IP₃-induced tension and IP₃-receptor expression in rat soleus muscle during postnatal development

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Talon, Sophie, Olivier Valloit, Corinne Huchet-Cadiou, Anne-Marie Lompré, and Claude Léoty. IP₃-induced tension and IP₃-receptor expression in rat soleus muscle during postnatal development. Am J Physiol Regulatory Integrative Comp Physiol 282: R1164–R1173, 2002; 10.1152/ajpregu.00073.2001.—The present study was designed to examine whether changes in Ca²⁺ release by inositol-1,4,5-trisphosphate (IP₃) in 8-, 15-, and 30-day-old rat skeletal muscles could be associated with the expression of IP₃ receptors. Experiments were conducted in slow-twitch muscle in which both IP₃-induced Ca²⁺ release and IP₃-receptor (IP₃R) expression have been shown to be larger than in fast-twitch muscle. In saponin-skinned fibers, IP₃ induced transient contractile responses in which the amplitude was dependent on the Ca²⁺-loading period with the maximal IP₃ contracture being at 20 min of loading. The IP₃ tension decreased during postnatal development, was partially inhibited by ryanodine (100 μM), and was blocked by heparin (20–400 μg/ml). Amplification of the DNA sequence encoding for IP₃R isoforms (using the RT-PCR technique) showed that in slow-twitch muscle, the type 2 isoform is mainly expressed, and its level decreases during postnatal development in parallel with changes in IP₃ responses in immature fibers. IP₃-induced Ca²⁺ release would then have greater participation in excitation-contraction coupling in developing fibers than in mature muscle.

sarcoplasmic reticulum Ca²⁺ release; inositol-1,4,5-trisphosphate; skinned slow-twitch fibers; mammalian muscles

INCREASED INTRACELLULAR CA²⁺-activity is an important factor in the contractile process of skeletal muscle, because Ca²⁺ binds to contractile proteins and triggers contraction. In adult skeletal muscle, the major pathway for increasing intracellular Ca²⁺ is the depolarization of transverse tubular system membranes, which induces the release of Ca²⁺ from the sarcoplasmic reticulum by opening Ca²⁺ channels/ryanodine receptors (RyR; see Ref. 8). The most accepted hypothesis for the coupling of Ca²⁺ release and the contractile responses to excitation in skeletal muscle is a direct interaction between the “voltage sensor” dihydropyridine receptor of tubular membranes and the RyR of the sarcoplasmic reticulum (15, 35). It has also been suggested that an intracellular messenger, inositol-1,4,5-trisphosphate (IP₃), contributes to excitation-contraction coupling in skeletal muscle (44, 45). Such chemical coupling is well established in smooth muscle (2, 39), but its role in skeletal muscle remains controversial. Although Posterino and Lamb (33) found that application of IP₃ failed to produce contractile force in fibers in which excitation-contraction coupling was functional, Lopez and Parra (25) have shown that local contraction can be induced by microinjection of IP₃ into intact skeletal fibers. Furthermore, recent experiments conducted on skinned skeletal muscle fibers have shown that IP₃ induces contractile responses (41, 42), which suggests that IP₃ may play a role in excitation-contraction coupling of skeletal muscle.

Although RyR appears to be the major Ca²⁺ channel of the sarcoplasmic reticulum that is expressed in skeletal muscle (47), a molecular and histochemical study has shown that IP₃ receptors (IP₃R) are also present in skeletal muscle (30). Rosembilt and colleagues (36) found that IP₃R are expressed at comparatively higher levels during early stages of striated muscle development in murine embryos, whereas the IP₃R level is much lower after birth and in adults (5, 30). Thus it is possible that the participation of IP₃R in excitation-contraction coupling of skeletal muscle is greater during muscular development than at mature stages.

The present study was designed to examine whether IP₃-mediated Ca²⁺ release changes during postnatal development in slow-twitch rat skeletal muscle and whether IP₃ contractile responses can be correlated with the level of IP₃R expression. Experiments were conducted in saponin-skinned soleus fibers, because IP₃-induced Ca²⁺ release and IP₃R expression were found to be larger in this type of muscle than in fast-twitch muscle (30, 41). In addition, molecular tech-
techniques were used to investigate the expression of IP₃R isoforms in neonatal muscles. The results show that the IP₃R are present and functional in immature slow-twitch skeletal fibers, and that IP₃-induced Ca²⁺ release and expression of IP₃R are greater in 8-day-old than in 30-day-old rat soleus muscles.

MATERIALS AND METHODS

Dissection

All procedures were carried out in accordance with the stipulations of the Helsinki Declaration for the care and use of laboratory animals. Newborn Wistar rats at different stages of postnatal development (7–8 days, 15–20 g; 14–15 days, 50–60 g; and 30 days, 80–100 g) were heavily anesthetized with ether vapor in a bell jar and killed by asphyxia. Soleus muscles were then excised and quickly frozen in liquid nitrogen for total RNA isolation or placed in oxygenated HEPES-buffered physiological solution in a dissecting dish at room temperature for skinning experiments.

The control physiological solution contained (in mM) 140 NaCl, 6 KCl, 3 CaCl₂, 2 MgCl₂, and 5 HEPES. The pH was adjusted to 7.4 with Tris base.

