J. Physiol. (1984), **348**, *pp.* 57–66
With 1 plate and 2 text-figures
Printed in Great Britain

IN VIVO DEVELOPMENT OF CHOLINESTERASE AT A NEUROMUSCULAR JUNCTION IN THE ABSENCE OF MOTOR ACTIVITY IN *XENOPUS LAEVIS*

BY M. W. COHEN, M. GRESCHNER AND M. TUCCI
From the Department of Physiology, McGill University, Montreal, Quebec, Canada

(*Received 8 June 1983*)

SUMMARY

1. Embryos of *Xenopus laevis* were selected prior to the onset of innervation and were raised for 2 days in the anaesthetic tricaine methanesulphonate (200 μg/ml). The gross development of these tricaine-reared animals appeared normal despite the absence of spontaneous motor activity and the lack of motor responses to prodding with a pin. Motor activity quickly appeared when the anaesthetic was withdrawn.

2. Intracellular recording from the myotomes of intact, tricaine-maintained animals failed to reveal any spontaneous muscle action potentials. Synaptic potentials increased in frequency and amplitude upon withdrawing tricaine, but resting potentials remained unchanged.

3. Cholinesterase activity, detected histochemically, was observed at the ends of the myotomes, the main site of innervation. The intensity of the histochemical reaction product at these sites appeared to be about as great in the myotomes of tricaine-reared animals as in control myotomes.

4. Miniature end-plate currents (m.e.p.c.s), examined by focal external recording, declined with a time constant of $2 \cdot 9 \pm 0 \cdot 2$ ms (mean $\pm$ S.E. of mean) in the myotomes of tricaine-reared animals (stages 40–41). The time constants in the myotomes of control animals were $1 \cdot 8 \pm 0 \cdot 1$ ms at stages 40–41 and $8 \cdot 7 \pm 0 \cdot 7$ ms at stages 24–26 (shortly after the onset of innervation).

5. The anticholinesterase neostigmine doubled m.e.p.c. time constants in the myotomes of tricaine-reared animals as well as in control myotomes at stages 40–41.

6. It is concluded that motor activity is not required for the *in vivo* development of physiological levels of synaptic cholinesterase in *Xenopus* myotomal muscle.

INTRODUCTION

Studies dating back to the beginning of this century have indicated that skeletal muscle can become functionally innervated in the absence of muscle impulses and contractile activity (Harrison, 1904; Matthews & Detwiler, 1926; Crain & Peterson, 1971; Cohen, 1972; Jansen & van Essen, 1975; Obata, 1977). A high density of acetylcholine (ACh) receptors and pre- and post-synaptic ultrastructural specializations can also develop at neuromuscular contacts in the absence of muscle activity (Steinbach, 1974; Anderson & Cohen, 1977; Anderson, Cohen & Zorychta, 1977; Weldon & Cohen, 1979; Rubin, Schuetze, Weill & Fischbach, 1980). On the other hand

the role played by muscle activity in the localization of cholinesterase (ChE) at the developing neuromuscular junction appears to be more complex. Studies on the reinnervation of rat muscle (Guth, Zalewski & Brown, 1966; Weinberg & Hall, 1979; Cangiano, Lømo, Lutzemberger & Sveen, 1980; Lømo & Slater, 1980) and on neuromuscular synaptogenesis in the chick, *in vivo* and in cell culture (Giacobini, Filogamo, Weber, Boquet & Changeux, 1973; Gordon, Perry, Tuffery & Vrbova, 1974; Oppenheim, Pittman, Gray & Maderdrut, 1978; Betz, Bourgeois & Changeux, 1980; Rubin *et al.* 1980), have indicated that muscle activity does contribute to the development of neuromuscular ChE. This dependence, however, is not absolute. For example, in the chick cultures, neuromuscular ChE can develop in the absence of muscle activity if the culture medium is supplemented with dibutyryl cyclic GMP (Rubin *et al.* 1980). Furthermore, in cultures derived from *Xenopus* embryos, ChE develops at neuromuscular synapses in the absence of muscle activity even though the culture medium is not supplemented with nucleotide (Moody-Corbett, Weldon & Cohen, 1982). Nor is muscle activity required for the development of sites of ChE activity on non-innervated, *Xenopus* muscle cells in culture (Moody-Corbett & Cohen, 1981; Weldon, Moody-Corbett & Cohen, 1981).

The studies on *Xenopus* cell cultures, in which ChE localization was demonstrated histochemically, raise at least two questions concerning the normal role of muscle activity in the development of neuromuscular ChE. Is ChE localization in the absence of muscle activity related to the conditions of the culture system, or can it also occur *in vivo*? Is the level of ChE activity that develops in the absence of muscle contraction sufficiently high to participate in terminating transmitter action? These questions were addressed in the present study by raising *Xenopus* embryos in anaesthetic in order to prevent muscle activity and then testing for the presence of neuromuscular ChE, histochemically as well as electrophysiologically.

