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N232S, G741R and D778G β-cardiac myosin mutants, implicated in familial hypertrophic cardiomyopathy, do not disrupt myofibrillar organisation in cultured myotubes

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First published online 29 November 2000

The heart disease familial hypertrophic cardiomyopathy (FHC) affects up to 0.2% of the population and is the largest cause of sudden death in young adults (reviewed in [1]). It is characterised by hypertrophy of cardiac myocytes, disarray of muscle fibres, and an increase in connective tissue. It is caused by a mutation in one of a number of cytoskeletal proteins that make up the muscle sarcomere, including myosin, C-protein, troponin-T and I, α-tropomyosin and the ventricular light chains.

About 15–30% of families with FHC carry mutations in the gene for β-cardiac myosin heavy chain (β-MHC), for which over 50 different point mutations are known. In common with other class II conventional myosins, β-cardiac myosin consists of two heavy chains and two pairs of light chains. The N-terminal globular region (catalytic domain) that binds nucleotide and actin is connected to the filament forming tail through a lever arm, consisting of an α-helical region to which the light chains bind. In FHC, mutation hot spots are found in regions of the myosin that bind actin and nucleotide, and in the converter domain, or fulcrum, thought to be important for transmitting conformational changes in the catalytic domain to the lever arm, which rotates during force production.

It is not clear whether the β-MHC mutants can directly cause myofibrillar disarray. Expression of the R403Q mutation, the best studied, causes myofibrillar disarray in cultured feline cardiomyocytes but not in rat [2]. Mutant mice carrying the equivalent mutation in α-cardiac myosin show the expected FHC phenotype, but do not develop myofibrillar disarray until about 15 weeks of age (reviewed in [1]). A second FHC mutation, R249Q, assembles normally into muscle sarcomeres in rat cardiomyocytes [2].

Given that we know that β-MHC mutants result in myofibrillar disarray, it is important to find out whether any of the known mutations can directly cause disarray as demonstrated for other myosin mutations not associated with FHC [3], by interfering with myofibrillogenesis in a dominant negative fashion. To address this question, we stably expressed wild type (WT) and three different FHC mutant myosins that have not been studied in vivo before, in cultured mouse myoblasts ($H2k^b$-tsA58, see [4]), and investigated their effects on myofibrillogenesis. Two of these were in the region of myosin thought to act as a fulcrum (G741R, D778G) and one was close to the nucleotide binding pocket (N232S). We used myoblasts that did not normally express β-MHC when differentiated into myotubes, as determined by Western blotting, immunofluorescence and enzyme-linked immunosorbent assay using β-MHC specific antibodies [5]. A full length β-MHC cDNA (kind gift of Prof. Vosberg) was used, and expressed using the CMV promoter. We transfected cells by electroporation, and, for WT and each of the mutants, we analysed one out of 24 clones recovered, which had the highest expression levels of β-MHC, in detail. Expression of WT β-MHC using a mouse embryonic MHC promoter, cloned in this laboratory (unpublished results), gave similar results to those using the CMV promoter in myotubes (data not shown).

The constitutive CMV promoter drove expression of β-MHC in proliferating myoblasts, which normally only express non-muscle myosins [4]. Immunostaining showed that each of the three FHC mutant myosins assembled into fibre like structures in the cytoplasm, demonstrating their ability to form filaments in a non-muscle environment (data not shown). However, the presence of β-MHC in proliferating myoblasts did not interfere with differentiation into myotubes.

