Mutations to Gly²³⁷⁰, Gly²³⁷³ or Gly²³⁷⁵ in malignant hyperthermia domain 2 decrease caffeine and cresol sensitivity of the rabbit skeletal-muscle Ca²⁺-release channel (ryanodine receptor isoform 1)

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Mutations G2370A, G2372A, G2373A, G2375A, Y3937A, S3938A, G3939A and K3940A were made in two potential ATP-binding motifs (amino acids 2370–2375 and 3937–3940) in the Ca²⁺-release channel of skeletal-muscle sarcoplasmic reticulum (ryanodine receptor or RyR1). Activation of [³H]ryanodine binding by Ca²⁺, caffeine and ATP (adenosine 5'-[β , γ -methylene]triphosphate, AMP-PCP) was used as an assay for channel opening, since ryanodine binds only to open channels. Caffeine-sensitivity of channel opening was also assayed by caffeine-induced Ca²⁺ release in HEK-293 cells expressing wild-type and mutant channels. Equilibrium [³H]ryanodine-binding properties and EC₅₀ values for Ca²⁺ activation of high-affinity [³H]ryanodine binding were similar between wild-type RyR1 and mutants. In the presence of 1 mM AMP-PCP, Ca²⁺-activation curves were shifted to higher affinity and maximal binding was increased to a similar extent for wild-type RyR1 and mutants. ATP sensitivity of channel opening was also similar for wild-type

and mutants. These observations apparently rule out sequences 2370–2375 and 3937–3940 as ATP-binding motifs. Caffeine or 4-chloro-*m*-cresol sensitivity, however, was decreased in mutants G2370A, G2373A and G2375A, whereas the other mutants retained normal sensitivity. Amino acids 2370–2375 lie within a sequence (amino acids 2163–2458) in which some eight RyR1 mutations have been associated with malignant hyperthermia and shown to be hypersensitive to caffeine and 4-chloro-*m*-cresol activation. By contrast, mutants G2370A, G2373A and G2375A are hyposensitive to caffeine and 4-chloro-*m*-cresol. Thus amino acids 2163–2458 form a regulatory domain (malignant hyperthermia regulatory domain 2) that regulates caffeine and 4-chloro-*m*-cresol sensitivity of RyR1.

Key words: ATP-binding site, 4-chloro-*m*-cresol, malignant hyperthermia regulatory domain II.

INTRODUCTION

Ca²⁺-release channels of sarcoplasmic reticulum (ryanodine receptors, or RyRs) are large homotetrameric molecules with molecular masses of $\approx$ 2.26 MDa [1–4]. These channels are modulated by endogenous and exogenous ligands such as Ca²⁺, ATP, caffeine, calmodulin, Mg²⁺, Ruthenium Red and ryanodine, but the extent of modulation varies among RyR1, the isoform expressed in skeletal muscle, RyR2, expressed in cardiac muscle, and RyR3, expressed more ubiquitously [5–7]. Ca²⁺ is a basic modulator of Ca²⁺-release channel function. At low concentrations, Ca²⁺ activates both skeletal- and cardiac-muscle Ca²⁺-release channels; at high concentrations, it inactivates the skeletal-muscle Ca²⁺-release channel. Ca²⁺ activation and inactivation sites are localized in the C-terminal one-fifth of the RyR1 molecule region [8–12]. This C-terminal region of RyR also forms the Ca²⁺-conducting pore and contains both high- and low-affinity binding sites for ryanodine [8,13–15]. The molecular mechanisms underlying the interactions of modulators with RyRs are poorly understood.

Adenine nucleotides activate Ca²⁺-release channels with EC₅₀ values in the millimolar range. Activation is probably through a molecular site different from, but interacting with, the Ca²⁺-activation or Mg²⁺-binding site [5–7]. Motifs identifying potential

nucleotide-binding sites [16,17] include consensus sequences GXGXXG, GXXXXGKT (Walker A), and (R/K)XXXGX-XXLhhD (Walker B), where h is a hydrophobic residue. The GXGXXG motif is present in amino acids 2–7, 699–704, 1195–1200, 2264–2269, 2370–2375, 4449–4454, 4452–4457 and 4602–4607 in RyR1. Amino acids 4449–4457 have also been predicted to be potential ATP-binding sites by Takeshima et al. [18], and amino acids 2660–2665 in RyR1 have been predicted to be a potential ATP-binding site by Zorzato et al. [19]. These RyR1 sequences do not conform to consensus Walker A and Walker B motifs, however. Two other amino acid sequences, 2126–2134 and 2369–2377, correspond to part of the Walker B sequence [(R/K)XXXGXXL]. The latter sequence includes amino acids 2370–2375, which conform to the GXGXXG motif. A peptide fragment which included amino acids 2370–2375 was heavily labelled with 8-azido[α -³²P]ATP photo-labelling in an earlier study [20], suggesting that an ATP-binding site might be located in this sequence.

Chaperonin proteins GroEL and GroES also possess binding sites for ATP with submillimolar affinity [21]. The consensus sequences are Y(G/A/S/T)(V/G)(K/T/Q/S/N) for GroES and GY(N/D)(A/T)(A/M/L/T/S) for GroEL. The consensus sequence for GroEL has not been found in the RyR family, but four sequences in RyR1, amino acids 1081–1084, 2935–2938,

Abbreviations used: RyR, ryanodine receptor; RyR1, skeletal-muscle RyR isoform; RyR2, cardiac-muscle RyR isoform; AMP-PCP, adenosine 5'-[β , γ -methylene]triphosphate; MH, malignant hyperthermia; CCD, central core disease; pCa, $-\log[\text{Ca}]$.

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3503–3506 and 3937–3940, conform to the consensus ATP-binding sites in GroES. Ogawa [22] has proposed that this motif might form a potential binding site for ATP in RyR proteins, since neither the ATP bound to this site in GroES nor the ATP which activates the Ca^{2+} -release channel is hydrolysed.

Caffeine has long been used as a tool for studying excitation–contraction coupling and the function of Ca^{2+} -release channels [23]. It activates the Ca^{2+} -release channel at concentrations below 2 mM by increasing Ca^{2+} sensitivity [5–7,24], but, at concentrations above 5 mM, the action of caffeine on the channel is more direct. Caffeine is also used in the clinical diagnosis for malignant hyperthermia (MH) in skeletal muscle [25]. RyR2 is more sensitive to caffeine activation than RyR1 [7,26–28], but the binding site for caffeine in these two molecules has not been defined. This functional difference might be due to differences in binding sites for caffeine, or to differences in other structures which determine caffeine sensitivity. For example, deletion of amino acids 4272–4535 in RyR1 increases the sensitivity of the mutant RyR1 channel to caffeine and Ca^{2+} [29]. Thus the deleted region might form an inhibitory domain for Ca^{2+} and/or caffeine activation of RyR1. Expression of the C-terminal one-fifth of the RyR1 sequence was sufficient to form a functional Ca^{2+} -release channel, but the channel was not caffeine-activated, demonstrating that upstream sequences are required for regulation of the Ca^{2+} -release channel by caffeine [30].

