Voltage-Dependent Calcium Release in Human Malignant Hyperthermia Muscle Fibers

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ABSTRACT Malignant hyperthermia (MH) results from a defect of calcium release control in skeletal muscle that is often caused by point mutations in the ryanodine receptor gene (RYR1). In malignant hyperthermia-susceptible (MHS) muscle, calcium release responds more sensitively to drugs such as halothane and caffeine. In addition, experiments on the porcine homolog of malignant hyperthermia (mutation Arg615Cys in RYR1) indicated a higher sensitivity to membrane depolarization. Here, we investigated depolarization-dependent calcium release under voltage clamp conditions in human MHS muscle. Segments of muscle fibers dissected from biopsies of the vastus lateralis muscle of MHN (malignant hyperthermia negative) and MHS subjects were voltage-clamped in a double vaseline gap system. Free calcium was determined with the fluorescent indicator fura-2 and converted to an estimate of the rate of SR calcium release. Both MHN and MHS fibers showed an initial peak of the release rate, a subsequent decline, and rapid turn-off after repolarization. Neither the kinetics nor the voltage dependence of calcium release showed significant deviations from controls, but the average maximal peak rate of release was about threefold larger in MHS fibers.

INTRODUCTION

Force activation in skeletal muscle fibers involves the rapid release of calcium ions from the sarcoplasmic reticulum (SR). This process is under direct control of the fiber membrane potential. The membrane potential is sensed by dihydropyridine receptors (DHP receptors) in the transverse tubular system that transmit information to the calcium release channels (ryanodine receptors) in the membrane of the terminal cisternae of the SR and cause them to open (for references see Melzer et al., 1995). Calcium release measured under voltage clamp conditions in isolated skeletal muscle fibers appears to be positively and negatively modulated by calcium (Schneider and Simon, 1988; Jacquemond et al., 1991; Csernoch et al., 1993; Chandler et al., 1995).

In the human skeletal muscle disorder malignant hyperthermia (MH), calcium release can be stimulated by clinical doses of anesthetics and depolarizing muscle relaxants commonly used in general anesthesia (Gronert, 1994; MacLennan and Phillips, 1992; Mickelson and Louis, 1996). Predisposed persons are at high risk to develop a condition of strongly increased muscle metabolism paired with muscle rigidity and massive hyperthermia during general anesthesia. If untreated, this condition is likely to be lethal. Standardized diagnostic tests have been developed in North America, Europe, and Japan to identify the susceptibility to malignant hyperthermia by measuring muscle force during application of triggering agents (Britt, 1989; European Malignant Hyperpyrexia Group, 1984; Kawana et al., 1992). In the European in vitro contracture test (IVCT), muscle bundles obtained from a skeletal muscle biopsy are subjected to increasing concentrations of caffeine and halothane. A significantly lowered threshold for the initiation of a contracture by both substances signals that the individual is malignant hyperthermia-susceptible (MHS).

Susceptibility to MH has been linked to several point mutations in the gene of the predominant skeletal muscle ryanodine receptor RYR1 (for a summary see Manning et al., 1998). Transfection of cDNA encoding mutant channels into muscle and nonmuscle cells led to expression of channels with higher caffeine and halothane sensitivity (Otsu et al., 1994; Tong et al., 1997; Censier et al., 1998). In a number of affected families, however, no linkage to the ryanodine receptor gene has been found (Deufel et al., 1992). One MH causing mutation in the α1-subunit of the DHP receptor, the putative voltage sensor for calcium release, has been published (Monnier et al., 1997). As has been the case in other channel diseases (Lehmann-Horn and Rüdel, 1996), naturally occurring mutations of the calcium release channel may provide important clues to molecular determinants for functional regions. Activation by calcium has been described to occur at lower concentrations for the human ryanodine receptors carrying mutation Gly2434Arg (Richter et al., 1997). Such altered calcium-dependent modulation (inactivation or activation) of the ryanodine receptor in MHS fibers might cause dynamic changes of the release rate measured during step depolarization in the absence of triggering agents.

One of the human RYR1 point mutations (Arg614Cys) is also found at a homologous location (Arg615Cys) in the porcine ryanodine receptor (Fujii et al., 1991) and causes a recessively inherited oversensitivity to halothane and stress (porcine stress syndrome). In porcine MH, a reduced sensitivity to inactivation by a low-affinity calcium/magnesium

Received for publication 26 February 1998 and in final form 8 August 1998.

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0006-3495/98/11/2402/09 $2.00

binding site of the ryanodine receptor has been reported (Laver et al., 1997). Work by Gallant and co-workers demonstrated a lowered concentration threshold for potassium-induced contractures in muscle from pigs carrying the mutation (Gallant and Lentz, 1992; Gallant and Jordan, 1996). The same group also showed a lowered voltage threshold for contraction in porcine myotubes (Gallant and Jordan, 1996). Voltage clamp experiments on myotubes from pigs homozygous for the Arg615Cys mutation showed activation of calcium release at lower voltages (Dietze et al., 1997) indicating altered voltage control caused by the mutation.

The present investigation is the first to study calcium transients in isolated single muscle fibers of human MHS individuals under voltage clamp conditions. It was carried out to identify possible alterations in the voltage dependence or time course of calcium release in this preparation.

METHODS

General procedure

Biopsies were obtained from the musculus vastus lateralis and used for MH diagnosis in the in vitro contracture test according to the European protocol. The use of this material is in agreement with the Helsinki convention and has been approved by the Ethics Commission of the University of Ulm. The response of bundles of fiber segments to increasing concentrations of caffeine and halothane served to classify subjects as normal (MHN), malignant hyperthermia equivocal (MHE), or malignant hyperthermia susceptible (MHS) as described by the European Malignant Hyperpyrexia Group (1984). Only MHN (controls) and MHS material were used for single fiber experiments. Segments of isolated muscle fibers were dissected from a bundle of the biopsy and voltage-clamped in a double vaseline gap system while intracellular calcium changes were recorded with the fluorescent indicator fura-2 (Fig. 1).

