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Fiber types in canine muscles: myosin isoform expression and functional characterization

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Toniolo L, Maccatrozzo L, Patruno M, Pavan E, Caliaro F, Rossi R, Rinaldi C, Canepari M, Reggiani C, Mascarello F. Fiber types in canine muscles: myosin isoform expression and functional characterization. *Am J Physiol Cell Physiol* 292: C1915–C1926, 2007. First published January 24, 2007; doi:10.1152/ajpcell.00601.2006.— This study was aimed to achieve a definitive and unambiguous identification of fiber types in canine skeletal muscles and of myosin isoforms that are expressed therein. Correspondence of canine myosin isoforms with orthologs in other species as assessed by base sequence comparison was the basis for primer preparation and for expression analysis with RT-PCR. Expression was confirmed at protein level with histochemistry, immunohistochemistry, and SDS-PAGE combined together and showed that limb and trunk muscles of the dog express myosin heavy chain (MHC) type 1, 2A, and 2X isoforms and the so-called “type 2dog” fibers express the MHC-2X isoform. MHC-2A was found to be the most abundant isoform in the trunk and limb muscle. MHC-2X was expressed in most but not all muscles and more frequently in hybrid 2A-2X fibers than in pure 2X fibers. MHC-2B was restricted to specialized extraocular and laryngeal muscles, although 2B mRNA, but not 2B protein, was occasionally detected in the semimembranosus muscle. Isometric tension (P_0) and maximum shortening velocity (V_0) were measured in single fibers classified on the basis of their MHC isoform composition. Purified myosin isoforms were extracted from single muscle fibers and characterized by the speed (V_f) of actin filament sliding on myosin in an in vitro motility assay. A close proportionality between V_0 and V_f indicated that the diversity in V_0 was due to the different myosin isoform composition. V_0 increased progressively in the order 1/slow < 2A < 2X < 2B, thus confirming the identification of the myosin isoforms and providing their first functional characterization of canine muscle fibers.

dog; muscle fiber types; single fiber mechanics; in vitro motility

AS “MAN’S BEST FRIENDS,” dogs now receive medical care comparable to that given to human beings. Canine diseases are studied with regard to their pathogenic mechanisms, and specific diagnostic tools and therapeutic treatments are developed. Several canine muscle diseases have been identified, and, among them, special interest has been focused on golden retriever muscular dystrophy, or GRMD (17), which is equivalent to the human Duchenne dystrophy (11) and has become an important model for understanding the pathogenic mechanisms and for testing genetic therapy (32). As with humans, the life style and the adaptations of skeletal muscle to disuse and sedentary life, exercise training, and aging find their equivalent in dogs. All this makes the study of canine muscles of great

interest as confirmed by the publication of several important papers in the last few years (1, 3, 10, 36, 38).

An essential feature of mammalian skeletal muscle is fiber heterogeneity. The diversity among fiber types reflects the motoneuron discharge pattern and the loading conditions on the muscles and is a major determinant of the contractile performance (34). Although virtually all structural and functional aspects of muscle fibers exhibit some degree of diversity, the composition in myosin isoforms has a special relevance, since myosin is the most abundant muscle protein and represents the molecular motor of muscle contraction. The initial investigations on fiber type heterogeneity in canine muscles led to controversial conclusions, since the myofibrillar ATPase (m-ATPase) staining did not permit an unambiguous identification of type 1, 2A, and 2B, and, because of the high oxidative metabolism of canine muscles, a clear separation of oxidative and glycolytic fibers was not possible (2, 6, 13, 15, 27, 42). By comparing different protocols of acid or alkali preincubation, Snow and coworkers (37) showed that in canine muscles two distinct types of fast fibers were present, the one corresponding to type 2A and the other not simply corresponding to type 2B. The specific features of this latter type were then carefully described by Latorre et al. (18), who proposed for such fibers the name “2dog fibers.” Other workers, however, preferred to maintain the more often-used term “2B” (29). The issue was recently taken up by Strbenc et al. (38) and by Smerdu et al. (36), who, on the basis of reactivity to a panel of monoclonal antibodies, concluded that 2dog fibers express myosin heavy chain (MHC) 2X. A similar conclusion was also reached by Acevedo and Rivero (1) who combined immunostaining with other histochemical approaches and showed that eight distinct fiber types can be identified in canine muscles, in relation to their MHC isoform composition, metabolic properties, SERCA isoforms, and capillary density. Acevedo and Rivero (1) emphasized the presence of hybrid fibers and the high oxidative metabolism of all fiber types. The abundance of hybrid fibers in canine muscles was also observed by Strbenc et al. (38), although their view was later revised after considering the limited specificity of the anti-myosin antibodies (36). The issue is very important since recent studies on fiber types in large mammals (40, 41) suggest that specificity of the monoclonal antibodies prepared to identify myosin isoforms in rodents (22, 33) cannot be transferred to all animal species without careful controls.

MHC isoforms are the markers of fiber types as they determine the pH sensitivity of m-ATPase staining and immunohis-

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tochemical reactivity (34). Electrophoretic separation of MHC isoforms in canine skeletal muscles shows three bands, which have been identified by comparison with other animal species as 2A-2X-1 from the slowest to the fastest migration rate (1, 36, 44). An additional, fourth band, likely corresponding to MHC-2B, has been described by Wu et al. (43) in laryngeal muscles. This finding has been recently confirmed by Bergin and coworkers (3), who used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for electrophoretic band identification, as the results obtained with Western blot were uncertain.

The uncertainties in the use of antibodies to identify dog muscles fiber types in immunohistochemistry (36) and myosin isoforms in immunoblots (3) can be overcome by an alternative approach made possible by the recent sequencing of dog genome (20) and the subsequent identification of the genes coding for MHC isoforms expressed in striated muscles (23). MHC expression can be studied by RT-PCR in muscle samples or even in single fibers and this, as discussed in our previous papers (40, 41), offers a unique chance to overcome all controversies about fiber types. Orthologous MHC isoforms can precisely be identified in various mammalian species by sequence comparison, and their expression can be determined at RNA level in muscle specimens without any ambiguity. RNA can be then associated with the corresponding protein identified in the same specimens with the various methods (histochemistry, immunohistochemistry, electrophoresis) discussed above.

The determination of the contractile properties of canine muscle fibers can further support the identification of the fiber types as a consistent relation exists between some kinetic parameters, such as maximum shortening velocity, and myosin isoforms (34). Little is presently known about the contractility of canine muscles at single fiber level, since data about shortening velocity or mechanical power output are available only for slow and fast 2A fibers (10, 25). The knowledge of the contractile parameters can furthermore provide a starting point for future studies on canine muscle adaptations in relation with development/aging, with disuse/training, or with diseases.

In this study we aimed to examine the open issues about MHC expression in canine skeletal muscle fibers, taking advantage of the combination of mRNA amplification with gel electrophoresis and histochemical/immunohistochemical methods. In particular, we wanted to confirm whether MHC-2X was the isoform expressed in the specific fiber type previously called "2dog" and assess whether the abundant presence of hybrid fibers was a true fact or the consequence of insufficient specificity of anti-myosin antibodies. We also wanted to confirm the presence of fibers expressing MHC-2B. For this reason

we extended our investigation to laryngeal and extraocular muscles, whereas masticatory muscles and the fiber expressing the specific masticatory myosin (2M) were deliberately excluded from this study. Moreover, the functional properties of single fibers and purified myosin isoforms were analyzed to further and independently verify the isoform identification and form the basis for future studies on adaptations and diseases in canine muscles.

METHODS

Sampling of dog muscles. Muscle samples were collected from adult dogs that had been euthanized at the Veterinary University Clinics, a separate department of the University of Padova. The dogs were afflicted with untreatable diseases or untreatable injuries following accidents or the dogs had been declared aggressive to human beings in accordance with Italian law. Six dogs were in good health at the time of the sampling, whereas two dogs had received a pharmacological therapy for their disease (antibiotics and anti-inflammatory drugs). The protocols for care and use of all animals in this project were approved by the institution. Eight dogs of different breeds (Labrador, German Shepherd, Shih Tzu, Rottweiler, cross breeds) and size, aged between 2 and 10 yr, were studied.

