Increased phosphorylation of myosin light chain associated with slow-to-fast transition in rat soleus

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Bozzo, Cyril, Laurence Stevens, Luana Toniolo, Yvonne Mounier, and Carlo Reggiani. Increased phosphorylation of myosin light chain associated with slow-to-fast transition in rat soleus. Am J Physiol Cell Physiol 285: C575–C583, 2003. First published May 14, 2003; 10.1152/ajpcell.00441.2002.—In striated muscles myosin light chain (MLC)2 phosphorylation regulates calcium sensitivity and mediates sarcomere organization. Little is known about the changes in MLC2 phosphorylation in relation to skeletal muscle plasticity. We studied changes in MLC2 phosphorylation in rats receiving three treatment conditions causing slow-to-fast transitions: 1) atrophy induced by 14 days of hindlimb suspension (HS), 2) hypertrophy induced by 14 days of clenbuterol administration (CB), and 3) 14 days of combined treatment (CB-HS). Three variants of the slow (MLC2s) and two variants of the fast MLC2 (MLC2f) isoform were separated with two-dimensional electrophoresis and identified with monoclonal and polyclonal antibodies specific for MLC2; their relative proportions were densitometrically quantified. In control soleus muscle MLC2s predominated over MLC2f (91.4 ± 3.9% vs. 8.5 ± 3.9%) and was separated into two spots, the less acidic spot being 73.5 ± 4.3% of the total. All treatments caused a decrease of the less acidic unphosphorylated spot of MLC2s (CB: 64.1 ± 5.6%, HS: 62.4 ± 6.8%, CB-HS: 56.4 ± 4.4%), the appearance of a third more acidic variant of MLC2s (representing 3.9–5.9% of total MLC2s), an increase of MLC2f (CB: 30.9 ± 3.1%, HS: 23.9 ± 3.3%, CB-HS: 25.3 ± 3.9%), and the phosphorylation of a large fraction of MLC2f (CB: 30.4 ± 6.7%, HS: 28.7 ± 6.5%, CB-HS: 21.8 ± 2.1%). Treatment with alkaline phosphatase catalyzed by the Ca2+/calmodulin-dependent kinase (4). In smooth muscle MLC2, the primary phosphorylation site is located at serine 19, and evidence for a second site of phosphorylation at serine 1–2 or at threonine 9 has been given (16). Phosphorylation at serine 19 is catalyzed by Ca2+/calmodulin-dependent MLC kinase (16) and by Rho kinase (1) with an activating effect on actin-myosin interaction, whereas phosphorylation at serine 1–2 or threonine 9 is catalyzed by PKC with an inhibitory effect on myosin function (17). Whether a second site of phosphorylation is also present in sarcomeric MLC2 is still controversial (14, 29).

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Changes in MLC2 phosphorylation pattern associated with variations of the functional state have been repeatedly demonstrated in cardiac and smooth muscles. In rat ventricular myocardium, MLC2 phosphorylation changes in connection with hypertrophy development (22, 28), is positively correlated with blood pressure (12), and shows seasonal variations (26) in hibernating mammals. In human ventricle, a recent analysis of cardiac muscle strain pattern in vivo has shown that gradients of phosphorylation correlate with gradients of strain on cardiac myocytes (9). In tracheal and bronchial smooth muscle, phosphorylation level increases in the respiratory airways of dogs sensitized to ragweed pollen (18), whereas in arterial smooth muscles, phosphorylation levels increase in relation to high-mechanical stress conditions (2). All these changes can be interpreted in light of a double role of MLC2 phosphorylation, as considered above: a functional role of modulating calcium sensitivity and a structural role of contributing to assembly of cytoskeleton and myofilament filaments.

Less information on long-term changes in MLC2 phosphorylation is available for skeletal muscles. It is well known that phosphorylation of MLC2 increases during repeated electrical stimulation, but long-term effects of electrical stimulation (chronic low-frequency stimulation; CLFS) seem to include a reduction in the degree of phosphorylation (14, 20, 21). This would agree with the observation that CLFS induces a fast-to-slow transition of fiber types and that slow muscles have a lower level of phosphorylation than fast muscles (25).

