by a positive clinical or family history and a positive caffeine contracture test (> 1 g contracture at < 2.0 mM caffeine) from a previous muscle biopsy.[16] After introduction of anesthesia, a 4-cm incision was made on the right leg. The sartorius muscle was identified, its surface was freed of connective tissue, and then the superficial muscle fibers were exposed and kept covered with a pool of warmed (36.3 ± 1° C) physiologic solution. The temperature was monitored by a thermistor placed in the pool. The pool was replenished before every measurement, and a constant temperature was maintained for the measurements. Any bleeding in the incision was controlled by careful ligation of individual blood vessels. Although this anesthesia technique provides excellent muscle immobilization, electrode movement artifacts were

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reduced by using flexible electrode holders as described previously.[3,4,6] Several separate impalements were made for control measurements and after each dose of dantrolene. The electrodes were removed from the cells after each measurement. When the measurements were completed, the incision was closed, and the subjects were observed in the recovery room for 8–10 h and then remained in the hospital for 24–48 h prior to discharge.

Calcium-selective Microelectrodes

Glass microelectrodes with an outside tip diameter of ~ 0.4 μM were pulled from cleaned single-barrel 1.5-mm-OD borosilicate capillary tubing with an internal filament (WPI IBI20F-4) that had been washed with HCl and then distilled water. Subsequently, after drying at 200° C for 2 h to remove any water adsorbed by the glass, the inner wall of the unbeveled glass microelectrodes were silanized near the tip by exposure to a small amount (15 μl) of N-(trimethylsilyl) dimethylamine (Fluka 41720). The microelectrodes then were cooled and 24 h later backfilled with the neutral Ca^{2+} resin ETH 129 (personal gift of D. Amman, Department of Organic Chemistry, Swiss Federal Institute of Technology, Zurich, Switzerland)[17,18] up to 1–2 mm from the tip. This high column of the neutral carrier provides better mechanical stability although it makes the microelectrodes slightly noisier. Their shanks were then backfilled with 100 nM $CaCl_2$ solution. The Ca^{2+}-selective microelectrodes were individually calibrated 24 h after they were made and were also calibrated before and after each series of determinations in Ca^{2+} solutions of different *p*Ca (negative log of the Ca^{2+} concentration) (*p*Ca 3–8) containing physiologic amounts of Na^+, K^+, and Mg^{2+}.[19] Capacitative charging was partially compensated for using electronic capacitance compensation; the remaining capacitance charge could not be altered because of the configuration of the microelectrode itself. The filled microelectrodes required storage for 36–48 h in nitrogen and argon. Only 25% of these microelectrodes acquired the Nernstian response needed to make accurate recordings (30.5 mV/pCa). The remainder of the electrodes had to be discarded. Usually four or five fibers could be sequentially impaled without appreciable changes in the microelectrode properties.

In a second set of experiments we used a double-barreled electrode to make measurements in three subjects. These electrodes were constructed using two borosilicate glass capillaries with internal filaments of 1.5 and 1.2 mm OD that had been glued together. The thinner tube then was softened in a vertical microelectrode puller (Narishige) and twisted 360° around the thicker tube. The two tubes then were pulled simultaneously to a short taper with a total outside tip diameter of 0.6 μm. During silanization the 1.5-mm-OD electrode barrel was exposed to dimethyldichlorosilane vapor while air pressure was applied to the other (1.2-mm-OD) electrode to prevent its silanization. The microelectrodes then were baked at 200° C for 2 h. After cooling, the silanized barrel was backfilled with a small column of neutral carrier in a manner similar to that used for the single-barreled electrodes, and the rest of the electrodes were backfilled with *p*Ca 7 reference solution. The reference electrode barrel was filled with 3 M KCl. These Ca^{2+} selective microelectrodes were calibrated in identical solutions as the single-barreled electrodes. The double-barreled electrodes required calibration after two measurements (as opposed to five or more) because they were not as stable in maintaining their Ca^{2+} sensitivity as the single-barreled electrodes. We cannot explain the difference in stability between the two types of electrodes other than to say that it might be due to the differences in silanization.

