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Fast fibres in a large animal: fibre types, contractile properties and myosin expression in pig skeletal muscles

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Summary

Little is known about the influence of Myosin Heavy Chain (MHC) isoforms on the contractile properties of single muscle fibres in large animals. We have studied MHC isoform composition and contractile properties of single muscle fibres from the pig. Masseter, diaphragm, longissimus, semitendinosus, rectractor bulbi and rectus lateralis were sampled in female pigs (aged 6 months, mass 160 kg). RT-PCR, histochemistry, immunohistochemistry and gel electrophoresis were combined to identify and separate four MHC isoforms: MHC-slow and three fast MHC (2A, 2X, 2B).

Maximum shortening velocity (V_0) and isometric tension (P_0) were measured in single muscle fibres with known MHC isoform composition. Six groups of fibres (pure: slow, 2A, 2X and 2B, and hybrid: 2A-2X and 2X-2B) with large differences in V_0 and P_0 were identified. Slow fibres had mean V_0=0.17±0.01 length s^{-1} and P_0=25.1±3.3 mN mm^{-2}. For fast fibres 2A, 2X and 2B, mean V_0 values were 1.86±0.18, 2.55±0.19 and 4.06±0.33 length s^{-1} and mean P_0 values 74.93±8.36, 66.85±7.58 and 32.96±7.47 mN mm^{-2}, respectively. An *in vitro* motility assay confirmed that V_0 strictly reflected the functional properties of the myosin isoforms.

We conclude that pig muscles express high proportions of fast MHC isoforms, including MHC-2B, and that V_0 values are higher than expected on the basis of the scaling relationship between contractile parameters and body size.

Key words: myosin heavy chain, isoform, shortening velocity, pig.

Introduction

In skeletal muscle fibres, myosin isoform composition is the main determinant of the contractile performance. Most information about the relationship between myosin isoform composition and contractile parameters such as maximum shortening velocity (V_o) and peak power (W_{max}) derive from studies on small laboratory animals such as rabbits (Sweeney et al., 1988), rats (Bottinelli et al., 1991) and mice (Pellegrino et al., 2003). Several studies have also investigated this relationship in human muscles in view of the relevance of muscle performance for neurology, gerontology and sport medicine (Larsson and Moss, 1993; Bottinelli et al., 1996; Widrick et al., 1996). Much less is known about how myosin isoforms determine muscle contractile parameters in large mammals. Several studies have described myosin isoforms and fibre types of equine muscles in connection with horse race performances, or in cattle and pig with respect to meat production. Only two studies have considered the impact of myosin isoform composition on maximum shortening velocity and peak power of single muscle fibres in large mammals (Rome et al., 1990; Seow and Ford, 1991). In these studies myosin isoforms were only partially identified and the analyses of the contractile properties were restricted to a limited number of fibre types.

Among large domestic mammals, the pig has received increasing attention in the last few years. From the most traditional point of view, pork meat is an important component of human food and several studies have examined the relationship between meat quality and muscle characteristics in various breeds (Huff-Lonergan et al., 2002; Davoli et al., 2003). The impact of feeding and breeding has been investigated and attention paid to the appearance of diseases related to intense selection, for example the halothane gene and malignant hyperthermia (MH) or porcine stress syndrome (PSS; Nelson, 2002; Depreux et al., 2002). Recently, the pig has become important in medicine, not only as a model for studying human disease, but also for the development of xenotransplantation (see, for example, Halperin, 2001; Dooldeniya and Warrens, 2003) and for the use of a small size variant (mini-pig) as a valuable pharmacological model (Bollen and Ellegaard, 1997). The amount of information on genes and

gene expresssion in the pig is quickly growing (Hawken et al., 1999; Yao et al., 2002) and makes the pig a candidate for complete genome sequencing.

Whereas excitation–contraction coupling in porcine muscles has been extensively investigated with respect to MHC (for reviews, see Mickelson and Louis, 1996; Meltzer and Dietze, 2001), no information is available on the relationship between contractile performance and myosin isoforms. Fibre typing has been done using several methods: histochemistry based on mATPase, immunohistochemistry, electrophoresis and, more recently, molecular biology. The results are partially controversial: expression studies based on RT-PCR clearly demonstrated that four distinct myosin heavy chain (MHC) isoforms (Chang and Fernandes, 1997; Chikuni et al., 2001; Lefaucheur et al., 2002), identified as slow, fast 2A, fast 2X and fast 2B for homology with other species, are expressed in adult pig skeletal muscles. Interestingly, the fast isoform 2B, which is generally considered typical of small mammals and marsupials (Zhong et al., 2001) and not expressed in large mammals such as man (Smerdu et al., 1994) and horse (Serrano et al., 1996), or in dog (Latorre et al., 1993), baboon and cat (Lucas et al., 2000; see also Schiaffino and Reggiani, 1996), is clearly expressed in pig muscles. However, electrophoresis has not achieved the separation of the corresponding four MHC isoforms (Bee et al., 1999), whereas the results from histochemistry and immunohistochemistry are controversial (Lefaucheur et al., 2002) due to the lack of reliable antibodies specific for pig MHC-2X and MHC-2B and the presence of a high proportion of hybrid fibres. The existence of large populations of hybrid fibres in pig muscles, where more than one MHC is expressed, was confirmed by *in situ* hybridization experiments, which also showed a mismatch between mRNA and protein expression (Lefaucheur et al., 2002).

The aim of this study was to analyse the contractile properties of pig muscle fibres in order to (i) extend our knowledge on the relationship between MHC isoforms and maximum shortening velocity in a large animal, and (ii) analyse, in a large animal, the contractile behaviour of fibres expressing MHC-2B. To this end we strengthened the identification criteria of myosin isoforms in pig skeletal muscle fibres by combining several approaches and established a clear relationship between each myosin isoform and the contractile parameters of the fibres where it is expressed. We also determined the speed at which actin filaments are translocated by each myosin isoform in an *in vitro* motility assay to assess whether single fibre shortening velocity is dependent only on myosin isoform composition or is also influenced by other myofibrillar proteins.

Materials and methods

Sampling of pig muscles

Muscle samples were collected from female pigs of the commercial breed ‘large white’ (*N*=7, age 6 months, mass 140–160 kg) killed in a slaughter house. The following muscles were sampled within 1 h *post mortem*: masseter, diaphragm, longissimus dorsi, semitendinosus (deep red and superficial white portions), rectus lateralis and retractor bulbi.

Single fibre mechanics

Single fibres were manually dissected under a stereomicroscope (10–60× magnification). At the end of the dissection, fibres were bathed for 1 h in a skinning solution containing 1% Triton X-100 to ensure complete membrane solubilization. Segments of 1–2 mm length were then cut from the fibres and light aluminium clips were applied at both ends. Skinning, relaxing, pre-activating and activating solutions employed for mechanical experiments with single fibres were prepared as previously described (Pellegrino et al., 2003). Protease inhibitors (E64 10 μmol l^{-1} and leupeptin 40 μmol l^{-1}) were added to all solutions.

Once the clips had been applied, the fibre segment was mounted in the experimental set-up in a drop of relaxing solution between the force transducer (AME-801 SensorOne, Sausalito, California) and the electromagnetic puller (SI, Heidelberg, Germany) equipped with a displacement transducer. All details of the set-up and the recording system were as described previously (Pellegrino et al., 2003). Diameters and sarcomere length were measured at 320× magnification. The contractile properties of each fibre were determined by measuring (i) isometric tension (P_o) during maximal activation (pCa=4.6), (ii) unloaded shortening velocity (V_o) and series compliance (SE) according to the ‘slack test’ procedure (Edman, 1979). All experiments were performed at 12°C. The experimental procedure has been described fully (Pellegrino et al., 2003). At the end of the experiment each fibre was immersed in Laemmli solution for electrophoretic analysis (see below).

