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Effect of Ryanodine Receptor Mutations on Interleukin-6 Release and Intracellular Calcium Homeostasis in Human Myotubes from Malignant Hyperthermia-susceptible Individuals and Patients Affected by Central Core Disease*

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In this study we report for the first time the functional properties of human myotubes isolated from patients harboring the native *RYR1* I4898T and R4893W mutations linked to central core disease. We examined two aspects of myotube physiology, namely excitation-contraction and excitation-secretion coupling. Our results show that upon activation of the ryanodine receptor (RYR), myotubes release interleukin-6 (IL-6); this was dependent on *de novo* protein synthesis and could be blocked by dantrolene and cyclosporine. Myotubes from the two patients affected by central core disease showed a 4-fold increase in the release of the inflammatory cytokine IL-6, compared with cells derived from control or malignant hyperthermia susceptible individuals. All tested myotubes released calcium from intracellular stores upon stimulation via surface membrane depolarization or direct RYR activation by 4-chloro-*m*-cresol. The functional impact on calcium release of *RYR1* mutations linked to central core disease or malignant hyperthermia is different: human myotubes carrying the malignant hyperthermia-linked *RYR1* mutation V2168M had a shift in their sensitivity to the RYR agonist 4-chloro-*m*-cresol to lower concentrations, whereas human myotubes harboring C-terminal mutations linked to central core disease exhibited reduced $[Ca^{2+}]_i$ increase in response to 4-chloro-*m*-cresol, caffeine, and KCl. Taken together, these results suggest that abnormal release of calcium via mutated RYR enhances the production of the inflammatory cytokine IL-6, which may in turn affect signaling pathways responsible for the trophic status of muscle fibers.

Central core disease (CCD)[1] is a congenital myopathy of autosomal dominant inheritance. Affected individuals often present with infantile hypotonia (floppy infant syndrome), weakness, hip dislocation, and a delay in achieving motor milestones. Later in life, the predominant symptom is a generalized muscle weakness affecting the proximal muscle groups more than the distal ones. The clinical severity is highly variable, but disease course is usually slow or nonprogressive (1–4). On the basis of clinical findings alone the diagnosis is difficult, and a histological examination of muscle tissue is essential. Typically, type I fibers are predominant and contain well demarcated and centrally located cores, which lack mitochondria and do not stain with histochemical dyes for oxidative enzymes. In longitudinal sections the core area is extensive and covers a considerable length of the fiber (4–6).

From a molecular point of view CCD is closely associated with malignant hyperthermia (MH), because both disorders are caused by mutations in the ryanodine receptor (*RYR1*), the calcium release channel of the sarcoplasmic reticulum. MH is a pharmacogenetic disease triggered by volatile anesthetics and the depolarizing muscle relaxant suxamethonium in predisposed (MH-susceptible (MHS)) individuals (7, 8). The clinical signs of an impending MH reaction are due to a hypermetabolic state and include muscle rigidity, metabolic acidosis, rhabdomyolysis, tachycardia, and/or an increase in body temperature. Histological examination of muscle fibers from MHS individuals does not reveal alterations of the status of the muscle.

To date more than 40 mutations have been identified in the ryanodine receptor gene (*RYR1*, human chromosome 19q13.1) and are associated with MH and/or CCD (for review see Refs. 9–11). The *RYR1* gene is comprised of 106 exons that code for one of the largest known proteins (5038 amino acids) (12, 13). The first 4000 amino acids are predicted to form the hydrophilic cytoplasmic domain, whereas the last 1000 amino acids form the hydrophobic membrane-spanning pore (14–16). The majority of *RYR1* mutations giving rise to the MHS phenotype appear to be clustered in the hydrophilic domain, whereas most mutations linked to CCD have been found in hydrophobic pore-forming domain 3 (17–20).

To understand the molecular mechanism underlying MH and CCD, it is essential to evaluate the impact of mutations on the functional properties of the RYR calcium channels and on

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[1] The abbreviations used are: CCD, central core disease; MH, malignant hyperthermia; RYR, ryanodine receptor; $[Ca^{2+}]_i$, intracellular calcium concentration; IL-6, interleukin-6; NF-κB, nuclear factor κB; JNK, c-Jun N-terminal kinase; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; NF-AT, nuclear factor of activated T cells.

excitation-contraction coupling. As far as MH-linked *RYR1* mutations are concerned, in most cases they cause an increased sensitivity of the RYR to pharmacological agonists such as caffeine, 4-chloro-*m*-cresol, and halothane (21–25). On the other hand, mutations causing CCD have been shown to lead to leaky calcium channels that can cause depletion of intracellular calcium pools (17, 20), or to uncoupled RYR calcium channels in which depolarization of the dihydropyridine receptor does not lead to opening of the RYR calcium channel (26, 27). Thus, the impact of most disease-causing *RYR1* mutations will necessarily affect calcium homeostasis of the muscle cell. To date, however, only few downstream effects of the altered intracellular calcium homeostasis have been investigated.

Calcium-dependent pathways regulate many aspects of muscle physiology, including growth and regeneration (28, 29). Increases in $[Ca^{2+}]_i$ have been shown to activate transcription of the pro-inflammatory regulators nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK), and nuclear factor of activated T cells (NF-AT) via activation of the calcium sensitive-phosphatase calcineurin (30). Skeletal muscle cells are capable of producing and responding to several cytokines such as IL-4, IL-6, IL-15, and tumor necrosis factor (31–34). Although the fine mechanism controlling cytokine release is not fully understood, muscle exercise has been shown to increase the plasma concentration of IL-6, which in turn has been proposed to exert a role on the maintenance of glucose homeostasis, its full physiological role, however, has yet to be established (for review see Ref. 32). When released in large quantities, IL-6 and IL-1 cause fever; *i.e.* they constitute an endogenous pyrogen and can induce the synthesis of acute phase plasma proteins and initiate metabolic wasting (35). Because the mechanism underlying CCD appears to be controversial, in the present report we examined how two endogenously expressed CCD-linked *RYR1* mutations affect $[Ca^{2+}]_i$ homeostasis and cytokine release from cultured human myotubes and compared their properties to those of myotubes from control individuals or MHS individuals carrying a *RYR1* mutation in a different domain of the calcium release channel.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium containing 4.5 mg/ml glucose, penicillin G, streptomycin, were all purchased from Invitrogen. Insulin was purchased from Eli Lilly and Co. (Indianapolis, IN). Cell culture plastic ware was from BD Biosciences. Glutamine, HEPES, bovine serum albumin, epidermal growth factor, cycloheximide, anti-α-actinin, anti-goat-FITC, anti-mouse-cy3, creatine, and dantrolene were from Sigma. Fura-2/AM and cyclosporine A were from Calbiochem. The calcium calibration kit was from Molecular Probes, Inc. A goat anti-α1.1 subunit of the skeletal muscle dihydropyridine receptor polyclonal antibodies was from Santa Cruz Biotechnology Inc. PeliKine-compact human IL-1β and IL-6 ELISA kits were from CLB, Amsterdam, The Netherlands. Mouse monoclonal antibody anti-IL-6R (CD130) was from BIOSOURCE International (Camarillo, CA). All other chemicals were reagent or highest available grade.

