Alteration of intracellular Ca^{2+} transients in COS-7 cells transfected with the cDNA encoding skeletal-muscle ryanodine receptor carrying a mutation associated with malignant hyperthermia

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Malignant hyperthermia (MH), an inherited neuromuscular disease triggered by halogenated inhalational anaesthetics and skeletal-muscle relaxants, appears to be due to an alteration of intracellular Ca^{2+} homoeostasis. MH occurs in 1 out of 20000 anaesthetized adults and is characterized by hypermetabolism, skeletal-muscle rigidity and elevation in body temperature, which is frequently fatal [MacLennan and Phillips (1992) Science **256**, 789–794]. The defect responsible for the disease may lie within the mechanism controlling the release of Ca^{2+} from sarcoplasmic reticulum via the ryanodine-receptor (RYR) Ca^{2+} channel; in fact a point mutation in the RYR has been associated with MH in some human families, as well as in the MH-susceptible pig. To date, however, no direct evidence has been obtained demonstrating that the point mutation is both necessary and sufficient to cause functional alterations in RYR-mediated Ca^{2+} release. In the present report we show that the presence of the Arg-to-Cys point mutation in the recombinant RYR expressed in COS-7 transfected cells causes abnormal cytosolic Ca^{2+} transients in response to 4-chloro-*m*-cresol, an agent capable of eliciting *in vitro* contracture of MH-susceptible muscles.

INTRODUCTION

The ryanodine receptor (RYR) is a homotetramer of four 565 kDa subunits that plays a crucial role in the release of Ca^{2+} from intracellular stores (Berridge, 1993). The functional properties of the RYR of terminal cisternae from malignant-hyperthermia (MH)-susceptible pig muscle displays significant differences in terms of Ca^{2+}-dependency of both [^{3}H]ryanodine binding and kinetics of single-channel activity in planar bilayer when compared with those of MH-normal muscles (Mickelson et al., 1988; Fill et al., 1990). Such differences have been associated with an Arg-to-Cys substitution at position 615 in the primary structure of the RYR cDNA from MH-susceptible pigs (Fujii et al., 1991). Although these results strongly suggest an important role for the RYR in the pathoaetiology of MH, it still remains possible that the MH phenotype is the result of alterations of both the RYR as well as other protein components of the sarcotubular membranes (Ervasti et al., 1989; Foster et al., 1989; Fletcher et al., 1991). We addressed this issue by studying the intracellular Ca^{2+} transients of COS-7 cells expressing either the wild-type or the mutated recombinant RYR. We provide evidence that substituting Cys for Arg^{615} in the primary structure of the RYR is sufficient to alter the intracellular Ca^{2+} homoeostasis of eukaryotic cells.

MATERIALS AND METHODS

Materials

The bluescript expression vector was from Stratagene, and the deaza T7 sequencing kit was from Pharmacia (Uppsala, Sweden); linkers, restriction enzymes and T4 DNA ligase were from Boehringer Mannheim; digitonin, fluorescein isothiocyanate- and peroxidase-conjugated anti-chicken IgG were from Sigma Chemicals (St. Louis, MO, U.S.A.); fura-2, Ca^{2+} standards, Texas Red–Dextran 10000 and ionomycin were from Molecular Probes (Eugene, OR, U.S.A.); 4-Chloro-*m*-cresol was from Fluka; the enhanced chemiluminescence (ECL) kit was from Amersham International. [^{3}H]Ryanodine was from du Pont. All other chemicals were of reagent (or highest available) grade.

