AN INVESTIGATION INTO THE MECHANISMS OF

SYMPATHETIC NEUROMUSCULAR TRANSMISSION

by

ALISTAIR MATHIE

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ABSTRACT

Stimulation of the sympathetic nerves innervating the rodent vas deferens elicits excitatory junction potentials (ejps). Intermittent transient accelerations in the rising phase of these ejps, termed discrete events (d.e.s.), were observed. D.e.s. were shown to reflect packets of transmitter acting on the recording cell. They vary in latency, amplitude and time course.

D.e.s. with similar latency and time course have been associated into 'families'. Families of d.e.s., generally, have multimodal amplitude distributions with clearly defined modes. Families were shown to reflect transmitter release from a single terminal nerve fibre. Variation in [Ca]_o altered the relative proportion of occurrence of large and small d.e.s., in a family, without affecting the modes themselves. Facilitation had the same effect. Therefore, families of d.e.s. represent the packeted, intermittent, release of transmitter from a single release site which may be either one varicosity or a group of varicosities on a single nerve fibre.

Spontaneous d.e.s. have also been observed. In most cells the amplitude distribution of spontaneous d.e.s. was unimodal and this mode was equal to the smallest evoked d.e. mode in the same cell.

Transmitter release from individual release sites could be modulated by α-adrenoreceptor agonists and antagonists. However, the effect of such drugs was not consistent with the presence of an endogenous system of modulation via prejunctional α-receptors. No evidence could be found for endogenous inhibitory modulation of release from pulse to pulse. Rather, short term facilitation appeared to occur.
Blockade of neuronal uptake of noradrenaline by desmethylimipramine had no effect on d.e.s. Higher concentrations of desmethylimipramine altered the time course of ejps and d.e.s. Finally d.e.s. were recorded in the rat tail artery. This may provide a more clinically relevant preparation for future studies.
INTRODUCTION

The sympathetic division of the autonomic nervous system innervates many peripheral structures. It plays a major role in the central control of circulation. The tone of blood vessels, for example, is dependent on the activity of the sympathetic nervous system and a large number of studies have been carried out to find ways of interfering with sympathetic control of blood pressure.

Unfortunately, there are many gaps in the knowledge of the mechanisms of sympathetic transmission, particularly in terms of release from individual release sites on the terminal nerve fibres. This lack of understanding of the mechanisms of transmitter release at the sympathetic neuromuscular junction can be contrasted with the somatic neuromuscular junction where such mechanisms are well understood.

Transmitter release at the somatic neuromuscular junction

In the early fifties, Fatt & Katz (1951) using intracellular microelectrodes recorded the potential change that occurs at skeletal muscle end plates (the end plate potential (e.p.p.)) in response to nerve stimulation. They also observed that, in resting muscle, spontaneous miniature discharges occurred at the junction region apparently at random (Fatt & Katz, 1950). Since these spontaneous potentials were found to be of uniform amplitude (Fatt & Katz, 1952) it was postulated that transmitter was released in uniformly sized packets.

Such an observation also suggested that prejunctional events at this junction could be assayed accurately using postjunctional intracellular electrophysiological techniques. These techniques were then widely used to gain understanding
of the transmission process.

Fatt & Katz (1952) hypothesised that, apart from their small size, the spontaneous occurrences were identical to epps and termed them miniature epps (mepps). This idea was substantiated by later studies (see del Castillo & Katz, 1954c; Liley, 1956a, b).

Convincing evidence supporting the view that transmitter was released in uniformly sized packets in response to nerve stimulation was obtained by del Castillo & Katz (1954a, b). These authors showed that the size of the epps could be varied by altering the concentration of either calcium or magnesium present in the perfusion fluid. By increasing the Mg^{++}/Ca^{++} ratio, they were able to decrease the amplitude of the epp until it became of the same order as the mepp. When the epp became so small, successive impulses produced a marked random fluctuation in amplitude with occasional total failures of response.

The amplitude histograms for epps and mepps were compared (del Castillo & Katz, 1954b; Boyd & Martin, 1956; Liley, 1956a). It was shown that the mepps represented the single unit of transmitter release and that the evoked response is made up of an integral multiple (0, 1, 2, 3) of that unit.

The amplitude distribution of packeted release under such conditions could be described by Poisson statistics (del Castillo & Katz, 1954a; Boyd & Martin, 1956; see also Katz, 1966).

From these studies it was suggested that in normal Ca^{++}/Mg^{++} solutions the epp is made up of a large integral multiple of the spontaneous unit, estimated as being between 100 and 300 units (Martin, 1955).
The use of postjunctional intracellular recording techniques at this junction to assay prejunctional release of transmitter was validated in 1965 when Katz & Miledi (1965a) were able to observe packeted transmitter release by recording extracellularly from presynaptic terminals in calcium free Ringer at a low temperature.

Similar studies at the sympathetic neuromuscular junction have been much less illuminating. This is because the structure of smooth muscle and its innervation is very different from that of skeletal muscle.

**Structure of skeletal muscle**

The structure of skeletal muscle and its innervation is straightforward and well established. Each muscle fibre is electrically isolated from its neighbours and is innervated by a single nerve fibre which terminates on that cell.

