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MEMBRANE PROPERTIES UNDERLYING SPONTANEOUS ACTIVITY OF DENERVATED MUSCLE FIBRES

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SUMMARY

We have examined the events underlying the initiation of spontaneous action potentials (fibrillation) in fibres of previously denervated rat diaphragm maintained in organ culture for up to 10 days.

1. Based on discharge pattern, two classes of spontaneously active fibres were found: rhythmically discharging fibres, and fibres in which action potentials occur at irregular intervals.

2. Sites of action potential initiation were located by exploration along the fibre length with two independent extracellular recording electrodes. The majority of sites of origin in both regular and irregular fibres were at the former end-plate zone; however, there was no region along the length that could not, at least in some fibres, be a site of origin.

3. Intracellular recording at or near sites of origin of action potential discharge showed two types of initiating events. Irregularly discharging fibres were brought to threshold by discrete depolarizations of up to 15 mV in amplitude, while regularly occurring action potentials were associated with oscillations of the membrane potential.

4. Discrete depolarizations (called fibrillatory origin potentials or f.o.p.s) at sites of origin in irregularly discharging fibres have the following properties: (*a*) random occurrence and nearly constant amplitude outside a refractory period during which both amplitude and probability of a second f.o.p. are reduced; (*b*) associated inward current flow which is localized to about 100 μm or less along the fibre length, and (*c*) dependence of amplitude and frequency on membrane potential.

5. Oscillation of membrane potential found at sites of origin of action

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potential discharge in regular fibres also occurred locally along the fibre length and was sensitive to changes in membrane potential.

6. Both f.o.p.s and oscillations of membrane potential were reversibly abolished by low Na^+–Ringer fluid or tetrodotoxin.

7. Neither type of initiating event was appreciably affected by concentrations of D-tubocurarine which blocked extrajunctional sensitivity to acetylcholine.

8. We conclude that spontaneous action potentials under these conditions arise from a localized Na^+-conductance change in the membrane of the active fibre; this conductance change is distinct from the increased Na^+-conductance which follows the interaction of acetylcholine with its receptor. Spontaneous activity in single, denervated muscle fibres is cyclical and self-inhibiting (Purves & Sakmann, 1974); thus the Na^+-conductance change underlying the initiation of spontaneous action potentials is affected by muscle fibre activity.

INTRODUCTION

Following denervation, mammalian skeletal muscle fibres generate spontaneous action potentials and visible contractions, a phenomenon called fibrillation (Tower, 1939). Recent experiments have shown that fibrillation in single fibres of rat diaphragm maintained in organ culture occurs cyclically rather than continually because spontaneous activity, over a period of many hours, is self-inhibiting (Purves & Sakmann, 1974). This finding implies that muscle fibre activity, or the lack of it, affects those properties of the fibre membrane leading to the initiation of spontaneous action potentials. The purpose of the present work was to determine the membrane properties underlying fibrillation and to investigate their relation to the extrajunctional acetylcholine supersensitivity of denervated mammalian muscle (Axelsson & Thesleff, 1959). The relationship between the membrane properties causing fibrillation and transmitter supersensitivity is important because of evidence that the level of muscle fibre activity exerts a marked influence on the distribution of acetylcholine (ACh)-sensitivity (Jones & Vrbová, 1970, 1971; Lømo & Rosenthal, 1972; Drachman & Witzke, 1972; Cohen & Fischbach, 1973; Purves & Sakmann, 1974). Our results show that the membrane conductance change which initiates spontaneous action potentials is different from the conductance change which follows the interaction of acetylcholine and its receptor. Thus the conductance change underlying fibrillation is an additional membrane property which can be altered by the activity level of the fibre.

METHODS

Preparation and maintenance of diaphragm strips in organ culture; methods of recording

The techniques of preparing, maintaining and recording from denervated adult rat diaphragm strips in organ culture are the same as those we have previously described (Purves & Sakmann, 1974). Left hemidiaphragms of rats weighing 150–250 g were denervated 6–16 days before being placed in organ culture; strips about 5 mm in width were maintained in Trowell's T8 medium (Flow Laboratories) directly bubbled with 95 % O_2 + 5 % CO_2 at 36–37° C for up to 10 days. Experiments were carried out in T8 medium under the same conditions as incubation unless otherwise stated. Micropipettes for intracellular recording were filled with 3 M-K acetate in most experiments; extracellular recordings were made with glass coated, platinized tungsten electrodes.

Location of sites of origin of action potential discharge

In some experiments we located the point along a fibre at which action potentials were being initiated. This was done by finding an active fibre with an extracellular electrode, and then placing a second independent extracellular electrode on the same fibre about 0·5 mm away. The sequence of action currents recorded at the two points indicated the direction of propagation. When one electrode was at the site of origin the action current appeared first at this electrode whether the second electrode was placed a few hundred microns to one side or the other. Proper location of the origin could be confirmed by the characteristic shape of the extracellularly recorded action potential at this point (see Fig. 2). Exploration of active fibres within 2–3 mm of the rib was often limited by a superficial layer of fatty connective tissue. In some cases we could only locate sites of origin to within this distance of the rib insertion. We also had difficulty in following some fibres closer than 1–2 mm from the central tendon. In many experiments when an origin had been localized a silver chloride mark about 50–100 μm in diameter was deposited on the muscle surface by passing current through a 50 μm diameter silver wire insulated to within about 30 μm of its tip.

Cholinesterase staining

Following location and marking of origins, strips were washed in 0·1 M maleate buffer at pH 6·0 for 10–20 min and stained for acetylcholinesterase by a method similar to that of Karnovsky (1964). After development of the stain, the distance of the AgCl marks was measured from the former end-plate region, costal insertion, and myotendinous junction. While the end-plate region is invariably located about midway along the fibre length in the rat diaphragm, the position of individual end-plates varies over a few hundred microns. Thus this method allows a resolution of only about $\pm$ 200 μm in judging the distance of the marked origin from the former end-plate. The total length of fibres in diaphragms from rats of this size is about 9–16 mm.

Changes of solution while recording from single fibres

In some experiments a flow chamber was used to allow application and washout of drugs while recording intracellularly from single fibres. These experiments were done in oxygenated mammalian Ringer solution at 25–35° C, temperature remaining constant ($\pm$ 1° C) during any one experiment. Solutions were changed by turning a tap, and flow was maintained at about 4–6 ml./min; the bath volume was 2 ml. In

experiments where the normal Na^+ concentration was reduced, NaCl was replaced with an equiosmolar amount of sucrose or Tris-Cl. To minimize liquid junction potentials, recording electrodes in these experiments were filled with 3 M-KCl instead of K acetate, and a 3 M-KCl-agar bridge used to ground the bath.

The composition (in m-mole/l.) of the Ringer fluid was: Na^+, 151·0; K^+, 5·0; Cl^-, 147·0; Mg^{2+}, 1·0; Ca^{2+}, 2·0; $H_2PO_4^-$, 1·0; HCO_3^-, 14; and D-glucose, 11·0. pH was maintained at about 7·2 by bubbling with 95 % O_2 + 5 % CO_2.

The peaks of extracellularly recorded spikes in Figs. 2, 5, 9 and 10 are retouched for clarity.

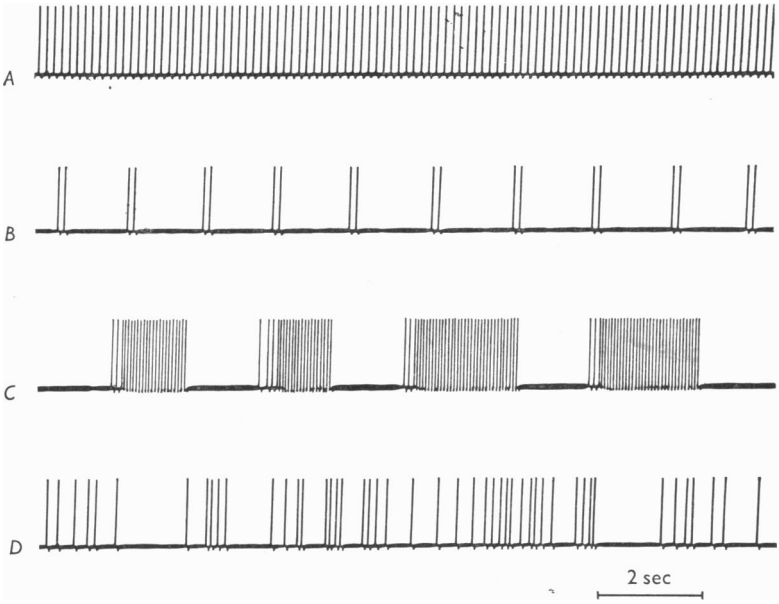

Fig. 1. Patterns of action potential discharge recorded extracellularly from single fibres (penwriter records indicating discharge rate only). *A*, single spikes occurring at fixed interval. *B*, doublets. *C*, bursts of action potentials occurring every few seconds. *D*, spikes occurring at apparently random intervals.