Because of the large amount of connective tissue and high fragility of the fibers, the yield was low relative to comparable experiments on frog fibers. Of a total of 108 experiments on human muscle fibers, 20 experiments (18 biopsies) could be used to determine the voltage dependence of calcium release. Of these, 11 experiments were on fibers of subjects diagnosed MHS (9 biopsies).

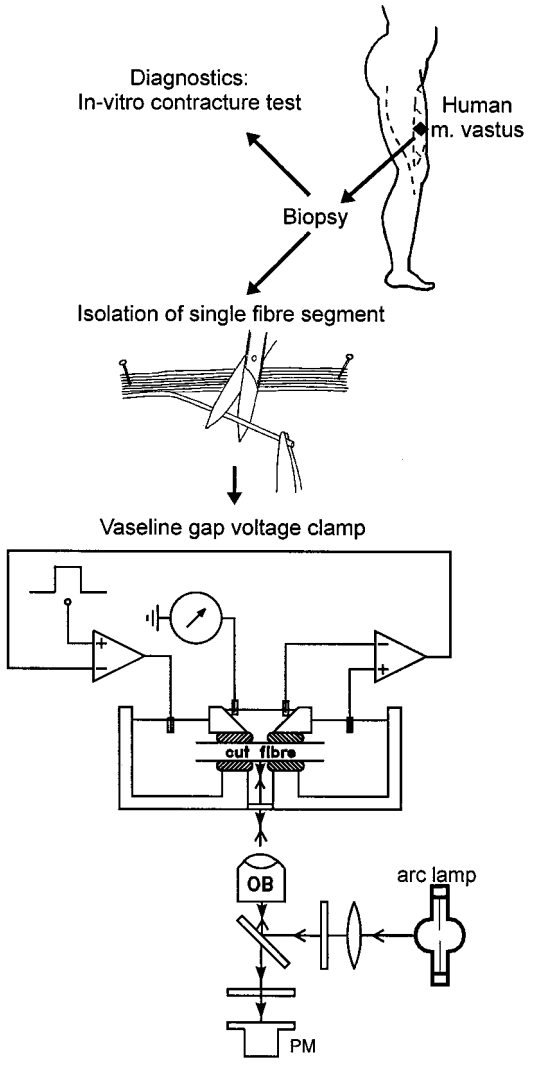

FIGURE 1 Experimental procedure. Muscle biopsies were split into small bundles and used primarily for IVCT diagnostics. A small portion of the material was used for dissecting single fiber segments for recording calcium-dependent fluorescence changes under voltage-clamp conditions.

Solutions

The following solutions were used for the experiments (concentrations in mM):

Storing and transfer of the biopsies: NaCl (145), KCl (5), $CaCl_2$ (2.5), $MgSO_4$ (1), HEPES (10), glucose (10), adjusted to pH 7.4 with NaOH.

Dissection of fiber bundles: NaCl (118), KCl (3.4), $CaCl_2$ (2.5), $MgSO_4$ (0.8), KH_2PO_4 (1.2), $NaHCO_3$ (25), glucose (adjusted to 290 mosmol/l), saturated with O_2 (95%) and CO_2 (5%).

Dissection of fiber segments: K_2SO_4 (95), HEPES (10), $MgCl_2$ (10), $CaCl_2$ (0.4), adjusted to pH 7.4 with NaOH, osmolarity adjusted to 300 mosmol/l with sucrose.

External experimental solution: CH_3SO_3H (140), $CaCl_2$ (2), $MgCl_2$ (2), HEPES (5), TTX (10^{-7} g/ml), adjusted to pH 7.4 with TEA-OH.

Internal (endpool) solution: glutamic acid (130), EGTA (0.1), HEPES (10), $CaCl_2$ (0,009), MgATP (5), (Na_2-) creatine phosphate (5), glucose (5), (K_5-)fura-2 (0.2), adjusted to pH 7.2 with CsOH.

Voltage clamping

Voltage clamping of cut fibers was carried out in a double vaseline gap system similar to the ones described in previous publications (Kovacs et al., 1983; Feldmeyer et al., 1990). Fibers were mounted in a recording chamber, which was constructed on a rectangular glass coverslip. The solution volume in each of the three pools (two endpools = internal, one middle pool = external) was ~250 μl. The voltage clamp electronics were connected to the solution pools of the chamber via silver-silver chloride electrodes bathed in 3 M KCl and via agar bridges (1.5%) equilibrated with the respective pool solutions (excluding glucose, ATP, creatine phosphate, and fura-2). For visual inspection and optical recording the chamber was mounted on an inverted fluorescence microscope (Axiovert 135 TV, Zeiss). Experiments were carried out at room temperature.

Calcium recording

Fibers were loaded from the cut ends with an artificial internal solution (see above) containing 0.2 mM of fura-2. While loading the fiber, the resting fluorescence at 500 nm was recorded during alternate excitation at 380 and 360 nm. Background fluorescence before fiber loading was subtracted. When a sufficiently high concentration of internal indicator was reached, calcium release was stimulated by depolarizing voltage steps. The fluorescence decrease at 380 nm excitation was recorded and divided by the

resting fluorescence at 360 nm excitation (isosbestic wavelength). The fluorescence ratio signal was converted to free calcium concentration using Eq. 1, which corrected for kinetic delays due to noninstantaneous calcium binding to the indicator dye (Klein et al., 1988).

$$[Ca^{2+}] = \frac{dR/dt + k_{off}(R - R_{min})}{k_{on}(R_{max} - R)} \quad (1)$$

The parameters $k_{on} = 1.44 \times 10^8\ M^{-1}\ s^{-1}$, $k_{off} = 26\ s^{-1}$, $R_{max} = 7.05$, $R_{min} = 1.93$ were obtained from calibration experiments on frog muscle fibers (Struk et al., 1998) using the same setup.

