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Surface plasmon resonance studies prove the interaction of skeletal muscle sarcoplasmic reticular Ca^{2+} release channel/ryanodine receptor with calsequestrin

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Abstract A high affinity molecular interaction is demonstrated between calsequestrin and the sarcoplasmic reticular Ca^{2+} release channel/ryanodine receptor (RyR) by surface plasmon resonance. K_D values of 92 nM and 102 nM for the phosphorylated and dephosphorylated calsequestrin have been determined, respectively. Phosphorylation of calsequestrin seems not to influence this high affinity interaction, i.e. calsequestrin might always be bound to RyR. However, the phosphorylation state of calsequestrin determines the amount of Ca^{2+} released from the lumen. Dephosphorylation of approximately 1% of the phosphorylated calsequestrin could be enough to activate the RyR channel half-maximally, as we have shown previously [Szegedi et al., Biochem. J. 337 (1999) 19].

Key words: Sarcoplasmic reticulum; Ca^{2+} release; Ryanodine receptor; Calsequestrin; Striated skeletal muscle; Surface plasmon resonance

1. Introduction

Skeletal muscle calsequestrin, the major Ca^{2+} storage protein within the cisternae of the sarcoplasmic reticulum (SR), binds and releases approximately 50 Ca^{2+} ions per molecule during each relaxation–contraction cycle [1]. This high capacity but low affinity Ca^{2+} binding protein is located in close proximity to the Ca^{2+} release channel/ryanodine receptor (RyR). Electron microscopy reveals calsequestrin as electron-dense material which is connected to RyR by 'anchoring strands' [2] or 'rope-like fibers' [3].

In recent years this structural organization has increasingly attracted attention and has led to assumptions of a functional significance of the above morphological arrangement and the existence of a coordinated process between luminal events and the cytosolic response.

In addition to the calcium buffering role of calsequestrin in the SR lumen, several strands of evidence predict that this acidic protein influences the Ca^{2+} release/RyR channel activity. Biochemical experiments have given first hints that calsequestrin is involved in regulating the released amount of Ca^{2+} through the RyR [4–6], which might occur indirectly by two further junctional integral membrane proteins, such as triadin [7,8] or junctin [9,10]. Murray and Ohlendieck, however, reported a direct complex formation between calsequestrin and RyR without detectable amounts of triadin being involved in the complex formation [11].

Our recent paper [12] confirms the effect of calsequestrin on the SR Ca^{2+} release channel. Moreover, we could show that calsequestrin, depending on its phosphorylation state, selectively controls the RyR channel activity. In the presence of 1 mM luminal Ca^{2+} only the dephosphorylated calsequestrin induces Ca^{2+} release from the SR lumen, whereas the phosphorylated form has no effect. Our results have demonstrated for the first time that the luminal phosphorylation/dephosphorylation system contributes to the regulation of the Ca^{2+} release process [12].

The existence of an endogenous phosphorylation system in the SR lumen has already been reported before (for review [13]): we described first [14] that calsequestrin can be phosphorylated and the isolated calsequestrin from rabbit skeletal muscle can be obtained in fully or partly phosphorylated forms [15]. Furthermore, two luminally located glycoproteins, sarcalumenin and the histidine-rich Ca^{2+} binding protein [16–19], can also be phosphorylated, thereby modulating the RyR activity [19,20]. The protein kinase responsible for the phosphorylation of these intraluminal proteins is casein kinase II, which has been shown to be located in the lumen [18,21]. The phosphate donor necessary for these phosphorylation processes is ATP which can be transported through an SR voltage-dependent anion channel/porin-like protein [22].

In the present work we will show a high affinity molecular interaction between the phosphorylated and dephosphorylated calsequestrin and RyR. However, no interaction could be observed between calsequestrin and the putative intraluminal RyR loops, M1/2 (aa 4580–4639) and M3/4 (aa 4860–4917).

2. Materials and methods

2.1. Preparations

Heavy SR vesicles and RyR were prepared exactly as described [12]. Mouse monoclonal IgM antibodies against rabbit skeletal muscle RyR (lot 16534) were purchased from Biomol (Germany).

