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EFFECT OF DENERVATION ON A STEADY ELECTRIC CURRENT GENERATED AT THE END-PLATE REGION OF RAT SKELETAL MUSCLE

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SUMMARY

1. An electric current flows continuously out of the synaptic region of rat lumbrical muscle fibres. It is generated apparently as a result of a non-uniform Cl^- conductance (G_{Cl}), with G_{Cl} being lowest at the end-plate.

2. We investigated the effects of denervation on this current. The current persisted with little change after denervation. This was somewhat unexpected, since G_{Cl} falls dramatically after denervation, and in acute experiments on normal muscles, the steady current is greatly reduced by agents which block G_{Cl}.

3. The steady current was blocked in denervated muscle, as in normal muscle, by low-Cl^- solutions, Na^+-free and K^+-free solutions, and treatment with furosemide and 9-anthracene-carboxylic acid. The current in denervated muscle appears to be generated by the same general mechanism as in normal muscle.

4. The results suggest that the $[Cl^-]_i$ is significantly higher in denervated than in normal muscle fibres. Preliminary experiments with Cl^--selective micro-electrodes have confirmed this: $[Cl^-]_i$ rises from about 12 mM to about 23 mM after denervation. This has the effect of moving the Cl^- equilibrium potential (E_{Cl}) in a positive direction, so that the driving force for passive Cl^- efflux is increased. The increased driving force compensates for the reduced G_{Cl}, allowing the steady current to persist in denervated fibres.

INTRODUCTION

Rat lumbrical muscle fibres generate a steady electric current which leaves the end-plate region of the cell and re-enters in the flanking extrajunctional regions (Caldwell & Betz, 1984; Betz, Caldwell & Kinnamon, 1984*b*; Betz, Caldwell, Harris & Kinnamon, 1984*c*). In effect, each cell creates around itself an electric field which is focused at the neuromuscular junction.

In previous studies, we examined the mechanism by which the steady outward current is generated, using ionic substitution and drugs which block certain ion conductance or transport pathways (Caldwell & Betz, 1984; Betz *et al.* 1984*b*). We

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proposed (Betz *et al.* 1984*b, c*) that Cl^- conductance (G_{Cl}) is non-uniformly distributed along the length of the muscle fibre, being lowest in the end-plate region. Since Cl^- appears to be actively accumulated by some muscle fibres (Bolton & Vaughan-Jones, 1977; Dulhunty, 1978; Vaughan-Jones, 1982; Betz *et al.* 1984*b*), the Cl^- equilibrium potential (E_{Cl}) is somewhat more positive than, and exerts a depolarizing action upon, the resting membrane potential (V_m). The relatively low G_{Cl} in the end-plate region causes V_m to be slightly more negative at that site than elsewhere (cf. Yoshioka & Miyata, 1983; Caldwell & Betz, 1984). The resulting intracellular voltage gradient thereby generates the steady outward current at the end-plate.

In order to learn more about the characteristics of the endogenous steady current in muscle, we have studied the effects of denervation. Since G_{Cl} falls dramatically after denervation (Camerino & Bryant, 1976; Lorkovic & Tomanek, 1977), and since blocking G_{Cl} in normal muscle with drugs abolishes the steady current (Betz *et al.* 1984*b*), one might predict that the current would be reduced or absent after denervation. We found that the current persisted undiminished for at least 6 weeks after denervation. Further investigation revealed that the current appears to be generated by essentially the same mechanism in denervated as in normal muscle. A brief account of these findings has appeared (Betz *et al.* 1984*c*).

METHODS

The second, third, and fourth deep lumbrical muscles of the rat hind foot were used interchangeably in all experiments. Muscles in one foot were denervated 1–46 days before the final acute experiment by removing a several millimetre section of sciatic nerve from the thigh of rats under ether anaesthesia. The nerve was resectioned 3 weeks after the initial denervation. This denervation produced a minimal disturbance of the animals' mobility in their environment and they resumed normal feeding and grooming behaviour. Control muscles were usually obtained from the contralateral, non-denervated foot. In some instances, control muscles were obtained from normal, unoperated animals. The choice of controls did not appear to affect the results. In experiments on isolated muscle fibres, the flexor digitorum brevis muscle was dissociated by collagenase treatment, as described by Bekoff & Betz (1977).

Methods for construction and calibration of micro-electrodes used in the vibrating probe as well as theoretical considerations have been presented previously (Jaffe & Nuccitelli, 1974; Betz & Caldwell, 1984). Briefly, a micro-electrode with a platinum black ball electroplated on its tip was attached to a piezo-electric reed and vibrated continuously in the extracellular fluid at a frequency of several hundred Hertz, with a peak-to-peak excursion of 20–30 μm. The electrode was vibrated perpendicular to the long axis of the muscle, and was moved to different positions close to the lateral margin of the muscle in order to record steady currents entering or leaving the muscle. The signal recorded by the probe was processed by a lock-in amplifier, and its output (the r.m.s. voltage difference between the excursion limits of the probe tip) was recorded on chart paper and later digitized. Knowing the resistivity of the medium (measured in a conductivity meter) and vibration distance (typically about 30 μm, measured visually during the experiment), we converted voltages to current density. Unless otherwise noted, all currents were measured at the end-plate region.

For studies of membrane potential, conventional intracellular micropipettes (20–40 MΩ) were filled with 3 M-potassium acetate. Voltages were recorded on chart paper.

Normal Krebs solution consisted of (mM): 136, NaCl; 5, KCl; 2, $CaCl_2$; 1, $MgCl_2$; 11, glucose and 2, PIPES (disodium salt) buffer. The pH was adjusted to 7·4 with H_2SO_4. For some experiments in which membrane potentials were measured the $CaCl_2$ concentration was elevated to 8 mM in order to achieve more stable recordings. For Na^+-free solutions, NaCl was replaced with choline chloride and muscles were pre-treated with α-bungarotoxin (4 μg/ml) in order to block activation of acetylcholine (ACh) receptors. K^+-free solutions were prepared by increasing the NaCl concentration to 141 mM and omitting KCl. Low-Cl^- (2 mM) solutions were ordinarily prepared by replacing NaCl

and KCl with the respective salts of isethionate or methanesulphonate and $CaCl_2$ with $CaSO_4$. Since $CaSO_4$ is relatively insoluble, free Ca^{2+} was lower than normal in this solution. However, Ca^{2+} has little or no effect on the steady current (Betz *et al.* 1984), and similar results were obtained in control experiments in which $CaCl_2$ was not altered ($[Cl^-]_o$ was 4 mM in these experiments). Thus, all ion-substituted solutions were isosmolar with normal Krebs solution. Solutions containing 9-anthracene-carboxylic acid (9-AC) were prepared from a stock solution of 22 mg 9-AC dissolved in 5 ml ethanol. Control muscles used in vibrating probe experiments were pre-treated with α-bungarotoxin (4 μg/ml; Sigma) to prevent any effects of neurally released ACh. For experiments involving low Cl^- or 9-AC, tetrodotoxin (TTX) (1 μg/l; Sankyo) was added to prevent muscle fibrillation. For recording membrane potentials during solution changes to low-Cl^- or to choline chloride solutions, the indifferent electrode consisted either of an agar–Ringer solution bridge or a micro-electrode filled with 3 M-KCl and positioned outside the fibre near the recording site. Recordings with a micro-electrode as reference gave smaller junction potentials, and agreed reasonably well with measurements made by repeatedly sampling V_m after the solution was changed (in which case, junction potential interference is avoided). In other experiments in which the membrane potential was recorded, a silver–silver chloride pellet served as reference electrode.