METHODS

Animals

Embryos of *Xenopus laevis* were selected between stages 15 and 17 (Nieuwkoop & Faber, 1967) and placed in 5% Ringer solution (diluted with water) containing gentamycin (100 μg/ml) and mycostatin (100 units/ml). Vitelline membranes were then removed by means of fine forceps and a sharp pin. It was usually not possible to tear the vitelline and free the embryo without piercing the embryo's skin. Freed embryos were accepted only if (a) the damage was minor and localized to the ventral surface of the animal and (b) the damaged area healed within 1 h. Once the damaged area had healed, the embryos appeared normal and continued to develop in a normal fashion. The embryos were then restaged and only those which were stage 18 or less were transferred to 5% Ringer solution containing tricaine (for concentration see Results). Stage 18 is about 3 h before the normal onset of innervation of the myotomes (Blackshaw & Warner, 1976*a*; Kullberg, Lentz & Cohen, 1977; Chow & Cohen, 1983). Control embryos were placed in tricaine-free 5% Ringer solution. The embryos were raised in these solutions in plastic Petri dishes, each dish containing about 12 ml solution and five embryos.

Embryos were transferred to fresh solution at intervals of 16 h or less. Just before being transferred they were examined with the aid of a dissecting microscope. Animals which exhibited motor responses or deformities were excluded from further experimentation.

Intracellular recording

Intracellular recordings were obtained from the myotomal muscle of intact anaesthetized animals. The animals were secured by two stainless-steel clips which were pushed into the Sylgard

184 (Dow Corning) floor of the recording bath. The animals were thus held firmly in place without rupturing their skin. The bathing solution (5% Ringer solution containing 200 μg/ml tricaine) was kept at room temperature for up to 16 h before use in order to mimic the situation under which the embryos were raised (see above). Solution was run continuously into the bath (volume: ~ 0·5 ml) at a rate of 1–2 ml/min. The bath was grounded through a silver/silver chloride electrode.

Glass micro-electrodes were filled with 3 M-KCl and had resistances of 30–60 MΩ. Electrode resistance was monitored continuously to aid in assessing that the electrode tip did not become plugged while crossing the skin or entering a muscle cell. A steady positive potential (40–80 mV) was observed across the skin. Recordings were displayed on an oscilloscope and photographed on moving film. Other features of the recording system have been described previously (Kullberg *et al.* 1977).

Focal external recording

Preparations of myotomal muscle and associated spinal cord were isolated from control and tricaine-reared animals and secured in the recording bath. The latter dissections were done in full-strength Ringer solution containing 200 μg/ml tricaine. Since this concentration of tricaine reduces the amplitudes of miniature end-plate potentials (m.e.p.p.s; M. W. Cohen, M. Greschner & M. Tucci, unpublished observations) the tricaine Ringer solution was then replaced by Ringer solution containing tetrodotoxin (0·4 μg/ml) for the remainder of the experiment. Tetrodotoxin was used in order to abolish spontaneous motor activity which can otherwise occur in these muscle–spinal cord preparations.

Focal external recordings were made as described previously (Kullberg *et al.* 1977; Kullberg, Mikelberg & Cohen, 1980), using glass micro-electrodes filled with 1 M-NaCl. Since extracellularly recorded m.e.p.p.s have the same wave form and time course as miniature end-plate currents (m.e.p.c.s; Castillo & Katz, 1956; Katz & Miledi, 1965, 1973) they will be referred to here as m.e.p.c.s. Filmed recordings were analysed with the aid of a computer-linked graphics tablet (Hewlett Packard). The system was programmed so that, by moving the cursor along the m.e.p.c., some thirty measurements were obtained of the declining phase between 10–90% of peak amplitude and expressed in terms of the best-fitting single exponential. The correlation coefficient was also obtained.

ChE histochemistry

Tricaine-anaesthetized animals were fixed for 30–60 min with 1·5% glutaraldehyde (in 0·1 M-sodium phosphate buffer, pH 7·3). After briefly rinsing with Ringer solution, the myotomal muscle on each side of the spinal cord and notochord was isolated (see Chow & Cohen, 1983) and incubated for 15 min in ChE-staining solution. This solution (Karnovsky, 1964) was prepared just before use and had the following composition in mM: acetylthiocholine iodide, 1·8; acetate buffer at pH 5·0, 65; sodium citrate, 15; copper sulphate, 3; potassium ferricyanide, 0·5. Subsequently the muscles were rinsed in Ringer solution and mounted in glycerol on glass slides.

Solutions

Tricaine methanesulphonate was prepared as a stock solution (10 mg/ml) at the beginning of each experiment and used for no more than the next 2 days. When diluting it to the desired concentration, Phenol Red was added (~ 10 μg/ml) and pH was adjusted to 7·2–7·4 with NaOH in order to obtain maximum potency and to avoid exposing the embryos to acidic solutions (Ohr, 1976; Smit & Hattingh, 1979). The stock solution of tricaine was stored in the refrigerator.

