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RELEASE OF TRANSMITTER FROM DEGENERATING LOCUST MOTORNEURONES

By P. N. R. USHERWOOD
From the Department of Zoology, The University, Glasgow, G12 8QQ

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INTRODUCTION

When locust motorneurones are severed their distal axon processes degenerate; but before degeneration is complete a number of interesting structural and physiological changes take place in the axon terminals at their synaptic contacts with skeletal muscle fibres (Usherwood, 1963*b*; Usherwood, Cochrane & Rees, 1968; Rees & Usherwood, 1970; Rees & Usherwood, 1972; Usherwood & Rees, 1972). First, impulse-linked release of transmitter from the degenerating axon terminals fails; then the pattern of spontaneous release of transmitter, i.e. the miniature discharge, becomes very non-random with the appearance of high-frequency bursts of miniature excitatory post-synaptic potentials (min. EPSPs), leading eventually to the production of 'giant' min. EPSPs up to 15 mV in amplitude. The appearance of 'giant' potentials signals an overall fall in miniature frequency followed eventually by complete cessation of the miniature discharge. The failure of impulse-linked release of transmitter and the alterations in the miniature discharge occur at a time when the spatial arrangement of synaptic vesicles within the degenerating nerve terminals is rapidly changing, with the vesicles (which are normally distributed in an approximately random fashion) aggregating to form variably sized clumps.

The main reason for studying degenerating insect nerve–muscle systems was to obtain further insight into the supposed relationship between synaptic vesicles and storage and release of transmitter. The idea that studies of degenerating nerve terminals might provide further clues concerning the function of synaptic vesicles is not new, of course (see, for example, Birks, Katz & Miledi, 1960), but to date the progressive alterations in function and fine structure which occur in vertebrate axon terminals after nerve section have not thrown much light on the parts played by vesicles in synaptic transmission. This publication is mainly concerned with the temporal distribution of min. EPSPs, which make up the miniature discharge at the nerve–muscle synapses on the retractor unguis muscle of the locust, at different times after transection of the motorneurones which innervate this muscle. The pattern of spontaneous transmitter release from normal retractor unguis motorneurones has been described recently by Usherwood (1972). The results of a parallel study of the changes in spatial organization of vesicles in normal and degenerating terminals of these motorneurones have been recently published (Rees & Usherwood, 1972; Usherwood & Rees, 1972).

METHODS

Over 200 metathoracic retractor unguis nerve–muscle preparations were examined during the period April 1968–June 1972. The two axons to the retractor unguis muscle of the left metathoracic leg were transected by severing left nerve 5 in the metathorax. Full details of this operation are given in an earlier publication (Usherwood, 1963*a*). The right retractor unguis muscle and its associated motorneurones were used as a control in each insect studied. Operated locusts were maintained at 30 °C. Twelve hours to 40 days after nerve section experimental and control nerve–muscle preparations were isolated from the insect, set up side-by-side in a small perfusion chamber (Usherwood & Machili, 1968) and perfused with locust saline (Usherwood & Grundfest, 1965). The nerve–muscle preparations were maintained at 20 °C. Recordings of synaptic events and muscle resting potentials were made with intracellular electrodes filled with 3 M-KCl, while electrodes filled with 2 M-NaCl were used to record extracellularly from retractor unguis nerve–muscle synapses. Active spots for extracellular DC recordings of synaptic events were located initially by stimulating the distal stump of the nerve containing the retractor unguis axons with a suction electrode (Usherwood, 1972), but after impulse-linked release of transmitter had failed, following degeneration of the axon terminals, a glutamate electrode was used to locate the synaptic sites (Usherwood, 1969). Methods for recording and analysing miniature discharges were identical to those described by Usherwood (1972), except that additional statistical techniques were used to investigate intervals between min. EPSPs. These techniques will be described in detail where appropriate.

RESULTS

Qualitative studies of transmitter release following nerve transection

The sequence of changes in the physiological properties of the nerve–muscle synapses on the white fibres (Usherwood, 1967) of the retractor unguis muscle, following transection of the retractor unguis axons, was basically similar to that described earlier for the locust extensor tibiae nerve–muscle system (Usherwood, 1963*a*, *b*).

In view of the length and multiterminal innervation of the retractor unguis muscle it is not surprising that failure of transmitter release did not occur simultaneously at all the synaptic sites on a muscle fibre. The nerve terminals at the distal end of a fibre were affected first, followed by terminals halfway along the length of the fibre. Changes at the proximal terminals were delayed by about 24 h compared with changes at distal terminals. One consequence of this progressive, apparently disto-proximal spread of synaptic transmission failure was that an intracellular electrode, on occasions, sampled activity at both relatively normal synapses and markedly degenerate synapses (Fig. 1). It also meant that the entire sequence of changes in impulse-linked release and spontaneous release of transmitter following nerve section could, on occasions, be observed in a single nerve–muscle preparation. Changes at synapses on the red muscle fibres were delayed by about 24 h compared with similarly positioned synapses on the white fibres. Regional variations in the time course of degeneration are not surprising, in view of the topography of the retractor unguis innervation and of the differences in diameter of the axons innervating the retractor unguis muscle. These physiological

