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IONIC CURRENTS AND CHARGE MOVEMENTS IN ORGAN-CULTURED RAT SKELETAL MUSCLE

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SUMMARY

1. The middle of the fibre voltage-clamp technique was used to measure ionic currents and non-linear charge movements in intact, organ-cultured (*in vitro* denervated) mammalian fast-twitch (rat extensor digitorum longus) muscle fibres.

2. Muscle fibres organ cultured for 4 days can be used as electrophysiological and morphological models for muscles *in vivo* denervated for the same length of time.

3. Sodium currents in organ-cultured muscle fibres are similar to innervated fibres except that in the temperature range 0–20 °C (a) in the steady state, the voltage distribution of inactivation in cultured fibres is shifted negatively some 20 mV; (b) at the same temperature and membrane potential, the time constant of inactivation in cultured fibres is about twice that of innervated fibres.

4. Potassium currents in innervated and cultured fibres at 15 °C can be fitted with the Hodgkin–Huxley n variable rasied to the second power. Despite the large rang we would estimate that the maximum value of the steady-state potassium conductance of cultured fibres is about one-half that of innervated fibres.

5. The estimated maximum amount of charge moved in cultured fibre is about one-third that in innervated fibres. Compared to innervated fibres, culturing doubles the kinetics of the decay phase of charge movement. The possibility of a negative shift of the voltage distribution of charge movements in cultured fibres is discussed.

INTRODUCTION

To date there have been a number of quantitative observations on the voltage dependence of ionic currents in innervated mammalian muscle fibres using a variety of voltage-clamp techniques (Adrian & Marshall, 1977; Duval & Leoty, 1978, 1980*a*, *b*; Pappone, 1980; Beam & Donaldson, 1983*a*, *b*). The observed voltage dependence of ionic conductances implies the movement of intramembranous charge particles (Hodgkin & Huxley, 1952). Recently, charge movements have also been observed in both fast- and slow-twitch mammalian muscle fibres (Hollingworth & Marshall, 1981; Dulhunty & Gage, 1983) and some support given to the association of these charge movements with excitation–contraction coupling.

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It has been known for some time that *in vivo* denervation induces marked membrane and mechanical changes in mammalian muscle fibres. For example, denervated fibres have surface membrane action potentials that show a partial resistance to tetrodotoxin (Redfern & Thesleff, 1971), they fire anode-break action potentials (Marshall & Ward, 1974), they fibrillate (Thesleff, 1963) and there is a general fall with time of the resting membrane potential (Albuquerque, Schuh & Kauffman, 1971). The effects of long- and short-term denervation on the mechanical properties of muscle have also been studied by a number of authors (e.g. Harris & Miledi, 1972; Gutmann, Melichna & Syrovy, 1976; Finol, Lewis & Owens, 1981). Finol *et al.* (1981) showed that short-term *in vivo* denervation of fast-twitch mammalian muscle caused a marked prolongation of the isometric twitch tension. In a further attempt to correlate charge movement with contractile activation, part of this study examines charge movements in cultured fibres.

The main aim of this study was to examine voltage-dependent ionic currents and charge movements in organ-cultured mammalian muscle (*in vitro* denervated) in an attempt to compare these cultured fibres with innervated and *in vivo* denervated mammalian fibres (Pappone, 1980; Duval & Leoty, 1984). We also set out to see if *in vitro* denervated, that is cultured fibres, were a good model for *in vivo* denervated fibres.

We show that action potentials generated in organ-cultured mammalian fibres have most of the above membrane properties of *in vivo* denervated fibres (see also Bekoff & Betz, 1977; Lee, Miledi & Ruzzier, 1982). The main findings of our voltage-clamp studies are that, when compared to innervated mammalian muscle fibres, the steady-state voltage distribution of the sodium inactivation variable in organ-cultured fibres is shifted negatively some 20 mV and that the maximum steady-state potassium conductance could be about one-half that of innervated fibres. The studies on charge movement indicate that the estimated maximum amount of charge measured in cultured fibres in isotonic solution is two to three times less when compared to innervated rat muscle fibres in hypertonic solution (Hollingworth & Marshall, 1981). Compared to innervated fibres cultured fibres also show an almost twofold increase in the kinetics of the decay phase of the charge movement.

METHODS

Electrophysiological methods

Current clamp. A two-micro-electrode technique was used to generate action potentials. With two micro-electrodes inserted in the same fibre ($\simeq 50$ μm apart) one was used to pass a constant current in order to change the membrane potential and the other was used to record the resulting potential changes. Voltage-recording electrodes were filled with 3 M-KCl (8–12 MΩ) and current-passing electrodes filled with 2 M-potassium citrate (6–7 MΩ).

Voltage clamp. The middle of the fibre voltage-clamp technique was used, and was the same as described by Adrian & Marshall (1977) for ionic currents and Hollingworth & Marshall (1981) for charge movements. Three micro-electrodes are employed in this method and the membrane current at time t, i_m (t) is calculated from

$$i_m(t) = (1/l) \cdot [I_0(t)/2 - \Delta V(t)/(r_1 l)], \quad (1)$$

where I_0 is the current delivered to a fibre from an electrode placed as nearly as possible to the centre of the fibre. $\Delta V(t)$ is the voltage difference between two voltage-recording electrodes V_1 and V_2. V_1 is placed at a measured distance $(l/2)$ from the I_0 electrode and V_2 is inserted at a distance l

($\simeq 500\ \mu m$) from the V_1 electrode. The longitudinal resistance, per unit length of the fibre, is r_i. The membrane capacity was obtained from the integral of the transient part of the membrane current (Adrian & Almers, 1976):

$$C_{eff} = 1/V_1(\infty) \int_0^\infty [i_m(t) - g_m V_1(t)]\, dt, \quad (2)$$

where $V_1(\infty)$ is the final value of $V_1(t)$ the clamped voltage at the V_1 micro-electrode and g_m is the time-independent membrane conductance per unit length of fibre. The command voltage pulse to the V_1 electrode was blunted exponentially with a time constant of 0·5 ms (Adrian & Marshall, 1977).

TABLE 1. Composition of solutions (mM)

Solution	Na^+	TEA^+	K^+	Rb^+	Ca^{2+}	Mg^{2+}	Cl^-	Glucose
A	150·8	—	4·0	—	2·0	1·0	160·8	11·0
B	38·4	112·4	—	4·0	2·0	1·0	160·8	11·0
C	96·0	54·8	—	4·0	2·0	1·0	160·8	11·0
D	—	150·8	—	4·0	2·0	1·0	160·8	11·0

Solutions were buffered with 2 mM-HEPES, titrated to pH 7·4 and gassed with 100% oxygen.

During an experiment a PDP 11/34 computer (DEC, Maynard) was used to calculate the muscle cable properties and so monitor the state of the fibre. Non-linear membrane currents were calculated from

$$i'_m(t) = i_m(t)_T - (V_1(\infty)_T / V_1(\infty)_c)\, i_m(t)_c, \quad (3)$$

where the subscripts T and c refer to test and control pulses. Usually a sequence of two control pulses followed by a test pulse was used. $i'_m(t)$ is the membrane current during and after the voltage-clamp test step to $V_1(\infty)_T$ from which has been subtracted the current in linear membrane elements from a voltage-clamp control step to $V_1(\infty)_c$. $V_1(\infty)_c$ was usually ± 20 mV from the holding potential. The solutions used for the experiments are given in Table 1. Rubidium was used to linearize cation current about the resting potential.

All experiments were made on isolated extensor digitorum longus (e.d.l.) muscle fibres dissected from 3–5 month old male and female Wistar rats (weight range 215–250 g) that had previously been killed by a swift blow to the head. For the organ-culture experiments, muscles were dissected under sterile conditions and kept moist during dissection with oxygenated Trowell medium.

Organ culture

A modification of the Trowell organ-culture technique was used (Trowell, 1959). Culture dishes (plastic Petri dishes, size 60 × 15 mm, Lux Scientific), were filled to a level of about 4 mm with an inert resin (Sylgard 184). Dissected muscles were placed on lens paper and rested on a stainless-steel grid platform, which had previously been embedded in the resin (Fig. 1). The muscles were slightly stretched and then pinned through the grid to about a 15% increase in their resting length. Trowell media (T-8 modified, Flow Laboratories) with added penicillin (50 i.u./ml) and streptomycin (50 μg/ml) was added to just cover the muscles. The culture dishes were kept in a large desiccator which had a moist sterile gaseous environment consisting of 95% oxygen and 5% carbon dioxide. Three-quarters of the culture medium, and all of the gas mixture, were changed daily under sterile conditions. Muscles were cultured at 37 °C and experiments were generally carried out on 4 day old cultures.

