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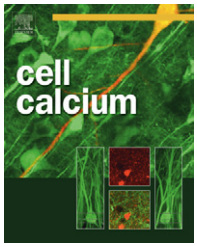

Phosphorylation of skeletal muscle calsequestrin enhances its Ca^{2+} binding capacity and promotes its association with junctin

Nicole A. Beard[a,*], Lan Wei[a], Stephanie N. Cheung[a], Takashi Kimura[a,1], Magdolna Varsányi[b], Angela F. Dulhunty[a]

[a] *John Curtin School of Medical Research, Australian Capital Territory, Australia*
[b] *Institut für Physiologische Chemie, Ruhr Universität, Bochum, Germany*

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Summary Calcium signaling, intrinsic to skeletal and cardiac muscle function, is critically dependent on the amount of calcium stored within the sarcoplasmic reticulum. Calsequestrin, the main calcium buffer in the sarcoplasmic reticulum, provides a pool of calcium for release through the ryanodine receptor and acts as a luminal calcium sensor for the channel via its interactions with triadin and junctin. We examined the influence of phosphorylation of calsequestrin on its ability to store calcium, to polymerise and to regulate ryanodine receptors by binding to triadin and junctin. Our hypothesis was that these parameters might be altered by phosphorylation of threonine 353, which is located near the calcium and triadin/junctin binding sites. Although phosphorylation increased the calcium binding capacity of calsequestrin nearly 2-fold, it did not alter calsequestrin polymerisation, its binding to triadin or junctin or inhibition of ryanodine receptor activity at 1 mM luminal calcium. Phosphorylation was required for calsequestrin binding to junctin when calcium concentration was low (100 nM), and ryanodine receptors were activated by dephosphorylated calsequestrin when it bound to triadin alone. These novel data shows that phosphorylated calsequestrin is required for high capacity calcium buffering and suggest that ryanodine receptor inhibition by calsequestrin is mediated by junctin.

Introduction

In skeletal muscle, Ca^{2+} release from the internal Ca^{2+} store, the sarcoplasmic reticulum (SR), triggers muscle contraction and this event is an integral part of the excitation–contraction-coupling process. Ca^{2+} flows through the Ca^{2+} release channel, the ryanodine receptor (RyR), which forms a luminal Ca^{2+} transduction machine with three

* Corresponding author at: John Curtin School of Medical Research, Australian National University, PO Box 334, Canberra, Australian Capital Territory 2601, Australia. Tel.: +61 2 6125 8391; fax: +61 2 6125 0415.
E-mail address: nicole.beard@anu.edu.au (N.A. Beard).
[1] Current address: Division of Neurology, Department of Internal Medicine, Hyogo College of Medicine, Hyogo, Japan.

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proteins within the SR lumen; calsequestrin (CSQ; the main SR Ca^{2+} storage protein) and triadin and junctin (anchoring proteins) [1]. Although the main role of CSQ is to maintain Ca^{2+} homeostasis by storing Ca^{2+} and providing Ca^{2+} for release through the RyR, it has recently been shown that CSQ also modifies Ca^{2+} signaling through its ability to regulate the activity of the RyR [2—7]. CSQ inhibits native skeletal RyR channels when associated with triadin and junctin [4,6] and ensures that RyR activity remains low when luminal Ca^{2+} concentrations are transiently reduced [8].