The following muscles were dissected to obtain samples with different composition in myosin isoforms: 1) longissimus dorsi (Ld), diaphragm (D), tibialis cranialis (Tc), and semimembranosus (Sm) were taken as examples of trunk and limb muscles; 2) rectus lateralis (Rl) and retractor bulbi (Rb) were sampled as representative extraocular muscles; 3) three laryngeal muscles were sampled: cricoarytenoideus dorsalis (Cad), arytenoideus transversus (At), and thyroarytenoideus, which was divided in pars ventricularis (Tve) and pars vocalis, in turn divided in the four portions of the caudal part (Tvc), cranial part (Tvc), lateral part (Tvl), and the medial part (Tvm). Temporalis (T) was sampled as a specific source of 2M myosin.

The samples were divided in parts which were either immersed in ice-cold skinning solution (see below) with 50% glycerol and used for muscle fiber mechanics, or frozen in isopentane cooled with liquid nitrogen for histochemistry, immunohistochemistry, and protein electrophoresis, or immersed in RNALater reagent (Ambion, Austin, TX) for RT-PCR analysis.

RNA expression analysis. Samples for RNA analysis were collected in vials containing RNA Later reagent and stored at -20°C . Total RNA was extracted from 100 mg of tissue in TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) and reverse transcribed with SuperScript II protocols (Invitrogen, Life Technologies) using as primers a mixture of random hexamers. The obtained cDNAs were used as templates for RT-PCR analysis. PCR reactions were performed using dog specific primers designed on the recently published dog MHC sequences (23) and listed in Table 1. PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized under UV light. A primer pair to amplify a fragment of β -actin gene was used to test the quality of the extracted RNA and the efficiency of reverse transcription (Table 1).

Table 1. Primers used for RT-PCR of specific canine MHC isoforms

Isoforms	Forward Primer (5'-3')	Reverse Primer (5'-3')	Ta	PCR
MHC-1	CACCAACCTGTCCAAGTTCC	CCGGGCAGATCAAGAGAAGATA	56	32
MHC-2A	AGTGCTATTGATATTTGGGCTTCA	TACAGCGCACAAAATGAGGATGGGT	60	30
MHC-2X	ATCACAGTCCCCAGCATTGATGACC	ACCGTACAAAGTGGGGGTGAGTGCT	60	30
MHC-2B	GCTGGGGGACAAAGTGCTGAAG	GCTTGAGTGGCGGTGATGAGTTGA	60	32
MHC-Neo	AGGCTGCAGGACTTGGTAGA	ATTGTGTTCACTCCGCACTGAT	54	32
MHC-EO	GCCAAACAGCAGATGTCCA	GCATCTGGGAAGGCGCTCATTC	55	32
β -Actin	CCATCTATGAGGGCTACGCGC	TTCTCAAAGTCCAAGGCCACGTA	50	32

MHC, myosin heavy chain. Ta, annealing temperature ($^{\circ}\text{C}$). PCR, number of PCR cycles.

Histochemistry and immunohistochemistry. Muscle samples or composite blocks with two or three different muscle samples were frozen in isopentane cooled with fluid nitrogen. Serial sections (10 μ m) were cut in a cryostat and stained for m-ATPase as previously described (18). M-ATPase staining followed either alkaline preincubation (*method 1* in Ref. 18, pH 10.4, 10.5, 10.6, and 10.7 with incubation time of 15 min) or acid preincubation (*method 3* in Ref. 18, 0.1 M sodium acetate, at pH 4.6, 4.55, 4.5, 4.45, or 4.4 for 10 or 15 min). All protocols distinguished reliably type 1 from type 2 fibers, whereas the separation of subgroups of fast type 2 fibers was more critical. Specific combinations of pH value and incubation time allowed the separation between type 2dog, type 2A, and type 2B (see RESULTS).

Additional serial sections were stained with the following monoclonal antibodies: BA-F8 [specific for MHC-1 or slow in several species from rat (5) to pig (41)], SC-71 [specific for MHC-2A in rat (5) and pig (41), but reactive also with MHC-2X in cow (24) and in dog (1, 36)], BF-35 new clone [specific for MHC-2X in pig (41) and cow (24), but reacting with all fast fibers in rat (41)], BF-35 old clone [positive for type 1, 2A, and 2B but not for 2X in rat (33)], BF-F3 [specific for MHC-2B in rat (33) and in pig (19)], 10F5 and 2F7 [reacting with type 2B and type 2A (22), respectively], and A4.74 [specific for type 2A in human and rat but staining all fast types in dog (36)]. A polyclonal antibody was used to identify fibers expressing neonatal myosin (26). The primary antibodies BA-F8, SC-71, BF-F3, and BF-35 new clone were purchased from DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), and the antibody A4.74 was from Alexis Biochemical; BF-35 old clone was a gift from Professor S. Schiaffino; and 10F5 and 2F7 were a gift of Professor J. Hoh.

The immunohistochemical staining was performed using the Envision method with the following secondary antibodies: rabbit anti-mouse immunoglobulins for monoclonal antibodies and goat anti-rabbit for polyclonal antibodies both conjugated to peroxidase-labeled complex (Dako, Glostrup, Denmark). The primary antiserum was applied overnight at room temperature using different dilutions ranging from 1:100 to 1:400 for the A4.74, SC-71, BA-F8, and BF-35 old and new clone antibodies and from 1:20 to 1:40 for the BF-F3 antibody. After rinsing in PBS buffer (pH 7.2–7.4), we incubated the sections for 30 min at room temperature in the Envision system, rinsed these in PBS, and visualized the immunoreactive sites using a freshly prepared solution of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) in 15 ml of a 0.05 M Tris buffer at pH 7.6, containing 1.5 ml of 0.03% H_2O_2 . The specificity of the immunostaining was verified 1) by incubating sections with PBS instead of the specific primary antiserum, 2) by incubating sections with preimmune serum instead of primary antiserum, or 3) by incubating sections with PBS instead of secondary antibody.

Gel electrophoresis. Muscle samples for protein electrophoresis were solubilized in Laemmli solution (62.5 mM Tris, pH 6.8, 10% glycerol, 2.3% SDS, 5% β -mercaptoethanol, with 0.1% E-64 and 0.1% leupeptin as anti-proteolytic factors). After heating for 5 min at 80°C, appropriate amounts of the protein suspension were loaded onto polyacrylamide gels (about 1 μ g of total protein/lane). For single fibers, a fiber segment of 1–3 mm was solubilized in 20 μ l of Laemmli solution, and 2–3 μ l were loaded onto gels.

The separation of MHC isoforms was obtained on 8% polyacrylamide slab gels with a protocol derived from Talmadge and Roy (39) with some modifications. Slabs 18 cm wide, 16 cm high, and 1 mm thick were used. Electrophoresis was run at 4°C for 24 h, at 70 V for 1 h and 230 V for the remaining time. Gels were silver stained (Bio-Rad Silver stain plus). The identification of the electrophoretic bands as distinct MHC isoforms was achieved by comparing gel electrophoresis with results obtained in RT-PCR, histochemistry, and immunohistochemistry (see RESULTS).

Single fiber mechanics. Muscle fiber bundles were kept at -20°C in skinning solution without ATP mixed with 50% glycerol until the

day of the experiment and in no case longer than 2 wk after sampling. On the day of the experiment the skinning-glycerol mixture was washed away and replaced with ice-cold skinning solution containing ATP, and single fibers were manually dissected under a stereomicroscope ($\times 10$ – 60 magnification). At the end of the dissection, fibers were bathed for 30 min in skinning solution containing 1% Triton X-100 to ensure complete membrane solubilization; then fiber segments of 1–2 mm length were cut, and light aluminum clips were applied at both ends.