Initial experiments were done with 1 mM internal free magnesium, which is commonly used for experiments in frog fibers. Under these conditions calcium transients were small and rose slowly upon depolarization. Mammalian fibers have been reported to contain lower concentrations of free magnesium (MacDermott, 1990; Westerblad and Allen, 1992). Reducing magnesium to obtain a free concentration of 0.5 mM improved the signal rise time and size and these conditions were therefore applied throughout.

RESULTS

In vitro contracture test

Fig. 2 shows the averaged results of the in vitro contracture test for the biopsies from which fibers were successfully used in our voltage clamp experiments. The result of the test, i.e., the force response to varying concentrations of caffeine and halothane, was used to distinguish the MHS group from controls (MHN). Clearly, MHS fiber bundles exhibited contracture force in a concentration range of halothane (Fig. 2 *B*) where MHN fibers showed no response. In MHS fibers, force developed at markedly lower concentrations of caffeine (Fig. 2 *A*).

Calcium release in voltage clamp experiments

Short segments of single muscle fibers were set up in a three-pool chamber as described in Methods. The cut ends were exposed to an artificial internal solution containing the fluorescent indicator dye fura-2. Before the dye reached the middle section of the fiber by diffusion, the membrane was clamped to a holding potential of −90 mV. Loading of the fibers as judged by the increase of resting fluorescence at 360 nm excitation was generally achieved 1.5–2 times faster than in frog fibers mounted in the same way in the same chamber and equilibrated with 100 μM fura-2. When loading approached a steady state, step depolarizations were applied to release calcium from the sarcoplasmic reticulum. The response to a 50-ms lasting depolarization (Fig. 3 *A*) is shown in Fig. 3 *B*. It shows the ratio R = F380/F360. The drop in the ratio value corresponds to a change in fractional calcium occupancy of the indicator dye from initially 46% to 75% at the end of the pulse. This is considerably smaller for this strength of depolarization than typically found in our experiments on frog fibers filled with comparable internal solutions. As in all other experiments, occupancy remained well below 100%, which justifies the use of the high-affinity indicator fura-2.

The result of converting the ratio signal to the transient of free myoplasmic calcium and correction for the noninstantaneous binding of calcium to the indicator by means of Eq. 1 is shown in Fig. 3 *C*. It should be noted that the relatively high concentration of the indicator dye certainly influences the time course of the free calcium transient, in particular the slow decay at the end of the pulse is partly due to buffering by the dye.

To get an estimate of the time course of calcium release from the sarcoplasmic reticulum we applied the method first described by Baylor et al. (1983). It uses reasonable estimates of the binding site concentrations in the fiber and literature values of rate constants for the main calcium binding sites determined in biochemical studies. With these data the time course of occupancy of each calcium compartment is calculated using the measured calcium transient. The sum of free and bound (or sequestered) calcium is the total amount of release and the time derivative of the total release is the rate of release from the SR. In the calculation we included fast calcium-specific sites on troponin C (T-sites), slow calcium-magnesium sites on troponin C (P-sites), EGTA, fura-2, and a contribution of the SR calcium pump (see Fig. 3 caption for references). Heizmann (1984) reported that m. vastus lateralis contains no parvalbumin. Therefore, parvalbumin was omitted from the calculation.

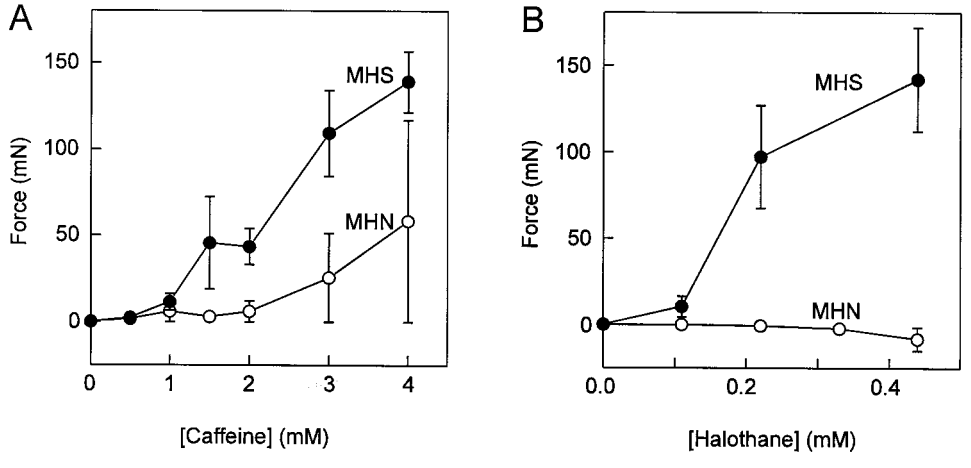

FIGURE 2 Results of the in vitro contracture test (IVCT). (*A*) Average cumulative force response of small biopsied muscle bundles to varying concentrations of caffeine. (*B*) Force response as a function of halothane concentration. *Open symbols:* MHN (nine biopsies); *closed symbols:* MHS (nine biopsies). Bundles had a diameter of ~3 mm and a length of 20 to 25 mm.

