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ORIGINAL PAPER

A two-gate model for the ryanodine receptor with allosteric modulation by caffeine and quercetin

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Abstract We have developed a model of the tetrameric ryanodine receptor—the calcium channel of the sarcoplasmic reticulum. The model accurately describes published experimental data on channel activity at various concentrations of Ca^{2+}, caffeine and quercetin. The proposed mechanisms involve allosteric regulation of Ca^{2+} affinity by both caffeine and quercetin, and the existence of two independent, A- and I-gates controlled by Ca^{2+} binding to an activating and an inhibitory module of the receptor. There are four different configurations of the receptor that affect ligand binding to the activation module, but not to the inhibition module. Consequently, there are four kinetic modes for the A-gate and one mode for the I-gate. At a certain moment, the receptor can be in any of the four possible conformations with equal probability. By fitting the data we are able to derive ligand affinities and Hill coefficients, to describe the observation that quercetin is an activating agent stronger than caffeine, and that caffeine and quercetin activate the channel at very low Ca^{2+} concentration ($\sim 10^{-11}$ M). We predict that the activation regime at saturating caffeine or quercetin should present four distinct regions at increasing Ca^{2+}, corresponding to the four different gating modes. Another interesting prediction is the enlargement of the activity domain toward higher Ca^{2+} concentrations in the presence of caffeine or quercetin.

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Introduction

Calcium signalling is employed in numerous physiological processes and in the cellular response to various external factors (Berridge et al. 1998, 2000; Clapham 1995). The complexity of intracellular calcium dynamics is governed mainly by the interplay between different Ca^{2+} fluxes and liberation of calcium ions from the sarcoplasmic/endoplasmic reticulum (SR/ER) through Ca^{2+} channels. In the skeletal or heart muscle, the excitation-contraction coupling is mediated by release of Ca^{2+} ions from the sarcoplasmic reticulum. This is a key event, which relates membrane depolarisation following an action potential, to the mechanical muscle contraction (Haddock et al. 1999; Berridge et al. 2000; Bootman et al. 2001).

Controlling the activity of the calcium ionic channel of the sarcoplasmic reticulum plays a dominant role in shaping the intracellular Ca^{2+} signal. There are two classes of SR/ER Ca^{2+} channels: the inositol 1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR). The first receptor type predominates in non-excitable cells, whereas the second type is the most abundant in excitable cells; some recent reviews on RyR can be found in Fill and Copello (2002), Hamilton (2005) and Meissner (2004). There are three known isoforms for both types of receptors and the relative distribution of the three different receptor types varies from tissue to tissue. RyR1 is the main RyR isoform in skeletal muscle, whereas RyR2 is the RyR dominant isoform in cardiac muscle. As for RyR3, it has been found to be expressed at relatively low levels in mammals, but its physiological role is still unclear. The three mammalian RyR isoforms share ~70% homology in their amino acid sequence and their three-dimensional structure is nearly identical (Ludtke et al. 2005; Serysheva et al. 1999; Sharma et al. 1998, 2000; Samso et al. 2005).

The ryanodine receptor is a homotetrameric structure with two distinct domains: the transmembrane assembly—the carboxyl-terminal hydrophobic domain forming a central conducting pore, and a large amino-terminal cytoplasmic domain referred to as the "foot structure" of the receptor (Ludtke et al. 2005; Meissner 2004; Serysheva et al. 1999; Sharma et al. 1998, 2000; Samso et al. 2005). There are four symmetrical, transmembrane helices (one per monomer) near the 4-fold axis of the channel that form a central, funnel-like cavity (the Ca^{2+} permeation pathway) in the tetrameric protein. These inner helices have a kinked structure as they bend away from the axis at a highly conserved glycine (G4934) in the hinge position. The ends of the lumenal halves of the four transmembrane helices delimit the lumenal vestibule of the channel, with an apparent opening of ~30 Å in diameter at the entrance. There is a further tapering to a constricted region of ~7 Å in diameter (the selectivity filter), determined by the projection into the channel cavity of four symmetrical, inner short helices (one per monomer) that point to the longitudinal axis of the channel, followed by a central cavity of ~15 Å in diameter (the inner vestibule) that extends up to the hinge region. Beyond the kinking point, the channel cavity enlarges further on toward the cytoplasmic mouth, where it reaches an apparent diameter of ~15 Å. The two inner helices in one receptor subunit have been assigned to as the pore-lining helix and pore helix, respectively. The pore helix is associated with the channel's selectivity filter (situated on the lumenal side of this receptor), whereas the centre of the pore-lining helix forms, together with its counterparts on the other three subunits, the gating hinge, or gating ring, which represents a putative ion gate of the channel. It is likely that this gate opens by translocation of the transmembrane pore-lining helices and opening of the hinge aperture, similarly to other channels (MthK, KcsA) that resemble RyR in the structure of their conducting pathway (Welch et al. 2004; Ludtke et al. 2005). However, it seems that kinking of the inner helix cannot open RyR1 by itself, so there must be some gating ring mechanism that should control the opening of the ion gate by inducing the movement of the pore-lining helices and thereby changing the diameter of the pore opening at the hinge level (Ludtke et al. 2005).

The activity of the ryanodine receptor is modulated by a number of cytosolic ligands, such as Ca^{2+}, ATP, Mg^{2+} and calmodulin (Smith et al. 1986; Meissner and Henderson 1987; Gyorke and Gyorke 1998; Meissner 2004). Experimental studies on these channels have proved that Ca^{2+} has a bimodal effect on the conducting activity of both IP_3R and RyR channels (Mak et al. 1998; Liu et al. 1998; Li and Chen 2001; Lee et al. 2002), and it is generally accepted that this behaviour is due to the existence of two antagonist classes (activating/inhibitory) of Ca^{2+} binding sites on the receptor protein. In the case of the ryanodine receptor, the precise location of these sites is not known, but there are evidences that they are presumably located on the large cytosolic foot region of the RyRs (Meissner 2004). In addition, the detailed mechanisms of pore opening/closing by Ca^{2+} binding to antagonist sites have remained unidentified as well. Recently, it has been proposed in analogy to other different channels—MthK and HCN, that Ca^{2+} bound to the activating site of RyR1 induces the expansion of a cytoplasmic gating ring thought to drive kinking of the inner helix and thereby opening the ion gate (Ludtke et al. 2005).

There are also other agents that can potently stimulate both calcium release and RyR activation by Ca^{2+}. Caffeine, a 1,3,7-trimethylxanthine, is a well known exogenous activator of the RyR (Rousseau and Meissner 1989; Sitsapesan and Williams 1990; Meissner et al. 1997; Liu et al. 1998; Xu and Meissner 1998) that increases the open probability of the channel (P_o) without modifying its conductance or ionic selectivity.

