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Expression of ACh-activated channels and sodium channels by messenger RNAs from innervated and denervated muscle

By I. Parker[1], K. Sumikawa[1], C. B. Gundersen[2] and R. Miledi[1], F.R.S.

[1] *Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, California* 92717, *U.S.A.*
[2] *Department of Pharmacology, U.C.L.A. School of Medicine, Los Angeles, California* 90024, *U.S.A.*

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Xenopus oocytes were used to express polyadenylated messenger RNAs (mRNAs) encoding acetylcholine receptors and voltage-activated sodium channels from innervated and denervated skeletal muscles of cat and rat. Oocytes injected with mRNA from denervated muscle acquired high sensitivity to acetylcholine, whereas those injected with mRNA from innervated muscle showed virtually no response. Hence the amount of translationally active mRNA encoding acetylcholine receptors appears to be very low in normally innervated muscle, but increases greatly after denervation. Conversely, voltage-activated sodium currents induced by mRNA from innervated muscle were about three times larger than those from denervated muscle; this result suggests that innervated muscle contains more mRNA coding for sodium channels. The sodium current induced by mRNA from denervated muscle was relatively more resistant to block by tetrodotoxin. Thus a proportion of the sodium channels in denervated muscle may be encoded by mRNAs different from those encoding the normal channels.

Introduction

Many characteristics of skeletal muscle are influenced by their innervating nerve fibres, as for example the number and distribution of acetylcholine receptors (AChRs) in the muscle membrane. Denervation causes the muscle to become supersensitive to ACh, because of the formation of many new AChRs located in extrajunctional regions of the fibres (Axelsson & Thesleff 1959; Miledi 1960; Fambrough 1979). Another influence of innervation is exerted on the voltage-gated sodium channels that are responsible for generating the muscle action potential. For example, slow muscle fibres of the frog are normally incapable of generating action potentials, but after denervation the membrane acquires sodium channels (Miledi *et al.* 1971). Moreover, after denervation the action potential of rat muscle becomes more resistant to blocking by tetrodotoxin (TTX) (Redfern & Thesleff 1971*b*; Harris & Thesleff 1971) because of the appearance of

a population of sodium channels with a lower affinity for TTX (Pappone 1980; Frelin *et al.* 1984; Rogart & Regan 1985; Weiss & Horne 1987).

The AChRs which appear after denervation are newly synthesized (Harris & Miledi 1968; Fambrough 1979). However, the control of AChR synthesis by the nerve might be exerted at any of several stages in the synthetic pathway, from the transcription of AChR genes to the incorporation of receptors into the plasma membrane. Previous experiments indicate that an increase in gene transcription may be a major factor, because denervation supersensitivity can be prevented by actinomycin D (Grampp *et al.* 1972; Fambrough 1979), and the levels of mRNA coding for the α subunit of the AChR were found to be increased by a factor of up to 100 after denervation (Merlie *et al.* 1984; Goldman *et al.* 1985; Klarsfield & Changeux 1985; Shieh *et al.* 1987; Evans *et al.* 1987).

We describe here experiments done some years ago, in which the mRNAs of innervated and denervated mammalian skeletal muscle were studied by injecting them into *Xenopus* oocytes, to cause the translation and functional expression of receptors and channels in the oocyte membrane (Barnard *et al.* 1982; Miledi & Sumikawa 1982; Miledi *et al.* 1982; Gundersen *et al.* 1983). This procedure allows the relative amounts of these messengers in denervated muscle to be estimated from the sizes of the current mediated by the receptors and channels; the characteristics of the membrane currents can also be examined.

Methods

Leg muscles of cat and rat were denervated by sectioning and removing a length of the sciatic nerve. Eleven to fourteen days later, the denervated and the contralateral innervated leg muscles were dissected out, and poly(A)$^+$ mRNA was isolated as previously described (Miledi & Sumikawa 1982). In some experiments, the mRNA was fractionated by sucrose-density centrifugation (Sumikawa *et al* 1984*a*, *b*). Samples of mRNA for injection were prepared at a concentration of *ca.* 1 mg ml^{-1} in HEPES buffer, and oocytes of *Xenopus laevis* were each injected with 40–50 nl of this solution. Electrophysiological recordings were made a few days after injection (Miledi 1982; Miledi & Sumikawa 1982), usually after collagenase treatment of oocytes to remove enveloping cells. During recording, oocytes were continuously superfused with normal frog Ringer solution at room temperature, and drugs were added to this bathing solution. Most of the experiments described here were carried out in the Department of Biophysics, University College London.