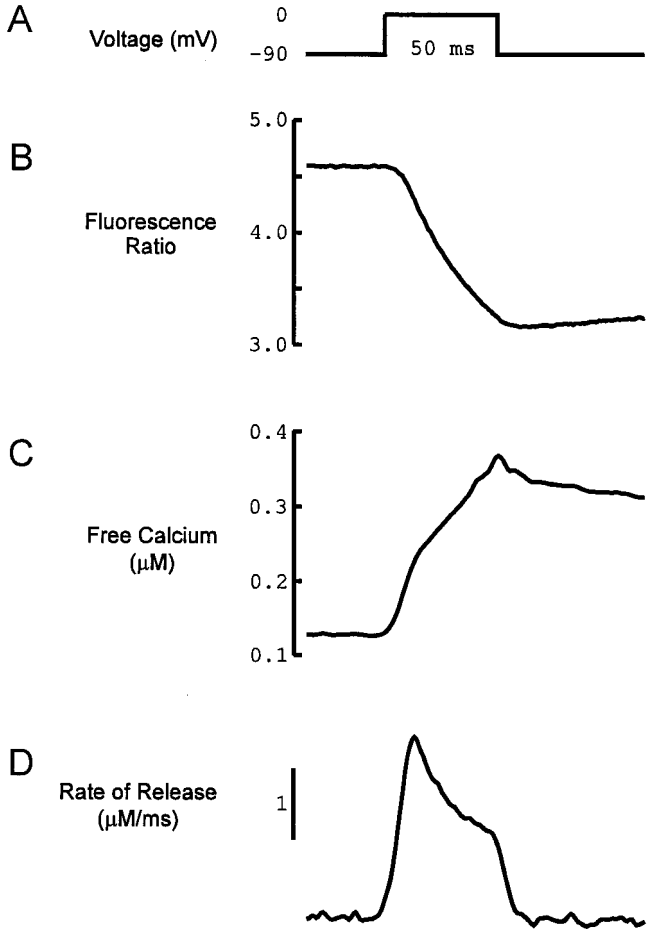

FIGURE 3 Estimation of the calcium release rate from fura-2 fluorescence transients measured during step depolarization. (*A*) Membrane depolarization (schematic) from −90 mV to 0 mV. (*B*) Fluorescence at 380 nm excitation divided by the resting fluorescence at 360 nm. (*C*) Free calcium calculated from (*B*) according to Eq. 1. (*D*) Rate of release, i.e., the time derivative of total calcium, calculated by solving the dynamic model equations of Brum et al. (1988) using the following set of parameters. On- and off-rate constants and total concentration of troponin C calcium-specific binding sites (T-sites; values according to model 1 of Pape et al., 1993): $k_{on} = 57.5\ \mu M^{-1}\ s^{-1}$, $k_{off} = 115\ s^{-1}$, [T] = 240 μM. On- and off-rate constants for calcium and magnesium and total concentration of parvalbumin-type binding sites (P-sites; values according to model 1 of Pape et al., 1993): $k_{on,Ca} = 125\ \mu M^{-1}\ s^{-1}$, $k_{off,Ca} = 0.5\ s^{-1}$, $k_{on,Mg} = 0.033\ \mu M^{-1}\ s^{-1}$, $k_{off,Mg} = 3\ s^{-1}$, [P] = 240 μM. On- and off-rate constants for calcium and total concentration of EGTA (values according to Pape et al., 1995): $k_{on} = 2.5\ \mu M^{-1}\ s^{-1}$, $k_{off} = 0.94\ s^{-1}$, [EGTA] = 100 μM. Sarcoplasmic reticulum pump (values according to Shirokova and Rios, 1996): pump stoichiometry $n = 2$, maximal pump rate M = 1 $\mu M\ ms^{-1}$, dissociation constant of individual pump site $K_D = 1\ \mu M$, concentration of pump sites 50 μM. Experiment 286.

When parvalbumin was included, the release rate estimates showed a constant non-zero level after the end of the pulse (not shown) while lack of parvalbumin was compatible with complete turn-off of release at repolarization. The result of the calculation is shown in Fig. 3 *D*.

The time course of the rate of release derived in this way exhibits the features previously described for amphibian (e.g., Melzer et al., 1984, 1987) and mammalian muscle (Garcia and Schneider, 1993; Delbono et al., 1995; Shirokova et al., 1996; Szentesi et al., 1997), i.e., an initial peak followed by a decay to a considerably lower value and a rapid turn-off after repolarization to the holding potential of −90 mV. Due to the sensitivity of the preparation, pulses had to be confined to relatively short durations (generally 50 ms). Therefore, we could not apply procedures to discriminate between inactivation of release and depletion of the SR to estimate SR permeability (Jacquemond et al., 1991), because they require long depolarizations.

Voltage dependence of calcium release

Free calcium was calculated according to Eq. 1. There is relatively close agreement among different groups on the value of the parameter k_{off}. Our own experiments on frog muscle fibers gave a value of 26 s^{-1}, which equals the value determined in rat fibers by Garcia and Schneider (1993). We used this value in Eq. 1. The estimates of k_{on} show a larger variance. Estimating the release rate requires the solution of a set of nonlinear differential equations. The time course of the release rate estimate might change depending on the scale of the calcium transient used for the calculation. In Fig. 4 we compare calculated release rates for two different choices of k_{on} leading to a difference in amplitude of the calcium transient by a factor of ~5. As can be seen, the general shape of the release rate waveforms was relatively invariant to this amplitude change. Furthermore, the uncertainties in scaling should be irrelevant for comparing MHN and MHS fibers, which were both subjected to the same calculation procedure. In the remaining part of this paper we used the fura-2 kinetic parameters introduced in Fig. 3.