Construction of the expression vectors

The full-length RYR cDNA was constructed by ligating the four overlapping RYR cDNA clones isolated from rabbit skeletal-muscle cDNA libraries (Zorzato et al., 1990). Replacement of cytosine-1843 with thymine was performed as described by Kunkel (1987). The clone $pfMH1^{Arg615Cys}$ was cut with the restriction enzymes *Stu*I (nucleotide 1681) and *Pst*1 (nucleotide 1909) and subcloned into the bluescript SK(−) vector. DNA sequencing was performed by the dideoxy method of Sanger et al. (1977). Mutated cDNA fragment *Bam*HI(843)–*Eco*RI(2396) was ligated into the plasmid Bluescript SK(−) to yield $pMH^{Arg615Cys}$. The cDNA fragment *Xba*I(vector)–*Bam*HI(843) from fZRR4 was ligated into $pMH^{Arg615Cys}$ to yield $pMH1^{Arg615Cys}$. The cDNA fragment *Sph*I(331)–*Nco*I(2389) from $pMH1^{Arg615Cys}$ was ligated into the Bluescript SK(−) plasmid containing fZRR4 (pfZRR4) to yield $pMHRR4^{Arg615Cys}$. From this step the construction of the full-length RYR clone was identical for both wild-type and mutated $RYR^{Arg615Cys}$. *Xba*I(vector)–*Aat*II(3335) fragment from pfZRR4 and $pMHRR4^{Arg615Cys}$ were ligated into pfZRR9 to yield pfZRR6 and $pMHRR6^{Arg615Cys}$. pfZRR9 was obtained by ligating the cDNA fragment *Bam*HI(4894)–*Xho*I(6470)–*Hin*dIII(vector) from pfZRR2 into pfZRR3. The *Xho*I(6470)–*Eco*RI(11625)–*Hin*dIII(vector) fragment from pfZRR10 was ligated into pfZRR6 and $pMHRR6^{Arg615Cys}$ to yield pfZRR8 and $pMHRR8^{Arg615Cys}$. pfZRR10 was obtained by ligating the *Stu*I(8821)–*Eco*RI(11625) fragment from pfZRR1 to pfZRR11 which contains the RYR cDNA defined

Abbreviations used: MH, malignant hyperthermia; RYR, ryanodine receptor; RSV LTR, Rous-sarcoma-virus long terminal repeat.

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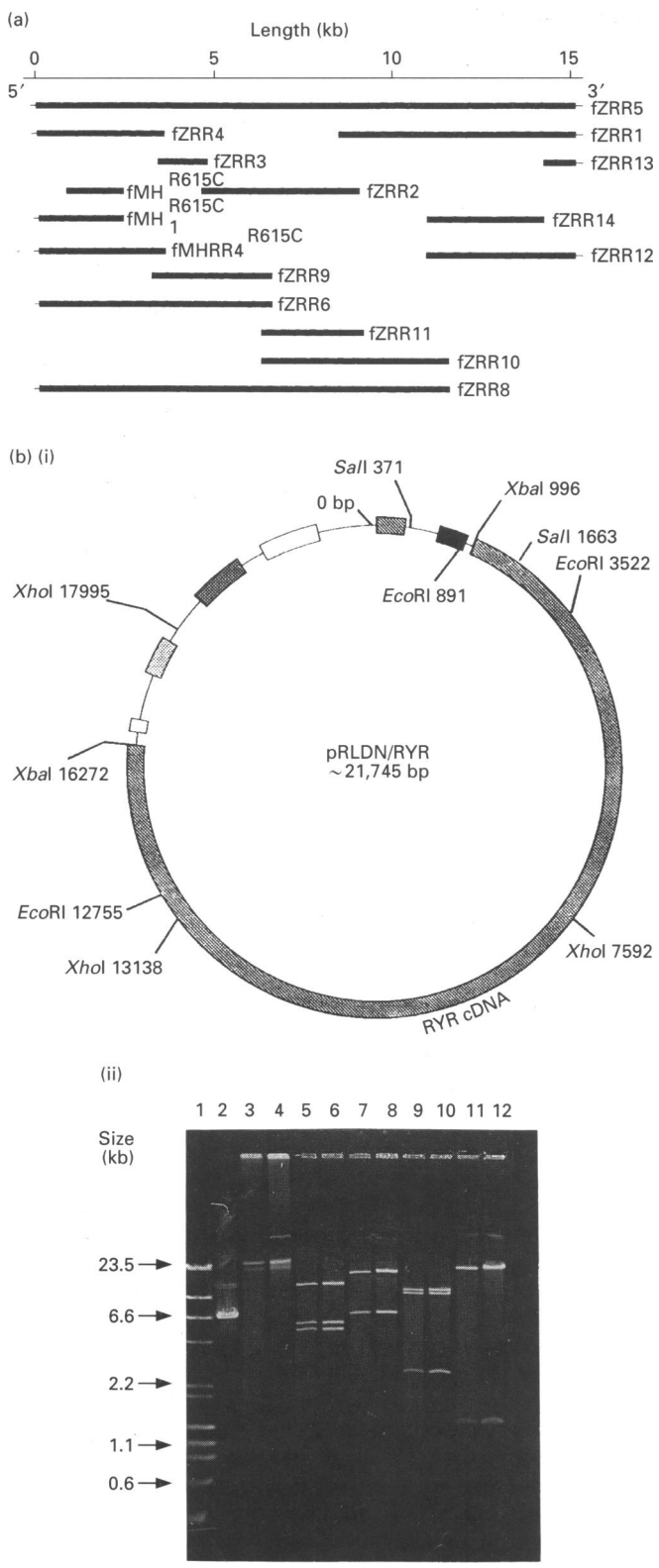