RESULTS

Patterns of action potential discharge

Spontaneous activity in single fibres recorded either intra- or extracellularly fell into two broad classes: activity with discernible regularity or rhythm of action potential discharge, and activity with no apparent pattern. Regularly active fibres usually displayed one of several characteristic patterns: single action potentials occurring at a fixed interval (to within a few percent of the average interval (Fig. 1A)), pairs of action potentials (doublets) or triplets (Fig. 1B), and bursts of action potentials with longer and somewhat irregular pauses between the bursts (Fig. 1C).

A fibre without obvious pattern of discharge (subsequently referred to as 'irregular') is shown in Fig. 1*D*. In some fibres with a very slow or very fast rate of firing, we could not be sure whether the discharge was regular or irregular.

Both regular and irregular fibres were found in most fibrillating muscle strips throughout the period of culture (up to 10 days). However, regularly discharging fibres tended to predominate during the first few days in culture, while irregular ones were prevalent in strips maintained for more than 4–5 days. About one-quarter to one-third of fibres were active in a strip at any one time (see Purves & Sakmann, 1974). In the following sections the nature of the events underlying these activity patterns are examined.

Location of sites of origin of action potentials along the fibre length

The origin of action potentials in many fibres could be located by exploring along the fibre with two independent extracellular electrodes. When recorded away from the site of origin the action current has an initial positive phase corresponding to outward current flow associated with the advancing action potential (Fig. 2*A*). At the origin, however, the positive phase is absent as current initially flows inward (Fig. 2*B*) (see also Lorente de Nó, 1947). The small step in the initial inward current in Fig. 2*B* is characteristically found at the site of origin of irregularly discharging fibres and is explained in a subsequent section.

The length of the muscle fibre initiating spontaneous action potentials was evaluated by moving one electrode away from the other positioned at the site of origin in increments of 50–100 μm. Separations of a few hundred microns were sufficient to appreciate an initial positive phase in the action current at the exploring electrode and a small delay due to conduction (Fig. 2*C* and *D*). In general, this was true for both regularly and irregularly discharging fibres, and indicates that the longitudinal extent of an active fibre which initiates action potentials at any one time is circumscribed.

Stability of sites of origin

In order to determine whether sites of origin are stable for long periods we recorded extracellularly from single active fibres for many hours (see Methods in Purves & Sakmann, 1974). An electrode was positioned at an origin determined by the criteria illustrated in Fig. 2; the chamber and electrode holder were then transferred to the incubator and discharge from the site of origin was monitored. Although a single electrode was used in this series of experiments, a change in the site of action potential initiation of 200 or 300 μm would cause a change in the shape of the action current (see Fig. 2*C* and *D*). Six fibres were followed in this way

for 4–11 hr, three with a regular pattern of discharge and three irregular fibres. There was no detectable change in the site of origin during the recording period in any of these fibres.

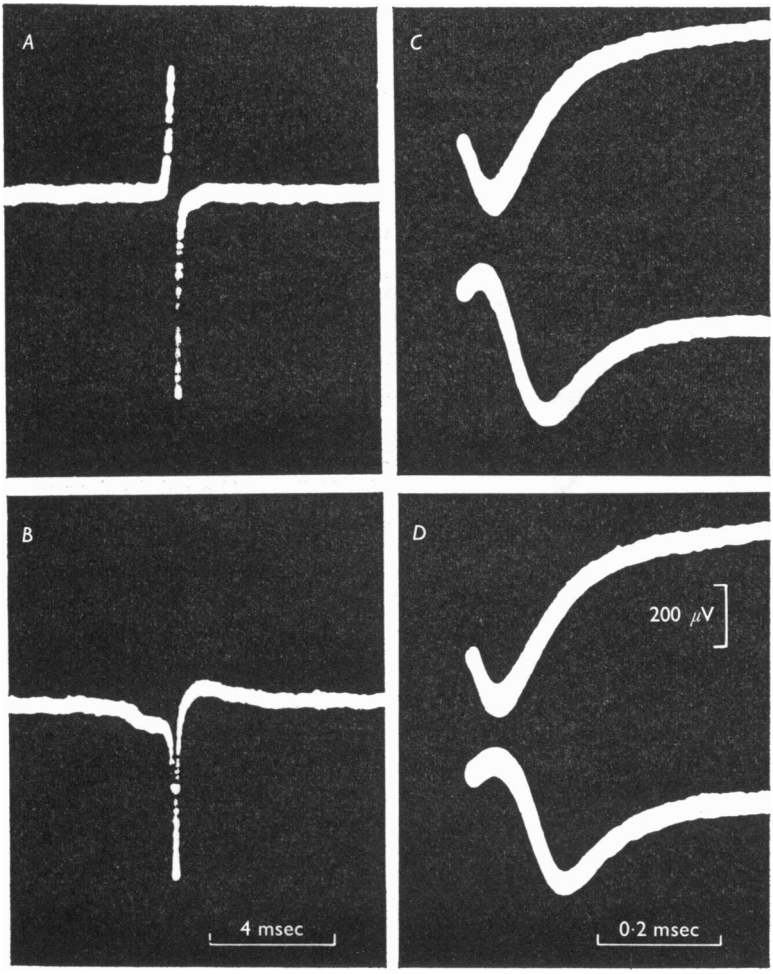

Fig. 2. Extracellular records of action currents from a single irregularly active fibre. Outward current flow leads to positive voltage at recording electrode (upward deflexion in oscillographs). *A*, action current recorded away from origin has initial positive phase due to outward current flow associated with advancing action potential; *B*, at the site of origin of action potential, positive phase is absent. *C*, oscilloscope sweep is triggered by action current at site of origin (upper trace); a second electrode is located 250 μm away along the fibre length in the direction of the rib (lower trace). At this distance action current has an initial positive phase. About ten traces are superimposed to intensify image. *D*, same as *C* but with second electrode positioned 250 μm away in the direction of the tendon.

During the course of other experiments, however, we sometimes encountered regularly discharging fibres where the site of origin shifted in a systematic way. The fibre shown in Fig. 3 was intermittently active in bursts lasting about 50 sec with about a 30 sec quiescent period between the bursts. Fig. 3*A* shows several superimposed action currents at the beginning of a burst recorded with two independent extracellular electrodes separated by 400 μm. In both traces the initial phase of the action current is positive and arrives first at the electrode located nearer the central tendon (lower trace). During the course of the burst the site of origin moves

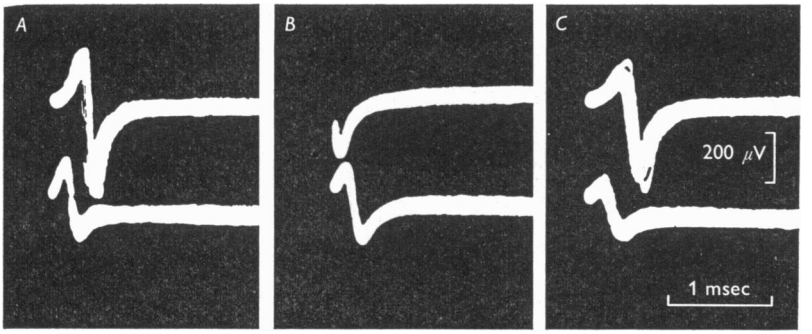

Fig. 3. Change in location of site of origin in a regular fibre discharging in bursts lasting about 50 sec. Two independent extracellular electrodes are positioned along the length of the fibre (upper trace 2·8 mm from myotendinous junction, lower trace 2·4 mm); action current in lower trace triggers sweeps which are superimposed to intensify image. *A*, at onset of burst origin is located to tendon side of electrode monitored in lower trace. *B*, during burst position of origin shifts to position near electrode monitored in the upper trace (untriggered sweeps showed initial inward current such as shown in Fig. 2*B*). By end of burst origin has returned to a position similar to *A*.

to a position near the other electrode (upper trace, Fig. 3*B*), and then returns to a location close to the initial site, (Fig. 3*C*). This change in the position of the origin occurred during each burst of activity. Thus in this fibre the site of origin shifts over a length of at least 600–700 μm.

Such systematic changes in the location of the origin were not found in irregularly discharging fibres, but often action currents with and without an initial positive phase were apparently recorded from the same fibre, suggesting more than one site of origin. This could be confirmed by simultaneous intra- and extracellular recording from the same fibre (see below).