**Implications for electrophysiological studies**

Intracellular electrophysiological studies, at this junction, provide an accurate assay for packets of released transmitter. However, one can also be certain that the entire packeted release of transmitter from a single nerve fibre in response to nerve stimulation can be measured, isolated from the response of any other fibres.

**Structure of smooth muscle and its innervation**

The structure of smooth muscle and its innervation is much more complex than that of skeletal muscle and not nearly so well established. The structure varies throughout the body and therefore it is worthwhile reviewing currently existing knowledge.
Structure of autonomically innervated smooth muscle and its innervation

Smooth muscle cells

Bundles. In most smooth muscle tissues the muscle cells are arranged as branching bundles (Merrillees, et al 1963; Merrillees, 1968; Yamauchi & Burnstock, 1969) each bundle surrounded by a connective tissue sheath, the épimysium; and containing about 20 cells (Prosser, Burnstock & Kahn, 1960).

The muscle cells in the bundle have been shown to have polyhedral profiles with an average of four to five sides (Bennett, 1972). When bundles are followed along their length using the technique of serial sectioning (Merrillees, 1968) they are frequently found to fuse to neighbouring bundles either by anastomosis or by bridges of small bundles. Thus the overall structure of a smooth muscle is dependent upon the patterns of interconnections and weaving together of individual bundles (Bennett, 1972).

The interweaving of bundles is accompanied by the interweaving of individual cells within the bundle. In this way each cell comes into close apposition with up to 12 other cells. The separation between cells is defined as being less than 80nm for 'close apposition' to be said to occur (Merrillees, 1968).

Interconnections. Merrillees (1968) has shown that muscle cells when in close apposition to one of their neighbours are, for most of their surface area, separated by a distance of 50 - 80nm. However, various authors have described small areas of very close contact between muscle cells either due to a direct fusion, defined by Dewey & Barr (1964) as a nexus (or 'tight junction') or because of an
intracellular bridge where the opposing membranes run apart from each other at a distance of ~10nm for 0.2 to 0.4μ (e.g. Richardson, 1962; Rhodin, 1962; Merrillees et al., 1963). Lane & Rhodin (1964a) showed that areas of close contact in the mouse vas deferens are extremely numerous such that 3-5% of the total cell area is within 10nm of an opposing cell.

In the 1960s a number of different groups showed that different tissues consisted of either tight junctions or intracellular bridges and there was much discussion as to whether different tissues did have different types of contact or whether such observations were an artefact of the particular methods of study used.

In the rodent vas deferens, for example, Richardson (1962, 1964); Lane & Rhodin (1964a) and Merrillees et al., (1963) all stated that intracellular bridges rather than tight junctions existed. Yamauchi & Burnstock (1969) showed, however, that tight junctions between some smooth muscle cells of the mouse vas deferens only appeared when certain fixatives were used. Subsequently, Uehara & Burnstock (1970) using high resolution electron microscopy, suggested that 'gap' junctions of about 2-3nm separation existed rather than true fusion.

It is now generally accepted (Burnstock, 1979) that these gap junctions or nexi represent the physical identity of the electrical conduction pathways between smooth muscle cells which give rise to the electrical syncytial nature of smooth muscle originally described by Bozler (1941).
Innervation

Postganglionic sympathetic axons are usually unmyelinated. Sjöstrand (1965) classified these axons into two groups, 'long' and 'short', the former being the neurons of the para- and pre-vertebral ganglia while the latter innervate the uro-genital organs.

These postganglionic axons enter the muscle in bundles of up to 100 axons and are already varicose (Merrillees, et al., 1963; Norberg & Hamberger, 1964). Hillarp (1959) described them as forming a "fine meshed" plexus.

Fluorescence histochemistry allowed the terminal apparatus of the noradrenergic fibres to be visualised (Falck, 1962; Falck, et al., 1962). The axons were seen to run parallel to muscle bundles and each axon was seen to branch. Malmfors (1969) and Olson & Malmfors (1970) described these terminal regions of the axons as resembling a 'spray of twigs'. In the terminal region the number of varicosities per unit length is at its most dense with up to 30 varicosities in the last 100μ of the axon (Norberg & Hamberger, 1964; Malmfors, 1965; Dahlström & Häggendal, 1966).

Studies of smooth muscle innervation using electron microscopy revealed areas of close apposition (70-200Å) between nerve and muscle membranes, which was termed the neuromuscular synapse (Richardson, 1962; Merrillees, et al., 1963). Furness & Iwayama (1972) have shown that axons are often deeply embedded in invaginations of the muscle cells when forming these close contacts.

However, it is not just the terminal varicosities which form close contacts with muscle fibres. Axons appear to run parallel alongside muscle fibres for a considerable length, occasionally coming into close contact with the muscle cells. This led Richardson (1962) to suggest that some form of
"en passage" release of transmitter from non terminal varicosities might occur.

Thus innervation of smooth muscle may occur in two different ways (Bennett, 1972). Transmitter is released either from the terminal varicosities of single axons at an area of close contact or alternatively "en passage" from the close contact non terminal varicosities in axon bundles still enveloped in Schwann cell processes.