Chemically Skinned Fiber Preparation

From freshly isolated soleus muscles, short-cut small bundles of 5–10 fibers (1–2 mm in length; 50–100 μm in diameter) were manually dissected with fine scissors and forceps with the aid of a microscope. Chemical skinning was carried out immediately after dissection. The experiments were conducted with fibers that had been skinned using two types of chemical detergents, saponin and Triton X-100.

For Triton X-100-skinned fibers, preparations were incubated for 1 h in a relaxing solution (pCa 9.0) containing 1% (vol/vol) Triton X-100 to solubilize membranes and were then transferred to relaxing solution without detergent. After fibers were skinned, some were stored at −20°C in relaxing solution containing 50% (vol/vol) glycerol. Saponin-induced skinning was performed by incubating the bundles for 30 min in relaxing solution containing 50 μg/ml saponin. This treatment permeabilizes the sarcolemmal and T-tubule membranes and leaves the sarcoplasmic reticulum intact (13). The mechanism of sarcoplasmic reticulum Ca²⁺ load and release could then be studied on these skinned fibers (14).

After the skinning procedure, fibers were transferred to a chamber and mounted between two stainless-steel tubes fixed to an assembly (21). One end of the fiber bundle was snared in a loop of fine hair pulled into a tube glued to a fixed rod. The other end was similarly snared to a tube glued to a flexible rod that supported a metal target facing the sensor of a displacement-measuring system transducer (KD 2300, 0.5 SU; Kaman, Colorado Springs, CO). The output voltage of the system was proportional to the distance between the sensor face and the target. The diameter and length of the skinned muscle fibers were measured using a binocular microscope. The preparation was adjusted to slack length and then stretched step by step (two or three times) until the tension (mN/mm²) developed at pCa 4.5 became maximal. Maximal tension (T_max) was generally reached when resting length was increased by 20%. The results were discarded if the decrease in T_max was >5%. All experiments were performed at 22°C.

Measurement of Ca²⁺-Activated Tension

For experiments on Triton X-100-skinned fibers, a full set of solutions containing different Ca²⁺ concentrations ([Ca²⁺]) was prepared, and the solution at each ([Ca²⁺]) was then divided into appropriate aliquots: one served as a control, and the others contained either IP₃ (100 μM), ryanodine (100 μM), or heparin (20, 100, or 400 μg/ml). The fiber was exposed sequentially to solutions of decreasing pCa until pCa 4.5, at which T_max developed, and then the fiber was returned to pCa 9.0. This control cycle was followed by a test cycle performed in the presence of IP₃, ryanodine, or heparin. Isometric tension was continuously recorded on chart paper (Linear Bioblock 1200, Reno, NV), and baseline tension was established at the steady state that was measured in the relaxing solution. To obtain the Ca²⁺-sensitivity curve, data for relative tensions were fitted using a modified Hill equation.

Relative tension = T/T_max = [Ca²⁺]/K_H + [Ca²⁺]

The pCa for half-maximal activation (pC₅₀ = −log₁₀ K_H) indicates the apparent Ca²⁺ sensitivity of contractile proteins, and the Hill coefficient (n_H) allows us to estimate the degree of cooperativity. These two parameters were calculated for each experiment using linear regression analysis. The value of n_H for each fiber type was calculated as the slope of the fitted straight lines. Resting tension was that in pCa 9.0, and T_max was obtained in pCa 4.5.

Ca²⁺ Uptake and Release in Sarcoplasmic Reticulum

For experiments on saponin-skinned fibers, the preparation was immersed sequentially in five different solutions to load the sarcoplasmic reticulum with Ca²⁺ and then release it with caffeine (10 mM; Table 1). The ionic compositions of these solutions were the same as those of the relaxing and activating solutions (pCa 9.0 and 4.5, respectively). However, the concentrations of EGTA, Mg²⁺, and Ca²⁺ differed, as is described here and in Table 1.

<table>
<thead>
<tr>
<th>Solution</th>
<th>pCa</th>
<th>[EGTA], mM</th>
<th>[Mg²⁺], mM</th>
<th>[Caffeine], mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Depleting</td>
<td>9.0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Washing</td>
<td>9.0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Loading</td>
<td>7.0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Washing</td>
<td>7.5</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>Releasing (CC)</td>
<td>7.5</td>
<td>0.4</td>
<td>0.1</td>
</tr>
</tbody>
</table>

CC, caffeine contracture; [EGTA], concentration of EGTA; pCa, −log₁₀ Ca²⁺ concentration.
a reduced sensitizing effect on contractile protein (46), whereas it provided a contracture amplitude identical to that obtained at larger caffeine concentrations. During a Ca\(^{2+}\) load-release cycle, the incubation time of skinned fiber was 1 min in solutions 1 and 2 and 2 min in solution 3, whereas solutions 4 and 5 were prolonged to allow recording of the complete transient tension developed by IP\(_3\) or caffeine. Caffeine induced a transient contracture for which the area (in mN-mm\(^{-2}\)-min\(^{-1}\)), amplitude (in mN/mm\(^2\)), time to peak (in s), and half-relaxation time (in s) were measured.

