J Physiol 586.19 (2008) pp 4763–4774

The Journal of Physiology

Expression of small-conductance calcium-activated potassium channels (SK3) in skeletal muscle: regulation by muscle activity

Morgana Favero[1,3], De-Jian Jiang[2], Christian Chiamulera[2,3], Alberto Cangiano[1,3] and Guido Francesco Fumagalli[2,3]

[1] *Department of Neurological and Visual Sciences, Section of Physiology, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy*
[2] *Department of Medicine and Public Health, Section of Pharmacology, University of Verona, P.le L.A. Scuro 10, 37134 Verona, Italy*
[3] *National Institute of Neuroscience, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy*

The type 3 small conductance calcium-activated potassium channel (SK3) is expressed in embryonic and adult denervated skeletal muscles where it contributes to hyperexcitability. This study aimed at determining the role of muscle activity in regulating SK3 channels. Soleus muscles of adult rats were denervated by cutting the sciatic nerve. In reinnervation studies, the soleus nerve was crushed: in one group, muscles were reinnervated with electrically silent axons, by chronic sciatic nerve perfusion with tetrodotoxin. Several groups of denervated muscles were subjected to chronic direct electrical stimulation, using either fast (100 Hz) or slower patterns (20 or 30 Hz). The SK3 mRNA and protein levels in soleus muscle were determined by reverse transcriptional-PCR, Western blot and immunofluorescence. Both denervated and reinnervated–paralysed soleus muscles displayed similar up-regulation of SK3 mRNA and protein. Reinnervation with electrically active axons instead inhibited SK3 up-regulation. Chronic muscle direct stimulation *in vivo,* irrespective of the pattern used, reversed the denervation-induced up-regulation of SK3 expression or prevented it when initiated at the time of denervation. Chronic electrical stimulation of denervated muscles also completely prevented the development of the after-hyperpolarization (AHP) following the action potential, normally induced in the muscle fibres by denervation. We conclude that action potential activity evoked by motor neurones in muscle fibres is both necessary and sufficient to account for the physiological down-regulation of SK3 channels in the non-junctional membrane of skeletal muscle.

(Received 9 May 2008; accepted after revision 12 August 2008; first published online 14 August 2008)
Corresponding author A. Cangiano: Dipartimento di Scienze Neurologiche e della Visione, Sezione di Fisiologia, Universitá di Verona, Strada Le Grazie 8, 37134 Verona, Italy. Email: alberto.cangiano@univr.it

Calcium-activated potassium channels are an heterogeneous family widely distributed in neurones and peripheral tissues, controlling repetitive firing in excitable cells and secretion in exo- and endocrine cells, activated by elevations in cytosolic calcium and by depolarization (for review: Blatz & Magleby, 1987; Sah, 1996; Vergara *et al.* 1998). A subset of this family are the small conductance ones (SK), first recorded at the single channel level by Blatz & Magleby (1986), voltage independent and found to underlie the long lasting after-hyperpolarization (AHP) following the action potential and its accompanying elevation in cytosolic calcium (Meech, 1978; Barrett *et al.* 1981; Sah, 1996; Xia *et al.* 1998). Based on these properties, SK channels transduce variations in intracellular calcium into changes in membrane potential and play diverse roles in modulating firing and responsiveness to synaptic input in different CNS neurone types (Hille, 2001; Cangiano *et al.* 2002; Bond *et al.* 2005).

SK channels are comprised of several types (SK1, SK2, SK3) encoded by specific genes (Kohler *et al.* 1996). While normally present in many neurone types, and well expressed in myofibres of developing muscles, they are down-regulated in normal, i.e. adult, muscle, to be up-regulated again following denervation (Schmid-Antomarchi *et al.* 1985). In particular, the channel type expressed in myotubes cultured alone (i.e. non-innervated) or in denervated muscle fibres is the SK3 (both mRNA and protein) (Pribnow *et al.* 1999; Roncarati

M. Favero and D.-J. Jiang contributed equally to this work.

DOI: 10.1113/jphysiol.2008.156588

et al. 2001). The calcium activating the SK channel of skeletal muscle, unlike in neurones, does not come from the outside through surface membrane channels, rather it is released from the sarcoplasmic reticulum when the action potential sweeps the muscle fibre (Neelands *et al.* 2001).

This SK3 channel type is characterized, along with the SK2 not expressed in muscle, by specific binding with the bee venom apamin (and thus also known as apamin receptor, Schmid-Antomarchi *et al.* 1985), resulting in a block of its conductance, as shown by suppression of single channel activity (Blatz & Magleby, 1986) and of the slow AHP following the action potential (Hugues *et al.* 1982; Romey & Lazdunski, 1984; Kohler *et al.* 1996; Pribnow *et al.* 1999). Denervated muscles are hyperexcitable as they display trains of spontaneous action potentials known as fibrillation (Tower, 1939; Purves & Sakmann, 1974*a*,*b*; Thesleff, 1974; Thesleff & Ward, 1975) and apamin also blocks this activity (Vergara *et al.* 1993; Jacobson *et al.* 2002). Similar hyperactivity causes muscle stiffness in human myotonic syndromes, in the most common of which (myotonic dystrophy, DM), apamin receptors are present (Renaud *et al.* 1986) and the spontaneous firing is blocked by apamin (Behrens *et al.* 1994).

Although, as explained above, it is clear that SK3 channel expression in the extra-junctional membrane of skeletal muscle is under physiological inhibitory control from the innervating motor neurones, the key question as to the nature of the neural signals involved, is still unresolved. As far as the non-junctional muscle properties are concerned, encompassing membrane receptors and ion channels as well as the contractile apparatus, rigorous experimental demonstrations have been presented about the essential role of electrical activity in their control (for review see Lømo, 2003; see also Methods and Discussion). In spite of this, for a long time the idea has persisted that action potential activity evoked in muscle by motor neurones, may not entirely explain the physiology of the regulation of non-junctional properties, and that unidentified chemical factors or the neurotransmitter itself, acetylcholine, take part in this regulation (see for review Lømo, 2003; Kalhovde *et al.* 2005).

In this paper we directly address this question by focusing on SK3 channels. Previous reports by one group of authors indeed suggested that both nerve-released 'trophic' factors and nerve-evoked muscle activity may participate in this control (Behrens & Vergara, 1992; Vergara *et al.* 1993; Ramirez *et al.* 1996) but were not conclusive. In our study, we specifically addressed the problem of whether or not muscle-evoked activity is the essential regulatory factor of SK3 mRNA and protein expression, through experiments of reinnervation, chronic nerve conduction block and direct electrical stimulation following denervation, using the rat soleus muscle.