There is substantial evidence that CSQ is phosphorylated within the SR of skeletal muscle and that kinases capable of phosphorylation and ATP transport pathways into the SR exist. CSQ can be isolated in dephosphorylated [9] or in fully and partially phosphorylated forms [4,10,11], and it undergoes autophosphorylation [12], so both phosphoforms exist *in vivo*. CSQ contains a consensus sequence for phosphorylation by casein kinase II and can be phosphorylated by the kinase, which has been located inside the SR lumen [13]. There is strong evidence that voltage-dependent ion channels in the SR membrane provide the pathway for ATP entry into the SR [14]. These reports provide compelling evidence for the existence of a CSQ phosphorylation/dephosphorylation cycle within the SR lumen. However, the way in which phosphorylation alters CSQ function and its ability to regulate native RyR activity in response to changes in luminal Ca^{2+} has not been explored. We have shown that both phosphorylated and dephosphorylated CSQ can inhibit the native skeletal RyR channel when they bind to triadin and junctin in the presence of 1 mM luminal Ca^{2+} [4]. In contrast, it has been reported that only dephosphorylated CSQ is able to activate the purified channel [3]. One residue, threonine 353 (^{353}Thr), on skeletal CSQ has been shown to be phosphorylated [9]. This residue sits just upstream of the acidic rich C-terminal residues 354—367, which contain the putative major Ca^{2+} binding region and the proposed interaction sites for triadin [15,16]. The binding site on CSQ for junctin has been examined only in cardiac muscle where the acidic rich C-terminus is believed to form at least part of this interaction site [17]. This may well be the same in skeletal muscle since the junctin isoform expressed is the same in both tissues [18]. Given the proximity of the phosphorylatable residue to the acidic C-terminus, phosphorylation may alter the Ca^{2+} binding properties of CSQ, or may disturb its interactions with triadin and junctin.

The structure of CSQ depends on Ca^{2+} concentration, with dimers and polymers forming at higher Ca^{2+} concentrations [8,15,19—21]. Given that skeletal CSQ binds more calcium ions than predictions based on net negative charge alone [20], it is apparent that dimer/polymer formation is essential for CSQs high Ca^{2+} binding capacity. The accepted model of CSQ polymerisation proposes that low Ca^{2+} concentration promotes folding of the three thioredoxin-like domains of CSQ into a folded monomer [20,21]. Back-to-back dimerisation promotes folding of the disordered C-terminus, forming a more structured Ca^{2+} binding pocket [22]. Further increases in Ca^{2+} promote polymerisation, which results in the formation of a highly charged surface onto which Ca^{2+} can be absorbed [20]. Given that polymerisation is a Ca^{2+}-dependent process, if CSQ phosphorylation alters the Ca^{2+} binding capacity of CSQ, it might also alter dimer and polymer formation. The effects of phosphorylation on the ability of CSQ to polymerise, to associate with Ca^{2+}, or to bind to triadin and junctin have not been previously investigated.

In this study, we tested whether CSQ phosphorylation alters its Ca^{2+} binding capacity, its ability to form multimers and its association with anchoring proteins triadin and junctin. We show for the first time that phosphorylation of CSQ results in a significant increase in its Ca^{2+} binding capacity, but does not affect the Ca^{2+}-induced polymerisation of the protein. Nor is the interaction between CSQ and triadin influenced by phosphorylation of CSQ. Curiously, only phosphorylated CSQ can bind to junctin when the Ca^{2+} concentration is low.

Materials and methods

SR vesicle isolation

SR vesicles were prepared from back and leg muscles of New Zealand white rabbits using the methods of Saito et al. [23] with minor changes [24].

Junctional face membrane preparation

Junctional face membrane was isolated from either heavy SR [25] or SR vesicles as previously described [26], with minor changes [5].

Skeletal muscle CSQ purification

CSQ isolated from rabbit skeletal muscle was purified from junctional face membrane, using the methodology reported previously [26] with minor changes [4].

Purification of triadin and junctin

Triadin and junctin were purified from junctional face membrane loaded onto a 10% SDS polyacrylamide gel and separated electrophoretically. Bands of triadin (~95 kDa) and junctin (~26 kDa) were excised and eluted from the gel matrix by resuspension in a buffer containing 0.5% CHAPS, 20 mM MOPS and 150 mM NaCl (pH 7.4) for 24—36 h at either room temperature or 37 °C, with gentle agitation. The supernatant (containing the protein) was concentrated and dialyzed against 20 mM MOPS and 150 mM NaCl, pH 7.4 (to remove CHAPS). Purification was enhanced by immunoselection with anti-junctin or anti-triadin.

Expression of rabbit skeletal muscle recombinant CSQ

cDNA encoding rabbit skeletal muscle CSQ was subcloned, expressed and purified according to Beard et al. [4].

Electrophoresis and Western blot

SDS-PAGE was performed using the Laemmli buffer system [27], with 6.5% or 10% polyacrylamide gels, whilst Western blot was as per Towbin et al. [28].