At the beginning of each experiment, two or three 10-mM caffeine contractures were generated, and similar challenges were performed regularly. During the experiments, the area, amplitude, time to peak, and half-relaxation time of control caffeine contracture were not significantly modified, which suggests that the sarcoplasmic reticulum remained in a suitable functional state after the saponin skinning. Results were discarded if the decrease in the area of transient tension was >5% at the end of the experiments. IP\(_3\) was used at a concentration of 100 \(\mu\)M to produce a marked effect and allow comparison of results with those previously reported for adult rats (41). The experimental protocol consisted of a Ca\(^{2+}\) load-release cycle in which solutions 3 and 4 were followed by a solution containing IP\(_3\) (100 \(\mu\)M) but was otherwise identical to solution 4 before the application of caffeine (solution 5). In subsequent experiments, the amplitude of the 10-mM caffeine contracture obtained (in mN/mm\(^2\)) was expressed as a percentage of \(T_{\text{max}}\). The difference between the tension achieved in solution 4 in the presence or absence of IP\(_3\) corresponded to the IP\(_3\) tension and was expressed as a percentage of \(T_{\text{max}}\). The effects of different loading periods (from 0 to 20 min in solution 3) were tested on the IP\(_3\) (100 \(\mu\)M) and caffeine (10 mM) responses. Heparin (20–400 \(\mu\)g/ml) applied for 2 min in solution 3 at the end of loading was also tested on IP\(_3\)-induced tension.

The effects of ryanodine (100 \(\mu\)M) were tested on the sarcoplasmic reticulum Ca\(^{2+}\) release induced by IP\(_3\) or caffeine. Ryanodine activates RyR at a low concentration (<10 \(\mu\)M) but blocks activated RyR at concentrations >10 \(\mu\)M (27). To block RyR, 100 \(\mu\)M of ryanodine were applied after a 20-min loading period during a contracture induced by 20 mM of caffeine (which activated ryanodine-sensitive Ca\(^{2+}\) channels). In the presence of ryanodine, three or four Ca\(^{2+}\) load-release cycles were needed to abolish caffeine contracture completely and allow Ca\(^{2+}\) release to be induced by IP\(_3\) (100 \(\mu\)M).

**Skinned-Fiber Solutions**

The [Ca\(^{2+}\)] of relaxing (pCa 9.0, solution A) and activating (pCa 4.5, solution B) skinned-fiber solutions was calculated using the computer program of Godt and Nosek (20). Solutions A and B, at pH 7.10, were calculated to contain 10 mM EGTA, 30 mM imidazole, 30.6 mM Na\(^+\), 1 mM Mg\(^2+\), 3.16 mM MgATP, 12 mM phosphocreatine, and 0.3 mM dithiothreitol. An ionic strength of 160 mM was achieved by adding KCl.

In saponin-skinned fiber experiments, solutions also contained phosphocreatine kinase (17.5 IU/ml) and sodium azide (1 mM). For Triton X-100- and saponin-skinned fiber experiments, solutions with intermediate [Ca\(^{2+}\)] were obtained by mixing relaxing and activating solutions in appropriate proportions. EGTA, phosphocreatine, heparin (low mol wt, 6,000 g/mol), and ryanodine were obtained from Sigma (St. Louis, MO), and IP\(_3\) was obtained from Calbiochem. IP\(_3\), heparin, and ryanodine were prepared as stock solutions (20, 16.6, and 5 mM, respectively) in deionized and distilled water.

**Statistical Analysis**

All values are expressed as means ± SE. Student’s unpaired t-test was used to compare the different parameters when groups were different. However, when experiments were performed on the same fiber, Student’s paired t-test was used (as specified in the text). Statistical significance was reached when \(P < 0.05\).

**Total RNA Isolation**

Skeletal tissues were homogenized at 4°C with RNA Insta-Pure (1–2 ml/50–100 mg of tissue; Eurogentec) with a few strokes in an Ultrarax homogenizer. Tissues were pooled from 6–9 rats for 1-, 7-, 8-, and 14–15-day-old animals. RNA was extracted using the protocol provided by the company. At the end of the procedure, the RNA pellet was dissolved in 100 \(\mu\)l of sterile water and was used immediately or stored in ethanol at −20°C.

**RT-PCR Analysis**

The procedures used for RT and PCR analysis in the present study were previously described by Perez and colleagues (32). Total RNA (1 \(\mu\)g) from rat skeletal muscle samples was used as a template for first-strand cDNA synthesis. Each 20-\(\mu\)l reaction contained 1 mM dNTPs (Promega), 40 U of RNase inhibitor (Promega), 50 pmol of random primers in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl\(_2\), and 50 U of murine leukemia virus reverse transcriptase (PerkinElmer). Reactions were incubated at room temperature for 10 min and then at 42°C for 40 min before being heat-inactivated for 5 min at 99°C.