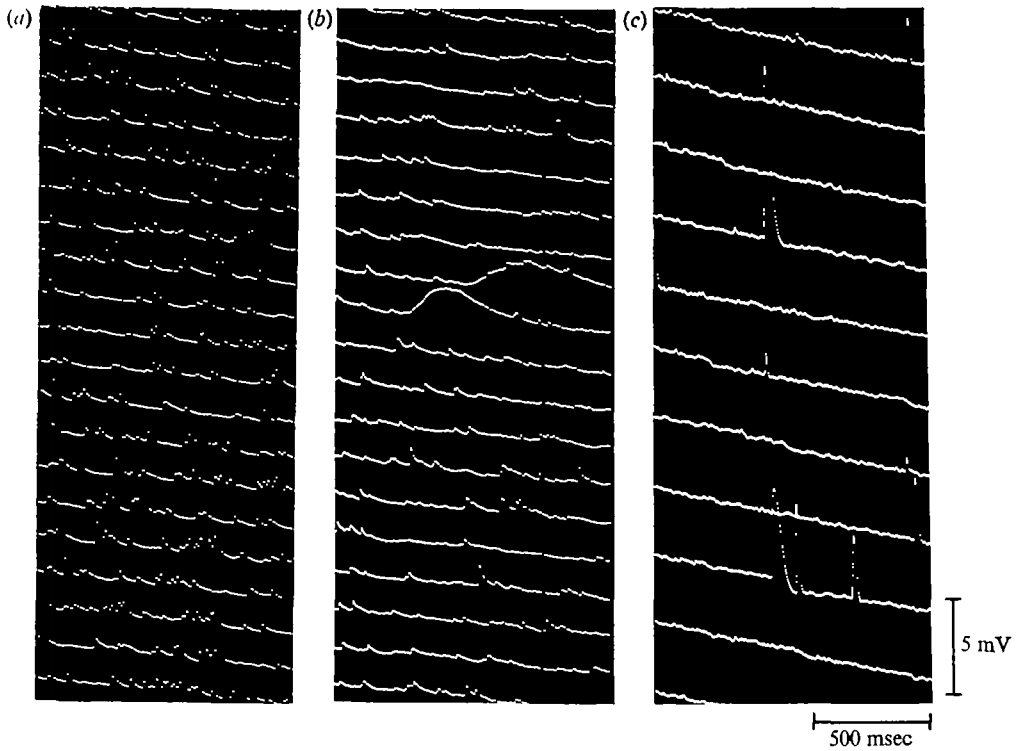

Fig. 1. Continuous intracellular recordings from three retractor unguis white muscle fibres of three different preparations. (*a*) 12 h, (*b*) 48 h, (*c*) 68 h after section of retractor unguis axons showing spontaneous miniature discharges at these times. The miniature discharge in (*a*) is relatively normal, being quite characteristic of a multiterminally innervated fibre with a length/length constant ratio > unity. The resting potential of this fibre was 59 mV. The recording electrode in (*b*) was positioned in the centre of a retractor unguis muscle fibre (resting potential = 58 mV). Synapses at distal terminals on this fibre were producing 'giant' min. EPSPs which were 'seen' by the recording electrode as large excursions with slow rise and decay times. The synapses close to the recording electrode were still able to generate EPSPs and the miniature discharges that they engendered were seemingly quite normal. In (*c*) the recording electrode was placed in a mid-fibre region and recorded 'giant' potentials which were presumably generated at different distances from the recording site. The resting potential of this fibre was 60 mV. Time and voltage calibrations same for (*a*)–(*c*).

findings are fully supported by earlier observations on the ultrastructural changes at retractor unguis nerve–muscle synapses following nerve section and do not conflict with accepted ideas concerning proximo-distal spread of axon degeneration following nerve transection (Rees & Usherwood, 1972; Usherwood & Rees, 1972).

'Giant' min. EPSPs invariably occurred after synaptic transmission had failed. Concomitant extracellular and intracellular recordings from degenerating nerve–muscle preparations clearly demonstrated that the 'giant' spontaneous potentials resulted from summation of normal-sized min. EPSPs (Fig. 2). In fact, it was possible to follow the development of these 'giant' potentials by taking a 42–72 h preparation and recording from a single active spot over a period of 6–12 h. This rather tedious procedure was followed on ten occasions with ten different preparations. After impulse transmission had failed, both the intracellular and extracellular miniature discharges

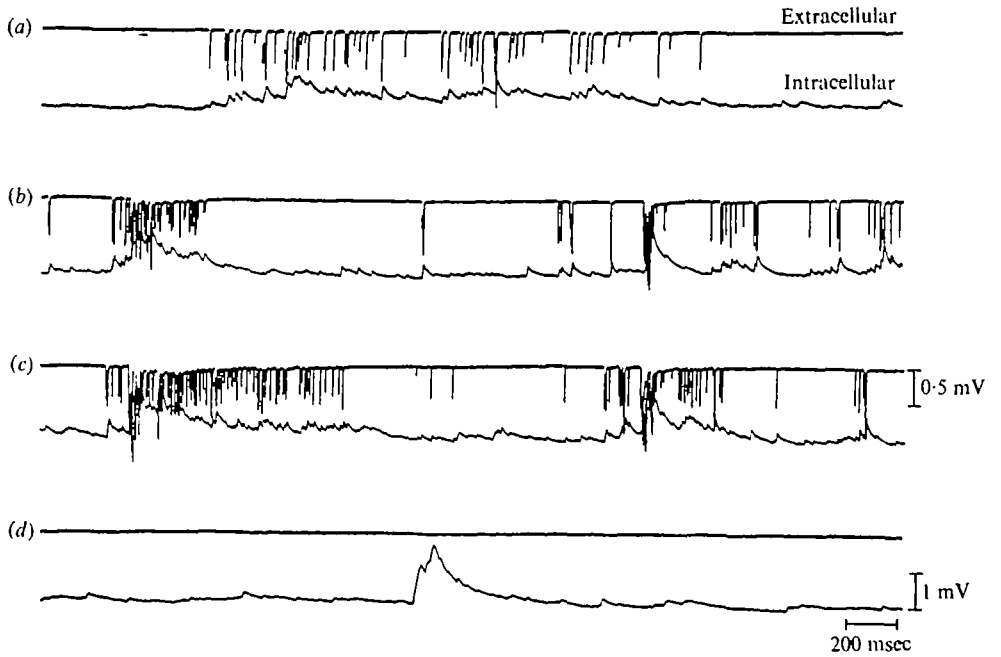

Fig. 2. Concomitant extracellular and intracellular data from the distal region of a retractor unguis white muscle fibre (*a*) 57 h, (*b*) 59 h, (*c*) 63 h and (*d*) 68 h after section of retractor unguis axons, illustrating the progressive development of 'giant' potentials. The extracellular bursts of min. EPSPs in (*a*)–(*c*) were preceded and followed by long periods (1–20 min) of almost complete inactivity. In (*d*) an irregularly shaped intracellular 'giant' potential, formed by incomplete summation of a burst of min. EPSPs, is not accompanied by extracellular min. EPSPs. Presumably the 'giant' potential in this instance occurred at a synapse distant from the extracellular recording electrode. The synapses on the terminal under the extracellular electrode had ceased to spontaneously release transmitter by this time. Resting potential of muscle fibre was initially 56 mV but rose during the course of the experiment to 60 mV. Voltage and time calibrations same for (*a*)–(*d*). Muscle was placed in perfusion bath 55 h after nerve section and maintained thereafter at 20 °C.