Skimming, relaxing, preactivating, and activating solutions employed for single fiber experiments were prepared as previously described (4). Their millimolar composition was as follows: 1) skinning solution contained 150 potassium propionate, 5 magnesium acetate, 5 ATP, 5 EGTA, and 5 KH_2PO_4 ; 2) relaxing solution contained 100 KCl, 20 imidazole, 5 MgCl_2 , 5 ATP, and 5 EGTA. Preactivating solution was similar to relaxing solution except that EGTA concentration was reduced to 0.5 mM and 25 mM creatine phosphate and 300 U/ml creatine phosphokinase were added, whereas activating solution was similar to relaxing solution with the addition of 5 mM CaCl_2 , 25 mM creatine phosphate, and 300 U/ml creatine phosphokinase. The pH of all solutions were adjusted to 7.0 at the temperature at which solutions were used (12°C). Protease inhibitors (10 μM E-64 and 40 μM leupeptin) were added to all solutions.

The fiber segments were transferred to the experimental setup and, with the help of the aluminum clips, mounted between the force transducer (model AME-801; SensorOne, Sausalito, CA) and the electromagnetic puller (SI, Heidelberg, Germany) equipped with a displacement transducer. The signals from the force and displacement transducers, after analog-to-digital conversion (interface CED 1401 plus; Cambridge Electronic Design, Cambridge, UK), were fed into a personal computer and stored in the hard disk. For data storage, recall, and analysis the software Spike2 (Cambridge Electronic Design) was used. The experimental setup consisted of an inverted microscope (Axiovert 25, Zeiss) on which a movable aluminum plate with three pedestals (height 0.5 mm) was placed on the stage: on each pedestal a drop of solution of 70 μ l volume was kept between a coverslip glued on the upper surface of the pedestal and a second coverslip connected with a movable arm. The distance between the upper surface of the pedestal and the coverslip was 2 mm. The three drops were composed of relaxing, preactivating, and activating solution, respectively. The upper surface of each pedestal had a central opening; through these openings the fiber segment could be seen via the objective piece of the inverted microscope ($\times 40$) combined with a $\times 10$ eyepiece. A stereomicroscope ($\times 10$ – 60) placed above the inverted microscope was used for mounting and removing the fiber.

The fiber segment was mounted in relaxing solution and, after measuring length, diameters, and sarcomere length at $\times 400$ magnification, was stretched by $\sim 20\%$. It was then transferred into the preactivating solution for at least 2 min and finally maximally activated by immersion in the activating solution (pCa 4.6). During maximal activation, isometric tension (P_0) was measured, and unloaded shortening velocity (V_0) and series elasticity were determined according to the slack test procedure (12). After the mechanical experiment, fibers were stored in Laemmli solution for electrophoretic analysis.

Myosin extraction and in vitro motility assay. Myosin was extracted and purified from single muscle fibers according to the procedure previously described in detail (9). A fragment of each fiber was cut and immersed in Laemmli solution for electrophoretic identification of MHC isoforms. The myosin sample, suspended in myosin-activity buffer (25 mM MOPS, 600 mM KCl, 2 mM MgCl_2 , 1 mM EGTA, and 4 mM DTT, pH 7.2), was placed as a drop on a coverslip coated with nitrocellulose. The coverslip was then used to construct a flow cell with a 30- μ l channel. The assay was carried out as previously described (28). Movement of the actin filaments was initiated by the infusion of 50 μ l of buffer containing 2 mM ATP into the flow cell. AF buffer composition was 25 mM MOPS, 25 mM KCl, 4 mM

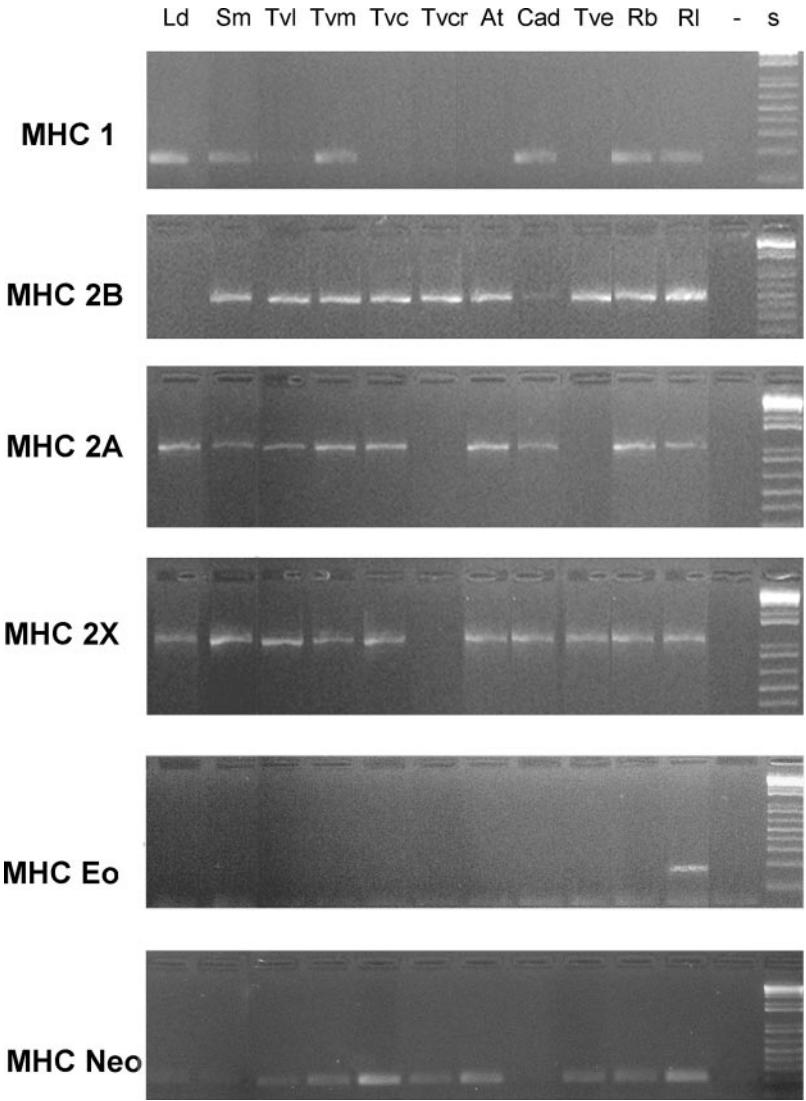
MgCl₂, 1 mM EGTA, 4 mM DTT, 2 mM ATP, 0.5 mg/ml BSA, 200 µg/ml glucose oxidase, 36 µg/ml catalase, and 5 µg /ml glucose (pH 7.2, T = 25°C). The flow cell was mounted on the stage of a fluorescence microscope (Zeiss Axiolab); actin filaments labeled with rhodamine phalloidin were visualized in epifluorescence, and images were recorded at a rate of 50 frames/s by an intensified CCD camera (Extended ISI, Photonic Science) that was connected with video tape recorder, TV monitor, and computer through a frame grabber interface (model PXC200F, Image Nation). Recorded images were analyzed by a software (written by A. Rubini, Pavia, Italy) that allowed us to minimize the background noise, select the rate of acquiring the frames (from 0.5 frames/s for very slow-moving filaments to 25 frames/s for very fast-moving filaments), and choose the duration of data acquisition (from 1 s to 100 s) (28). For each myosin sample the velocities of 50–100 filaments were measured and their distribution characterized according to parametric statistics. Velocity was expressed in micrometers per second.

Statistical analysis. Data are expressed as means and standard errors. One-way ANOVA was used for comparison among fiber types. Linear regression analysis was used to study the correlation between parameters determined in single fibers mechanics and in in vitro motility assay.

