The effect of the intramuscular injection of bupivacaine hydrochloride on selected morphological characteristics and contractile properties of adult rat extensor digitorum longus muscle was studied. Recovery of normal fiber size was already present 30 days after bupivacaine injection and at 90 days after injection, values of the normalized twitch tension (mN/mg of tissue) and of the fatigue index approached those measured in control muscle, whereas the normalized tetanic tension remained 57% of control. At 7–30 days postinjection, twitch force was decreased by reducing $[Ca^{2+}]_0$ (substituted by Mg^{2+}) or adding Co^{2+} (5 mmol/L^{-1}). By contrast potentiation of the twitch was recorded in the presence of Cd^{2+} (2 mmol/L^{-1}). Glycerol treatment only reduced, but did not eliminate twitches developed by muscles 7 days after injection. Present results emphasize the importance of the recovery process in the loss of the susceptibility of the contractile responses to extracellular calcium in bupivacaine-injected muscles. These data may be of interest in the evaluation of functional aspects of muscles in which injections of viral vector or autologous myoblasts have been performed.
Key words: extracellular calcium • muscle regeneration • mammalian skeletal muscle

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EXTERNAL CALCIUM DEPENDENCE OF EXTENSOR DIGITORUM LONGUS MUSCLE CONTRACTILITY DURING BUPIVACAINE-INDUCED REGENERATION

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During the excitation–contraction (EC) coupling process of adult skeletal muscle fiber, asymmetric charge movements (voltage sensors) located in the transverse tubules (T-tubules) membranes lead to opening of calcium (Ca^{2+}) release channels in the sarcoplasmic reticulum (SR). Although in adult skeletal muscle fibers, extracellular Ca^{2+} is not involved in the twitch, external Ca^{2+} may play a role by modulating the inactivated states of the voltage sensor (for review, see Ref. 30). It has also been shown that variations of the extracellular calcium concentration ($[Ca^{2+}]_0$) or blockade of Ca^{2+} entry could differentially influence the contractile responses elicited in extensor digitorum longus (EDL) muscles of newborn and adult rats.[6,29] The contractile strength of developing skeletal muscle is significantly more diminished than in mature muscles when extracellular Ca^{2+} is reduced, suggesting that the features of EC coupling change during development.

Muscle regeneration recapitulates the steps of normal myogenesis[12,13] and we have recently shown that in notexin-injected soleus (SOL) muscle, the amplitudes of the twich and tetanic tensions were greatly reduced in nominally calcium-free solution.[25] The inoculation of the crude venom of the Australian tiger snake, *Notechis scutatus scutatus*, induces a degeneration of the SOL muscle as a result of the presence of a number of myotoxic phospholipases of the A2 type.[20] However, the observations from this slow-twitch muscle cannot be extrapolated to a predominantly fast-twitch muscle, like EDL, as maturation of muscular cells appears to be muscle-specific.[13] Fur-

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thermore the action of notexin is more effective on soleus muscle than EDL muscle.[20] Bupivacaine hydrochloride (BPVC) is also a well-known chemical agent that causes acute muscle fiber necrosis and massive cellular infiltration of macrophages, followed by rapid regeneration of muscle fibers. Myotoxic effects of local anesthetics,[10] such as bupivacaine, allowed BPVC intramuscular injection to be routinely used for producing degeneration of muscle in rats. Muscle fibers respond to the insults not only by degenerating but by regenerating in a remarkably complete way.[2] In fact, myotoxic action of bupivacaine provides a unique opportunity for studying regeneration of muscle in an environment conducive to optimal recovery. In contrast to the damage caused by the various grafting procedures, bupivacaine does not damage the muscle's satellite cell population,[17,18] vasculature,[15] basal lamina or endomysial tubes,[16] or intramuscular nerves,[34] all these being elements which influence the rate and extent of muscle regeneration.

Thus, the aim of the present study was to characterize the influence of the external Ca^{2+} upon the contractile responses elicited in regenerated EDL muscles following bupivacaine injection. Concomitantly, a morphological study was performed to follow the steps of regeneration in EDL muscle.

MATERIALS AND METHODS

In vitro experiments were carried out on EDL muscles from Wistar male adult rats weighing 250 g. Prior to surgical intervention, rats were anesthetized with Ketamin (Ketalar, 50 mg, Parke-Davis and Co.). At various times after surgical intervention, the animals were heavily anesthetized by an ether vapor flow and after respiratory arrest, the EDL muscle was removed, placed in a Petri dish, and bathed in an oxygenated *N*-2-hydroxyethylpiperazine-*N*'-2 ethanesulphonic acid (HEPES)-buffered physiological solution at room temperature (19–22°C).