Patients, Identification of RYR1 Mutations, and Human Skeletal Muscle Cell Culture—Primary human muscle cell cultures were established after obtaining informed consent from muscle biopsies of patients undergoing the *in vitro* contracture test for the diagnosis of MH susceptibility, in 10 control patients from 3 different families, 9 patients with a positive family history for MH in which the V2168M mutation in the *RYR1* gene was identified (from 5 different families), and from 2 patients affected by CCD. In these patients, exons 98–103 of the *RYR1* gene, the major mutation hot spot, were screened for mutations by single strand conformation analysis and direct sequencing as described previously (20).

Human skeletal muscle cell cultures were established as previously described (22). To differentiate cells into myotubes, the medium was switched to Dulbecco's modified Eagle's medium plus 4.5 mg/ml glucose, 0.5% bovine serum albumin, 10 ng/ml epidermal growth factor, 0.15 mg/ml creatine, 5 ng/ml insulin, 200 mM glutamine, 600 ng/ml penicillin G and streptomycin, and 7 mM HEPES, pH 7.4 (36).

Intracellular $[Ca^{2+}]$ Measurements—Cells were transferred onto glass coverslips (5 cm in diameter) and allowed to proliferate in growth medium until visible groups of >10 cells were formed. The medium was then switched to differentiation medium, and $[Ca^{2+}]$ measurements were performed 7–10 days later. The free cytosolic $[Ca^{2+}]$ was determined using the fluorescent Ca^{2+} indicator Fura-2 as described (22). Coverslips were mounted onto a 37 °C thermostatted chamber, which was continuously perfused with Krebs Ringer medium; individual cells were stimulated with a 12-way 100-mm diameter quartz micromanifold computer-controlled microperfuser (ALA Scientific) as previously described (37). On-line (340 nm, 380 nm, and ratio) measurements were recorded using a fluorescent Axiovert S100 TV inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a ×20 water-immersion FLUAR objective (0.17 numerical aperture) with filters (BP 340/380, FT 425, and BP 500/530) and attached to a Hamamatsu multiformat charge-coupled device camera. The cells were analyzed using an Openlab imaging system, and the average pixel value for each cell was measured at excitation wavelengths of 340 and 380 nm. Ca^{2+} calibration was performed following the instructions provided with the calcium calibration kit.

Release of Cytokines—Human skeletal muscle cells were placed in the wells of a 24-microtiter plate and allowed to grow until visible aggregates of >10 cells were seen. The medium was then switched to differentiation medium for 7–10 days. On the day of the experiment the medium was changed, and cells were stimulated at 37 °C under the specified conditions. The amount of IL-1β or IL-6 released into the supernatant was determined by using the CLB PeliKine Compact indirect ELISA kit following the manufacturer's instructions. All tests were performed in duplicate.

Immunofluorescence—Undifferentiated myoblasts, growing in growth medium or myotubes 7–10 days after being grown in differentiation medium, were rinsed, fixed in ice-cold methanol:acetone (1:1) for 20 min, and processed as previously described (22). Fluorescence was detected using a fluorescence Axiovert S100 TV inverted microscope (Carl Zeiss GmbH, Jena, Germany) equipped with a ×20 FLUAR objective and Zeiss filter sets (BP 475/40, FT 500, and BP 530/50; and BP 546, FT 560, and BP 575–640) for detection of FITC and Cy3 fluorescence, respectively.

Statistical Analysis—Statistical analysis was performed using the Student's *t* test for paired samples when there were two groups and ANOVA for more than two groups. Origin computer program (Microcal Software, Inc., Northampton, MA) was used for statistical analysis and curve generation.

RESULTS

RYR1 Mutation Analysis—RYR1 mutations of the nine MH patients have been identified and described earlier (38). In the two patients diagnosed clinically and histologically as having CCD we screened exons 98–103 of the *RYR1* gene by SCCA. We concentrated on this region, because it is part of the highly conserved hydrophobic C-terminal domain of the *RYR1* protein and has been found to be a hot spot for CCD-linked mutations (19, 20). Aberrant bands were identified, and direct DNA sequencing confirmed the presence of mutations I4898T in patient CCD1 and of R4893W in patient CCD2.

Differentiated Cultured Human Skeletal Muscle Cells Express Proteins Characteristic of Differentiated Skeletal Muscle—To demonstrate that under our experimental culture conditions human primary muscle cells differentiate sufficiently, we performed immunofluorescence studies. Fig. 1 shows the appearance of human myoblasts cultured under growth conditions (Fig. 1, *A–E*) or myotubes after 7–10 days of culture in differentiation medium (Fig. 1, *F–J*). The expression of cytoskeletal proteins specific for skeletal muscle was demonstrated using an anti-α-sarcomeric actinin mouse monoclonal antibody. This protein is expressed only in striated muscle cells and is used to identify cells or tissues of striated muscle lineage (39). Fig. 1 (*B* and *G*) shows the fluorescence pattern of cultured human muscle cells: undifferentiated myoblasts are immunonegative, while differentiated myotubes exhibit a fluorescent pattern that is compatible with that of a cytoskeletal protein. Fig. 1 (*C–I*) shows the immunofluorescent patterns obtained using anti-RYR1 (Fig. 1, *C* and *H*) and anti-α1.1 dihydropyri-

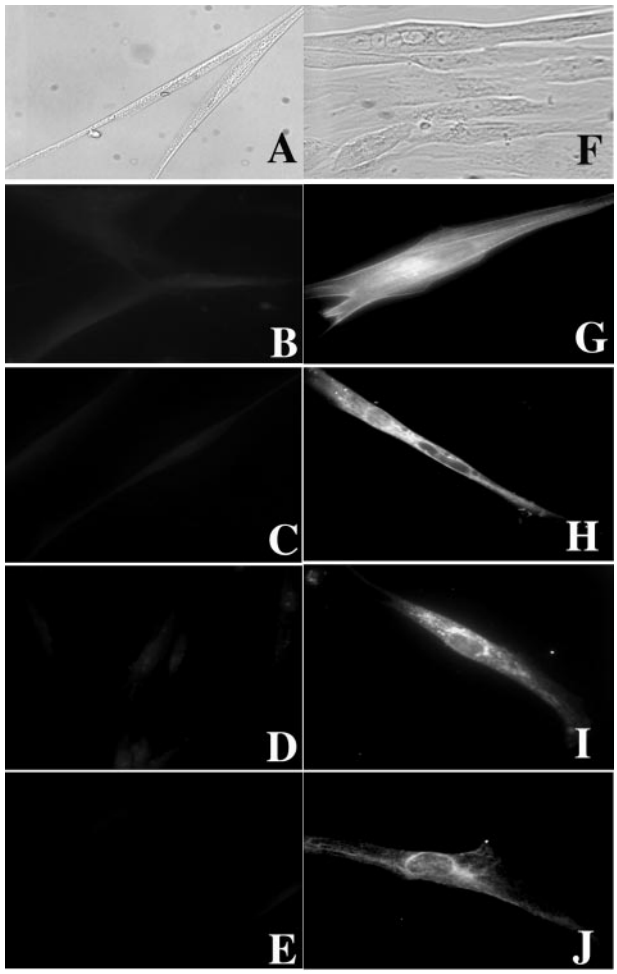

FIG. 1. **Photomicrograph of cultured human myoblasts and myotubes.** *A–E*, myoblasts cultured in growth medium. *F–J* myotubes cultured for 7–10 days in differentiation medium. *A* and *F*, phase-contrast micrograph. Note the presence of >5 nuclei in the uppermost cell of *panel F*. *B–J*, indirect immunofluorescent staining for sarcomeric α-actinin (*B* and *G*), skeletal muscle RYR (*C* and *H*), α1.1 subunit of the dihydropyridine receptor (*D* and *I*), and IL-6 receptor (*E* and *J*). ×20 Zeiss FLUAR objective; magnification, ×500).

dine antibodies (Fig. 1, *D* and *I*), confirming that myotubes express the two main protein components of skeletal muscle excitation-contraction coupling (40–42). We also verified whether cultured muscle cells express the IL-6 receptor, because IL-6 is released in large quantities during intense muscle activity (32). Of interest, immature myoblasts did not express the IL-6 receptor (Fig. 1*E*), whereas differentiated cells were immunopositive (Fig. 1*J*).

