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# Myosin heavy chain isoforms in histochemically defined fiber types of rat muscle

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**Summary.** Combined histochemical and biochemical analyses were performed on rat skeletal muscles in order to determine the myosin heavy chain patterns in specific fiber types. Four myosin heavy chain isoforms were separated by gradient polyacrylamide gel electrophoresis of extracts from single fibers and whole muscle homogenates. Their electrophoretic mobility increased in the order HCIIa, HCIIb, and HCI. HCIIa, HCIIb and HCI were present as unique isoforms in histochemically defined fiber types IIA, IIB and I, respectively. The isoforms HCI and HCIIa coexisted at variable ratios in type IC and IIC fibers. An additional fast myosin heavy chain isoform with an electrophoretic mobility between HCIIa and HCIIb was designated as HCIId because of its abundance in fast fibers of large diameter in the diaphragm. With the exception of slight differences in mATPase staining intensity after acid preincubation, these fibers were almost indistinguishable from type IIB fibers. In view of their specific myosin heavy chain composition (HCIId), these fibers were named type IID. In the extensor digitorum longus muscle, type IID fibers were of smaller size than type IIB and differed from the latter by higher NADH tetrazolium reductase activities. Circumstantial evidence suggests that type IID fibers are identical with the 2X fibers, previously described by Schiaffino et al. (1986).

## Introduction

With the use of an improved electrophoretic method we have previously been able to separate three fast myosin heavy chain isoforms in rat skeletal muscles (Bär and Pette 1986). As judged from their electrophoretic mobility and distribution in muscles of defined fiber type composition, two of the fast heavy chains were tentatively assigned to fiber types IIA and IIB. A third isoform was found to be predominant in diaphragm and other muscles adjusted to sustained contractile activity. This isoform was tentatively designated as HCIId. The distribution of this new isoform in specific fiber types remained unclear. In the present study we have addressed this question by analysing electrophoretically microdissected fragments of histochemically classified fiber types. The fiber classification was based upon differences in myofibrillar actomyosin ATPase (mATPase) activity and, in addition, on metabolic characteristics as judged from the histochemically assessed activity of NADH tetrazolium reductase. With this approach, we were able to assign the three fast myosin heavy chain isoforms to specific fiber types of rat muscle.

## Materials and methods

Extensor digitorum longus, soleus and diaphragm were excised from adult male Wistar rats. Additional analyses were performed on 28-day-stimulated tibialis anterior and normal soleus muscles from Sprague-Dawley rat. Chronic low-frequency stimulation (10 Hz, 10 h daily) was performed as previously described (Simoneau and Pette 1988). Muscles were excised and immediately frozen in a slightly stretched position in melting isopentane (−160° C). For histochemistry, serial cross-sections were cut on a microtome in a cryostat at −25° C, mounted on coverslips, air-dried and processed for mATPase according to Brooke and Kaiser (1970) as described (Staron and Pette 1986). One cross section was stained for NADH tetrazolium reductase according to Farber et al. (1956). Six to eight thick (approximately 100 μm) cross sections, cut from the same block, were transferred to vacuum recipients and freeze-dried. Fragments of histochemically (mATPase and NADH tetrazolium reductase) classified fibers were dissected from the thick cross sections under a stereomicroscope in a humidity- and temperature-controlled room. The fiber fragments (6–8 from the same fiber) were transferred under the stereomicroscope into a glass capillary and lysed during 10 min at 60° C in 10 μl of a lysis buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% sodium dodecyl sulfate, 60 m*M* *Tris*-HCl, pH 6.8). A total of 15 fibers from extensor digitorum longus, 30 fibers from diaphragm and 31 fibers from soleus (Sprague Dawley) were analyzed.

