Journal of Physiology (1992), **454**, *pp.* 9–26
With 9 figures
Printed in Great Britain

PROBABILISTIC SECRETION OF QUANTA FROM VISUALIZED SYMPATHETIC NERVE VARICOSITIES IN MOUSE VAS DEFERENS

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(*Received 14 August 1991*)

SUMMARY

1. Sympathetic varicosities on the surface of smooth muscle cells of the mouse vas deferens were visualized with the fluorescent dye 3-3 Diethyloxardicarbocyanine iodide ($DiOC_2(5)$) and quantal secretion recorded from these with both small diameter (4–6 μm) and large diameter (20–50 μm) microelectrodes. Small diameter electrodes were placed over one to three varicosities and large diameter electrodes over three to seven varicosities.

2. The size and distribution of varicosities along individual terminal branches was about the same when these were fluoresced with $DiOC_2(5)$ (length $1{\cdot}09 \pm 0{\cdot}40$ μm (mean $\pm$ S.D.); intervaricosity distance $5{\cdot}53 \pm 2{\cdot}68$ μm) as when they were stained for catecholamines using Faglu fluorescence (length $1{\cdot}05 \pm 0{\cdot}43$ μm; intervaricosity distance $5{\cdot}12 \pm 2{\cdot}79$ μm) suggesting that $DiOC_2(5)$ does allow for identification of the catecholamine-containing varicosities.

3. The spontaneous excitatory junctional currents (EJCs) recorded from visualized varicosities with small diameter electrodes (amplitudes 59–67 μV) were much larger than those recorded with large diameter electrodes (amplitudes 25–29 μV). The frequency of evoked EJCs as well as the amplitude–frequency distribution of these EJCs varied greatly between sets of visualized varicosities recorded along individual branches, either with a small or large diameter electrode. These amplitude–frequency distributions typically followed Poisson statistics, in which the mean quantal content of the EJC ($\bar{m}$) varied by over threefold for different sets of varicosities on the same branch ($\bar{m}$ was 0·07–0·21 for small electrodes whereas $\bar{m}$ was 1–3 for large electrodes).

4. Although $\bar{m}$ varied considerably for a constant number of varicosities beneath the electrode at different sites along a single branch, there was an overall correlation between $\bar{m}$ and the number of varicosities, $\bar{m}$ increasing on average 0·25 for each additional varicosity in a $[Ca^{2+}]_o$ of 4·0 mM.

5. The frequency of evoked EJCs at visualized sets of varicosities along some branches was sufficiently high to allow binomial statistics to predict the amplitude–frequency distributions of evoked EJCs. In these cases $\bar{m}$ was again shown to vary considerably along single terminal branches, and this was primarily

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due to variation in the probability of secretion (p) between sets of varicosities and not to variation in binomial parameter n.

6. In one case a relatively isolated varicosity, over 3 μm from adjacent varicosities, was recorded for 30 min with a 4 μm diameter electrode. The mean and variance of the evoked EJC was similar to that of the spontaneous EJCs suggesting that this varicosity secreted at most one quantum on arrival of the nerve impulse.

7. There was a correlation between the number of varicosities beneath the recording electrode and binomial parameter n, but not with the probability for secretion, p. Parameter n increased on average 0·8 for each additional varicosity.

INTRODUCTION

The probability of evoked quantal secretion at different release sites along single terminal branches in the amphibian neuromuscular junction is non-uniform (Bennett & Lavidis, 1979; Bennett & Lavidis, 1982; D'Alonzo & Grinnell, 1985; Zefirov, 1985; for a review see Robitaille & Tremblay, 1987). The extent of non-uniformity is best determined by recording the electrical signs of transmission (the endplate current) from small groups of release sites with an external microelectrode (del Castillo & Katz, 1956), after the terminal branches have been visualized with the fluorescent dye 3-3 Diethyloxardicarbocyanine iodide ($DiOC_2(5)$; Bennett, Jones & Lavidis, 1986). Using this approach, it has been shown that relatively low probability release sites exist in close juxtaposition to relatively high probability release sites which themselves decline in probability along the length of terminal branches (Bennett & Lavidis, 1989). Our ability to resolve quantal secretion from single release sites or even a small number of release sites is limited by the fact that release sites are spaced at 1 μm intervals along somatic motor nerve terminal branches (Couteaux & Pecot-Dechavassine, 1968; Miller & Heuser, 1984). We have therefore sought a preparation in which release sites are separated by greater distances, allowing for the possibility of recording from individual sites.

The autonomic neuromuscular junction offers some advantages over the somatic neuromuscular junction in the analysis of the mechanisms which determine the probability of quantal secretion at release sites (Burnstock & Holman, 1961; Bennett, 1972). Autonomic motor nerve terminals possess release sites confined to varicosities that are several micrometres apart (Bennett & Merrillees, 1966; Merrillees, 1968), allowing for greater spatial resolution in the attempt to record from small groups of release sites and single release sites with an external electrode (Brock & Cunnane, 1987). In the present work varicose sympathetic nerve terminals on smooth muscle cells have been visualized with $DiOC_2(5)$ and the electrical signs of quantal secretion recorded with an external microelectrode positioned at different sites along the length of individual terminals. Using this approach it has been shown that different varicosities along a single terminal branch have different probabilities for secretion as do the release sites along a single branch at the somatic neuromuscular junction.

METHODS

Tissue preparation

Vasa deferentia of mice (strain Balb/c), 5–6 weeks postnatal were used in these experiments. Animals were killed by a cervical fracture and an incision made into the lower abdomen. Both vasa

deferentia, together with connective tissue containing the fine hypogastric nerves, were dissected free from other organs and fat tissue. Careful stripping of the sheath of epimysium from the surface of the vas deferens was then performed by gently teasing the sheath from the prostatic end for a distance of about 5 mm along the vas deferens. The tissue was then placed in a Perspex chamber of 3 ml capacity and pinned through the seminal vesicle end to the sheet of Sylgard at the bottom of the chamber. The stimulation glass electrode filled with the bathing modified Krebs–Henseleit solution was used to suck up the prostatic end of the vas deferens. The bathing solution had the following composition (mM): Na^+, 141; K^+, 4·7; Mg^{2+}, 1·2; Cl^-, 157; $H_2PO_4^-$, 0·4; HCO_3^-, 2·5; Ca^{2+}, 4·0 and glucose 11·2. The solution was gassed continuously with 95% O_2 and 5% CO_2 so that the pH was maintained between 7·2 and 7·4. The tissue was perfused with warm (31–36 °C) Krebs–Henseleit solution at the rate of 5 ml min^{-1}.