Mutations causing MH and central core disease (CCD) are clustered in three regions lying between amino acids 35 and 614 (MH domain 1), 2163 and 2458 (MH domain 2) and 4800 and 4900 (MH domain 3) [25,31]. Mutations in MH domains 1 and 2 increase the sensitivity of the channel to caffeine and halothane [32–35], suggesting that both domains are involved in caffeine activation and thus contain potential caffeine-binding site(s). A CCD mutation found in MH region 3, however, had normal caffeine and halothane sensitivity when expressed as a heterozygote [31].

In an attempt to identify potential ATP-binding sites in the Ca^{2+} -release channel, we have carried out alanine-scanning mutagenesis of amino acids in two motifs: amino acids 2370–2375, located in MH domain 2, and 3937–3940, by creating the mutants G2370A, G2372A, G2373A, G2375A, Y3937A, S3938A, G3939A and K3940A. Since [^3H]ryanodine only binds to open channels, high-affinity [^3H]ryanodine binding to the mutants was used as a means of detecting channel activation by adenosine 5'-[β,γ -methylene]triphosphate (AMP-PCP), Ca^{2+} and caffeine. We also used Ca^{2+} photometry to measure Ca^{2+} release induced by caffeine and 4-chloro-*m*-cresol (a skeletal-muscle Ca^{2+} -release channel agonist) in HEK-293 cells expressing wild-type and mutant RyR1. Mutations of any these residues had no effect on ATP activation of RyR1, but mutations G2370A, G2373A and G2375A in MH domain 2 lowered sensitivity to caffeine and 4-chloro-*m*-cresol activation.

MATERIALS AND METHODS

Materials

Plasmid bluescript KS(+), restriction endonucleases and other DNA-modifying enzymes were from Stratagene, Boehringer Mannheim, New England Biolabs, Promega and Amersham Pharmacia Biotech; fura 2 acetoxymethyl ester was from Molecular Probes; caffeine, AMP-PCP and protease inhibitors were from Sigma; [^3H]ryanodine was from DuPont NEN; unlabelled ryanodine and CHAPS were from Calbiochem; phosphatidylcholine was from Northern Lipids. Monoclonal antibody 34C was a kind gift from Dr Judith Airey (University of Nevada

School of Medicine, Reno, NV, U.S.A.) [36]. All other reagents were of reagent grade or the highest grade available.

Oligonucleotide-directed mutagenesis

Site-directed mutagenesis of RyR1 cDNA was carried out in cassettes which were excised from the full-length sequence in pBluescript KS(+) (pBS-RyR1) using unique restriction endonucleases engineered into the molecule [34]. In this study, mutagenesis involved cassettes C6 (*SpeI*–*SpI*) and C9 (*NdeI*–*NheI*) in vector pBS2 [34]. Site-specific mutagenesis was performed according to the method of Kunkel [37]. The complete sequence of the mutated fragment was confirmed by DNA sequencing. Fragments with the desired mutations were subcloned back into their original positions in pBS-RyR1, and then the entire sequence was excised with *XbaI* and *HindIII* (blunt-ended) and subcloned into *XbaI* and *SmaI* sites in modified pMT2 vector, in which additional multiple cloning sites were created (*EcoRI*–*XbaI*–*NotI*–*SmaI*–*KpnI*–*SalI*–*EcoRI*) for expression in HEK-293 cells.

Cell culture and DNA transfection

Culture of HEK-293 cells and cDNA transfection by the calcium phosphate precipitation method [38] were carried out as described previously [39].

Fluorescence measurements

A microfluorimetry system (Photon Technology International) was used to monitor the fura 2 acetoxymethyl ester fluorescence changes in transiently transfected or non-transfected HEK-293 cells, as described previously [28].

[^3H]Ryanodine binding

Transfected HEK-293 cells grown in 100 mm Petri dishes were solubilized with 1% CHAPS and 5 mg/ml phosphatidylcholine at 4 °C for 1 h. After precipitation at 8000 *g* (Sorvall ss34 rotor) for 20 min, supernatants were centrifugated at 45000 *g* for 1 h in a Beckman Ti70.1 rotor. The resulting pellets were dissolved in 250 mM sucrose, 150 mM KCl and 25 mM Hepes, pH 7.1. Channel opening was then analysed using the [^3H]ryanodine-binding assay described previously [28]. In brief, 10 μl aliquots of protein were added in 0.25 ml of binding buffer composed of 0.5 M KCl, 1 mM ATP, 100 μM free Ca^{2+} , 0.2 mM EGTA, 50 mM Hepes, pH 7.1, a protease-inhibitor mix {0.1 mM AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride], 1 mM benzamide, 1 $\mu\text{g}/\text{ml}$ of leupeptin, pepstatin and aprotinin, and E64} and various concentrations of [^3H]ryanodine. Non-specific binding was determined using a 1000-fold excess of unlabelled ryanodine. After 2 h at 37 °C, the samples were diluted with 1 ml of ice-cold washing buffer composed of 25 mM Hepes, pH 7.1, and 0.25 M KCl and placed on Whatman GF/B membrane filters soaked with 1% polyethyleneimine in washing buffer. Filters were washed three times with 6 ml of washing buffer. [^3H]Ryanodine bound to the filter was quantified by liquid scintillation counting. All binding assays were carried out in duplicate.

To assess the effects of Ca^{2+} and ATP on high-affinity ryanodine binding, different concentrations of Ca^{2+} were added in the presence of 5 nM [^3H]ryanodine and in the presence or absence of 1 mM AMP-PCP, a non-hydrolysable ATP analogue. To test the sensitivity of ATP activation of the mutants, different concentrations of AMP-PCP were included in the binding buffer {*pCa* (–log[Ca]) 6.0}. To test caffeine sensitivity in the mutants, caffeine concentrations, ranging from 0 to 100 mM, were added

to the binding buffer ($p\text{Ca}$ 6.7). The incubation time was 1 h for all. Free Ca^{2+} was calculated using the apparent binding constants described by Fabiato [40].

SDS/PAGE, immunoblotting and protein assay

About 50 μg of protein from cells lysed with CHAPS were separated by 6% SDS/PAGE [41]. RyR protein was detected by immunoblotting [42], as described previously [39].

