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Expression of eight distinct MHC isoforms in bovine striated muscles: evidence for MHC-2B presence only in extraocular muscles

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Summary

This study aimed to analyse the expression of myosin heavy chain (MHC) isoforms in bovine muscles, with particular attention to the *MHC-2B* gene. Diaphragm, longissimus dorsi, masseter, several laryngeal muscles and two extraocular muscles (rectus lateralis and retractor bulbi) were sampled in adult male *Bos taurus* (age 18–24 months, mass 400–500 kg) and analysed by RT-PCR, gel electrophoresis and immunohistochemistry. Transcripts and proteins corresponding to eight MHC isoforms were identified: MHC-α and MHC-β/slow (or MHC-1), two developmental isoforms (MHC-embryonic and MHC-neonatal), three adult fast isoforms (MHC-2A, MHC-2X and MHC-2B) and the extraocular isoform MHC-Eo. All eight MHC isoforms were found to be co-expressed in extrinsic eye muscles, retractor bulbi and rectus lateralis, four (β/slow, 2A, 2X, neonatal) in laryngeal muscles, three (β/slow, 2A and 2X) in trunk and limb muscles and two (β/slow and α) in masseter. The expression of MHC-2B and MHC-Eo was restricted to extraocular muscles. Developmental MHC isoforms (neonatal and embryonic) were only found in specialized muscles in the larynx and in the eye. MHC-α was only found in extraocular and masseter muscle. Single fibres dissected from masseter, diaphragm and longissimus were classified into five groups (expressing, respectively, β/slow, α, slow and 2A, 2A and 2X) on the basis of MHC isoform electrophoretical separation, and their contractile properties [maximum shortening velocity (v_0) and isometric tension (P_0)] were determined. v_0 increased progressively from slow to fast 2A and fast 2X, whereas hybrid 1–2A fibres and fibres containing MHC-α were intermediate between slow and fast 2A.

Key words: myosin, MHC isoforms, cattle, skeletal muscles, extraocular muscles, laryngeal muscles, RT-PCR, electrophoresis, immunohistochemistry.

Introduction

The genes of four ‘adult’ skeletal muscle myosin heavy chain (MHC) isoforms have been found by genomic analysis in all mammalian species considered until now: one slow (or type 1) isoform and three fast isoforms called 2A, 2X and 2B. In small rodents such as mice and rats, in rabbits and in marsupials (Schiaffino and Reggiani, 1996; Zhong et al., 2001), all four MHC isoforms are expressed, giving origin to four distinct fibre types. By contrast, in humans, the *MHC-2B* gene identified in the genome is not expressed, and only 1, 2A and 2X MHC isoforms are expressed in skeletal muscles. The fibre type originally classified as 2B (therefore often called ‘conventional 2B’) contains MHC-2X (Smerdu et al., 1994). In some specialised human muscles, such as masseter, the expression of the *2B* gene has been demonstrated by *in situ* hybridisation, but the corresponding protein has not been found (Horton et al., 2001). The MHC-2B RNA is abundant in masseter fibres, histochemically and immunohistochemically identified as type 2A fibres, but is rare in limb muscle and present in few fibres of the abdominal external oblique muscle (Horton et al., 2001).

The inter-species diversity of the expression of the MHC-2B isoforms between humans and laboratory animals has prompted further studies to assess the expression in various species of veterinarian or experimental interest, from monkey to dog, sheep and llama (for a review, see Reggiani and Mascarello, 2004). In all these species, only three fibre types and three MHC isoforms have been identified in skeletal muscles, two of them (types 1 and 2A) being very similar to the corresponding ones in rat and mouse and the third one being similar to 2X. Thus, the 2B fibre type seems to be specific only for small mammals and marsupials. A recent study suggests that in the genome of some species, such as the horse, only a pseudogene with a sequence similar to that of the *MHC-2B* gene is present (Chikuni et al., 2004a). There are, however, exceptions: in pigs, the 2B isoform is expressed at the protein level in several skeletal muscles, generally in hybrid 2X/B fibres (Da Costa et al., 2002; Toniolo et al., 2004), and in cattle we have recently shown the expression of MHC-2B at the mRNA level in extraocular muscles (Maccatrozzo et al., 2004).

The reliable identification of the presence of an MHC isoform as protein is often more difficult in large mammals than in laboratory animals because of the high percentage of hybrid fibres that express two or more isoforms. For example, in pig skeletal muscles, 2A/X and 2X/B hybrid fibres are abundant in trunk and limb muscles, whereas the pure 2B fibres are present only in specialised muscles such as extraocular muscles (Toniolo et al., 2004). It is also important to note that antibodies normally used in laboratory animals may give different results when employed in large animals, making it necessary to check their specificity by a combination of several independent approaches. For example, SC-71 monoclonal antibody (mAb), specific for MHC-2A in all mammalian species studied so far, was not able to distinguish the two types of fast fibres in bovine muscles (Duris et al., 2000). It is likely that the lack of specificity of the SC-71 antibody is due to the sequence similarity between bovine MHC-2X and MHC-2A (Maccatrozzo et al., 2004). In our view (Reggiani and Mascarello, 2004; Toniolo et al., 2004), to identify reliably 2X and 2B MHC in skeletal muscles in different species, the histochemical and immunohistochemical results need to be confirmed by (1) MHC gene expression analysis based on RT-PCR and (2) identification of the corresponding proteins by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

Our previous studies on bovine skeletal muscles (Maccatrozzo et al., 2004) have shown that the three fibre types identified by m-ATPase staining as type 1, 2A and conventional 2B express slow, 2A and 2X MHC isoforms, respectively, and that the *2B* gene is expressed at the mRNA level only in specific muscles, such as retractor bulbi and extraocular muscles. The study of *2B* expression in these specific muscles has allowed us to sequence a fragment of the bovine *2B* gene. It is still unknown whether the protein corresponding to the gene coding for MHC-2B is present in bovine skeletal muscles and, in particular, in bovine specialised muscles such as extraocular muscles, where the corresponding mRNA is present. The reactivity of some fibres with the antibody BF-F3 can only be considered as an indication of the presence of the protein, in view of the above-mentioned limitations in the reliability of anti-myosin antibodies. In the present study, we aimed to demonstrate whether MHC-2B protein is present in bovine muscles and to extend the investigation to laryngeal muscles, a group of muscles where expression of MHC-2B can be expected. As recently reviewed by Hoh (2005), laryngeal muscles and, in particular, thyreoarytenoideus muscle express, in several species, MHC isoforms different from those found in the limb skeletal muscles. In rat and rabbit, thyreoarytenoideus muscle expresses MHC-2B and MHC-Eo (extraocular); there is also evidence in favour of the presence of MHC-2B in cat (Hoh, 2005) and possibly also in dog (Wu et al., 2000). In bovine thyreoarytenoideus muscle, fibres show an unusual histochemical profile (Mascarello and Veggetti, 1979) and this suggests the presence of some isoforms different from those present in limb muscles.

