Troponin I converts the skeletal muscle ryanodine receptor into a rectifying calcium release channel

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Abstract The goal of our present studies has been to find novel ryanodine receptor (RyR1) interacting polypeptides that modulate the channel activity from the luminal side of RyR1. Using K^+ as charge carrier for recording of single channel events here we demonstrate a very unexpected observation that troponin I substantially alters RyR's gating behavior, and that RyR1 in association with troponin I becomes a rectifying Ca^{2+} release channel. Troponin I rapidly locks the RyR1 in a non-conducting state only at a negative holding potential, and only when applied to the luminal side; switching to a positive holding potential results in the channel returning to its original activity, immediately. A hypothesis is proposed to account for how an intraluminally located, positively charged molecule might function as a RyR1 regulator under physiological conditions.

Key words: Ryanodine receptor; Calcium release; Rectifying channel; Troponin I; Yeast two-hybrid screen; Skeletal muscle

1. Introduction

The skeletal muscle ryanodine receptor (RyR1) in the terminal cisternae of the sarcoplasmic reticulum (SR) has an essential role in intracellular signalling by mediating the rapid Ca^{2+} release from the SR. RyR1 belongs to a superfamily of intracellular Ca^{2+} release channels and is one of the key proteins involved in the excitation–contraction coupling of skeletal muscle. As a response to depolarization of the surface membrane RyR1 initiates Ca^{2+} release from the lumen into the cytosol [1,2]. The functional unit of RyR1 consists of four identical 560 kDa proteins and four 12 kDa FK506 binding proteins [3,4]. Each RyR1 monomer forms membrane spanning segments in the C-terminal region [5,6] and the luminal loop between transmembrane regions may serve as part of the ion conduction pore [7,8]. The regulation of this channel from the cytoplasmic (*cis*) side has been studied extensively [9,10]. Phosphorylation on Ser 2843, which also occurs endogenously to a significant extent [11], leads to an enhanced open probability (P_o) by increasing the sensitivity towards ATP_{cis} and Ca^{2+}_{cis} [12].

During the last years accumulating evidence has shown that also events occurring within the SR lumen are essential for modulating the Ca^{2+} release process. The existence of an endogenous phosphorylation system in the lumen has been reported by Shoshan et al. [13–15]. Later we could show that calsequestrin, depending on its phosphorylation state, selectively controls the SR Ca^{2+} release channel activity [16] and surface plasmon resonance studies stated the high affinity molecular interaction of RyR1 with calsequestrin [17]. All these results emphasize the potential and specificity of phosphorylation-induced changes in protein interactions as signal transfer mechanism [18].

The goal of our present studies was to unveil further novel RyR1 interacting polypeptides that modulate the channel activity from the luminal side of RyR1. Our experiments presented here show that, when applied to the luminal side and only at negative holding potential, troponin I inactivates the SR Ca^{2+} release channel. This inactivation is a result of a non-conducting state, occurring in response to the negative holding potential and can immediately be restored by switching the holding potential to a positive value, i.e. troponin I rectifies the Ca^{2+} gating of RyR1 very specifically.

2. Materials and methods

2.1. Yeast two-hybrid screen

The M1/M2 loop (bp 13740–13920) and the M3/M4 loop (bp 14577–14751) of the RyR1 [6] were amplified via reverse transcription-PCR from rabbit skeletal muscle mRNA. For the first strand synthesis the primer 5′-CACTTGCTCCTGCTGGT-3′ was used. The primers selected for the PCR (for M1/M2 loop 5′-CGGAATTC-AAGGTCTCAGACTCTCCAC-3′ and 5′-AAAGGTCGACCTCCA-TGTAGCCCGTGCTC-3′; for M3/M4 loop 5′-CGGAATTCCGCA-AGTTCTACAACAAGAG-3′ and 5′-AAAGGTCGACGTCGAAG-ACCACCCGGTA-3′) also introduced the restriction sites *Eco*RI and *Sal*I. These restriction sites allowed the cloning of the loops into the PAS2-1 yeast two-hybrid vector from Clontech. With the M3/M4 loop containing construct as bait a human skeletal muscle Matchmaker cDNA library using the yeast cells CG1945 from Clontech was screened. In the first step 365 clones were positive-related to the activity of the first reporter gene (grown on His-deficient medium). In the second step 156 positive clones were detected which were successful in activating the second reporter gene (expression of β-galactosidase). The following yeast mating step (exclusion of the housekeeping genes in the reporter gene activation) resulted in 19 positive clones.