For preparing crude myosin extracts from whole muscle homogenates, aliquots from extensor digitorum longus, soleus (Wistar), soleus (Sprague-Dawley), diaphragm, and 28-day-stimulated tibialis anterior were pulverized under liquid $N_2$ and homogenized 1:7 (w/v) in the following medium: 0.3 *M* KCl, 0.1 *M* $KH_2PO_4$, 50 m*M* $K_2HPO_4$, 10 m*M* EDTA, pH 6.5. After stirring for 15 min on ice, the homogenate was centrifuged at $10000 \times g$. The supernatant fraction was diluted two-fold with glycerol and stored at −20° C. Protein was determined according to Lowry et al. (1951). Myosin heavy chain isoforms were electrophoretically separated after applying the total lysed probe of the dissected fiber fragment or 0.1–0.5 μg protein of muscle extract to a polyacrylamide gel. Electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) was carried out using a 0.75-mm-thick 5%–8% gradient separating gel and a 3.5% stacking gel (Bär and Pette 1988). Electrophoresis lasted 24 h at 120 V. Gels were silver-stained according to Oakley et al. (1980).

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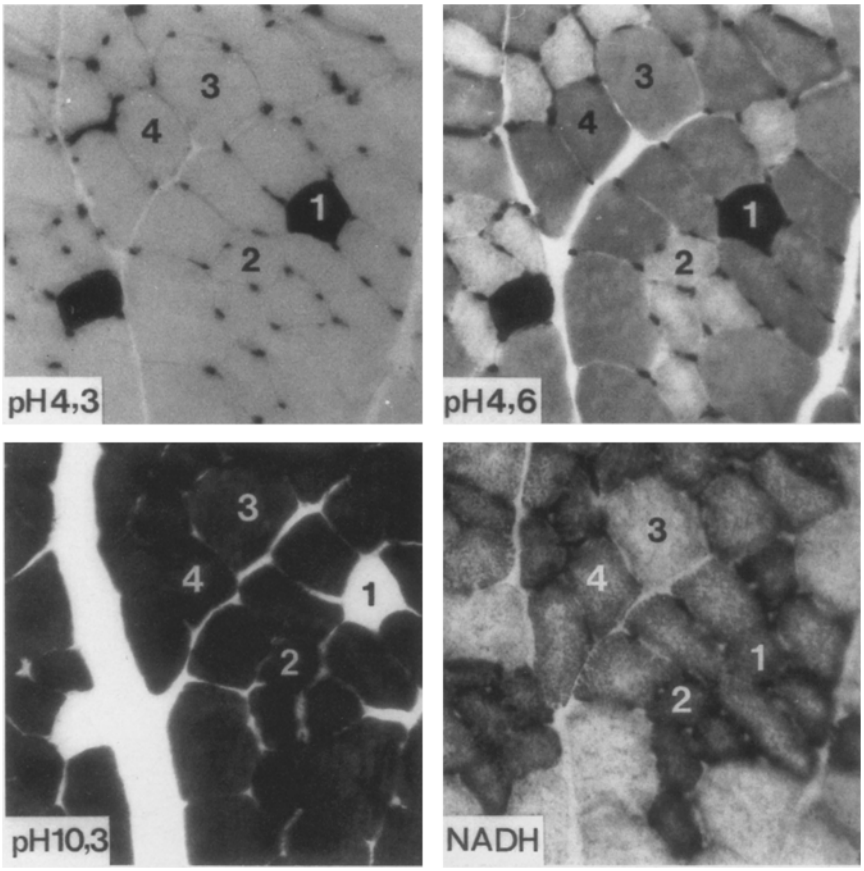


**Fig. 1.** Serial cross sections of rat extensor digitorum longus muscle stained for myofibrillar actomyosin ATPase after preincubation at pH values 4.3, 4.6 and 10.3, and for NADH tetrazolium reductase. The numbers designate single fibers analysed for their myosin heavy chain composition in Fig. 2. Fiber 1 is type I, fiber 2 is type IIA, fiber 3 is type IIB, fiber 4 is type IID. ×200