The lack of information on the variation in MLC2 phosphorylation during muscle adaptations involving changes in fiber size and fiber type, and the relevance of MLC2 phosphorylation to the regulation of contractile performance, prompted us to study MLC2 phosphorylation in two well-known models of muscle plasticity, the atrophy induced by muscle disuse during hindlimb suspension in two well-known models of muscle plasticity, the atrophy induced by muscle disuse during hindlimb suspension (22, 28), is positively correlated with blood pressure (12). All these changes can be interpreted in light of a double role of MLC2 phosphorylation, as considered above: a functional role of modulating calcium sensitivity and a structural role of contributing to assembly of cytoskeleton and myofilament filaments.

The three experimental treatments (clenbuterol, hindlimb suspension, and combined treatment) represent an optimal condition to assess 1) whether a slow-to-fast transition implies an increased phosphorylation of MLC2, as one might expect from the comparison between fast and slow muscles (25) and from the observation that CLFS causes a lower phosphorylation level associated with the appearance of a slow phenotype (see above), and 2) whether atrophy and hypertrophy are associated with different patterns of phosphorylation, as one might expect from studies on cardiac muscles (see above) and from the opposite effects on calcium sensitivity (decrease by hindlimb suspension and increased by clenbuterol treatment, see above). The results obtained showed that slow-to-fast transition is associated with increased levels of phosphorylation regardless of the development of atrophy or hypertrophy.

**MATERIALS AND METHODS**

**Animals and muscles.** Adult male Wistar rats (initial body wt of -300 g) were randomly divided into four groups: control (Cont), clenbuterol administration (CB), hindlimb suspension (HS), and clenbuterol combined with hindlimb suspension (HS-CB). Hindlimb suspension was applied for 14 days as previously described (44, 45) according to the protocol proposed by Morey (30). Clenbuterol (Sigma, St. Louis, MO), freshly prepared each day, was administered via drinking water (30 mg/l) for 14 days (37). At the end of treatment, the animals were anesthetized by intraperitoneal injection of ethylcarbamate and killed by exsanguination, and soleus muscles were dissected, blotted, and weighed. In control rats, extensor digitorum longus (EDL) muscle was also dissected. The body and soleus muscle weights of the four groups at the end of the experimental period are shown in Table 1. After dissection, the muscles were frozen in liquid nitrogen, pulverized in a small steel mortar, and stored at -80 °C until being analyzed. The experiments received authorization from both the French Ministry of Agriculture (Veterinary Service of Health and Animal Protection) and the French Ministry of Education (authorization no. 59-00996).

**Protein extraction and sample preparation.** Myofilament proteins were extracted from 7–10 mg of dry muscle powder as described previously (50), washing first with a solution containing 6.3 mM EDTA (pH 7.0), 0.1% pepstatin, and 1% phenylmethylsulfonyl fluoride (PMSF) and then with a second solution containing 50 mM KCl, 0.1% pepstatin, and 1% PMSF. The myofilament proteins were resuspended in 500 μl of milliQ water, and their concentration was determined with a protein assay kit (DC Protein Assay, Bio-Rad) to prepare samples with a final quantity of 50 μg. The proteins were then precipitated for 2 h with acetone (8 vol (aceton/vol (sample)), followed by centrifugation for 1 h at 13,000 rpm. The pellet was dissolved in Laemmli solution for SDS-PAGE or in rehydration buffer for two-dimensional gel electrophoresis.

**Protein desphosphorylation.** The samples (proteins suspended in water, see Protein extraction and sample preparation).

**Table 1. Muscle weight, body weight, and muscle weight-to-body weight ratio of soleus muscles**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Muscle Weight, mg (n = 6)</th>
<th>Body Weight, g (n = 6)</th>
<th>Muscle Weight/Body Weight, mg/g (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>139.34 ± 4.44</td>
<td>322.8 ± 14.8</td>
<td>0.469 ± 0.02</td>
</tr>
<tr>
<td>CB</td>
<td>164.86 ± 4.77*</td>
<td>380.4 ± 22.2*</td>
<td>0.437 ± 0.015</td>
</tr>
<tr>
<td>HS</td>
<td>82.16 ± 0.96*</td>
<td>311.5 ± 10.9</td>
<td>0.264 ± 0.008*</td>
</tr>
<tr>
<td>HS-CB</td>
<td>101.81 ± 10.45†</td>
<td>336.0 ± 21.7</td>
<td>0.320 ± 0.015†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Cont, control group; CB, clenbuterol-treated group; HS, hindlimb suspension-exposed group; HS-CB, combined treatment group. Significant differences (P < 0.05). *vs. Cont, †vs. HS.
tion) were incubated for 4 h at 37°C either with calf intestinal alkaline phosphatase (10 U/50 μg protein; Sigma P6772) or with serine/threonine phosphatase (PP1) extracted from rabbit skeletal muscle (1 U/50 μg protein; Upstate Cell Signaling Solutions). At the end of the incubation proteins were precipitated with acetone, centrifuged, and resuspended in rehydration buffer (see Protein extraction and sample preparation).