Calsequestrin was isolated from the protein–glycogen complex as described previously [14,23]. Phosphorylation of calsequestrin by casein kinase II was carried out as reported before [12].

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Abbreviations: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; GST, glutathione *S*-transferase; SPR, surface plasmon resonance; RU, response unit; aa, amino acids; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

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2.2. Cloning, overexpression and purification of the two putative luminal loops of the RyR

The two putative luminal loops M1/2 (aa 4580–4639) and M3/4 (aa 4860–4917) were amplified via RT-PCR from skeletal muscle mRNA. First strand synthesis was carried out using a primer (5′-TGCTCCTGCTGGT-3′) derived from bases 14 832–14 850. The PCR of the M1/2 loop DNA was done using two primers (5′-GGAATTCCTCCATGTAGCCCGTGCTCT-3′, 5′-CGGATCCAG-GTCTCAGACTCTCCACC-3′) and PCR of the M3/4 loop DNA was carried out with the primers 5′-CGGGATCCGCAAGTTCTA-CAACAAGAG-3′ and 5′-GGAATTCGTCGAAGACCACCCGG-TAGA-3′ introducing the restriction sites *Bam*HI and *Eco*RI into both PCR products. The loops were cloned into the pGEX-5X-1 (Pharmacia) via the restriction sites. They were transformed into competent *Escherichia coli* BL21DE3 cells and plated on ampicillin containing Luria–Bertani/agar plates (Difco). 500 ml ampicillin containing Luria–Bertani broth medium was inoculated from the plates and peptide overexpression was induced using 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma) at 37°C. The glutathione *S*-transferase (GST) fusion peptides were purified using glutathione-Sepharose according to the instructions of Pharmacia.

2.3. Surface plasmon resonance (SPR)

SPR studies were carried out using a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). The ligands were immobilized onto a CM5 sensor chip (Biosensor) either directly, covalently via primary amines or indirectly, site specifically onto an α-GST antibody surface via a GST fusion tag.

Calsequestrin was immobilized using a standard amine coupling in a buffer containing in 500 mM NaCl, 100 μM $CaCl_2$ and 50 mM HEPES pH 8.2. The association and dissociation of RyR were determined on a surface containing 800 response units (RU) of calsequestrin. The running buffer for the interaction studies between RyR and calsequestrin contained 150 mM KCl, 50 mM HEPES pH 7.2, 100 μM $CaCl_2$, 0.5% CHAPS and 1 mM DTE. RyR in a concentration range of 5–160 nM was injected as analyte onto the calsequestrin loaded surface with a flow rate of 10 μl/min at 20°C. Both the injection and dissociation times were 700 s. The regeneration of the calsequestrin surface, i.e. the removal of the bound RyR, was performed using 0.2% SDS resulting in a completely regenerated surface. The surface could be used three times without losing its binding activity. After three experiments a new sensor surface was prepared.

To determine whether the calsequestrin bound protein is really the RyR, antibodies against the RyR were injected during the dissociation phase (for details see the legend of Fig. 3). The increase in response is due to an enhancement of the surface concentration and is the evidence for binding of antibodies to the RyR bound on the immobilized calsequestrin.

Chase experiments were performed to check if rebinding affects the dissociation kinetics. For these chase experiments first RyR was injected onto surfaces containing immobilized dephosphorylated or phosphorylated calsequestrin. Then, during the dissociation phase dephosphorylated or phosphorylated calsequestrin (1–20 μM)) was injected.

The data evaluation was carried out using the software program BIAevaluation version 3.0.

The kinetic constants were calculated by non-linear regression of data using the pseudo first order rate equation.

$$R = k_{ass} C R_{max}/(k_{ass} C + k_{diss}) \times (1 - e^{-(k_{ass} C + k_{diss})t})$$

where R is the SPR signal in RU, k_{ass} is the association rate constant, k_{diss} is the dissociation rate constant and C is the concentration of the injected analyte.

The dissociation rate constant was calculated using the following equation:

$$R_t = R_0 \, e^{-k_{diss}(t - t_0)}$$

where R_t is the response signal at a time t and R_0 is the response signal at the start time t_0.

