Skeletal and cardiac ryanodine receptors bind to the Ca^{2+}-sensor region of dihydropyridine receptor α_{1C} subunit

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Abstract In striated muscles, excitation–contraction coupling is mediated by the functional interplay between dihydropyridine receptor L-type calcium channels (DHPR) and ryanodine receptor calcium-release channel (RyR). Although significantly different molecular mechanisms are involved in skeletal and cardiac muscles, bidirectional cross-talk between the two channels has been described in both tissues. In the present study using surface plasmon resonance spectroscopy, we demonstrate that both RyR_1 and RyR_2 can bind to structural elements of the C-terminal cytoplasmic domain of α_{1C}. The interaction is restricted to the CB and IQ motifs involved in the calmodulin-mediated Ca^{2+}-dependent inactivation of the DHPR, suggesting functional interactions between the two channels. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Calcium channel; Dihydropyridine receptor; Ryanodine receptor; Excitation–contraction coupling

1. Introduction

Excitation–contraction (EC) coupling is initiated by a massive elevation of the cytoplasmic Ca^{2+} concentration. This process is controlled by the functional interplay of two types of Ca^{2+} channels: dihydropyridine receptor L-type calcium channels (DHPR), the high-voltage-activated channels of the transverse tubule membrane, perceive the depolarizing signal and induce the opening of ryanodine receptor calcium-release channel (RyR), the Ca^{2+}-release channels of the sarcoplasmic reticulum membrane. Skeletal and cardiac muscles differ both in the subtypes of channels involved and in their mechanism of coupling.

In skeletal muscles, the α_{1S} subunit of the DHPR behaves as a voltage sensor and conveys directly the information to the RyR_1 subtype, without a requirement for Ca^{2+} influx [1,2]. This directly coupled calcium-release mechanism (DCCR) is assumed to require a physical contact between the two partners. Evidence of this interaction has been provided by coimmunoprecipitation of the α_{1S} containing DHPR and RyR_1 solubilized from skeletal muscles [3]. The II–III cytoplasmic loop of α_{1S} has been identified as a major domain involved in DCCR [4,5].

In cardiac muscles, extracellular Ca^{2+} enters upon the depolarization-induced opening of the α_{1C} subunit of the DHPR and induces the opening of RyR_2 [6]. This calcium-induced calcium release (CICR), also requires a close apposition of the two partners, since Ca^{2+} must reach RyR_2 before diffusion and buffering occur and reduce the Ca^{2+} wave efficacy.

Since α_{1C} is assumed to be mainly engaged in a CICR coupling, the possibility of other interactions of α_{1C} with RyRs has not deserved much attention. However, indirect evidence of a strong and bidirectional coupling between DHPR and RyR in cardiac muscle [7–10] and neurons [11] has been reported. These results suggest that a direct or indirect physical interaction between α_{1C} and RyR_2 may exist and allows RyR_2 to control the activity of the DHPR. On the molecular side, Slavik et al. [12] demonstrated that the CB peptide of the C-terminal region of α_{1S} was able to inhibit binding of [^{3}H]ryanodine to both RyR_1 and RyR_2 and reduced the activity of RyR_1 incorporated into planar lipid bilayers. The CB motif has been recently demonstrated to be part of the locus involved in the Ca^{2+}-dependent inactivation of DHPR [13–15], along with the IQ motif [16,17] present at a 30-residues distance on the C-side. Both motifs bind calmodulin (CaM) during the inactivation process.

Considering the hypothesis that CICR also implicates a cross-talk of both partners by direct protein–protein contact, we characterized the interaction of the C-terminal region of α_{1C} with RyR1 and RyR2. We showed by experiments of surface plasmon resonance spectroscopy (SPR) that both RyR_1 and RyR_2 bind strongly to the proximal part of the tail on the motifs CB and IQ. Our results suggest that this cytoplasmic C-terminal region is involved in the bidirectional coupling of these channels.

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Abbreviations: DHPR, dihydropyridine receptor L-type calcium channel; RyR, ryanodine receptor calcium-release channel; InsP3, inositol triphosphate; EC, excitation–contraction; DCCR, directly coupled calcium release; CICR, calcium-induced calcium release; SPR, surface plasmon resonance spectroscopy; RU, resonance units; VSRM, voltage-sensitive release mechanism

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2. Materials and methods

2.1. Fusion proteins and peptides

The construction and purification of the 6 His-tagged pF2, pF3, pF4 and pF2ΔΔ fusion constructs and the synthesis of N-terminally biotinylated peptides CB (NEELRAIIKKIWKRTSMKLL, residues 1585–1604) and IQ (YATFLIQEYFRKFKKRKEQG, residues 1622–1641) were described [15]. Residues are mapped according to the rat sequence of α_{1C} (GenBank accession number M67515).

