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Expression of slow skeletal myosin binding C-protein in normal adult mammalian heart

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Abstract

Myosin binding C-protein exists in three main isoforms in striated muscle. While expression of cardiac muscle type C-protein is detected in skeletal muscle during early fetal development, there have not so far been any reports of the expression of the skeletal muscle isoforms of this protein in either developing or adult vertebrate heart. The present study demonstrates slow skeletal muscle type C-protein in moderate amount in right atrium and interatrial septum of adult human, rabbit, rat and bovine hearts using both immunocytochemical and immunoblotting procedures.

Introduction

C-protein is one of the major myosin-binding proteins located at the A-bands of the myofibril in striated muscle stabilizing thick filaments during sarcomere assembly (Winegrad, 1999). Regulatory phosphorylation of cardiac C-protein by cAMP-dependent protein kinase upon adrenergic stimulation is linked to modulation of cardiac contraction (Gautel *et al*., 1995) and has thus been proposed to have both structural and regulatory functions (Winegrad, 1999). It has been described to exist in three main isoforms encoded by three distinct genes, fast skeletal, slow skeletal, and cardiac muscle type. In addition, it generates further isoforms by alternative splicing of the cardiac C-protein gene (Kojima *et al*., 1990; Sato *et al*., 2003). The distribution of the skeletal muscle isoforms of the C-protein, unlike some other myofibrillar proteins, however, does not show a strict correlation with the fibre type distribution in adult skeletal muscle. For example, although certain type I and type II fibres contain only the slow and fast skeletal muscle C-proteins, respectively, as expected, some fibres, clearly type II, by the normal histochemical criteria, contain both slow and fast isoforms of this protein (Dhoot *et al*., 1985). Thus although fast and slow isoforms of myosin binding C-protein are generally expressed in a tissue-specific manner, both fast and slow isoforms are transcribed in a subset of skeletal muscle fibres as well. In contrast, myocardial cells have been reported to contain only the cardiac isoform of C-protein throughout development and in the adult, not only in avian and rodent but also human heart (Kawashima *et al*., 1986; Gautel *et al*., 1998; Kurasawa *et al*., 1999). The skeletal muscle type isoforms of a number of myofibrillar proteins are often expressed in cardiac muscle during early fetal or neonatal development. For example, slow skeletal muscle type troponin I and troponin T isoforms are expressed in early developing heart, the expression of which is downregulated during neonatal development becoming undetectable in the normal adult heart (Sabry and Dhoot, 1989a, b; Saggin *et al*., 1989; Bhavsar *et al*., 1991; Krishan *et al*., 2000). Unlike troponins, no skeletal muscle isoforms of C-protein, however, have been described in either the fetal or postnatal hearts. This is significant since multiple mutations in cardiac C-protein lead to familial hypertrophic cardiomyopathy.

It was noted in our earlier study (Dhoot *et al*., 1985) that although the antibodies to skeletal C-protein did not react with the majority of the rabbit myocardial cells, a small proportion of the myocardial cells stained with the antibody to C-protein from slow skeletal muscle. Further investigation now confirms the expression of slow skeletal muscle type C-protein in a number of myocardial cells of the right atrium and the interatrial septum in all mammalian species studied so far including man that could be significant to its regulation in the myopathic heart. For example, mutations in the cardiac C-protein gene cause familial hypertrophic cardiomyopathy (Lin *et al*., 1996; Watkins *et al*., 2001; Yang *et al*., 2001). Since heart has been reported to express only the cardiac C-protein, the nondetection of all other isoforms of C-protein in cardiac muscle has led to a conclusion that transcomplementation of mutated or truncated cardiac C-protein with other

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isoforms is thus not possible (Gautel *et al*., 1998). The present study, however, reports the expression of slow skeletal muscle type C-protein in easily detectable levels in both the right atrium and the interatrial septum, indicating that mutation of cardiac C-protein disrupting the synthesis of full-length functional protein may up regulate the expression of slow C-protein as a compensatory mechanism and may be one of the reasons for much milder hypertrophy associated with mutations in cardiac C-protein although such a hypothesis needs to be confirmed by experimental analysis of affected hearts to determine if it occurs in practice.