became characterized by bursts of min. EPSPs. Initially, the bursts contained only a few potentials (5–10) but later bursts of 100+ min. EPSPs occurred. The frequency of occurrence of these bursts then gradually decreased as the burst length increased. With further degeneration of the terminal under the recording electrode, the frequency of the min. EPSPs in each burst progressively increased and the burst length began to decrease. At the same time the frequency of min. EPSPs in the interburst periods declined. As the frequency of the min. EPSPs in a burst increased, the intracellular potentials summated (Figs. 2, 3) so that eventually single large potentials were obtained. The extracellular miniatures at this time were still obviously unitary events, showing only slight summation. However, following further increases in burst frequency the extracellular min. EPSPs in the bursts also began to summate, with the eventual appearance of 'giant' extracellular miniatures (Fig. 4). It was noticeable that both extracellular and intracellular min. EPSP bursts often contained a high proportion of min. EPSPs of almost identical amplitudes, suggesting that these potentials resulted from the action of transmitter released spontaneously from a single synaptic site on a

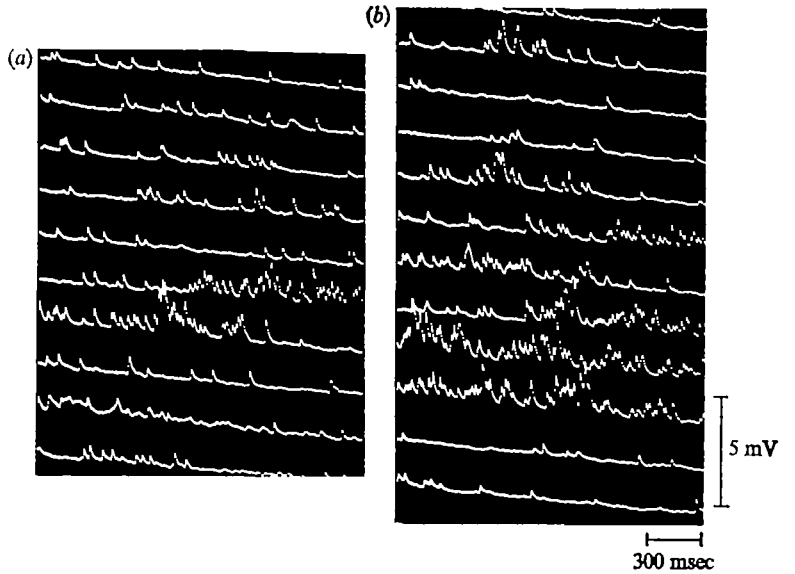

Fig. 3. Continuous intracellular recordings from the distal regions of two white retractor unguis muscle fibres 45 h after section of the retractor unguis axons. The miniature discharges recorded from these fibres were characterized by long bursts of min. EPSPs. Voltage and time calibrations same for (a)–(b). Resting potential (a) 57 mV, (b) 59 mV.

nerve terminal (Usherwood, 1972). Some of the 'giant' potentials were accompanied by electrically excited responses.

The extracellular min. EPSP bursts or 'giants' were usually accompanied by an intracellular discharge, whereas intracellular min. EPSP bursts or 'giants' frequently occurred without concomitant activity at an extracellular recording site (Figs. 2*d*, 4*d*). Furthermore, 'giant' potentials recorded at an active spot on one fibre were not recorded by an intracellular electrode in another fibre or by an extracellular electrode at a second, but distant active spot on either that fibre or another fibre. These observations clearly reflect the multiterminal nature of the innervation of the retractor unguis muscle fibres and provide support for the contention that the 'giant' potentials have a synaptic origin. The local nature of the min. EPSP bursts and 'giants' make it unlikely that these phenomena arise from electrical disturbances within the preterminal regions of the degenerating axons, especially since the axonal membrane is still capable of conducting action potentials at this time, unless, of course, such disturbances are strictly small local events which could not be seen with the extracellular electrodes. Since 'giant' potentials and min. EPSP bursts were recorded both extracellularly and intracellularly, and since these phenomena could be seen at single recording sites for periods of 6–12 h, it is also highly unlikely that they arose from some mechanical disturbance of the degenerating nerve terminals. The possibility that the 'giant' potentials occurred following some interaction between degenerating axon terminals and saline was eliminated by demonstrating that these potentials could also be recorded from retractor unguis muscle fibres *in situ*, with the fibres bathed in haemolymph.

Addition of 25 mM/l Mg^{2+} to saline bathing a degenerating preparation exhibiting either min. EPSP bursts or 'giant' potentials, markedly reduced the frequency of