RESULTS

MHC isoform expression studied with RT-PCR. Figure 1 shows the results of the RT-PCR on samples from two trunk and limb muscles (semimembranosus and longissimus dorsi); seven samples of laryngeal muscles (cricothyroideus dorsalis, arytenoideus transversus, pars ventricularis of thyroarytenoideus; and four regions, caudal, cranial, medial, and lateral, of the pars vocalis of thyroarytenoideus); and two extraocular muscles (retractor bulbi and rectus lateralis). The messengers corresponding to eight distinct MHC isoforms were amplified using the primers listed in Table 1 (see METHODS). As can be seen, only three MHC isoforms (1, 2A, 2X) were expressed in the longissimus dorsi sample, a condition shared by diaphragm and tibialis cranialis muscles not shown in Fig. 1. The semimembranosus sample represented a unique condition among the limb muscles explored, since the RNA of a fourth isoform, MHC-2B, was detected in two of eight dogs (not shown). The expression of all four adult MHC isoforms (1, 2A, 2X, and 2B) was consistently observed in some laryngeal muscles (medial region of pars vocalis of thyroarytenoideus, Tvm) where also

Fig. 1. MHC isoform expression determined with RT-PCR in canine skeletal muscles. RT-PCR was carried out on samples from 2 trunk and limb muscles (semimembranosus and longissimus dorsi, Sm and Ld), 7 distinct laryngeal muscle locations [cricothyroideus dorsalis (Cad); arytenoideus transversus (At); ventricularis portion of thyroarytenoideus (Tve); and 4 portions, caudal, cranial, medial, and lateral, of the vocalis portion of thyroarytenoideus (Tvc, Tvcr, Tvm, and Tvl, respectively)], and 2 extraocular muscles [retractor bulbi and rectus lateralis (Rb and Rl, respectively)]. The messengers corresponding to 8 distinct MHC isoforms were amplified using the primers listed in Table 1. “S” in the far right lane indicates the standards, for comparison.



neonatal MHC was expressed without any substantial difference among individual dogs. Most laryngeal muscles, however, expressed only the three fast MHC isoforms associated with neonatal MHC. Among extraocular muscles, in retractor bulbi all four adult isoforms and neonatal MHC were expressed, whereas expression of extraocular MHC was restricted to rectus lateralis.

MHC isoform expression studied with SDS-PAGE. In the same muscles where MHC expression was analyzed by RT-PCR, the presence of the corresponding proteins was investigated by SDS-PAGE. The analysis of single fibers or small fiber bundles where only few MHC isoforms were expressed helped to identify the electrophoretic bands based on the assumption of a general correspondence between RNA and protein. As can be seen in Fig. 2, three bands were detectable in longissimus dorsi corresponding to MHC-1, 2A, and 2X, a condition common to most of trunk and limb dog muscles. Three bands (1, 2A, 2X) were also observed in semimembranosus, even in the two cases where MHC-2B RNA was detected (see above). Apparently, either translation was suppressed by posttranscriptional mechanisms or the level of protein expression was below the detection of the gel electrophoresis. Only two bands (1 and 2A) were generally detected in diaphragm.

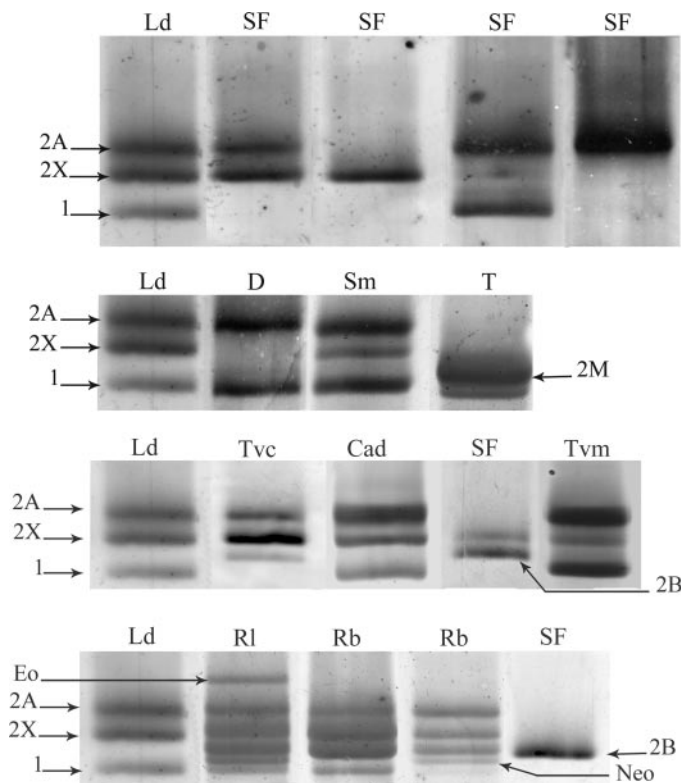


Fig. 2. Electrophoretic separation of MHC isoforms in canine skeletal muscles. From top to bottom: the first and the second panels show the three adult isoforms of MHC (1, 2A, 2X) expressed in trunk and limb muscles (Ld, Sm, D) and in several single fibers (SF) containing either one (pure) or two (hybrid) MHC isoforms. The third panel shows MHC isoforms in laryngeal muscles where either 1-2A-2X or 2A-2X-2B are expressed, and the fourth panel shows MHC isoforms in extrinsic eye muscles, RI where six MHC isoforms are detectable (Eo-2A-2X-2B-Neo-1) and Rb where 2A-2X-2B are accompanied by MHC-Neo or by MHC-1.

Electrophoresis in specialized muscles, laryngeal and extraocular, revealed the presence of the isoforms expected on the basis of RT-PCR. Five bands were detected in retractor bulbi (1, 2A, 2X, 2B, and neonatal) and six bands in rectus lateralis, where extraocular (MHC-EO) was also present. With the aim of a more complete analysis, a sample of temporalis was also examined to identify the location of all MHC isoforms, included MHC-2M or masticatory, abundantly expressed in dog temporalis (31). Under the experimental conditions used (see METHODS) the order of migration speed from the fastest to the slowest was: slow, neonatal, 2M, 2B, 2X, 2A, extraocular. In laryngeal muscles, the three fast MHC isoforms (2A, 2X, 2B) were detectable in all samples. Slow MHC was found in samples from cricoarytenoideus dorsalis (Cad) and from the medial region of the pars vocalis of thyroarytenoideus (Tvm) and neonatal MHC only in two dogs over 8 yr.

A total of about 250 single fibers were analyzed with SDS-PAGE with respect to their MHC isoform composition, most of them being also characterized for their contraction parameters or in *in vitro* motility assay (see below). Their distribution in seven groups based on MHC isoform composition (1, 1-2A, 2A, 2A-2X, 2X) is shown in Table 2 and points to type 2A as the most abundant fiber type in trunk and limb canine muscles. Examples of electrophoresis on pure and hybrid fibers are shown in Fig. 2.

Histochemistry and immunohistochemistry. The expression of MHC isoforms in individual muscle fibers was also studied using histochemistry and immunohistochemistry. In semimembranosus muscle three major fiber types were clearly identified (see Fig. 3 and Table 3): 1) Type 1 fibers were acid resistant (even below 4.4) and alkali labile, i.e., not stained if incubated at pH higher than 10.4; these fibers were positive for BA-F8, which is specific for MHC-1. 2) Type 2A were moderately alkali and acid resistant, i.e., still positive at pH 4.45 and pH 10.45 or higher; these fibers were specifically stained by antibody 2F7 only when used at high dilution and stained in the same way as type 2dog fibers by SC-71 and A4.74; these were not stained by antibody BF-35 new clone, but were stained by the BF-35 old clone. 3) Type 2dog fibers were the most acid labile (negative below 4.5) and positive only at moderately alkali pH (not higher than 10.5); these fibers were not stained by BF-35 old clone, but specifically stained by BF-35 new clone and stained by SC-71 and A4.74 as type 2A fibers. Both type 2A and type 2dog were not reactive with the antibodies BF-F3 and 10F5. In addition to the above-described three types, a variety of hybrid fibers could be identified based on intermediate levels of alkali and acid sensitivity of the ATPase reaction or of the reactivity with antibodies. Similar results were obtained in other trunk and limb muscles. Among the three muscles examined (Sm, Ld, D), pure 2X fibers, negative with BF-35 old clone, were found in semimembranosus, whereas hybrid 2A-2X fibers, positive to BF-35 new clone, SC-71, A4.74 were found in semimembranosus and longissimus dorsi.