RESULTS

Cable constants. Linear fibre constants obtained in innervated fibres and fibres cultured for 4 days in various solutions at different temperatures are shown in Table 2. After 4 days in culture few changes in linear cable properties occured although, like *in vivo* denervated fibres (Albuquerque *et al.* 1971), the resting membrane potential had fallen to $-61{\cdot}1 \pm 0{\cdot}7$ mV (mean ± S.E. of mean, $n = 50$, five muscles).

Muscles with resting membrane potentials positive to −60 mV generally did not generate action potentials in response to pulses of depolarizing current from their resting potential.

Current-clamp experiments

To test for similarities between organ-cultured and *in vivo* denervated fibres, action potentials were generated using the two-micro-electrode techniques (see Methods) in the presence of 1 μM-tetrodotoxin (TTX). If the membrane of an *in vivo* denervated fibre is first locally hyperpolarized to −90 to −100 mV and then a brief pulse of

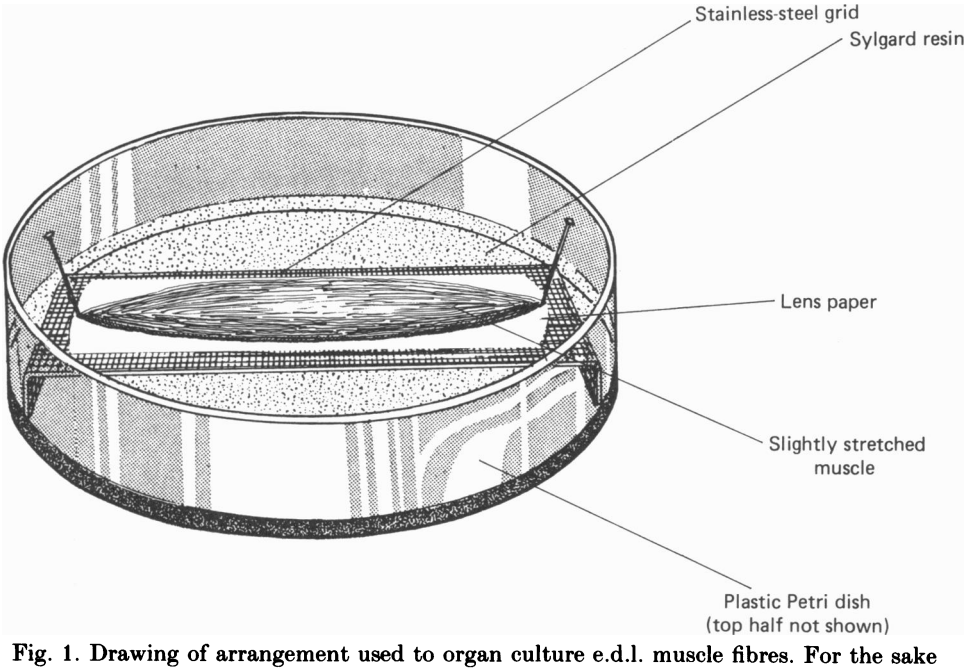

Fig. 1. Drawing of arrangement used to organ culture e.d.l. muscle fibres. For the sake of clarity the culture medium is not shown.

depolarizing current passed to generate an action potential, in contrast to innervated fibres, TTX at a concentration of 1 μM does not block action potential generation (for details, see Redfern & Thesleff, 1971). About one-half of the fibres cultured for 4 days that were examined in this way showed this 1 μM-TTX resistance (Fig. 2*A*; the action potentials in the other fibres were blocked by 1 μM-TTX). Reduced TTX sensitivity has been similarly observed in cultured rat diaphragm muscles from 1–2 month old rats (Lee *et al.* 1982).

A feature of fibres denervated *in vivo* for 4 days is that they fire anode-break action potentials (Marshall & Ward, 1974). Fig. 2*B* shows an anode-break action potential from a cultured fibre and like fibres denervated *in vivo* for 4 days, all fibres cultured for 4 days or more fired anode-break action potentials.

In contrast to innervated fibres, the rate of rise, the overshoot and threshold of an action potential of *in vivo* denervated fibres increases with membrane hyperpolarization (Redfern & Thesleff, 1971; Marshall & Ward, 1974). These parameters

TABLE 2. Mean values of constants for innervated and 4 day organ cultured fibres

	Solution	Temperature (°C)	n	λ (mm)	R_i (Ω cm)	R_m (kΩ cm^2)	Diameter (μm)	C_{eff} (μF cm^{-2})
Innervated	D	3·1±0·3	10	1·34±0·07	288·3±2·8	4·16±0·53	56·1±3·8	4·14±0·38
Cultured	D	4·2±0·2	9	1·20±0·09	278·0±1·9	3·63±0·65	48·8±2·3	5·13±0·38
	A	14·0±0·3	10	1·02±0·02	204·6±1·6	1·87±0·26	48·7±2·7	4·79±0·48
	B	9·3±0·2	8	1·07±0·09	237·0±1·4	2·18±0·32	51·9±2·2	4·62±0·46

Constants obtained at the beginning of each experiment. Values are means ±S.E. of mean. Cable constants and R_i calculated as described by Adrian & Marshall (1977).

TABLE 3. Constants for potassium currents in innervated and cultured fibres

			Results of n^2 analysis				
Type	Temperature (°C)	n	$\bar{V}_n$ (mV)	$\bar{\alpha}_n$ (ms^{-1})	$V_{\alpha n}$ (mV)	$\bar{\beta}_n$ (ms^{-1})	$V_{\beta n}$ (mV)
Innervated	14·7±0·1	9	−41·6±2·2	0·0044±0·0006	4·0±0·6	0·0370±0·0031	35·3±1·8
4 day cultured	14·1±0·4	5	−41·9±4·0	0·0057±0·0011	4·0±0·9	0·0342±0·0030	41·5±3·1

Muscles in solution A plus 7 μM-TTX. For innervated fibres, solution A made hypertonic with 350 mM-sucrose. Numbers of fibres in parentheses. Values are means ±S.E. of mean. $\alpha_n = \bar{\alpha}_n\,(V-\bar{V}_n)/(1-\exp[-((V-\bar{V}_n)/V_{\alpha n})])$ and $\beta_n = \bar{\beta}_n \exp[-((V-\bar{V}_n)/V_{\beta n})]$. For innervated fibres (15 °C) V_K, −73±2 mV (5); $\bar{G}_K$, 29·9±8·8 mS cm^{-2} (9), range 13·7–91·8. For fibres organ cultured for 4 days (14 °C) V_K, +−75·6±3·0 mV (5); $\bar{G}_K$, 7·1±1·0 mS cm^{-2} (5), range 5·6±10·9.

measured in fibres cultured for 4 days showed a similar dependence on membrane hyperpolarization (Fig. 2*C*). Thus, in solution A (Table 1) at 20 °C and at a membrane potential of −75 mV the mean value of the maximum rate of rise was 34·7 ± 10·1 V s^{-1} (± S.E. of mean, three fibres). At a holding potential of −80 mV a mean value of 166·8 ± 52·1 V s^{-1} (± S.E. of mean, three fibres) was obtained, while at −110 mV the