In preliminary experiments, two of us (G.L.H. and W.J.B.) have used Cl^--selective micro-electrodes to measure intracellular Cl^- activity in normal and denervated muscles. The methods and results will be published in detail (G. L. Harris & W. J. Betz, in preparation). Briefly, electrodes were fabricated according to a modified design given by Saunders & Brown (1977), filled with Resin 170 (W.P.I., Inc.). Slopes of 55–59 mV/decade change in Cl^- activity in KCl solutions were routinely obtained. Cl^- concentration was calculated assuming an activity coefficient of 0·76. Intracellular activities were calculated from the difference between the potential recorded by the Cl^--selective electrode and a conventional intracellular pipette filled with 0·5 M-K_2SO_4 plus 0·2 M-KCl.

Statistical significance values (P) were calculated according to the student's two-tailed t test, assuming equal standard deviations (Snedecor & Cochran, 1980). Means are given ±1 S.E. of the mean.

RESULTS

Persistence of the outward current after denervation

The steady current was measured with a vibrating micro-electrode (Jaffe & Nuccitelli, 1974) as described in Methods. The electrode was vibrated continuously, perpendicular to the long axis of the muscle. As illustrated in Fig. 1, the peak amplitude of the outward current showed no dramatic change after denervation (controls *versus* all denervated: $P = 0{\cdot}28$). In animals denervated for 10–20 days, the average current was significantly larger than controls ($P < 0{\cdot}03$). However, because of the large amount of scatter in the data, it is not clear whether this represents a real increase in the size of the current during this period.

Spatial maps of currents from typical muscles are shown in Fig. 2*A* (control muscles, cf. Caldwell & Betz, 1984) and 2*B* (muscles denervated for 10–20 days). For each muscle, distance zero represents the position of maximum outward current. The most obvious effect of denervation was that the outward current could be recorded over a longer distance along the edge of the muscle (1·01 mm before and 1·58 mm after denervation; $P < 0{\cdot}01$). This may have resulted from the increased length constant, due to the increased input resistance of individual muscle fibres, which occurs after denervation (Nichols, 1956; Albuquerque & McIsaac, 1970; Westgaard, 1975). Accordingly, the integrals of the outward currents were significantly greater in denervated than in control muscles ($P < 0{\cdot}03$). The peak inward currents recorded in the flanking regions were also somewhat larger after denervation (control $= -1{\cdot}26 \pm 0{\cdot}12$ μA/cm² and 10–20 days post-denervation $= -2{\cdot}64 \pm 0{\cdot}37$ μA/cm²; $P < 0{\cdot}005$).

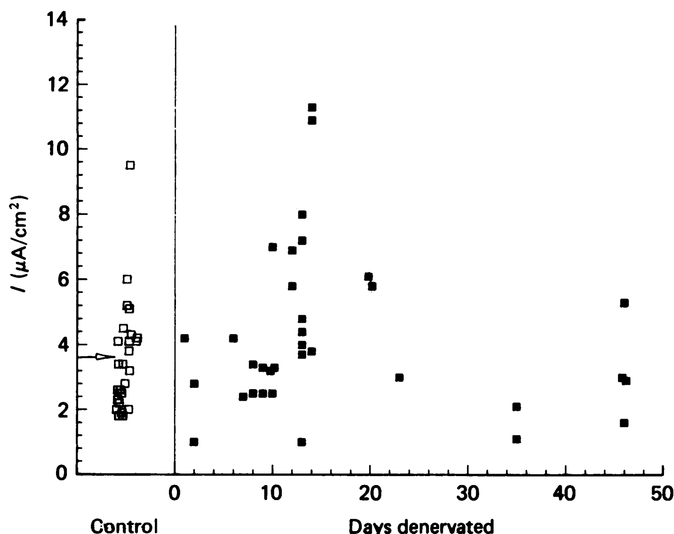

Fig. 1. Peak outward currents recorded from control muscles (open symbols) and muscles denervated for different periods of time (filled symbols). The arrow marks the mean amplitude of all control currents.

In normal muscle, the location of the peak outward current is found precisely at the end-plate region (Caldwell & Betz, 1984). In order to test whether this was also the case for denervated muscles, carbachol (165 μM) was added to the bathing solution. The steady current promptly reversed, becoming large and inward. As shown in Fig. 3*A*, mapping experiments revealed that the locations of the normal peak outward current (filled squares) and peak inward current in carbachol (open squares) coincided. The large inward current could be blocked with α-bungarotoxin (4 μg/ml). Since ACh receptor density is greatest at the end-plate, the position of peak inward current corresponds to the end-plate region. Thus, it appears that the outward current in denervated muscle is focused at the end-plate region, as in normal muscle. This was confirmed in a separate series of experiments, in which the current generated by isolated muscle fibres (enzymically dissociated from chronically denervated flexor digitorum brevis muscles; Bekoff & Betz, 1977) was examined with the vibrating probe. An example is shown in Fig. 3*B*. The peak outward current corresponded exactly to the position of the end-plate, which could be directly visualized in the isolated fibre.