Results

ACh-*sensitivity induced by denervated and innervated muscle* mRNAs

Native (non-mRNA-injected) *Xenopus* oocytes do not show nicotinic responses to ACh, but oocytes from many donors do possess muscarinic ACh receptors (Kusano *et al.* 1982). To avoid complications from these native muscarinic responses, all experiments were done in the continued presence of atropine (10^{-6} M).

ACh was applied by bath perfusion at high concentration (usually 10^{-4} M), in order to elicit near maximal responses. Oocytes injected with mRNA from denervated cat and rat muscles gave large inward currents in response to ACh (see, for example, figure 1*b*), like those we had previously described (Miledi & Sumikawa 1982; Miledi *et al.* 1982). In contrast, oocytes injected with preparations of mRNA from the contralateral innervated muscles usually failed to give any clear response (figure 1*a*). Mean response sizes measured in oocytes from a single donor injected with innervated and denervated rat muscle mRNAs are shown in figure 4*a*, and data from other donors and mRNA preparations are included in table 1. Small currents of a few nanoamperes were sometimes seen in the oocytes injected with innervated mRNA, but these responses were close to our limit of resolution and might have been artefacts of the solution change. Thus the mean values for ACh responses with innervated mRNA in table 1 are very likely an overestimate.

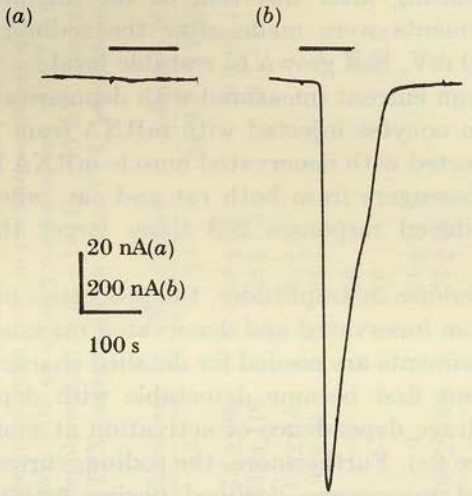

FIGURE 1. Responses to ACh in oocytes injected with mRNA fraction 18*S* derived from innervated (*a*) and denervated (*b*) cat muscle. Traces show membrane currents recorded at a clamp potential of −60 mV in response to ACh (10^{-4} M) applied by bath superfusion for the times indicated by the bars. In this and other figures, downward deflections correspond to inward membrane currents. Atropine (10^{-6} M) was present in ACh and wash solutions, beginning a few minutes before the records were obtained.

Better discrimination was obtained with a preparation of cat muscle mRNA (number 4 in table 1), where oocytes injected with denervated mRNA gave a mean ACh-activated current of nearly 2.5 μA, while those injected with innervated mRNA gave no obvious response. Currents of more than about 2 nA would have been clearly detectable; these results indicate that any sensitivity to ACh induced by the innervated mRNA must have been less than 0.1 % of that with denervated mRNA. In an attempt to increase further the size of the responses, oocytes were injected with fractions of cat muscle mRNA in which the mRNAs coding for the different subunits of the AChR are expected to be relatively enriched (Sumikawa *et al* 1984*a*, *b*). However, in this case the mean response size induced by the

fractionated mRNA from denervated muscle (preparation 7 in table 1) was smaller than obtained with the whole poly(A)$^+$ mRNA; so, although this experiment confirmed the earlier result, it did not give any improvement.