Methods

Animals and surgery

The experiments were done on adult male Wistar rats (300–350 g body weight, Charles River, Italy). The experiments were authorized by the Istituto Superiore di Sanita′ and the Ministry of Health of Italy and all procedures were supervised by the local veterinary. Surgery was performed under equithesin anaesthesia (0.4 ml 100 g^{-1}, 9.7 mg/ml sodium pentobarbital and 42.5 mg/ml cloralium hydrate, I.P.), and was used both initially to establish the various conditions described below, and again at the time of the acute experiment (after several days of chronic treatment *in vivo*) when the soleus muscles were dissected out for biochemical or electrophysiological measurements. After muscle removal, while maintaining the anaesthesia, the rats were killed by cervical dislocation. For denervation purposes, a ∼10 mm segment of the sciatic nerve was removed, at the mid-thigh level. For blocking conduction, a sciatic nerve segment was freed from surrounding tissues and placed inside a silicone cuff to which tetrodotoxin (TTX) was continuously delivered (see next section for details). For reinnervation experiments, the soleus nerve was crushed with thin forceps at its entrance into the muscle belly. Reinnervation started quickly, around the third to fourth day after the crush, and proceeded rapidly so that on day 12 almost the entire soleus muscle was reinnervated: this is based both on previous data (Sala *et al.* 1995) and on a short series of preliminary experiments.

Electrical stimulation and TTX treatment

In planning the experiments, we took into account the indications of previous work about the ability of different patterns of stimulation in counteracting the changes induced by denervation in the membrane of the muscle fibres (Lømo & Westgaard, 1975) and about the efficacy of pure muscle paralysis in reproducing those changes. This planning also included attention to the fact that denervation causes, in addition to paralysis, the transient appearance in the interstitium between the muscle fibres of the breakdown products of the degenerating axons, which were shown to have a synergistic effect with inactivity in inducing the membrane changes (Jones & Vrbová, 1974; Lømo & Westgaard, 1975, 1976; Cangiano & Lutzemberger, 1977, 1980; Brown *et al.* 1978; Cangiano *et al.* 1984; Arancio *et al.* 1992; Pasino *et al.* 1996). To avoid interference from the effects of nerve degeneration, which are rapid and transient, one must: (1) for the chronic stimulation experiments, continue the procedure up to a time point which is beyond the duration of the bulk of these effects (in practice 12 days were sufficient) (Lømo & Westgaard, 1976), and (2) for the experiments comparing denervation with chronic conduction block,

to apply the latter to axons regenerating after a crush: this way the blocked side is quickly reinnervated, without return of muscle activity, and one can compare the effect of conduction block *per se* with that of denervation (Pasino *et al.* 1996).

For the chronic *in vivo* electrical stimulation experiments, rats were bilaterally denervated and electrodes were implanted on one denervated leg and connected to an external stimulator as previously described (Windisch *et al.* 1998). The contralateral side served as denervated control. Briefly, the end of two multi-stranded steel wires (AS-632, Cooner Wire, Chatsworth, CA, USA) with their Teflon insulation removed for the distal 20 mm, were placed in contact with the soleus in a proximo-distal direction, one on the anterior and the other on the posterior side. A Master 8 pulse generator (AMPI, Israel) delivered the stimulus pattern to a 4-channel constant current stimulator (Basile, Comerio, Italy), whose output were rectangular pulses 200 μs in duration of alternating polarity. No drop in current intensity during the train of stimuli, due to electrode polarization, was ever observed with the values used (20–25 mA in 80% of the rats; up to 50 mA, in the remainder). No signs of inflammation were generally observed, except in two muscles that were not included; lack of damage was also confirmed by the electrophysiological recordings from surface muscle fibres and by contractile tension measurements (see Results). The value used in each rat was 4 × the threshold (Th) intensity for inducing the extension of the foot, checked and re-adjusted daily. In fact, in preliminary experiments a ∼3 × Th intensity was found to induce maximal twitch and tetanic contractions of denervated soleus muscles for a few days, as measured with a strain gauge *in vivo* (Cyber Amp 320, Axon Instruments, CA, USA). The patterns of stimulation used were: (i) a fast pattern, i.e. 100 pulses at 100 Hz for 1 s every 100 s, (ii) a slow pattern, i.e. 100 pulses at 30 Hz for 3.33 s every 100 s; both applying 86 400 stimuli day^{-1}, and (iii) a second slow pattern, using a somewhat lower frequency and twice as many stimuli per day, 172 800, i.e. 100 pulses at 20 Hz for 5 s every 50 s. The stimulation lasted 6 or 12 days, as detailed in the Results.

Paralysis of leg muscles was obtained by continuously (12 days) infusing a TTX solution in saline (9 μg day^{-1}) into a silicone cuff (length, 8 mm; i.d., 1.5 mm; o.d. 6.5 mm) placed around the sciatic nerve, through a tubing connected to a subcutaneous osmotic pump (Alzet, Palo Alto, model 2002): the cuff allows optimal nerve perfusion (details in Pasino *et al.* 1996). Simultaneously the soleus nerve was crushed at the muscle entrance. Nerve block and muscle paralysis were checked daily once reinnervation had begun: spread of the digits or foot flexion and extension were absent, as well as any reaction to pinching of the foot. Further controls were made at the end of the chronic block (see Results).

Electrophysiology

In one group of rats, soleus muscles of both sides (12 days denervated + stimulated on one side and denervated alone on the other side), were dissected free and placed in a chamber under oxygenated saline solution. Recordings were made using a Duo 773 Electrometer (WPI): each myofibre was impaled with two micropipettes, a short distance apart (∼50 μm), one used to inject current and the other to record the electrical response (details in Pasino *et al.* 1996). The action potential was evoked by anodal break stimulation (hyperpolarizing pulse of 300 ms duration) and the resting membrane potential (RMP) was maintained at −70 mV in each fibre by injecting DC current. Some fibres were also recorded after bath application of 1 μM apamin (Sigma, Milano, Italy). Recordings were made from first layer myofibres, and data stored on computer with Axotape and processed by Clampfit.

