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SPONTANEOUS RELEASE OF MULTIQUANTAL MINIATURE EXCITATORY JUNCTION POTENTIALS INDUCED BY A *DROSOPHILA* MUTANT

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SUMMARY

1. Intracellular recordings were made from muscle fibre No. 6 of the dorsal longitudinal flight muscle (DLM) of *Drosophila melanogaster* in both wild-type flies and the temperature-sensitive paralytic mutant, *shibire*$^{ts-1}$ (*shi*).

2. Continuous recordings of the miniature excitatory junction potentials (MEJPs) in this fibre were made as the temperature was changed from 19 to 29 °C, and back to 19 °C. In *shi* flies, synapses become depleted of vesicles at 29 °C due to a temperature-dependent blockage in the recycling process, while transmitter release proceeds normally. When the temperature is lowered to 19 °C, recycling is allowed to proceed and recovery of the full complement of synaptic vesicles gradually occurs in about 20 min.

3. It was observed that the MEJP amplitude distribution in *shi* flies was typically unimodal at 19 °C prior to heating (as was wild-type), but during recovery from 8 min exposure to 29 °C became multimodal, with peaks at roughly integral multiples of the original peak prior to heating. This effect was never seen in wild-type flies.

4. Also, during recovery, the MEJP did not occur randomly, but rather occurred in a clustered fashion.

5. It is concluded that during recovery from depletion in *shi* neuromuscular junctions, a condition exists which causes the synchronization of spontaneous release, causing multiquantal MEJPs or clustering of MEJPs, depending on the degree of synchronization.

6. The possible role of Ca^{2+} in this phenomenon is discussed.

INTRODUCTION

The amplitudes of spontaneous synaptic potentials under normal conditions were originally reported by Fatt & Katz (1952) to fit a normal distribution. Thus, the idea of the unitary potential, the quantum, came into being (del Castillo & Katz, 1954). However, there have been many reports since that time of non-Poisson distributions for spontaneous potentials under normal conditions (Martin & Pilar, 1964; Rotschenker & Rahamimoff, 1971; Dennis, Harris & Kuffler, 1971; Cohen, Kita & Van der Kloot, 1974; Brenner & Martin, 1976; Bevan, 1976; Kriebel, Llados &

Matteson, 1976; Bornstein, 1978; Kelly & Robbins, 1984). Often these suggest a multimodal distribution of amplitudes, with peaks which are roughly integral multiples of the smallest peak (Martin & Pilar, 1964; Dennis *et al.* 1971; Kriebel & Gross, 1974; Wernig & Stirner, 1977; Bornstein, 1978).

Often associated with the observance of multiple peaks in these miniature end-plate potential (MEPP) amplitude histograms is the observance of 'clustering'. In these cases, the spontaneous release process is not random and independent as originally suggested by Fatt & Katz (1952), but rather, the probability of occurrence of a unitary spontaneous potential is greatly enhanced immediately following any given spontaneous potential (Usherwood, 1972; Cohen *et al.* 1974; Rees, 1974; Bennett & Pettigrew, 1975; Kriebel & Stolper, 1975; Bornstein, 1978). Thus, the multiunit potentials, which are a result of synchronous or near-synchronous release of two or more unit-sized events, might be the result of the same phenomenon which causes the clustering, the only difference being how closely in time one MEPP follows the next.

In this paper it is shown that the miniature excitatory junction potentials (MEJPs) in the dorsal longitudinal flight muscle (DLM) of the temperature-sensitive endocytosis mutant, *shibire*$^{ts\text{-}1}$ (*shi*), of *Drosophila* become multiquantal and/or occur in a clustered manner during the early stages of recovery from vesicle depletion induced by exposure to 29 °C.

