COMMENTARY

Unravelling calcium-release channel gating: clues from a ‘hot’ disease

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Ryanodine receptors (RyRs) are a family of intracellular channels that mediate Ca^{2+} release from the endoplasmic and sarcoplasmic reticulum. More than 50 distinct point mutations in one member of this family, RyR1, cause malignant hyperthermia, a potentially lethal pharmacogenetic disorder of skeletal muscle. These mutations are not randomly distributed throughout the primary structure of RyR1, but are grouped in three discrete clusters. In this

issue of the *Biochemical Journal*, Kobayashi et al. present evidence that interdomain interactions between two of these mutation-enriched regions play a key role in the gating mechanism of RyR1.

Key words: calcium channel gating, malignant hyperthermia, ryanodine receptor.

Controlled release of ionized Ca^{2+} from intracellular stores such as the endoplasmic and sarcoplasmic reticulum (ER/SR) plays a key role in the regulation of a plethora of cellular processes, ranging from muscle contraction to fertilization. Conversely, dysregulated Ca^{2+} signalling can lead to cellular pathology. An example of this is the pharmacogenetic disorder malignant hyperthermia (MH), in which administration of certain triggering volatile anaesthetics or depolarizing muscle relaxants to susceptible individuals results in hyperthermia, skeletal muscle rigidity, tachycardia, arrhythmia and metabolic acidosis. Unless treated with the muscle relaxant sodium dantrolene, an inhibitor of SR Ca^{2+} release, fulminant MH crises can be fatal. The underlying physiological defect in this disorder is aberrant Ca^{2+} mobilization from the SR of skeletal muscle, leading to excessive myofibrillar contraction, increased respiration and the generation of heat. In many cases, MH is linked to one of more than 50 distinct point mutations in the gene encoding the type 1 ryanodine receptor (RyR1), a Ca^{2+} -release channel protein [1]. The opening of RyR1 channels from MH-susceptible individuals is hypersensitive to endogenous and pharmacological activators such as caffeine, halothane, Ca^{2+} and polylysine. Although the clinical incidence of MH episodes is low, epidemiological data indicate that the frequency of MH susceptibility in human populations is approx. 1 % [2]. Other conditions associated with mutations in the *RYR1* gene include central core disease (CCD), a disorder characterized by MH susceptibility along with moderate-to-severe muscle weakness and ‘cores’ in the muscle fibre revealed by histochemical staining, foetal akinesia, multi-minicore disease and exercise-induced rhabdomyolysis [1]. Analogous mutations of the *RYR2* gene expressed in heart can cause arrhythmogenic ventricular cardiomyopathy type 2 and stress-induced polymorphic ventricular tachycardia, two potentially fatal familial cardiomyopathies [3].

In order to understand fully their roles in disease processes, the mechanisms by which RyR channels are gated under normal circumstances need to be elucidated. RyRs are formed by exceptionally large macromolecular complexes, comprising four monomers of approx. 5000 amino acid residues each, in combination with an array of accessory proteins [4]. Each monomer is predicted to consist of a cytoplasmic N-terminal region constituting more than 80 % of the protein, multiple membrane-spanning

