Journal of Physiology (1988), **401**, *pp.* 657–670
With 10 text-figures
Printed in Great Britain

TIME COURSE OF TRANSMITTER ACTION AT THE SYMPATHETIC NEUROEFFECTOR JUNCTION IN RODENT VASCULAR AND NON-VASCULAR SMOOTH MUSCLE

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(*Received 9 December 1987*)

SUMMARY

1. Transmitter release from sympathetic postganglionic nerve terminals innervating the guinea-pig and mouse vas deferens and the rat tail artery has been studied *in vitro* by focal extracellular recording with particular emphasis on the time course of transmitter action underlying the intracellular potential changes.

2. In the absence of stimulation, spontaneous excitatory junction currents (SEJCs) were recorded with amplitudes up to 500 μV and durations between 40 and 100 ms. SEJCs were unaffected by the competitive α-adrenoceptor antagonist prazosin but 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.

4. SEJCs were similar in amplitude and time course to EJCs evoked by low-frequency stimulation in the same attachment in all three tissues.

5. SEJCs recorded using either a conventional AC amplifier or a patch clamp amplifier had the same time course.

6. These studies show that the time course of the current underlying the excitatory junction potential is brief and essentially the same in three different tissues. The prolonged time course of the excitatory junction potential in different tissues can be accounted for by the passive membrane properties.

INTRODUCTION

At the autonomic neuroeffector junction (ANJ), the mechanisms governing transmitter release and the time course of its postjunctional action are not well understood. This can be largely attributed to the morphological complexity of the ANJ and to the way in which postjunctional electrical activity spreads. In general, smooth muscle cells are electrically coupled to one another and multiply innervated, and consequently the excitatory junction potential (EJP) recorded with an intracellular microelectrode measures transmitter release from many different

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varicosities (Burnstock & Holman, 1961). EJPs recorded from guinea-pig and mouse vas deferens and rat tail artery are known to have widely differing time courses with durations ranging from 200 ms to 2 s, but the time course of transmitter action underlying the EJP cannot be stated with any degree of confidence (see Holman, 1970 and Discussion).

In this study the time course of transmitter action in vascular and non-vascular smooth muscle has been measured with focal electrodes extracellularly (see Brock & Cunnane, 1987*a*, *b*, 1988). The aim of the investigation was to compare the time course of transmitter action in tissues innervated by short (guinea-pig and mouse vas deferens) and long (rat tail artery) postganglionic sympathetic neurones. There is good reason to believe that measurements of this type reflect the time course of the current underlying the intracellular potential change (cf. del Castillo & Katz, 1956; Stühmer, Roberts & Almers, 1983). To assess the validity of the extracellular recording method the time course of the current was measured using a patch clamp technique.

METHODS

The methods used are largely the same as those previously reported (Brock & Cunnane, 1987*a*, 1988). Briefly, guinea-pigs (Duncan Hartley, 350–600 g), mice and rats were killed by a blow to the head and bled. Tissues 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^{-1} with Krebs solution of the following composition (mM): NaCl, 118·4; $NaHCO_3$, 25·0; NaH_2PO_4, 1·13; $CaCl_2$, 1·8 or 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 guinea-pig vas deferens was electrically stimulated indirectly through the hypogastric nerve trunk (Ag–AgCl suction electrode) and mice vasa by field stimulation of the prostatic end. The rat tail artery was stimulated using a Ag–AgCl suction electrode applied to the proximal end. The stimulus parameters were varied as required but in general the pulse width was set between 0·01 and 0·1 ms (7–30 V).

A window was cut in the connective tissue sheath enveloping the tissues, and a bevelled glass electrode (tip diameter $\leqslant 50\ \mu m$) 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. Electrical activity was recorded in two ways: (1) through an AC-coupled amplifier (Neurolog NL104, low-frequency cut-off 0·1 Hz); (2) through a List EPC-7 amplifier in voltage clamp mode. The outputs of both amplifiers were fed through a low-pass filter (Neurolog NL125) with the cut-off set at 1·5 kHz.

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). The mean base line was determined by averaging the initial part of the digitized signal (points 15–45) 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 base line. The 10–90% rise time and time constant of decay was determined by linear-regression analysis. The rise time from 10–90% of the peak value was assumed to be linear and the decay from 90–10% of the peak value monoexponential. Selected records were measured manually using cursor controls from time to time to assess the accuracy of the fit.

Drugs. α,β-Methylene adenosine 5′-triphosphate was obtained from Sigma and prazosin hydrochloride from Pfizer. α,β-Methylene adenosine 5′-triphosphate was made up as a concentrated stock solution in distilled water. Prazosin was dissolved in *N*,*N*-dimethylacetamide (Sigma). Stock solutions of drugs were serially diluted in Krebs solution to the required final bath concentration.