After differentiating the myoblasts for 5 days, we found that myofibrillogenesis in myotubes that expressed N232S, G741R or D778G β-MHC mutants was indistinguishable from those that expressed WT β-MHC. Staining with an anti-all skeletal myosin antibody (A1025) showed that regular arrays of muscle sarcomeres were visible in many of the myotubes (data not shown). Furthermore, the expressed WT and mutant β-MHCs assembled into muscle sarcomeres, and co-localised with the endogenous embryonic myosin (Fig. 1), in a manner indistinguishable from each other. The staining for embryonic myosin showed a single band for each sarcomere, as expected, as the epitope for this antibody is in the tail domain (see [5]). The staining for the expressed β-MHC showed two bands for each sarcomere, with a gap in staining in the middle of the sarcomere even though the epitope for this antibody is also in the tail domain. This may suggest that the cardiac myosin is preferentially adding in at the ends of the embryonic myosin filaments.

These data also show that heterologous expression of β-cardiac MHC in itself does not disrupt sarcomere assembly, as might be expected, as expression of β-MHC increases the overall levels of myosin expressed in the myotubes. Semi-quantitative estimates of the approximate levels of β-MHC expression by Western blotting showed that expression levels were roughly similar for all of the clones, and less than 0.5% of the total protein (amount of β-cardiac myosin (μg) per 100 μg total protein, mean ± S.E.M. (*n*) was as follows: WT, 0.17 ± 0.05 (6); N232S, 0.34 ± 0.11 (6); G741R, 0.29 ± 0.08 (6); D778G, 0.39 ± 0.15 (5)).

In summary, the data presented here suggest that none of the three mutant β-MHCs investigated (N232S, G741R and D778G) disrupts myofibrillogenesis, and all three mutants are capable of assembling into muscle sarcomeres. This suggests that they are unlikely to cause the myofibrillar disarray seen in FHC, by directly interfering with myofibrillogenesis. Together with the results from a similar, previous study [2], for the two FHC mutations R204Q and R403Q, we can conclude that it is unlikely that the majority of the FHC mutants cause myofibrillar disarray in this way.