One-dimensional electrophoresis (SDS-polyacrylamide gel electrophoresis). The isoform composition of myosin heavy chain (MHC) and MLC was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separation of MHC isoforms was achieved by the method previously described (46) with a 4.5% stacking gel and a 7.5% separating gel. Electrophoresis was run for 18 h at low temperature with 180 V (constant voltage; current ~13 mA/gel). Separation of MLC isoforms was achieved as described in Two-dimensional gel electrophoresis for the second dimension of two-dimensional gel electrophoresis. Gels were silver stained (31) and digitized with an Epson 1650 scanner at a resolution of 200 dpi. Identification of MHC and MLC isoforms was based on the migration rate. The areas under the peaks corresponding to the MHC or MLC isoforms were measured, and the area of each peak was expressed as a fraction of the total area of the peaks corresponding to MHC, to alkali MLC, or to regulatory MLC.

Two-dimensional gel electrophoresis. Proteins were separated by two-dimensional gel electrophoresis with a procedure similar to those previously used for MLC2 separation in sarcomeric muscles by Morano et al. (27) and Gonzalez et al. (14). For the first dimension, isoelectric focusing (IEF), proteins were solubilized in a buffer containing 8 M urea, 2% CHAPS, 0.01 M dithiothreitol (DTT), and 2% carrier ampholites (Amersham Biosciences) and then separated with the Ettan IPGphor Isoelectric Focusing System (Amersham Biosciences) on 3.5% acrylamide strips with immobilized pH gradients (4–7) (Amersham Biosciences). Strips were rehydrated at 50 V for 12 h, and proteins were focused under the following voltage conditions: 500 V for 1 h, 500–1,000 V for 1 h, 5,000 V until reaching 100,000 V-h. Temperature was kept constant at 20°C.

After reduction with a buffer containing 6 M urea, 30% glycerol, 0.375 M Tris-HCl (pH 6.8), and 2% DTT and alkylation with the same buffer with the addition of 2.5% iodoacetamide, the strips were embedded in 4% polyacrylamide stacking gels and the proteins were separated in 12% polyacrylamide gels (SDS-PAGE) for 8 h at 150 V and low temperature (4°C). After electrophoresis gels were silver stained according to Oakley et al. (31).

Image analysis and quantification. Two-dimensional gels were digitized with an Epson 1650 scanner at a resolution of 1,200 dpi. The spots were analyzed densitometrically, determining brightness-area product (BAP) with a constant threshold after black/white inversion with Adobe Photoshop software. In each gel, the BAP values of the spots identified as slow and fast MLC2 were summed to give a total for slow MLC2 and a total for fast MLC2: the value of each spot identified as slow MLC2 was expressed as a percentage of total slow MLC2, and the same was done for the spots identified as fast MLC2. From percentage values obtained in different gels means ± SE were calculated. The quantification procedure was validated by running mixtures of a constant amount of purified actin and increasing amounts of purified slow MLC2 (kindly donated by Dr. Monica Canepari and Dr. Daniela Romano, University of Pavia, Pavia, Italy) on separate gels and determining the ratio between the BAP values of MLC2 and actin. Examples of the linear relation

![Fig. 1. Identification of the spots corresponding to myosin light chain (MLC2) and validation of the densitometric quantification procedure. A: immunostaining after blotting of a 2-dimensional gel in which soleus and extensor digitorum longus (EDL) samples from control rats were run together. The antibody FL-172ex15370 (Santa Cruz) was used with a dilution of 1:200. Only 4 spots were recognized by the antibody: 3 easily detectable spots (from left to right: s, f, f1; see also Figs. 3 and 4) and a fourth spot, s1, hardly detectable just above the spot f. B: correlation between the ratios of the brightness-area products (BAP) of slow MLC2 spots over actin spots and the amounts of purified slow MLC2 loaded. In each experiment 3 increasing amounts of purified slow MLC2 were run with a constant amount of actin. The amount of actin was higher in experiment 1 (top) and lower in experiment 2 (bottom).](http://ajpcell.physiology.org/)
between the ratio of MLC2 and actin BAP values and the actual amounts of MLC2 loaded are shown in Fig. 1B.

