

# Skeletal and cardiac ryanodine receptors bind to the $\text{Ca}^{2+}$ -sensor region of dihydropyridine receptor $\alpha_{1C}$ 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<sub>1</sub> and RyR<sub>2</sub> can bind to structural elements of the C-terminal cytoplasmic domain of  $\alpha_{1C}$ . The interaction is restricted to the CB and IQ motifs involved in the calmodulin-mediated  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  concentration. This process is controlled by the functional interplay of two types of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ -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  $\alpha_{1S}$  subunit of the DHPR behaves as a voltage sensor and conveys directly the information to the RyR<sub>1</sub> subtype, without a requirement for  $\text{Ca}^{2+}$  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  $\alpha_{1S}$  containing DHPR and RyR<sub>1</sub> solubilized from skeletal muscles [3]. The II–III cytoplasmic loop of  $\alpha_{1S}$  has been identified as a major domain involved in DCCR [4,5].

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

Since  $\alpha_{1C}$  is assumed to be mainly engaged in a CICR coupling, the possibility of other interactions of  $\alpha_{1C}$  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  $\alpha_{1C}$  and RyR<sub>2</sub> may exist and allows RyR<sub>2</sub> 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  $\alpha_{1S}$  was able to inhibit binding of [<sup>3</sup>H]ryanodine to both RyR<sub>1</sub> and RyR<sub>2</sub> and reduced the activity of RyR<sub>1</sub> incorporated into planar lipid bilayers. The CB motif has been recently demonstrated to be part of the locus involved in the  $\text{Ca}^{2+}$ -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  $\alpha_{1C}$  with RyR<sub>1</sub> and RyR<sub>2</sub>. We showed by experiments of surface plasmon resonance spectroscopy (SPR) that both RyR<sub>1</sub> and RyR<sub>2</sub> 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; InsP<sub>3</sub>, 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

## 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 (NEELRAIKKIWKRTSMKLL, residues 1585–1604) and IQ (YATFLIQEYFRKFKKRKEQG, residues 1622–1641) were described [15]. Residues are mapped according to the rat sequence of  $\alpha_{1C}$  (GenBank accession number M67515).

### 2.2. RyR purification

Rabbit RyR<sub>1</sub> was purified on sucrose gradient after solubilization of heavy sarcoplasmic reticulum membranes with CHAPS as described [18]. Canine cardiac ryanodine receptor RyR<sub>2</sub> 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  $\mu$ l/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<sup>2</sup> for the peptides, and ranged between 0.4 and 1.2 ng/mm<sup>2</sup> 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<sub>2</sub> 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  $\mu$ M CaCl<sub>2</sub>, 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  $\mu$ l and washed three times with the same volume of buffer T. RyR (50 pmol) was added to the beads in a volume of 400  $\mu$ l, the suspension was incubated 1 h at 25°C under gentle agitation and the beads were washed twice with 400  $\mu$ l 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  $\alpha_{1C}$  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<sup>2+</sup>-release channels bound to pF2, which covers the most proximal part of 225 residues of the  $\alpha_{1C}$  C-terminal tail. The kinetics of binding were comparable for RyR<sub>1</sub> and RyR<sub>2</sub> 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