For direct interaction tests the selected positive clones were isolated and cotransformed with the M1/M2 loop – cloned before in PAS2-1 – into the yeast cells CG1945 and the transformants were then handled as described above.

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Abbreviations: SR, sarcoplasmic reticulum; RyR1, ryanodine receptor; P_o, open probability of the RyR1

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DNA sequencing was carried out with the DNA sequencing kit in ABI PRISM and the software Lasergene of DNASTAR was used for analyzing the sequence data.

2.2. Preparations

Heavy SR vesicles were isolated from rabbit (New Zealand white) longissimus dorsi and were used immediately for the purification of the solubilized RyR channel complex as described previously [16].

Holotroponin was prepared from rabbit skeletal muscle [19] and its subunits, troponin T, I and C, were obtained as previously described [20].

2.3. Single channel measurements

The CHAPS (3-[(3-cholamido-propyl)dimethylammonio]-1-propane-sulfonate)-solubilized RyR1 was incorporated into a Müller–Rudin type planar lipid bilayer [21]. Bilayers were formed across a 250 μm aperture on the delrin cup (Warner Instruments, Campden, NJ, USA) using a mixture of phosphatidylethanolamine, phosphatidylserine and L-α- phosphatidylcholine at a weight ratio of 5:4:1 dissolved in *n*-decane up to a final concentration of 20 mg/ml. Incorporation of the RyR1 into the bilayer was initiated in symmetric buffer solution (250 mM KCl, 100 μM EGTA, 150 μM $CaCl_2$, 20 mM PIPES, pH 7.2). Small aliquots of the solubilized receptor were added to one side of the bilayer chamber defined as the *cis* (cytoplasmic) side. Orientation of the incorporated receptor was verified at the last step of each single experiment using ryanodine or ruthenium red additions. Successful incorporation was detected as a step like increase of membrane current. Electric potential is given as potential of the *cis* chamber relative to the potential of the *trans* chamber, which was kept at ground potential. Current signals were filtered at 1 kHz using an eight pole low-pass Bessel filter, the filtered signal was digitized at 3.3 kHz, at 12 bit resolution using an Axopatch 200 amplifier with Digidata 1200 interface (Axon Instruments Inc., Union City, CA, USA). After changing the conditions in the chamber, at least 5 min were allowed to reach the new equilibrium and total recording time was more than 5 min for each experimental condition. P_o values were calculated from representative data segments of 50–100 s duration using the pClamp 6.02 software package of Axon Instruments. Free [Ca^{2+}] was calculated according to [22].

3. Results and discussion

The search for intraluminally located RyR1 interacting proteins by means of the yeast two-hybrid method [23] resulted in 19 positive clones. cDNA sequencing of these clones revealed six independent clones of the skeletal muscle troponin, two positive clones were identified as skeletal muscle protein C and the remaining 11 clones were disregarded because they did not represent muscle specific proteins. These results first draw our attention to troponin. To analyze this surprising observation first we performed direct two-hybrid tests also with the M1/M2 loop of RyR1. The results of these tests stated the above observation, both loops, M1/M2 and M3/M4, interact specifically with skeletal muscle troponin. To see whether this peculiar finding could have consequences on the RyR1 channel activity, electrophysiological experiments were carried out.

Following isolation of the subunits of the skeletal muscle troponin complex, first, the effect of troponin I on RyR1 was studied in single channel measurements. The SR calcium channel is not permeable to Cl ions, but it conducts monovalent cations more efficiently than Ca^{2+} [24]. Thus K^+ instead of Ca^{2+} was selected as current carrier for recording of single channel events in these experiments.