**Protein transfer and immunoblotting.** Two-dimensional gels were transferred to nitrocellulose membrane to identify MLC2 spots by immunostaining. Transfer was obtained by a semidry transfer procedure by applying a current of 0.8 mA/cm² for 6 h. Nitrocellulose sheets were reacted first with a primary antibody directed against MLC2 and then with a peroxidase-conjugated secondary antibody. The spots were visualized by an enhanced chemiluminescence (ECL) method in which luminol was excited by peroxidase in the presence of H₂O₂. Two antibodies specific for MLC2 were used: the polyclonal antibody FL-172sc15370 (Santa Cruz) and the monoclonal antibody F109.3E1 (Alexis). A typical example of the results is shown in Fig. 1A: the spots corresponding to slow and fast MLC2 are clearly identified.

**Statistical analysis.** Data are expressed as means ± SE and were compared by one-way ANOVA followed by Bonferroni test. The level of significance was set at P < 0.05.

**RESULTS**

**Effects of experimental treatments: slow-to-fast transformation, atrophy, and hypertrophy.** Samples of myofibrillar proteins from each experimental group were analyzed by one-dimensional PAGE followed by densitometric quantification of MHC and MLC isoforms. MHC isoforms were analyzed because they are reliable indicators of fiber type transformation (40). The results are shown in Fig. 2 and Table 2. As expected (43), both clenbuterol and hindlimb suspension induced a slow-to-fast transition that also occurred with the combined treatment. The proportions of the fast MLC2 isoform increased and the proportion of slow MLC2 isoform decreased compared with controls in all three experimental groups, as shown in Table 2 and Fig. 2. It is important to emphasize, however, that the changes in MLC2 isoform distribution were accompanied by opposite changes in muscle size: hindlimb suspension induced soleus atrophy, whereas clenbuterol administration induced hypertrophy (see Table 1). The combined treatment reduced the muscle atrophy induced by inactivity. When normalized to actin, no difference in the total amount of MLC2 isoforms was detectable between the control group and each experimental group (data not shown). This suggests that no selective loss or accumulation of this myosin subunit occurred in atrophic or hypertrophic muscles.

**MLC2 isoform identification.** The positions of slow and fast isoforms of MLC2 on two-dimensional gels were determined according to their molecular weight, using appropriate markers of molecular weight in the second dimension, and to their isoelectric point in the first dimension (IEF) and were confirmed by immunoblotting (see Fig. 1A). The spots of MLC2 on the two-dimensional gel detectable in soleus and EDL of control rats are shown in Fig. 3 and indicated as s and s1 and f and f1. In the soleus of control rats, slow MLC2 was the predominant regulatory light chain and appeared separated into two spots: s and s1 (see Fig. 3, left); a third almost undetectable more acidic spot was occa-

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**Fig. 2.** Typical example of separation of MLC isoforms on SDS-polyacrylamide gel electrophoresis (PAGE) of control (Cont) rat soleus; soleus of a rat treated with clenbuterol (CB), soleus of a rat exposed to hindlimb suspension (HS), and soleus of a rat exposed to both treatments (HS-CB). EDL, sample of EDL muscle of a control rat taken as a reference for fast MLC isoforms.
sionally present. Only a minor amount of fast MLC2 was present in the soleus and it appeared as a single spot, located close to the s1 spot of slow MLC2 in a position corresponding to the spot indicated as f in the EDL. In the EDL, the fast isoform of MLC2 was predominant and appeared composed of two spots (f and f1) of similar size with lower molecular weights and more acidic isoelectric points than the spots of the slow isoform (see Fig. 3, right).