Solutions. The normal physiological solution contained (mmol/L^{-1}): Na^+, 140; K^+, 6; Ca^{2+}, 3; Mg^{2+}, 2; Cl−, 156; HEPES, 5; pH was adjusted to 7.4 with Tris-aminomethane. The solutions were equilibrated with 100% O_2. Calcium was added as a 1 mol/L^{-1} $CaCl_2$ solution (B.D.H. Poole, GB, volumetric standard, Analar grade) to give a concentration of 3 mmol/L^{-1}, unless its concentration was varied as part of the experiment. When the bathing Ca^{2+} concentration was reduced, an equivalent amount of $MgCl_2$ was added. Divalent cations [cobalt (Co^{2+}), cadmium (Cd^{2+}), or strontium (Sr^{2+})], as chloride salts (Sigma) were also used. Caffeine (Sigma) was dissolved in the appropriate medium to obtain the required final concentration. In some experiments, isolated bundles of fibers were subject to glycerol treatment according to the method described by Eisenberg et al.[9] The experiments were conducted at room temperature (19–22°C).

Bupivacaine Injection. The right EDL muscle was surgically exposed and injected with 0.5 mL of 0.75% bupivacaine hydrochloride in 0.9% w/v NaCl (Sigma, L'Isle d'Abeau Chesnes, France). A single injection was performed. Experiments were carried out at different times (7, 14, 30, 60, and 90 days) after BPVC injection. Contralateral EDL muscle was used as control.

Contractile Experiments. Bundles of 5–10 fibers were isolated and dissected along their whole length under a binocular microscope. The preparation was then transferred to the experimental chamber and the two ends of the muscle were carefully snared by fine platinum wire loops: one fixed in the experimental bath and the other to the tip of a force transducer (displacement measuring system Kaman KD 2300, Colorado Springs, CO). Twitches were displayed on an oscilloscope (Tektronix 5115 N, Beaverton, OR) upon stimulation by current pulses (0.5 ms duration, twice the threshold value, frequency 0.07 Hz) applied between two platinum plate electrodes on either side of the channel and the maximum twitch tension (Pt) determined. Tetanic contractions were elicited with trains of stimuli at 100 Hz for the time necessary to get a steady-state tension (duration of trains: 0.7–1.5 s) and estimate the maximum tetanic tension (Po). According to previously reported protocol,[25] posttetanic potentiation (PTP) was then measured by stimulating muscle with a single pulse (pretwitch) followed 60 s later by a tetanus (duration:1 s). After 20 s, posttwitch was elicited. The fatigue profile of the muscle was measured by stimulating the muscle every 30 s, 10 times with 8-s tetani.[25] The tension of the last tetanus divided by the tension of the initial tetanus was taken as an index of fatigue (Fi). The fast-flow perfusion stream (20 mL/min), coupled to the small chamber volume allowed a total bath medium change in less than 0.2 s. Resting tension, twitches, and tetanus were recorded continuously on a chart recorder (SE 120, BBC Goerz Metrawatt, Nürnberg, Germany). The preparation was weighed and the developed forces, expressed in mN, were normalized to muscle weight in mg.

Microscopic Studies, Fiber Typing, and Morphometry. For the morphological characterization and for histochemical and immunocytochemical studies,

standard procedures have been essentially used. Transverse cryostat sections (10 μm thick) were stained with hematoxylin and eosin, Gomori's trichrome, Sudan black and red, and Alizarin red (pH 5.4) for myosin adenosine triphosphatase (ATPase) activity (pH 4.35, 4.63, and 9.4) and for the mitochondrial oxydative enzymes (DPNH-TR) activity. Muscle fibers were classically classified into type I [or slow oxidative (SO) fibers], IIA [or fast-oxidative-glycolytic (FOG) fibers], and IIB [or fast-glycolytic (FG) fibers.][3]

SO, FOG, and FG fibers and fibers exhibiting central myonuclei were counted in cross-sections of muscle appropriately stained and the number of each was calculated as a percentage of at least 200 fibers from five different regions within a section of muscle. Morphometry was performed on transverse sections obtained from the middle part of the muscle. Cross-sectional area (CSA) and diameter were measured on ATPase-stained sections. At least 200 fibers from five different regions within a section of muscle were examined under light microscopy using an image analyzer (Summagraphics/Tandon PC AT).

Neuromuscular junction formations were stained either by a combined stain for AChE activity and silver impregnation or by TMR-αBTX binding to the acetylcholine receptor.

Statistical Analysis. Results were expressed as mean $\pm$ SD. Statistical evaluation was performed with the nonparametric Mann–Whitney test between different groups. The test was considered to be significant at $P < 0.05$.