It has been shown that the bioflavonoid quercetin (3,3′,4′,5,7-pentahydroxyflavone) reversibly inhibits the SR Ca^{2+}- ATP-ase and the uptake of Ca^{2+} ions by the sarcoplasmic reticulum, inducing a slow increase of muscle tension (Shoshan et al. 1980, 1981; Fewtrell and Gomperts 1977). In addition, it has been found that quercetin can stimulate calcium release from SR, in the presence of an inorganic phosphate (Kim et al. 1983; Palade et al. 1983). At the channel level, both caffeine (Caf) and quercetin (Que) activate the RyR incorporated in planar bilayers (Lee et al. 2002), with quercetin being a stronger agonist of the receptor than caffeine. In the study of Lee et al., low concentrations of quercetin affected only the ascending phase of the activity curve of the ryanodine receptor, represented by the Ca^{2+} dependence of the open probability of the channel, $P_o([Ca^{2+}])$. The rise phase of this 'bell-shaped' curve corresponds to activating calcium concentrations (up to about 20 μM), whereas the descending phase corresponds to the domain of inhibitory concentrations of Ca^{2+} (higher than about 20 μM). Unlike quercetin, caffeine in low concentration modifies both the rising and descending phases of the $P_o([Ca^{2+}])$ curve, and it has been argued (Lee et al. 2002) that this difference is not determined by variations in the solubility in the lipid bilayer, because caffeine binds to the cytosolic domain of the receptor. It has been observed that ryanodine binding to RyR stabilizes the open state conformation of the channel, but the conductance of the channel is lower than normally (Rousseau et al. 1987; Buck et al. 1992). Instead, the current/voltage relations show that quercetin does not interfere with the ryanodine effect on channel conductivity (Lee et al. 2002). Although the skeletal ryanodine receptor proved to have a weak, but significant voltage dependence (Ma 1995), it appears that during calcium release the membrane of the sarcoplasmic reticulum remains close to its resting, nearly neutral state (~0 mV) (Somlyo et al. 1977). Therefore, there is an ongoing effort in studying the ryanodine receptor with concentrated focus on its regulation by various ligands and to a much lesser extent on its voltage dependent activity.

Despite continuing efforts, the gating mechanisms of the sarcoplasmic reticulum calcium channel in the presence/absence of caffeine or quercetin remain unknown at the moment, and the available experimental data are quite scarce (Meissner et al. 1997; Li and Chen 2001; Lee et al. 2002; Meissner 2004; Hamilton 2005; Zahradnik et al. 2005). Therefore, further theoretical and experimental investigations are needed to clarify the processes of regulating the activity of the ryanodine receptor by calcium, caffeine and quercetin. Among various existent theoretical models (Keizer and Levine 1996; Stern et al. 1999; Wang et al. 2005; Zahradnik et al. 2005) that have approached the activation mechanisms of the RyR, only few models (discussed by Zahradnik et al. 2005) take into consideration the specific structure of the receptor and therefore describe realistically a number of experimental data and observations related to RyR regulation by Ca^{2+}. Understanding the function and regulation of the Ca^{2+} release channel is an important step in elucidating the mechanisms of excitation–contraction coupling, since many intracellular calcium signalling phenomena are relying on the proper function of this channel, and a number of pathological states are associated with mutations in the skeletal or cardiac ryanodine receptors, such as in malignant hyperthermia or the central core disease (Hamilton 2005). Therefore, theoretical models can provide new insights into the molecular mechanisms of Ca^{2+} sensing by RyR and offer important clues regarding the action of important pharmacological agents.

At the time of our investigations, single-channel data that describe the effects of the flavonoid quercetin on RyRs were not available, to our knowledge, in the literature, except the data provided by Lee et al. (2002). Moreover, since they compared the effects of quercetin with the effects of a typical RyR modulator (caffeine) under otherwise similar conditions, the results obtained by fitting their data can provide a unitary framework for describing native RyR regulation by Ca^{2+}, caffeine and quercetin, whereby further complications associated with other frequently used ligands, with mutated channels or with variable environments are avoided; for example, ryanodine is known for its ability to modify channel conductance (Rousseau et al. 1987; Buck et al. 1992), whereas mutations in the RyR amino acid sequence may induce long-range structural changes that affect channel function (Meissner 2004). Therefore, our model analysis relies entirely on the data presented by Lee et al. (2002) and offers a new interpretation for RyR regulation by Ca^{2+}. Moreover, quercetin is known as one of the most abundant dietary flavonoids present in fruits and vegetables and its average human daily intake is estimated to be about 20 mg, with doses in dietary supplements reaching as high as 1,000 mg/day (Haghiac and Walle 2005). Quercetin has been found to have a broad spectrum of potentially beneficial biological, pharmacological, and medicinal properties. The molecular properties underlying these effects are thought to be due to antioxidant properties and effects on enzymes and signal transduction pathways. Some of these effects may be facilitated by quercetin insertion into lipid membranes, which leads to an increase in membrane conductance (Ionescu et al. 2007). Since quercetin seems to be a strong stimulator of calcium release in muscle cells through direct effects on the Ca^{2+}-ATP-ase and the SR Ca^{2+} channel, the mode of action of this abundant plant flavonoid on RyR or other cell membrane structures should be investigated in more detail.

It is well established that both RyR and IP_3R are tetrameric receptors (Fill 2002; Meissner 2004; Hamilton 2005; Mak et al. 1998; Falcke 2004), and their activity is currently considered to be modulated by two classes of functional cytosolic sites for binding calcium ions, of which one is activating (denoted here S_a) and one is inhibitory (S_i). Consequently, RyRs are first activated by Ca^{2+} concentrations in the micromolar range, with a peak in activity at about 10 μM, and then they are turned off by high Ca^{2+} levels. The RyR1 channel is almost entirely inhibited by 1 mM Ca^{2+}, whereas substantially higher Ca^{2+} levels (~10 mM) are required to inhibit the RyR2 and RyR3 channels, but the physiological relevance of such a requirement is not evident since levels of ~10 mM Ca^{2+} are probably never reached in the cytosol (Fill and Copello 2002). The bell-shaped open probability of the IP_3R1 channel in the presence of 10 μM IP_3 has been described by a simplified biphasic formula of the Hill-type (Mak et al. 1998), whereby Ca^{2+} binds to S_a with an activation constant $K_a \sim 210$ nM and Hill coefficient $h_a \sim 1.9$ and binds to S_i with $K_i \sim 54$ μM and Hill coefficient $h_i \sim 3.9$. When studied in the artificial lipid bilayer environment, the same receptor preserves the biphasic shape of the curve $P_o([Ca^{2+}])$ even if it shows a quantitatively different behaviour (Bezprozvanny et al. 1991; Kaftan et al. 1997).

