Journal of Physiology (1988), **399**, *pp.* 607–632
With 1 plate and 21 text-figures
Printed in Great Britain

ELECTRICAL ACTIVITY AT THE SYMPATHETIC NEUROEFFECTOR JUNCTION IN THE GUINEA-PIG VAS DEFERENS

BY JAMES A. BROCK AND THOMAS C. CUNNANE
From the University Department of Pharmacology, South Parks Road, Oxford OX1 3QT

(*Received 28 October 1987*)

SUMMARY

1. The relationship between the nerve terminal action potential and transmitter release from sympathetic postganglionic nerve terminals has been studied *in vitro* by focal extracellular recording.

2. In the absence of stimulation, 'spontaneous excitatory junction currents' (SEJCs) were recorded with amplitudes up to 500 μV, durations of 50–80 ms and frequencies of occurrence of 0·3–0·05 Hz; SEJCs of unusually long time course were also observed. The SEJCs were not recorded in tissues pre-treated with 6-hydroxydopamine to destroy sympathetic nerves, were unaffected by tetrodotoxin (TTX), the competitive α-adrenoceptor antagonists, prazosin and phentolamine, the irreversible α-adrenoceptor antagonist benextramine but were blocked by α,β-methylene ATP which desensitizes P_2-purinoceptors.

3. During trains of supramaximal stimuli at 0·1–4 Hz stimulus locked 'excitatory junction currents' (EJCs) were evoked intermittently from the population of varicosities located under the suction electrode with a probability of occurrence of 0·005–0·8. Although EJCs occurred intermittently, they were always preceded by an associated, non-intermittent, nerve impulse (delay $\leqslant$ 3 ms).

4. The EJCs reflect transmitter release from nerves because they were abolished by TTX, removal of calcium from the bathing medium, exposure to α,β-methylene ATP and exhibited frequency-dependent facilitation.

5. Amplitude distributions of SEJCs and EJCs recorded in the same attachment were similar and skewed towards low-amplitude events. Individual SEJCs and EJCs could be found which were identical in amplitude and time course.

6. Locally applied TTX blocked impulse propagation and transmitter release in the terminal regions; electrotonic invasion of the terminals from the point of block did not activate the transmitter release process.

7. These studies indicate that (1) intermittence of transmitter release is caused by a low probability of release in the invaded varicosity and is not caused by conduction failure in the terminal regions, (2) only a single quantum is normally secreted when the release mechanism of a varicosity is activated by the nerve impulse and (3) active invasion of the terminals is necessary for transmitter release to occur.

INTRODUCTION

For a number of reasons, it has been difficult to analyse the transmitter release mechanism at the level of the individual varicosity at the autonomic neuroeffector junction. Burnstock & Holman (1961, 1962) were the first to demonstrate using intracellular recording techniques that transmitter release was quantal at the sympathetic neuroeffector junction. However, because of multiple innervation of smooth muscle and electrical coupling between cells the question of the size of the transmitter quantum remained unanswered. Biochemical studies of noradrenaline release from sympathetic nerve terminals revealed a disparity between the amount of transmitter released from a varicosity and that estimated to be contained in a single vesicle (Folkow, Häggendal & Lisander, 1967). Assuming that the major part of the tissue noradrenaline is vesicular, then estimates of the number of vesicles per varicosity indicate that the 'average' varicosity when stimulated releases the equivalent of only 1% of the contents of a single vesicle (see Smith & Winckler, 1972). To explain these findings two conflicting hypotheses were put forward: either the vesicle was not the basic unit of transmission and the quantum was a fraction of the transmitter content of one vesicle (Folkow *et al.* 1967) or alternatively individual varicosities do not normally release transmitter when stimulated. In the latter case release is intermittent and the entire transmitter content of a vesicle is the quantum (Bevan, Chesher & Su, 1969; Folkow & Häggendal, 1970). This issue was resolved when it was demonstrated that transmitter release from the sympathetic nerve terminals in the vas deferens of the guinea-pig occurred intermittently (Blakeley & Cunnane, 1978, 1979). Several questions remained unanswered because techniques were not available to measure impulse propagation in sympathetic nerve terminals (Cunnane, 1984; Cunnane & Stjärne, 1984*a*). In particular, it has not been possible to distinguish between the two possible causes of intermittence, namely a low probability of transmitter release in the depolarized terminals or intermittent invasion (Alberts, Bartfai & Stjärne, 1981).

At the skeletal neuromuscular junction, much useful information about the mechanisms involved in the release of transmitter has been obtained using focal extracellular recording (Fatt & Katz, 1952; Del Castillo & Katz, 1956). Recently we have developed a method of focal extracellular recording to study the relationship between the nerve terminal action potential and transmitter release at the sympathetic neuroeffector junction and have demonstrated that invasion failure does not occur (Brock & Cunnane, 1987*a*). The aim of the present investigation was to carry out further studies on impulse propagation and the transmitter release process in postganglionic sympathetic nerve terminals. Preliminary reports of some of these findings have been published previously (Brock & Cunnane, 1987*a*, *b*).

METHODS

Male guinea-pigs (Duncan Hartley, 350–600 g) were killed by a blow to the head and bled. Vasa deferentia were removed and individual preparations pinned to the Sylgard (Dow Corning)-covered base of a 3 ml glass organ bath, which was mounted on the stage of a Zeiss ACM microscope. The organ bath was perfused continuously at 1–3 ml/min with Krebs solution of the following composition (mM): NaCl, 118·4; $NaHCO_3$, 25·0; NaH_2PO_4, 1·13; $CaCl_2$, 2·6; KCl, 4·7; $MgCl_2$, 1·3;

and glucose, 11·0. The solution was gassed with a mixture of 95% O_2 and 5% CO_2 (to pH 7·4) and maintained at 36–37 °C. The vas deferens was electrically stimulated indirectly through the hypogastric nerve trunk (Ag–AgCl suction electrode) or by field stimulation of the prostatic end of the vas deferens (Ag–AgCl ring electrodes, o.d. 2 mm). The stimulus parameters were modified as required throughout the experiment (pulse width 0·01–0·1 ms, 7–30 V).

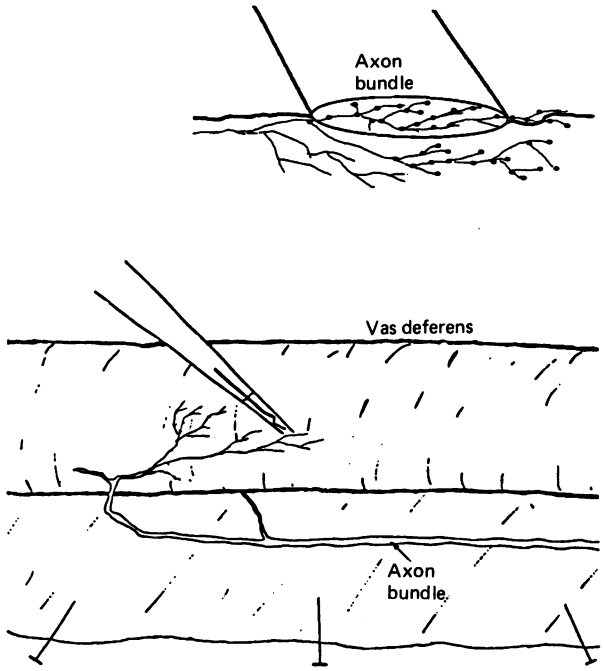


Fig. 1. Schematic illustration of extracellular recording using a suction electrode from the surface of the guinea-pig vas deferens. The upper panel shows an expanded view of the electrode tip.

A window was cut in the connective tissue sheath enveloping the vas deferens, and a bevelled glass electrode (tip diameter $< 50\ \mu m$) applied to the muscle surface with slight suction (seal resistance $< 1\ M\Omega$). A sintered Ag–AgCl pellet in the organ bath served as the indifferent electrode. A schematic illustration of the experimental set up is shown in Fig. 1. Electrical activity was recorded through an AC amplifier (Neurolog NL104, low-frequency cut-off 0·1 Hz) and the output fed through a low-pass filter (Neurolog NL125) with the cut-off set at 1·5 kHz. There is good reason to believe that this method of recording measures the current underlying the intracellular potential changes (Brooks & Eccles, 1947; Del Castillo & Katz, 1956). It should be remembered that the absolute size of any record depends on many factors (e.g. seal resistance, size of the nerve fibre and relationship of the transmitter release site to the smooth muscle cell) and therefore absolute comparisons between different attachments have little quantitative meaning. However, we have included a calibration bar in microvolts on all records to indicate the size of the signals obtained in different attachments.

Spontaneous excitatory junction potentials (SEJPs) and excitatory junction potentials (EJPs) were also recorded intracellularly using glass microelectrodes filled with 5 M-potassium acetate (resistances 30–60 MΩ) (Cunnane & Stjärne, 1984*b*).

Electron microscopy. Tissue was prepared for electron microscopy essentially as previously described (Totterdell & Smith, 1986). Briefly, guinea-pigs were anaesthetized with sodium pentobarbitone (240 mg/kg. I.P.) and the aorta cannulated. After an initial 2 min perfusion with 0·9% (w/v) NaCl, guinea-pigs were perfused with 2% glutaraldehyde and 0·1% paraformaldehyde in 0·1 M-phosphate buffer (pH 7·4). Fixed vasa deferentia were dissected and placed for 1 h in fresh

fixative. A piece of vas was taken which had not been handled and blocks of tissue dissected out and thoroughly washed in phosphate buffer (0·1 M). Following washing, blocks were placed in 1% osmium tetroxide in 0·1 M-phosphate buffer for 40 min. Blocks were then dehydrated in a graded alcohol series and embedded in resin (Durcupan, ACM, Fluka). Ultrathin sections were cut and collected on Formvar-coated single slot grids and examined in a Philips 201C electron microscope at 80 kV. Contrast of the sections was enhanced by uranyl acetate (1% in 70% ethanol for 40 min) and lead citrate (Reynolds, 1963).

6-Hydroxydopamine pre-treatment. Guinea-pigs were pre-treated with 6-hydroxydopamine according to the following protocol: the animals were injected (I.P.) with 6-hydroxydopamine, 150 mg/kg on day 1 and 250 mg/kg on day 2; the animals were killed on day 3.

Histochemistry. The extent of the degeneration of sympathetic nerves was assessed histochemically using a modification of the Falck–Hillarp technique (Gillespie & Kirpekar, 1966). Sections (6 μm) were cut and mounted on a Carl Zeiss ACM photomicroscope, equipped with a IV Fl epifluorescence system. The light source was an Osram HB50 mercury lamp; the filters used were: exciter-interference BP405/8; barrier-LP418; dichromatic beam splitter FT420.