In view of our goal, samples from adult and foetal bovine skeletal muscles and from atrial and ventricular bovine myocardium were analysed by combining immunohistochemistry, RT-PCR and gel electrophoresis to identify all expressed MHC isoforms. The results obtained showed that MHC-2B is present as protein in extraocular muscles but not in laryngeal muscles. The second aim of the study was to describe the contractile properties of bovine single muscle fibres. In this respect, the identification of the electrophoretic bands corresponding to MHC isoforms was a pre-requisite to classify the fibres whose isometric tension and maximum shortening velocity were measured. On the whole, the results provide a complete picture of bovine fibre types with regard to their MHC isoform composition and their contractile properties.

Materials and methods

Sampling of bovine muscles

Muscle samples were collected from adult male *Bos taurus* L. (age 18–24 months, mass 400–500 kg) of commercial breeds killed in a slaughter house. Several skeletal muscles were sampled and used for RNA and protein expression analysis and for immunohistochemistry: masseter (M), diaphragma pars costalis (D), longissimus dorsi (Ld), extensor carpi radialis (Ecr) pectoralis (P), specialized extraocular muscles [rectus lateralis (Rl); retractor bulbi (Rb)] and some laryngeal muscles [cricoarytenoideus dorsalis (Cd); arytenoideus transverses (At); thyreoarytenoideus (T)]. In bovine larynx, T muscle is not divided in ventricular and vocal muscles, but histochemical staining allows a separation between rostral and caudal portions that corresponds to ventricular and vocalis muscles of other species (Mascarello and Veggetti, 1979). On these grounds, T was dissected into a rostral (Tvr) and a caudal part (Tvc) corresponding to the ventricularis portion and into a caudal part corresponding to the vocalis portion (Tvo). Samples of cardiac muscle, ventricular and atrial portions were collected to identify cardiac-specific myosin isoforms. Finally, diaphragma pars costalis (D-f), longissimus dorsi (Ld-f) and masseter (M-f) were dissected from a bovine foetus (age 120 days) to characterize also developmental myosin isoforms. Unless otherwise stated, the samples were divided into four different portions: one for RNA preparation, one for protein electrophoresis, one for immunohistochemistry and one for single fibre mechanics.

RNA extraction and RT-PCR

Fragments (~100 mg) of the muscle samples to be used for RNA analysis were immersed as quickly as possible in RNA Later Reagent (Ambion, Austin, TX, USA). Total RNA was extracted from muscle tissue using TRIZOL® reagent (Gibco-BRL, Gaithersburg, MD, USA) and reverse-transcribed with Superscript II protocols (Invitrogen, Life Technologies, Paisley, UK) using a mixture of random hexamers as primer. The obtained cDNAs were used as template for RT-PCR

Table 1. *Primers used for RT-PCR of MHC-Eo (extraocular) and MHC-Neo (neonatal)*

Isoforms	Forward primer (5′→3′)	Reverse primer (5′→3′)	Temperature (°C)	PCR cycles
MHC-Eo	TCGGGAGGAAGCAAGAAGGGCGGGA	ATCACGCCAGGTGTCTTAGTCTCATTGGG	64	32
MHC-Neo	CGTAATGCTGAGGCTATTAAAGGCTT	ACAAAGCAAGTGACCCAAAATAGCA	55	32

analysis. PCR reactions and bovine-specific primers used to amplify MHC-1, 2A, 2X and 2B isoforms were described in a previous study (Maccatrozzo et al., 2004). Primers for MHC-1, 2A and 2X were kindly provided by Prof. K. Chikuni; the primers for MHC-2B were designed by our group (Maccatrozzo et al., 2004); new primers were prepared for extraocular and neonatal MHC; their sequences are shown in Table 1. Primers for neonatal MHC were designed from sequences available in GenBank (accession number AB090156), whereas for extraocular MHC a first cloning was obtained using degenerate primers and, from the sequence obtained with degenerate primers, new specific primers were designed (Table 1).

Gel electrophoresis

Muscle samples for protein electrophoresis were solubilized at 90°C for 5 min in Laemmli buffer solution [Tris 62.5 mmol l^{-1}, pH 6.8, glycerol 10%, SDS 2.3%, β-mercaptoethanol 5%, with E-64 0.1% and leupeptin 0.1% (Sigma, St Louis, MO, USA) as antiproteolytic factors].

Two distinct protocols were used to determine the composition of MHC isoforms in muscle samples. A first protocol was used to separate all MHC isoforms except the two cardiac isoforms (MHC-β and MHC-α). Samples were analysed on 9% polyacrylamide slab gels after denaturation in SDS (SDS–PAGE) with a procedure derived from Blough et al. (1996), modifying stacking gel composition with 29% glycerol. Slabs 18 cm wide, 16 cm high and 1 mm thick were used. Electrophoresis was carried out at 4°C for 46 h, at 100 V for the first 3 h and at 230 V for the remaining time. Gels were silver stained (Silver stain plus, Biorad, Hercules, CA, USA). Six bands were separated in the region of 200 kDa, corresponding, in order of migration rate from the fastest to the slowest, to MHC-1, MHC-Eo, MHC-2B, MHC-Neo and MHC-2X, which co-migrated, MHC-Emb and MHC-2A.

A second protocol was used to separate the two cardiac isoforms (MHC-β and MHC-α). The separation was obtained on 8% polyacrylamide slab gels after denaturation in SDS (SDS–PAGE) and was based on the procedure described by Talmage and Roy (1993), modifying run parameters. Electrophoresis was carried out at 4°C for 40 h, at 70 V for the first 2 h and 170 V for the remaining time. Gels were silver stained (Bio-Rad Silver stain plus).

The identification of the electrophoretic bands in the gel region corresponding to 200 kDa as distinct MHC isoforms was obtained by comparing RT-PCR results, immunohistochemistry assay and protein electrophoresis, as described in the Results.