The RyR1 calcium channel presents remarkable similarities with the IP_3R1 channel. For example, ryanodine binding to the RyR1 receptor (which is a measure of channel's activity because ryanodine binds the receptor in the open configuration) depends on the Ca^{2+} level in the same biphasic manner as discussed above, so that it can be fitted as well to a biphasic Hill-type formula, with similar apparent Ca^{2+} activation and inhibition constants $K_a \sim$ 420–920 nM, $h_a \sim 1.4$–1.7, $K_i \sim 49$–5635 μM and $h_i \sim 1.0$–1.8, depending on the KCl level (Meissner et al. 1997). Having in view the similarities between IP_3R1 and RyR1, in this study we use and adapt our previous IP_3R model (Baran 2003, 2005) to the specificity of some data obtained by reconstitution of the skeletal RyR in planar lipid bilayers (Lee et al. 2002). We assume that binding of Ca^{2+} to the activating site of one monomer is independent of calcium binding to the inhibitory site on the same monomer, and vice-versa, but depends on binding to the receptor of the ligands caffeine and quercetin. Our model considers that the activity of the RyR1 calcium channel is determined by the opening of two independent gates, each of them being operated by a certain region of the receptor, termed *module*. One of the two gating modules is activated by Ca^{2+}, and is therefore named activation module, whereas the second module is inhibited by Ca^{2+}, and is referred to as the inhibition module. The experimental data analysed in this paper (provided by the study of Lee et al. 2002) are accurately reproduced if we consider that the activities of these two gates (gate A, activation gate, and gate I, inhibition gate) are independent one of another, and that gating is allosterically modulated by Ca^{2+}, Caf and Que, according to the scheme presented in Fig. 1. From a structural point of view, gate A is most likely the putative ion gate at the hinge region of the pore-lining helix, and Ca^{2+} binding to the activating site would produce a possibly long-range signal, transmitted to the gate via a conformational change, as proposed (Ludtke et al. 2005). At this point, the nature of gate I remains speculative. It is worth mentioning, however, the existence of other tetrameric or even pentameric channels (such as the Na^+ channel, the acetylcholine receptor AChR, the *Shaker* Kv channels or the K^+ channel Kir3.1/Kir3.4) presenting two or three functional distinct gates that may be correlated or not (Armstrong 2006; Claydon et al. 2003; Panyi and Deutsch 2006; Purohit and Grosman 2006). The RyR1 share some similarities to the potassium KcsA channel (Ludtke et al. 2005), as does the Kir3.1/Kir3.4 channel (Claydon et al. 2003). There are evidences that in the tetrameric Kir3.1/Kir3.4 channel one gate is formed by the selectivity filter, whereas the second gate may be formed by a ring of hydrophobic residues near the permeation pore, acting as a barrier to ion permeation. In addition, it has been inferred that there are at least four important residues per monomer, at different sites along the permeation pathway of the RyR, that impose strong kinetic barriers for permeation and thereby can effectively stop the ion translocation through the pore (Welch et al. 2004). It is conceivable that the I-gate assumed in the present RyR1 model could open and close following local changes in such a gating ring formed of residues from all the monomers. Another possibility comes from the same Kir3.1/Kir3.4 channel, where mechanical transduction of ligand binding on the cytoplasmic domain may propagate to the hinge point to induce subtle conformational changes within the selectivity filter and thereby change the ion flux through the channel (Claydon et al. 2003). Similarly, the Kv channels have an activation gate formed by a four-helix bundle at the cytoplasmic end of the pore, a fast inactivation gate formed by the cytosolic N terminus that can enter the channel cavity and block permeation, and a slow inactivation gate that closes after rearrangements in some regions in the outer mouth of the pore and selectivity filter (Panyi and Deutsch 2006). So, our interpretation of RyR1 regulation by two independent gates agrees with several possible mechanisms of channel gating that were proposed previously for other channels. In particular, a likely candidate for a key component in I-gating is a short helix situated relatively close to the pore-lining helix, at the interface between the cytosol and the lipid bilayer and oriented parallel to the membrane surface (helix 3 in the notation of Ludtke et al. 2005). There are indications (Ludtke et al. 2005) that this helix could be a previously identified sequence (amino acids 4300–4363) that makes part of a negative regulatory module for channel opening (amino acids 4274–4535) (Du et al. 2000, 2004). Du et al. (2000) also have presented evidence that part of the Ca^{2+} inhibition site lies in the RyR1 region of amino acids 4187–4381 and that the inhibition module does not involve the activation sites for Ca^{2+} and caffeine. Taken together, all these experimental data provide a good support for our two-gate model for RyR1.

The experimental investigations of the RyR1 activity in planar bilayers support the present idea of a manifest interplay between caffeine and quercetin, with clear effects on the opening kinetics of the channel (Lee et al. 2002). So, we assume that there exist two classes of RyR binding sites

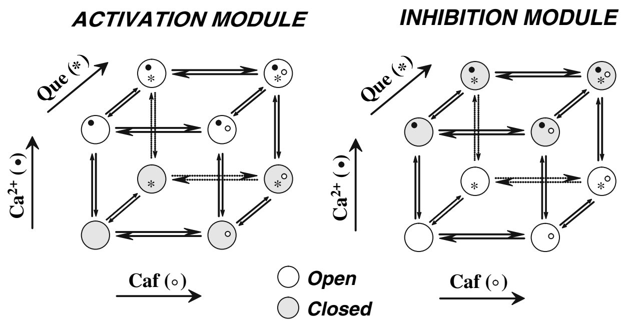

Fig. 1 States and state transitions within the activation and inhibition modules of the ryanodine receptor calcium channel. *White* and *grey circles* represent states of the respective module for which the gate is open or closed, respectively. The ligands bound to the module are represented by *small symbols* inside the circles

for each of the three ligands (Ca^{2+}, Caf and Que), with one class of sites belonging to the activation module (sites S_a, $S_{a,Caf}$, $S_{a,Que}$), and the other class of sites belonging to the inhibition module (sites S_i, $S_{i,Caf}$, $S_{i,Que}$). In order for the channel to be in an open conformation, both gates must be activated, otherwise the channel is closed. Ca^{2+} bound to the activation module opens the channel, whereas Ca^{2+} bound to the inhibition module shuts the channel. We should stress here that our attempts to reproduce in a satisfactory manner the data of Lee et al. (2002) with a one-gate model assuming a single site for caffeine and a single site for quercetin, each regulating allosterically both the activation and inhibition Ca^{2+} sites, have failed, whereas we found that the two-gate model presented here can provide a very good description of the data. Moreover, besides the recognized regulation by caffeine of Ca^{2+} affinity toward its activating sites on RyR, there are some indications that channel inhibition by Ca^{2+} is also regulated by caffeine, without correlation to the activation module (Du et al. 2000).

Methods

The open probability of the channel in the presence of Ca^{2+} and Caf, or Ca^{2+} and Que

Because the reduced number of available data does not allow us to derive all the transition rates of the system described in Fig. 1, in this study we analyse only data obtained in the presence of either Ca^{2+} and Caf, or Ca^{2+} and Que (Lee et al. 2002). If in the cytosolic medium only one of the two exogenous ligands is present, namely Caf or Que, each module will have only four possible states, as represented in Fig. 2.

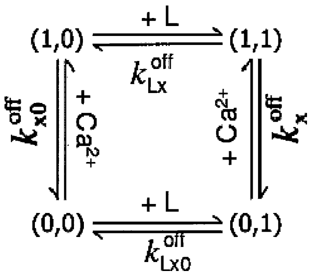

Fig. 2 Representation of states and transitions within activation/inhibition modules of the RyR calcium channel, in the presence of a single modulator ligand (Caf or Que) in the cytosolic medium. The scheme is representative for each of the two modules (with the notations x ≡ a and X ≡ A for the activation module; x ≡ i and X ≡ I for the inactivation module). L represents the exogenous modulator ligand (Caf or Que). The four possible states in the cycle are characterized by different combinations (n_{Ca}, n_L) of Ca^{2+}- and L-site occupancies; n = 1 (or 0) if the site is (or is not) bound

Like in our previous IP_3R model (Baran 2003, 2005), the receptor is considered as a unitary system, and binding of the ligands was considered as being characteristic to the whole tetrameric complex rather than assuming separate monomers. Therefore, our RyR1 model describes how various ligands bind globally to a certain module, not to the corresponding portion of each monomer within that module. So, we consider that each module provides a single equivalent binding site for each of the three ligands in part (Ca^{2+}, Caf, Que). Then the Hill coefficient that defines ligand binding to such a site gives the mean number of molecules that bind at an instant the receptor, at the locus of that binding site.