Mechanism of generation of steady outward current

In order to determine the mechanisms responsible for the generation of the steady outward current in denervated muscle, we examined the effects of various drugs and ions on membrane potential (V_m), recorded with a conventional intracellular micro-electrode, and on the peak outward current, measured with the vibrating probe. As shown by others (e.g. Ware, Bennett & McIntyre, 1954; Albuquerque & Thesleff, 1968; Albuquerque & McIsaac, 1970) and confirmed in the present study, resting

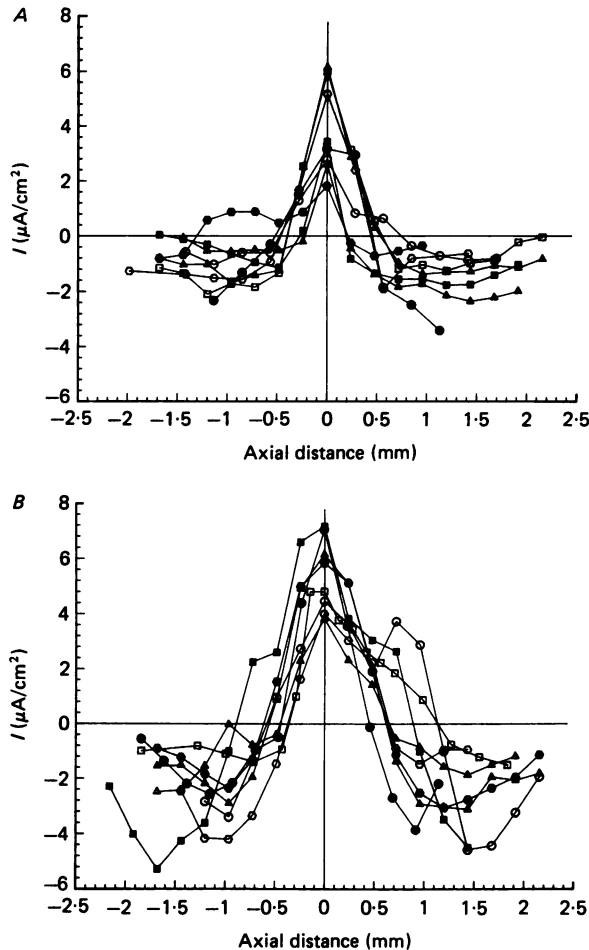

Fig. 2. Typical spatial maps of currents recorded from several control muscles (*A*) and muscles denervated for 10–20 days (*B*). Different symbols represent different muscles. Distance zero represents the point of maximum outward current for each muscle. One notable difference is that outward current was recorded over a longer distance in denervated than in control muscles.

membrane potentials were depolarized by about 10 mV in denervated muscles (control $= -69.2 \pm 0.4$ mV; denervated $= -60.0 \pm 0.5$ mV; $P < 0.001$).

Cl^- substitution: effects on V_m (Fig. 4A). Recordings of V_m from a control and denervated muscle fibre before, during, and after replacement of Cl^- with isethionate are shown in Fig. 4*A*. The control fibre response (continuous line) is typical of certain mammalian muscle fibres (Dulhunty, 1978; Betz *et al.* 1984*b*). The initial depolarization reflects the large positive shift of E_{Cl} when Cl^- is removed, the recovery of V_m reflects depletion of internal Cl^- (Hodgkin & Horowicz, 1959), and the steady-state hyperpolarization in low-Cl^- Krebs solution may reflect the presence of an inwardly directed Cl^- 'pump' in normal muscle. When normal Krebs solution was restored (open arrow), V_m transiently hyperpolarized before returning to its normal

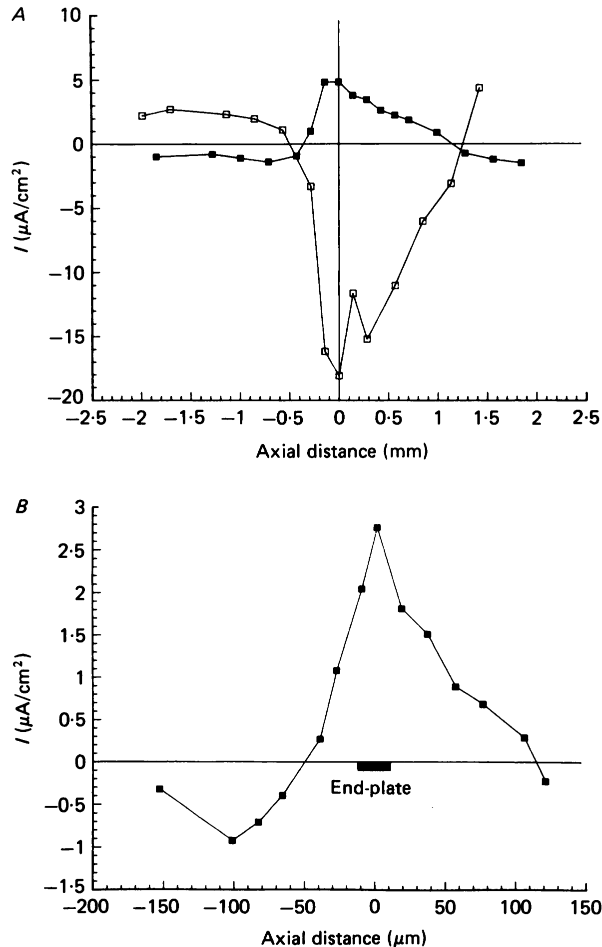

Fig. 3. Spatial maps recorded from a denervated whole muscle (*A*) and from an isolated, denervated muscle fibre (*B*). In *A*, the muscle was denervated for 13 days. Filled symbols were obtained before, and open symbols during the application of 165 μM-carbachol. Note that the peak inward current in carbachol, which marks the location of end-plates, coincides with the position of the peak outward current, indicating that in denervated muscle, as in normal muscle, the steady outward current is centred at the end-plate. *B*, spatial map obtained from a fibre enzymically isolated from a muscle denervated for 21 days. The end-plate was visible in the microscope.

level. In other experiments, multiple samples of V_m in different fibres were taken; the results were consistent with continuous recordings like those in Fig. 4*A*.

In the denervated muscle fibre (Fig. 4*A*, dotted line), exposure to low Cl^- Krebs solution caused a rapid hyperpolarization, without a transient depolarization. Upon returning to normal Krebs solution (filled arrow), V_m recovered quickly, without a transient hyperpolarization. These rapid, monotonic responses were somewhat unexpected, since it is unlikely that internal Cl^- could equilibrate faster than in normal fibres, given the reduced conductance of denervated fibres (see Discussion).