Fluorescence

The vas deferens was perfused for 20 s with the fluorescent dye $DiOC_2(5)$ (0·02–0·5 μM) and then washed in Krebs solution for 5 min (Bennett *et al.* 1986). $DiOC_2(5)$ fluorescence of the axons and varicosities was observed during excitation at 540 nm using an Olympus rhodamine filter set (Olympus, BH-2 microscope). Preparations were viewed using 20 × and 50 × long working distance objectives (Olympus, LWD-C20 and ULWD-MS Plan 50). The images of fluorescent varicose axons found on the surface of the smooth muscle cells were captured by a low light TV camera (Panasonic, WV1900/B) and displayed on a TV monitor (National, WV5410).

Stimulation and recording

The prostatic end of the vas deferens (2–3 mm) was sucked lightly into a modified bilbate which was filled with Krebs solution. Silver–silver chloride electrodes were used to stimulate the axons with current pulses of 0·05 ms duration and 10–20 V amplitude. The frequency of stimulation was 0·17 Hz and 200 stimulus trials were recorded for each sample.

Recording microelectrodes filled with Krebs solution and having a tip diameter of between 4 and 50 μm were placed over the area of a group of visualized varicosities, care being taken not to deeply indent the surface of the vas deferens with the electrode. Suction was not applied to the electrode in order to form a loose seal. The extracellular field potential of the nerve impulse in the varicose axons could be observed under these recording conditions, along with the electrical signs of the excitatory junctional currents (EJCs) due to the secretion of quanta. Following each stimulation, 60 ms of the recording was filtered at 1 kHz, digitized and stored on hard disk in an IBM-AT computer.

It is uncertain how the excitatory junctional current decays in the extracellular space surrounding a varicose axon. At the amphibian neuromuscular junction endplate current decays in the extracellular space around visualized terminal branches, such that an external electrode with tip diameter less than 1 μm records quantal secretion originating over about 12 μm (Bennett *et al.* 1986; see their Fig. 2; see also del Castillo & Katz, 1956). In order to determine the spatial decay of the EJC with distance from varicose axons, the microelectrode (4–20 μm diameter) was moved about 5 μm to either side of a visualized varicose axon. Without the application of suction in an electrode, EJCs could only be recorded if the electrode was placed over the surface of a set of varicosities; this 5 μm movement of the electrode away from these varicosities resulted in a loss of the EJCs.

Effect of $DiOC_2(5)$ and fluorescence on quantal secretion

Low concentrations (0·5 μM) of $DiOC_2(5)$ do not alter neurosecretion at amphibian neuromuscular synapses (Yoshikami & Okun, 1984; Bennett *et al.* 1986; Herrera & Banner, 1990). The effect of $DiOC_2(5)$ on secretion at sympathetic nerve terminals was checked using large diameter (20–40 μm) electrodes to search for EJCs. Following the recording of 200 trials, $DiOC_2(5)$ (5 μM) was applied to the preparation for a period of 5 min, the preparation washed for 5 min with Krebs solution and fluoresced for 5 min. There was no change in the evoked secretion of quanta following this treatment in four preparations. Longer exposures of fluorescence (greater than 15 min) did increase the frequency of spontaneous EJCs and spontaneous contractions of the vas deferens were observed. In order to avoid this, the concentration of $DiOC_2(5)$ was kept to less than 0·5 μM and the period of excitation kept to less than 2 min. The field of excitation was restricted to the region being examined. If necessary, preparations were treated with $DiOC_2(5)$ and fluoresced a second time.

The appropriate positioning of an electrode and determination of the rates of secretion at

different points along the length of a terminal branch often took longer than 2 h. To avoid prolonged periods of $DiOC_2(5)$ excitation, the field of fluorescing varicose axons was hand drawn on the TV monitor screen as soon as their fluorescent profiles had been identified; excitation was then terminated and transmitted light used to view the same area. Structures such as blood vessels

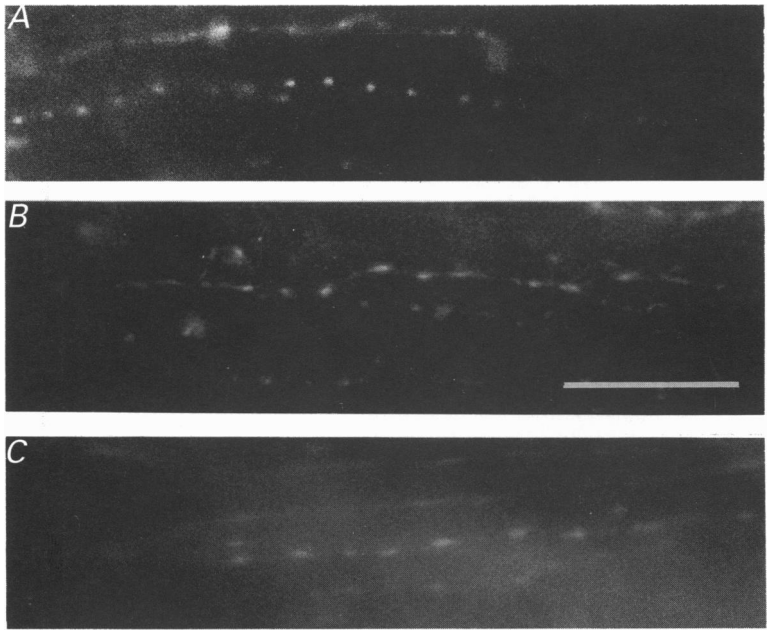

Fig. 1. Distribution of $DiOC_2(5)$-fluoresced nerve terminal varicosities (*A* and *B*) and Faglu-fluoresced catecholamine-containing nerve terminal varicosities (*C*) on the surface of the mouse vas deferens. Calibration for *A* and *B* is 20 μm and for *C* is 12 μm.

and connective tissue were drawn onto the TV monitor; the glass microelectrode was then moved into the field of the drawing of the varicose axons. Any shift of the varicose axons with respect to the drawing could finally be checked by refluorescing the varicose axons at the termination of the experiment. Electrophysiological study was only made of fluorescing varicose axons on the surface of the muscle that were at least 50 μm distant from any other surface axons.

