Embryonic lethality and abnormal cardiac myocytes in mice lacking ryanodine receptor type 2

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The ryanodine receptor type 2 (RyR-2) functions as a Ca2+-induced Ca2+ release (CICR) channel on intracellular Ca2+ stores and is distributed in most excitable cells with the exception of skeletal muscle cells. RyR-2 is abundantly expressed in cardiac muscle cells and is thought to mediate Ca2+ release triggered by Ca2+ influx through the voltage-gated Ca2+ channel to constitute the cardiac type of excitation–contraction (E–C) coupling. Here we report on mutant mice lacking RyR-2. The mutant mice died at approximately embryonic day (E) 10 with morphological abnormalities in the heart tube. Prior to embryonic death, large vacuolate sarcoplasmic reticulum (SR) and structurally abnormal mitochondria began to develop in the mutant cardiac myocytes, and the vacuolate SR appeared to contain high concentrations of Ca2+. Fluorometric Ca2+ measurements showed that a Ca2+ transient evoked by caffeine, an activator of RyRs, was abolished in the mutant cardiac myocytes. However, both mutant and control hearts showed spontaneous rhythmic contractions at E9.5. Moreover, treatment with ryanodine, which locks RyR channels in their open state, did not exert a major effect on spontaneous Ca2+ transients in control cardiac myocytes at E9.5–11.5. These results suggest no essential contribution of the RyR-2 to E–C coupling in cardiac myocytes during early embryonic stages. Our results from the mutant mice indicate that the major role of RyR-2 is not in E–C coupling as the CICR channel in embryonic cardiac myocytes but it is absolutely required for cellular Ca2+ homeostasis most probably as a major Ca2+ leak channel to maintain the developing SR.

Keywords: Ca2+ store/caffeine/gene targeting/ryanodine/ryanodine receptor

Introduction

Ca2+ signalling is crucial to the regulation of a wide variety of cellular functions, and intracellular Ca2+ stores play an essential role in the regulation of the cytoplasmic Ca2+ concentration. The ryanodine receptor (RyR) constitutes a major class of intracellular Ca2+ release channels in the Ca2+ stores (Berridge, 1993) and mediates Ca2+-induced Ca2+ release (CICR) which is a mechanism of Ca2+ release from the stores, the rate of release being enhanced by increasing cytoplasmic Ca2+ concentrations (Endo, 1977; Fabiato and Fabiato, 1978). RyR was first identified as the CICR channel and has been best characterized in skeletal muscle. The purified RyR protein was shown to form a homotetramer with the characteristic ‘foot’ structure which spans the gap between the membranes of the sarcoplasmic reticulum (SR) and transverse tubule (Fleisher and Inui, 1989; Franzini-Armstrong and Jorgensen, 1994). It was deduced by cloning cDNA that the monomeric RyR is composed of ~5000 amino acid residues with the C-terminal channel region containing transmembrane segments and the remaining large cytoplasmic portion constituting the foot structure (Takeshima et al., 1989). Recent cDNA expression studies identified several functional domains in the primary structure of the RyR molecule. For example, the C-terminal ~20% of the RyR is sufficient to form a functional Ca2+ release channel retaining the Ca2+- and ryanodine-binding sites for channel opening but lacking the low-affinity Ca2+-binding site(s) for channel inactivation (Bhat et al., 1997); also the segment of ~100 amino acid residues flanking residue 1350 in the foot region is one of the critical determinants for excitation–contraction (E–C) coupling in skeletal muscle (Yamazawa et al., 1997).