## Results

Three fiber types were delineated by mATPase histochemistry in rat extensor digitorum longus muscle and designated according to Brooke and Kaiser (1970) as type I, type IIA and type IIB (Fig. 1). Type I fibers stained dark after preincubation at pH 4.3 and pH 4.6 and light after preincubation at pH 10.3 (Fig. 1, no. 1). This fiber type displayed the fastest migrating heavy chain (Fig. 2, no. 1). The mobility of this heavy chain, designated as HCI, corresponded to that of the single band detected in crude myosin preparations from soleus muscle of Wistar rat (Fig. 2, $S_{wi}$). Type IIA fibers reacted strongly after preincubation at pH 10.3 and showed little or no activity after preincubations at pH 4.6 and pH 4.3, respectively (Fig. 1, no. 2). The heavy chain of type IIA fibers (HCIIa) exhibited the lowest electrophoretic mobility (Fig. 2, no. 2) and corresponded to the slowest migrating band in whole muscle extracts from normal extensor digitorum longus and 28-day-stimulated tibialis anterior muscles (Fig. 2, EC and TS). Type IIB fibers were unreactive after preincubation at pH 4.3 and reacted intermediately after preincubations at pH 4.6 and strongly at pH 10.3 (Fig. 1, no. 3). The myosin heavy chain of type IIB fibers (HCIIb) exhibited a slightly lower mobility than HCI (Fig. 2, no. 3).

Some fibers appeared distinct from the fiber types described so far. These fibers resembled type IIB with regard to their mATPase reactivity after preincubation at pH 4.3 and 10.3. However, at pH 4.6 these fibers appeared slightly darker than type IIB fibers. Moreover, they were smaller in size and exhibited higher NADH tetrazolium reductase activities than type IIB fibers (Fig. 1, no. 4). The myosin heavy chain of these fibers migrated between HCIIa and HCIIb (Fig. 2, no. 4). This isoform (HCIId) was also detected in chronically stimulated tibialis anterior muscle (Fig. 2, TS) and in fibers of rat diaphragm.

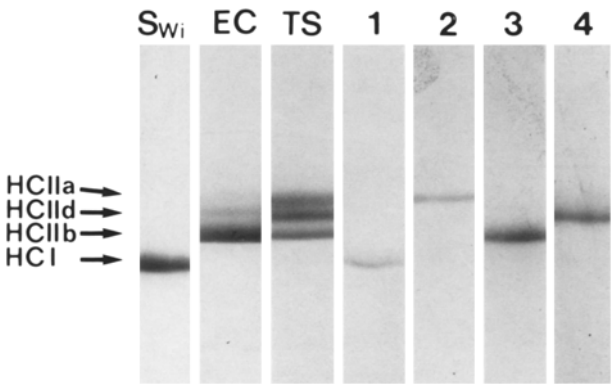


**Fig. 2.** Gradient PAA (5%–8%) gel electrophoresis of microdissected fragments from histochemically classified single fibers (Fig. 1) from rat extensor digitorum longus muscle. The numbers correspond to the same fibers marked in Fig. 1. Electrophoreses of whole muscle extracts are included for comparison: $S_{wi}$, soleus muscle Wistar strain; *EC*, normal extensor digitorum longus; *TS*, 28-day-stimulated tibialis anterior

Applying the criteria of Brooke and Kaiser (1970), a major fraction of the fast fiber population in rat diaphragm had the histochemical appearance of type IIB fibers (Fig. 3, no. 3–5). However, as judged from myosin heavy chain analysis these fibers were biochemically distinct from the