Mycostatin and gentamycin were obtained from Grand Island Biological Co., tetrodotoxin from Calbiochem, and acetylthiocholine iodide, neostigmine bromide and tricaine methanesulphonate from Sigma Chemical Co.

The Ringer solution had the following composition, in mM: NaCl, 111; KCl, 3; $CaCl_2$, 1·8; HEPES, 10. It was adjusted to pH 7·3 with NaOH. All experiments were carried out at room temperature (21–24 °C).

RESULTS

Abolition of myotomal muscle activity by tricaine

Tricaine concentration. When *Xenopus* embryos were raised in 400 μg/ml tricaine they continued to develop, but by the second day they often exhibited gross abnormalities such as no heart beat, a poorly developed ventral fin, a ruptured cloaca and other deformities which prevented proper staging of the animals. Such abnormalities did not arise when the embryos were raised in tricaine (200 μg/ml). In this concentration their development appeared normal and by the end of 2 days they had reached stages 40–41, the same as control animals. Accordingly this concentration was chosen for all subsequent experiments. A concentration of 1 mM (261 μg/ml) was found to be effective in preventing the developmental loss of electrical coupling between *Xenopus* myotomal muscle cells (Armstrong, Turin & Warner, 1983).

Spontaneous and evoked motor activity. In acute experiments on animals between stages 26 (29·5 h) and 49 (12 days), tricaine (200 μg/ml) abolished spontaneous as well as evoked (see below) motor activity within 15 min, and no sign of contractile activity could then be seen in the myotomes even though the animals were viewed through a dissecting microscope at a magnification of 30–60 times. Similar tests were also made on all tricaine-reared animals and on control animals at intervals of 16 h or less, just before transferring the animals to fresh solution. The control animals exhibited spontaneous swimming behaviour and always responsed by briskly swimming away when prodded with a fine pin. By contrast, spontaneous motor activity was never observed in tricaine-reared animals. When prodded with a pin about 30% of these animals did exhibit a faint motor response which consisted of a small single movement of the tail. Tricaine-reared animals which exhibited any hint of a motor response were eliminated from further experimentation.

When tricaine-reared animals which had never shown any sign of motor activity were transferred to tricaine-free solution they began to swim within 20 min and their motor behaviour was then indistinguishable from that of control animals.

Intracellular recording. As an additional check for muscle activity, intracellular recordings were made from the myotomal muscle of intact tricaine-reared animals (see Methods). Recordings were obtained from two animals during the first day in tricaine (stages 22–26), from five animals after 1 day in tricaine (stages 34–36), and from two animals after 2 days in tricaine (stage 40). The recording times in tricaine totalled 134, 138 and 76 min respectively and varied from 12 to 68 min in the twelve cells from which continuous recordings were made. Not one muscle action potential was observed. Unitary synaptic potential occurred at a low frequency, about 1–10/min. They were usually less than 2 mV in amplitude and never more than 6 mV (Fig. 1*A* and *C*). Summated potentials also occurred sporadically during the first day in tricaine but were never more than 10 mV in peak amplitude (Fig. 1*A*). Resting potentials were unaffected by tricaine (see also Armstrong *et al.* 1983). They varied between 65 and 95 mV (mean: 76 mV), similar to the range found in previous studies (Blackshaw & Warner, 1976*b*; Kullberg *et al.* 1977). When tricaine-free solution was introduced there was no significant change in resting potential ($P > 0{\cdot}3$), but synaptic potentials quickly became more frequent and larger, often reaching amplitudes of 15 mV or more (Fig. 1*B* and *D*). Motor activity also became apparent in tricaine-free

solution and prevented further recording. These findings indicate that synaptic activity in the myotomal muscle remained subthreshold for generating action potentials as long as the animals were maintained in tricaine and that this muscle inactivity was unaccompanied by a fall in resting potential.