METHODS

Four-day-old adult *shibire*$^{ts\text{-}1}$ and wild-type (Oregon-R) *Drosophila melanogaster* were used. The *shi* mutation is in a single gene which codes for a protein involved in the process of endocytosis. As a result of the base-pair change in the DNA, the amino acid sequence of the protein is altered, so that it becomes unstable at high temperature (29 °C), making the protein non-functional, which blocks the process of endocytosis at the stage where pits form on the plasma membrane (Kosaka & Ikeda, 1983). The fly was mounted dorsal side up in Tackiwax over an opening in a plastic tube so that when covered with saline (128 mM-NaCl; 4·7 mM-KCl; 1·8 mM-$CaCl_2$; buffered to pH 7·4 with 5 mM-Tris-aminomethane HCl), the ventral side of the thorax, including the posterior spiracles and the entire abdomen, remained exposed to the air in the tube, allowing the fly to remain in good respiratory condition throughout the experiment. The temperature was regulated using a Peltier heating-cooling device, and monitored by a thermistor placed in the bath.

MEJPs were recorded intracellularly from fibre No. 6 (Mihályi, 1936) of the DLM using a glass micropipette filled with 2·5 M-potassium acetate inserted through the anterior end of the muscle fibre where it attaches to the tergum (see Koenig & Ikeda, 1980*a*, for details of this recording technique). Fibre No. 6 was chosen as a material because it is the smallest of the six DLM fibres and is multiterminally innervated by a single excitatory motoneurone (Ikeda, Koenig & Tsuruhara, 1980). Since this muscle fibre is isopotential, the length constant (3 mm) far exceeding the length of the entire fibre (350 μm), all MEJPs from this neurone were observed without spatial decay. It is estimated that thousands of active sites exist on this muscle (K. Ikeda, unpublished observations), making the frequency of spontaneous release quite high (see Results section).

Continuous recordings beginning at 19 °C, raising the temperature to 29 °C, and lowering it back to 19 °C, were made in order to observe the recovery process from exposure to high temperature. At about 26 °C in this mutant, the motor output pattern (flight pattern) to this muscle from the central nervous system occurs, the result of disinhibition of the motoneurones as transmission becomes blocked (Koenig & Ikeda, 1980*b*). This activity causes the gradual depletion of synaptic vesicles from the DLM terminals. In some cases, the recordings were made from dissected preparations in which the posterior dorsal mesothoracic nerve (PDMN), which innervates the DLM, was cut and sucked into a suction electrode for stimulation (see Koenig, Saito & Ikeda, 1983,

for details of this recording technique). In these experiments, the motoneurones were depleted by stimulating the nerve. Data were taken only from flies which showed at least −90 mV resting potentials both at the beginning and the end of the experiment.

Measurements of MEJP amplitudes and measurements of intervals between MEJPs were done with a micrometer attached to a stereomicroscope from film taken from the oscilloscope using a Grass kimograph camera. For the distribution analyses (Table 1), it was necessary to select those fibres which exhibited a stable MEJP frequency which was low enough to clearly distinguish between individual events. Only about 10 % of the *shi* fibres observed during recovery at 19 °C were usable for the present purpose, the rest having too high, or too unstable, an MEJP frequency for analysis. No distribution analyses could be made on wild-type flies at any temperature or *shi* fibres at 19 °C, due to the high frequency of spontaneous release (see Fig. 1 *A*). In scoring events, those larger MEJPs which were clearly made up of two or more near-simultaneous events (i.e. with notches on their rising phases), were scored as separate events occurring a finite time apart. Those larger MEJPs with fast, unnotched rising phases were scored as single events.