Cl^- substitution: effects on the outward current (Fig. 4B). Replacement of Cl^- with

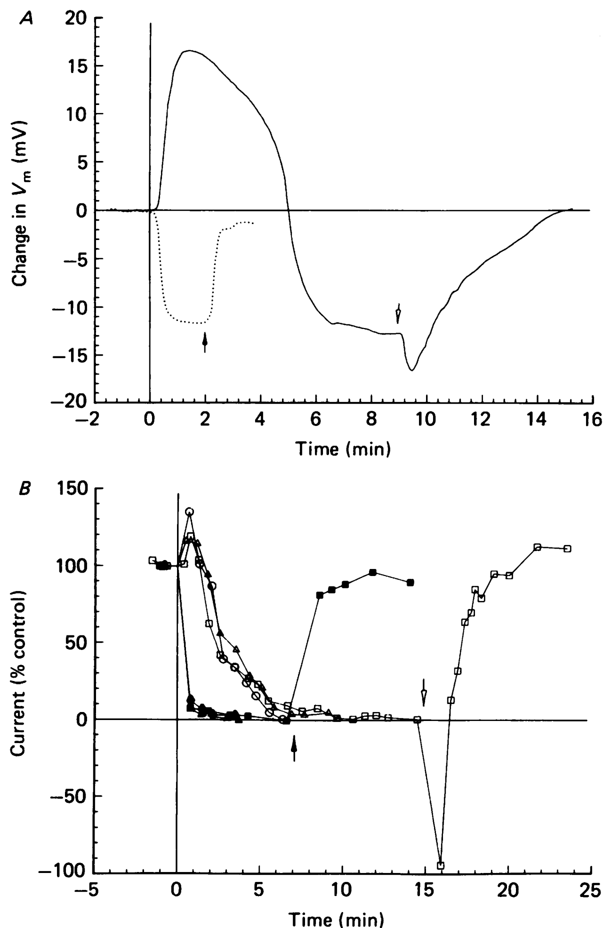

Fig. 4. Effects of low-Cl^- Krebs solution, applied at time zero, on V_m and the outward current. *A*, typical recordings of V_m from a control (continuous line) and an 8-day-denervated (dotted line) muscle fibre. While both fibres were hyperpolarized by low-Cl^- Krebs solution, the denervated fibre did not show the preceding transient depolarization, which is characteristic of normal fibres. *B*, the steady outward current in control muscles (open symbols) and 13-day-denervated muscles (filled symbols) was abolished by low-Cl^- Krebs solution, with a time course similar to the change in V_m. In both *A* and *B*, arrows mark return to normal Krebs solution.

isethionate reversibly abolished the outward current in both control (open symbols) and denervated (filled symbols) muscles. The two most notable differences were that the transient responses seen in control muscles when solutions were changed were not observed in denervated muscles, and that the steady current was abolished more rapidly in the denervated muscles.

K^+-free solutions: effects on V_m. K^+-free solutions had very different effects on V_m in control and denervated fibres. Typical results are shown in Fig. 5*A*. The fibre from a control muscle (continuous line) was depolarized by about 5 mV in K^+-free Krebs (cf. Betz *et al.* 1984*b*), while the fibres from a denervated muscle (dotted and dashed

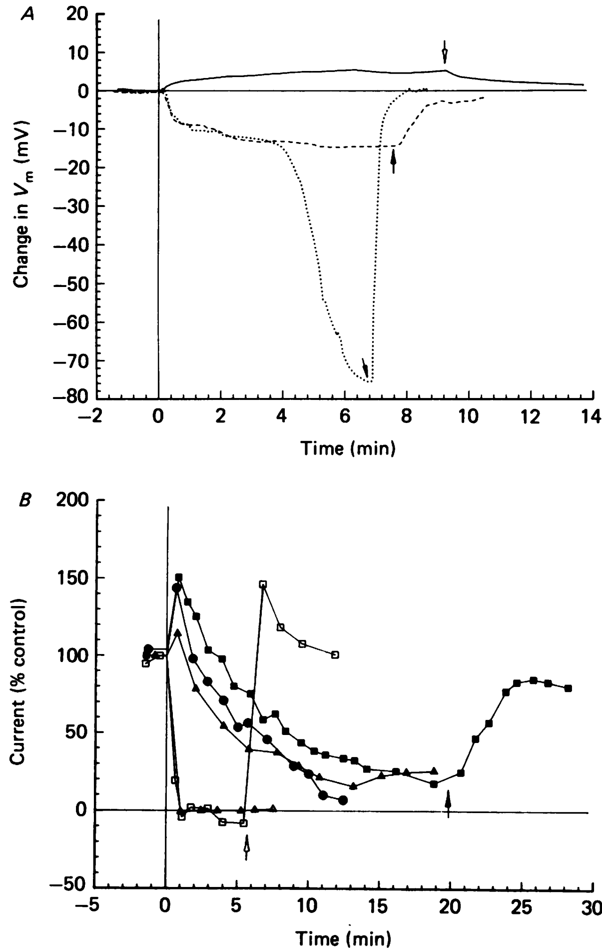

Fig. 5. Effects of K^+-free Krebs solution, applied at time zero, on V_m and the outward current. *A*, typical recordings of V_m. The control fibre (continuous line) was depolarized, while 12-day-denervated fibres (dashed and dotted lines) were hyperpolarized. In one case (dotted line), a second, delayed hyperpolarization occurred after several minutes in the K^+-free solution. *B*, the outward current in control muscles (open symbols) was rapidly abolished, while in 10–13-day-denervated muscles (filled symbols), the current transiently increased, and then slowly was reduced to a low level. In both *A* and *B*, arrows mark return to normal Krebs solution.

lines) were hyperpolarized by 10–15 mV. Moreover, the initial hyperpolarization of denervated fibres was often followed, within several minutes, by a further hyperpolarization which moved V_m to about -140 mV (dashed line). When normal Krebs solution was reintroduced (arrows), V_m recovered fully. The delayed hyperpolarization was a puzzling phenomenon. Such negative membrane potentials were never observed immediately upon impaling a cell, even after prolonged soaking in K^+-free solutions; they always required several minutes to appear after a fibre was impaled. The delay depended on $[Ca^{2+}]_o$, being shortened when $[Ca^{2+}]_o$ was raised from the normal 2 mM to 8 mM. One explanation for this result is that damage, produced by the impalement,

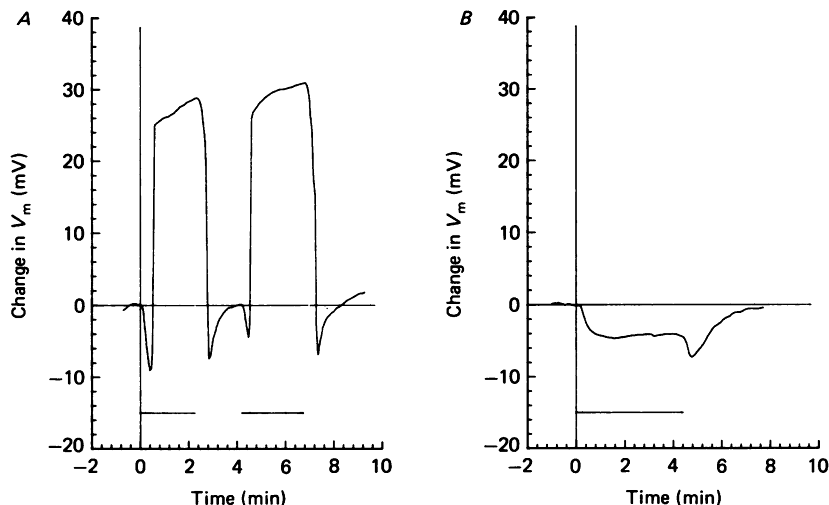

Fig. 6. Effects on V_m of low-Cl^-, K^+-free solutions. Low Cl^- was present throughout the recording period. During the times marked by the horizontal lines, K^+ was also removed. In the control fibre (*A*), V_m was depolarized by about 25 mV when K^+ was removed. In the 8-day-denervated fibre (*B*), V_m was hyperpolarized by removal of K^+.

allowed Ca^{2+} to enter the cell and activate a Ca^{2+}-dependent K^+ conductance, which drove V_m close to E_K. Such responses were also seen occasionally in control muscle fibres after a fibre had been impaled for 20–30 min.