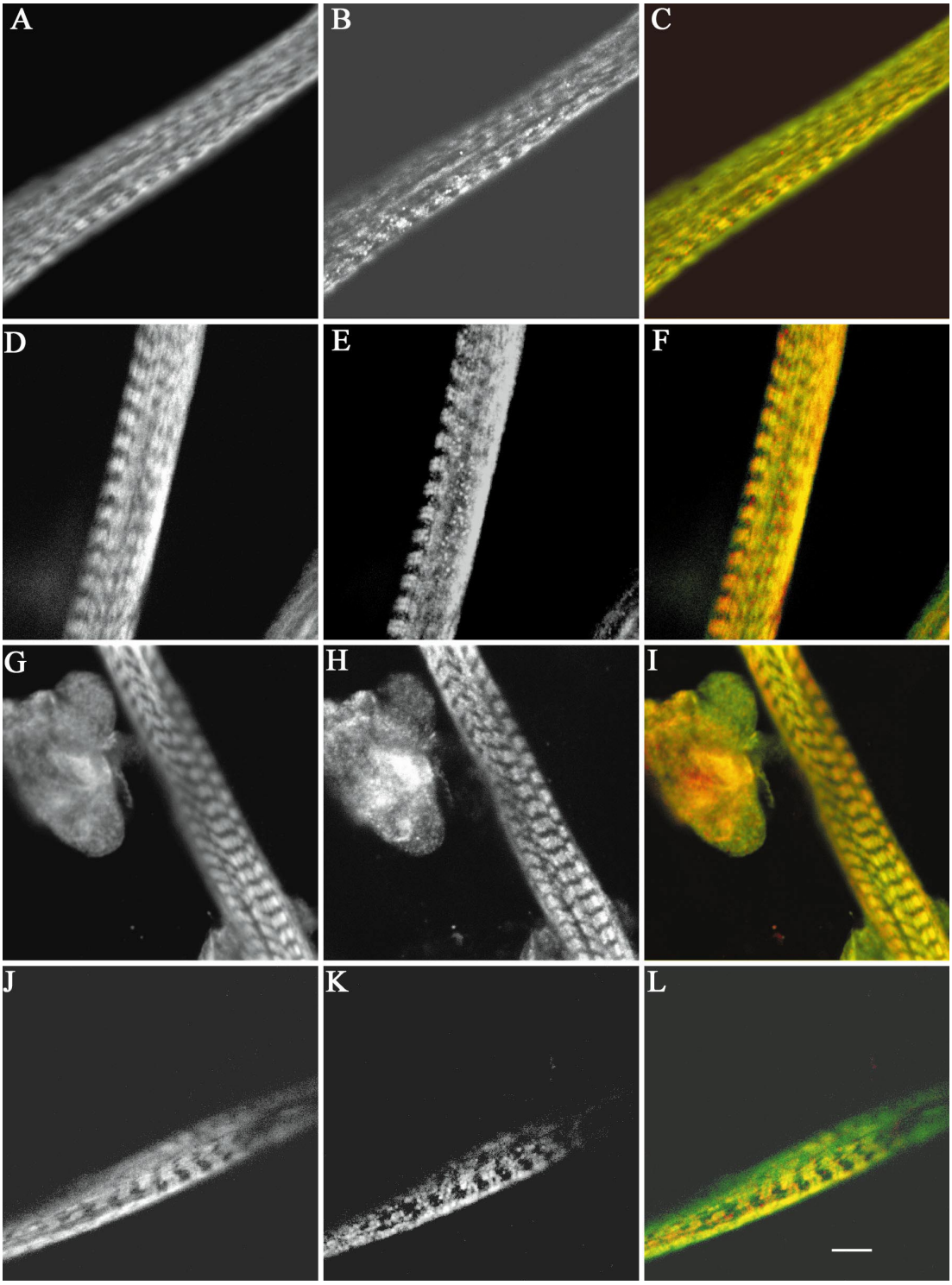

Fig. 1. Confocal images for myosin staining in 5 day old myotubes, transfected with WT β-MHC (A–C), the mutant N232S (D–F), the mutant N741R (G–I) and the mutant D778G (J–L). The left hand column (A, D, G and J) shows staining with anti-embryonic myosin antibody (F1652; IgG1), the middle column (B, E, H and K) staining with anti-cardiac myosin antibody (A4840; IgM) and the right hand column (C, F, I and L) shows the resultant merged images, where F1652 is shown as green, and A4840 as red (areas of co-localisation show up as yellow/orange). Myotubes were fixed using ice-cold methanol, and staining procedures, fluorescent and confocal microscopy were as described [4]. IgG1 and IgM specific secondary antibodies (Serotec) were used to detect F1652 and A4840 staining, respectively. Scale bar 10 μm.

As none of the mutants so far looked at interfered with nascent myofibrillogenesis, we think that myofibrillar disarray is more likely to arise from a change in the contractile properties of the cardiomyocytes. However, it is not clear how this occurs. Some evidence suggests that the contractile properties of FHC mutants are reduced compared to WT myosin. This 'hypo-contractility' would lead to a compensatory hypertrophy (reviewed in [1]). Other evidence suggests that cardiac myocytes become hyper-contractile, directly leading to hypertrophy (reviewed in [1]). A smooth muscle MHC carrying a

mutation equivalent to D778G, and expressed and purified using a baculovirus system [6], increased the actin filament velocity in an in vitro motility assay by 50% compared to WT. Its actin-activated ATPase activity was also higher suggesting that hypertrophy is an adaptive response to increased ATP consumption [6]. One difficulty in interpreting many of these studies is that the FHC mutations are often duplicated into smooth muscle MHC, and do not use β-MHC.

In conclusion, our results support the idea that the FHC myosin mutants do not directly disrupt myofibrillogenesis. It is more likely that a change in contractile function causes the myofibrillar disarray observed in FHC. It will be important to confirm whether particular FHC mutations cause hypo- or hyper-contractility.

Acknowledgements: This work was supported by a British Heart Foundation Grant PG97094. M.C. was an MRC Ph.D. student.

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PII: S0014-5793(00)02237-7