Figure 1 Construction of expression plasmids

(**a**) The numbering is positive, beginning at the first nucleotide of the initiator methionine. fZRR4 (−130 to 3519 bp), fZRR3 (3234–4916 bp), fZRR2 (4541–9142 bp), fZRR1 (8621–15218 bp) indicate cDNA fragments of the original clones. fZRR5 indicates the RYR cDNA cloned into the expression plasmid pRLDN. Closed boxes indicate the coding sequence; 5′ and 3′ non-coding regions are shown as a continuous line. Arg-to-Cys substitution is indicated in the single-letter code. (**b**) Restriction-endonuclease digestion of the pPRLDN/RYR constructs. The circular construct (i) shows the restriction-endonuclease sites. DNA fragments were separated in a 0.6% agarose gel and stained with ethidium bromide (ii). Lane 1, DNA standards; lane 2, pRLDN digested with *Eco*RI; lanes 3–12, wild-type RYR lanes 3, 5, 7, 9 and 11, and RYR^{Arg615Cys} (lanes 4, 6, 8, 10 and 12) inserted in the pRLDN expression vector with undigested constructs (lanes 3 and 4), constructs digested with *Xho*I (lanes 5 and 6), *Xba*I (lanes 7 and 8), *Eco*RI (lanes 9 and 10) and *Sal*I (lanes 11 and 12).

by *Sma*(6420)–9142 bp/*Eco*RI(vector). The *Nde*I(11151)–*Sma*(15166)/*Hin*dIII(vector) fragment from pfZRR12 was ligated into pfZRR8 and pMHRR8^{Arg615Cys} to yield pfZRR5 and pMHRR5^{Arg615Cys}. pfZRR12 was obtained by ligating the *Cla*I(14300)–*Sma*I(15166)–*Hin*dIII fragment from pfZRR13 into pfZRR14. pfZRR13 was obtained by ligating the *Cla*I(14300)–*Sma*I(15166) fragment from pfZRR1 into Bluescript KS(−), which was then cloned into the *Cla*I–*Xba*I sites of pTZ19. *Bam*HI(10971)–*Cla*I(14300) from pFZRR1 was cloned into a Bluescript KS(−) in which the *Xho*I site was transformed into *Hin*dIII by the addition of linkers. The fZRR5 and MHRR5^{Arg615Cys} cDNAs were excised from the vector by *Xba*I digestion and cloned into the pRLDN expression vector.

Immunological identification of the recombinant RYR

Immunofluorescence

COS-7 cells were transfected by the scrape-loading technique (McNeil, 1989). After 48 h, cells were fixed in 4% paraformaldehyde for 60 min, washed three times with PBS and permeabilized with 0.1% Triton X-100. Cells were rinsed with PBS, preincubated with 1% BSA for 60 min and incubated with 30 μg/ml chicken anti-(rabbit skeletal-muscle RYR) antibodies (Treves et al., 1993) in 0.1% Tween. After 60 min, the coverslips were washed five times with PBS containing 0.1% Tween (PBST) and incubated with fluorescein isothiocyanate-conjugated anti-chicken IgG in 0.1% Tween (1:32 dilution) for 30 min. The coverslips were washed five times with PBST mounted in glycerol and examined with a Zeiss Axioskop fluorescence microscope.