Previous cDNA cloning studies have defined three subtypes of RyR (RyR-1, RyR-2 and RyR-3) that are coded by distinct genes in vertebrates (Meissner, 1994). Briefly, as clarified using subtype-specific probes, RyR-1 is expressed abundantly in skeletal muscle cells, RyR-2 is present predominantly in cardiac muscle cells and at moderate levels in most excitable cells and RyR-3 is expressed at low levels in a wide variety of cell types including most excitable cells and certain non-excitable cells (e.g. Giannini et al., 1995). The knockout mice lacking RyR-1 die perinatally with abnormalities of skeletal muscle, probably due to respiratory failure. In the RyR-1-deficient muscle the contractile response to electrical stimulation under physiological conditions is totally abolished, demonstrating that RyR-1 mediates Ca2+ release during E–C coupling in skeletal muscle cells (Takeshima et al., 1994). The knockout mice lacking RyR-3 show apparently normal growth and reproduction with no gross abnormalities. Skeletal muscle from the RyR-3-deficient neonates retained the normal E–C coupling mechanism (Takeshima et al., 1996) but showed reduced contractile responses in comparison with control muscle (Bertocchini et al., 1997). An increased locomotion activity was found in RyR-3-deficient mice, suggesting that Ca2+ release via RyR-3 is essential for the function of certain neurons in the central nervous system.
Fig. 1. Mutations introduced into the mouse RyR-2 gene by homologous recombination and Cre-loxP recombination. Restriction enzyme maps of the wild-type allele, targeting vector, targeted mutant allele (crrm1) and Cre-recombined mutant allele (crrm2) are illustrated. The first exon in the gene (E1), GFP, Neo and virus thymidine kinase gene (TK) are indicated by open boxes; the directions of transcription are indicated by arrows. In the targeting vector, the loxP sequence was introduced into the BspEI site of the 5'-untranslated region in exon 1, and the loxP–GFP–Neo cassette was inserted into the ScaI site in intron 1. The genomic DNA probes and PCR primers for detection of the mutations are indicated by hatched boxes and opened arrows, respectively; predicted sizes of DNA fragments in the Southern blot analysis and the PCR are also shown. The nucleotide sequence of the exon 1 and intron 1 in the gene was submitted to the DDBJ/EMBL/GenBank database with the accession No. AB012003.

Results and discussion

Generation of mutant mice lacking RyR-2

A genomic DNA clone carrying the first exon of the mouse RyR-2 gene was isolated, and the DNA insert was used to construct a targeting vector (Figure 1). In the vector, the loxP sequence for the Cre recombinase-mediated DNA recombination (Sauer and Henderson, 1988) was introduced into the 5'-untranslated region, and another loxP sequence, linked to the green fluorescence protein-coding sequence (GFP) and the neomycin resistance gene without the polyadenylation signal (Neo), was inserted into the first intronic region of the gene. J1 embryonic stem (ES) cells were transfected with the targeting vector, and ES clones containing the homologously recombined gene, designated crrm1, were isolated. The ES cells carrying the crrm1 allele were further treated with adenovirus vectors for transient expression of Cre recombinase to establish ES clones carrying the recombinated mutant gene referred to as crrm2. In the crrm2 allele the first protein-coding sequence of 48 base pairs and the partial sequence of the first intron are deleted from the RyR-2 gene. The ES clones harbouring the introduced mutant genes showed the expected pattern of arrangement in genomic DNA by Southern blot hybridization analysis using several restriction enzymes and specific probes for the gene (Figure 2A; Materials and methods).

Chimeric male mice were generated using the ES cells...
we could not generate neonates homozygous for M.Nishi, unpublished observation). On the other hand, reproduction over a 1 year period (H.Takeshima and of the RyR-2-deficient mutant mice with the E10.5 in the homozygous state. The characteristic features RyR-2 gene associating an embryonic lethality at around

However, the embryos homozygous for the mutation of RyR-2-deficient embryos showed no significant

during E8.5–9.5 and is interrupted at E9.5. Significant histological abnormalities in the mutant embryos at E9.5 were found in the heart (Figure 3). In control E9.5 hearts, the myocardium and trabeculae composed of one to three myocyte layers were developing well and the epicardium, the single cell layer surrounding the heart, was organized. In the mutant hearts on the other hand, the myocardium and trabeculae were arranged irregularly and the epicardium was not well-organized. The results indicate that RyR-2 deficiency interferes with the maturation and development of the heart.

