Correlation of parvalbumin concentration with relaxation speed in mammalian muscles

(Ca²⁺-binding proteins/muscle physiology/HPLC)

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ABSTRACT The physiological role of the Ca²⁺-binding protein parvalbumin in skeletal muscle has been investigated by measuring the parvalbumin content by HPLC in a variety of mammalian muscles, including man, and comparing the results with the respective muscle relaxation properties and fiber type compositions. The parvalbumin concentrations were highest in the skeletal muscles of the smallest animal investigated (mouse, gastrocnemius; 4.9 g/kg), which has the highest relaxation speed, and lowest in the larger animals (horse, deep gluteal muscle: ≤0.001 g/kg) and man (vastus, triceps: ≤0.001 g/kg), which have much lower relaxation speeds. Analysis of three type-homogeneous muscles of the guinea pig revealed highest parvalbumin concentrations (0.25 g/kg) in sartorius (type IIB) and lowest concentrations (≤0.007 g/kg) in soleus (type I), consistent with the different half-relaxation times and slowest relaxation speeds. Analysis of the rat extensor digitorum longus, which increases the half-relaxation time from 0.4 to 19 msec, resulted in a 20% decrease of the parvalbumin content. Given this close correlation between parvalbumin content and relaxation speed in a variety of muscles and species, we suggest that parvalbumin is involved directly in the relaxation process in fast muscles.

Two opposing hypotheses coexist about the role of the Ca²⁺-binding protein parvalbumin in the contraction-relaxation cycle of skeletal muscle. On the basis of kinetic experiments it was proposed that muscle parvalbumin is a relaxing factor capable of removing Ca²⁺ ions from the myofibrillar Ca²⁺-binding subunit of troponin, troponin C, and then passing the Ca²⁺ ions to the sarcoplasmic reticulum (1–4). On the other hand, it was concluded that parvalbumin cannot be involved directly in the rapid decay of tension in a single twitch of muscle essentially because of the slow Ca²⁺ "off" rate (resulting from the high affinity of these sites for Ca²⁺) (5–7). Thus, despite a similar experimental approach there is disagreement about the in vivo Ca²⁺-binding properties and the functional role of parvalbumin.

We chose to approach this problem from a different point of view by comparing the parvalbumin concentrations, determined by HPLC and two-dimensional (2-D) gel electrophoresis, with the contraction and relaxation properties of a variety of muscles of several mammalian species, including man.

If parvalbumin is involved in the relaxation process of muscles, its distribution and concentration must fulfill the following criteria:

(i) Within any single species of animal more parvalbumin should be present in muscles known to have fast relaxation times than in muscles that relax slowly.

(ii) Similarly, on a much more general level, within each species, the parvalbumin content of individual muscles should be related to their fiber type composition, with more parvalbumin present in muscles with a high proportion of fast-twitch (type II) fibers (because these also relax faster than slow-twitch fibers).

(iii) Muscles of the same fiber type composition but from animals of different size should contain more parvalbumin in the smaller animal. This follows directly from Hill’s argument (8) concerning running speeds of animals of different sizes. As he pointed out, animals of similar design but different size have very similar top running speeds. Therefore, it follows (9) that at the same running speed, small animals must move their legs to and fro much faster and have muscles with much higher shortening velocities. For obvious reasons, this faster contraction time is accompanied by shorter relaxation times.

(iv) In any one species, muscles that have similar fiber type compositions but different relaxation times might have correspondingly different parvalbumin contents. There are two well-documented situations of this kind in the rat: (a) denervated muscles, which develop a much slower relaxation time within a few days after denervation (10, 11) (before any substantial changes can occur in the fiber type composition); and (b) the extraocular muscles (EOM), which have a shorter relaxation (and contraction) time than hind leg muscles of similar composition (12).

Quantitative analysis of various muscle extracts for parvalbumin by HPLC and 2-D gel electrophoresis showed a direct correlation between parvalbumin concentration and half-relaxation time, strongly suggesting that parvalbumin has a role to play in the relaxation process.