RESULTS

Spontaneous electrical activity

When small suction electrodes were applied to the surface of the guinea-pig and mouse vas deferens, and to the rat tail artery, transient negative-going potentials were recorded in the absence of nerve stimulation (Fig. 1). There is good reason to believe that this method of recording faithfully measures the time course of the current underlying the intracellular potential changes (Brooks & Eccles, 1947; del

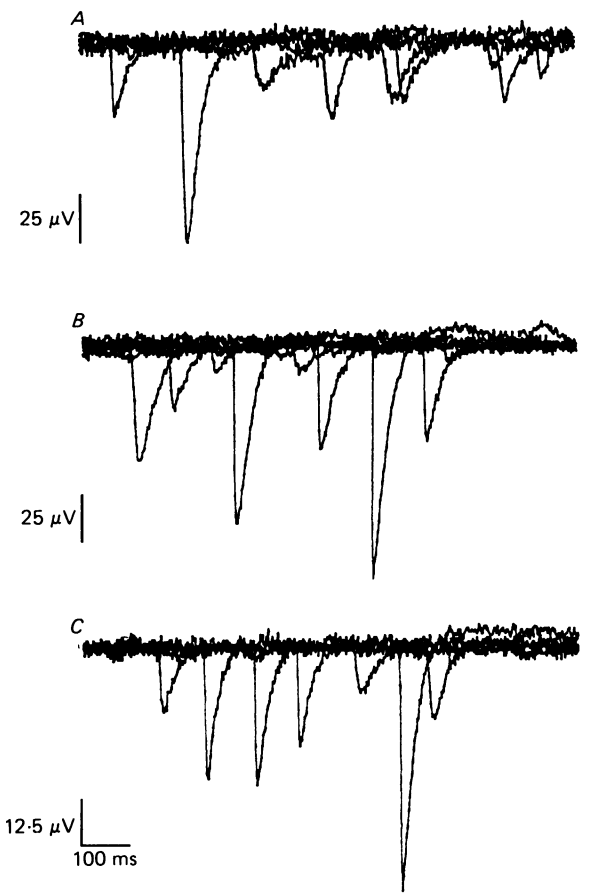

Fig. 1. Spontaneous activity recorded with an extracellular suction electrode from guinea-pig vas deferens (*A*), mouse vas deferens (*B*) and rat tail artery (*C*). A series of random traces are superimposed to show the characteristic features of SEJCs.

Castillo & Katz, 1956; Stühmer *et al.* 1983) and for this reason these extracellular events have been termed spontaneous excitatory junction currents (SEJCs) (Brock & Cunnane, 1987*a*, 1988). The features of SEJCs recorded from the three tissues have been characterized with regard to amplitude, time course and frequency of occurrence.