2.2. RyR purification

Rabbit RyR_1 was purified on sucrose gradient after solubilization of heavy sarcoplasmic reticulum membranes with CHAPS as described [18]. Canine cardiac ryanodine receptor RyR_2 was obtained by a similar protocol [19]. Purified RyR preparations were stored in liquid nitrogen and their concentrations routinely determined by the Folin method relative to bovine serum albumin.

2.3. SPR

Experiments were performed on a BIAcore 2000 biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden). All experiments were performed at 25°C with a constant flow rate of 10 μl/min. Synthetic peptides were directly coupled to a streptavidin-coated dextran matrix (SA sensor chip, Pharmacia Biosensor). Fusion proteins were labeled with Sulfo-NHS-LC-Biotin (Pierce, Rockford IL, USA) essentially according to the supplier's specifications. The reaction was performed at pH 7.0 with a two-fold molar excess of reagent in order to favor N-terminal labeling. The density of immobilized ligand on the sensor chip surface was 0.2 ng/mm^2 for the peptides, and ranged between 0.4 and 1.2 ng/mm^2 for fusion proteins. Immobilization was monitored using SPR spectroscopy. Purified RyRs, dialyzed overnight at 4°C against a buffer containing 10 mM HEPES, pH 7.4, and 150 mM NaCl were injected as the mobile phase in the same buffer containing 1 mg/ml CM-dextran, 0.005% polysorbate 20 (running buffer) and the appropriate concentration of $CaCl_2$ or EGTA. Non-specific binding was measured in parallel on an uncoated matrix and subtracted. Regeneration was achieved by adding 0.4% CHAPS to the running buffer which released RyRs but not the immobilized peptides or fusion proteins.

2.4. Immobilization on streptavidin biomagnetic beads

All incubations and washes were performed in buffer T at pH 7.4 and pCa 7.0 (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 600 μM $CaCl_2$, 0.005% Tween 20). Streptavidin Dynabeads M-280 (Dynal, France) were washed three times. The fusion proteins (2 mg) were incubated 30 min at 25°C with 1 mg of beads in a volume of 200 μl and washed three times with the same volume of buffer T. RyR (50 pmol) was added to the beads in a volume of 400 μl, the suspension was incubated 1 h at 25°C under gentle agitation and the beads were washed twice with 400 μl of buffer. Bound proteins were eluted with gel loading buffer by a 5-min incubation at 95°C and were subjected to SDS–PAGE on 7.5% gels. Western blots on nitrocellulose membranes were analyzed using the ACter anti-RyR antibody as previously described [20]. Aliquots of the input proteins and of the washes were analyzed in parallel with the eluates.

3. Results

Three non-overlapping segments covering the entire 670-residues C-terminal cytoplasmic tail of α_{1C} were expressed as 6 His-tagged fusion proteins, biotinylated (Fig. 1A). They were immobilized on streptavidin-coated sensor chips, and their interaction with RyRs was assessed by SPR spectroscopy (Fig. 1B,C). In these conditions, both Ca^{2+}-release channels bound to pF2, which covers the most proximal part of 225 residues of the α_{1C} C-terminal tail. The kinetics of binding were comparable for RyR_1 and RyR_2 displaying slow association and dissociation. Variations in dissociation rates were observed depending on the batch of RyRs. For comparative purpose, experiments presented in Fig. 1B,C have been consistently performed with the same preparation of RyRs and