The histological abnormalities of the heart may or may not be the cause of lethality of the mutant mice in the early developmental stages, because RyR-2 is expressed in most excitable cells except for skeletal muscle cells in adult mice and may participate in as yet unknown essential functions. However, in the E9.5 mutant embryos, we could not detect clear histological abnormalities in the developing neural tubule, blood vessels or primitive digestive organs, that contain immature neurons or smooth muscle cells. It is therefore likely that RyR-2 deficiency primarily damages embryonic cardiac myocytes.

Ultrastructural abnormalities in cardiac myocytes from mice lacking RyR-2

We examined the ultrastructure of cardiac myocytes from the RyR-2-deficient embryos (Figure 4). The rough endoplasmic reticulum (rER) was partly swollen at E8.5 and many vacuoles were generated at E9.5 and E10.5 in the mutant cardiac myocytes. The vacuoles were developing in size during the embryonic stages; the diameters of the swollen rER or the vacuoles were <0.2 μm at E8.5, 0.2–0.8 μm at E9.5 and 2–5 μm at E10.5. No such swollen rER or vacuoles were detected in control myocytes. These observations, together with the fact that the SR is derived from rER (Flucher, 1992), indicate that the abnormal vacuoles observed in the mutant myocytes correspond to developing SR vesicles. This conclusion is supported by the observation that the rER disappeared in the E9.5 and E10.5 mutant myocytes. Moreover, most mitochondria exhibited tubular cristae and were structurally abnormal in the mutant myocytes; they were smaller than those in controls at E9.5 and exhibited swollen and round structures at E10.5. The abnormalities of the mitochondrial organelles could not be found in other tissues examined in the mutant embryos, including epiderm, mesenchyme and neural tube. The ultrastructural abnormalities in the RyR-2-deficient embryos. At E11.5 the mutant embryos were white in colour and their bodies showed autolysis. Thus, the embryonic development of the RyR-2-deficient mice is inhibited during E8.5–9.5 and is interrupted at E9.5.

Table I. Genotype of pups obtained by crosses between +/crrm2 mice

<table>
<thead>
<tr>
<th>genotype</th>
<th>E8.5</th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
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<tr>
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<td>3</td>
<td>24</td>
<td>18</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
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<td>38</td>
<td>31</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>E10.5</td>
<td>8</td>
<td>32</td>
<td>30</td>
<td>9</td>
<td>3</td>
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<tr>
<td>E11.5</td>
<td>32</td>
<td>32</td>
<td>30</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>E12.5</td>
<td>32</td>
<td>32</td>
<td>30</td>
<td>9</td>
<td>3</td>
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*Embryos exhibiting cardiac arrest and congestive peripheral tissues.
*Embryos exhibiting autolysis.

Histological abnormalities in mice lacking RyR-2

The RyR-2-deficient embryos showed no significant morphological abnormalities at E8.5 but the mutants exhibited slightly delayed development in size and appearance compared with control embryos at E9.5 (Figure 3). In both mutant and control E8.5 embryos, the looped heart tubes were formed but no spontaneous contractions were detected. At E9.5 spontaneous rhythmic contractions of the heart were observed in embryos of both genotypes (see section below). However, the E10.5 mutant embryos exhibited no heart beats and congestive peripheral tissues, and their anatomical features were similar to those of E9.5.
cardiac myocytes might cause the histological derangement of the heart described in the above section.

To examine the localization of Ca\(^{2+}\), cardiac myocytes were treated with fixatives containing oxalate and ferricyanide and analysed under an electron microscope equipped with an X-ray microanalyser (Figure 5). In E9.5 mutant myocytes the abnormal vacuoles contained electron-dense deposits that were identified as precipitates of calcium oxalate by the X-ray microanalysis. The vacuoles therefore contained enough Ca\(^{2+}\) to form precipitates with oxalate. However, in control experiments using wild-type myocytes we did not detect such electron-dense deposits in intracellular organelles (data not shown). The results suggest that Ca\(^{2+}\) overloading results in the generation of abnormal vacuoles from the developing SR in mutant cardiac myocytes.