Analysis of data

At least twenty spontaneous EJCs and 200 stimulation trials were used to construct amplitude–frequency histograms of the spontaneous EJCs and evoked EJCs respectively. Most histograms of evoked secretion were best predicted by Poisson statistics, and an estimate of the mean quantal content of the EJC ($\bar{m}$) and its standard error was determined (see Robinson, 1976). On some occasions the histograms of evoked EJCs could only be predicted by binomial statistics, allowing estimates to be made of $\bar{m}$, the mean probability of secretion (p) and the binomial parameter n. Estimates of p were made from:

$$p = (1-(S^2/m\gamma)+\sigma^2/\gamma^2),$$

where m and S^2 are the mean and variance of the EJC amplitudes, and γ and σ^2 are the mean and variance of the spontaneous EJCs. The amplitude–frequency histograms were predicted from:

$$P(x) = \sum_{r=0}^{n} {}^nC_r p^r q^{n-r} \frac{\lambda^{kr}}{\Gamma(kr)} e^{-\lambda k} x^{kr-1},$$

derived by Robinson (1976), where $P(x)$ is the expected frequency of EJCs with the amplitude of x mV, $r(= 0, 1, 2, \ldots, n)$ is the possible quantal content value of the EJC, Γ is the gamma function

and $\lambda = \gamma/\sigma^2$, $k = \gamma^2/\sigma^2$. The distribution of spontaneous EJCs was assumed to follow a gamma distribution (Bennett, Florin & Pettigrew, 1976). Equations for the standard errors of the means of p and n are given by Robinson (1976).

Histology

A comparison was made between the distribution of varicosities stained with $DiOC_2(5)$ and those stained with Faglu for amines (4% paraformaldehyde and 0·5% glutaraldehyde in phosphate buffer, see Furness, Costa & Llewellyn-Smith, 1987). The distance between varicosities was measured along with the length of varicosities following staining with either of these techniques.

RESULTS

The distribution of $DiOC_2(5)$ stained varicosities

When part of the epimysium of the mouse vas deferens is carefully removed so as to form a window clear of this sheath, varicosities can be easily visualized on the surface of the smooth muscle after fluorescing with $DiOC_2(5)$ (Fig. 1*A*). These varicosities have a length of $1{\cdot}09 \pm 0{\cdot}40$ μm ($N = 354$; mean $\pm$ S.D.) and are spaced $5{\cdot}53 \pm 2{\cdot}68$ μm ($N = 734$) apart; the dimensions of these varicosities fall into the size range of varicosities which form synaptic contacts with smooth muscle cells (Luff &

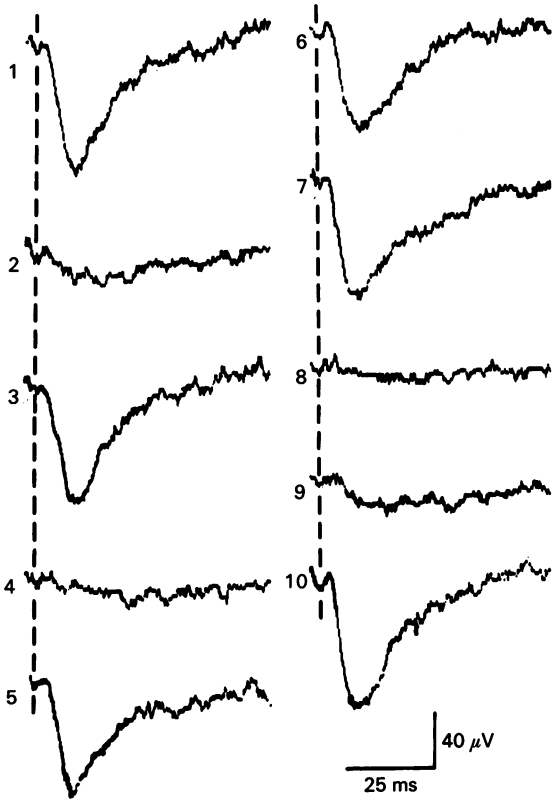

Fig. 2. Recordings of EJCs from a visualized ($DiOC_2(5)$-stained) varicose branch recorded with a 30 μm diameter microelectrode that contained five varicosities. The results of ten successive stimuli to the nerves in the intact epimysium at the proximal end of the vas deferens are shown, numbered 1 to 10. Stimuli 2, 4, 8 and 9 gave no EJCs but still evoked a nerve action potential (indicated by the vertical dashed lines).

McLachlan, 1988). In order to confirm that the $DiOC_2(5)$ has stained all the varicosities on the surface, a comparison was made between the size and distribution of catecholamine-containing varicosities determined using the Faglu method. This method showed catecholamine-containing structures on the surface of the vas