Protein concentration was determined by dye binding using BSA as standard [43].

Data analysis

Data were analysed with Microcal Origin software (Microcal Software, Northampton, MA, U.S.A.). Scatchard analysis was used to determine the dissociation constant (K_d) and maximal binding capacity (B_{max}) from equilibrium binding data. EC_{50} values were obtained by fitting the curves with an equation for logistic dose-response. Data are expressed as means $\pm$ S.E.M. Paired or unpaired Student t tests were used for evaluation of the mean values. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Mutagenesis and expression of mutant RyR1 cDNAs

Amino acids 1081–1084, 2370–2375 and 3937–3940 are conserved within the RyR family. The alignments for the last two motifs in the RyR family are shown in Figure 1(A). Mutants G2370A, G2372A, G2373A, G2375A, Y3937A, S3938A, G3939A and K3940A were constructed and expressed in HEK-293 cells.

Western blots of whole-cell lysates with monoclonal antibody 34C are presented in Figure 1(B). No expression of RyR1 was observed in non-transfected or vector-transfected HEK-293 cells, but wild-type RyR and all of the mutant RyR1 cDNAs were expressed at readily detectable levels. There was also no significant difference in apparent molecular mass between wild-type and mutant RyR1 proteins.

Figure 1 Selected ATP-binding motifs in the RyR family and expression of wild-type and mutant RyR1 in HEK-293 cells

(A) Alignment of two potential ATP-binding motifs, which are conserved in the RyR family. GenBank accession numbers are given in parentheses. The underlined residues were selected for mutagenesis. (B) Expression of RyR1 and mutants in HEK-293 cells. CHAPS lysates from HEK-293 cells (25 μL containing about 50 μg of protein), harvested 48 h after transfection with pMT2, RyR1 and mutated RyR1 cDNAs were subjected to SDS/PAGE (6% gels), transferred to nitrocellulose filters and probed with monoclonal antibody 34C and secondary horseradish peroxidase-conjugated anti-mouse IgG.

High-affinity equilibrium binding of [^3H]ryanodine to mutants

The equilibrium properties of [^3H]ryanodine binding to the mutant RyR proteins were measured to determine whether the high-affinity ryanodine-binding site was preserved in the presence of 100 μM Ca^{2+} . Scatchard analysis showed a single binding site in all of the chimaeras (Figure 2). K_d values ranging from 3.4 to 6.4 nM for the mutants were similar to the K_d of 3.3 nM for wild-type RyR1 (inset to Figure 2), indicating that the high-affinity

binding site for ryanodine was retained in all of the mutants. B_{max} values ranged from 0.3 to 0.49 pmol/mg of protein in the mutants (Figure 2), confirming that all of the mutants were expressed to about the same level. There was no [^3H]ryanodine binding in samples isolated from pMT2-transfected HEK-293 cells (results not shown).

Ca^{2+} activation of high-affinity [^3H]ryanodine binding to mutants and the effect of 1 mM AMP-PCP

Typical dose-response curves for Ca^{2+} activation of [^3H]ryanodine binding to wild-type RyR1 and to mutant G2370A are shown in Figure 3. At Ca^{2+} concentrations below $p\text{Ca}$ 7.0, there was little binding of [^3H]ryanodine to the recombinant RyR proteins. [^3H]Ryanodine binding was activated by increasing Ca^{2+} concentrations, with maximal binding occurring between $p\text{Ca}$ 5.7 and 4.0. EC_{50} values for wild-type and mutant RyR1, expressed in $p\text{Ca}$ units, were similar (Figures 3 and 4). When 1 mM AMP-PCP was present, the Ca^{2+} -activation curve was shifted to the left in wild-type RyR1 and in all of the mutants (Figures 3 and 4). EC_{50} values in the presence of AMP-PCP were increased over those in the absence of AMP-PCP, and the magnitude of the change was similar for wild-type RyR1 and mutant RyR1 (Figure 4 and Table 1). Optimal [^3H]ryanodine binding was observed between $p\text{Ca}$ 5.7 and 4.0 in the presence of 1 mM AMP-PCP. Data obtained from curve fitting show that [^3H]ryanodine binding was increased between 23% and 44% in both wild-type and mutant RyR1 (Table 1). These data indicate that Ca^{2+} activation and the effect of AMP-PCP on Ca^{2+} activation were normal in these mutants. Slopes for Ca^{2+} activation ranged between 0.9 and 1.6 (Table 1). These values are lower than those observed in our previous experiments with recombinant RyR1, in which the slopes ranged from 1.5 to 2.1 [28]. Sample preparation was different in this study, in that we excluded soluble cellular components and much of the detergent CHAPS by sedimentation.

AMP-PCP activation of high-affinity [^3H]ryanodine binding to mutants

Dose-response curves for AMP-PCP activation of [^3H]ryanodine binding to wild-type and mutant RyR1 are presented in Figure 5. At $p\text{Ca}$ 6.0, [^3H]ryanodine binding was increased in the presence of incremental AMP-PCP concentrations for wild-type and mutant RyR1. Saturation was reached at 1–3 mM AMP-PCP. EC_{50} values for wild-type and RyR1 mutants were similar (inset to Figure 5), ranging from 0.2 to 0.31 mM. These data indicate that the mutations do not alter ATP activation of the channel.

Caffeine activation of high-affinity [^3H]ryanodine binding to mutants

Dose-response curves for caffeine activation of [^3H]ryanodine binding are presented in Figure 6. At $p\text{Ca}$ 6.7, [^3H]ryanodine binding was negligible at 0.3 mM caffeine when compared with

Figure 2 Scatchard analysis of [³H]ryanodine binding to wild-type and mutant RyR1 expressed in transfected HEK-293 cells

Sample preparation is described in the Materials and methods section. Aliquots of 10 μ l samples were incubated with various concentrations of [³H]ryanodine (0.031–20 nM) in binding buffer (pCa 4) containing 1 mM ATP at 37 °C for 2 h. Specific [³H]ryanodine binding was determined by filtration, as described in the Materials and methods section. Bound/free [³H]ryanodine (B/F, pmol per mg of protein/nM) was plotted as a function of bound [³H]ryanodine (pmol/mg of protein). Linear fitting yielded K_d and B_{max} values, which are presented in the inset.

Figure 3 Ca^{2+} and Ca^{2+} plus AMP-PCP dependence of [³H]ryanodine binding

(A) Wild-type RyR1; (B) mutant G2370A. Aliquots of 10 μ l protein samples from transfected HEK-293 cells were incubated for 1 h at 37 °C with 5 nM [³H]ryanodine in a buffer containing different Ca^{2+} concentrations, in the absence (■) or presence (●) of 1 mM AMP-PCP, in a total volume of 0.25 ml. The experiments were carried out as described in the Materials and methods section.