K^+-free solutions: effects on the outward current. In control muscles (Fig. 5*B*, open symbols), the steady outward current was rapidly and reversibly abolished in K^+-free Krebs solution (cf. Betz *et al.* 1984*b*). The same effect was seen in muscles denervated for 1 day (not shown). With longer periods of denervation, however, the response changed (Fig. 5*B*, filled symbols). When exposed to K^+-free Krebs solution, the current transiently increased, and then decreased to a low level. The decrease was much slower than in control muscles. The effect was reversible (arrow), and often a transient inward current preceded the reappearance of the normal outward current when normal Krebs was restored.

Effects of combined removal of Cl^- and K^+. The effects of Cl^- and K^+ removal on V_m were very different in denervated muscle fibres and controls. It was thus of interest to examine the effects of their combined removal. First, low Cl^- Krebs ($[Cl^-]_o = 2$ mM) was applied, as above. Then a low Cl^-, K^+-free solution was applied. In normal muscles, this caused a very large, rapid depolarization which was rapidly reversible (Fig. 6*A*; cf. Lorkovic, 1976). This depolarization probably reflects a reduction in K^+ permeability in the absence of external K^+ (Katz, 1949; Hodgkin & Horowicz, 1959; Almers, 1972), as has been demonstrated in heart muscle (Gadsby & Cranefield, 1977). A very different response was seen in denervated muscle (Fig. 6*B*). As before, low $[Cl^-]_o$ was present throughout the experiment. When K^+ was also removed, V_m hyperpolarized by about 5 mV. The effect was less rapid than the depolarization observed for normal muscle, but fully reversible.

Effects of Na^+ substitution. Membrane potentials were hyperpolarized when Na^+ was

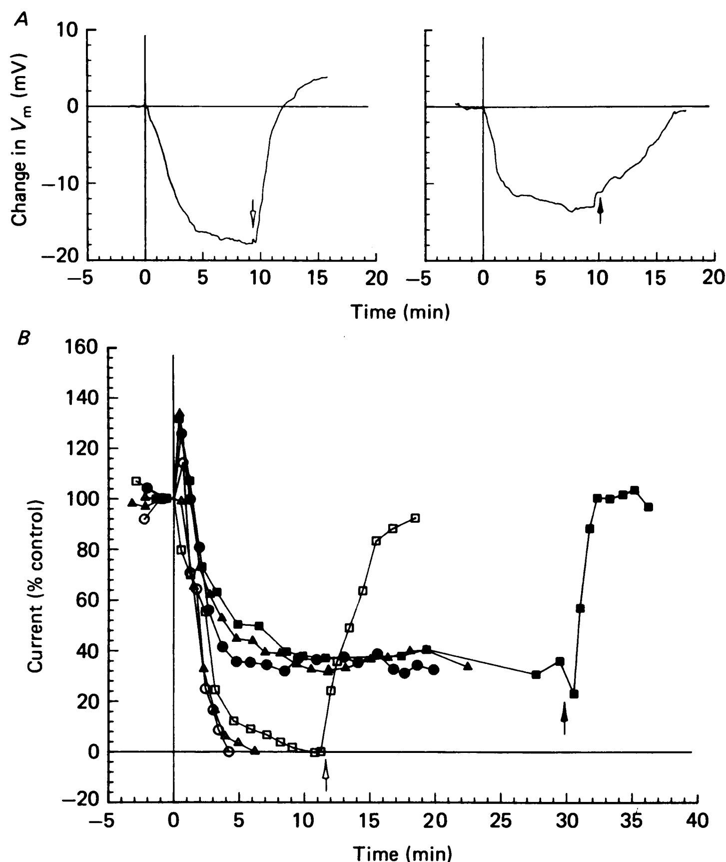

Fig. 7. Effects of Na^+-free Krebs solution, applied at time zero, on V_m and the outward current. Na^+ was replaced with choline. *A*, typical recordings in a control (left side) and 14-day-denervated (right side) fibre. In both, V_m was hyperpolarized. *B*, effects of Na^+ removal on the outward current in control (open symbols) and 14–20-day-denervated (filled symbols) muscles. The current in controls was abolished, while that in denervated muscles was reduced to about one-third of normal values. In both *A* and *B*, arrows mark return to normal Krebs solution.

replaced with choline in both control (Fig. 7*A*, left side) and denervated (Fig. 7*A*, right side) muscle fibres. The steady outward current also was affected similarly in control (Fig. 7*B*, open symbols) and denervated (Fig. 7*B*, filled symbols) muscles. The outward current transiently increased and then decreased within several minutes after exposure to choline Krebs solution. The time course of decrease in the outward current paralleled the membrane hyperpolarization recorded intracellularly. When Na^+ was reintroduced (Fig. 7*B*, arrows), the outward current recovered. The only consistent difference between control and denervated muscles was that Na^+ substitution completely abolished the current in control muscles, while in denervated muscles the current was reduced to about one-third of its resting value.

