This is the pre-peer-reviewed version of the following article: Murphy RM, Dutka TL, Horvath D, Bell JR, Delbridge LM and Lamb GD. (2013) Ca^{2+}-dependent Proteolysis of Junctophilin 1 and Junctophilin 2 in Skeletal and Cardiac Muscle JP in muscle weakness. Journal of Physiology, 591: 719-729, 2013, which has been published in final form at http://jp.physoc.org/content/591/3/719.full.pdf+html

Ca^{2+}-dependent Proteolysis of Junctophilin 1 and Junctophilin 2 in Skeletal and Cardiac Muscle

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Keywords: excitation-contraction coupling; muscle weakness; Ca^{2+} release

Word count: 4997

Table of Contents category: Skeletal muscle and exercise

Key Points

- If skeletal muscle fibres are subjected to excessive activation, or stretched whilst contracting, they subsequently display long term reductions in their force response, apparently due in part to structural or molecular changes at the triad junction, where excitation of the surface membrane triggers Ca^{2+} release from the internal Ca^{2+} store.
- The changes appear to be due to excessive or prolonged increases in intracellular Ca^{2+} levels, which activate Ca^{2+}-dependent proteases known as calpains, but their target proteins are currently unknown.
- This study shows that excessive muscle stimulation, or directly raising intracellular Ca^{2+} levels, causes calpain activation in tandem with proteolysis of junctophilin, a key protein thought to hold the triad junction together.
- Proteolysis of junctophilin is also seen in muscle of mice with muscle dystrophy and in cardiac muscle following ischaemic damage.
- Proteolysis of junctophilin may be a major factor causing muscle weakness and cardiac dysfunction in a range of circumstances.

Word count: 150

Abstract

Excessive increases in intracellular [Ca^{2+}] in skeletal muscle fibres cause failure of excitation-contraction (EC-) coupling by disrupting communication between the dihydropyridine receptors (DHPRs) in the transverse tubular (T-) system and the Ca^{2+} release channels (RyRs) in the sarcoplasmic reticulum (SR), but the exact mechanism is unknown. Previous work suggested a possible role of Ca^{2+}-dependent proteolysis in this uncoupling process, but found no proteolysis of the DHPRs, RyRs or triadin. Junctophilin-1 (JP1) (~90 kDa) stabilizes close apposition of the T-system and SR membranes in adult skeletal muscle; its C-terminal end is embedded in the SR and its N-terminal associates with the T-system membrane. Exposure of skeletal muscle homogenates to precisely set [Ca^{2+}] revealed that JP1 undergoes Ca^{2+}-dependent proteolysis over the physiological [Ca^{2+}] range in tandem with autolytic activation of endogenous μ-calpain. JP1 cleavage occurs close to the C-terminal, yielding a ~75 kDa diffusible fragment and a fixed ~15 kDa fragment. Depolarization-induced force responses in rat skinned fibres were abolished following 1 min exposure to 40μM Ca^{2+}, with accompanying loss of full-length JP1. Supra-physiological stimulation of rat skeletal muscle in-vitro by repeated tetanic stimulation in 30 mM caffeine also produced marked proteolysis of JP1 (and not RyR1). In dystrophic *mdx* mice, JP1 proteolysis is seen in limb muscles at 4 and not at 10 weeks of age. Junctophilin-2 in cardiac and skeletal muscle also undergoes Ca^{2+}-dependent proteolysis and JP2 levels are reduced following cardiac ischaemia-reperfusion. Junctophilin proteolysis may contribute to skeletal muscle weakness and cardiac dysfunction in a range of circumstances.

Abbreviations List

CrP, creatine phosphate; DHPR, dihydropyridine receptor; EDL, extensor digitorum longus; HDTA, hexa-methylene-diamine-tetraacetate; EC-coupling, excitation-contraction coupling; I/R, ischaemia-reperfused; JP1, junctophilin-1; JP2, junctophilin-2; LVDP, left ventricular developed pressure; MORN, membrane occupation and recognition nexus; n, number of fibres; RT, room temperature; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T-system, transverse tubular system; TA, tibialis anterior; wt, wild type

Introduction

Excitation-contraction (EC-) coupling in adult skeletal muscle depends on signal transmission across the triad junction, where the transverse tubular (T-) system abuts the terminal cisternae of the sarcoplasmic reticulum (SR). Action potentials activate the dihydropyridine receptors (DHPRs) in the T-system, which by some protein-protein interaction(s) open the Ca^{2+} release channels/ryanodine receptors (RyRs) in the adjacent SR, leading to a rise in cytoplasmic [Ca^{2+}] and consequent contraction. A range of experiments have shown that excessive or prolonged increases in cytoplasmic [Ca^{2+}] can lead to disruption of coupling between the DHPRs and the RyRs. Elevated intracellular [Ca^{2+}] completely abolishes depolarization-induced and action potential-induced force responses in skinned muscle fibres of rat and mouse in a concentration- and time-dependent manner, with half-maximal effects seen either by applying comparatively high Ca^{2+} levels for a short time (e.g. ~25 μM for 10 s) or lower levels for a longer time (e.g. ~5 μM for 180 s) (Lamb *et al.*, 1995; Verburg *et al.*, 2006). The failure is evidently caused by disrupted coupling between the DHPRs and the RyRs, as the T-system shows no indication of chronic depolarization and Ca^{2+} can still be released from the SR by directly activating the RyRs with caffeine or low cytoplasmic [Mg^{2+}]. Furthermore, electron microscopy shows structural alterations in the triad junctions in the Ca^{2+}-treated fibres (Lamb *et al.*, 1995). Coupling can also be disrupted both in intact (Chin & Allen, 1996) and skinned fibres (Lamb *et al.*, 1995; Verburg *et al.*, 2006) by evoking excessive or prolonged release of SR Ca^{2+}, such as by stimulating fibres simultaneously with caffeine and depolarization. Eccentric (or lengthening) contraction in muscle fibres also produces disrupted coupling between the DHPRs and RyRs (Warren *et al.*, 1993; Balnave & Allen, 1995; Ingalls *et al.*, 1998; Corona *et al.*, 2010), and is associated with increased influx of extracellular Ca^{2+} and raised resting [Ca^{2+}] (Ingalls *et al.*, 1998; Allen, 2004). Furthermore, eccentric contractions cause Ca^{2+}-dependent disruption of EC-coupling more readily in dystrophic *mdx* mice than in wild type mice (Yeung *et al.*, 2005), and interestingly AP-induced Ca^{2+} release in isolated *mdx* muscle fibres is impaired to some degree even without eccentric stimulation (Woods *et al.*, 2004).