Sodium currents induced by muscle mRNAs

Xenopus oocytes occasionally show appreciable 'native' sodium currents on depolarization (Parker & Miledi 1987), but such responses are rare, and were not present in the oocytes used here. However, oocytes injected with mRNA from innervated or denervated rat and cat muscles showed a transient inward current on depolarization to potentials beyond about −40 mV (figure 2), which arose from activation of a voltage-gated sodium conductance (Gundersen *et al.* 1983; Methfessel *et al.* 1986; Sigel 1987). This current increased progressively in size over a few minutes after clamping the membrane potential at −100 mV, probably reflecting a recovery from inactivation at the normal resting potential, or at the lowered potential resulting after insertion of the microelectrodes. Therefore, quantitative measurements were made after the sodium current, elicited by depolarization to −10 mV, had grown to a stable level.

The size of the sodium current (measured with depolarization to −10 mV) was consistently greater in oocytes injected with mRNA from innervated muscle as compared to those injected with denervated muscle mRNA (figures 2 and 4). This was the case with messengers from both rat and cat, where the mRNAs from innervated muscle-induced responses 2–3 times larger than from denervated muscle (table 1).

Apart from the difference in amplitudes, the properties of the sodium currents induced by mRNA from innervated and denervated rat muscle appeared similar, although further experiments are needed for detailed characterization. With both messengers, the current first became detectable with depolarization to about −40 mV, and the voltage dependence of activation at more positive potentials matched closely (figure 2*c*). Furthermore, the sodium currents induced by innervated and denervated messengers declined during maintained depolarization and followed similar time courses, which became more rapid with increasing depolarization (figure 2*a*, *b*).

Blocking of Na^+ *currents by* TTX

Quantitative measurements of the action of tetrodotoxin (TTX) on the sodium current were made by first obtaining control records of depolarization to −10 mV in normal Ringer, and then repeating the depolarization after equilibrating the oocytes in various concentrations of TTX for a few minutes (figure 3). Mean values are given in table 1, and are illustrated graphically in figure 4*c*, *d*.

At a concentration of 9.4 nM, TTX reduced the sodium current in oocytes injected with either innervated or denervated rat muscle mRNA by about 30% (figure 4 and table 1) and there was no significant difference between the innervated and denervated messengers. If we assume that the binding of a single molecule of TTX is sufficient to block a sodium channel (cf. Pappone 1980), this degree of block corresponds to an apparent dissociation constant of about 20 nM, similar to the value of about 11 nM previously reported for rat muscle sodium channels in the

TABLE 1. ACh-CURRENTS AND Na^+ CURRENTS EXPRESSED BY SKELETAL MUSCLE mRNA

(Expression of ACh-activated currents and voltage-activated sodium currents in oocytes injected with poly(A)$^+$ mRNA from innervated (I) and denervated (D) rat and cat muscles. Data are shown from seven experiments, each done with oocytes from a different donor. Measurements are given as mean and standard error of mean, with number of oocytes given in parentheses where different from that listed in column 4. Responses to ACh were recorded at a clamp potential of −60 mV, with an agonist concentration of 10^{-4} M (except where noted) and in the presence of atropine (10^{-6} M). Sodium currents were elicited by depolarization from −100 to −10 mV. The percentage reduction of the sodium current in 9.4 nM TTX, and the percentage remaining in 313 nM TTX (compared with 31 μM TTX) were estimated as in figure 4. The final column indicates the amounts of mRNA injected into each oocyte.)

expt	type of mRNA and date made	innervated or denervated	no. of oocytes	ACh current nA	Na^+ current nA	reduction of N^+ by 9.4 nM TTX(%)	Na^+ remaining in 313 nM TTX(%)	mRNA injected ng
1	rat	I	11	2.1±0.8	414±68	29.9±3.1	6±0.6 (5)	55
	13 Apl 1984	D	10	244±119	142±1.5	28.7±1.5	10.7±1.2 (4)	55
2	rat	I	8	1.4±0.5	202±21	—	—	55
	13 Apl 1984	D	7	93±44	91±35	—	—	55
3	rat	I	3	—	356	36	5.5	55
	13 Apl 1984	D	2	—	110	35.5	11.2	55
4	cat	I	5	0	121±71	—	—	30
	3 Jun. 1983	D	9	2460±1033	37±16	—	—	60
5	cat	I	7	0	—	—	—	30
	3 Jun. 1983	D	4	122±20 (ACh 2±10^{-6} M)	—	—	—	60
6	cat	I	5	0	86±36	—	—	35
	16 Dec. 1983	D	6	604±201	19±4	—	—	45
7	car fr. 18*S*	I	5	1.4±0.8	—	—	—	—
		D	4	835±213	—	—	—	—