Myosin extraction and in vitro *motility assay*

Myosin was extracted and purified from single muscle fibres dissected from pig diaphragm and longissimus muscle as previously described (Canepari et al., 1999). A fragment of each fibre was immersed in Laemmli solution for electrophoretic identification of MHC isoforms (see below). Sliding velocity of actin filaments labelled with rhodamine–phalloidin was determined for the myosin prepared from each fibre in an *in vitro* motility assay, as previously described (Pellegrino et al., 2003).

Histochemistry and immunohistochemistry

Muscle samples alone or combined into composite blocks were frozen in isopentane cooled with fluid nitrogen, and serial sections (10 μm) cut in a cryostat. Serial sections were stained for myofibrillar ATPase (mATPase) as previously described in detail (Latorre et al., 1993). ATPase staining followed either alkaline pre-incubation at increasing pH values (method 1, pH 10.2, 10.3, 10.4 and 10.5, incubation times 7–15 min) or acid pre-incubation (method 2, sodium acetate 0.2 mol l^{-1}; method 3, sodium acetate 0.1 mol l^{-1} at pH 4.6, 4.5, 4,4); see Table 1. All methods well distinguished type 1 from type 2

fibres, but the separation of the different fast type 2 fibres was critical. mATPase activity after alkaline pre-incubation at increasing pH values allowed us to distinguish fast (positive) from slow (negative) fibres at pH 10.2, to separate 2A (weakly positive) from other fast fibres (positive) at pH 10.3–10.4 with a preincubation time of 7–10 min and to achieve an uncertain and weak differentiation between 2X and 2B fibres (pH 10.4 or 10.5 for 7–10 min). After 15 min of pre-incubation at pH 10.5, the mATPase activity disappeared in all fibres. The best results with mATPase activity after acid pre-incubation were obtained using method 2 (0.2 mol l^{-1} sodium acetate adjusted with acetic acid at pH 4.6, 4.55, 4.5, 4.4 for 5 min, see Table 1). In any case, the conditions in which 2X fibres are stained differently from 2B fibres are very critical.

Additional serial sections were stained with the following monoclonal antibodies: BAF8, BFD5, SC71, BF35, BFF3. The binding of the primary antibody was detected with a peroxidase-conjugated secondary antibody and visualized with the Envision method (Dako, Milano, Italy). The antibodies were purchased at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and had been previously characterized in rat muscles (Schiaffino et al., 1989; Bottinelli et al., 1991). The specificity of the mATPase reaction and the reactivity of the antibodies are shown in Table 1.

Electrophoretical analysis and western blot

The MHC isoform composition of muscle samples (fibre bundles) or single muscle fibres was determined on 8% polyacrylamide slab gels after denaturation in SDS (SDS-PAGE) using a procedure derived from Talmadge and Roy (1993). Slabs 18 cm wide, 16 cm high and 1 mm thick were electrophoresed in the cold room (4°C) for a total of 26 h (at 70 V for the first 1.5 h and 230 V for the remaining time). Whereas the compositions of the stacking and separating gels were identical to those described by Talmadge and Roy (1993), the increased gel thickness (1 mm instead of 0.75 mm, in order to reduce resistance) and the lower voltage associated with a prolonged running time allowed us to achieve the resolution required for the separation of all four adult MHC isoforms. Gels were stained using Bio-Rad silver stain plus. For western blot, proteins were transferred to nitrocellulose sheets according to the semidry transfer procedure (Towbin and Gordon, 1984) by applying a current of 0.8 mA cm^{-2} for 6 h. Nitrocellulose sheets were first reacted with primary anti-MHC antibodies and then with a peroxidase-conjugated secondary antibody. MHC bands were visualized by an enhanced chemiluminescence method in which luminol was excited by peroxidase in presence of H_2O_2 (ECL Amersham Products, Milano, Italy).

Table 1. *Specificity of the mATPase staining in relation to pH values and reactivity of the monoclonal antibodies used to identify MHC isoforms and fibre types*

	MHC isoform					
	1	2A	2A/X	2X	2X/B	2B
mATPase						
pH 10.3	–	+++	+++	+++	+++	+++
pH 10.5	–	++	++	+++	+++	+++
pH 4.6	+++	–	+	++	++	++
pH4.55/4.5	+++	–	–/+	+	+/++	++
pH 4.4	+++	–	–	–	–	–
Antibodies						
BAF8	+++	–	–	–	–	–
BFD5	+++	–	–	–	–	–
SC71	–	+++	++/+	–	–	–
BF35	–	–	+/++	+++	++/+	–
BFF3	–	–	–	–	–	+

Preparation of cDNAs and RT-PCR

RNA samples were extracted from pig muscle samples using TRIZOL Reagent (GibcoBRL, Life Technologies, Paisley, UK). The first-strand cDNAs were synthesised with random hexamers using Superscript RNase H-reverse transcriptase (GibcoBRL, Life Technologies) from the same amount of total RNA (1.5 μg). Qualitative PCR reactions were standardised for each single isoform (i.e. cycle numbers and annealing temperature). The following optimised PCR conditions (27 cycles for 45 s at 94°C, 45 s at 55°C and 45 s at 72°C) were adopted for the MHC isoform specific primers shown in Table 2. Forward primers were designed from the coding regions (sequences available on GenBank, accession numbers: MHC- 2A, AB025260; MHC-2B, AB025261; MHC-2X, AB025262; MHC-1, AB053226). Reverse primers were designed on the 3′ untranslated regions (UTRs), which have been sequenced by Chikuni et al. (2001, 2002). The sequence of the 3′ UTR of MHC-1 is available on GenBank (accession number, AB053226); the sequences of the 3′UTR of the fast isoforms were kindly provided to us by the authors

All PCR were performed in 20 μl of PCR mix: 1× PCR buffer (Gibco, BRL), 1.8 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} of each dNTPs, 0.5 μmol l^{-1} of each primer, 0.5 U Taq DNA polymerase and 1 μl cDNA. PCR products were electrophoresed on a 1.8% agarose gel, stained with ethidium bromide, and visualised under UV light. A fragment of the pig α-actin gene was amplified as an internal control to test the quality of extracted RNA and the efficiency of RT reaction (data not shown).

Table 2. *Primers used for RT-PCR of MHC isoforms*

MHC-2A	for 5′AGCCGGGAGGTTCACACAAA3′
	rev 5′CTTTGCAATAGGGTAGGATAGGC3′
MHC-2B	for 5′TGAAAATGAACAGAAGCGCAATG3′
	rev 5′TTTCCTTGACATCACATGACATATAAC3′
MHC-2X	for 5′TGAAGAAGAACCTGGAGCAGACG3′
	rev 5′CATTAAGTACAAAACAGAGTGACAAAGATT3′
MHC-slow	for 5′TGGAGCGCATGAAGAAGAACA3′
	rev 5′AGTGCTTTATTCTGCTTCCTCCAA3′

for, forward; rev, reverse.

Statistical analysis

Data are expressed as means ± S.E.M. 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

The first step towards a functional and structural characterization of single muscle fibres was the unambiguous identification of the myosin heavy chain (MHC) isoforms, which are the main determinants of contractile performance (see Schiaffino and Reggiani, 1996). The strategy to reach this goal was based on (1) sampling of muscles with different MHC isoform composition and (2) determination of MHC isoform composition in each muscle sample using three distinct approaches: (i) RT-PCR to identify the expressed MHC on the basis of their nucleotide sequence and establish a precise link with corresponding orthologous isoforms in different species, (ii) histochemistry and immunohistochemistry to confirm the expression of MHC isoforms as proteins and provide information on the muscle tissue architecture (fibre size, fibre distribution, pure and hybrid fibres), (iii) electrophoresis and western blot to separate and identify MHC isoforms. Electrophoresis of single muscle fibres enabled their classification into groups and interpretation of the mechanical experiments.

