RESEARCH COMMUNICATION

Calsequestrin: more than ‘only’ a luminal Ca^{2+} buffer inside the sarcoplasmic reticulum

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In striated muscle, the sarcoplasmic reticulum (SR) Ca^{2+} release/ryanodine receptor (RyR) channel provides the pathway through which stored Ca^{2+} is released into the myoplasm during excitation–contraction coupling. Various luminal Ca^{2+} -binding proteins are responsible for maintaining the free $[\text{Ca}^{2+}]$ at 10^{-3} – 10^{-4} M in the SR lumen; in skeletal-muscle SR, it is mainly calsequestrin. Here we show that, depending on its phosphorylation state, calsequestrin selectively controls the RyR channel activity at 1 mM free luminal $[\text{Ca}^{2+}]$. Calsequestrin exclusively in the dephosphorylated state enhanced the open probability

by approx. 5-fold with a Hill coefficient (h) of 3.3, and increased the mean open time by about 2-fold, i.e. solely dephosphorylated calsequestrin regulates Ca^{2+} release from the SR. Because calsequestrin has been found to occur mainly in the phosphorylated state in the skeletal-muscle SR for the regulation of RyR channel activity, the dephosphorylation of calsequestrin would appear to be a quintessential physiological event.

Key words: calcium ryanodine receptor, skeletal muscles.

INTRODUCTION

In skeletal muscle, the ryanodine receptor (RyR) channel present in the terminal cisternae of the sarcoplasmic reticulum (SR) mediates the rapid release of Ca^{2+} from intracellular stores [1,2]. The regulation of this channel from the cytoplasmic (*cis*) side has been studied extensively [3,4]. Phosphorylation on Ser²⁸⁴², which also occurs endogenously to a significant extent [5,6], leads to an enhanced open probability (P_o) by increasing sensitivity [7] towards *cis*-ATP and *cis*- Ca^{2+} .

Calsequestrin, a highly acidic glycoprotein with a high capacity for binding Ca^{2+} [8], but only an intermediate affinity for this cation [9], is concentrated in the SR lumen, and plays a major role in lowering the amount of free Ca^{2+} inside the SR. The distribution of calsequestrin is not even throughout the lumen, but is markedly concentrated in close proximity to the RyR [10,11]. Following Ca^{2+} binding to calsequestrin, conformational changes in the RyR occur and these can be reversed by dissociation of the calsequestrin–RyR complex [12]. Calsequestrin and the calcium-channel protein are mutually coupled, such that the conformational changes of one protein are transmitted to the other [13,14]. One role of calsequestrin in regulating the SR Ca^{2+} release has been suggested previously [12–14]. Very recently, the complex formation between calsequestrin and the RyR in fast- and slow-twitch rabbit skeletal muscle has been demonstrated [15]. Sarcalumenin and the histidine-rich Ca^{2+} -binding protein are also located in the lumen [16–18]; all these glycoproteins can be phosphorylated [19–21]. ATP transport into the SR lumen, necessary for these phosphorylation processes, might occur through an SR voltage-dependent anion channel/porin [22].

For the first time, the data in the present study demonstrate the physiological relevance of an intraluminal phosphorylation/dephosphorylation system. Calsequestrin, depending on its phosphorylation state, selectively controls the RyR channel

activity: in the presence of 1 mM free luminal $[\text{Ca}^{2+}]$, only calsequestrin in the dephosphorylated state induces Ca^{2+} release from the SR lumen, whereas the phosphorylated one has no effect.

EXPERIMENTAL

Heavy SR vesicles (HSRV)

HSRV were isolated from rabbit longissimus dorsi, as described previously [7], with the following modifications. Homogenization was carried out in 100 mM NaCl/20 mM EGTA/20 mM sodium Hepes, pH 7.5, containing 200 μM Pefabloc, 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A and 1 mM benzamidine. Cell organelles were removed at 4500 *g* (25 min centrifugation) and, from the resulting supernatant, crude microsomes were collected by centrifugation at 40000 *g* for 30 min. Actomyosin was extracted with a buffer containing 600 mM KCl, 10 mM potassium Pipes, 250 mM sucrose, 0.1 mM EGTA and 90 μM CaCl_2 , pH 7.0. After 1 h of incubation at 4 °C, the microsome fraction was collected at 109000 *g* (30 min centrifugation). The pellet was resuspended in a buffer containing 100 mM NaCl, 10 mM potassium Pipes and 485 mM sucrose, pH 7.0. This suspension was loaded on to a linear 20–45% sucrose gradient, and centrifuged for 16 h at 86000 *g* (4 °C) using an SW-27 Beckman rotor. The visible protein ring corresponding to the HSRV fraction was extracted from the 36–38% region of the gradient, and collected at 124000 *g* for 60 min. The pellet, resuspended in 0.3 M sucrose/10 mM potassium Pipes, pH 7.0, was used straightaway for RyR purification.