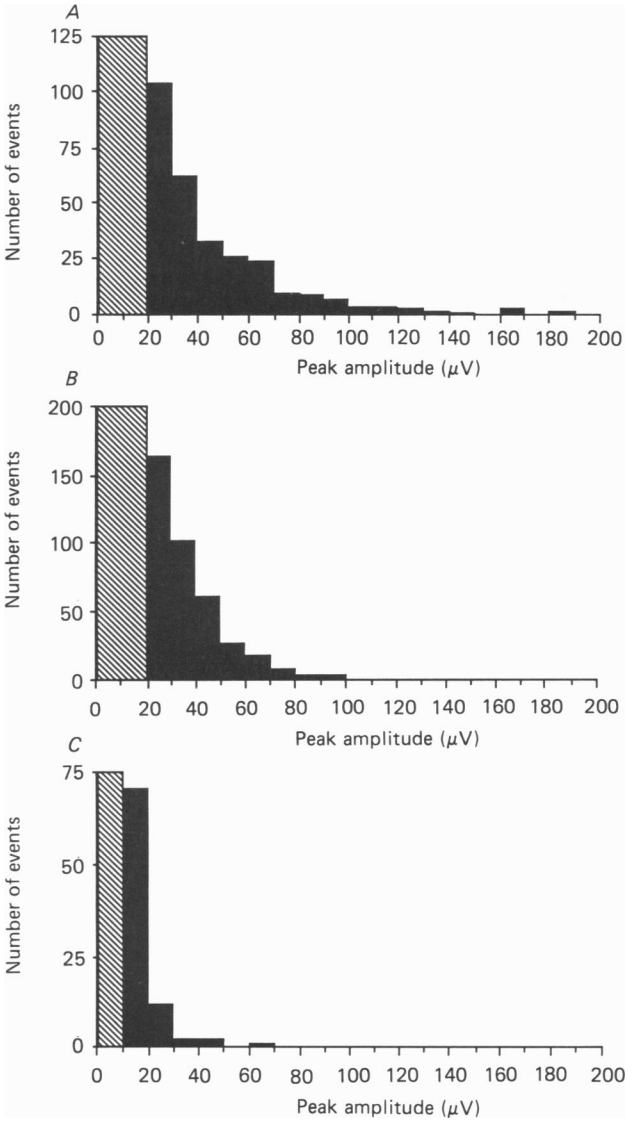

Fig. 2. Amplitude distributions of SEJCs in the guinea-pig vas deferens (*A*), the mouse vas deferens (*B*) and the rat tail artery (*C*). Several hundred SEJCs were recorded in a single attachment to construct each distribution. The histograms show that SEJC distributions are normally skewed towards 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 determined with any degree of confidence.

Amplitude of the SEJC

The amplitude of individual events varied from tissue to tissue and from attachment to attachment but could be as large as 500 μV. It should be remembered that the absolute size of any event will depend on many factors (for example, the seal

resistance and the relationship of the transmitter release site to the smooth muscle cell) and therefore absolute comparisons between different attachments have little quantitative meaning. A calibration bar has been included on all records to indicate the size of the potentials recorded in separate attachments. However, it is only meaningful to compare amplitudes within the same attachment. Examples of amplitude distributions of SEJCs (events larger than twice baseline noise) recorded from one attachment in each tissue are shown in Fig. 2. It is clear that amplitude

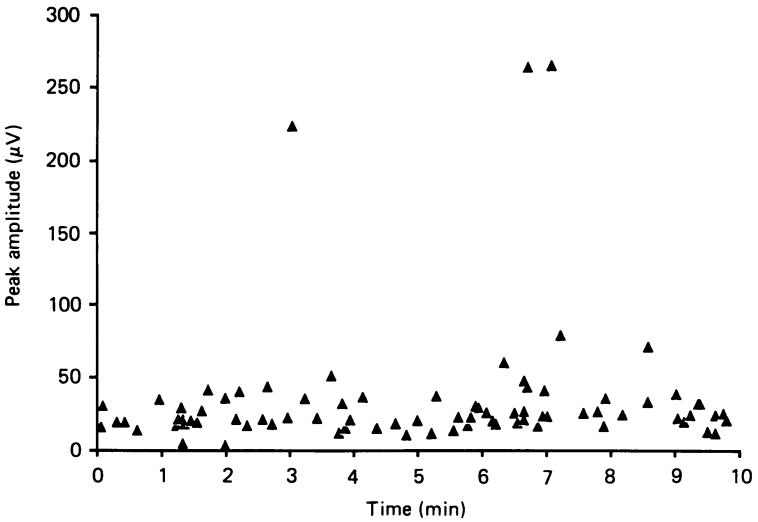

Fig. 3. Serial plot of all SEJCs recorded from the surface of the guinea-pig vas deferens. The Figure shows the amplitudes of all SEJCs recorded for 10 min. Note that occasional very large SEJCs were recorded.

distributions are normally skewed towards the lowest-amplitude events but occasional 'giant' spontaneous EJCs with a normal time course were observed. A serial plot of all SEJCs recorded during a 10 min attachment to the guinea-pig vas deferens reveals the presence of three 'giant' SEJCs (Fig. 3). The characteristics of these large SEJCs will require further investigation and may represent the release of the transmitter contents of a large vesicle which represents about 3% of all vesicles in varicosities innervating the guinea-pig vas deferens.

Rise-time of SEJCs

The mean 10–90% rise time was $7{\cdot}9 \pm 1{\cdot}0$ ms ($n = 751$ in ten attachments) (mean $\pm$ standard deviation of the group mean) in the guinea-pig vas, $4{\cdot}3 \pm 1{\cdot}5$ ms ($n = 924$ in five attachments) in the mouse vas and $6{\cdot}9 \pm 0{\cdot}5$ ms ($n = 250$ in five attachments) in the rat tail artery.