The open probability P_o of the channel in steady state is calculated by considering first-order kinetics for the reactions of association/dissociation, mass balance equations, and the conditions for thermodynamical equilibrium (Baran 2003, 2005):

$$E_{Lx} \times E_{x0} = E_{Lx0} \times E_x \tag{1}$$

where $E_{(L)x(0)} = k^{off}_{(L)x(0)}/k^{on}_{(L)x(0)}$, with x = a or i, and L = Caf or Que, is the equilibrium constant of the transition from the unoccupied to the occupied state of the site $S_{(L)x}$, and

$$k^{on}_x = k^{off}_x \times ([Ca^{2+}]/K_x)^{h_x} \tag{2}$$

$$k^{on}_{x0} = k^{off}_{x0} \times ([Ca^{2+}]/K_{x0})^{h_x} \tag{3}$$

$$k^{on}_{Lx} = k^{off}_{Lx} \times ([L]/K_{Lx})^{h_{Lx}} \tag{4}$$

$$k^{on}_{Lx0} = k^{off}_{Lx0} \times ([L]/K_{Lx0})^{h_{Lx}}. \tag{5}$$

Equations (1)–(5) can be written for all the four-state cycles appearing in the full scheme (Fig. 1); however, we shall use only the cycles presented in Fig. 2. Here and all over the paper, [Y] represents the concentration of Y species, k^{on} the constant of the binding rate, k^{off} the dissociation rate constant, K the dissociation constant, and h the Hill coefficient. As it will be shown later on, a plausible interpretation of our results is that driving of the inactivation gate may involve some domains from all the subunits of the receptor to act in a concerted manner, whereas the activation gate may require participation of other domain(s) from one, two, three or four monomers at a time. In the particular case where a single monomer would be engaged in opening the A-gate, the coefficients h appearing in (2)–(5) would not be Hill coefficients in the narrower sense, but would denote generically the number of ligand molecules that bind the monomer at the activation site. However, we cannot rule out the possibility that the binding site can be composed by several domains requiring cooperative interactions, or, alternatively, multiple binding sites, acting cooperatively, could exist on a single monomer, as it has been suggested for Ca^{2+} ions (Du and MacLennan 1999).

It is easy to show that the four states (Fig. 2), denoted $X_1 = (0,0)$, $X_2 = (0,1)$, $X_3 = (1,1)$, and $X_4 = (1,0)$, of each X-type module (where $X \equiv A$ for the activation module, and $X \equiv I$ for the inactivation module), are occupied in steady state with the probabilities:

$$P_{X1} = 1 - P_{X2} - P_{X3} - P_{X4} \tag{6}$$

$$P_{X2} = (\gamma_4^X - \gamma_2^X)/\delta^X \tag{7}$$

$$P_{X4} = (\gamma_1^X - \gamma_3^X)/\delta^X \tag{8}$$

$$P_{X3} = (P_{X2}\lambda_{23}^X + P_{X4}\lambda_{43}^X)/\lambda_3^X \tag{9}$$

where

$$\gamma_1^X = 1 + \frac{\lambda_{21}^X}{\lambda_1^X} + \frac{\lambda_{23}^X}{\lambda_3^X} \tag{10}$$

$$\gamma_2^X = 1 + \frac{\lambda_{41}^X}{\lambda_1^X} + \frac{\lambda_{43}^X}{\lambda_3^X} \tag{11}$$

$$\gamma_3^X = 1 + \frac{\lambda_{23}^X}{\lambda_3^X}\left(1 - \frac{\lambda_{34}^X}{\lambda_{14}^X}\right) \tag{12}$$

$$\gamma_4^X = 1 + \frac{\lambda_{43}^X}{\lambda_3^X}\left(1 - \frac{\lambda_{34}^X}{\lambda_{14}^X}\right) + \frac{\lambda_4^X}{\lambda_{14}^X} \tag{13}$$

$$\delta^X = \gamma_1^X\gamma_4^X - \gamma_2^X\gamma_3^X \tag{14}$$

under the restriction that $\delta^x \neq 0$. Here

$$\lambda_1^X = \lambda_{12}^X + \lambda_{14}^X \tag{15}$$

$$\lambda_3^X = \lambda_{32}^X + \lambda_{34}^X \tag{16}$$

$$\lambda_4^X = \lambda_{41}^X + \lambda_{42}^X \tag{17}$$

where λ_{ij}^X is the rate of the transition $X_i \to X_j$, $(i, j = 1,\ldots, 4;\ i \neq j)$.

The activation module has an open conformation when it is either in state A_3 or A_4, whereas the inhibition module is in open conformation in either state I_1 or I_2. The probability of the state (q_a, q_{La}, q_i, q_{Li}) in which the sites $S_{(L)x}$ have the occupation degree $q_{(L)x}$ equal to 0 if the site is not occupied or equal to 1 if the site is occupied, is obtained as the product between the open probabilities of the two independent gates:

$$P(q_a,\ q_{La},\ q_i, q_{Li}) = P(q_a, q_{La}) \ \times \ P(q_i, q_{Li}) \tag{18}$$

It follows then that the open probability of the channel in steady state in the presence of Caf or Que is:

$$P_o = P_A \times P_I \tag{19}$$

where

$$P_A = P_{A3} + P_{A4} \tag{20}$$

$$P_I = P_{I1} + P_{I2} \tag{21}$$

Results

Several constraints imposed by the data

By applying the calculation method described above, we have analysed the experimental data of Lee et al. (2002) and come to the conclusion that these data cannot be described satisfactorily if the activation module has unique binding properties, i.e. fixed values of the affinities and Hill coefficients, for the three ligands altogether. This outcome is a natural consequence of the strongly nonuniform character of the $P_o([Ca^{2+}])$ curves in the activation domain, as observed from the data, which indicate the existence of several activation phases (Figs. 3, 4). We then assumed that there may be multiple possible regimes of the activation module, with different binding properties for the three ligands. Then, the probability that gate A is open reads as:

$$P_A = \sum_{j=1}^{N} p_j P_A^{mj} \tag{22}$$

where N is the number of possible regimes (modes) of the activation module, p_j is the probability to found the activation module in the "j" mode, and P_A^{mj} is the open probability of gate A under the regime "j" [P_A^{mj} is calculated according to (20) with a specific set of binding constants]. The fitting results obtained with one, two or three different modes of activation ($N = 1$, 2 or 3) were far from being satisfactory. Instead, when four different activation modes were assumed ($N = 4$), the data could be very well reproduced. It is important to stress that the best fit of the experimental data lying within the activation range of cytosolic calcium concentrations has been obtained under the constraint that each mode contributes in proportion of 25% to gating of the activation module (all the four fractions p_j, with $0 < p_j < 1$, have been taken as freely variable parameters in the fit and the eventually selected values $p_1 = p_2 = p_3 = p_4 = 0.25$ have been obtained by best fit to the data, see below).

Bearing in mind the symmetrical, tetrameric structure of the ryanodine receptor, this result suggests three possible scenarios. First, at a certain moment one single subunit could access the gate sensor, and the selection of that monomer is random, so that the probability for a certain receptor subunit to control the gate would be 1/4. Alternatively, at a moment, a single monomer may expose the activating Ca^{2+} site to the cytosolic medium, and the exposure of various monomers is random. However, in both cases it would mean that the ligand binding properties vary among the four monomers, making these scenarios quite unrealistic. The third possibility could be linked to the observation that purified RyR can exhibit four equally

