Comparative changes of levels of nitrendipine Ca^{2+} channels, of tetrodotoxin-sensitive Na^+ channels and of ouabain-sensitive $(Na^+ + K^+)$-ATPase following denervation of rat and chick skeletal muscle

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Three major ion transport systems, the nitrendipine-sensitive Ca^{2+} channels, the tetrodotoxin-sensitive Na^+ channel and the ouabain-sensitive $(Na^+ + K^+)$-ATPase, have been studied in skeletal muscle from rat and chick after chronic denervation. It is shown that the situation found for the Ca^{2+} channel differs dramatically from that found for the Na^+ channel and the $(Na^+ + K^+)$-ATPase and that regulation of the nitrendipine-sensitive Ca^{2+} channel in denervated muscle also differs widely from that of the tetrodotoxin-sensitive Na^+ channel and the ouabain-sensitive $(Na^+ + K^+)$-ATPase which show a quite similar evolution.

Denervation *Skeletal muscle* *Nitrendipine receptor* *Tetrodotoxin receptor* *$(Na^+ + K^+)$-ATPase*

1. INTRODUCTION

The best known effects of denervation on membrane properties of mammalian skeletal muscle fibers are a reduction of the resting membrane potential [1,2], the appearance of extrajunctional cholinergic receptors [3,4] and the development of an action potential resistant to tetrodotoxin (TTX) [5–7]. The generation of action potentials in skeletal muscle involves the participation of voltage-dependent Na^+ and K^+ channels [8]. Calcium channels have also been described in frog skeletal muscle [9,10] and recent work has shown that most, if not all, calcium channels are localized in the transverse tubular membrane system [11,12].

The purpose of this study is to examine the comparative effects of denervation in rat and chick skeletal muscle on biochemical properties of 3 major ion transport systems of the membrane. The Ca^{2+} channel properties were studied by using a potent Ca^{2+} channel blocker, [^{3}H]nitrendipine; the properties of the tetrodotoxin-sensitive Na^+ channel were studied using a tritiated TTX derivative and variations affecting the $(Na^+ + K^+)$-ATPase were studied using [^{3}H]ouabain.

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Abbreviations: TTX, tetrodotoxin; [^{3}H]en-TTX, tritiated ethylenediamine tetrodotoxin

2. MATERIALS AND METHODS

All experiments were performed on adult male Wistar rats weighing approx. 200 g and on 7-day-old chicks weighing approx. 50 g. Under sodium pentobarbital anaesthesia one leg was denervated by cutting the sciatic nerve at the thigh level and 4 mm of the sciatic nerve was removed. This transection totally denervated all the muscles of the lower leg. The contralateral leg of operated animals or unoperated animals of the same age served as controls. At various times after denervation, denervated and control muscles were isolated and prepared for binding assays.

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2.1. Tissue preparation

Whole skeletal muscles from lower legs were dissected, weighed and washed in ice-cold 20 mM Tris–HCl buffer containing 0.25 M sucrose and 1 mM EDTA at pH 7.4 (TSE buffer). Homogenization was performed at 4°C in 10 vols of the TSE buffer using a polytron apparatus (Brinckman instruments) at setting 5 with three 5-s bursts. Homogenates were washed twice by centrifugation at 25 000 × *g* for 10 min followed by resuspension of the pellet in fresh TSE buffer to 100 mg original tissue weight/ml TSE buffer. Subsequently, the homogenates were filtered through 4 layers of cheese cloth.

2.2. Binding assays

[^{3}H]Nitrendipine binding assays were carried out as follows: 0.5–1 mg homogenate protein was equilibrated in 1 ml standard incubation medium containing 20 mM Tris–Cl, 50 mM choline chloride and 10^{-4} M of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), at pH 7.4 and 4°C in the absence (total binding) and presence (non-specific binding) of 1 μM unlabelled nitrendipine. After 40 min incubation at 4°C binding was stopped by filtering, in duplicate, aliquots of 400 μl incubation medium through Whatman GF/B glass fiber filters under reduced pressure. [^{3}H]en-TTX binding assays were carried out at 4°C as described for nitrendipine, using increasing amounts of [^{3}H]en-TTX in the absence (total binding) and presence (non-specific binding) of 5 μM TTX. After 20 min incubation at 4°C binding was stopped by filtering in duplicate aliquots of 400 μl incubation medium as described above. [^{3}H]ouabain-binding assays were carried out at 20°C in a medium containing 100 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 0.1 mM PMSF and 50 mM triethanolamine buffer at pH 7.4, supplemented with increasing concentrations of [^{3}H]ouabain (total binding) and 10 mM ouabain (non-specific binding). After 30 min incubation at 20°C binding was stopped by filtering in duplicate aliquots of 400 μl incubation medium as described above. Filters were rapidly washed twice, with 5 ml of an ice-cold solution containing 200 mM choline chloride and 20 mM Tris–Cl buffer at pH 7.4 for [^{3}H]nitrendipine and [^{3}H]en-TTX binding assays and with a medium containing 150 mM NaCl, 5 mM $MgCl_2$ and 10 mM Hepes–Tris at pH 7.4 for [^{3}H]ouabain binding assays. In all cases, aliquots of 100 μl of the incubation mixture were taken for measurement of the concentration of tritiated ligands present. Experiments with [^{3}H]nitrendipine were carried out under dim light because of the light sensitivity of the dihydropyridine derivative. Protein content was determined using bovine serum albumin as a standard [13].