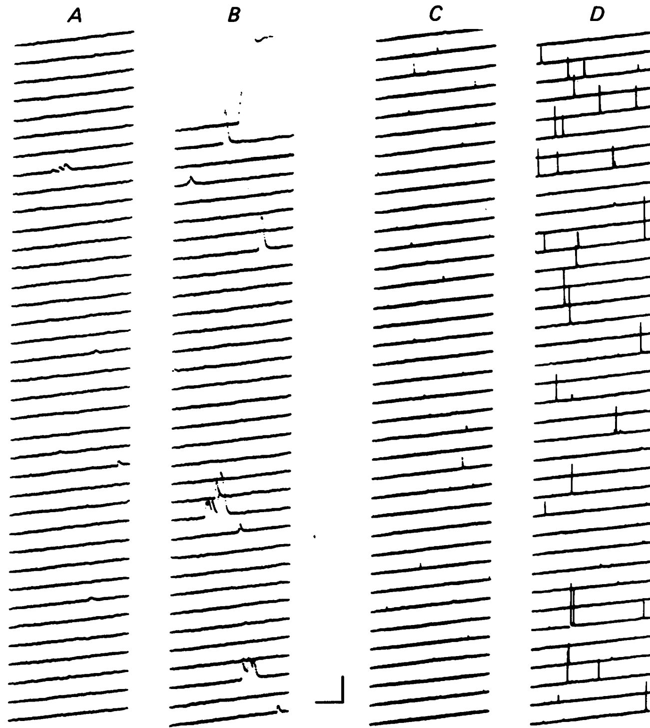


Fig. 1. Intracellular recordings from myotomal muscle cells in intact tricaine-reared animals. *A*, from a stage 25 animal, with tricaine present. *B*, same cell as *A*, 2 min after switching to tricaine-free solution. *C*, from a stage 36 animal, with tricaine present. *D*, same cell as *C*, after 4 min in tricaine-free solution. The potentials in *D* have been retouched. Voltage calibration is 20 mV for *A*, *B* and *D* and 10 mV for *C*. Time calibration is 1 s in all cases. In *B* the impalement was lost during a large summated potential. The failure of 30 mV potentials in *D* to generate muscle action potentials may be due to incomplete recovery from the depressed membrane excitability which tricaine is known to produce (see Frazier & Narahashi, 1975).

ChE histochemistry in muscle from tricaine-reared animals

ChE was examined histochemically in control muscles and in muscles from animals (stages 40–41) which had been reared in tricaine for 2 days without any sign of motor activity. In both cases the ChE reaction product was localized almost entirely at the ends of the myotomes (Pl. 1*A* and *B*), the main site of innervation. There was no consistent difference in staining intensity between control muscles and muscles from tricaine-reared animals, suggesting that the lack of motor activity did not substantially reduce the development of ChE.

When viewed at higher magnification most of the reaction product at the ends of the myotomes appeared as narrow bands, less than 2 μm wide. These bands were

generally oriented transversely with respect to the long axis of the muscle cells but often extended for short distances in a longitudinal orientation as well (Pl. 1*B* and *C*). This pattern of ChE localization is similar to the pattern of innervation and to the pattern of ACh receptor localization at the ends of the myotomes (Chow & Cohen, 1983). It is likely therefore that much of the reaction product was generated by neuromuscular ChE. This is in contrast to the situation at later stages of development where myotendinous ChE also contributes prominently to the staining pattern (Cohen, 1980).

Short narrow bands of ChE reaction product were also observed scattered at low density elsewhere along the myotomes (Pl. 1*B* and *D*). Their occurrence was highly variable but appeared to be more frequent in muscles from tricaine-reared animals than in control muscles. These 'centrally-located' sites of ChE activity may reflect the presence of neuromuscular junctions away from the ends of the muscle cells. Indeed some nerve fibres and sites of ACh receptor localization have been observed in these regions of the myotome (Chow & Cohen, 1983). Alternatively they may reflect the presence of non-innervated, post-synaptic-like sites similar to those which occur on embryonic muscle cells in culture (Moody-Corbett & Cohen, 1981; Weldon *et al.* 1981) and on denervated muscle cells (Harris, 1981; Steinbach, 1981). In either case it is apparent that motor inactivity did not prevent the development of ChE in *Xenopus* myotomal muscle.

Prolongation of m.e.p.c.s by neostigmine in muscle from tricaine-reared animals

To test further for the development of neuromuscular ChE in tricaine-reared animals, focal external recording was employed to measure the duration of m.e.p.c.s in the presence and absence of the anticholinesterase, neostigmine bromide (50 μg/ml). In previous work it was found that the half-decay times of synaptic currents in *Xenopus* myotomal muscle decrease about 8-fold during normal development, that much of this shortening has already occurred by stage 40, and that neostigmine is effective in prolonging synaptic currents by stage 40, but not at stages 24–26, indicating that neuromuscular ChE is important for the termination of transmitter action by the later stage (Kullberg *et al.* 1980). In the present study we measured the time constant of the declining phase of m.e.p.c.s in the myotomes of animals (stages 40–41) which had been reared in tricaine for 2 days and which showed no sign of motor activity. For comparison, similar measurements were made in the myotomes of control animals at the same stage and at stages 24–26, a few hours after the onset of innervation. The declining phase of each m.e.p.c. was measured at 30 or more points between 10 and 90% of peak amplitude and the time constant was estimated by the best fit for a single exponential. The correlation coefficients were 0·98 or greater for more than 92% of the m.e.p.c.s, confirming that the declining phases behave as single exponentials.

The results are summarized in Table 1 and reveal two main points. The time constants in tricaine-reared animals at stage 40–41 (mean: 2·9 ms), although greater than in control animals at the same stage (mean: 1·8 ms), were 3-fold less than the values at stage 24–26 (mean: 8·7 ms). This shows that a substantial developmental shortening of m.e.p.c. duration occurred in tricaine-reared animals, but not as much as in control animals. Secondly, neostigmine prolonged the m.e.p.c.s of tricaine-reared

animals by about 2-fold, as in control animals (Table 1; Fig. 2). These findings indicate that the neuromuscular ChE which developed in the myotomes of tricaine-reared animals was effective in terminating transmitter action.