Skeletal muscle cDNAs were used as templates for amplification of DNA sequences encoding both the IP\(_3R\) and the RyR. For IP\(_3R\) and RyR amplification, 18 and 2 \(\mu\)l, respectively, of cDNA were used in final volumes of 100 and 50 \(\mu\)l, respectively. The PCR reaction contained 200 \(\mu\)M dNTPs, 50 pmol each of sense and antisense oligonucleotides, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM MgCl\(_2\), and 2.5 U of AmpliTaq DNA polymerase (PerkinElmer). Control templates for types 1, 2, and 3 IP\(_3R\) were from brain, heart, and colon carcinoma cells (Caco cells), respectively. The 5′ and 3′ oligonucleotide primers common to all receptor types used in the PCR assays were AGGAATGAGCTTCTATCTGTTTACCTGTTTCTC and GCAGGCTCTCGCTCTTCAAGCGCGTGAAGCA, respectively. Reaction conditions consisted of 1 cycle at 95°C for 2 min; 40 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 7 min. The 5′ and 3′ primers to the type 1 RyR used in the PCR assays were GTTCTAGAGAAAGGTGTGCTGGACAAAAACAC and CCAAAGTCTTGCTCTTTGTTAGATT, respectively. Reaction conditions consisted of 1 cycle at 95°C for 2 min; 28 cycles at 95°C for 20 s, 52°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 7 min.

**Southern Blotting and Hybridization**

PCR products derived from rat skeletal muscle total RNA primed with RyR oligonucleotides and IP\(_3R\) consensus oligonucleotides were resolved on 8% acrylamide gels. RyR product was visualized using Vistra Green. The IP\(_3R\) products were loaded on three gels and blotted onto individual nylon membranes. Blots were hybridized to receptor type-specific \(^{32}\)P-labeled oligonucleotides and washed at high stringency in solutions containing a saline sodium citrate buffer (SSC, 10×) and sodium dodecyl sulfate (SDS, 20%). All membranes were washed for 5 min in a SSC (6×)-SDS (0.5%) solution at room temperature. The membranes were then washed for 1 h.
in a SSC (2x)-SDS (0.1%) solution at 50°C for types 1 and 2 and at 40°C for type 3. The last washing of membranes was performed for 2 h in SSC (0.5x)-SDS (0.1%) solution at 50°C for types 1 and 2 and at 40°C for type 3. The receptor type-specific oligonucleotides were as follows: type 1, CGCATCGATGCTTGTGGCCTCTTTGGATGGCTTCCT; type 2, CCTCCCTACCGGCATTCGAAGA; and type 3, AGGGGCTTGCTTAGAATGTCGC.

RESULTS

Changes in Maximal Ca^{2+}-Activated Tension and IP_3 Contractile Responses During Postnatal Development

In saponin-induced skinning experiments, immature slow-twitch fibers were loaded for different periods (2, 5, 10, and 20 min) with Ca^{2+} (pCa 7.0, solution 3). After a given loading period, fibers were bathed in a solution at pCa 7.5, and an application of an identical solution with or without IP_3 (100 μM) was subsequently applied. Caffeine (10 mM) was then applied to release Ca^{2+} from the sarcoplasmic reticulum, which resulted in transient contracture (see Table 1). Caffeine contracture amplitude was used as an index of sarcoplasmic reticulum Ca^{2+} content to ensure the integrity of the sarcoplasmic reticulum.

A series of measurements was first made to estimate the maximal Ca^{2+}-activated tension (T_{max}) of newborn rat slow-twitch fibers. Results showed the amplitude of T_{max} increased significantly during development [61.5 ± 2.7 mN/mm^2 (n = 14), 86.3 ± 2.7 mN/mm^2 (n = 12; P < 0.01), and 120.3 ± 7.4 mN/mm^2 (n = 11; P < 0.001)] in 8-, 15-, and 30-day-old rat muscles, respectively.

Figure 1 illustrates the effects of a solution at pCa 7.5 (0.1 mM Mg^{2+}) with (B) or without (A) 100 μM IP_3 on the tension achieved at pCa 7.5 (0.1 mM Mg^{2+}) in newborn rats. Figure 1B shows that after 20 min of loading in solution 3, IP_3 (100 μM) applied after the tension produced at pCa 7.5 in solution 4 had reached a steady level resulted in transient tension. IP_3-induced tension decreased during postnatal development (Fig. 1B). No increase in tension was observed in the absence of IP_3 (Fig. 1A); however, the tension achieved at pCa 7.5 in the absence of IP_3 (Fig. 1, A and B) increased with postnatal development. For example, after 20 min of loading, the amplitude of this tension was 2.1 ± 0.4 mN/mm^2 (n = 14), 3.7 ± 0.7 mN/mm^2 (n = 21), and 7.6 ± 1.7 mN/mm^2 (n = 19; P < 0.01) in fibers from 8-, 15-, and 30-day-old rats, respectively.
R1168 DEVELOPMENT OF IP₃ TENSION AND IP₃ RECEPTORS

To compare the IP₃ responses obtained between immature fibers, the IP₃-induced enhancements in tension achieved at pCa 7.5 were expressed as a percentage of Tₘₐₓ for each stage of postnatal development. Figure 2 illustrates the changes in IP₃ tension for different loading periods in 8-, 15-, and 30-day-old rat soleus muscle fibers. The results show that a 2-min loading period was sufficient to produce significant IP₃ response in immature fibers (Fig. 2). The peak tension induced by IP₃ at 2 min was not significantly different in neonatal muscles at 8, 15, and 30 days of development [0.94 ± 0.13% (n = 14), 1.11 ± 0.16% (n = 12), and 0.87 ± 0.11% of Tₘₐₓ (n = 11), respectively]. For 5 and 10 min of loading, the amplitude of the IP₃ response was similar in fibers from 8- and 15-day-old rat muscles, but greater than that obtained at 30 days of development. For example, the peak tension induced by IP₃ after 10 min of loading was 1.90 ± 0.3% (n = 8), 1.93 ± 0.54% (n = 7), and 1.26 ± 0.54% of Tₘₐₓ (n = 8), respectively, 8, 15, and 30 days after birth. At 20 min, fibers from 8- and 15-day-old rat soleus muscle developed a similar IP₃ tension that was greater than that obtained in fibers from 30-day-old animals (Table 2).