Western blots

For Western blots, 100–150 mg of muscle tissue was homogenized at 4°C using a tissuemizer in 3 ml homogenization buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA) supplemented with 2 mM phenylmethanesulphonylfluoride (PMSF), 2 μg ml^{-1} aprotinin, pepstatin and leupeptin. The sample was centrifuged for 30 min at 20,800g. at 4°C and the supernatant was discarded. The pellet was resuspended in 100 μl of extraction buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 2 mM EDTA, 2 mM EGTA) freshly supplemented with 2 mM PMSF, 2 μg ml^{-1} aprotinin, pepstatin and leupeptin. Triton X-100 was added to a 1% final concentration; the sample was incubated for 1 h at 4°C and then centrifuged for 30 min at 20,800g at 4°C. The supernatant was retained for analysis. Total protein concentration was determined using the Dc protein assay kit. Samples containing equal amounts (100 μg) of total protein were loaded and separated on 8% SDS-polyacrylamide gel electrophoresis and then transferred on to PVDF membrane. The blots were then washed with Tris-buffered saline–Tween (TBST, 50 mM Tris base, 150 mM NaCl, and 0.05% Tween 20) containing 5% dry milk powder. The same solution was used to dilute antibodies. All incubations were done at room temperature. Blots were incubated for 2 h with the rabbit polyclonal antibody for SK3 (1 : 1000 dilution); after 30 min washes, blots were incubated for 1 h with goat anti-rabbit horse-radish peroxidase (1 : 2000 dilution) and then washed before processing with the ECL kit (Amersham). Band intensities were acquired with a densitometer and data were analysed using the Scion image software (NIH). Values of SK3 expression at day 6 or day 12 after denervation were set to 100.

Immunofluorescence

For immunofluorescence analysis, anaesthetized animals were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Muscles were dissected, infiltrated with 12–18% sucrose and frozen in liquid nitrogen. Twelve micrometre-thick sections were cut in a cryostat and collected on gelatin-coated slides. Sections on slides were washed in PBS at room temperature and permeabilized in PBS containing 0.5% Triton X-100 and 0.5% bovine serum albumin. The same solution was used to dilute immunoreagents and to rinse specimens. All incubations were done at room temperature. The rabbit polyclonal antibody for SK3 was used at a 1 : 1000 dilution. Samples were incubated with primary antibodies for 2 h; after a 30 min rinse, samples were incubated with Cy3-conjugated goat anti-rabbit antibodies (1: 1000 dilution). For a better visualization of the membrane expression of the SK3 channels, we used fluorescein isothiocyanate (FITC)-conjugated concanavalin A (Con A; 1 : 5000 dilution) to label membrane glycoproteins, which is independent of the state of innervation of muscle. After final washes in PBS, preparations were mounted on the anti-fading DABCO (Sigma) in PBS containing 50% glycerol. All preparations were viewed with a Zeiss LSM 510 confocal microscope equipped with argon (488 nm) and helium–neon (543 nm) excitation beams.

Reverse transcriptional-PCR (RT-PCR)

Total RNA was extracted from tissue using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of RNA were determined by the absorbance at 260 and 280 nm. Semi-quantitative RT-PCR was performed according to standard techniques. The specific primer pairs and the PCR fragment size of SK3 were as followed: 5′-CAAGTGTCCCTGTCCATCTTCTG-3′ (forward) and 5′-CCAAGTGGTCATTGAGATTTAGC-3′ (reverse) (356 bp). The PCR amplification profiles consisted of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 20 s. The linear exponential phases for SK3 PCR were 25 cycles. Equal amounts of corresponding SK3 RT-PCR products were loaded on 2% agarose gels. Optical densities of ethidium bromide-stained DNA bands were quantified and the values of INN were set to 1.

Statistical analysis

Results are expressed as mean ± S.E.M. Data were analysed by one-way ANOVA followed by Dunnett's or unpaired Student's *t* test for multiple comparisons. The significance level was chosen as $P < 0.05$.

Results

Effect of denervation on SK3 expression in soleus muscle

The soleus muscles from both denervated and intact control sides were removed at different time points after denervation (at least 3 subjects for each time). mRNA and protein levels of SK3 were detected by RT-PCR and Western blots, respectively. As shown in Fig. 1*A*, SK3 mRNA had a low level in innervated (INN)muscle and was significantly elevated after denervation, reaching a steady level at day 6 after denervation (∼2.5 × INN). Similarly, expression of the SK3 protein, which was undetectable in innervated control muscle, was markedly elevated after denervation and reached a steady level at day 12 after denervation (Fig. 1*B*).

Effect of reinnervation on SK3 expression in soleus muscle

As in our previous report (Sala *et al.* 1995), early signs of reinnervation were detected 3–4 days after the nerve crush; approximately 90% of the fibres were reinnervated at the ninth day and complete reinnervation was seen at day 16. The expression of SK3 protein was significantly increased up to the sixth day after the crush and then sharply declined. At the ninth day, the expression of SK3 protein was only ∼6% of that at the sixth day ($P < 0.01$, Fig. 2). Thus, reinnervation by electrically active axons markedly down-regulated SK3 expression.

Effect of nerve block by TTX on SK3 expression in reinnervated soleus muscle

The type of nerve block used in this group of experiments has been specifically investigated by us in a previous study, and shown to insure a complete blockade of impulse conduction without any signs of axonal damage or of impairment of axonal transport (Pasino *et al.* 1996). Furthermore, in the three rats in which the reinnervation–paralysis protocol was investigated for SK3 expression on day 12 after soleus nerve crush, a complete TTX block and a well developed reinnervation were detected *in vivo* on the same day: electrical stimulation of the nerve proximal to the cuff evoked no contractions while distal stimulation evoked vigorous contractions of leg muscles including the soleus. The amount of reinnervation was estimated by measuring in these muscles the isometric tetanic contraction following supramaximal nerve stimulation distal to the block (1 s train, 50 μs pulses at 20 Hz), and comparing it with similar contraction elicited by direct stimulation (same type of train, 200 μs pulses) in denervated soleus muscles (of this and other groups). The tension developed was similar,

indicating that an essentially complete reinnervation by the impulse-blocked axons had occurred and that no damage was induced by the cuff (69.01 ± 2.87 and 63.51 ± 9.48 g, 3 reinnervated–paralysed and 6 denervated muscles, respectively; amount of atrophy also comparable, muscle weight being 118.33 ± 4.37 and 125.33 ± 6.11 mg, respectively; differences not significant. By comparison, three denervated 12-day-stimulated muscles at 20 Hz, had a tetanic contraction of 152.88 ± 8.13 g and muscle weight of 158.33 ± 2.03 mg; difference significant at $P < 0.05$ with respect to both preceding groups). These contractile and atrophy data confirm those obtained in a previous study (Buffelli *et al.* 1997).

As shown by RT-PCR, the SK3 mRNA level was significantly increased in the soleus muscles examined 12 days after nerve crush and reinnervated with axons subjected to chronic TTX conduction block (TTX-REINN), with respect to innervated (INN) muscles (Fig. 3*A*). The SK3 protein expression was also increased in the same muscles, as shown by Western blot and immunofluorescence (Fig. 3*B* and *C*). Most important, both mRNA and protein levels were comparable to those after 12 day denervation (contralateral muscles, DEN). Thus, reinnervation by electrically silent axons had no effect on SK3 expression (in contrast to the marked down-regulation caused by electrically active axons, Fig. 2).