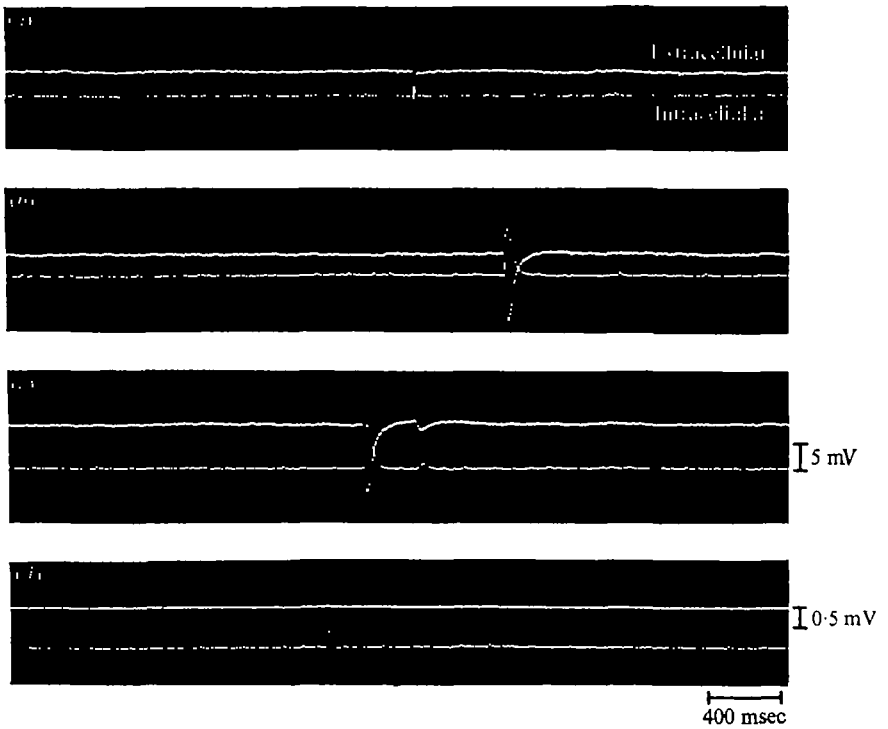

Fig. 4. Intracellular and extracellular spontaneous potentials recorded from mid-regions of four retractor unguis muscle white fibres, one from each of four muscle preparations; (*a*) 72 h, (*b*) 75 h, (*c*) 75 h, (*d*) 77 h after section of retractor unguis axons. Active spots on these fibres were located by a glutamate electrode. The extracellular potentials in (*b*) and (*c*) were very large, prolonged and slightly diphasic, possibly because of involvement of electrically excitable muscle membrane. In view of this it is difficult to be certain that they originated at a synapse on the terminal under the extracellular electrode or indeed that they had a synaptic origin. Resting potentials: (*a*) 56 mV, (*b*) 58 mV, (*c*) 57 mV, (*d*) 56 mV. Voltage and time calibrations same for (*a*)–(*d*).

occurrence of both bursts and 'giants' and also reduced the amplitude of the 'giants', but did not completely abolish the miniature discharge. However, the 'giants' were completely abolished with 40 mM/l Mg^{2+}. Indeed the impression gained from studies with Mg saline was that this saline converted the abnormal miniature discharge, which characterizes the degenerating synapses, to a pattern more consistent with that seen at normal synapses, albeit at a lower overall discharge frequency (Usherwood, 1963*c*, 1972).

With continued degeneration of the nerve terminals the overall miniature frequency and the frequency of the 'giant' potentials gradually declined to zero. The complete cessation of the miniature discharge was correlated with either complete loss of synaptic vesicles in the degenerating terminals or engulfment of the terminals by glial cells (Rees & Usherwood, 1972).

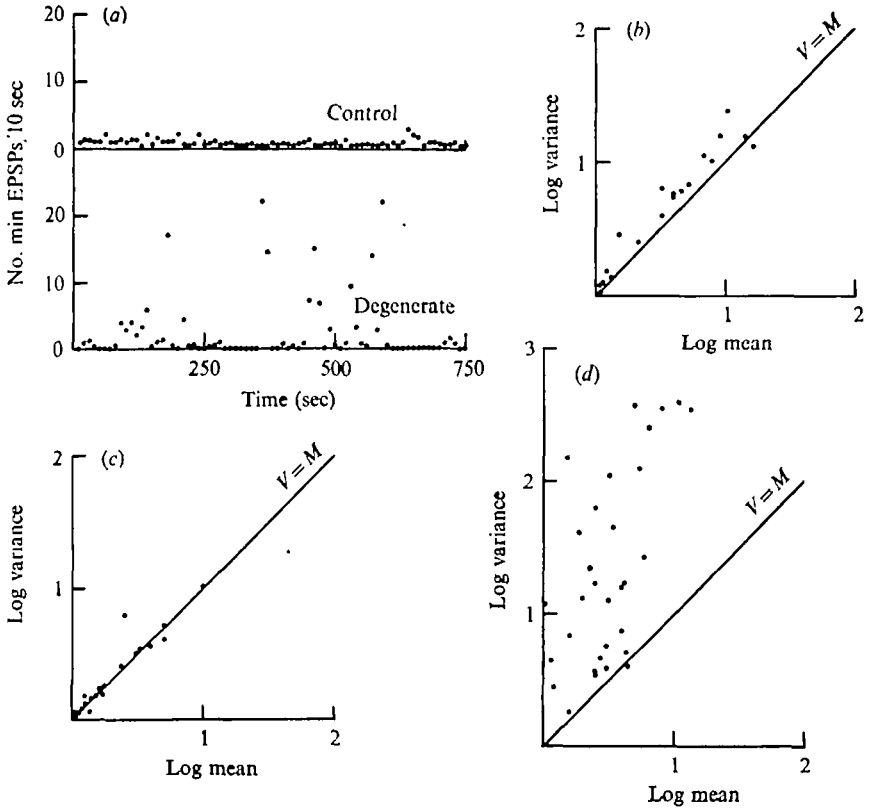

Fig. 5. Frequency analyses of miniature discharges recorded extracellularly from synapses at axon terminals on normal retractor unguis white muscle fibres and on fibres at different times after section of retractor unguis axons. (*a*) Plot of min. EPSP frequency per 10 sec period against time for a control nerve terminal and for a distal terminal 51 h after nerve section. (*b*), (*c*) Log variance/log mean plot of the number of min. EPSPs occurring per unit time for (*b*) control terminals, (*c*) distal degenerating terminals 24 h after nerve section, (*d*) distal degenerating terminals 48 h after nerve section. Note that data in (*c*) indicate closer agreement with 45° line, indicating pattern of miniature discharge is more random at this time than for control terminals in (*b*). The miniature discharges used to construct the plot illustrated in (*d*) were mostly characterized by bursts of min. EPSPs and, as expected, are markedly non-random. Each point on graphs (*b*)–(*d*) represents data obtained from a single extracellular recording site on one retractor unguis preparation. Graphs constructed using data from 70 preparations.