TABLE 1. Time constant of decay of m.e.p.c.s and prolongation by neostigmine. Recordings were made in the presence or absence of neostigmine bromide (50 μg/ml). Between 13 and 65 m.e.p.c.s (mean: 38) were measured at each recording site. Numbers in parentheses indicate the number of recording sites and the number of animals respectively

		Time constant (ms)		Factor of increase by neostigmine	
Stage	Rearing condition	Neostigmine absent (mean ± S.E. of mean)	Neostigmine present (mean ± S.E. of mean)	All sites	Same site (mean ± S.E. of mean)
24–26	Normal	8·7 ± 0·7 (7, 6)	—	—	—
40–41	Normal	1·8 ± 0·1 (14, 5)	3·7 ± 0·3 (8, 4)	2·06	—
40–41	Tricaine	2·9 ± 0·2 (16, 5)	5·9 ± 0·2 (17, 5)	2·03	2·23 ± 0·15 (4, 4)

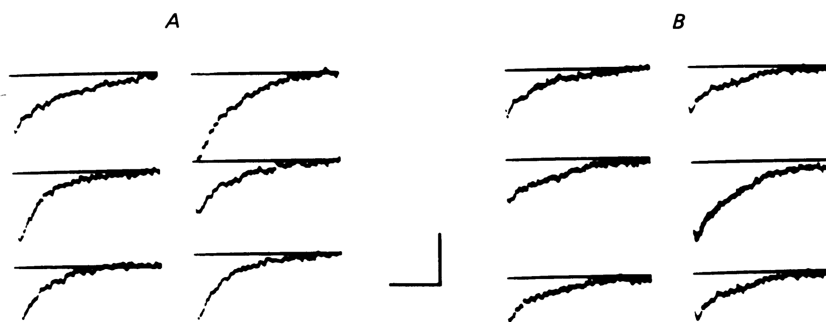


Fig. 2. Focal external recordings of m.e.p.c.s in a muscle from a tricaine-reared animal. *A*, before the addition of neostigmine bromide. *B*, same recording site after 20 min in neostigmine (50 μg/ml). Voltage scale is 0·4 mV. Time scale is 4 ms for *A* and 10 ms for *B*. Note that neostigmine prolonged the m.e.p.c.s. Initial portions of the m.e.p.c.s are not seen because they served to trigger the oscilloscope beam.

DISCUSSION

Since the tricaine-reared *Xenopus* embryos were not monitored continuously it could be argued that some muscle contraction did occur but went undetected. Our observations make this possibility unlikely. The animals were tested at intervals of 16 h or less, just before being transferred to fresh tricaine solution. Unlike controls, they never exhibited spontaneous motor activity during these periods of observation and those which were accepted for examination of ChE development remained immobile even when prodded with a pin. In view of this lack of a motor response it seems unlikely that the animals could have generated spontaneous motor activity while in tricaine. Further support in this regard comes from our finding that synaptic potentials in the myotomal muscle of intact tricaine-reared animals were too small

to generate muscle impulses. Taken together, therefore, the evidence indicates that motor activity was completely eliminated in tricaine.

That physiological levels of ChE developed at the *Xenopus* myotomal neuromuscular junction *in vivo* under these conditions of muscle inactivity, and without any decline in muscle resting potential, extends previous findings which indicated that muscle activity is not required for the development of histochemically detectable levels of ChE at *Xenopus* myotomal neuromuscular junctions formed in cell culture (Moody-Corbett *et al.* 1982). Furthermore, comparison of histochemical staining intensities and of the prolongation of m.e.p.c.s by neostigmine in control and tricaine-reared animals suggests that the level of ChE activity may have been about the same in both groups of animal. However, m.e.p.c. durations were longer in tricaine-reared animals. One interpretation is that the development of neuromuscular ChE was retarded in these animals. An alternative possibility is that prolonged treatment with tricaine altered other determinants of m.e.p.c. time course, such as the kinetics of transmitter release or the open times of synaptic ion channels.

Several studies on rat and chick muscle have indicated that muscle inactivity inhibits the development of neuromuscular ChE (Guth *et al.* 1966; Giacobini *et al.* 1973; Gordon *et al.* 1974; Oppenheim *et al.* 1978; Weinberg & Hall, 1979; Betz *et al.* 1980; Cangiano *et al.* 1980; Lømo & Slater, 1980; Rubin *et al.* 1980). Nevertheless, *Xenopus* myotomal muscle may not be unique in its development of neuromuscular ChE in the absence of muscle activity. Recent evidence suggests that neuromuscular ChE can also develop in inactive cat muscle, although the rate of development may be very slow (Eldridge, Liebhold & Steinbach, 1981). It is also interesting to note that even in rat and chick muscle, as in *Xenopus* myotomal muscle, other synaptic specializations including a high density of ACh receptors and transmitter release mechanisms do form in the absence of muscle activity (Jansen & Van Essen, 1975; Obata, 1977; Betz *et al.* 1980; Cangiano *et al.* 1980; Lømo & Slater, 1980; Rubin *et al.* 1980). Activity in rat and chick muscle is thought to influence the development of neuromuscular ChE by stimulating synthesis of the synaptic form of ChE, by triggering its secretion from the muscle cells, or by permitting it to bind in the synaptic cleft (Lømo & Slater, 1980; Rieger, Koenig & Vigny, 1980; Rubin *et al.* 1980). One possibility is that activity regulates these processes by altering the level of intracellular calcium (Walicke & Patterson, 1981). If this is the case then it follows that in *Xenopus* myotomal muscle the level of intracellular calcium is sufficient to permit these processes to operate even in the absence of muscle activity.