Distribution of sites of origin along the fibre length

The position of origins along the length of active fibres could be determined by marking the site of initiation (see Methods) and measuring its distance from the central tendon, costal insertion and former end-plate zone which was stained for cholinesterase at the end of the experiment. Fig. 4 shows the distribution of sites of origin in a series of 142 fibres. More than half the origins (61 %) were located at the former end-plate

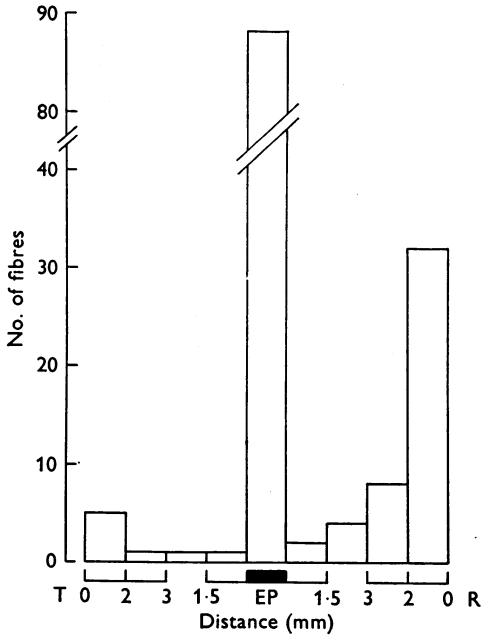

Fig. 4. Distribution of sites of origin of action potentials along the length of single fibres. Location of origins was measured from either the central tendon (T), rib insertion (R) or former end-plate region (EP, dark bar). Distances represented by bars are not equal; since the length of muscle strips varied, two bars represent an unspecified number of mm corresponding to origins located more than 3 mm from the insertions or 1·5 mm from the former end-plate region.

($\pm$ 200 μm) while 39 % were found elsewhere along the fibre length. Of those outside the end-plate region, most were within a few mm of the rib insertion, although there was no region along the fibre at which occasional origins were not found. There was no obvious difference in the distribution along regularly and irregularly discharging fibres (regular fibres made up about one-third of the sample).

Intracellularly recorded events at sites of origin

Having located the site of origin of action potential discharge, we could insert a micro-electrode into the fibre at or near that point to study the membrane events leading to spike initiation.

(*a*) *Irregularly discharging fibres.* Action potentials recorded near the site of origin in irregularly discharging fibres were preceded by characteristic prepotentials (Fig. 5*A*). In some fibres these prepotentials occasionally failed to reach threshold (Fig. 5*B*), while in others most of these events remained subthreshold (Fig. 5*C*). Prepotentials were found at all sites of origin of irregularly discharging fibres. We have called these events fibrillatory origin potentials (f.o.p.s) to distinguish them from the membrane oscillations found at sites of origin in regularly discharging fibres (see below).

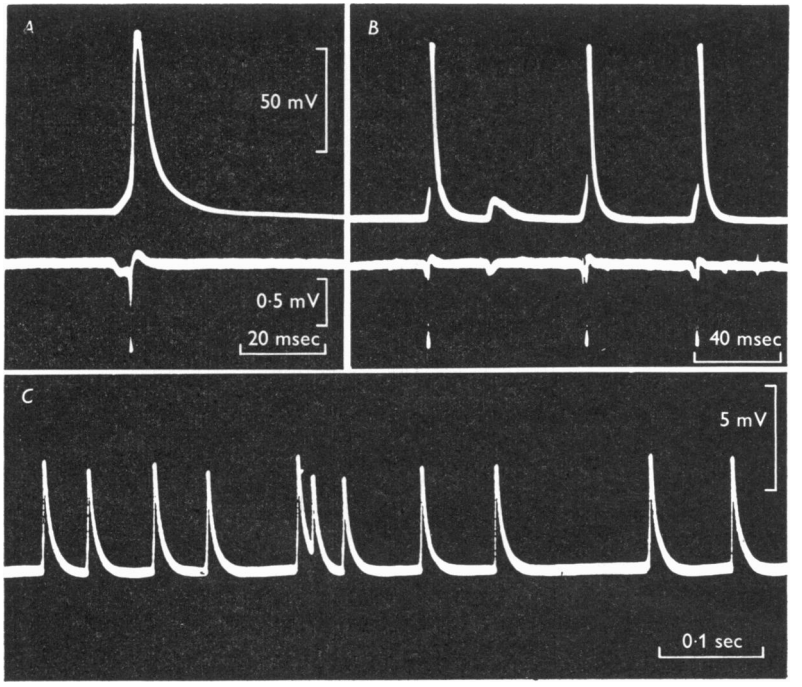

Fig. 5. Discrete depolarizations (f.o.p.s) at sites of origin of irregularly discharging fibres. *A*, simultaneous intracellular (upper trace) and extracellular (lower trace) record of an action potential at its origin. Action potential arises from a preceding depolarization about 10 mV in amplitude corresponding to step in extracellular record. Resting potential is −68 mV. *B*, same fibre as (*A*). One f.o.p. fails to bring fibre to threshold. *C*, intracellular record from site of origin of a different fibre at higher gain; all f.o.p.s are subthreshold. Resting potential −69 mV.

In many fibres the amplitude of f.o.p.s decreased during the first minute or two of intracellular recording, probably as a result of local damage (although this occurred without apparent loss of resting potential). F.o.p.s were also recorded in some fibres which probably were not fibrillating. In fibres where f.o.p. amplitude remained near the firing level (for example Fig. 5*B*) we could determine the threshold. In twenty fibres threshold was $-59{\cdot}3 \pm 0{\cdot}29$ mV (mean ± S.E.) which was, on average, 9·5 mV more positive than the resting potential in this sample. Thus in most of these fibres f.o.p.s had to depolarize the membrane locally by

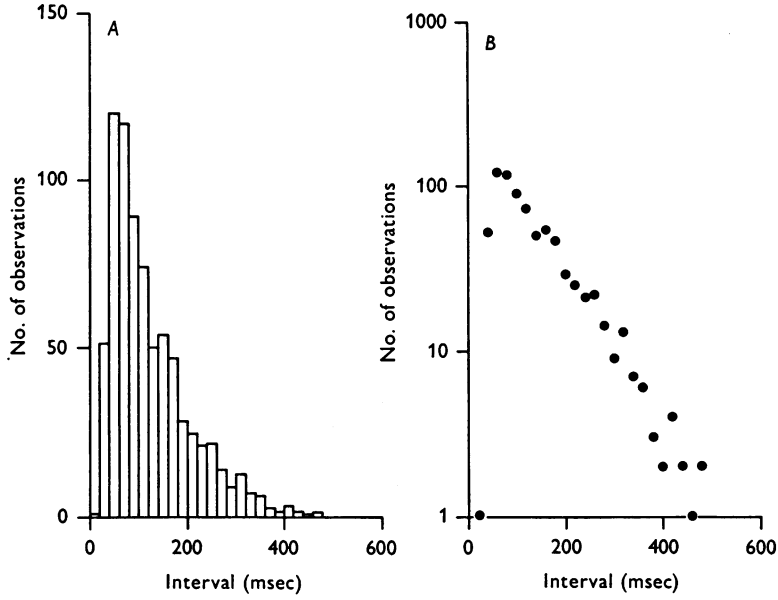

Fig. 6. Distribution of intervals between f.o.p.s. *A*, interval histogram of a series of 780 consecutive f.o.p.s. Following a f.o.p. there is a 'dead' period of about 20 msec during which the probability of a second event is very low (see also Fig. 7*A*). Thereafter the probability of a second event reaches a maximum and declines. *B*, same as *A* with ordinate representing log of the number of observations in each class. Approximately exponential fall is consistent with random occurrence of f.o.p.s outside a refractory period.

about 10 mV for fibrillation to occur. Resting potentials at or near the site of origin and elsewhere along the fibre length were similar. The mean resting potential at the origin in irregular fibres maintained in culture for 3–6 days was $68{\cdot}4 \pm 0{\cdot}38$ mV (± S.E., $n = 60$); the mean resting potential determined away from sites of origin (usually 2–4 mm from the central tendon) after 3–6 days in culture was $69{\cdot}0 \pm 0{\cdot}28$ mV ($n = 350$).

The frequency of f.o.p.s varied from fibre to fibre, but in general covered

the same range as spontaneous action potentials, that is about 0·1–24/sec (Purves & Sakmann, 1974). Frequency and amplitude of f.o.p.s could be studied statistically by recording a large number of sequential events in fibres where f.o.p.s remained subthreshold (Fig. 5*C* is a small portion of such a record). Three fibres were examined in detail by analysing 650–1000 consecutive f.o.p.s. Recording was begun at least several minutes after penetration and resting potentials did not change by more than 1–2 mV during the recording. Interval histograms were similar for the three series (Fig. 6*A*). Immediately after a f.o.p. there is a period of up to 20 msec during which the probability of a second f.o.p. occurring is very low. Following this 'dead' period the probability of a second event increases and reaches a maximum. The interval distribution thereafter falls approximately exponentially (Fig. 6*B*). These findings suggest that at these three sites of origin f.o.p.s occurred randomly outside a refractory period. A few fibres were encountered where f.o.p.s occurred in showers or at regular intervals. A possible explanation is interaction between adjacent fibres (see below). F.o.p.s were not seen during action potentials.