Time constant of decay of SEJCs

The majority of the SEJCs recorded decayed monoexponentially (Fig. 4). In the guinea-pig vas, the mean time constant of decay was $24{\cdot}9 \pm 3{\cdot}2$ ms ($n = 751$ in ten

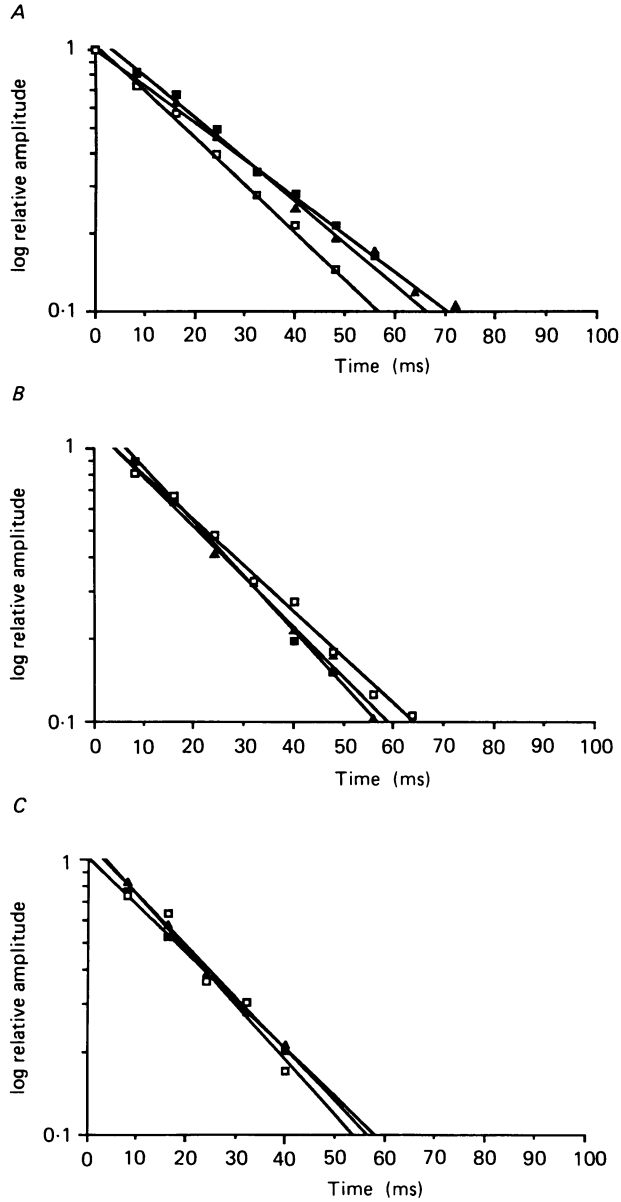

Fig. 4. Semilogarithmic plots of the decay phases of three representative SEJCs in the guinea-pig vas deferens (*A*), the mouse vas deferens (*B*) and the rat tail artery (*C*).

attachments), in the mouse vas 24·6 ± 2·3 ms ($n = 924$ in five attachments) and 21·8 ms ± 2·2 ($n = 250$ in five attachments) in the rat tail artery. It is noteworthy that the time constant of decay was similar in all three tissues studied. The range of values was similar whether distributions were compared within one attachment or when data from several different attachments were pooled. Distributions of decay time constants are shown in Fig. 5 for guinea-pig and mouse vas deferens and the rat

