J. exp. Biol. (1980), 87, 195–201
With 4 figures
Printed in Great Britain

THE STATISTICAL ANALYSIS OF SPONTANEOUS TRANSMITTER RELEASE AT INDIVIDUAL JUNCTIONS ON COCKROACH MUSCLE

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(*Received* 5 *November* 1979)

SUMMARY

1. Miniature excitatory post-synaptic potentials (MEPSPs) were recorded extra- and intracellularly from the coxal depressor muscle of the cockroach, *Periplaneta americana*.

2. Statistical analysis of the time intervals between MEPSPs showed that the variance-to-mean curve for the extracellular data sets lay significantly above the line predicted for a Poisson process. A shuffling procedure, however, made the series almost random.

3. The serial correlation coefficients for the extracellular data sets exceeded the confidence limits for various lags and most of them were positive. In comparison with the intensity function, the departure from the random process appeared to be mainly due to large positive correlation of intervals.

4. It is concluded that in insect neuromuscular junctions, the spontaneous release of transmitter at individual sites is more clustered than that predicted by a Poisson process.

INTRODUCTION

In the pioneering work of Fatt & Katz (1952), the spontaneous release of transmitter was shown to occur at random intervals indicating no interaction between consecutive potentials in frog neuromuscular junctions. Later, Gage & Hubbard (1965), by counting the number of miniature endoplate potentials (MEPPs) per unit time intervals, showed that spontaneous transmitter release followed to a Poisson distribution under a variety of experimental conditions at the mammalian neuromuscular junction.

However, more sophisticated analysis (Cox & Lewis, 1966) indicated some departure from a Poisson process in the mode of transmitter release at vertebrate (Hubbard & Jones, 1973; Cohen, Kita & Van der Kloot, 1974*a*) and invertebrate (Cohen *et al.* 1974*b*) neuromuscular junctions and at the mammalian ganglionic synapse (Bornstein, 1978). In insect neuromuscular junctions (Usherwood, 1972; Rees, 1974; Hodgkiss & Usherwood, 1978) it has also been shown that the spontaneous transmitter release is rarely a random process, bursts of miniature excitatory post-synaptic potentials (MEPSPs) being frequently recorded. Our previous work (Washio & Inouye, 1975) on

the series of MEPSPs recorded intracellularly from insect muscle showed that significant departure from a Poisson process resulted from short irregular fluctuations in the rate of the transmitter release. We also found that there was no significant deviation from a random process, if the frequency of the MEPSPs did not change with time.

Because an insect muscle is innervated multiterminally, intracellular recordings integrate many junctional potentials. Therefore, the mode of spontaneous transmitter release would be better obtained by recording exclusively from a single neuromuscular junction. In the present work the time series of MEPSPs detected at insect neuromuscular junctions with focal extracellular recordings was analysed by the methods of Cox & Lewis (1966).

MATERIALS AND METHODS

The coxal depressor muscle number 178 (Carbonell, 1949) from metathoracic legs of cockroach, *Periplaneta americana*, was used in all experiments. The properties of the muscle have been described in previous publications (Pearson & Iles, 1971; Washio & Inouye, 1978). The preparation was mounted in 1·5 ml acrylic bath and illuminated from below.

Spontaneous miniature potentials were recorded extra- and intracellularly together with conventional techniques. Extracellular recordings were made with microelectrodes filled with 2 M-NaCl having a resistance of 2–5 MΩ; intracellular recordings with microelectrodes filled with 3 M KCl having a resistance of 5–10 MΩ. The details of the methods are similar to those used by Washio & Inouye (1978).

The standard bathing solution had the following composition (in mM): NaCl 158·0, KCl 10·8, $CaCl_2$ 5·0, $NaHCO_3$ 1·0, $NaHPO_4$ 0·1 (Becht, Hoyle & Usherwood, 1960). Experiments were all done at 20–24 °C.

Statistical analysis

Records of MEPSPs were converted into series of intervals between events. Frequencies in 1 min were plotted against time in order to eliminate a series which had a time-dependent change. Non-parametric procedures for analysing statistical properties of time series of events were employed, according to Cox & Lewis (1966).