Figure 4 Comparison of EC_{50} values for Ca^{2+} dependence of [³H]ryanodine binding, obtained in the absence and presence of 1 mM AMP-PCP

Values for EC_{50} were obtained from three independent experiments by fitting the curves, examples of which are shown in Figure 3, with an equation for logistic dose–response. ^a $P < 0.05$ when compared with the data obtained before the addition of 1 mM AMP-PCP.

binding in the absence of caffeine, but was increased as the concentration increased for both wild-type and the mutants. [³H]Ryanodine binding reached a peak at 30 mM caffeine (Figure 6) and declined at higher concentrations (results not shown), demonstrating the desensitizing effect of high concentrations of

caffeine. EC_{50} values were obtained by fitting curves between 0.3 and 30 mM caffeine, after normalization to the value at 30 mM. The data are summarized in Table 2. Mutants G2370A, G2373A and G2375A, but not G2372A, Y3937A, S3938A, G3939A and K3940A, had higher EC_{50} values, indicating that mutants

Table 1 Effect of 1 mM AMP-PCP on Ca^{2+} dependence of high-affinity [^3H]ryanodine binding

$\Delta p\text{Ca}$ is the difference in EC_{50} values obtained before and after the addition of 1 mM AMP-PCP, as shown in Figure 4, by fitting each single dose–response curve. The increase in [^3H]ryanodine binding in the presence of 1 mM AMP-PCP, compared with that in its absence, was determined using the highest value in the fitted curve at optimal Ca^{2+} concentrations ($p\text{Ca}$ 5.5–4.0). Experiments were done three times independently and data are presented as means $\pm$ S.E.M.

	$\Delta p\text{Ca}$	Slope		[^3H]Ryanodine binding (+ AMP-PCP / – AMP-PCP ratio)
		– AMP-PCP	+ AMP-PCP	
RyR1	0.37 $\pm$ 0.03	0.9 $\pm$ 0.2	1.0 $\pm$ 0.1	1.32 $\pm$ 0.04
G2370A	0.41 $\pm$ 0.09	1.1 $\pm$ 0.2	1.6 $\pm$ 0.4	1.44 $\pm$ 0.09
G2372A	0.40 $\pm$ 0.08	1.3 $\pm$ 0.1	1.0 $\pm$ 0.3	1.39 $\pm$ 0.07
G2373A	0.34 $\pm$ 0.04	1.5 $\pm$ 0.2	1.0 $\pm$ 0.1	1.23 $\pm$ 0.01
G2375A	0.35 $\pm$ 0.04	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.28 $\pm$ 0.08
Y3937A	0.44 $\pm$ 0.01	1.0 $\pm$ 0.2	0.9 $\pm$ 0.1	1.41 $\pm$ 0.10
S3938A	0.37 $\pm$ 0.01	1.5 $\pm$ 0.2	1.1 $\pm$ 0.1	1.38 $\pm$ 0.04
G3939A	0.39 $\pm$ 0.12	1.2 $\pm$ 0.2	1.0 $\pm$ 0.1	1.34 $\pm$ 0.03
K3940A	0.38 $\pm$ 0.03	1.3 $\pm$ 0.3	1.2 $\pm$ 0.1	1.37 $\pm$ 0.02

Figure 5 Sensitivity of [^3H]ryanodine binding to AMP-PCP activation

Aliquots of 10 μl of protein from transfected HEK-293 cells were incubated for 1 h at 37 $^{\circ}\text{C}$ with 5 nM [^3H]ryanodine in a buffer containing different AMP-PCP concentrations at $p\text{Ca}$ 6.0 in a total volume of 0.25 ml. The experiments were carried out as described in the Materials and methods section. EC_{50} and slope values were obtained by sigmoidal fitting of each curve for three independent experiments and are presented as means $\pm$ S.E.M. in the inset. Solid line, RyR1; dashed lines, mutants.

G2370A, G2373A and G2375A had decreased sensitivity to caffeine activation.

Ca^{2+} photometric measurement of caffeine-induced Ca^{2+} release in transfected HEK-293 cells

To confirm the diminished caffeine sensitivity of mutants G2370A, G2373A and G2375A, we used Ca^{2+} photometry to measure the properties of caffeine-induced Ca^{2+} release in HEK-293 cells transfected with wild-type and mutant RyR1 cDNAs [28]. Figures 7(A) and 7(B) show representative fluorimetric recordings for intracellular Ca^{2+} transients in response to incremental applications of caffeine (0.03–30 mM) in HEK-293 cells transfected with RyR1 and mutant G2375A cDNA. Caffeine up to 30 mM did not cause any significant Ca^{2+} release in pMT2-transfected cells (results not shown). Ca^{2+} transient traces in

Figure 7(A) show that cells transfected with RyR1 cDNA responded to low concentrations of caffeine, and similar traces were obtained for mutants Y3937A, S3938A, G3939A and K3940A. Figure 7(B) shows that cells transfected with G2375A cDNA responded only to higher concentrations of caffeine, and similar traces were obtained for mutants G2370A, G2372A and G2373A.

Threshold values for caffeine activation of Ca^{2+} release for wild-type and mutant RyR1 are summarized in Table 2. Threshold values for the four mutants G2370A, G2372A, G2373A and G2375A ranged from 1.0 to 5.2 mM caffeine. Threshold values for RyR1 mutants Y3927A, S3938A, G3939A and K3940A ranged from 0.3 to 0.5 mM caffeine. These results indicate that the sensitivity to caffeine in the mutants between amino acids 2370 and 2375 is lower than in the wild type.