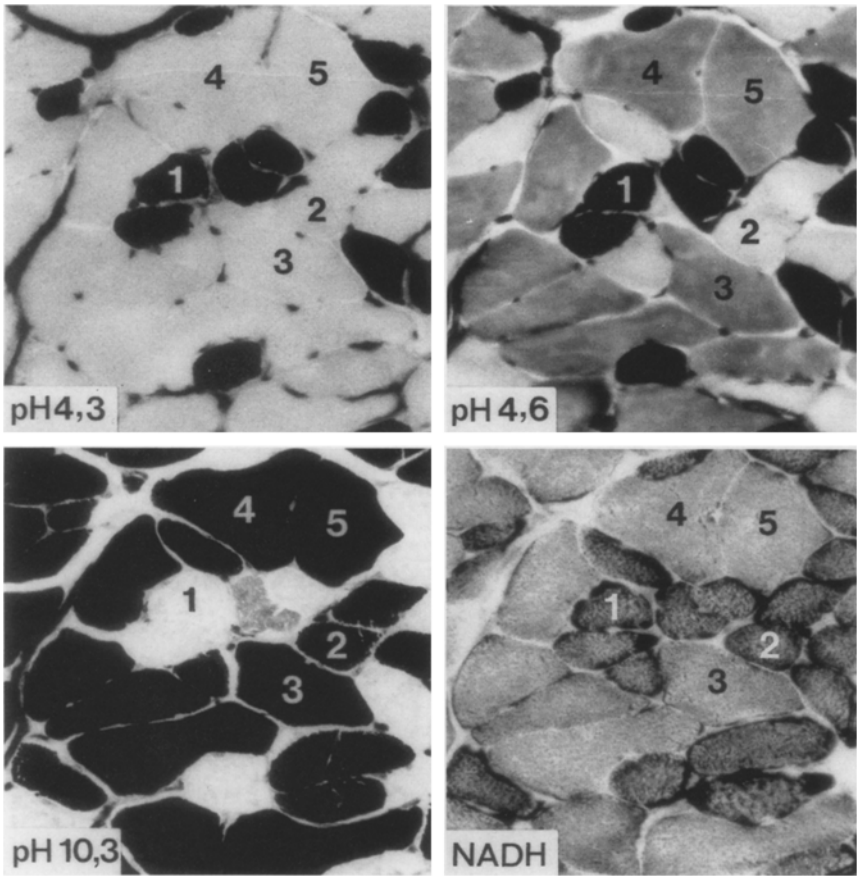


**Fig. 3.** Serial cross sections of rat diaphragm stained for myofibrillar actomyosin ATPase after preincubation at pH values 4.3, 4.6 and 10.3, and for NADH tetrazolium reductase. The numbers designate single fibers analyzed for their myosin heavy chain composition in Fig. 4. Fiber 1 is type I, fiber 2 is type IIA, fibers 3–5 are type IID. ×200

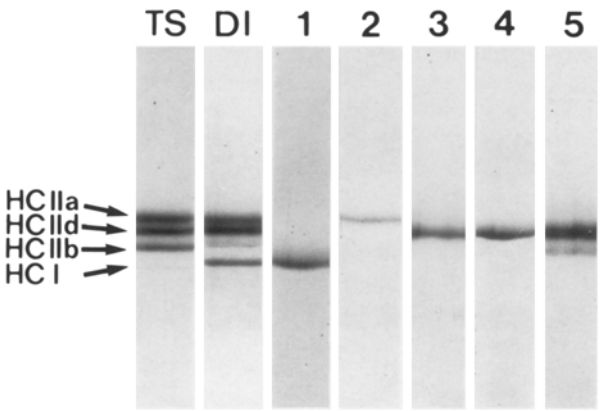


**Fig. 4.** Gradient PAA (5%–8%) gel electrophoresis of microdissected fragments from histochemically classified single fibers (Fig. 3) from rat diaphragm. The numbers correspond to the same fibers marked in Fig. 3. Electrophoreses of whole muscle extracts are included for comparison: *TS*, 28-day-stimulated tibialis anterior; *DI*, diaphragm

type IIB. Their myosin heavy chain migrated electrophoretically between heavy chains HCIIb and HCIIa (Fig. 4, no. 3–5) and thus corresponded to the specific isoform identified in the above-mentioned subgroup of fast fibers in the leg muscle. However, contrary to the corresponding fibers in the extensor muscle, these fibers were larger in size. Their tetrazolium reductase activity was lower than that of type I and type IIA fibers. Because of their abundance in the diaphragm, these fibers were tentatively designated as type IID. Consequently, their myosin heavy chain isoform was named HCIId. Electrophoretic analyses performed on 30 single fibers from rat diaphragm showed that type IID could be identified biochemically by its specific heavy chain isoform. However, some fibers were detected which, in addition to HCIId, contained HCIIb as a second myosin heavy chain isoform (Fig. 4, no. 5). The HCIId to HCIIb ratio was found to vary in this fraction of hybrid fibers. Among the investigated fibers only one fiber could be identified which only contained HCIIb (not shown). On the basis of these biochemical results it became evident that the majority of the fast fibers which usually are classified as type IIB in rat diaphragm, were of the type IID.