The variance-to-mean curve represents variance ($V(N(t))$), of a number of events in a time interval, t, versus mean number, $E(N(t))$, of events in that time interval. It is a good measure of randomness because a Poisson process yields a relation $V(N(t)) = E(N(t))$ for any time interval. Significant limits of the deviation from a Poisson process were estimated by the method of Cox & Lewis (1966). The intensity function is a kind of auto-correlation function. It represents a probability of the event when the event occurs at time zero. The function was calculated by superposition of the time series given that an event occurred at time zero. Serial correlation coefficients of the intervals were computed in a standard manner. These coefficients evaluate the extent of the mutual dependency of the intervals.

Shuffling was performed with a series of permutation between the interval and entry assigned by a random number. The procedure randomizes the correlation between intervals leaving the intensity function unaffected. Thus, the shuffli

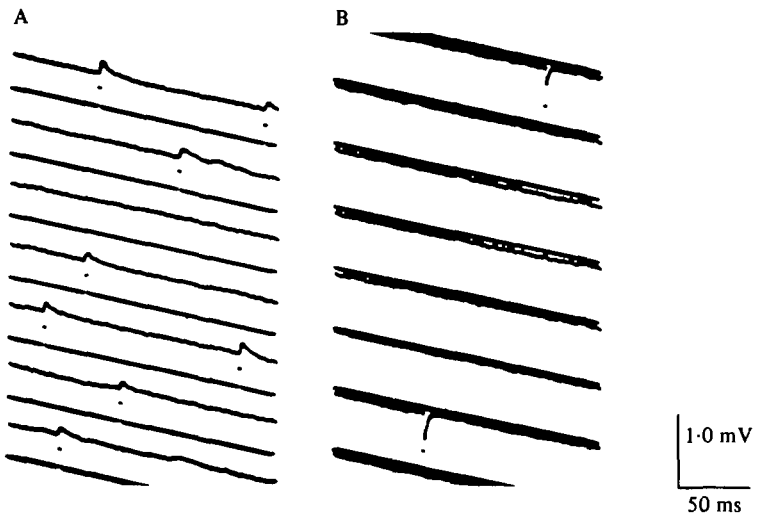

Fig. 1. Internal (A) and external (B) records of MEPSPs. The occurrence of the potentials (upper trace) just above the position of the noise level was detected by a capacity-coupled discriminator and triggered a pulse (lower trace) that coincided with the potential.

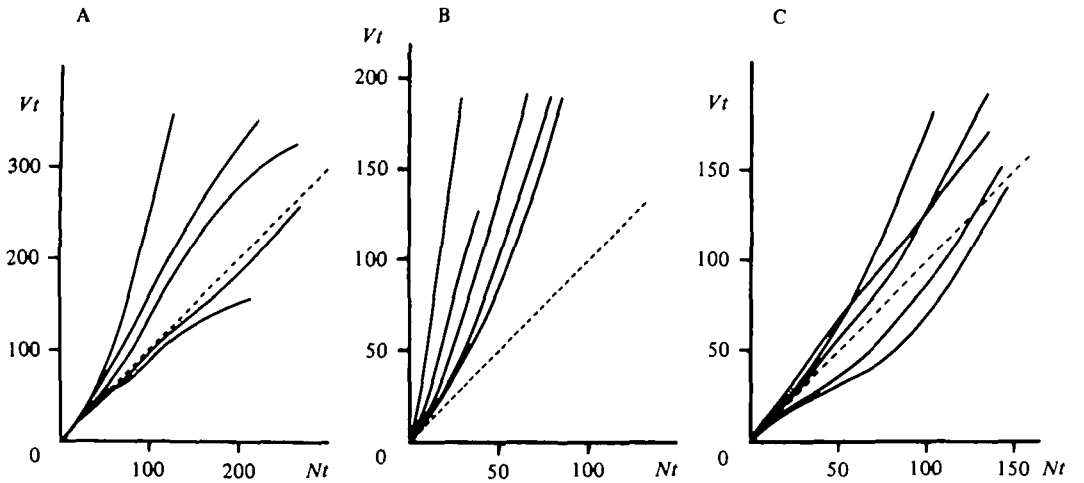

Fig. 2. The variance-to-mean curves calculated from the data recorded intracellularly from five cells (A), recorded extracellularly at five active spots before (B) and after (C), applying a shuffling procedure. The dashed lines show the relationship expected for a Poisson process. The abscissa is the mean number, Nt, of the events occurring in any duration of time t. The ordinate is the variance, Vt, of the event in that duration.

procedure might be a useful tool for discriminating several causes of the deviation from a Poisson process. Superposition of a series was performed on the assumption that each new series that is generated in the series is independent of the original series. The new series which was obtained by displacing the sequence of the original series randomly was summated into a superposition of the series. The procedure was repeated a specific number of times.