Data collection and analysis. A system based on an IBM.AT microcomputer and a Data Translation DT2801A analog-to-digital converter card was used to digitize and analyse electrophysiological signals previously recorded on tape. Records were normally digitized at 1–8 kHz, 512 points) and the amplitude, rise time and time constant of decay of individual events measured using a computer program written in compiled BASICA (DVIEWTC). Baseline noise was determined by averaging the initial part of the digitized signal (points 15 to 45) and subsequently overlapping groups of five points were scanned to determine the point with a peak value. Peak amplitude of individual excitatory junction currents was calculated by subtracting the mean of three points about this peak value from the mean background noise. The 10–90% rise time and time constant of decay were determined by linear regression analysis. The rise time from 10 to 90% of the peak value was assumed to be linear and the decay from 90 to 10% of the peak value monoexponential. Selected records were measured manually using cursor controls from time to time to check the accuracy of the fit.

Drugs. These were benextramine tetrahydrochloride (Sigma), bretylium tosylate (Wellcome), desipramine hydrochloride (Sigma), 6-hydroxydopamine hydrobromide (Sigma), α,β-methylene adenosine 5′-triphosphate (Sigma), prazosin hydrochloride (Pfizer), phentolamine mesylate (Ciba), tetrodotoxin (Sigma) and sodium pentobarbitone (Sagital, May & Baker). The 6-hydroxydopamine was made up in saline immediately before use. Prazosin was made up as a stock solution in *N*,*N*-dimethylacetamide (Sigma) and diluted as required in Krebs solution. The remaining drugs were made up as concentrated stock solutions in distilled water and serially diluted in Krebs solution to the required final concentration.

RESULTS

Spontaneous electrical activity

When a small suction electrode was applied to the surface of the vas deferens, in the absence of stimulation, spontaneous negative-going potentials were recorded. Figure 2*A* shows examples of extracellularly and intracellularly recorded spontaneous events (for comparison). The characteristic features of spontaneous extracellular events can be summarized briefly. First, the amplitude of individual events varied between 500 μV and the noise level of the recording system (maximum 10 μV peak-to-peak). In twenty-six attachments the mean amplitude was 36 ± 21 μV (mean $\pm$ S.D. of the group mean), $n = 3023$ events. Secondly, the mean frequency of occurrence was $0{\cdot}14 \pm 0{\cdot}001$ Hz (mean $\pm$ S.D), $n = 26$ attachments. Thirdly, the mean rise time, measured as the time taken for the signal to rise from 10 to 90% of its maximum amplitude was $8{\cdot}7 \pm 1{\cdot}1$ ms (mean $\pm$ S.D. of the group mean), $n = 3023$ and the time constant of decay was $26{\cdot}8 \pm 2{\cdot}9$ ms, $n = 3023$. Individual events normally had a total duration of 50–80 ms but a class of spontaneous event was observed with

an unusually long time course lasting several hundred milliseconds. An example of a spontaneous event with a prolonged time course is shown in Fig. 2*B* together with a spontaneous event of normal duration. The distribution of amplitudes of the spontaneous events in each recording was skewed towards the low-amplitude events (Fig. 3).

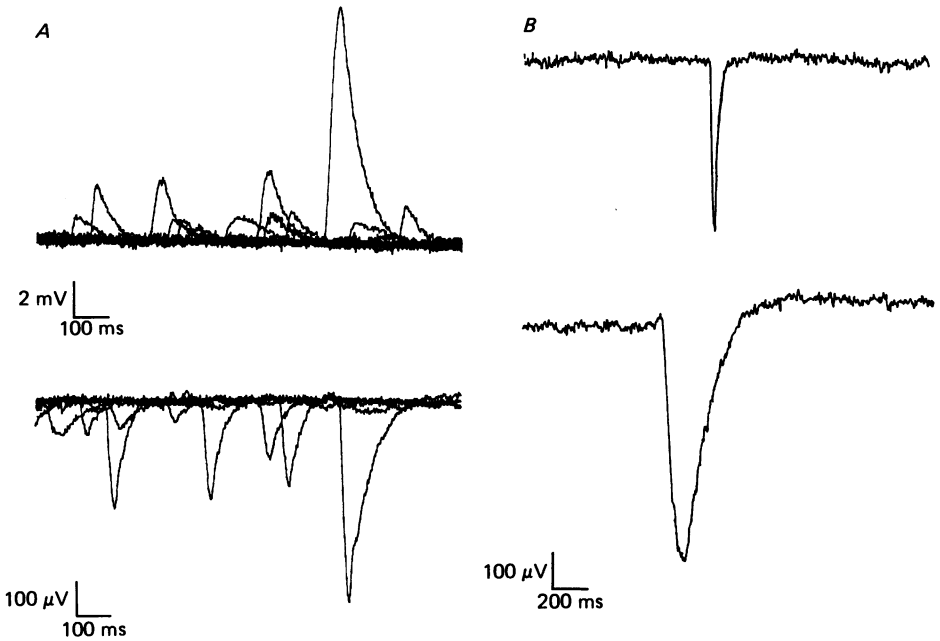


Fig. 2. *A*, spontaneous activity recorded with an intracellular microelectrode (upper panel) and an extracellular suction electrode (lower panel) from the guinea-pig isolated vas deferens. A series of random traces are superimposed to show the characteristic features of both types of spontaneous event. *B*, example of one unusually slow spontaneous excitatory junction current (lower panel) displayed together with one of normal time course (upper panel).

A number of pharmacological agents and procedures were used to establish that these extracellularly recorded events corresponded to the intracellularly recorded spontaneous excitatory junction potentials (SEJPs). The extracellularly recorded SEJCs were unaffected by the competitive α-adrenoceptor antagonists, prazosin and phentolamine (10^{-7} to 10^{-5} M), the irreversible α-adrenoceptor antagonist, benextramine, but blocked by α,β-methylene ATP (3×10^{-6} M) (Fig. 4), which desensitizes P_2-purinoceptors (Kasakov & Burnstock, 1982). They were also unaffected by tetrodotoxin (3×10^{-7} to 10^{-6} M), suggesting that they are not caused by spontaneously generated sodium-dependent nerve impulses in the terminals. Spontaneous events were still recorded when the calcium content of the Krebs had been reduced to 'zero' and in the presence of the inorganic calcium entry blocker, cobalt (0·1–2 mM). In tissues from guinea-pigs which had been treated with 6-hydroxydopamine to destroy the sympathetic nerves the majority of spontaneous events were abolished. Sympathetic denervation was confirmed using Falck histochemistry. Our results suggest that these spontaneous potentials are the

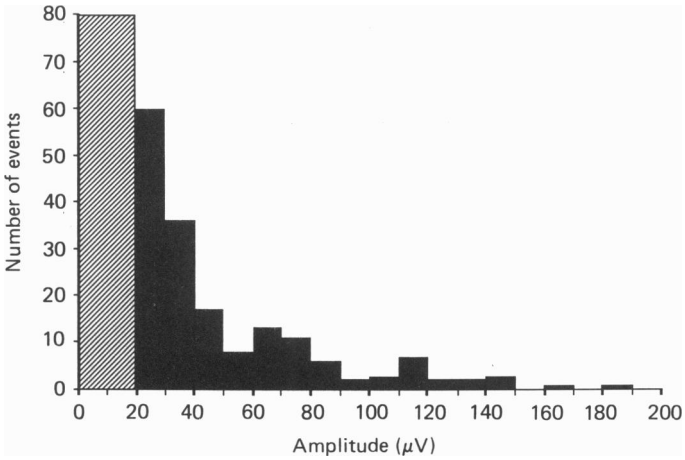


Fig. 3. Amplitude distribution of SEJCs. Recording was made of 182 SEJCs in a single attachment for 12 min. The histogram shows that the SEJC distribution is normally skewed towards low-amplitude events. The shaded area represents twice background noise level and is the level where the presence or absence of events could not be stated with any confidence.

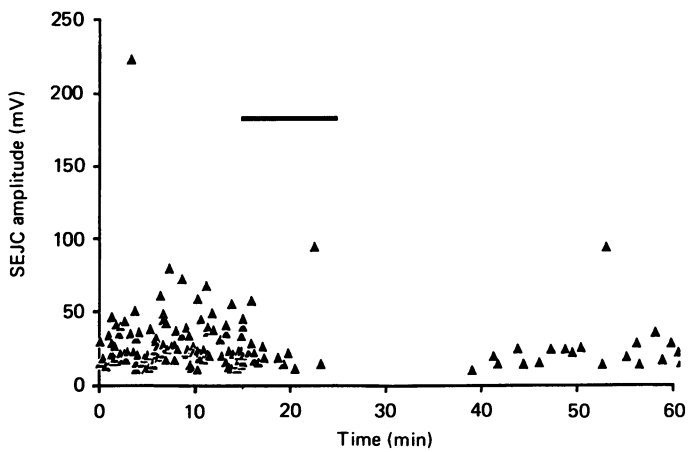


Fig. 4. Effects of α,β-methylene-ATP on SEJCs recorded from the surface of the guinea-pig vas deferens. The Figure shows the amplitudes of all SEJCs recorded before, during the application of 1 μM-α,β-methylene-ATP for 10 min (bar denotes period of application) and after wash for 35 min.

extracellular equivalent of the SEJP recorded intracellularly in smooth muscle cells. By analogy with focal recording at the skeletal neuromuscular junction (Fatt & Katz, 1952; Del Castillo & Katz, 1956), these potentials are likely to be a good measure of the time course of the current underlying the SEJP and have been termed 'spontaneous excitatory junction currents' (SEJCs). For technical reasons it has not yet been possible to record simultaneously the same event both intracellularly and extracellularly but it is clear from separate studies that the mean total duration of the SEJP is similar to that of the SEJC. Histograms of the distributions of time

constants for SEJCs and SEJPs are shown in Fig. 5*A*. Semilogarithmic plots of the decay of representative SEJCs and SEJPs are shown in Fig. 5*B*.