Activation of the RYR Leads to IL-6 Release from Cultured Human Myotubes—We next carried out experiments aimed at determining whether activation of the RYR leads to IL-6 release from cultured human myotubes. On the day of the experiment, differentiation medium was replaced with fresh differentiation medium containing no agonist or 4-chloro-*m*-cresol or caffeine. Cells from three control individuals were incubated at 37 °C for different time periods, and the amount of IL-6 released into the supernatant was determined by ELISA. Fig. 2 shows the time course of IL-6 release after treatment with 600 μM 4-chloro-*m*-cresol (Fig. 2*A*) and 10 mM caffeine (Fig. 2*B*). Maximal release was obtained 4–6 h after addition of either agonist, indicating that activation of the RYR most likely does not result in the release of pre-formed cytokine but stimulates activation of transcription and protein synthesis. The total amount of IL-6 released into the supernatant after 6 h of

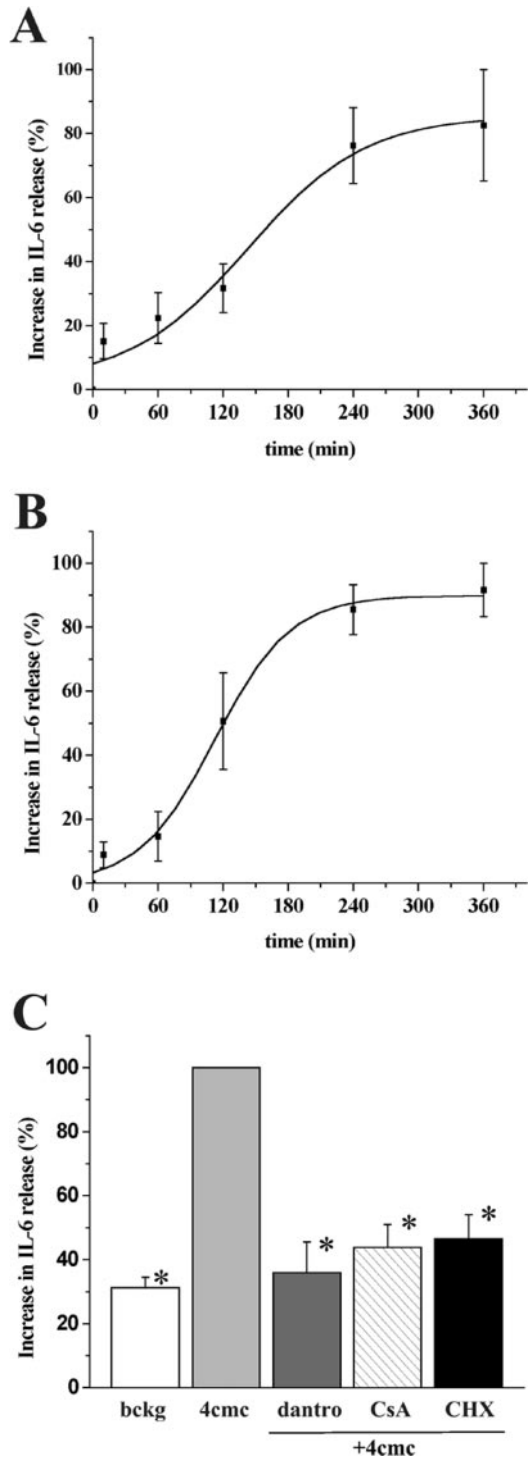

FIG. 2. **Time course of IL-6 release by human myotubes from normal individuals, induced by pharmacological activation of the ryanodine receptor.** *A* and *B*, cells were incubated in differentiation medium plus 600 μM 4-chloro-*m*-cresol (*A*) or 10 mM caffeine (*B*) at 37 °C for the indicated times. The amount of IL-6 released into the supernatant was determined by ELISA. The results are expressed as percent increase in IL-6 released; the maximal amount of IL-6 released by the RYR1 agonists was considered 100%. The results are the mean ± S.E. of three experiments carried out in duplicate on cultures established from three different control individuals. The mean maximal IL-6 released by 600 μM 4-chloro-*m*-cresol and 10 mM caffeine was 5.0 ± 0.7 and 7.5 ± 1.2 ng/10^6 cells, respectively. *C*, IL-6 release induced by a 5-h incubation with 600 μM 4-chloro-*m*-cresol is significantly reduced in the presence of dantrolene (*dantro*, 20 μM), cyclosporine A (*CsA*, 100 nM), and cycloheximide (*CHX*, 3 μg/ml). *Bckg* represents the background IL-6 release of myotubes incubated for 5 h in the absence of added agonist. Results represent the mean (± S.E.) of at least four separate experiments carried out in duplicate (Student's *t* test; *, $p < 0.00001$).