The amplitudes of individual f.o.p.s in a series of consecutive events varied (see Fig. 5*C* for example). This variability is largely due to reduction in the amplitude of potentials occurring shortly after a preceding f.o.p. In Fig. 7*A* amplitude is plotted as function of time after a preceding 'primary' f.o.p. (defined as occurring at least 100 msec after the previous f.o.p. in the series). The amplitude distribution of primary f.o.p.s from these three series is shown in Fig. 7*B–D*. The narrow range in any one series suggests an all or none event.

In many instances the shape of intracellularly recorded f.o.p.s was more complex, with one or more inflexions (Fig. 8). Such complex potentials were usually seen in the first minute or so after impalement and tended to assume a simpler shape as their amplitude decreased. At any particular origin the inflexions were often uniform in size and position, and were not associated with activity in nearby fibres. In the absence of more precise knowledge about the membrane area giving rise to f.o.p.s (see Discussion), the interpretation of these inflexions remains uncertain.

Intracellularly recorded f.o.p.s were often biphasic, depolarization being followed by a small hyperpolarization (see for example Fig. 7*A* and Fig. 17). Current flow associated with the depolarizing phase could frequently be recorded extracellularly, either as a step preceding the action current (Fig. 9*A*; see also Figs. 2*B* and 5*A*) or by itself if a f.o.p. failed to reach threshold (Fig. 9*B*). Recording f.o.p. current required placement of the electrode within about 100 μm of the site of origin (100 μm is approximately the limit of resolution with our technique).

The occurrence of multiple sites of origin in some irregularly discharging

fibres could be confirmed by intracellular recording (Fig. 10). In these fibres some action potentials arose from prepotentials, while others were conducted from one or more sites of origin at least a length constant away from the recording electrode.

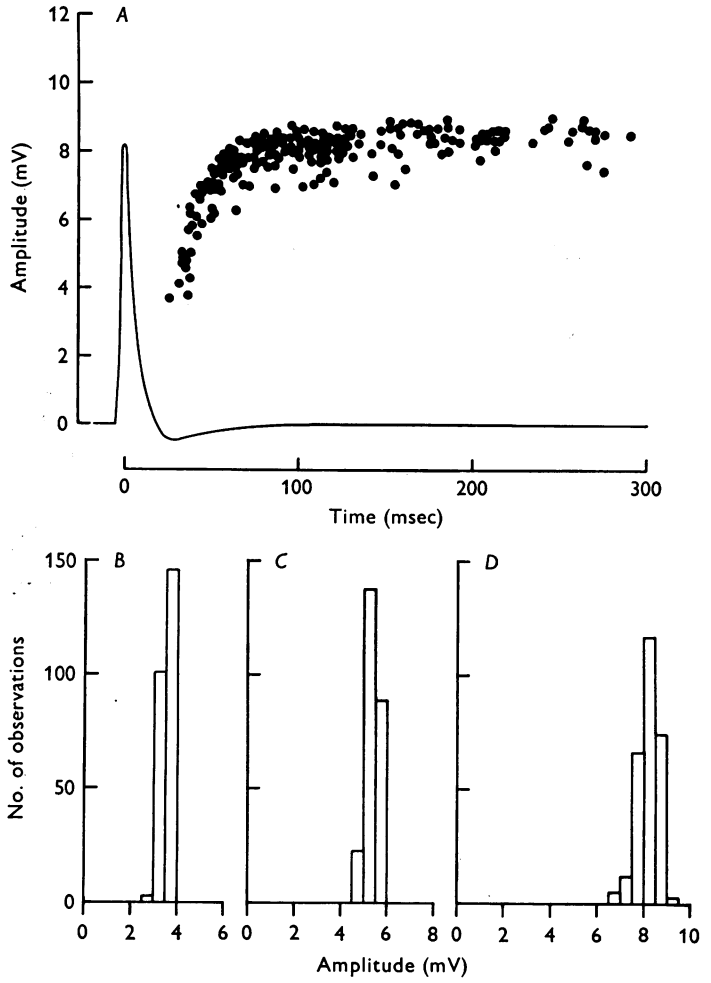

Fig. 7. F.o.p. amplitude. *A*, amplitude of f.o.p.s in a fibre as a function of time after a preceding 'primary' f.o.p., defined as occurring at least 100 msec after the previous f.o.p. in this series. Tracing is of a typical primary f.o.p. (its amplitude corresponds to the mean of all primary f.o.p.s in the series). Amplitude of a second f.o.p. is reduced for up to about 100 msec. Resting potential −70 mV. *B–D*, amplitude distribution of primary f.o.p.s in the three series studied in detail (*D* corresponds to series in *A*). Narrow distribution in each series is consistent with an all or none event. Mean amplitude 3·5 mV (*B*), 5·3 mV (*C*), 8·1 mV (*D*).

(*b*) *Regularly discharging fibres.* In many regularly active fibres the site of origin was more labile than in irregular fibres (see, for example, Fig. 3). Although an origin could remain at one location for many hours, impalement often resulted in cessation of discharge or a shift of the site of origin to another nearby locus. For this reason some intracellular records in this section are from impalements a millimetre or more from the actual site of origin, but sufficiently close to record the events associated with action potential discharge.

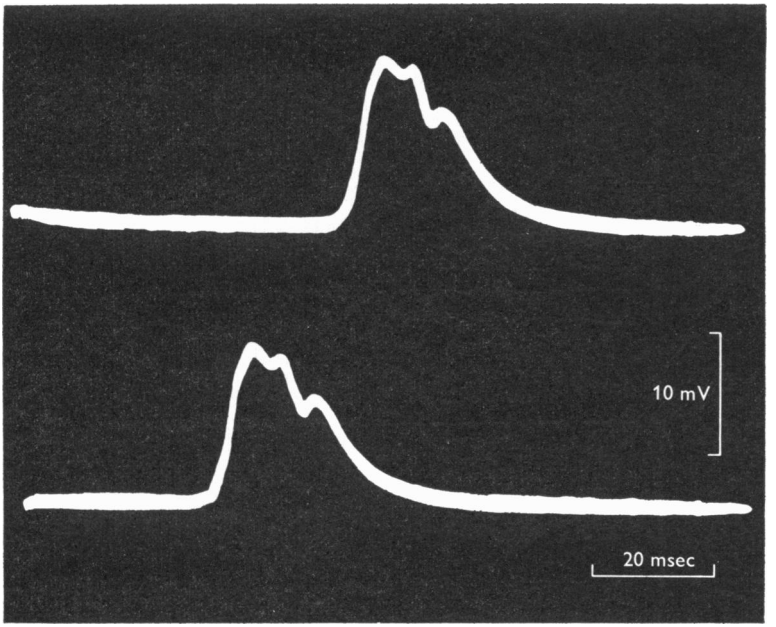

Fig. 8. Complex f.o.p.s at site of origin in an irregular fibre. At any one origin shape of each f.o.p. is similar. Resting potential −69 mV.

Intracellular recordings at or near sites of origin in regularly discharging fibres did not show discrete depolarizations, nor was a step seen in the action current (Fig. 11); rather action potentials were associated with oscillations of the membrane potential. In fibres where the pattern of discharge was continuous (for example, Fig. 11*A*; see also Fig. 1*A*) membrane oscillation was seen when occasional action potentials failed as in Fig. 12*A*. In fibres where action potentials occurred as a slow continuous train, or as doublets or triplets (see Fig. 1*B*) oscillations tended to wax and wane slightly between spikes (Fig. 12*B*, *C*). In fibres where action potentials occurred in bursts (Fig. 1*C*) oscillations built up gradually from a flat base line and damped down again after the discharge (Fig. 12*D*).

In fibres such as the one shown in Fig. 12*D* the firing level was at least several millivolts more positive than the resting potential. It was difficult to study the extent of the site of origin as critically as in irregularly discharging fibres where current associated with f.o.p.s could be recorded. By making multiple intracellular recordings along single regular fibres we

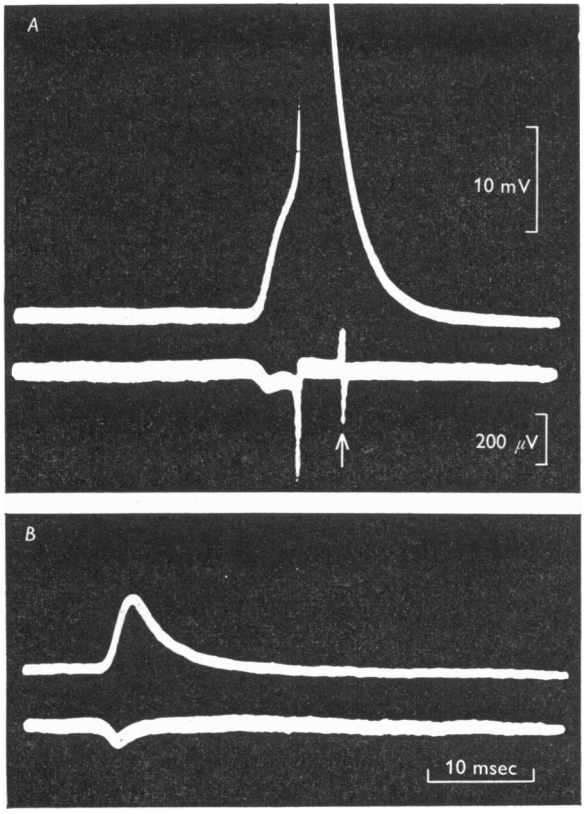

Fig. 9. Current flow associated with f.o.p. *A*, simultaneous intracellular (upper trace) and extracellular (lower trace) records from site of origin of an irregularly discharging fibre. *A*, step of inward current flow is associated with f.o.p. that gives rise to action potential (not fully shown at this gain; arrow indicates unrelated action current in nearby fibre). *B*, same as *A* but f.o.p. subthreshold. Inward current flow occurs during rising phase of intracellularly recorded f.o.p. (upper trace). Resting potential −71 mV.