Spontaneous electrical activity recorded from two adjacent sites

An attempt was made to determine the extent of the spread of the extracellular field by recording spontaneous activity simultaneously from two extracellular

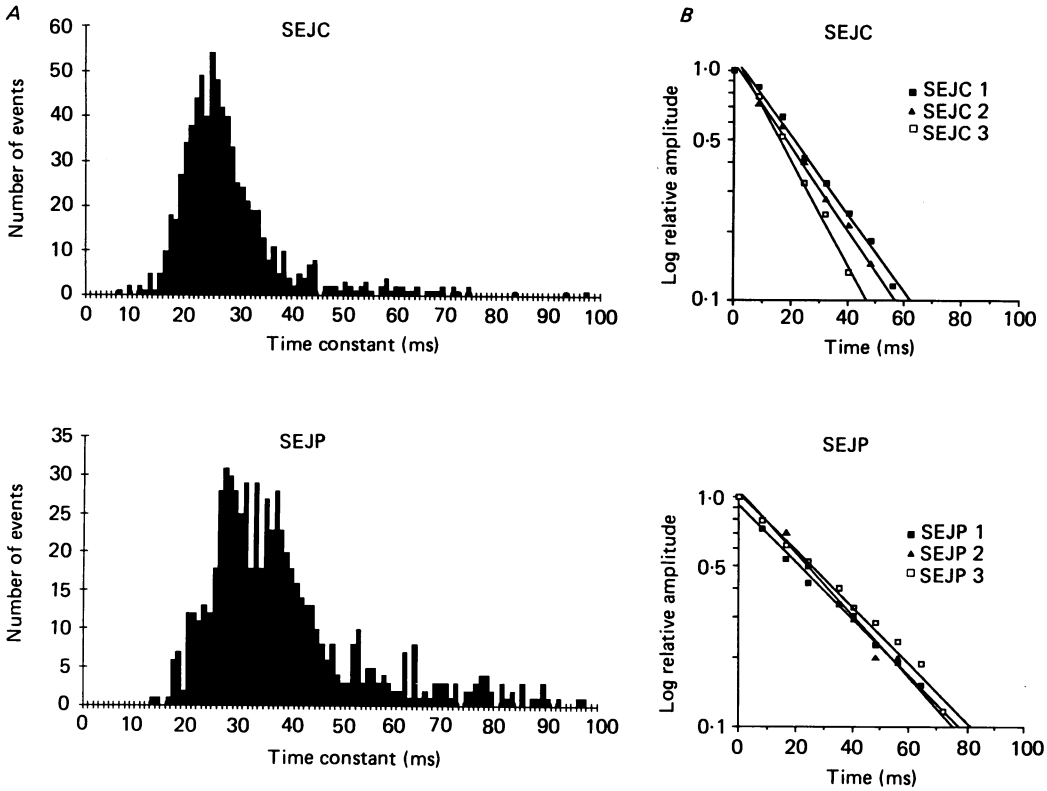


Fig. 5. Time course of spontaneous events. *A*, time constant distribution of SEJCs recorded in a single attachment ($n = 720$) (upper panel) and of SEJPs (lower panel). The SEJP distribution shows the time constants of 699 SEJPs recorded in ten separate impalements. In any one impalement the overall form of the distribution was similar. *B*, semilogarithmic plots of the decay of representative SEJCs (upper panel) and SEJPs (lower panel).

electrodes placed next to one another. In each electrode SEJCs were recorded of comparable amplitude and time course but they were largely independent of one another (Fig. 6). However, occasionally coincident SEJCs were recorded in both electrodes. When using extracellular suction electrodes a spontaneous event in one electrode will normally be recorded with an inverted polarity with respect to the same event in a second extracellular electrode. Inspection of Fig. 6 shows that whenever simultaneous SEJCs were recorded, there was a marked attenuation of the SEJC in one electrode with respect to the other, suggesting that the length constant of the extracellular field is of the order of 50 μm or less.

Evoked electrical activity

The patterns of postjunctional electrical activity recorded depended to a large extent on the position of the suction electrode and the stimulation parameters employed but could in general be divided into three classes. The first consisted of

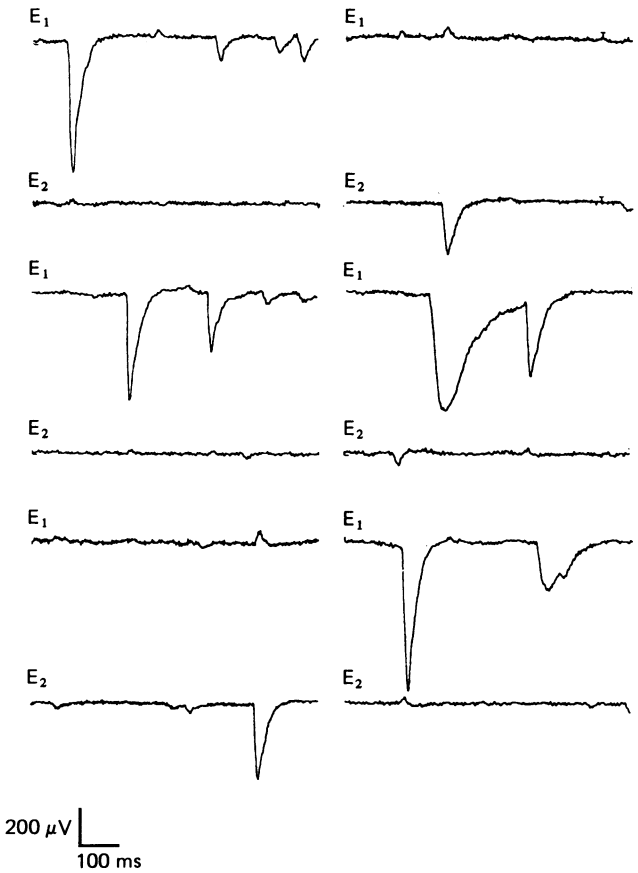


Fig. 6. Simultaneous recording of spontaneous excitatory junction currents (SEJCs) from the surface of the guinea-pig vas deferens using two adjacent extracellular suction electrodes (E_1 and E_2). The SEJCs recorded in each electrode were of comparable amplitude and time course. However, it is important to note that SEJCs recorded with one electrode were not normally detected by the second electrode.

stimulus-locked negative-going potentials (Fig. 7) whose amplitude and time courses were similar to those of the SEJCs in the same attachment. It was often necessary to move the suction electrode to several locations before this pattern of activity was recorded. This pattern was usually observed following stimulation at low frequencies and stimulation intensities when only one or a few vas deferens nerve fibre(s) were excited. At all frequencies of stimulation (0·1–4 Hz) evoked events occurred intermittently. The characteristics of these potentials were similar to those of the intracellularly recorded discrete events previously described in the same tissue

(Blakeley & Cunnane, 1979) in that at a constant latency they (1) occurred intermittently, (2) varied in amplitude and (3) had similar amplitudes and time courses to the SEJCs recorded in the same attachment. These brief negative-going potentials have been termed excitatory junction currents (EJCs) and probably reflect the release of transmitter from varicosities located under the suction

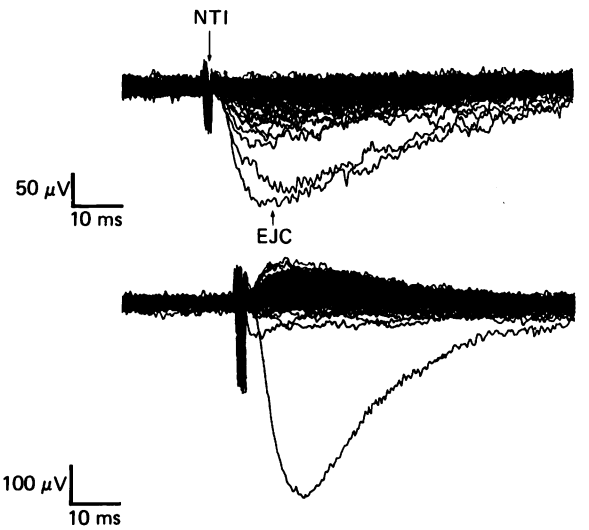


Fig. 7. Simultaneous measurement of the nerve terminal impulse (NTI) and evoked excitatory junction currents (EJCs) recorded extracellularly (continuous train of fifty impulses at 1 Hz) in two different attachments, showing that transmitter release is intermittent and that the pattern of activity varies from attachment to attachment.

electrode. It is worth noting at this stage that all EJCs were preceded by a non-intermittent nerve impulse (Fig. 7) which will be considered in some detail in a later part of the Results section. Figure 8 shows semilogarithmic plots of the decay of a representative SEJC, an evoked EJC and an intracellularly recorded SEJP showing their time constants to be very similar and brief. The decay of a representative EJP is also shown for comparison. Note that the time constant of decay of an EJP is very long, presumably because the major contributions to the EJP arise from the electrotonic spread of activity from many distant release sites.

The second pattern of postjunctional electrical activity was observed when the stimulation intensity was increased further so that more nerve fibres were recruited, and differed from the first in that this pattern was non-intermittent and positive going (Fig. 9), graded with stimulus strength and usually exhibited frequency-dependent facilitation in a manner reminiscent of the intracellularly recorded EJP. These positive-going potentials are interpreted to be the extracellular equivalent of the EJP representing the integrated activity of transmitter released from many varicosities acting on smooth muscle cells outside the suction electrode. The recorded signal is now positive-going since local current is flowing outward as a result of transmitter-induced membrane activity at adjacent sites outside the recording electrode. In some circumstances it is clear that the net current flow will reflect the

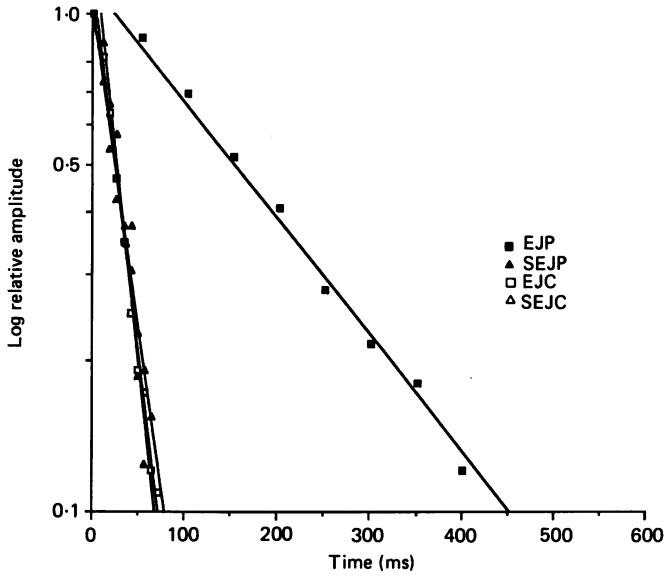


Fig. 8. Semilogarithmic plots of the decay of a representative SEJP, SEJC, EJC and EJP. The plot shows that the time course of the intracellularly recorded SEJP is similar to that of the extracellularly recorded SEJC and the evoked EJC, suggesting that the time course of the current underlying the EJP is brief compared to its duration.

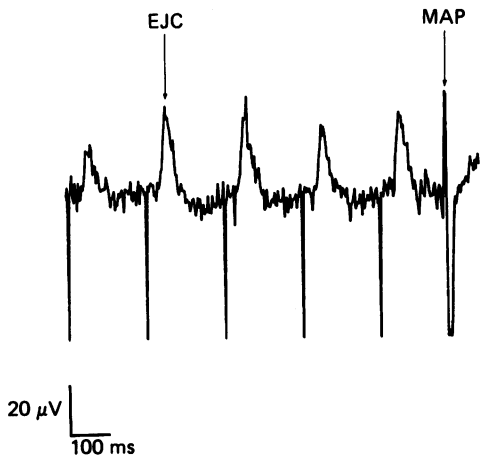


Fig. 9. Positive-going EJCs recorded following stimulation at high intensity showing the EJCs triggering the smooth muscle action potential MAP. The negative potential preceding each EJC is the stimulus artifact. It is important to note that at this frequency of stimulation no summation of EJPs was recorded (unlike intracellularly recorded EJPs).

difference between the inward and outward components which can lead to errors in estimating the true time course of the current.