RyR isolation

The isolation of the RyR was on the basis of a method described previously [23]. HSRV (3 mg/ml) were solubilized for 2 h in a buffer containing 1% CHAPS, 1 M NaCl, 100 μM EGTA,

Abbreviations used: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; P_o , open probability of the RyR; HSRV, heavy SR vesicles; DTT, dithiothreitol.

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150 μM CaCl_2 , 5 mM AMP, 0.45 % (w/v) phosphatidylcholine, 20 mM sodium Pipes, pH 7.2, at 4 °C, also containing the protease inhibitors in concentrations identical with those described in the HSRV preparation, and 1 μM calpain inhibitor I (*N*-acetyl-Leu-Leu-norleucinal) and 1 μM calpain inhibitor II (*N*-acetyl-Leu-Leu-methionial). To localize the receptor in the gradient, a sample of solubilized vesicles was labelled with 8 nM [^3H]ryanodine, as described previously [7]. RyR-containing fractions were collected, frozen in liquid nitrogen and stored at -70 °C.

Planar lipid bilayer

Measurements were carried out as described previously [7] using a bilayer chamber manufactured by Warner Instruments Inc. (Hamden, CT, U.S.A.). Lipids were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The holding potential of the membrane was adjusted and the bilayer currents were measured using an Axopath 200 amplifier (Axon Instruments, Foster City, CA, U.S.A.). Records of the current obtained were analysed using PCLAMP 6.2 software. At the final point of each measurement, ryanodine or Ruthenium Red was added in order to lock the channel into its half-inhibited, or closed, state. These records of the current were taken to determine the noise level of the given record. Membrane currents that exceeded the baseline of the noise amplitude by more than 3-fold were designated as representing an open state of the channel.

Calsequestrin was isolated from the protein–glycogen complex, as described previously [19,24].

Calsequestrin phosphorylation by casein kinase II

The phosphorylation of calsequestrin by casein kinase II was carried out mainly as described previously [25], but with some minor modifications. Calsequestrin (2 mg) was phosphorylated with 10 m-units of casein kinase II in a phosphorylation assay {20 mM Tris/HCl (pH 7.5)/15 mM sodium 2-glycerophosphate/1 mM dithiothreitol (DTT)/0.2 mM EGTA/10 mM MgCl_2 /1 mM [γ - ^{32}P]ATP}. Phosphorylation was initiated by adding Mg^{2+} after a 2 min preincubation at 30 °C. During the reaction, samples were removed and analysed for [^{32}P]P_i incorporation [20]. The reaction was stopped by the addition (at one-tenth of the incubation volume) of buffer A [500 mM Mops/5 M NaCl/10 mM DTT/2 μM okadaic acid/10 ml of phenyl-Sepharose, pre-equilibrated with 10 mM Mops/500 mM NaCl/1 mM DTT/0.1 mM EGTA/0.02 % (w/v) sodium azide]. After gentle stirring at room temperature for 1 h, the suspension was loaded on to a column. Following intensive washing with buffer A, calsequestrin was eluted [26] with 10 mM ATP containing buffer A. The experiments presented here were performed with three charges of phosphorylated calsequestrin with an average of 0.92 ± 0.12 mol (mean $\pm$ S.E.M) of phosphate/mol of protein. Both phosphorylated and dephosphorylated calsequestrin was stored in 20 mM potassium Pipes, pH 7.0, containing 150 mM KCl.

RESULTS AND DISCUSSION

Changes in the intraluminal free [Ca^{2+}] markedly affect both the conductance and the gating behaviour of the RyR channel, and also the Ca^{2+} release from the SR [27]. Figure 1 shows that increasing the luminal (*trans* side of the chamber; [28]) free [Ca^{2+}] from 50 μM to 1 mM led to a greater-than-one-order-of-magnitude decrease in P_o . The subsequent addition of dephosphorylated calsequestrin to the *trans* side activated

Figure 1 Effect of luminal calcium on the P_o of the RyR channel

Isolated RyR was incorporated into planar lipid bilayer in a symmetrical bathing medium containing 250 mM KCl, 100 μM EGTA, 150 μM CaCl_2 and 20 mM Pipes, pH 7.2. P_o values were obtained from single-channel recordings ($n = 5-8$) in the presence of the indicated free [Ca^{2+}]_{trans}, while the free [Ca^{2+}]_{cis} was held constant at 50 μM . Data were fitted to a single exponential curve, resulting in a half-maximal inhibition of P_o at 138 μM [Ca^{2+}]_{trans}.