We thank Dr J. Mortola for generously sharing his computer-linked graphics tablet, Ms Anna Noworay for designing the computer program, Ms E. Soro for measuring m.e.p.c. time constants and Mr C. Cantin for technical assistance. We also thank Dr E. J. Cooper for helpful comments on the manuscript. The work was supported by the Medical Research Council of Canada.

REFERENCES

ANDERSON, M. J. & COHEN, M. W. (1977). Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 757–773.

ANDERSON, M. J., COHEN, M. W. & ZORYCHTA, E. (1977). Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 731–756.

ARMSTRONG, D. L., TURIN, L. & WARNER, A. E. (1983). Muscle activity and the loss of electrical coupling between striated muscle cells in *Xenopus* embryos. *J. Neurosci.* **3**, 1414–1421.

BETZ, H., BOURGEOIS, J.-P. & CHANGEUX, J.-P. (1980). Evolution of cholinergic proteins in developing slow and fast skeletal muscles in chick embryo. *J. Physiol.* **302**, 197–218.

BLACKSHAW, S. E. & WARNER, A. E. (1976*a*). Onset of acetylcholine sensitivity and endplate activity in developing myotome muscles of *Xenopus*. *Nature, Lond.* **262**, 217–218.

BLACKSHAW, S. E. & WARNER, A. E. (1976*b*). Low resistance junctions between mesoderm cells during development of trunk muscles. *J. Physiol.* **255**, 209–230.

CANGIANO, A., LØMO, T., LUTZEMBERGER, L. & SVEEN, O. (1980). Effects of chronic nerve conduction block on formation of neuromuscular junctions and junctional AChE in the rat. *Acta physiol. scand.* **109**, 283–296.

CHOW, I. & COHEN, M. W. (1983). Developmental changes in the distribution of acetylcholine receptors in the myotomes of *Xenopus laevis*. *J. Physiol.* **339**, 553–571.

COHEN, M. W. (1972). The development of neuromuscular connexions in the presence of D-tubucurarine. *Brain Res.* **41**, 457–463.

COHEN, M. W. (1980). Development of an amphibian neuromuscular junction *in vivo* and in culture. *J. exp. Biol.* **89**, 43–56.

CRAIN, S. M. & PETERSON, E. R. (1971). Development of paired explants of fetal spinal cord and adult skeletal muscle during chronic exposure to curare and hemicholinium. *In Vitro* **6**, 373–389.

DEL CASTILLO, J. & KATZ, B. (1956). Localization of active spots within the neuromuscular junction of the frog. *J. Physiol.* **132**, 630–649.

ELDRIDGE, L., LIEBHOLD, M. & STEINBACH, J. H. (1981). Alterations in cat skeletal neuromuscular junctions following prolonged inactivity. *J. Physiol.* **313**, 529–545.

FRAZIER, D. T. & NARAHASHI, T. (1975). Tricaine (MS-222): effects on ionic conductances of squid axon membranes. *Eur. J. Pharmacol.* **33**, 313–317.

GIACOBINI, G., FILOGAMO, G., WEBER, M., BOQUET, P. & CHANGEUX, J.-P. (1973). Effects of a snake α-neurotoxin on the development of innervated skeletal muscles in chick embryo. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1708–1712.

GORDON, T., PERRY, R., TUFFERY, A. R. & VRBOVA, G. (1974). Possible mechanisms determining synapse formation in developing skeletal muscles of the chick. *Cell & Tissue Res.* **155**, 13–25.

GUTH, L., ZALEWSKI, A. A. & BROWN, W. C. (1966). Quantitative changes in cholinesterase activity of denervated sole plates following implantation of nerve into muscle. *Expl Neurol.* **16**, 136–147.

HARRIS, A. J. (1981). Embryonic growth and innervation of rat skeletal muscles. II. Neural regulation of muscle cholinesterase. *Phil. Trans. R. Soc. B* **293**, 279–286.

HARRISON, R. G. (1904). An experimental study of the relation of the nervous system to the developing musculature in the embryo of the frog. *Am. J. Anat.* **3**, 197–220.

JANSEN, J. K. S. & VAN ESSEN, D. C. (1975). Re-innervation of rat skeletal muscle in the presence of α-bungarotoxin. *J. Physiol.* **250**, 651–667.