RESULTS

Morphological Description of Muscle Regeneration after Bupivacaine Injection. At the different periods of time chosen for the study, no abnormality was observed in contralateral (control) muscles and morphological features were essentially similar to those already reported.[1,16,17,19,31,32] Shortly after injection of bupivacaine (6 h), muscle fibers were necrotic and were partly invaded by macrophages and polymorphonuclear cells. In 3 days postinjected muscles, many colonies of myotubes, mainly with central myonuclei (84%), were detected. Figure 1 summarizes the percentages of fibers with centrally situated nuclei in bupivacaine-injected EDL muscle. In bupivacaine-treated muscles, 7 days after injection, the muscles contained small-diameter fibers, with central nuclei. From 14 to 30 days after bupivacaine injection, diameters of fibers increased progressively. The proportion of the different types of fibers are summarized in Table 1. In control EDL muscles,

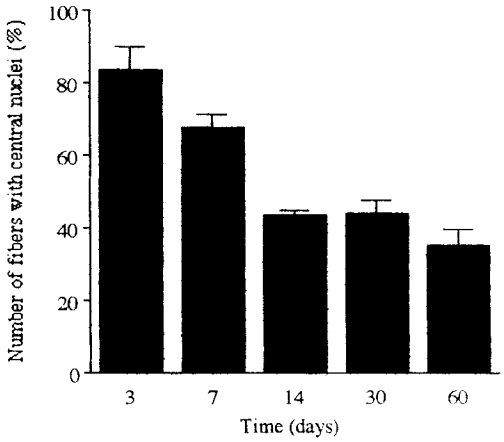

FIGURE 1. Distribution–frequency histogram of centrally nucleated fibers from EDL muscles at 3, 7, 14, 30, and 60 days after bupivacaine injection.

the percentage of type I and type II fibers was 5–7% and 93–94.5%, respectively. Histoenzymological differentiation of fibers was not apparent 3 and 7 days after the injection. By 14 days after the injection, the differentiation of fibers was complete and the number of type I fibers represented 71%, 134%, and 155% of control 14, 30, and 60 days after injection, respectively.

Mean diameter and mean CSA reached control values by 60 days and there was no difference in diameter and CSA when normal EDL was compared with bupivacaine-injected EDL at 60 days. The evolution of the distribution of the CSA of the fibers from bupivacaine-injected muscles is summarized in Figure 2.

By 30 days after the injection, combined AchE–silver staining and TMR-αBGTx gave a perfect matching of the aspects of the subneural apparatus. The neuromuscular junctions were of typical, mature-animal size and morphology.

Twitch and Tetanic Tensions. In the control EDL muscles, normalized Pt and Po were 3.3 $\pm$ 0.2 mN/mg (control Pt) and 18.3 $\pm$ 0.9 $\pm$ 0.05 mN/mg (control Po) ($n = 6$), respectively (Table 2). In bupivacaine-treated muscles, 7 days after injection, Pt was already 26.5% of the value measured in control adult muscles ($P < 0.01$). Pt and Po increased progressively with time; 90 days after the intervention, Pt was almost 79% of control in injected muscles ($P < 0.05$) and Po was 57% of control (Table 2, $P < 0.02$) (Fig. 3).

Effects of Calcium Withdrawal on Twitch and Tetanic Characteristics. External calcium withdrawal affected the amplitude of the twitch developed by 7

Table 1. Proportions of the different types of muscle fibers in contralateral and bupivacaine-injected EDL muscles.

Time (days)	Type I		Type II		Type IIA		Type IIB	
	BPVC	Control	BPVC	Control	BPVC	Control	BPVC	Control
3	—	5.9 ± 1.1	—	94 ± 1.1	—	55.2 ± 5.2	—	44.7 ± 4.2
7	—	6.2 ± 2.3	—	93.4 ± 2.3	—	55.5 ± 3.1	—	44.4 ± 3.1
14	4.8 ± 3.6	6.8 ± 0.4	95.1 ± 3.6	93.1 ± 0.5	55.5 ± 5.2	60.3 ± 5.4	44.4 ± 5.3	39.5 ± 5.4
30	9.4 ± 2.1	7.1 ± 0.7	90.6 ± 2.2	92.9 ± 0.8	52.2 ± 5.7	56.1 ± 3.8	47.6 ± 5.7	43.9 ± 4.5
60	8.4 ± 1.8	5.4 ± 0.9	91.6 ± 1.9	94.5 ± 2.1	50.2 ± 5.7	56.2 ± 2.3	49.7 ± 4.9	43.7 ± 3.5

days postinjected EDL muscles. After 10 min in nominally Ca^{2+}-free solution, compared with developed tension in control medium, twitch force was decreased by 57 ± 8% ($n = 7, P < 0.01$) (Table 3). This reduction of twitch amplitude might be due to an increase of relaxation rate of the contractile response despite the activation process being maximum. Consequently the effects of nominally Ca^{2+}-free solution on twitch duration have been investigated. Values of time to peak (49 ± 11 ms) and half relaxation time (66 ± 15 ms) became slightly smaller in the absence of external Ca^{2+} and decreased to 43.5 ± 10 ms and 65.5 ± 16 ms ($n = 9$, ns). No modification of time to peak and half relaxation time was observed from 14 to 60 days after the injection of bupivacaine-injected EDL muscles, in the absence of Ca^{2+}.