Materials and methods

Electrophoresis

Immediately after death, the animal hearts analysed by immunoblotting procedure were divided into right and left atria, ventricle, and interatrial and interventricular septa. A small number of biopsied post-mortem human tissue samples provided by the Pathology department, university of Birmingham, obtained within 10–12 h of death, were also analysed by both immunoblotting and immunohistochemical analyses. Tissue samples were analysed by SDS elelectrophoresis in 10% polyacrylamide made in 0.1 M Tris–Bicine buffer, pH 8.3 (Weeds *et al*., 1975). Extracts for electrophoresis were prepared by homogenising freshly excised muscle in 10 volumes of either 9 M urea, 75 mM Tris–HCl (pH 8.3), 2 mM $CaCl_2$, 15 mM mercaptoethanol or 2%SDS, 0.1 M Tris–Bicine buffer (pH 8.3), 50 mM 2-mercaptoethanol. After mixing with 0.2 volume of 50% glycerol containing a trace of bromophenol blue as tracing dye, 10 µl of each tissue extract was applied to the gel. Western blots were prepared by transferring the protein bands to nitrocellulose sheets by the procedure of Towbin *et al*. (1979) and stained with peroxidase-linked antibody by the sandwich technique using 3,3-diaminobenzidine and H_2O_2 as substrate (De Blas and Cherwinski, 1983). To quantify the levels of slow C-protein in different tissue samples, the immunoblots were scanned on a densitometer before staining them with amido black to quantify the total amount of this protein by densitometer scanning.

The preparation of C-protein for immunisation to produce the antibody and the specificity of the monoclonal antibody 83B24 used in this study has already been described in detail (Dhoot *et al*., 1985).

Immunocytochemistry and histochemistry

Frozen sections (6 micron thick) were stained by the immunoperoxidase procedure as described previously (Dhoot *et al*., 1985). Some sections were also processed for myosin ATPase after preincubation at pH 4.1 as described in Dubowitz and Brooke (1973).

Results and discussion

Expression of slow skeletal muscle type C-protein in the heart investigated by immunoblotting procedure

When extracts of fractions of the rat heart were examined by the immunoblotting procedure using antibody 83B24 to slow C-protein, a protein band of approximately 140,000 molecular weight, constituting approximately 5% of the total C-protein, stained in all samples of the interatrial septum. Weaker staining of this band was also observed in about one third of the samples of the right atrium, constituting approximately 1% of the total C-protein, while no staining was observed in extracts from any other regions of the heart such as left atrium or any parts of the ventricle (Figure 1). In other mammalian species studied such as the man and the rabbit, staining was also observed in only certain fractions of the interatrial and right atria (not shown). With larger animals such as the cow, different regions of the interatrial septum and right atrium were individually analysed because of their larger size. In these samples, only about half of the interatrial samples stained with antibody to slow C-protein clearly implying that this isoform was not evenly distributed throughout the interatrial septum. The nondetection of this isoform of C-protein in some samples may also reflect low levels of expression in this tissue that will not be detected by immunoblotting procedure. In every heart studied, however, reaction of slow C-protein antibody was obtained with some fractions.

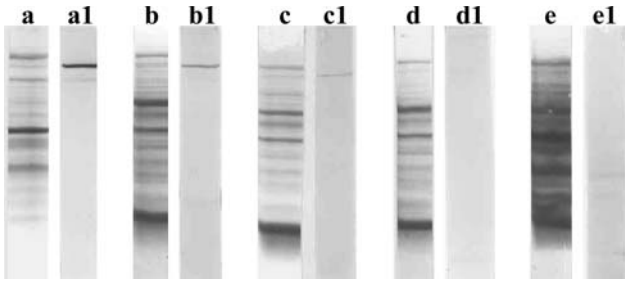

Fig. 1. Western blots of electrophoretograms of skeletal and cardiac muscle extracts. Electrophoresis in SDS carried out as described in the Materials and methods section. Stained with amido black (a, b, c, d, e) and antibody 83B24 to slow isoform of C-protein (a1, b1, c1, d1, e1) by immunoperoxidase procedure. All extracts from rat tissues: a, a1 = Soleus; b, b1 = interatrial septum; c, c1 = right atrium; d, d1 = left atrium; e, e1 = ventricle. The highest level of slow C-protein expression is apparent in Soleus with moderate level of expression in the interatrial septum and low levels in the right atrium. No expression is observed in either the left atrium or the ventricle. All samples presented here were selected from the same gel and immunoblot staining developed for the same length of time under similar conditions.