In some smooth muscles (e.g. mouse vas deferens, Richardson (1962)) the innervation is said to be completely by single axon terminal varicosities, in others (e.g. ileum, uterus, Gabella, 1976) there are no single axons, or at least they are very rare. Many other smooth muscles have some intermediate form where both are present (e.g. guinea pig vas deferens, Bennett, 1972).

In a muscle which has an intermediate form of innervation the relative physiological importance, if any, of the two forms is unclear.

One factor which adds to the confusion of trying to describe the innervation is the terminology applied to varicose regions of the sympathetic nerves.

Burnstock (1970) attempted to clarify the situation by describing terminal varicosities as being restricted to the last varicosity of the nerve and dividing preterminal varicosities into three groups:

a) varicosities on the nerve before the nerve enters the effector organ - completely enveloped in Schwann cell processes.

b) varicosities lying within the effector organ - partially enclosed in Schwann cell processes.

c) the last few preterminal varicosities before the nerve terminates - completely naked of Schwann cells.
On the other hand Hillarp (1959) and Gabella (1976) described all varicosities lying within the effector organ as being terminal varicosities.

This had led to difficulty in interpreting a number of publications dealing with innervation, in particular, the paper by Merrillees (1968) which is probably the most complete structural study of a sympathetically innervated smooth muscle made using electron microscope studies of serial sections. Merrillees showed that axon terminations were bare for about 10-60μ (containing 10-20 varicosities) and that many of these terminations made close contact with muscle fibres. However, less than half of the muscle cells made close contact (<20nm) with one of these bare terminations.

Merrillees also showed that preterminal varicosities frequently made 'en passage' contacts of less than 10000μ, with anything from 0 to 75 occurring to each smooth muscle cell. Close examination of the data in Merrillees' paper suggests that these occur more or less at a random distance from the muscle cell, and therefore each cell receives from 0 to about 40 'en passage' contacts within about 50nm (although, as stated above, less than half the cells have contacts closer than 20nm). Those "semi-close" varicosities, therefore could also provide a functional innervation of the cells (see Bennett & Merrillees, 1966) particularly as Malmfors (1965) has shown that transmitter is depleted from all varicosities in the region of the effector cell during intense stimulation.

Thus in the guinea pig vas deferens, it may be that either less than half the cells or alternatively all the cells receive some form of direct functional innervation.

In the vas deferens of other rodents individual innervation of all the smooth muscle cells has been shown to
occur (Richardson, 1962; Lane & Rhodin, 1964a; Yamauchi & Burnstock, 1969) with at least one and possibly up to six close neuromuscular junctions (Furness & Burnstock, 1969).

The neuromuscular synapse differs in size from species to species. In the guinea pig and rat vas deferens it is said to be 180-250\(\Omega\) (Richardson, 1962; Merrillees et al., 1963) while in the mouse it is said to be 70-80\(\Omega\) (Lane & Rhodin, 1964a).

**Implications for electrophysiology**

Because smooth muscle cells are connected together by low resistance pathways, the electrical signal recorded in response to nerve stimulation represents the summed response of transmitter from a number of release sites acting on a number of cells. Furthermore the amplitudes of potentials generated by transmitter action on muscle cells are rapidly attenuated across the couplings between cells as the separation between synapse and recording point increases (Tomita, 1970; Bennett, 1972). Spontaneous potentials (mejps), therefore, have amplitudes which vary in size down to the level of the recording noise (Burnstock & Holman, 1962). Mejps also have a much shorter time course than evoked potentials (ejps) because the electrical response of the muscle depends on whether it is activated at a single point or at a number of sites along its length (see Tomita, 1970). Thus (in contrast to the skeletal neuromuscular junction) comparison of spontaneous and evoked release is meaningless; the number of nerve fibres which are contributing to the response is unknown and it is impossible to show that transmitter release is packeted at this junction using conventional intracellular electrophysiological recording.
While the structure of the two muscles and their innervation varies immensely, there is one structural feature common to both sympathetic and somatic nerves. In the terminal regions of both types of fibre, vesicles have been shown to occur. They occur in specialised regions of the terminal fibre termed varicosities in sympathetic nerves and active zones in somatic nerves. The development of the vesicle hypothesis for transmitter release is peculiar in that it relies on combined evidence from both the sympathetic and somatic nervous systems.

The Vesicle Hypothesis

Around the same time as the initial work was carried out showing that acetylcholine was released in distinct packets at the skeletal neuromuscular junction, electron microscopists found that at a number of junctions the nerve terminals contained synaptic vesicles (de Robertis & Bennett, 1955; Robertson, 1956) adjacent to the synaptic cleft. Liley (1956a) and Birks et al. (1960) showed that degeneration of nerve terminals caused spontaneous quantal release (measured electrophysiologically) and synaptic vesicles (observed using electron microscopy) to disappear, while regeneration caused both to reappear at the same time. Similarly Heuser & Reese (1973) showed that the number of vesicles in the nerve terminals of the frog skeletal neuromuscular junction was reduced following nerve stimulation of the motor nerves and, following a period of rest, the number of vesicles returned to normal.