Regarding the 10-mM caffeine-induced contractile responses obtained in skeletal fibers of newborn rats, the contracture amplitude, when expressed as a percentage of Tₘₐₓ amplitude at each stage of development, was not significantly different between the immature slow-twitch muscles (Table 2). Furthermore, the increase in the loading period from 2 to 20 min induced no significant variations in the amplitude of caffeine contracture at each stage of postnatal development tested. The maximal response induced by caffeine was obtained at 2 min of loading, whereas 20 min were required to reach the maximum of IP₃ tension.

The IP₃ tension observed in the present study could have been related to Ca²⁺ release from sarcoplasmic reticulum or an increase in the Ca²⁺ sensitivity of the contractile apparatus. To investigate the latter possibility, the effects of IP₃ were estimated on maximal Ca²⁺-activated tension and the apparent Ca²⁺ sensitivity of contractile proteins in Triton X-100-skinned fibers from newborn rats.

Effects of IP₃ on Ca²⁺-Activated Tension on Immature Triton X-100-Skinned Fibers

The results obtained showed that the tension developed by the contractile apparatus when maximally activated by Ca²⁺ (pCa 4.5) was not affected by IP₃ (100 μM) at each stage of postnatal development tested. Moreover, no significant differences in apparent Ca²⁺ sensitivity were observed between the relative tension-pCa curves recorded in the absence or presence of IP₃ (100 μM). Thus in immature fibers, IP₃ failed to

Table 2. Caffeine contracture, IP₃ tension, and effects of ryanodine and heparin on IP₃ tension in slow-twitch skinned fibers from rats

<table>
<thead>
<tr>
<th>Stage of Postnatal Development</th>
<th>8 Days</th>
<th>15 Days</th>
<th>30 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine contracture, % of Tₘₐₓ (2 min Ca²⁺-loading period)</td>
<td>64.9 ± 2.6 (9)</td>
<td>75.4 ± 1.7 (12)</td>
<td>73.2 ± 3.1 (6)</td>
</tr>
<tr>
<td>IP₃ tension, % of Tₘₐₓ (20 min Ca²⁺-loading period)</td>
<td>2.16 ± 0.27 (14)</td>
<td>2.19 ± 0.36 (21)</td>
<td>1.64 ± 0.13 (19)</td>
</tr>
<tr>
<td>Ryanodine effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.10 ± 0.50</td>
<td>2.05 ± 0.20</td>
<td>1.74 ± 0.20</td>
</tr>
<tr>
<td>Ryanodine (100 μM)</td>
<td>0.83 ± 0.33 †</td>
<td>1.01 ± 0.26 †</td>
<td>0.89 ± 0.17 †</td>
</tr>
<tr>
<td>Heparin effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.47 ± 0.22</td>
<td>2.57 ± 0.87</td>
<td>0.80 ± 0.21</td>
</tr>
<tr>
<td>Heparin (10 μg/ml)</td>
<td>0.97 ± 0.07 ‡</td>
<td>1.54 ± 0.42 ‡</td>
<td>0.81 ± 0.20</td>
</tr>
<tr>
<td>Control 2</td>
<td>1.48 ± 0.02 ‡</td>
<td>1.23 ± 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>Heparin (400 μg/ml)</td>
<td>0.28 ± 0.07 ‡</td>
<td>0.27 ± 0.37</td>
<td>0.07 ± 0.07 ‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; no. of skinned fibers is in parentheses. Effects of ryanodine and heparin were tested on IP₃ tension after 20 min of Ca²⁺ loading. Tₘₐₓ, maximal Ca²⁺-activated tension; IP₃, inositol-1,4,5-trisphosphate. *P < 0.05 vs. IP₃ tension at each stage of development; †P < 0.05 vs. IP₃ tension from control in ryanodine experiments; ‡‡P < 0.05 vs. control 1 and control 2, respectively, in heparin experiments; ND, data not determined.

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affect pCa$_{50}$ (the amount of Ca$^{2+}$ required to produce 50% of $T_{\text{max}}$). For example, pCa$_{50}$ in the absence or presence of IP$_3$ (100 μM) was 6.41 ± 0.03 versus 6.42 ± 0.03 (n = 3), 6.45 ± 0.03 versus 6.47 ± 0.02 (n = 3), and 6.47 ± 0.03 versus 6.45 ± 0.02 (n = 3), respectively, in 8-, 15-, and 30-day-old rat soleus fibers.

Thus the IP$_3$ tension previously observed in saponin-skinned preparations could have been related to an effect of IP$_3$ on Ca$^{2+}$ release from the sarcoplasmic reticulum. Accordingly, the effects of ryanodine and heparin (inhibitors of RyR and IP$_3$R, respectively) were tested on IP$_3$-induced contractile responses.