The third pattern of postjunctional activity recorded from the surface of the vas deferens was observed when the frequency of stimulation was increased from 1 to 2–5 Hz. Under these conditions, the integrated positive-going EJCs (representing the

summed activity of many distant release sites) exhibited frequency-dependent facilitation and were observed to trigger a brief potential associated with contraction of the vas which we have interpreted as the smooth muscle action potential (Fig. 9).

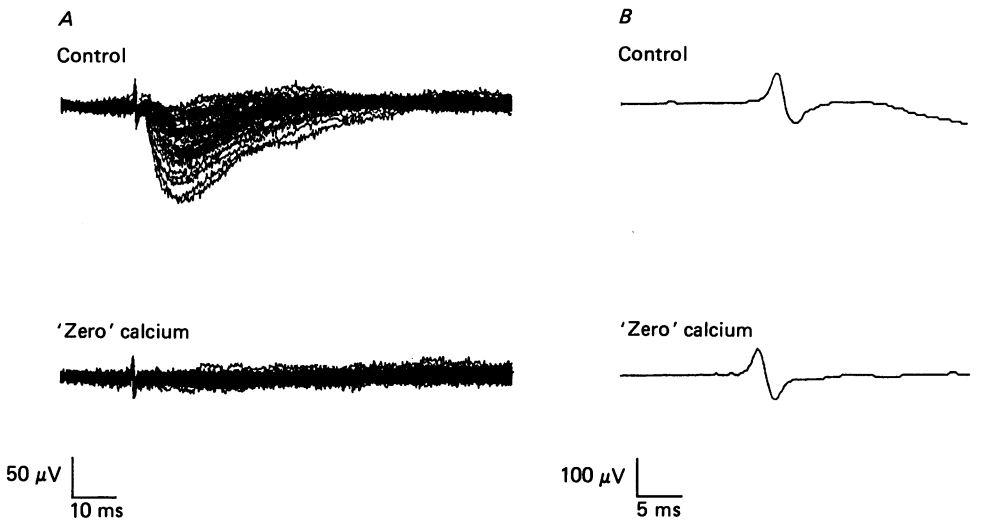


Fig. 10. Effects of 'zero' calcium on evoked electrical activity recorded extracellularly from the surface of the guinea-pig vas deferens. *A*, nerve impulses and EJCs evoked by trains of fifty pulses at 1 Hz recorded before and 10 min after the removal of calcium from the Krebs solution perfusing both the inside and outside of the suction electrode. *B*, averages of eighty nerve impulses recorded in the same attachment before, and during perfusion with Krebs solution containing no added calcium. At a time when transmitter release was abolished there was only a small effect on the shape of the nerve terminal impulse. However, progressively with time, the nerve terminal impulse became briefer.

Pharmacology of the excitatory junction current

In all respects the pharmacology of the EJC was similar to that of the SEJC (see above) with the exception that evoked transmitter release was absolutely dependent on the presence of extracellular calcium (Fig. 10*A*). The effects of 'zero' calcium on the shape of the nerve terminal impulse are shown in Fig. 10*B*. When release was completely inhibited there was only a small effect on the nerve terminal impulse but with time this effect became more marked (20 min), the nerve terminal impulse becoming briefer (not shown).

Nerve terminal impulse and transmitter release

On every occasion that EJCs have been recorded they were preceded by a non-intermittent nerve impulse (see Fig. 7). We believe this nerve impulse was generated in the varicose fibres from which transmitter release was evoked. The link between the arrival of the nerve impulse in the secretory terminals and transmitter release was established by deliberately setting the stimulus strength close to threshold. In this way we could ensure that not every stimulus evoked a nerve impulse in the parent axon so that during trains of several hundred stimuli at 1 Hz the nerve impulse

arrived 'intermittently' in the terminals; on every occasion that an EJC was elicited it was always preceded by a nerve impulse.

On many occasions nerve impulses were recorded from small nerve bundles running on the surface of the vas deferens but these impulses were not associated with local transmitter release; this activity originating in nerve fibres running to innervate more distal regions of the vas deferens.

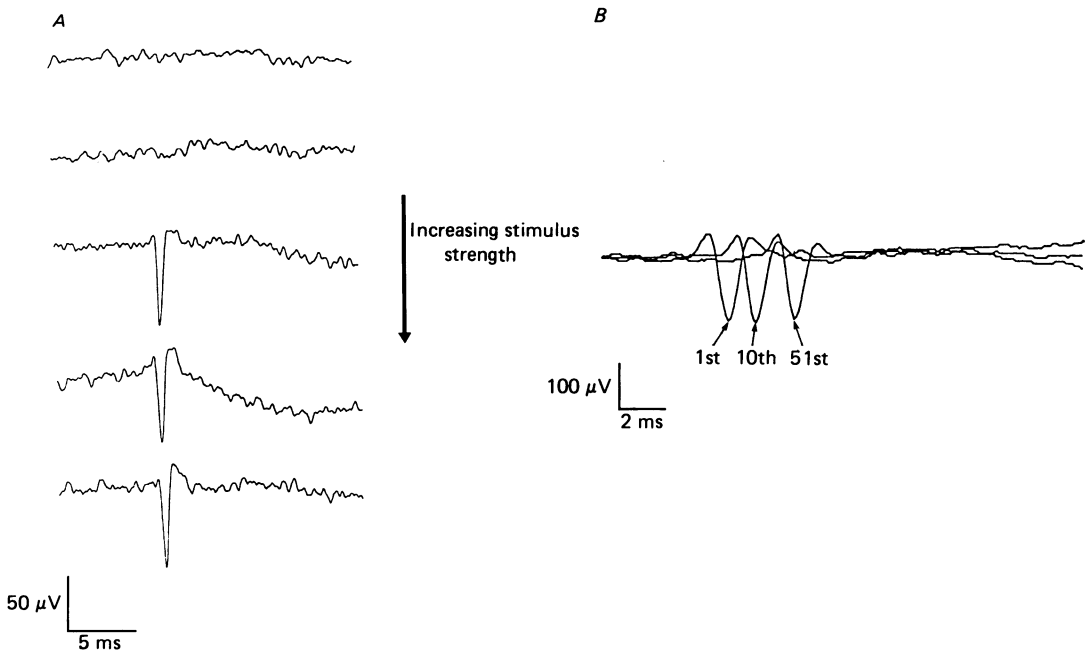


Fig. 11. *A*, effect of small increases in stimulus intensity on the activity of a 'single unit'. The nerve impulse was an all-or-none event of constant amplitude and latency. *B*, increase in the latency of the nerve terminal impulse during a train of stimuli at 4 Hz. Although the latency increased by several milliseconds, the nerve impulse retained its shape, suggesting that it was not a compound 'spike'. The number refers to the position of the nerve terminal impulse in the train.

It was of particular interest to determine whether there was a variation in the size of the invading nerve impulse when transmitter release occurred and when it did not, which might have accounted for intermittence. Apparent variation in the size of the nerve impulse could be accounted for by the variance in the background noise. Thus variation in the size of the nerve impulse cannot account for failure of release because there were no detectable differences between averages of nerve impulses which evoked transmitter release and those which did not (see Fig. 20).

The question naturally arises whether the nerve impulses recorded were single-unit or compound 'spikes'. We believe they are single-unit recordings because they were evoked in an all-or-none manner about the stimulus threshold (Fig. 11*A*) and retained the same shape during repetitive stimulation (Fig. 11*B*). When impulses were recorded from fibres with similar thresholds and conduction velocities the 'multiunit' response could usually be broken down into separate components by

these procedures. In preliminary studies we have demonstrated using electron microscopy that single varicose fibres run on the surface of the vas deferens (see Plate 1). However, it will be necessary to correlate the electrical activity recorded with the microanatomy of the region under the suction electrode to determine how many varicosities might have contributed to the electrical activity recorded.

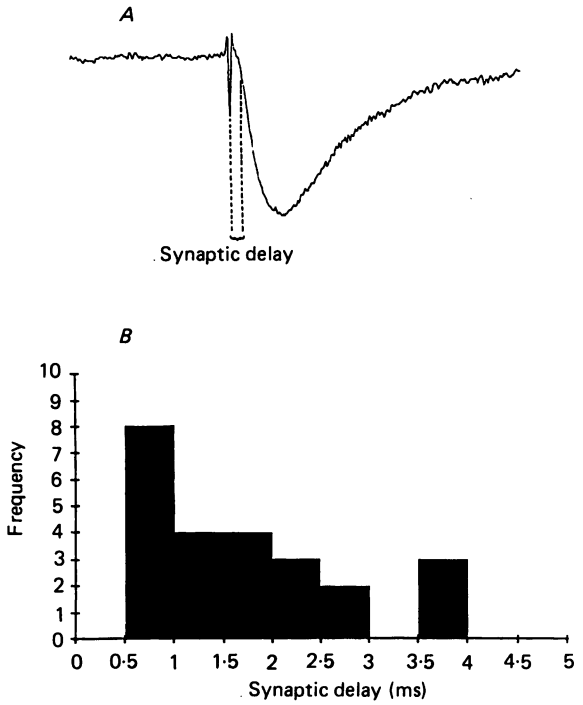


Fig. 12. *A*, schematic illustration of the measurement of synaptic delay. *B*, histogram of the spread of latencies in one attachment at 36 °C.

Synaptic delay at the sympathetic neuroeffector junction

The delay between the arrival of the nerve impulse and transmitter release was taken to be the interval between the peak negative value of the nerve impulse and the start of the EJC; the time taken for the EJC to reach 10% of its peak value was found to be the most reliable measure of the start of the EJC (Fig. 12*A*). Typically the mean synaptic delay at 36 °C was found to be between 1 and 3 ms but there was a spread of latencies for any series of evoked EJCs. A histogram of the spread of synaptic delays measured in one experiment when EJCs were evoked at 1 Hz is shown in Fig. 12*B*. The significance of the spread of latencies requires further investigation.

Pharmacology of the nerve terminal action impulse

Effects of adrenergic neurone blockers. Pharmacological evidence also indicates that the electrical impulse preceding the EJC is likely to be the extracellular equivalent of the nerve terminal action potential. The nerve impulse was blocked locally by the adrenergic neurone blocking agent bretylium (Fig. 13*A*), at a concentration 100

times lower than that required to block impulse propagation when the drug was applied to more proximal regions of the nerve. The specificity of this agent arises from the fact that it enters sympathetic nerve terminals by a specific neuronal uptake process for catecholamines (Iversen, 1967). The uptake$_1$ inhibitor desipramine (10^{-6} M) protected the terminals in this region from the effects of bretylium (Fig. 14).