RT-PCR and gel electrophoresis on fibres and fibre bundles

Small muscle fragments (<10 mg), bundles of fibres (2–10 fibres) and single fibres were manually dissected from samples of rectus lateralis (Rl), retractor bulbi (Rb) and diaphragm (D) muscles in skinning solution (see below) under a stereomicroscope (10–60× magnification). Fibre bundles and single fibres were transversally cut into two parts (each of them ~2 mm long), one half of which was immersed in 200 μl TRIZOL® reagent and used for total RNA extraction whereas the other half was immersed in 20 μl of Laemmli solution to solubilize proteins for MHC electrophoretic analysis.

RNA was extracted with the same protocol used for larger samples, scaling down the reaction. The cDNA reverse-transcribed from extracted total RNA was used to test the expression of adult MHC isoforms (1, 2A, 2X and 2B), extraocular (Eo) and neonatal (Neo) MHC isoforms. The products obtained with PCR were used as templates in a second round of PCR with the same conditions because of the small amount of starting sample. To assess the quality of the RNA and the efficiency of the RT reaction, a fragment of the bovine β-actin cDNA was amplified for each sample. From the Laemmli solution containing solubilized proteins, a 3 μl sample was used for electrophoretic separation of MHC isoforms under the condition described above.

Immunohistochemistry

A specialized extraocular muscle (rectus lateralis) was combined with a skeletal muscle (longissimus dorsi) into a composite block and frozen in isopentane cooled with fluid nitrogen. Serial sections (10 μm) were cut in a cryostat and stained according to the protocol described by Maccatrozzo et al. (2004) with the following antibodies: monoclonal BF-F3, which is specific for MHC-2B in rat and in pig (Lefaucheur et al., 2002; Toniolo et al., 2004), polyclonal anti-MHC-Eo (Sartore et al., 1987) and polyclonal anti-MHC-Neo (Mascarello and Rowlerson, 1992). The procedures to assess the specificity of the reactions were described in Maccatrozzo et al. (2004).

Single fibre mechanics

Muscle samples from masseter, diaphragm and longissimus dorsi were immersed in ice-cold skinning solution (see below), divided into small fibre bundles and stored at –20°C in a mixture of skinning solution and 50% glycerol. On the day of the experiment, the bundles were removed from the freezer and repeatedly washed with skinning solution. Single fibres were manually dissected from fibre bundles under a stereomicroscope (10–60× magnification). At the end of the dissection, fibres were bathed for 1 h in skinning solution

containing 1% Triton X-100 to ensure complete membrane solubilization. Segments of 1–2 mm in length were then cut from the fibres, and light aluminium clips were applied at both ends.

Skinning, relaxing, pre-activating and activating solutions utilized for mechanical experiments with single fibres were prepared as previously described (Bottinelli et al., 1996). Preactivating solution had a composition similar to the relaxing solution except that EGTA concentration was reduced to 0.5 mmol l^{-1}, and 25 mmol l^{-1} creatine phosphate and 300 U ml^{-1} creatine phosphokinase were added. Activating solution was similar to the relaxing solution except for the addition of 5 mmol l^{-1} $CaCl_2$, 25 mmol l^{-1} creatine phosphate and 300 U ml^{-1} creatine phosphokinase. The pH of all solutions was adjusted to 7.0 at the temperature at which solutions were used (12°C). Protease inhibitors [E64 (10 μmol l^{-1}) and leupeptin (40 μmol l^{-1})] were present in all solutions.

In each fibre segment, isometric tension (P_0) and unloaded shortening velocity (v_0) were measured during maximal activations at 12°C, pCa=4.8 under the conditions described in previous studies (Pellegrino et al., 2003; Toniolo et al., 2004). The kinetics of tension redevelopment was measured from the time course of tension development after a fast (<1 ms) manoeuvre of shortening to reduce tension to zero and fast re-lengthening back to the initial length after an interval of 30 ms. Tension redevelopment was recorded and characterised by the time required to redevelop two-thirds of isometric tension. Images of each fibre were taken with a camera connected to the microscope at 360× magnification. Cross-sectional area (CSA) was calculated from the average of three diameters, spaced at equal intervals along the length of the fibre segment, assuming a circular shape. Sarcomere length was determined by counting sarcomeres at several intervals of 30 μm.

Statistical analysis

Data were expressed as means and standard errors. Statistical significance of the differences between means was assessed by analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. A probability of less than 5% was considered statistically significant.

Results

MHC isoform expression in bovine muscles

Samples of bovine muscles were analysed with 9% SDS–PAGE to detect MHCs described in the Materials and methods. One MHC band was found in masseter, two bands were found in most samples of diaphragm and three bands were found in longissimus dorsi, extensor carpi radialis and pectoralis (Fig. 1A). Comparison with the results of RT-PCR in the same muscles (Fig. 1B) identified the band present in masseter as MHC-1 (also called β/slow), the upper band present in diaphragm as MHC-2A, and the middle band detectable in longissimus dorsi, extensor carpi radialis and pectoralis as MHC-2X.

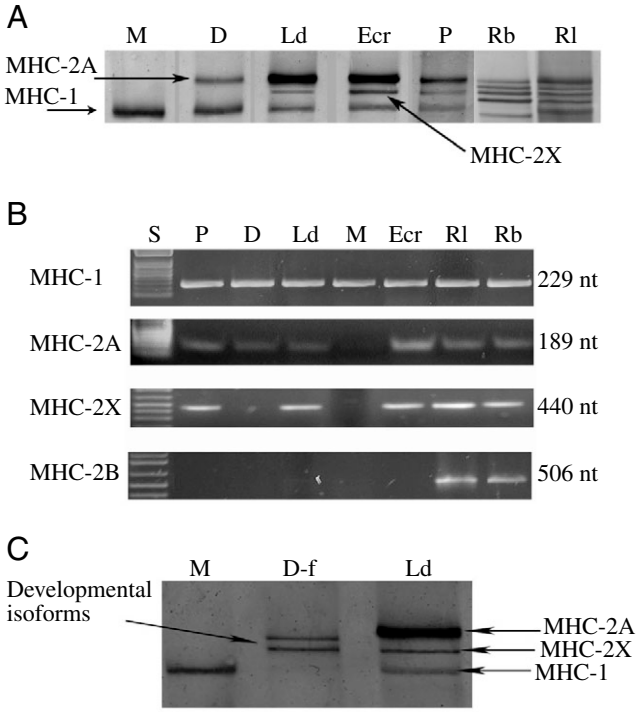

Fig. 1. MHC isoform expression in bovine muscles. (A) Electrophoretic separation of MHC isoforms in various adult bovine muscles: M, masseter; D, diaphragm; Ld, longissimus dorsi; Ecr, extensor carpi radialis; P, pectoralis; Rb, retractor bulbi; Rl, rectus lateralis. (B) RT-PCR analysis of MHC isoform expression in the same muscles as in A. S, size standard. (C) Electrophoretic separation of MHC isoforms in foetal diaphragm (D-f) compared with adult masseter and longissimus dorsi muscle.