After incorporation – at symmetrical 50 μM [Ca^{2+}] on both the cytosolic (*cis*) and the lumenal (*trans*) side of the chamber, resulting in a close to maximal channel activation – RyR1 displayed polarity-independent gating characteristics with a $P_o = 0.405 \pm 0.11$ and $\tau = 0.72 \pm 0.12$ ms (mean ± S.E.M., $n = 7$) (Fig. 1a). Following addition of troponin I to the *trans* side – but not to the cytosolic (*cis*) side – the gating characteristics of RyR1 are dramatically altered. In the presence of 200 nM troponin I_{trans} the channel retains its original gating behavior at a positive holding potential. At a negative bilayer potential (*cis* relative to *trans*), the channel, after a period of regular gating, suddenly locks into a closed state (Fig. 1b). The channel still functions even though its P_o is almost zero, as demonstrated at the left side of the trace in Fig. 1c. Changing the holding potential back to the positive sign resulted in the channel promptly opening and its gating pattern and conductance were immediately restored for the given current direction (compare Fig. 1c with the right part of Fig. 1a). Once developed, this polarity-dependent gating behavior was maintained indefinitely, i.e. so long as the membrane remained intact.

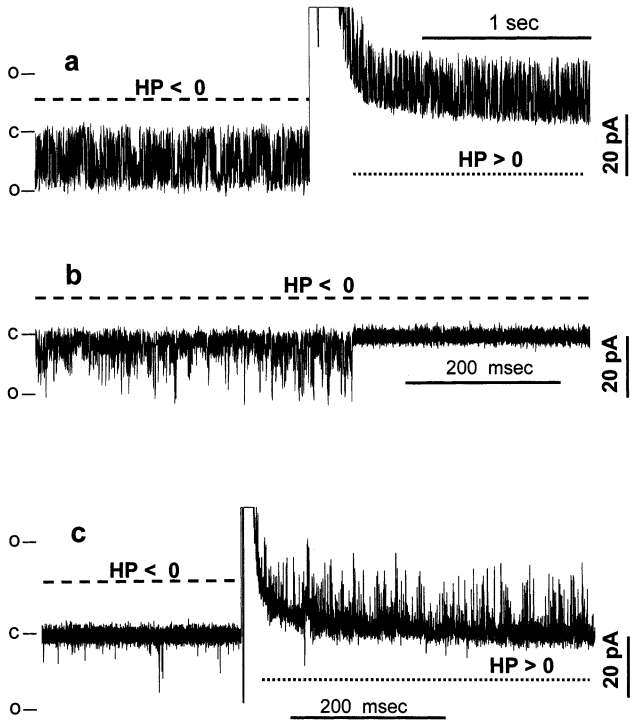

Fig. 1. Effect of troponin I_{trans} on RyR1 channel gating. Charge carrier 250 mM K^+, free [Ca^{2+}] of 50 μM at *cis* and *trans* sides. a: Current traces obtained after incorporation of the channel, at a holding potential of $H_p = -40$ mV (left side of a, marked with dashed line) and at $H_p = +40$ mV (right side of a, marked with dotted line) (overshot is due to the polarity change, consequence of the membrane capacity recharge to the new potential). The channel gating is identical at both polarities. b: Current traces at negative holding potential ($H_p = -40$ mV) after the addition of 200 nM troponin I_{trans}. Following the mixing, the channel enters a new gating mode, resulting in an apparent closed state which is distinguished from the ruthenium red-blocked channel only from the noise spectrum. c: This peculiar gating mode is characterized by an apparent closed state at negative holding potential only (left side of c, dashed line), since changing the holding potential to positive, the channel gating is similar to that before addition of troponin I (compare right sides of c and a, dotted line). Transition time required for the gating mode change is less than the time required for the capacitive transient to diminish (about 25 ms). Note the very rare 'extinguished' opening events, showing that the channel is still present in the bilayer and attempts to open (left side of c).

Fig. 2 shows that at a positive holding potential the channel exhibited 'control like' gating, while switching the holding