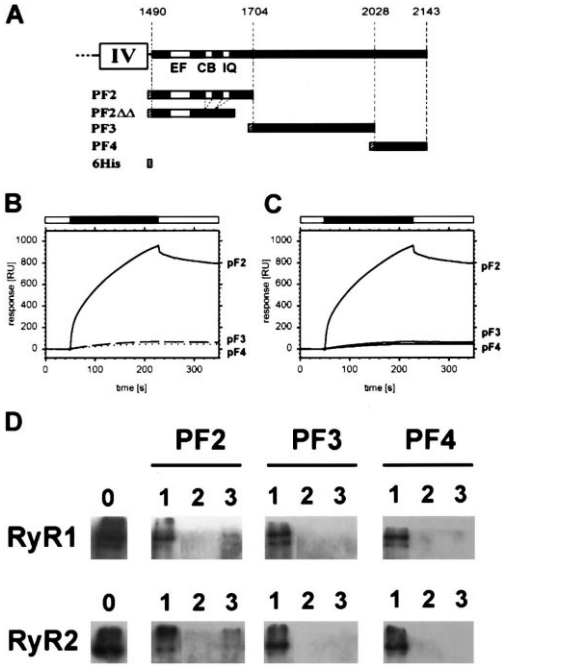

Fig. 1. Interactions of RyRs with fusion proteins from α_{1C}. A: Scheme of the fusion proteins derived from the C-terminal part of rat α_{1C} on the C-side of the fourth transmembrane domain (IV). Positions of the EF-hand, CB and IQ motifs are indicated. Map coordinates refer to GenBank accession number: M67515. B: SPR sensorgrams of the interaction of RyR_1 (50 nM) with immobilized fusion proteins in the presence of 1 mM Ca^{2+}. C: SPR sensorgram of the interaction of RyR_2 (25 nM) in the same conditions. Filled bars above the plots indicate when RyR was applied, empty bars denote running buffer alone. D: Binding of RyR_1 and RyR_2 to streptavidin fusion protein-coated paramagnetic beads. Aliquots were loaded on 7.5% SDS–PAGE gels and analyzed by Western blotting with anti-RyR antibody ACter; lanes 0: input RyR; lanes 1: supernatant of the incubation; lanes 2: second wash; lanes 3: eluate.

on the same coated chips. The same precautions were applied to the experiments presented in the next figures. Neither pF3 nor pF4, the more distal fragments were able to retain the RyRs. As pF2 contains the CB and IQ motifs interacting with CaM during Ca^{2+}-induced inactivation of the DHPR, we also tested the construct pF2ΔΔ, where both motifs were deleted. This deletion construct was unable to bind the RyRs. We concluded that the region containing the CaM-binding motifs was also essential for the interaction with RyR_1 and RyR_2. The interaction was not dependent on Ca^{2+}, since sensorgrams recorded in 1 mM EDTA yielded essentially the same result (data not shown). The binding of RyR_1 and RyR_2 with the fusion proteins was verified by a pull-down assay with streptavidin-coated paramagnetic beads at 100 nM Ca^{2+} concentration, corresponding to the resting state of muscle cells (Fig. 1D). In agreement with the SPR experiments, RyR_1 and RyR_2 were bound to pF2-coated beads, but not to pF3 nor pF4.

The absence of binding to pF2ΔΔ prompted us to test the binding of the RyRs on the individual CB and IQ peptides in SPR. Fig. 2 (left panels) illustrates the sensorgrams obtained with immobilized CB at increasing concentrations of RyR in the mobile phase. Both RyR_1 and RyR_2 displayed a concentration-dependent interaction with CB, but with slightly different binding profiles. Due to both the unusual slow kinetics of

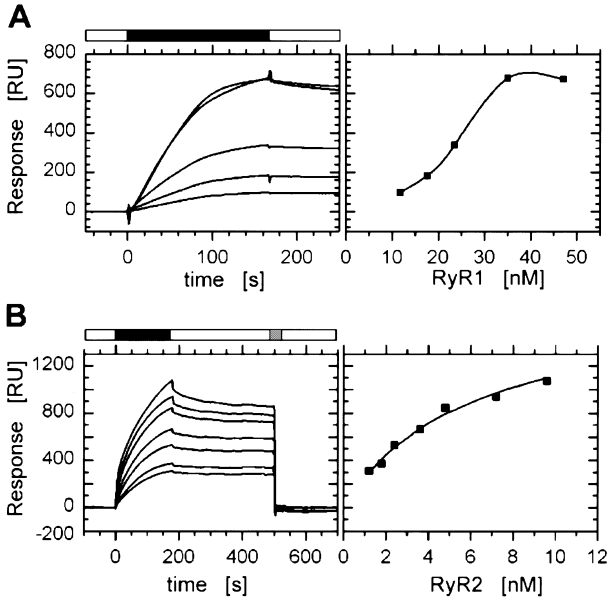

Fig. 2. Interaction of RyR_1 an RyR_2 with immobilized CB peptide in the presence of 1 mM Ca^{2+}. Left panels are sensorgrams of increasing concentrations of RyR run over immobilized CB. Empty bars above the plots: buffer alone; filled bars: RyR in the same buffer; hatched bar: wash with 0.4% CHAPS. Right panels: dependence of the signal on RyR concentration as measured at the end of RyR injection (3 min). A: RyR_1, concentrations are 11.75, 17.5, 23.5, 35 and 47 nM. B: RyR_2, concentrations are 1.2, 1.8, 2.4, 3.6, 4.8, 7.2 and 9.6 nM.