RT-PCR

Qualitative RT-PCR performed on several samples of masseter, diaphragm, red and white portions of semitendinosus, longissimus dorsi and rectractor bulbi showed clear differences in MHC isoform expression (Fig. 1). All four adult sarcomeric MHC isoforms were expressed in adult porcine muscles; in particular (i) the fast type 2A (fragment of 142 bp) was found in all muscle samples analysed, (ii) the slow type 1 (fragment of 573 bp) was expressed in every muscle sample tested except for the retractor bulbi, (iii) the fast MHC-2X (fragment of 541 bp) was expressed in all muscles examined with the exception of the masseter, (iv) the isoform 2B (fragment of 416 bp) was absent in masseter and diaphragm but was expressed in longissimus dorsi, white semitendinosus and retractor bulbi.

Histochemistry and immunohistochemistry

Histochemistry and immunohistochemistry were used to confirm the results of RT-PCR at the protein level. Samples of masseter, diaphragm, longissimus dorsi and red and white portions of semitendinosus were stained for mATPase after acid and alkaline pre-incubation, using appropriate incubation time, pH and molarity of pre-incubation solution, which permitted identification of the different fibre types (see Table 1).

Type 1 (strong acid-stable) and 2A (the most acid-labile fibre type, negative at pH lower than 4.6) fibres were easily identified, as can be seen in Fig. 2A, left (masseter at pH 4.55). The presence of type 1 and type 2A fibres in the masseter was in full agreement with RT-PCR data. In Fig. 2A, right (longissimus dorsi) another type of fibre was clearly detectable. This type, histochemically classified as conventional 2B (2* in the figure), showed a moderate acid-stable and strong alkaline-stable mATPase activity. This third type of fibre (conventional 2B or 2*) was also found in the red semitendinosus and in diaphragm (not shown). Whereas in longissimus dorsi RT-PCR revealed expression of both MHC-2X and MHC-2B, in

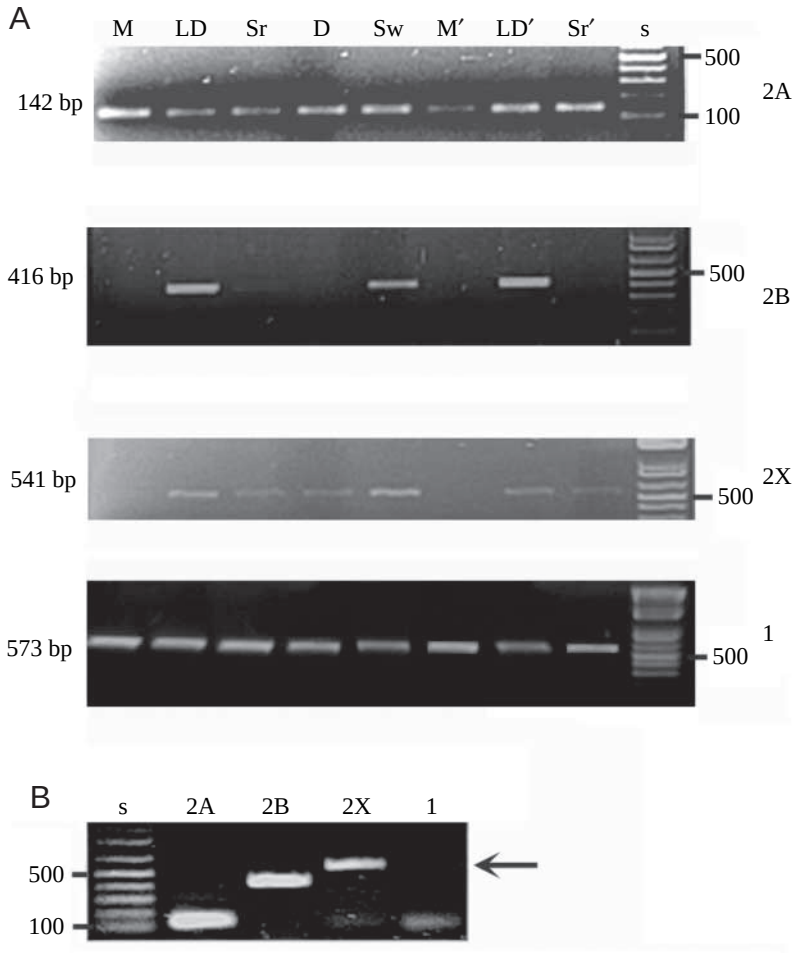

Fig. 1. RT-PCR expression analysis of pig myosin isoforms (1, 2A, 2B, 2X) on different muscle tissue. (A) Samples were loaded onto agarose gel in the following order: masseter (M), longissimus dorsi (LD), semitendinosus red portion (Sr), diaphragm (D), white semitendinosus (Sw) and duplicated masseter (M′), longissimus dorsi (LD′), red semitendinosus (Sr′). s, standards; 1Kb Plus DNA Ladder (Invitrogen). The brightest band (500 bp) and bottom band (100 bp) of the molecular size markers are indicated. (B) The same isoforms were analysed in the retractor bulbi muscle (Rb). All MHC isoforms are expressed except for the slow isoform (type 1). The arrow indicates the position of the amplification of the slow isoform fragment (of 573 bp, see A) that is not expressed in this muscle. The band at 100 bp is due to primer aspecific anealing. s, markers as in A.