Western-blot analysis of microsomal fractions

Total microsomal fractions from COS-7 transfected cells were obtained by differential centrifugation as described previously (Vilsen et al., 1989). Terminal-cisternae microsomes were purified from rabbit skeletal muscle as described by Saito et al. (1984). Protein concentration was determined as described by Bradford (1976). Immunodetection was carried out using anti-RYR Ab as previously described (Treves et al., 1993). Western blots were probed with 5 μg/ml chicken anti-(skeletal muscle RYR) antibody, followed by peroxidase-conjugated anti-chicken IgG (1:6000). The blots were developed with the ECL kit (Amersham).

Intracellular Ca^{2+} measurement of transfected COS-7 cells

COS-7 cells were transfected (McNeil, 1989) in the presence of 250 μM Texas Red–Dextran 10000 (Molecular probes). The cells were allowed to adhere to polylysine-treated coverslips (1 mg/ml in PBS) for 5 min, after which non-adherent cells were washed off with PBS. After 48 h, single-cell calcium measurements were performed using the FL-4000 imaging system (Georgia instruments, Roswell, GA, U.S.A.) attached to a Zeiss Axiovert fluorescent microscope. Coverslips were affixed to a PDMI-2 culture chamber (Medical System Corp., Greenvale, NY, U.S.A.) thermostatically controlled at 37 °C; 5–10 cells were identified in each field and the average pixel (32 frames averaged and 'ratioed' every 5 s) value for each cell was measured at excitation wavelengths of 340 and 380 nm at various times. Cells were exposed to increasing concentrations of 4-chloro-*m*-cresol. Ca^{2+} cali-

bration was performed by measuring the fluorescence of known standards (Molecular Probes). Texas Red fluorescence was detected using an excitation wavelength of 590 nm and a 635 nm-bandpass emission filter.

Ryanodine binding

Ryanodine binding was examined in digitonin (5 μM)-permeabilized cells as described by Fleischer et al. (1985), in the presence of an anti-proteolytic cocktail (Treves et al., 1993).

Statistical analysis

Statistical analysis was performed using the Student t test for paired samples.

RESULTS AND DISCUSSION

We constructed the full-length wild-type (RYR) and mutated RYR (RYR^{R615C}) cDNAs and inserted them into the pRLDN mammalian expression vector under the control of Rous-sarcoma-virus long terminal repeat (RSV LTR) (Figure 1a). The identity and integrity of the wild-type and mutated constructs was confirmed by partial restriction-endonuclease mapping. As shown in Figure 1(b), the undigested constructs migrate as bands of 23 kb (lanes 3 and 4). In order to insert the RYR cDNAs into the pRLDN expression vector, the cDNAs were tailed with *Xba*I linkers; thus digestion with *Xba*I yields two fragments, one having the same size as the linearized vector (compare lane 2 with lanes 7 and 8), while the RYR cDNAs have an electrophoretic mobility which corresponds to the expected one, i.e. approx. 16 kb. Digestion with *Xho*I (lanes 5 and 6), *Eco*RI (lanes 9 and 10) and *Sal*I (lanes 11 and 12) gives rise to DNA fragments of the expected size. Skeletal RYR cDNA contains a unique *Sal*I site located at the 5′ end, thus digestion with this restriction endonuclease indicates that the RYR cDNAs were positioned in the 5′-to-3′ orientation with respect to the RSV LTR promoter.

The presence of the mutation at position 1843 of the RYR cDNAs was confirmed by partial sequencing of the two cDNAs (Figure 2). The expression and localization of the recombinant RYR was monitored by a combination of immunocytochemical and biochemical techniques. Immunofluorescence staining for RYR in both COS-7 transfectants revealed similar diffuse granular perinuclear fluorescence (Figure 3, panels B and C) that was not detected in mock-transfected cells (Figure 3, panel A). This staining was consistent with localization of RYR to intracellular membranes. This conclusion was also supported by Western-blotting analysis on the microsomal fraction of COS-7 transfected cells. As shown (Figure 4, lane 1), these antibodies recognize the skeletal-muscle RYR. Furthermore, the microsomal fraction of COS-7 cells transfected either with the cDNAs encoding wild-type RYR or RYR^{R615C} (Figure 4, lanes 2 and 3 respectively), contained an immunopositive band having the same apparent M_r as the skeletal-muscle RYR. As expected no immunological reaction was detected in the microsomes of mock transfected cells (results not shown). In addition, Western-blot analysis suggests that the content of expressed wild-type and mutated RYR is similar. A result also supported by [^{3}H]ryanodine