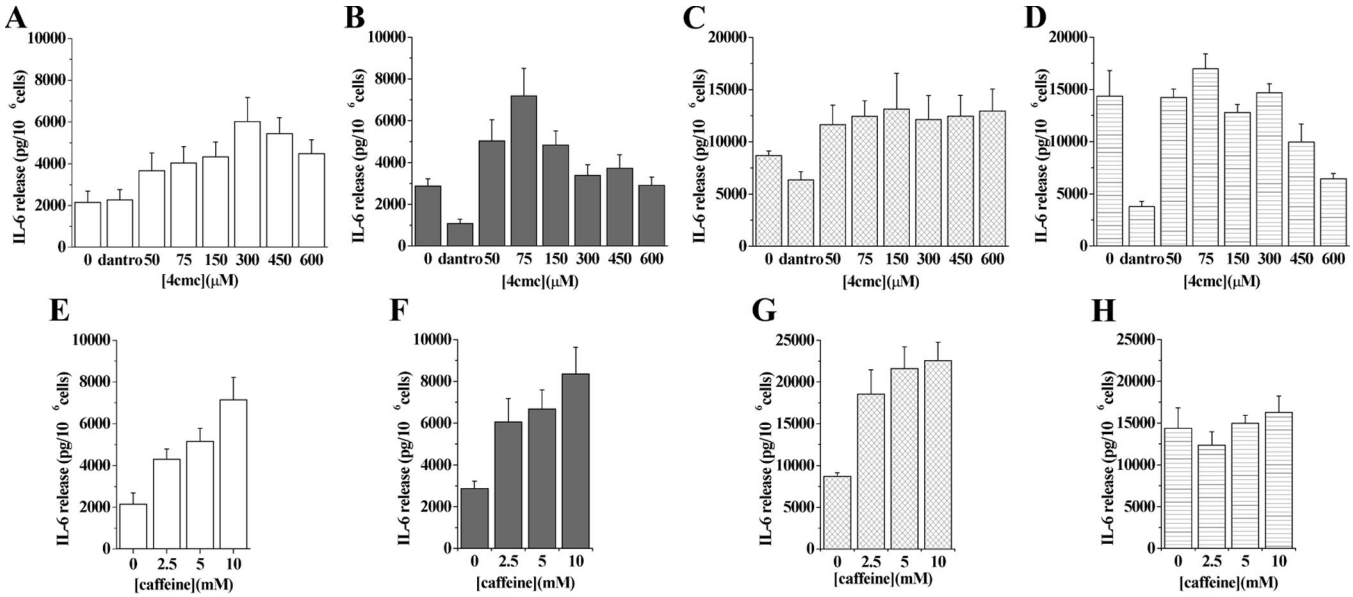

FIG. 3. **Dose-dependent 4-chloro-*m*-cresol and caffeine-induced IL-6 release from human myotubes from control individuals and patients bearing MH and CCD-linked *RYR1* mutations.** Cells were incubated for 5 h in differentiation medium plus the indicated concentrations of 4-chloro-*m*-cresol (*A–D*) or caffeine (*E–H*); the amount of IL-6 released in the supernatant was determined by ELISA. Data represent the mean (± S.E.) of experiments carried out at least three times in duplicate. *Empty boxes*, cells from controls (at least four different individuals; *A* and *E*); *filled boxes*, cells from MHS individuals bearing the V2168M *RYR1* mutation (eight different individuals; *B* and *F*); *crossed boxes*, cells from the CCD individual bearing the I4898T *RYR1* mutation (*C* and *G*); *lined boxes*, cells from the CCD individual bearing the R4893W *RYR1* mutation (*D* and *H*). *dantro*, cells incubated with 20 μM dantrolene plus 600 μM 4-chloro-*m*-cresol.

incubation of muscle cells from control individuals with 600 μM 4-chloro-*m*-cresol and 10 mM caffeine was 5.0 ± 0.7 and 7.5 ± 1.2 ng/10^6 cells, respectively.

To verify that this IL-6 release was (i) dependent on protein synthesis and (ii) dependent on activation of the RYR1, we performed experiments on cells stimulated with 600 μM 4-chloro-*m*-cresol, which had been pre-treated with cycloheximide to inhibit protein synthesis (3 μg/ml, 30 min) (43), dantrolene (20 μM, 30 min) (44) to prevent activation of RYR, or cyclosporine (100 nM, 30 min) (45) to block calcium-dependent calcineurin activation of the transcription factor NF-AT. Pre-treatment with any of the three compounds resulted in a significant inhibition (>50%, $p < 0.00001$, Student's t test) of 4-chloro-*m*-cresol-activated IL-6 release (Fig. 2*C*). Background IL-6 release after 5 h of incubation of the myotubes at 37 °C in the absence of any RYR agonist was 31.3% ± 3.2 of the maximal release (mean ± S.E.; $n = 6$). Contrary to what was observed in lymphocytes (46), treatment of human myotubes with 4-chloro-*m*-cresol or caffeine did not result in the release of significant amounts of IL-1 (<50 pg/10^6 cells).

We next determined the dose-response curves of 4-chloro-*m*-cresol-induced and caffeine-induced IL-6 release from human myotubes obtained from control individuals. Fig. 3 shows that treatment of cells with either agonist resulted in the significant release of IL-6 ($p < 0.0018$; ANOVA). The release could be blocked by pre-treatment with 20 μM dantrolene. In cells from control individuals, the maximal release was obtained at a concentration of 300 μM 4-chloro-*m*-cresol and 10 mM caffeine: the mean (± S.E.) IL-6 released was 6.0 ± 1.1 ng/10^6 cells and 7.1 ± 1.1 ng/10^6 cells, respectively (Fig. 3, *A* and *E*). These results represent the mean of eight different experiments carried out on muscle cells from at least four different individuals. The amounts of IL-6 released by 10 mM caffeine or 300 μM 4-chloro-*m*-cresol were not significantly different ($p = 0.497$). Of interest, treatment with either agonist resulted in a larger amount (1.4-fold) of cytokine release compared with that obtained by treating the cells with 1 μM ionomycin (mean ± S.E.; IL-6 released was 5.3 ± 0.8 ng/10^6 cells; not shown).

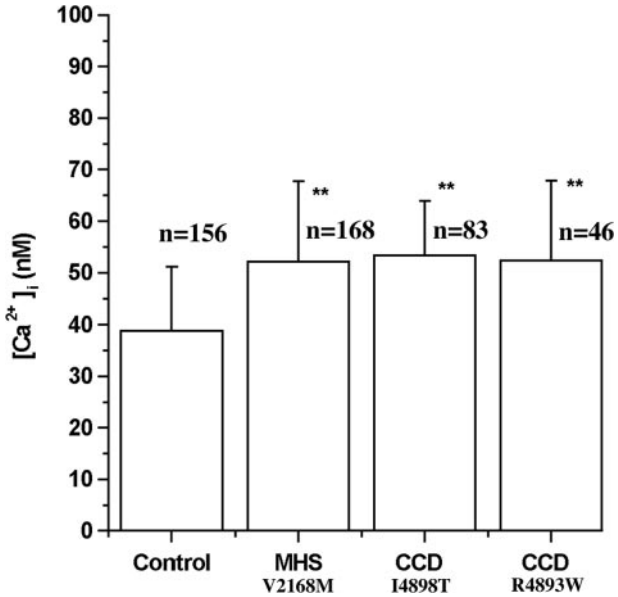

FIG. 4. **Resting calcium concentration of human myotubes from control individuals and individuals bearing *RYR1* mutations.** Average resting myoplasmic [Ca^{2+}] of cells from control, MHS individuals, and CCD individuals bearing the indicated *RYR1* mutation. Values are the mean (± S.E.) of the indicated number of analyzed cells. The $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fura-2, in Krebs Ringer containing 1 mM $CaCl_2$. (Student's t test, **, $p < 0.0001$).

When the same experiment was performed on cells from MHS individuals carrying the *RYR1* V2168M mutation, addition of 4-chloro-*m*-cresol and caffeine resulted in the significant release of IL-6 ($p < 0.0001$; ANOVA). Maximal release was obtained at a concentration of 75 μM 4-chloro-*m*-cresol and 10 mM caffeine: the mean (± S.E.) IL-6 released was 7.2 ± 1.3 ng/10^6 cells and 8.4 ± 1.3 ng/10^6 cells, respectively (Fig. 3, *B* and *F*). Pre-treatment of the cells with 20 μM dantrolene inhibited IL-6 release, whereas treatment with 1 μM ionomycin