The dissociation constant was calculated according to the following equation:

$$K_D = k_{diss}/k_{ass}$$

3. Results and discussion

The molecular interaction between an immobilized component, referred to as the ligand, and a molecule in the mobile phase, designated the analyte, was determined by SPR studies in the BIAcore instrument. Changes in surface concentration are proportional to changes in the refractive index on the surface which results in changes in the SPR signal; these are plotted as RU as a function of time. 1000 RU corresponds to a surface concentration of 1 ng/mm^2 [24].

Representative sensorgrams in Fig. 1 demonstrate the interaction between calsequestrin and RyR. Panels A and B show the binding curves that are the result of an interaction between the dephosphorylated (A) or the phosphorylated calsequestrin (B) and RyR in different concentrations. Panels D (refers to A) and E (refers to B) represent the corrected data after determining and subtracting the control values shown in panel C. During the binding of RyR to dephosphorylated or phosphorylated calsequestrin, a quasi saturation phenomenon was observed at concentrations higher than 40 nM RyR. Therefore, association rate constants were calculated with RyR concentrations between 5 and 40 nM. Covalently, 800 RU calsequestrin (17.8 fmol/mm^2) was immobilized to the chip surface and this immobilized calsequestrin bound up to 2200 RU monomer RyR (3.9 fmol/mm^2).

These measurements were carried out at a fixed [Ca^{2+}] of about 100 μM. This [Ca^{2+}] should induce a defined conformation state of calsequestrin which allows the protein to participate in protein–protein interactions [25]. Moreover, at rest the in situ free [Ca^{2+}] of SR was calculated to be about 3.6 mM, thus, calsequestrin is largely saturated with Ca^{2+} in the SR lumen [26].

Using a series of different concentrations of RyR, the association rate constant was calculated as described in Section 2. For the interaction of the phosphorylated calsequestrin with RyR a k_{ass} of 1.23×10^5 M^{-1} s^{-1} and for that of dephosphorylated calsequestrin a k_{ass} of 1.35×10^5 M^{-1} s^{-1} was obtained.

Rebinding is known to be a common problem in the analysis of the dissociation phase. Therefore, additional chase experiments were carried out by injecting dephosphorylated or phosphorylated calsequestrin during the dissociation phase. Fig. 2A,B shows a representative set of these chase experiments. Addition of 1 μM calsequestrin indeed increases the dissociation rate approximately two-fold, there is only a marginal further increase by enhancing [calsequestrin] up to 20 μM. There is essentially no difference between the effects of phosphorylated and dephosphorylated calsequestrin as ligand or as chasing agent, respectively (compare Fig. 2A and B). The upper curves without label show the unchased dissociation phase. As can be seen during the dissociation phase RyR itself seems to rebind to the calsequestrin surface. Therefore, to calculate the dissociation rate constants the chase experiments were chosen: k_{diss} values of 0.0113 s^{-1} and 0.0137 s^{-1} for the phosphorylated and dephosphorylated calsequestrin, respectively, were calculated.

In order to prove that the bound protein is RyR on the immobilized calsequestrin, RyR antibodies were injected during the dissociation phase. Fig. 3 shows the interaction of anti-RyR with the RyR–calsequestrin complex. The binding curves indicate that the calsequestrin bound protein is the RyR (for experimental details see legend of Fig. 3).