Fig. 5 compares calculated release rates for depolarizing pulses of different amplitude both in a control muscle fiber (*A*) and in a fiber of a patient diagnosed MHS (*B*). In both cases the release rate exhibits the same general phasic time course (>−30 mV). For rat fibers it has been reported that the ratio of peak to steady level is approximately constant at different potentials (Shirokova et al., 1996). Even though at 50 ms the final level was not attained in all cases, it became apparent that generally the peak increased in relation to the level that the release rate decayed to during the pulse.

At the bottom of the figure the peak rates of release are plotted versus pulse voltage and the relation is fitted by a Boltzmann distribution. The parameters describing the voltage dependence differ only slightly. Half-maximal activation is attained at −14 mV (control) and −9 mV (MHS) respectively, and the steepness parameter k is 7.8 and 7.1 mV, respectively. The amplitude is more than three times larger in the MHS fiber.

Fig. 6 shows the average results regarding the voltage dependence of all MHN and MHS fibers. Each set of data from individual fibers was fitted separately by a Boltzmann equation. The results of the individual fits are summarized in Table 1 (MHN) and Table 2 (MHS). The plots in Fig. 6 show the data normalized to the maximal value obtained by

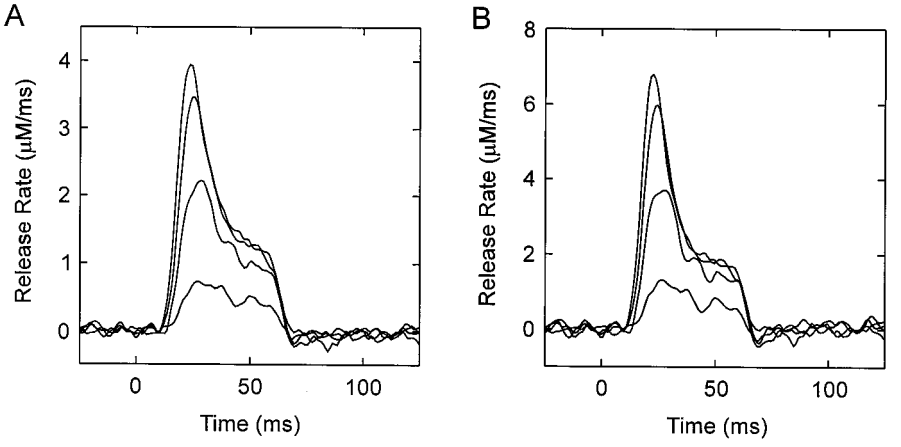

FIGURE 4 Calculated release rates assuming two different absolute scales of free calcium. A set of experimental records for voltage pulses to −20, −10, 0, and +10 mV (50 ms) was used. Two different assumptions for $k_{on\text{-}fura}$ were used in deriving free calcium (Eq. 1) leading to a difference in absolute scale by a factor of ~5: (*A*) $k_{on\text{-}fura} = 1.44 \cdot 10^8\ \mu M^{-1}\ s^{-1}$; (*B*) $k_{on\text{-}fura} = 0.3 \cdot 10^8\ \mu M^{-1}\ s^{-1}$. Experiment 304 (MHS fiber).

the fit and voltages displayed as deviations from the mean voltage of half-maximal activation. This voltage and the average steepness parameter k of the Boltzmann relation

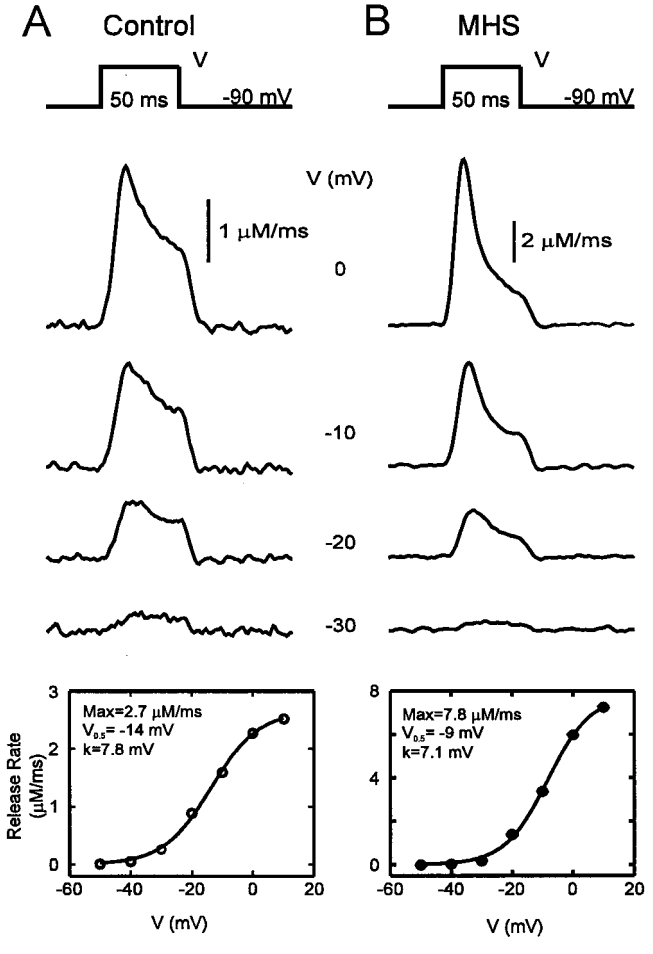

FIGURE 5 Voltage dependence of the rate of calcium release in an MHN (control) and an MHS fiber. Step depolarizations of 50 ms duration to voltages between −50 and 0 mV were applied. The figure shows the superthreshold responses. Insets at the bottom of the figure: voltage dependence of peak release rates and the parameters obtained from a least-squares fit of a Boltzmann function to the data. (*A*) Experiment 251 (MHN); (*B*) experiment 309 (MHS)