MATERIALS AND METHODS

Individual muscles (see Table 1) were taken from adult mice (ICRZ), rats (SIV-50), guinea pigs (Himalayan spotted white), horse, and man. Muscle pieces (0.03–0.3 g) were ground to a powder in liquid N₂ and weighed; they then were added to an equal volume of a 4 mM EDTA solution (pH 7.0) containing 1 μM pepstatin, 0.4 mM phenylmethylsulfonil fluoride, 0.15 mM N-tosylphenylalanine chloromethyl ketone, and 1 μM leupeptin as protease inhibitors. The suspension then was sonicated six times for 10 sec each (100 W, Branson ultrasonic) and was centrifuged. The pellets were reextracted with 1 vol of the same medium and were centrifuged again. Both supernatants were combined and heated for 30 min at 85°C. The heat-treated extracts then were centrifuged and the pellets were resuspended in 1 vol of the above EDTA solution and were centrifuged again; the wash was combined with the heat-stable supernatant.

Abbreviations: 2-D, two-dimensional; EDL, extensor digitorum longus; EOM, extraocular muscle(s); Gast., gastrocnemius; TA, tibialis anterior; EHB, extensor hallucis brevis; BB, retractor bulbii; 1st DI, first dorsal interosseus (hand); troponin C, Ca²⁺-binding subunit of troponin.
Table 1. Correlation of parvalbumin content of various muscles with physiological data*

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Fiber type composition, %</th>
<th>Contraction time, msec Motor units</th>
<th>Whole muscle</th>
<th>Half-relaxation time, msec Motor units</th>
<th>Whole muscle</th>
<th>Parvalbumin, g/kg wet weight$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gast. (sup.)$^a$</td>
<td>IIB, 100</td>
<td>$=$50; IIA, =50</td>
<td>I and IIA, 37–7$^c$; I and IIA, 17.7$^c$; I, IIA, and IIB, 20.9$^c$</td>
<td>I, 31$^c$; IIA, 10$^c$; I and IIA, 22.5$^d$</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Soleus$^b$</td>
<td>IIB, 100</td>
<td>I, 85; IIA, 15</td>
<td>I and IIA, 34$^m$; I and IIA, 38$^{lm}$; I, 26–44$^i$; IIA, 15–20$^i$</td>
<td>I, 49$^k$; I, 51$^{61}$; I, 45; IIA, 11</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>EDL$^c$</td>
<td>I, =5; IIA and IIB, $&gt;$95</td>
<td>IIA and IIB, 11$^k$; IIA and IIB, 10.5$^k$; IIA and IIB, 8.4$^n$</td>
<td>IIA and IIB, 10.5$^k$; IIA and IIB, 8.4$^n$</td>
<td>IIA and IIB, 8.6$^f$</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>TA$^d$</td>
<td>I, =5; IIA and IIB, $&gt;$90</td>
<td>IIB, 5$^p$</td>
<td>IIA and IIB, 6.3$^p$</td>
<td>IIA and IIB, 6.3$^p$</td>
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</tr>
<tr>
<td>EOM$^e$</td>
<td>I, 20–30; IIB and IIB, 70–80</td>
<td>IIB, 5$^p$</td>
<td>IIA and IIB, 19–22$^e$</td>
<td>IIA and IIB, 19–21$^e$</td>
<td>1.8</td>
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</tr>
<tr>
<td>RB</td>
<td>IIB, 100**</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Masseter$^h$</td>
<td>IIB, 100</td>
<td>I, 82$^f$</td>
<td>I, 113.8$^f$</td>
<td></td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Soleus$^g$</td>
<td>IIB, 100</td>
<td>I, 100</td>
<td>IIA and IIB, 96–82</td>
<td>IIA and IIB, 19–22$^e$</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Sartorius$^i$</td>
<td>IIB, 100</td>
<td>I, 100</td>
<td>IIA and IIB, 19–22$^e$</td>
<td>IIA and IIB, 19–21$^e$</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Fast muscles$^j$</td>
<td>I, 4–18; IIA and IIB, 96–82</td>
<td>IIA and IIB, 19–22$^e$</td>
<td>IIA and IIB, 19–22$^e$</td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Deep gluteal$^k$</td>
<td>I, 22; IIA, 43; IIB, 35</td>
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<td>Unknown</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Vastus$^l$</td>
<td>I, =50; IIA and IIB, =50</td>
<td>I and IIA, 37$^c$; I and IIA, 17.7$^c$; I, IIA, and IIB, 20.9$^c$</td>
<td>I and IIA, 37$^c$; I and IIA, 17.7$^c$; I, IIA, and IIB, 20.9$^c$</td>
<td>I and IIA, 37$^c$; I and IIA, 17.7$^c$; I, IIA, and IIB, 20.9$^c$</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Triceps$^l$</td>
<td>I, =50; IIA and IIB, =50</td>
<td>I and IIA, 37$^c$; I and IIA, 17.7$^c$; I, IIA, and IIB, 20.9$^c$</td>
<td>I and IIA, 37$^c$; I and IIA, 17.7$^c$; I, IIA, and IIB, 20.9$^c$</td>
<td>I and IIA, 37$^c$; I and IIA, 17.7$^c$; I, IIA, and IIB, 20.9$^c$</td>
<td>0.001</td>
<td></td>
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<tr>
<td>EHB$^m$</td>
<td>I, 79$^f$; IIA and IIB, 43$^f$</td>
<td>I and IIA, 57$^f$; I and IIA, 85.7$^f$; I and IIA, 57$^f$</td>
<td>I, IIA, and IIB, 50$^o$</td>
<td>I and IIA, 40$^o$</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>1st DI$^{1,n}$</td>
<td>I, IIA, and IIB, range 100–30$^i$</td>
<td>I, IIA, and IIB, 45$^f$; range</td>
<td>I, IIA, and IIB, 45$^f$; range</td>
<td>I, IIA, and IIB, 45$^f$; range</td>
<td>0.001</td>
<td></td>
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<td>0.001</td>
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<td>0.001</td>
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</tbody>
</table>
| Gast. (sup.), gastrocnemius (superficial portion); EDL, extensor digitorum longus; TA, tibialis anterior; RB, retractor bulbis; EHB, extensor halucis brevis; 1st DI, first dorsal interosseus (hand).