Expression of slow skeletal muscle C-protein in the heart investigated by immunocytochemical procedure

Study of the distribution of the slow skeletal muscle type C-protein reacting with antibody 83B24 in sections of cardiac tissue confirmed the findings from the immunoblotting analysis of tissue extracts. Staining of the myocardial cells with antibody 83B24 was only observed in the interatrial septum and right atrium even with this technique that could detect isolated positive cells in other regions. The number of myocardial cells stained was a small proportion of the total and varied in sections from different blocks and between different regions of the same block. The number of myocardial cells staining in the right atrium was usually much lower when compared with the interatrial septum. The staining of cells also varied considerably in intensity from dark to intermediate levels (Figure 2–4).

The myocardial cells that stained with antibody 83B24 did not show uniform morphology and ranged from very small cells to those similar in size to neighbouring unstained cells. The variation in staining intensity implied as has been shown to be the case in skeletal muscle (Dhoot *et al*., 1985) that a proportion of the myocardial cells contained another isoform of the C-protein, presumably the cardiac muscle type C-protein in addition to that reacting with antibody 83B24. The distribution of the protein reacting with antibody 83B24 in myocardial cells did not correlate with the distribution of myosin isoforms as revealed by ATPase staining (Figure 3) or immunochemical studies reported by other workers (Gorza *et al*., 1986; Bouvagnet *et al*., 1987).

The distribution of the protein reacting with antibody 83B24 implies heterogeneity of the cell types of the interatrial septum and the right atrium that has not been previously reported. The precise identity of the myocardial cells containing 83B24 positive antigen, however, is not known. Its distribution in the right atrium is somewhat similar to that of the conductive tissue although it appears to be present in considerably greater proportion of cells than are presumed to be associated with the latter system. The conduction system contains heterogeneous muscle cell populations specialized for the generation and propagation of the electrical impulse. The myocardial cells expressing slow isoform of C-protein also appear quite heterogeneous in size and morphology. Although some cells stained with antibody 83B24 could include a proportion of cells representing parts of the conduction system responsible for the coordinated activation of myocardium (Schiaffino, 1997), this certainly did not include all cells of the conduction tissue. For example, no staining of purkinje fibres that have been reported to express skeletal myosin binding H-protein (Alyonycheva *et al*., 1997) was observed in ventricle. Furthermore, the myocardial cells of conduction system make up only a small proportion of the total myocardial mass (less than 1%), the protein constituents of which are usually not detectable by immunoblotting procedure. The slow C-protein expression by contrast was easily detectable in most samples from the interatrial septum and right atrium using not only immunocytochemical but also immunoblotting procedure. While the expression of a number of conductive tissue markers such as slow myosin and skeletal muscle type myosin binding H-protein as well as the expression of myoD, a myogenic transcription factor