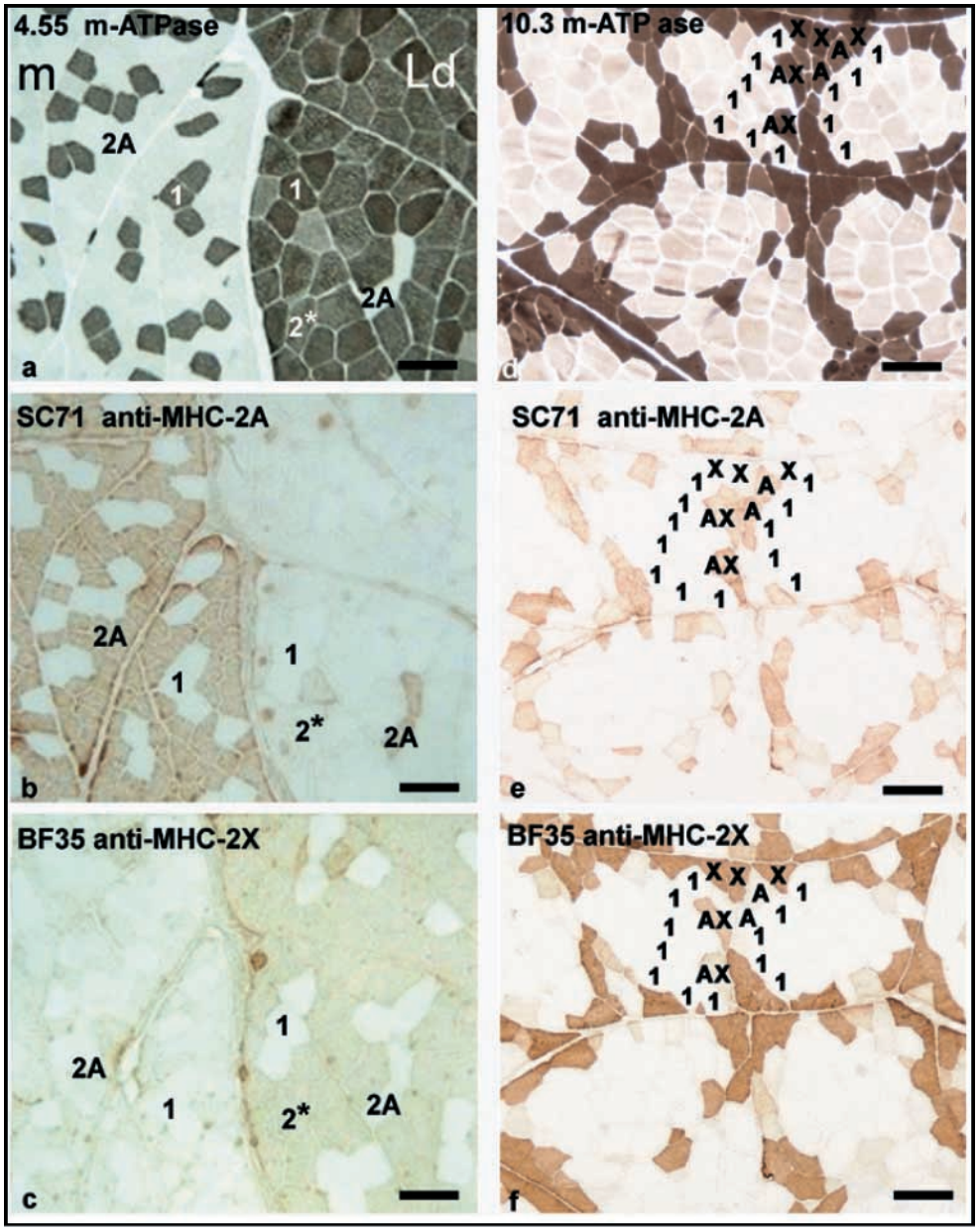

Fig. 2. Histochemistry and immunohistochemistry on a composition of masseter (m) and longissimus dorsi (Ld) (a–c) and on a sample of red semitendinosus (Str) (d–f). Consecutive sections were stained for mATPase with acid (pH 4.55; a) or alkali (pH 10.3; d) preincubation and with monoclonal antibodies specific for MHC-2A (SC71; b,e) and MHC-2X (BF-35; c,f). Slow fibres (type1) are dark with mATPase staining after acid preincubation and white after alkali preincubation. In masseter, only type 1 and 2A are present, whereas in red semitendinosus type 2X and 2A/X fibres are also detectable. In Ld muscle, the conventional 2B fibre type (2*) might be either 2B or 2X or hybrids (2A/X or 2X/B). Scale bars, 40 μm.

diaphragm and in most samples of the red semitendinosus RT-PCR did not show MHC-2B expression (see above and Fig. 1), implying that histochemical methods could not distinguish 2X from 2B fibres, as both appeared as conventional 2B. One sample of longissimus dorsi muscle (see Fig. 3A,D), taken from a very deep portion, near the lumbar vertebra and multifidus lumborum, showed fibres with gradual levels of acid or alkaline stable mATPase activity, probably indicating a distinction of the conventional 2B fibres in 2X and 2B (see Materials and methods): it was not possible, however, to distinguish 2X and 2B pure fibres from hybrid 2A/X, 2X/B fibres.

The immunohistochemical staining with the monoclonal antibodies BAF8 and the SC71 confirmed the histochemical identification of slow and 2A fibres and the results of RT-PCR (see Figs 2B,E and 3B,E). Staining with BF35 gave results similar to those reported in a previous study (Sciote and Rowlerson, 1998): BF35 did not react with type 1 and 2A (see masseter, longissimus dorsi and semitendinosus in Figs 2C,F and 3F) but was reactive with other type 2* fibres classified histochemically as conventional 2B. These fibres, positive to BF35, might be either 2B (in longissimus and white semitendinosus muscles) or 2X (in red semitendinosus and diaphragm) or hybrid 2A/X and 2X/B. The 2B conventional fibres were also generally negative to BFF3 specific for MHC-2B in rat muscles. However, in the sample of longissimus dorsi (taken from a very deep portion, near the lumbar vertebra, described before, see Fig. 3), some fibres were not reactive with BF35 and showed a moderate acid stable and strong alkaline stable mATPase activity. These latter fibres were also moderately positive to BFF3 and probably corresponded to pure 2B type (fibres indicated as B

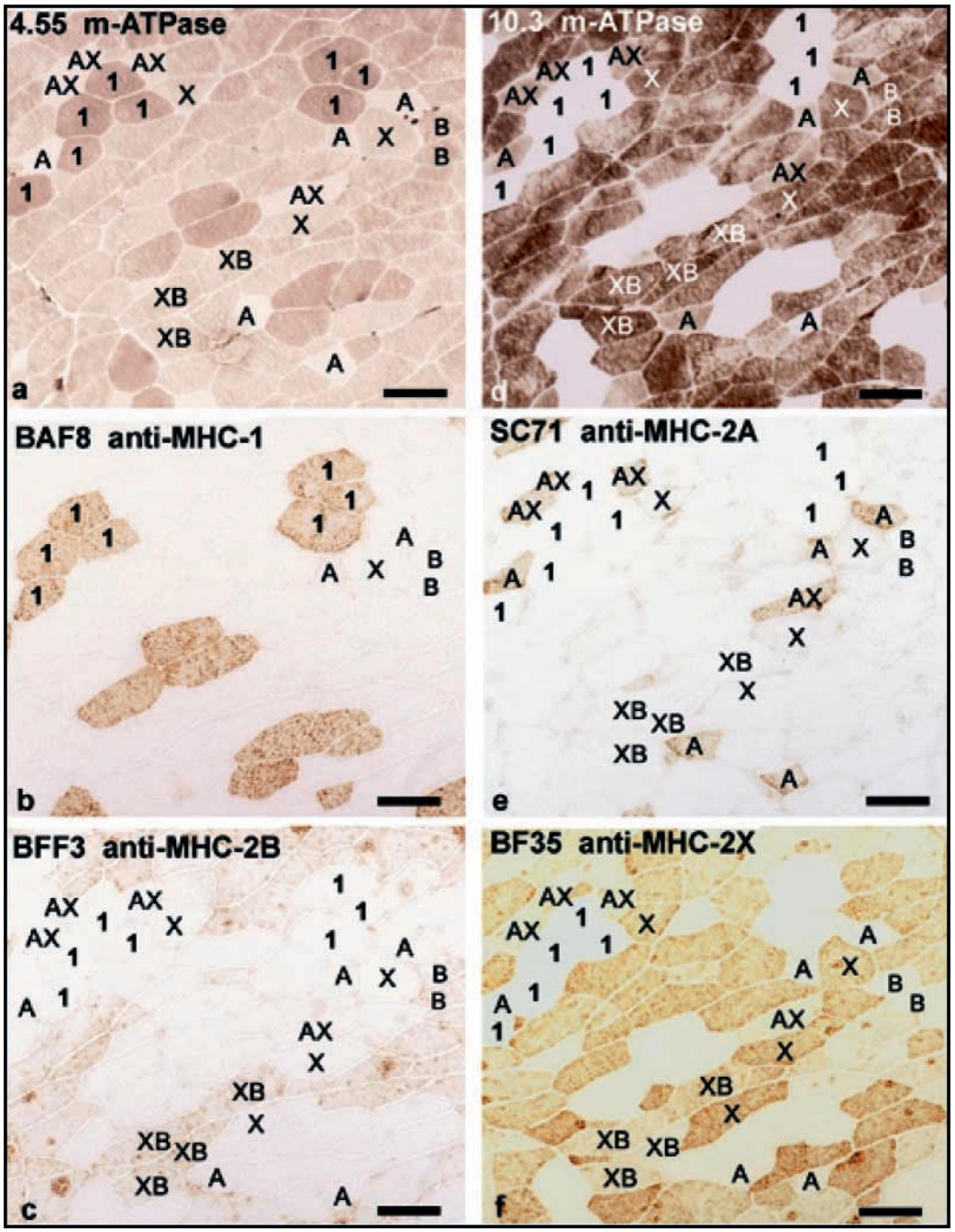

Fig. 3. Histochemistry and immunohistochemistry on longissimus dorsi muscle. Consecutive sections were stained for mATPase after acid (a) or alkali (d) preincubation and with four monoclonal antibodies specific for MHC isoforms (b,c,e,f). The typical organization of islets of slow fibres surrounded by fast fibres is visible. Among fast fibres, hybrid fibres 2A/X and 2X/B are very abundant, while only few pure 2A and 2X are present. In the deep portion of the muscle, next to multifidus lomborum muscle, some pure 2B fibres are detectable (indicated as B). Scale bars, 40 μm.