When electrophoretic protocol was applied to extraocular muscles (Rl and Rb), a high number of bands (up to six) was detected in the region of 200–220 kDa (Fig. 1A). RT-PCR showed that all four adult MHC isoforms (i.e. 1, 2A, 2X and 2B) were expressed (Fig. 1B; see also Maccatrozzo et al., 2004). To identify other bands detectable in the MHC region, we first analysed immature bovine muscles. Fig. 1C shows MHC isoform separation in samples of foetal muscles: two bands, one migrating above the MHC-2X band and one co-migrating with the MHC-2X band, are clearly detectable and correspond to neonatal (MHC-Neo) and embryonic (MHC-Emb) isoforms. As expected (Picard et al., 1994), at 120 days after conception no other MHC isoforms are expressed.

Using a specific protocol (8% gels, see Materials and methods) for separation of cardiac MHC α from MHC-1 or β/slow, the two cardiac isoforms were resolved. Fig. 2 shows the two cardiac MHC isoforms in bovine atrial and ventricular myocardium, which contain α and β MHCs, respectively (Gorza et al., 1986). Among the muscles examined, a weak band of α MHC was detected only in masseter (Figs 2, 6), whereas both rectus lateralis and retractor bulbi muscles showed the presence of α cardiac MHC isoform (data not show).

Specific primers were prepared for MHC-Neo and MHC-Eo

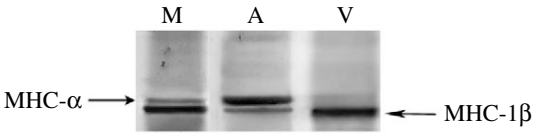

Fig. 2. Electrophoretic analysis of the expression of α and β MHC isoforms in atrial myocardium (A), ventricular myocardium (V) and masseter (M).

(see Table 1), and RT-PCR showed that both extraocular and neonatal isoforms were expressed in rectus lateralis (data not shown) and retractor bulbi (see Fig. 4A) in addition to the four major adult MHC isoforms. The results of RT-PCR together with the immunohistochemical staining (see Fig. 3) confirmed the complex pattern of MHC isoform expression in extraocular muscles without giving any clue to the identity of the specific MHC isoforms corresponding to the bands separated by the 9% SDS–PAGE.

Fig. 3 shows immunohistochemical staining of a composite block with rectus lateralis and longissimus dorsi. Serial sections showed that individual fibres of rectus lateralis were reactive with the antibodies anti-MHC-Neo, anti-MHC-Eo and anti-MHC-2B (BF-F3), whereas longissimus dorsi was completely negative when exposed to the same antibodies. It is interesting to note that, in the extraocular muscle, some fibres react with all three antibodies (asterisks in Fig. 3), many fibres react with two of them (circles in Fig. 3) and one fibre reacts only with anti 2B (hash mark in Fig. 3). As described in previous studies (Jacoby et al., 1990; Lucas and Hoh, 1997), hybrid fibres are particularly abundant in extraocular muscles.

The unambiguous identification of electrophoretic bands required the parallel analysis of MHC isoforms at the RNA and protein level in samples with different MHC isoform composition. RNA and protein identification from individual fibres was first validated on diaphragm fibres (N=10) and then applied to bundles of few (2–10) fibres from extraocular muscles, since fibres were very thin in these muscles and did not yield enough material to determine protein and RNA in a single fibre. The analysis of such fibre bundles permitted us to completely identify the relative positions of MHC isoforms in electrophoretic migration. Fig. 4 shows three typical small bundles with increasing numbers of electrophoretic bands. Fibre bundle 10Rb exhibited two electrophoretic bands and two RT-PCR products: MHC-2A, with the slowest electrophoretic migration (see above), and MHC-Eo. Thus, the second band in 10Rb could be identified as MHC-Eo with a migration position above MHC-slow (compare with the larger samples of retractor bulbi, where five bands are visible). The migration position of MHC-Emb was determined in fibre bundle 7Rl. At RNA level, two products corresponding to 2A and Eo were amplified. A third band showed a migration position identical to one of two developmental isoforms (just below MHC-2A; see Fig. 1C). This band was identified as MHC-Emb, because in this sample no MHC-Neo was amplified with RT-PCR. A similar analysis on bundle 7Rb