The mechanism underlying the Ca^{2+}-dependent disruption of DHPR-RyR communication is unknown, but a range of evidence suggests possible involvement of the Ca^{2+}-dependent protease, μ-calpain. Firstly, the [Ca^{2+}] range over which the disruption occurs is similar to that for

activation of μ-calpain (Murphy *et al.*, 2006a; Murphy *et al.*, 2006b). Secondly, low temperature (3°C) and acidic conditions (pH 5.8) greatly slow the rate of coupling disruption (Lamb *et al.*, 1995), similar to their effects on μ-calpain activity (Inomata *et al.*, 1984; Ono *et al.*, 2004), and Sr^{2+} activates both processes at a similar concentration which is ~10 to 20 fold higher than for Ca^{2+} in both cases (Inomata *et al.*, 1984; Lamb *et al.*, 1995). Thirdly, calpain has been observed to localize near the triadic SR (Gilchrist *et al.*, 1992), and applying exogenous μ-calpain cleaves triad junctions (Kim *et al.*, 1990), and disrupts EC-coupling in a Ca^{2+}-dependent manner (Verburg *et al.*, 2009). However, to date no triadic protein has been identified as undergoing Ca^{2+}-dependent proteolysis in tandem with the coupling disruption, and in particular the DHPR, RyR and triadin were all found to remain intact in fibres where coupling had been fully disrupted (Lamb *et al.*, 1995).

It is currently thought that the junctophilin family of proteins are primarily responsible for mediating the close contact between the SR and the cell surface/T-tubules in both skeletal and cardiac muscle cells (Takeshima *et al.*, 2000; Ito *et al.*, 2001; Komazaki *et al.*, 2002; Hirata *et al.*, 2006). Both the junctophilin-1 (JP1) and junctophilin-2 (JP2) isoforms are localized at the triad junction in skeletal muscle fibres, whereas it is primarily JP2 that is present at diad/triad junctions in cardiac muscle cells (Takeshima *et al.*, 2000; Ito *et al.*, 2001). Knockout of JP2 is embryonically lethal because the SR in the cardiac cells fails to form normal diadic junctions and the mice experience cardiac arrest (Takeshima *et al.*, 2000). Mice with JP1 knocked out have abnormal skeletal muscle triad formation and die at birth despite the presence of JP2, leading to the proposal that JP2 is sufficient for some degree of coupling between the SR and the cell surface/T-tubule whereas JP1 is needed for proper formation and maintenance of the tight triad junctions characteristic of mature skeletal muscle (Ito *et al.*, 2001; Komazaki *et al.*, 2002). The structures of JP1 and JP2 are highly conserved across mammalian species, with the proteins observed to run on SDS-PAGE at ~90 kDa and ~100 kDa, respectively (Takeshima *et al.*, 2000; Phimister *et al.*, 2007; Golini *et al.*, 2011). In addition to having a structural role at the triad junction in skeletal muscle, JP1 interacts with the RyRs in a conformationally-sensitive manner (Phimister *et al.*, 2007), and both JP1 and JP2 interact with the DHPRs (Golini *et al.*, 2011). Both JP1 and JP2 insert into the SR membrane via a transmembrane region in their C-terminal, and their N-terminal ends associate with the surface/T-tubule membrane via so-called ‘MORN’ (Membrane Occupation and Recognition Nexus) motifs.

The present study examines whether JP1 and JP2 undergo Ca^{2+}-dependent proteolysis, and whether this occurs in conjunction with the disruption of EC-coupling in Ca^{2+}-treated skinned fibres and in other intact muscle situations such as following supra-physiological stimulation of skeletal muscle and ischaemia-reperfusion in heart.

Methods

All animal experiments were carried out in accordance with the Australian NHMRC's 'Australian code of practice for care and use of animals for scientific purposes', and with approval of the Animal Ethics Committees of La Trobe University, Victoria University and University of Melbourne. Male Long-Evans hooded rats (12, 5-12 months old) were killed by isoflurane overdose (4% v/v) in a glass chamber and the extensor digitorum longus (EDL) muscle and heart removed. C57BL/10 and mdx (C57BL/10ScSn-Dmdmdx) mice (20 in total, Animal Resource Centre Western Australia) were killed by cardiac excision while anaesthetized with pentobarbitone sodium (Rhone Merieux, Queensland, Australia) (40mg/kg i.p.) and the tibialis anterior (TA) and diaphragm muscles removed. For whole heart preparations, 16 additional adult (16 wk) Sprague Dawley rats were anaesthetized with sodium pentobarbitone (60mg/kg i.p.) and injected with sodium heparin (200IU) via the femoral vein, and the hearts rapidly excised (see below).

Human vastus lateralis biopsy samples from four males aged 18-40 years (2 healthy and 2 diagnosed as having limb girdle muscular dystrophy 2A with dysfunctional calpain-3) were obtained from the Neurogenetic Biospeciman Bank at The Children's Hospital at Westmead, Sydney; the results described later were similar for all four cases. All procedures conformed to the *Declaration of Helsinki*, informed consent was obtained in writing from all subjects, and the Human Research Ethics Committees of the Children's Hospital at Westmead and La Trobe University approved the studies.

Skinned fibre solutions

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. K-HDTA solution contained (mM): HDTA, 50; EGTA, 0.05; total ATP, 8; creatine phosphate (CrP), 10; Na^+, 36; K^+, 126; total Mg, 8.6; total; Hepes, 90; pH 7.1 and 0.1 μM free Ca^{2+}. Skinned fibres depolarized in Na-HDTA solution (K-HDTA solution with all K^+ replaced by Na^+). Rigor solution similar to K-HDTA solution but with 66 mM HDTA, 0.25 mM EGTA and no ATP or CrP, and Ca-rigor solution had 40 μM free Ca^{2+}.

Skinned fibre preparations

Rat EDL muscles were pinned at resting length under paraffin oil at ~10°C. Individual fibre segments were mechanically skinned and mounted at 120% of resting length on a force transducer (AME801, SensoNor, Horten, Norway), placed in a bath with K-HDTA solution at room temperature (RT), and depolarized and Ca^{2+} treated as previously (Lamb *et al.*, 1995). Force responses recorded using Powerlab 4/20 hardware (ADInstruments, Sydney, Australia).