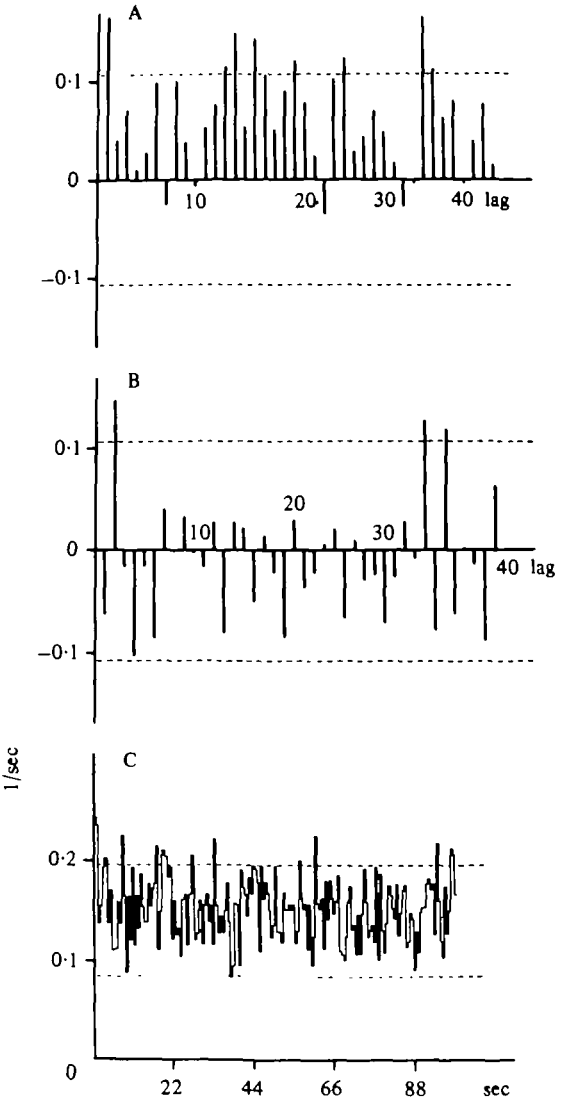

Fig. 3. (A) Serial correlation coefficients for the first 40 lags for the data recorded extracellularly. The horizontal dashed lines show the values expected for individual estimates showing the 0·05 significance limits. The abscissa is the number of intervening events between intervals being considered. The ordinate is the serial correlation coefficients. (B) Serial correlation coefficients for the first 40 lags for the data after shuffling procedure of the same data set as shown in A has been made. The axes are as for A. (C) Intensity function for the same data as shown in A. The horizontal dashed lines show 0·05 significance limits for individual estimates for a Poisson process. The abscissa is time (s) after an event. The ordinate is the probability of occurrence of events in 1 s.

RESULTS AND DISCUSSION

The frequency of MEPSPs recorded extracellularly was much lower than that recorded intracellularly in this tissue (Fig. 1). Other characteristics of the potentials were reversed polarity, brief time course, and critical location.

Sets of interval data, free from distinct trends, were obtained from five focal site

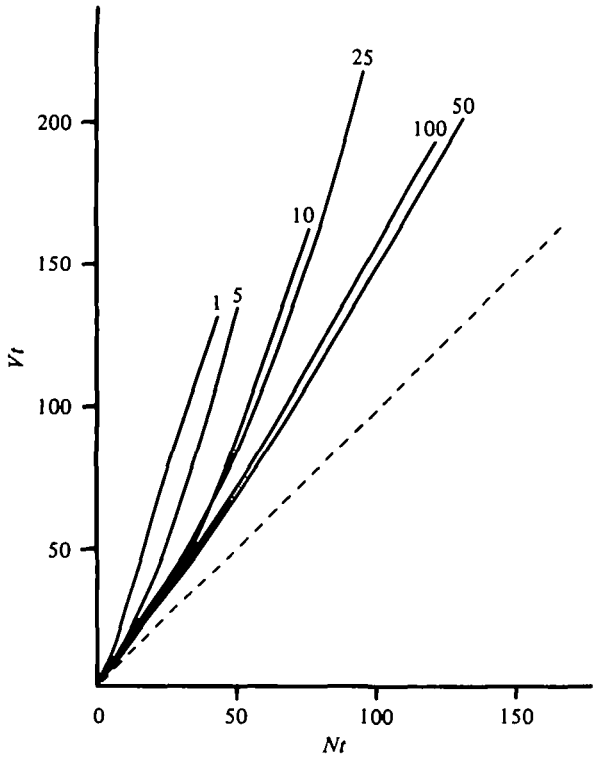

Fig. 4. The variance-to-mean curves for computer-generated data series simulating the pooled output of a finite number (5, 10, 25, 50 and 100) of release sites. The axes and dashed line are as for Fig. 2.