These results raised the question as to the possible expression of HCIId in other fiber types. In this conjunction, analyses were performed on single fibers from slow-twitch soleus muscle of Sprague-Dawley rats (Figs. 5 and 6). As compared to the Wistar strain, soleus muscle of Sprague-Dawley rats had previously been shown to contain a relatively high amount of fast myosin heavy chains (Bär and Pette 1988). Electrophoretic analyses of whole soleus muscle extracts from Sprague-Dawley rats (Fig. 6, $S_{SD}$) showed, in addition to the predominant HCI, the presence of the fast heavy chain HCIIa and minute amounts of HCIIb. These isoforms were not detected in soleus muscle from Wistar rats (Fig. 6, $S_{Wi}$). The fiber population of soleus muscle from Sprague-Dawley rat was heterogeneous and contained, in addition to the predominant type I fibers (Fig. 5, no. 1, 2), type IIA fibers (Fig. 5, no. 6, 7). According to their throughout positive mATPase reactivity after preincubations at pH values 4.1, 4.3, 10.3, a few fibers were

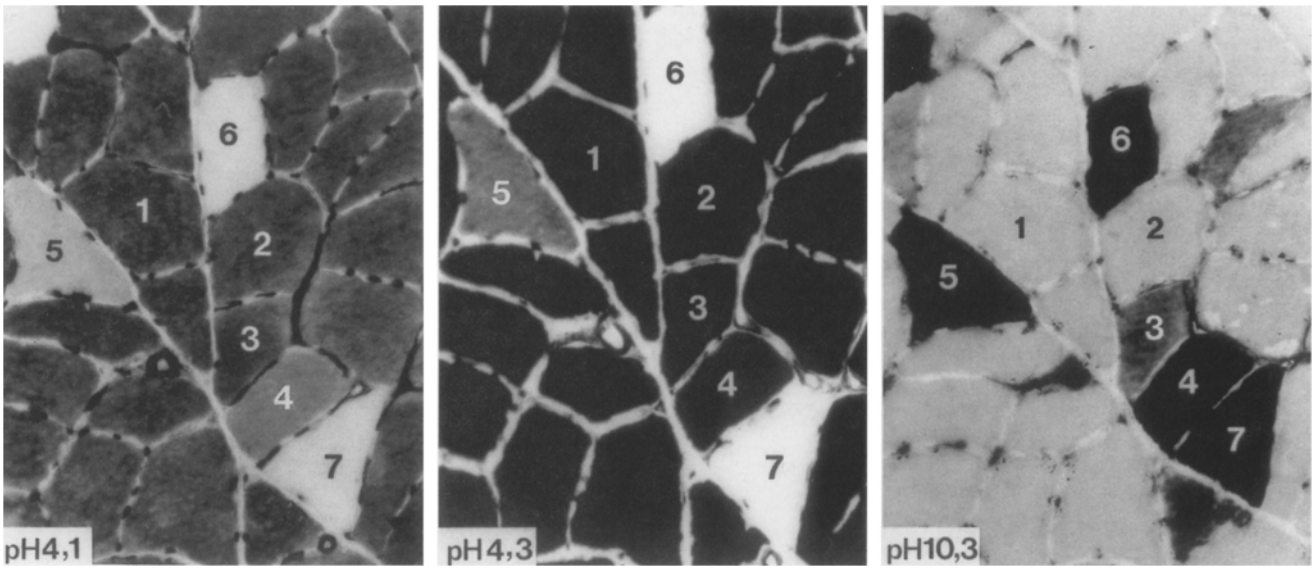


**Fig. 5.** Serial cross sections of rat soleus muscle (Sprague Dawley strain) stained for myofibrillar actomyosin ATPase after preincubation at pH values 4.1, 4.3 and 10.3, and for NADH tetrazolium reductase. The numbers designate single fibers analyzed for their myosin heavy chain composition in Fig. 6. Fibers 1 and 2 are type I, fiber 3 is type IC, fibers 4 and 5 are type IIC, fibers 6 and 7 are type IIA. ×200