In-vitro stimulation of rat muscles

EDL muscles were attached to a force-transducer in a bath with Krebs-Ringer solution bubbled with 95% O_2 and 5% CO_2 (pH ~7.4) at RT, and stimulated by field stimulation (1 ms pulses). Isometric force responses were recorded as for skinned fibres. Supra-physiological stimulation was elicited by adding 30 mM caffeine to bathing solution and 1 min later applying repeated 50 Hz stimulation (for 0.5 s every 2 s) until tetanic force decreased to <10% of initial (~3 to 4 min). Superficial fibres dissected and Western blotting performed on groups of three fibre segments. JP1 diffusibility examined as in (Murphy *et al.*, 2006b; Murphy *et al.*, 2011) by skinning fibres and bathing a group of 3 segments in 10 µl of relaxing solution for 5 min, and analyzing the contents of the solution and fibre samples by Western blotting (Fig. 3E).

Exposure of muscle homogenate to elevated [Ca^{2+}]

Thin (10µm) cyrosections of human skeletal muscle biopsies (6-8 sections per sample) were prepared in solutions with free Ca^{2+} strongly buffered to set levels in the range ~10 nM to 500 µM with 50 mM CaEGTA-EGTA) (vol. to muscle mass ≥10:1) and kept at RT for 60 min. Pieces of fresh or frozen EDL muscle of rat were homogenized and treated similarly.

Isolated heart preparations

Hearts (n=8) were retrogradely perfused with oxygenated (95% O_2, 5% CO_2) bicarbonate buffer (37˚C, pH 7.4) in the non-recirculating Langendorff mode at a constant pressure equivalent to 73 mmHg using an STH pump controller (ADInstruments). Bicarbonate buffer contained (in mM) 118.5 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 25.0 $NaHCO_3$, 1.2 $MgCl_2$, 1.4 $CaCl_2$, and 11.1 glucose. Left ventricular pressure measurements were performed using a fluid-filled balloon connected to a pressure transducer (MLT844) and recorded on a MacLab data acquisition system

(ADInstruments). Following an aerobic stabilization period, hearts were subjected to 20 min global ischemia (37˚C) and 60 min reperfusion, and allowed to beat spontaneously throughout. Control hearts were perfused aerobically throughout. Hearts were frozen in liquid nitrogen at the end of the perfusion protocols and subsequently homogenized (10% wt/vol) in buffer containing (in mM) 50.0 Tris-HCl, 5.0 EGTA, 5.0 EDTA, 5 NaF, 0.5 Na_3VO_4 and protease inhibitors (Roche, Basel, Switzerland) at 4˚C, and added to 2x SDS sample buffer.

Western blotting

Western blotting was performed as previously described (Murphy *et al.*, 2011). Primary antibodies used were: rabbit anti-JP1 (mid-region, 1 in 300 or C-terminal, 1 in 250), rabbit anti-JP2 (1 in 250), mouse anti-μ-calpain antibody (1 in 1000) (all from Invitrogen, Sydney, Australia), mouse anti-RyR1 (34C, Development Studies Hybridoma Bank; 1 in 100) and mouse anti-RyR2 (Badrilla, UK; 1 in 300). Protein bands were visualized using west-femto chemiluminescent substrate (Thermoscientific, Australia) using a Chemidoc MP (BioRad, Sydney, Australia) with ImageLab software (BioRad) for collection of images and data analyses. A three or four point standard curve was generated on every gel by running a range of homogenate samples encompassing the test sample range (e.g. Fig. 5A) to ensure any differences in band densities were detectable.

Results

Ca^{2+}-dependent proteolysis of junctophilins in skeletal muscle

JP1 in both human and rat skeletal muscle underwent Ca^{2+}-dependent proteolysis by endogenous proteases when exposed to elevated [Ca^{2+}]. Fig. 1A. (middle panel) shows a Western blot for JP1 in Ca^{2+}-treated human muscle cryosections. Using an antibody to the mid-region of JP1, skeletal muscle tissue maintained in low [Ca^{2+}] conditions (e.g. <10 nM Ca^{2+}) for 60 min displayed only a single band running at ~90 kDa (normal full-length form of JP1), whereas muscle exposed to [Ca^{2+}] higher than ~0.5 μM showed progressively larger loss of the 90 kDa band and appearance of a ~75 kDa band, which was evidently a proteolytic fragment. Western blotting for μ-calpain in these same preparations showed a matching increase in autolytic activation of μ-calpain over the same [Ca^{2+}] range (Fig. 1A&B). Similar results were seen in 4 independent human muscle preparations, and also in rat skeletal muscle (not shown). Proteolysis of JP1 could also be detected using a C-terminal antibody, which showed the same Ca^{2+}-dependent loss of the 90 kDa full-length band accompanied by formation of a ~15 kDa C-terminal fragment (see later). Junctophilin-2 (JP2) in skeletal muscle underwent similar Ca^{2+}-dependent proteolysis, with complete loss of full-length JP2 observed following a 60 min exposure to 500 μM Ca^{2+} at RT in four independent rat muscle preparations (e.g. Fig. 1C), though no proteolytic fragment was detected with the JP2 C-terminal antibody used, possibly because the proteolysis affected the antibody binding site.

Ca^{2+}-mediated disruption of EC-coupling in skinned muscle fibres

As described previously (Lamb *et al.*, 1995; Verburg *et al.*, 2006), depolarization-induced force responses in skinned fibres were completely and irreversibly abolished following a 1 min exposure to a free [Ca^{2+}] of 40 μM or above (e.g. Fig. 2A). Western blotting of such Ca^{2+}-treated fibres (Fig. 2B) revealed significant loss of full-length JP1 in fibre segments treated with 40 μM Ca^{2+} compared to other fibres treated similarly but exposed to a solution with [Ca^{2+}] kept at <10 nM (Fig. 2B&C). The presence of a 75 kDa proteolytic fragment of JP1 was not detected in the Western blotting of these single fibre segments, possibly owing to detection limitations in these small samples or to further proteolysis or diffusional loss of the 75 kDa fragment (see later). Figure 2D illustrates the possible role of JP1 proteolysis in Ca^{2+}-dependent disruption of EC-coupling.