Measurement of EC_{50} values confirmed an alteration of caffeine

Figure 6 Sensitivity to caffeine activation of [³H]ryanodine binding

Aliquots of 10 μ l of protein from transfected HEK-293 cells were incubated for 1 h at 37 °C with 5 nM [³H]ryanodine in a buffer containing caffeine concentrations ranging from 0 to 100 mM at pCa 6.0 in a total volume of 0.25 ml. The experiments were carried out as described in the Materials and methods section. ■, RyR1; ●, G2370A; ▲, G2372A; ▼, G2373A; ◆, G2375A; □, Y3937A; ○, S3938A; △, G3939A; ▽, K3940A. Solid line, RyR1; dotted lines, mutants. EC₅₀ and slope values for each experiment were obtained by sigmoidal fitting and are summarized in Table 2.

sensitivity in the 2370–2375 mutants. Peak amplitude for each caffeine application was normalized to the peak amplitude for maximal Ca²⁺ release induced by 30 mM caffeine in each trace, and the resultant dose–response curves were obtained from three to four independent experiments, like those shown in Figures 7(A) and 7(B). EC₅₀ values for RyR1 and the mutants were calculated by fitting the caffeine dose–response curves with an equation for logistic dose–response. These values are listed in Table 2. The EC₅₀ values for G2370A, G2372A, G2373A and G2375A were significantly higher than the EC₅₀ values for wild-type RyR1 or mutants Y3937A, S3938A, G3939A or K3940A, confirming the decreased sensitivity to caffeine activation of mutants G2370A, G2373A and G2375A. The slope for caffeine activation was about 2 (range of 1.6–2.9) for RyR1 and all mutants (Table 2). The slope obtained for RyR1 and mutants G2370A, G2372A, G2373A and G2375A from this assay differed

Figure 7 Fluorescence measurements of Ca²⁺ release from internal stores into the cytoplasm of HEK-293 cells transfected with RyR1 and mutant cDNA, induced by incremental concentrations of caffeine

Representative Ca²⁺ transients in HEK-293 cells transfected with RyR1 (A) and G2370A (B) cDNAs were induced by incremental concentrations of caffeine. Cells on a coverslip were treated as described previously [28]. Individual peak amplitudes of 340/380 nm ratio (fluorescence ratio at the highest response to caffeine minus the ratio at rest) for wild-type and mutant RyR1 were collected and normalized to the maximal amplitude of the peak response in fluorescence ratio (340/380 nm) induced by 30 mM caffeine. Dose–response curves, expressed as means $\pm$ S.E.M., for wild-type (solid line) and mutants (dashed lines) are shown in (C). EC₅₀ and slope values for each independent experiment are summarized in Table 2. Symbols are as in Figure 6.

from those obtained from the [³H]ryanodine-binding assay, a probable reflection of the different nature of the assays.

Table 2 Comparison between the caffeine sensitivity of [³H]ryanodine binding and Ca²⁺ release in HEK-293 cells expressing wild-type and mutant RyR1 transiently

EC₅₀ values and Hill coefficients were obtained by fitting each single dose–response curve, as shown in Figures 6 and 7(C), with an equation for logistic dose–response and then averaging from three to five separate experiments for the mutants. The data for caffeine-induced Ca²⁺ release for RyR1 have been shown elsewhere [28].

	Caffeine-induced Ca ²⁺ release			Caffeine sensitivity of [³ H]ryanodine binding	
	Threshold value (mM)	EC ₅₀ (mM)	Slope	EC ₅₀ (mM)	Slope
RyR1	0.3–0.5	2.8 $\pm$ 0.2	1.9 $\pm$ 0.1	2.7 $\pm$ 0.4	1.5 $\pm$ 0.1
G2370A	1.7–3.0	9.1 $\pm$ 1.4*	2.4 $\pm$ 0.4	4.4 $\pm$ 0.5*	1.5 $\pm$ 0.2
G2372A	1.0–1.7	4.3 $\pm$ 0.3*	2.9 $\pm$ 1.4	4.2 $\pm$ 1.1	1.5 $\pm$ 0.1
G2373A	3.0–5.2	7.2 $\pm$ 1.0*	2.1 $\pm$ 0.1	5.9 $\pm$ 0.6*	1.2 $\pm$ 0.1
G2375A	1.0–3.0	8.7 $\pm$ 1.6*	2.6 $\pm$ 0.2	5.4 $\pm$ 0.7*	1.2 $\pm$ 0.1
Y3937A	0.3–0.5	3.3 $\pm$ 1.2	2.1 $\pm$ 0.5	3.1 $\pm$ 0.4	1.5 $\pm$ 0.1
S3938A	0.3–0.5	2.9 $\pm$ 0.4	1.6 $\pm$ 0.03	3.2 $\pm$ 0.2	1.4 $\pm$ 0.2
G3939A	0.3–0.5	3.2 $\pm$ 0.5	2.0 $\pm$ 0.3	3.0 $\pm$ 0.1	1.5 $\pm$ 0.1
K3940A	0.3–0.5	3.9 $\pm$ 1.4	1.6 $\pm$ 0.1	3.2 $\pm$ 0.3	1.6 $\pm$ 0.1

* $P \leq 0.05$ when compared with RyR1.

Figure 8 Sensitivity of 4-chloro-*m*-cresol activation of wild-type RyR1 and mutants expressed in HEK-293 cells measured with Ca²⁺ photometry

Ca²⁺ fluorescence measurements were the same as those described for caffeine-induced Ca²⁺ release in Figure 7. Individual peak amplitudes of 340/380 nm ratio (fluorescence ratio at the highest response to 4-chloro-*m*-cresol minus the ratio at rest) for wild-type and mutant RyR1 were collected and normalized to the maximal amplitude of the peak response in fluorescence ratio (340/380 nm) induced by 0.5 mM 4-chloro-*m*-cresol. Dose-response curves, expressed as means ± S.E.M., for wild-type (solid line) and mutants (dotted lines) are shown. EC₅₀ and slope values from three to four experiments for each mutant are summarized in the inset. **P* < 0.05 when compared with the EC₅₀ value for RyR1.

The effect of mutations in amino acids 2370–2375 and 3937–3940 on 4-chloro-*m*-cresol-induced Ca²⁺ release in HEK-293 cells expressing wild-type or mutant proteins was determined by measuring changes in photometrical Ca²⁺ fluorescence. 4-Chloro-*m*-cresol at concentrations of more than 0.5 mM could induce Ca²⁺ release with a slow release rate in untransfected HEK-293 cells (results not shown). The peak amplitude for each 4-chloro-*m*-cresol application was normalized to the peak amplitude for maximal Ca²⁺ release induced by 0.5 mM 4-chloro-*m*-cresol in each trace, and the resultant dose-response curves (Figure 8) were obtained from three to four independent experiments. EC₅₀ values for RyR1 and the mutants are listed in the inset to Figure 8. The EC₅₀ values for G2370A, G2373A and G2375A were significantly higher than that for wild-type RyR1, whereas values for other mutants G2372A, Y3937A, S3938A, G3939A and K3940A were similar to that for the wild-type, indicating the decreased sensitivity to 4-chloro-*m*-cresol activation of mutants G2370A, G2373A and G2375A. The slope for 4-chloro-*m*-cresol activation was similar between RyR1 and all the mutants (inset to Figure 8).