* Citations from the literature are indicated by superscript letters as follows: a, ref. 13; b, ref. 14; c, ref. 15; d, ref. 16; e, ref. 17; f, D. Bateson and D. Parry, personal communication; g, D. Parry, personal communication; h, ref. 18; i, ref. 19; j, ref. 20; k, ref. 21; l, ref. 22; m, ref. 23; n, ref. 24; o, ref. 11; p, ref. 25; q, ref. 26; r, ref. 27; s, ref. 28; t, ref. 29.

† Average of three independent determinations; values =0.01 g/kg varied ±5%; values below 0.01 g/kg varied up to 50%.

‡ Determined for this study.

§ Proportion of type I fibers was sufficiently low for whole muscle properties to reflect only fast fiber properties.

Ⅰ Young (12-week-old) rats.

Ⅰ Inferior rectus, properties of twitch fibers only.

** RB only.

†† Not strictly comparable with the other species for purposes of the Hill argument because man is bipedal, whereas other species run on four legs.

The protein concentrations of these solutions, measured with the micro-biuret method (30), were 0.9–4.5 mg of protein per ml. Parvalbumin used for calibration was prepared from rat leg muscles (31, 32). The pH, M., and hydrophobicity of rat, mouse, guinea pig, human parvalbumin are very similar. Antiserum raised against rat parvalbumin (33) crossreacted with mouse and human muscle extracts but not with guinea pig, cat, or horse muscle extract (not shown). Single muscle fibers from freeze-dried samples of rat soleus and Gast. (superficial portion) muscles were prepared and typed as described (18, 34). Muscle extracts and single fibers were $^{14}$C-labeled by reductive methylation (35) by using NaCNBH$_3$ and $[^{14}$C]formaldehyde (specific
activity = 52 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels; New England Nuclear).