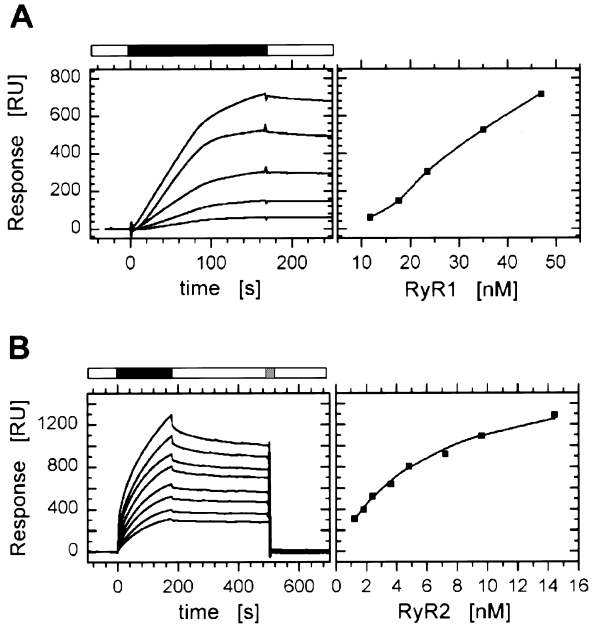

Fig. 3. Interaction of RyR_1 an RyR_2 with immobilized IQ peptide in the presence of 1 mM Ca^{2+}. Left panels: sensorgrams of increasing concentrations of RyRs run over immobilized IQ. Empty bars above the plots: buffer alone; filled bars: RyR in the same buffer; hatched bar: wash with 0.4% CHAPS. Right panels: dependence of the signal on RyR concentration as measured at the end of RyR injection (3 min). A: RyR_1, concentrations are 11.75, 17.5, 23.5, 35 and 47 nM. B: RyR_2, concentrations are 1.2, 1.8, 2.4, 3.6, 4.8, 7.2, 9.6 and 14.4 nM.

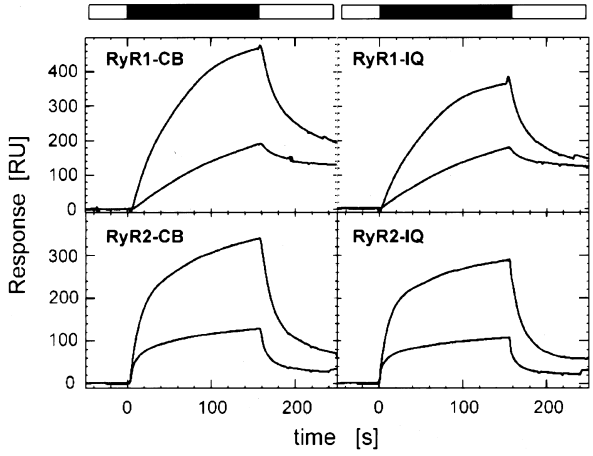

Fig. 4. Interactions of RyR with immobilized peptides in the absence of Ca^{2+}. Sensorgrams of RyRs run over immobilized CB or IQ. Running buffer contained 1 mM EDTA. Concentrations of RyR_1 were 4.0 and 10.0 nM. Concentrations of RyR_2 were 0.5 and 1 nM. Filled bars: injection of RyR.

binding and the large size of the RyRs tetramers ($\sim 2\times 10^3$ kDa), reaching saturation proved unfeasible. In place we relied on measurements in an approach to steady-state in order to compare the affinities of RyR_1 and RyR_2. The resonance unit (RU) value obtained after 3 min of injection of the RyRs was taken as an index of binding and plotted against the concentration of RyR (Fig. 2, right panels). RyR_2 displayed a regular saturation curve suitable for Scatchard analysis and yielded an apparent K_D of 6.0 nM with a pseudo-B_{max} of 1800 RU ($r = 0.94$). The saturation curve obtained with RyR_1 was not regular, precluding a Scatchard analysis. Moreover, it apparently saturated around 40 nM with a value of 700 RU. Since RyR_1 and RyR_2 are of the same size, one would expect to obtain comparable RU values at saturation. As the saturation observed for RyR_1 is 2.5 times lower than that computed for RyR_2 we suspected possible artefacts at the highest concentrations of RyR_1. When using 700 RU as the saturation value of RyR_1, we obtained a EC_{50} of 25 nM, whereas using 1800 RU as the saturation level yielded an EC_{50} of 50 nM. In any case, RyR_1 displayed a significantly lower affinity for CB than RyR_2.

The binding of RyRs to immobilized IQ peptide were similar to the binding to CB (Fig. 3). The binding of RyR_2, as evaluated by approach to steady-state, yielded an apparent K_D of 6.0 nM ($r = 0.98$), almost identical to the affinity for CB. Using the computed B_{max} for RyR_2–IQ binding, the EC_{50} of the RyR_1–IQ interaction was in the range of 70 nM.