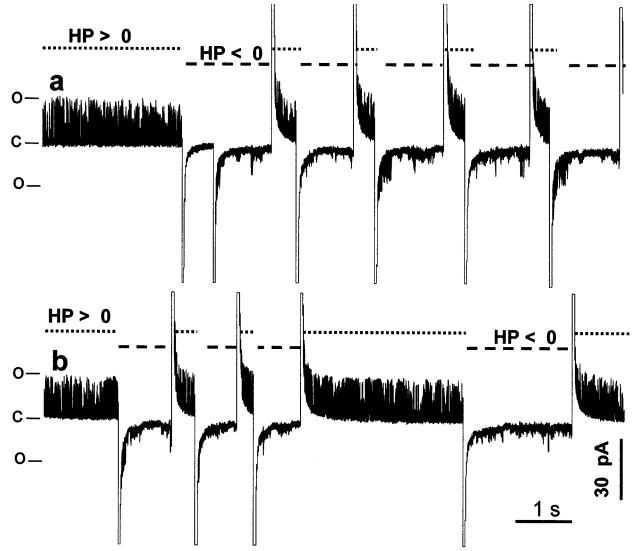

Fig. 2. Polarity-dependent gating mode sustained. The unique fashion of gating is maintained indefinitely, independently of duration of the applied potentials nor the frequency of polarity changes. Holding potential set to 43 mV. Note that the reported behavior is independent of the value of the applied holding potential, as tested in the range of 10 mV to 90 mV (absolute values). The lowest possible holding potential is determined by the noise of the current amplifier, limiting it to about 10 mV.

potential to a negative value resulted in the channel immediately locking into a closed state where it remained. The onset of this blocking behavior was delayed by a few seconds after addition of troponin I. This delay might be due to a diffusion-controlled movement of troponin I to the ion conducting pore. At constant negative holding potential the channel is locked and becomes inactivated, however, that is still present in the bilayer as it can be deduced from the observed rare opening attempts. The formation of this rectifying state is concentration-independent in the range between 0.2 μM and 2.7 μM troponin I.

To further clarify this surprising effect of troponin I the influences of troponin T, troponin C and histone were analyzed exactly as described above (Fig. 3).

None of these agents affected RyR1 activity at all, nor did they induce any polarity- or concentration-dependent channel gating.

To avoid the possibility of a contaminating component in the isolated troponin I – which might be responsible for the modulation of RyR1 gating – exactly the same single channel measurements were carried out in the absence and presence of human cardiac recombinant troponin I.

In the absence of the recombinant cardiac troponin I the channel gating was identical at positive and at negative holding potentials (Fig. 4a,b) and characterized by $P_o = 0.391 \pm 0.074$ and $\tau = 0.70 \pm 0.15$ ms ($n = 5$). Addition of the recombinant troponin I did not alter the gating of the RyR1 at a positive holding potential (Fig. 4c), however, at negative holding potential the channel was locked into a non-conducting state, and again short opening events could be observed. When the holding potential was switched to the positive range the channel gating was promptly and completely restored (Fig. 4d). On applying repetitive polarity changes, the RyR1 went into a long-standing closed state exclusively at negative holding potential, with a P_o value of almost zero.

These experiments show for the first time that, when applied to the luminal side and only at negative holding poten-

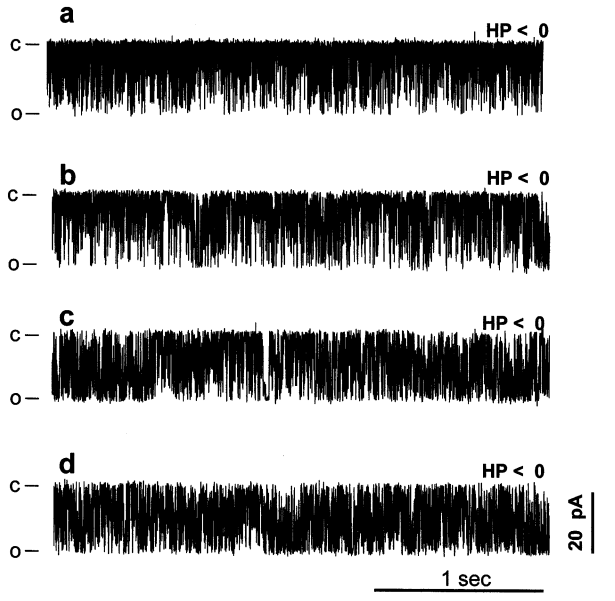

Fig. 3. Effect of troponin C, troponin T and histone on the RyR1 channel activity. Channel current recorded at positive and at negative holding potential of 40 mV. Charge carrier 250 mM K^+, free [Ca^{2+}] of 50 μM at *cis* and *trans* sides using Ca^{2+}-EGTA buffer. a: Representative current trace in the absence of modulators. b: Representative current record in the presence of 2 μM troponin C_{trans}. c: Representative current record in the presence of 2 μM troponin T_{trans}. d: Representative current record in the presence of 20 μM histone$_{trans}$.