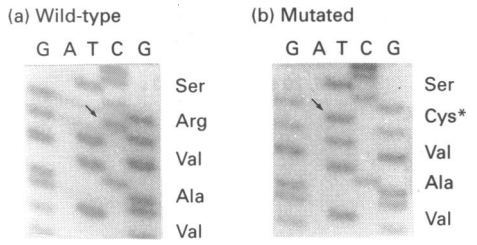

Figure 2 Partial sequence of the RYR cDNA constructs

(**a**) Shows the partial sequence of the wild-type RYR, while the sequence of the mutated RYR is shown in (**b**). The point mutation (replacement of thymine by cytosine) is indicated by the arrow; the amino acid replacement is indicated by the asterisk.

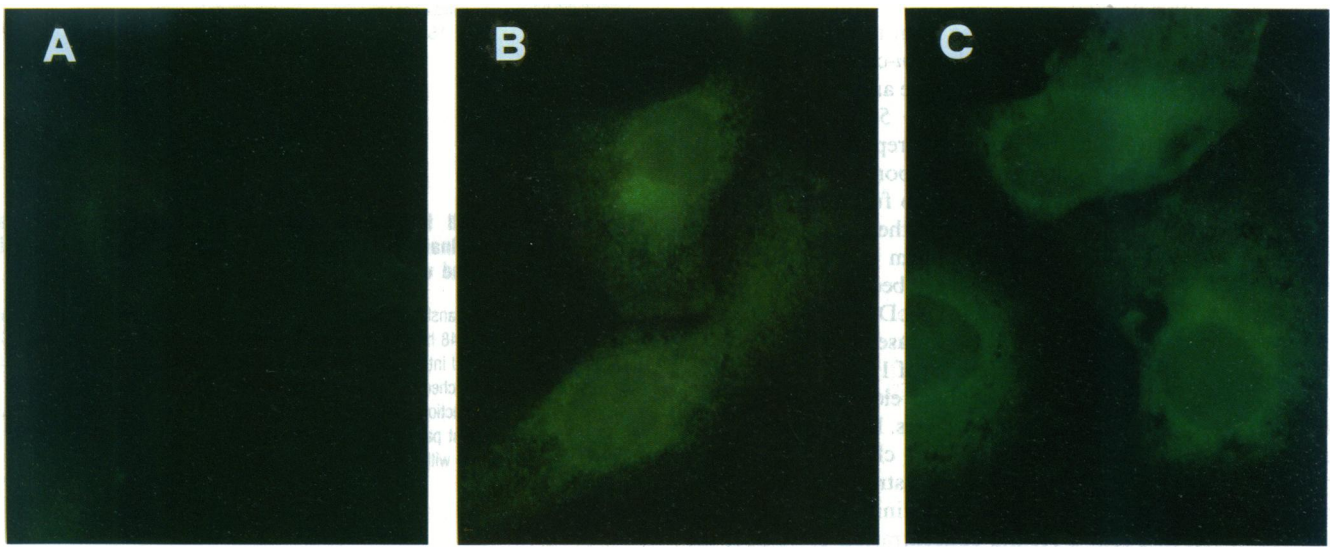

Figure 3 Indirect immunofluorescence of COS-7 cells transfected with wild-type and mutated RYR cDNA

COS-7 cells were mock-transfected (panel A), transfected with the expression plasmid containing the cDNA encoding the wild-type RYR (panel B) or the $RYR^{Arg615Cys}$ cDNA (panel C) (magnification 460 ×).