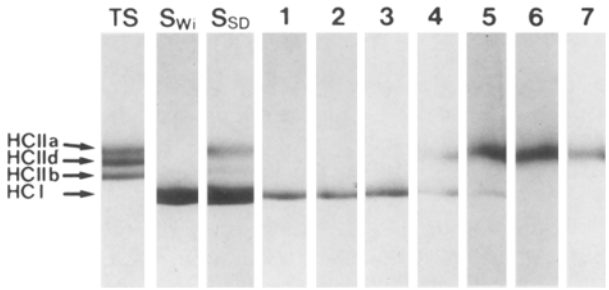


**Fig. 6.** Gradient PAA (5%–8%) gel electrophoresis of microdissected fragments from histochemically classified single fibers (Fig. 5) from rat soleus muscle. The numbers correspond to the same fibers marked in Fig. 5. Electrophoreses of whole muscle extracts are included for comparison: *TS*, 28-day-stimulated tibialis anterior; $S_{wi}$, soleus Wistar; $S_{SD}$, soleus Sprague Dawley

classified as type C fibers (Staron and Pette 1986, 1987a, b). Type IC stained intensely after acid preincubation and moderately after alkali preincubation (Fig. 5, no. 3). Type IIC fibers stained strongly at pH 10.3 and moderately after acid preincubation (Fig. 5, no. 4, 5).

According to electrophoretic analyses soleus type I fibers contained the slow heavy chain isoform HCI (Fig. 6, no. 1, 2). Type IIA fibers contained the slowest migrating isoform, i.e. HCIIa. The heavy chain composition of the C fibers was complex and consisted of isoforms HCI and HCIIa in variable ratios. Type IC fibers (Fig. 5, no. 3) contained higher amounts of HC I than of HCIIa (Fig. 6, no. 3), whereas HCIIa was the predominant isoform in type IIC fibers (Figs. 5 and 6, no. 4, 5). The HCIIa content of fiber 3 in Fig. 5 was extremely low and was insufficiently documented (Fig. 6, no. 3).

## Discussion

The assignment of myosin heavy chain isoforms to specific muscle fiber types can be achieved either by immunohistochemistry or by microbiochemical analyses of microdissected and histochemically identified single fibers. As previously shown, four different myosin heavy chain isoforms can be separated by gradient gel electrophoresis of extracts from various skeletal muscles of the adult rat (Bär and Pette 1988). In the present study, these myosin heavy chain isoforms can be definitely assigned to histochemically classified fiber types. Fiber types I, IIA, and IIB express myosin heavy chains HCI, HCIIa, and HCIIb, respectively. An additional fast fiber type (type IID), abundant in the diaphragm, can be delineated by its specific myosin heavy chain, HCIId. With the exception of slight differences in the mATPase staining after preincubation at pH 4.6, type IID fibers are almost indistinguishable from type IIB fibers. Thus, using the criteria of histochemical fiber typing according to Brooke and Kaiser (1970), it is evident that in rat skeletal muscle and diaphragm the classical type IIB fiber population is heterogeneous and can be subdivided biochemically by differences in myosin heavy chain composition into types IIB and IID. In the investigated leg muscle, type IID fibers are of intermediate size and can be separated from type IIB fibers by their higher NADH-tetrazolium reductase activities. In the diaphragm, type IID fibers represent a major fast fiber subgroup with large diameter and relatively low NADH tetrazolium reductase activities.

According to the results of the present study, the four myosin heavy chain isoforms detected in rat skeletal mucle are characterized by an order of increasing electrophoretic mobilities: HCIIa < HCIId < HCIIb < HCI (Fig. 5). The assignment of heavy chains HCIIa and HCIId is in contrast to our previous results which were derived from electrophoretic analyses performed on whole muscle extracts. In our previous work, HCIId was tentatively identified as the slowest migrating isoform (Bär and Pette 1988). This designation has to be revised in view of the results of the present study. Thus, HCIIa is the slowest migrating isoform and not HCIId which migrates electrophoretically between heavy chains HCIIa and HCIIb (Fig. 7). This renaming