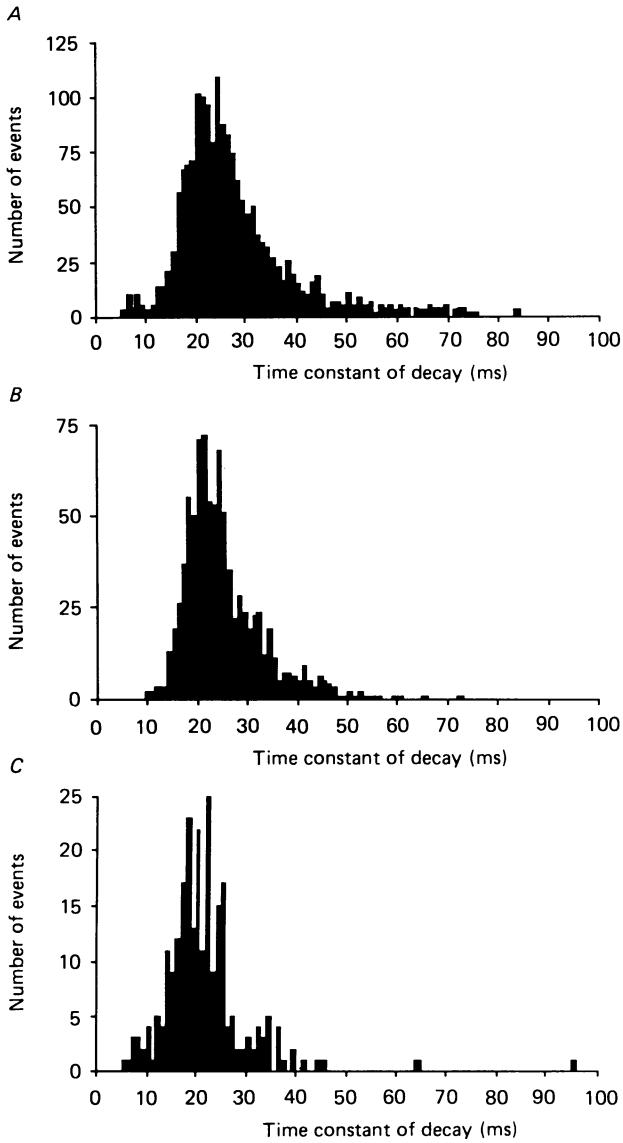

Fig. 5. Time constant of decay distributions of SEJCs in the guinea-pig vas deferens (*A*), the mouse vas deferens (*B*) and the rat tail artery (*C*). Several hundred SEJCs were recorded in a few attachments and the data pooled. The distributions were similar for data from single attachments.

tail artery. No obvious correlation was found between the amplitude of the SEJC and the time constant of decay.

SEJC frequency

The absolute frequency of occurrence of SEJCs recorded varied considerably from attachment to attachment for a number of reasons which include the tip diameter of

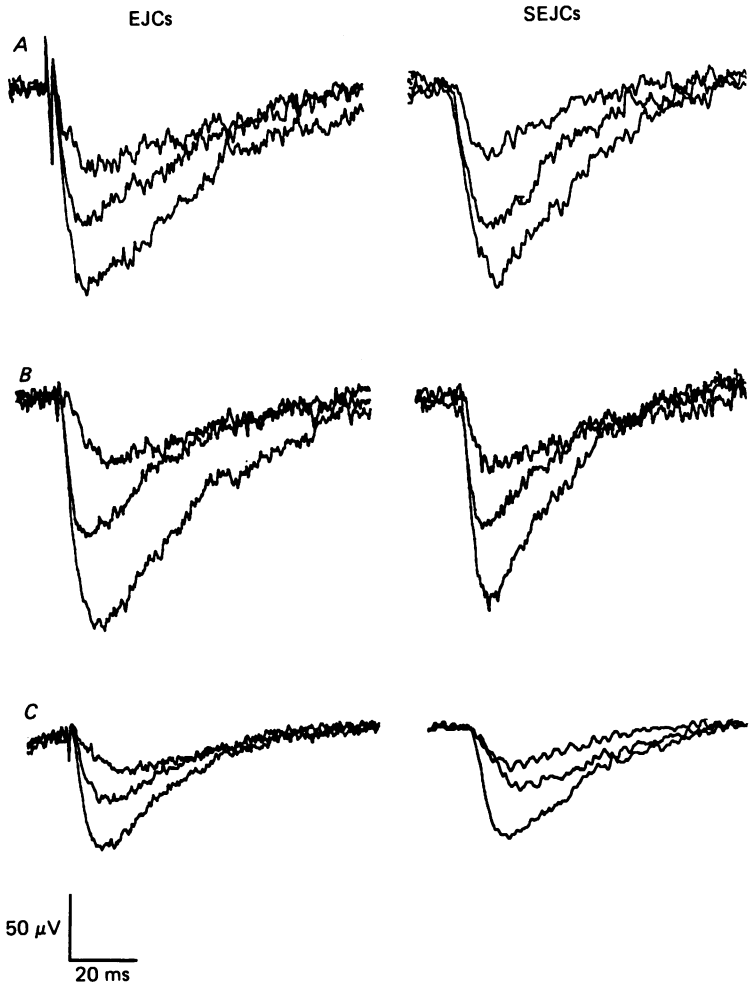

Fig. 6. Comparison of evoked EJCs and SEJCs in several tissues. Postjunctional electrical activity was recorded extracellularly from the surface of the guinea-pig vas deferens (*A*), the mouse vas deferens (*B*) and the rat tail artery (*C*). In all tissues EJCs and SEJCs were of comparable amplitude and time course.

the recording electrode and the seal resistance. In general SEJC frequency ranged between 5 and 20 min^{-1} in the guinea-pig vas deferens, 20 and 40 min^{-1} in the mouse vas deferens and 5 and 20 min^{-1} in the rat tail artery.

Pharmacology of the SEJC

Application of α,β-methylene ATP (10^{-6} to 10^{-5} M) markedly depressed SEJC amplitude in all three preparations within 15–20 min. This effect was reversible by wash-out. Normal SEJCs were recorded after more than 40 min exposure to 10^{-6} M-prazosin showing that they are not generated by noradrenaline acting through α_1-adrenoceptors.