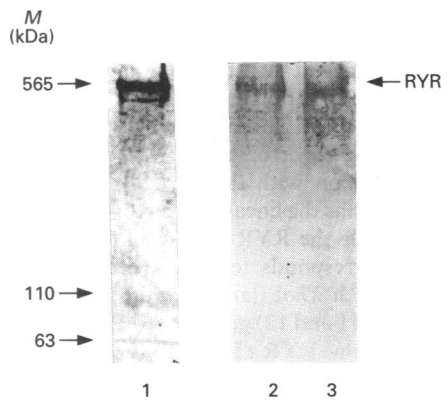

Figure 4 Western-blot analysis of COS-7 cells transfected with the cDNA encoding the skeletal-muscle RYR

Lane 1, 10 μg of skeletal-muscle cisternae; lanes 2 and 3, 150 μg of total microsomal fraction isolated from COS-7 cells which were transfected with the expression vector containing the wild-type RYR cDNA (lane 2) or the $RYR^{Arg615Cys}$ cDNA (lane 3). The arrow indicates the RYR.

binding studies. We found that, at 200 nM ryanodine, the COS-7 cells transfected with wild-type and mutated RYR cDNAs bound 300 and 275 fmol of ryanodine/mg protein ($n = 2$) respectively. Under the same experimental conditions, mock-transfected cells bound 23 fmol/mg of protein.

We next examined whether the RYR expressed by COS-7 cells was (i) functional and (ii) whether cells transfected with the mutated RYR cDNA had altered intracellular Ca^{2+} transients. Semi-confluent cells were scrape-loaded simultaneously with the RYR constructs and Texas Red–Dextran. It was assumed that those cells which had incorporated the fluorescent macromolecule had also incorporated pRLDN/RYR DNA. Intracellular Ca^{2+} measurements were carried out in those cells which were positive for Texas Red fluorescence (lowest part Figures 4a, 4b and 4c). Cells were stimulated with increasing concentrations of 4-chloro-*m*-cresol, a potent RYR agonist (Zorzato et al., 1994), in Ca^{2+}-free medium supplemented with 2 mM EGTA, thus the resulting changes in $[Ca^{2+}]_i$ were due to release from intracellular stores. The threshold concentration for 4-chloro-*m*-cresol-induced Ca^{2+} transients in cells transfected with wild-type and $Arg^{615}Cys$ RYR cDNA were $166+12$ μM ($n = 21$) and $56+3$ μM ($n = 24$; $P < 0.001$) respectively. Figure 5 shows a representative experiment. Mock-transfected cells did not respond to either a first addition of 50 μM 4-chloro-*m*-cresol or to further additions of the same compound up to 150 μM, yet in these cells, ionomycin (1 μM) was capable of releasing Ca^{2+} from intracellular stores (Figure 5a). When COS-7 cells which had been transfected with the plasmid containing the wild type RYR cDNA were subjected to the same protocol, we observed an increase in the $[Ca^{2+}]_i$ from 80 nM to approx. 400 nM in the presence of 150 μM 4-chloro-*m*-cresol (Figure 5b). The $[Ca^{2+}]_i$ remained elevated for approx. 30 s, after which it returned to resting values. In contrast with the cells transfected with the wild-type RYR cDNA, COS-7 cells transfected with RYR^{R615C} cDNA demonstrated a marked increase in cytosolic Ca^{2+} in response to the initial dose of 50 μM 4-chloro-*m*-cresol. From a resting concentration of 80 nM, the $[Ca^{2+}]_i$ rose to 600 nM, returning to resting levels within 20 s. One may argue that these results reflect a different content of recombinant RYR in the single cells examined. We think that this explanation is unlikely, since such differences should not affect the threshold sensitivity to 4-chloro-*m*-cresol. The ab-