Figure 1 shows a representative calibration curve from a single-barreled Ca^{2+} microelectrode prepared with ETH 129 before and after five measurements were made. As can be seen in figure 1, the voltage deflection was linear (30.5 mV per *p*Ca unit—the Nernstian response) to *p*Ca 7 and then became nonlinear between *p*Ca 7 and 8 (about 20–25 mV) and even more nonlinear between *p*Ca 8 and 9 (12.5 mV). It can be seen that the electrode's response did not change significantly before and after five uses to measure $[Ca^{2+}]_i$. The properties of ETH 129 are about two orders of magnitude more sensitive than ETH 1001 on which we have reported before.[15]

The interference of Mg^{2+}, Na^+, and H^+ on the Ca^{2+} signal was explored in some microelectrodes prior to making Ca^{2+} measurements *in vivo*. These experiments were done in a solution of very low Ca^{2+} (*p*Ca 8). Elevation

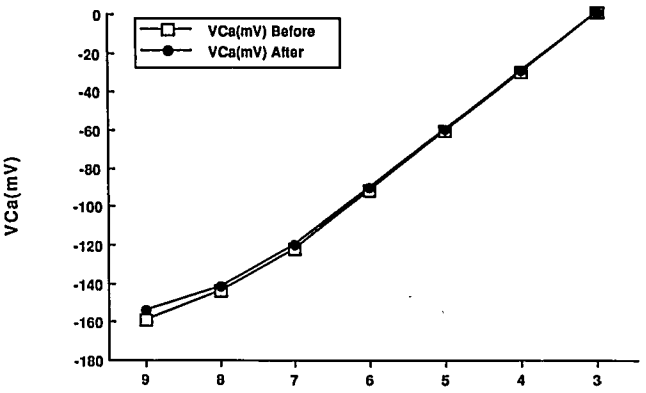

FIG. 1. Calibration curve of a representative Ca^{2+}-selective microelectrode using ETH 129 as the neutral carrier. The relationship between voltage (V_{Ca}) and *p*Ca follows the predicted Nernst relationship from *p*Ca 3 to *p*Ca 7 (30.5 mu per *p*Ca unit) and shows a modest loss of linearity between *p*Ca 7 and *p*Ca 8 (20–25 mV) and an even greater loss between *p*Ca 8 and *p*Ca 9 (15–20 mV). Note that there was no significant difference in the calibration curve between the preuse and the postuse calibration.

of Mg^{2+} from 0 to 6 mM altered the microelectrode potential by 2 mV (full-scale deflection ~ 160–240 mV). Increments of Na^+ concentration from 0 to 20 mM altered the microelectrode potential by 4 mV. Changes in *p*H between 6.2 and 7.2 changed the potential by 2 mV. The response of the microelectrodes was not altered by dantrolene up to 10^{-5} M. The response of our Ca^{2+}-selective microelectrodes in the calibration solutions indicates that they can measure the Ca^{2+} concentration to below *p*Ca 7 and in the presence of those cations that are present in the myoplasm. Therefore, the values of $[Ca^{2+}]_i$ reported should not be limited by the response of the microelectrodes or by interference from intracellular cations.

Recording Procedure

In seven MH-susceptible and six control subjects, a superficial muscle fiber was impaled first with a conventional 3 M KCl microelectrode to measure the resting membrane potential (V_m) and an adjacent fiber was impaled with the Ca^{2+}-selective microelectrode to measure the myoplasmic free Ca^{2+} concentration ($V_{Ca}E$). The measurements of these two parameters were carried out in different muscle fibers because the experimental arrangement prevented the use of single fibers *in vivo*. We have shown previously that the use of different muscle cells for the two measurements did not invalidate the quantitative aspects of our measurements because of the small variability observed in the membrane potential values.[4] In addition, we have made similar measurements *in vitro* where both electrodes were placed in a single muscle fiber, which yielded results similar to those we found when two fibers were used.

In one MH-susceptible individual and two control individuals, measurements of both V_m and $V_{Ca}E$ were made in the same fiber *in vivo* using the double-barreled microelectrode arrangement, to eliminate any question of the validity and accuracy of the single-barreled technique.

For the single-barreled electrodes the Ca^{2+}-specific potential (V_{Ca}) was calculated after the recording procedure by subtracting the mean of the V_m measurements for a single individual for a given treatment condition from each measurement of the output of the Ca^{2+} microelectrode ($V_{Ca}E$) for that condition. The actual $[Ca^{2+}]_i$ value for a particular muscle cell was obtained from the calibration curve of the Ca^{2+} microelectrode used in that cell. For the double-barreled experiments each individual measurement of V_m was electrically subtracted from $V_{Ca}E$ to yield V_{Ca}, and as above, the $[Ca^{2+}]_i$ value for a particular muscle cell was obtained from the calibration curve of the Ca^{2+} microelectrode used in that cell.