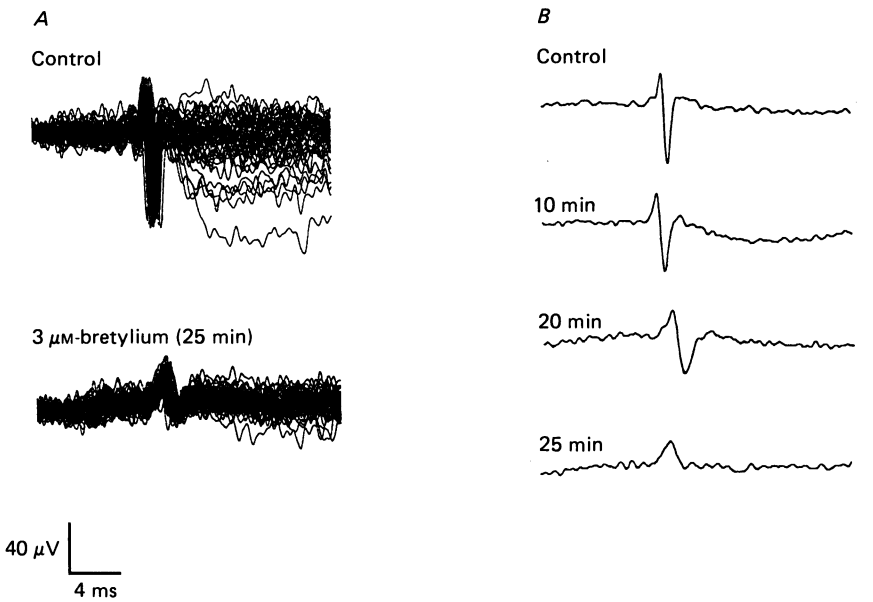


Fig. 13. Effects of bretylium on impulse propagation and transmitter release from sympathetic nerve terminals. *A*, nerve impulses and EJCs evoked by trains of fifty pulses at 1 Hz recorded before and 25 min after the addition of 3×10^{-6} M-bretylium to the bathing solution. *B*, averages of twenty nerve impulses recorded in the same attachment before and at various times following the addition of bretylium. At a time when transmitter release was abolished, the active propagation of the nerve impulse was blocked, leaving a positive-going potential which represents the electrotonic spread of potential from the point of block.

Local application of tetrodotoxin. In order to determine the characteristic features of impulse propagation in sympathetic nerve terminals, the method was modified to allow internal perfusion of the suction electrode so that the effects of local application of substances to the site of recording could be studied. It was of particular interest to apply tetrodotoxin (TTX) to determine whether the action potential actively invades sympathetic nerve terminals. The tip of the suction electrode was perfused at a rate of 50 μl/min with either Krebs solution or Krebs solution containing TTX. Tetrodotoxin (10^{-6} M) blocked the inward current of the nerve terminal impulse and abolished transmitter release; spontaneous transmitter release was unaffected. The recorded electrotonic depolarization of the varicosities spreading from the point of local block was insufficient to trigger the transmitter release mechanism (Fig. 15). The effects of TTX were readily reversed by wash. Release from varicosities located

outside the electrode was unaffected by TTX applied within the electrode, demonstrating that significant leakage to the exterior did not occur.

In general, three shapes of nerve impulse were recorded which may well reflect recordings made from different regions of the terminals: first, the classical triphasic, positive–negative–positive signal reflecting the extracellular equivalent of the

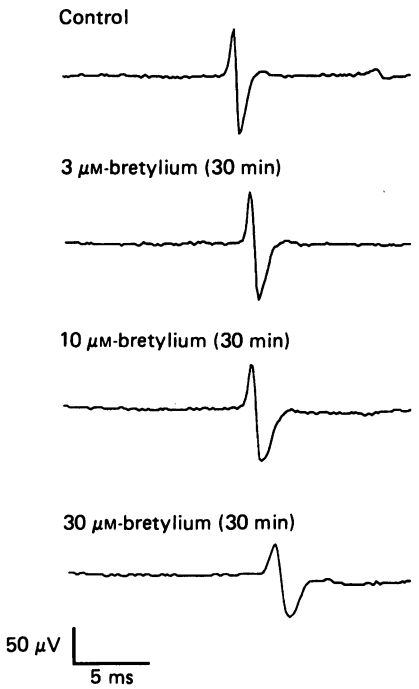


Fig. 14. Effects of bretylium in the presence of 10^{-6} M-desipramine. When neuronal uptake was inhibited the terminals were protected from the inhibitory effects of bretylium at concentrations up to 10 times those normally required to inhibit impulse propagation.

propagating nerve impulse; secondly, a more diphasic, positive–negative-going nerve impulse; thirdly, an impulse which was largely positive going with only a small negative-going component. Tetrodotoxin powerfully inhibited transmitter release regardless of the shape of the nerve impulse. Examples of each type of nerve impulse recorded are shown in Fig. 16.

Quantal content of the excitatory junction current. The relationship of the evoked EJC to the SEJC was established by comparing the amplitude distributions of a series of evoked and spontaneous currents in the same attachment. Amplitude distributions of SEJCs recorded either during the intervals between individual stimuli or in the absence of stimulation were similar. The amplitude distributions of EJCs evoked at four different frequencies of nerve stimulation (500 stimuli at 0·5, 1, 2 and 4 Hz) in the same attachment are shown in Fig. 17 together with a superimposed amplitude distribution of SEJCs recorded in the same attachment. The distribution of amplitudes of SEJCs and of EJCs evoked at 0·5–2 Hz were

virtually identical. At 4 Hz there was a shift to the right in the amplitude range, indicating summation of releases from different varicosities. A consistent finding was that the magnitude of this shift was often small and was rarely more than double. If the probability of release is very low (per varicosity), and the size of the population of varicosities not very large, this is perhaps not surprising.

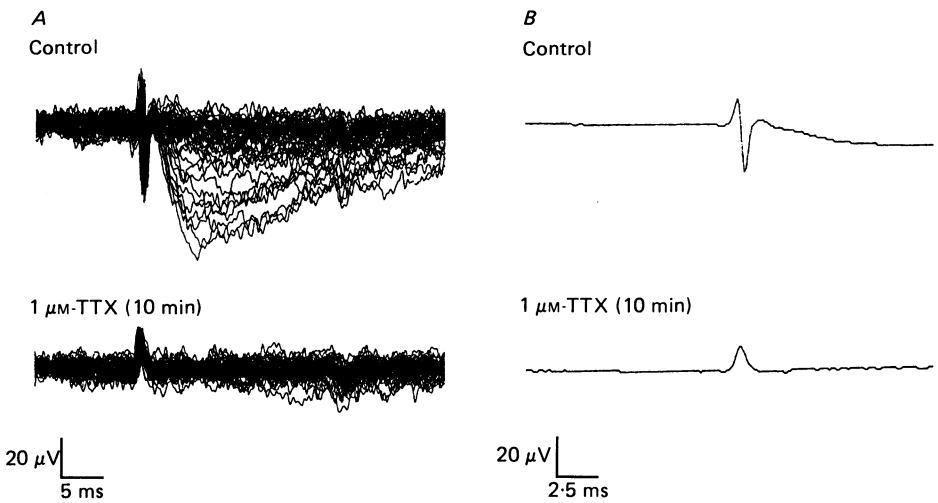


Fig. 15. Effects of local application of TTX inside the suction electrode on impulse conduction in sympathetic nerve terminals. *A*, nerve impulses and EJCs evoked by a train of fifty pulses at 1 Hz before and after perfusion of the suction electrode with 1 μM-TTX for 10 min. *B*, averages of eighty nerve impulses recorded in the same attachment before and during the addition of TTX. Although the nerve impulse still electrotonically invaded the terminals the residual depolarization was insufficient to trigger the transmitter release mechanism.

In addition, during trains of stimuli it was possible to record EJCs which repeat at the same latency with virtually identical amplitude and time course (Fig. 18). It was also possible to match individual EJCs with selected SEJCs recorded in the same attachment, showing them to be identical (Fig. 19). The simplest interpretation of these observations is that the same or closely related release site was activated and released a single quantum of transmitter.

Effects of frequency. The probability of recording an EJC was dependent on the frequency of stimulation; the number of EJCs recorded per train of stimuli increased when the frequency of stimulation was raised (Fig. 20*A*). A histogram of the number of EJCs evoked during trains of 500 stimuli at 0·5, 1, 2 and 4 Hz in a single attachment is shown in Fig. 21. Thus it can be clearly demonstrated that transmitter release is frequency dependent. Using extracellular recording it is possible to extend the frequency range over which evoked transmitter release can be studied. In particular it is remarkable to see the difference between transmitter release at 1 Hz when 100 nerve impulses failed to activate the release mechanism and 4 Hz where many releases occur (Fig. 20).

Configuration of the nerve terminal impulse. One possible explanation for facilitation of transmitter release from sympathetic nerves is that there may be a frequency-

dependent increase in the size or duration of the nerve terminal impulse (Brown & Holmes, 1956). Increasing the frequency of stimulation increased the probability of observing an evoked EJC but there was no detectable alteration in the size or shape of the nerve terminal impulse recorded with this method. (Fig. 20*B*).

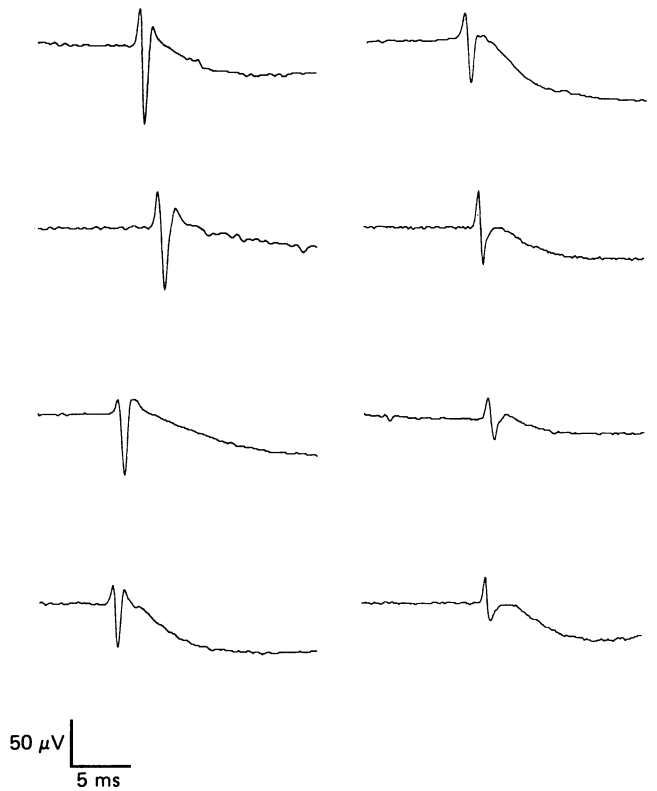


Fig. 16. Examples of the different shapes of nerve impulses that were recorded in various attachments. Because of technical difficulties, it has not been possible to relate the shape of the nerve impulse to the region of the nerve terminal from which the recordings were made.