With longer time after bupivacaine injection in adult rat, twitch tension was less affected by the absence of external Ca^{2+} and was reduced by 30 ± 13% ($n = 10, P < 0.05$) and only by 6 ± 0.3% ($n = 6$, ns) at 14–30 days and 60 days postinjection, respectively. In solutions containing no added calcium, a decrease of tetanic tension was also observed (Fig. 4A). Compared with tetanic tension developed in control medium, the amplitude of the response was diminished by 74 ± 19% ($P < 0.03, n = 4$) in 7 days postinjected muscles. At 60 days after bupivacaine injection, tetanic tension was only reduced by 20 ± 4% (ns).

Effects of Divalent Cations on Twitch and Tetanic Tensions. Substitution of 2 mmol/L^{-1} Mg^{2+} for Ca^{2+} in the external solution ($[Ca^{2+}]_0$ = 1 mmol/L^{-1}) resulted in a decrease of 38% ($P < 0.01$, 7 days postinjected) of the twitch maximum tension compared to the one recorded in control medium ($[Ca^{2+}]_0$ = 3 mmol/L^{-1}, $[Mg^{2+}]_0$ = 2 mmol/L^{-1}) 7 days after the injection of bupivacaine. The decrease of the amplitude of the twitch tension was of 15% 14 days after the injection and slight modifications of the amplitude of the twitch were observed after this date.

In developing rat EDL muscle, the use of Sr^{2+} as a substitute for Ca^{2+} allows the muscle to develop tension. The replacement of Ca^{2+} (3 mmol/L^{-1}) by Sr^{2+} (3 mmol/L^{-1}) was tested in regenerating muscles. The amplitude of the contractile response elicited in nominally Ca^{2+}-free medium (Sr^{2+}-substituted) was decreased by 18% ($P < 0.05$) in 7 days injected muscles.

In all groups of muscles whatever the time after the injection, increasing $[Ca^{2+}]_0$ to 6 mmol/L^{-1} (no Mg^{2+} added) resulted in a decline of twitch amplitude of 10–21%. This depression probably resulted from a reduced mechanical inactivation and could be attributed to a less negative surface potential due to the higher divalent cation concentrations.[11]

The possibility that inflow of Ca^{2+} occurred upon depolarization was tested by studying the effect on the contractile response of perfusing EDL muscle preparations in the presence of inorganic cations: cobalt (Co^{2+}, 5 mmol/L^{-1}) and cadmium (Cd^{2+}, 2 mmol/L^{-1}). Addition of 5 mmol/L^{-1} Co^{2+} to the perfusing solution resulted in a reduction of twitch amplitude by 71 ± 7% ($P < 0.01, n = 5$) in 7 days injected muscles. In those muscles, the presence of Co^{2+} induced a depression of the tetanic tension by about 60% ($P < 0.01$) (Fig. 4B).

In the presence of 2 mmol/$L^{-1}$$Cd^{2+}$, both twitch (not illustrated) and tetanic (Fig. 4C) tensions were potentiated in 7–14 days postinjected muscles by 66 ± 9% ($P < 0.01$) and 22 ± 4% ($P < 0.05$), respectively.

Posttetanic Potentiation. The measured values of PTP (108 ± 6%, $n = 5$) in control EDL muscles were characteristic of fast-twitch skeletal muscles. Bupivacaine-injected muscles presented no significant difference for PTP compared to control EDL muscles. PTP was indeed 104 ± 9% ($n = 15$), 105 ± 2% ($n = 10$), and 109 ± 7% ($n = 7$) in EDL muscles at 7, 14, and 60 days after BPVC injection, respectively.

Fatigue. The tension of the 10th successive tetanus was considerably reduced compared to the initial one in control and in 90 days injected EDL muscles (Fi:22 ± 13% and 29 ± 20%, $n = 5$, ns, respectively).