3. RESULTS

3.1. Changes in the number of nitrendipine-sensitive Ca^{2+} channels in chronically denervated skeletal muscle from rat and chick

[^{3}H]Nitrendipine has been used as a biochemical marker of the voltage-dependent Ca^{2+} channel in denervated rat and chick skeletal muscle. Fig.1A,B shows typical Scatchard plots of the specific binding of [^{3}H]nitrendipine from whole homogenates of innervated contralateral muscle and of chronically

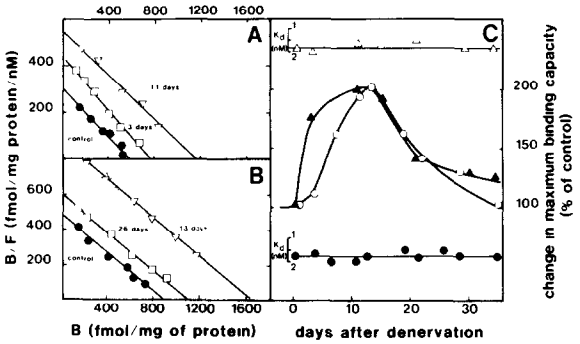

Fig.1. Effects of chronic denervation on lower leg muscle of rat and chick on the nitrendipine-sensitive Ca^{2+} channel. Lower legs of adult rats and 7-day-old chicks were denervated by section of the sciatic nerve and whole homogenates of skeletal muscles were prepared as described in section 2. Contralateral unoperated legs and animals of the same age served as control. (A,B) Typical Scatchard plots for the specific [^{3}H]nitrendipine binding to whole homogenates from muscles of unoperated legs (●) and from skeletal muscles at different times after denervation for rat (A) and chick (B). (C) Time course of the change in maximum binding capacity for [^{3}H]nitrendipine after denervation of lower muscle leg of rat (▲) and chick (○). K_d values are obtained at each stage of denervation from Scatchard plot analysis for rat (△) and chick (●) muscles. K_d and B_{max} values are derived from 3–4 series of experiments at times indicated after denervation.

denervated rat and chick muscles at two stages of denervation. Fig.1C shows the evolution of the total number of [^{3}H]nitrendipine binding sites of chronically denervated skeletal muscle from rat and chick measured at various intervals of time after denervation. Results are expressed in percentages of control values. Control values (100%) were obtained from the contralateral unoperated leg. For unoperated rat skeletal muscle, the maximum binding capacity (B_{max}) and the dissociation constant of the nitrendipine–receptor complex (K_d) are 608 ± 50 fmol/mg protein and 1.7 ± 0.2 nM, respectively. For chick muscle these values are 880 ± 70 fmol/mg protein and 1.8 ± 0.2 nM, respectively. In rat muscle as soon as 3 days after denervation there is a significant increase of about 75% in the number of nitrendipine binding sites. The maximum increase reaches 200% of control values and is observed 11 days after denervation. This initial increase is followed by a slow drop of the number of nitrendipine binding sites. The number of [^{3}H]nitrendipine binding sites remains about 30% higher than control values at 35 days after denervation. K_d values of the nitrendipine–receptor complex remained at 1.8 ± 0.2 nM which is similar to that found for control values. In chick muscle an increase of [^{3}H]nitrendipine binding is also observed which reaches 200% above the control value 15 days after denervation. This change is also followed by a slow decrease in the number of nitrendipine binding sites which return to control values 35 days after denervation. At all stages studied, the K_d value of the nitrendipine–receptor complex remained at 1.8 ± 0.1 nM which is very similar to that of the control value (K_d = 1.8 ± 0.2 nM) and to that reported for the rat.