segments and a small cytoplasmic C-terminus. Despite the significance of this channel family in physiology and pathology, unravelling of the molecular mechanism of RyR channel gating has been hampered by the enormous size of this protein complex. In particular, X-ray crystallographic and spectroscopic techniques are poorly suited to the analysis of large, transmembrane protein structures. Consequently, investigation of RyR structure–function relationships has necessitated the development of alternative methodologies. Cryoelectron microscopy coupled with computer-assisted image reconstruction has been employed to map the three-dimensional structure of purified RyR complexes, as well as the sites of interaction of certain accessory proteins and of sequence-specific antibodies. This approach has permitted some assignment of the positions of key motifs in the primary structure of RyRs to their three-dimensional architecture, at a resolution of 20–30 Å (1 Å = 0.1 nm). Comparison of reconstituted images of RyR1 in pharmacologically induced ‘open’ and ‘closed’ conformations has revealed that channel activation is accompanied by rotation of the transmembrane segments relative to the cytoplasmic domain, as well as numerous mass translocations in the cytoplasmic region. [5]. Generation of chimaeric and truncated RyR complexes using recombinant DNA technology has also generated insights into the structure–function relationships of these channel proteins. For example, construction of an RyR2 chimaera with green fluorescent protein inserted into its so-called ‘divergent region 1’ generated a functional channel that could release Ca^{2+} in response to caffeine in transfected cells. Purification, cryoelectron microscopy, image analysis and comparison with wild-type complexes indicated that the divergent region 1 is located in the ‘handle’ domain of the three-dimensional structure of RyR2 [6]. However, data obtained by analysing such chimaeric proteins should be interpreted with some caution. It has been demonstrated that modification of the primary structure of RyR1 by insertion of fluorescent protein ‘tags’ can alter their pharmacological properties, presumably via perturbation of three-dimensional architecture [7]. Generation of amino acid substitutions in RyR primary structures using recombinant DNA techniques has provided invaluable information on the mechanism of RyR gating. For example, mutation of a highly conserved glutamate residue at position 3885 in RyR3 produced a channel that has reduced sensitivity to activation by Ca^{2+} [8]. Screening of

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the effect of substituting each amino acid of RyR structures on their electrophysiological properties could provide a powerful approach to understanding channel structure–function relationships. However, the time and financial cost of substituting every amino acid of a 5000-amino-acid primary sequence, then analysing the functional consequences, would be prohibitive. As a result, any conceptual approach that can limit the number of residues to be analysed before any experimentation is of considerable value. Nature has provided many clues as to which amino acid residues are key players in RyR gating. Bioinformatic approaches have identified amino acid residues in RyR structures that are highly conserved throughout evolution and so might play crucial roles in channel function. Comparison of RyR primary structures with those of other channel families has also generated insights into the structure–function relationships of these proteins. Using this approach, it is suggested that certain RyR transmembrane helices might form a three-dimensional structure analogous to that of the *Streptomyces lividans* KcsA potassium channel pore [9]. Mutations in the *RYR1* gene are expressed as susceptibility to MH by virtue of their influence on the function of the channel protein product. The 50 or so *RyR1* mutations appear to be grouped into three clusters in the primary structure of this protein: one near the N-terminus, one central and the other near the C-terminus. Provided that it does not arise from biases in polymorphism detection methods and analyses, the distribution of these mutations implies that these three regions are likely to be important determinants of *RyR1* channel gating.

In this issue of the *Biochemical Journal*, as well as in a number of recently published observations summarized in their excellent recent review [10], Kobayashi et al. [11] exploit the apparent clustering of MH mutations into discrete domains of *RyR1* to explore the roles these regions play in channel gating. They propose that, while in its resting state, the *RyR1* channel is blocked by interdomain interactions between regions corresponding to the N-terminal and central MH mutation clusters. Factors that destabilize these interactions, such as endogenous and pharmacological *RyR* activators or MH mutations, ‘unzip’ the interacting domains, thereby unblocking the channel pore (Figure 1). In order to test this hypothesis further, Kobayashi et al. [11] generated sequence-specific antibodies against the synthetic peptides ‘domain peptide 1’ (DP1, *RyR1* residues 590–609) and ‘domain peptide 4’ (DP4, residues 2442–2477). These antibodies specifically recognized their target sequences and did not interact with peptides corresponding to other regions of *RyR1*. Purified anti-DP1 and anti-DP4 immunoglobulins both enhanced [^{3} H]ryanodine binding to skeletal muscle microsomes in a concentration-dependent manner, whereas non-specific IgG had no effect. Since interaction of the plant alkaloid ryanodine with *RyRs* is favoured by conditions that activate the channel [9], it was suggested that anti-DP1 and anti-DP4 antibodies promote opening of this permeation pathway. Indeed, Kobayashi et al. [11] demonstrate that these immunoglobulins trigger Ca^{2+} release from skeletal muscle microsomes in their own right, as well as enhancing Ca^{2+} -mobilization elicited by polylysine. On the basis of these observations, the authors suggest that anti-DP1 and anti-DP4 antibodies mimic MH mutations by destabilizing interdomain interactions within the *RyR* structure. In order to test this hypothesis, a fluorescent moiety was introduced into the N-terminal MH mutation cluster of *RyR1* via binding of this domain to a labelled DP4 peptide. Neither anti-DP1 nor anti-DP4 antibodies influenced quenching of this fluorescent moiety by a small molecular probe, QSY. However, both antibodies enhanced quenching by a macromolecular form of QSY conjugated to BSA. It was suggested that anti-DP1 and anti-DP4 ‘unzip’ interdomain contacts between the *RyR1* N-terminal and central