could, however, determine that the length of the fibre which oscillates is limited. Fig. 13*A* is an intracellular recording near the origin of a regular fibre discharging in doublets, triplets and short bursts. The impalements in Fig. 13*B* and *C* are from the same fibre at increasing distances from the

origin; lower amplitude oscillation can still be seen between spikes. In Fig. 13*D* the recording electrode is 4·3 mm from its location in Fig. 13*A*; at this distance from the origin no oscillation of membrane potential is recorded between spikes. Fig. 13*E* is a record from another fibre in which some bursts are associated with oscillations while others are triggered by an action potential conducted from a more distant site of origin. This is analogous to multiple origins in which discharge is initiated by f.o.p.s (Fig. 10). That oscillations associated with action potentials are limited to a fraction of the length of an active fibre was repeatedly confirmed in other experiments where penetrations made 2–4 mm from the central

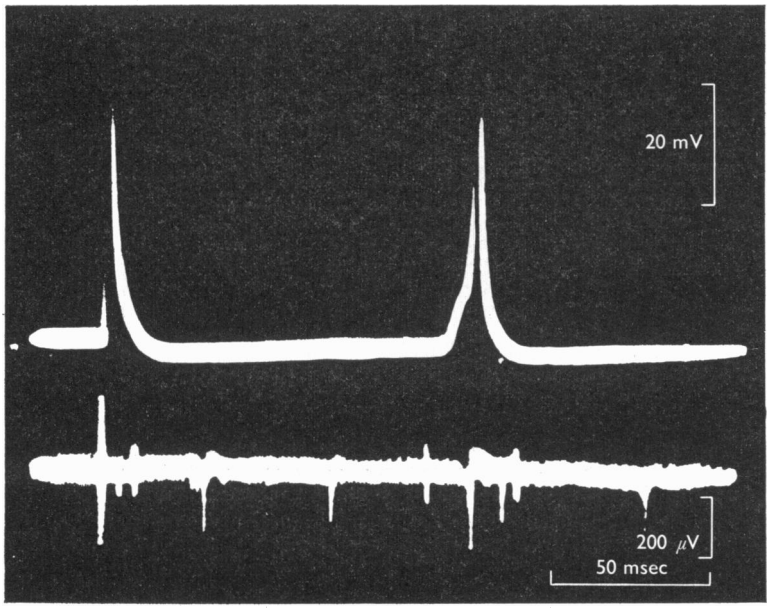

Fig. 10. More than one site of origin in an irregularly discharging fibre. Simultaneous intracellular (upper trace) and extracellular (lower trace) records: first action potential arises from flat base line and corresponding action current shows initial positive deflexion; second action potential arises from f.o.p. and corresponding action current is typical of waveform recorded at origin. Thus fibre has at least two sites of origin more than a length constant apart. Action potentials not fully shown at this gain. Resting potential −67 mV.

tendon usually showed regularly occurring action potentials arising without preceding depolarization (see, for example, Fig. 7, Purves & Sakmann, 1974).

Mean resting potentials of regular and irregular fibres determined away from sites of origin (usually 2–4 mm from the central tendon) were similar on days 3–8 of culture (see also Purves & Sakmann, 1974). As in irregularly

discharging fibres, resting potentials of regular fibres at and away from sites of origin were not significantly different.

In the course of these experiments we did not encounter f.o.p.s and oscillations occurring simultaneously in the same fibre, although there is no obvious reason why this should not be possible (see Discussion).

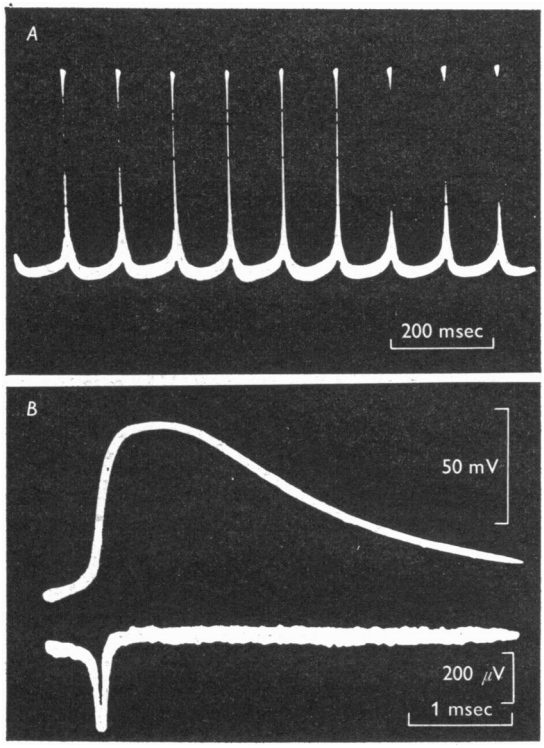

Fig. 11. Intracellular recording at site of origin of a regularly discharging fibre. *A*, intracellular record shows action potentials arising from rhythmic change in membrane potential. *B*, simultaneous intracellular (upper trace) and extracellular (lower trace) records from same origin; there is no initial step in the action current (compare with Fig. 2*B* and Fig. 9*A*). About ten triggered sweeps are superimposed to intensify image.

Response of initiating events to changes in membrane potential

In order to study the response of f.o.p.s to changes in membrane potential we inserted a current passing electrode within 150 μm of an intracellular recording electrode located at a site of origin where sub-threshold f.o.p.s were occurring (as in Fig. 5*C*). By passing current pulses of both polarities we obtained a series of records relating f.o.p. amplitude and frequency to membrane potential. Injected current was monitored so

that a current–voltage curve for the fibre could be constructed over the same potential range. The result of this experiment at ten origins was similar: shifting the membrane potential towards zero increased the frequency of f.o.p.s and decreased their amplitude, while hyperpolarization decreased the frequency of these events and increased their amplitude

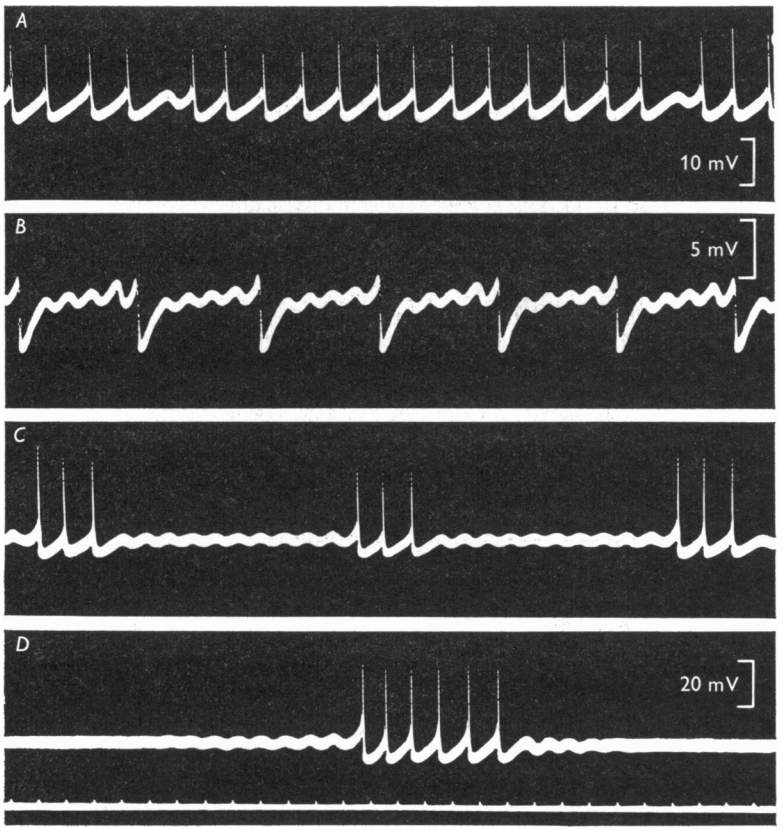

Fig. 12. Oscillation of membrane potential near sites of origin of regularly discharging fibres. *A*, single action potentials occurring at fixed interval; when spike fails, subthreshold fluctuation of membrane potential is seen. Resting potential about −66 mV. *B*, single action potentials in a different fibre occurring at fixed interval but at slower rate than *A*; four oscillations of membrane potential between each action potential appear to build slightly in amplitude during the interspike interval. Resting potential about −62 mV. *C*, action potentials discharging in triplets. Resting potential about −69 mV. *D*, action potentials occurring in bursts associated with progressively larger membrane oscillations which build up from flat base line. Resting potential −67 mV. Time marks = 100 msec. Voltage calibration in *C* same as *D*. Full amplitude of action potentials not shown.