KARNOVSKY, M. J. (1964). The localization of cholinesterase activity in rat cardiac muscle by electron microscopy. *J. Cell Biol.* **23**, 217–232.

KATZ, B. & MILEDI, R. (1965). The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc.* B **161**, 483–495.

KATZ, B. & MILEDI, R. (1973). The binding of ACh to receptors and its removal from the synaptic cleft. *J. Physiol.* **231**, 549–574.

KULLBERG, R. W., LENTZ, T. L. & COHEN, M. W. (1977). Development of the myotomal neuromuscular junction in *Xenopus laevis*: an electrophysiological and fine-structural study. *Devl Biol.* **60**, 101–129.

KULLBERG, R. W., MIKELBERG, F. S. & COHEN, M. W. (1980). Contribution of cholinesterase to developmental decreases in the time course of synaptic potentials at an amphibian neuromuscular junction. *Devl Biol.* **75**, 255–267.

LØMO, T. & SLATER, C. R. (1980). Control of junctional acetylcholinesterase by neural and muscular influence in the rat. *J. Physiol.* **303**, 191–202.

MATTHEWS, S. A. & DETWILER, S. R. (1926). The reactions of *Amblystoma* embryos following prolonged treatment with chloretone. *J. exp. Zool.* **45**, 279–292.

MOODY-CORBETT, F. & COHEN, M. W. (1981). Localization of cholinesterase at sites of high acetylcholine receptor density on embryonic amphibian muscle cells cultured without nerve. *J. Neurosci.* **1**, 596–605.

Moody-Corbett, F., Weldon, P. R. & Cohen, M. W. (1982). Cholinesterase localization at sites of nerve contact on embryonic amphibian muscle cells in culture. *J. Neurocytol.* **11**, 381–394.

Nieuwkoop, P. D. & Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin)*, 2nd edn. Amsterdam: North-Holland Publ. Co.

Obata, K. (1977). Development of neuromuscular transmission in culture with a variety of neurons and in the presence of cholinergic substances and tetrodotoxin. *Brain Res.* **119**, 141–153.

Ohr, E. A. (1976). Tricaine methanesulfonate. I. pH and its effects on anaesthetic potency. *Comp. Biochem. Physiol.* **54C**, 13–17.

Oppenheim, R. W., Pittman, R., Gray, M. & Maderdrut, J. L. (1978). Embryonic behaviour, hatching and neuromuscular development in the chick following a transient reduction of spontaneous motility and sensory input by neuromuscular blocking agents. *J. comp. Neurol.* **179**, 619–640.

Rieger, F., Koenig, J. & Vigny, M. (1980). Spontaneous contractile activity and the presence of the 16S form of acetylcholinesterase in rat muscle cells in culture: reversible suppressive action of tetrodotoxin. *Devl Biol.* **76**, 358–365.

Rubin, L. L., Schuetze, S. M., Weill, C. L. & Fischbach, G. D. (1980). Regulation of acetylcholinesterase appearance at neuromuscular junctions *in vitro*. *Nature, Lond.* **283**, 264–267.

Smit, G. L. & Hattingh, J. (1979). Anaesthetic potency of MS222 and neutralized MS222 as studied in three freshwater fish species. *Comp. Biochem. Physiol.* **62C**, 237–241.

Steinbach, J. H. (1974). Role of muscle activity in nerve–muscle interaction *in vivo*. *Nature, Lond.* **248**, 70–71.

Steinbach, J. H. (1981). Neuromuscular junctions and α-bungarotoxin-binding sites in denervated and contralateral cat skeletal muscles. *J. Physiol.* **313**, 513–528.

Walicke, P. A. & Patterson, P. H. (1981). On the role of Ca^{2+} in the transmitter choice made by cultured sympathetic neurons. *J. Neurosci.* **1**, 343–350.

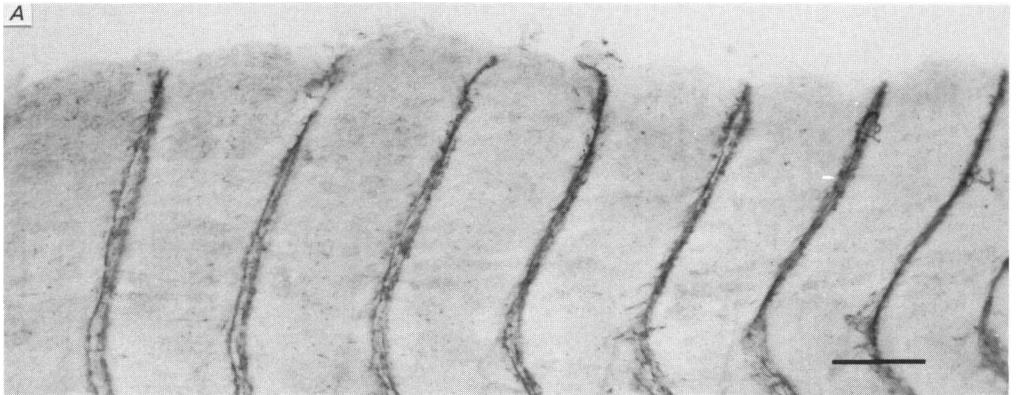
Weinberg, C. B. & Hall, Z. W. (1979). Junctional form of acetylcholinesterase restored at nerve-free endplates. *Devl Biol.* **68**, 631–635.