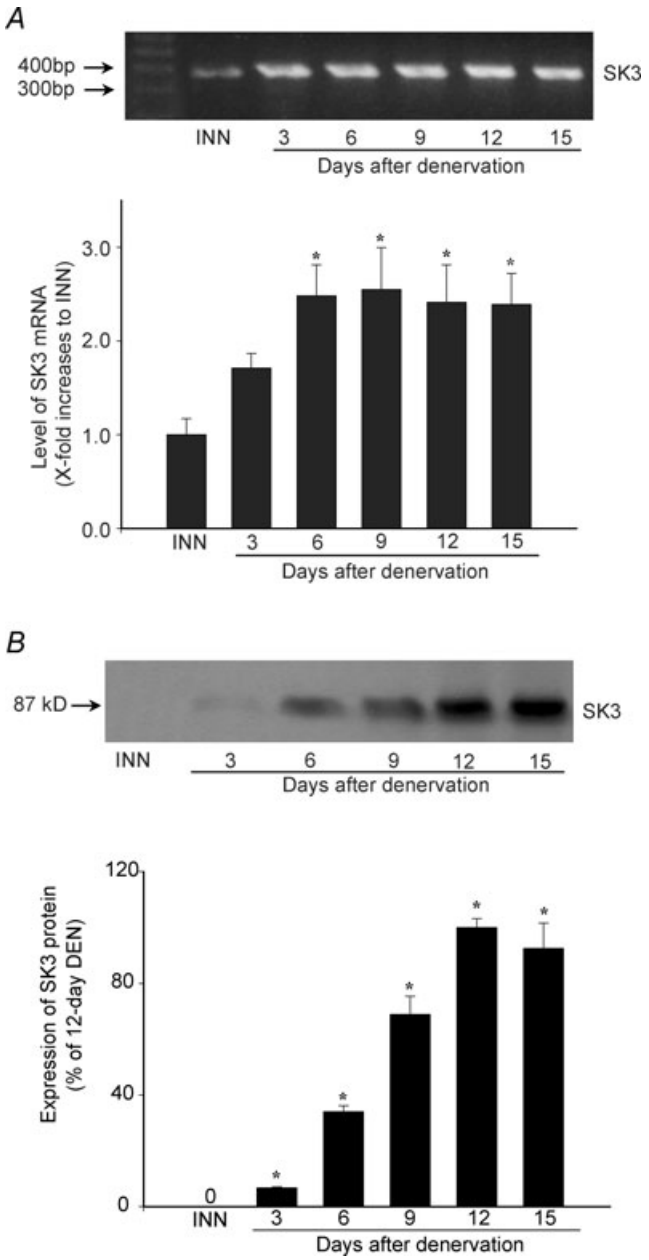

Figure 1. Effect of denervation on SK3 channel expression in soleus muscle
A, time course of the changes induced by denervation in mRNA levels. Representative result and quantification of RT-PCR are shown. The values of contralateral innervated muscles (INN) are set to 1. *B*, time course of the changes induced by denervation in SK3 protein expression. Representative result and quantification of Western blots are shown. The mean value of 12-day-denervated muscles is set to 100. The quantifications of both RT-PCR and Western blot bands were done by analysis of optical densities. Mean ± S.E.M., 3–4 animals per time point. $^{*}P < 0.01$ *versus* INN.

Effect of direct electrical stimulation *in vivo* on SK3 expression in denervated soleus muscle

Stimulation starting on the day of denervation. We prepared initially a series of muscles in which we started

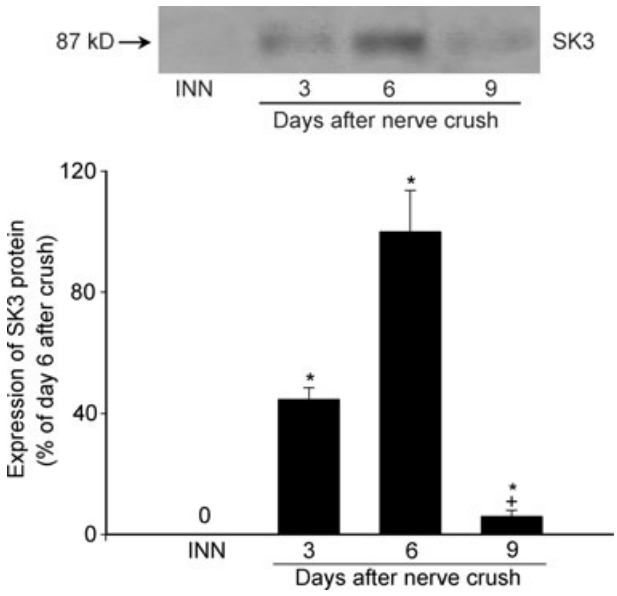

Figure 2. Time course of the changes induced by denervation followed by reinnervation in SK3 protein expression
Denervation performed through soleus nerve crush. Representative result and quantification of Western blots are shown. The mean value of the sixth day after crush was set to 100. Mean ± S.E.M., 3–4 animals per time point. $^{*}P < 0.01$ *versus* mean value of contralateral innervated muscles (INN), $^{+}P < 0.01$ *versus* day 6.

the stimulation at the time of denervation, applying the more powerful fast pattern (100 pulses at 100 Hz for 1 s every 100 s, 86 400 stimuli day^{-1}) and terminated it after 6 days. As shown by Western blot and immunofluorescence (Fig. 4*A* and *B*), the fast pattern stimulation was very effective in preventing the up-regulation of SK3 induced in soleus muscle by denervation, as the value at day 6 was only ∼2% of that induced by denervation alone at the same time distance.