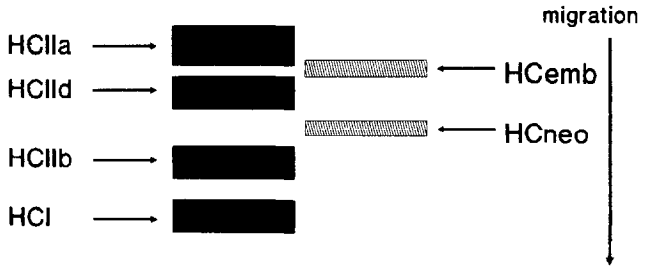


**Fig. 7.** Schematic illustration of the electrophoretic mobilities of embryonic ($HC_{emb}$), neonatal ($HC_{neo}$), adult fast (HCIIa, HCIId, HCIIb) and slow myosin heavy chain (HCI) isoforms of rat skeletal muscle

makes it also necessary to correct our previous results on the percentage distribution of HCIId and HCIIa in various skeletal muscles of the rat (Bär and Pette 1988).

The delineation of heavy chain HCIId raises the question as to the functional significance of this new isoform and its corresponding fiber type IID. The present results suggest that this isoform is intermediate between HCIIb and HCIIa. In normal extensor digitorum longus and diaphragm of the rat it is expressed as the only isoform in a specific fast fiber subgroup which can be separated in the leg muscle from type IIB and type IIA fibers by an intermediate aerobic-oxidative metabolic capacity. The oxidative potential of type IID fibers in the diaphragm is difficult to estimate by enzyme histochemistry because the content of pure type IIB fibers (as judged from myosin heavy chain analyses) is extremely low in this muscle. Thus, a comparison of the oxidative potential of type IID fibers with that of type IIB fibers is difficult in diaphragm.

It appears that the HCIId isoform is preferentially expressed in fast fibers subjected to sustained activity. This follows from the high content of type IID fibers in the diaphragm. It also follows from the previously observed increase in HCIId and HCIIa content of chronically stimulated rat fast-twitch muscle (Bär and Pette 1988). The present results indicate that HCIId may coexist with HCIIb in fast fibers. According to the single fiber analyses of soleus muscle, HCIId is not expressed in soleus fibers. The hybrid fibers detected in soleus muscle, i.e. type IC and IIC fibers, coexpress HCI together with HCIIa. However, a coexistence of HCIId together with HCIIa follows from unpublished single fiber analyses on chronically stimulated extensor digitorum longus muscle of the rat. Taken together, these results corroborate the suggested intermediate position of HCIId or fiber type IID between heavy chains IIb and HCIIa or fiber types IIB and IIA, respectively.

Our single fiber analyses provide evidence that fiber type IID may be identical with the previously detected fiber type 2X identified by Schiaffino et al. (1985, 1986, 1988, 1989). With the use of several monoclonal antibodies, these authors were able to immunohistochemically delineate in rat skeletal muscles a new fiber type, designated type 2X. This fiber type was characterized by an acid stability of the mATPase similar to type IIB fibers. It was shown to differ from the type IIB fibers by a higher activity of succinate dehydrogenase. An additional difference between type IIB and type 2X fibers was that the mATPase of type 2X fibers is more resistant to formaldehyde preincubation than type IIB fibers (Schiaffino et al. 1986, 1989). Preliminary studies in our laboratory indicate a similar difference between types IID and IIB (unpublished observations). Although Schiaffino et al. (1989) were not able to electrophoretically separate HC2x and HCIIa isoforms in whole muscle myosin preparations, it is evident from their published immunoblot analyses that their slowest migrating band contained two immunochemically distinct components, most likely corresponding to HCIIa and HC2x. Taken together, these independent findings suggest that the myosin heavy chain isoforms of type IID and type 2X fibers are the same.

Schiaffino et al. (1988) investigated contractile properties of rat soleus muscle that had been transformed into a faster contracting muscle by chronic stimulation with a phasic, high-frequency impulse pattern. The shortening velocity of the transformed soleus was intermediate between that of normal soleus (predominance of type I fibers) and that of EDL (predominance of type IIB fibers). Because the majority of the fiber population of the transformed soleus muscle consisted of type 2X fibers, these authors concluded that the HC2x is responsible for the intermediate shortening velocity.

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