Among laryngeal muscles, the thyroarytenoideus muscle, which in the dog is composed of two distinct components, indicated as pars vocalis and pars ventricularis, was analyzed using histochemical and immunohistochemical methods. Serial sections from the lateral portion of the pars vocalis are shown in Fig. 4. Type 1 fibers, i.e., acid resistant and positive with

Table 2. Percent distribution of single muscle fibers on the basis of their MHC isoform composition as determined by gel electrophoresis

	Ld	D	Sm	Tc	Tvm	Tve
1	25%	33%	15%	27%	5%	0
1-2A	4%	5%	5%	5%	0	9%
2A	50%	62%	64%	32%	14%	6%
2A-2X	17%	0	3%	2%	30%	57%
2X	4%	0	13%	34%	33%	28%
2X-2B	0	0	0	0	10%	0
2B	0	0	0	0	8%	0
	100%	100%	100%	100%	100%	100%
Fiber number	24	23	81	40	58	21

Single muscle fibers were dissected from six distinct muscles and classified in seven groups on the basis of their MHC isoform composition as determined by gel electrophoresis. Ld, longissimus dorsi; D, diaphragma; Sm, semimembranosus; Tc, tibialis cranialis; Tvm and Tve, vocalis medial part and ventricular part of thyroarytenoideus.

BA-F8, were not detected in this region in agreement with the results of RT-PCR and SDS-PAGE. Three populations of fast fibers were identified in vocalis muscle: 1) a few fibers were moderately acid resistant (positive at pH > 4.4) and alkali resistant (negative at pH > 10.6); these fibers were likely type 2A. 2) among the remaining fibers, the most acid-labile fibers were negative to BF-35 old clone and positive to BF-35 new clone; they were also positive to SC-71 unless used at very low concentration and very likely contained MHC-2X. 3) the fibers that were resistant to pH 4.55 (weakly acid resistant) but negative at pH 4.45 were positive to BF-F3 and 10F5, positive to BF-35 old clone, and negative to BF-35 new clone, SC-71, and 2F7 and likely expressed MHC-2B. In addition to the three groups here described, other fibers had intermediate features and likely were hybrid fibers or fibers expressing neonatal MHC, as demonstrated by specific antibodies in two dogs in full agreement with electrophoresis (not shown). The pars ventricularis portion of thyroarytenoideus muscle was similar to the lateral regions of the pars vocalis, mainly composed of fast 2X and 2B fibers. Arytenoideus transversus muscle was also mainly composed of fast 2X and 2B fibers with few 2A and no slow fibers. The cricoarytenoideus muscle was clearly different, since slow fibers were abundant, up to 50%, and fast 2B fibers, i.e., fibers positive to BF-F3 or 10F5, were not found.

In retractor bulbi muscle, histochemical and immunohistochemical staining showed that fibers positive to BF-F3 and 10F5, likely containing MHC-2B, and fibers positive to BF-35 new clone and negative to BF-35 old clone, thus likely expressing MHC-2X, were predominant. Rectus lateralis muscle was much more complex, containing virtually all types of muscle fibers, in full agreement with wealth of MHC isoforms detected with RT-PCR and SDS-PAGE (see above).

Contractile parameters of single canine muscle fibers containing distinct MHC isoforms. A total of 146 single muscle fibers, dissected from 5 muscles (fiber numbers in parentheses), diaphragma ($n = 23$), semimembranosus ($n = 49$), longissimus dorsi ($n = 24$), tibialis cranialis ($n = 22$), and pars vocalis of thyroarytenoideus ($n = 28$), were successfully analyzed in their contractile properties and classified on the basis of their MHC isoform composition as determined by SDS-PAGE in the following groups (number of fibers in parentheses): slow or 1

($n = 24$), mixed 1-2A ($n = 9$), fast 2A ($n = 72$), mixed 2A-2X ($n = 8$), fast 2X ($n = 23$), mixed 2X-2B ($n = 6$), and fast 2B ($n = 4$).

The slack sarcomere length did not show significant differences among fiber types, and, after stretching by $\sim 20\%$, the sarcomere length at which contraction was induced was (means $\pm$ SE) $2.848 \pm 0.017 \mu\text{m}$ for slow fibers, $2.86 \pm 0.051 \mu\text{m}$ for 2A fibers, $2.839 \pm 0.058 \mu\text{m}$ for 2X fibers, and $2.645 \pm 0.113 \mu\text{m}$ for 2B fibers.

The average values of cross-sectional area (CSA) of the single fibers grouped according to their MHC isoform composition and muscle of origin are shown in Fig. 5A. Large variations occurred between muscles and fiber types, with the fibers from the hind limb muscles tibialis cranialis and semimembranosus being larger than the fibers from diaphragma and longissimus dorsi regardless of the myosin expressed. Fibers from vocalis muscle were smaller.

The contractile performance of single fibers was characterized by two parameters, isometric tension (P_o) and unloaded shortening velocity (V_o), measured during maximal calcium activations. Whereas the values of P_o did not show significant differences among fiber groups (Fig. 5B), V_o showed as the expected large diversity among fibers with different MHC isoform composition (Fig. 5C). V_o increased progressively from slow to 2A, 2X, and 2B fibers, although the difference between 2X and 2B did not reach statistical significance. Hybrid fibers (1-2A, 2A-2X, and 2X-2B) had intermediate values of V_o (not shown). No significant difference was found in the values of series elasticity, which were calculated with the slack test maneuver and expressed in percent of fiber segment length (means $\pm$ SE): slow fibers, $5.37 \pm 0.46\%$; 2A fibers, $4.89 \pm 0.28\%$; 2X fibers, $6.13 \pm 0.48\%$; and 2B fibers, $7.24 \pm 0.41\%$.

Canine myosin isoforms analyzed with in vitro motility assay. Myosin was prepared from a total of 48 fibers dissected from semimembranosus and tibialis cranialis muscles and identified through SDS-PAGE as slow fibers (13), pure fast 2A fibers (21), and pure fast 2X fibers (14). The determination of the sliding velocity of actin filaments (V_f) in the motility assay yielded the following values (means $\pm$ SE, in $\mu\text{m/s}$): 0.495 ± 0.022 for slow myosin, 1.282 ± 0.064 for fast 2A myosin, and 1.664 ± 0.091 for fast 2X myosin. Variance analysis showed that the differences between the three groups were statistically significant. No significant difference was found between the average values of sliding velocity of myosin of the same type extracted from these two muscles: in semimembranosus, V_f for slow myosin was 0.503 ± 0.025 ($n = 10$), for 2A myosin 1.205 ± 0.055 ($n = 14$), and for 2X myosin 1.757 ± 0.167 ($n = 6$); whereas in tibialis cranialis, V_f for slow myosin was 0.45 ± 0.016 ($n = 3$), for 2A myosin 1.459 ± 0.154 ($n = 7$), and for 2X myosin 1.605 ± 0.109 ($n = 8$).

When the values of V_f were plotted against the V_o values measured in fibers with the same MHC isoform composition and expressed in micrometers per second per half sarcomere (see Fig. 6), the linear regression analysis showed a highly significant correlation ($P = 0.0226$) with a slope of 0.495 ± 0.020 , not significantly different from that (0.529 ± 0.015) calculated by Pellegrino and coworkers (28) for several myosin isoforms extracted from single fibers of mouse, rat, rabbit, and human muscles.