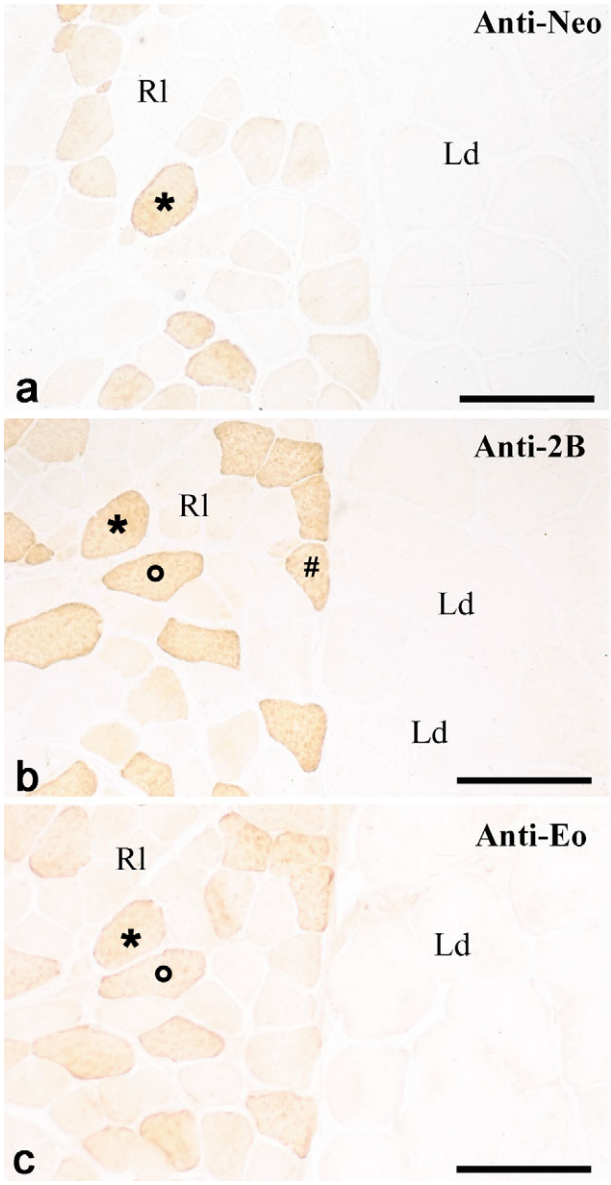

Fig. 3. Immunohistochemistry on a composite block of longissimus dorsi (Ld) and rectus lateralis (Rl) muscles. Serial sections are stained with anti-neonatal MHC (a), anti-2B MHC (b) and anti-extraocular MHC (c) antibodies. In one skeletal muscle (Ld), all fibres are negative whereas in extraocular muscle (Rl) some fibres are positive to the antibodies tested. The 'hybrid' fibres are numerous and positive either to all antibodies (asterisk) or to two of them (circle); only a few fibres are 'pure' and positive with anti-2B MHC antibody (hash mark). Scale bar, 100 μm.

from retractor bulbi confirmed the migration positions of MHC-2A, MHC-Emb, MHC-2X and MHC-Eo. Finally, the analysis of larger samples of retractor bulbi (Rb in Fig. 4), where neonatal, Eo, 2A, 2X, 2B and slow MHC isoforms were expressed, made it possible to determine the migration position of MHC-2B. MHC-2B migration was intermediate between MHC-Eo and MHC-2X, which co-migrated with MHC-Neo (see also Fig. 1C). Thus, the immunohistochemical

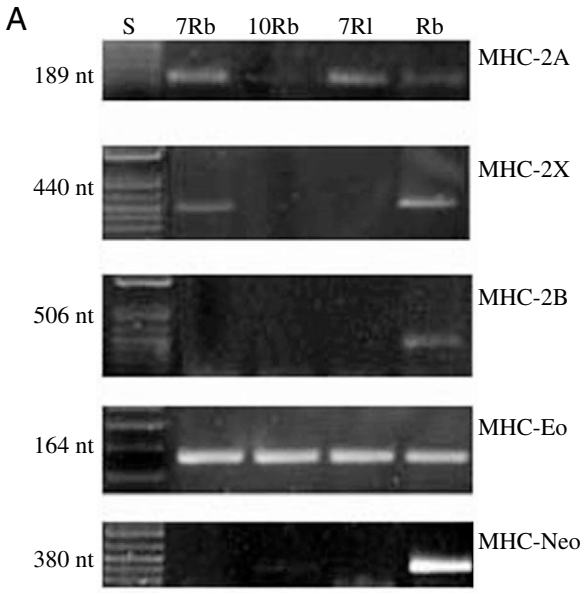

Fig. 4. MHC isoform expression in bundles of fibres from rectus lateralis (7Rl) and from retractor bulbi (7Rb, 10Rb) and in two larger samples of retractor bulbi muscle (Rb). The same MHC isoforms were detected by RT-PCR (A) and SDS–PAGE (B). Two samples of Rb are shown because it is not possible to detect all six bands in the same sample due to the different proportion of each isoform and to the distribution of the isoforms inside the muscle. S, size standards.

(Fig. 3) and the electrophoretic (Fig. 4) results both support the presence of MHC-2B protein in rectractor bulbi and rectus lateralis.

Since MHC-2B might be expressed also in laryngeal muscles (see for a review Hoh, 2005), we extended the analysis to bovine laryngeal muscles. Results are shown in Fig. 5. All specialized laryngeal muscles expressed MHC-1 and MHC-2A, both at the RNA (data not shown) and the protein level (Fig. 5B). MHC-2X was present in arytenoideus transversus (At) and in thyreoarytenoideus (Tvr, Tvc, Tvo). MHC-Neo was expressed in rostral ventricularis portion (Tvr) and in the vocalis portion (Tvo) of thyreoarytenoideus muscle (see Fig. 5A,B, where the band corresponding to MHC-Neo is superimposed on the MHC-2X band). MHC-2B and MHC-Eo isoforms were absent in all laryngeal samples, both at the RNA and the protein level (see Fig. 5A,B).

Contractile performance of single muscle fibres

Single fibres, dissected from masseter (N=25), diaphragm (N=36) and longissimus dorsi (N=35) were analysed with regard to their contractile properties and classified on the basis of their MHC isoform composition, as determined by SDS–PAGE.

The values of v_0 determined in the fibres dissected from masseter showed a clear bimodal distribution, as can be seen in Fig. 6. The electrophoretic analysis on 8% gels showed the presence of MHC-α at various proportions in a group of fibres dissected from masseter. In this way, two groups could be formed: one containing only MHC-β/slow and showing lower average v_0 ($0.25\pm0.02\ L\ s^{-1}$) and a second also containing MHC-α and characterised by higher average v_0

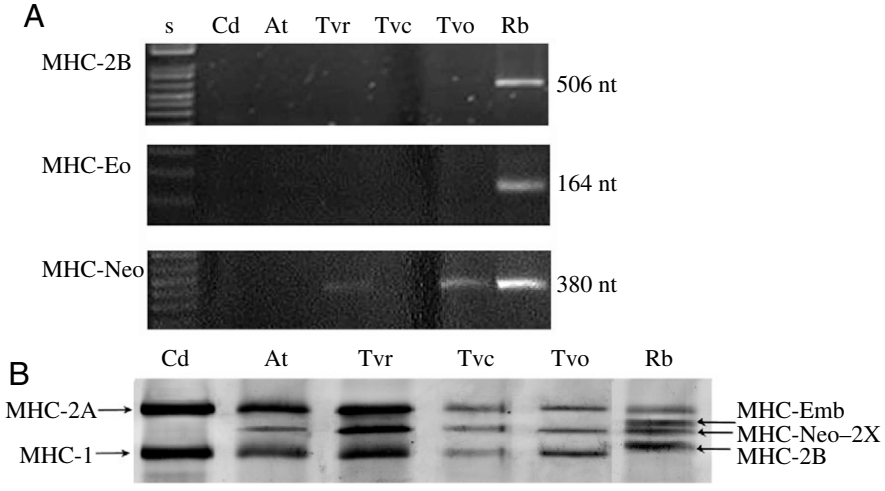

Fig. 5. MHC isoform expression in bovine laryngeal muscles. (A) RT-PCR using specific primers for MHC-2B, MHC-Eo and MHC-Neo. (B) Electrophoretic separation of MHC isoforms; the migration order is (from top to bottom): MHC-2A, MHC-Emb, MHC-2X and MHC-Neo (comigration), MHC-2B, MHC-1. Cd, cricoarytenoideus dorsalis; At, arytenoideus transverses; T, thyreoarytenoideus [Tvr, Tvc (pars rostralis and caudalis of ventricular part) and Tvo (pars vocalis)]; Rb, retractor bulbi (used as a control; note that MHC-1 is not detectable in this sample).