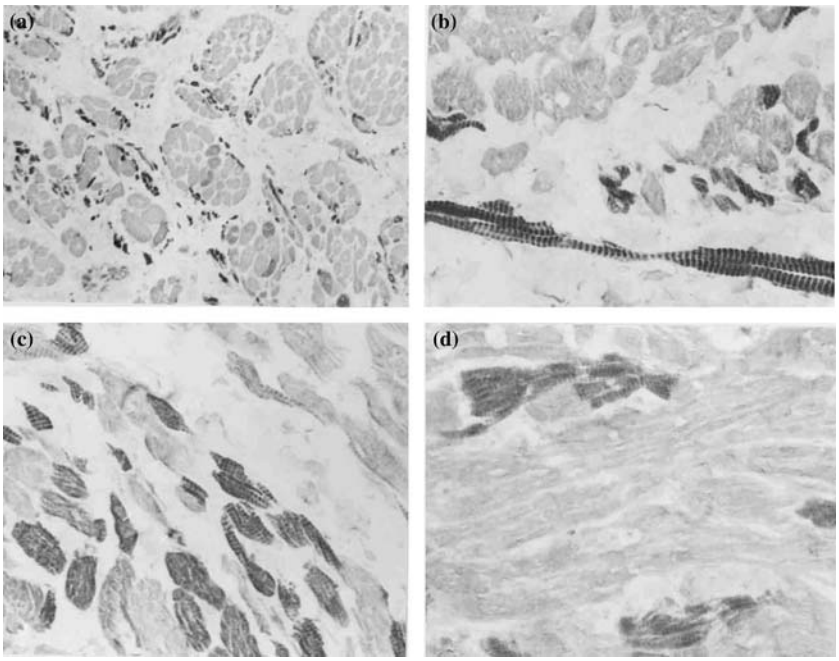

Fig. 2. Immunoperoxidase staining of frozen sections of bovine cardiac muscle with antibody 83B24 to slow isoform of C-protein. A, B and C represent different regions of interatrial septum; D = a region of right atrium. Magnification a = ×48; b–d = ×193.

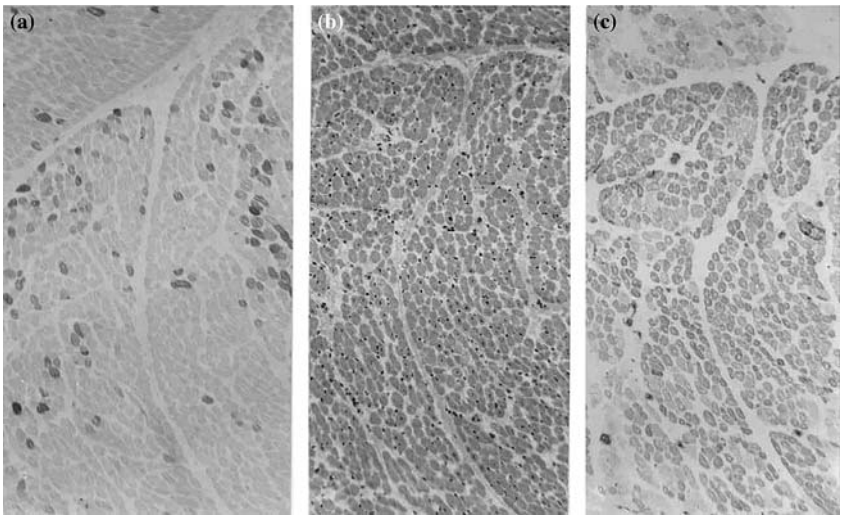

Fig. 3. Immunoperoxidase and histochemical staining of frozen sections of bovine interatrial septum. Sections are through the same region of interatrial septum but not serial. A = antibody 83B24 using immunoperoxidase procedure; B = Haemotoxylin and Eosin; C = myosin ATPase after preincubation at pH 4.1. Magnification = ×80.