Table 1 summarizes the association and dissociation rate

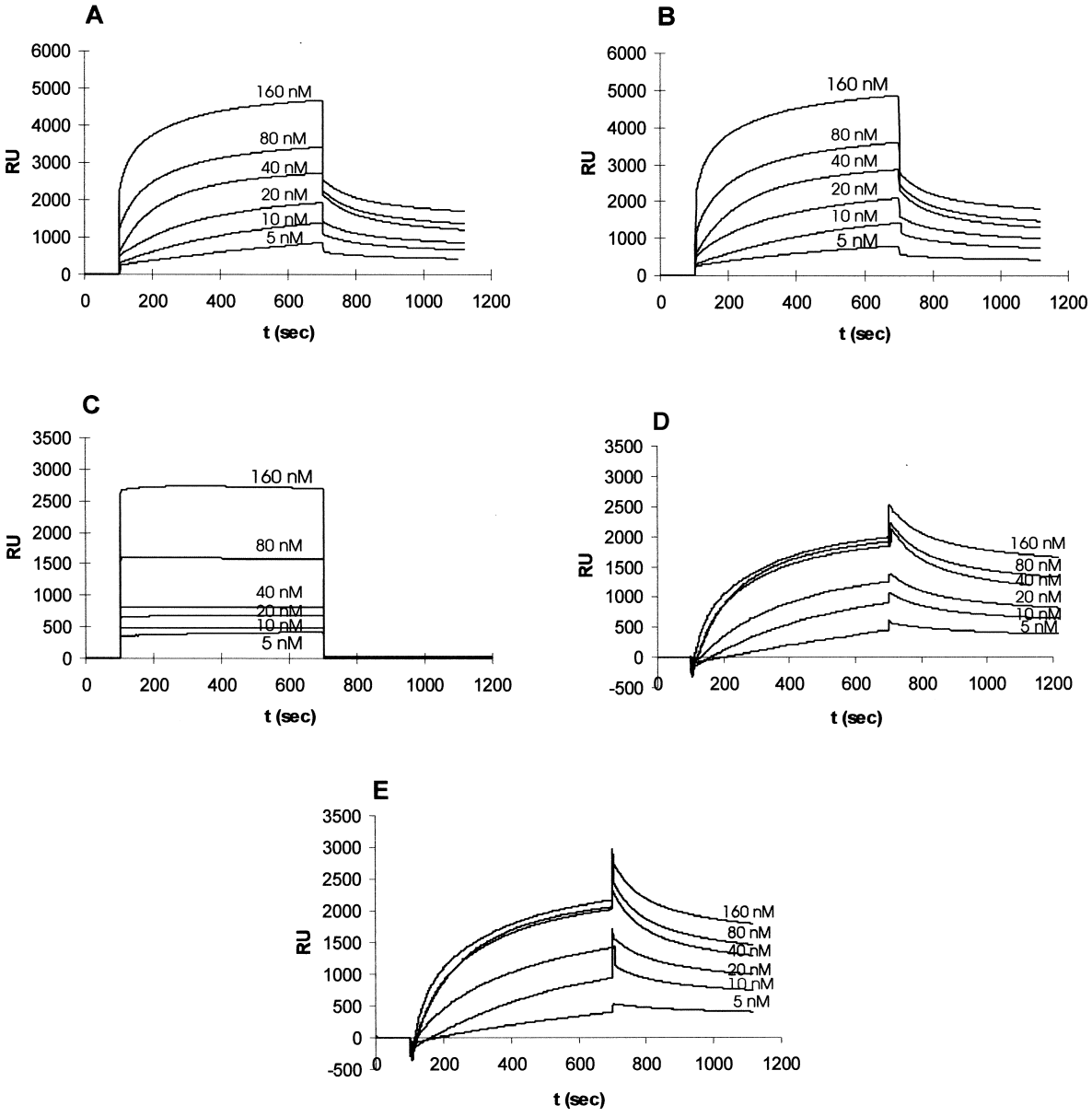

Fig. 1. RyR binds with a high affinity to phosphorylated and dephosphorylated calsequestrin. Phosphorylated and dephosphorylated calsequestrin were immobilized on a CM5 chip as described in Section 2. The association and dissociation phases of the interaction with RyR were monitored by following changes in the SPR signal, given in RU. The upper curves show the interaction of the RyR with dephosphorylated (A) and phosphorylated (B) calsequestrin. For a negative control the RyR was injected onto an activated and then deactivated CM5 surface (C). No non-specific interaction was detected between the RyR and the CM5 chip surface. After subtracting the bulk changes (C) from A or B, respectively, the specific interactions between the dephosphorylated (D) or phosphorylated (E) calsequestrin and RyR are shown.