Quantitative studies of transmitter release following nerve transection

Miniature frequency

When the miniature discharges, recorded extracellularly from normal terminals and from degenerating terminals at which impulse transmission had failed, are compared by plotting the number of miniatures per unit time against time, the differences between the two discharge patterns are quite striking (Fig. 5*a*). At normal terminals few deviations from the mean miniature frequency are evident, although the pattern is not exactly random (Usherwood, 1972); at degenerating terminals the miniature discharge fluctuates considerably. In a previous publication Usherwood (1972) demonstrated that for extracellular recordings of miniature discharges at normal locust nerve terminals, the variance (V) of the number of min. EPSPs occurring in a set time

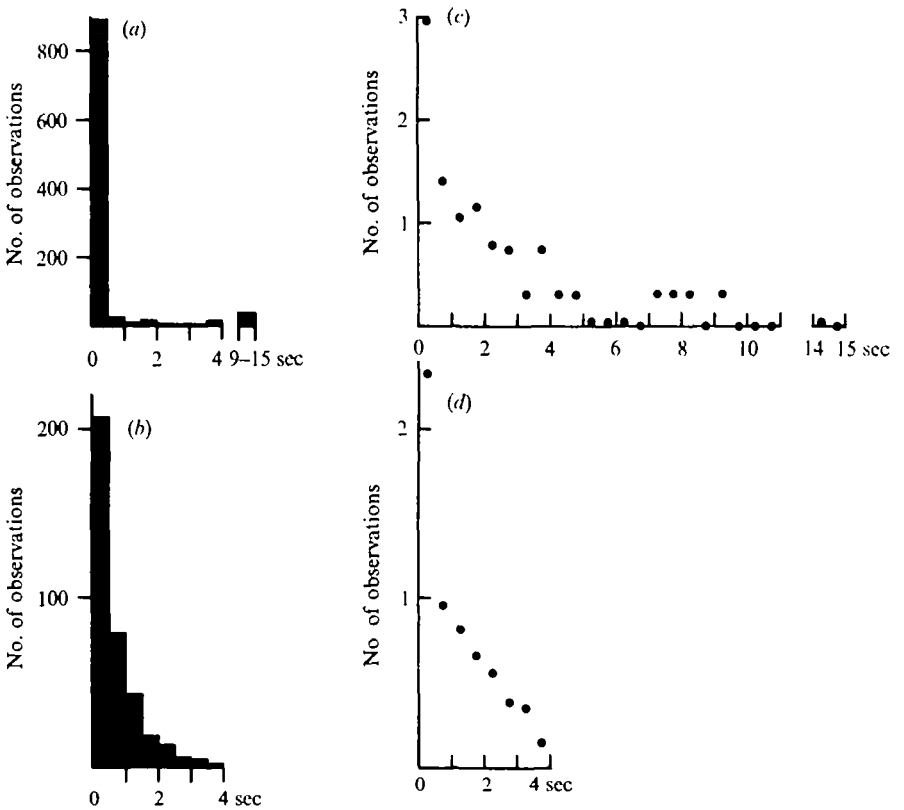

Fig. 6. Frequency distribution of intervals between extracellular min. EPSPs for (*a*) a distal degenerating terminal on a red muscle fibre 65 h after section of retractor unguis axons and (*b*) a control terminal on a red muscle fibre. The non-exponential distribution of amplitudes for the degenerating terminal is clearly apparent. (*c*), (*d*) Semilogarithmic plots of interval histograms of (*c*) a degenerating terminal on the mid-region of a white fibre 69 h after neurotomy, and (*d*) a control terminal on a white fibre. The control plot in (*d*) is approximately linear for all intervals except the shortest indicating that the miniature discharge approximates a stochastic process. The plot in (*c*) is markedly non-linear reflecting the non-random nature of the miniature discharge.

interval (t) was usually greater than the mean (M) number of min. EPSPs occurring in t (see also Fig. 5*b*). Twenty-four hours after section of the retractor unguis axons the ratio V/M approaches unity much more closely, which indicates that the miniature discharge at this time is almost random (Fig. 5*c*). It is perhaps significant that the synaptic vesicles in nerve terminals 24 h after nerve transection are more randomly distributed than they are in normal terminals (Usherwood & Rees, 1972). With the appearance of min. EPSP bursts the miniature discharge becomes very non-random and V/M ratios obtained at this time are therefore much greater than unity (Fig. 5*d*).

Histograms of the intervals between successive extracellular min. EPSPs, for discharges recorded from normal terminals (Fig. 6*b*) and from degenerating terminals (Fig. 6*a*) at different times after nerve section, were constructed and demonstrate a clear-cut difference between these discharges. The histograms for the normal terminals appear unimodal and asymmetric, as would be expected for a quasi-random discharge. When the data in these histograms were plotted semilogarithmically most of the points