**Effects of Ryanodine on IP$_3$ Tension**

Ryanodine experiments were performed as previously described in adult muscle (41). Briefly, after a 20-min loading time, 100 μM ryanodine was applied during a caffeine contracture (caffeine activates ryanodine-sensitive Ca$^{2+}$ channels). The transient caffeine contractures in the presence of ryanodine were irreversibly abolished after three or four Ca$^{2+}$-load-release cycles, which suggests that all caffeine-sensitive Ca$^{2+}$-release sites were blocked by ryanodine in immature fibers. After fibers were loaded for 20 min, IP$_3$ (100 μM) was applied in solution at pCa 7.5 (solution 4; see Table 1). The results showed that application of IP$_3$ after ryanodine treatment can induce an increase in the tension achieved at pCa 7.5 in immature fibers. The IP$_3$ tension generated by immature fibers 8, 15, and 30 days after birth was significantly decreased in the presence of ryanodine by 65.9 ± 8.1% (n = 3), 51.1 ± 11.8% (n = 4), and 47.9 ± 9.1% (n = 4), respectively (see Table 2). It is noteworthy that the tension obtained at pCa 7.5 in the absence of IP$_3$ was also reduced by ryanodine [33.3 ± 1.5% (n = 3), 73.0 ± 1.0% (n = 4), and 64.1 ± 4.0% (n = 4)] in 8-, 15-, and 30-day-old rat muscle fibers, respectively.

Because the ryanodine-induced decrease in IP$_3$ tension could have been related to the action of this inhibitor on contractile proteins, the effects of ryanodine were investigated in Triton X-100-skinned slow-twitch fibers from newborn rats. No significant changes in $T_{\text{max}}$ and apparent Ca$^{2+}$ sensitivity were found at any stage of development in the presence of ryanodine (100 μM). For example, in the absence or presence of ryanodine, pCa$_{50}$ was 6.49 ± 0.02 versus 6.48 ± 0.02 (n = 3), 6.48 ± 0.02 versus 6.49 ± 0.04 (n = 3), and 6.47 ± 0.04 versus 6.44 ± 0.01 (n = 3), respectively, in 8-, 15-, and 30-day-old rat slow-twitch preparations. These results suggest that the ryanodine-induced decrease in IP$_3$ tension observed in immature saponin-skinned fibers was related to the inhibition of IP$_3$-induced Ca$^{2+}$ release by ryanodine.

**Effects of Heparin on IP$_3$ Tension**

At 20 μg/ml, heparin decreased IP$_3$ tension by 59.9 ± 6.0% (n = 3) and 35.3 ± 10.1% (n = 3) in 8- and 15-day-old rat slow-twitch fibers, respectively, whereas no changes were observed in fibers from 30-day-old animals (see Table 2). The application of higher concentrations of heparin (100 and 400 μg/ml) induced larger reductions in IP$_3$ tension at each stage of development (see Table 2). For example, at 400 μg/ml, heparin-induced inhibition of IP$_3$ tension was almost maximal in immature fibers [87.9 ± 1.8% (n = 3), 85.0 ± 15.0% (n = 3), and 92.5 ± 7.5% (n = 3) from soleus muscles in 8-, 15-, and 30-day-old rats, respectively; see Table 2]. It is noteworthy that the effects of heparin were not fully reversible. For example, in soleus muscles of 8- and 15-day-old rats, after application of 100 μg/ml of heparin and a return to control conditions for 20 min, IP$_3$ tension was still reduced by 39.3 ± 6.4% (n = 3) and 17.5 ± 2.5% (n = 3), respectively (see Table 2).

Because the heparin-induced decrease in IP$_3$ tension could have been related to an effect on the contractile apparatus, $T_{\text{max}}$ and the apparent Ca$^{2+}$ sensitivity of contractile proteins were estimated in the absence or presence of 20, 100, or 400 μg/ml of heparin in immature Triton X-100-skinned fibers. At each tested stage of development, $T_{\text{max}}$ was not affected by 20 μg/ml of heparin. However, heparin at a concentration of 100 μg/ml reduced $T_{\text{max}}$ by 6.3 ± 0.5% (n = 3), 15.0 ± 1.4% (n = 3), and 10.2 ± 8.2% (n = 3) in 8-, 15-, and 30-day-old rat fibers, respectively. This effect on $T_{\text{max}}$ was more pronounced when a larger concentration of heparin was used. For example, at 400 μg/ml, $T_{\text{max}}$ was decreased by 29.4 ± 12.1% (n = 3), 30.2 ± 3.1% (n = 3), 21.1 ± 0.7% (n = 3) at 8, 15, and 30 days after birth, respectively. At each stage of postnatal development, 20 and 100 μg/ml of heparin induced no significant changes in relative tension-pCa relationships, which indicates that apparent Ca$^{2+}$ sensitivity was unaffected by low concentrations of heparin in immature fibers. However, heparin at 400 μg/ml induced a shift to the right in relative tension-pCa curves (Fig. 3), thereby reducing pCa$_{50}$ below the control value (ApCa$_{50}$): −0.05 ± 0.01 (n = 3; P < 0.05), −0.06 ± 0.01 (n = 3; P < 0.05), and −0.08 ± 0.01 (n = 3; P < 0.05) from soleus muscles in 8-, 15-, and 30-day-old rats, respectively.