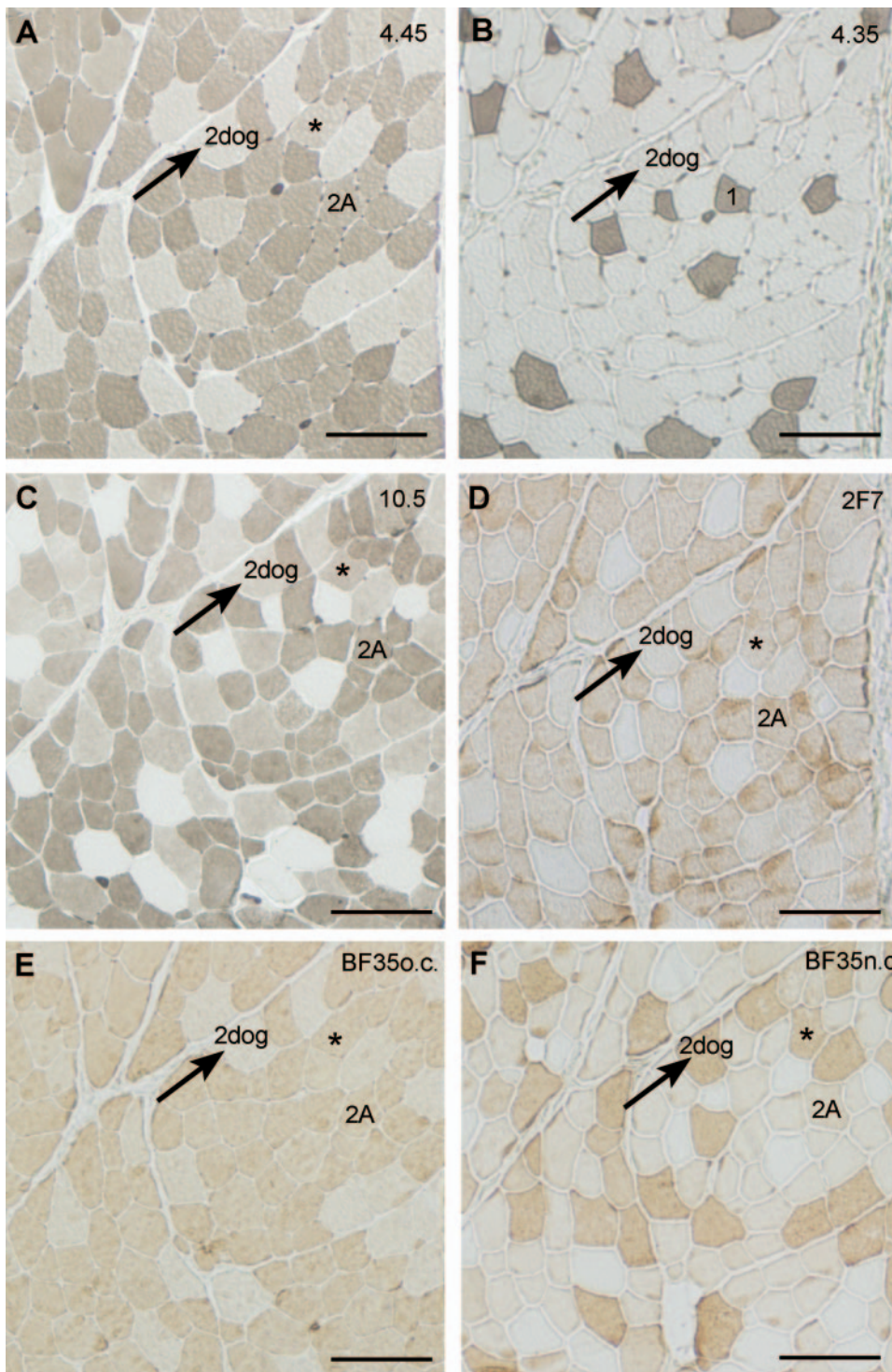


Fig. 3. Histochemistry and immunohistochemistry on semimembranosus muscle. Serial sections were stained for myofibrillar ATPase (m-ATPase) activity after preincubation at pH 4.45 (A), 4.35 (B), or 10.5 (C) and by indirect immunoperoxidase using the monoclonal antibodies (mAbs) 2F7 (D), BF-35 old clone (E), and BF-35 new clone (F). The “2dog” type fibers (arrow) are easily distinguished from type 1 (B) and type 2A fibers (A and C) after acid (A) or alkaline (C) preincubation before m-ATPase staining. “Pure” 2dog type fibers (arrow) were negative to the BF-35 old clone (o.c., E), whereas these were strongly reactive with the BF-35 new clone (n.c., F), as these expressed only MHC-2X. Hybrid fibers 2A-2X (*) were positive to both clones (E and F). The 2F7 clone was moderately positive in type 2A and hybrid fibers 2A-2X (D). Scale bars = 100 μ m.

DISCUSSION

The last 2 years have seen not only the sequencing of dog genome (20) but also a growing interest in dog muscles from the point of view of muscle biology (see Refs. 1, 3, 36, 38) as well as of muscle pathology (10, 32). Some basic issues, however, required further work, in particular the full and definitive characterization of the fiber types in canine muscles, and this implies a correct analysis of the expression of MHC isoforms, generally used as molecular markers for fiber type

classification, and the determination of the contractile parameters of the main fiber types.

Although it is clear that one slow and two fast fiber types are detectable in canine trunk and limb skeletal muscles, the precise identification of the second fast type has been controversial for a long time. This is due to the peculiar histochemical and immunohistochemical characteristics that make this type different not only from the slow fibers and the fast 2A fibers but also from fast 2B fibers (18). The identification of the third

Table 3. Criteria of histochemical and immunohistochemical identification of fiber types in dog skeletal muscles

	Type 1	Type 2A	Type 2dog/X	Type 2B
m-ATPase				
pH 4.35	+++	—	—	—
pH 4.45	+++	++	—	—
pH 4.55	+++	+++	+	+++
pH 10.4	—	+++	+++	+++
pH 10.5	—	++	++	—
pH 10.6	—	+	—	—
Monoclonal antibodies				
BA-F8	+++	—	—	—
2F7	—	++	+/-	—
SC-71	—	++	++	—
SC-71 high dilution	—	+	—	—
BF-35 old clone	+++	+++	—	+++
BF-35 new clone	—	-/+	+++	—
BF-F3 and 10F5	—	—	—	++

Myofibrillar ATPase (m-ATPase) staining followed either alkaline preincubation (*method 1*: pH 10.4, 10.5, 10.6, or 10.7) or acid preincubation (*method 3*: 0.1 M sodium acetate, at pH 4.6, 4.55, 4.5, 4.45, 4.4, or 4.35); see Ref. 18. Symbols: “+” indicates staining; “—” indicates lack of staining.

MHC isoform with MHC-2X has been first suggested by Zhang et al. (44) based on the electrophoretic pattern and recently confirmed using antibodies in Western blot and immunohistochemistry by (1, 36, 38). Since no antibody specific for MHC-2X was available, identification has been based on the principle of exclusion using the antibody BF-35, which, in the rat (33), reacts with all MHC isoforms except MHC-2X. Antibody specificity changes, however, from species to species: for example, SC-71 is highly specific for MHC-2A in rat and mouse, but reacts also with MHC-2X in cow and dog. To overcome the limits of the antibody specificity in Western blot or in immunohistochemistry, recently MALDI-TOF-MS has been used to study MHC isoforms in canine laryngeal muscles (3). In the present study the RT-PCR has been used to confirm without ambiguity the identification of the canine isoforms with the orthologs present in other species. RT-PCR can be used only when genes coding for specific MHC isoforms have been sequenced and identified. Since MHC isoforms sequences are very similar, with identity often above 90%, a detailed analysis based on BLAST and construction of phylogenetic trees is required. In this case, the identification of canine MHC-2X is supported by 96% identity with human ortholog and 97% with mouse ortholog (23). In addition, the monoclonal antibody BF-35 new clone (see also Ref. 41) has allowed a specific staining of fibers expressing MHC-2X.

Electrophoretic separation of MHC isoforms in canine laryngeal muscles (3, 43) has revealed the presence of a fourth band with migration speed between MHC-2X and MHC-1. The identification of this MHC isoform with MHC-2B has been proposed on the basis of electrophoretic migration and antibody reactivity (3, 43). In this study, RT-PCR combined with electrophoresis and histochemical and immunohistochemical methodologies has demonstrated the expression of MHC-2B in laryngeal muscles and also in extraocular muscles. Occasional presence of MHC-2B messenger has been observed in a limb muscle, the semimembranosus muscle, although the presence of the corresponding protein has not been documented in any muscle samples from limb and trunk examined in this study. The occasional inconsistency between MHC mRNA as de-

tected with RT-PCR and MHC protein identified with gel electrophoresis has been reported previously by us (40). The presence of MHC-2B messenger without the corresponding protein has been described in human masseter (16). A possible explanation is given by the higher sensitivity of the RT-PCR compared with gel electrophoresis, but it is also possible that some posttranscriptional controls inhibit the translation to protein. Why only in two dogs of eight MHC-2B expression was detected remains an intriguing question and possible relation to breed or physiological state might be considered. Interestingly, in both cases, 2B expression was observed in dogs of large body size.