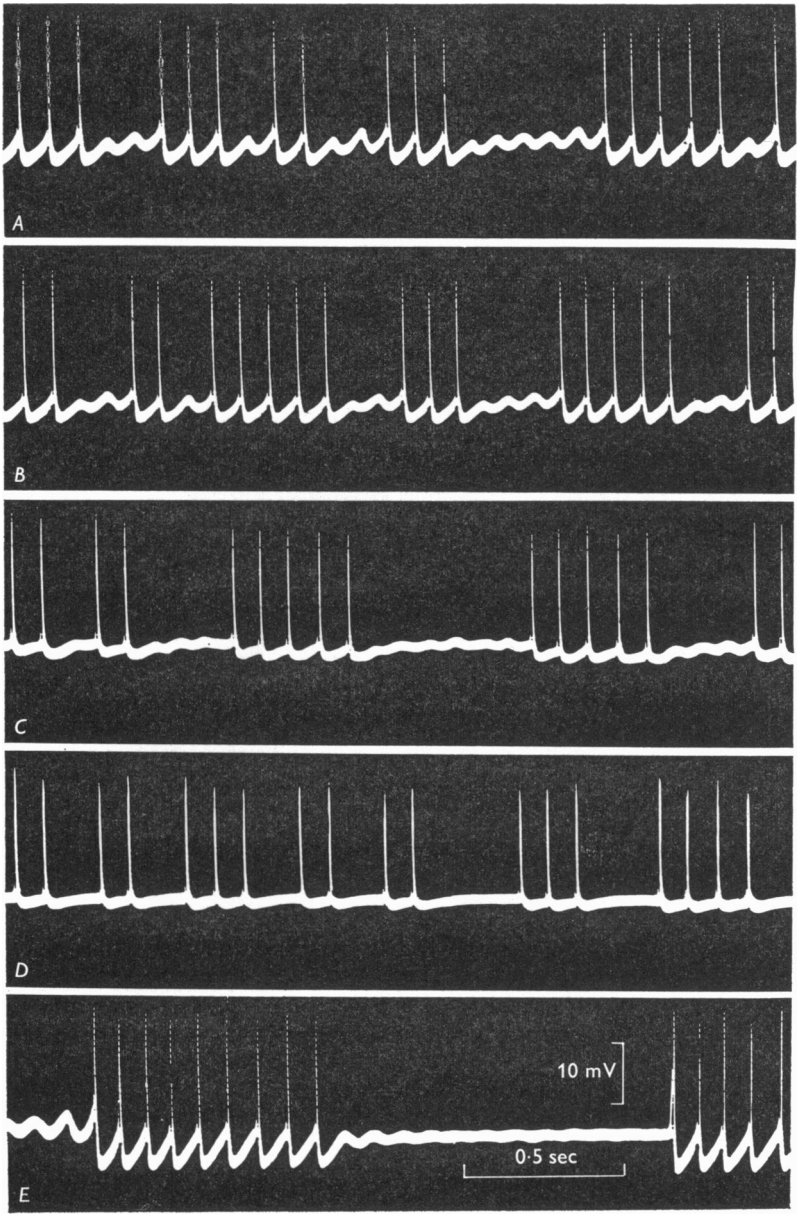

Fig. 13. For legend see facing page.

(Figs. 14 and 15). The severalfold change in f.o.p. amplitudes with small changes in membrane potential suggests that the conductance change giving rise to these events is sensitive to voltage. This behaviour is different from the conductance change underlying an end-plate potential which is largely insensitive to voltage (Takeuchi & Takeuchi, 1960); thus the end-plate potential changes in approximate proportion to the membrane potential (Fatt & Katz, 1951). In some fibres sufficient current could be passed to depolarize the membrane by about 20 mV; although firing of the fibre occurred, very small f.o.p.s could still be seen between spikes. In other fibres large depolarizations caused f.o.p.s to stop abruptly. Reversal of f.o.p.s was not seen.

Hyperpolarizing pulses reduced the rate of action potential discharge when injected near sites of origin in regular fibres leaving a residual oscillation if spikes were blocked; depolarization increased the frequency of oscillation (or discharge) (Fig. 16). Current injections of either polarity several millimetres away from the origin had no effect on discharge frequency.

Response of initiating events to reduced Na^+ *or tetrodotoxin*

In these experiments a flow chamber was used so that the preparation could be bathed in low Na^+ Ringer fluid (9 % of normal concentration) or Ringer fluid containing tetrodotoxin $2–5 \times 10^{-7}$ g/ml., while recording from a site of origin where subthreshold f.o.p.s were occurring. Results with low Na^+ fluid (with either sucrose or Tris-Cl substituting for NaCl) or fluid containing tetrodotoxin were similar: over a period of one or a few minutes f.o.p.s decreased in both amplitude and frequency and then stopped altogether (Fig. 17). Following the disappearance of f.o.p.s, the bath could again be perfused with normal Ringer fluid; f.o.p.s usually returned within

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Fig. 13. Localization of membrane oscillation in a regularly discharging fibre. *A*, impalement near site of origin shows typical oscillation of the membrane potential; origin is located between recording electrode and costal insertion 1–2 mm away and could not be approached more closely because of overlying connective tissue. Resting potential approximately −70 mV. *B*, same fibre impaled 0·7 mm away; oscillation is slightly smaller. Resting potential about −70 mV. *C*, impalement 2·2 mm from *A*. Resting potential −67 mV. *D*, impalement 4·3 mm from *A*. At this distance oscillation is no longer appreciable. Resting potential −70 mV. Typically the 'undershoot' of the action potential was larger near the origin. *E*, record from another fibre in which some bursts of action potentials are associated with membrane oscillation while others are probably triggered by an action potential from one or more sites of origin at least a length constant from the recording electrode. Resting potential −68 mV. Full amplitude of action potentials not shown.

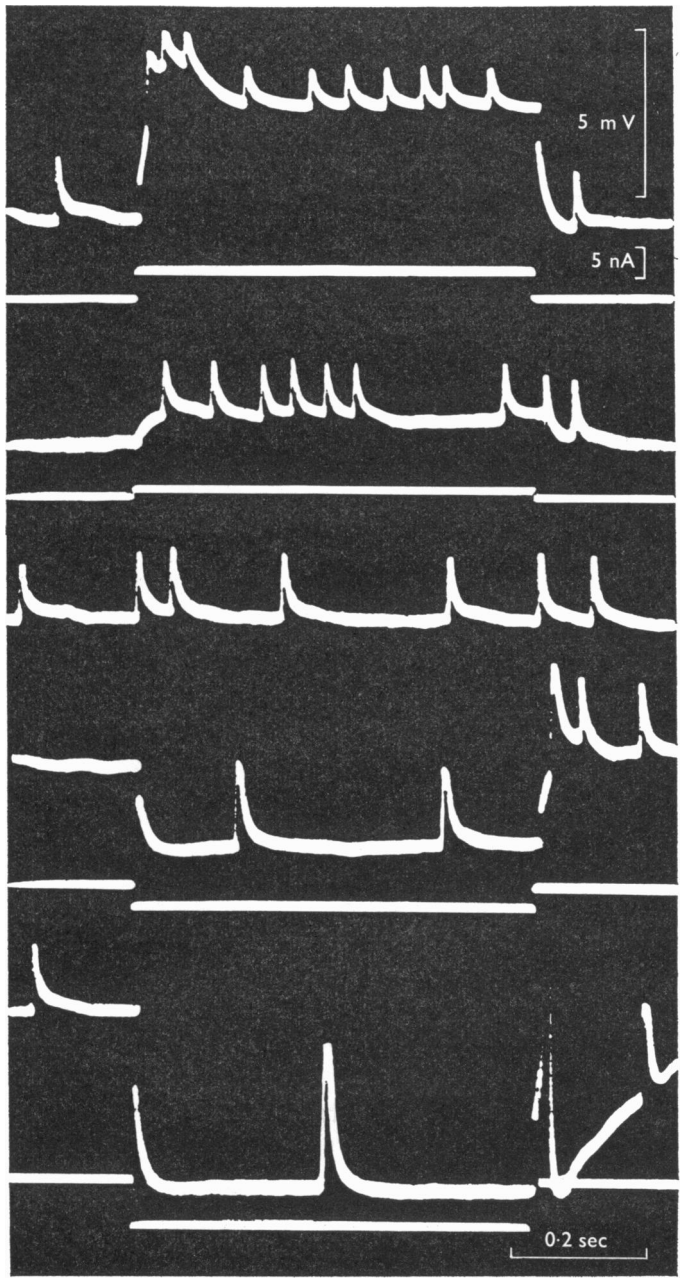

Fig. 14. For legend see facing page.

a few minutes, although after tetrodotoxin fluid this could take up to an hour or two.