RESULTS

A typical intracellular recording from DLM fibre No. 6 of a *shi* fly at 19 °C is shown in Fig. 1*A*. It is characterized by many small (0·4–0·6 mV) spontaneous potentials,

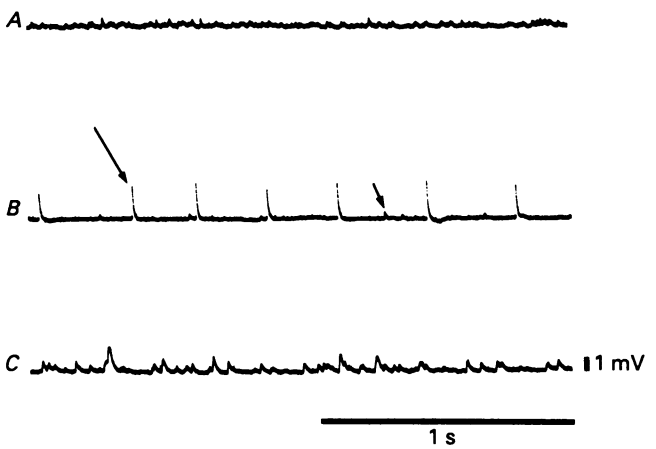

Fig. 1. Examples from a continuous recording from DLM fibre No. 6 of a *shi* fly. *A*, 19 °C prior to heating. Note high frequency of MEJP's, which gives the impression of a noisy recording, but actually represents many small MEJPs. *B*, after 8 min at 29 °C. Note decrease in frequency of MEJPs (short arrow) which exposes actual baseline (signal-to-noise ratio = 3 : 1). Also note small-amplitude EJPs (long arrow), which are occurring as a result of the motor flight pattern induced at high temperature in this mutant. *C*, recovery from exposure to 29 °C: 1 min at 19 °C after 8 min at 29 °C. Note change in size of MEJPs in comparison to top trace.

which give the impression of a noisy recording (actual baseline can be seen in Fig. 1*B*). The frequency of these MEJPs is quite high (in this case, ≈ 40/s), a result of the fact that this fibre is isopotential and is innervated by a single motoneurone which makes thousands of synapses on it. This recording is also typical of wild-type flies at 19 °C.

A typical example of an intracellular recording from DLM fibre No. 6 of a *shi* fly after 8 min exposure to 29 °C is seen in Fig. 1*B*. Only a few MEJPs are now observed, and the EJP is diminished from about 60 mV to about 3 mV as a result of the

activated flight pattern which has occurred while recycling is blocked. These decreases in MEJP frequency and EJP amplitude have been correlated with a large reduction in the number of synaptic vesicles in the terminals (Koenig *et al.* 1983).

Figure 1*C* shows a typical recording from a *shi* DLM fibre which has been allowed to recover for 1 min at 19 °C, after having been exposed to 29 °C for 8 min. The examples in Fig. 1 are taken from a continuous recording from a single fly. During