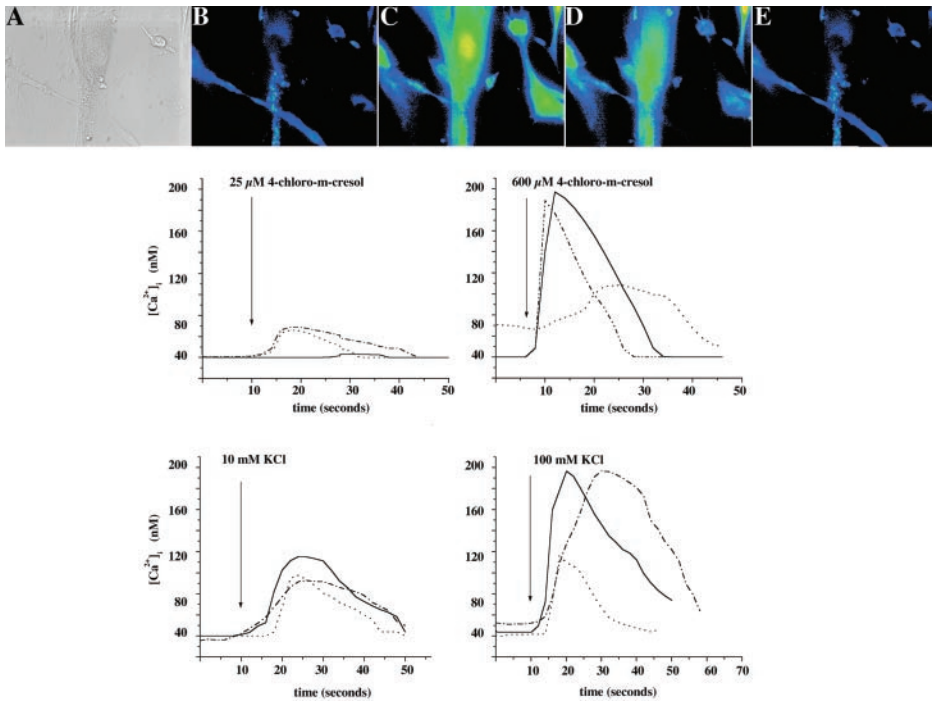

FIG. 5. **Calcium release stimulated by 4-chloro-*m*-cresol and KCl in human myotubes from control individuals and individuals bearing *RYR1*-mutations.** *B–E*, single cell intracellular Ca^{2+} measurements of Fura-2-loaded human myotubes. *A*, phase contrast; *B*, resting $[Ca^{2+}]_i$ before the application of 100 mM KCl and 10 (*C*), 30 (*D*), and 40 (*E*) s after the application of 100 mM KCl. Myotubes were individually stimulated by addition of the agonist in Krebs-Ringer buffer containing 0.5 mM EGTA, 100 μM La^{3+}, thus the increase in $[Ca^{2+}]_i$ represents only release of calcium from intracellular stores. Experiments were as described under "Experimental Procedures." The *bottom traces* show representative curves obtained after stimulation of single cells (*arrow*) with 4-chloro-*m*-cresol (25 μM, *top left*; 600 μM, *top right*) and KCl (10 mM, *bottom left*; 100 mM, *bottom right*). *Solid lines*, control; *dashed and dotted lines*, MHS; and *dotted lines*, CCD.

resulted in the release of 5.5 ± 0.8 ng/10^6 cells. Background release of IL-6 in the absence of added RYR agonist was similar in myotubes from control and MHS individuals (mean ± S.E.; release was 2.1 ± 0.5 and 2.99 ± 0.2 ng/10^6 cells, respectively).

Fig. 3 (*C*, *D*, *G*, and *H*) summarize the results obtained when experiments were performed on myotubes from the CCD patients harboring the I4898T and R4893W *RYR1* mutations. The presence of either mutation in the C-terminal region of *RYR1* caused a significant increase in the background levels of IL-6 released in the absence of an exogenous pharmacological activator of the RYR: the mean (± S.E.) amount of IL-6 released was 8.7 ± 0.4 and 14.7 ± 2.4 ng/10^6 myotubes carrying the *RYR1* I4898T and R4893W mutations, respectively ($p < 0.0001$, ANOVA; compared with the background release of myotubes from control individuals). This background release of IL-6 blunted the stimulatory effect of 4-chloro-*m*-cresol to myotubes from the CCD patient harboring the I4898T mutation; the release induced by 600 μM 4-chloro-*m*-cresol was inhibited by pre-treatment with 20 μM dantrolene (Student's *t* test $p < 0.0012$; Fig. 3*C*). On the other hand, addition of caffeine caused a significant increase in IL-6 release, compared with background release ($p < 0.004$; ANOVA, Fig. 3*G*). 4-chloro-*m*-cresol (figure 3 panel D) and caffeine (figure 3 panel H) induced IL-6 release in myotubes from the CCD patient harboring the R4893W mutation were not significantly different; pre-treatment of these cells with 20 μM dantrolene, caused a significant reduction in IL-6 release by 600 μM 4-chloro-*m*-cresol (Student's *t* test $p < 0.007$).

$[Ca^{2+}]_i$ Homeostasis in Cultured Human Myotubes Carrying RYR1 Mutations—We assessed the intracellular calcium homeostasis of myotubes from individuals carrying different mutations in *RYR1* by studying: (i) resting myoplasmic free calcium concentration; (ii) KCl-induced (depolarization) calcium release and (iii) 4-chloro-*m*-cresol induced calcium release. All mutations caused a small but significant increase in the $[Ca^{2+}]_i$ (Student's *t* test, $p < 0.0001$); the difference between the mean resting $[Ca^{2+}]_i$ of muscle cells from control and *RYR1*-mutation bearing individuals was ~13 nM. However no difference was found when comparing $[Ca^{2+}]_i$ from muscle cells bearing the different *RYR1* mutations ($p = 0.937$; ANOVA, Fig. 4).

In the next series of experiments we determined whether cells carrying different *RYR1* mutations respond differently to treatment with RYR1 agonists. Fig. 5 shows a representative single cell $[Ca^{2+}]_i$ measurement experiment: addition of the agonists resulted in an increase in the myoplasmic $[Ca^{2+}]_i$, which varied in extent depending on the mutation present and agonist concentration used. Fig. 6 shows the changes in fluorescence induced by the addition of different concentrations of KCl to myotubes from controls, MHS and CCD individuals. In control cells the EC_{50} for KCl-induced calcium release was 30 mM KCl, and maximal release was observed at 60 mM KCl, whereas in cells from MHS individuals harboring the V2168M mutation the EC_{50} for KCl-induced calcium release was 10 mM. In cells from the CCD patient harboring the *RYR1* I4898T mutation the EC_{50} for KCl-induced calcium release was 40 mM, whereas cells harboring the R4893W *RYR1* mutation were more sensitive to KCl-induced calcium release (EC_{50} for KCl was 10 mM). Cells from both CCD patients, however, released significantly less calcium compared with cells from control or MHS individuals.

We next assessed cells from the different patients for their response to the RYR-specific activator 4-chloro-*m*-cresol (Fig. 7); muscle cells from control individuals had an EC_{50} of 120 μM for 4-chloro-*m*-cresol-induced calcium release, whereas all cells harboring *RYR1* mutations were more sensitive to the agonist and had a lower EC_{50} for 4-chloro-*m*-cresol-induced calcium release (the EC_{50} values were 10 and 50 μM for cells from MHS individuals harboring the V2168M mutation and cells from CCD individuals, respectively). Of interest the cells from the CCD patient harboring the R4893W mutation released very little calcium when challenged even with high (up to 600 μM) concentrations of 4-chloro-*m*-cresol. As observed with KCl, cells from patients affected by CCD, irrespective of the mutation released less calcium than cells from MHS or control individuals.