The response of regularly discharging fibres was similar to irregular ones: within several minutes of the application of tetrodotoxin or low Na^+ Ringer fluid action potentials failed and the residual oscillations decreased in amplitude and then disappeared altogether. Oscillations and action potentials reappeared after further perfusion with normal Ringer fluid.

Response of initiating events to curare

Both f.o.p.s and membrane oscillations at sites of origin could be recorded in the presence of high levels of D-tubocurarine (5×10^{-5}–10^{-4} g/ml.) even after several hours incubation. Extrajunctional iontophoretic application of acetylcholine (see Methods in Purves & Sakmann, 1974) after incubation in curare showed complete block of acetylcholine receptors.

Interaction between spontaneously active fibres

Not infrequently a spontaneously active fibre was encountered which was time-locked to an action potential in an adjacent fibre (Fig. 18). We did not find evidence of electrical coupling between fibres in penetrations of about 100 pairs of adjacent fibres in the region of the former end-plate where most sites of origin are located (see Fig. 4). Interaction between fibres might be due either to mechanical effects or transient changes in electrical fields such that an action potential in one fibre might influence the occurrence of initiating events (f.o.p.s or oscillations) in a neighbouring one (Arvanitaki, 1942). Synchronization of active fibres has also been described in fibrillating muscles *in vivo* (Harvey & Kuffler, 1943).

Effect of activity on fibrillation in regularly and irregularly discharging fibres

Because of the different events associated with the initiation of action potentials in regularly and irregularly discharging fibres, it was of interest to ask whether the self-inhibiting effect of activity (Purves & Sakmann, 1974) on the two types of spontaneous discharge is similar. Of the twenty fibres from which we recorded for prolonged periods with extra-

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Fig. 14. Change in amplitude and frequency of f.o.p.s as a function of membrane potential. Impalement is at site of origin of irregular fibre; on successive sweeps membrane potential is shifted from the resting level by depolarizing or hyperpolarizing current pulses. Depolarization decreases amplitude of f.o.p.s and increases frequency, while hyperpolarization increases amplitude and decreases frequency. Anode break spike follows larger hyperpolarizing current pulse. Lower traces monitor injected current. Resting potential −66 mV.

cellular electrodes in previous experiments (Purves & Sakmann, 1974), nine had a regular pattern of discharge while eleven were irregular. The type of discharge was usually unchanged throughout an active period as

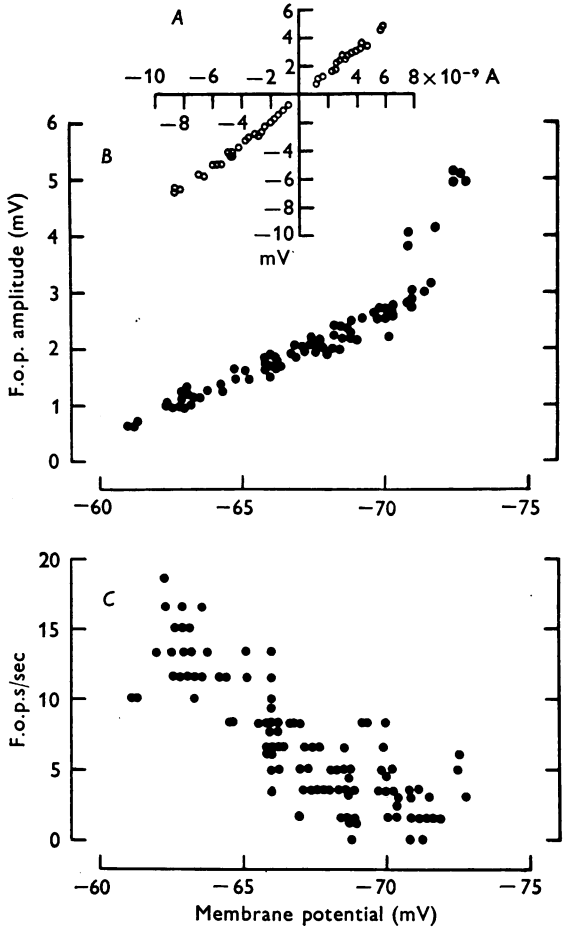

Fig. 15. F.o.p. amplitude and frequency as a function of membrane potential (same fibre as Fig. 14). *A*, current–voltage relation over range of membrane potential examined shows no appreciable rectification. *B*, amplitude of f.o.p.s plotted as a function of membrane potential. Small changes in potential cause severalfold changes in f.o.p. amplitude. Resting potential −66 mV. Each point on graph represents mean amplitude of f.o.p.s occurring during the steady-state portion of current pulse (see Fig. 14); scatter increases as membrane is hyperpolarized in part because frequency decreases. *C*, same fibre as *A* and *B*. F.o.p. frequency during current pulse as a function of membrane potential. In other fibres rectification was apparent when membrane potential was changed over a larger range.

was the case in these twenty fibres, although in other experiments we sometimes observed a transition from regular to irregular activity. The mean observed period of activity in regular fibres was 12 h 43 min ± 2 h 3 min (mean ± S.E.) while for irregular fibres it was 9 h 14 min ±

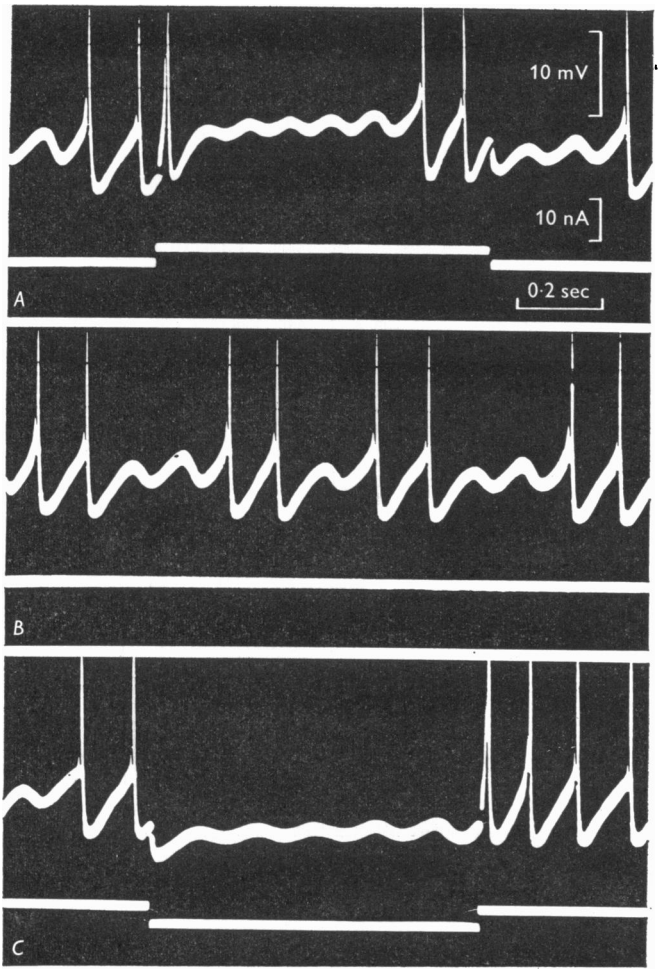

Fig. 16. Typical effect of changes in membrane potential near site of origin of a regular fibre. *A*, depolarization increases the frequency of oscillation but decreases its amplitude. *B*, spontaneous discharge in absence of injected current. *C*, hyperpolarization reduces the frequency of oscillation slightly and also decreases the amplitude. Current injections more than a few mm from the origin have no effect on frequency of spike discharge. Lower trace monitors injected current. Resting potential about −67 mV. Action potentials not fully shown at this gain.

2 h 38 min. This difference is not significant. Since the onset of recording occurs at a random time after the onset of activity in the fibre, the full active period is probably about twice the observed mean value.