Supra-physiological stimulation of skeletal muscles also leads to JP1 proteolysis

Figure 3 shows that JP1 proteolysis can also be brought about by raising the intracellular Ca^{2+} level within intact muscle fibres by stimulation of whole muscles in-vitro. Rat EDL muscles were subjected to repeated 50 Hz simulation for ~4 min in the presence of 30 mM caffeine, in order to elevate the intracellular [Ca^{2+}] substantially for a prolonged period (Chin & Allen, 1996). Western blotting of superficial fibres from these stimulated muscles revealed clear loss of full-length (90 kDa) JP1, accompanied by formation of both the 75 kDa and 15 kDa proteolytic fragments (Fig. 3A, lower two panels; Western blotting performed with 3 fibre segments in each lane to improve detection limits). The amount of 75 kDa fragment detected in a given sample, expressed relative to the sum of the 90 kDa and 75 kDa bands, was ~43% in fibre samples from the stimulated muscles and <5 % in fibre samples from the non-stimulated contralateral muscles (Fig. 3B). Western blotting of the lower portion of the same gels with the JP1 C-terminal antibody showed greatly increased amounts of the 15 kDa band in the fibres from the stimulated muscle relative to those from the non-stimulated muscles (Fig. 3A&C), with the amount of the 15 kDa band being highly correlated with the appearance of the 75 kDa band detected with the mid-region antibody in the same fibre samples (R^2 =0.70, P<0.001, n=17 fibres from 3 muscles, not shown). In contrast to the very substantial level of JP1 proteolysis, Western blotting of a subset of the same samples showed no evidence of proteolysis of RyR1 (e.g. Fig. 3A, only full-length RyR1 was apparent) nor significant decrease in the amount of RyR1 present in the stimulated samples (Fig. 3D). It is possible nevertheless that some proteolysis of RyR1 occurred but that it fell below detection limits.

75 kDa JP1 proteolytic fragment is diffusible

In order to investigate whether JP1 and its proteolytic fragments were diffusible or fixed inside muscle fibres, fibre segments from a supra-stimulated muscle were skinned under paraffin oil and bathed in an intracellular-like solution ('wash solution') for 5 min, and then Western blotting used to examine the presence of JP1 and its fragments in the wash solution and fibre segments (see Methods and Murphy *et al*. (2006b)). Fig. 2E displays two examples showing ~15 and 40% of the total 75 kDa fragment present had diffused into the wash solution without any detectable washout of either full-length JP1 or the 15 kDa C-terminal fragment. The MHC bands in the Stain Free gel (top of Fig. 3E) confirm that the fibre segments were fully removed from the wash.

These findings are consistent with full-length JP1 being embedded in the SR membrane at its C-terminal end and with proteolysis creating a potentially diffusible 75 kDa N-terminal fragment and a 15 kDa fixed C-terminal fragment (see Fig. 2D).

Proteolysed JP1 and JP2 in limb and diaphragm muscle in *mdx* mice

In *mdx* dystrophic mice, limb muscles display an acute period of degeneration and weakness at ~3 weeks of age but then recover to near normal by ~12 weeks, whereas the diaphragm muscle shows progressively greater deterioration and weakness throughout adulthood (Dupont-Versteegden & McCarter, 1992). In 28 day *mdx* mice, ~60% of the JP1 in TA muscle was in the 75 kDa proteolysed form, whereas in wild type mice of the same age the percentage was only ~10% (Fig. 4A&B). There was also marked activation of the μ-calpain in the 28 day *mdx* muscle (not shown). At 70 days the extent of JP1 proteolysis in the TA muscles of the *mdx* mice had dropped back to levels close to that in wild type. Similarly, there was a marked loss of full-length JP2 in the TA muscle of *mdx* mice at 28 days, but it recovered to wild type levels by 70 days of age (Fig. 4C&D). The amounts of both JP1 and JP2 in TA muscles of wild type mice (expressed relative to MHC) increased ~2 fold between day 28 and 70 (e.g. Fig. 4D), possibly owing to the relative increase in the extensiveness of the T-system in muscle fibres as they become larger with maturity. In contrast to the recovery of JP1 levels in the TA muscle of *mdx* mice, marked JP1 proteolysis was observed in the diaphragm at 7 months of age, as well as activation of μ-calpain (Fig. 4E).

Loss of JP2 in heart muscle following ischaemia-reperfusion

Treatment of rat cardiac ventricular tissue with 0.5 mM Ca^{2+} for 60 min at RT resulted in ~50% loss of JP2 (Fig. 5A&B). Interestingly, the loss of JP2 in cardiac muscle was less than that in skeletal tissue run in parallel (e.g. Fig. 1C), perhaps due to differences in the level or location of calpain or its inhibitor calpastatin in the two types of tissue. Following ischaemia-reperfusion in rat hearts, left ventricular developed pressure (LVDP) decreased to ~25% of initial level (Fig. 5C) and the amount of full-length JP2 found in the hearts was significantly lower (~25%) than in matched control hearts (Fig. 5D&E). In contrast, there was no detectable proteolytic cleavage or loss of RyR2 in the same samples (e.g. Fig. 5D).

Discussion

This study demonstrates that both JP1 and JP2 in skeletal muscle undergo Ca^{2+}-dependent proteolysis by endogenous proteases when the intracellular [Ca^{2+}] is raised within the physiological range for a sustained period (Fig. 1). This is the first demonstration that any members of the junctophilin family undergo such proteolysis. The proteolysis was likely brought about by the ubiquitous calpains, in particular by μ-calpain, which was found to be autolytically activated over the same Ca^{2+} range in the samples (Fig. 1), in agreement with our previous studies (Murphy *et al*., 2006a,b). It was further shown that JP1 in skeletal muscle fibres could be proteolysed by raising the intracellular [Ca^{2+}] by supra-physiological stimulation of muscles in-vitro (Fig. 3). JP1 proteolysis was seen in rat, mouse and human skeletal muscle and seemingly occurs at a single site near the C-terminal end, leading to formation of ~75 kDa N-terminal and ~15 kDa C-terminal fragments, with the latter remaining embedded in the SR membrane. The N-terminal end of JP1 is thought to bind via MORN motifs to the T-tubule membrane (Takeshima *et al.*, 2000), but following proteolysis the 75 kDa fragment evidently becomes diffusible to some extent (Fig. 3E), and in such a situation JP1 could no longer be functioning as a physical link between the T-tubule and SR membranes (see Fig. 2D).