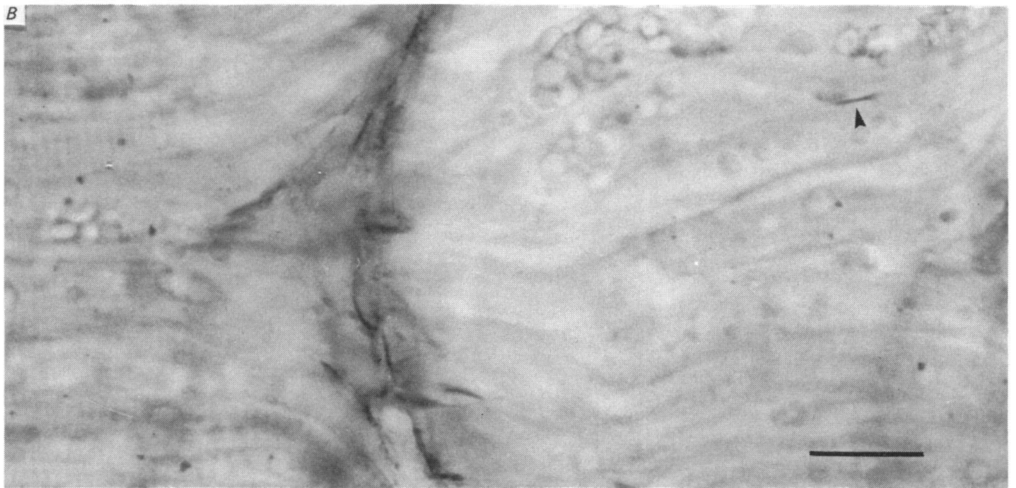
Weldon, P. R. & Cohen, M. W. (1979). Development of synaptic ultrastructure at neuromuscular contacts in an amphibian cell culture system. *J. Neurocytol.* **8**, 239–259.

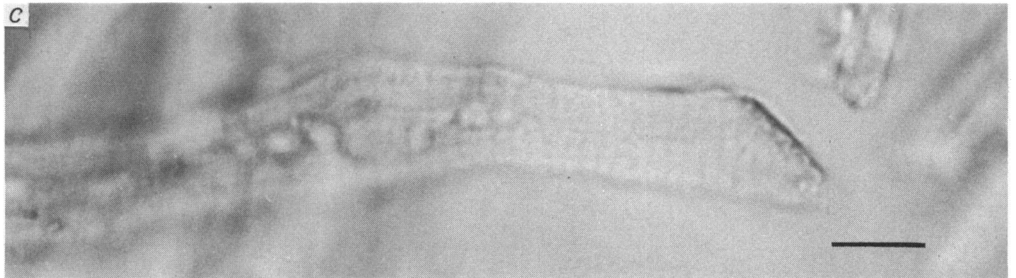
Weldon, P. R., Moody-Corbett, F. & Cohen, M. W. (1981). Ultrastructure of sites of cholinesterase activity on amphibian embryonic muscle cells cultured without nerve. *Devl Biol.* **84**, 341–350.

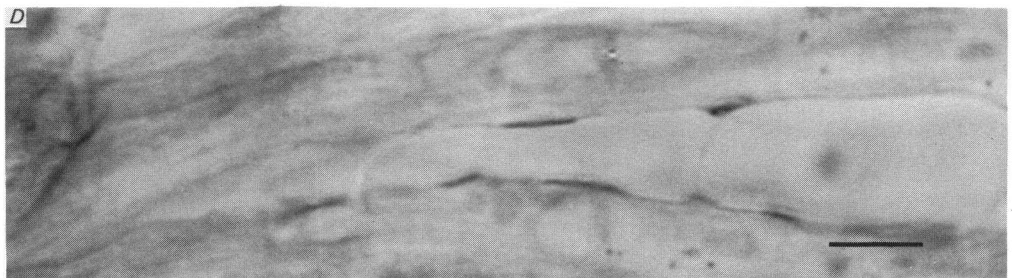
EXPLANATION OF PLATE

ChE histochemistry in myotomal muscle from tricaine-reared animals. *A*, low-power view showing stain located at the ends of myotomes, the main site of innervation. *B*, higher magnification showing discrete bands of stain at the ends of the two adjacent myotomes. Also seen is a short band of stain in the central region of one of the myotomes (arrowhead). *C*, ChE stain at the end of a teased muscle fibre. *D*, discrete bands of stain in the central region of two teased muscle fibres. Scale bars are 100 μm in *A*, 20 μm in *B*, and 10 μm in *C* and *D*.


A


B


C


D