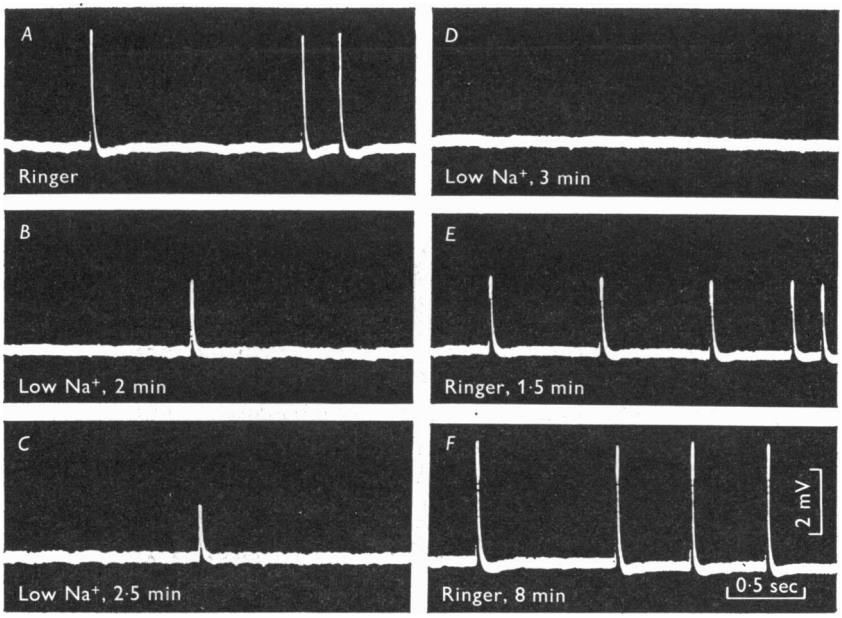

Fig. 17. Effect of low-Na^+ Ringer fluid on f.o.p.s. Temperature is 28° C throughout. *A*, preparation bathed in normal Ringer fluid. *B*, after 2 min in Ringer fluid in which 91 % of the Na^+ has been replaced with an equi-osmolar amount of sucrose; amplitude and frequency of f.o.p.s has declined. Initial resting potential of fibre −69 mV. During first half minute in low-Na^+ Ringer there was a gain of about 4 mV in resting potential. *C*, after 2½ min in low-Na^+ Ringer fluid. *D*, after 3 min in low-Na^+ Ringer f.o.p.s are completely abolished. *E*, 1½ min after return to normal Ringer fluid; f.o.p.s have reappeared although they remain somewhat reduced in amplitude. *F*, 8 min after return to normal Ringer f.o.p. frequency and amplitude are similar to control values (*A*).

DISCUSSION

Depolarizing events of two types are associated with the initiation of action potentials under the conditions of organ culture: discrete depolarizations of up to 15 mV in amplitude (fibrillatory origin potentials) found in irregularly discharging fibres, and oscillations of the membrane potential in fibres with a regular or rhythmic pattern of discharge.

Fibrillatory origin potentials (*f.o.p.s*)

The discrete depolarizations found at sites of origin of action potentials in irregularly discharging fibres are of nearly constant amplitude outside of a refractory period, are reversibly abolished by tetrodotoxin or by removal of most of the Na^+ from the bathing fluid, and are sensitive to voltage: both amplitude and frequency are affected by small changes in membrane potential. These properties suggest that their mechanism is related to the regenerative Na^+ conductance change associated with the normal action potential. Unlike the action potential, however, f.o.p.s are not conducted

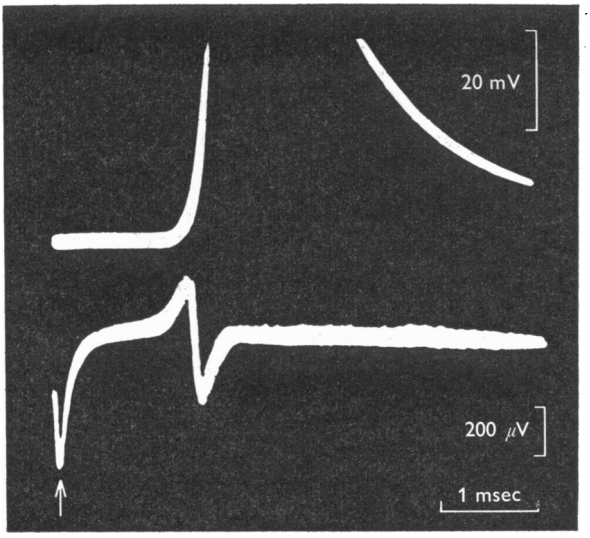

Fig. 18. Interaction between adjacent fibres. Simultaneous intracellular (upper trace) and extracellular (lower trace) records from an active fibre. About ten sweeps are superimposed, and are triggered by action currents from an adjacent fibre (arrow) recorded with the same extracellular electrode. Action currents in the adjacent fibre are unfailingly followed by an action potential in the impaled fibre. Resting potential −71 mV. Amplitude of action potential not fully shown.

but are restricted to highly localized regions probably less than 100 μm in length. This might be because the site of origin is somehow electrotonically separated by a high resistance connexion from the rest of the fibre, or because it is limited to a very small patch of abnormally excitable membrane. Two morphological features of denervated muscle could provide a basis for electrotonic separation. Within 15 days of denervation some fibres in rat diaphragm begin to 'fragment' (Miledi & Slater, 1969); as

pieces of the parent fibre split off they sometimes appear to remain attached to it by a fine strand. Action potentials occurring in the fragment might be sufficiently attenuated by a narrow, high resistance connexion to be recorded as subthreshold events in the parent fibre. Another possibility, suggested to us by A. F. Huxley, is that f.o.p.s may represent spontaneous regenerative activity within transverse tubules. The system of internal tubules (T-system) remains intact in denervated mammalian muscle fibres, although it undergoes some morphological changes (Pellegrino & Franzini, 1963; Miledi & Slater, 1969; Gauthier & Dunn, 1973), and recent work by Costantin (1970) has shown that, in frog at least, the membranes of the T-system are capable of a regenerative Na^+ conductance change. In this case the small area of active membrane provided by a tubule might not always be sufficient to elicit an action potential from the rest of the fibre membrane. It does not seem possible from our experiments to decide between an abnormal patch of surface membrane or a more remote source of f.o.p.s such as membrane fragments or T-tubules. The reason for the prevalence of sites of origin at the region of the former end-plate (and to a lesser extent at the costal insertion) is also uncertain. We only note the specialized nature of these areas compared to the rest of the fibre, for example the presence of cholinesterase and the increased infolding of the surface membrane (Gerebtzoff, 1954; Schwarzacher, 1960).

Oscillations of membrane potential

Oscillations of membrane potential found at sites of origin of action potentials in regularly discharging fibres have several properties in common with f.o.p.s: they are reversibly abolished by low Na^+ or tetrodotoxin, are sensitive to small changes in membrane potential, are localized, and have roughly the same preference as f.o.p.s for the former end-plate region and the costal insertion (though they can both occur at any point along the fibre). Unlike f.o.p.s, however, the area giving rise to the depolarization is less sharply defined. Thus we often found that the site of origin changed location in regularly discharging fibres; this could occur after the fibre was impaled near an origin, or during bursts of activity (Fig. 3).

Subthreshold oscillations similar to those reported here have been found in other excitable cells, notably molluscan neurons (Arvanataki, 1943; Tauc, 1966), denervated mammalian muscle *in vivo* (Li, Shy & Wells, 1957), and avian muscle maintained in organ culture (Li, Engel & Klatzo, 1959). Indeed, oscillation of the membrane potential is predicted from the Hodgkin–Huxley equations under conditions of low Ca^{2+} or depolarization (Huxley, 1959). The simplest view is that the oscillatory potential changes in regularly discharging fibres occur across the surface

membrane and are facilitated by the depolarization of fibres which follows denervation (Albuquerque & Thesleff, 1968).

Spontaneous activity of denervated muscle in vivo

Several characteristics of fibrillation *in vivo* have been established and can be compared with our results in organ culture. Both regularly and irregularly discharging fibres have been described (Belmar & Eyzaguirre, 1966; Thesleff, 1963), as well as subthreshold oscillations of membrane potential (Li *et al.* 1957), and discrete subthreshold depolarizations (Thesleff, 1963). Belmar & Eyzaguirre (1966) have shown that most spontaneous action potentials in denervated rat anterior gracilis muscle originate in the region of the former end-plate, and that the rate of discharge increases with depolarization and decreases with hyperpolarization. Qualitatively at least, spontaneous activity of mammalian muscle maintained in organ culture is similar to fibrillation *in vivo*.

Spontaneous activity and extrajunctional acetylcholine-sensitivity

The Na^+ conductance change underlying the initiating events of spontaneous activity (either f.o.p.s or oscillations of the membrane potential) is distinct from the conductance change following the interaction of acetylcholine with its receptor. Thus the conductance change which gives rise to fibrillation is blocked by tetrodotoxin, is insensitive to doses of curare sufficient to block ACh receptors (see also Rosenblueth & Luco, 1937; Belmar & Eyzaquirre, 1966), and is affected by small changes in membrane potential. It seems unlikely therefore that fibrillation is directly related to the phenomenon of denervation supersensitivity. Consistent with this view is the finding that periods of spontaneous activity in single fibres lasting somewhat less than a day, on average, are self-inhibiting, but have only a small effect on extrajunctional ACh-sensitivity (Purves & Sakmann, 1974). Taken together the results of these experiments indicate that, in addition to its influence on extrajunctional ACh sensitivity (Jones & Vrbová, 1970, 1971; Lømo & Rosenthal, 1972; Drachman & Witzke, 1972; Cohen & Fischbach, 1973; Purves & Sakmann, 1974), activity affects another type of Na^+ conductance mechanism which is responsible for the spontaneous activity of denervated muscle fibres. The way in which activity alters these membrane properties is not known.

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