Figure 2 Effect of dephosphorylated calsequestrin on the RyR channel

Single-channel current records obtained at a holding potential of -40 mV using K^+ ions as the charge carrier in a buffer containing 250 mM KCl and 20 mM Pipes, pH 7.2. Free calcium concentrations were adjusted using an EGTA/ Ca^{2+} buffer system containing 100 μM EGTA (see the Experimental section). Open events are shown as downward deflections, where c and o represent the closed and open states of the channel respectively. (A) Record of the current at 50 μM free [Ca^{2+}] present on both sides of the channel. (B) Effect of high luminal calcium at 50 μM free [Ca^{2+}]_{cis} and 1 mM free [Ca^{2+}]_{trans}. (C) Effect of dephosphorylated calsequestrin at 50 μM free [Ca^{2+}]_{cis} and 1 mM free [Ca^{2+}]_{trans} + 3.74 μM [calsequestrin]_{trans}. (D) Ryanodine still binds to the channel–calsequestrin complex. Addition of ryanodine locks the channel into the characteristic ‘half-open’ state at 50 μM free [Ca^{2+}]_{cis} and 1 mM free [Ca^{2+}]_{trans} + 3.74 μM calsequestrin_{trans} + 1.6 μM ryanodine_{cis}.

Figure 3 Effect of phosphorylated and dephosphorylated calsequestrin on the channel parameters

P_o and mean open time of the reconstituted RyR channel were determined from representative records of the current. The bathing solution contained 250 mM KCl/20 mM Hepes, pH 7.2, on both sides, while calsequestrin and calcium concentrations were adjusted as indicated. Upper panel: concentration dependence of the P_o values was determined using data from representative single-channel recordings in the presence of 50 μM $[\text{Ca}^{2+}]_{\text{cis}}$ and 1 mM $[\text{Ca}^{2+}]_{\text{trans}}$ at indicated dephosphorylated (●) or phosphorylated (■) calsequestrin. Both forms of calsequestrin were applied on the *trans* side. P_o values were calculated from current records of 20–60 s duration, as described in the Experimental section. Upper panel: mean open time was calculated using the same current records, as described for the upper panel. The experimental data shown for dephosphorylated calsequestrin (●) were fitted as a hyperbolic curve, resulting in a 'basic' mean open time of 301 μs and a half-maximal activation at 3.48 μM dephosphorylated calsequestrin with $dt = 0.279$ ms. The P_o and mean open time of the RyR were not modified significantly by the phosphorylated calsequestrin.

the channel (Figures 2A–2C). It is remarkable, however, that the channel-opening characteristics differ greatly from those of the 'classical activators', e.g. Ca^{2+} or ATP, when calsequestrin is present. Figure 2(C) shows that the number of occasions in which the channel was open ('short-open events') increased considerably upon addition of dephosphorylated calsequestrin. This effect, which is characteristic of dephosphorylated calsequestrin acting on the RyR channel, was highly specific. Neither the phosphorylated calsequestrin nor BSA, used as an unspecific control protein, enhanced the number of these short-open events; furthermore, ryanodine irreversibly closed the calsequestrin-reactivated Ca^{2+} release channel and stabilized it into a characteristic sub-conductance state (Figure 2D). These observations confirm the measurement of the incorporated RyR channel activity in the planar lipid bilayer exclusively.

Figure 4 Hill plot of the dephosphorylated-calsequestrin-induced activation of the RyR channel

P_o values of the RyR channel at different calsequestrin concentrations were calculated from the records shown in Figure 3. Mean values for several curves ($n = 4$ –8) were used for the calculations. Numerical analysis revealed a strong co-operativity, with a Hill coefficient (h) of 3.34 ($r = 0.98$).