In all cases multiple measurements were made for each treatment condition in each individual. Data that conformed to the following criteria were accepted:

1. The microelectrode calibration curve performed before and after each five impalements matched within 4 mV from *p*Ca 6 to 7 (this was done after two impalements for the double-barreled electrode).
2. A stable V_m was not more positive than −79 mV.
3. The membrane potential or Ca^{2+} potential could not shift by more than 4 mV during the Ca^{2+} microelectrode impalements.

Measurements of myoplasmic Ca^{2+} concentration were carried out as follows: 1) after induction of anesthesia, before dantrolene administration; 2) within 10 min after the administration of intravenous dantrolene 0.5 mg/kg; 3) within 10 min after the administration of the second dose of intravenous dantrolene, 1 mg/kg (total dose 1.5 mg/kg); and 4) within 10 min after the third dose of intravenous dantrolene 1 mg/kg (total dose 2.5 mg/kg). Three of the control subjects and one MH-susceptible subject did not wish to receive dantrolene because of the expected side effects, and therefore only measurements of resting $[Ca^{2+}]_i$ without dantrolene were performed on these subjects.

To make certain that the high $[Ca^{2+}]_i$ values reported were not due to a leak around the microelectrodes from the extracellular fluid, in all subjects during the control period, two or three additional Ca^{2+} measurements were performed after the $[Ca^{2+}]$ in the physiologic solution was increased from 1.8 mM to 9 mM.

Statistical analysis was done using a Kruskall-Wallis and Mann-Whitney U test because the data did not conform to a normal distribution. Significance was accepted at the $P < 0.05$ level.

Results

Resting $[Ca^{2+}]_i$ Measurements

Using the single-barreled electrodes the mean resting $[Ca^{2+}]_i$ was 0.112 ± 0.004 μM, mean ± SEM (n = 31) in the control subjects and 0.485 ± 0.022 μM (n = 32) in the MH-susceptible subjects ($P < 0.001$). The mean resting potential was a −84 ± 0.36 mV in the control subjects and −83 ± 0.39 mV in the MH-susceptible subjects (*P* not significant). Using the double-barreled electrodes the mean resting $[Ca^{2+}]_i$ was 0.103 ± 0.002 μM (n = 14) in the control subjects and 0.420 ± 0.015 μM (n = 9) in the MH-susceptible subject. Mean resting potential in the same cells was −82 ± 0.4 mV in the control subjects and −82 ± 0.4 mV in the MH-susceptible subject. These results were not different than the results obtained using the single-barreled electrodes ($P > 0.60$). The results of the individual measurements of the single-barreled electrodes are provided in tables 1 and 2, and those from the double-barreled electrode are provided in table 3. In the

TABLE 1. Individual and Mean Measurements of Membrane Potential, Calcium Potential, and Intracellular Calcium Concentration for Control Subjects at All Dantrolene Doses