Heuser et al (1979) developed a technique which involved quick-freezing preparations a few milliseconds after
stimulation and then producing freeze-fracture replicas of the nerve terminals. In this way they were able to show vesicles in the act of exocytosis. They also found a good correlation between the estimated quantal content and the number of vesicle openings. This paper is probably the most convincing direct evidence so far produced relating quantal release of transmitter to the exocytosis of the contents of vesicles.

Before the studies of Heuser et al., the most convincing evidence relating the packeted release of transmitter seen at the skeletal neuromuscular junction to the release of transmitter from vesicles was obtained from biochemical studies of the sympathetic neuromuscular junction.

Vesicles in the sympathetic nervous system

In both the preterminal and terminal varicosities of sympathetic nerves, vesicles are found to occur (e.g. Richardson, 1962; see Geffen & Livett, 1971). Unlike the vesicles in somatic nerves, which occur in a single form, they occur mainly in two forms.

a) **small granular vesicles (SGVs)**. These vesicles vary in diameter from 250 to 600Å and have a dense central core of about 150Å (Burnstock, 1970).

b) **large granular vesicles (LGVs)**. These vesicles vary in diameter from 700 to 1600Å and have a dense central core from anything between 300 to 900Å (Burnstock, 1970).
The vesicular hypothesis and sympathetic transmission

In 1971 Geffen & Livett stated that the majority of noradrenaline in sympathetic nerves is located in the varicosities of the terminal region and that it is stored in SGVs. Previously, Potter & Axelrod (1963) had shown that fractionation by centrifugation of the vas deferens left the bulk of endogenous noradrenaline and pre-incorporated $^3$H-noradrenaline in the vesicular microsomal fraction. It had also been shown that reserpine depleted SGVs of their dense core (de Robertis & Pellegrino de Iraldi (1961).

Electron microscopy studies have shown that vesicles fuse with the membrane during nerve stimulation (Fillenz, 1971). de Potter et al (1969) showed that the enzyme dopamine-$eta$-hydroxylase and the protein chromogranin A are released with noradrenaline and ATP during nerve stimulation. This is convincing evidence that nerve impulses release vesicular noradrenaline as both proteins are found only in preterminal vesicles. Since by far the majority of vesicles (80-90%) in the varicosities were found to be of the small granular type and LGVs were assumed to be deficient of noradrenaline and occur mainly in the axon body, Smith (1972) stated that SGVs were mainly involved in neurotransmitter release while LGVs had no major functional significance in this respect.

This view has, however, been challenged. Klein & Lagercrantz (1981) have shown that the ratios of small to large vesicles in varicosities varies from tissue to tissue and species to species, varying from 5%-95% SGV/LGV up to 50%/50%. Generally the larger the species, the larger the percentage of large vesicles. Indeed in large animals, contrary to the view of Geffen & Livett (1971) the greater
percentage of noradrenaline is stored in LGVs. Thureson-Klein & Stjärne (1981) have shown that both LGVs and SGVs can fuse with the nerve terminal both in human omental vein (% LGV = 30%) and in guinea pig vas deferens (% LGV = 5%). These authors also stated that dopamine-β-hydroxylase is only released from LGVs. On the other hand Basbaum & Heuser (1979) have shown that in the mouse vas deferens, where the percentage of LGVs is admittedly low (5%), intense stimulation depletes only SGVs.

Klein & Lagercrantz (1981) have stated that LGVs at least in the tissues of large animals may be the main source of released transmitter. However, this view is difficult to reconcile with the fact that there are no LGVs in the CNS of any animals so far studied. It seems more likely that both types of vesicles are equally capable of release and that the relative contribution of the two depends on their relative proportion of occurrence.

It is also considered likely that LGVs supply SGVs with transmitter (see Lagercrantz, 1981).

Thus the vesicle hypothesis at present is dependent on combining information obtained from both the sympathetic and somatic junctions. The structural evidence from both junctions is good. However, to build up a convincing argument, the electrophysiological evidence obtained at the somatic junction has to be combined with biochemical evidence at the sympathetic junction.

A number of authors have disputed the validity of the vesicular hypothesis, e.g. Dunant (1981); Uvnäs (1984). Perhaps the most serious criticism of the vesicular hypothesis at the sympathetic junction arose from the experiments of Folkow et al (1967). They observed that the fractional release of transmitter from sympathetically innervated tissues
per pulse is low (about 1 part in 50,000) while the number of vesicles per varicosity is generally estimated at about 1000. It follows that the average release corresponds to less than one vesicle per varicosity. This was said by Folkow & Häggendal (1970) to be evidence against the vesicular hypothesis.

Before any further insight into the mechanisms of sympathetic transmitter release could be obtained it had to be established whether release at this junction was packeted.

In an attempt to resolve this problem two separate techniques were developed with a view to extracting more information from intracellular electrophysiological studies of sympathetic transmission.

Adapted electrophysiological studies of sympathetic transmission

Hirst & Neild (1978, 1980) have used short isolated segments of arteriole which can be shown to remain isopotential throughout their length during current injection at any point on them. Thus data obtained with an intracellular electrode positioned anywhere will not be attenuated to propagation from variable electrical distances.

This preparation is technically difficult to use, mainly due to a high background recording noise level and few meaningful results have been obtained so far (see Hirst & Neild, 1980). However, it does allow the correct measurement of transmitter release from the relatively few release sites which innervate such segments.