Stimulation starting on day 6 after denervation. In subsequent series we started the stimulation with a delay of 6 days with respect to denervation and terminated it on day 12 post-denervation. In addition to the fast pattern

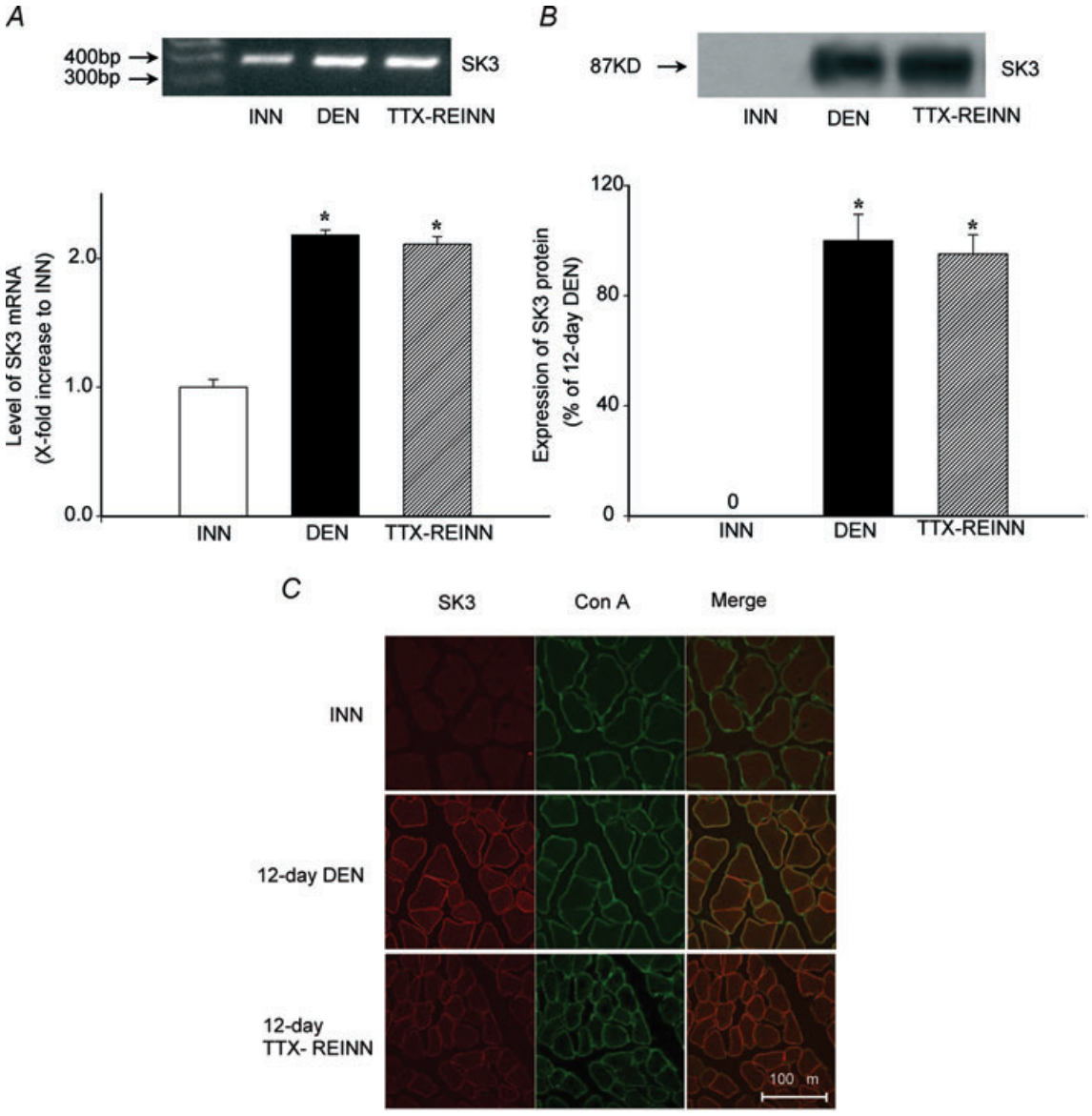

Figure 3. Effect of reinnervation with tetrodotoxin-inactive nerves on SK3 channels in soleus muscle
A, expression level of SK3 mRNA 12 days after nerve crush at the point of entry into soleus muscle, followed by quick reinnervation (starting at day 3–4) under conditions of TTX conduction block. Representative result and quantification of RT-PCR are shown. The mean value of innervated muscles (INN) was set to 1. DEN, 12-day-denervated contralateral muscles. *B*, expression level of SK3 protein in the same muscles as in *A*, determined by Western blot. Representative result and quantification of Western blots are shown. The mean value of denervated muscles was set to 100. *C*, expression of SK3 protein determined by immunofluorescence, in the same muscles as in *A* and *B*. Representative immunofluorescence images with red (SK3) and green (Con A) colour are shown. INN, innervation; DEN, denervation for 12 days; TTX-REINN, 12 days reinnervation with TTX-blocked axons (perfusion of sciatic nerve with saline solution delivering 9 μg day^{-1}); Con A, concanavalin A. Mean ± S.E.M., 3 muscles per group. $^{*}P < 0.01$ *versus* INN.

(see above) we also used slow patterns (100 pulses at 30 Hz for 3.33 s every 100 s, 86 400 stimuli day^{-1}; 100 pulses at 20 Hz for 5 s every 50 s, 172 800 stimuli day^{-1}). The reason was twofold: (1) to see whether evoked muscle activity was able to repress an already established SK3 expression; (2) to test the efficacy of the slow pattern, physiological for soleus, while circumventing the transient effect of the products of nerve degeneration, by carrying the stimulation up to a point in time (day 12) much later than that attained (6 days) in the initial stimulation series with the fast pattern. All the above indicated stimulation patterns were effective in reversing the denervation-induced up-regulation of SK3 protein (Fig. 5*A* and *C*) and so was the 20 Hz slow pattern for mRNA expression (fast and 30 Hz patterns not tested) (Fig. 5*B*). With respect to the protein expression value of denervated muscles, taken as 100, the fast stimulated muscles are 4.87 ± 2.52%, the slow 30 Hz 6.42 ± 3.06%, the slow 20 Hz 6.78 ± 2.56% (no statistically significant difference between the stimulated groups; highly significant difference between each of them and the denervated group). Electrodes implanted in control innervated muscles and left *in vivo* for 6 days (sham animals) did not induce the expression of the SK3 channel (data not shown).

Effect of direct electrical stimulation *in vivo* on AHP following action potential in denervated soleus muscle fibres

A separate group of rats was investigated for this experiment, using the slow pattern at 20 Hz (172 800 stimuli day^{-1}). In order to test this pattern in the prevention protocol we started the stimulation at day 0 (the time of denervation), while to circumvent the products of nerve degeneration effect we carried it *in vivo* until day 12 (the time of the *in vitro* electrophysiological measurement of the AHP). In three experimental muscles, i.e. denervated + stimulated (43 fibres), we did not detect a slow AHP following the action potential elicited by anodal break stimulation, using a current injecting electrode inserted in the myofibre close to the recording one. These fibres thus behaved as normal fibres. On the contrary, a deep and long-lasting AHP was always present in five denervated, non-stimulated, muscles (31 fibres) that underwent a major block after application of 1 μM apamin. All these results are illustrated both as electrophysiological records (Fig. 6*A*) and as a histogram displaying the measured AHP areas (Fig. 6*B*).

The mean RMP of denervated fibres was 59.8 ± 0.69 mV ($n = 73$) and that of denervated + stimulated fibres 81.4 ± 0.45 mV ($n = 71$). Thus, direct electrical stimulation of denervated muscle fibres maintains RMP values similar to those of normal fibres, as previously demonstrated (Lømo & Westgaard, 1975).