constants and the equilibrium binding constants for the interaction of dephosphorylated or phosphorylated calsequestrin with RyR. The calculated K_D for the interaction of the RyR with the dephosphorylated calsequestrin is 102 nM using a k_{ass} of 1.35×10^5 M^{-1} s^{-1} and a k_{diss} of 0.0137 s^{-1}; for the interaction with the phosphorylated calsequestrin it is 92 nM, applying a k_{ass} of 1.23×10^5 M^{-1} s^{-1} and a k_{diss} of 0.0113 s^{-1}. Both sets of rate constants, for the dephosphorylated and for the phosphorylated calsequestrin, were in the same range. This observation indicates that calsequestrin phosphorylation has no effect on the molecular interaction of calsequestrin with RyR. In contrast, phosphorylation of calsequestrin controls the amount of released Ca^{2+} from the lumen through the RyR as our recent electrophysiological measurements have revealed [12]; i.e. calsequestrin phosphorylation does not change the overall affinity of calsequestrin to RyR, it exerts a much more subtle effect on the interaction of these two proteins.

To see whether the two putative luminal RyR loops, M1/M2 and M3/M4, are involved in the specific interaction of the RyR with calsequestrin these RyR luminal loops (Fig. 4) were expressed as GST fusion proteins and were immobilized on a CM5 chip. Calsequestrin was injected as an analyte onto these surfaces with the aim of invoking changes in the refractive index as a result of a potential protein–protein interaction. No specific interaction has been observed (not shown). There

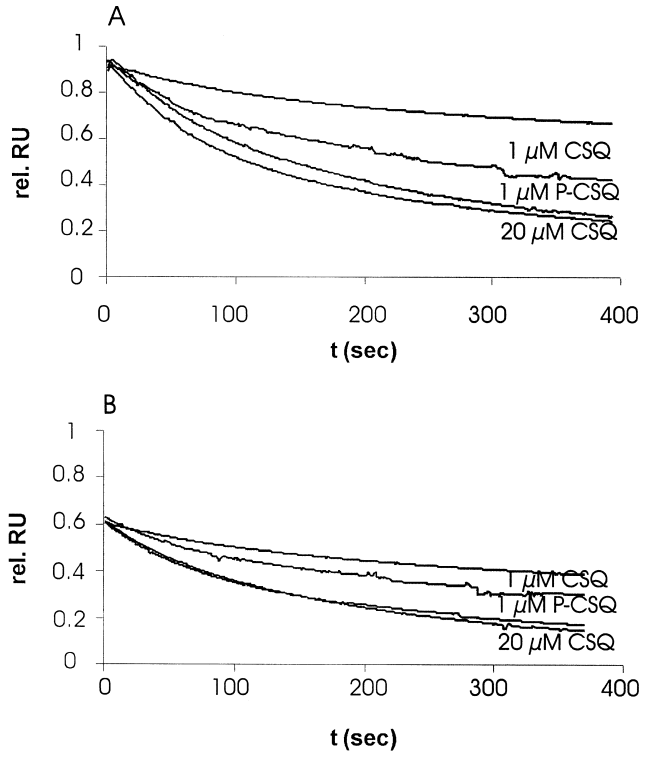

Fig. 2. Chase experiments reveal the rebinding of the RyR to calsequestrin. Approximately the same amount of RyR (40 nM) was injected onto a CM5 chip with immobilized dephosphorylated (A) and phosphorylated (B) calsequestrin on it. Different concentrations of dephosphorylated (CSQ) and phosphorylated calsequestrin (P-CSQ) were included during the dissociation phase. As a control the RyR was injected onto an activated and then deactivated surface. Panels A and B show the corrected binding, i.e. following subtraction of the control values. The upper curves without label show the unchased dissociation phase. A relative RU of 1 corresponds to 800 RU or 700 RU dephosphorylated or phosphorylated calsequestrin–RyR complex on the surface, respectively.