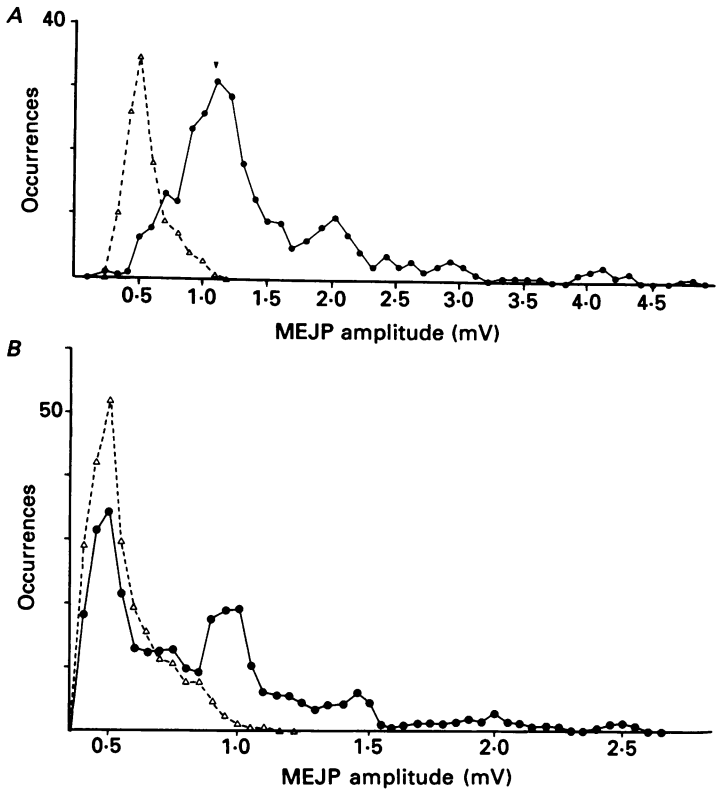

Fig. 2. MEJP amplitude distributions (DLM, fibre No. 6) from two different *shi* flies, showing MEJP amplitudes at 19 °C prior to heating (△) and at 19 °C after 1 min recovery from 8 min exposure to 29 °C (●). Note shift to large amplitudes during recovery phase.

the 1 min recovery period at 19 °C, the frequency of MEJPs, which was greatly reduced at 29 °C, gradually increases. Also, the amplitude of the EJP gradually increases to ≈ 40 mV, although no motor activity occurs at this time. It is immediately apparent that the MEJPs during this early stage of recovery from exposure to 29 °C are much larger in amplitude than they were prior to heating. This phenomenon was observed in over fifty *shi* flies, but was never observed in the wild-type controls which were treated identically. If the synapse is allowed to recover for about 20 min, the amplitude of the MEJPs returns to what it was prior to heating.

Since no change in input resistance or resting membrane potential before and after this exposure to high temperature was observed, the larger-sized MEJPs must be due

to a change either in quantal size or the number of quanta making up the MEJPs. Amplitude histograms of the MEJPs prior to heating and during recovery help to distinguish between these two possibilities. Two typical histograms from two different *shi* flies are shown in Fig. 2. As can be seen, prior to heating (19 °C) the MEJP amplitude histogram is unimodal (dashed line), while during recovery from exposure to high temperature (19 °C–recovery) the histogram is multimodal

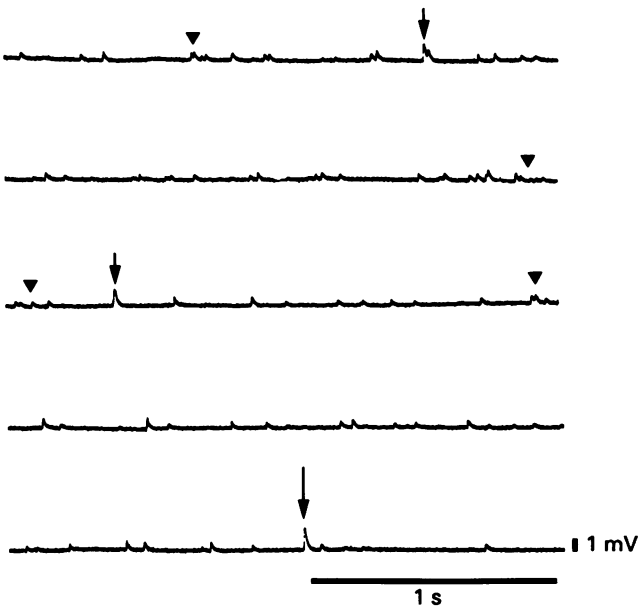

Fig. 3. Continuous recording of MEJPs recorded from DLM fibre No. 6 of a *shi* fly after 1 min recovery at 19 °C from 8 min exposure to 29 °C. Note synchronous release (long arrow), near-synchronous release (short arrows) and clusters (arrow-heads) of MEJPs.

(continuous line), with peaks which appear to occur at integral multiples of the single peak seen prior to heating. Thus, the MEJPs appear to be multiquantal. The percentage of multiquantal MEJPs relative to unitary MEJPs during early recovery varied from specimen to specimen. Thus, it can be seen in Fig. 2*A* that most of the MEJPs are made up of two or more quanta during recovery, while in Fig. 2*B* only about one-half of the MEJPs are multiquantal during recovery.