Effects of furosemide (*Fig. 8*). This drug has been shown to block active Cl^- transport

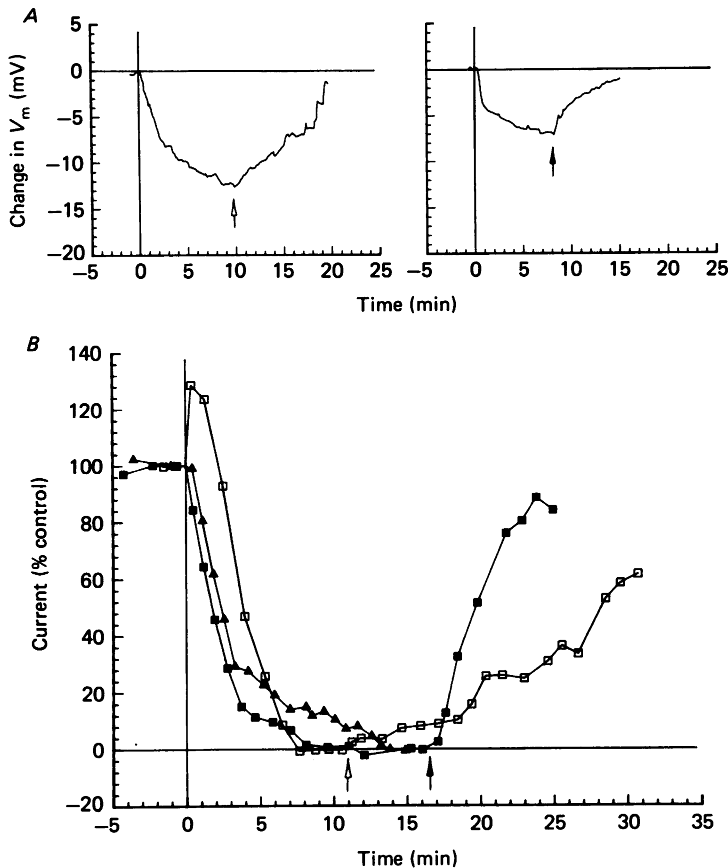

Fig. 8. Effects of furosemide (10 μM), applied at time zero, on V_m and the outward current. *A*, typical recordings in a control (left side) and 10-day-denervated (right side) fibre. In both, V_m was hyperpolarized by furosemide. *B*, effects on the outward current in control (open symbols) and 13–14-day-denervated (filled symbols) muscles. In both cases, the current was abolished. In both *A* and *B*, arrows mark the return to normal Krebs solution.

in a variety of tissues (Musch, Orellana, Kimberg, Field, Halm, Krasny & Frizzell, 1982; Russell, 1983). The effects of furosemide were virtually indistinguishable in control and denervated muscles. V_m hyperpolarized by about the same amount, with similar time courses (Fig. 8*A*). The steady current was reversibly reduced or abolished, with a time course that paralleled the change in V_m (Fig. 8*B*).

Effects of 9-AC. This drug has been shown to block G_{Cl} in skeletal muscle (Palade & Barchi, 1977; Betz *et al.* 1984*b*). As with furosemide, its effects were very similar in control and denervated muscles. V_m hyperpolarized by 10–15 mV (Fig. 9*A*) and the steady current was greatly reduced or abolished with a similar time course (Fig. 9*B*). Both effects were reversible with prolonged washing.

Effects of TTX. Na^+ channels are non-uniformly distributed in the muscle membrane, with a higher Na^+ conductance found at the end-plate than in extra-junctional regions (Betz, Caldwell & Kinnamon, 1984*a*). This pathway undergoes several changes after denervation. One of the earliest signs of denervation in skeletal

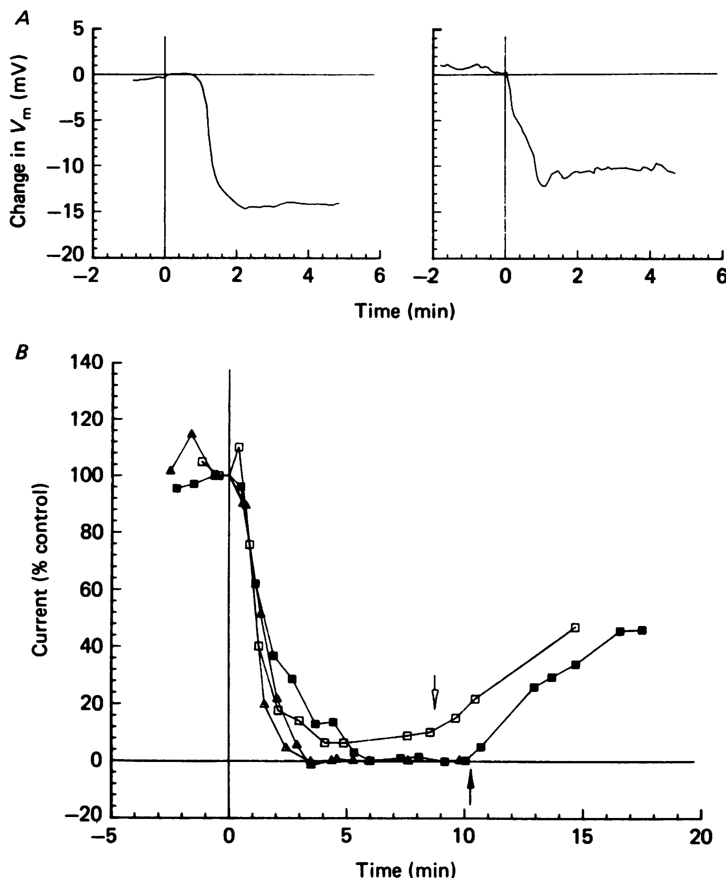

Fig. 9. Effects of 9-AC (100 μM), applied at time zero, on V_m and the outward current. *A*, typical recordings of V_m in a control (left side) and 12-day-denervated (right side) fibre. In both, V_m was hyperpolarized by 9-AC. *B*, effects on the outward current in control (open symbols) and 10–13-day-denervated (filled symbols) muscles. In both cases, the current was greatly reduced. Arrows mark return to normal Krebs solution.

muscle is a decrease in the resting membrane potential (Albuquerque, Schuh, & Kauffman, 1971). Robbins (1977) gave evidence that the depolarization is due to an increase in resting sodium conductance (G_{Na}). Consistent with this, TTX has been reported to cause membrane hyperpolarization in fibres denervated for up to two weeks (Grampp, Harris & Thesleff, 1972; Albuquerque & Warnick, 1972; Sellin & Thesleff, 1980). We observed a similar effect in denervated lumbrical muscle. An example is shown in Fig. 10, inset. During the first week after denervation, TTX (1 μM) hyperpolarized muscle fibres by as much as 5 mV. The effect was quantified by sampling V_m in twenty to twenty-five fibres from each of two control muscles and two muscles denervated for 9 days, before and after the addition of TTX (1 μM). In denervated fibres, V_m hyperpolarized from a resting $-52{\cdot}6 \pm 1{\cdot}9$ mV to $-55{\cdot}6 \pm 1{\cdot}7$ mV ($P < 0{\cdot}001$); there was no significant effect on V_m in control fibres.