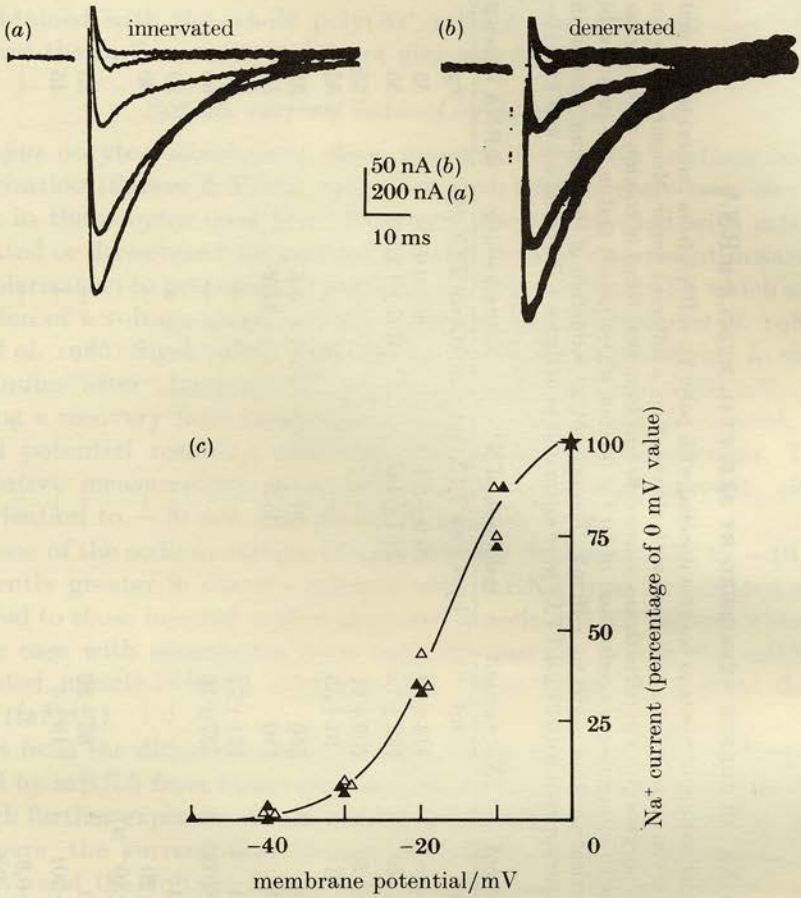

FIGURE 2. Inward sodium currents elicited by depolarizations to various voltages in oocytes injected with mRNA from innervated (*a*) and denervated (*b*) rat muscle. The oocytes were held at a potential of −100 mV, and stepped briefly to potentials of −40, −30, −20, −10 and 0 mV. In both frames, increasing depolarizations elicited progressively greater inward currents (downward deflections). Note the difference in recording gains between (*a*) and (*b*). (*c*) Voltage dependence of activation of sodium currents in two oocytes injected with innervated rat muscle mRNA (open symbols) and two oocytes injected with denervated rat muscle mRNA (filled symbols). Measurements were obtained from records like those in (*a*) and (*b*), and are scales as a percentage of the response at 0 mV in each fibre.

oocyte (Methfessel *et al.* 1986). When the concentration of TTX was raised to 313 nM, the sodium current substantially reduced but a small part remained, which could be distinguished from capacitative and other currents by its inactivation with repeated pulses (cf. Gundersen *et al.* 1983). The size of the residual sodium current in 313 nM TTX was estimated by further increasing the concentration of TTX to 31 μM (figure 3*b*, *d*), so as to obtain a virtually complete block. Oocytes injected with mRNA from innervated rat muscle showed a small reduction in current on going from 313 nM to 31 μM TTX, which corresponded to 5.8 ± 0.4 %