Evoked electrical activity

Stimulus-locked negative-going excitatory junction currents (EJCs) were recorded in all three preparations following nerve stimulation (Fig. 6). The pattern of activity recorded was dependent not only on the frequency of stimulation but also on the intensity. With careful positioning of the stimulating and recording electrodes it was possible to isolate the activity of a few release sites, presumed to be varicosities. In

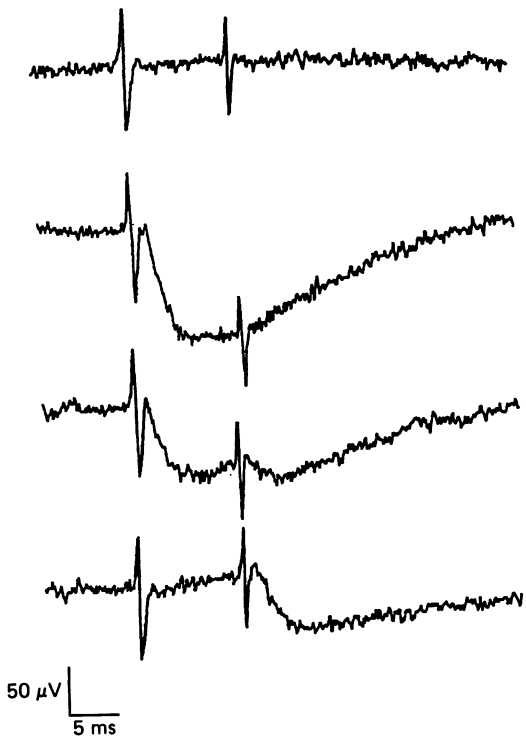

Fig. 7. Simultaneous measurement of the nerve terminal impulse and evoked EJCs in the guinea-pig vas deferens. Nerve terminal action potentials were recorded at two different latencies, showing how the asynchronous arrival of nerve impulses in the terminals contributes to the prolonged rising phase of the EJP recorded intracellularly. Records were selected during trains of stimuli at 1 Hz and show that release is intermittent.

these cases the evoked EJCs occurred at a constant latency, intermittently and, on all occasions, were preceded by a non-intermittent nerve impulse which was tied to transmitter release (Brock & Cunnane, 1987*a*, 1988). The characteristics of the intermittent transmitter release process will not be discussed here and have been reported elsewhere (Brock & Cunnane, 1987*a*, 1988).

The amplitude and time course of SEJCs were similar to those of EJCs recorded in the same attachment following low-frequency nerve stimulation (Fig. 6). When several nerve fibres were activated at higher stimulus intensities, groups of EJCs were evoked at different latencies each associated with a corresponding nerve impulse. This pattern probably reflected transmitter release from several sympathetic nerve fibres with different conduction velocities (Fig. 7). In the mouse vas

deferens the time course of the negative-going EJC was often distorted by the simultaneously occurring positive-going potentials. These positive-going potentials represent local current flowing outwards (i.e. from the muscle cells) as a result of depolarization of smooth muscle cells located outside the suction electrode (see Brock & Cunnane, 1987*a*). However, in cells where the stimulus intensity was low and the probability of transmitter release was low, EJCs without apparent distortion could be recorded (see Fig. 6). Distributions of the time constant of decay of EJCs and SEJCs were similar in all tissues.

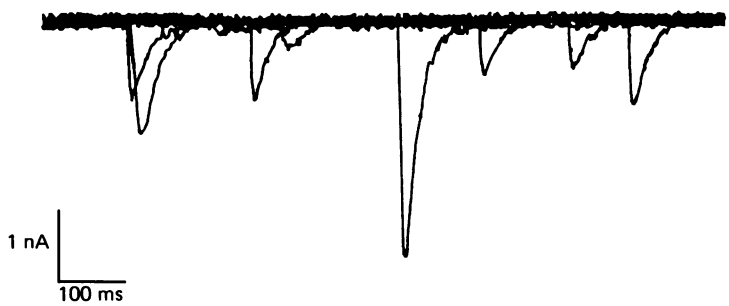

Fig. 8. Spontaneous currents recorded from the surface of the guinea-pig isolated vas deferens using an extracellular suction electrode connected to a patch clamp amplifier. A series of random traces are superimposed to show the characteristic features of SEJCs.