In observing the recordings of MEJPs during recovery, it was immediately apparent that many of the large MEJPs had notches on their rising phases, i.e. they were obviously made up of more than one quantum (these were scored as separate events). These almost-synchronous MEJPs were much too frequent to be a result of random overlapping of events, as will be shown below. Also, it was apparent that 'clusters' of unitary MEJPs occurred. Some examples of this can be seen in Fig. 3. Thus, the spontaneous release process during recovery does not appear to be random, but rather, there is a preponderance of clustered events. This is seen in Table 1, which shows that the appearance of MEJPs during early recovery from exposure to 29 °C in *shi* DLM does not fit a Poisson distribution (data taken from same recording as that shown in Fig. 3). Using the χ^2 test as a measure of agreement between the

TABLE 1. Fit of MEPP occurrences to a Poisson distribution during recovery

MEPP in sample (x)	N_x	
	Observed	Expected
(A) With synchronized MEPPS scored as single events		
0	1366	1340
1	180	227·6
2	33	19·3
3	9	1·1
4		
5		
6		
(B) With synchronized MEPPs scored as multiple events		
0	1366	1171
1	58	357·2
2	91	52·4
3	59	5·5
4	6	4·1
5	6	0·025
6	2	0·001

Fit of MEJP occurrence to a Poisson distribution after exposure to 29 °C for 8 min followed by 1 min recovery at 19 °C in DLM fibre No. 6 of a *shi* fly. The expected values were derived from the equation $N_x = N_T e^{-\alpha} \alpha^x / x!$ where N_T is the total number of samples (10 ms bins), N_x is the number of bins containing x number of MEJPs and α is the mean number of MEJPs per bin, calculated by dividing the total number of MEPPs by the total number of bins (1588). A, MEJP's with unnotched rising phases scored as single events regardless of amplitude. Total number of MEJPs = 273, $\alpha = 0{\cdot}17$. B, same data as A, but with the larger than unitary size MEJPs with unnotched rising phases scored as multiple events, depending on amplitude. One MEJP = 0·3–0·7 mV, two synchronized MEJPs = 0·8–1·4 mV, three synchronized MEJPs = 1·7–2·1 mV, etc., based on amplitude histogram for this recording. Thus, total number of MEJPs is increased to 483, and α is 0·304.

experimental distribution and that predicted by the Poisson theorem, a probability value of $< 0{\cdot}001$ was obtained, whether the large MEJPs were scored as single events (A) or as multiquantal events (B). Thus, the probability that a given MEJP will fire either simultaneously with or soon after another MEJP is much higher than would occur by chance during early recovery of *shi*. Since the MEJP frequency was so high in wild-type flies at 19 °C, it was not possible to do a distribution analysis on these flies. However, very few multiquantal or near-synchronous events occurred in wild-type flies at 19 °C, even though the frequency was higher.

DISCUSSION

The results show that during early recovery from depletion of synaptic vesicles induced by exposure to 29 °C in the DLM fibres in *shi* flies, the MEJPs are larger in amplitude than they were prior to heating. Amplitude histograms show a multimodal distribution with the smallest peak being equivalent in amplitude to that of the MEJPs prior to heating, and the other peaks appearing to be integral multiples of this smallest peak. In addition, it was observed that many near-simultaneous events occurred, as well as a clustering of unitary events. Thus, it appears that during

recovery there is an increased tendency for the synchronized release of MEJPs, the degree of synchronization determining whether the MEJPs appear as multiquantal events, near-synchronous events or as a cluster of unitary events.

One condition which has been shown to affect 'clustering' and multiquantal release is extracellular Ca^{2+}. It has been shown that increasing the extracellular Ca^{2+} concentration increases the clustering of classical MEJPs and the probability of multiquantal release (Rotshenker & Rahamimoff, 1970; Dennis *et al.* 1971; Bornstein, 1978). In our experiments using the neuromuscular junction of the larval body wall muscle of wild-type *Drosophila* (Oregon-R), the same phenomenon has been observed (Yamaoka & Ikeda, 1985). Thus, the amount of clustering and the percentage of synchronized (multiquantal) MEJPs increased with elevated levels of external Ca^{2+}, and decreased with reduced levels of external Ca^{2+}. In the experiments presented here, it is unlikely that the *shi* mutation itself would cause an increase in internal Ca^{2+}, since the *shi* effect is very specific to the process of endocytosis. It might be possible, however, that an increase in internal Ca^{2+} could result from depletion of synaptic vesicles, if vesicles normally have a function of sequestering Ca^{2+}, as has been suggested (Tauc, 1982).