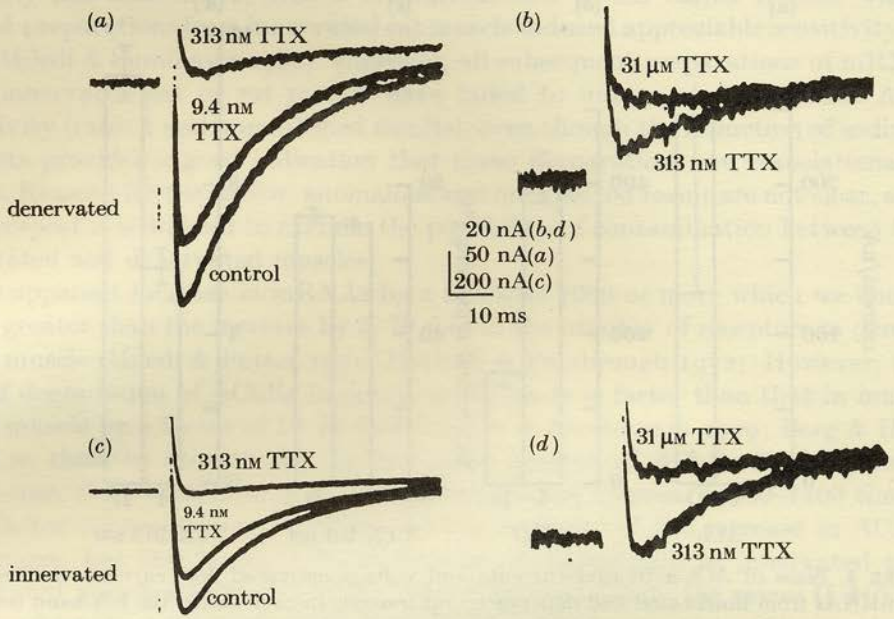

FIGURE 3. Blocking of sodium currents by different concentrations of TTX. Records from one oocyte injected with mRNA from denervated rat muscle (*a*, *b*) and from another oocyte injected with innervated rat muscle mRNA (*c*, *d*). Each frame shows superimposed traces of currents elicited by depolarization from −100 to −10 mV. (*a*, *c*) Records with the oocytes equilibrium in normal Ringer, or in TTX at the concentrations indicated; (*b*, *d*) records at a higher gain, showing currents in 313 nM and 31 μM TTX.

(s.e. of mean, eight oocytes from two donors) of the total sodium current in normal Ringer (figure 4*d*). From the dissociation constant estimated above, the fraction of sodium channels remaining unblocked in 313 nM TTX is expected to be 6.5% of the total (assuming 1:1 binding), so that this result is consistent with the innervated mRNA expressing mainly a single population of channels with uniform TTX binding. Different to this, Thesleff *et al.* (1974) found a proportion of sodium channels at the endplate of innervated muscles to be TTX-resistant, but if these turn over slowly they would need only a small amount of mRNA, which might escape our detection. In contrast to the oocytes injected with mRNA from innervated muscle, those injected with denervated muscle mRNA showed a proportionally larger reduction in sodium current on going from 313 nM to 31 μM TTX, equivalent to 10.9 ± 0.8% (six oocytes) of the current in normal Ringer (figure 4*d*). Thus, a fraction of the sodium current induced by denervated muscle mRNA is more resistant to relatively high concentrations of TTX (313 nM) than that induced by innervated mRNA.