were almost identical in the MHN and MHS population of fibers (−12.0 ± 1.5 mV and 6.9 ± 0.3 mV versus −12.5 ± 1.5 and 6.3 ± 0.4 mV). A significant difference, however, was found in the mean values of the absolute amplitudes of the release rates: 2.1 ± 0.8 (n = 9) in normal fibers versus 5.6 ± 1.0 (n = 11) in MHS fibers; i.e., on average release flux was 2.7-fold larger in MHS fibers. A similar result was obtained when the raw fluorescence signals normalized to resting fluorescence were fitted as a function of voltage (see relative fluorescence change in Tables 1 and 2). In the same way we analyzed the end values of the release rates (average of the last 10 points during the stimulus interval). The variances were considerably larger but the result was equivalent. Maximum value, $V_{0.5}$, and k of the Boltzmann fit were 0.71 ± 0.26 μM/ms, −9.0 ± 10.0 mV, and 9.0 ± 5.5 mV (MHN) and 2.1 ± 0.7 μM/ms, −17.0 ± 7.0 mV, and 6.0 ± 2.1 mV (MHS), respectively. Again, only the maximal values were significantly different.

Inspecting the individual maximal release rates revealed that all MHS fibers ranged between 2 and 11 μM/ms while about half the MHN fibers had considerably lower amplitudes (below 1 μM/ms). Fig. 7 shows that this amplitude variation correlates with the age of the subjects. The smaller amplitudes were exclusively found in fibers from subjects older than 40.

Time course of calcium release

The examples chosen for Fig. 5 suggest a difference in the time course of calcium release rates, i.e., a faster decay after the peak in the MHS fiber. We tried to substantiate a possible difference in time course by averaging all calculated release rates from different fibers for a depolarization to 0 mV. The result is shown in Fig. 8. The bold line presents the average (normalized to the peak) and the thin lines indicate the standard error for each mean value. The two normalized curves were not significantly different.

For a second comparison of the time course, the decay phase of each individual rate of release record was fitted by a single exponential plus a constant. This analysis was

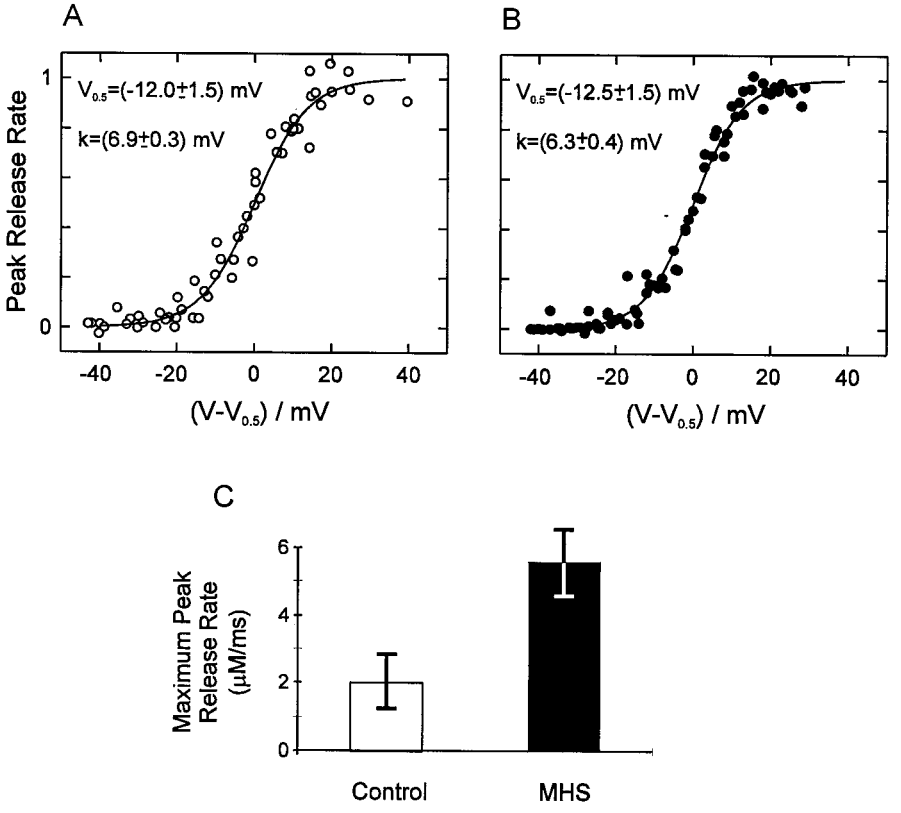

FIGURE 6 Average normalized voltage dependence of the peak rate of release. (*A*) MHN (control); (*B*) MHS; (*C*) average maximal amplitude (obtained from Boltzmann fits).

carried out for pulses to −10, 0, and +10 mV. The time constants were 18 ± 6 ms (n = 4), 11 ± 1 ms (n = 7), 10 ± 3 ms (n = 4) for MHN, and 15 ± 3 ms (n = 10), 12 ± 1 ms (n = 10), 12 ± 1 ms (n = 6) for MHS, respectively. The time constants of decay after the peak did not differ significantly at the three potentials. The same result was found when comparing time course and decay time constants separately for the age groups below and above 40 years. Thus, an alteration in the time course of the calcium release rate in MHS fibers could not be confirmed.