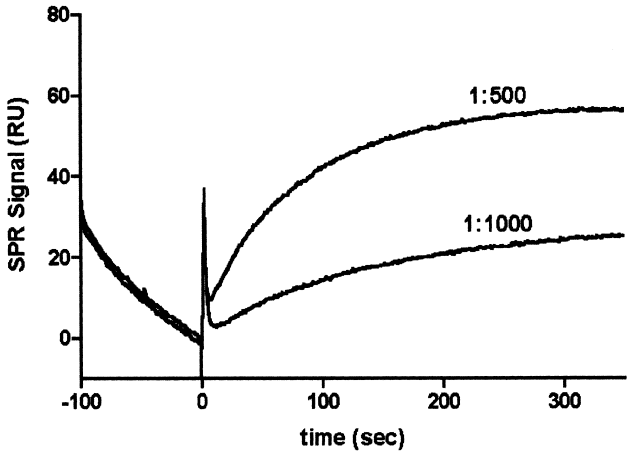

Fig. 3. Antibody against the RyR proves the interaction of the RyR with calsequestrin. During each cycle approximately the same amount of RyR (40 nM) was injected onto a CM5 chip with immobilized dephosphorylated calsequestrin on it. After the end of the injection anti-RyR antibodies were included during the dissociation phase in 500- or 1000-fold dilution. To control for non-specific binding, the RyR antibody was injected over a calsequestrin loaded surface: no non-specific binding could be detected (data not shown). The curves before addition of antibodies show the initial dissociation phase in the absence of RyR antibodies.

are several possibilities that could explain these results: the expressed polypeptides might fold differently in comparison to the native protein and thus the binding domain is either not in the right conformation or not accessible. Other possibilities are that the calsequestrin interaction site might not be localized on the tested putative loops, or the interaction of the calsequestrin with the RyR occurs indirectly, via an as yet unknown and unidentified component which could be present in our RyR preparation.

One of the potential candidates might be triadin which has been intensively studied and controversially discussed in the literature as a possible link in the interaction between calsequestrin and RyR. Fan et al. [27] have proposed a model in which the cytoplasmic region of triadin, aa 110–163, interacts with RyR at the cytoplasmic side. This was further supported by Ohkura et al. [28]. Recently, Groh et al. [29] reported that a small cytoplasmic region of triadin (aa 18–46) interacts with RyR; a direct molecular interaction of this domain with RyR was confirmed by overlay assays, SPR studies and by RyR channel activity measurements in planar lipid bilayers. On the other hand, Froemming et al. [30] reported that although triadin exists in the near vicinity of the RyR channel tetramer, it does not seem to be directly linked to the other main triadic components; the proposed role of triadin should be the maintenance of the overall triadic architecture. Actually, Murray and Ohlendieck [11] have shown that calsequestrin is directly coupled to the RyR. Also morphological studies provide strong evidence for a direct connection of calsequestrin to RyR [31].

Our RyR, prepared according to standard procedure, is enriched with the channel protein to approximately 90% and the main component of the 10% contamination is the SR Ca^{2+} transport ATPase. Coomassie brilliant blue stained SDS–PAGE gels show in the range of 95k polypeptide(s) present in an amount of ca. 1% which could include triadin, however, its presence alone would not allow us to draw conclusions about its interaction with other triadic proteins. On the basis of SPR studies here we demonstrate an interaction with high affinity in the nM range without being able to decide whether the interaction of calsequestrin with RyR occurs directly or indirectly. Obviously, phosphorylation of calsequestrin has no influence on this high affinity molecular interaction. It means that calsequestrin might always be bound on the SR Ca^{2+} release channel and actually, the phosphory-

Table 1
Kinetic parameters determined by SPR measurements

Interaction of RyR with	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	K_D (nM)
Dephosphorylated calsequestrin	1.35×10^5	0.0137	101.5
Phosphorylated calsequestrin	1.23×10^5	0.0113	91.9

Data of representative sensorgrams on the interaction between calsequestrin and RyR were evaluated as described in Section 2.

M1/2-Loop
AA 4580-4640
KVSDSPPGEDDMEGSAAGDLAGAGSGGGSGWGSGAGEEAEGDEDENMVYYFLE
ESTGYME

M3/4-Loop
AA 4859-4917
RKFYNKSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGDEIEDPAGDEYELYRV
VFD

Fig. 4. Amino acid sequences of the expressed putative M1/M2 and M3/4 RyR loops.

lation state of calsequestrin could determine the amount of Ca^{2+} released from the lumen. On the basis of our electrophysiological studies in [12] the dephosphorylation of approximately 1% of phosphorylated calsequestrin might be enough to activate the RyR channel half-maximally presumably without dissociating from it.

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