Discussion

In the present study we investigated the neural mechanism regulating the expression of the SK3 channel in rat soleus muscle, by assessing the role of evoked muscle activity. The main results are: (i) denervation caused

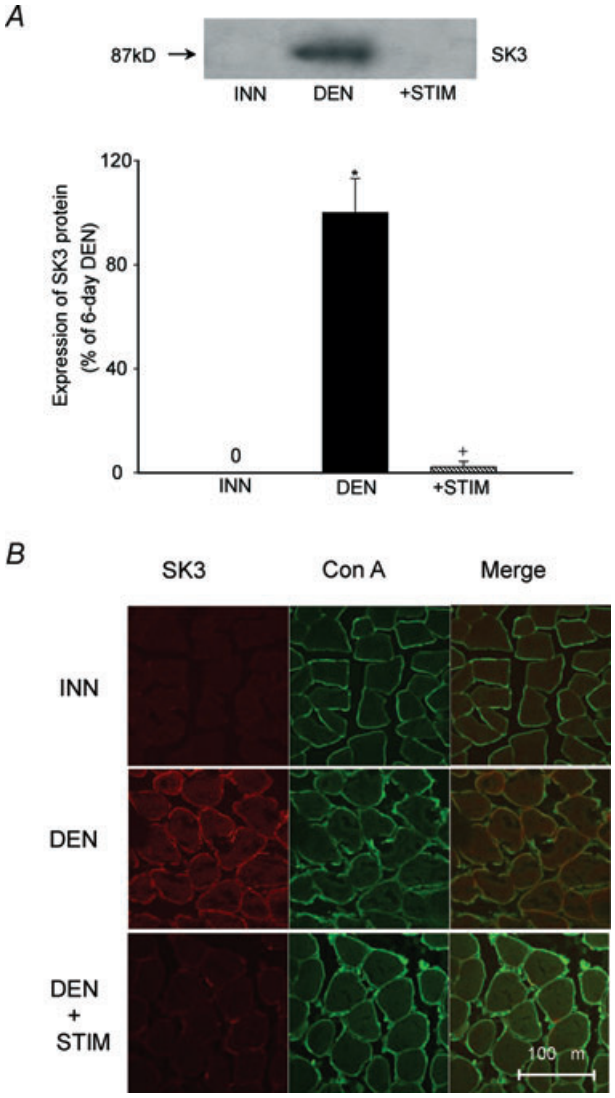

Figure 4. Effect of electrical stimulation starting on the day of denervation on SK3 channel expression in soleus muscle
The experimental muscles were electrically stimulated with a fast pattern (100 Hz for 1 s every 100 s). *A*, representative result and quantification of the Western blots are shown. The mean value of 6 day denervation was set at 100. *B*, representative immunofluorescence images with red (SK3) and green (Con A) colour are shown. INN, innervation; DEN, denervation for 6 days; DEN + STIM, 6 days electrical stimulation starting on the day of denervation. Mean ± S.E.M., 3–4 muscles per group. $^{*}P < 0.01$ *versus* INN, $^{+}P < 0.01$ *versus* DEN.

the up-regulation of SK3 mRNA and protein in soleus muscle, while reinnervation inhibited it; (ii) paralysis of reinnervated muscle, induced by nerve conduction block by TTX, maintained the SK3 channel expression induced by previous denervation; (iii) direct muscle electrical stimulation *in vivo* completely prevented or suppressed the denervation-induced up-regulation of SK3 expression; and (iv) the stimulation also completely prevented the development of the AHP following the action potential in denervated muscle.