The steady current in denervated muscles was reversibly increased by TTX. This

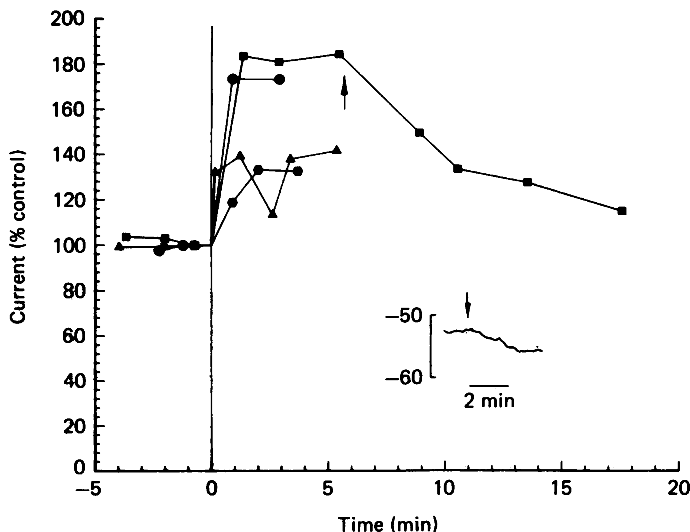

Fig. 10. Effects of TTX (1 μM) in denervated muscles. The outward current was increased by exposure to TTX. Muscles were denervated for 1 day (hexagons), 2 days (triangles), 8 days (circles), and 9 days (squares). TTX applied at time zero; arrow marks return to normal Krebs solution. Inset: V_m was hyperpolarized by a few millivolts in this 9-day-denervated fibre; TTX was applied at arrow.

may be explained as follows: the magnitude of the steady current depends in part on the driving force on Cl^- ($V_m - E_{Cl}$). Since V_m hyperpolarized in TTX, the driving force on Cl^-, and therefore the steady current were increased. By about two weeks after denervation, the effect of TTX on the steady current became less prominent than in muscles denervated for shorter periods, consistent with its effect on V_m. In control muscles, TTX did not affect the outward current (cf. Caldwell & Betz, 1984).

Ba^{2+}: effects on V_m. Ba^{2+} has been reported to block resting G_K in skeletal muscle fibres (Sperelakis, Schneider & Harris, 1967; Standen & Stanfield, 1978). Consistent with this, control lumbrical fibres were reversibly depolarized by 5–10 mV in the presence of 50 μM-$BaCl_2$. An example is shown in Fig. 11*A*, continuous line (50 μM-Ba^{2+} applied at time zero; cf. Betz *et al.* 1984*b*). In a denervated fibre (Fig. 11*A*, dashed line), 50 μM-Ba^{2+} produced a very small depolarization; when [Ba^{2+}] was raised by 10-fold, to 500 μM (Fig. 11*A*, double arrows), a further depolarization ensued.

Ba^{2+}: effects on the outward current. In control muscles (Fig. 11*B*, open squares), 50 μM-Ba^{2+} caused the steady outward current to reverse, becoming inward. Then, even in the continued presence of Ba^{2+}, the current reversed again, becoming outward once more (cf. Betz *et al.* 1984*b*). In denervated muscles, the same concentration had little effect on the steady current (triangles). Higher concentrations of Ba^{2+} (500 μM, circles) caused a transient reduction in the steady current, and 1 mM-Ba^{2+} (filled squares) briefly abolished the steady current.

Intracellular Cl^- activity measurements. The persistence of the outward current in denervated muscle could result from an increased $[Cl^-]_i$. In order to test this, we have recently measured internal Cl^- activity with Cl^--selective micro-electrodes before and after denervation. Our preliminary results show that $[Cl^-]_i$ approximately doubles

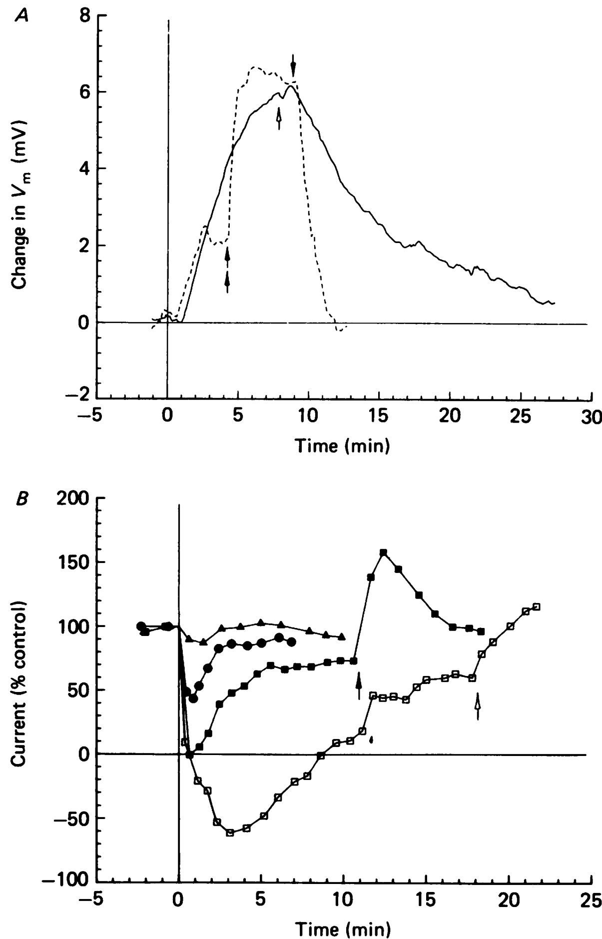

Fig. 11. Effects of Ba^{2+}, applied at time zero, on V_m and the outward current. *A*, superimposed recordings of the effect of Ba^{2+} on V_m in a typical control fibre (continuous line) and in a 10-day-denervated fibre (dashed line). The initial response was to 50 μM-Ba^{2+}. At the time marked by the double arrows, the denervated fibre was exposed to 500 μM-Ba^{2+}. Thus, the denervated fibre was considerably less sensitive to the depolarizing action of Ba^{2+} than was the control fibre. Single arrows mark return to normal Krebs solution. *B*, the steady outward current in the control muscle (open symbols) transiently reversed sign, becoming inward. It then became outward again, even in the continued presence of Ba^{2+}. The current in the 14-day-denervated muscle (triangles) was hardly affected by the same concentration of Ba^{2+} (50 μM). Higher concentrations of Ba^{2+} (circles, 500 μM; filled squares, 1 mM) produced larger effects. Arrows mark return to normal Krebs solution.

during the 1–2 weeks after denervation, rising from 12.1 ± 0.3 mM ($n = 144$ fibres in thirteen muscles) to 22.1 ± 0.8 mM ($n = 132$ fibres in nine muscles; $P < 0.01$).

DISCUSSION

The steady current persists with relatively little change after denervation. Small changes, especially in the magnitude of the current, cannot be ruled out, because the

vibrating probe records the contributions of currents from many individual muscle fibres (Caldwell & Betz, 1984). Nevertheless, it is clear that the steady current is not abolished, and may even be somewhat enhanced by denervation.