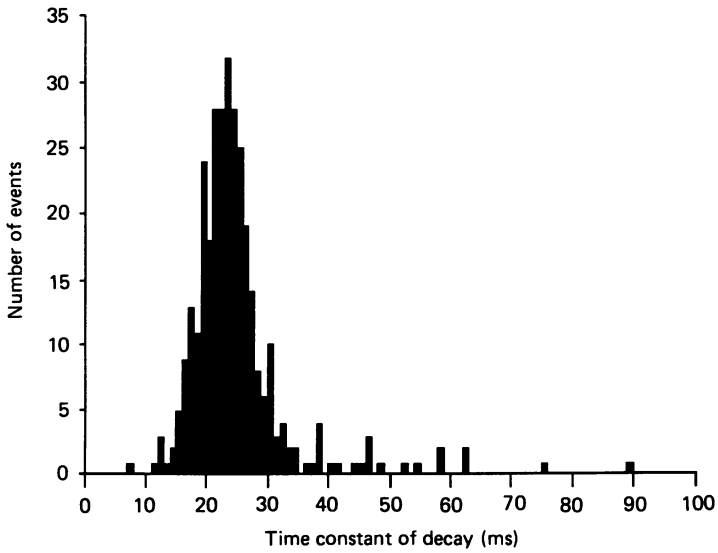

Fig. 9. Histogram of the time constant of decay distributions of SEJCs in the guinea-pig vas deferens recorded through a patch clamp amplifier (compare with Fig. 5*A*).

Time course of the SEJC measured with a 'loose' patch clamp

The time course of SEJCs was measured in the guinea-pig vas deferens using a List EPC-7 patch clamp amplifier (voltage clamp mode) in order to obtain a second measurement of the time course of current (Fig. 8). In general, recordings made with

the 'loose' patch clamp had a better signal-to-noise ratio than those made through a conventional AC-coupled amplifier. Decay (time constant) distributions of a series of SEJCs recorded by both methods were similar (see Fig. 5*A*, Fig. 9). The average size of the current recorded in one attachment was about 0·4 nA ($n = 321$) with values ranging from mean baseline up to 2 nA. This compares with average values of approximately 1–5 nA for the miniature end-plate current at the skeletal neuromuscular junction in a number of species (Gage & Armstrong, 1968; Cull-Candy, Miledi & Trautmann, 1979). Clearly, the absolute value of the currents

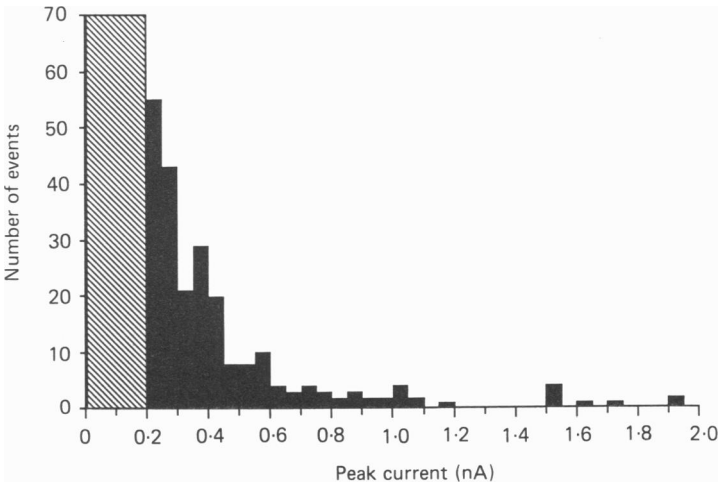

Fig. 10. Amplitude distributions of SEJCs recorded through a patch clamp amplifier in the guinea-pig vas deferens. Several hundred SEJCs were recorded in a single attachment to construct the distribution. The histograms show that SEJCs distribution is skewed towards low-amplitude events (compare with Fig. 2*A*). 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.

obtained should be treated with extreme caution owing to the application of a large, loose patch-clamp electrode to a three-dimensional syncytial tissue, but this provides the best estimate at present. An amplitude distribution of the peak currents of the SEJCs measured using the EPC-7 is shown in Fig. 10.

DISCUSSION

The basic recording conditions encountered when point electrodes are used to detect transmitter release extracellularly have previously been thoroughly discussed (del Castillo & Katz, 1956). The principles involved in the use of a suction electrode can be briefly summarized; a resistance is created at the junction between the edge of the electrode and the smooth muscle surface. A potential change proportional to the postjunctional current is created over this 'seal' resistance (Stühmer *et al.* 1983). The polarity of the potential is determined by the direction of current flow over the 'seal' resistance. A negative-going EJC reflects postjunctional current originating inside the suction electrode, while transmitter action outside the electrode generates a positive-going signal (Brock & Cunnane, 1987*a*, 1988).