DISCUSSION

ATP acts as a non-hydrolysed ligand for the activation of several receptors and channels located in cell-surface and intracellular membranes. Extracellular ATP activates P2X receptors (ligand-gated cation channels) and a family of seven transmembrane G-protein-coupled receptors (P2Y receptors) [44]. Intracellular ATP also activates ion channels such as ATP-sensitive K⁺ channels [45] and ryanodine receptors [6,7]. In contrast with the knowledge base for ATP-sensitive K⁺ channels [45], the structural and functional basis for ATP modulation of RyR and the physiological importance of ATP modulation are poorly understood.

Millimolar concentrations of adenine nucleotides have been shown to activate Ca²⁺ release from sarcoplasmic reticulum [46–50], increase channel-opening probability in single-channel measurement [39,49,51] and enhance [³H]ryanodine binding to RyR [28,52–54]. CTP, GTP, ITP and UTP had no significant effect on Ca²⁺ release [46,48]. Adenine nucleotides are most likely to bind to an interaction site to activate the channel rather than to participate in phosphorylation to modify channel function. Nevertheless, phosphorylation of RyR1 and RyR2 modulates channel function [5–7].

The existence of binding sites for adenine nucleotides has been confirmed in native RyR1 through photoaffinity labelling of ATP analogues [4,20,55,56]. The binding domains have been localized following proteolysis to several fragments, two of which were as small as 27 and 13 kDa [20,55]. Unfortunately, amino acid sequencing was not carried out, so the more detailed location of the binding sites was not determined.

To carry out structure/function analysis of potential ATP-binding sites in RyR, we used site-directed mutagenesis, expression and functional analysis to test the effect of mutagenesis of specific amino acids on activation of the ryanodine receptor. Since there is no typical nucleotide-binding site in the primary sequence of RyR1, we carried out mutagenesis on two consensus sequences, GXGXXG and Y(G/A/S/T)(V/G)(K/T/Q/S/N), for potential ATP-binding sites. From a number of consensus sequences, we selected conserved amino acid sequences 2370–2375 and 3937–3940 for this study. We detected a 1.3-fold activation of [³H]ryanodine binding by AMP-PCP in the presence of limiting Ca²⁺ and an EC₅₀ value of 0.24 mM for AMP-PCP activation of [³H]ryanodine binding for the wild-type channel (Figure 4, Figure 5 and Table 1), but we were unable to detect any defect in ATP activation of [³H]ryanodine

binding to any of our mutants, demonstrating that these amino acids do not provide sites for ATP binding and ATP activation of the channel. Although our observations were negative, we have demonstrated that the [^3H]ryanodine-binding assay used in this study provides an efficient means for measuring ATP activation of the channel and could be used for the future screening of mutants in ATP-binding motifs.

An unexpected finding in this study was that mutations in amino acids 2370–2375 caused a marked decrease in caffeine and 4-chloro-*m*-cresol sensitivity. This sequence lies in MH regulatory domain 2, consisting of amino acids 2163 and 2458 [25]. Throughout this domain, naturally occurring mutations such as R2162C, R2162H, V2167M, T2205M, G2434R, R2435H, R2458C and R2458H cause MH and/or CCD. These mutant RyRs, when expressed in HEK-293 cells, enhance caffeine sensitivity (decreased caffeine EC_{50} and threshold values), in line with results obtained by clinical caffeine halothane contracture testing *in vitro* [34,35]. The mutant G2434R has been characterized by isolating sarcoplasmic-reticulum vesicles from skeletal-muscle biopsies followed by [^3H]ryanodine binding [33]. This mutant had increased sensitivity to activation by Ca^{2+} , caffeine and 4-chloro-*m*-cresol. These data strongly suggest that MH and CCD mutations in MH region 2 alter the conformation of RyR1, leading to an increased affinity for caffeine and 4-chloro-*m*-cresol.

In the present study, mutants G2370A, G2373A, G2375A and probably G2372A in MH domain 2 were found to decrease caffeine sensitivity and threshold values in both [^3H]ryanodine-binding and caffeine-induced Ca^{2+} -release assays, the opposite to that observed with MH/CCD mutations. These results suggest that amino acids 2370–2375 are involved in caffeine activation, and may form part of the caffeine-binding site. They also imply that the caffeine-binding site is very complex, perhaps with different domains having distinct functions; some inhibitory, others stimulatory. Since caffeine activates the Ca^{2+} -release channel by sensitizing it to Ca^{2+} activation, it is possible that amino acids 2370–2375 participate in association of the caffeine-binding site with the Ca^{2+} -activation site. Mutations G2370A, G2373A and G2375A may impair this link, leading to a diminished response at low caffeine concentrations. It will be difficult, however, to define the caffeine interaction site in RyR, because of the low affinity of binding of caffeine to RyR proteins.

RyR1 carrying MH mutation G2434R had an increased sensitivity to activation, in a [^3H]ryanodine-binding assay, by 4-chloro-*m*-cresol, a skeletal-muscle Ca^{2+} -release channel agonist [33,57]. 4-Chloro-*m*-cresol has been identified recently as a trigger of MH in susceptible swine [58,59] and has been recommended for use in a supplementary test for diagnosis of MH [60,61]. In the present study, mutants G2370A, G2373A and G2375A had decreased 4-chloro-*m*-cresol sensitivity of activation, in parallel with decreased caffeine sensitivity, suggesting that MH domain 2 is an important regulatory domain for both caffeine and 4-chloro-*m*-cresol. Similar to the case of caffeine, interaction site(s) in RyR Ca^{2+} -release channels for 4-chloro-*m*-cresol remain to be defined.

In conclusion, mutations in two predicted ATP-binding sites (amino acids 2370–2375 and 3937–3940) of RyR1 did not alter ATP activation of [^3H]ryanodine binding to the RyR channel, most likely ruling out the possibility of these two regions being ATP-binding sites. However, mutations in amino acids 2370–2375, which are located in MH domain 2, resulted in decreased caffeine and 4-chloro-*m*-cresol sensitivity. Recently several mutations in the corresponding region of RyR2 have been associated with catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy type 2 [62,63]. This, together with the fact that MH

domain 2 is a hot spot for MH and CCD mutations, highlights the importance of this domain in channel function.