Finally we compared the calcium released from the intracellular stores of myotubes by 150 mM KCl, 600 μM 4-chloro-*m*-cresol, or 10 mM caffeine (Fig. 8). Addition of the agonists caused similar increases in the myoplasmic $[Ca^{2+}]_i$ of the myotubes obtained from control and MHS individuals; on the other hand, irrespective of which mutation was present in the C-

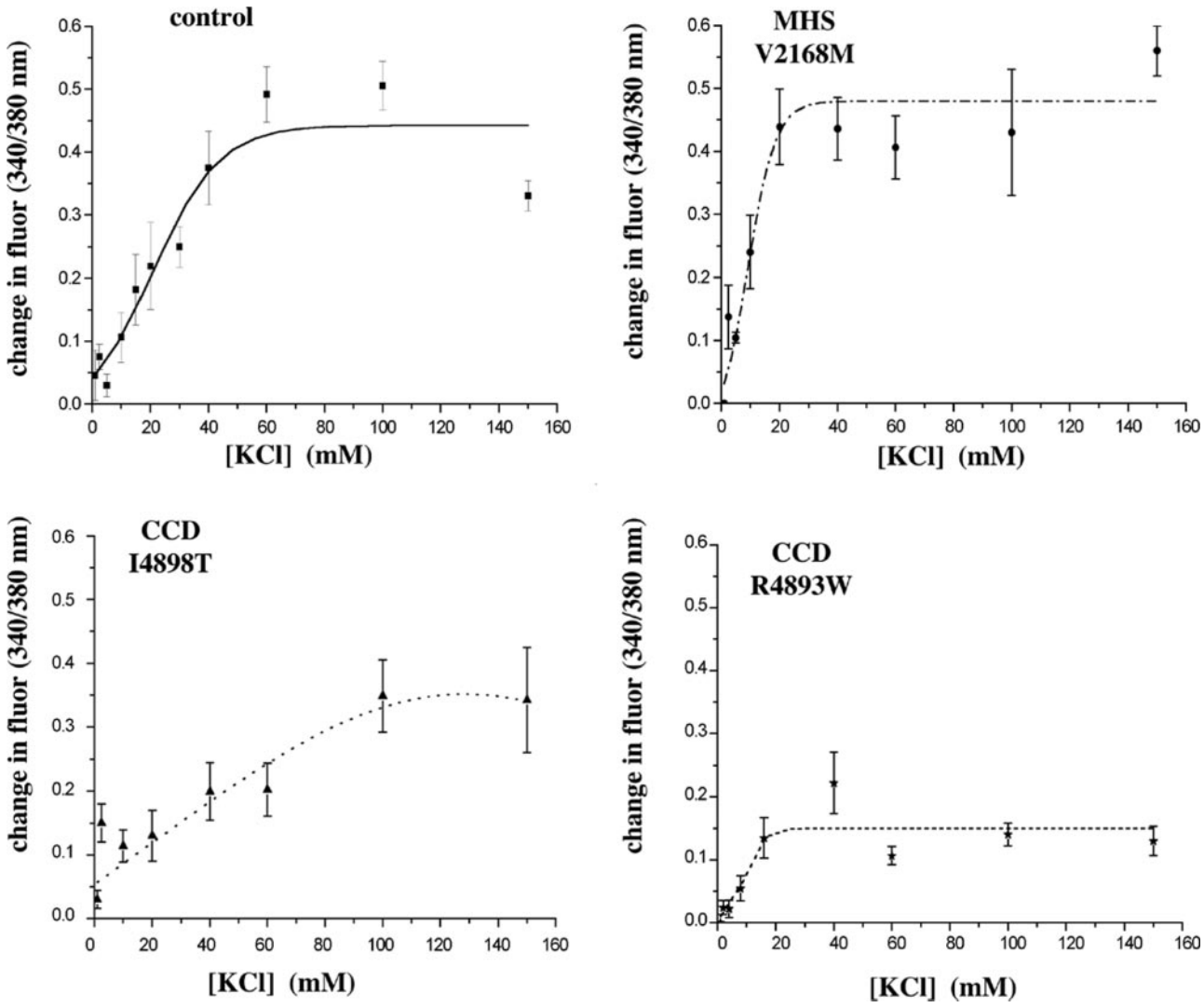

FIG. 6. **Calcium release stimulated by KCl in human myotubes.** Calcium imaging was performed as described for Fig. 5 and under "Experimental Procedures": the *curves* show the KCl dose-dependent change in calcium, expressed as a change in fluorescence ratio (peak ratio 340/380 nm; resting ratio 340/380 nm). Each *point* represents the mean (±S.E.) of the change in fluorescence of 5–10 measurements. Dose-response curves were generated using the Origin software.

terminal domain of the *RYR1*, myotubes from CCD individuals were always less responsive, because stimulation with KCl, 4-chloro-*m*-cresol, or caffeine induced significantly smaller changes in the $[Ca^{2+}]_i$.

DISCUSSION

This is the first report in which some of the physiological properties of myotubes harboring endogenous mutations in the C-terminal domain of *RYR1* obtained from individuals affected by CCD were investigated. We (i) compared the calcium release properties of myotubes from patients bearing the different *RYR1* mutations and (ii) investigated whether cultured human myotubes are capable of releasing IL-6 after activation of the ryanodine receptor and whether there are differences between cells from normal individuals and individuals bearing MH and CCD-linked *RYR1* mutations.

During the past years a number of studies have addressed the role of growth factors and inflammatory cytokines in muscle physiology (31, 47). IL-6 is a member of pro-inflammatory cytokines, which also include IL-1β and tumor necrosis factorα; these cytokines are also known as endogenous pyrogens, because they are capable of increasing body temperature and stimulating the release of acute phase proteins from the liver (35). Although IL-6 is released by cells of the immune system, other cell types, including skeletal muscle cells can produce and release this cytokine (32, 48, 49). Of interest, the plasma levels of IL-6 have been shown to increase by almost 100-fold after a marathon race, and a role for changes in the myoplasmic calcium concentration in IL-6 release from human muscle cells has been put forward (32, 48). Available data indicate that in skeletal muscle this cytokine may act as a hormone in the maintenance of glucose homeostasis during muscle contraction (32). However, IL-6 must also have an autocrine effect, because muscle cells express the IL-6 receptor and up-regulation of its transcript has been demonstrated following muscle injury (50).

The results of the present investigation are relevant to demonstrate an important role of calcium signaling in excitation-transcription coupling. Our results show that human myotubes are capable of releasing IL-6 several hours after RYR1 activation and that the presence of a *RYR1* mutation affects the amount of IL-6 released. RYR1 activation specifically caused IL-6 release from cultured human myotubes since: (i) IL-6 release was elicited by both 4-chloro-*m*-cresol and caffeine, two agonists that activate calcium release via RYR1 (24, 46); (ii) IL-6 release could be blocked by pre-treatment with dantrolene, a substance that specifically closes the calcium channel, thereby blocking calcium release from the sarcoplasmic