Taken together, the present results indicate that the heparin-induced decrease in IP$_3$ tension in saponin-skinned immature fibers was mainly related to an inhibition of IP$_3$-induced Ca$^{2+}$ release. This suggests that IP$_3$R were present and functional in immature skeletal muscle. To complete the results obtained with Ca$^{2+}$ release inhibitors, a molecular technique (RT-PCR) was used to investigate the presence of IP$_3$R.

**Analysis of IP$_3$R Expression**

To determine the expression pattern of IP$_3$R mRNA in skeletal muscle, RT-PCR was performed on total RNA from slow-twitch muscles isolated from rats at different stages of postnatal development. Primers common to all three types of IP$_3$R cDNA sequences were used, and IP$_3$R-specific isoforms were detected by Southern blotting using specific internal primers. The results (Fig. 4, middle) show that type 2 receptor isoform transcript was the predominant IP$_3$R expressed
in adult and developing skeletal muscle and that the expression of this isoform decreased during postnatal development in skeletal muscle. Type 2 IP₃R (IP₃R2) was highly expressed in brain and heart. Furthermore, type 1 isoform (IP₃R1), mainly present in brain, was also amplified in skeletal muscle during the first stages of development (Fig. 4, top). It is noteworthy that type 3 IP₃R (IP₃R3) was not detected in skeletal muscle regardless of the stage of development studied (data not shown).

Compared with IP₃R mRNA, type 1 RyR (RyR1) mRNA was highly present at each stage of postnatal development, but was not expressed in brain and heart (Fig. 4, bottom).

DISCUSSION

This study shows that in newborn slow-twitch fibers, a tension is produced by the application of IP₃, an activator of IP₃R. The results obtained from Triton X-100-skinning experiments indicate that contractile responses are not related to an IP₃-induced increase in the Ca²⁺ sensitivity of the contractile apparatus. It could be proposed that the IP₃ contractures observed in immature skeletal muscle fibers were due to the release of Ca²⁺ from the sarcoplasmic reticulum through IP₃R. This hypothesis is supported by experiments in saponin-skinning fibers where it was found that heparin, a specific inhibitor of IP₃R, blocks IP₃-induced contractile responses. Furthermore, the IP₃ contractures could also be generated in the presence of ryanodine, a specific inhibitor of RyR. Taken together, these results suggest that in immature slow-twitch fibers, IP₃ induces Ca²⁺ release from the sarcoplasmic reticulum and probably through IP₃R. The results presently found are similar to those previously obtained in adult skeletal muscle showing that IP₃R were present and functional in soleus fibers (41, 42). Then, the Ca²⁺ release from the sarcoplasmic reticulum in mammalian skeletal muscle could be elicited through two types of receptors: one sensitive to IP₃ and heparin and the other to caffeine and ryanodine.

The IP₃R (a tetramer with a molecular mass of ≈1.2 million Da) is a ligand-activated channel that requires IP₃ to function (12). Three forms of IP₃R have been identified. IP₃R1, which is expressed in various tissues such as smooth muscle or brain, has been extensively characterized (28). IP₃R2 and IP₃R3 isoforms share 69 and 64% identity, respectively, with the amino acid sequence of IP₃R1. IP₃R3 is predominant in non-neural tissues (4), whereas IP₃R2 is the main isoform expressed in cardiomyocytes (24, 32). Most tissues express at least two subtypes although in varying ratios (43). The expression of IP₃R subtypes could be altered in particular circumstances such as during differentiation in some cell types, which suggests an involvement in cell development (18, 40). In our study, IP₃R2 was the main form found in all skeletal muscle samples tested, a result that is similar to those previously reported by De Smedt and colleagues (10) but different from those of Moschella and co-workers (30). Furthermore, our results show that the expression of this isoform decreases during postnatal development (see Fig. 4, middle) in parallel with changes in IP₃ tensions in immature saponin-skinning fibers. De Smedt and colleagues (10) have found that a type 2 isoform persisted in adult muscle, whereas the expression of IP₃R2 in slow-twitch muscle was below the detection level in our study. Futatsugi and colleagues (19) detected a novel isoform of IP₃R2 specifically expressed in skeletal muscle and suggested that the protein encoded by this isoform could be involved in the regulation of IP₃-mediated Ca²⁺ release in skeletal muscle. It is still unclear whether this protein is present in immature fibers. Rosemblit and colleagues (36) showed that high levels of the type 1 isoform are expressed during embryogenesis. Our results indicate that IP₃R1 mRNA is present at 8 days and decreases during the postnatal period. IP₃R3 was not detected regardless of the post-
nental stages studied, whereas De Smedt and colleagues (10, 11) found high levels of this isoform in undifferentiated mouse skeletal muscle cell lines, which were decreased when the cells were induced to differentiate. These differences could be due to the type of preparation and/or to the animal species used and/or to a different sensitivity in the technique used, particularly in the oligonucleotide primer sequences.