Thus, the loss of EC-coupling occurring in skinned fibres exposed to raised [Ca^{2+}] (Lamb *et al.*, 1995) can be accounted for at least in part by the apparent Ca^{2+}-dependent proteolysis of JP1 occurring in such fibres (Fig. 2). This is also consistent with previous observations that i) the Ca^{2+} treatment activates μ-calpain in the skinned fibres and activated μ-calpain both disrupts EC-coupling (Verburg *et al.*, 2009) and cleaves triad junctions (Kim *et al.*, 1990), and ii) supra-physiological stimulation of isolated muscles or fibres activates μ-calpain (Murphy *et al.*, 2006b) and disrupts EC-coupling (Lamb *et al.*, 1995; Chin & Allen, 1996) without proteolysis of the DHPRs or RyRs themselves. RyRs in skinned fibres can be proteolysed by application of exogenous m-calpain in 2 mM Ca^{2+} for 60 min (Iino *et al.*, 1992), but the findings here (Fig. 3) indicate that activation of the endogenous calpains in intact muscle fibres appears to have proportionately greater effect on JP1 than on the RyRs. Disruption of coupling nevertheless may be determined by the summed effect of proteolysis of a number of different junctional proteins. The postulated change at the triad junction occurring with JP1 proteolysis (Fig 2D) may also

account for the increased SR Ca^{2+} leakage seen after Ca^{2+} treatment (Lamb & Cellini, 1999), brought about by the loss of the normal inhibitory actions of the DHPRs on the RyRs.

Ca^{2+}-dependent proteolysis of JP1 likely also contributes to the disruption of EC-coupling occurring after eccentric contractions (see Introduction). Corona et al (2010) found that levels of full-length JP1 and JP2 were reduced following eccentric contractions in mouse, though they attributed this to mechanical damage to the proteins rather than to Ca^{2+}-dependent proteolysis because their previous experiments had found that the force loss was not prevented by calpain inhibitors or removal of extracellular Ca^{2+}. However, using a similar preparation another group has observed that μ-calpain becomes activated with eccentric contractions (Zhang *et al.*, 2012), and that the force loss can be reduced both by Ca^{2+} removal and the calpain inhibitor leupeptin (Zhang *et al.*, 2008). We have previously shown that Ca^{2+}-disruption of EC-coupling in skinned fibres can be reduced with calpain inhibitors, but only if the uncoupling occurs at a low rate (Lamb *et al.*, 1995), likely owing to the difficulty of controlling Ca^{2+}-dependent processes within the triad junction, particularly given that the inhibitors only act on calpain after it is activated.

Ca^{2+}-dependent proteolysis of JP1 may also underlie reductions in Ca^{2+} release seen in dystrophic muscle (Woods *et al.*, 2004; Yeung *et al.*, 2005). In *mdx* mice, limb muscles display a transient period of degeneration and weakness at 3 to 4 weeks of age, at which time there is calpain activation and major proteolytic loss of both JP1 and JP2 (Fig. 4). In contrast to the limb muscles, the diaphragm shows progressively greater dystrophy and weakness into and throughout adulthood (Dupont-Versteegden & McCarter, 1992), and marked calpain activation and junctophilin proteolysis can be seen in *mdx* mice at 7 months (Fig. 4). Stretch readily induces elevated intracellular [Ca^{2+}] in *mdx* muscle fibres (Yeung *et al.*, 2005), probably due to stretch-induced production of reactive oxygen species (Khairallah *et al.*, 2012) leading to both increased influx of extracellular Ca^{2+} (Whitehead *et al.*, 2010) and increased release of SR Ca^{2+} (Bellinger *et al.*, 2009). The findings here suggest that increases in intracellular [Ca^{2+}] by either mechanism would readily lead to calpain activation and proteolysis of the junctophilins.

Finally, our results suggest that Ca^{2+}-dependent proteolysis of JP2 may contribute to the impaired SR Ca^{2+} release and contractility occurring in heart muscle after ischaemia-reperfusion.

Ischaemia-reperfusion in rat hearts sufficient to induce marked deterioration of LVDP is accompanied by increased calpain activity (Singh *et al.*, 2012), and calpain inhibitors reduce both calpain activity and the decrease in LVDP (Singh & Dhalla, 2010). In the present study a loss of JP2 following ischaemia-reperfusion was observed (Fig. 5), which might be expected to have had deleterious effect on SR Ca^{2+} release, given that cardiac-specific knockdown of JP2 has been shown to result in disrupted junctional membrane complexes and reduced EC-coupling gain (van Oort *et al.*, 2011).

Concluding remarks

Our findings show that both JP1 and JP2 undergo Ca^{2+}-dependent proteolysis in skeletal and cardiac muscle and suggest that such proteolysis may be a significant factor in reduced Ca^{2+} release and consequent muscle weakness and cardiac dysfunction in a range of circumstances.

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Author contributions

RMM and GDL conceived and designed most experiments and drafted the manuscript. All authors contributed to collection, analysis and interpretation of data. All authors have approved the final version of the submitted manuscript. Biochemical analysis of all tissue and physiological measurements on skinned muscle fibres were performed at La Trobe University and the whole heart preparation performed at University of Melbourne.

Acknowledgments

We thank Maria Cellini and Heidy Latchman for technical assistance, Professor Kathy North for facilitating access to the Neurogenetic Biospeciman Bank, and the National Health & Medical Research Council of Australia for financial support (Grant number 602538). The monoclonal antibody directed against RyR1 was developed by Drs J. Airey and J. Sutko and obtained from the Development Studies Hybridoma Bank, under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA.