0 mg/kg Dantrolene				0.5 mg/kg Dantrolene				1.5 mg/kg Dantrolene				2.5 mg/kg Dantrolene			
Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)	Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)	Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)	Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)
A	85	120	0.11	C	86	122	0.10	C	83	126	0.07	C	83	128	0.06
A	82	118	0.13	C	84	121	0.10	C	84	126	0.07	C	82	124	0.09
A	83	119	0.12	C	83	121	0.10	C	83	123	0.09	C	82	126	0.07
A	84	122	0.10	C	85	121	0.10	D	85	126	0.07	C	83	128	0.06
A	82	123	0.09	C	86	124	0.09	D	86	127	0.06	C	84	127	0.06
B	82	118	0.13	C	85	121	0.10	D	82	125	0.07	C	86	128	0.06
B	86	120	0.11	D	86	126	0.07	D	81	124	0.09	C	80	124	0.09
B	88	124	0.09	D	83	123	0.09	D	83	126	0.07	D	82	124	0.09
B	89	126	0.07	D	84	122	0.10	D	83	124	0.09	D	83	129	0.05
B	86	122	0.10	D	82	121	0.10	E	85	125	0.07	D	83	126	0.07
B	80	115	0.16	D	83	123	0.09	E	86	129	0.05	D	82	125	0.07
C	84	119	0.12	D	82	120	0.11	E	84	126	0.07	D	83	125	0.07
C	86	123	0.09	E	86	124	0.09	E	83	125	0.07	D	84	130	0.05
C	83	121	0.10	E	84	120	0.11	E	82	122	0.10	D	82	126	0.07
C	85	120	0.11	E	85	122	0.10	E	84	124	0.09	D	80	124	0.09
C	84	118	0.13	E	86	123	0.09	F	86	126	0.07	E	82	128	0.06
C	85	119	0.12	F	82	120	0.11	F	84	122	0.10	E	80	127	0.06
D	86	117	0.14	F	83	121	0.10	F	85	125	0.07	E	82	127	0.06
D	86	119	0.12	F	82	123	0.09	F	85	122	0.10	E	83	130	0.05
D	85	116	0.15	F	84	126	0.07	F	86	126	0.07	E	80	127	0.06
D	86	119	0.12	F	83	122	0.10	F	85	126	0.07	E	82	131	0.05
E	83	119	0.12									F	84	127	0.06
E	83	123	0.09									F	85	125	0.07
E	83	121	0.10									F	83	128	0.06
E	86	124	0.09									F	82	124	0.09
E	85	122	0.10									F	80	121	0.10
E	84	120	0.11									F	82	127	0.06
E	86	122	0.10									F	83	126	0.07
F	85	119	0.12												
F	82	119	0.12												
F	81	119	0.12												
Mean ± SEM															
A	83 ± 0.58	120 ± 0.93	0.11 ± 0.007												
B	85 ± 1.40	121 ± 1.60	0.11 ± 0.013												
C	84 ± 0.42	120 ± 0.73	0.11 ± 0.006	C	85 ± 0.50	122 ± 0.50	0.098 ± 0.002	C	83 ± 0.33	125 ± 1.00	0.077 ± 0.007	C	83 ± 0.70	126 ± 0.69	0.070 ± 0.005
D	84 ± 0.45	120 ± 0.60	0.11 ± 0.004	D	83 ± 0.61	122 ± 0.85	0.093 ± 0.006	D	83 ± 0.84	125 ± 0.58	0.076 ± 0.006	D	82 ± 0.42	126 ± 0.79	0.070 ± 0.005
E	84 ± 0.40	120 ± 0.73	0.11 ± 0.006	E	85 ± 0.50	122 ± 0.85	0.098 ± 0.005	E	84 ± 0.58	125 ± 0.95	0.075 ± 0.007	E	81 ± 0.05	128 ± 0.72	0.057 ± 0.002
F	83 ± 1.20	119 ± 0.00	0.12 ± 0.000	F	83 ± 0.37	122 ± 1.03	0.094 ± 0.007	F	85 ± 0.31	124 ± 0.81	0.080 ± 0.006	F	82 ± 0.61	125 ± 0.90	0.073 ± 0.006
Grand mean	84 ± 0.36	120 ± 0.44	0.11 ± 0.003	Grand mean	84 ± 0.32	122 ± 0.38	0.096 ± 0.002	Grand mean	84 ± 0.32	125 ± 0.38	0.077 ± 0.003	Grand mean	82 ± 0.28	126 ± 0.42	0.068 ± 0.003

All measurements were made with single-barreled microelectrodes placed in separate cells. V_m = membrane potential; $V_{Ca}E$ = calcium potential (individual V_{Ca} − the mean V_m for that individual, for that treatment); $[Ca^{2+}]_i$ = calculated intracellular free calcium concentration using the calculated $V_{Ca}E$ and the calibration curve for the electrode used for that measurement. More than one electrode may have been used for a given set of measurements, which would allow the same value for $V_{Ca}E$ to yield a different value of $[Ca^{2+}]_i$.

TABLE 2. Individual and Mean Measurements of Membrane Potential, Calcium Potential, and Intracellular Calcium Concentration for Malignant Hyperthermia–susceptible Patients at All Dantrolene Doses