The induction of multiquantal MEJPs and clustering may indeed be a result of vesicle depletion, since many reports exist of 'giant' MEPPs occurring after various treatments which cause depletion (Fritz, Atwood & Jahromi, 1980 (black widow spider venom); Augustine & Levitan, 1983 (Erythrosin B); Heuser, 1974 (tetanic stimulation, lanthanum); Molenaar, Oen & Polak, 1987 (high K^+)). Often the amplitudes of these giant MEPPs are many times the amplitude of the 'classical' MEPPs, so that it would not be possible to determine if they were made up of many normal-sized quanta or one oversized quantum. It has been reported, however, that during recovery from stimulation with a high-K^+ medium, which causes excessive release and ultimate depletion, giant MEPPs and also 'bursts' of MEPPs are observed. This suggests that in this case at least, the giant MEPPs may indeed be a synchronized release of many quanta, rather than one oversized quantum (Molenaar *et al.* 1987).

One explanation for these giant events has been that oversized vesicles – possibly intermediates during the membrane recycling process – are responsible (Heuser, 1974; Pécot-Dechavassine & Couteaux, 1972; Fritz *et al.* 1980). However, not all treatments which cause giant MEPPs show abnormal synapses with oversized vesicles (Pécot-Dechavassine & Molgó, 1982 (4-aminoquinoline); Pécot-Dechavassine, 1976 (vinblastine)). Also, oversized vesicles do not explain the 'clustering' aspect of release which was seen in these experiments. Thus, this explanation seems unlikely in this case.

It is interesting to note that the drug, vinblastine, apparently causes a very similar effect on release to the one seen here (Pécot-Dechavassine, 1976). Thus, many multiquantal MEPPs were observed soon after application of this drug. Although vesicle depletion is not observed, it has been shown that, in addition to other effects, vinblastine can bind to sites which can also bind Ca^{2+} ions (Wilson, Bkyan, Ruby & Maria, 1970). Thus, one possibility is that it might bind Ca^{2+} receptors on the vesicles and block Ca^{2+} binding to the vesicle membrane, thus increasing the intracellular Ca^{2+} concentration.

It has been observed that Cl^- is necessary for the increase in giant MEPP frequency observed during recovery from stimulation with a high-K^+ medium (Molenaar *et al.* 1987). A possible link with accelerated vesicle recycling was sought and it was reported as 'unpublished results' that vesicle depletion persists in the absence of Cl^-. Again, this observation is compatible with the idea that vesicle depletion *itself* may be involved in producing this phenomenon.

In summary, we present here an observation which may link various previous observations regarding the occurrence of multiquantal miniature potentials. On the one hand are observations of miniature potentials which occur as clusters, or occur as larger MEPPs apparently made up of several synchronously released quanta, and which are stimulated by increased extracellular Ca^{2+}. On the other hand are observations on giant MEPPs which are usually many times the amplitude of the classical MEPPs and which are observed during recovery from various methods causing massive transmitter release and vesicle depletion. In our experiments, we induce vesicle depletion by blocking recycling with a mutation specific to the recycling process, thus eliminating the necessity for excessive stimulation. During recovery from this treatment, clustering and multiquantal MEJPs were observed. Thus, it is suggested that the giant MEPPs seen as a result of depletion in some preparations, the MEPPs resulting from the synchronized release of a few quanta, and the clusters of unitary MEPPs may all result from the same phenomenon. An increase in intracellular Ca^{2+} might be the underlying factor linking these various phenomena, though this has yet to be proven.

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