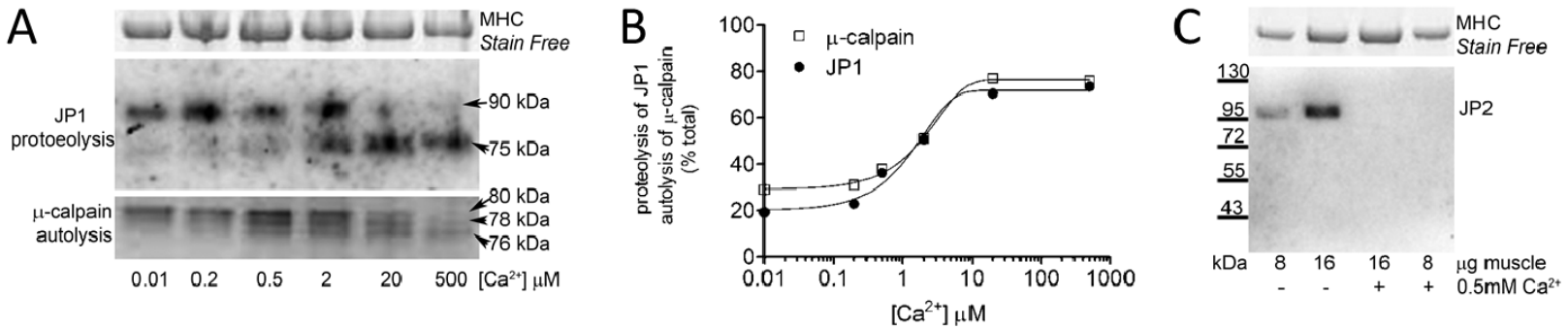

Figure 1. JP1 and JP2 undergo Ca^{2+}-dependent proteolysis by endogenous proteases. *A*, Western blot of human skeletal muscle cryosections following exposure to various set [Ca^{2+}] for 60 min at RT. Whole muscle separated on 10% Stain Free gels and probed using a mid-region JP1 antibody, which detects full-length 90 kDa JP1 as well as proteolysed 75 kDa JP1. MHC bands in Stain Free gel used as loading control (top panel). Separate gel run to detect μ-calpain in same homogenate samples (lower panel). Full-length μ-calpain is detected at 80 kDa, and autolyses to 78 and 76 kDa forms, indicative it has been activated. *B*, Percentage proteolysis (JP1) or autolysis (μ-calpain) following exposure to indicated [Ca^{2+}]; data are mean values from two repeats on separate gels. *C*, Western blot of JP2 in rat EDL muscle homogenates treated with or without 0.5 mM Ca^{2+} for 60 min at RT, separated on 4-15% Stain Free gel.

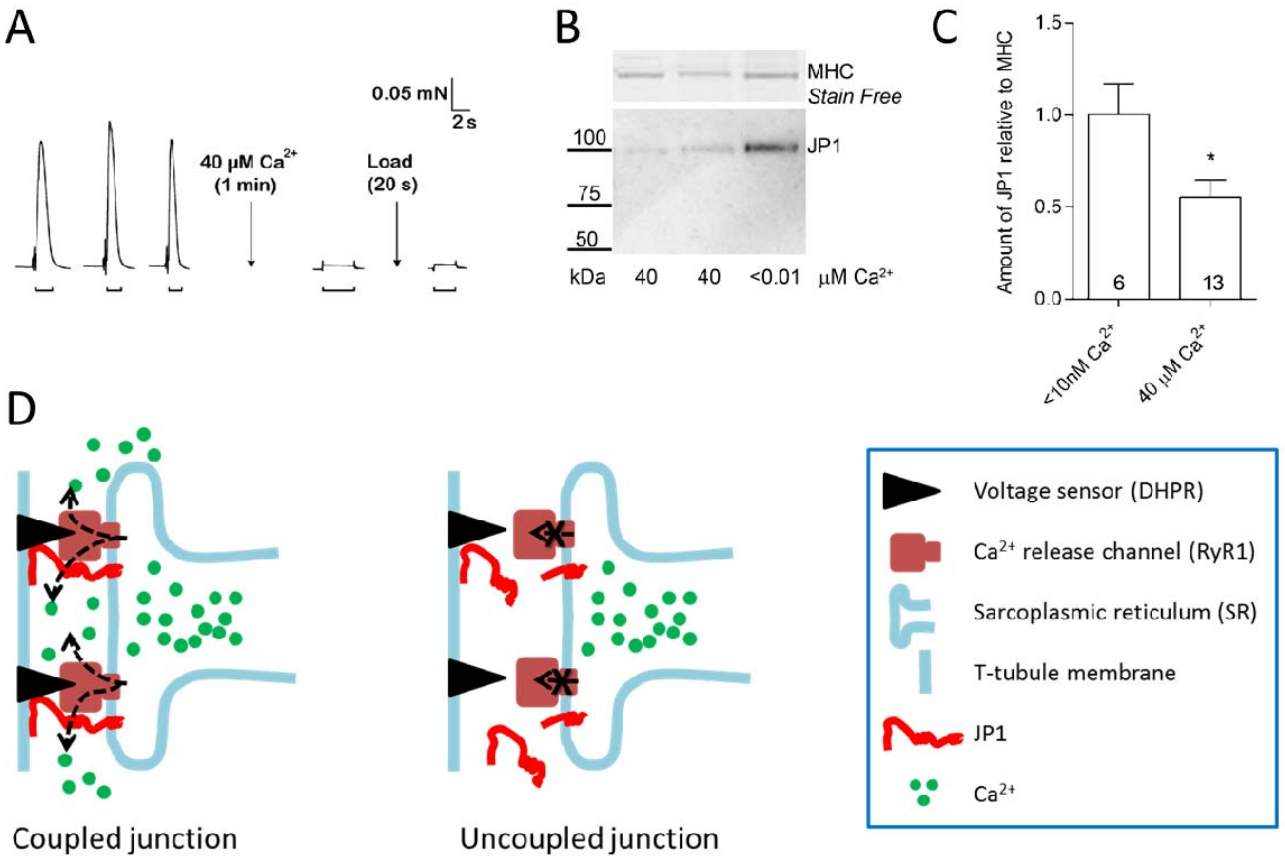

Figure 2. Proteolytic loss of JP1 in skinned fibres after Ca^{2+} disruption of EC-coupling. *A*, Depolarization-induced force responses in a skinned EDL fibre of rat were abolished after 1 min exposure to 40 µM free Ca^{2+} (applied in rigor conditions, see Methods) and were not restored by additional SR Ca^{2+} loading. *B*, Western blot for JP1 in single fibre segment shown in *A* (run in lane 1) and for two other segments treated with 40 µM Ca^{2+} or similarly with Ca^{2+} at <10 nM. 10% Stain Free gel blotted with mid-region JP1 antibody. *C*, Mean (+SEM) amount of JP1 detected in individual fibre segments exposed to indicated Ca^{2+} (number of fibres shown in bars). JP1 signal for each fibre normalised to MHC band and expressed relative to mean value for fibres exposed to 10 nM Ca^{2+} on same blot (3 on each gel) (* $P<0.05$, Student's 2-tailed unpaired t-test). *D*, Diagram depicting a coupled (functional) triad junction in skeletal muscle. After Ca^{2+} treatment the triad junction becomes dysfunctional (uncoupled), possibly owing to cleavage of JP1 and loss of normal communication between the DHPRs and RyRs.