Single muscle fibers and muscle extracts were analyzed by 2-D gel electrophoresis (36). The isoelectric focusing gel contained a mixture of 20% Ampholine at pH 2.5–4 and 80% Ampholine at pH 3.5–10.0 (LKB), which resulted in a linear gradient from 4.0 to 6.1. After electrophoresis in the second dimension (15% acrylamide), the gels were processed for fluorography (37, 38).

The fiber type composition of some of the muscles was determined by staining frozen sections for myofibrillar ATPase activity (18). EDL muscles of 3-week-old rats were denervated by cutting the muscle nerve. After 4 days, these muscles and the EDL muscles of the unoperated side were examined for parvalbumin content as described above. The parvalbumin content of the various muscle extracts was measured by HPLC. The apparatus consisted of two Altex (Berkeley, CA) model 110 pumps, an ASI 45-sample auto-injector with a 50-μl sample loop (Kontron), an Altex model 420 microprocessor, and a Uvicron 725 spectrophotometer with an 8-μl flow-through cell connected to a W+W Tarkan-600 recorder. An Aquapore RP-300 (4.6 × 250 mm) column (RP-8, 10-μm particle size, Brownlee Labs) was used for all chromatographic runs. Flow rates of 1 ml/min at room temperature were standard conditions. The following buffer systems were used in all HPLC runs: buffer A (50 mM Tris-HCl, pH 7.5/0.1 M CaCl₂) and buffer B (buffer A with 60% (vol/vol) acetonitrile).

RESULTS

A variety of muscles (mixed or homogeneous in fiber type composition) from mouse, rat, guinea pig, horse, and man (several with known relaxation times) was analyzed for parvalbumin content by 2-D gel electrophoresis and HPLC on reversed-phase supports. Parvalbumin from rat skeletal muscle, which has been described (31, 32), was used as a protein standard. Parvalbumin, troponin C, S-100, and calmodulin were well separated under these conditions used for HPLC and 2-D gel electrophoresis (unpublished data).

A large parvalbumin spot was found in all 2-D gels of single, fast-twitch type IIB fibers (from rat Gast. muscle) but not in any type I (slow-twitch) fibers (Fig. 1). The identification of this spot as parvalbumin was confirmed by comigration experiments with homogeneous parvalbumin (not shown).

The heat-stable muscle extracts were examined for the presence of parvalbumin by 2-D gel electrophoresis (Fig. 2). Samples of the same extracts then were analyzed by HPLC to determine the quantity of parvalbumin present, from which it was possible to calculate the parvalbumin concentration in the muscles (see Table 1). Fig. 2 shows the 2-D gels of heat-stable extracts from two "pure" type IIB muscles: guinea pig sartorius (white portion) and the superficial portion of Gast. of the mouse. The amount of parvalbumin present is highest in the mouse muscle and least in the guinea pig (identical quantities of heat-stable proteins were applied on these gels, so a direct comparison is quite valid).

Human vastus and triceps muscles, although consisting of about 50% type II fibers, showed a very faint parvalbumin spot, but only when the fluorograms were exposed for a much longer time period (over 1 month) than that for the other samples (usually 24 hr). The deep portion of the medial gastrocnemius muscle of the horse, with a 35% content of type IIB fibers, gave no detectable parvalbumin spot on 2-D gels (not shown).

A quantitative analysis of the heat-treated muscle extracts was performed by HPLC. It had been shown earlier that this method could be used for the separation and quantitation of a series of Ca^{2+}-binding proteins, including parvalbumin, according to their distinct hydrophobicities (ref. 31; unpublished data). The highest parvalbumin concentration, 4.9 g/kg of wet weight, was found in the superficial portion of the Gast. muscle of the mouse, which was the smallest animal investigated; in the mouse, this muscle has the highest speeds of muscle contraction and half-relaxation times (see Table 1 and Fig. 3). The same muscle in the rat contains less parvalbumin (6.4 g/kg) and the sartorius of the guinea pig contains only 0.25 g/kg. Because these three muscles contain only type IIB fibers, it is clear that type IIB muscle fibers of the guinea pig contain only ≈5% of the parvalbumin of those muscle fibers of the mouse.