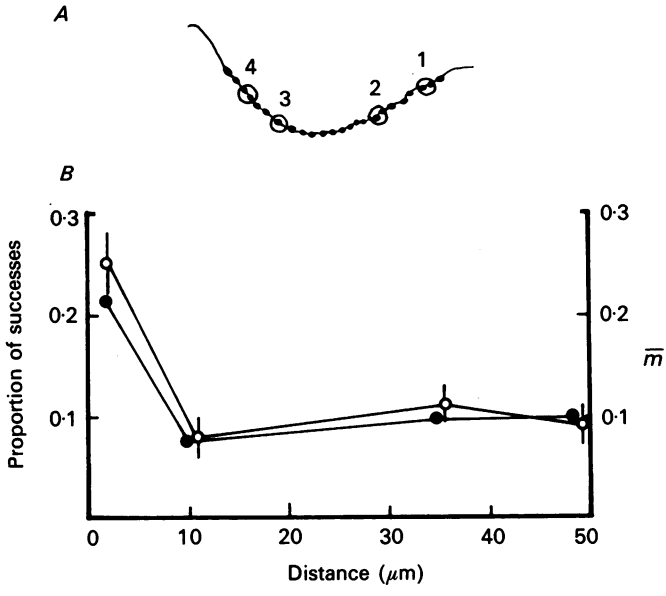

Fig. 3. Changes in the secretion of transmitter and $\bar{m}$ at different positions along a varicose axon recorded with a small diameter microelectrode of 4 μm. The upper panel gives a drawing of the varicose terminal, derived from the $DiOC_2(5)$-fluoresced nerve; ellipses give the outline of the recording electrode tip and the numbers indicate the sequence in which recordings were made. The lower panel gives the proportion of stimuli that gave an EJC at different positions along the length of the terminal axon (●) as well as $\bar{m}$ (○; bars give S.E.M.); the origin of the distance is taken as the extreme left-handed or right-handed recording site on the axon; all graphs of the proportions of successes and of $\bar{m}$ have been plotted in the direction along the axon branch which gives a declining proportion of successes.

deferens smooth muscle in much the same way as did $DiOC_2(5)$ (Fig. 1*B*). These varicosities had a similar length ($1{\cdot}05 \pm 0{\cdot}43$ μm ($N = 376$)) and spacing along the length of axons ($5{\cdot}12 \pm 2{\cdot}79$ μm ($N = 751$)) as did the $DiOC_2(5)$-stained varicosities. It has not been possible to counterstain catecholamine-fluorescing varicosities with $DiOC_2(5)$ so as to show directly that both stains identify the same set of varicosities.

Electrophysiological recordings of EJCs from visualized varicosities

Observations described by Poisson statistics

Glass microelectrodes filled with Krebs solution were used to record from $DiOC_2(5)$-fluoresced varicosities. These electrodes were of two different size classes: large diameter electrodes varied from 20 to 50 μm whereas small diameter electrodes were from 4 to 6 μm. Either of these size classes were easily manipulated to within a micrometre or so of the visualized varicosities using the monitor screen attached to the low-light video camera. If the nerves were then stimulated with 200 impulses at

0·17 Hz, occasional EJCs were evoked following the electrical signs of the nerve impulse, as shown in Fig. 2. Fluctuations in the size and dispersion of the nerve impulse between trials were not due to differences in the number of axons excited as changes in the strength of stimulation did not change the size of the nerve impulse

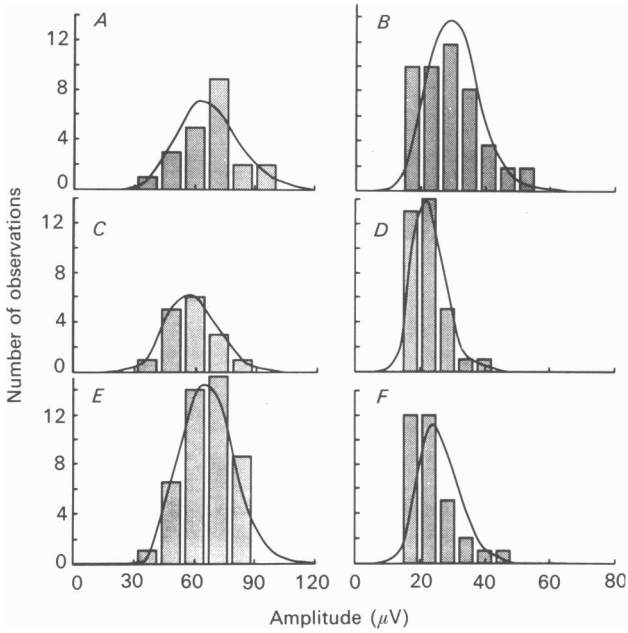

Fig. 4. Amplitude–frequency distribution of spontaneous EJCs recorded from visualized varicose axons with a small diameter (4 μm) electrode (*A*, *C* and *E*) and with a large diameter (30 μm) electrode (*B*, *D* and *F*). The different histograms have the following means and variances for small electrodes: *A*, 67 mV $\pm$ 226 mV^2; *C*, 59 mV $\pm$ 151 mV^2; *E*, 64 mV $\pm$ 105 mV^2. For large electrodes: *B*, 29 mV $\pm$ 51 mV^2; *D*, 25 mV $\pm$ 33 mV^2; *F*, 25 mV $\pm$ 223 mV^2. The extent of skewness of these distributions is given by $[\frac{1}{n}\Sigma (x-\bar{x})^3]/[\frac{1}{n}\Sigma (x-\bar{x})^2]^{3/2}$, where $\bar{x}$ is the mean of a distribution. The values of this factor are $-0{\cdot}2$, $+1{\cdot}0$, $+0{\cdot}25$, $+1{\cdot}2$, $-0{\cdot}26$, and $+1{\cdot}35$ for *A* to *F* respectively. *A*, *C* and *E* are therefore approximately Gaussian whereas *B*, *D* and *F* are positively skewed.

averaged over several trials at each stimulus strength. The best spatial resolution was obtained with recording electrodes of about 4 μm diameter. Figure 3 shows the results for recordings made with such an electrode along the length of a single visualized varicose branch. This electrode was placed over two varicosities at four different positions along the branch (Fig. 3). At position 4 the proportion of trials which successfully gave rise to an EJC was three times greater than for positions 3, 2 and 1, indicating considerable non-uniformity in the secretion probability. The question arises as to whether recordings were made from varicosities outside the end of the electrode. If this was the case then the variations in successful secretions could be attributed to variations in the number of varicosities recorded from. However, the branch in Fig. 3 shows an equal density of varicosities along its length, making it unlikely that the relatively high rates of successful secretions recorded at position 4 can be explained in this way.