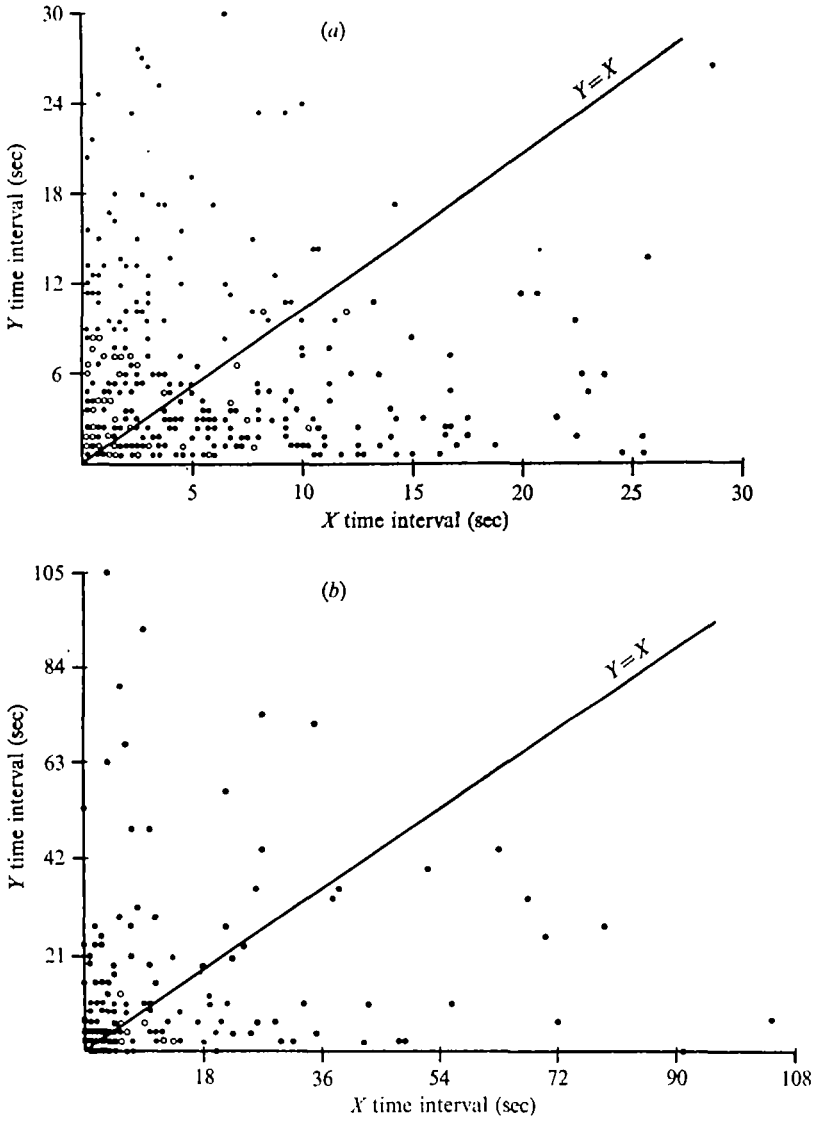

Fig. 7. Joint-interval histograms of extracellular min. EPSPs of (*a*) a control nerve terminal on a retractor unguis white muscle fibre, and (*b*) a degenerating terminal located proximally on a white fibre. Recording made 79 h after nerve section. The miniature discharge recorded at the degenerating terminal was characterized by min. EPSP bursts. See text for further explanation.

for the normal terminals, except those for the smallest intervals, fell approximately on a straight line (Fig. 6*d*). This indicates once again that the normal discharge approaches a stochastic process. Multimodal histograms were obtained for degenerating terminals which were characterized by 'bursty' discharges (Fig. 6*a*), clearly reflecting the non-random nature of the discharges recorded from these structures. Semilogarithmic plots of these histograms highlight the non-stochastic nature of the miniature discharge at this time (i.e. about 56 h after nerve section) (Fig. 6*c*).

While the interval histogram gives the range and distribution of time intervals it

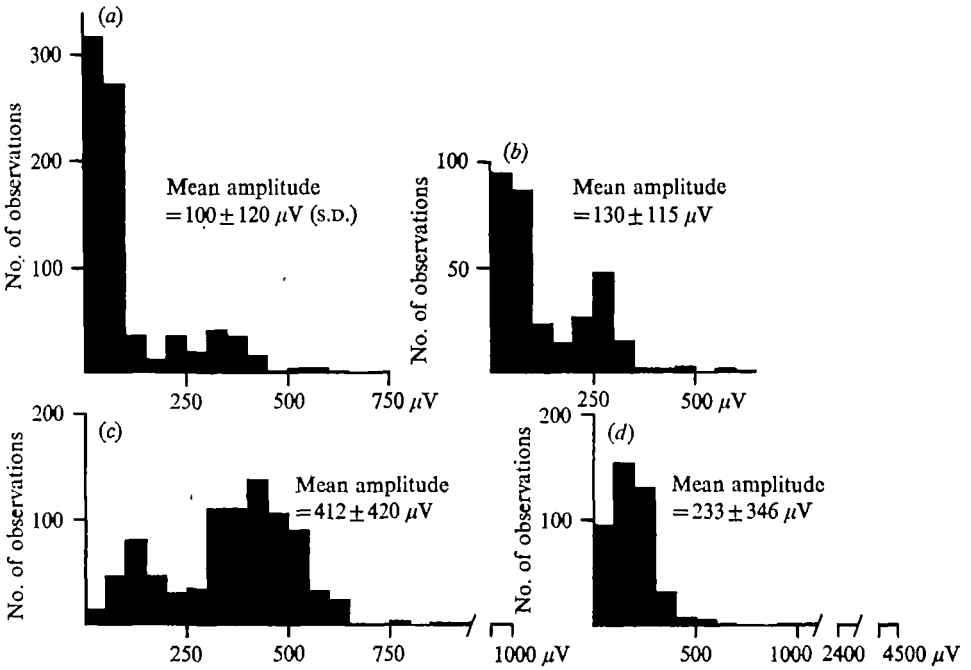

Fig. 8. Amplitude histograms for extracellular min. EPSPs (*a*)–(*c*) and selected ('marked') intracellular min. EPSPs (*d*). (*a*), (*b*) from a distal recording site 48 h after nerve section; (*d*) distal intracellular recording 53 h after nerve section. The histogram in (*c*) was obtained from a distal nerve terminal on a white fibre 60 h after nerve section. The min. EPSPs recorded at this site presumably originated from two release sites on the nerve terminal, close to the extracellular recording electrode. Resting potential of fibre in (*d*) was 59 mV.

does not allow for detection of any ordered pattern of intervals (Rodieck, Kiang & Gerstein, 1962). To do this, joint-interval histograms, i.e. histograms of the joint distribution of two successive intervals between min. EPSPs, were constructed and displayed as two-dimensional plots (Fig. 7), with the abscissae representing the duration of the first pair of intervals and the ordinates representing the duration of the second pair of intervals. Joint-interval histograms for normal terminals approach the 45° symmetry test (Fig. 7*a*), i.e. successive intervals are independent, except that there is usually a tendency for a short interval to be preceded by a short interval. There is also a tendency for a long interval to precede a short interval and vice versa. A dependency between short intervals is, of course, an obvious characteristic of the 'bursty' miniature discharge recorded extracellularly from degenerating terminals (Fig. 7*b*). Assessment of the above data must take into account the fact that an extracellular electrode located on a single active spot usually samples activity at more than one synapse or site of transmitter release on a nerve terminal (Rees & Usherwood, 1972; Usherwood, 1972). As a result, any departures from randomness in the miniature discharge recorded from a nerve terminal by an extracellular electrode may reflect much larger deviations from randomness at a single release site on that terminal.