Figure 1 Regulation of *RyR1* gating by ‘unzipping’ of interdomain interactions

Mutations in *RyR1* that underlie MH are clustered into three discrete regions located near the N-terminus (‘MH-N’, Cys³⁵–Arg⁶¹⁴), in the centre (‘MH-Cent’, Asp²¹²⁹–Arg²⁴⁵⁸) and near the C-terminus (‘MH-C’, Ile³⁹¹⁶–Ala⁴⁹⁴²) of the protein. Kobayashi et al. [11] hypothesize that interactions between the MH-N and MH-Cent domains are destabilized by endogenous or pharmacological activators of *RyR1*, or by MH mutations, thereby ‘unblocking’ the channel. Anti-DP1 and anti-DP4 antibodies, which bind to epitopes within these interacting domains, mimic the effects of activators or mutations, thereby opening the *RyR1* channel.

MH mutation clusters, making the former more accessible to the macromolecular quenching, whereas the small molecular quencher could access the fluorescent label equally well in both ‘zipped’ and ‘unzipped’ conformations. Although available data strongly support the notion that interdomain interactions between the regions defined by DP1 and DP4 are critical in *RyR1* gating, intramolecular contacts between other regions are also likely to be important. Studies by Zorzato et al. [12], employing a monoclonal antibody that recognizes an epitope located between *RyR1* residues 335–620, also demonstrated that interdomain interactions regulate the gating of the channel. However, a fusion protein containing this epitope interacted with regions containing residues 799–1172 and residues 3010–3225 of *RyR1* in overlay assays [12]. These regions are distinct from that encompassing DP4 and one of them (residues 3010–3225) is a calmodulin-binding domain, indicating that accessory proteins might modulate pivotal interdomain interactions within *RyR1*.

Although the findings presented by Kobayashi et al. [11] represent a significant step forward in resolving the molecular detail of *RyR* channel gating, there is considerable scope for further investigation. For example, most mutations that cause CCD are located in the C-terminal cluster of *RyR1*. There is debate over whether such mutations generate channels that are excessively leaky and deplete Ca^{2+} in SR stores, or do not form fully functional channel complexes due to modification of the transmembrane pore [13,14]. Approaches similar to those employed by Kobayashi et al. [11], examining the influence of ‘CCD domain’-specific peptides and antibodies on *RyR1* channel gating, might help to clarify this issue. Indeed, during preparation of this Commentary, George et al. [15] published

data using FRET (fluorescence resonance energy transfer) techniques to demonstrate that the transmembrane channel-forming domain of RyR2 interacts functionally with a cytoplasmic region corresponding to amino acid residues 3722–4610. The interacting domains defined by DP1 and DP4 are of suitable size to permit resolution of their solution structures by use of NMR imaging. Such approaches could be employed to define the critical interacting residues between these domains, the conformational changes that result from their binding and the influence of incorporating MH mutations on these molecular contacts. Cryoelectron microscopy and image reconstitution could be used to assign the position of epitopes recognized by domain-specific antibodies to the three-dimensional structure of RyR1, as well as to examine the influence of these probes on channel ultrastructure. We anticipate that interplay between the fields of molecular genetics and channel biochemistry, such as that presented by Kobayashi et al. [11], will continue to increase appreciation of the molecular basis of RyR channel gating and its participation in human disease processes.

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