RyRs can be identified as foot structures by electron microscopic observation clustered in junctional gaps between the cell surface and intracellular vesicular membranes (Franzini-Armstrong and Jorgensen, 1994). However, in cardiac myocytes from E9.5 or E10.5 control embryos we could not observe either clustered foot structures or junctional structures between the SR and cell-surface membranes. The results in the embryonic myocytes indicate not only the low content of RyR-2 but also the absence of co-localization of the L-type Ca\(^{2+}\) channel and the RyR-2. The co-localization of the channel proteins is thought to be required for E–C coupling of mature cardiac muscle cells (Sun et al., 1995).

Surplus cells are removed by apoptotic cell death during embryonic development, and apoptosis is known to accompany morphological changes including cell shrinkage and condensation of nuclei (Jacobson and Raff, 1997). However, mutant cardiac myocytes from the E9.5 or E10.5 embryos did not show such characteristic features of apoptosis (data not shown). It is probable that the mode of cell death in the mutant cardiac myocytes is necrosis.

**Ca\(^{2+}\) signalling in cardiac myocytes from mice lacking RyR-2**

We investigated Ca\(^{2+}\) signalling in embryonic myocytes using Fluo-3 as a Ca\(^{2+}\) indicator. Cardiac myocytes from both mutant and control embryos at E9.5 showed spontaneous Ca\(^{2+}\) oscillations (Figure 6). This is consistent with the presence of rhythmic contractions of hearts in both genotypes. The control myocytes showed Ca\(^{2+}\) transients upon application of caffeine, an activator of RyR subtypes, in a Ca\(^{2+}\)-free bathing solution. In contrast, no response to caffeine was detected in the mutant myocytes. These results indicate that the mutant cardiac myocytes do not express any known RyR subtypes which are sensitive to caffeine and also suggest that RyR-2 exists in control myocytes at E9.5.

To determine the possible contribution of RyR-2 to E–C coupling in the embryonic hearts, cardiac myocytes from E10.5 wild-type embryos were treated with ryanodine, which binds to activated RyRs to lock them in the open state and hence irreversibly depletes the intracellular Ca\(^{2+}\) stores. The increase in intracellular Ca\(^{2+}\) concentration during spontaneous contractions was still retained.
Ryanodine receptor type 2 knockout mice

Fig. 4. Ultrastructural abnormalities in cardiac myocytes from E8.5–10.5 RyR-2-deficient embryos. Electron micrographs were obtained from cardiac myocytes in (A) E8.5 wild-type, (B) E8.5 mutant, (C) E9.5 wild-type, (D) E9.5 mutant and (E) E10.5 mutant embryos. Abnormally large vacuoles were found in mutant myocytes, and the growth of the vacuoles in size were observed during the embryonic development in the mutant mice. The normal rER (or developing SR) in control myocytes and the rER carrying swelling parts and abnormal vacuoles in mutant myocytes are indicated by arrows. The majority of mitochondria with abnormal tubular cristae were smaller at E9.5 and swelling at E10.5 in the mutant myocytes (insets in D and E). Scale bars: 5 μm in (A)–(E); 1 μm in insets of (D) and (E). Representative data are shown from the observation of wild-type (E8.5, n = 3; E9.5, n = 5; E10.5, n = 6), +/-crrm2 (E8.5, n = 3; E9.5, n = 7; E10.5, n = 6) and crrm2/crrm2 (E8.5, n = 3; E9.5, n = 8; E10.5, n = 6) embryos.
were obtained from the electron-dense deposit (shown from observations of wild-type (2) embryos). These data therefore suggest that the loss of the CICR via RyR-2 does not abolish E–C coupling in the early embryonic stages. In accordance with this notion, previous studies in rat have suggested that Ca\(^{2+}\) sparks and waves (Yamazawa et al., 1996). This observation is thought to reflect the opening of the RyR-2 channel at resting intracellular Ca\(^{2+}\) levels and that RyR-2 has the highest Ca\(^{2+}\) sensitivity among the RyR subtypes. The RyR-2 channel may open occasionally, even at resting intracellular Ca\(^{2+}\) levels, in fetal cardiac myocytes, although Ca\(^{2+}\) release does not primarily contribute to E–C coupling. On the other hand, mutant cardiac myocytes losing functional CICR channel activity contain the large vacuoles of the SR and abnormal mitochondria. The ultrastructural defects of the cytoplasmic organelles are essentially the same as those found in skeletal muscle cells from double-mutant mice lacking both RyR-1 and RyR-3 (Ikemoto et al., 1997). Skeletal muscle cells contain RyR-1 and RyR-3 as the predominant and minor components of the Ca\(^{2+}\) release channels, respectively, but the mutant myocytes lacking either RyR-1 or RyR-3 do not exhibit such severe ultrastructural defects as in the double-mutants (Takeshima et al., 1994, 1996). Based on two independent examples, the mutant cardiac myocytes lacking RyR-2 and the double-mutant skeletal muscle cells lacking both RyR-1 and RyR-3, it seems reasonable to conclude that both abnormalities of the organelles are caused by the complete loss of the Ca\(^{2+}\) release channel as a safety valve for intracellular Ca\(^{2+}\) stores. Without the Ca\(^{2+}\) release channel, Ca\(^{2+}\) stores may become overloaded with cytoplasmic Ca\(^{2+}\). Such Ca\(^{2+}\) overloading is thought to be more severe in mutant cardiac myocytes than in double-mutant skeletal muscle cells, because continuous Ca\(^{2+}\) oscillations accompany greater Ca\(^{2+}\) influxes through cardiac L-type Ca\(^{2+}\) channels,
Ryanodine receptor type 2 knockout mice