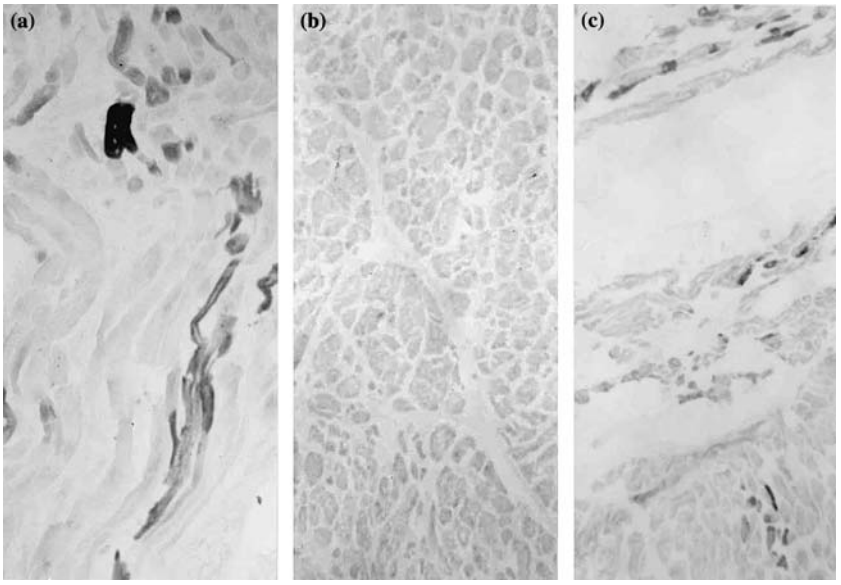

Fig. 4. Immunoperoxidase staining of frozen sections of atrial and ventricular tissues stained with antibody 83B24 to slow isoform of C-protein. A = Human right atrium; B = Human ventricle; C = rabbit right atrium.

associated with activation of skeletal muscle programme has been described in embryonic heart (Alyonycheva *et al.*, 1997; Takebayashi-Suzuki *et al.*, 2001), we did not detect slow C-protein expression in either fetal or neonatal hearts (not shown).

In the adult, the cardiac C-protein is usually restricted to the heart but its presence has been demonstrated in embryonic skeletal muscle during early muscle development (Kawashima *et al.*, 1986). Apart from our preliminary observation (Dhoot *et al.*, 1985), there have as yet, however, been no reports of the presence of the skeletal isoforms of C-protein in cardiac muscle. Our results suggest that a small fraction of certain myocardial cells in the interatrial and right atrial regions of the mammalian heart contain varying amounts of a protein that is very similar if not identical to the slow skeletal muscle isoform of C-protein. In this context, the observation that this antigen has the same molecular weight as the slow skeletal muscle isoform of C-protein suggests that protein reacting with antibody 83B24 in certain myocardial cells closely resembles the mammalian slow skeletal muscle C-protein. This protein is unlikely to represent an embryonic form of cardiac C-protein as we did not detect its presence in either the fetal or neonatal rat hearts while in contrast, all myofibres in fetal and neonatal rat skeletal muscle stained with this antibody to slow C-protein (not shown).

The expression of slow C-protein in the adult heart may be clinically relevant since the cardiac C-protein

mutations cause familial hypertrophic cardiomyopathy in man (Lin *et al*., 1996; Watkins *et al*., 2001; Yang *et al*., 2001). A study of transgenic mice in which cardiac troponin I gene was inactivated, however, failed to maintain the expression of slow skeletal troponin I during postnatal development even though mice lacking cardiac troponin I were healthy during early development due to compensation by the slow isoform (Huang *et al*., 1999). While slow troponin I is normally expressed only during fetal and early neonatal development of the heart, slow C-protein expression in the heart is different as it is normally expressed in certain regions of the adult heart and the possibility therefore exists that slow skeletal muscle type C-protein may be able to compensate for the mutated cardiac isoform of C-protein. It remains, however, to be determined whether the mutation of cardiac C-protein can lead to the induction of an alternative C-protein isoform in the non-slow C-protein expressing myocardial cells or its up regulation in those myocardial cells that originally expressed only a low level of slow isoform. Indeed, the observation that most patients carrying a mutation in cardiac C-protein show only mild cardiac hypertrophy until much later in life (Yang *et al*., 2001) may relate to some compensation by up regulation of slow skeletal muscle type isoform of C-protein. Unlike cardiac muscle, no myopathy associated with C-protein mutations has been reported for skeletal muscle believed to result from compensatory up regulation of the other isoform of C-protein since a considerable proportion of adult normal skeletal muscle fibres express both fast and slow isoforms of C-protein in the same cell (Dhoot et al., 1985). Although symptomatic familial hypertrophic cardiomyopathy associated with cardiac C-protein mutations or truncations occurs much later in life compared with mutations in myosin, a recent study (Yang *et al*., 2001) of a mouse mutation demonstrated reduction in power output that again could indicate changes in isoform composition that have not been investigated so far. It will therefore be interesting to determine whether the expression of slow skeletal muscle type C-protein is upregulated in diseased myocardium of patients with C-protein associated familial hypertrophic cardiomyopathy.

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