0 mg/kg Dantrolene				0.5 mg/kg Dantrolene				1.5 mg/kg Dantrolene				2.5 mg/kg Dantrolene			
Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)	Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)	Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)	Patient	V_m (mV)	$V_{Ca}E$ (mV)	$[Ca^{2+}]_i$ (μM)
A	85	106	0.33	A	80	108	0.26	A	83	114	0.16	A	81	123	0.09
A	88	107	0.30	A	88	110	0.23	A	82	111	0.22	A	80	120	0.11
A	80	97	0.63	B	83	108	0.26	A	88	118	0.12	A	83	126	0.07
A	81	97	0.63	B	85	104	0.35	B	86	115	0.16	A	80	123	0.09
A	84	97	0.63	B	83	112	0.20	B	83	111	0.22	B	82	124	0.09
B	83	94	0.72	B	86	113	0.19	B	84	109	0.30	B	82	120	0.11
B	88	100	0.50	C	85	105	0.33	B	82	110	0.23	B	84	125	0.07
C	86	104	0.35	C	85	108	0.26	C	81	107	0.30	C	83	123	0.09
C	81	101	0.46	C	83	108	0.26	C	82	106	0.33	C	85	125	0.07
C	81	100	0.50	D	83	103	0.41	C	83	112	0.20	C	84	125	0.07
C	82	103	0.41	D	84	101	0.46	C	84	110	0.23	C	83	126	0.07
D	81	101	0.46	D	85	104	0.35	D	82	104	0.35	D	82	124	0.09
D	81	99	0.56	D	85	104	0.35	D	85	109	0.30	D	85	126	0.07
D	84	102	0.44	E	83	107	0.30	D	83	106	0.33	D	80	124	0.09
D	83	98	0.58	E	83	106	0.33	E	86	116	0.15	D	83	128	0.06
D	83	99	0.56	E	82	101	0.46	E	85	114	0.16	E	83	122	0.10
E	82	103	0.41	F	84	109	0.30	E	82	112	0.20	E	85	127	0.06
E	84	110	0.23	F	83	106	0.33	E	82	107	0.30	E	86	126	0.06
E	82	101	0.46	G	84	105	0.33	F	83	115	0.16	E	82	119	0.11
E	83	101	0.46	G	86	105	0.33	F	84	116	0.15	E	84	121	0.10
E	82	99	0.56	G	85	101	0.46	G	82	108	0.26	F	86	128	0.06
F	80	100	0.50	G	83	103	0.41	G	82	106	0.33	F	84	125	0.07
F	83	102	0.44					G	83	113	0.19	F	83	122	0.10
F	82	99	0.56									G	84	113	0.19
F	83	103	0.41									G	84	125	0.07
F	84	103	0.41									G	82	108	0.26
F	85	103	0.41									G	82	121	0.10
G	85	100	0.50									G	86	130	0.05
G	82	93	0.72												
G	86	99	0.56												
G	85	100	0.50												
G	87	100	0.50												
Mean ± SEM															
A	83 ± 1.43	100 ± 2.33	0.50 ± 0.077	A	84 ± 4.00	109 ± 1.00	0.25 ± 0.015	A	84 ± 1.85	114 ± 2.03	0.170 ± 0.029	A	81 ± 0.71	123 ± 1.22	0.090 ± 0.008
B	85 ± 2.50	97 ± 3.0	0.61 ± 0.110	B	84 ± 0.75	109 ± 2.06	0.25 ± 0.037	B	84 ± 0.85	111 ± 1.31	0.228 ± 0.029	B	83 ± 0.67	123 ± 1.53	0.090 ± 0.012
C	83 ± 1.19	102 ± 0.91	0.43 ± 0.032	C	84 ± 0.67	107 ± 1.00	0.28 ± 0.023	C	83 ± 0.65	108 ± 1.38	0.265 ± 0.030	C	84 ± 0.48	125 ± 0.63	0.075 ± 0.005
D	82 ± 0.45	99 ± 0.73	0.52 ± 0.029	D	84 ± 0.61	103 ± 0.71	0.39 ± 0.027	D	83 ± 0.88	106 ± 1.45	0.327 ± 0.015	D	82 ± 1.04	125 ± 0.96	0.078 ± 0.008
E	83 ± 0.40	103 ± 1.91	0.42 ± 0.054	E	83 ± 0.33	105 ± 1.85	0.36 ± 0.049	E	84 ± 1.03	112 ± 1.93	0.202 ± 0.034	E	84 ± 0.71	123 ± 1.57	0.086 ± 0.006
F	83 ± 0.70	101 ± 0.72	0.45 ± 0.025	F	83 ± 0.50	108 ± 1.50	0.32 ± 0.015	F	84 ± 0.50	115 ± 0.50	0.155 ± 0.005	F	84 ± 0.88	125 ± 1.73	0.077 ± 0.012
G	85 ± 0.84	98 ± 1.36	0.56 ± 0.043	G	85 ± 0.65	104 ± 0.96	0.38 ± 0.032	G	82 ± 0.33	109 ± 2.08	0.260 ± 0.040	G	84 ± 0.75	119 ± 3.98	0.134 ± 0.040
Grand mean	83 ± 0.38	100 ± 0.60	0.49 ± 0.020	Grand mean	84 ± 0.32	105 ± 0.71	0.33 ± 0.017	Grand mean	83 ± 0.35	110 ± 0.81	0.233 ± 0.015	Grand mean	83 ± 0.33	123 ± 0.85	0.092 ± 0.008

All measurements were made with single-barreled microelectrodes placed in separate cells. V_m = membrane potential; $V_{Ca}E$ = calcium potential (individual V_{Ca} − the mean V_m for that individual, for that treatment); $[Ca^{2+}]_i$ = calculated intracellular free calcium concentration using the calculated $V_{Ca}E$ and the calibration curve for the electrode used for that measurement. More than one electrode may have been used for a given set of measurements, which would allow the same value for $V_{Ca}E$ to yield a different value of $[Ca^{2+}]_i$.