Fig. 8. Proposed role of RyR-2 on developing Ca\(^{2+}\) stores in embryonic cardiac myocytes. Major intracellular Ca\(^{2+}\) flow is represented schematically. Even though only a slight contribution to the Ca\(^{2+}\) signalling during E–C coupling can be detected, RyR-2 probably functions as a safety valve in developing Ca\(^{2+}\) stores for the release of overloaded luminal Ca\(^{2+}\). Without RyR-2, the overloading of Ca\(^{2+}\) may induce vacuole formation of the developing SR, then the disfunction of the SR may cause mitochondrial abnormalities and finally myocytes may not contribute to the body-fluid circulation.

which have a higher conductance than skeletal muscle counterparts.

On the basis of the above observations, we propose that RyR-2 does not participate principally in Ca\(^{2+}\) signalling during E–C coupling in the embryonic heart but functions as a major leak Ca\(^{2+}\) channel to maintain the normal range of luminal Ca\(^{2+}\) levels in the developing SR. In cardiac myocytes lacking RyR-2, cytoplasmic Ca\(^{2+}\) derived from extracellular fluid during E–C coupling may be gradually accumulated in the SR lacking RyR-2 as a leak Ca\(^{2+}\) channel, which cannot be sequestered by the overloaded SR may flow into mitochondria, and finally defective organelles and/or abnormal Ca\(^{2+}\) homeostasis may cause dysfunction of mutant cardiac myocytes (Figure 8). Thus, our present study indicates a novel physiological role for the RyRs in developing intracellular Ca\(^{2+}\) stores. Local intracellular Ca\(^{2+}\) transients caused by the activation of several RyR channels on the SR can be detected as Ca\(^{2+}\) sparks in cardiac muscle cells and Ca\(^{2+}\) sparks are proposed to be the unitary events underlying cardiac E–C coupling (Cannell et al., 1995; Lopez-Lopez et al., 1995). In addition, Ca\(^{2+}\) sparks might reflect RyR-mediated leak Ca\(^{2+}\) release for the maintenance of Ca\(^{2+}\) concentration in stores. It is an attractive hypothesis that in order to prevent store overloading, RyR channels may be activated in response to elevation of luminal Ca\(^{2+}\) levels. This idea may be supported by previous bilayer studies which indicate the luminal Ca\(^{2+}\) dependency of RyR activation (Sitsapesan and Williams, 1997).