DISCUSSION

In the present study we investigated calcium release in muscle fibers of malignant hyperthermia-susceptible human

TABLE 1 Voltage dependence of fluorescence signal and calculated calcium release rate in control fibers

		Relative fluorescence change (ΔF/F)			Peak rate of release		
Fiber	Age (years)	Maximum	$V_{0.5}$ (mV)	k (mV)	Maximum (mM/s)	$V_{0.5}$ (mV)	k (mV)
124	34.2	0.416	−13.4	6.1	4.5	−10.2	6.4
133	34.4	0.496	−13	4.3	6.8	−8.0	5.7
243	43.8	0.039	−11.1	8.4	0.17	−14.6	6.6
251	33.4	0.360	−14.6	8.2	2.9	−11.3	8.02
256	39.2	0.230	−7.1	8.8	2.0	−7.1	7.38
263	56.9	0.044	−24	4.7	0.32	−24.2*	4.9*
274	40.4	0.030	−7.0	13	0.15	−19.5	8.13
286	33.6	0.180	−14.6	7.2	1.63	−9.9	7.07
312	48.1	0.094	−9.5	9.6	0.4	−15.7	6.1
Mean	40.4	0.210	−12.7	7.8	2.1	−12.0	6.9
S.E.M.		0.059	1.7	0.9	0.8	1.5	0.3
S.D.	8	0.177	5.1	2.7	2.3	4.2	0.9
n	9	9	9	9	9	8	8
p	–	0.01	n.s.	n.s.	0.01	n.s.	n.s.

n, Number of experiments; p, WILCOXON significance level for comparison with mean values of the MHS ensemble (Tab 2); n.s., not significant.
*Bad fit, not included in average.

TABLE 2 Voltage dependence of fluorescence signal and calculated calcium release rate in MHS fibers

		Relative fluorescence change ΔF/F			Peak rate of release		
Fiber	Age (years)	Maximum	$V_{0.5}$ (mV)	k (mV)	Maximum (mM/s)	$V_{0.5}$ (mV)	k (mV)
114*	37.0	0.473	−18	5.1	6.9	−13	7
168	21.5	0.457	−6.5	4.5	4.4	−6	{3.9}
224#	32.8	0.234	−16.4	8.5	2.0	−18	6.9
226#	sic	0.355	−15.0	6.9	3.0	−22	5.8
240§	51.9	0.434	−9.4	8.8	4.6	−8	8.1
241§	sic	0.686	−25.4	5.1	11.4	−15	5.9
272¶	41.6	0.351	−11.1	6.7	3.2	−10	5.7
304	60.5	0.362	−13.3	6.2	3.7	−11	5.9
305	34.3	0.470	−18.9	5.0	5.1	−15.5‖	4.14‖
309*	41.1	0.541	−16.8	5.94	11.4	−8.8	6.8
310	56.5	0.435	−19.8	3.84	6.03	−12.9	4.4
Mean	42.0	0.436	−15.5	6.1	5.6	−12.5	6.3
S.E.M.		0.035	1.6	0.5	1.0	1.5	0.4
S.D.	11.9	0.116	5.3	1.6	3.2	4.9	1.1
n	11	11	11	11	11	10	9

*Mutation Gly2434Arg.
#Mutation Gly2434Arg; fibers from same patient.
§Fibers from same patient.
¶Mutation Arg614Cys.
‖Bad fit, not included in average.

subjects. We used microfluorimetry to record calcium signals stimulated with voltage clamp depolarizations from isolated cut-open muscle fiber segments. The experiments allowed assessment of both voltage dependence and time course of calcium release in normal and MHS muscle fibers.

We confirmed the phasic time course of voltage-dependent calcium release that has been shown in studies on frog and rat fibers (Melzer et al., 1984, 1987; Garcia and Schneider, 1993; Shirokova et al., 1996) and in one report on human muscle (Delbono et al., 1995). In both normal and MHS fibers the release rate reached a peak soon after depolarization and declined rapidly thereafter. Release was strongly reduced (probably completely turned off) almost instantly after repolarization to the holding potential.

Compared to experiments on frog fibers carried out with the same methods in the same setup the average peak release rate amplitudes determined in human fibers were smaller by a factor of >10. Extrapolating to the physiological temperature of 37°C by using Q_{10} values between 3 and 4 determined for the peak release rate in rat fibers (Shirokova et al., 1996) leads to scaling factors between 5.6 and 8.8 for the human fibers. Following the arguments of Shirokova et al. (1996), remaining differences between frog and human fibers in their release rates at the respective physiological temperatures may be a result of the fact that mammalian fibers have two release regions per sarcomere, while frog fibers have only one. Furthermore, in the vastus lateralis fibers the apparent lack of parvalbumin may require less release to reach similar calcium levels. Another reason for finding low release rates in our human fibers may be a low loading state of the SR resulting from the biopsy procedure: the fibers had to be cut during surgery and to be maintained in the cut state for a much longer time before use in the experiment than frog fibers.

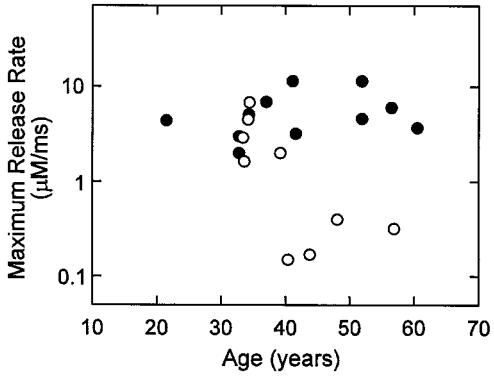

FIGURE 7 Maximum release rate plotted as a function of age. *Open symbols:* MHN (controls); *filled symbols:* MHS.