3.2. *Changes in the number of the TTX-sensitive Na^+ channels in chronically denervated skeletal muscle from rat and chick*

Fig.2A,B shows typical Scatchard plots of the specific binding of [^{3}H]en-TTX at two stages of denervation in rat and chick skeletal muscle and for innervated contralateral muscle. The evolution of voltage-dependent Na^+ channels characterized as high affinity binding sites for TTX is shown in Fig.2C. A very rapid drop in the maximum binding capacity for [^{3}H]en-TTX was observed between 3 and 10 days after denervation. At this time after denervation less than 40 and 50% of the initial

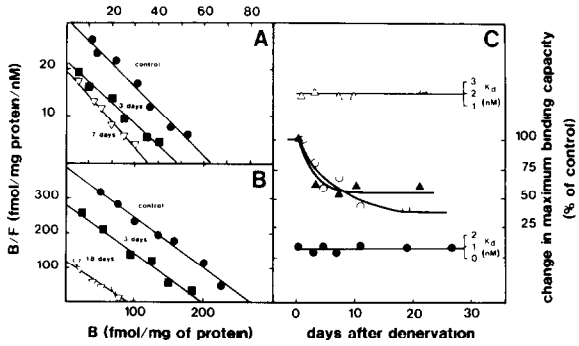

Fig.2. Effects of chronic denervation on lower leg muscle of rat and chick on TTX-sensitive Na^+ channels. (A,B) Typical Scatchard plots for the specific [^{3}H]en-TTX binding to muscle of unoperated legs (●) and to skeletal muscle at different times after denervation for rat (A) and chick (B). (C) Time course of the change in maximum binding capacity for [^{3}H]en-TTX after denervation of lower leg muscle of rat (▲) and chick (○). K_d values are obtained at each stage of denervation from Scatchard plot analysis for rat (△) and chick (●). K_d and B_{max} values are derived from 3–4 series of experiments at indicated times after denervation.

binding were detectable in rat and chick muscle, respectively. These values remained relatively stable until 21 (rat) and 26 (chick) days of denervation. At all stages between 3 and 21–26 days after denervation, K_d values of the TTX–receptor complex remained at 1.8 ± 0.2 nM for rat muscle and 0.8 ± 0.1 nM for chick muscle.

3.3. *Changes in the number of ouabain-sensitive ($Na^+ + K^+$)-ATPases on chronically denervated skeletal muscle from rat and chick*

Binding of [^{3}H]ouabain has been carried out on rat and chick skeletal muscles to measure the changes in the number of ($Na^+ + K^+$)-ATPases after denervation. Fig.3A,B shows typical Scatchard plots for the specific binding of [^{3}H]ouabain to rat and chick skeletal muscle at two stages after denervation and to controls. Fig.3C shows a rapid decrease in the number of ($Na^+ + K^+$)-ATPases to about 60% (rat) and 40% (chick) below the control values between 7 and 10 days after denervation. At all stages studied, the dissociation constant of the ouabain–ATPase complex remained at 175 ± 24 nM (rat) and 100 ± 20 nM (chick). These values are very similar to those observed for control values

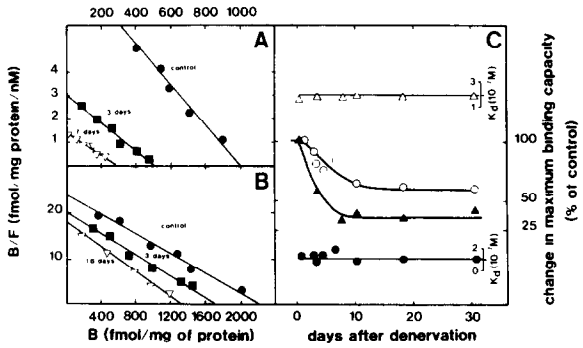

Fig.3. Effects of chronic denervation on the [^{3}H]ouabain binding sites of the lower leg muscle of rat and chick. (A,B) Typical Scatchard plots for the specific [^{3}H]ouabain binding on unoperated leg muscle (●) and on skeletal muscles at different times after denervation for rat (A) and chick (B). (C) Time course of the change in maximum binding capacity for [^{3}H]ouabain after denervation of lower leg muscle of rat (▲) and chick (○). K_d values are obtained at each stage of denervation from Scatchard plot analysis for rat (△) and chick (●) muscles. K_d and B_{max} values are derived from 3–4 series of experiments at indicated times after denervation.

(190 ± 22 nM and 130 ± 20 nM for rat and chick, respectively).