TABLE 3. Individual Measurements of Resting Membrane Potential and Intracellular Calcium Concentration with a Double-Barreled Electrode in Control and Malignant Hyperthermia–susceptible Subjects before Administration of Dantrolene Sodium

Patient (control)	V_m (mV)	$[Ca^{2+}]_i$ μM	Patient (Malignant Hyperthermia–susceptible)	V_m (mV)	$[Ca^{2+}]_i$ μM
G	−81	0.106	H	−81	0.389
G	−83	0.110	H	−84	0.420
G	−81	0.106	H	−82	0.436
G	−84	0.109	H	−83	0.380
G	−81	0.110	H	−82	0.379
G	−83	0.103	H	−82	0.453
G	−81	0.98	H	−81	0.476
G	−81	0.102	H	−81	0.412
G	−82	0.96	H	−83	0.432
H	−83	0.98			
H	−82	0.105			
H	−82	0.103			
H	−83	0.97			
H	−85	0.108			
Mean ± SEM	−82 ± 0.34	0.10 ± 0.001	Mean ± SEM	−82 ± 0.35	0.419 ± 0.011

In this case both measurements were made in a single muscle cell.

experiments performed after the $[Ca^{2+}]$ in the physiologic solution was increased from 1.8 to 9 mM, to test for possible leakage around the microelectrodes, $[Ca^{2+}]_i$ was not increased by increasing extracellular $[Ca^{2+}]$ (data not shown).

Effects of Dantrolene on Resting Myoplasmic $[Ca^{2+}]_i$

Figure 2 shows the summary of the changes in the mean resting $[Ca^{2+}]_i$ before and after dantrolene was administered in MH-susceptible subjects and normal controls. Thus, from an initial resting $[Ca^{2+}]_i$ of 0.485 ± 0.021 μM (n = 32), it decreased to 0.325 ± 0.017 μM (n = 22) after the administration of dantrolene 0.5 mg/kg, to 0.233 ± 0.015 μM (n = 23) after dantrolene 1.5 mg/kg, and to 0.092 ± 0.008 μM (n = 28) after dantrolene 2.5 mg/kg. Similar qualitative effects on $[Ca^{2+}]_i$ were observed in the control subjects who received dantrolene starting at the lower initial resting $[Ca^{2+}]_i$. All subjects who received dantrolene complained of significant muscle weakness for 2–3 days after the procedure. This complaint was significant enough to necessitate their planned admission to the hospital for 24–48 h after completion of the study.

Discussion

MH is a hereditary syndrome in genetically predisposed humans and swine. Its transmission is autosomal dominant in humans and autosomal recessive in swine. This syndrome can be triggered by volatile anesthetics such as halothane and/or depolarizing muscle relaxants such as succinylcholine. It is characterized by arrhythmias, muscle rigidity, metabolic and respiratory acidosis, and acute elevation of the body temperature.[1,2] The primary defect in MH subjects is believed to be in skeletal muscle tissue. In this regard a higher sensitivity to caffeine and halothane as well as a faster Ca^{2+} release by the sarcoplasmic reticulum (SR) have been described in MH muscle fibers compared to control.[16,20–22] In addition, a malfunction of intracellular Ca^{2+} homeostasis in skeletal muscle has been shown in MH-susceptible patients and swine.[3–6,15,19]