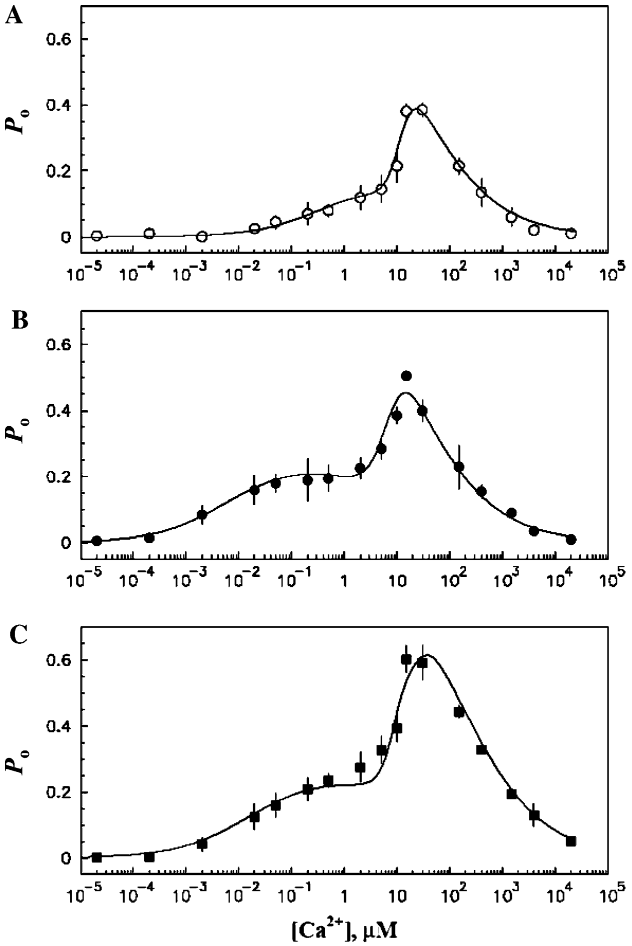

Fig. 3 The open probability of the channel (P_o) in function of the cytosolic calcium concentration, in the absence of the ligands (**a**) or in the presence of 3 mM caffeine (**b**) or 10 μM quercetin (**c**). The experimental data are estimated from the literature (Lee et al. 2002); theoretical curves are obtained by best fit of the present model to the data

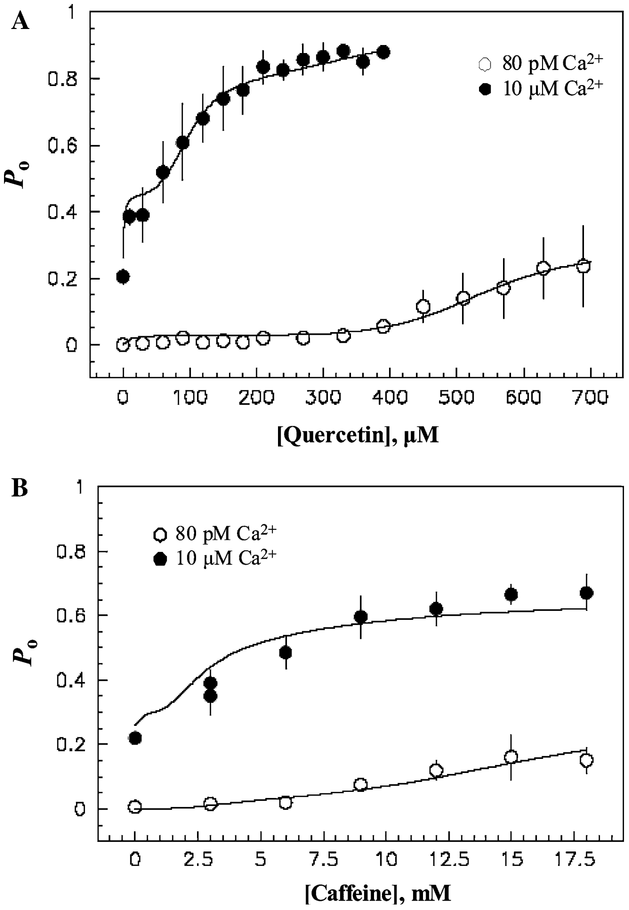

Fig. 4 The open probability of the RyR calcium channel (P_o) in function of the concentration of the ligand quercetin (**a**) or caffeine (**b**), at two different concentrations of cytosolic [Ca^{2+}]: 80 pM (*open circles*), or 10 μM (*solid circles*). The experimental data are estimated from the literature (Lee et al. 2002); theoretical curves are obtained by best fit of the present model to the data

spaced subconductance levels (~0.25, ~0.5, ~0.75 and ~1.00 of the maximal channel conductance) in the absence of the FK506-binding protein (FKBP12) (Brillantes et al. 1994; Ahern et al. 1997), which is known to associate with each monomer of the receptor, thereby stabilizing the channel in the full-conductance state. In another RyR model (Wang et al. 2005), it has been assumed that each receptor subunit is responsible for one-quarter of the maximum current via a conformational change. It is then possible that an intrinsic mechanism exists that regulates the diameter of the pore at the release site, by equally co-opting one, two, three or all the monomers, most likely by movements of their pore-lining helices on the cytosolic side of the channel. A further 2-fold reduction in all the four conductance levels can be induced by ryanodine binding to the receptor, suggesting an additional conformational change that reduces the diameter of the permeation pathway at a different point (Ahern et al. 1997), possibly at the level of the selectivity filter (Welch et al. 2004). Nonetheless, whatever the details of these regulatory mechanisms, our results indicate that the activation module can switch between four distinct, but equally probable modes with different gating kinetics.

In both cases, one can easily find that the open probability of the channel is:

$$P_o = (0.25\ P_A^{m1} + 0.25\ P_A^{m2} + 0.25\ P_A^{m3} + 0.25\ P_A^{m4}) \times P_I \tag{23}$$

where $P_A^{mj} = P_{A3}^{mj} + P_{A4}^{mj}$ represents the open probability of gate A in the mode j ($j = 1,\ldots, 4$), and is calculated according to (8), (9), (20), written for that mode with a characteristic set of binding parameters. Our fitting procedure selected the best fit to seven different data sets that were fitted simultaneously to their corresponding analytical functions, according to (23). Taken together, all the seven sets of experimental data that we used in this study (see below) have restricted all the possibilities to the unique case of random switch between four different configurations.

Unfortunately, the existent data are not sufficient to draw a similar conclusion for the inhibition module; however, no such differences are apparent in the inhibition domain (Figs. 3, 4). Therefore, we assume that the inhibition sites on the tetramer interact cooperatively to drive the I gate and the features of I gating are not altered by changes in the configuration of the receptor, so that there is a unique mode of gating in the inhibition module, associated with a single characteristic set of binding parameter values.

Agreement of the model with the data

We have obtained a very good agreement of this model with the experimental data related to the P_o dependence on the cytosolic calcium concentration, measured in the absence or presence of caffeine or quercetin (Fig. 3). We have also tested our model by stochastic simulations of channel opening with a relative occurrence of every activation mode equal to 0.25 (not shown), at various concentrations of cytosolic calcium, and the agreement with the analytical solution described by (23) was very good.

Other data employed in this study have been obtained (Lee et al. 2002) by an experimental procedure slightly modified from the one used in obtaining the data presented in Fig. 3. More exactly, the open probability P_o has been determined for a fixed concentration of cytosolic calcium, but with different levels of the ligand caffeine/quercetin that were achieved by successive addition of precise quantities of caffeine/quercetin in the cytosolic medium. We suspect that this difference in the experimental method could affect the channel behaviour by altering the affinity of various binding sites of the receptor. However, we have further assumed that the activity of the channel within the lipid bilayer remains independent of the method of measurement, so that we fitted simultaneously all the seven sets of experimental data with the same values of the free parameters of the model. It is worth mentioning that if the different data sets obtained by different experimental procedures (namely the three data sets in Fig. 3 and the four data sets in Fig. 4) are fitted separately, with different parameter values, a much better fit of the model to the data can be obtained (not shown). However, such an approach would introduce arbitrarily a variation in the binding affinity of the ligands toward the receptor. At the moment, the available data are not sufficient to resolve the problem of separate data sets. In both Fig. 4a and b, we have added a supplementary data point from such separate sets of data, taken from the data presented in Fig. 3b and c, respectively, namely the P_o value obtained at 10 μM Ca^{2+} and 10 μM quercetin or 3 mM caffeine.