Materials and methods

Generation of mutant mice

The rabbit RyR-2 cDNA fragment (nucleotide residues –54 to 49; Nakai et al., 1990) was amplified by PCR using synthetic primers. A mouse genomic DNA library was screened with the amplified fragment as a probe to yield AMHRRG153 carrying the 5’-terminal region of the RyR-2 gene. The targeting vector was constructed using the genomic DNA fragments obtained, synthetic linkers carrying the foxP sequence (Shibata et al., 1997), the GFP-coding region from pGreen Lantern-1 (Gibco-BRL), the neomycin-resistance gene from pMC1 Neo (Stratagene), the virus thymidine kinase gene (Takehisa et al., 1994) and pBluescript SK(–) (Stratagene). The short arm of the vector is the 1.0 kb SpH–BspEI fragment containing the putative promoter and 5’-untranslated sequences, and the long arm is the 6.5 kb fragment containing the first intronic sequence (Figure 1).

J1 ES cells (Li et al., 1992) were transfected with the linearized targeting vector and selected using G418 and FIAU (Takehisa et al., 1994). Of ~300 clones isolated, Southern blotting analysis identified four clones carrying the homologous mutation (crr\(^{m1}\)) including the ES clone numbered 154. The #154 cells were inoculated with the recombinant adenovirus (AxSR\(_{crr}\)) for transient expression of Cre recombinase (Shibata et al., 1997) and several clones, including the clone numbered A7, were selected as the ES cells containing the recombined mutant gene (crr\(^{m2}\)). The mutations introduced into the clones were further confirmed by Southern blot analysis using several restriction enzymes (EcoRI, XhoI, BamHI and XhoI) and two different probes from the 5’-flanking region (probe 1) and first intron (probe 2) as shown in Figure 1. The production of chimeric mice using ES clones and mutant mice homozygous for crr\(^{m1}\) or crr\(^{m2}\) was carried out essentially as described previously (Takehisa et al., 1994). To determine the genotypes of the mutant mice, PCR was carried out using primers from the genomic sequence, the forward primer (Ex1-P6D, 25mer: GAGCCCGTCA-GAACATCCCTGGTTTAC) and the reverse primer (Ala6-668, 25mer: GCTGAGAAGGCTGCCCAGGGTGC). The expected sizes of amplified DNA fragments from the wild-type, crr\(^{m1}\) and crr\(^{m2}\) alleles are shown in Figure 1.

Anatomical analyses

Mouse embryos were treated with a pre-fixative buffer containing 3% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate, pH 7.5. After washing with the buffer solution, they were fixed with a post-fixative solution containing 1% OsO\(_4\), 0.1 M sodium cacodylate, pH 7.5, and then the fixed embryos were dehydrated and embedded in Epon. For light-microscopy observation, 0.5 μm sections were prepared and stained with 0.1% toluidine blue solution. For ultrastructural analysis
using an electron microscope (JEOL, JEM-200CX), 80–90 nm sections were prepared and stained with uranyl acetate and lead citrate.

In an attempt to determine the ultrastructural localization of Ca$^{2+}$, fetal hearts were treated with the pre-fixative buffer supplemented with 50 mM potassium oxalate and then post-fixed in a solution containing 1% OsO$_4$, 0.1 M potassium ferricyanide and 0.1 M sodium cacodylate, pH 7.5. After dehydration, the tissues were embedded in Epon, and 100–150 nm sections were prepared and observed without staining. The X-ray microanalysis was performed on sections using a Delta Level-IV X-ray analyser (Kevec Co., Foster City, USA) as described previously (McGrew et al., 1980; Komazaki and Hiruma, 1994).

**Ca$^{2+}$ measurements**

Mouse embryonic hearts were isolated and fixed on silicone rubber with stainless steel pins. The tissue was submerged in a physiological salt solution (PSS; 150 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, 5.6 mM glucose, pH 7.4) containing 5 μM Fluo-3 AM and 0.1% bovine serum albumin for ~1 h. The heart mounted on silicone rubber was placed on a glass-bottomed culture dish (MatTek Corp., USA). A cooled CCD camera (Photometrics) mounted on the microscope (IX-70, Olympus), equipped with a polychromatic illumination system (TILL Photonics, Germany), was used to capture the fluorescence images with excitation at 470 nm and emission at >510 nm at 4 frames/s. The experiments were carried out at room temperature.

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### References


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