in Fig. 3). Taken together these results suggest that BF35 is specifically reactive with MHC-2X since: (i) it is negative in type 1 and 2A fibres (as seen in masseter), (ii) it is negative in 2B pure fibres as shown in one sample of longissimus dorsi, (iii) it is positive with fibres of diaphragm and red semitendinosus where MHC-2X but not MHC-2B is expressed. This view is well supported by results in longissimus dorsi and white semitendinosus muscles, where the MHC-2X and MHC-2B are expressed. In fact the co-expression of hybrid 2X/B hybrid was the rule, as shown by single fibre electrophoresis (see below). Pure 2B fibres were very rare. Hybrid 2A/X fibres were frequent: actually some SC71 positive fibres were also moderately positive or positive to BF35 (see longissimus dorsi and red semitendinosus, Fig. 2C).

Electrophoresis and western blot

The improved electrophoretic protocol designed for separation of porcine MHC isoforms (see Materials and methods) was validated by comparing the results obtained in various muscle samples (i.e. fibre bundles) with the results of RT-PCR and immunohistochemistry in the same samples.

Two bands in the MHC region were found in most masseter samples (see Fig. 4A) in full agreement with the results from RT-PCR and immunohistochemistry that masseter only contains MHC-1 or slow and MHC-2A. In accordance with the

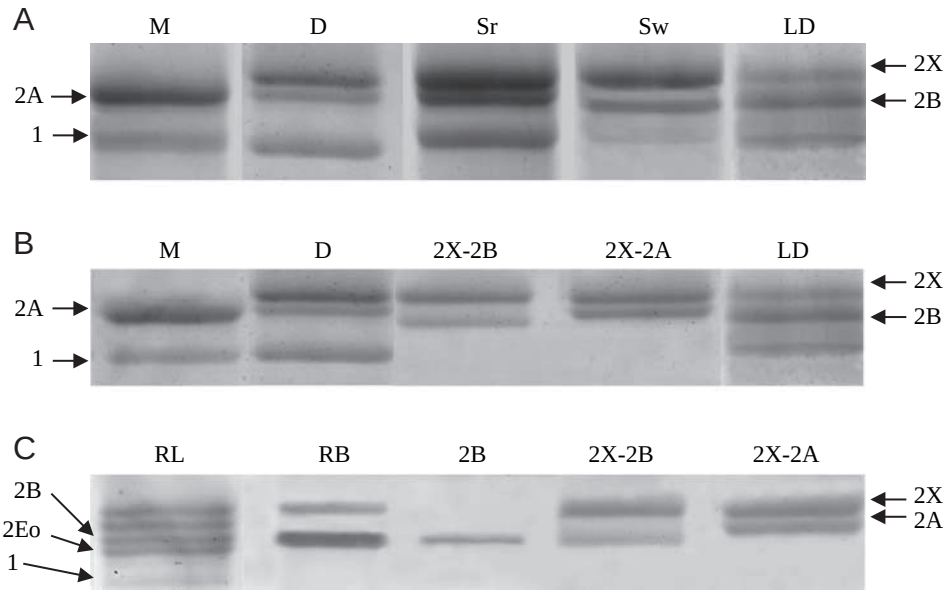

Fig. 4. Electrophoretic separation of MHC isoforms in muscle samples and single fibres. (A) Two bands (MHC-1 and MHC-2A) can be seen in masseter (M), three bands (MHC-1 MHC-2A and MHC-2X) in diaphragm (D) and (MHC-1 MHC-2B and MHC-2X) in red semitendinosus (Sr), white semitendinosus (Sw) and longissimus dorsi (LD). (B) Two hybrid single fibres (2X-2B and 2X-2A) are compared with masseter (M), diaphragm (D) and longissimus (L). (C) Five bands (three fast MHC-2B, -2X and -2A, extraocular (Eo) and MHC-1, barely detectable) are separated in the extraocular rectus lateralis (RL) and two bands (MHC-2X and MHC-2B) in rectractor bulbi (RB). Three single fibres (pure 2B and hybrid 2X-2B and 2X-2A) are also shown.

migration rates observed in other animal species (Pellegrino et al., 2003), the fast migrating isoform was identified as MHC-1 and slow migrating with 2A. The identification was fully confirmed by western blot (see below) and by the measurements of maximum shortening velocity (see below). A third band located above the 2A band was observed in the diaphragm and in the red semitendinosus where, from RT-PCR and immunohistochemistry, MHC-slow, MHC-2A and MHC-2X are expressed. Thus, this third band was identified as MHC-2X. RT-PCR and immunohistochemistry showed that white semitendinosus and longissimus dorsi expressed four different MHC isoforms. The comparison of the migration patterns clearly showed the presence of a band slightly but clearly lower than the intermediate band detected in diaphragm or in red semitendinosus. This band found in longissimus and in white semitendinosus was therefore identified as MHC-2B. In conclusion the four MHC isoforms migrate in the following order: the fastest migrating, MHC-slow (type 1), followed by MHC-2B, MHC-2A and MHC-2X, the slowest migrating.

Attempts to display all three fast isoforms together in the same gel in longissimus and white semitendinosus were not successful, because resolution decreased if the amount of protein loaded was increased and, if the amount loaded was reduced, the less abundant isoform, MHC-2A, became undetectable. The separation was, however, clearly confirmed by electrophoresis of single fibres. Many fibres (see Fig. 4B) were hybrid and in these fibres the isoform associations were 2A-2X or 2X-2B and never 2A-2B, in accordance with the 'nearest neighbour rule' based on sequential MHC isoform transition: 1→2A→2X→2B (Schiaffino and Reggiani, 1996).

Although hybrid fibres were very abundant in porcine muscles (see also Lefaucheur et al., 2002), many 'pure' fibres, i.e. fibres containing only one MHC isoform, were found, particularly fibres containing MHC-slow and fibres containing MHC-2A in masseter and in diaphragm, and fibres containing MHC-2X in longissimus (not shown). Surprisingly, no pure fibres containing MHC-2B were found in longissimus or in white semitendinosus, although the total amount of this isoform was high (25–30%) and some 2B fibres were detected by immunohistochemistry (see Fig. 3F).

To find pure 2B fibres expressing only MHC-2B, extrinsic eye muscles were examined. Previous studies had shown that in many animal species retractor bulbi is a completely fast muscle (Sartore et al., 1987; Mascarello and Rowlerson, 1992): in porcine retractor bulbi (Fig. 1B) RT-PCR revealed the expression of all three fast MHC isoforms and SDS-PAGE showed two bands corresponding to MHC-2X and MHC-2B and an additional band corresponding to MHC-Eo or extra-ocular. The band corresponding to MHC-2A was undetectable due to the low amount of this isoform: some single fibres containing MHC-2A were, however, identified (not shown). The identification of the extra-ocular isoform was confirmed by the comparison with rectus lateralis, where MHC-Eo was expressed together with all three fast MHC isoform and MHC-slow (see Fig. 4C). Several pure 2B fibres were dissected from retractor bulbi (see one example in Fig. 4C).