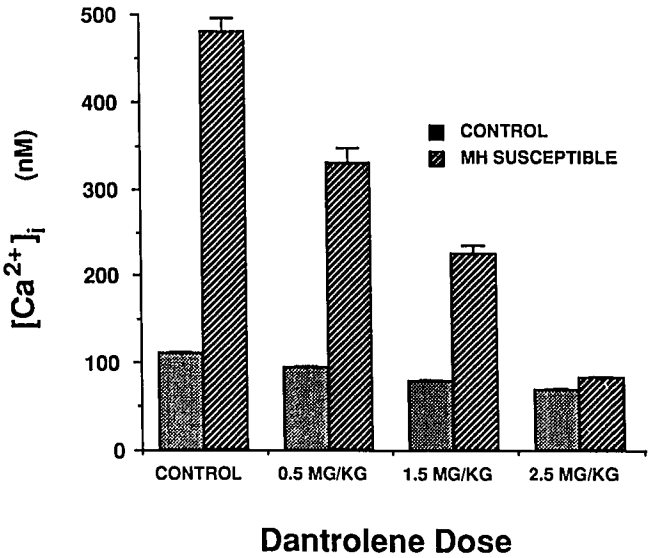

FIG. 2. Average values using single-barreled microelectrodes (mean ± SE) of $[Ca^{2+}]_i$ for six control (four with dantrolene) and 7 malignant hyperthermia (MH)–susceptible patients from a cumulative intravenous dantrolene dose of 0, 0.5, 1.5, and 2.5 mg/kg. For control patients these were 103 ± 2.7 nM, 96 ± 1.5 nM, 79 ± 1.7 nM, and 66 ± 1.6 nM, and for MH-susceptible patients these were 456 ± 17 nM, 305 ± 18 nM, 220 ± 11 nM, and 86 ± 2.4 nM.

The pathophysiology of this syndrome has been related to an alteration in the ryanodine receptor localized in the SR membrane.[9] It has been reported that exposure of human normal and MH-susceptible skeletal muscle to ryanodine at low concentrations (0.001–0.1 μM) produced an increment in the $[Ca^{2+}]_i$, and at concentrations higher than 0.1 μM this increment in the $[Ca^{2+}]_i$ was accompanied by a slow contracture. The ryanodine concentrations required to induced either increment in the $[Ca^{2+}]_i$ or muscle contracture were much lower in the MH muscle than in control.[19] An altered Ca^{2+} dependence of single channel inactivation in MH SR high-conductance cation channels (which are ryanodine sensitive and believed to be the SR Ca^{2+} release channel or ryanodine receptor) has also been reported.[8] It has been shown that there is a greater spontaneous leak in SR vesicles from MH-susceptible swine, and an altered rate and amount of Ca^{2+} release in response to Ca^{2+}, halothane, or depolarization.[20]

All of this evidence is consistent with the possibility that one candidate for the defective gene in MH-susceptible humans and swine is the SR Ca^{2+}-release channel/ryanodine receptor. Linkage studies with caffeine contracture data have shown significant linkage between abnormal caffeine contracture and a defect on chromosome 19 close to the location of the ryanodine receptor gene.[23,24] A point mutation in the coding region of this gene, which alters a single amino acid, has been found in all breeds of swine that are susceptible to MH.[24] Whether this point mutation is responsible for the alterations in the physiology of the channel remains to be seen.

Dantrolene is a unique muscle relaxant that has therapeutic and prophylactic effects in MH-susceptible patients and swine.[10–12] Dantrolene is a direct muscle relaxant that reduces force development in skeletal muscle.[25,26] It has no effect on neuromuscular transmission, on the electrical activity, or on the inward spread of activation in skeletal muscle.[27,28] The relaxant effect of dantrolene appears to be mediated by an action on inhibition of Ca^{+} release by the SR rather than on the sarcolemma.[29,30] In addition, dantrolene has been shown to block the conductance of Ca^{2+} channels in purified SR membranes studied with patch-clamp in artificial bilayers.[30] We have reported previously that the prophylactic effect of dantrolene in MH-susceptible swine is associated with its effect on reducing $[Ca^{2+}]_i$[15] and that the effect of a single dose can be observed for more than 1 h. The current data extend our *in vitro* studies in intact intercostal fibers[6] on the effect of dantrolene on $[Ca^{2+}]_i$ in MH-susceptible human skeletal muscle. The present data indicate that dantrolene reduces skeletal muscle $[Ca^{2+}]_i$ in a dose-dependent manner between 0.5–2.5 mg/kg, without any apparent change in V_m. Our previous finding in swine,[15] that a dose of dantrolene of 2.5 mg/kg and its associated reduction in $[Ca^{2+}]_i$ is able to prevent the onset of MH during a halothane/succinylcholine challenge, appears to be extendable to MH patients, since we have found similar qualitative and quantitative effects induced by dantrolene in both species. These results agree with the observation by Flewellen *et al.*,[31] who, using measures of muscle strength, concluded that 2.4 mg/kg dantrolene should be effective in producing prophylaxis. The present data also suggest that 1.5 mg/kg may be effective as a prophylactic dose, since this concentration lowered the $[Ca^{2+}]_i$ to 0.22 μM, which is below the 0.25 μM triggering threshold in swine.[12]