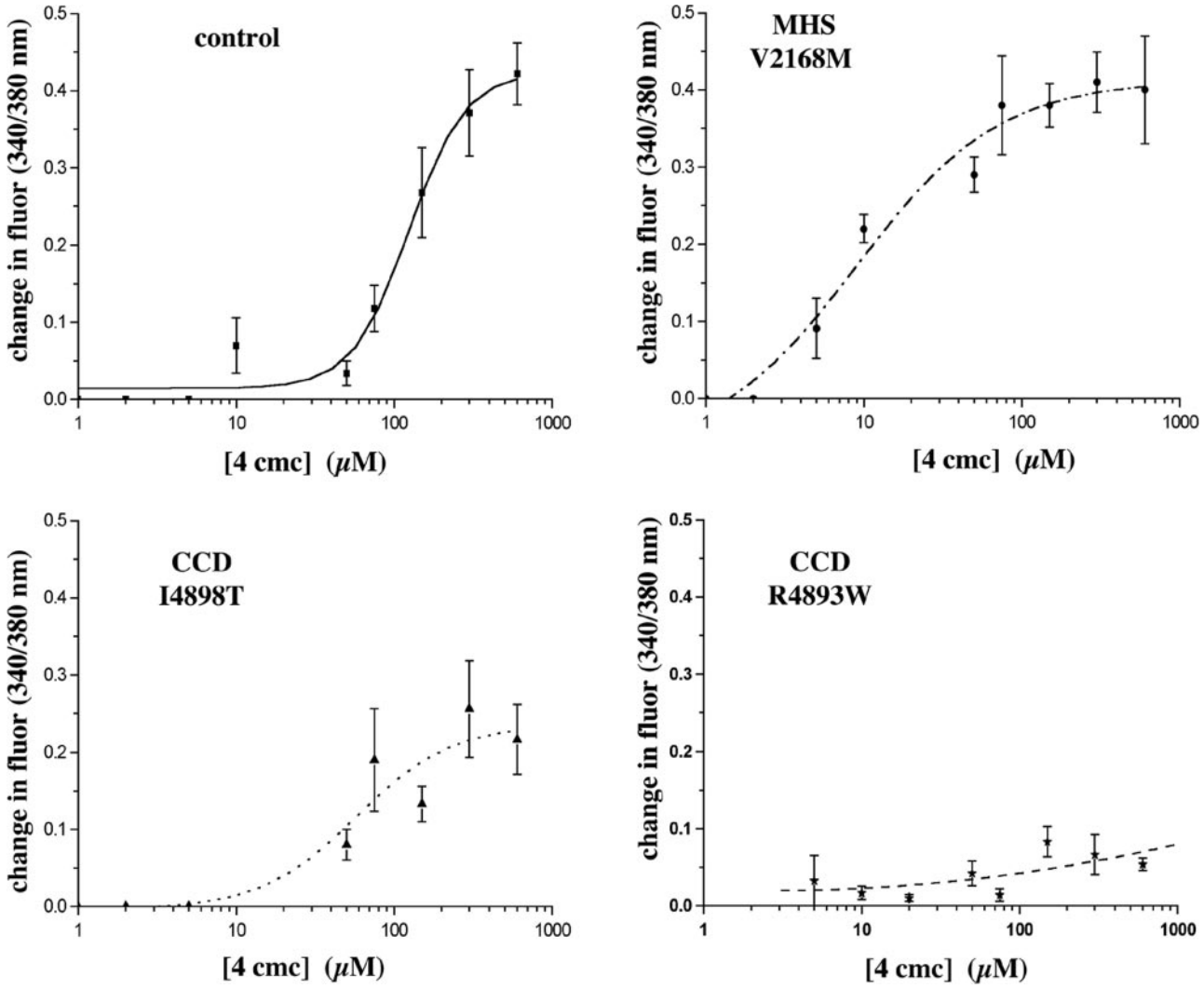

FIG. 7. **Calcium release stimulated by 4-chloro-*m*-cresol in human myotubes.** Curves show the 4-chloro-*m*-cresol dose-dependent change in calcium, expressed as change in fluorescence ratio (peak ratio 340/380 nm; resting ratio 340/380 nm). Each *point* represents the mean (±S.E.) of the change in fluorescence of 5–10 measurements. Dose-response curves were generated using the Origin software. Conditions as described for Fig. 6.

reticulum (44). Of interest, in cells from controls and MHS individuals, the concentration of 4-chloro-*m*-cresol causing maximal IL-6 release paralleled its maximal calcium releasing effect. On the other hand, IL-6 release from myotubes from CCD patients appears to be more complex. The most intriguing observation is that myotubes harboring *RYR1* mutations in the C-terminal domain of the RYR exhibited a 4-fold increase in the background level of IL-6 released compared with cells from controls or MHS individuals. Such a background IL-6 release likely attenuates the IL-6-releasing effect of 4-chloro-*m*-cresol, because it would be difficult to overstimulate the IL-6-releasing pathway. The IL-6 release observed in the presence of 4-chloro-cresol is sensitive to the RYR blocker dantrolene indicating that it involves RYR activation. Thus, it is plausible that the background IL-6 release may result, at least in part, from "unprompted calcium release events" via RYR. The hypothesis of unprompted release via RYR is consistent with (i) data obtained in CCD lymphocytes and with (ii) the observation that calcium release from intracellular stores of myotubes from CCD patients is decreased in the presence of EGTA in the extracellular medium, a condition that does not allow the full recharge of the calcium store (Fig. 8). The latter condition is in line with previous reports (15, 51) showing that recombinant RYR1 channels in which Ile-4898 was replaced by either Ala, Val, or Leu, or carrying a 5-amino acid deletion in the C-terminal region, failed to close completely for long time periods even in the presence of mM EGTA. We measured calcium release in the presence of EGTA to evaluate the release from the stores to exclude calcium signals generated by influx of calcium from the extracellular medium. During the determination of IL-6 release, the presence of extracellular calcium would facilitate recharging of calcium stores, enhancing the driving force of calcium release through mutated RYR via unprompted events or via RYR agonist activation. For the above reasons, we believe that the presence of extracellular calcium during the IL-6 release assay accounts for the apparent miss-correlation between the effect of 4-chloro-cresol on calcium release (Fig. 8) and on IL-6 release (Fig. 3). Altogether these results are consistent with the idea that the one or more calcium signals generated via activation of RYR1 is relevant to the signal transduction pathway leading to IL-6 release in human myotubes, but we can not exclude the possibility that other signals might also be involved. In this context it should be kept in mind that calcium-independent mechanisms have also been implicated in IL-6 release (32, 52, 53).

In many cases cytokine production is regulated by the intracellular calcium concentration, via the phosphatase calcineurin and the nuclear factors of activated T cells (NF-AT)-signal