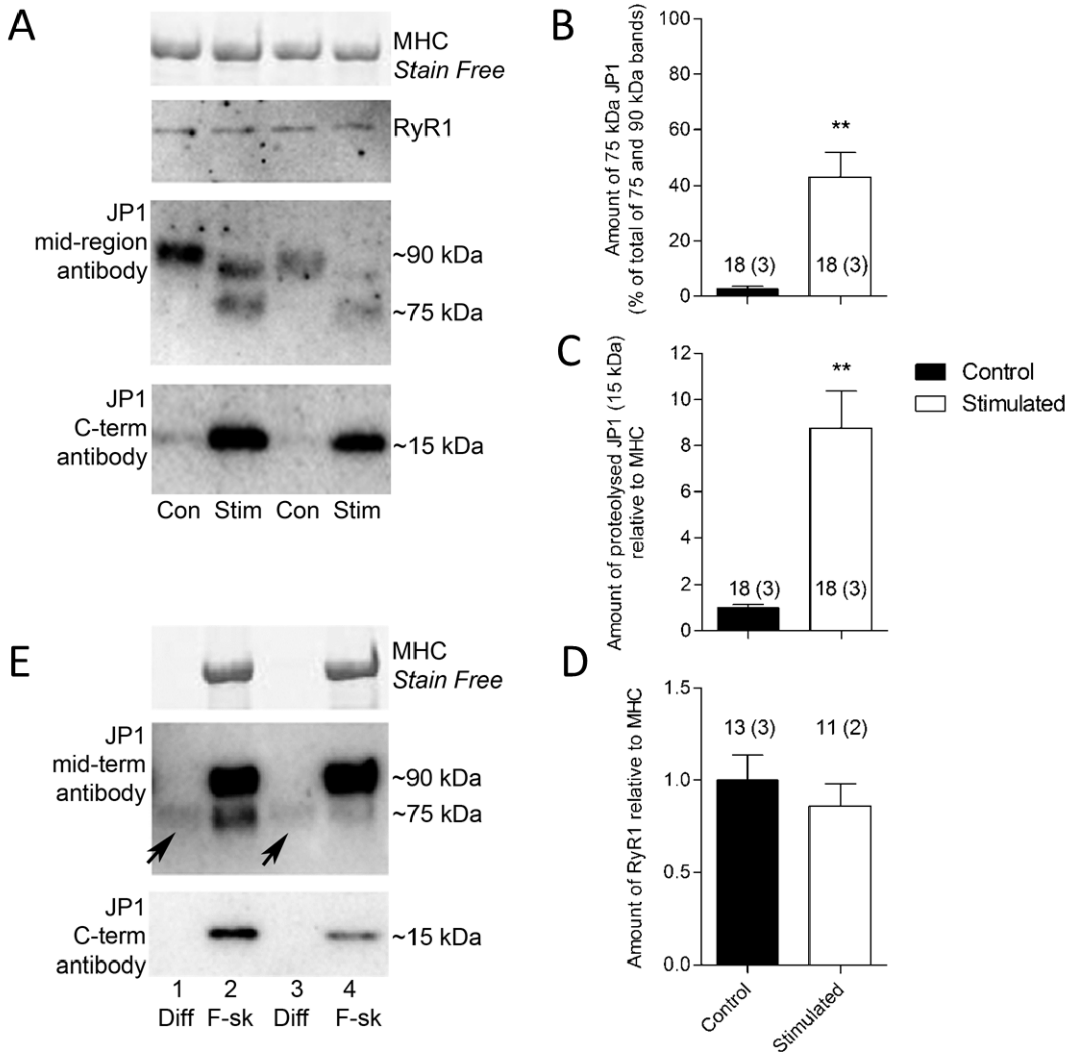

Figure 3. Proteolysis of JP1 in intensively stimulated muscles. *A*, Western blot of JP1 in fibres from rat EDL muscles stimulated intensively in-vitro in presence of 30 mM caffeine (Stim) and from non-stimulated contralateral muscles (Con); 3 fibre segments pooled in each lane. Proteins separated on 4-15% Stain Free gels and probed for JP1 using mid-region or C-terminal antibody, and also for RyR1. *B*, Mean (+SEM) amount of 75 kDa JP1 fragment in fibre samples from Stim and Con muscles, expressed as percentage of total JP1 detected (i.e. sum of 75 and 90 kDa bands). Number of samples shown with each bar, number of muscles in brackets (** $P<0.01$, Mann-Whitney 2 tailed test). *C*, Mean (+SEM) amount of 15 kDa JP1 fragment in samples (each value first normalised to MHC). *D*, Mean (+SEM) amount of RyR1 in samples. *E*, Western blot of JP1 and 75 kDa and 15 kDa proteolytic fragments remaining in skinned fibre (F-sk) or diffusing into wash solution (Diff) after bathing skinned fibre segments in wash solution for 5 min (see Methods). Fibres obtained from stimulated muscle; 3 skinned segments used in each of two wash-fibre sets shown. A substantial proportion of the 75 kDa proteolytic fragments diffused into wash solution (arrows).