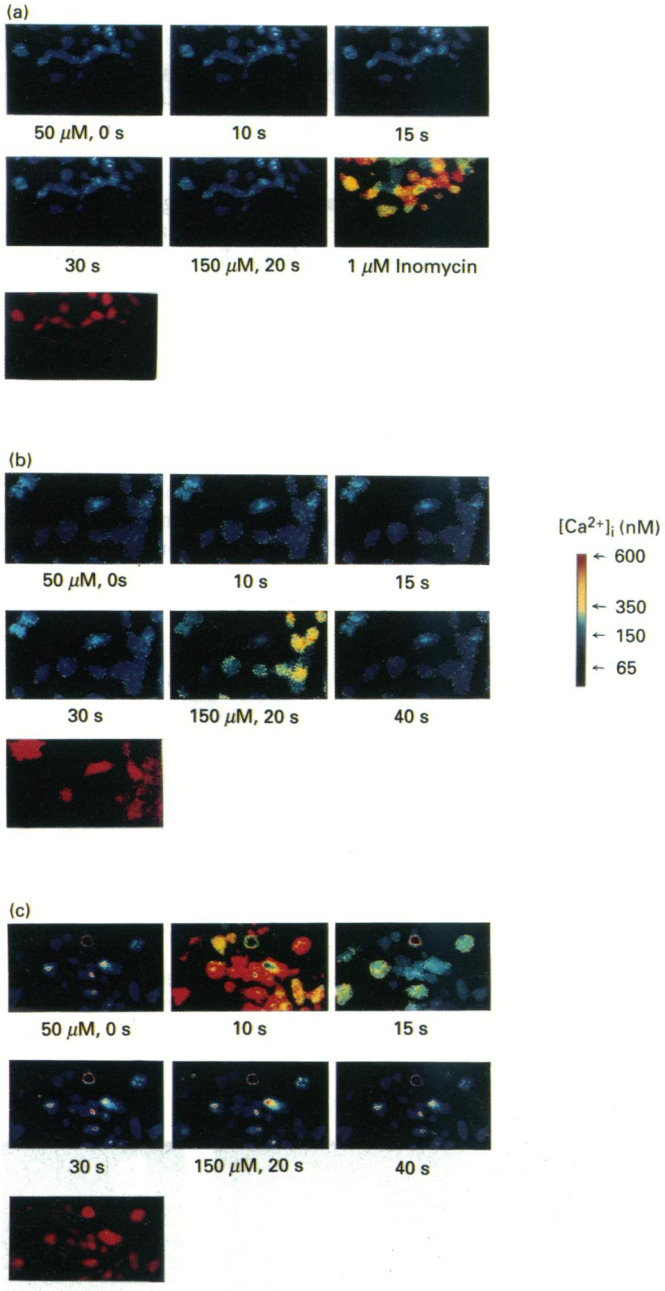

Figure 5 Single-cell intracellular Ca^{2+} measurements of COS-7 cells expressing a recombinant wild-type skeletal-muscle RYR or a RYR carrying a mutation associated with MH

COS-7 cells were mock-transfected (**a**), transfected with the wild-type RYR cDNA (**b**) or with $RYR^{Arg615Cys}$ (**c**). Cells 48 h after transfection were loaded with the fluorescent Ca^{2+} indicator fura-2; single-cell intracellular Ca^{2+} measurements were performed using the FL-4000 Ca^{2+} imaging system attached to a Zeiss Axiovert fluorescent microscope as described in the Materials and methods section. Cells were stimulated with 50 μM 4-chloro-*m*-cresol aliquots up to 150 μM. The lowest part of each figure in each panel shows a fluorescent micrograph of the same cells stained with Texas Red.

normal Ca^{2+} transients we observed in COS-7 may rather be due to intrinsic differences of the gating properties of the mutated RYR. One must point out that, in intact fibres from MH-susceptible muscles, the threshold concentration for 4-chloro-*m*-cresol-induced contracture is approx. 2.5 times lower than for

normal fibres (Galloway and Denborough, 1986), a value that closely matches the one that we observed with the recombinant RYRs. We provide direct and definitive proof that the $Arg^{615}Cys$ mutation in the primary sequence of the RYR is alone sufficient to confer functional properties in the Ca^{2+} release channel which are characteristic of the MH-susceptible RYR.

A general, yet unsolved, question concerning the RYR Ca^{2+} channels is the identification of the functional domains involved in the regulation of these endocellular Ca^{2+} channels. Our experiments also give a clue in this direction. Activation of Ca^{2+} release by 4-chloro-*m*-cresol is mediated by a Ca^{2+}-induced Ca^{2+} release mechanism, since its effect is prevented by Ruthenium Red, an agent known to block this type of Ca^{2+} release (Palade et al., 1989). The $Arg^{615}Cys$ mutation may lead to the suppression of an inhibitory binding site of the Ca^{2+}-induced Ca^{2+} release mechanism, an event consistent with an alteration of the equilibrium between open and closed states of the channel. This would make the RYR Ca^{2+} channel hypersensitive to 4-chloro-*m*-cresol. This interpretation is in agreement with the model proposed by Iino et al. (1992), which predicts that the N-terminal portion of the RYR protomer is involved in the suppression of Ca^{2+}-induced Ca^{2+}-release activity.

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