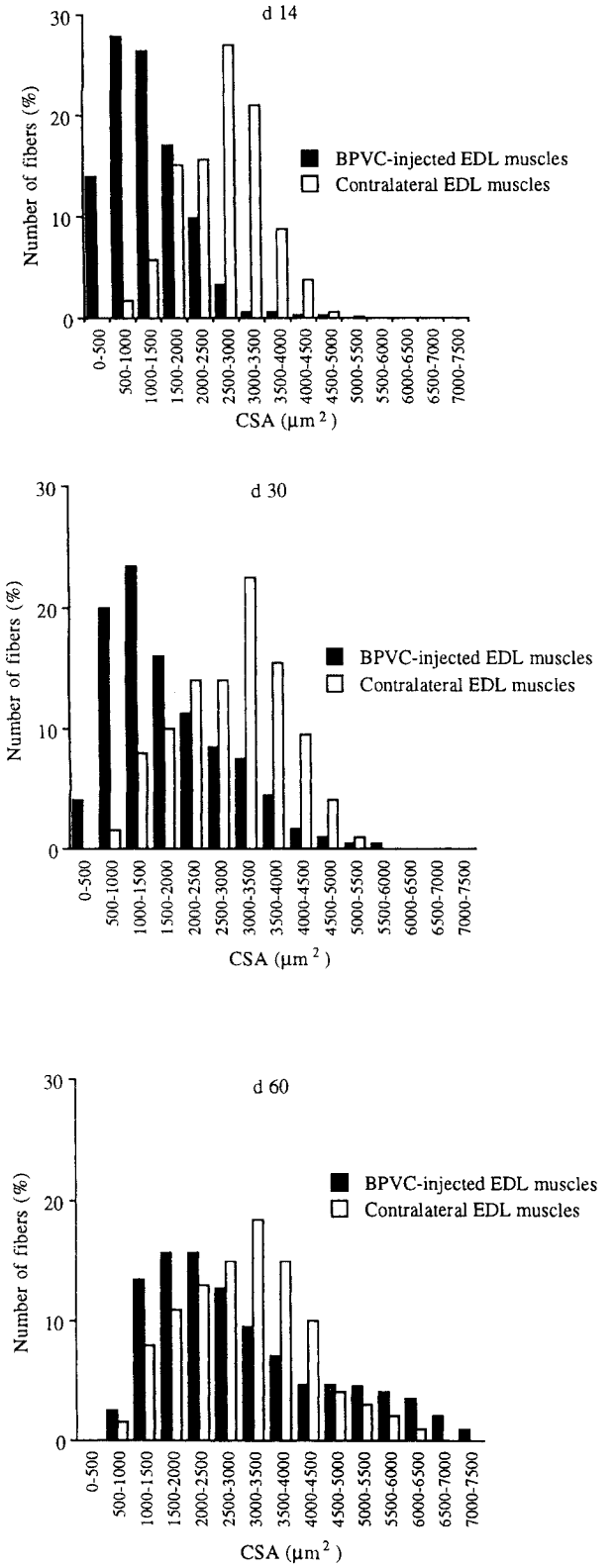

FIGURE 2. Frequency distribution of fiber cross-sectional area (CSA) in control and bupivacaine-injected EDL muscles. Diameter distribution of bupivacaine-injected EDL muscle was shifted toward smaller fiber CSA respectively 14 and 30 days after the injection. By 60 days, after the injection, the frequency distribution of the fiber CSA was not different from the control one.

Table 2. Normalized tetanic (Po) and twitch (Pt) tension developed by bupivacaine (BPVC)-injected EDL muscles at different times postinjection ($5 < n < 10$).

Time (days)	Po (mN/mg muscle weight)		Pt (mN/mg muscle weight)	
	Control	BPVC	Control	BPVC
7	14.7 ± 0.5	7.6 ± 0.3	2.7 ± 0.3	0.9 ± 0.1
14	15.3 ± 0.3	8.9 ± 0.9	2.8 ± 0.2	1.8 ± 0.2
30	15.7 ± 0.3	9.4 ± 1.1	3.1 ± 0.1	2.3 ± 0.1
60	16.9 ± 0.9	10.3 ± 0.7	3.2 ± 0.3	2.5 ± 0.2
90	18.3 ± 0.9	10.5 ± 0.9	3.3 ± 0.2	2.7 ± 0.3

Control values were measured on the controlateral muscle. Time is expressed in days postinjection.

These low values of Fi are a normal characteristic of fast-twitch muscle because of their lack of resistance to fatigue and furthermore suggest that this property was present after 90 days postinjection.

Glycerol-Treated Muscles. After the perfusion with control solution containing 400 mmol/L^{-1} glycerol (hypertonic medium), a transient contracture occurred in all muscles. In 14–60 days post-injected muscles, the amplitude of this contracture was reduced by 87% compared to the one developed at 7 days postinjection ($P < 0.01$).

In control muscles perfused in hypertonic solution for 60 min, the amplitude of the twitch was reduced by 98% and on return to isotonic solutions, twitch tension was abolished. In injected muscles perfused in hypertonic solution for 60 min, the amplitude of the twitch was either enhanced by 32% ($P < 0.01$), or reduced by 55% ($P < 0.01$) and 92% ($P < 0.01$) in 7, 14, and 60 days post bupivacaine injected muscles, respectively.

In regenerating muscles, on return to isotonic solutions (detubulated fibers), twitch tension was abolished except in 7 days postinjected muscles where twitches could still be elicited with amplitude about 39% of the control ($P < 0.01$).