In order to examine the statistics of quantal secretion from different sets of varicosities along terminal branches it is necessary to have a measure of the quantal size from spontaneous EJCs. In general, spontaneous EJCs were infrequent during the 33 min taken to record the 200 stimulation trials. It was found that the

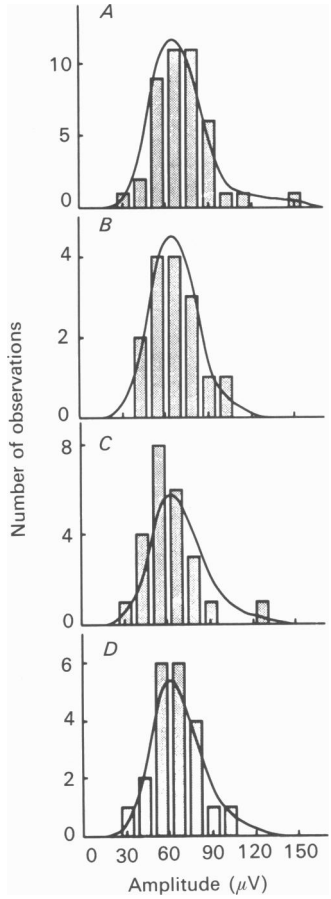

Fig. 5. Amplitude–frequency distribution of EJCs recorded with a small diameter electrode from the visualized branch shown in Fig. 3. *A*, *B*, *C* and *D* give the distributions for electrode positions 4, 3, 2 and 1 respectively, shown in Fig. 3. The continuous line gives the predictions of the Poisson distribution. The values of $\bar{m}$ (and the predicted number of failures) were as follows: *A*, $\bar{m} = 0{\cdot}252 \pm 0{\cdot}038$, failures 154 (157); *B*, $\bar{m} = 0{\cdot}076 \pm 0{\cdot}020$, failures 185 (185); *C*, $\bar{m} = 0{\cdot}110 \pm 0{\cdot}023$, failures 178 (176); *D*, $\bar{m} = 0{\cdot}099 \pm 0{\cdot}022$, failures 179 (179). The mean and variance of the spontaneous EJC was taken as 66 μV $\pm$ 300 μV^2. Number of trials was 200 in each case.

amplitude–frequency distributions of spontaneous EJCs were quite different for recordings using small diameter electrodes (4 to 6 μm; Fig. 4*A*, *C* and *E*) compared with those using large diameter electrodes (> 20 μm; Fig. 4*B*, *D* and *F*): in the former case the amplitude and variance of the spontaneous EJCs were larger (means in the range 59–67 μV and variances in the range 105–226 μV^2; Fig. 4*A*, *C* and *E*)

than in the latter case (means in the range 25–29 μV and variances 23–51 μV^2; Fig. 4*B*, *D* and *F*). The small electrodes gave Gaussian distributions (Fig. 4*A*, *C* and *E*) whereas the larger electrodes gave positively skewed distributions (Fig. 4*B*, *D* and *F*). The amplitudes and variances of the smallest evoked EJCs were about the same

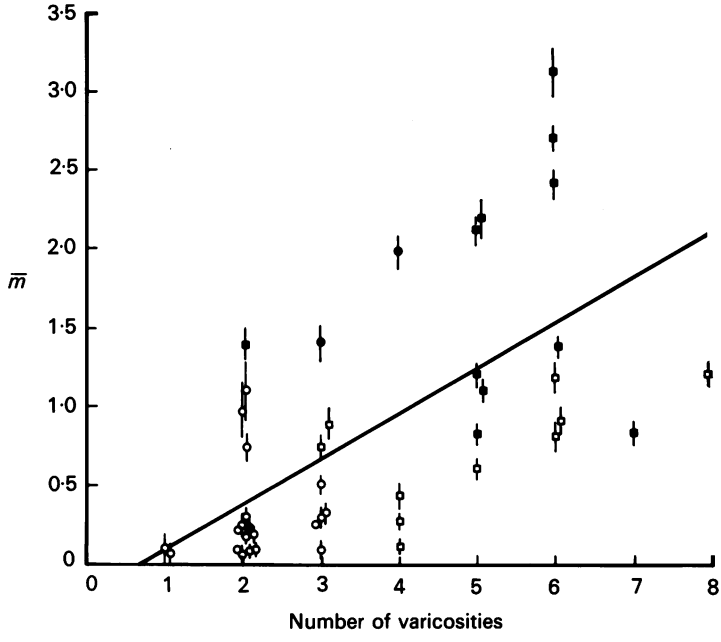

Fig. 6. Comparison between the number of visualized varicosities beneath an electrode and the value of $\bar{m}$. The circles give the results for small diameter electrodes and squares for large diameter electrodes; open symbols give $\bar{m}$ for observations that could only be fitted with Poisson distributions and filled symbols those that could be fitted with binomial distributions; vertical bars on the symbols give $\pm$ S.E.M. The regression line has a correlation coefficient of 0·45.

as those of the spontaneous EJCs. Estimates of the mean amplitude and variance of spontaneous EJCs were made from amplitude–frequency histograms as shown in Fig. 4. The mean amplitude and variance of spontaneous EJCs for large diameter electrodes were estimated as 27 μV and 30 μV^2 respectively while that for small diameter electrodes were 66 μV and 300 μV^2 respectively. Using these values it was then possible to conduct a statistical analysis of secretion at visualized varicosities. Amplitude–frequency distributions of EJCs recorded with small electrodes from the varicosities shown in Fig. 3 are given in Fig. 5. There is good agreement between the experimental observations and the predictions according to Poisson statistics. Estimates of $\bar{m}$ for the various sets of varicosities recorded from are shown in Fig. 3: these also vary about threefold even though the number of varicosities beneath the electrode was constant.

The variability of $\bar{m}$ for different sets of visualized varicosities is summarized for a number of different axons in Fig. 6. For electrodes of a given size class, whether large diameter or small diameter, the value of $\bar{m}$ was not constant for a given number

of varicosities. For example the variation in $\bar{m}$ for six varicosities, shown in Fig. 6, was 0·8 to 3·0. Despite this variation in the size of $\bar{m}$ for different sets of varicosities, there was an increase in $\bar{m}$ as the number of varicosities beneath the recording electrode increased for both small electrodes and large electrodes (Fig. 6).