The other technique is the one used in this study (see Chapter 3). It was developed by Blakeley & Cunnane (1979) and involves close examination of the rising phase of the excitatory junction potential (ejp) which provides information about release from a few release sites close to
the recording electrode.

The results obtained from experiments using these techniques have suggested that release of transmitter from varicosities is both packeted and intermittent (Blakeley & Cunnane, 1979; Hirst & Neild, 1980). The observation that transmitter release from varicosities is intermittent provides an alternative explanation to that of Folkow & Häggendal (1970) for the low fractional release of noradrenaline per pulse seen by Folkow et al (1967).

While these studies have shown that sympathetic transmitter is released in packets, they do not provide information as to how many release sites are contributing to the recording potential, what the relative activity of each release site is, and how different release sites may interact to control transmitter release.

Blakeley & Cunnane (1979) proposed that the multipacketed release of transmitter they observed following certain stimuli in a train, reflected the ability of a single release site (which they assumed to be a single varicosity) to release many packets of transmitter at the same time - multiquantally. More recently, however, it has been suggested that release from any particular varicosity innervating certain 'simple' cells is monoquantal and extremely intermittent (Cunnane & Stjärne, 1982, 1984). It is fair to say however that information concerning the release processes seen most often when recording from sympathetically innervated smooth muscle cells (the 'complex' cells of Cunnane & Stjärne, 1982) is inconclusive. The number of times release occurs onto these cells in a train of stimuli is much greater than that seen in 'simple' cells and the amount of transmitter released, per pulse, from varicosities surrounding these cells can also be much greater (see Cunnane & Stjärne, 1982).
the number of transmitter releases in a train of stimuli may be due either to more varicosities affecting these cells or to each varicosity having an increased rate of secretion (decreased intermittency). The increase in the amount released per pulse may be due to co-incident monoquantal release from a number of varicosities or multi-quantal release from a few particular varicosities.

The first part of this thesis, therefore, will employ the technique of Blakeley & Cunnane (1979) to carry out a detailed study of the mechanisms of transmitter release from individual varicosities in an attempt to propose a model of the patterns of transmitter release from the varicosities surrounding a particular smooth muscle cell.

Once such a model is established it will be used to investigate other areas of the sympathetic transmission process where conflicting hypotheses exist.

**Other features of the sympathetic nervous system**

One area of sympathetic transmission which has been extensively investigated is ways in which transmitter release can be modified pharmacologically. Such studies have revealed a large number of chemicals which can modify sympathetic transmitter release. While many of these actions are undoubtedly purely pharmacological, a number of substances known to be released in increased quantities into the synaptic cleft during sympathetic nerve activity, have also been shown to modify release and one or all of these may have a physiological role in modulating transmitter release. The most widely studied of these chemicals is the reputed excitatory transmitter, noradrenaline which is said to control its own release, physiologically by means of a negative feedback loop (\(\alpha\)-autoinhibition).
None of the proposed systems of modulation have any
direct proof of existence, however, and there has been
little work carried out to determine where exactly on the
terminal nerve net they act. There is also much
contradictory evidence as to what circumstances must exist
for them to operate physiologically.

The evidence regarding each of the possible systems of
physiological modulation of sympathetic transmitter release
will be reviewed below. Before doing that, however, it is
important to point out that while the most favoured system
of modulation is that of α-autoinhibition by released
noradrenaline, considerable doubts exist, at least in some
sympathetic nerves, as to the very nature of the excitatory
transmitter. Firstly, then, the evidence and current
hypotheses concerning the nature of the excitatory
transmitter in sympathetic nerves will be considered.

The nature of the excitatory transmitter of the sympathetic
nervous system

This section concentrates on the nature of the
excitatory transmitter in the rodent vas deferens with
reference to other sympathetic junctions where appropriate.

There were two main reasons for doing so. Firstly, the
mouse vas deferens is the preparation used in the majority of
experiments in this thesis and secondly, the rodent vas
deferens is probably the sympathetic junction where the nature
of the excitatory transmitter has been investigated most.

The excitatory transmitter of the rodent vas deferens

Ever since the introduction by Huković (1961) of the
isolated guinea-pig hypogastric nerve vas deferens preparation
doubts have been raised as to whether noradrenaline is the
excitatory transmitter, despite the fact that this tissue contains the highest noradrenaline content of any in the body (Sjöstrand, 1965; Blakeley, Dearnaley & Harrison, 1970). Boyd, Chang & Rand (1960) had previously shown that cholinesterase inhibitors potentiated the response of the vas deferens to nerve stimulation and that this effect could be abolished by atropine. They also showed that the motor response was resistant to α-adrenoreceptor antagonists. These results and others led Burn & Rand (1960) to suggest that some cholinergic link was involved in the release of adrenergic transmitter.

Sjöstrand (1962a, b) found that a number of ganglion blockers blocked the response of the vas deferens to hypogastric nerve stimulation and he suggested that there existed a peripheral ganglionic relay close to the vas deferens. Subsequent histochemical studies (Falck, Owman & Sjöstrand, 1965; Sjöstrand, 1965) confirmed this view. Sjöstrand's results were assumed to explain the observed effects of acetylcholinesterase inhibitors and atropine by Boyd et al (1960). However, there is some evidence of a small cholinergic innervation of the vas deferens (Bell, 1967; Burnstock & Robinson, 1967) the significance of which is unknown. The resistance of the mechanical response to α-antagonists could also not be fully explained.