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REFERENCES

- Inui, M., Saito, A. and Fleischer, S. (1987) Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *J. Biol. Chem.* **262**, 1740–1747
- Inui, M., Saito, A. and Fleischer, S. (1987) Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. *J. Biol. Chem.* **262**, 15637–15642
- Imagawa, T., Smith, J. S., Coronado, R. and Campbell, K. P. (1987) Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+} -permeable pore of the calcium release channel. *J. Biol. Chem.* **262**, 16636–16643
- Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q. Y. and Meissner, G. (1988) Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature (London)* **331**, 315–319
- Coronado, R., Morrisette, J., Sukhareva, M. and Vaughan, D. M. (1994) Structure and function of ryanodine receptors. *Am. J. Physiol.* **266**, C1485–C1504
- Meissner, G. (1994) Ryanodine receptor/ Ca^{2+} release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.* **56**, 485–508
- Zucchi, R. and Ronca-Testoni, S. (1997) The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. *Pharmacol. Rev.* **49**, 53–98
- Bhat, M. B., Zhao, J., Takeshima, H. and Ma, J. (1997) Functional calcium release channel formed by the carboxyl-terminal portion of ryanodine receptor. *Biophys. J.* **73**, 1329–1336
- Chen, S. R. W., Ebisawa, K., Li, X. and Zhang, L. (1998) Molecular identification of the ryanodine receptor Ca^{2+} sensor. *J. Biol. Chem.* **273**, 14675–14678
- Du, G. G. and MacLennan, D. H. (1998) Functional consequences of mutations of conserved, polar amino acids in transmembrane sequences of the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **273**, 31867–31872
- Du, G. G. and MacLennan, D. H. (1999) Ca^{2+} inactivation sites are located in the COOH-terminal quarter of recombinant rabbit skeletal muscle Ca^{2+} release channels (ryanodine receptors). *J. Biol. Chem.* **274**, 26120–26126
- Nakai, J., Gao, L., Xu, L., Xin, C., Pasek, D. A. and Meissner, G. (1999) Evidence for a role of C-terminus in Ca^{2+} inactivation of skeletal muscle Ca^{2+} release channel (ryanodine receptor). *FEBS Lett.* **459**, 154–158
- Callaway, C., Seryshev, A., Wang, J. P., Slavik, K. J., Needleman, D. H., Cantu, 3rd, C., Wu, Y., Jayaraman, T., Marks, A. R. and Hamilton, S. L. (1994) Localization of the high and low affinity [^3H]ryanodine binding sites on the skeletal muscle Ca^{2+} release channel. *J. Biol. Chem.* **269**, 15876–15884
- Witcher, D. R., McPherson, P. S., Kahl, S. D., Lewis, T., Bentley, P., Mullinix, M. J., Windass, J. D. and Campbell, K. P. (1994) Photoaffinity labeling of the ryanodine receptor/ Ca^{2+} release channel with an azido derivative of ryanodine. *J. Biol. Chem.* **269**, 13076–13079
- Wang, J. P., Needleman, D. H., Seryshev, A. B., Aghdasi, B., Slavik, K. J., Liu, S. Q., Pedersen, S. E. and Hamilton, S. L. (1996) Interaction between ryanodine and neomycin binding sites on Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **271**, 8387–8393
- Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1**, 945–951
- Wierenga, R. K. and Hol, W. G. (1983) Predicted nucleotide-binding properties of p21 protein and its cancer-associated variant. *Nature (London)* **302**, 842–844
- Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature (London)* **339**, 439–445
- Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., Meissner, G. and MacLennan, D. H. (1990) Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **265**, 2244–2256
- Brandt, N. R., Caswell, A. H., Brandt, T., Brew, K. and Mellgren, R. L. (1992) Mapping of the calpain proteolysis products of the junctional foot protein of the skeletal muscle triad junction. *J. Membr. Biol.* **127**, 35–47