Figure 3 shows the effect of *trans* calsequestrin on the P_o (upper panel) and the mean open time (lower panel) of the RyR channel activity, which is dependent on the phosphorylation state of this luminal protein. Increasing the concentration of dephosphorylated calsequestrin led to an approx. 5-fold enhancement of P_o , with an EC_{50} value of 3.4 μM . In comparison, phosphorylated calsequestrin did not influence the P_o at all (Figure 3, upper panel). This enhancement of P_o due to the dephosphorylation of calsequestrin is significant ($P = 0.95$) at concentrations of this protein equal to or higher than 2.3 μM (measured by Student's t test).

The dephosphorylated-calsequestrin-induced enhancement in P_o is due to prolonged-open events and/or the increased number of short-open events. Phosphorylated calsequestrin abolishes this pronounced activating effect on the RyR channel.

The net calcium flux depends equally on the P_o and the mean open time. By increasing the luminal free $[\text{Ca}^{2+}]$ from 50 μM to 1 mM, the mean open time constant decreased slightly from 0.449 ± 0.081 ms to 0.283 ± 0.059 ms ($n = 8$). Figure 3 (lower panel) shows the changes in mean open time as a function of $[\text{calsequestrin}]_{\text{trans}}$. Adding dephosphorylated calsequestrin enhanced significantly ($P = 0.95$) this value by approx. 2-fold with an EC_{50} of 3.6 μM . Again, the phosphorylated calsequestrin had no influence on the mean open time of the RyR channel. Figure 4 demonstrates that the binding of dephosphorylated calsequestrin on the RyR is strongly co-operative, with a Hill coefficient (h) of 3.3.

The observed activating effect of the dephosphorylated calsequestrin is highly specific, and cannot be due to the lowering of the free luminal $[\text{Ca}^{2+}]$. The maximal P_o was achieved using 7.5 μM calsequestrin (see Figure 3, upper panel); it corresponds to approx. 340 μM bound Ca^{2+} , resulting in a lowering of the free luminal $[\text{Ca}^{2+}]$ from 1 mM to about 660 μM [8]. In this case, this calcium concentration change will not significantly modify the P_o (Figure 1). Moreover, phosphorylated calsequestrin in the same concentration range has no detectable effect on the RyR channel activity. These results suggest a physiological role of calsequestrin, and the potential importance

of its phosphorylation state in regulating Ca^{2+} release through the RyR channel.

On the basis of available morphological data [29,30], we calculated possible calsequestrin concentrations in the terminal cisternae. Assuming a homogeneous distribution of the calsequestrin within the cisternae resulted in a calsequestrin concentration of approx. $630 \mu\text{M}$. Furthermore, we had observed previously [19,20] that the phosphorylation state of the isolated calsequestrin obtained from rabbit skeletal muscle changed from preparation to preparation between fully and partly phosphorylated forms: this shows that the calsequestrin has already been phosphorylated in the SR terminal cisternae.

The concentrations of the dephosphorylated calsequestrin required for half-maximal enhancement of P_o and the mean open time are $3.4 \mu\text{M}$ and $3.6 \mu\text{M}$ respectively, i.e. dephosphorylation of a small fraction of the intraluminal calsequestrin could possibly be sufficient to activate the RyR. Consequently, the dephosphorylation of calsequestrin seems to be the physiologically essential event in RyR channel regulation. The existence of a luminal protein phosphatase (presumably a Mg^{2+} -dependent one) has been described previously [31]. More information on the regulation of this lumenally located SR phosphatase would enable us to gain further insights into how the dephosphorylation of calsequestrin controls the Ca^{2+} -release process through the RyR on the luminal side of the channel.

The way in which dephosphorylated calsequestrin affects RyR activity, but the phosphorylated protein does not, still remains to be elucidated. Both the Ca^{2+} -binding capacity and the affinity for Ca^{2+} of calsequestrin is not changed upon phosphorylation-dephosphorylation [25], thus showing that this post-translational modification exerts its influence on the calsequestrin-RyR interaction directly, and not through the changes in the Ca^{2+} -binding properties of calsequestrin. One possible explanation is that conformational changes might occur when calsequestrin is phosphorylated/dephosphorylated, and these conformational changes either directly or indirectly monitor Ca^{2+} release from the lumen.

Dephosphorylated-calsequestrin-induced Ca^{2+} release from the lumen could be of physiological relevance for RyR molecules that are not directly coupled to the voltage-sensing molecule located in the tubular invaginations of the surface membrane, the dihydropyridine receptor, i.e. they are not controlled by the voltage sensor. Such an interaction of calsequestrin with the luminal domain(s) of RyR could be a possible mechanism for controlling the SR Ca^{2+} -release channel activity.

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