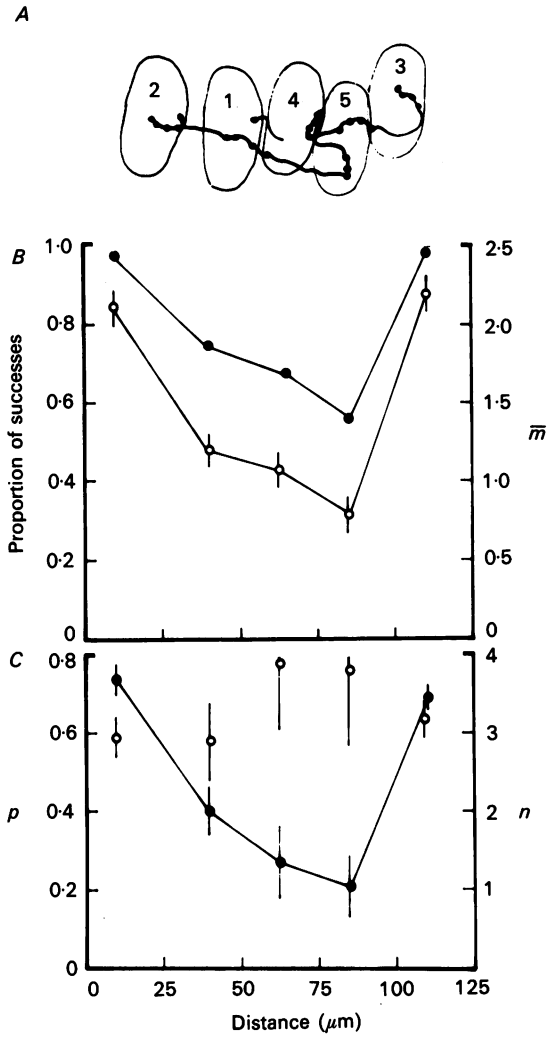

Fig. 7. Changes in the binomial parameters p and n along the length of a varicose axon recorded using a large diameter (20 μm) electrode. The upper panel (A) gives a drawing of the varicose terminal, derived from the $DiOC_2(5)$-fluoresced nerve; the ellipses give the outline of the recording-electrode tip and the numbers indicate the sequence in which recordings were made. The middle panel (B) gives the proportion of stimuli that gave an EJC at different positions along the length of the terminal axon (●) as well as $\bar{m}$ (○); the origin of the distance is determined as in Fig. 3. The bottom panel (C) gives the binomial parameters p and n for each recording site. Note that there is no significant change in the value of n whereas p follows the changes in $\bar{m}$. The vertical bars on the symbols give ± S.E.M.

Electrophysiological recordings of EJCs from visualized varicosities: observations described by binomial statistics

In some cases, the probability for secretion from sets of varicosities along a terminal branch was sufficiently high that secretion was best described by binomial

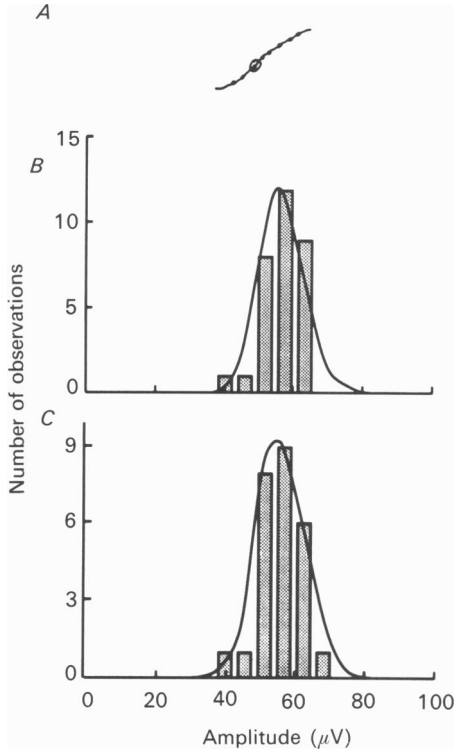

Fig. 8. Amplitude–frequency distribution of spontaneous EJCs (*B*) and EJCs (*C*) recorded with a very small diameter electrode (4 μm) from a single visualized varicosity ($DiOC_2(5)$-fluoresced), situated over 3 μm distant from other varicosities (*A*). The spontaneous EJC distribution in *B* had a mean and variance of $56 \pm 44\ \mu V^2$. The binomial fit to the distribution in *C* gave $\bar{m} = 0{\cdot}131 \pm 0{\cdot}024$, $p = 0{\cdot}124 \pm 0{\cdot}051$, $n = 1{\cdot}06 \pm 0{\cdot}43$ and number of failures was 174 (170). Number of trials was 200.

rather than Poisson statistics, allowing estimates to be made of p and n. Figure 7 shows such an example using a large electrode, in which the proportion of successes at each of five recording positions along a branch was high (range from 0·84 at position 2 to 0·32 at position 5). The amplitude–frequency distribution of EJCs at each of these recording sites was predicted by binomial statistics, allowing evaluation of $\bar{m}$, p and n at each site (Fig. 7). The decline in $\bar{m}$ along the length of the branch for the first four sets of varicosities (positions 2, 1, 4 and 5) was due to a decline in the probability of secretion p from $0{\cdot}74 \pm 0{\cdot}04$ to $0{\cdot}27 \pm 0{\cdot}08$; binomial parameter n remained reasonably constant (range $3 \pm 0{\cdot}5$ to $4 \pm 0{\cdot}8$) as did the number of varicosities beneath the electrode which was four to five for the first three sites (Fig.