Miniature amplitudes

Histograms of amplitude distributions of miniature potentials recorded extracellularly from retractor unguis nerve terminals 1–3 days after nerve section are

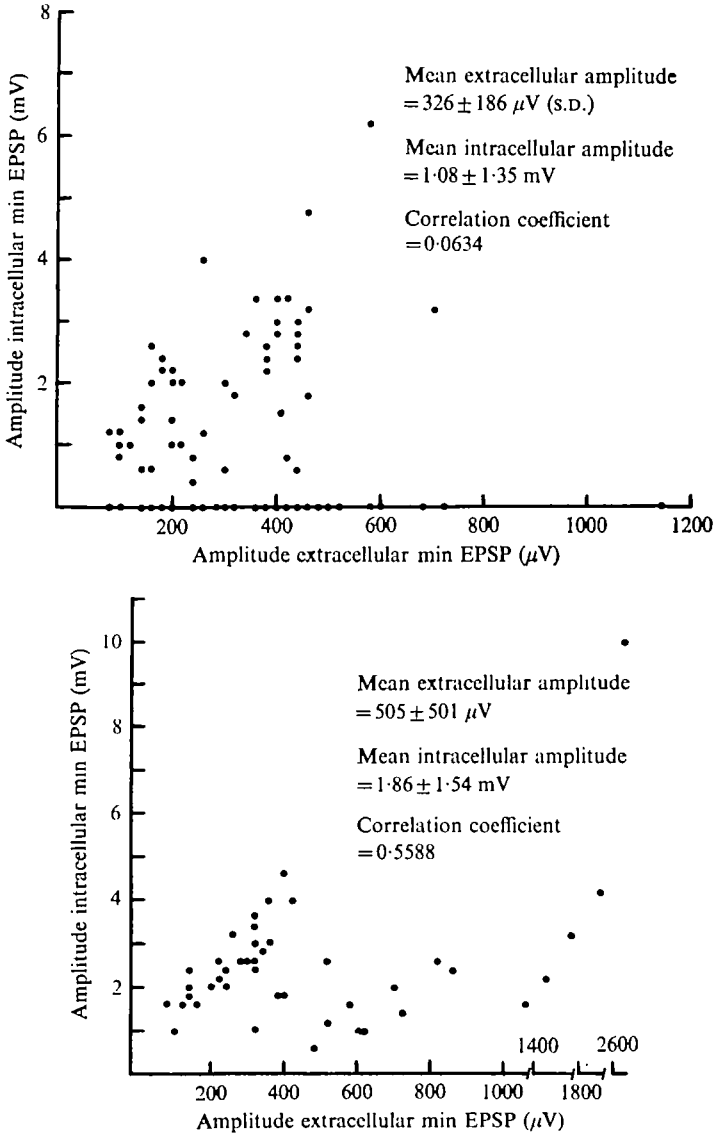

Fig. 9. Scattergrams of extracellular and corresponding ('marked') intracellular min. EPSP amplitudes from recording sites at which the miniature discharges were characterized by some 'giant' potentials. Many of the extracellular potentials in (*a*) were not accompanied by intracellular responses probably because the extracellular electrode sampled from synapses on more than one muscle fibre. As a result the correlation coefficient for these data is not particularly meaningful.

illustrated in Fig. 8. In view of the variable amplitude distributions for extracellular min. EPSPs for normal terminals (Usherwood, 1972) these data are not particularly instructive (Fig. 8*a–c*). The multimodal nature of the amplitude distributions is clearly evident and probably reflects the complex arrangement of the synaptic contacts on the retractor unguis muscle fibres. By inserting an intracellular electrode into a retractor unguis muscle fibre close to an extracellular electrode located on an active spot on that fibre, and selecting only those intracellular min. EPSPs which correspond

to extracellular miniatures, it is possible to obtain intracellular records of the miniature discharge at synapses on a single nerve terminal (Bittner & Harrison, 1970). Usherwood (1972) used this technique for investigating the spontaneous release of transmitter at normal retractor unguis nerve-muscle synapses and found that the distribution of the selected or 'marked' intracellular min. EPSP amplitudes was approximately Gaussian. However, the occurrence of 'giant' min. EPSPs following nerve section results in skew amplitude histograms (Fig. 8*d*), the smallest class in many cases being the greatest.

Scattergrams of extracellular and corresponding 'marked' intracellular min. EPSPs for discharges recorded from degenerating preparations showing 'giant' miniature activity were sometimes like those for normal terminals (Usherwood, 1972) in that they showed no correlation. However, unlike the scattergrams for normal terminals the points on scattergrams from many degenerating terminals were aggregated into discrete populations and in these instances the amplitudes of the extracellular and intracellular potentials were correlated (Fig. 9). In view of the multi-synaptic nature of the locust retractor unguis axon terminal it seems reasonable to assume that each population represents responses at one synaptic site. If this is true it is clear that there is a good correlation between extracellular and intracellular events at each site. Possibly the multisynaptic nature of the miniature discharge is more evident at degenerating terminals because the extracellular current fields for the synapses on a terminal do not overlap so much as at normal terminals. This may be due to the fact that many of the synaptic sites on the degenerating terminal are no longer releasing transmitter spontaneously.

DISCUSSION

Following section of the nerve supply to locust muscle there is a period when nerve-muscle transmission is maintained, the duration of this period depending upon the length of the peripheral nerve stump, the diameter of the motor axons in this stump and the temperature at which the operated animals are maintained (Usherwood, 1963*b*; Usherwood *et al.* 1968). The degenerating locust nerve–muscle system is therefore somewhat similar to vertebrate nerve–muscle systems following motor nerve section (e.g. Birks *et al.* 1960; Miledi & Slater, 1968; Slater, 1966). However, in the frog (Birks *et al.* 1960) and in the rat (Miledi & Slater, 1970) impulse-linked release and spontaneous release of transmitter from motor endplates usually fail simultaneously following neurotomy, whereas the miniature discharge at degenerating locust nerve-muscle synapses outlasts impulse transmission by many hours (Usherwood, 1963*b*). The reason for this difference is best understood by comparing the structural changes at degenerating locust and vertebrate nerve–muscle synapses, especially with regard to the role of the glial cells which normally accompany the motorneurones at these sites. At vertebrate motor end-plates the glial or Schwann cells frequently engulf the degenerating nerve terminals before the terminal mitochondria and synaptic vesicles are profoundly affected by axonal degeneration. This could account for the dramatic and simultaneous failure of impulse-linked release and spontaneous release of transmitter at these sites. At the degenerating nerve terminal on locust retractor unguis muscle, however, the presynaptic organelles within the axon terminal undergo marked structural and spatial changes before the terminal is phagocytosed by glial tissue. In

this case impulse-linked release of transmitter presumably fails because of the changes in arrangement of the synaptic vesicles, changes which would (according to the vesicle hypothesis, Katz, 1962) alter, but not abolish, the miniature discharge. The alterations in spatial arrangement of vesicles in degenerating locust nerve terminals occur gradually and one might reasonably anticipate, therefore, that failure of impulse-linked release of transmitter would also be a gradual process. This is indeed the case.