Spontaneous electrical activity

SEJCs recorded from guinea-pig and mouse vasa deferentia and the rat tail artery were similar in many respects when analysed with respect to amplitude, rise time and time constant of decay. The overall time course of SEJCs in each tissue was similar, with time to peak values of less than 10 ms and a monoexponential decay phase with a time constant of 15–30 ms. The total duration of the majority of SEJCs was about 80 ms (range 40–100 ms).

The frequency distributions of SEJC amplitudes are skewed to the left in a similar manner to intracellularly recorded SEJPs in the guinea-pig (Burnstock & Holman, 1962) and mouse vas deferens (Holman, 1970). This indicates that electrotonic decrement of SEJPs with varying distance from the intracellular recording electrode is not likely to be the major factor causing the skewed SEJP amplitude distribution. The most likely explanations include (1) different relationships between individual varicosities and the smooth muscle cells which they innervate, (2) variation in the size of the quantum of transmitter released from different sites, and/or (3) variation in the density of the postjunctional receptors. It is interesting to note that the 'average' SEJC amplitude in general was larger in the vasa than in the rat tail artery. This might be explained simply by the additional surface connective tissue in the rat tail artery, thus preventing the formation of an adequate seal. However, it could also reflect the number of 'close-contact' (< 20 nm junctional cleft width) varicosities innervating the smooth muscle cells of the vas deferens as compared to the rat tail artery.

Electrically evoked responses

The main concern of the present paper is the time course of transmitter action underlying the EJP. Changes in membrane potential (EJPs) evoked by field stimulation of the sympathetic nerves exhibit widely differing time courses, the decay phases of EJPs recorded from guinea-pig vas deferens and rat tail artery (Surprenant, 1980; Sneddon & Burnstock, 1984*a*, *b*) being several times longer than in the mouse vas deferens (Holman, 1970). It is clear however, that the time course of the SEJC and the EJC recorded extracellularly, and the time course of the SEJP recorded intracellularly are similar in all tissues (see also Brock & Cunnane, 1988). The similarities in SEJC and EJC time constants suggest that the difference in EJP time course can be accounted for by different passive membrane properties of the respective smooth muscle cells and not by differences in the time course of transmitter action. Another factor influencing the time course of the EJP, particularly the rising phase, is variation in the temporal spread of the action potential volley (see Fig. 7) invading the nerve terminals (see Blakeley & Cunnane, 1979).

Previously it has not been possible to state with any confidence the duration of transmitter action generating the EJP (Holman, 1970). Several groups have suggested that the conductance change underlying the junctional potential is brief when compared with the total duration of the potential (Hirst & Neild, 1978; Blakeley & Cunnane, 1979; Bywater & Taylor, 1980; Finkel, Hirst & Van Helden, 1984; Blakeley, Dunn, & Petersen, 1986). Our measurements using focal electrodes

extracellularly, strongly support these conclusions. Thus we have found that the time course of the current in several different tissues is similar even though the EJPs have widely different time courses.

Number of transmitter molecules in a quantum

The transmitter generating the EJP is likely to be ATP because in all preparations, α,β-methylene ATP, a stable analogue of ATP which desensitizes P_2-purinoceptors (Kasakov & Burnstock, 1982), abolished SEJPs and EJPs (Sneddon, Westfall & Fedan, 1982; Sneddon & Burnstock, 1984*a*; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1984; Allcorn, Cunnane & Kirkpatrick, 1985). Assuming that the effects of α,β-methylene ATP are specific for P_2-purinoceptors (see Byrne & Large, 1986; Cunnane, Muir & Wardle, 1987), then it is interesting to note that when ATP acts on P_2-purinoceptors in several different tissues, the time course of transmitter action is identical. Thus, the molecular events associated with receptor activation in several different sympathetically innervated tissues appear to be similar.

It has always been difficult at the ANJ to estimate the number of transmitter molecules in a single quantum. The noradrenaline–ATP ratio in a sympathetic vesicle may be as low as 50:1 (Fried, 1981; Stjärne & Åstrand, 1985), the vesicle containing perhaps only twenty molecules of ATP. If only twenty molecules of ATP are released and this results in a 2 nA current then the individual channel current would be 100 pA. Assuming that each molecule of ATP opens a single channel and that the driving voltage is approximately −60 mV, a single-channel conductance of 1·66 nS would result and this seems highly improbable. This suggests either that the estimates of the ATP content in a vesicle are wrong, or, alternatively, that the transmitter acts indirectly via intermediate messengers and possibly an amplifying sequence of enzymatic steps and that only a small number of molecules are required to initiate a substantial depolarization.

This work was supported by a grant from the British Heart Foundation and the Wellcome Trust. The authors are grateful to Mr J. Dempster, Strathclyde University, for providing extremely useful computer programs.

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