Effect of clenbuterol administration on MLC2 isoform distribution.
As reported in Table 2 and shown in Fig. 4 (left), clenbuterol administration stimulated the expression of fast MLC2 in the soleus. In control soleus muscles the slow MLC2 isoform showed the following distribution: s represented 74%, s1 represented 26%, and the third more acidic spot, s2, was almost undetectable (see Table 3 and Fig. 4). Fast MLC2 was scarcely expressed and was represented by the less acidic form f. In CB soleus muscles, the most acidic form (s2) was consistently present and reached a relative proportion of ~6%. The proportion of s1 was not significantly modified. Furthermore, clenbuterol treatment induced not only the expression of fast MLC2 but also the concomitant appearance of the acidic form f1, which represented 30% of the total vs. 70% of the less acidic form f (see Table 3 and Fig. 4). The incubation with alkaline phosphatase removed the most acidic forms of both slow and fast MLC2 isoforms (see Fig. 4, right), making the pattern of CB soleus in two-dimensional electrophoresis similar to that of the control soleus, except for the greater presence of the fast MLC2 isoform.

Effect of hindlimb suspension on MLC2 isoform distribution.
The inactivity caused by hindlimb suspension induced the appearance of s2 as observed in CB muscles (see Table 3 and Fig. 4). The proportions of s and s1, however, were not significantly reduced in HS

### Table 2. Distribution of slow and fast MHC and MLC isoforms of rat soleus muscles determined by one-dimensional gel electrophoresis

<table>
<thead>
<tr>
<th></th>
<th>Cont (n = 5)</th>
<th>CB (n = 5)</th>
<th>HS (n = 5)</th>
<th>HS-CB (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC I</td>
<td>95.05 ± 2.80</td>
<td>72.55 ± 1.28*</td>
<td>73.05 ± 2.25*</td>
<td>65.09 ± 0.10*†</td>
</tr>
<tr>
<td>MHC IIa</td>
<td>4.95 ± 1.99</td>
<td>22.00 ± 2.81*</td>
<td>9.34 ± 1.75*</td>
<td>15.6 ± 0.6†</td>
</tr>
<tr>
<td>MHC IIx</td>
<td>2.91 ± 0.42*</td>
<td>8.33 ± 1.01*</td>
<td>11.75 ± 0.74*†</td>
<td></td>
</tr>
<tr>
<td>MHC IIb</td>
<td>2.54 ± 1.26*</td>
<td>9.26 ± 1.43*</td>
<td>7.55 ± 0.25*</td>
<td></td>
</tr>
<tr>
<td>Alkali MLC</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>MLC 1s</td>
<td>92.60 ± 4.14</td>
<td>80.18 ± 4.06*</td>
<td>76.05 ± 4.13*</td>
<td>73.54 ± 5.86*</td>
</tr>
<tr>
<td>MLC 1f + 3f</td>
<td>7.40 ± 0.65</td>
<td>19.42 ± 3.92*</td>
<td>23.95 ± 2.56*</td>
<td>26.46 ± 2.78*</td>
</tr>
<tr>
<td>Regulatory MLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLC 2s</td>
<td>91.44 ± 3.95</td>
<td>69.12 ± 3.05*</td>
<td>76.06 ± 2.5*</td>
<td>74.71 ± 3.89*</td>
</tr>
<tr>
<td>MLC 2f</td>
<td>8.56 ± 3.95</td>
<td>30.88 ± 3.05*</td>
<td>23.94 ± 2.5*</td>
<td>25.29 ± 3.89*</td>
</tr>
</tbody>
</table>

Values are means ± SE. The amount of each myosin heavy chain (MHC) isoform is expressed as % of total MHC. Each alkali [myosin light chain (MLC) 1 and/or 3] or regulatory (MLC2) isoform is expressed as % of total of alkali or regulatory MLC. s, Slow; f, Fast. Significant differences (P < 0.05). *vs. Cont, †vs. HS.
suspension caused muscle atrophy slightly but significantly, and both s1 and s2 increased. Two spots corresponding to the fast MLC2 isoform, f and f1, were present (see Fig. 4 and Table 3). As in the two treatments described above, the phosphatase action removed the more acidic spots, thus determining a distribution reminiscent of that of control soleus (see Fig. 4).