Swedin (1971) re-investigated the mechanisms of chemical transmission in the vas. He was able to show that the mechanical response of the vas to nerve stimulation could be split into two components - a fast initial 'twitch' and a slower second phase of contraction. Swedin found that the twitch was resistant to α-blockade in adult animals but not in the vas of animals in the first 10-15 days post partum. The second slower phase of contraction was always inhibited by
α-blockers. Swedin interpreted these results as meaning that
the twitch was induced by the initial release of
noradrenaline reaching a high local concentration within the
close neuromuscular junction. This was said to be inaccessible
to α-antagonists in high enough concentrations to inhibit the
twitch. The second phase was said to be mediated by lower
concentrations of noradrenaline acting on receptors located
outside the neuromuscular junction and thus easily accessed
by α-antagonists.

Ambache & Zar (1971) carried out a similar study. They
showed the existence of two tetrodotoxin susceptible components
in the motor response of the vas deferens which responded
maximally to difference pulse widths and were attributed to
the possible existence of two different groups of nerve fibres.
These authors also found similar results to Swedin (1971) in
terms of resistance of the twitch component of the contractile
response to α-antagonists. They also found that exogenous
noradrenaline inhibited the twitch response. Ambache & Zar
(1971) suggested on the basis of this evidence that motor
transmission was 'non-adrenergic'. In a later paper Ambache et
al (1972) suggested that noradrenaline was actually an
inhibitory transmitter acting prejunctionally on the excitatory
nerve endings.

Similar results to those of Ambache & Zar (1971) were
obtained when the biphasic response of the vas deferens to a
single stimulus was examined (Anton & McGrath, 1977; McGrath,
1978).

In 1976 Burnstock discussed the possibility that nerves
could release more than one transmitter. Hökfelt et al (1977)
produced the first convincing demonstration of such an
occurrence when they showed that somatostatin co-existed with
noradrenaline in a population of peripheral sympathetic
ganglia in the guinea pig. Since then many reports have claimed that nerves can release more than one transmitter and the idea of co-transmission is now well accepted (see recent reviews by Lundberg & Hökfelt, 1983; Iversen, 1984).

Sneddon, Westfall & Fedan (1982) showed that both the twitch response of the vas deferens and the electrical response to nerve stimulation (ejp) could be blocked by a reputed ATP antagonist ANAPP. Sneddon & Westfall (1984) extended these observations to show that the addition of ATP locally, using pressure ejection from a micropipette, produced a depolarisation similar in magnitude and time course to the ejp, while noradrenaline added in a similar manner produced no such response. Furthermore, exogenous ATP mimicked the first phase of the contractile response while exogenous noradrenaline mimicked the second phase. As in previous studies, (Ambache & Zar, 1971; Swedin, 1971) α-antagonists and reserpine affected the second and not the first component of contraction.

On the basis of these observations Sneddon & Westfall (1984) proposed that ATP or a related purine and noradrenaline are co-transmitters in the guinea pig vas deferens. Similar results have been found in other preparations, e.g. rat vas deferens (French & Scott, 1983); rat tail artery (Burnstock & Sneddon, 1984).

Studies of the structure and contents of vesicles in the nerve terminal varicosities tend to support this hypothesis. Lagercrantz (1981) noted that chromaffin cell vesicles contain a higher percentage of ATP than any other organelle of the body and questioned its role in such vesicles. Blaschko et al (1956) and Falck et al (1956) found that a molar ratio of 4:1 (noradrenaline:ATP) existed in granular vesicles. This ratio would provide a negative charge sufficient to bind noradrenaline.
Originally, then, ATP was said to be present in nerve terminals purely to fulfil this role and therefore to allow a large concentration of noradrenaline into the vesicles. More recent studies, however, have shown that in some nerve terminal vesicles the noradrenaline:ATP ratio may be as high as 60:1 (Fried, 1981). Thus the ATP concentration would be too small to bind noradrenaline and an alternative role must be sought. Lagercrantz (1981) stated that the possibility that it acts as a secondary transmitter "cannot be excluded".

At present, the hypothesis is a powerful one and few observations can be cited which argue against it. Ambache & Zar (1971), after hypothesising that noradrenaline was not the excitatory transmitter in the guinea pig vas deferens, suggested and then rejected a number of alternatives. ATP was one of those. They showed that addition of exogenous ATP mimicked the twitch response. However the tissue soon became completely de-sensitised to exogenous ATP while still showing a normal response to nerve stimulation. On the other hand, Meldrum & Burnstock (1983) showed that de-sensitisation of the tissue using α-β methylene ATP tachyphylaxis blocked both the response to exogenous ATP and the twitch response to nerve stimulation.

Booth et al (1978) and Brown et al (1983) showed that chemical destruction of the sympathetic nerve terminals with 6-hydroxy-dopamine eliminated the second component of the contractile response but left the twitch response. This observation is difficult to reconcile with an hypothesis of co-transmission.