It is, of course, very tempting to assume that there is a causal relationship between synaptic vesicles and storage and release of transmitter. Indeed, the fact that deviations from randomness in the distribution of vesicles at normal synapses (Usherwood & Rees, 1972) are accompanied by more or less equivalent deviations from randomness in the miniature discharge at these synapses (Usherwood, 1972) lends some support to this idea. It may also be significant that the miniature discharge recorded from degenerating locust nerve–muscle synapses about 24 h after nerve section becomes almost random, at a time when the vesicle population is more randomly distributed than normal. Since the synaptic vesicles eventually aggregate following nerve transection and since this change from a quasi-random distribution to a non-random distribution of vesicles is clearly accompanied by an approximately equivalent deviation of the miniature discharge from a Poisson process, it could be argued that vesicle arrangement and miniature discharge pattern are causally related phenomena. According to Vere-Jones (1966) irregularities in the stream of vesicles moving into the region of release on the presynaptic membrane will cause irregularities in the output which will tend to reflect those of the input. Vesicle aggregation might be expected, therefore, to be accompanied by bursts of min. EPSPs and possibly even 'giant' potentials. Vesicle aggregation might also be expected to result, eventually, in failure of impulse-linked release of transmitter, since the availability of vesicles to occupy the additional release sites induced by an invading nerve impulse would be seriously reduced. If this is the case then during the initial phase of vesicle aggregation, which occurs in locust motor nerve terminals following nerve section at a time when the EPSP amplitude is slowly declining, the distribution of EPSP amplitudes might be expected to deviate much more than is normally the case (Usherwood, 1972) from a Poisson distribution. It would certainly be worth while, if somewhat difficult, to examine this possibility.

'Giant' min. EPSPs which form part of the miniature discharge recorded at some normal mammalian end-plates (e.g. Liley, 1956) are either double the size of the normal min. EPSPs or multiples thereof, and possibly represent responses to more than one quantum of transmitter released at a time (Liley, 1956). Martin & Pilar (1964) suggested that these 'giants' may be an indication that the quanta are not truly independent in their release, there being a tendency for events to snowball. Hubbard (1970) suggested that the 'giants' recorded from some normal mammalian end-plates result from the release of large quanta, possibly resulting from fusion of two or more vesicles, and cited evidence of Jones & Kwanbunbumpen (1968) for a relationship between vesicle volume and amplitude of quanta (i.e. min. EPSPs). Although there is some doubt about the origin of 'giant' min. EPSPs at vertebrate synapses, there seems little doubt that the 'giants' recorded from degenerating locust synapses result from the more or less simultaneous release of many packets of quanta with varying degrees of synchrony. The progressive aggregation of the synaptic vesicles which

takes place as these 'giant' min. EPSPs develop could account for the appearance of these potentials. It is possible, of course, that the bursts of miniatures and 'giants' seen at degenerating synapses on locust muscle fibres result from changes in the properties of the presynaptic membrane as it degenerates, rather than from changes in vesicle arrangement, although the membrane of the distal axon stump is still capable of conducting action potentials at a time when this abnormal miniature activity is occurring.

Perhaps some additional clues as to the origin of min. EPSP bursts and 'giant' potentials can be obtained from the results of recent studies by Katz & Miledi (1969) on the release of transmitter from frog nerve–muscle preparations after 4–7½ h in a solution containing 83 mM-$CaCl_2$. When such preparations were returned to normal frog Ringer the miniature discharge was characterized by a wide amplitude distribution and low discharge frequency. They concluded that the prolonged exposure to Ca^{2+} Ringer had somehow disorganized the motor nerve endings and produced an abnormal mode of spontaneous transmitter release. Rotshenker & Rahaminoff (1970) found that under normal conditions the appearance of min. EPSPs at the frog end-plate obeys a Poisson formulation, but that in Ringer containing 15 mM/l Ca^{2+} this no longer holds, the miniature potentials now occurring in bursts. Quantitative studies of this abnormal discharge showed that the spontaneous release of a quantum increased the probability of release of another quantum for several hundred msec. It is perhaps interesting to note that Banks (1966) found that adrenal medullar vesicles clump together in the presence of a high Ca^{2+} concentration, possibly due to neutralization of the net negative charges on these vesicles. Perhaps the free Ca^{2+} content of axon terminals increases as they degenerate, possibly because of failure of some Ca^{2+} sequestration process normally performed by presynaptic organelles. The ability of Mg^{2+} to abolish the 'giant' potentials recorded at degenerating locust nerve–muscle synapses could have some relevance to this, and it would certainly be of interest to determine the effect of this ion on the spatial arrangement of vesicles in degenerating locust nerve terminals. It is possible, of course, that Mg^{2+} abolishes the 'giant' min. EPSPs by virtue of its action on the presynaptic membrane (Usherwood, 1963*c*, 1972). Although studies of degenerating locust nerve–muscle synapses do not demonstrate unequivocally that synaptic vesicles store and release quanta of transmitter, the observations made on the spatial relationships of synaptic vesicles in locust nerve terminals and their behaviour during degeneration of the axon, together with the changes in spontaneous and impulse-linked release of transmitter, are all consistent with the concept that the vesicles represent the quantal unit of transmitter postulated by Fatt & Katz (1952).

SUMMARY

1. When the motorneurones to the femoral part of the retractor unguis muscle of the locust *Schistocerca gregaria* are severed, the changes in impulse-linked release and spontaneous release of transmitter which take place at the synapses between the motorneurones and the retractor unguis muscle fibres can be related to known changes in the numbers and distribution of synaptic vesicles at these synapses.

2. Impulse-linked transmitter release fails when the synaptic vesicles aggregate to form clumps of vesicles, and at this time the miniature discharge recorded from the

muscle fibres become characterized, initially, by bursts of miniature excitatory post-synaptic potentials (min. EPSPs) and, eventually, by 'giant' intracellular min. EPSPs up to 15 mV in amplitude.

3. The appearance of 'giant' potentials is accompanied by an overall fall in the frequency of the miniature discharge, which continues to decline thereafter until spontaneous transmitter release fails. The miniature discharge ceases at a time when the axon terminals are engulfed by glial tissue or become devoid of synaptic vesicles.

4. The parallel changes in temporal arrangement of min. EPSPs and spatial arrangement of synaptic vesicles seen during degeneration of locust nerve-muscle synapses provide further support for the 'vesicle hypothesis'.

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