7). Parameter n did not then give a reliable quantitative measure of the number of varicosities beneath the recording electrode. In one case (Fig. 8) a visualized varicose branch was found in which the varicosities were separated sufficiently for a 4 μm diameter electrode to record the secretion of quanta from a relatively isolated

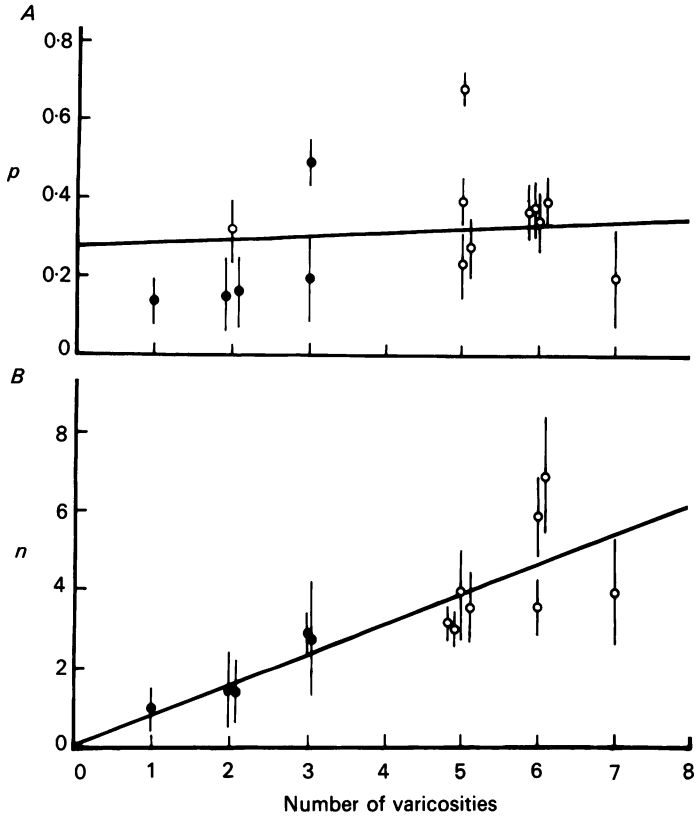

Fig. 9. Changes in the binomial parameters p and n with the number of varicosities recorded from. Filled symbols give observations from small diameter electrodes and open symbols give observations from large diameter electrodes. Vertical bars give $\pm$ S.E.M. There is no correlation between p and the number of varicosities (regression line has correlation coefficient of 0·27) whereas there is a correlation between binomial parameter n and the number of varicosities (regression line has a correlation coefficient of 0·74).

varicosity for over 2 h. Both spontaneous EJCs and evoked EJCs were recorded. The amplitude–frequency distribution of spontaneous EJCs formed a normal distribution (Fig. 8*B*) as did the distribution of evoked EJCs (Fig. 8*C*), and both had similar mean and modal values, indicating that the varicosity secreted at most one quantum per trial.

A comparison was made between the value of binomial parameter p and n, and the number of varicosities beneath both large- and small-diameter electrodes (Fig. 9). No correlation was found between p and the number of varicosities beneath the electrode (Fig. 9*A*) whereas n increased with the number of varicosities beneath the electrode

(Fig. 9*B*). The parameter n was nearly always less than the number of varicosities beneath the electrode, a result to be expected if the electrode recorded from a set of varicosities with a non-uniform probability for secretion (Bennett & Robinson, 1990).

DISCUSSION

Recording the extracellular signs of quantal transmission

Placing either small diameter or large diameter electrodes over the surface of a set of visualized varicosities with sufficient pressure to produce a small indentation of the muscle surface allows for the recording of EJCs. These are always of negative polarity, like the endplate currents recorded at the neuromuscular synapses (del Castillo & Katz, 1956). Such EJCs are not recorded when the electrode is displaced 5 μm laterally from the visualized varicosities, so it is likely that these alone give rise to the EJC, without contamination from varicosities buried a smooth muscle cell diameter or so beneath the surface of the tissue. The amplitude–frequency distributions of spontaneous EJCs using this technique is Gaussian, with a modal value of about 66 μV if small diameter microelectrodes (4–6 μm diameter) are used; the distribution is distinct from the noise level of about 10–20 μV. This is in contrast to the results when using a recording technique in which an external electrode of 30–80 μm diameter is placed on the muscle surface and suction applied to a pressure of 30 mmHg so as to produce a seal resistance of about 1 MΩ (Brock & Cunnane, 1991). In this case EJCs of positive polarity are often recorded together with those of negative polarity (Brock & Cunnane, 1987, 1988). Positive polarity EJCs are not observed when an electrode is placed over the surface of visualized varicosities and no suction is applied. These positive-polarity EJCs have been interpreted as due to transmitter secretion outside the electrode generating a potential change across the seal resistance between the edge of the electrode and the smooth muscle surface as a consequence of postjunctional current flow (Åstrand, Brock & Cunnane, 1988); negative polarity EJCs are then due to transmitter secretion from release sites within the electrode (Stuhmer, Roberts & Almers, 1983). The spontaneous negative polarity EJCs recorded with such suction electrodes placed randomly on the surface of the smooth muscle have an amplitude–frequency distribution skewed towards low amplitudes, with the smaller values lost in the noise level of 10–20 μV and the largest values at about 60 or 100 μV (Fig. 2 in Åstrand & Stjärne, 1988; Fig. 2 in Åstrand *et al.* 1988). Such amplitude–frequency distributions of spontaneous EJCs are similar to those observed with large diameter (20–50 μm) electrodes placed over visualized sets of varicosities, although in some cases the modal value of these distributions was observed to occur at amplitudes greater than the noise level. These skewed distributions of spontaneous EJCs have been attributed to either differences in the distances between varicosities and smooth muscle cells giving different diffusion distances for the transmitter, or to variations in the amount of transmitter in a quantum, or to variations in the density of receptors beneath the varicosities (Åstrand *et al.* 1988). However, the existence of Gaussian distributions of spontaneous EJCs when recorded with a small diameter electrode from visualized varicosities suggests that this is not the case: the skewed

distributions recorded with large diameter electrodes, especially of the suction type, may occur as a consequence of the spatial attenuation of currents arising from distant sources.

Poisson statistics of secretion at varicosities

The number of secretory events observed during 200 stimulation trials varied greatly for different sets of varicosities along single visualized branches, even when the number of varicosities within a set was approximately constant. The smaller evoked EJCs recorded with a small diameter electrode had the same modal size as did the spontaneous EJCs with a Gaussian amplitude distribution, suggesting that these were due to the secretion of individual quanta. The smallest amplitude EJCs recorded with a large diameter electrode had an amplitude distribution skewed into the noise level as did the spontaneous EJCs. Such amplitude distributions of spontaneous EJCs and evoked EJCs, skewed towards low amplitudes, have been observed by Brock & Cunnane (1988), using large diameter suction electrodes placed on the smooth muscle surface at random.