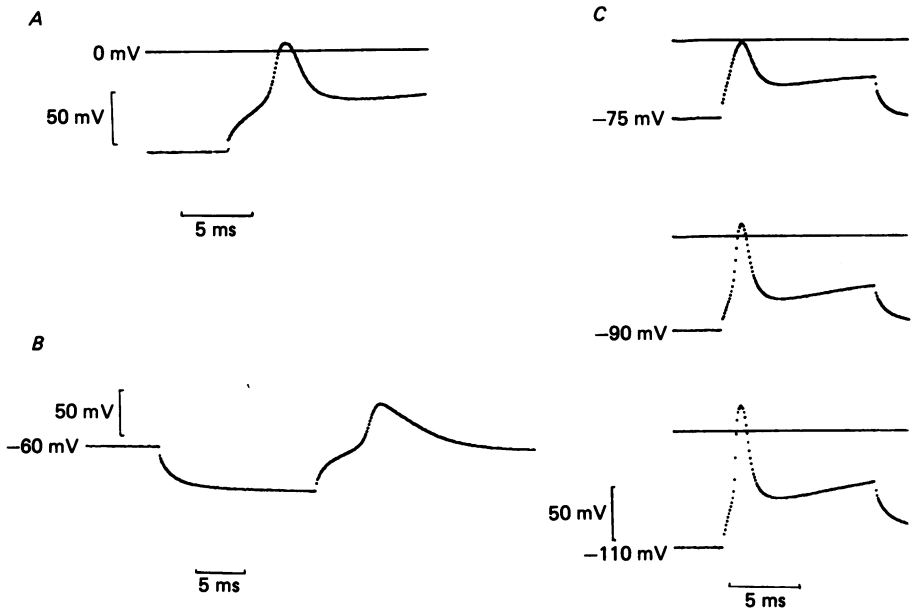

Fig. 2. Action potentials generated in a fibre cultured for 4 days using the two-micro-electrode current-clamp technique. *A*, TTX-resistant action potential. Solution A (Table 1) with 1 μM-TTX, resting potential −58 mV, holding potential −90 mV, temperature 24 °C. *B*, anode-break action potential in the absence of TTX. Arrows indicate the start and finish of the hyperpolarizing constant-current pulse. Solution A, resting potential −60 mV, holding potential −60 mV before hyperpolarizing pulse, temperature 20 °C. *C*, action potentials in the absence of TTX at holding potentials of −75, −90 and −110 mV. Resting potential −58 mV, temperature 21 °C.

mean value was 314·7 ± 35·3 V s^{-1} (± S.E. of mean, three fibres). These results were obtained from three *in vitro* denervated muscles.

Although cultured diaphragm muscles show spontaneous activity (e.g. Purves & Sakmann, 1974) we rarely saw similar activity in our organ-cultured e.d.l. muscles.

Voltage-clamp experiments

Sodium-current activation. Fig. 3*A* shows non-linear membrane currents at different levels of clamped membrane potential in isotonic solution B (Table 1). Currents at more positive levels of potential were not obtained as the fibres contracted in the isotonic Ringer solution. Isotonic solutions were generally used throughout this study as cultured fibres did not survive well in hypertonic solutions.

In Fig. 3*A* the early inward current is reduced as most of the external sodium is replaced with tetraethylammonium (TEA) ions and the late outward current is almost

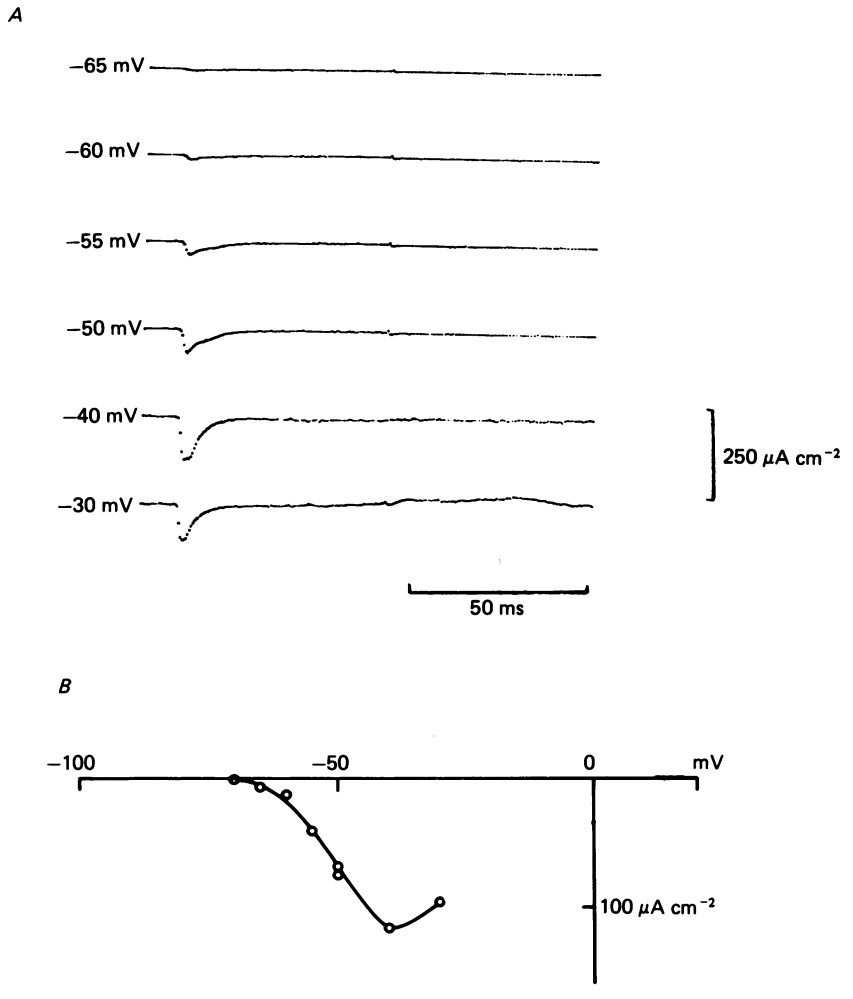

Fig. 3. *A*, records of sodium current obtained in a fibre cultured for 4 days in isotonic solution B (Table 1, external sodium concentration 38·4 mM). Holding potential −90 mV, 20 mV negative control pulses. Resting potential −56 mV, temperature 12·5 °C. *B*, maximum values of sodium current obtained from the fibre in *A* as a function of membrane potential. Abscissa: membrane potential at V_1. Ordinate: maximum inward current density.

absent as it is blocked by these ions (Stanfield, 1970). From a holding potential of −90 mV, about 50 % of the sodium channels are switched off (see inactivation results below). Thus the sodium currents that we measured will have smaller inward currents than if we had first hyperpolarized the membrane to more negative values and decreased inactivation. However, we found that holding muscle fibres at large negative potentials (in excess of −120 mV) for long periods often caused membrane breakdown, so generally we held at −90 mV. Fig. 3*B* shows the peak inward current obtained from each record in Fig. 3*A* as a function of membrane potential. In every fibre examined the early inward current was abolished by 7 μM-TTX, confirming that

it is a sodium current. Similar current–voltage curves were obtained in three other cultured fibres (two at 10 °C and one at 13 °C) and the maximum inward currents occurred between −30 and −40 mV. These values of membrane potential are in the same range as those found by Adrian & Marshall (1977) for innervated fibres. In 38 mM-external sodium, the largest values of measured currents in the four cultured fibres (three muscles) ranged between 0·025 and 0·122 mA cm^{-2}.

The sodium reversal potential in innervated and *in vivo* denervated fibres is the same (Pappone, 1980) and *in vivo* denervation does not change the internal sodium concentration (Robbins, 1977). If we make these two assumptions for *in vitro* denervated fibres (there was some evidence to suggest that the zero-current potential was unchanged after culturing) then it is possible to convert peak sodium currents into permeabilities (Adrian & Marshall, 1977) and normalize these permeabilities to the permeabilities at their maximum current value. Plots were made of relative permeability against voltage for four fibres, including the fibre in Fig. 3, and the mid-point of each curve ranged between −42 and −49 mV with a mean value of −45·5 ± 1·4 mV (± S.E. of mean). The voltage dependence of these relative peak permeabilities was similar to that reported for innervated mammalian muscle fibres in hypertonic solutions (Adrian & Marshall, 1977).