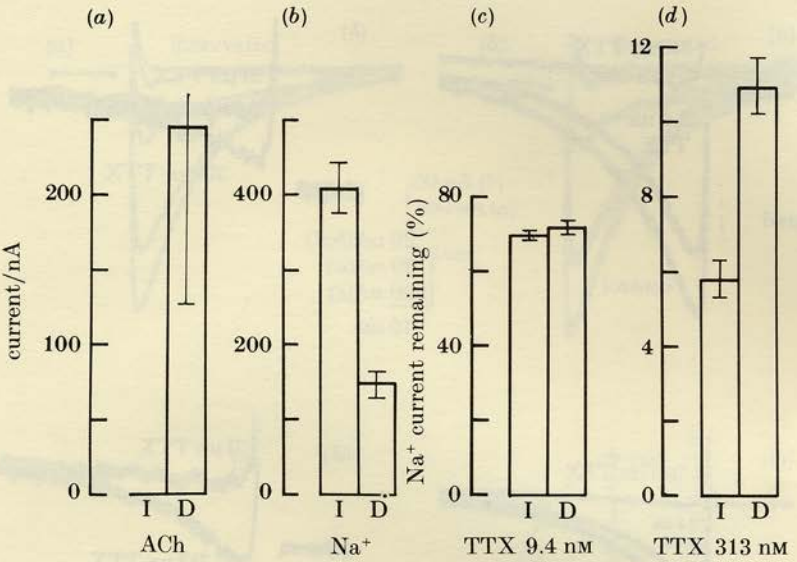

FIGURE 4. Sizes of ACh-activated currents and voltage-activated Na^+ currents induced by mRNAs from innervated and denervated rat muscle. In each block, the left-hand column refers to oocytes injected with mRNA from innervated muscle, and the right-hand column to mRNA from denervated muscle. Error bars indicate ± 1 standard error of mean. Data in (*a*) and (*b*) were obtained from 8–12 oocytes from a single donor. Results in (*c*) and (*d*) are from 6–11 oocytes from two donors. (*a*) Mean size of currents elicited by ACh (10^{-4} M + 10^{-6} M atropine), measured at a clamp potential of -60 mV from records like those in figure 1. (*b*) Mean size of inward sodium currents activated by depolarization from -100 to -10 mV. Measurements were taken as the difference between currents in normal Ringer and Ringer containing 313 nM TTX. (*c*) Sodium current remaining in 9.4 nM TTX, expressed as a percentage of that in normal Ringer. (*d*) Sodium current remaining in 313 nM TTX.

DISCUSSION

The main findings were: (i) mRNA from innervated muscles induced virtually no ACh-sensitivity in the oocytes, whereas mRNA from contralateral denervated muscles caused them to acquire high sensitivity; (ii) mRNA from innervated muscle induced larger sodium currents than mRNA from denervated muscle; and (iii) sodium currents induced by mRNA from denervated muscle showed a relatively greater resistance to TTX. We have previously shown that the expression of ACh receptors in the oocyte (measured by α-bungarotoxin binding) increases linearly with the amount of mRNA injected (Miledi & Sumikawa 1982), and this is also the case for the induction of voltage-activated sodium currents (Sumikawa *et al.* 1986). The oocytes in the present experiments were injected with roughly similar amounts of total poly(A)$^+$ mRNA from innervated or denervated muscle. Thus the differences in expression of ACh receptors and sodium channels probably reflect the relative abundances of the mRNAs coding for these receptors and channels in innervated and denervated muscle.

From the results in table 1, the amount of mRNAs coding for AChRs in innervated muscle is at most only 1% of that in denervated muscle, and more

probably less than 0.1%. This is in contradiction to our earlier results, where mRNA preparations from innervated cat muscle induced appreciable sensitivity to ACh (Miledi & Sumikawa 1982). However, all subsequent preparations of mRNA from innervated cat or rat muscle have failed to induce any significant ACh sensitivity (table 1 and unpublished results), even though the induction of sodium currents provides a good indication that these preparations are translationally active. Reasons for the earlier, anomalous and unexpected result are not clear, and in retrospect it is difficult to exclude the possibility of contamination between the innervated and denervated muscles.