in five fibres. More than 500 MEPSPs were recorded extracellularly from each site. For comparison with extracellular recordings, intracellular recordings of MEPSPs were made, more than 2000 MEPSPs being subjected to the statistical analysis.

First we estimated the accumulation of total variance as a function of time. For a Poisson process the variance, Vt, of the event in an observation period of time t is equal to the mean number, Nt, of events occurring in time t. Variance-to-mean curves for the extracellular data sets were always found to lie above the line predicted for a Poisson process (Fig. 2B). In contrast with the curves for the extracellular data, those for the intracellular data sets in which the frequency of the MEPSPs did not change with time were found to lie around the line for a Poisson prediction (Fig. 2A), in agreement with the earlier finding (Washio & Inouye, 1975).

For further analysis, we calculated the serial correlation coefficient and the intensity function. A typical example is shown in Fig. 3A, in which 9 of the 40 correlation coefficients exceeded the 0·05 significant limits and most of the coefficients were positive. This implies that short intervals are followed more frequently by short ones and the long by subsequent long ones. However, shuffling procedures which randomize the order of intervals made the series almost random (Fig. 3B). This was also clearly shown in variance-to-mean curves for shuffled series. The curves indeed lay around the straight line for a Poisson prediction (Fig. 2C). These results suggest

that the intervals between the MEPSPs recorded at individual sites are not independent but are autocorrelated. On the other hand, the intensity function (Fig. 3C) showed that most of the probabilities were inside the 0·05 significance limits. There was no significant initial increase in the function. By comparing the serial correlation coefficient and the intensity function of the events, it seems likely that the departure from a random process is due mainly to large positive serial correlation of the intervals rather than to the correlation of the probability of events (intensity function). The fact that the probabilities are inside the significance limits implies that the probability of encountering any event as function of time after a given event does not change significantly. In other words, there is no 'drag' effect in the sequence of the MEPSPs at the insect neuromuscular junction as was postulated by Martin & Pilar (1964), since such an effect implies a transient increase in the probability of resultant release of quanta after release of one quantum. The above findings are consistent with the fact that the synaptic vesicles at synaptic foci in the nerve terminals of insect motoneurones are not randomly distributed (Usherwood & Rees, 1972), indicating a causal relationship between vesicle distribution and sequence of spontaneous transmitter release (Vere-Jones, 1966, Usherwood, 1972).

Each fibre of the insect coxal depressor muscle receives multiterminal innervation from the same excitatory axon (Pearson & Iles, 1971). It seems likely that the extracellular electrode monitors spilling of quanta released from only one terminal. Mathematically, time series of MEPSPs recorded intracellularly may be the pooled output of the potentials recorded extracellularly. Therefore, it may be possible to represent the time series of the potentials recorded with the intracellular electrode when we superpose a finite number of series recorded with the extracellular electrode, assuming independence of each terminal. The simulation revealed that the superposition of the series made it more random process, as shown in Fig. 4. Thus, the magnitude of the deviation from a Poisson prediction decreased as the number of component sites increased, but the degree to which the series approached to a Poisson process depended on the strength of the serial correlation. The number of transmitter release sites over a whole fibre was estimated to be several tens to a hundred on the basis of this model. The value was roughly comparable with that obtained morphologically (Hoyle, 1957; Edwards, 1959; Rees & Usherwood, 1972).

In conclusion our results strongly suggest that observed transmitter release may be the pooled output of a finite number of transmitter release sites (Fatt & Katz, 1952; Cox & Lewis, 1966; Vere-Jones, 1966; Hubbard & Jones, 1973), each of which release quanta in a manner more clustered than Poisson in insect neuromuscular junctions. We could not find any signs of an ordered process of transmitter release at the junctions, similar to that reported in the rat neuromuscular junction (Hubbard & Jones, 1973).

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