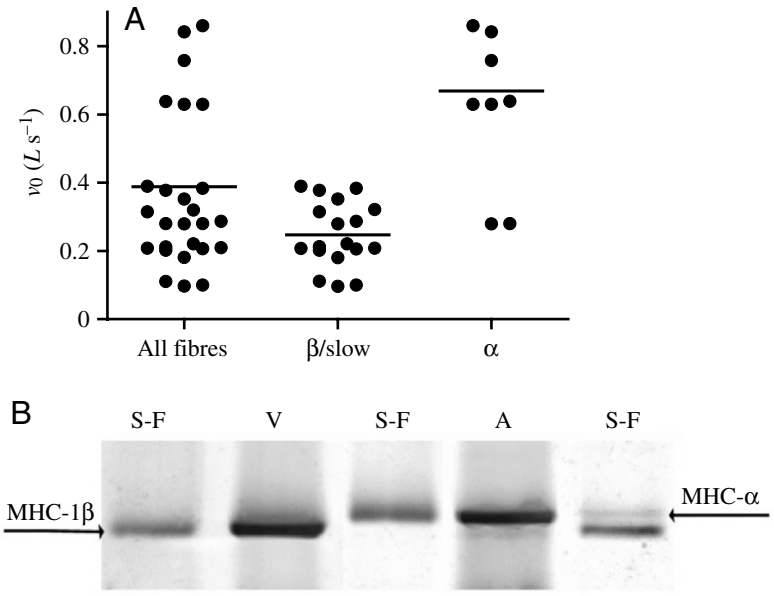

Fig. 6. Single fibre analysis in bovine masseter. (A) Distribution of maximum shortening velocity (v_0) values of 35 fibres isolated from masseter and studied for their mechanical properties and their MHC isoform composition. Each dot corresponds to a single fibre: first column shows all fibres together; second column shows fibres expressing only MHC-β/slow; third column shows fibres also expressing MHC-α. (B) Examples of electrophoretic separation of MHC isoforms in bovine masseter fibres: S-F, single fibre; V, ventricular myocardium (expressing MHC-β); A, atrial myocardium (expressing mainly MHC-α).

(0.668±0.08 L s^{-1}; P<0.01 compared with the former group). P_0 was not significantly different between the two groups (53.8±6.8 *vs* 54.5±7.0 mN mm^{-2}), and the same was true for cross-sectional area (1401±89 *vs* 1536±182 μm^2). Slow fibres (i.e. fibres containing only MHC-β/slow) from masseter showed v_0 values similar to those isolated from diaphragm (0.29±0.03 L s^{-1}); this was in agreement with our previous observations (Toniolo et al., 2004) that fibres with the same MHC isoform composition have similar values of v_0 regardless the muscle of origin. It was thus possible to form a large homogeneous group of slow fibres, whose parameters (v_0, P_0 and CSA) are reported in the histograms of Fig. 7 together with other groups of fibres, formed on the basis of their MHC isoform composition regardless of the muscle of origin.

The mean CSA values of single fibres grouped according to their MHC isoform composition are shown in Fig. 7A. Slow fibres were much thinner than fast fibres: this was true for both slow fibres from masseter (1401±89 μm^2, N=25) and slow fibres from diaphragm (2050±274 μm^2, N=13). Fast 2A and 2X fibres were significantly thicker.

Slack sarcomere lengths were not significantly different among fibre types: 2.38±0.04 μm for slow fibres, 2.42±0.08 μm for 2A fibres, 2.44±0.09 μm for 2X fibres. Since fibres were stretched by approximately 20% after mounting, the activation was induced at sarcomere lengths of 2.78±0.04 μm in slow fibres, 2.73±0.08 μm in 2A fibres and 2.78±0.11 μm in 2X fibres.

Fibres with different MHC isoform composition exhibited large diversity in maximum v_0 and, to a lesser extent, in P_0 (see Fig. 7 where statistical comparisons are reported). v_0 values

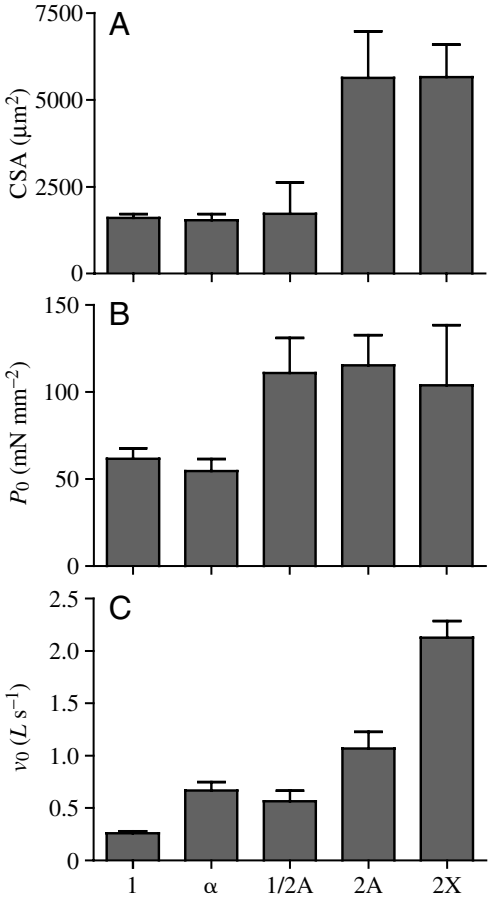

Fig. 7. Mean values (+ S.E.M.) of cross-sectional area (A), isometric tension (B) and maximum shortening velocity (C) in five fibre types from bovine muscles. Variance analysis showed that (1) cross-sectional area values of 2A and 2X fibres were significantly different from those of type 1, 1–2A and α fibres, (2) isometric tension of 1 and α types was significantly different from that of 1–2A, 2A and 2X, (3) maximum shortening velocity values of each fibre type were significantly different from those of all other types, except α and 1–2A.

increased from slow to fast 2A and 2X fibres, with intermediate values in fibres containing MHC-α and hybrid 1–2A fibres. Fast 2X fibres had a v_0 value significantly higher than fast 2A fibres. Series compliance (expressed relative to fibre segment length) was not significantly different among the fibre groups, ranging from 5.3±1.4% in slow fibres to 6.6±0.9% in 2X fibres.