The apparent increase in mRNAs by a factor of 1000 or more which we find is much greater than the increase by 5–70-fold in the number of receptors in denervated muscle (Miledi & Potter 1971; Hartzell & Fambrough 1972). However, the rate of degradation of AChRs in denervated muscle is faster than that in innervated muscle by a factor of 10–20-fold (Linden & Fambrough 1979; Berg & Hall 1975) so that, to account for the increased number of AChRs in the muscle membrane, the overall rate of AChR synthesis must increase by 50–1400 times. This factor corresponds more closely to our estimate of the increase in AChR messengers, but this is true only if the rate of degradation of innervated and denervated muscle receptors in the oocyte is approximately the same. If AChRs expressed by denervated muscle mRNA were to turn over at a rapid rate in the oocyte, as they do in the muscle, then our figure for the relative increase in messengers coding for AChRs following denervation would be a considerable underestimate. However, preliminary results with labelled α-bungarotoxin indicate that AChRs expressed in the oocyte by mRNA from denervated muscle degrade much slower than in the muscle (K. Sumikawa & R. Miledi, unpublished data), and this is supported by the finding that high ACh-sensitivity can still be found in oocytes several weeks after injection of mRNA from denervated cat muscle.

The AChR is formed from four different subunits, each encoded by a distinct mRNA. Thus the availability of any one of these subunit transcripts could serve to regulate the expression of functional AChRs. Studies with cDNA hybridization probes indicate that the levels of all four subunit mRNAs increase following muscle denervation, but that some subunit transcripts increase more than others (Evans *et al.* 1987). Quantitative measurements have been made on the α-subunit mRNA, which has been found to increase following denervation by factors of between 7 and 100 times in different muscles from rat, mouse and chick (Merlie *et al.* 1984; Goldman *et al.* 1985; Klarsfield & Changeux 1985; Shieh *et al.* 1987). Our results are in general agreement with this, in showing that levels of AChR and mRNA increase greatly following denervation, and further suggest that the availability of the α-subunit transcript may not be the limiting factor.

Sodium currents were induced more effectively in the oocyte by mRNA from innervated rather than denervated muscle, suggesting that the level of mRNAs coding for sodium channels is 2–3 times higher in innervated muscle, or that there are differences in the stability of the mRNA, or in the electrical characteristics or rates of turnover of sodium channels expressed in the oocyte by mRNA from denervated and innervated muscle. It is not yet clear, however, how the different

expressional potency of the muscle mRNAs relates to the numbers of sodium channels in the muscle membrane. The maximal rate of rise of the action potential is slowed after denervation (Redfern & Thesleff 1971*a*); this result suggests that there are fewer sodium channels. On the other hand, the number of channels estimated from saxitoxin binding (Ritchie & Rogart 1977), or from the maximal conductance change during depolarization (Pappone 1980), has been reported to change little with denervation. It was shown recently that sodium channels are more concentrated near the endplate in innervated fibres, whereas in the absence of innervation they are more mobile and distribute evenly throughout the muscle membrane (Beam *et al.* 1985; Angelides 1986). It will be interesting to see if this localization and immobilization of sodium channels at the endplate is associated with an increased local transcription of mRNAs encoding the channels. Furthermore, after denervation there are changes in the structure of the muscle that may be accompanied by changes in the relative amounts of sodium channels in the surface and T-tubule membranes (Miledi & Slater 1969).

Sodium currents induced by mRNA from denervated muscle were slightly more resistant to blocking by TTX than those from innervated muscle, and at a concentration of 313 nM TTX the proportion of the total sodium current remaining with denervated muscle mRNA was about twice that with mRNA from innervated muscle. This suggests that the denervated-muscle mRNA induced an additional population of relatively TTX-resistant channels. From our data this would amount to about 5% of the total numbers expressed, a smaller proportion than that (*ca.* 25%) reported for sodium channels in 'native' denervated muscle (Pappone 1980). An attractive explanation for the appearance of TTX-resistant channels in the oocyte is that they were encoded by messengers different from those encoding the normal channels; but there are other possibilities. For example, it is known that slight modifications of the sodium channel can abolish its sensitivity to TTX (Caterall 1986), so that a messenger from the denervated muscle might code for a substance which modifies the sodium channels so as to make them less susceptible to TTX. It is known that inhibition of transcription in muscle by actinomycin D blocks the appearance of TTX-resistant sodium channels (Grampp *et al.* 1972; Ruzzier *et al.* 1982), but this also fails to discriminate between the above possibilities. However, as a result of the cloning of sodium-channel genes (Noda *et al.* 1984), this question should soon be answered.

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