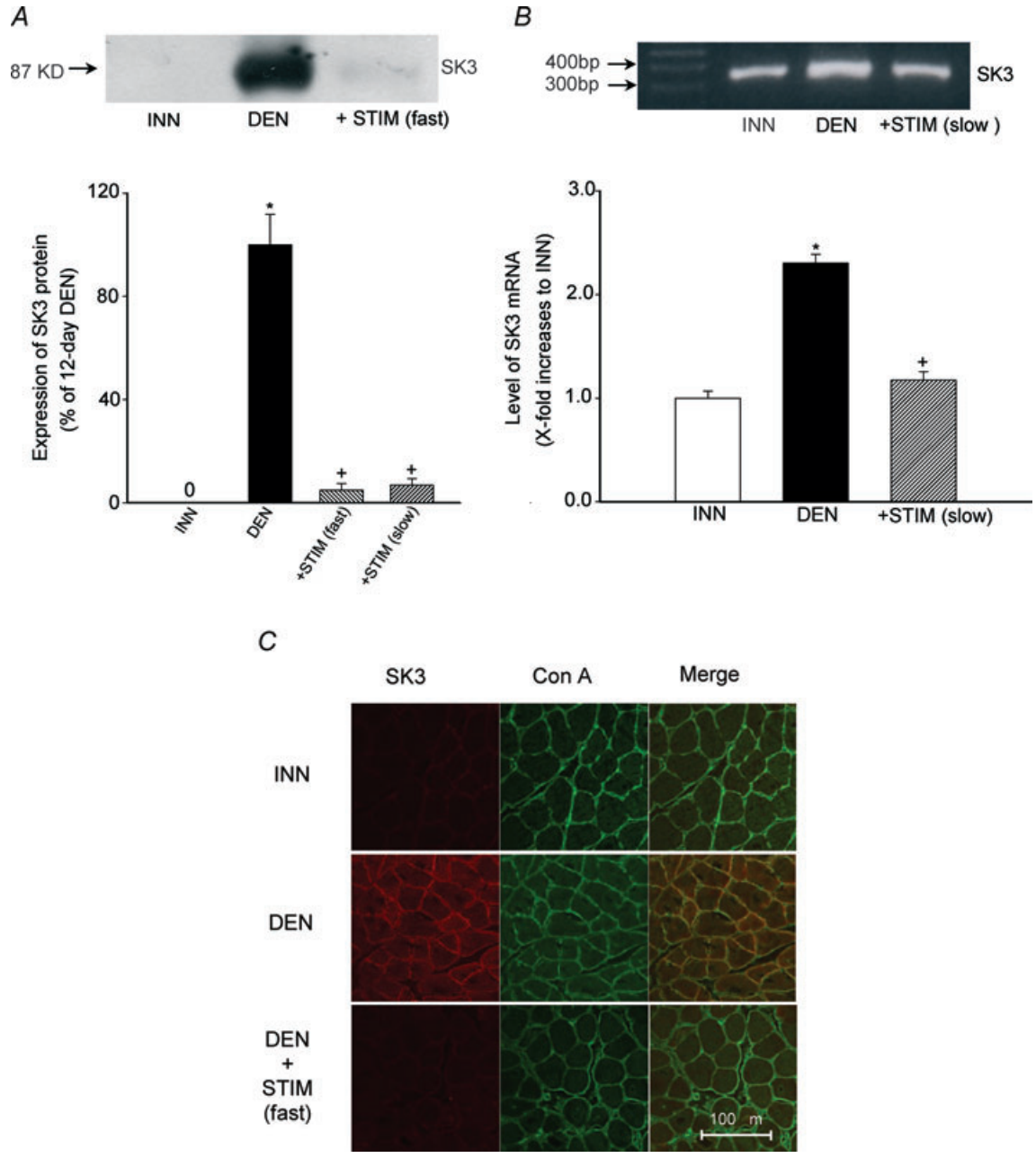

Figure 5. Effect of electrical stimulation on up-regulation of SK3 channels in denervated soleus muscle
The experimental muscles were denervated for 6 days and then electrically stimulated for 6 additional days with either a fast pattern (100 Hz) or a slow pattern (20 Hz). *A*, representative result and quantification of the Western blots are shown. The mean value of 12 day denervation was set to 100. *B*, representative result and quantification of RT-PCR are shown. The mean value of INN was set to 1. *C*, representative immunofluorescence images with red (SK3) and green (Con A) colour are shown. INN, innervation; DEN, denervation for 12 days; DEN + STIM (fast): 6 days of electrical stimulation starting 6 days after denervation, using a fast pattern (100 Hz for 1 s every 100 s, 86 400 stimulations day^{-1}); + STIM (slow): same, but using a slow 20 Hz pattern (20 Hz for 5 s every 50 s, 172 800 stimulations day^{-1}). Mean ± S.E.M., 3–4 muscles per group. $^{*}P < 0.01$ *versus* INN, $^{+}P < 0.01$ *versus* DEN.

We first confirmed that denervation induces a significant increase in the protein expression of the SK3 channel. Moreover, immunofluorescence analysis showed that the extrajunctional expression of the SK3 channel was evenly distributed in the surface membrane of muscle fibres. The time delay to attain the steady-state level of SK3 expression was 12 days after denervation, which is in agreement with a previous study (Roncarati *et al.* 2001). The mRNA level of SK3 was also increased, the steady-state level being attained 6 days after denervation. The difference in these delay times may reflect the translation and membrane insertion time for protein. We also confirmed the nerve dependence of SK3 channel protein expression, by observing its down-regulation in denervated soleus muscle after reinnervation of the original endplates by motor axons. This is in agreement with a previous *in vitro* study in which the apamin-sensitive AHP of cultured rat myotubes disappeared when they were co-cultured with and innervated by nerve cells from the spinal cord (Schmid-Antomarchi *et al.* 1985).

The fact that denervation up-regulated, and reinnervation down-regulated the extrajunctional expression of the SK3 channel in skeletal muscle, implies that the motor nerve exerts an inhibitory influence on SK3 channels. A long-standing interest has been to identify the neural factors that regulate the extrajunctional properties of muscle fibres. The two main candidates considered have been neurotrophic factors released from motor nerves and muscle-evoked activity (see introduction). With regard to SK3 channel expression, our experiments focused on the role of the latter factor and the results fully support the conclusion that the expression is controlled by muscle activity, that appears to inhibit it already at the transcript level. On the one hand, impulse-blocked reinnervating axons could not down-regulate, to any degree, the mRNA and protein of SK3 channels expressed in denervated soleus muscles. On the other hand,

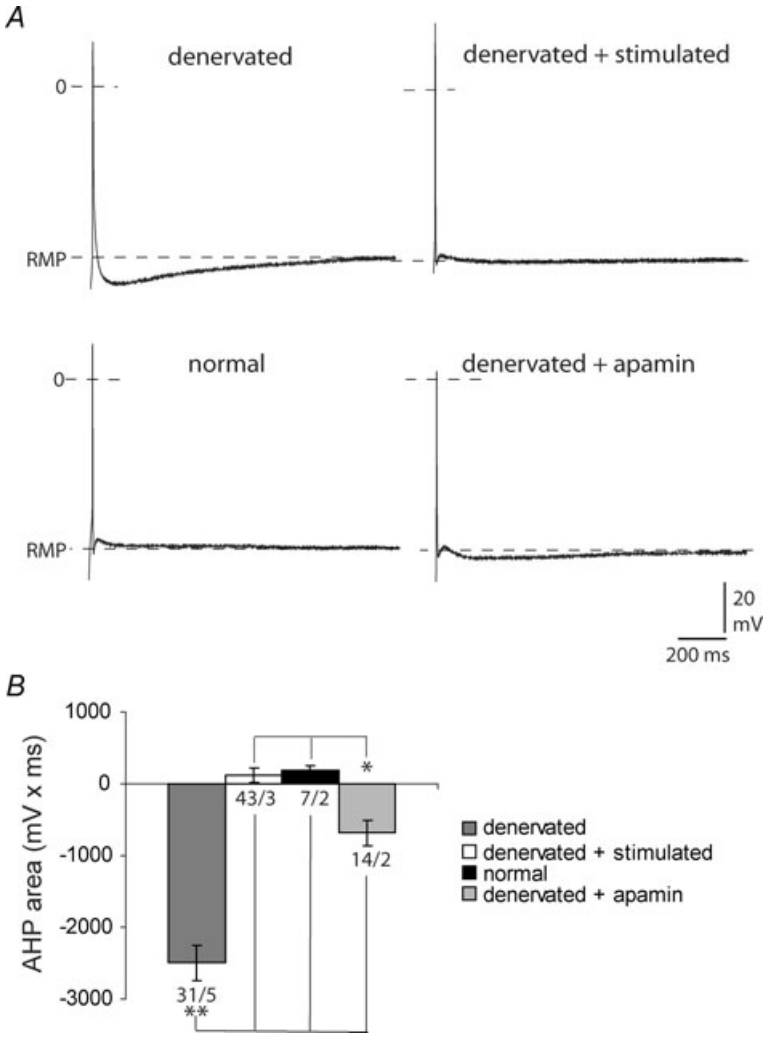

Figure 6. After-hyperpolarization (AHP) following the action potential evoked by anodal break stimulation, present in denervated soleus muscle fibres, but absent when they are kept active by chronic direct stimulation
In muscles isolated *in vitro*, each myofibre was impaled by two microelectrodes, one used to inject current and the other to record the electrical response (DC recording). See Methods for further details. *A*, a 300 ms hyperpolarizing pulse (ending just at the start of each trace) elicits immediately after its termination an action potential, which is followed by a long-lasting AHP provided the muscle fibres are chronically denervated (records taken at day 12 post-denervation). Chronic electrical stimulation (slow pattern, 20 Hz for 5 s every 50 s, 172 800 stimulations day^{-1}) starting at the time of denervation and also lasting 12 days, completely prevents the AHP development. Also shown is the major block of the AHP by 1 μM apamin. *B*, mean ± S.E.M. values of the AHP areas, under the different, indicated conditions. No. of fibres/No. of muscles, shown below each column. $^{*}P < 0.01$, $^{**}P < 0.001$.