At most sets of varicosities recorded from with small diameter electrodes, the amplitude–frequency distribution of evoked EJCs could be predicted by Poisson statistics using the Gaussian distribution of spontaneous EJCs as a measure of the quantal unit size. The value of $\bar{m}$ determined in this way varied from one set of varicosities to another in much the same way as did the proportion of successes, indicating that the latter did give an indication of the secretory capacity of a set of varicosities. For large diameter electrodes, the skewed distribution of spontaneous EJCs was fitted by a gamma distribution; using this as a measure of the unit size gave reasonable fits to the amplitude distribution of evoked EJCs recorded with the electrodes. The $\bar{m}$ obtained in this way also varied from one set of varicosities to another in much the same way as did the proportion of successes.

There was considerable variation in $\bar{m}$ for a similar number of varicosities along individual terminal branches, suggesting differences in the probability of secretion from different varicosities. However, it is not known how many release sites occur in each of these varicosities. Many varicosities on arterioles come into close apposition to smooth muscle cells, with only basal lamina intervening between the two structures (Luff, McLachlan & Hirst, 1987). Some varicosities which come into close apposition with smooth muscle cells show no prejunctional specializations while most show one specialization (Luff & McLachlan, 1988). The number of such prejunctional specializations per varicosity in the vas deferens is not known. It is possible then that fluctuations in $\bar{m}$ between varicosities indicates a difference in the number of prejunctional specializations per varicosity rather than in the probability of secretion from each prejunctional specialization or release site. Another difficulty in relating $\bar{m}$ to the number of varicosities is that it is possible that not all varicosities secrete the transmitter which gives rise to the EJC. In the vas deferens the EJC is probably due to the secretion of ATP (Sneddon, Westfall & Fedan, 1982; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Allcorn, Cunnane & Kirkpatrick, 1986; McKenzie, Kirkpatrick & Burnstock, 1988), since α,β-methylene ATP totally blocks the EJC (Brock & Cunnane, 1988) as well as the response to ionphoretically applied ATP (Cunnane & Manchanda, 1988). Ionphoretically applied noradrenaline does not produce membrane potential changes (Cunnane & Manchanda, 1988) and is

thought to be a co-transmitter acting through second messenger systems to evoke a tonic contraction (Burnstock, 1988). Some varicosities may secrete ATP and others noradrenaline, as has been suggested (Ellis & Burnstock, 1989), so that the varicosities beneath an electrode may constitute a heterogeneous group, some giving rise to an EJC and others failing to do so. This seems unlikely since the distance between varicosities was the same when measured either with the $DiOC_2(5)$ technique or with the Faglu technique for identifying catecholamines, indicating that all varicosities contain noradrenaline. Whether all varicosities contain ATP that can be secreted remains to be determined (Fried, 1981).

Binomial statistics of secretion at varicosities

Some recordings from sets of varicosities gave EJCs with amplitude–frequency distributions that were best described by binomial statistics rather than Poisson statistics, as is the case for recordings from some sets of release sites at the amphibian neuromuscular junction (Bennett & Lavidis, 1988). Changes in $\bar{m}$ along single visualized branches were primarily due to changes in p rather than n, and this was the case whether small or large diameter electrodes were used. There was a positive correlation between the size of n and the number of varicosities under the electrode, as occurs for the number of release sites at inhibitory terminals on Mauthner neurones and n (Korn & Faber, 1987). However, unlike the terminals on Mauthner neurones, the regression line relating n to the number of varicosities underestimated these (see Fig. 9*B*). This could be due to non-uniformity in the probability of secretion for the different varicosities beneath the recording electrode. In this case $p = \bar{p} + (\sigma_p^2/\bar{p})$ where $\bar{p} = \Sigma p_j/N$, p_j is the probability of secretion from each varicosity and σ_p^2 is the variance in p_j form the N varicosities beneath the recording electrode (Bennett & Lavidis, 1979, 1989). Binomial p then increases with the variance of the p_j's so that an underestimate of binomial determined n is obtained if the p_j's are highly non-uniform. This is likely to be happening for most release sites of somatic motor nerve terminals as well as for those in the central nervous system (Bennett & Lavidis, 1979; Redman, 1990).

The quantal secretion from sets of varicosities which had p values in the range 0·15–0·68 was best predicted by binomial statistics. When $\bar{m}$ values of less then 0·2 were obtained from a set of varicosities, Poisson statistics best predicted quantal secretion. These estimates of the probability of secretion of a quantum from a varicosity are higher than those previously made for sympathetic varicosities in the rat tail artery (0·002–0·02; Åstrand & Stjärne, 1988), in arterioles (0·01–0·02; Hirst & Edwards, 1989; Hirst & Neild, 1980) and in the guinea-pig vas deferens (about 0·03; Brock & Cunnane, 1988). One reason for the higher probabilities recorded in the present work may be the higher calcium concentration of 4 mM compared with the 1·8–2·6 mM used in previous studies. Differences in probability may also arise from the different methods of estimation. In the present study small numbers of visualized varicosities were recorded from whereas in the study on arterioles an unknown number of varicosities gave rise to the recorded secretions and independent estimates of this number had to be made in order to calculate the probability. In the studies on the rat tail artery and guinea-pig vas deferens estimates of the probability of secretion involves identifying evoked EJCs of like amplitude as originating from the same release site (Brock & Cunnane, 1988). However, the present work shows that

the variance of the spontaneous EJCs recorded from two varicosities can be several times larger than the mean, suggesting the possibility that EJCs of considerably different amplitude can originate from a single site. One possibility is that some injury occurs to the visualized release sites when a small diameter electrode is placed in close proximity to them and that this has artifactually increased the probability for secretion. This problem will not be satisfactorily resolved until greater spatial resolution of the recording technique has been achieved so that secretions from single varicosities can be determined without placing the electrode on the varicose nerves themselves.

This work was supported by a National Health and Medical Research Council project grant and a Clive and Vera Ramaciotti equipment grant.

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