The role of the steady outward current is unknown. One possibility is that the electric field which it creates may be a useful synaptic specialization. Several studies have shown that applied steady electric currents can affect cell growth and differentiation, including direction of axon growth in culture (Hinkle, McCaig & Robinson, 1981; Patel & Poo, 1982, 1984), movement of membrane receptors on myotubes grown in culture (Poo, Lam, & Orida, 1979; McLaughlin & Poo, 1981), migration of neural crest cells in *Xenopus* embryos (Stump & Robinson, 1983), and fibroblast motility and orientation (Erickson & Nuccitelli, 1984). These observations raise the possibility that the endogenous electric field associated with the steady current may perform similar functions. For instance, the steady current in denervated muscle might serve as a guidance mechanism for regenerating neurites, although at present there is no direct evidence for such a role.

The persistence of the steady current in denervated muscle was somewhat unexpected. Since blocking G_{Cl} in normal muscle with 9-AC greatly reduces the steady current (Betz *et al.* 1984*b*), and since G_{Cl} falls 2–5-fold after denervation (Camerino & Bryant, 1976; Lorkovic & Tomanek, 1977), one might have predicted that the current would be reduced or abolished by denervation. All of the results suggested that the mechanism is essentially unchanged after denervation. Thus, the current was greatly reduced or abolished by all of the procedures which reduce or abolish it in normal muscle. In normal muscle, these treatments were interpreted as interfering with active Cl^- accumulation via a Na^+- and K^+-dependent co-transport system for Cl^- entry, similar to a process shown to operate in squid axon (Russell, 1983) and epithelial cells (Musch *et al.* 1982). In addition, 9-AC, which blocks G_{Cl} in skeletal muscle (Palade & Barchi, 1977; Betz *et al.* 1984*b*), reduced the steady current similarly in normal and denervated muscle.

These observations suggest that $[Cl^-]_i$ is significantly elevated in denervated muscle. That is, in order to generate the same current in the face of a reduced conductance, the driving force on Cl^- efflux ($V_m - E_{Cl}$) must be correspondingly greater in denervated than in normal muscle. In normal muscle, we estimated from indirect evidence that E_{Cl} is about 5 mV positive to the resting V_m (Betz *et al.* 1984*b*). Thus, for example, to compensate for a uniform 3–4-fold reduction in G_{Cl}, E_{Cl} would have to be about 15–20 mV positive to V_m in denervated muscle, in order to generate the same steady current. Since V_m is about −60 mV after denervation, this suggests that E_{Cl} is about −45 to −40 mV after denervation. According to the Nernst equation, at room temperature this corresponds to a $[Cl^-]_i$ of 25–30 mM. This prediction has recently been confirmed in preliminary experiments (G. L. Harris & W. J. Betz, unpublished); $[Cl^-]_i$ rises from about 12 mM to about 23 mM after denervation.

It thus appears that the steady outward current persists after denervation because, as G_{Cl} falls, Cl^- accumulation continues, and drives E_{Cl} further from the resting membrane potential, thereby increasing the driving force on Cl^- by about the same amount as G_{Cl} is reduced.

While the steady-state effects of all treatments on the outward current were similar

in control and denervated muscles, there were some notable differences in transient responses and in the time course of responses. For example, low-Cl^- solutions (Cl^- replaced with isethionate or methanesulphonate) produced no transient depolarization in denervated muscles; instead the membrane rapidly hyperpolarized. Given the reduced G_{Cl} in denervated muscle, one might have predicted a smaller, more prolonged depolarization, reflecting the slower efflux of Cl^-. The unexpected rapid hyperpolarization resembled that observed in cardiac muscle (Vaughan-Jones, 1982). One possible explanation is that, in denervated muscle, Cl^- permeability is blocked by isethionate and methanesulphonate (cf. the blocking action of NO_3^- on Cl^- permeability in normal frog skeletal muscle; Hodgkin & Horowicz, 1959). The effect of Cl^--free solutions on the steady current was consistent with this explanation; the current was very rapidly abolished, as if Cl^- permeability were greatly reduced.

Another difference concerns the effects of K^+-free solutions. In normal muscle, the membrane was depolarized by 5–10 mV in K^+-free solutions. If the experiment was performed in low-Cl^- Krebs solution, a much larger depolarization (about 25 mV) was observed. These changes probably reflect a reduction in G_K when K^+ is removed (cf. Gadsby & Cranefield, 1977). In denervated muscles, K^+-free solutions caused a hyperpolarization, even in the presence of low Cl^-. The reason for this difference is unknown; perhaps K^+ conductance becomes insensitive to removal of external K^+ after denervation, so that V_m follows changes in E_K more closely (cf. Lorkovic & Tomanek, 1977).

The steady current in control and denervated muscles exposed to K^+-free solutions behaved in a way predicted by the observed change in V_m. In control muscles, the current was rapidly abolished. In earlier work (Betz *et al.* 1984*b*), this was interpreted as reflecting the rapid depolarization (so that $V_m \simeq E_{Cl}$), plus a blocking effect of K^+ removal on active Cl^- accumulation by the fibre (cf. Russell, 1983). In denervated muscles, the steady outward current transiently increased in K^+-free Krebs solution as the driving force on Cl^- increased with membrane hyperpolarization. Then the steady current slowly decreased to a very low level. This presumably reflects the blocking action of the K^+-free solution on active Cl^- accumulation, and a slow drift to equilibrium as Cl^- leaks out of the relatively impermeable denervated fibre.

Finally, in normal muscle, 50 μM-Ba^{2+} caused a large membrane depolarization, due apparently to its blocking action on resting G_K (Sperelakis *et al.* 1967; Standen & Stanfield, 1978). The steady current in 50 μM-Ba^{2+} transiently reversed sign, becoming inward, and then became outward again. This response was explained (Betz *et al.* 1984*b*) as follows: when V_m depolarized and crossed E_{Cl}, the steady current reversed sign. Then, with continued intracellular accumulation of Cl^-, E_{Cl} was moved in a positive direction. When E_{Cl} re-crossed V_m, the current became outward again. In denervated muscle , Ba^{2+} also depolarized the membrane, but the steady current was affected much less than in normal muscle. This result may reflect a large positive shift in E_{Cl} in denervated muscle.

In summary, the steady outward current persists as an end-plate specialization after denervation. Its mechanism is qualitatively unchanged, owing apparently to two offsetting events, namely a reduction in G_{Cl} and a compensatory increase in internal Cl^- concentration, which increases the driving force on Cl^- efflux, $V_m - E_{Cl}$. It thus remains as a steady electrical landmark of the location of the denervated end-plate.

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