The parameter values summarized in Table 1 were derived from the best fit, obtained by the least-square method, to all the data sets shown in Figs. 3 and 4. It is to be mentioned that in the fitting procedure, we have assumed that allosteric modulation of channel activity does not alter the Hill coefficients of the ligands, since the reduced number of data does not allow for such a detailed analysis.

Proposed mechanisms for channel regulation

In conclusion, based on our results, we propose that the following mechanisms for regulation of the RyR1 calcium channel activity can well describe a number of data obtained with the channel incorporated in planar lipid bilayers, in the absence or presence of caffeine or quercetin.

Two independent gates, the activation (A) gate and inhibition (I) gate, can be associated with the channel that are controlled by the activation module and the inhibition module, respectively. Each module has one equivalent class of binding sites for Ca^{2+} ions, which are allosterically regulated by caffeine and quercetin binding to their sites in the respective module. There are four different configurations of the receptor that affect ligand binding to the activation module, but not to the inhibition module. At a certain moment, the receptor can be found in any of the four possible conformations with equal probability. The channel is open when the functional activating Ca^{2+} binding site in the current configuration is occupied and the inhibitory Ca^{2+} sites of the receptor are unoccupied.

Within the activation module, binding of Ca^{2+} ions presents negative cooperativity ($h = 0.65$) in one configuration (mode 1), and strong cooperativity in the other three configurations ($h = 3$ or 3.5).

In three of the four configurations, caffeine increases the affinity of activating Ca^{2+} ions by decreasing the calcium dissociation constant from 800 to 2 nM in mode 1, from 13 to 7.9 μM in mode 2, and from 11 μM to 10 pM in mode 3. In mode 4, caffeine lowers the affinity of Ca^{2+} by increasing the dissociation constant of calcium from 13 to 170 μM. Moreover, the affinity of caffeine depends on the occupation degree of the Ca^{2+} binding site. If this site is not bound, the affinity of caffeine toward the activation module in the modes 1, 2 and 4 is moderate, with a dissociation constant of ~ 1 mM, whereas in mode 3 the caffeine binding site has a low affinity, with a dissociation constant of 50 mM that does not satisfy for the condition of thermodynamical equilibrium. If this condition were fulfilled, the site would be virtually unoccupied, meaning that the site is

Table 1 Parameter values obtained by best fit to the data in Figs. 3–4

Ligand	Parameter	Description	Value
Activation module			
Mode 1			
Ca^{2+}	K_{a0}	$S_{a,Caf}$ unoccupied	0.8 μM
	h_{a0}	$S_{a,Que}$ unoccupied	0.65
	K_{aC}	$S_{a,Caf}$ occupied	2 nM
	h_{aC}	$S_{a,Que}$ unoccupied	0.65
	K_{aQ}	$S_{a,Caf}$ unoccupied	2 nM
	h_{aQ}	$S_{a,Que}$ occupied	0.65
Caffeine	K_{1a0}	S_{a} unoccupied	3.971 mM[a]
	h_{1a0}	$S_{a,Que}$ unoccupied	4
	K_{1a}	S_{a} occupied	1.5 mM
	h_{1a}	$S_{a,Que}$ unoccupied	4
Quercetin	K_{2a0}	S_{a} unoccupied	10.5 μM[a]
	h_{2a0}	$S_{a,Caf}$ unoccupied	2
	K_{2a}	S_{a} occupied	1.5 μM
	h_{2a}	$S_{a,Caf}$ unoccupied	2
Mode 2			
Ca^{2+}	K_{a0}	$S_{a,Caf}$ unoccupied	13 μM
	h_{a0}	$S_{a,Que}$ unoccupied	3
	K_{aC}	$S_{a,Caf}$ occupied	7.9 μM
	h_{aC}	$S_{a,Que}$ unoccupied	3
	K_{aQ}	$S_{a,Caf}$ unoccupied	0.5 μM
	h_{aQ}	$S_{a,Que}$ occupied	3
Caffeine	K_{1a0}	S_{a} unoccupied	422 μM[a]
	h_{1a0}	$S_{a,Que}$ unoccupied	2
	K_{1a}	S_{a} occupied	200 μM
	h_{1a}	$S_{a,Que}$ unoccupied	2
Quercetin	K_{2a0}	S_{a} unoccupied	8.8 mM[a]
	h_{2a0}	$S_{a,Caf}$ unoccupied	1
	K_{2a}	S_{a} occupied	0.5 μM
	h_{2a}	$S_{a,Caf}$ unoccupied	1
Mode 3			
Ca^{2+}	K_{a0}	$S_{a,Caf}$ unoccupied	11 μM
	h_{a0}	$S_{a,Que}$ unoccupied	3.5
	K_{aC}	$S_{a,Caf}$ occupied	0.01 nM
	h_{aC}	$S_{a,Que}$ unoccupied	3.5
	K_{aQ}	$S_{a,Caf}$ unoccupied	0.01 nM
	h_{aQ}	$S_{a,Que}$ occupied	3.5
Caffeine	K_{1a0}	S_{a} unoccupied	50 mM[b]
	h_{1a0}	$S_{a,Que}$ unoccupied	2
	K_{1a}	S_{a} occupied	5.5 mM
	h_{1a}	$S_{a,Que}$ unoccupied	2
Quercetin	K_{2a0}	S_{a} unoccupied	1 mM[b]
	h_{2a0}	$S_{a,Caf}$ unoccupied	4
	K_{2a}	S_{a} occupied	300 μM
	h_{2a}	$S_{a,Caf}$ unoccupied	4
Mode 4			
Ca^{2+}	K_{a0}	$S_{a,Caf}$ unoccupied	13 μM
	h_{a0}	$S_{a,Que}$ unoccupied	3
	K_{aC}	$S_{a,Caf}$ occupied	170 μM
	h_{aC}	$S_{a,Que}$ unoccupied	3
	K_{aQ}	$S_{a,Caf}$ unoccupied	5 μM
	h_{aQ}	$S_{a,Que}$ occupied	3
Caffeine	K_{1a0}	S_{a} unoccupied	1.057 mM[a]
	h_{1a0}	$S_{a,Que}$ unoccupied	2
	K_{1a}	S_{a} occupied	50 mM
	h_{1a}	$S_{a,Que}$ unoccupied	2
Quercetin	K_{2a0}	S_{a} unoccupied	10.5 μM[a]
	h_{2a0}	$S_{a,Caf}$ unoccupied	1
	K_{2a}	S_{a} occupied	0.6 μM
	h_{2a}	$S_{a,Caf}$ unoccupied	1
Inhibition module			
Ca^{2+}	K_{i0}	$S_{i,Caf}$ unoccupied	15 μM
	h_{i0}	$S_{i,Que}$ unoccupied	0.55
	K_{ic}	$S_{i,Caf}$ occupied	4 mM
	h_{iC}	$S_{i,Que}$ unoccupied	0.55
	K_{iQ}	$S_{i,Caf}$ unoccupied	10 mM
	h_{iQ}	$S_{i,Que}$ occupied	0.55
Caffeine	K_{1i0}	S_{i} unoccupied	2.155 mM[a]
	h_{1i0}	$S_{i,Que}$ unoccupied	3
	K_{1i}	S_{i} occupied	6 mM
	h_{1i}	$S_{i,Que}$ unoccupied	3
Quercetin	K_{2i0}	S_{i} unoccupied	85.9 μM[a]
	h_{2i0}	$S_{i,Caf}$ unoccupied	4
	K_{2a}	S_{i} occupied	210 μM
	h_{2i}	$S_{i,Caf}$ unoccupied	4

[a] Values calculated from the condition of thermodynamical equilibrium

[b] Values that do not respect the condition of thermodynamical equilibrium, suggesting the need for a cytoplasmic energy source, such as ATP, to allow the access to the ligand binding site

not accessible. According to our investigations, this scenario cannot reproduce the experimental data. A possible explanation is that the caffeine site in mode 3 could be regulated by another cytoplasmic factor, such as ATP, that provides the energy for rendering this site accessible. In modes 1 and 2, the affinity of caffeine increases about two times, whereas in mode 4 the caffeine affinity decreases about 50 times. In all four conformations, binding of caffeine to the site belonging to the activation module shows positive cooperativity, with a Hill coefficient equal to 4 in mode 1, and equal to 2 in the other modes.