Hybrid fibers, i.e., fibers where more than one MHC isoform is expressed, likely represent an important form of regulation of contractile properties (8). Immunohistochemistry might encounter difficulties in distinguishing hybrid fibers from pure fibers, i.e., fibers where only one MHC isoform is expressed. In fact, the principle of exclusion applied to BF-35 (old clone) antibody allows unambiguous identification of pure 2X fibers (36), provided that its specificity is preserved in dog as in rat muscles, but the lack of an antibody specific for MHC-2A makes it virtually impossible to recognize pure 2A fibers, as both SC-71 and A4-74 react with both MHC-2A and MHC-2X (36). Our data with single fiber electrophoresis, although limited in the number of fibers analyzed, confirmed that pure 2X fibers are frequent in some muscles as semimembranosus and Tc, in agreement with previous studies (1, 36), whereas in other muscles hybrid 2A-2X fibers are more abundant (for example, longissimus dorsi) or even no fibers expressing 2X can be found (for example, diaphragm). As discussed above, the presence of MHC-2X either alone or associated with MHC-2A gives origin to the fiber type indicated as type 2dog (18). Our data suggest that fast 2A fibers are, by far, the most abundant fiber type in dog trunk and limb muscles. Fast 2A fibers are generally associated with aerobic oxidative metabolism (7), and dog muscles are known to rely heavily on mitochondrial ATP production, a metabolic feature that contributes to the high resistance to fatigue in long-lasting exercise (37). Immunohistochemical analysis (1, 38) has suggested that 2A fibers in canine muscles are predominantly pure in fast muscles and represent 30–50% of the total fibers according to Acevedo and Rivero (1) and even more according to Strbenec et al. (38). The electrophoretic analysis on single fibers confirms that pure 2A fibers are probably more abundant than hybrid 1-2A and 2A-2X fibers. The distribution is totally different in laryngeal muscles and particularly in thyroarytenoideus, where hybrid 2A-2X and 2X-2B fibers are abundant and pure fibers, especially pure 2B fibers, although rare, are present, in agreement with the findings of Berggrin et al. (3).

In this study we report the first determination of the kinetic parameters, in the dog, of all four myosin isoforms typical of adult skeletal muscle fibers: 1 or slow, 2A, 2X, and 2B. In a recent study (25), values of maximum shortening velocity of canine slow and fast 2A fibers have been obtained at 15°C, sarcomere length 2.8 μm . Such V_0 values are perfectly consistent with the values obtained in this study assuming a Q_{10} value of 4, similar to that previously determined by us in human skeletal muscle fibers (4). The values of tension developed in maximal isometric activation obtained in this study are virtually identical to those reported by Childers et al. (10) but lower

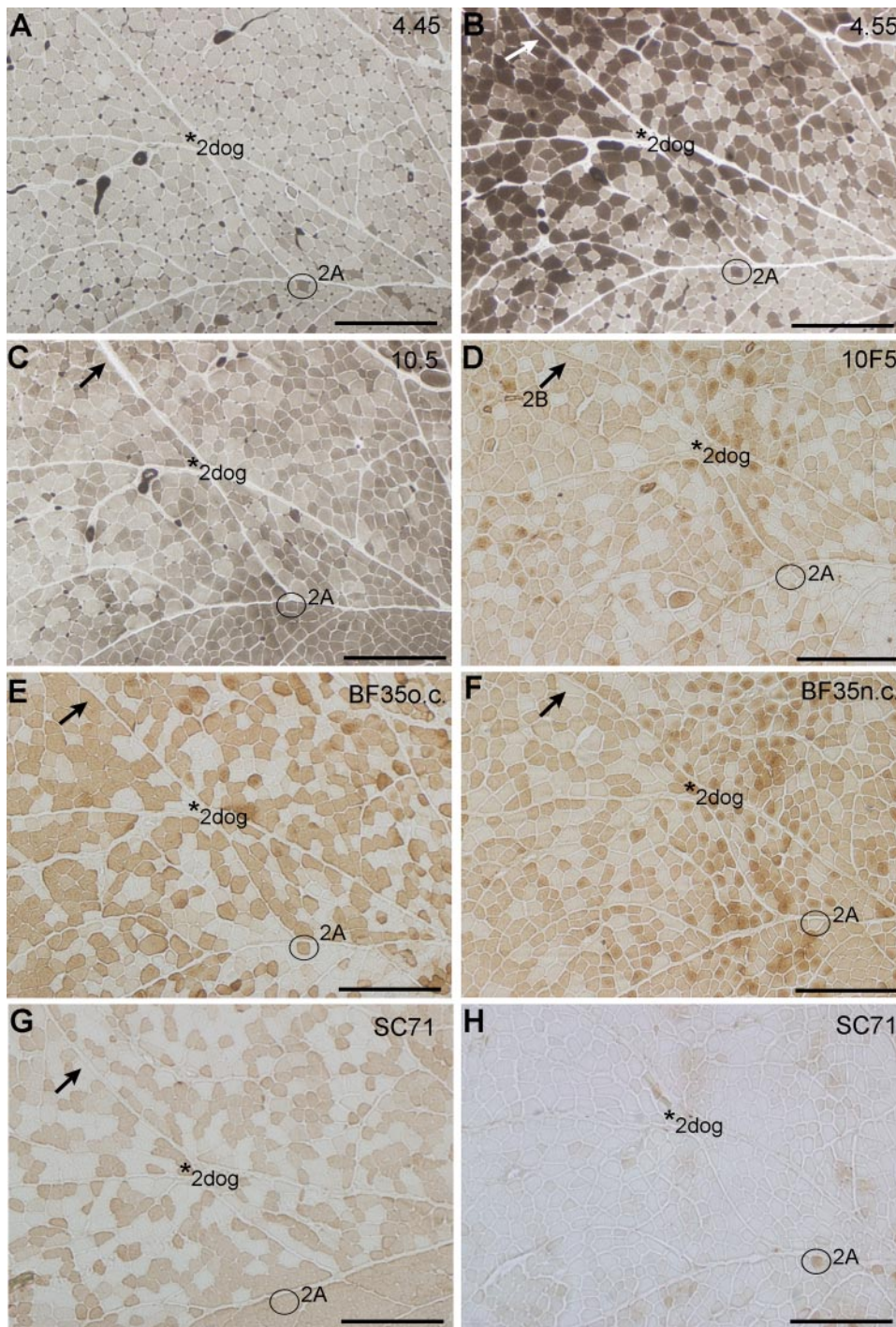


Fig. 4. Histochemistry and immunohistochemistry on pars vocalis of thyroarytenoideus. Serial sections were stained for m-ATPase activity after preincubation at pH 4.45 (A), 4.55 (B), or 10.5 (C) and by indirect immunoperoxidase using the mAbs 10F5 (D), BF-35 old clone (E), BF-35 new clone (F), and SC-71 at low (G) and high dilution (H). Preincubation at pH 4.45 allowed identification type 2A fibers, which were moderately acid resistant (A, circle) and other fibers more acid labile. These latter fibers are subdivided in two populations: some fibers are acid labile from pH 4.55 (B, *) and correspond to type 2dog expressing MHC-2X, whereas other fibers are fully stained and acid resistant at this pH as type 2A (white arrow). After alkaline preincubation at pH 10.5 (C) the first group (2dog/2X) and the second group (2A) are positive, whereas the third group is unstained, i.e., is more alkali labile (arrow). Fiber of the third group (arrow) are positive to 10F5 mAb (D), specific for MHC-2B, positive for BF-35 old clone (E), and negative to BF-35 new clone (F), specific for MHC-2X. Thus, fibers of the third group express MHC-2B. Note that mAb SC-71 used at low dilution (G) was positive for type 2A (circle) and 2X fibers (*) but not for the type 2B (arrow), whereas at very high dilution it was positive only for 2A fibers (H, circle). The compartmentalization of the vocalis muscle is visible: at the bottom of the section, type 2A and 2X fibers are present, while in the middle and top part of the section only type 2X and 2B (in both pure and hybrid forms) are present. Scale bars = 200 μ m.

than those measured by Marx and coworkers (25). The likely reason of the difference can be found in the CSA values, employed to normalize force and calculate tension. CSA values determined by Marx et al. (25) are about 50% lower than those measured in our specimens. The difference depends on the procedure followed by Marx et al. (25) who measured the depth (vertical diameter) of the fibers, whereas three diameters in the horizontal plane were utilized for calculation in our study.