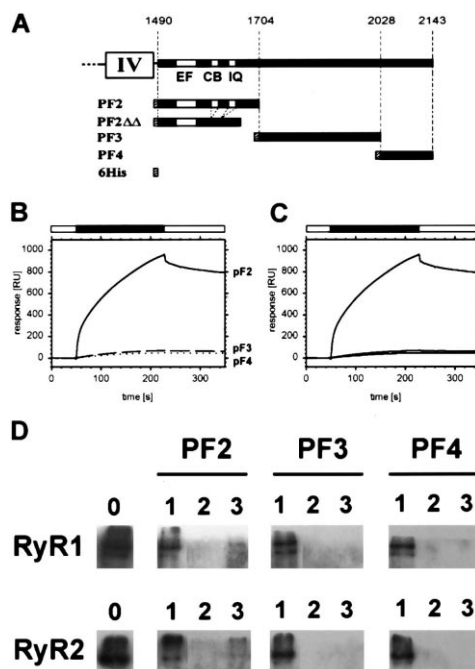


Fig. 1. Interactions of RyRs with fusion proteins from  $\alpha_{1C}$ . A: Scheme of the fusion proteins derived from the C-terminal part of rat  $\alpha_{1C}$  on the C-side of the fourth transmembrane domain (IV). Positions of the EF-hand, CB and IQ motifs are indicated. Map coordinates refer to GenBank accession number: M67515. B: SPR sensorgrams of the interaction of RyR<sub>1</sub> (50 nM) with immobilized fusion proteins in the presence of 1 mM Ca<sup>2+</sup>. C: SPR sensorgram of the interaction of RyR<sub>2</sub> (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<sub>1</sub> and RyR<sub>2</sub> 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<sup>2+</sup>-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<sub>1</sub> and RyR<sub>2</sub>. The interaction was not dependent on Ca<sup>2+</sup>, since sensorgrams recorded in 1 mM EDTA yielded essentially the same result (data not shown). The binding of RyR<sub>1</sub> and RyR<sub>2</sub> with the fusion proteins was verified by a pull-down assay with streptavidin-coated paramagnetic beads at 100 nM Ca<sup>2+</sup> concentration, corresponding to the resting state of muscle cells (Fig. 1D). In agreement with the SPR experiments, RyR<sub>1</sub> and RyR<sub>2</sub> 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<sub>1</sub> and RyR<sub>2</sub> displayed a concentration-dependent interaction with CB, but with slightly different binding profiles. Due to both the unusual slow kinetics of

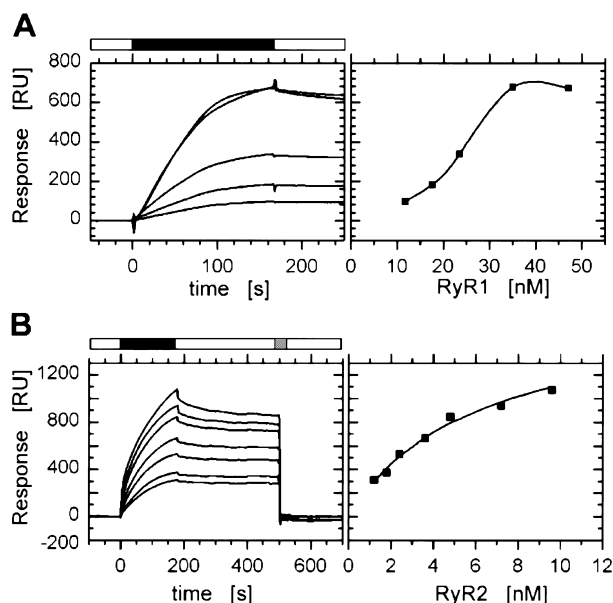


Fig. 2. Interaction of RyR<sub>1</sub> and RyR<sub>2</sub> with immobilized CB peptide in the presence of 1 mM Ca<sup>2+</sup>. Left panels are sensorgrams of increasing concentrations of RyR run over immobilized CB. Empty bars above the plots: buffer alone; filled bars: RyR in the same buffer; hatched 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<sub>1</sub>, concentrations are 11.75, 17.5, 23.5, 35 and 47 nM. B: RyR<sub>2</sub>, concentrations are 1.2, 1.8, 2.4, 3.6, 4.8, 7.2 and 9.6 nM.

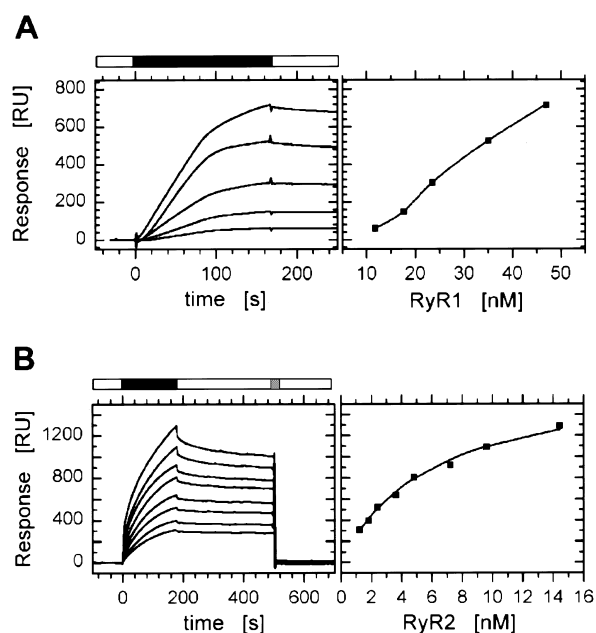


Fig. 3. Interaction of RyR<sub>1</sub> and RyR<sub>2</sub> with immobilized IQ peptide in the presence of 1 mM Ca<sup>2+</sup>. Left panels: sensorgrams of increasing concentrations of RyRs run over immobilized IQ. Empty bars above the plots: buffer alone; filled bars: RyR in the same buffer; hatched 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<sub>1</sub>, concentrations are 11.75, 17.5, 23.5, 35 and 47 nM. B: RyR<sub>2</sub>, concentrations are 1.2, 1.8, 2.4, 3.6, 4.8, 7.2, 9.6 and 14.4 nM.