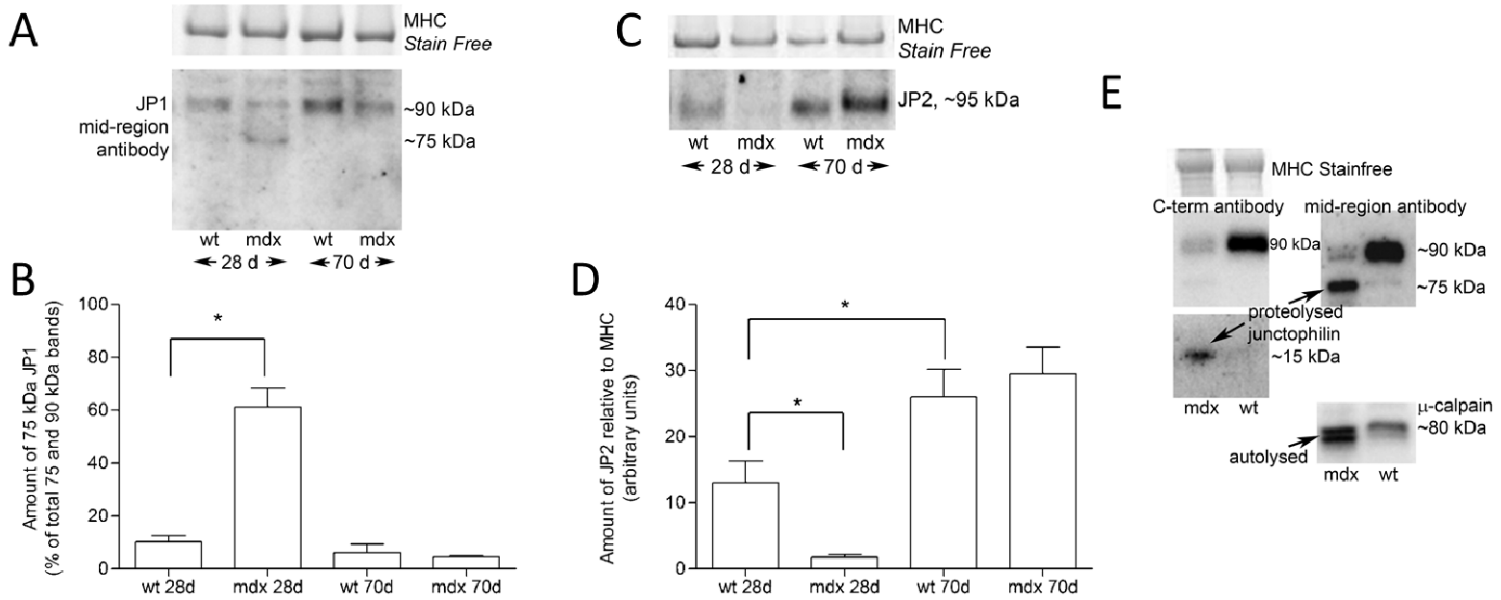

Figure 4. Proteolysis of JP1 and JP2 in limb mucles of 28 day old *mdx* mice. *A*, Western blot of JP1 in TA muscle of *mdx* and age-matched wild type (wt) mice at 28 and 70 days of age. *B*, Mean +SEM percentage of JP1 found as 75 kDa proteolytic fragment (n=5 mice in each group). *C & D*, Western blot and mean amounts of JP2 in same samples. *E*, Western blot of JP1 and μ-calpain in diaphragm of *mdx* mouse and wt mouse at 7 months. * indicates significantly different ($P<0.05$, Student's 2-tailed unpaired t-test).

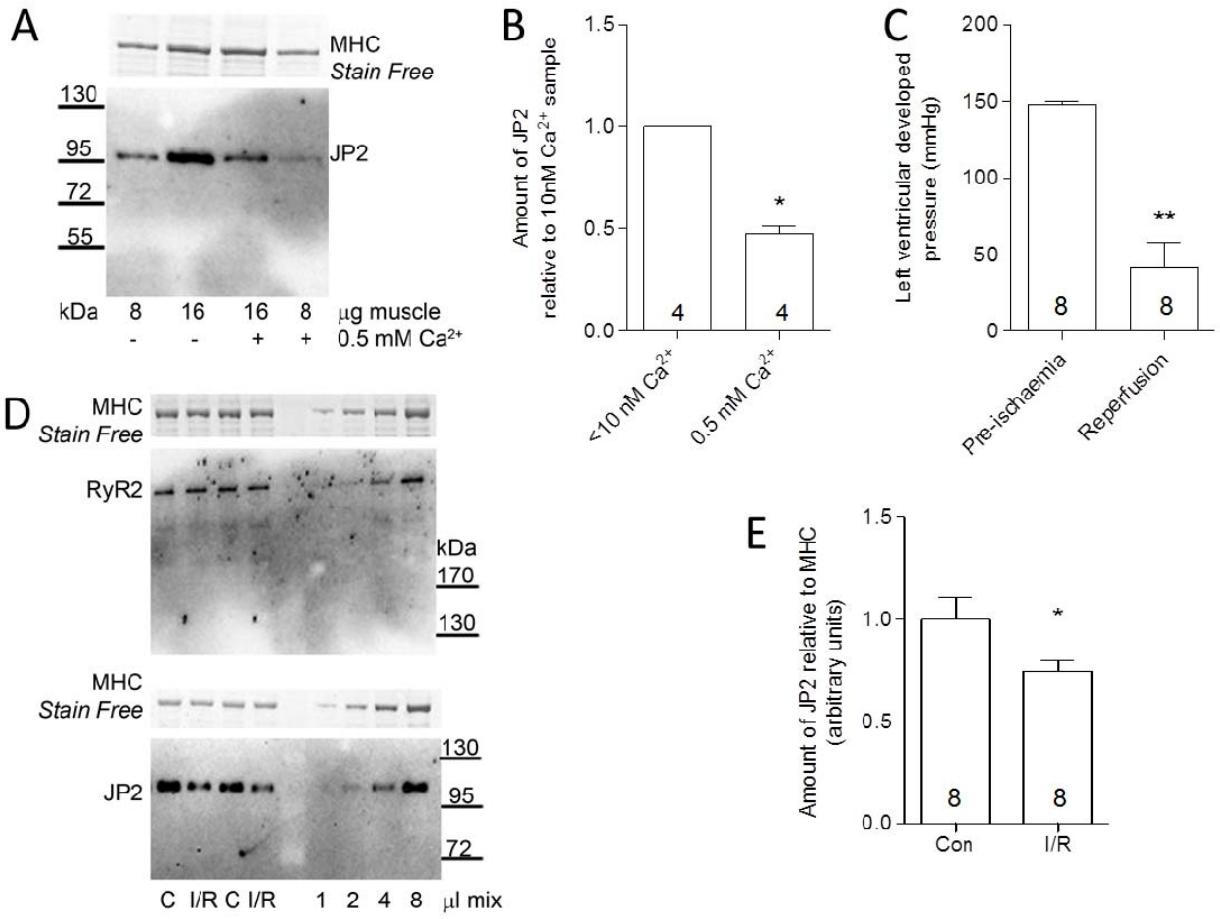

Figure 5. JP2 is proteolysed in cardiac muscle with ischaemia-reperfusion. *A*, JP2 in rat cardiac ventricular muscle homogenised in presence or absence of 0.5 mM free Ca^{2+} for 60 min at RT (4-15% Stain Free gel). *B*, Mean (+SEM) amount of JP2 in ventricular samples remaining following treatments. Data from 4 independent gels (* $P<0.01$, Student's 1-tailed paired t-test). *C*, LVDP in rat hearts before and after ischaemia-reperfusion (I/R); ** $P<0.001$, Student's 1-tailed paired t-test. *D*, Western blots of RyR2 and JP2 in separate portions of same homogenates of ventricular muscle from control (C) and I/R hearts (4-15% and 10% Stain Free gels). *E*, Mean amount of JP2 in control (Con) and I/R hearts; * $P<0.05$, Student's 1-tailed unpaired t-test.