electrical stimulation of denervated muscles suppressed the expression in the extrajunctional regions of muscle fibres of SK3 mRNA and protein once already developed, or prevented their development when stimulation started at the time of denervation. This was also true for the slow patterns utilized which are close to the physiological range for soleus (especially the slowest one: 20 Hz trains, 172 800 stimulations day^{-1}), whose motor neurones have been recorded to fire *in vivo* between 300 000 and 500 000 action potentials per day (Hennig & Lømo, 1985). The efficacy of the stimulation is also indicated by the short time required (6 days) to down-regulate SK3 mRNA and protein, once they have been up-regulated by a period lasting several days of denervation without stimulation.

Our results are in agreement with previous data in the literature indicating that other extrajunctional membrane properties (such as non-junctional AChRs, TTX-insensitive Na^+ channels, RMP depolarization, receptivity to synapse formation, fibrillatory activity) are only subjected to an inhibitory control by action potential activity transmitted to muscle fibres by motor neurones (Lømo & Rosenthal, 1972; Jansen *et al.* 1973; Purves & Sakmann, 1974*a*; Lømo & Westgaard, 1975; Pasino *et al.* 1996). For the inhibitory regulation of SK3 channels, however, previous reports stated that neurotrophic factors released from the nerve also play an important role (Behrens & Vergara, 1992; Vergara *et al.* 1993; Ramirez *et al.* 1996). This contention was based on the following data. First, chronic electrical stimulation of denervated rat muscles was found to reduce the expression of SK3 channels (measured as apamin binding sites) only partially (Ramirez *et al.* 1996) and not completely as in our study. However, the stimulation was very infrequent (2 trains of stimuli per day, separated by ∼12 h of electrical silence), the frequency in the train very low (1 Hz) and the number of stimuli per day also low (1200), a set of parameters inappropriate for either slow or fast muscle. In fact, too long pauses in activity result in little or no effect in down-regulating the extrajunctional membrane changes of denervated muscles (Lømo & Westgaard, 1975). Furthermore, as direct evidence in support of the role of a neurotrophic factor carried by axonal transport, the following data were presented: (1) in denervated muscles with a short nerve stump, apamin receptors develop earlier than in muscles with a long stump (Ramirez *et al.* 1996), and (2) nerve treatment with axonal transport blockers such as colchicine or vinblastine, is followed by expression of apamin receptors in the innervated muscles (Behrens & Vergara, 1992; Vergara *et al.* 1993; Ramirez *et al.* 1996). As far as the length of the nerve stump effect is concerned, multiple evidence in the literature has shown that this phenomenon is well accounted for by the earlier appearance in the interstitium between the myofibres of the 'nerve degeneration products' when the stump is short (see Methods, also for citations). On the other hand the effects of the application to nerves of colchicine and vinblastine have been shown to induce several unforeseen side-effects: (1) by actions at the site of application, chronic nerve conduction block or axonal damage followed by muscle partial denervation; (2) by systemic actions, effects on distant musculature (Cangiano & Fried, 1974; Lømo, 1974; Cangiano & Fried, 1977). These effects entirely explain the changes observed in muscle, making it impossible to attribute them to a selective block of transport of a hypothetical trophic factor for muscle. A completely different line of investigation in support of the existence of a neural trophic factor controlling SK3 expression, is the isolation from the chick spinal cord of a soluble factor (< 4000 Da) that blocks the AHP following the spike in cultured rat muscle cells (Suarez-Isla *et al.* 1986). However, another investigation has shown that pig and rat neural tissues contain a factor that induces a fast block of the post-spike AHP (minutes), and has therefore been identified as an apamin-like factor possibly playing a physiological role as a neuromodulator of the Ca^{2+}-activated K^+ channel conductance (Fosset *et al.* 1984).

In our study, we have also confirmed that denervation induces in muscle a post-spike AHP and that it is blocked by apamin (Romey & Lazdunski, 1984; Schmid-Antomarchi *et al.* 1985). In addition we contributed the new finding that activity down-regulates this property: in fact, direct electrical stimulation of denervated soleus muscles for 12 days completely prevented the AHP development. Since fibrillation of denervated muscle is also blocked by apamin (Vergara *et al.* 1993; Jacobson *et al.* 2002; our observations in the present experiments), a role for SK3 channels and AHP in the origin of fibrillation appears well based. The fact that electrical stimulation suppresses not only the AHP (present work) but also fibrillation (Purves & Sakmann, 1974*a*; our observations in the present experiments), is also in line with this conclusion. One must also consider, however, that fibrillatory spike activity of denervated muscle originates from subthreshold potentials (Purves & Sakmann, 1974*b*) and that these are suppressed by direct electrical stimulation as well as cyclically by the firing itself of fibrillatory spikes (Purves & Sakmann, 1974*a*). It is therefore clear that activity affects, in an inhibitory manner, at least two, rather than only one property underlying fibrillation: origin potentials and SK3 channels. The first property is the primary cause of fibrillation, while the role of the second is probably that of a permissive factor, as the AHP would rhythmically remove, after each spike, Na^+ channel inactivation (Thesleff & Ward, 1975; Blatz & Magleby, 1987; Kimura *et al.* 2000). Depolarization of the resting membrane potential induced by denervation is also likely to make a contribution. That SK3 channels are a necessary but not sufficient condition to induce fibrillation is also elegantly shown by experiments

using a transgenic mouse model (Jacobson *et al.* 2002).

In conclusion, the present experiments, based on chronic stimulation of denervated muscles and on reinnervation with electrically silent axons, demonstrate that action potential activity evoked by motor neurones in muscle fibres can entirely explain the suppression of SK3 channels and of the post-spike AHP, characterizing normally innervated adult muscles. Up-regulation of these channels in not yet innervated muscle fibres during development and after denervation in the adult, is therefore the consequence of the absence of evoked muscle activity.

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Acknowledgements

This study was supported by grants from the Italian Ministry for Universities and the Scientific and Technological Research (MURST) to A.C. and to G.F.F.

Author's present address

D.-J. Jiang: Department of Pharmacology, School of Pharmaceutical Sciences, Central South University, Changsha 410078, China.