Effect of Caffeine. The caffeine contracture technique bypasses the electrical excitation mechanism and directly tests the ability of the SR to release calcium and the contractile response. By 7 days after bupivacaine injection, exposure of the regenerating EDL muscles to solutions containing 2.5 mmol/L caffeine resulted in a contracture that was about 7.7% of Po, while the 60 days postinjected EDL muscle developed a contracture that was only 2.2% of Po. To avoid any damage in the muscle cell structure

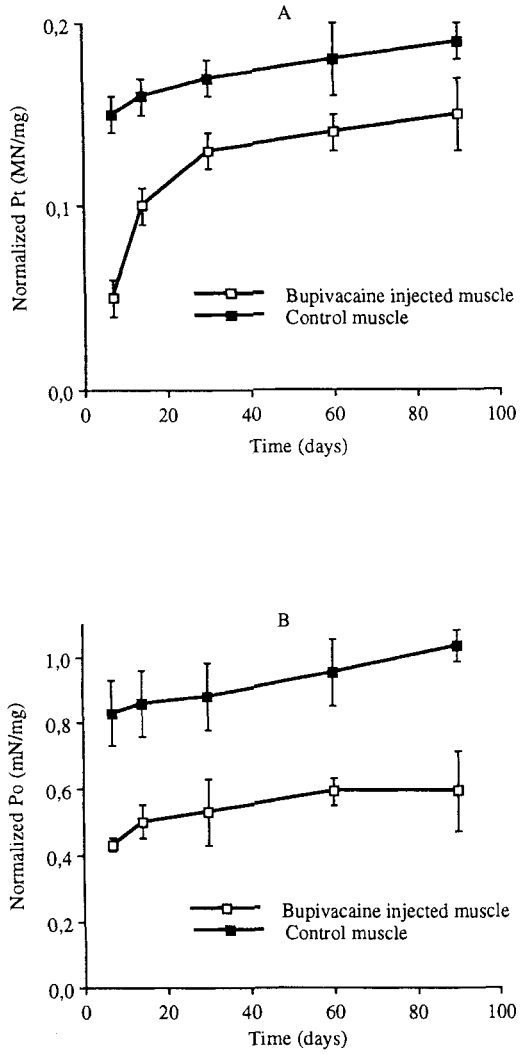

FIGURE 3. Evolution of the normalized twitch **(A)** and tetanic **(B)** tensions in control and bupivacaine-injected EDL muscles.

due to the application of high caffeine concentration, the dose–response curve was obtained only for concentrations lower than 20 mmol/L. However, the fact that on linear coordinates, the strength of the contractures was related to the caffeine concentration by a rectangular hyperbola, and on double reciprocal coordinates by a straight line, suggested that caffeine was involved in a first order saturable reaction.[28] Then assuming Michaelis–Menten kinetics, the double reciprocal plot yielded a mean half-saturation constant (K_{caf}) that could be estimated.[28] The calculated K_{cat} of the regenerating EDL muscle 7 days postinjection (13.7 ± 5.1 mmol/L) was reduced compared to muscles 60 days postinjection (36.1 ± 1.6 mmol/L, $P < 0.01$). Even if such an approach might be subject to criticism, it could give a quantitative aspect of the degree of sensitivity to caffeine and be used to compare data. The results obtained 60 days after bupivacaine injection were characteristic of adult muscle. When EDL muscle was exposed to 2.5 mmol/L caffeine, twitch was potentiated by 250 ± 13% and 150 ± 10% of initial tension 7 days and 60 days after injection, respectively. The study of iterative caffeine contractures (elicited every 20 min) has shown that, as in the contralateral muscle, whatever the caffeine concentration (2.5–20 mmol/L) used, there was little variation between the amplitude of the third and the first contracture (−18 ± 11%) in 60 days postinjected muscles. By contrast, in 7 days postinjected EDL muscles, the tension developed by the third contracture was markedly decreased and averaged 38 ± 3% of the first response. For the same period of time (7 days) after injection, the 10 mmol/L caffeine contracture tension was reversibly reduced by 67 ± 4% when withdrawing external Ca^{2+} (10 min exposure). By 60 days postinjection, only a slight diminution of the contractile response in the absence of external Ca^{2+} (16 ± 1%) could be observed (contralateral muscle: 15.2 ± 1.5%).

DISCUSSION

The present work has clearly shown that, by contrast with the situation in mature EDL muscle, in regenerating muscles, removal of Ca^{2+} led to reversible depression of twitch and tetanic tensions. As it has been recently reported[6,29] that twitch tension of 1–7

Table 3. Effects of 0 mmol/L Ca on twitch and tetanic tensions in bupivacaine-injected EDL muscle.