Since both CB and IQ motifs of α_{1C} were shown to display Ca^{2+}-dependent interactions with CaM [18], we tested the binding of RyR_1 and RyR_2 to each of the peptides in the absence of Ca^{2+} (1 mM EDTA). As shown in Fig. 4, RyR_1 and RyR_2 also displayed interactions with the peptides in a concentration-dependent manner.

4. Discussion

In the present study, we show by SPR spectroscopy that both RyR_1 and RyR_2 interact with the C-terminal CB and IQ motifs of the DHPR $\alpha 1_C$ subunit, which are the major structural elements mediating its Ca^{2+}-dependent inactivation [13–17]. The inactivation is promoted by complex interactions

with CaM. The interaction of IQ with CaM strictly depends on micromolar Ca^{2+} concentrations [16], whereas CB has been reported to bind CaM at concentrations as low as 50 nM [14]. We recently showed that CB and IQ form a ternary complex with CaM at the Ca^{2+} concentration reached during the opening of the channel [15]. Indirect evidence was provided that the domain covering these two motifs interacts with CaM at low concentrations of Ca^{2+} corresponding to the resting state, suggesting that this domain could also be the constitutive binding site for CaM [14,15,21].

Whereas the role of Ca^{2+} is prominent in the interaction between CaM and the peptides, our results showed that the RyR–peptide interactions do not depend on the cation concentration. Competitions of CaM and RyRs for binding to this DHPR domain were precluded in the present study by the large size of the RyR and the slow reversibility of the interaction. Furthermore, working with the whole RyRs is complicated by the well known presence of intrinsically bound CaM to the channel, and the direct contribution of this cofactor on the RyR needs to be evaluated in order to interpret competition experiments. The use of fragments of RyR expressed as fusion proteins should resolve this issue.

Our results suggest that RyRs can interfere with the Ca^{2+}-dependent inactivation of the DHPR channel in a retrograde fashion by interaction with the motifs CB and IQ. On the other hand, it was shown that CB inhibits both binding of ryanodine and the activity of RyR_1, providing hints of an anterograde inhibitory control [12].

Such bidirectional controls have been described in skeletal muscle DCCR involving the couple α_{1S}/RyR_1. In myotubes of dyspedic mice lacking RyR_1, L-type currents are decreased by a factor of 30 and injection of RyR_1-encoding cDNA restores the normal density of current [7]. This retrograde activating effect involves at least partly the II–III loop of the DHPR [22]. An anterograde inhibitory effect of the DHPR to the RyR has also been described: Repolarization of the membrane reduces the transient Ca^{2+}-elevation promoted by caffeine and this phenomenon is inhibited by dihydropyridine antagonists [8].

In cardiac muscle, the CICR mechanism involving α_{1C}/RyR_2 also displayed bidirectional interplay. Application of Bay K 8644, a DHP agonist, reduced Ca^{2+} release upon stimulation to 50% of the control value, while enhancing the Ca^{2+} entry [9]. It also appears that the opening of RyR_2 is partly due to a voltage-sensitive release mechanism (VSRM) implying a direct cross-talk between α_{1C} and RyR_2 [10].

In neurons, activation of type-1 metabotropic glutamate receptor enhances Ba^{2+} currents through the DHPR [23]. This effect, independent of inositol triphosphate (InsP3), is mimicked by caffeine and inhibited by ryanodine suggesting the implication of RyR [11]. Moreover, the phenomenon is still observed after patch excision, a feature indicative of a robust physical interaction.

In the present study, the observed lower affinity of RyR_1 for CB and IQ as compared to RyR_2 may reflect cross-regulations that differ in DCCR and CICR. Alternatively, since the IQ sequence of α_{1S} differs slightly from the sequence of α_{1C} used in this study (five residues divergence), the lower affinity displayed with this peptide may be due to partial unmatching of the two partners.

It is worth noticing that the emerging picture of the interplay between DHPRs and RyRs, bears striking analogies with the recently described functional interaction between InsP3 receptors and the TRP family of putative store-operated channels, where Ca^{2+}-CaM inhibits TRP opening by binding to its C-terminal region and the InsP3 receptor relieves the inhibition by competing with CaM for the same site of TRP [24–26]. Since neither DHPR and TRP, nor RyR and InsP3, display significant sequence homology, the strong functional similarity between the two systems seems to reflect a convergent evolution promoting a tight concerted control of the influx and release of Ca^{2+} in excitable cells.

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