Sodium-current inactivation. A steady-state inactivation curve (see Hodgkin & Hurley, 1952 for definition of h_∞) is shown in Fig. 4*A* and was obtained by preceding the test pulse with a 200 ms pre-pulse. The mid-point of the curve in Fig. 4*A* is −85 mV. In three other individually analysed fibres using either 100 or 200 ms pre-pulses the mid-point potentials ranged from −82 to −96 mV. The mean value of the four fibres was −88·5 ± 3·1 mV (± S.E. of mean) and this value is some 20 mV more negative than that found for innervated intact fibres in hypertonic solutions (Adrian & Marshall, 1977). The steady-state inactivation curves in cultured fibres all saturated at about −110 mV. Fig. 4*B* shows the voltage dependence of the mean values of the rate constants of inactivation of the sodium current from five individually analysed muscle fibres cultured for 4 days. Rate constants were obtained by fitting a single exponential to the decay phase of the current. The values obtained in cultured muscles at −60 mV are twice those obtained from innervated e.d.l. fibres in the same isotonic solution and at the same temperature. For comparative purposes we have replotted the mean values of the results obtained by Adrian & Marshall (1977) for innervated e.d.l. muscles in hypertonic solution at 14 °C.

Potassium currents. Adrian & Marshall (1977) observed potassium currents in intact, innervated rat e.d.l. muscle fibres and, since then, potassium currents in cut e.d.l. and other innervated types of mammalian muscle fibre have been studied in some detail (Duval & Leoty, 1978, 1980*a*, *b*; Pappone, 1980; Beam & Donaldson, 1983*a*, *b*). As a control for our cultured muscles and as a detailed study of potassium currents in intact, innervated e.d.l. muscles did not exist, a Hodgkin & Huxley (1952) analysis of innervated e.d.l. fibres was carried out.

Innervated fibres. Fig. 5*A* shows potassium currents in an innervated fibre in solution A (Table 1) with 7 μM-TTX and made hypertonic by the addition of sucrose (350 mM). From the tail-current experiment shown in Fig. 5*B* an instantaneous current–voltage (I–V) relation for potassium channels can be plotted and estimates made of the zero-current potential (V_K). V_K measured in this way in five fibres ranged between

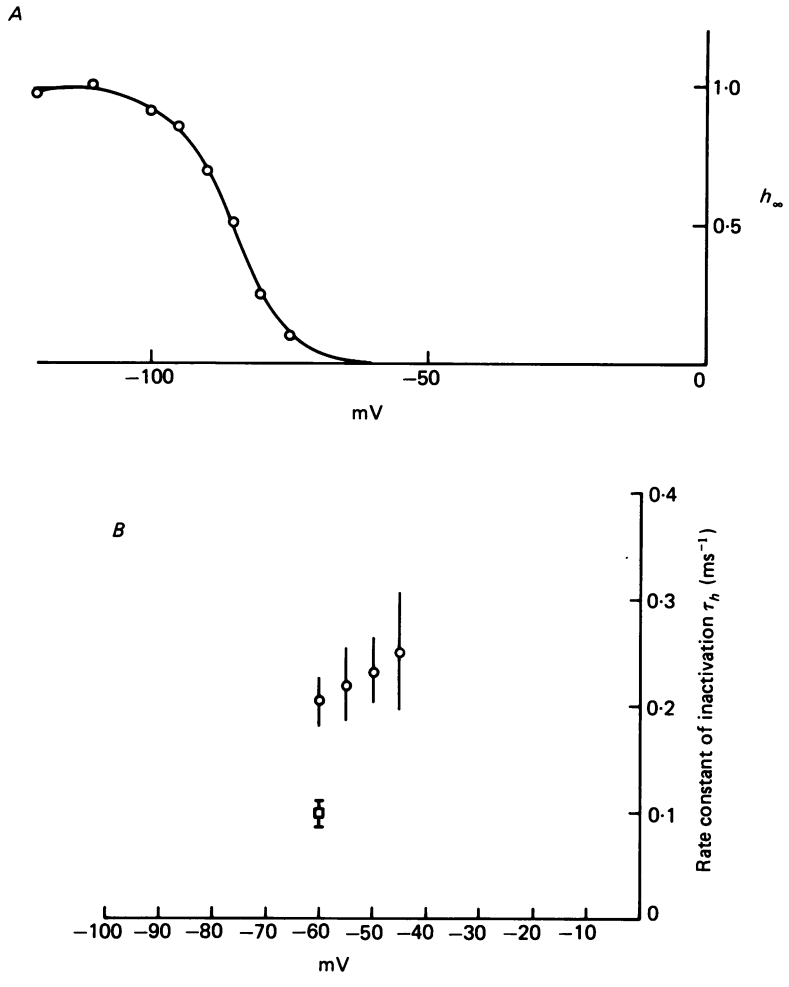

Fig. 4. *A*, relation between h_∞ (see text) and membrane potential in isotonic solution B (Table 1) for a fibre cultured for 4 days, resting potential −57 mV, temperature 9 °C. Curve drawn by eye. Abscissa: membrane potential at V_1 of a pre-pulse that lasted 200 ms. Ordinate: ratio of inward current with pre-pulse to inward current with V_1 −110 mV. Test pulse to −50 mV. *B*, relation between rate constants of inactivation (τ_h) of the inward current and membrane potential at V_1. Open circles, fibres cultured for 4 days, in isotonic solutions B or C (Table 1) at 10 °C; square, innervated fibres in isotonic solution B at 10 °C. Each point is the mean value of at least seven fibres. Vertical bars ±S.E. of mean. Filled circles for mean values of innervated fibres at 14 °C in hypertonic solution (replotted from Adrian & Marshall, 1977, Fig. 14).

−67 and −78 mV, and the mean value was −73±2 mV (±S.E. of mean). It is possible that this is an incorrect estimate of V_K as tubular capacity current and potassium accumulation in the transverse tubular system could shift V_K about 10 mV positively.

The currents shown in Fig. 5*A* rose with less delay than is shown in frog potassium currents measured in the middle of a fibre (Fig. 5*C*) (S. Hollingworth & M. W. Marshall,

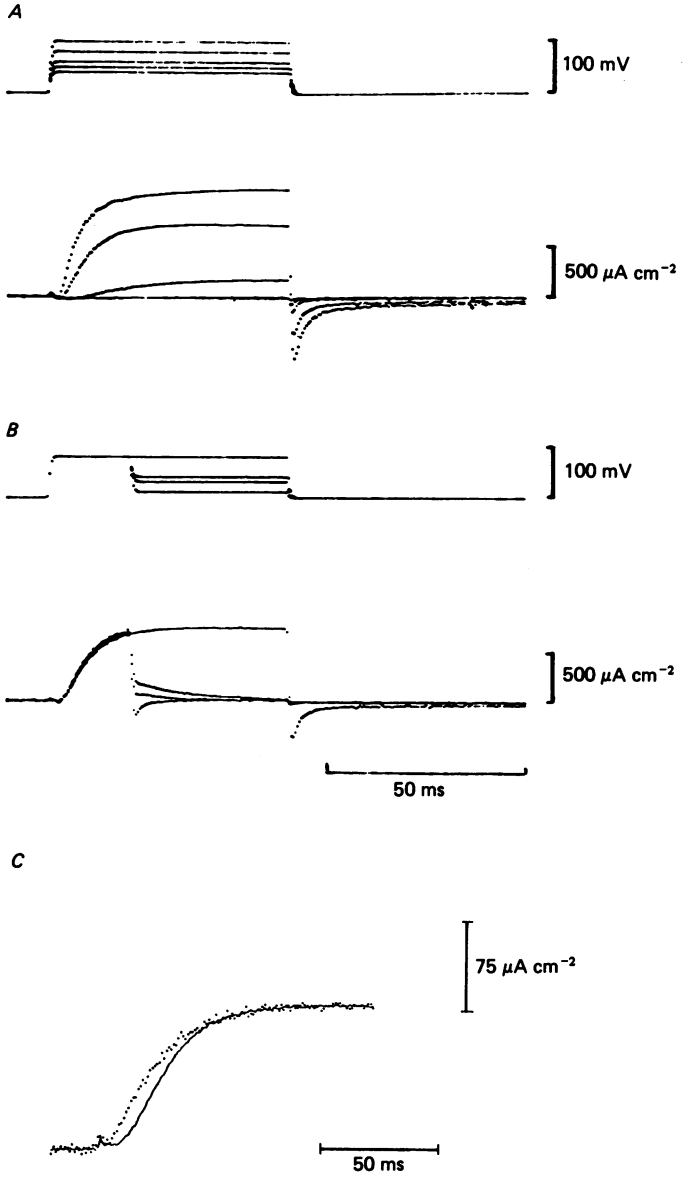

Fig. 5. *A*, potassium currents (lower) recorded in response to depolarizations (upper) in an innervated fibre in solution A with 7 μM-TTX (Table 1). *B*, membrane currents (lower) and membrane potential (upper) in the same fibre as *A*, where a second pulse repolarized the membrane after 20 ms. Resting potential −79 mV, holding potential −90 mV and temperature 15 °C. *C*, dotted curve is the potassium current from a rat e.d.l. fibre at 15 °C following a voltage-clamp pulse to −40 mV. Continuous curve is from a frog (*Rana temporaria*) sartorius fibre at 2 °C in which the size of a voltage dependence of $G_{K\infty}$ was similar to the rat fibre. Frog curve (scaled by 0·75 so that the two curves are the same height) was best fitted with a n^4 variable, while the rat curve was best fitted with a n^2 variable.