4. DISCUSSION

This paper deals with the effects of denervation of skeletal muscle from rat and chick on 3 membrane components which are essential for the electrical activity and coupling between excitation and contraction in muscle. They are the nitrendipine-sensitive Ca^{2+} channel which may be implicated in the excitation–contraction coupling mechanism that triggers contraction [8], the voltage-dependent Na^+ channel which is responsible for the rapid phase of depolarization of the action potential [8] and the $(Na^+ + K^+)$-ATPase which is essential for the maintenance of ionic gradients across the membrane and of the resting membrane potential [14].

The situation found for the Ca^{2+} channel differs dramatically from that found for the Na^+ channel and the $(Na^+ + K^+)$-ATPase. During the first phase of denervation in both rat and chick muscle, the Ca^{2+} channel level increases up to 200% from the control level whereas levels of the TTX-sensitive Na^+ channel and of the $(Na^+ + K^+)$-ATPase drop during the same period of time to 40–60% of their initial level. After day 11 (rat) or day 15 (chick) of denervation the amount of [^{3}H]nitrendipine receptors declined slowly to reach a value that is near the initial control value after 30–35 days of denervation. During this second phase of evolution of the nitrendipine-sensitive Ca^{2+} channel levels, amounts of the Na^+ channel and $(Na^+ + K^+)$-ATPase in the membrane remained relatively stable. In controls, the respective levels of ouabain-receptor [$(Na^+ + K^+)$-ATPase], nitrendipine receptors (Ca^{2+} channels) and TTX receptors (Na^+ channels) are of a ratio of 1:0.6:0.06 in rat, and 2.2:0.9:0.3 in chick muscle. After the first phase of denervation, when Ca^{2+} channels reach a maximum level these ratios become 0.3:1.2:0.03 for the rat muscle and 1.3:1.8:0.09 for chick muscle. At a longer time after denervation (>20 days) the ratios are 0.3:0.7:0.03 for rat muscle and 1.3:0.9:0.09 for chick muscle.

The formation of the transverse tubule system and its maintenance in differentiated skeletal muscle is one of the important events in skeletal muscle differentiation [15,16]. Biochemical techniques using [^{3}H]nitrendipine [12] as well as electrophysiological techniques [11] have shown that Ca^{2+} channels are preferentially localized in the transverse tubule system. It has been reported that denervation of rat muscle fibers induces the formation of an abnormal transverse tubule system structure including an overproduction of the tubule system [17]. This observation probably explains the initial increase in the number of nitrendipine binding sites following denervation. The decrease in number of nitrendipine receptors that starts 11 and 15 days after denervation for rat and chick skeletal muscle, respectively, may be due to muscle atrophy which becomes evident 2 weeks after denervation.

Na^+ channel levels measured as high affinity sites for TTX (K_d = 0.8–1.8 nM) decrease after denervation in a similar way for chick and rat skeletal muscle. A decrease of 33 to 43% for rat muscle had previously been reported using [^{3}H]saxitoxin to titrate Na^+ channels [18,19]. Although the situation in terms of TTX receptors is similar for chick and rat muscle, it is different when one looks at the functionality of Na^+ channels. Functional Na^+ channels are of a high affinity for TTX

(K_d = 0.8 nM) in innervated chick muscle and denervation does not change the TTX sensitivity of the channels [20]. Conversely, whereas functional Na^+ channels are of a high affinity for TTX (K_d = 1.8 nM), in innervated rat skeletal muscle denervation induces the appearance of a family of voltage-sensitive Na^+ channels that are resistant to TTX (K_d = 1 μM) [5–7]. TTX-resistant Na^+ channels are also observed in cultures of rat skeletal myotubes [21]. They coexist with sites which have a high affinity for TTX (K_d = 1 nM) but are not electrically functional [21,22].

The ouabain-sensitive ($Na^+ + K^+$)-ATPase followed an evolution similar to that of the TTX-sensitive Na^+ channel in denervated conditions with a decline in [^{3}H]ouabain binding sites soon after denervation which is followed by a further drop to a plateau value at about 60% (rat) and 40% (chick) below the control values. This result is in agreement with those reported for denervated mouse skeletal muscle [23] and also with the observation showing that the maximum number of ouabain binding sites in in vitro cultures of chick myotubes was about 4-times less than in muscle fibers that have developed in vivo [24]. This decrease in the number of ($Na^+ + K^+$)-ATPase following denervation is likely to be in direct relation to the membrane depolarization which follows denervation and the change of the resting membrane potential of the rat muscle cells by 10–12 mV [1,2]. It is known that in cardiac cells inhibition by ouabain of 50–60% or more of the total Na^+-pump creates severe changes in membrane properties resulting in the generation of arhythmia [25]. In conclusion, our results show that innervation exerts a regulatory influence on the expression and/or synthesis of important membrane proteins which are involved in the excitability of the muscle and the coupling between excitation and contraction.

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