- 21 Martin, J., Geromanos, S., Tempst, P. and Hartl, F. U. (1993) Identification of nucleotide-binding regions in the chaperonin proteins GroEL and GroES. *Nature* (London) **366**, 279–282
- 22 Ogawa, Y. (1994) Role of ryanodine receptors. *Crit. Rev. Biochem. Mol. Biol.* **29**, 229–274
- 23 Herrmann-Frank, A., Luttgau, H. C. and Stephenson, D. G. (1999) Caffeine and excitation-contraction coupling in skeletal muscle: a stimulating story. *J. Muscle Res. Cell Motil.* **20**, 223–237
- 24 Franzini-Armstrong, C. and Protasi, F. (1997) Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol. Rev.* **77**, 699–729
- 25 Loke, J. and MacLennan, D. H. (1998) Malignant hyperthermia and central core disease: disorders of Ca^{2+} release channels. *Am. J. Med.* **104**, 470–486
- 26 Meissner, G. and Henderson, J. S. (1987) Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+} , adenine nucleotide, and calmodulin. *J. Biol. Chem.* **262**, 3065–3073
- 27 Zimanyi, I. and Pessah, I. N. (1991) Comparison of [^3H]ryanodine receptors and Ca^{2+} release from rat cardiac and rabbit skeletal muscle sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther.* **256**, 938–946
- 28 Du, G. G., Imredy, J. P. and MacLennan, D. H. (1998) Characterization of recombinant rabbit cardiac and skeletal muscle Ca^{2+} release channel (ryanodine receptors) with a novel [^3H]ryanodine binding assay. *J. Biol. Chem.* **273**, 33259–33266
- 29 Du, G. G., Khanna, V. K. and MacLennan, D. H. (2000) Mutation of divergent region 1 alters caffeine and Ca^{2+} sensitivity of the skeletal muscle Ca^{2+} release channel (ryanodine receptor). *J. Biol. Chem.* **275**, 11778–11783
- 30 Bhat, M. B., Zhao, J., Zang, W., Balke, C. W., Takeshima, H., Wier, W. G. and Ma, J. (1997) Caffeine-induced release of intracellular Ca^{2+} from Chinese hamster ovary cells expressing skeletal muscle ryanodine receptor. Effects on full-length and carboxyl-terminal portion of Ca^{2+} release channels. *J. Gen. Physiol.* **110**, 749–762
- 31 Lynch, P. J., Tong, J., Lehane, M., Mallet, A., Giblin, L., Heffron, J. J., Vaughan, P., Zafra, G., MacLennan, D. H. and McCarthy, T. V. (1999) A mutation in the transmembrane/luminal domain of the ryanodine receptor is associated with abnormal Ca^{2+} release channel function and severe central core disease. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 4164–4169
- 32 Otsu, K., Nishida, K., Kimura, Y., Kuzuya, T., Hori, M., Kamada, T. and Tada, M. (1994) The point mutation Arg615 $\rightarrow$ Cys in the Ca^{2+} release channel of skeletal sarcoplasmic reticulum is responsible for hypersensitivity to caffeine and halothane in malignant hyperthermia. *J. Biol. Chem.* **269**, 9413–9415
- 33 Richter, M., Schleithoff, L., Deufel, T., Lehmann-Horn, F. and Herrmann-Frank, A. (1997) Functional characterization of a distinct ryanodine receptor mutation in human malignant hyperthermia-susceptible muscle. *J. Biol. Chem.* **272**, 5256–5260
- 34 Tong, J., Oyama, H., Demarex, N., Grinstein, S., McCarthy, T. V. and MacLennan, D. H. (1997) Caffeine and halothane sensitivity of intracellular Ca^{2+} release is altered by 15 calcium release channel (ryanodine receptor) mutations associated with malignant hyperthermia and/or central core disease. *J. Biol. Chem.* **272**, 26332–26339
- 35 Tong, J., McCarthy, T. V. and MacLennan, D. H. (1999) Measurement of resting cytosolic Ca^{2+} concentrations and Ca^{2+} store size in HEK-293 cells transfected with malignant hyperthermia or central core disease mutant Ca^{2+} release channels. *J. Biol. Chem.* **274**, 693–702
- 36 Airey, J. A., Beck, C. F., Murakami, K., Tanksley, S. J., Deerinck, T. J., Ellisman, M. H. and Sutko, J. L. (1990) Identification and localization of two triad junctional foot protein isoforms in mature avian fast twitch skeletal muscle. *J. Biol. Chem.* **265**, 14187–14194
- 37 Kunkel, T. A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488–492
- 38 Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745–2752
- 39 Chen, S. R., Vaughan, D. M., Airey, J. A., Coronado, R. and MacLennan, D. H. (1993) Functional expression of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum in COS-1 cells. *Biochemistry* **32**, 3743–3753
- 40 Fabiato, A. (1988) Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol.* **157**, 378–417
- 41 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**, 680–685
- 42 Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- 43 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- 44 MacKenzie, A. B., Surprenant, A. and North, R. A. (1999) Functional and molecular diversity of purinergic ion channel receptors. *Ann. N.Y. Acad. Sci.* **868**, 716–729
- 45 Miki, T., Nagashima, K. and Seino, S. (1999) The structure and function of the ATP-sensitive K^{+} channel in insulin-secreting pancreatic beta-cells. *J. Mol. Endocrinol.* **22**, 113–123
- 46 Morii, H. and Tonomura, Y. (1983) The gating behavior of a channel for Ca^{2+} -induced Ca^{2+} release in fragmented sarcoplasmic reticulum. *J. Biochem. (Tokyo)* **93**, 1271–1285
- 47 Nagasaki, K. and Kasai, M. (1983) Fast release of calcium from sarcoplasmic reticulum vesicles monitored by chlortetracycline fluorescence. *J. Biochem. (Tokyo)* **94**, 1101–1109
- 48 Meissner, G. (1984) Adenine nucleotide stimulation of Ca^{2+} -induced Ca^{2+} release in sarcoplasmic reticulum. *J. Biol. Chem.* **259**, 2365–2374
- 49 Smith, J. S., Coronado, R. and Meissner, G. (1985) Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature* (London) **316**, 446–449
- 50 Meissner, G., Darling, E. and Eveleth, J. (1986) Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+} , Mg^{2+} , and adenine nucleotides. *Biochemistry* **25**, 236–244
- 51 Smith, J. S., Coronado, R. and Meissner, G. (1986) Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+} . *J. Gen. Physiol.* **88**, 573–588
- 52 Pessah, I. N., Stambuk, R. A. and Casida, J. E. (1987) Ca^{2+} -activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+} , caffeine, and adenine nucleotides. *Mol. Pharmacol.* **31**, 232–238
- 53 Michalak, M., Dupraz, P. and Shoshan-Barmatz, V. (1988) Ryanodine binding to sarcoplasmic reticulum membrane; comparison between cardiac and skeletal muscle. *Biochim. Biophys. Acta* **939**, 587–594
- 54 Chu, A., Diaz-Munoz, M., Hawkes, M. J., Brush, K. and Hamilton, S. L. (1990) Ryanodine as a probe for the functional state of the skeletal muscle sarcoplasmic reticulum calcium release channel. *Mol. Pharmacol.* **37**, 735–741
- 55 Zarka, A. and Shoshan-Barmatz, V. (1993) Characterization and photoaffinity labeling of the ATP binding site of the ryanodine receptor from skeletal muscle. *Eur. J. Biochem.* **213**, 147–154
- 56 Hohenegger, M., Herrmann-Frank, A., Richter, M. and Lehmann-Horn, F. (1995) Activation and labelling of the purified skeletal muscle ryanodine receptor by an oxidized ATP analogue. *Biochem. J.* **308**, 119–125
- 57 Zorzato, F., Scutari, E., Tegazzin, V., Clementi, E. and Treves, S. (1993) Chlorocresol: an activator of ryanodine receptor-mediated Ca^{2+} release. *Mol. Pharmacol.* **44**, 1192–1201
- 58 Izzo, P. A., Johnson, B. A., Nagao, K. and Gallagher, W. J. (1999) 4-Chloro-*m*-cresol triggers malignant hyperthermia in susceptible swine at doses greatly exceeding those found in drug preparations. *Anesthesiology* **90**, 1723–1732
- 59 Wappler, F., Scholz, J., Fiege, M., Kolodzie, K., Kudlik, C., Weisshorn, R. and Schulte am Esch, J. (1999) 4-Chloro-*m*-cresol is a trigger of malignant hyperthermia in susceptible swine. *Anesthesiology* **90**, 1733–1740
- 60 Ording, H., Glahn, K., Gardi, T., Fagerlund, T. and Bendixen, D. (1997) 4-Chloro-*m*-cresol test – a possible supplementary test for diagnosis of malignant hyperthermia susceptibility. *Acta Anaesthesiol. Scand.* **41**, 967–972
- 61 Baur, C. P., Bellon, L., Felleiter, P., Fiege, M., Fricker, R., Glahn, K., Heffron, J. J., Herrmann-Frank, A., Jurkat-Rott, K., Klingler, W. et al. (2000) A multicenter study of 4-chloro-*m*-cresol for diagnosing malignant hyperthermia susceptibility. *Anesth. Analg.* **90**, 200–205
- 62 Priori, S. G., Napolitano, C., Tiso, N., Memmi, M., Vignati, G., Bloise, R., Sorrentino, V. and Danieli, G. A. (2001) Mutations in the cardiac ryanodine receptor gene (*hRyR2*) underlie catecholaminergic polymorphic ventricular tachycardia. *Circulation* **103**, 196–200
- 63 Tiso, N., Stephan, D. A., Nava, A., Bagattin, A., Devaney, J. M., Stanchi, F., Lardet, G., Brahmabhatt, B., Brown, K., Baucé, B. et al. (2001) Identification of mutations in the cardiac ryanodine receptor gene in families affected with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2). *Hum. Mol. Genet.* **10**, 189–194