The interpretation of the electrophoretic separation was based on muscle sample comparison and was further supported by western blot analysis. The fastest migrating band was stained with BFD5, which is specific for MHC-slow. The upper bands corresponding to fast MHC isoforms were stained by SC71, which reacted with MHC-2A, and BF35, which reacted with MHC-2X. However, under denaturating conditions, the specificity was reduced and SC71 also crossreacted with MHC-2X and BF35 with MHC-2B (data not shown).

Contractile properties of single muscle fibres

A total of 172 single fibres, dissected from five muscles (masseter, diaphragm, red and white semitendinosus, longissimus dorsi and rectractor bulbi), were analysed in mechanical experiments and classified on the basis of their MHC isoform composition as determined by SDS-PAGE. The

origin of the fibres was as follows: masseter 30, diaphragm 59, semitendinosus 47, longissimus 16, retractor bulbi 20. On the basis of their MHC isoform composition the following groups (number of fibres in parentheses) were formed: slow (36), fast 2A (43), mixed 2A-2X (23), fast 2X (19), mixed 2X-2B (36) and fast 2B (11); four fibres from retractor bulbi contained more than two MHC. In agreement with the indications of histochemistry and immuno-histochemistry, single fibre electrophoresis showed that (i) fast fibres were more abundant than slow fibres, (ii) hybrid fibres were very abundant, and (iii) no mixed 1-2A fibres were found. Although MHC-2B was abundantly expressed in longissimus and semitendinosus and few pure 2B fibres were immunohistochemically detected in the deepest part of longissimus (see Fig. 3), pure 2B fibres were only dissected from retractor bulbi. Large groups of 2X-2B fibres were obtained from longissimus and semitendinosus.

The mean values of cross sectional area (CSA) of single fibres grouped according to their MHC isoform composition are shown in Fig. 5A. For each fibre type cross sectional area was rather variable, depending on the muscle of origin. For example, for slow fibres (type 1) CSA (mean ± S.E.M.) ranged from 6523±246 μm^2 in masseter to 9150±531 μm^2 in diaphragm and to 17368±3579 μm^2 in the red portion of semitendinosus. The CSA of fast 2A fibres was 3362±274 μm^2 in masseter, 7823±837 μm^2 in diaphragm and 8167±789 μm^2 in semitendinosus. The thickness of fast 2X fibres was 16074±2851 μm^2 in diaphragm, 14441±1367 μm^2 in red semitendinosus and 9559±1591 μm^2 in white semitendinosus. Among the fibres and the muscles studied the largest fibres were 2X-2B type: 19474±1576 μm^2 in red semitendinosus, 11093±1129 μm^2 in white semitendinosus and 14862±1088 μm^2 in longissimus, whereas the thinnest fibres were pure 2B fibres dissected from retractor bulbi (1097±131 μm^2).

The slack sarcomere length was not significantly different among fibre types: slow fibres 2.28±0.06 μm, 2A fibres 2.35±0.05 μm, 2X fibres 2.22±0.09 μm, 2B fibres 2.43±0.06 μm. The fibres were stretched by approximately 20% at rest and activation was induced at sarcomere lengths between 2.77±0.09 μm for 2X fibres and 2.89±0.08 μm for 2B fibres.

The determination of the isometric tension (P_o) and of the unloaded shortening velocity (V_o) revealed large diversity among fibres with different MHC isoform composition. Slow fibres developed significantly less tension than fast fibres, with the exception of pure 2B fibres, which also developed low isometric tension (see Fig. 5B). In accordance with previous observations in other species (Pellegrino et al., 2003), V_o values increased from slow to fast 2A, 2X and 2B fibres (see Fig. 5C). The difference between fast 2A and fast 2X fibres was statistically significant, but hybrid fibres 2A-2X and 2X-2B fibres had V_o values similar to those of pure 2X fibres. Fibres with the same MHC isoform composition had similar values of V_o (measured in fibre segment length s^{-1}) regardless of the muscle of origin. V_o of slow fibres was 0.172±0.049 (mean ± S.E.M.) in masseter, 0.176±0.017 in diaphragm, 0.163±0.025 in red semitendinosus. V_o of fast 2A fibres ranged from 1.798±0.283 in diaphragm to 2.024±0.326 in masseter and to 2.208±0.224 in white semitendinosus. V_o of fast 2X fibres was 2.611±0.415 in diaphragm, 2.67±0.236 and

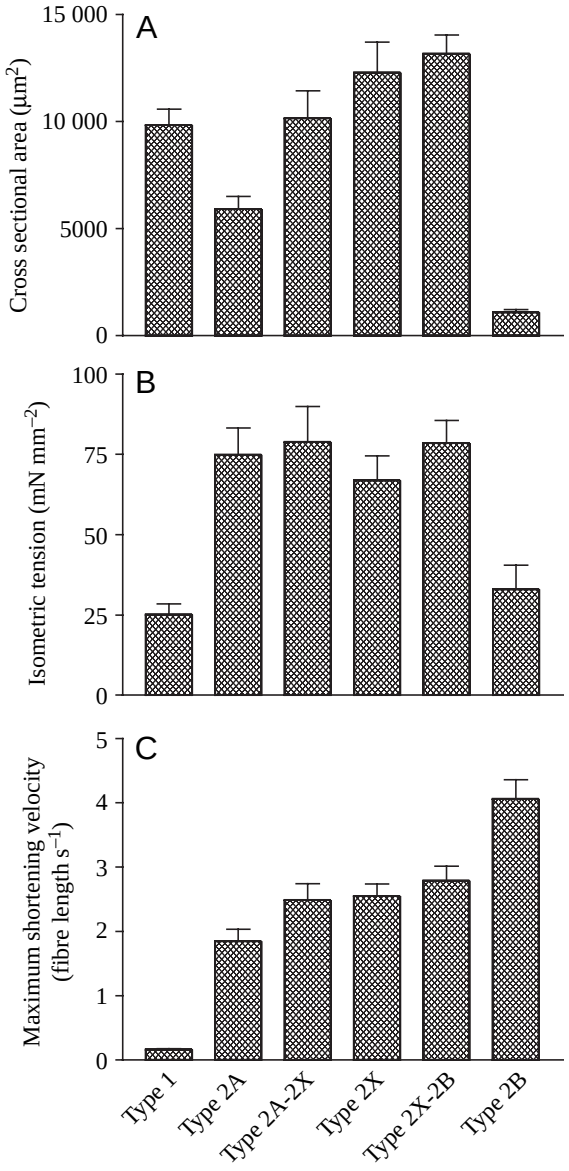

Fig. 5. Mean values of cross sectional area (CSA) (A), isometric tension P_o (B) and maximum shortening velocity V_o (C) of the six fibre types identified in pig muscles. Values are means ± S.E.M. ANOVA showed that (i) CSA values of slow fibres (type 1) were significantly different from those of 2A and 2B fibres, values of 2A fibres were significantly different from those of all other fibre types, values of 2B fibres were significantly different from those of all other fibre types; (ii) P_o values of slow fibres (type 1) were significantly different from those of all other fibre types except 2B fibres and P_o values of 2B fibres were significantly different from those of all fibre types except slow fibres; (iii) V_o values of slow fibres (type 1) were significantly different from those of all other fibre types, V_o values of 2A fibres were significantly different from those of all other fibre types, V_o values of 2B fibres were significantly different from those of all other fibre types.