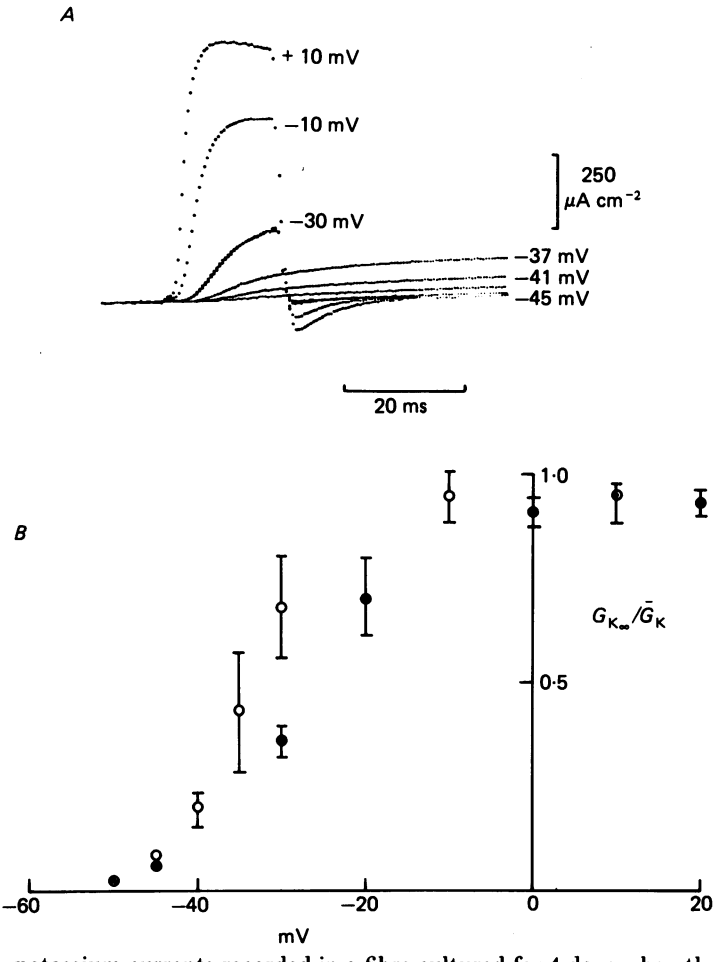

Fig. 6. *A*, potassium currents recorded in a fibre cultured for 4 days when the membrane was depolarized to the value shown alongside each trace. The −30 mV membrane potential used to bracket all the records. Solution A (Table 1) with 7 μM-TTX, resting potential −57 mV, holding potential −90 mV and temperature 15 °C. *B*, relation between the ratio $G_{K\infty}/\bar{G}_K$ and the membrane potential at V_1. Innervated fibres (filled circles) at 15 °C; cultured fibres (open circles) at 14 °C. Solution A (Table 1) made hypertonic with 350 mM-sucrose for innervated fibres. Holding potentials −90 mV, means ± S.E. of means. Each mean has a minimum of five observations.

unpublished observations). Unlike frog fibres this and all other innervated e.d.l. fibres examined at 15 °C were better fitted by the Hodgkin–Huxley (1952) n variable raised to a second rather than a fourth power (see also Beam & Donaldson, 1983*a*). In order to test how well n^2 analysis described the measured potassium currents, the currents in Fig. 5 were recalculated using n^2 analysis and the measured currents at 15 °C were reasonably well described by the second power. Table 3 shows the mean values of the voltage dependence of the Hodgkin–Huxley rate constants (α_n, β_n) obtained from this fibre and eight other innervated fibres all individually analysed using n^2 kinetics.

Cultured fibres (in vitro denervated). Fig. 6*A* shows potassium currents measured

in a fibre cultured for 4 days at 15 °C in isotonic solution A (Table 1) with 7 μM-TTX. The currents are similar to those measured in the innervated fibre shown in Fig. 5*A* except that the two records following the most positive depolarizing voltage step show slow changes in potassium current. These changes, which often occurred in innervated fibres, could be due to either inactivation of the potassium current or potassium accumulation in the transverse tubular system or a combination of both mechanisms (see also Duval & Leoty, 1980*b*; Beam & Donaldson, 1983*b*). Tail-current experiments in the same fibre of Fig. 6 were carried out and the instantaneous *I–V* curve for this fibre gave a zero-current potential, V_K of −85 mV. In five fibres, including this fibre, the mean was −75±3 mV (±S.E. of mean; Table 3). Because of the difficulties of obtaining accurate V_K values (due to problems such as tubular potassium accumulation and tubular capacity currents) it is difficult to say if there is a significant difference in V_K between innervated and cultured fibres.

Fig. 6*B* shows the voltage dependence of the steady-state potassium conductance ($G_{K\infty}$) normalized to the maximum value obtained ($\bar{G}_K$) (see also Table 3). Fig. 6*B* is included as it is model independent and, for comparison, the Figure also shows the mean values of normalized potassium conductance obtained in innervated e.d.l. fibres at the same temperature. Table 3 shows that, although the range in innervated fibres is large, the mean maximum potassium conductance for innervated fibres is over three times that of cultured fibres. However, innervated fibres were in hypertonic solution and as the tonicity is about two times isotonic there will be about a twofold increase in the internal activity of the potassium ions. If, as in squid axon (Chandler, Hodgkin & Meves, 1965), the potassium current is proportional to the internal potassium concentration the measured value of $\bar{G}_K$ will correspond to a value of about 15 mS cm^{-2} in isotonic solution. This value is still almost twice as large as that obtained from cultured fibres. From Fig. 6*B* it is also possible that the cultured-fibre curve is shifted negatively by about 5 mV when compared to the innervated fibres.

The currents shown in Fig. 6*A* (and also for four other analysed fibres) were all reasonably well fitted with the Hodgkin–Huxley model using n^2 analysis. The analysis was reasonable up to membrane potentials of around −30 mV. Further depolarization resulted in less-good fits and this was probably due to the apparent slow changes in potassium conductance which were not modelled. Table 3 shows the mean values of voltage dependence of the rate constants of five fibres cultured for 4 days, all individually analysed using n^2 kinetics.

Charge movements. Fig. 7*A* shows charge movement records obtained in solution D (Table 1) with 7 μM-TTX at 5 °C from a fibre cultured for 4 days. Fig. 7*B* shows that there was good equality between the amount of 'on' and 'off' charge. Fig. 7*C* shows the amount of charge moved as a function of membrane potential ($Q(V)$). The continuous line in Fig. 7*C* is a least-squares best fit to the data assuming a Boltzman distribution for charge between two membrane locations $(Q(V)) = Q_{max}/(1+\exp(-(V-\bar{V})/_k))$; Schneider & Chandler, 1973). The mean value of the saturating amount of charge (Q_{max}) estimated from an unconstrained least-squares fit of the $Q(V)$ curve to this and two other fibres at 5 °C was 12·1±2·2 nC μF^{-1} (±S.E. of mean). The mean value of the membrane potential at which charge was equally located between the two locations ($\bar{V}$) was −26·5±6·0 mV (±S.E. of mean) and

the mean value of k (a factor related to the steepness of the curve) was $13{\cdot}3 \pm 3{\cdot}1$ mV ($\pm$S.E. of mean).