**Effects of PP1 dephosphorylation.** Samples of soleus muscles from rats exposed to the experimental treatments were incubated with PP1. The results obtained in HS soleus are shown in Fig. 5: after PP1 incubation two acidic spots, s2 and f1, were completely removed, whereas the proportion of the intermediate acidic form s1 was reduced (to ~10% of total slow MLC2) but not eliminated. Similar results were obtained with CB and CB-HS soleus samples (data not shown). The specificity of the phosphatase action was tested by incubating the samples with heat-inactivated PP1: no change in spot distribution occurred after incubation with heat-inactivated PP1 (see Fig. 5).

**DISCUSSION**

The results obtained in this study show, for the first time, that an increase of MLC2 phosphorylation is associated with the hypertrophy caused by clenbuterol administration and the atrophy caused by hindlimb suspension. The two treatments have in common the transformation of the muscle phenotype with a marked slow-to-fast transition. The analysis of MLC2 phosphorylation was performed with two-dimensional electrophoresis, which revealed enhanced expression of the fast MLC2 isoform and the appearance of phosphorylated variants of both fast and slow MLC2 in the soleus of CB, HS, and HS-CB rats compared with control animals. Previous studies had shown that, whereas the immediate effect of repetitive electrical stimulation is the increase of the MLC2 phosphorylation that explains the posttetanic twitch potentiation (23, 25), long-term CLFS reduces the level of phosphorylation (14, 20, 21). To the best of our knowledge, no other condition of muscle plasticity has been analyzed in terms of MLC2 phosphorylation until now.

Most of the classic studies on MLC2 phosphorylation in skeletal muscles have been based on isoelectrofocusing (IEF) on polyacrylamide gels after nondenaturing pyrophosphate electrophoresis of myosin (41). Two-dimensional electrophoresis has been applied first to smooth muscle (11), then to cardiac muscle (27), and only more recently to skeletal muscle (10, 14). The use of two-dimensional electrophoresis has definitely improved the resolution of the protein separation, particularly with the use of narrow and immobilized pH

| Table 3. Relative distribution of the variants of the slow and fast isoforms of MLC2 present in rat soleus muscles |
|---|---|---|---|---|
| s | s1 | s2 | f | f1 |
| Cont | 73.56 ± 4.35 | 26.44 ± 4.35 | 0 ± 0 | 100 ± 0 | 0 ± 0 |
| CB | 62.83 ± 5.69 | 34.38 ± 5.87 | 5.89 ± 0.92 | 69.57 ± 6.67 | 30.43 ± 6.67 |
| HS | 56.41 ± 4.43 | 39.60 ± 2.81 | 3.99 ± 0.99 | 78.23 ± 2.12 | 21.77 ± 2.12 |
| HS-CB | 56.41 ± 4.43 | 39.60 ± 2.81 | 3.99 ± 0.99 | 78.23 ± 2.12 | 21.77 ± 2.12 |

Values (in % of total isoform) are means ± SE of 5–10 samples. *Significantly different (P < 0.05) from cont.
gradients (39). The two isoforms of MLC2 were unambiguously identified on the basis of their molecular weight and their reactivity with specific antibodies; they appeared divided into different spots, among which the more acidic forms could be considered as phosphorylated forms. In rat skeletal muscles fast MLC2 appeared to be composed of two spots and slow MLC2 of three spots (14). Interestingly, if we assume that the more acidic spots represent the phosphorylated forms, the picture emerging from two-dimensional electrophoresis is slightly different from that obtained with more classic methods (41). Separation on two-dimensional gels indicates that in the rat ~30% of the slow MLC2 isoform in soleus and 30–40% of the fast MLC2 isoform in EDL are in phosphorylated forms (see also Refs. 10 and 14). By contrast, no significant amount of phosphorylated MLC2 in the rat soleus and only 0.1 mol/mol in rat white gastrocnemius were found by Moore and Stull (25). Similar ratios (0.10 and 0.19 mol/mol, respectively) have been reported for rat gastrocnemius (21, 23).