	Twitch tension (mN/mg)		Tetanic tension (mN/mg)	
Time (days)	3 mmol/L Ca	0 mmol/L Ca	3 mmol/L Ca	0 mmol/L Ca
7	0.9 ± 0.1	0.4 ± 0.1	7.6 ± 0.3	1.9 ± 0.3
14	1.8 ± 0.2	1.2 ± 0.2	—	—
30	2.3 ± 0.1	1.6 ± 0.2	—	—
60	2.5 ± 0.2	2.4 ± 0.3	10.3 ± 0.7	8.2 ± 0.7
90	2.7 ± 0.3	2.6 ± 0.2	10.5 ± 0.9	8.4 ± 0.9
Control values	3.3 ± 0.2	3.2 ± 0.2	18.3 ± 0.9	17.8 ± 1.6

Time is expressed in days postinjection. Control values were measured in contralateral muscles. Data are given as mean ± 1 SD.

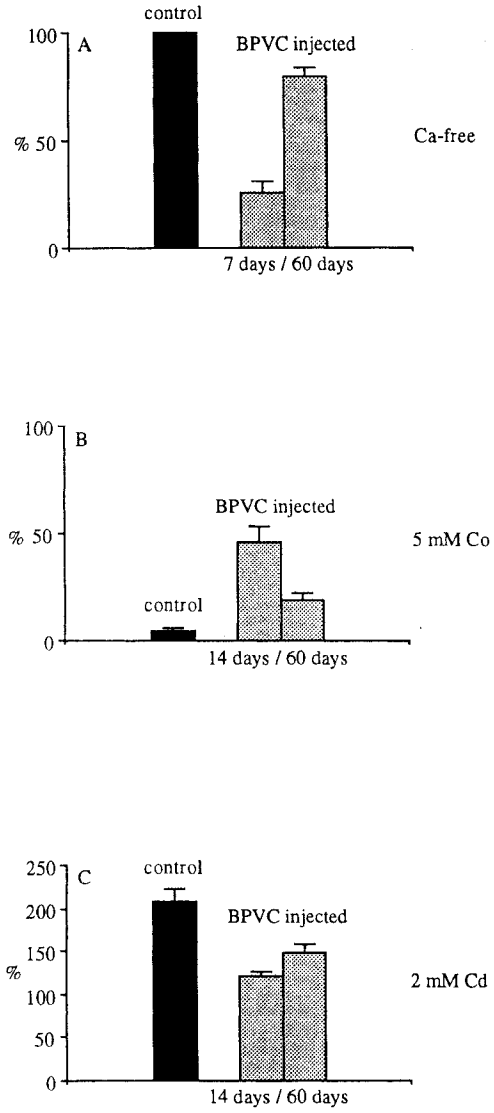

FIGURE 4. Effects of Ca-free (5 mmol/L^{-1} $[Mg]_o$, **A**), 5 mmol/L^{-1} Co^{2+} **(B)**, and 2 mmol/L^{-1} Cd^{2+} **(C)** on the tetanic tension developed by EDL muscles after bupivacaine injection at 7 days **(A)**, 14 days **(B, C)**, and 60 days **(A, B, C)** postinjection. Results are compared with those obtained in control (black columns) muscles. Each muscle was used as its own control. Thus reference to a percentage decrease relates to the results obtained in normal medium. Error bars show SD of the mean.

days postnatal muscles was diminished by reducing $[Ca^{2+}]_o$ or adding inorganic divalent cations, whereas in the oldest animals (14–90 days postnatal) twitches were unaffected, our functional data support the idea that muscle regeneration reiterates the steps of normal myogenesis. The external Ca^{2+} dependence of the contraction was also observed in the model of muscle regeneration presently studied. Nevertheless this $[Ca^{2+}]_o$ dependence was transitory in bupivacaine-injected muscles and, thus, could involved muscle innervation in this phenomenon. By contrast, an external Ca^{2+} dependence of the contraction was also observed in notexin-injected soleus muscle, a model in which the terminal innervation is distroyed by the venom. Indeed, in bupivacaine-injected preparations, intramuscular nerve remained intact and neuromuscular junctions appear normal.[19,22,34] Furthermore, in EDL muscles denervated at 1 day postnatal, depression of twitch tension following Ca^{2+} withdrawal was more prolonged after sciatic nerve transection than after nerve crush where reinnervation was faster (Louboutin JP, unpublished results). It could then be suggested that temporary removal of neural influence during muscle regeneration might lead to a persistent susceptibility to $[Ca^{2+}]_o$ of contractile responses developed by regenerated fibers.

The present study confirmed that normal fiber size could be observed 30 days after bupivacaine injection and that completed recovery of regenerated fiber size was observed 2 months postinjection.[16,31,33] In the present study, differentiation of regenerating fibers into type I and II was observed 14 days after bupivacaine injection.[1,19,32] However, from 30 to 90 days (longest time presently studied) after injection, the percentage of type I fibers remained larger than in control muscles. In the work reported by Hall-Craggs and Seyan,[19] the distribution of slow and fast histochemical fiber type was considered similar in control and injected muscles. However, these authors did not perform morphometric studies and the small difference (less than 3%) of the number of type I fibers between both groups of muscles (Ref. 32, present study) is probably difficult to appreciate by simple observation in a cross-section of an entire muscle. Furthermore, the results concerning the posttetanic potentiation and the fatigue in 90 days postinjected EDL muscles were characteristic of fast-twitch skeletal muscle.