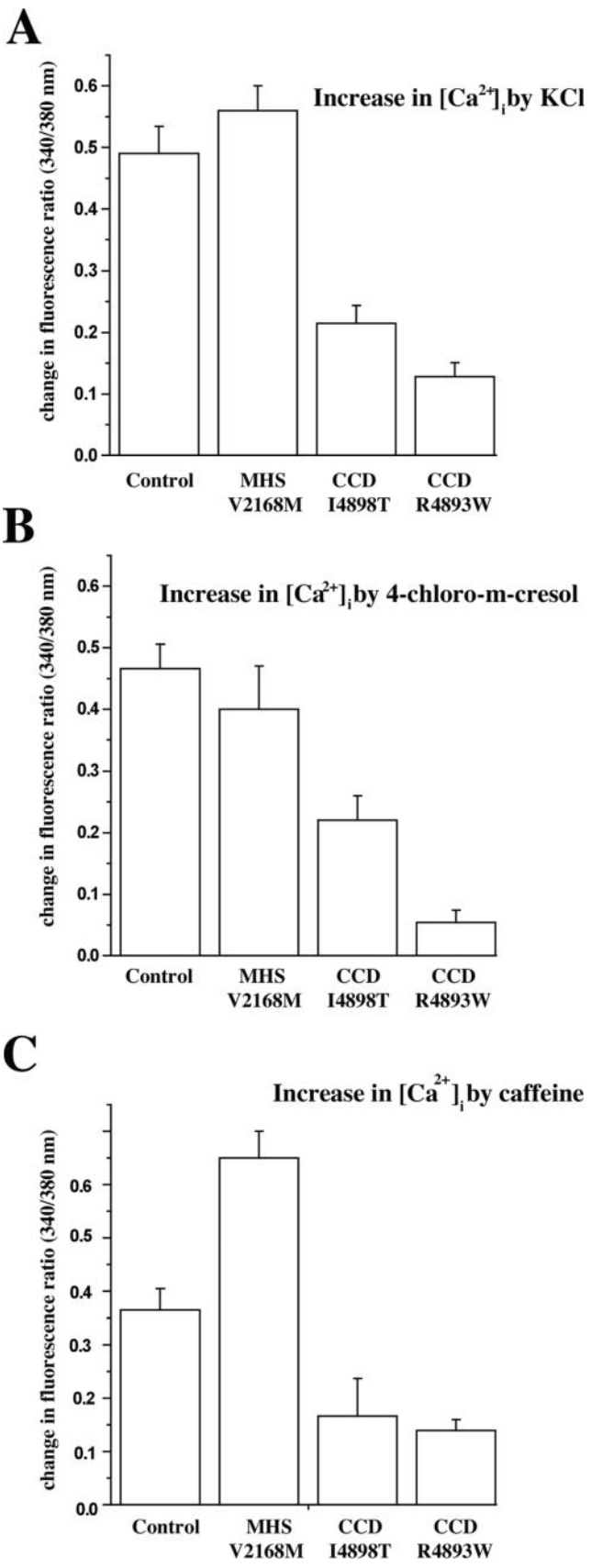

FIG. 8. **Increase in $[Ca^{2+}]_i$ induced by KCl, 4 chloro-*m*-cresol, and caffeine in human myotubes from control, MHS, and CCD individuals.** Change in fluorescence ratio (peak ratio 340/380 nm; resting ratio 340/380 nm) induced by 150 mM KCl, 600 μM 4-chloro-*m*-cresol, and 10 mM caffeine in Krebs-Ringer containing 0.5 mM EGTA and 100 μM La^{3+}. Results are the mean (±S.E.) of *n* (6–34) independent measurements.

transduction pathway (30). The results of the present investigation show that activation of the RYR leads to a calcineurin-dependent IL-6 release, because pre-treatment with cyclosporine blocked release of this cytokine from human myotubes. It will be interesting to study whether release of other muscle-derived cytokines such as IL-4, IL-15, and IL-13 whose transcription is regulated by the NF-AT pathway can also be elicited by RYR activation. The release of cytokines via activation of RYR1 also has important implications in MH. This condition is characterized by hypermetabolism and a rapid increase in body temperature following the administration of trigger agents (7–9). Point mutations in *RYR1* not only affect the amount of calcium released from the sarcoplasmic reticulum but also, in view of the present findings, the amount of IL-6 that can potentially be released. Because muscle mass constitutes more than 40% of total body weight, part of the downstream effects of mutations in *RYR1* may include an increase in the circulating levels of IL-6, which may result in fever, muscle wasting, and fiber type switching (via Myf5 gene expression) (54). From a histological point of view MH does not induce major changes in muscle fiber appearance, whereas CCD is associated with fiber atrophy/hypotrophy (1–6). Thus alterations of calcium homeostasis caused by RYR-linked mutations in CCD patients, could potentially influence the signaling pathways responsible for the trophic status of muscle fibers and account for the different phenotypes (MH *versus* CCD), which are associated with mutations of the same molecule. Cytokine release experiments on a larger number of cells from patients affected by CCD may help clarify the clinical relevance of this novel observation.

During the past decade a number of studies have appeared concerning the functional impact of mutations in the *RYR1* gene associated with CCD and/or the MHS phenotype (9–11). Most MH-linked mutations are clustered within two domains of the large hydrophilic region of the RYR protomer and cause a shift in the dose-response curve to agonists such as caffeine, 4-chloro-*m*-cresol, and halothane (9–11, 22–24). As far as the MH-linked *RYR1* V2168M mutation is concerned, the results of the present investigation are consistent with our previous studies using immortalized lymphoblastoid cells of the same genotype (46) and those of Yang *et al.* (25) using dyspedic mouse myotubes transduced with wild type or mutated *RYR1* cDNA. The presence of the mutation resulted in a shift in the EC_{50} of calcium release stimulated by 4-chloro-*m*-cresol from ~130 to 28 μM, in wild type and mutation carrying myotubes, respectively. Similarly, the dose-response curve for KCl-induced calcium release was shifted in myotubes from MHS individuals carrying the V2168M *RYR1* mutation. These results confirm those of Yang *et al.* (25) and Gallant *et al.* (55) except that under our experimental conditions the EC_{50} for KCl-induced calcium release ~2-fold lower; the differences most likely reflect differences in the experimental approaches and the use of different calcium indicators.

CCD-linked *RYR1* mutations in the C-terminal transmembrane domain have been shown to cause reduced calcium release following RYR activation (17, 20). This observation has been explained by the "leaky channel" hypothesis, which states that the presence of C-terminal CCD-linked *RYR1* mutations result in a reduction of the intracellular calcium stores, which in turn cause muscle weakness, because the contractile force in normal excitation-contraction coupling is proportional to activating Ca^{2+}. For some mutations, however, a second hypothesis has been put forward: the presence of C-terminal *RYR1* mutations reduces voltage-dependent calcium release even in the absence of sarcoplasmic reticulum calcium store depletion, *i.e.* there is excitation-contraction uncoupling (26, 27). The

results of the present investigation support the overall view that mutations in the C-terminal domain of *RYR1* reduce the amount of calcium that is released via the RYR calcium channel, whereby cells expressing mutant C-terminal channels have an altered excitation-contraction coupling. As to the differences in agonist-induced calcium release between our results and those of Avila *et al.* (27), they probably reflect the different experimental models used; *i.e.* dyspedic mouse myotubes reconstituted with recombinant homozygous mutated rabbit RYR1 on a homogeneous genetic background, *versus* expression of the endogenous heterozygous mutation in cultured human myotubes on the patient's individual genetic background.

As far as the resting myoplasmic calcium concentration is concerned, cells carrying any *RYR1* mutation had a small but significantly higher resting calcium concentration, supporting previous observations that the presence of *RYR1*-linked mutations does not grossly alter the resting calcium concentration of cells expressing the native endogenous mutation (22, 27). This is expected, because resting calcium is the net balance between efflux from the sarcoplasmic reticulum via the RYR, re-uptake into the sarcoplasmic reticulum via the sarcoplasmic reticulum Ca^{2+}-ATPase, and the activity of other calcium extrusion pathways. A high myoplasmic calcium concentration would be detrimental to the cells, possibly activating calcium-dependent proteases and eventually leading to cell death.

In conclusion our data show that skeletal muscle cells respond to RYR activation by releasing IL-6, and that the presence of *RYR1* mutations not only affects calcium homeostasis but, indirectly, also the amount of cytokine released. Our data also show that myotubes from patients affected by CCD release significantly more IL-6 compared with cells from control and MHS individuals and point toward a complex and heterogeneous pathogenetic pathway leading to the myopathy in CCD patients with different mutations.

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