The major alternative view to ATP/noradrenaline co-transmission comes from the work of Hirst & Neild (1980) on guinea pig mesenteric arterioles. These authors showed that the response to locally applied exogenous noradrenaline onto
extrajunctional sites could be blocked by prazosin (an α-antagonist) while the response produced by locally applied noradrenaline to junctional sites could not.

Hirst & Neild (1980) stated that noradrenaline must be acting on receptors in the junction which were different from those outside the junction. These were later classed as γ receptors (see Neild & Hirst, 1984). This idea is similar to that of Swedin (1971) described earlier, except that noradrenaline is said to act on a different type of receptor rather than at a receptor inaccessible to exogenously applied drugs. Sneddon & Westfall (1984) have compared the evidence regarding both the above hypothesis and their own co-transmission hypothesis. They found that most of the available evidence can be explained by both hypotheses (although generally more easily by the co-transmitter hypothesis) with the exception of the effects of reserpine. EJPs and the 'twitch' contraction were resistant to reserpine while the second phase of contraction is not. If noradrenaline, as Hirst & Neild suggest, mediates all three responses each should be similarly effected by noradrenaline depletion caused by reserpine. Sneddon & Westfall (1984), therefore, suggested that the current available evidence is best explained by the co-transmitter hypothesis for sympathetic transmission, at least at sympathetic junctions where it has been shown to exist (guinea pig and rat vas deferens and rat tail artery - for references see above). Whether the hypothesis can be extended to all sympathetic junctions is open to question. In the mouse vas deferens, the preparation used for the major part of this study, there has always been much less doubt as to the nature of the excitatory transmitter, (see for example Illes & Starke, 1983) and it has recently been suggested that noradrenaline is the primary transmitter responsible for the ejp and 'twitch'
contraction responses in the mouse vas with ATP responsible for only a small component of each (Stjärne & Åstrand, 1984).

As far as this thesis is concerned, whichever of the two current hypotheses as to the nature of the excitatory transmitter is correct, the interpretation of the results obtained will not be altered to any significant degree. The patterns of transmitter release from individual release sites will be the same regardless of the transmitter. In the studies of modulation of transmitter release all the chemicals which will be considered are released in increased quantities during sympathetic nerve activity regardless of what the transmitter is. The only difference might be in terminology. If ATP is in fact the major excitatory transmitter, noradrenaline, if it modulates transmitter release, can no longer be said to be acting via a negative feedback loop. Instead, it must be regarded as an inhibitory modulator of transmitter release.

Modulators of transmitter release

A. α-adrenoreceptor mediated inhibition

It is now well established that presynaptic α-adrenoreceptors exist in the autonomic nervous system. Suggestions of their existence were based mainly on observations of unusual effects of classical α-adrenoreceptor agonists and antagonists on transmitter overflow. In a number of preparations α-antagonists like phenoxybenzamine were found to decrease the postjunctional response produced by noradrenaline (normally contraction of smooth muscle) while increasing the concentration of noradrenaline in the overflow. Brown & Gillespie (1956, 1957) showed that dibenamine and
phenoxybenzamine greatly increased stimulation evoked noradrenaline overflow. This was the first observation that drugs could actually increase transmitter release. At the time Brown & Gillespie interpreted their results as showing that a greater proportion of noradrenaline overflowed because it was prevented from binding to the postjunctional α-receptors by the α-antagonists.

This view was challenged when it was shown that phenoxybenzamine was able to inhibit both neuronal and extraneuronal uptake of noradrenaline (Hertting et al 1961; Hertting, 1965; Iversen, 1965, 1967). This alternative view as to the action of phenoxybenzamine on transmitter overflow also had problems, however. Cocaine and desipramine two proven blockers of neuronal uptake produced little or no increase in transmitter overflow during nerve stimulation (Blakeley, Brown & Ferry, 1963; Geffen, 1965). Conversely, phentolamine increased transmitter overflow during nerve stimulation but had no effect on neuronal uptake (Kirpekar & Cervoni, 1963). de Potter et al (1969) reported that dopamine-β-hydroxylase found solely in adrenergic vesicles and not inactivated or subject to uptake was released in increased concentrations in the presence of phenoxybenzamine during nerve stimulation while cocaine did not increase its release. This was convincing evidence that the amount of transmitter released was actually increased by phenoxybenzamine.

In 1971 a number of groups independently suggested that α-adrenoreceptors were present in the terminal varicosities of adrenergic neurones (Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Langer, Adler, Enero & Stefano, 1971; Starke, 1971) and that activation of these receptors by α-agonists inhibited transmitter release.
The evidence for such an hypothesis has received frequent reviews (e.g. Langer, 1977; Starke, 1977, 1981; Westfall, 1977; Gillespie, 1980). It is now so substantial that there can be little doubt that α-adrenoreceptor agonists and antagonists can modify the amount of transmitter released by an action on prejunctional α-receptors.