2.762±0.207 in red and in white semitendinosus respectively. As mentioned above, pure fast 2B fibres were only found in one muscle: the retractor bulbi. Series compliance (expressed relative to fibre segment length) was not significantly different among the six groups of fibres, ranging from 0.045±0.006 in fast 2B fibres to 0.057±0.008 in fast 2A fibres.

In vitro *motility assay*

Myosin was prepared from slow fibres, pure fast 2A fibres and pure fast 2X fibres. The velocity of actin filaments (V_f) in the motility assay showed large and significant differences. V_f values (means ± S.E.M.) were 0.26±0.01 μm s^{-1} (N=11) for slow myosin, 0.99±0.10 μm s^{-1} (N=8) for fast 2A myosin and 1.31±0.12 μm s^{-1} (N=7) for fast 2X myosin, all differences being statistically significant (P<0.01). Linear regression analysis showed a highly significant correlation (P=0.0018) between V_f and V_o of corresponding fibre types: the slope (with V_o expressed in μm s^{-1} half sarcomere and V_f expressed in μm s^{-1}) was 0.525±0.022, a value not significantly different from 0.529±0.015 calculated by Pellegrino et al. (2003) for various myosin isoforms from mouse, rat, rabbit and human muscles.

Discussion

In a recently published study (Pellegrino et al., 2003), the relationship between muscle contractile parameters and body size was investigated in four species, mouse, rat, rabbit and human, which cover a body size range of approximately 2000-fold. The results of that study showed that (i) for slow and fast fibres, shortening velocity and peak power decreased inversely with respect to body mass, and (ii) fast myosin expression was higher in smaller than in larger animals. In the present study, the contractile properties and myosin isoform composition of pig single muscle fibres were characterized for the first time. The picture emerging was surprising as pig muscles were found to be unexpectedly rich in fast myosins, including 2B myosin, and have V_o values higher than predicted from body size on the basis of the previously determined scaling equation (Pellegrino et al., 2003).

As demonstrated by many previous studies (for a review, see Schiaffino and Reggiani, 1996), myosin isoforms are the main determinants of the contractile performance: maximum shortening velocity, peak power output and ATP consumption rates all depend on myosin isoform composition and particularly on MHC isoforms. Thus, the characterization of the contractile properties of single muscle fibres becomes significant only if myosin isoform composition is precisely defined. The best method for myosin isoform identification in single fibres is gel electrophoresis (for a discussion, see Pette et al., 1999). Previous attempts to separate all four adult MHC isoforms in porcine skeletal muscles were not successful (Bee et al., 1999). In the present study a modification of the electrophoretic protocol proposed by Talmadge and Roy (1993) and combination of histochemical and molecular methods of MHC isoform analysis led to the reliable separation and identification of all four adult sarcomeric MHC isoforms.

The need to combine several methods arises from the limitations that each method presents. Histochemical mATPase determination allows a clear identification of slow and pure fast 2A fibres, but cannot unambiguously separate 2X and 2B fibres, a situation that is further complicated by the presence of a large number of hybrid fibres. Attempts to identify all four fibre types have been performed by combining the mATPase reaction with metabolic enzyme reactions (Gil et al., 2001), but the separation between 2X and 2B fibres was only indirectly correlated with their myosin isoform composition.

Immunohistochemistry is limited by the lack of antibodies specific for each MHC isoform. Monoclonal antibodies are powerful tools for MHC isoform identification, but they have two major limitations. First, their specificity is species-dependent, as corresponding orthologous isoforms may show structural diversities that affect the antigenic site, and second, their use is made more difficult by the presence of a large fraction of hybrid fibres. Antibodies specific for all fast MHC isoforms of rat, cat, rabbit and marsupials (Lucas et al., 2000) have been recently described; however, they are not commercially available and have not been tested in pig muscle fibres. Among the antibodies used in this study, the highly specific reactivity of BFD5 and BAF8 with MHC-slow myosin was confirmed in pig muscles and SC71 was found to be specific for MHC-2A. The slight cross reactivity of SC71 with MHC-2X reported by Lefaucheur et al. (2002) might be explained by the abundant presence of hybrid 2A-2X fibres. In the present study, however, a cross reactivity of SC71 with MHC-2X was observed under denaturating conditions in immunoblotting. In complete agreement with Lefaucheur et al. (2002), BFF3 showed a weak but consistent reactivity with 2B fibres. The monoclonal antibody BF35 was surprisingly found to react with MHC-2X, as indicated by immunohistochemical staining, where pure 2X and hybrid 2A-2X and 2X-2B fibres were stained by this antibody. The unexpected reactivity was confirmed also with rat muscles, where BF35 was negative only with type 1 fibres (data not shown). The antibody BF35 was originally characterized in rat muscles as reactive with all MHC isoforms except 2X (Schiaffino et al., 1989; Bottinelli et al., 1991), and the inversion of specificity observed in this study has already been reported elsewhere (Sciote and Rowlerson, 1998). Preliminary unpublished results have shown that BF35 exhibits a similar reactivity pattern in dog, cow, monkey and tiger muscles. The presence of fibres reactive with BF35 in red semitendinosus (where MHC 2X is expressed, but not MHC 2B) and the lack of reactive fibres masseter (where MHC 2X is not expressed) confirm the above view.

RT-PCR is a reliable tool used to determine which MHC genes are transcribed in a given tissue sample (for a discussion, see Pette et al., 1999). Expression at mRNA level, however, does not guarantee that the protein is present in the tissue. Mismatch between mRNA and protein in porcine muscle fibres

has been recently reported (Lefaucheur et al., 2002). In the present study, the comparison between the results of RT-PCR and the results of immunohistochemistry and electrophoresis on large fibre bundles revealed the close correlation between the mRNAs and the proteins in muscles with distinct patterns of MHC expression. For example, both mRNA and protein determination indicated that masseter expressed only the types 1 and 2A, whereas longissumus dorsi expressed all four MHC. In view of the electrophoretic demonstration of the presence of a high number of hybrid fibres (2A/X, 2X/B), it would be interesting to extend the comparison between mRNA and protein to single fibres. Electrophoretic separation of myosin isoforms is the method most suited for single fibre characterization and also for definitive assessment of myosin isoforms present in the tissue. The resolution of a second less-abundant isoform is limited to 1% (Bottinelli et al., 1994), but such an amount has hardly any detectable effects on the contractile properties of muscle fibres (Reiser et al., 1985; Larsson and Moss, 1993). The combination of electrophoresis with the other methods helps to solve the uncertainties which arise from the facts that (i) the sequential order of myosin isoform migration is species-specific and (ii) different isoforms might migrate with the same speed and overlap each other.

The picture emerging from the combined use of the various methods shows that (i) only two isoforms, MHC-slow and MHC-2A, are expressed in masseter, with the possible presence of limited amounts of MHC-2X in the most superficial layers; (ii) three MHC isoforms, namely slow, 2A and 2X are expressed in the diaphragm and the red portion of semitendinosus; (iii) four isoforms, MHC-slow and three fast MHC isoforms (2A, 2X and 2B) are expressed in longissimus dorsi and white superficial part of semitendinosus. Retractor bulbi contains three fast MHC isoforms and extraocular MHC but lacks MHC-slow, whereas rectus lateralis contains all five MHC isoforms. The analysis of rectus lateralis and retractor bulbi is far from complete, since retractor bulbi was used only as a source of pure 2B fibres and *ad hoc* designed studies are required for a definitive analysis of pig extrinsic eye muscles.