Although cooling mammalian muscle to 5 °C markedly reduced twitch tension (Hill, 1972) local muscle contractions were obtained with large depolarizing voltage-clamp pulses and so recording charge movements at positive membrane potentials in order to obtain an accurate Q_{max} was difficult. The mean estimated value of Q_{max} of unconstrained $Q(V)$ fits in cultured fibres was less than one-third the mean value obtained by Hollingworth & Marshall (1981) in innervated fibres in hypertonic solutions at 3 °C (where $Q_{max} = 46{\cdot}2$ nC μF^{-1}; $\overline{V} = -22{\cdot}8$ mV; $k = 13{\cdot}4$ mV). This difference might arise if substantial amounts of charge were moved in cultured fibres at membrane potentials more positive than those explored. To test for this, the data from each cultured fibre were also fitted with a constrained fit of the $Q(V)$ curve. Thus, Q_{max} was fixed at either 25 or 50 nC μF^{-1}. These fits (although always less good than those in which Q_{max} was free; see, for example, the legend to Fig. 8*C*) were adequate. The three cultured fibres gave mean values ($\pm$S.E. of mean) of $\overline{V}$ and k for a Q_{max} fixed at 25 nC μF^{-1} of $-0{\cdot}3 \pm 4{\cdot}2$ mV ($\overline{V}$) and $19{\cdot}7 \pm 1{\cdot}8$ mV (k) and for Q_{max} fixed at 50 nC μF^{-1} of $22{\cdot}5 \pm 4{\cdot}9$ mV ($\overline{V}$) and $22{\cdot}5 \pm 1{\cdot}5$ mV (k). These results might suggest that the cultured data could be fitted without a reduced Q_{max} if $\overline{V}$ were shifted positively some 20–40 mV and if the $Q(V)$ curve rose considerably less steeply. However, a measure of the steepness of the rise of the curve can be estimated from the exponential rise of charge at the foot of the $Q(V)$ curve. In innervated e.d.l. muscles this e-fold rise is close to the value of k obtained from unconstrained $Q(V)$ fits to the whole curve (see Hollingworth & Marshall, 1981, Table 3). The mean e-fold rise in the three cultured fibres was $14{\cdot}1 \pm 0{\cdot}8$ mV ($\pm$S.E. of mean), close to the value obtained from fitting the whole curve with Q_{max} free ($k = 13{\cdot}3$ mV), and consistent therefore with a reduced Q_{max}. The voltage intercept of the semilogarithmic plot used to obtain the e-fold rise was $-56{\cdot}1 \pm 0{\cdot}34$ mV ($\pm$S.E. of mean) and is similarly more compatible with charge-movement parameters obtained with a reduced Q_{max}.

$\overline{V}$ of charge movement in innervated e.d.l. fibres in isotonic solution is probably shifted positively by about 6 mV compared to $\overline{V}$ in hypertonic solution (Hollingworth & Marshall, 1981) and would be close, therefore to -17 mV. The $\overline{V}$ measured in cultured fibres is about 10 mV negative to the value indicating a possible negative shift of the $Q(V)$ curve obtained from cultured fibres.

The decay phase of charge movement in fibres cultured for 4 days was reasonably well fitted by a single exponential, although occasionally fitting was difficult as only small amounts of charge were moved. Fig. 7*D* shows that the time constant of the decay phase of charge movement in cultured fibres is about twice that found in innervated e.d.l. fibres (Hollingworth & Marshall, 1981).

DISCUSSION

Our measurements on action-potential generation in surface fibres from cultured *in vitro* denervated muscle suggest that these fibres have membrane properties similar to both *in vivo* denervated fibres and fibres in developing rats where the adult innervation pattern has yet to be established (e.g. Harris & Marshall, 1973).

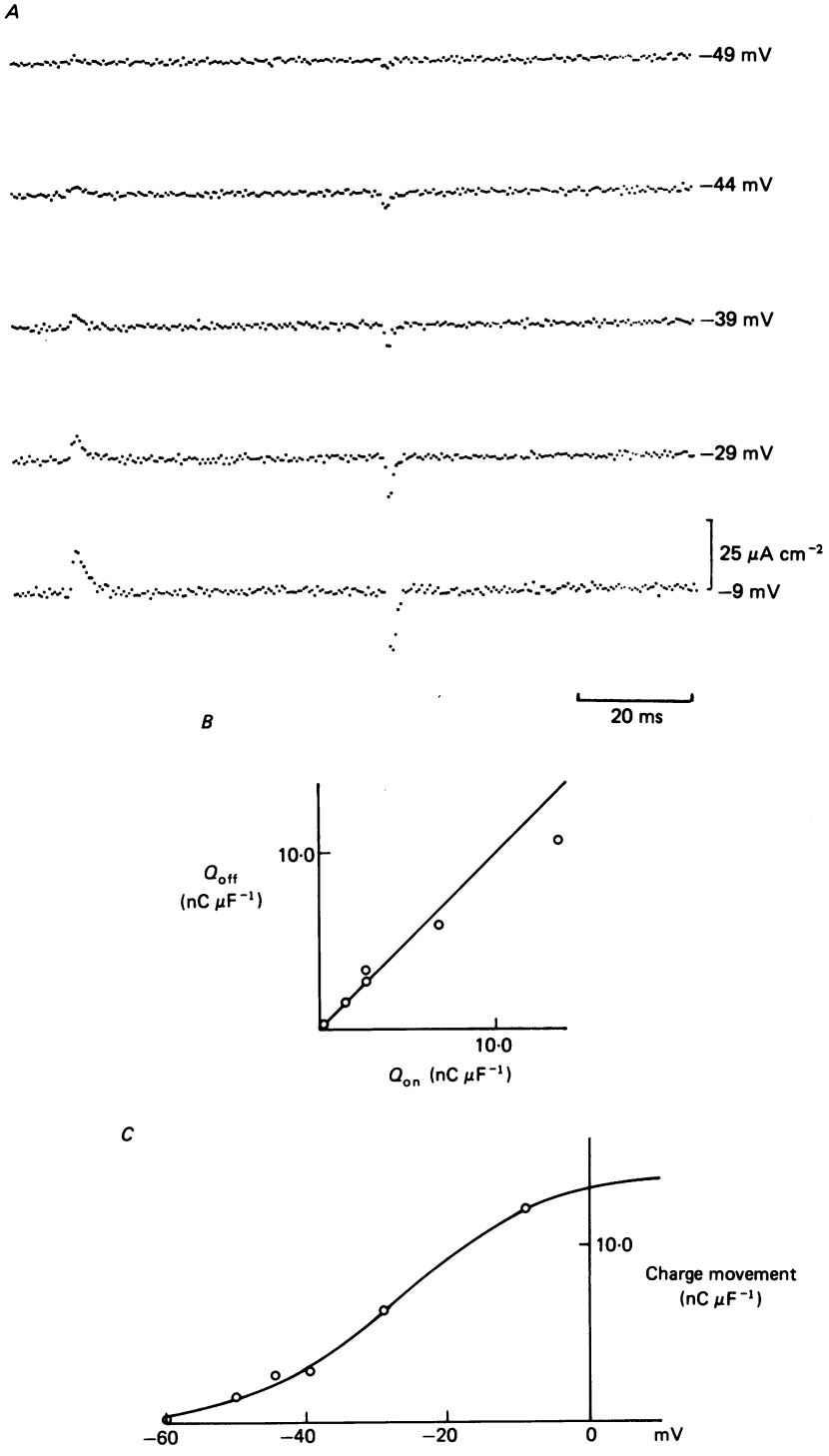

Fig. 7. *A–C*, for legend see opposite.