Although IP$_3$R are present and their expression is associated with IP$_3$-induced contractile responses, IP$_3$ contractures are small compared with the responses due to caffeine. This raises the question about the role of IP$_3$R in skeletal muscle. Recently, Lipp and co-workers (24) have shown that in cardiomyocytes, IP$_3$R are colocalized with junctional RyR in the subsarcomcial space and could modulate excitation-contraction coupling. Indeed, their IP$_3$ ester-induced activation provokes spontaneous Ca$^{2+}$ release and increases the frequency of Ca$^{2+}$ spark. Such Ca$^{2+}$ sparks, which are also present in skeletal muscle, could be involved in an amplification mechanism of Ca$^{2+}$ release initially induced by “voltage sensor” during membrane depolarization (7, 31). Thus it could be imagined that as in cardiac myocytes, the IP$_3$R could participate in excitation-contraction of skeletal muscle by increasing the small subcellular Ca$^{2+}$ release evoked by RyR. Such a possibility could then explain the decrease in IP$_3$ contractile responses presently observed in the presence of ryanodine (see Table 2).

At birth, mammalian skeletal muscle cells are not fully differentiated. During embryonic and postnatal development, important changes occur in enzyme activities, innervation patterns, and membrane systems that could account for changes in mechanisms involved in contraction. These modifications correspond to the gradual formation of T tubules and the sarcoplasmic reticulum and to the establishment of an adult-specific protein pattern (16, 37). The contractile properties of skeletal muscle depend mainly on the expression of fiber-type protein isoforms constituting thin and thick filaments (myosin, troponin, and tropomyosin). The synthesis of slow myosin, the major component of thick filaments in slow-twitch fibers, is an early event in skeletal myogenesis. D’Albis and co-workers (9) have shown that the level of this isoform increases continuously in soleus muscle from 40% of total myosin a few days after birth to 80% at 1 mo of age. Adams and colleagues (1) have shown that soleus muscle at birth contains mainly a mixture of embryonic and neonatal myosin heavy chain (MHC) isoforms, which are rapidly repressed and progressively replaced by adult isoform during postnatal development. In skeletal muscle, the adult MHC phenotype is reached in the first 4 wk after birth (1). More recently, Krishan and colleagues (23) have shown that the slow troponin T, a regulatory protein of contractile apparatus, is highly expressed in all embryonic muscle masses, whereas in late development stages its expression is restricted to the slow-twitch fibers. Thus in the present study, the increase in $T_{max}$ observed during the postnatal development could also be related to higher levels of contractile protein expression as well as to a transition from an immature to an adult protein pattern, particularly in the MHC and troponin phenotypes.

Previous investigations have shown that the mechanical activity (by interfering with excitation-contraction coupling) could delay the muscle development. In the poorly developed sarcoplasmic reticulum from immature skeletal muscles, the Ca$^{2+}$ storage capacity as well as the calsequestrin content is low (17). Furthermore, it is important for correct function of the adult muscle that protein-protein interactions become more and more complex during development (17). A tight correlation between the mechanical factors and the organization of myofibrillar protein subunits into sarcomeres has been previously established, and some of the processes involved at the myofibrillar level are Ca$^{2+}$ dependent (6). The expression and the participa-
tion of the IP$_3$R in Ca$^{2+}$ homeostasis could represent an additional mechanism to release Ca$^{2+}$ and favor the development of mechanical activity. Then the IP$_3$R could have a role comparable to that of RyR type 3 (RyR3) that was previously shown to be required for an efficient contraction in neonatal skeletal muscles (3). It could be proposed that IP$_3$R as RyR3 could contribute to a secondary component of excitation-contraction coupling which creates an amplification mechanism for regulation of skeletal muscle contraction through a "Ca$^{2+}$-induced Ca$^{2+}$-release" mechanism, the latter being particularly important in neonatal muscles where the development of the triad structure is not complete (3,16).

In summary, the present work clearly shows that the application of IP$_3$ to skeletal muscle induced contractile responses related to Ca$^{2+}$ release from the sarcoplasmic reticulum. The complementary use of pharmacological tools (ryanodine and heparin) and molecular techniques indicated that IP$_3$R are present and functional in slow-twitch muscle and that the changes in expression during postnatal development were associated with those observed in IP$_3$ tension. Taken together, these results suggest a larger participation of IP$_3$ in excitation-contraction coupling in developing slow-twitch skeletal muscle than in mature muscle.

Perspectives

Although the physiological function of IP$_3$R in skeletal muscle remains unclear, changes in the IP$_3$R pattern occurring in immature skeletal muscle could be involved in the maintenance and/or the control of Ca$^{2+}$ homeostasis during normal muscle development. Besides, in brain and heart, a dysfunction of IP$_3$R and/or of IP$_3$-induced Ca$^{2+}$ release contributes to the pathogenesis of Alzheimer's disease and arrhythmia, both disorders being characterized by alterations of Ca$^{2+}$ homeostasis (29,38). Abnormalities in Ca$^{2+}$ homeostasis also occur in various pathological conditions of skeletal muscle. For example, the resting intracellular [Ca$^{2+}$] is increased in soleus muscle after hindlimb unloading and reloading (22). It was also proposed that Ca$^{2+}$ homeostasis alterations would be involved in the processes of cell death in Duchenne muscular dystrophy (26). Therefore, some investigations of the effects of IP$_3$ in pathological skeletal muscle would be helpful toward clarifying and improving our understanding of the role of this intracellular messenger in skeletal muscle. Furthermore, as previously reported with RyR3 (3), knockout mice may represent an interesting animal model for further studying the role of IP$_3$R in skeletal muscle.

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