In accordance with previous observations (Bee et al., 1999; Lefaucheur et al., 1991), fast myosins were more abundant than slow myosins in all muscles examined and 80% of the fibres characterized in mechanical experiments were fast fibres. The high proportion of hybrid fibres seems to be a typical feature of porcine muscles (see also Lefaucheur et al., 2002). In particular, the hybrid fast fibres, 2A-2X and 2X-2B, were very abundant, whereas the fast-slow or 1-2A fibres were hardly found. The latter fibres are also called 2C and are frequent in small laboratory animals such as rat or rabbits (Staron and Pette, 1987a,b). Pure 2B fibres were very rare or absent in trunk and limb muscles, although MHC-2B is abundantly expressed. Fibres containing MHC-2B alone were only found in retractor bulbi. The functional significance of hybrid fibres is not yet completely understood. As discussed in a recent review (Stephenson, 2001), hybrid fibres can be seen (i) as a means to obtain a fine functional tuning since their contractile properties lie between those of pure fibres, (ii) as the results of incomplete differentiation, (iii) as an indication of differential responsiveness of individual myonuclei to contrasting signals. The last interpretation is intriguing as in pigs raised for meat production muscles appear well developed and almost hypertrophic although virtually no physical activity is allowed.

Pig single muscle fibres showed an impressive heterogeneity in contractile properties (V_o values ranged from 0.1 to 5 fibre segment length s^{-1} in individual fibres), which could be largely resolved by grouping the fibres on the basis of their MHC isoform composition. Large differences between slow and fast fibres were found not only in unloaded shortening velocity but also in isometric tension.

The values of V_o and P_o obtained in pig muscle fibres can be compared with recently published data (Pellegrino et al., 2003) obtained in other animal species (mouse, rat, rabbit and man) under the same experimental conditions. The comparison is shown in Fig. 6. Whereas isometric tension of pure 2A and 2X fibres is not significantly different from that measured in corresponding fibres of other animal species, both slow fibres and fast 2B fibres exhibit lower tension values. The low isometric tension of slow fibres seems to be the result of a trend to decreasing tension with increasing animal size: actually the highest value for slow fibres is reached in the mouse (see Fig. 6B and Pellegrino et al., 2003). The low value of tension developed by 2B fibres might be related to the specific features of retractor bulbi, a muscle with very limited functional tasks. Previous studies have found that isometric tension developed by extraocular muscles is lower than that developed by limb muscles (Close and Luff, 1974; Asmussen and Gaunitz, 1981), although a recent paper has reached opposite conclusions (Frueh et al., 2001). Interestingly, whereas hybrid 2X-2B were the largest fibres found in pig muscle samples, the pure 2B fibres from retractor bulbi were the thinnest and the 2B fibres identified by immuno-histochemistry in sections of longissimus dorsi (see Fig. 3F) were also rather thin.

Hill (1950) first observed that, comparing different animal species, the maximum speed of locomotion is largely independent of animal size. This requires that the speed at which muscles shorten during locomotion be inversely related to limb length or to the cubic root of body mass. If the shortening velocity at which muscles are used represents a constant fraction of maximum shortening velocity (V_o),V_o will also scale in proportion to a power of body size. The scaling relation between V_o and body mass (Fig. 6) shows that pig slow fibres perfectly conform to the value expected from the scaling equation obtained with other animal species: in other words, the scaling equation predicts the variation of V_o of slow fibres from mouse (about 30 g) to pig (about 160 kg) without exceptions. The results concerning fast fibres are surprising as the values measured in the pig are higher than those obtained in human fibres and similar to those of rabbit fibres (Pellegrino et al., 2003), i.e. pig fast fibres do not follow the scaling equation. To our knowledge, shortening velocity was measured in muscle fibres of only three species among large mammals: horse (Rome et al., 1990), sheep and cow (Seow and Ford,

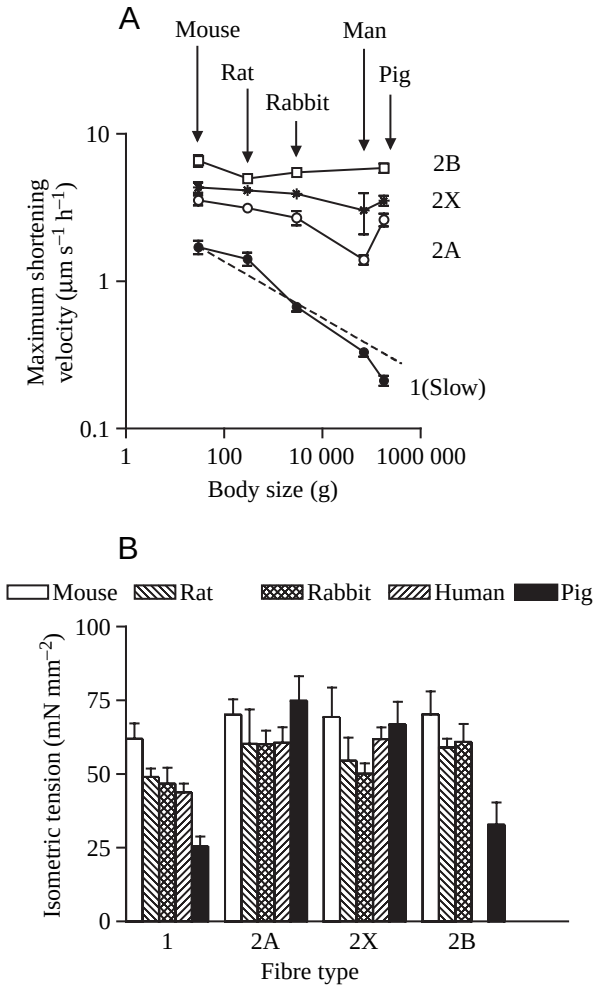

Fig. 6. Interspecies variations of V_o and P_o. (A) Relationship between maximum shortening velocity V_o (expressed in μm h^{-1} s^{-1} for correct comparison among species) and body size. A regression line for slow fibres was obtained by fitting the experimental data with the scaling equation $y=ax^{-b}$; the parameters were a=3.309±0.614 and b=0.192±0.013. (B) Values of isometric tension P_o measured in various fibre types in five animal species. Statistically significant differences were found for slow fibres (mouse and pig different from all other species) and for 2B fibres (pig different from all other species). Data for mouse, rat, rabbit and man from Pellegrino et al. (2003).

1991). Although the orthologous myosin identification and the comparison between corresponding fibre types were not completely precise, the trend to decrease shortening velocity with increasing body size was found in all cases. The pig seems therefore to represent a unique exception. The results of the *in vitro* motility assay confirmed the conclusions based on V_o measurements. Actin filament sliding velocity (V_f) was strictly proportional to V_o of fibres containing the same MHC isoform, in agreement with the view that V_o of single fibres is directly determined by myosin isoforms. Statistical comparison of V_f values between pig and human corresponding myosins showed that V_f was significantly lower in pig slow myosin than in human slow myosin, whereas for fast 2A myosin no significant difference was present and for fast 2X myosin V_f was significantly higher in pig than in man.

In conclusion, the present results represent the first complete and systematic analysis of the relationship between muscle fibre contractile properties and myosin isoform composition in the pig. They also provide the first determination of the contractile properties of fast fibres expressing MHC-2B in a large mammal. The results raise interesting biological questions: why do pig muscles not only express the fastest myosin isoform 2B, which is not present in other large mammals, but are also composed of fibres that are faster than expected from the scaling equation? A possible answer is that the pig varieties available today, including the 'large white' examined in this study, are the results of a selective pressure aiming only to increase fertility and muscle size, without any attention to locomotion activity. In this respect the pig might be different from the cow and the sheep where the breeding conditions (grazing) require movement and, more obviously, from the horse, a species where the locomotion performance has become the reference parameter for selection.

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