The interpretation of the three spots observed in rat slow MLC2 is still controversial. A convincing demonstration that all three spots correspond to MLC2 has been given by Gonzalez et al. (14), using specific antibodies in Western blots. The three spots (indicated as s, s1, and s2 in this study) might correspond to two variants of slow MLC2, each of them under unphosphorylated and phosphorylated states, with the assumption that the intermediate spot (s1) contains one unphosphorylated and one phosphorylated form (14). Evidence in favor of the existence of two distinct variants of slow MLC2 was first given for the ventricular myocardium (27, 35). Two variants of slow MLC2 have also been observed in human vastus lateralis muscle (15). It is worthwhile to recall that only one gene coding for the slow isoform of MLC2 is expressed in ventricular myocardiun and in slow skeletal muscle fibers (40). The presence of two variants must therefore be attributed to posttranscriptional or posttranslational modifications. In the latter alternative the hypothesis of double phosphorylation can be considered, especially if we refer to the demonstration of several phosphorylation sites in smooth muscle MLC2 (16). In this case, the increase of phosphorylation observed in this study might be explained by phosphorylation of the fast MLC2 and a second phosphorylation of the phosphorylated slow MLC2. Against the hypothesis of the double phosphorylation of slow MLC2 is the observation that only the most acidic spot (s2) of the slow MLC2 is completely removed by phosphatase action, whereas the intermediate acidic spot (s1) is greatly reduced but does not disappear. The spot of fast MLC2 considered as phosphorylated (f1) is completely removed (see also Ref. 14). Both alkaline phosphatase and PP1 were tested and were found insufficient to completely dephosphorylate slow MLC2. The specificity of PP1 is determined by the regulatory subunit (7), and the commercially available muscle PP1 used in this study contains a mixture of regulatory subunits, as stated by the manufacturer. Thus the partial effect of phosphatase might be due to a limitation to the access of the enzyme, for instance, by a structural constraint: only a very specific phosphatase, for example, PP1-M containing the myosin-specific regulatory subunit (MYPT2; Ref. 7) might produce a pattern with only one spot for the slow MLC2 and one spot for the fast MLC2.

The increase in MLC2 phosphorylation was associated with the slow-to-fast transition induced by all three treatments considered in this study; this is in agreement with the fact that MLC2 phosphorylation is higher in fast than in slow muscles, the difference being in turn dependent on different levels of calmodulin-dependent kinase activity (25). An inhibition of kinase gene expression (20) has been reported in relation to the decrease of the phosphorylation level observed after CLFS (14, 20, 21). One might speculate that, if the fast-to-slow transition induced by CLFS implies the inhibition of the calmodulin-dependent MLC kinase, the slow-to-fast transition determined by clenbuterol or by hindlimb suspension could include the activation of the same gene, as a part of a general “fast muscle fiber” program. This is, however, a simplified view: there are possibly also different isoforms of MLC kinase and regulatory mechanisms based on phosphorylation and autophosphorylation. The auto-phosphorylation reaction depends on calcium/calmodulin activation (13) with a rate of phosphorylation different from that of MLC2 phosphorylation but does not seem to modulate the catalytic function of the kinase. It is yet not known whether Rho kinase (1) can phosphorylate MLC2 in skeletal muscle. A recent study, however, showed variations of Rho kinase in relation to muscle atrophy (6). Finally, an increase of MLC2 phosphorylation might be caused by a decreased activity of specific phosphatase: the possible regulation of the MLC phosphatase (or PP1-M) in relation with β-adrenergic stimulation was recently discussed by Decostre et al. (10).

There is convincing evidence that phosphorylation of MLC2 increases the calcium sensitivity of the myofibrils and therefore force development at submaximal activation (33, 47, 49). As mentioned in the introduction, previous work by our group (44, 45) showed that calcium sensitivity decreased at atrophy induced by hindlimb suspension, whereas it increased after clenbuterol administration and after the combined treatment (37). Surprisingly, in both cases the phosphorylation level was found to be increased. This suggests that the decrease in calcium affinity in muscle fibers atrophied by hindlimb suspension in the presence of a high degree of MLC2 phosphorylation is due to other factors.

In conclusion, this study represents one of the first demonstrations that the long-term adaptation mechanisms of the contractile performance of skeletal muscles, although mainly based on regulation of myosin isoform expression and therefore operated at the transcriptional level, can be based partly on posttranslational modifications, phosphorylation in this case or glycosylation in other cases (36), as discussed in a recently published review (5). This finding is of special
interest considering that phosphorylation represents the way of regulation used by a large number of intracellular signaling pathways.

DISCLOSURES
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