Quercetin decreases the affinity of Ca^{2+} ions toward the activation site in all configurations, by decreasing the calcium dissociation constant from 800 to 2 nM in mode 1, from 13 to 0.5 μM in mode 2, from 11 μM to 10 pM in mode 3 and from 13 to 5 μM in mode 4. In addition, quercetin affinity depends on whether Ca^{2+} is bound to its activation site. If the calcium site is unoccupied, the quercetin dissociation constant is 10.5 μM in modes 1 and 4, its affinity is low in mode 2 ($K \sim 9$ mM) and in mode 3 ($K = 1$mM). It is likely that the quercetin-binding site in mode 3 is not exposed to the cytosolic medium, or the access of quercetin to that site is blocked in the absence of ATP. In the same mode, calcium binding to its activation site increases the affinity for quercetin, with a dissociation constant reaching 300 μM. We should note that in modes 1, 2 and 4, binding of Ca^{2+} ions to the activating site determines a very high affinity of quercetin for the activation module, with a dissociation constant ranging between 0.5 and 1.5 μM. Binding of quercetin to the sites of the activation module presents positive cooperativity in two modes, with a Hill coefficient equal to 2 in mode 1, and equal to 4 in mode 3. In modes 2 and 4, the Hill coefficient is 1, indicating that a single molecule of quercetin binds to the activation module of the receptor in these configurations.

In the inactivation module there are no differences in affinity between different configurations of the receptor. Binding of calcium ions shows negative cooperativity ($h = 0.55$), and when the sites for caffeine and quercetin are unoccupied, the Ca^{2+} dissociation constant is 15 μM. Caffeine consistently decreases the affinity of Ca^{2+} ions toward the inactivation module, by increasing the calcium dissociation constant to 4 mM. Caffeine binding shows strong positive cooperativity ($h = 3$) suggesting that three molecules of caffeine bind cooperatively to the inactivation module, presumably one molecule per each of three monomers of the receptor. When bound to the inactivation site, Ca^{2+} decreases about three times the affinity of caffeine for the receptor, increasing its dissociation constant from about 2 to 6 mM.

Quercetin consistently decreases the affinity of Ca^{2+} ions for the inactivation module, by increasing the calcium dissociation constant from 15 μM to 10 mM. Quercetin shows strong positive cooperativity ($h = 4$), suggesting that four molecules of quercetin bind cooperatively to the inactivation module of the receptor, possibly with one molecule per each monomer. Ca^{2+} decreases the affinity of quercetin about 2.5 times, increasing the dissociation constant of quercetin from about 86 to 210 μM.

Discussion

As we have shown above, the gating mechanisms of the RyR1 proposed in this study accurately reproduce a number of experimental data obtained with the receptor reconstituted in lipid bilayers. Our model can be tested experimentally by assessing the open probability of the channel at various concentrations of cytosolic calcium, caffeine and quercetin. In particular, several theoretical predictions of the model, regarding the activity curve $P_o([Ca^{2+}])$ of the channel at different concentrations of caffeine or quercetin are presented in Figs. 5 and 6.

In the experiments of Lee et al. (2002), the channel retained its full conductance indicating that FKBP12 was associated with the receptor protein. Moreover, ryanodine was able to lock the channel into the typical subconductance state (~50%), as expected. Based on our results, we suggest that under their experimental conditions, the cytoplasmic bundle of four inner pore-lining helices of RyR1 undergoes specific changes in its configuration and flickers in four different conformations that seem to affect the overall channel sensitivity to Ca^{2+}, caffeine and quercetin. Consequently, Ca^{2+} binding to the activating site opens the hinge gate with different kinetics in different conformations. However, none of these conformational changes reduces the size of the permeation pathway, which remains stabilized at its maximal value due to FKBP12 association with the receptor. It is likely that loss of FKBP12 relaxes the pore structure at the release site and the four distinct arrangements of the inner helix bundle correspond to the four observed conductance levels. The most appealing and logical explanation would be that, in the absence of FKBP12, the pore-lining helices are able to move themselves or to move some hydrophobic or positively charged residues closer to the longitudinal axis of the channel, in a new position that would obstruct the cation flow along that direction, thereby reducing by 25% the total flux with every displaced helix. Similarly to K^+ channels, it has been suggested that in the open configuration of the RyR, the apposition of four alanine residues (A4939) present in the hinge motif GLIIDA (G4934–A4939)

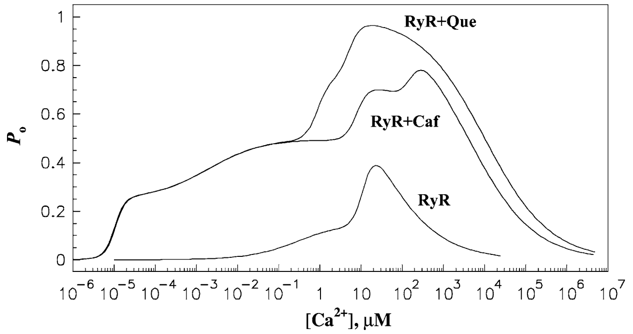

Fig. 5 Model prediction for the open probability of the RyR channel (P_o) in function of the cytosolic $[Ca^{2+}]$, in the absence of the ligands (RyR), in the presence of saturating caffeine (RyR+Caf) or in the presence of quercetin at saturation (RyR+Que)

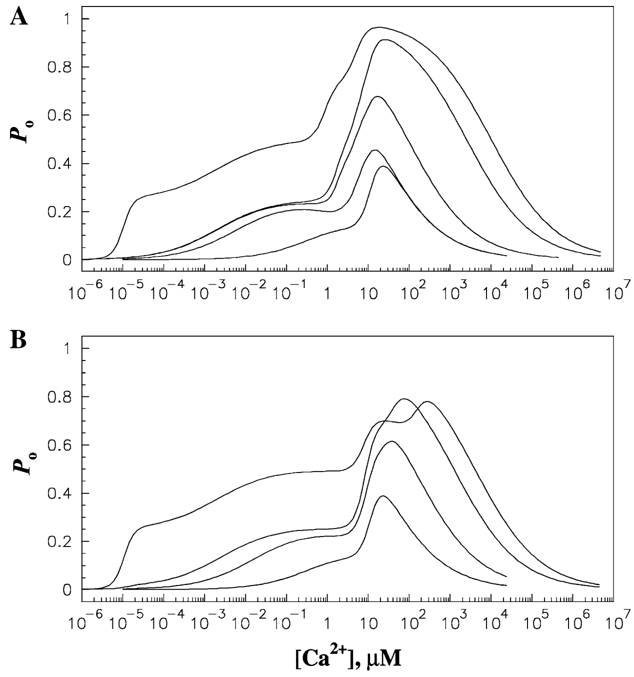

Fig. 6 Model prediction for the open probability of the RyR channel (P_o) in function of the cytosolic [Ca^{2+}], at different concentrations of quercetin or caffeine. The curves correspond, from the lowermost to the uppermost, to 0, 2, 100, 200 and 2,000 μM quercetin, respectively (**a**), and to 0, 6, 50 and 100 mM caffeine, respectively (**b**)