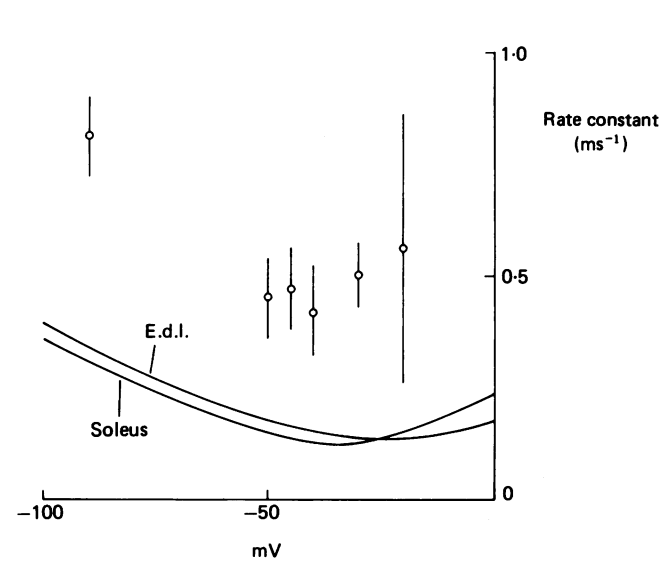

Fig. 7. *A*, single-sweep records of charge movements in solution D (Table 1) with 7 μM-TTX in fibres cultured for 4 days. The membrane potential at V_1 during the test pulse given alongside each trace. Resting potential −59 mV, holding potential −90 mV, control pulses to −110 mV, temperature 4·8 °C. Muscles held for at least 5 min at holding potential before the start of experiment. *B*, equality of 'on' and 'off' charge (Q_{on} and Q_{off}). The line drawn represents absolute equality of charge. Charge transients normalized to membrane capacity. *C*, charge movement as a function of voltage at V_1. Each point is the average of 'on' and 'off' charge. Smooth curve is unconstrained best fit of $Q(V) = Q_{max}/(1+\exp(-(V-\bar{V})/k))$, where best-fit parameters were k, a steepness factor, = 10·2 mV, $\bar{V} = -27{\cdot}2$ mV and $Q_{max} = 14{\cdot}1$ nC μF^{-1}, and the root-mean-square error of the data points (r.m.s.) = 0·33. With Q_{max} fixed at 20 nC μF^{-1}, r.m.s. error = 0·53 and with Q_{max} fixed at 50 nC μF^{-1}, r.m.s. error = 0·74. *D*, rate constants from organ-cultured muscle as a function of membrane potential at V_1. Each point is the mean of between three and seven observations. Vertical bars ±S.E. of mean, temperature 4 °C. For comparison we include curves from innervated e.d.l. and soleus fibres. These continuous curves are drawn through the mean values for rate constants for both e.d.l. and soleus fibres at 2 °C (replotted from Hollingworth & Marshall, 1981, Figs. 4 and 6).

Examination of the ultrastructure of the surface fibres of the organ-cultured muscles showed that the surface membrane, transverse tubular system and sarcoplasmic reticulum were well preserved and maintained their normal innervated spatial relations which are a feature of short-term *in vivo* denervated fibres (Cullen & Pluskal, 1972; Cullen, Hollingworth & Marshall, 1984). In particular the organization of the triads, with the double row of junctional 'feet' opposing the upper and lower surfaces, was unaltered (Pl. 1). The other components of the muscle fibre were also well maintained after 4 days in culture. There were small areas of myofilament disorganization but over the greater part of the muscle the banding pattern and filament alignment were well preserved (M. Cullen, unpublished observation). The triads had not yet moved to longitudinal orientation, a feature of long-term *in vivo* denervation (Cullen & Pluskal, 1972). We would thus conclude that our cultured fibres

can be used as electrophysiological and morphological models for the *in vivo* denervation-induced changes in muscle fibre properties.

Sodium and potassium currents and charge movements were present in the cultured fibres and were not greatly different from innervated e.d.l. fibres. Over the same temperature range (0–20 °C) two differences in the sodium currents between cultured and innervated fibres were found. The voltage dependence of the steady-state inactivation curve (h_∞) is shifted about 20 mV negatively when compared to the same curve in innervated muscle in hypertonic solution. Hypertonic solutions shift the voltage dependence of tension development (Gordon & Godt, 1970) and charge movements (Hollingworth & Marshall, 1981) about 5–10 mV negatively. Hypertonicity then will cause an increase in the observed shift of the h_∞ curve, and it is thus likely that there is a real negative shift of at least 20 mV. At the same temperature, and in isotonic solution, the rate constant of inactivation of the inward current is about twice that of an innervated-fibre e.d.l. This difference in the rate constants for the cultured and innervated fibres might reflect the observed negative shift of the h_∞ curve. Thus, if the rate-constant curve for innervated fibres was shifted negatively by about 20 mV this would effectively double the rate constants at any given membrane potential. This negative shift in the steady-state inactivation curve could explain why *in vivo* and *in vitro* denervated fibres form anode-break action potentials and why the rate of rise and overshoot of the action potential depend upon the level of membrane hyperpolarization. Thus, hyperpolarizing the membrane makes more sodium channels available for the inward current.

In contrast the steady-state activation curve in cultured fibres (m_∞) is probably not much different from that found in innervated e.d.l. fibres. Allowing for the effects of tonicity it is likely that there is no more than a 5 mV shift in cultured fibres.

Pappone (1980) studied sodium currents in cut, innervated and *in vivo* denervated e.d.l. muscles. Unlike the results presented here in intact fibres, h_∞ curves measured by Pappone in either cut innervated or denervated e.d.l. fibres did not saturate. With cut fibres, denervation caused a negative shift in the h_∞ curve but not a similar shift in the time constant of inactivation. These cut fibres showed a 10 mV negative shift of the m_∞ curve after *in vivo* denervation. These differences between Pappone's results and ours may be due to the effects of cutting the muscle fibres before voltage clamping the fibres.

Unlike potassium currents from the middle of frog fibres, our innervated rat e.d.l. muscle fibres at 15 °C are not well fitted by n^4 kinetics, better fits being obtained with n^2 kinetics. A similar result was found by Beam & Donaldson (1983*a*) who found that the ability of n^4 kinetics to describe the potassium currents in intact innervated rat omohyoid muscles was a function of temperature. Pappone (1980) found that potassium currents in innervated cut fibres at 24 °C, when present, were well fitted by n^4 kinetics with a time delay.

In isotonic solutions at 15 °C, potassium currents were always observed in our cultured fibres and were, like innervated fibres at the same temperature, reasonably well fitted by n^2 kinetics. After about 4 days in culture there would appear to be a fall in the maximum potassium conductance to about one-half that found in innervated fibres. Recently Duval & Leoty (1984) have suggested that fibres denervated *in vivo* for 5 days have outward currents with a slow component that is

TEA insensitive. Since our fibres denervated *in vitro* for 4 days had potassium currents that were blocked by TEA, it is possible that before mucles develop this TEA resistance they may have to be denervated for at least 5 days.

Charge movements were observed in cultured fibres and denervation is difficult to assess in paraplegia as the muscle fibres are still connected to their ventral horn cells but it would appear, from the Dulhunty & Gage (1983) study, that spinal cord transection, and hence reduced muscle activity, resulted in a decreased maximum amount of charge moved. Compared to charge movements measured in the middle of innervated rat muscle in hypertonic solution, the estimated maximum amount of charge moved *in vitro* denervated cultured fibres would seem to be about one-third less.

Culturing seems to have almost doubled the kinetics of charge movement decay, and the voltage distribution of the charge movement could be shifted negatively by about 10 mV. It is possible that muscle inactivity in our cultured fibres results in a fall in Q_{max} and the negative shift in the voltage distribution and increase in the kinetics of charge movement. Hollingworth & Marshall (1981) noted that compared to fast-twitch fibres, slow-twitch fibres showed about a one-third reduction in Q_{max} and the voltage distribution of the charge was also shifted negatively by about 10–15 mV. In addition, Finol *et al.* (1981) noted that short-term *in vivo* denervation of fast-twitch fibres resulted in a prolongation of the isometric twitch tension. Thus, our results might suggest that short-term culturing of the fast-twitch e.d.l. fibres causes them to develop some properties similar to slow-twitch fibres, and this would be an interesting line of investigation to follow.

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REFERENCES

ADRIAN, R. H. & ALMERS, W. (1976). The voltage dependence of membrane capacity. *Journal of Physiology* **254**, 317–338.

ADRIAN, R. H. & MARSHALL, M. W. (1977). Sodium currents in mammalian muscle. *Journal of Physiology* **268**, 223–250.

ALBUQUERQUE, E. X., SCHUH, F. T. & KAUFFMAN, F. C. (1971). Early membrane depolarisation of the fast mammalian muscle after denervation. *Pflügers Archiv* **328**, 36–50.

BEAM, K. G. & DONALDSON, P. L. (1983*a*). A quantitative study of potassium channel kinetics of rat skeletal muscle from 1 to 37 °C. *Journal of General Physiology* **81**, 485–512.