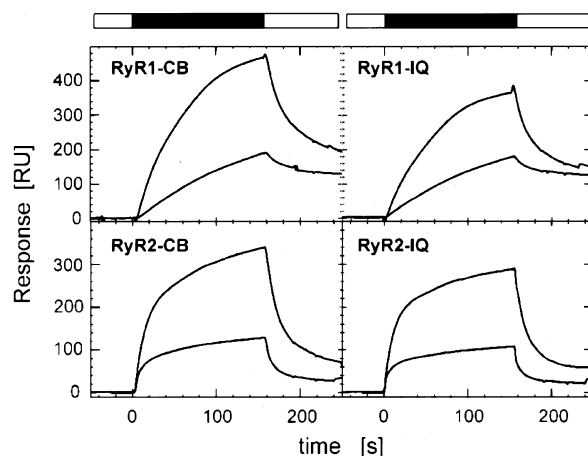


Fig. 4. Interactions of RyR with immobilized peptides in the absence of Ca<sup>2+</sup>. Sensorgrams of RyRs run over immobilized CB or IQ. Running buffer contained 1 mM EDTA. Concentrations of RyR<sub>1</sub> were 4.0 and 10.0 nM. Concentrations of RyR<sub>2</sub> were 0.5 and 1 nM. Filled bars: injection 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<sub>1</sub> and RyR<sub>2</sub>. 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<sub>2</sub> 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<sub>1</sub> was not regular, precluding a Scatchard analysis. Moreover, it apparently saturated around 40 nM with a value of 700 RU. Since RyR<sub>1</sub> and RyR<sub>2</sub> are of the same size, one would expect to obtain comparable RU values at saturation. As the saturation observed for RyR<sub>1</sub> is 2.5 times lower than that computed for RyR<sub>2</sub> we suspected possible artefacts at the highest concentrations of RyR<sub>1</sub>. When using 700 RU as the saturation value of RyR<sub>1</sub>, 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<sub>1</sub> displayed a significantly lower affinity for CB than RyR<sub>2</sub>.

The binding of RyRs to immobilized IQ peptide were similar to the binding to CB (Fig. 3). The binding of RyR<sub>2</sub>, 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<sub>2</sub>–IQ binding, the  $EC_{50}$  of the RyR<sub>1</sub>–IQ interaction was in the range of 70 nM.

Since both CB and IQ motifs of  $\alpha_{1C}$  were shown to display Ca<sup>2+</sup>-dependent interactions with CaM [18], we tested the binding of RyR<sub>1</sub> and RyR<sub>2</sub> to each of the peptides in the absence of Ca<sup>2+</sup> (1 mM EDTA). As shown in Fig. 4, RyR<sub>1</sub> and RyR<sub>2</sub> 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<sub>1</sub> and RyR<sub>2</sub> interact with the C-terminal CB and IQ motifs of the DHPR  $\alpha_{1C}$  subunit, which are the major structural elements mediating its Ca<sup>2+</sup>-dependent inactivation [13–17]. The inactivation is promoted by complex interactions

with CaM. The interaction of IQ with CaM strictly depends on micromolar  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 co-factor 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  $\text{Ca}^{2+}$ -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<sub>1</sub>, providing hints of an anterograde inhibitory control [12].

Such bidirectional controls have been described in skeletal muscle DCCR involving the couple  $\alpha_{1S}$ /RyR<sub>1</sub>. In myotubes of dyspedic mice lacking RyR<sub>1</sub>, L-type currents are decreased by a factor of 30 and injection of RyR<sub>1</sub>-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  $\text{Ca}^{2+}$ -elevation promoted by caffeine and this phenomenon is inhibited by dihydropyridine antagonists [8].

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

In neurons, activation of type-1 metabotropic glutamate receptor enhances  $\text{Ba}^{2+}$  currents through the DHPR [23]. This effect, independent of inositol triphosphate (InsP<sub>3</sub>), 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<sub>1</sub> for CB and IQ as compared to RyR<sub>2</sub> may reflect cross-regulations that differ in DCCR and CICR. Alternatively, since the IQ sequence of  $\alpha_{1S}$  differs slightly from the sequence of  $\alpha_{1C}$  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 inter-

play between DHPRs and RyRs, bears striking analogies with the recently described functional interaction between InsP<sub>3</sub> receptors and the TRP family of putative store-operated channels, where  $\text{Ca}^{2+}$ -CaM inhibits TRP opening by binding to its C-terminal region and the InsP<sub>3</sub> receptor relieves the inhibition by competing with CaM for the same site of TRP [24–26]. Since neither DHPR and TRP, nor RyR and InsP<sub>3</sub>, 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  $\text{Ca}^{2+}$  in excitable cells.

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