(Ludtke et al. 2005) of the four inner helices forms the narrowest portion of the open cavity (Welch et al. 2004). Consequently, the four different configurations in our model may correspond to four bundle arrangements, in which one, two, three or four helices move coordinately, with or without relocation of the A4939 residues (depending on whether FKBP12 is bound or not to the receptor). According to our model, in the FKBP12-bound receptor, the (local) movements of individual pore-lining helixes are not random, since the four distinct combinations have equal probabilities to produce. Therefore, there must be some sort of selective mechanism, possibly involving cooperative interactions among monomers, for example a sequential chain-type interaction in which one, two, three and four monomers are consecutively engaged. In agreement with our interpretation, it has been previously proposed that FKBP12 is crucial in coordinating the RyR tetramer, so that it promotes the closed conformation and stabilizes the maximum channel conductance (Ahern et al. 1997; Brillantes et al. 1994). More importantly, Brillantes et al. (1994) found an even distribution of activity to the three submaximal conductance levels in recombinant FKBP12-deficient RyR channels activated by caffeine, which is consistent with our evenly probable modes of activation. However, as one can see from the values given in Table 1, the different modes of activity predicted by the present model may be not readily distinguishable within the sequence of openings and closings observed in single channel recordings, since there are several evident similarities in ligand sensitivity among the four distinct configurations. However, under favourable circumstances, depending also on various binding rate constants (Baran 2003, 2005) at least some evident differences should be observed. Lee et al. (2002) observed two clearly distinct modes of activity in the presence of both caffeine and quercetin, and there are also several other reports of modal gating in RyRs under different conditions (Ma 1995; Zahradnikova and Zahradnik 1995). In the cardiac ryanodine receptor three gating modes have been observed at ~10 μM Ca^{2+}, with relative occurrence of 0.20 ± 0.06 (I-mode), 0.56 ± 0.09 (L-mode) and 0.22 ± 0.08 (H-mode), respectively (Zahradnikova and Zahradnik 1995). These values would be in excellent agreement with our predictions if the L-mode actually comprised two modes with closely similar kinetics, and our results strongly favours this scenario. Moreover, it has been proven that transitions between I (inactivity), L (low-activity) and H (high-activity) modes are not random, in agreement with our interpretation. Within our model, the I-mode should correspond to the configuration with the lowest affinity for Ca^{2+} toward the activation module, the L-mode to two closely similar modes with intermediate affinity for Ca^{2+}, whereas the H-mode would be associated to the conformation with the highest affinity for Ca^{2+}. Taken together, all these findings lend further support for our model.

By studying ryanodine binding to the cardiac receptor RyR1, it has been proposed previously that Ca^{2+} binds cooperatively to the activation site in the ryanodine-bound form of the receptor, with apparent Hill coefficient ~1.4–1.7 (Meissner et al. 1997). To describe the data of Lee et al. (2002), we find strong cooperativity ($h = 3$ or 3.5) in three configurations of the activation module, and negative cooperativity in one configuration ($h = 0.65$), which is in overall agreement with the respective observations. In the same study, the apparent Ca^{2+} activation constant ranged between 0.42 and 0.92 μM for KCl between 0.1 and 1 mM, whereas our figures describing the current data are 0.8, 11 and 13 μM for different configurations, in agreement with the derived Ca^{2+} concentration for half-maximal activation of 5.3 μM (Lee et al. 2002). The differences in the actual experimental conditions established in different studies may influence the affinities and Hill coefficients of the ligands, since RyR activity is sensitive to ionic strength and composition of the medium (Meissner et al. 1997). Nevertheless, the most important cause for this discrepancy might result from the assessment method, namely determination of ryanodine binding to the receptor versus measurement of single-channel activity in the absence of ryanodine, since there is evidence that ryanodine sensitizes Ca^{2+} activation of the Ca^{2+} release channel and desensitizes Ca^{2+} inactivation through an allosteric interaction (Du et al. 2001). Variations in activation and inhibition constants are therefore to be expected when data obtained by these two particular methods are compared. The difference appears even larger for the values describing the inhibition site. We find $K_{i0} = 15\ \mu M$ and $h_{i0} = 0.55$, while Meissner et al. derived $K = 5.6$ mM and $h = 1.5$ from ryanodine binding data at the same level of KCl, and the Ca^{2+} concentration for observed half-maximal inhibition is 186 μM (Lee et al. 2002, and Fig. 3a here).

In the presence of 0.25 M KCl, 20 mM caffeine stimulates activation of the RyR1 by increasing the apparent affinity of the Ca^{2+} activation site for Ca^{2+} (Meissner et al. 1997). In the similar RyR2 cardiac isoform of the ryanodine receptor, caffeine decreases the affinity of the inactivation site under the same conditions (Liu et al. 1998). The measurements of Lee et al. (2002) also indicate that 3 mM caffeine in 1 M KCl increase the activation site affinity and decrease the affinity of the inactivation site for Ca^{2+}. At the moment, the caffeine sites on the Ca^{2+} release channel protein have not yet been identified, but it has been suggested that the regions responsible for caffeine effect reside in the amino-terminal domain of the RyR (Bhat et al. 1997). Our model is consistent with these findings (Figs. 5, 6, Table 1) and, moreover, predicts that the caffeine stimulating effect on the activation site manifests in three configurations of the activation module, while in the fourth configuration caffeine decreases the affinity of the activating site for Ca^{2+}. Interestingly, in the presence of 20 mM caffeine, the Hill coefficient characterizing Ca^{2+} binding to the inactivation site of the RyR2 was found to be 0.6 (Liu et al. 1998), closely similar to our value here of 0.55. In addition, the apparent Ca^{2+} inhibition constant K_i's is of similar order: 4 mM in this study versus 14.8 mM found by Liu et al., but however, note that there a different KCl level was used.

In agreement with the conclusions of other previous studies (Lee et al. 2002), the current model predicts that quercetin is a stronger activator of the RyR calcium channel than caffeine (Fig. 5), so that the maximal open probability of the channel is about 0.97 at saturating concentrations of quercetin, as compared with about 0.87 in the presence of saturating concentrations of caffeine. In addition, high values of the open probability P_o (>0.5) can be obtained in a calcium domain that is about three times larger in the presence of quercetin than in the presence of caffeine. Moreover, according to our model, caffeine or quercetin in large concentrations determine the activation of the RyR channel even at extremely low concentrations of calcium ($>10^{-11}$ M). Another interesting prediction is that the channel activation phase in the presence of saturating quercetin or caffeine presents four distinct domains when the cytosolic calcium is increased progressively, corresponding to the four different activation modes (Fig. 5). In particular, the four relative maximum levels of P_o with saturating quercetin should be clearly visible as four plateau regions at about 0.25, 0.5, 0.75 and 1. Such kind of experimental determinations would be a feasible test of the hypotheses advanced in this study.

The results presented here suggest that both quercetin and caffeine can consistently decrease the affinity of the RyR1 inactivation site for Ca^{2+} (Fig. 6). We suppose that the enlargement of the activity domain toward larger concentrations of calcium under the action of caffeine or quercetin (Fig. 6) has not been observed in previous studies (Lee et al. 2002) because there were used relatively low concentrations of caffeine and quercetin. An immediate test of our predictions could be done by measuring the open probability of the channel at a fixed concentration of calcium, of the order of 1 mM, for different concentrations of quercetin or caffeine. According to the results presented in Fig. 6, the curve that would be obtained by this procedure should increase with the ligand concentration, for both quercetin and caffeine.

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