The time course of tension redevelopment after a manoeuvre of fast shortening and re-lengthening, with an interval of 30 ms, was recorded and characterised by the time required to redevelop two-thirds of isometric tension. Slow fibres showed the longest redevelopment time (1.94±0.16 s), 2X fibres showed the shortest (0.29±0.02 s), and 2A fibres showed an intermediate value (0.60±0.22 s).

Discussion

The first major result of this study was that, based on careful electrophoretic analysis combined with RT-PCR and immunohistochemistry, eight proteins corresponding to MHC isoforms were identified in adult bovine striated muscles. The eight MHC isoforms are two cardiac isoforms, namely MHC-α and MHC-β, which is also called type 1 or slow, two developmental isoforms, i.e. MHC-embryonic and MHC-neonatal, three adult fast isoforms, MHC-2A, MHC-2X and MHC-2B, and the extraocular isoform MHC-Eo. All eight MHC isoforms were found co-expressed in extrinsic eye muscles, retractor bulbi and rectus lateralis. In other muscles, fewer isoforms were expressed, in particular three (1, 2A and 2X) in trunk and limb muscles and only two (β/slow and α) in masseter. The expression of MHC-2B and MHC-Eo was restricted to extraocular muscles. Developmental MHC isoforms (neonatal and embryonic) were only found in specialized muscles in the larynx and in the eye. MHC-α was only found in masseter and extraocular muscles.

The procedure adopted for MHC isoform identification was similar to that utilized in our recent study on pig muscles (Toniolo et al., 2004). The specificity of monoclonal antibodies needs to be validated in each species, and the order of electrophoretic migration of MHC isoforms might change from species to species (Reggiani and Mascarello, 2004). Thus, MHC isoforms were first identified at the mRNA level, since this allows an unambiguous identification based on the comparison of base or amino-acid sequences with orthologous isoforms in other animal species. Then, immunohistochemistry and electrophoresis were employed on those samples that had been previously analysed with RT-PCR, and the presence of electrophoretic bands was considered the ultimate proof of the existence of a protein product. Importantly, electrophoresis could be applied to determine MHC isoform composition in single muscle fibres that had been characterised with regard to their contractile properties, and this provided a functional validation of the MHC identification.

The expression of MHC-2B is of special interest as it represents a controversial issue. This isoform is abundantly expressed in small rodents, rabbit and marsupials (Schiaffino and Reggiani, 1996; Zhong et al., 2001). In human muscles, it is present as mRNA but not as protein (Smerdu et al., 1994; Horton et al., 2001). Recent studies (Chang and Fernandes, 1997; Chikuni et al., 2001; Toniolo et al., 2004) have shown MHC-2B expression in porcine skeletal muscles. Controversial results have been obtained with gel electrophoresis in bovine muscles, as two (Totland and Kryvi, 1991; Jurie et al., 1995) or three (Picard et al., 1999) fast MHC isoforms have been separated in adult skeletal muscles. RT-PCR has unambiguously shown that only MHC-2A and MHC-2X are expressed in trunk and limb muscles of cattle (Tanabe et al., 1998; Chikuni et al., 2004b). Our results confirm that only 1, 2A and 2X MHC isoforms are expressed in trunk and limb muscles (Maccatrozzo et al., 2004) but also show (present study) the presence of MHC-2B as mRNA and protein in specialized extraocular muscles. This finding is clearly in contrast with the conclusion of Chikuni and co-workers (Chikuni et al., 2004b) that a functional gene coding for MHC-2B is not present in the genome of all ungulates examined, including the water buffalo (*Bubalus bubalis*), a species strictly related to cattle (*Bos taurus*). Our results are in accordance with the information recently made available in GenBank showing the sequence of a gene coding for MHC-2B in the cattle genome (accession number XM_615303).

Interestingly, our finding that MHC-2B is present in specialized muscles as mRNA and protein is at variance with the observation that, in some human muscles, MHC-2B is present as mRNA but not as protein (Horton et al., 2001). The lack of a protein in fibres where the corresponding mRNA is present implies a post-transcriptional regulation that either inhibits the translation of the protein or induces a prompt degradation, making accumulation in the cytosol or in the myofibrils impossible. An example of incomplete transcription followed by degradation is the MHC-2M isoform in human masticatory muscles (Stedman et al., 2004). If the observations by Horton and co-workers raise a question about post-transcriptional regulation, our finding that MHC-2B expression is restricted to extraocular muscles raises a question about transcriptional regulation. Why is expression restricted to such specialized muscles? Several MHC isoforms are expressed in extraocular muscles, as suggested by published evidence on various animal species [mouse (Porter et al., 2001), rat (Wieczorek et al., 1985; Rubinstein and Hoh, 2000), rabbit (Briggs and Schachat, 2002; Lucas and Hoh, 2003)]. In particular, MHC-Eo (Sartore et al., 1987), developmental MHCs (Jacoby et al., 1990) and α cardiac MHC (Wieczorek et al., 1985; Lucas and Hoh, 2003) can be expressed in extraocular muscles. Whether such peculiar expression should be related to the embryological origin or to the specialized contractile requirements of extraocular muscles is a matter of speculation. MHC-2B and MHC-Eo are expressed both in rectus lateralis and retractor bulbi. Whereas rectus lateralis is involved in a variety of motor tasks, such as fixation, pursuit and saccades, which might require fibres expressing different MHC isoforms, retractor bulbi does not have any known motor function. The mechanisms leading to stabilization of the expression of developmental isoforms in specialized laryngeal

and extraocular muscles are also unknown. Bovine laryngeal muscles are rather similar to trunk and limb muscles in their MHC isoform expression, as MHC-Eo and MHC-2B are not present. In thyreoarytenoideus, a fourth MHC isoform was present and was identified as MHC-Neo. The expression of an additional MHC isoform is a typical feature of thyreoarytenoideus as discussed by Hoh (2005) and may need further investigation.