Information on the voltage dependence of release in human fibers has not been described yet. In our experiments, the ratio of peak release to the value attained at the end of the depolarizing pulse (50 ms) often increased with increasing depolarization. In rat muscle, this ratio had been reported to be independent of voltage in the range −60 to +10 mV, while frog fibers exhibited a maximum at ~−40 mV (Shirokova et al., 1996). Compared to rat fibers, the human fibers may have had a higher contribution from depletion at early times during large depolarization, possibly due to a less efficient reuptake or insufficient luminal buffering. Inasmuch as the mechanisms determining the complex time course of the release rate are not yet satisfactorily understood, other reasons regarding the control mech-

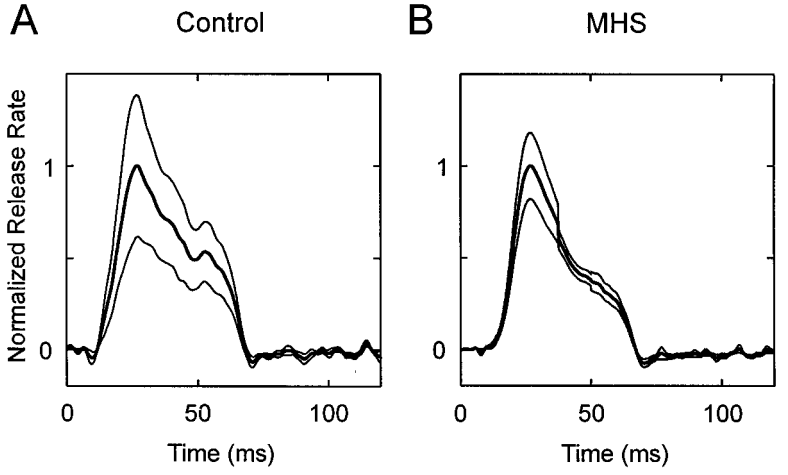

FIGURE 8 Time course of the rate of calcium release. Average of individual responses obtained in different experiments for a 50-ms depolarization to 0 mV. *Bold lines:* mean values; *thin lines:* SEM interval. (*A*) MHN (control), $n = 9$; (*B*) MHS, $n = 11$.

anism of the release channels are possible. For instance, with larger depolarization more and more release channels may be activated indirectly by calcium rather than directly by voltage (see the dual control model of calcium release presented by Shirokova et al., 1996). In contrast to our experiments, those of Shirokova et al. (1996), who had obtained a voltage-independent ratio, had been done with 8 mM EGTA in the internal solution, which reduces cross talk between adjacent ryanodine receptors (Pape et al., 1995).

The release exhibited a sigmoidal dependence on voltage that could be well described by a single Boltzmann relation. The result was consistent with a gating mechanism that displayed a dipole moment change equivalent to the displacement of four elementary charges across the membrane electrical field. Threshold activation was ~ -30 mV, which corresponds to experiments in rat fibers by Garcia and Schneider (1993), whereas Shirokova et al. (1996) obtained detectable calcium transients in rat fibers already at -60 mV.

The average peak release rate was markedly higher in the MHS fibers, which could be the result of enhanced calcium-dependent activation or reduced inactivation [for instance, reduced steady-state inactivation by myoplasmic magnesium as proposed by Owen et al. (1997) and Laver et al. (1997)]. A decrease in release activity with age as seen in the control fibers has also been reported by Delbono et al. (1995), yet to a considerably smaller extent. They attributed their effect to a reduced density of voltage sensors. In the MHS fibers the negative correlation of release with age was absent. Possibly a higher contribution of secondary, i.e., calcium-induced, release could make overall release less sensitive to a downregulation of voltage sensors. Significant alterations in kinetics could not be identified in our experiments. However, due to the relatively large variability of the signals, small changes in the decline rate cannot be ruled out.

A change in the voltage dependence of activation as reported by Gallant and Lentz (1992), Gallant and Jordan (1996), and Dietze et al. (1997) could not be found in our human fibers. Both steepness and half-maximal voltage for activation were the same. One reason for the difference between human and porcine muscle could be the nature of the mutation. In the studies on pig muscle, a genetically homogeneous group including only preparations with the Arg615Cys mutation, was studied. The common characteristic of our MHS fibers was the positive result of the diagnostic test, i.e., the higher sensitivity to caffeine and halothane. However, the genetic basis of MH in humans is heterogeneous. A higher sensitivity of the ryanodine receptor to the voltage sensor input may be restricted to only certain types of mutations (among them porcine Arg615Cys). In our experiments only one fiber was from a carrier of the Arg615Cys mutation; it showed no obvious deviation from the control results. The dominating mutation was Gly2434Arg (4 of 11 fibers, see Table 2). The best-fit parameters of this subgroup, taken separately, were not significantly different from those of the whole ensemble of MHS fibers. For the remaining fibers a causative mutation has not yet been identified.

In summary, we compared calcium release characteristics under voltage clamp conditions of muscle fiber segments isolated from normal human subjects with those of patients diagnosed as malignant hyperthermia-susceptible. We found no significant difference in voltage dependence nor in time course of calcium release between the two populations of fibers. Instead, the average release rate at maximal activation was markedly increased in MHS fibers.

We thank the surgery staff of the Rehabilitationskrankenhaus Ulm for carrying out the diagnostic biopsies, Dr. Ch. Baur, U. Mohr, and E. Schoch for providing us with results of the in vitro-contracture test, and Dr. L. Schleithoff and A. Brandt for information on the genotyping of MHS patients. We are grateful to Dr. G. Szücs for help and stimulating suggestions in the initial phase of the experiments, U. Richter for technical help, and B. Dietze for valuable discussions. This work was supported by the IZKF ULm.

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