DISCUSSION

In the present investigation a new approach has been used to establish the relationship between the nerve impulse and transmitter release in postganglionic sympathetic nerve terminals. This method allows many interesting questions to be asked of the transmitter release process at the autonomic neuroeffector junction.

Electrical activity recorded from the surface of the vas deferens

We believe that the extracellular potentials recorded in the absence of stimulation (SEJCs) are produced by the spontaneous release of transmitter from the varicose nerve terminals, since they have similar characteristics to SEJPs recorded intracellularly (Burnstock & Holman, 1962; Allcorn, Cunnane & Kirkpatrick, 1986; Brock & Cunnane, 1987*a*). First, SEJCs were unaffected by TTX, suggesting that

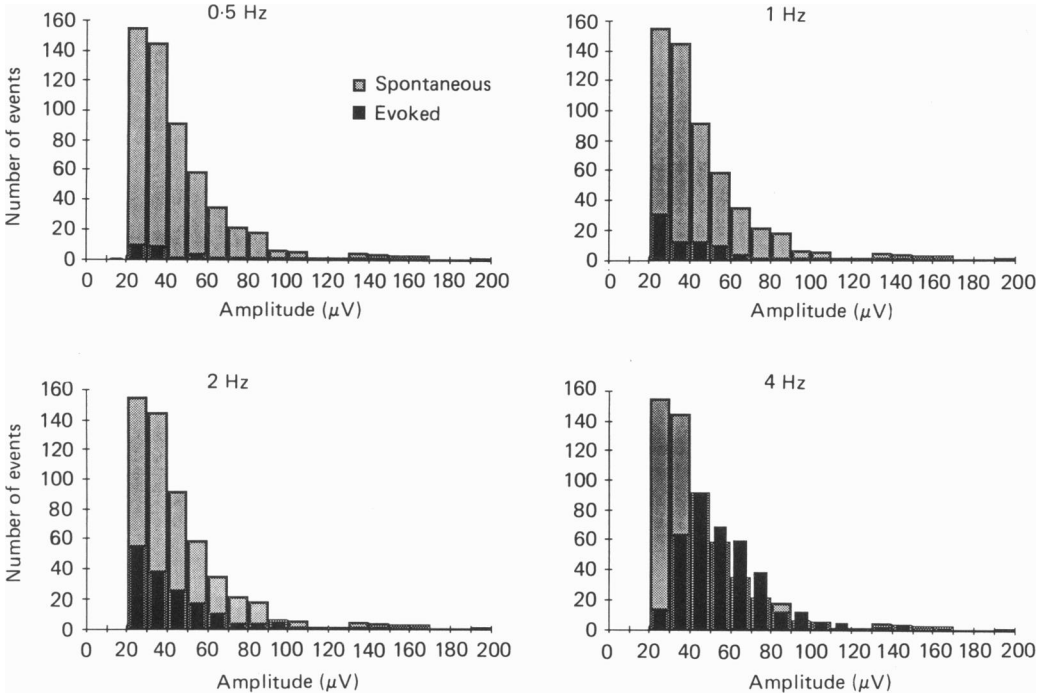


Fig. 17. Comparison of amplitude distributions of SEJCs and EJCs recorded in the same attachment. The EJCs were evoked by trains of 500 stimuli at 0·5, 1, 2 and 4 Hz. The amplitude distribution of SEJCs is superimposed on that of the EJCs at each frequency for ease of comparison.

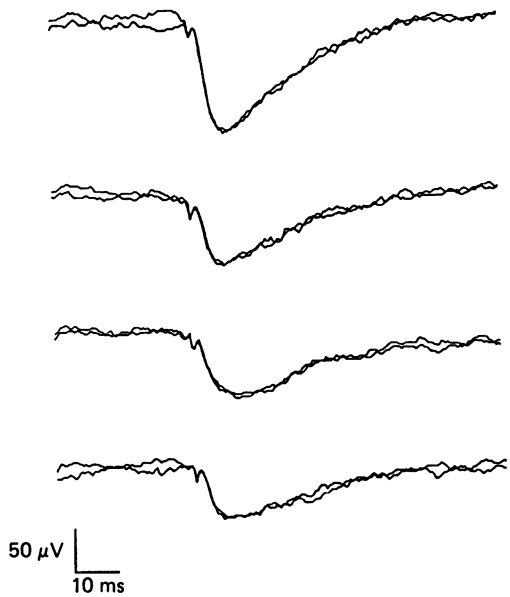


Fig. 18. Examples of paired EJCs repeating during long trains of stimuli with virtually identical amplitude and time course.

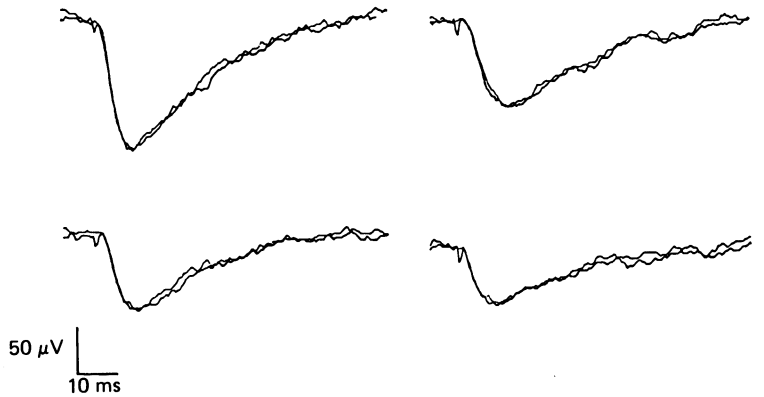


Fig. 19. Examples of SEJCs and EJCs of similar amplitude and time course recorded in the same attachment. Individual records are superimposed for ease of comparison.

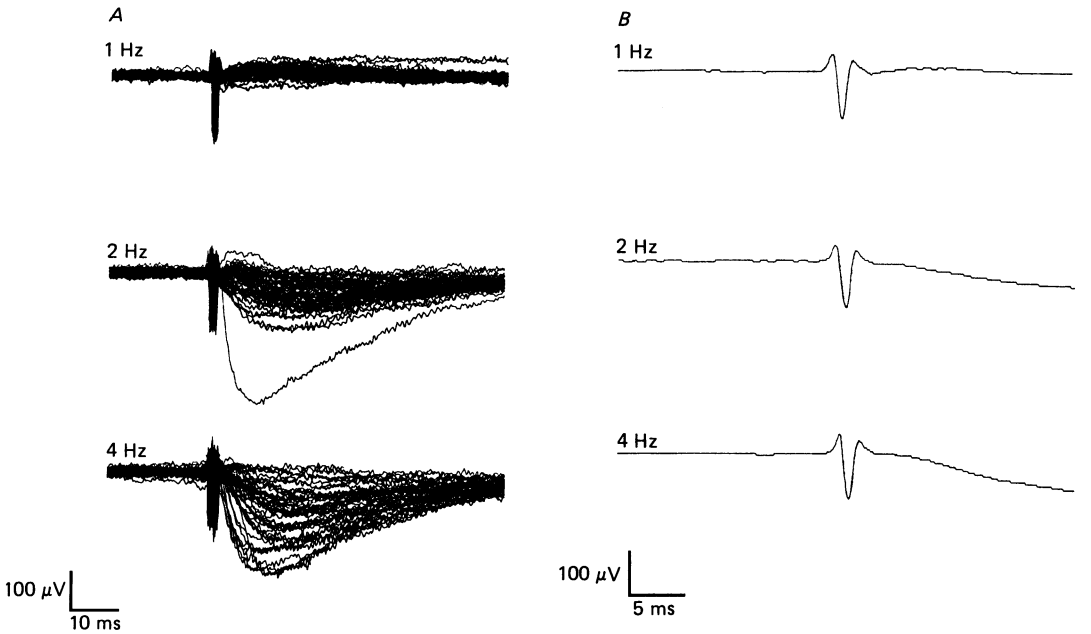


Fig. 20. Frequency-dependent facilitation of transmitter release in the guinea-pig vas deferens. *A*, extracellular recording of nerve terminal impulses and EJCs evoked by stimulation of the hypogastric nerve with trains of 100 suprathreshold stimuli at 1, 2 and 4 Hz. *B*, averages of twenty nerve terminal impulses and associated EJCs in the same attachment, showing that the facilitation is not associated with a detectable alteration in the configuration of the nerve terminal impulse.

they were not elicited by the generation of spontaneous, sodium-dependent nerve impulses in the terminals; in fact, spontaneous events were never associated with preceding nerve impulses. Secondly, a reduction in the external calcium concentration or the addition of the inorganic calcium entry blocker cobalt had little effect on the frequency of SEJCs. Thirdly, SEJCs were rarely recorded in tissues from

guinea-pigs which had been treated with 6-hydroxydopamine to destroy the sympathetic nerves. Fourthly, SEJCs were resistant to the action of the competitive α-adrenoceptor antagonists, prazosin and phentolamine, the irreversible α-adrenoceptor antagonist, benextramine, but were abolished by α,β-methylene ATP, an agent which selectively desensitizes P_2-purinoceptors (Kasakov & Burnstock, 1982).

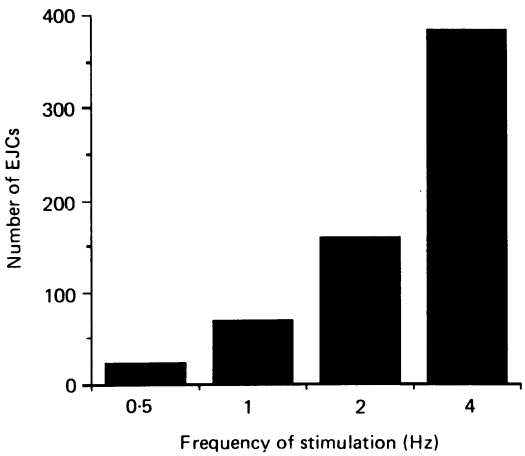


Fig. 21. Histogram of the number of EJCs evoked by trains of 500 stimuli at 0·5, 1, 2 and 4 Hz in a single attachment.

The evoked potentials recorded extracellularly (EJCs) also had a similar pharmacological profile to the intracellularly recorded EJP in that they were calcium dependent and readily abolished by TTX. Thus the electrical activity recorded extracellularly from the surface of the guinea-pig vas results from the release of transmitter from sympathetic nerves.

No invasion failure

Using extracellular recording we have confirmed that electrically evoked transmitter release from individual varicosities is intermittent (Blakeley & Cunnane, 1979). It is now clear that the nerve impulse propagates faithfully to the secretory terminals; intermittence of transmitter release therefore results from a low probability of release in the invaded varicosity and is not caused by failure of the nerve impulse to reach the secretory terminals.