BEAM, K. G. & DONALDSON, P. L. (1983*b*). Slow components of potassium tail currents in rat skeletal muscle. *Journal of General Physiology* **81**, 513–530.

BEKOFF, A. & BETZ, W. (1977). Properties of isolated adult rat muscle fibres maintained in tissue culture. *Journal of Physiologuy* **271**, 537–547.

CHANDLER, W. K., HODGKIN, A. L. & MEVES, H. (1965). The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *Journal of Physiology* **180**, 821–836.

CULLEN, M. J., HOLLINGWORTH, S. & MARSHALL, M. W. (1984). A comparative study of the transverse tubular system of the rat extensor digitorum longus and soleus muscle. *Journal of Anatomy* **138 (2)**, 297–308.

CULLEN, M. J. & PLUSKAL, M. G. (1972). Early changes in the ultrastructure of denervated rat skeletal muscle. *Experimental Neurology* **56**, 115–131.

DULHUNTY, A. P. & GAGE, P. (1983). Asymmetrical charge movement in slow- and fast-twitch mammalian muscle fibres in normal and paraplegic rats. *Journal of Physiology* **341**, 213–231.

DUVAL, A. & LEOTY, C. (1978). Ionic currents in mammalian fast skeletal muscle. *Journal of Physiology* **278**, 403–423.

DUVAL, A. & LEOTY, C. (1980*a*). Ionic currents in slow twitch skeletal muscle in the rat. *Journal of Physiology* **307**, 23–41.

DUVAL, A. & LEOTY, C. (1980*b*). Comparison between delayed outward current in slow and fast twitch skeletal muscle in the rat. *Journal of Physiology* **307**, 43–57.

DUVAL, A. & LEOTY, C. (1984). Effects of denervation on ionic currents in mammalian fast and slow twitch skeletal muscle. *Journal of Physiology* **350**, 50*P*.

FINOL, H. J., LEWIS, D. M. & OWENS, R. (1981). The effects of denervation on contractile properties of rat skeletal muscle. *Journal of Physiology* **319**, 81–92.

GORDON, A. M. & GODT, R. E. (1970). Some effects of hypertonic solution in contraction and excitation–contraction coupling in frog skeletal muscles. *Journal of Physiology* **55**, 254–275.

GUTMANN, E., MELICHNA, J. & SYROVY, I. (1976). Independence of changes in contraction properties and myofibrillar ATPase activity of denervated mammalian muscle on length of nerves stump. *Physiologia bohemoslovenica* **25**, 43–50.

HARRIS, J. B. & MARSHALL, M. W. (1973). Tetrodotoxin resistant action potentials in newborn rat muscle. *Nature, New Biology* **243**, 191–192.

HARRIS, A. J. & MILEDI, R. (1972). Study of frog muscle maintained in organ culture. *Journal of Physiology* **221**, 207–226.

HILL, D. K. (1972). Resting tension and the form of the twitch of rat skeletal muscle at low temperature. *Journal of Physiology* **221**, 161–171.

HODGKIN, A. L. & HUXLEY, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *Journal of Physiology* **117**, 500–544.

HOLLINGWORTH, S. & MARSHALL, M. W. (1981). A comparative study of charge movements in rat and frog skeletal muscle fibres. *Journal of Physiology* **321**, 583–602.

LEE, S., MILEDI, R. & RUZZIER, F. (1982). *In vitro* development of TTX resistant action potentials in rat muscle. *Journal of Physiology* **327**, 32*P*.

MARSHALL, M. W. & WARD, M. R. (1974). Anode break excitation in denervated rat skeletal muscle fibres. *Journal of Physiology* **236**, 413–420.

PAPPONE, P. A. (1980). Voltage-clamp experiments in normal and denervated mammalian skeletal muscle fibres. *Journal of Physiology* **306**, 377–410.

PURVES, D. & SAKMANN, B. (1974). The effect of contractile activity on fibrillation and extrajunctional acetylcholine-sensitivity in rat muscle maintained in organ culture. *Journal of Physiology* **237**, 157–182.

REDFERN, P. & THESLEFF, S. (1971). Action potential generation in denervated rat skeletal muscle. Part II. The action of tetrodotoxin. *Acta physiologica scandinavica* **82**, 70–78.

ROBBINS, N. (1977). Cation movements in normal and short-term denervated rat fast-twitch muscle. *Journal of Physiology* **271**, 605–624.

SCHNEIDER, M. E. & CHANDLER, W. K. (1973). Voltage dependent charge movements in skeletal muscle. *Nature* **242**, 224–246.

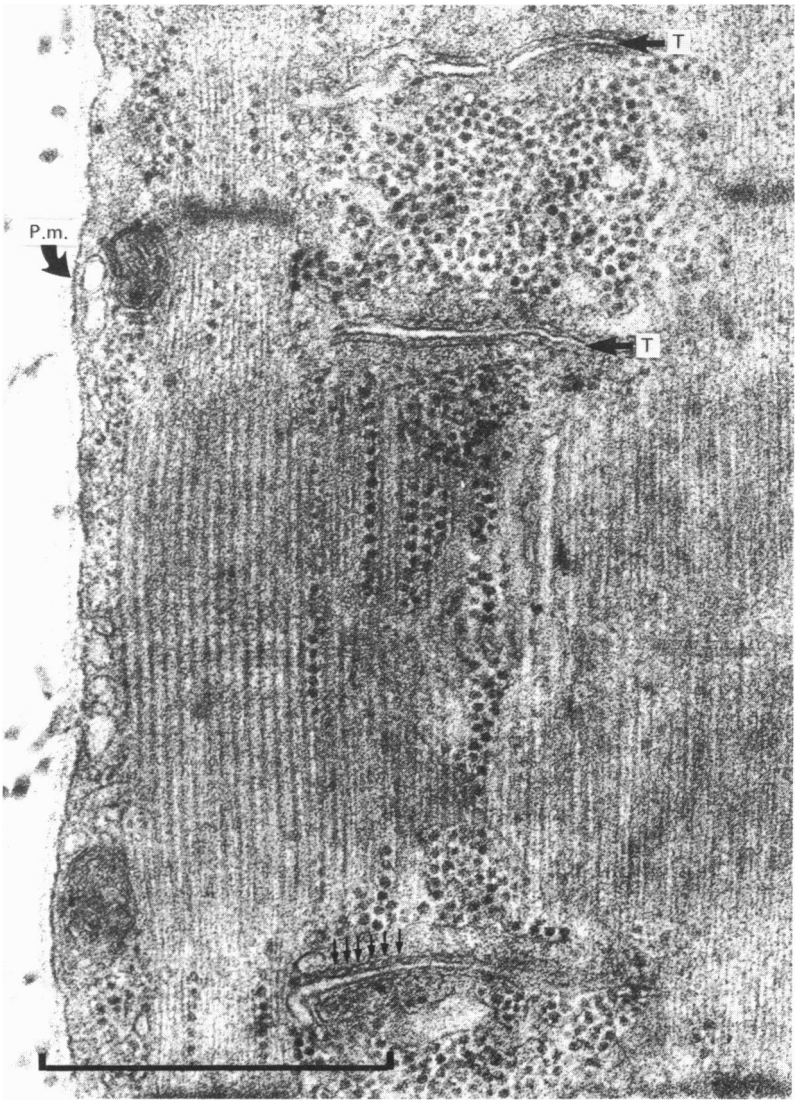
STANFIELD, P. R. (1970). The effect of tetraethylammonium ions in the delayed currents of frog skeletal muscle. *Journal of Physiology* **209**, 209–229.

THESLEFF, S. (1963). Spontaneous electrical activity in denervated rat skeletal muscle In *The Effect of Use and Disease in Neuromuscular Function*, ed. GUTMANN, E. & HNIK, P., pp. 41–51. Prague: Czechoslovak Academy of Sciences.

TROWELL, O. A. (1959). The culture of mature organs in a synthetic medium *Experimental Cell Research* **16**, 118–147.

EXPLANATION OF PLATE

Longitudinal section of the edge of a peripheral fibre of a rat e.d.l. muscle fibre after 4 days in culture. A row of 'feet' is arrowed. T = transverse tubular system, p.m. = plasma membrane. Bar = 1 μm. Electron-dense-staining particles are glycogen granules.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T