Central location of myonuclei is usually considered a marker of fiber damage and regeneration.[32,35] In the present work, a progressive decrease in the percentage of central myonuclei from 84% to 35% was observed between 3 and 60 days after bupivacaine injection. With a single injection of 0.5% bupivacaine, less numerous central myonuclei (14%) have been reported 2 months postinjection.[31,32] In that last study, the rate of regeneration was faster than in the present report. Eighty days postinjection, the normalized isometric tension developed by injected muscles was already 73% of the one measured in control EDL muscles, whereas in the present work, 90 days after injection, normalized tetanic tension was only 57% of control. By contrast, addition of hyaluronidase to

bupivacaine resulted in a vast majority of fibers with central nuclei still present 3 weeks postinjection.[16]

In the present study, an attempt was made to characterize some of the contractile properties of regenerated EDL muscles following bupivacaine injection. In muscles 90 days after bupivacaine injection, although the normalized twitch tension (Pt) was already about 80% of the control, the normalized tetanic tension (Po) was significantly less than in control EDL muscles. In that respect, regeneration of bupivacaine-injected muscles is confirmed to be more efficient than in grafted muscles, where revascularization and reinnervation have to occur concomitantly. In autotransplanted and sliced muscles, both parameters, Pt and Po, were markedly reduced even after 3 months of regeneration.[4,5]

In the present experiments, potentiation of twitch and tetanic tensions developed by bupivacaine-injected muscles was observed in the presence of Cd^{2+}. Similar results were obtained in adult EDL muscles.[29] Although Cd^{2+} could reduce the negative charged site density on the external surface of the membrane, the enhancement of contraction cannot be interpreted by a positive shift in the tension–voltage relationship. In adult rat soleus, Dulhunty and Gage[8] suggested that divalent cation could bind a "precursor" molecule accessible from the T-tubule lumen which modulates conformational changes to active and inactive states, or alternatively induce a direct activation of the contractile proteins (for detailed discussion, see Louboutin et al.[25] and Péréon et al.[29]).

In the present study, twitch tensions of long-term regenerated EDL muscles were abolished after glycerol treatment. By contrast, this procedure only reduced, but did not eliminate contractions developed by muscles recently injected (7 days previously). Different changes of skeletal muscle internal structure have been reported to occur following glycerol treatment, such as rupture and constriction of the T-tubules. Furthermore, the disruption of the junction between T-tubules and terminal cisternae of the SR, essential for EC coupling,[7,21,27] may be involved in contractile abolition. The persistence of developed tension in 7 days postinjection muscles after glycerol treatment might be due to the presence of peripheral coupling not affected by the detubulating procedure and/or to a reduced susceptibility of the T-tubules to the hypertonic medium. In that respect, in EDL muscles 7 days postnatal, twitches could still be elicited following glycerol treatment (Louboutin JP, unpublished results) and it is known that during development, direct peripheral couplings between the SR and the sarcolemma are transiently formed.[23]

The present work demonstrates that caffeine contractures could be elicited in rat EDL muscles during the regenerative processes following BPVC injection. Furthermore, immature muscles were more sensitive to caffeine at 7 days postinjection than at 60 days. Caffeine is known to potentiate the twitch and to induce contracture via SR Ca^{2+} release and increased Ca^{2+} sensitivity of the myofilaments.[36] SR membranes are poorly developed in immature skeletal muscle fibers[12,13,26] and although myofibrillar Ca^{2+} responsiveness is constant during development of fast-twitch muscle,[24] it cannot be excluded that a greater sensitivity of the myofilaments to caffeine is present in immature muscles. On the other hand, the increase in apparent caffeine sensitivity of immature EDL muscles could be related to the slowing of the Ca^{2+} uptake by the SR,[14] resulting in a reduced efficiency at buffering the Ca^{2+} load evoked in the presence of caffeine. This may also account for the marked decrease in the amplitude of contractures when caffeine is repeatedly applied to immature muscles at the early stage of regeneration, whereas there is no significant change in the amplitude of iterative caffeine contracture after a longer time postinjection. The present work shows that caffeine contractures behave in the same way as twitch and tetanic tensions and are dependent upon $[Ca^{2+}]_o$ during muscle regeneration. In conclusion, during early stages of regeneration, external Ca^{2+} may play a fundamental role due to the poorly developed and not yet fully differentiated state of the SR or to different isoforms of the dihydropyridine receptor expressed in the T-tubule membrane that might conduct Ca^{2+} to directly activate the contraction.

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