Is active invasion necessary for transmitter release to occur?

One question we can address with the present technique is whether impulse propagation in the terminal varicose regions is an active or a passive process. Following internal perfusion of the suction electrode with TTX active invasion of the varicosities was blocked but electrotonic invasion from the point of block still occurred. The results show unequivocally that active invasion by a TTX-sensitive nerve impulse is required for evoked transmitter release to occur.

The shape of the nerve terminal impulse recorded in separate attachments varied and this may well reflect recordings made from different regions of the terminals.

Brooks & Eccles (1947) demonstrated theoretically that in a nerve terminal which is actively invaded by an impulse, an extracellular focal electrode would record a triphasic, positive–negative–positive, impulse in the more proximal regions of the nerve fibre, this becoming diphasic, positive–negative, in the terminal region of the nerve fibre. This interpretation is supported by the studies of Katz & Miledi (1965) in which they studied impulses recorded using focal electrodes from the 'closed end' of a muscle fibre. In this study they demonstrated that the diphasic impulse recorded close to the muscle–tendon junction becomes predominately positive going at the junction with the tendon, although both sites were actively invaded. It is possible that the diphasic impulses that we recorded were due to block of impulse propagation beyond the site of the recording, resulting from damage to the nerve fibre. However, on occasion diphasic impulses were associated with release at sites close to but not within the electrode, as indicated by an intermittent positive-going potential. These were sometimes found to occur at latencies which suggested propagation of the impulse beyond the site of recording. Tetrodotoxin powerfully inhibited transmitter release irrespective of the impulse shape, suggesting that even the most distal regions of the sympathetic varicose nerve terminals are actively invaded. Similar observations were made using the adrenergic neurone blocker bretylium. These results are less clear, however, because bretylium may have had direct effects on depolarization–secretion coupling in addition to its effects on the nerve impulse. Nevertheless, bretylium has powerful effects on impulse propagation in sympathetic nerve terminals and these effects probably account for its ability to inhibit transmitter release.

Quantal release from individual varicosities

The fundamental question raised is whether the varicosity secretes a single quantum or several quanta when the release process is activated by the nerve impulse (see Blakeley & Cunnane, 1986). Previous studies of the first time differential of the rising phases of a series of EJPs (discrete events) demonstrated that transmitter release occurred intermittently and was packeted. The question remained whether the discrete event at a single latency represents the activity of a single release site or of several release sites summed together. Since only the latency was available to distinguish transmitter release sites, determination of the probability of release from any one site was likely to be an overestimate (Blakeley & Cunnane, 1979). Focal extracellular recording has allowed this issue to be resolved in the guinea-pig vas deferens. In part, this is because an extracellular electrode 'sees' far fewer release sites. At the skeletal neuromuscular junction, two extracellular focal electrodes placed 15–20 μm apart record totally independent events, whereas the length constant for intracellular recording is 0·5–1 mm (Del Castillo & Katz, 1956; Katz & Miledi, 1965). When recordings were made with two adjacent electrodes simultaneous events were observed rarely. Sometimes coincident SEJCs were recorded but in this case the SEJC in one electrode was recorded in the second with greatly attenuated amplitude and inverted polarity (see Fig. 6). Thus the negative-going signals recorded with a suction electrode are highly localized and do not spread for any appreciable distance.

The evidence obtained in the present inquiry supports the view that only a single

quantum is secreted by a varicosity. First, the amplitude distributions of SEJCs and EJCs recorded in the same attachment were similar; multimodal distributions of the amplitudes of evoked events were not observed. Secondly, during trains of stimuli EJCs were recorded with identical amplitude and time course; indeed, pairs of identical EJCs were often recorded within a few stimuli of each other, suggesting that previous release facilitates subsequent release from the same site. Similar observations have been made elsewhere and this may be a characteristic feature of transmitter release from closely associated release sites (Cunnane & Stjärne, 1984*b*; Robitaille & Tremblay, 1987). Thirdly, individual EJCs, regardless of their amplitude, could be matched with selected SEJCs in the same attachment. Taking the simplest interpretation of the quantal hypothesis that the SEJC represents the postjunctional response to the release of a single quantum, then individual varicosities normally secrete only one quantum, intermittently, when the release mechanism is activated during trains of stimuli at low frequency. It seems unlikely in a low-probability system that evoked EJCs of identical form, which occur within a few stimuli of each other, represent multiquantal releases from the same varicosity; in this case the matching SEJC would also have to be the result of a multiquantal release. At present we cannot rule out the possibility that individual release sites on a varicosity may occasionally release more than one quantum. To resolve the problem of the *uni- versus multi*-quantal release capability of a single varicosity, one needs to know the number of varicosities and the maximum obtainable quantal content of the response from them, before reaching a firm conclusion.

There is some agreement that release sites on other nerve terminals in the peripheral and central nervous system behave in a similar manner, transmitter release being intermittent and monoquantal (Jack, Redman & Wong, 1981; Korn, Mallet, Triller & Faber, 1982).

Probability of transmitter release

In general the frequency of occurrence of spontaneous and evoked EJCs depended on the tip diameter of the recording electrode. One can make a rough calculation about the number of varicosities covered in a suction electrode with a tip diameter of about 50 μm. Assuming that varicosities have similar release characteristics and that they are no more than 5 μm apart, then at least ten may contribute to the evoked activity recorded by an electrode with 50 μm tip diameter. If the axon branches and recordings are made from several branches, the nerve impulse having the same latency in all branches, then this will also be an underestimate. When recordings were made using point electrodes (micropipettes, 1–3 μm diameter, filled with 2 M-NaCl) resolution of release sites was further increased and the probability of transmitter release from some site was observed to be lower still (J. A. Brock & T. C. Cunnane, unpublished observations). Using both methods of recording, probabilities of the order of 0·03 or less were observed at frequencies of stimulation $\leqslant 1$ Hz, but detailed microanatomical studies of the recording site are required before making definitive statements about the probability of release from an individual varicosity.

Nature of the transmitter

There now appears to be a good general agreement between the electrophysiological results presented here and previous biochemical studies of the release process in the same tissue (Alberts *et al.* 1981). However, this conclusion may be tenuous in that the transmitter generating the EJP (and therefore the EJC) may not be noradrenaline but ATP or a related purine co-released with noradrenaline from the sympathetic nerve terminals (Sneddon, Westfall & Fedan, 1982; Sneddon & Burnstock 1984; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1984; Allcorn *et al.* 1986). The pharmacological profile of the extracellularly recorded EJC closely parallels that of the intracellularly recorded EJP and probably reflects the quantal release of ATP from sympathetic nerve terminals. It is unlikely that the EJCs result from the release of ATP from sensory fibres, because when preganglionic stimulation was employed they were abolished by the ganglion blocking agent hexamethonium.

Times course of transmitter action

One of the difficulties in relating the intracellularly recorded SEJP to the EJP has been the wide variation in the time course of the two processes. Thus it has not been possible to state with any confidence the duration of transmitter action generating these potentials (Holman, 1970). A study of discrete events suggested that the time course of transmitter action underlying the EJP in the guinea-pig vas deferens was brief (Blakeley & Cunnane, 1979). Hirst & Neild (1978), from an analysis of excitatory junctional potentials in arterioles, also concluded that the conductance change underlying the junctional potential is brief when compared with the total duration of the potential. Bywater & Taylor (1980) concluded from a study of excitatory junction potentials and electrotonic potentials in the guinea-pig vas deferens that the estimated time course of the current is similar to that of the intracellularly recorded SEJP, which has a time constant of decay of approximately 30 ms. Our measurements using extracellular electrodes, which gives a good approximation of the time course of the current underlying the intracellular potential change, strongly support these conclusions. Blakeley, Dunn & Petersen (1986), using single intracellular micro-electrode current and voltage clamp techniques, have also demonstrated that the decay constant of the EJC in the mouse vas deferens is about 25 ms. In preliminary studies, we have found that the time course of the current in several different tissues was similar even though the EJPs had widely different time courses. This suggests that the difference in the time course of the EJP in different smooth muscles is caused by differences in the passive membrane properties of the respective smooth muscle cells and not by differences in the time course of transmitter action (J. A. Brock, T. C. Cunnane & P. Åstrand, unpublished).

Frequency-dependent facilitation

No detectable changes in the configuration of the nerve terminal impulse were found at frequencies of stimulation up to 4 Hz, which might have accounted for facilitation (see Fig. 20 and Brock & Cunnane, 1987*a*). It is worth noting that

extracellular recording will tend to emphasize the high-frequency components in the nerve impulse and it is quite possible that important slower conductance changes may have been overlooked. At present, however, it seems that the increased probability of release associated with facilitation is due to some mechanism acting at the level of the individual release site, presumed to be the varicosity. In all our experiments facilitation was observed as a change in the number of EJCs evoked per train of stimuli. Facilitation does not, however, appear to involve the same release site secreting a variable number of quanta. It is our impression to date that facilitation involves either the recruitment of previously silent varicosities or more release sites on the same varicosities, each capable of secreting a single quantum or a combination of these mechanisms.

Thus the secretory mechanism in the guinea-pig vas deferens filters out stray impulses, and a train of stimuli is therefore required to activate synchronously the release mechanism in many varicosities and therefore contraction. It is noteworthy that sympathetic nerves discharge *in vivo* with a characteristic bursting pattern (Hallin & Torebjörk, 1974).

The techniques described in the present study offer a new approach to the study of transmitter release from sympathetic nerve terminals and provide new insights into the fundamental mechanisms involved in depolarization–secretion coupling in varicose nerve fibres.

This work was supported by a grant from the British Heart Foundation. The authors are grateful to Mr J. Dempster, Strathclyde University, for providing computer programs which greatly facilitated data collection and analysis and Dr S. Totterdell for expert assistance in the preparation of tissues for electronmicroscopy.

REFERENCES

ALBERTS, P., BARTFAI, T. & STJÄRNE, L. (1981). Sites(s) and ionic basis of α-autoinhibition and facilitation of [^{3}H]noradrenaline secretion in the guinea-pig vas deferens. *Journal of Physiology* **312**, 297–334.

ALLCORN, R. J., CUNNANE, T. C. & KIRKPATRICK, K. (1986). Actions of α,β-methylene ATP and 6-hydroxydopamine on the sympathetic neurotransmission in the vas deferens of the guinea pig, rat and mouse: support for co-transmission. *British Journal of Pharmacology* **89**, 647–659.

BEVAN, J. A., CHESHER, G. B. & SU, C. (1969). Release of adrenergic transmitter from terminal plexus in artery. *Agents and Actions* **1**, 20–26.

BLAKELEY, A. G. H. & CUNNANE, T. C. (1978). Is the vesicle the quantum of sympathetic transmission? *Journal of Physiology* **280**, 30–31*P*.