Similarly, when comparing EDL of the mouse and the rat, both muscles consist of approximately ≈5% type I and ≈95% type II fibers; however, the parvalbumin concentration in the mouse muscle is approximately twice as much as that in the rat. The direct comparison of three different muscles of the rat (Fig. 3e)—Gast., EDL, and soleus—demonstrates a wide range of parvalbumin concentrations within a single species, depending on the fiber type composition of the individual muscles (Table 1).

Masseter, soleus, and sartorius muscles of the guinea pig have the useful characteristic of being homogeneous type IIA, I, and IIB muscles, respectively. The HPLC analysis (Fig. 3b) reveals that the soleus muscle (pure type I) contains only ≈0.007 g of parvalbumin per kg, masseter (pure type IIA) contains 10 times as much (0.08 g/kg), and the sartorius (pure type
IIA) contains the highest parvalbumin concentration (0.25 g/kg), consistent with the known differences in the half-relaxation times of fast and slow muscles of the guinea pig (see Table 1). Horse and human muscles have very low concentrations of parvalbumin, estimated to be ≤1 mg/kg of wet muscle tissue.

Denervation is known to affect the contractile properties of fast-twitch muscles—e.g., EDL of the rat (11). Major changes occurred over a time period from 2 to 6 days after denervation, affecting mostly the twitch time to peak and half-relaxation, both of which are prolonged. The parvalbumin concentration was determined in the denervated and control muscle extracts by HPLC. The concentration of parvalbumin in the denervated EDL muscle was 20% lower than in the control muscle. Longer denervation periods, which lead to atrophy of the muscle fibers and a leaking out of the soluble parvalbumin, were not examined.

**DISCUSSION**

Although our previous immunohistochemical results (39) and the single fiber analysis (described here) demonstrated an association of parvalbumin mainly with the type IIB (fast-twitch, glycolytic) muscle fibers, a quantitative approach was necessary to gather evidence in favor of the hypothesis that parvalbumin is a soluble relaxation factor in fast skeletal muscle fibers.

As mentioned above (see Introduction), if parvalbumin does play a role in muscle relaxation, its distribution in muscle must fulfill a number of criteria, and our results seem to meet these requirements satisfactorily.

(i) From the results presented in Table 1, the parvalbumin concentration within single species correlated well with the half-relaxation time values when these are known from the literature.

(ii) Comparison of the parvalbumin content and fiber type composition of muscles of rats, mice, and guinea pigs shows that not only is more parvalbumin found in the muscles with a higher fast-twitch (type II) fiber content but also more parvalbumin is found in a type IIB muscle than in a type IIA muscle (sartorius vs. masseter of the guinea pig), in very good agreement with the results of our previous immunohistochemical study (39). Unfortunately, physiological data supporting the difference in the half-relaxation times of types IIA and IIB fibers are not available for pure type IIA and IIB muscles of the guinea pig. However, in other species, motor unit data are available, with the advantage that the vast majority of muscle fibers in a single motor unit is of the same fiber type. The most complete physiological data are for cat muscles (for review see ref. 40), in which motor units composed of type IIB fibers relax on average faster than units composed of type IIA fibers (though with a large overlap); unfortunately, the heat-stable protein found in cat fast-contracting muscles is distinct in Mr, pI, and hydrophobicity to rat parvalbumin and therefore cannot be classified as a parvalbumin with absolute certainty before it is fully characterized. However, Ashton (24) has found that in the rat, fast-fatigue-resistant units (composed of type IIA fibers) also relax slower on average than fast-fatiguing units (composed of type IIB fibers). Therefore, it seems reasonable to assume similar differences in the half-relaxation time between the types IIA and IIB fibers occur in all species, and thus the differences we observed in the amounts of parvalbumin between types IIA and IIB fibers of guinea pig muscles would seem to be significant.