The present *in vivo* study confirms the findings of our previous *in vitro* reports[3,5,6,19] that the resting $[Ca^{2+}]_i$ is higher in MH-susceptible than in control subjects. This point seems of particular importance because it indicates a long-term imbalance of the intracellular Ca^{2+} regulation in MH subjects, since they were not exposed to any MH-triggering drugs before the measurements were carried out. The short-term regulation of $[Ca^{2+}]_i$ in skeletal muscle is controlled largely by the SR Ca^{2+} ATPase, whereas the long-term regulation is controlled by the sarcolemmal Ca^{2+} pump and Na^{+}/Ca^{2+} exchange. The activity of the SR Ca^{2+} ATPase has been shown to be normal in pigs and is presumed to be so in humans, although this last point is somewhat controversial.[1] Given a normal SR Ca^{2+} ATPase, it is at first difficult to explain why the SR is not capable of keeping myoplasmic Ca^{2+} normal. In MH-susceptible individuals the SR Ca^{2+} ATPase is capable of rapidly pumping $[Ca^{2+}]_i$ from a contraction level of over 1 μM to a level below the contraction threshold. This contraction level is much higher than the resting levels we have observed. If one assumes that there is no passive leak for the normal SR and that the pump is set to keep myoplasmic levels at 100 nM, then in MH-susceptible individuals the higher resting level of $[Ca^{2+}]_i$ would keep the pump constantly working to achieve the normal level. This in turn should increase resting basal metabolic rate. However, no increase in basal metabolic rate in MH-susceptible individuals has been shown.

In fact, however, there is a leak or passive release of Ca^{2+} from SR, and the resting $[Ca^{2+}]_i$ is an equilibrium between leak and uptake from the SR, as well as other factors. The Michaelis constant (K_m) for the SR Ca^{2+} ATPase is about 5 μM,[32] and it is likely that with such a high K_m the increased leak from the SR in MH-susceptible individuals would not cause a significant increase in pump activity, but rather a new equilibrium "set point." The data in this study leaves no doubt that the increase in $[Ca^{2+}]_i$ in MH-susceptible individuals comes from an increased basal release from the SR, and that if this increased leak is stopped, that the SR Ca^{2+} ATPase is capable of pumping the level of $[Ca^{2+}]_i$ down to normal. In the long term, high resting $[Ca^{2+}]_i$ should be compensated for by the long-term regulators of $[Ca^{2+}]_i$ in the sarcolemma. It has been reported that in sarcolemmal vesicles obtained

from MH-susceptible swine, the total accumulating capacity of the Ca^{2+} ATPase pump is significantly reduced from control.[33] Experiments conducted in a similar preparation obtained from MH patients showed a similar deficiency in this Ca^{2+} regulatory mechanism.[19] This raises the possibility that the altered Ca^{2+} homeostasis in MH individuals may be multifactorial.

The value of resting $[Ca^{2+}]_i$ in MH-susceptible subjects is in disagreement with the reports by Iaizzo *et al.*[34,35] in which fura-2 was used as a Ca^{2+} indicator. In these experiments the authors found no difference in resting $[Ca^{2+}]_i$ between MH-susceptible and control human and swine muscle fibers. They suggested that our finding of a higher resting $[Ca^{2+}]_i$ in MH subjects must be related to unnoticed membrane damage occurring only in the MH fibers during the microelectrode impalement. They based this hypothesis not only on the difference between our measurements but also on their assertion that fibers with a resting $[Ca^{2+}]_i$ in the range that we found for MH fibers should show microscopic damage from the action of Ca^{2+} similar to those seen in muscular dystrophy.[36] We believe this discrepancy is related only to limitations of fura-2 as a Ca^{2+} indicator. The measurements with double-barreled electrodes prove that there is no cell damage as is indicated by cells' ability to maintain a normal resting potential during electrode impalement. On the other hand, because a large fraction of fura-2 is bound to cellular structures, calibration of dye signals *in vitro* is almost impossible to extrapolate to *in vivo* absolute levels of $[Ca^{2+}]_i$,[37] and finally fura-2 is a high-affinity Ca^{2+} buffer and can itself directly lower absolute and measured $[Ca^{2+}]_i$ in a nonlinear fashion.[38]

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