BLAKELEY, A. G. H. & CUNNANE, T. C. (1979). The packeted release of transmitter from the sympathetic nerves of the guinea-pig vas deferens: an electrophysiological study. *Journal of Physiology* **296**, 85–96.

BLAKELEY, A. G. H. & CUNNANE, T. C. (1986). Junction potentials in smooth muscle: a potent electrophysiological tool. In *Trends in Autonomic Pharmacology*, vol. 3, ed. KALSNER, S., pp. 99–111. London, Philadelphia: Taylor & Francis.

BLAKELEY, A. G. H., DUNN, P. M. & PETERSEN, S. A. (1986). Properties of high-resistance smooth muscle cells in the isolated mouse vas deferens. *Journal of Physiology* **382**, 167*P*.

BROCK, J. A. & CUNNANE, T. C. (1987*a*). Relationship between the nerve action potential and tramsmitter release from sympathetic postganglionic nerve terminals. *Nature* **326**, 605–607.

BROCK, J. A. & CUNNANE, T. C. (1987*b*). Focal extracellular recording of the nerve terminal action potential and transmitter release in the guinea-pig vas deferens. *Journal of Physiology* **392**, 116*P*.

BROOKS, C. MC. C. & ECCLES, J. E. (1947). Electrical investigation of the monosynaptic pathway through the spinal cord. *Journal of Neurophysiology* **10**, 251–274.

BROWN, G. L. & HOLMES, O. (1956). The effects of activity on mammalian nerve fibres of low conduction velocity. *Proceedings of the Royal Society* B **145**, 1–14.

BURNSTOCK, G. & HOLMAN, M. E. (1961). The transmission of excitation from autonomic nerve to smooth muscle. *Journal of Physiology* **155**, 115–133.

BURNSTOCK, G. & HOLMAN, M. E. (1962). Spontaneous potentials at the sympathetic nerve endings in smooth muscle. *Journal of Physiology* **160**, 446–460.

BYWATER, R. A. R. & TAYLOR, G. S. (1980). The passive membrane properties and excitatory junction potentials of the guinea-pig vas deferens. *Journal of Physiology* **300**, 303–316.

CUNNANE, T. C. (1984). The mechanism of neurotransmitter release from sympathetic nerves. *Trends in Neuroscience* **7**, 248–253.

CUNNANE, T. C. & STJÄRNE, L. (1984*a*). Frequency dependent intermittency and ionic basis of impulse conduction in postganglionic sympathetic fibres of guinea-pig vas deferens. *Neuroscience* **11**, 211–229.

CUNNANE, T. C. & STJÄRNE, L. (1984*b*). Transmitter secretion from individual varicosities of guinea-pig and mouse vas deferens: highly intermittent and monoquantal. *Neuroscience* **13**, 1–20.

DEL CASTILLO, J. & KATZ, B. (1956). Localization of active spots within the neuromuscular junction of the frog. *Journal of Physiology* **132**, 630–649.

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *Journal of Physiology* **117**, 109–128.

FOLKOW, B., HÄGGENDAL, J. & LISANDER, B. (1967). Extent of release and elimination of noradrenaline at peripheral adrenergic nerve terminals. *Acta physiologica scandinavica* **72**, suppl., 307.

FOLKOW, B. & HÄGGENDAL, J. (1970). Some aspects of the quantal release of the adrenergic transmitter. In *Bayer Symposium II*, pp. 91–97. Berlin, Heidelberg, New York: Springer-Verlag.

GILLESPIE, J. S. & KIRPEKAR, S. M. (1966). The histological localization of noradrenaline in the cat spleen. *Journal of Physiology* **187**, 69–79.

HALLIN, R. G. & TOREBJÖRK, H. E. (1974). Single unit sympathetic activity in human skin nerves during rest and various manoeuvres. *Acta physiologica scandinavica* **92**, 303–317.

HIRST, G. D. S. & NEILD, T. O. (1978). An analysis of excitatory junctional potentials recorded from arterioles. *Journal of Physiology* **280**, 87–104.

HOLMAN, M. E. (1970). Junctional potentials in smooth muscle. In *Smooth Muscle*, ed. BULBRING, E., BRADING, A., JONES, A. & TOMITA, T., pp. 244–289. London: Edward Arnold.

IVERSEN, L. L. (1967). *The Uptake and Storage of Noradrenaline in Sympathetic Nerves*. Cambridge: Cambridge University Press.

JACK, J. J. B., REDMAN, S. J. & WONG, K. (1981). The components of synaptic potentials evoked in cat spinal motoneurones by impulses in single group Ia afferents. *Journal of Physiology* **321**, 65–96.

KASAKOV, L. & BURNSTOCK, G. (1982). The use of the slowly degradable analog α,β-methylene ATP to produce desensitization of the P_2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea pig bladder. *European Journal of Pharmacology* **86**, 291–294.

KATZ, B. & MILEDI, R. (1965). Propagation of electrical activity in motor nerve terminals. *Proceedings of the Royal Society* B **161**, 453–482.

KORN, H., MALLET, A., TRILLER, A. & FABER, D. S. (1982). Transmission at a central inhibitory synapse. II. Quantal description of release, with a physical correlate for binomial n. *Journal of Neurophysiology* **48**, 679–707.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.

ROBITAILLE, R. & TREMBLAY, J. P. (1987). Non-uniform release at the frog neuromuscular junction: evidence of morphological and physiological plasticity. *Brain Research Reviews* **12**, 95–116.

SMITH, A. D. & WINKLER, H. (1972). Fundamental mechanisms in the release of catecholamines. In *Catecholamines*, ed. BLASCHKO, H. & MUSCHOLL, E., pp. 538–617. Berlin, Heidelberg, New York: Springer-Verlag.

SNEDDON, P. & BURNSTOCK, G. (1984). Inhibition of excitatory junction potentials in guinea-pig vas deferens by α,β-methylene-ATP: further evidence for ATP and noradrenaline as co-transmitters. *European Journal of Pharmacology* **100**, 85–90.

SNEDDON, P. & WESTFALL, D. P. (1984). Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens. *Journal of Physiology* **347**, 561–580.

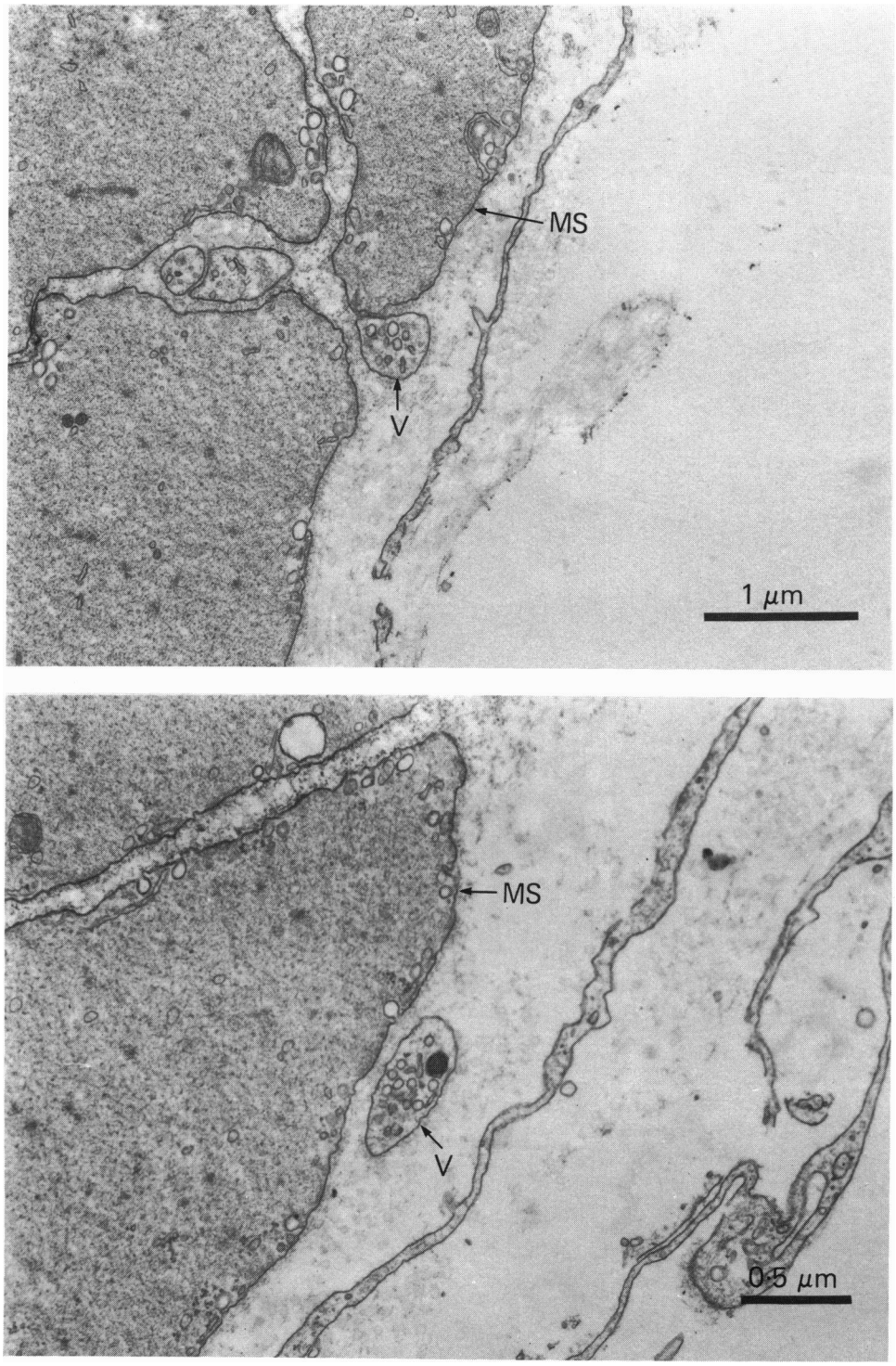
SNEDDON, P., WESTFALL, D. P. & FEDAN, J. S. (1982). Co-transmitters in the motor nerves of the guinea-pig vas deferens: electrophysiological evidence. *Science* **218**, 693–695.

STJÄRNE, L. & ÅSTRAND, P. (1984). Discrete events measure single quanta of adenosine 5′-triphosphate secreted from sympathetic nerves of guinea-pig and mouse vas deferens. *Neuroscience* **13**, 21–28.

TOTTERDELL, S. & SMITH, A. D. (1986). Cholecystokinin-immunoreactive boutons in synaptic contact with hippocampal pyramidal neurons that project to the nucleus accumbens. *Neuroscience* **19**, 181–192.

EXPLANATION OF PLATE

Electron micrographs of transverse sections through the guinea-pig vas deferens showing single varicose fibres (V) running on the muscle surface (MS).


MS
V
1 μm
MS
V
0·5 μm