(iii) Directly following predictions of Hill's argument (ref. 8; outlined in the Introduction), the concentration of parvalbumin was highest in the smallest animal, which has the highest contraction–relaxation speed, and lowest in the larger animals in

![Figure 2](image1.png)

**Fig. 2.** 2-D gel electrophoresis of heat-stable extracts of two homogeneous type IIB muscles. (a) Mouse superficial portion of Gast.; (b) guinea pig, sartorius, white portion. Fifteen micrograms of protein was applied on each gel and 14C-labeled proteins were visualized by fluorography (20-hr exposure). Mouse Gast. muscle contains the highest concentrations of parvalbumin (PV) (see also Table 1). TM, tropomyosin.

![Figure 3](image2.png)

**Fig. 3.** Quantitation of parvalbumin in heat-stable muscle extracts by HPLC. Protein extracts from 16.5 mg of muscle tissue (wet weight) were injected. (a) Numbers 1, 2, 3, and 4 correspond to Gast. (rat), EDL (rat), soleus (rat), and purified rat muscle parvalbumin as standard (10.6 µg), respectively. A gradient from 0% to 100% buffer B was applied (solid diagonal line). (b) Only a part of the chromatogram is shown. Numbers 1, 2, 3, and 4 correspond to sartorius (guinea pig), masseter (guinea pig), soleus (guinea pig), and purified rat muscle parvalbumin as standard (2.6 µg), respectively. A gradient from 20% to 75% buffer B was applied (solid diagonal line). Short thick arrows (+) indicate sample injection and long thin arrows (−) indicate position of parvalbumin. The difference in the pen position of the absorbance and % B (buffer B) recording channel corresponds to 5 min, or 11% buffer B.
which the contraction–relaxation speeds are slower. This point is particularly well illustrated by comparing three type IIB muscles of mouse, rat, and guinea pig, in which parvalbumin content decreases as body size increases.

Denervation is known to affect the contractile properties of rat skeletal muscle (10). The half-relaxation time of EDL increases from 9.4 to 19 msec only 4–5 days after denervation (11) and, during this time period, the concentration of parvalbumin was found to decrease by 20%, which is consistent with parvalbumin having a role in muscle relaxation.

Although the contraction and relaxation times of the EOM are particularly fast, their parvalbumin content was less than in EDL. Because of the higher amount of nonmuscle tissue in EOM, the measured parvalbumin content of this muscle is probably underestimated.

As already pointed out, the physiological role of parvalbumin in mammalian muscles is still a matter of controversy. Recently, it has been suggested (5–7) that parvalbumin cannot be directly involved in the rise or decay of tension in a single twitch, as proposed earlier (1–4), but rather, is involved in a tetanus in which there is a constant Ca$^{2+}$ flux coming into the muscle cell (5–7). This assumption is based mainly on in vitro measurements with isolated Ca$^{2+}$-binding proteins, suggesting that in the relaxed state, the two Ca$^{2+}$, Mg$^{2+}$-binding sites of parvalbumin are fully saturated with Mg$^{2+}$ and that the off rate for Mg$^{2+}$ is too slow for the subsequent Ca$^{2+}$ uptake to be of use during the contraction–relaxation cycle of a single twitch. On the other hand, our results do indicate a very good correlation between parvalbumin content and relaxation speed. Furthermore, given the observed parvalbumin distribution in different muscles, it is difficult to explain why parvalbumin should be involved in a tetanus, because both fast and slow muscles can produce a tetanus, with the duration of the tetanus being dependent upon the fatigue resistance of the muscle fibers (and neuromuscular junction), which is quite unrelated to parvalbumin content. Therefore, our results lead us to suggest that these in vitro measurements on the isolated protein components do not perfectly mimic the physiological conditions within a muscle cell. Furthermore, high parvalbumin contents also have been found in fish muscle consisting mainly of fast-contracting muscle fibers (41–44).

In summary, we suggest that the close correlation established between the parvalbumin content and relaxation speed of a variety of muscles is fully compatible with the hypothesis that parvalbumin is involved in the fast relaxation process of mammalian muscle fibers. The strength of the correlation supports further investigations into the activity of parvalbumin under physiological conditions.

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