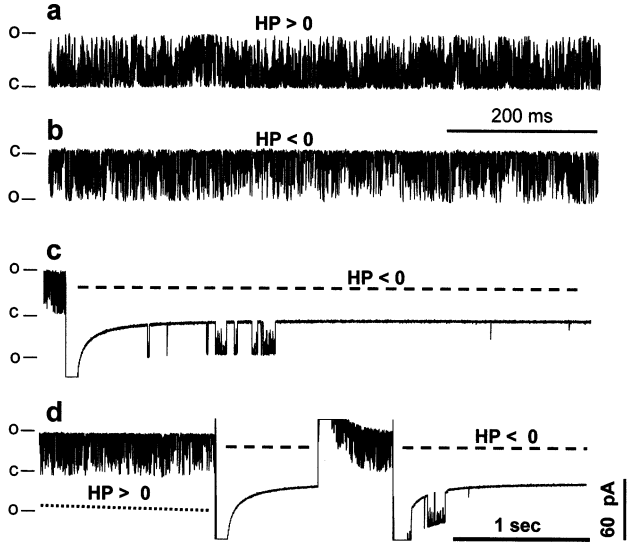

Fig. 4. Effect of human recombinant cardiac troponin I_{trans} on RyR1 channel activity. a,b: Channel current recorded at positive (a) and negative (b) holding potential of 80 mV. Channel gating is polarity- and potential (not shown)-independent. Charge carrier 250 mM K^+, free [Ca^{2+}] of 50 μM at *cis* and *trans* sides. c,d: Current records after the addition of 200 nM recombinant cardiac troponin I to the *trans* side of the chamber. Note the unique rectifier type behavior of the channel, which is basically identical to the unique rectifying character of the native skeletal muscle troponin I, composed of 'normal' gating at positive holding potential, while 'apparently closed' state at negative potential, containing only occasionally a few attempts to open (compare to Fig. 1).

tial, troponin I inactivates the SR Ca^{2+} release channel. This inactivation is a result of a non-conducting state, occurring in response to the negative holding potential, and can immediately be restored by switching the holding potential to a positive value. In our experiments troponin I – in the role of a luminally acting positively charged inactivating molecule – rectifies the Ca^{2+} gating of RyR1 very specifically. Our finding is very surprising and its physiological relevance is completely open. The transient inactivation of the RyR1 channel by a functional interaction with troponin I leads to a transient prompt inhibition of the Ca^{2+} release from the SR lumen into the cytosol. Troponin I is a strong basic protein (p*I* 8.9) and under physiological conditions it might interact with the acidic residues of the pore forming segment, which exhibits an estimated p*I* value of ca. 4.1 using its amino acid sequence information reported in [7]. These electrostatic interactions might help troponin I to find the right orientation for locking the channel into a non-conducting state. However, exclusively at negative holding potential the troponin I_{trans} will be 'fixed' and can interrupt the Ca^{2+} release process rapidly and very effectively. Furthermore, the transition of the Ca^{2+} release channel into a non-conducting state is very specific for troponin I, neither the basic protein histone, nor other subunits of the troponin complex, troponin C or troponin T are capable of inducing the same substate.

One might speculate as to the physiological role of these findings: Ca^{2+} release through the RyR channel generates a transient negative inside potential of the SR lumen. Relaxation caused by the reuptake of Ca^{2+} by the Ca^{2+} transport ATPase reduces continuously this transient negative potential. Even during Ca^{2+} release the calcium pump starts to work, and the membrane potential of the SR is determined by the net calcium flux.

At negative potential a troponin I like intraluminally located regulator, a polypeptide with similar structure and charge characteristics, locks the RyR1 into a non-conducting state, allowing no Ca^{2+} flux through the channel. It forces the channel to stay closed until the myoplasmic [Ca^{2+}] declines below a certain level thereby accelerating the relaxation process. Unfortunately, the noise associated with our bilayer measurements did not allow the determination of the voltage threshold at which the troponin I-induced effect starts.

This model provides a possible mechanism for an essential step, in the effective, reversible inhibition of Ca^{2+} fluxing through RyR1, which should be fast enough to terminate Ca^{2+} release during relaxation.

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