The determination of the kinetic parameters of all four MHC isoforms provides further support the identification of the

isoforms, since the sequence of progressive increase in V_o values found in dog fibers was $1 < 2A < 2X < 2B$, similar to that of all other mammalian species until now examined (34). Available evidence suggests that the surface loops of the myosin heads, i.e., *loop 1* at the opening of the nucleotide binding site and *loops 2* and *3* on the actin-binding interface, play a critical role in determining the differences in V_o between myosin isoforms (see Ref. 23 and references therein). Similar amino acid sequences are present in the surface loops of orthologous myosins in various animal species and guarantee that the relative functional diversity is maintained. The corre-

lation of V_o with the speed of filament translocation in the *in vitro* motility assay (V_f) confirms the view that, under the experimental conditions used, V_o values are genuine expressions of the functional properties of myosin molecules incorporated in the sarcomeric structure (28). Interestingly, the slope of the regression lines between V_f and V_o is virtually identical in the seven mammalian species up to now analyzed (see Refs. 28, 40, 41).

It can be of interest to compare the values of V_o of canine single muscle fibers with those of other animal species of different body size. It is well known that V_o tends to decrease with increasing body mass (28, 30, 35, 40, 41) or, as recently proposed (25), with increasing limb length. The values of V_o in canine fast fibers fit rather well with the prediction of the scaling relation, being lower than values measured in rat and mouse and lower or similar to values measured in rabbit (Fig. 7). More complex is the scaling relation for slow fibers where canine fibers represent a second exception after the recently published bovine slow fibers (40). The comparison with rabbit, a species that was not considered in the recent paper by Marx and coworkers (25), shows that the average V_o value of dog slow fibers is higher than the V_o value of rabbit slow fibers. The use of femur length (25) would not resolve the inconsistency, since body size and limb length are quite variable in dogs; however, the adults of most canine varieties have heavier body mass and longer limbs than rabbits. Also, the gray wolf, from

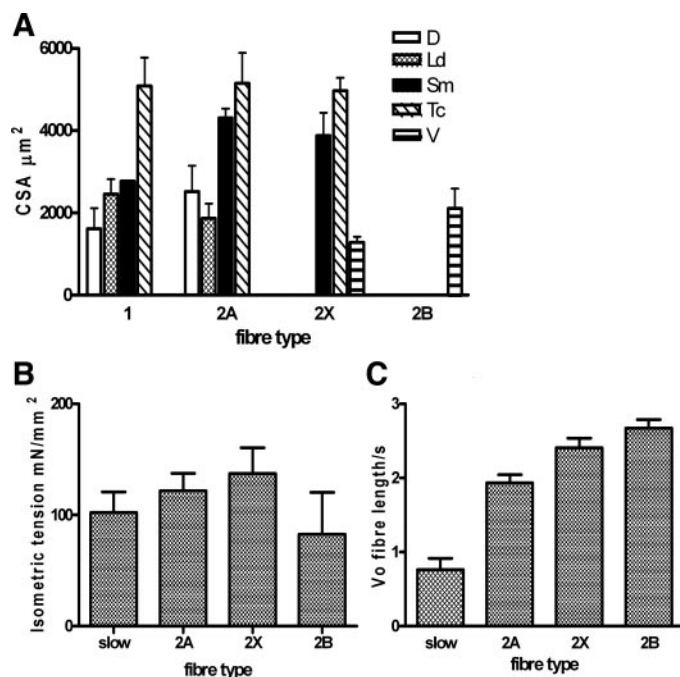


Fig. 5. Maximum shortening velocity (V_o), isometric tension (P_o), and cross-sectional area (CSA) of canine single muscle fibers. Fibers are grouped on the basis of their MHC isoform composition determined by gel electrophoresis. Only pure fibers, i.e., fibers containing one MHC isoform are shown. A: fiber groups are further divided according to the muscle of origin and fibers from Sm (semimembranosus) and Tc (tibialis cranialis) are significantly thicker than fibers with the same MHC isoform content from D (diaphragm), Ld (longissimus dorsi), and V (vocalis pars thyroarytenoideus). B: no significant difference is present between values of P_o developed by the four groups of fibers. In C, variance analysis shows that all differences in V_o between fiber groups are statistically significant, except the difference between 2X and 2B fibers. V_o is expressed in segment length per second. Values are means $\pm$ SE.

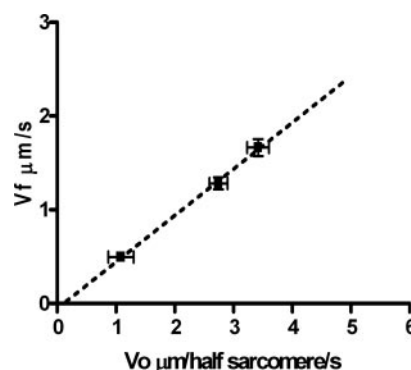


Fig. 6. Correlation between speed of actin filament translocation on purified myosin isoforms (V_f) and single fiber maximum shortening velocity (V_o). Data (means $\pm$ SE) from three groups of pure fibers (1 or slow, fast 2A, and fast 2X) are reported together with the regression line ($V_f = 0.051 + 0.495 \cdot V_o$, $r^2 = 0.998$). V_o is expressed in micrometers per half sarcomere per second.

which the dog branched about 9,000 generations ago due to domestication (see discussion in Ref. 20), has an adult size of ~ 35 – 45 kg. The consistent values of the slope of the linear relation between V_o measured in fibers with intact sarcomeric structure and V_f measured with purified myosins strongly support the view that the differences in V_o among orthologous myosins of different animal species are not due to the interference of other myofibrillar protein or to structural constraints of the sarcomere architecture. It is thus reasonable to conclude that the scaling relation of V_o , although valid in general terms and on a large range, can show exceptions related to specific and still unknown details of the evolutionary history of individual species. In regard to this, it is important to note that slow or beta myosin is also abundantly expressed in ventricular myocardium of the dog (21), and its functional features might be influenced by the requirement of cardiac activity as recently suggested (15).

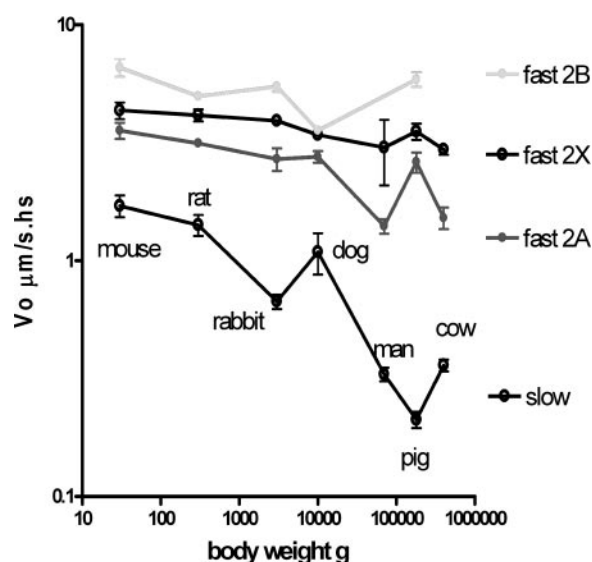


Fig. 7. Scaling relation between maximum shortening velocity and body size in 7 animal species. V_o is expressed in micrometers per half sarcomere per second, for correct comparison among species. Data for mouse, rat, rabbit, and human come from Ref. 28, data for pig from Ref. 41, and data for cow from Ref. 38.

In conclusion, the data reported in this study provide a definitive answer to the issue of myosin isoform and fiber type identification in canine muscles and make available tools for the expression studies at mRNA and protein levels. This study furthermore provides a first systematic description of the functional properties of the four adult skeletal muscle myosin isoforms. These results represent the starting point for future studies on canine muscle adaptations, which in our opinion find two very interesting perspectives, i.e., the diversity between canine breeds in relation to their body size and physiological features and, more interesting for medicine, the impact of life style on muscles, and the effects of aging.

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