A last 'open issue' concerns the presence of MHC-α in masseter. Although this is the first demonstration of the expression of MHC-α in bovine masseter, the presence of this isoform in masticatory muscles is not surprising as it has been found in human, rabbit and marsupial masseter (Bredman et al., 1991; Hoh et al., 2000). As discussed by Hoh et al. (2000), fibres expressing MHC-α are particularly suited for the diet and the chewing action of wallaby and kangaroo, where masseter is homogeneously composed of this isoform. The masticatory function of cattle, i.e. the slow, rhythmic and long-lasting rumination, seems to fit well with the expression of MHC-slow. It is likely that for other types of movement, e.g. grasping grass and grazing, a small group of fast motor units is needed and, in those fast fibres, MHC-α might replace MHC-2A, which is not expressed in bovine masticatory muscles.

The second novel result of this study is the characterisation of contractile properties of single skeletal muscle fibres in relation to their MHC isoform content. Fibres containing the three major MHC isoforms – β/slow/1, 2A, 2X and the less-studied MHC-α – have been characterised. In a previous study (Seow and Ford, 1991), only two types of fibres were described – fast and slow – without any further identification of fast subtypes. Here, five types (1, α, 1–2A, 2A, 2X) were characterised on the basis of a large pool of fibres dissected from masseter, diaphragm and longissimus dorsi. Until now, only one study has measured maximum shortening velocity in skeletal muscle fibres expressing MHC-α (Sciote and Kentish, 1996). Fibres were dissected from rabbit masseter and their v_0 was intermediate between slow and fast 2A fibres. Similar conclusions were reached in a recent study (Andruchov et al., 2004) where tension transients after a small and fast elongation were analysed. Our results indicate that fibres containing MHC-α are similar to hybrid 1–2A fibres in their speed of shortening, tension development and, thus, in power output. The functional advantage given by the presence of MHC-α is not clear (see above), but one might speculate that fibres expressing MHC-α alone or together with MHC-β represent more stable units than hybrid 1–2A, which are considered transition fibres.

Interestingly, bovine fibres dissected for this study from cows of more than 400 kg were rather thin (range 1700 μm^2 for slow fibres and 5600 μm^2 for 2X fibres, after swelling due to chemical skinning) compared with those dissected from other mammalian species in the same experimental conditions [see, for example, human fibres (Bottinelli et al., 1996) and pig fibres (Toniolo et al., 2004)]. Extraocular and laryngeal muscles provided fibres too thin to be successfully analysed in mechanical experiments. The low values of diameters of bovine skeletal muscle fibres were observed also by Ford and co-workers (Seow and Ford, 1991), who proposed an inverse correlation between isometric tension and diameters. Actually, we confirmed that values of isometric tension developed by fast bovine fibres are rather high in comparison with corresponding fibres from other animal species (see Fig. 8).

The determination of v_0 values in fibres with distinct MHC isoform composition leads to the usual trend, with v_0 increasing from slow to fast 2A to fast 2X MHC. This trend confirms the identification based on the base sequence from the functional point of view. In addition, the values of v_0 obtained in bovine fibres can be compared with corresponding values determined in other species. The values of v_0 obtained in this study can be represented on the scaling diagram published as fig. 6 by Toniolo et al. (2004), and the resulting relationship between v_0 values and body size for six species and four fibre types is shown in Fig. 8. v_0 values measured in bovine fibres were

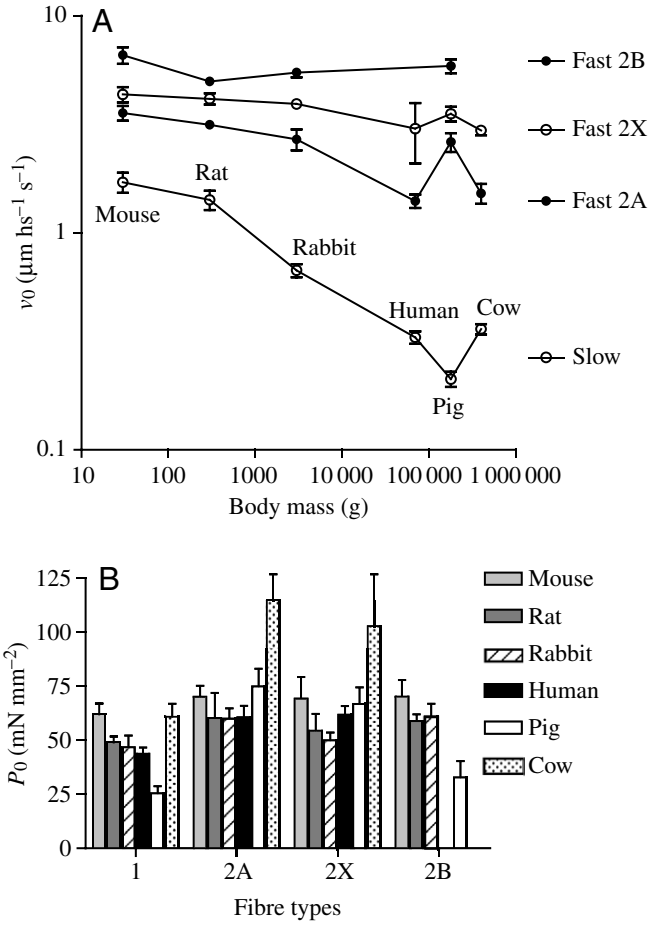

Fig. 8. Interspecies variations of maximum shortening velocity (v_0) and isometric tension (P_0). (A) Relationship between v_0 (expressed in μm hs^{-1} s^{-1}, where hs is half sarcomere, for correct comparison among species) and body size. (B) Values of P_0 measured in various fibre types in six animal species. Data for mouse, rat, rabbit and human come from Pellegrino et al. (2003); data for pig come from Toniolo et al. (2004).

similar to those measured in human fibres under identical conditions and, compared with those measured in porcine fibres, were higher for slow fibres but lower for fast fibres. Pig muscles, however, represent an exception also for the abundance of MHC-2B in trunk and appendicular muscles, which offers a further broadening of functional possibilities (Toniolo et al., 2004). The present results on bovine fibres confirm that the scaling rule apparently holds true even to 500 kg body mass (i.e. ~20 000-fold heavier than a mouse), at least in general terms, although some minor discrepancies are clearly present.

In conclusion, our results provide a general and complete description of bovine striated muscles at the fibre level, particularly with regard to specialized muscles such as masseter, extraocular and laryngeal muscles, myosin isoform expression and contractile properties. The most intriguing finding of this study is the evidence of MHC-2B expression at the protein level in those muscles where the corresponding mRNA is present, i.e. extraocular muscles. This finding raises new and unanswered questions about signalling pathways and gene expression regulation concerning the MHC-2B isoform.

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