The hypothesis that released noradrenaline inhibits subsequent noradrenaline release by such an action on prejunctional α-receptors has followed almost automatically. This hypothesis is based entirely on the notion that if antagonists have an effect on transmitter release it must be because they are interrupting an action that already exists; namely endogenous noradrenaline inhibiting subsequent transmitter release. This is to say the action of α-antagonists is a purely passive one. This hypothesis is undoubtedly attractive. Rand et al (1982) stated that "this has proved to be a useful unifying hypothesis. It links together a number of observations on the effects of drugs on responses to sympathetic nerve stimulation that do not fit within previous concepts of noradrenergic transmission; it supplies a logical basis for understanding these observations and it has considerable predictive capacity".

Unfortunately, until recently, few studies have been carried out with the specific intention of testing the physiological relevance of prejunctional α-receptors. Almost without exception, those that have been published have required that the negative feedback hypothesis be, at the very best, adapted. Its "predictive capacity" has proven to be very low indeed.

Chan & Kalsner (1979) measured the effect of phenoxybenzamine on noradrenaline overflow from cattle renal arteries following a fixed number of stimuli at various frequencies between 1 and
These authors found that the enhancing effect of phenoxybenzamine on transmitter release was greatest at low frequencies and was in fact inversely proportional to the frequency. This effect had been seen in previous studies (e.g. Dubocovich & Langer, 1974; Langer et al, 1975; Henderson, et al., 1975) but never, previously, had the implications of such a finding been fully discussed.

Gillespie (1980) pointed out that it cannot be argued that the concentrations of released noradrenaline are so high as to competitively displace the blocking agent since the effect is as marked with a non equilibrium blocking drug like phenoxybenzamine added before stimulation as it is with a competitive antagonist such as phentolamine.

Kalsner (1979, 1981, 1983) carried out a further series of experiments where the amount of noradrenaline released was altered by a number of methods such as varying the $[\text{Ca}^{++}]_o$ and altering the width of the stimulus pulse. In each case he found no predictable relationship between the concentration of antagonist and the amount of transmitter release which occurred.

Angus & Korner (1980) and Angus et al (1984) have shown in cardiac tissue that auto-inhibitory feedback plays little role in the modulation of transmitter release under physiological conditions because of the existence of a very efficient neuronal uptake process. Angus et al (1984) did however see evidence of autoinhibition when the stimulus frequency was set very high. Story et al (1981) confirmed this point when they repeated the experiments of Angus & Korner (1980) and showed that stimulation had to be applied in longer trains than those used by Angus & Korner at their frequency, in order for autoinhibition to appear.

Electrophysiological studies have the advantage that modulation from pulse to pulse can be examined fairly accurately.
Holman & Surprenant (1980) in an electrophysiological study of a number of different arteries found that α-adrenoreceptor antagonists such as phentolamine and phenoxybenzamine not only increased the amplitude of steady-state ejps but also the amplitude of ejps evoked by a single stimulus. Other studies using both electrophysiological (Kuriyama & Makita, 1983) and overflow studies (e.g. Kalsner, 1979) have found similar effects of certain α-antagonists on the first pulse in a train of stimuli.

Such results are difficult to explain in terms of a negative feedback loop and have led to suggestions that at least part of the action of α-adrenoreceptor antagonists is active and not merely due to passive blockade of α-adrenoreceptors (Kajiwara, Kitamura & Kuriyama, 1981).

Another possible explanation for the lack of correlation between drug concentrations and the actions of an endogenous feedback loop is that, physiologically, the feedback system only operates to combat dramatic increases in transmitter release such as is caused by facilitation when sympathetic nerves initially become active after a period of rest.

Electrophysiological studies in the vas deferens of mice and guinea pigs have shown that after facilitation occurs, subsequent stimuli cause a secondary depression. This secondary depression was found to be abolished by α-antagonists (Bennett & Middleton, 1975). Similarly it can be shown that α-adrenoreceptor agonists such as clonidine cause decay of facilitation to occur much more rapidly than in control situations while yohimbine and piperoxan, two α-adrenoreceptor antagonists increase the time constant of decay of facilitation (Blakeley et al., 1984).
B. Other possible modulators of sympathetic transmitter release

A vast array of different drugs and chemicals have now been established as having a prejunctional action modifying the release of sympathetic transmitter (see Langer, 1977). At present four of these, apart from noradrenaline, are known to be released into the extracellular space in increased concentrations during sympathetic nerve stimulation.

First, however, an alternative action of noradrenaline prejunctionally, has been considered. Presynaptic $\beta$ receptors are known to exist on sympathetic nerve terminals (Langer, Adler-Graschinsky & Eneroth, 1974; Stjärne & Brundin, 1975). During periods of stimulation which releases low concentrations of noradrenaline it can be shown that activation of these presynaptic $\beta$ receptors increases the amount of transmitter release (Stjärne & Brundin, 1976). Langer (1977) has postulated that when release is high noradrenaline acts on prejunctional $\alpha$ receptors to reduce transmitter release. When release is low, however, he suggests that noradrenaline acts on prejunctional $\beta$ receptors to increase transmitter release. Gillespie (1980) has suggested that the observed facilitation of noradrenaline release at the beginning of a train of stimuli may be due to presynaptic $\beta$ receptor activation.

1. Dopamine

Dopamine, a possible CNS neurotransmitter, is known to be present in a number of sympathetic nerve endings in varying concentrations (Bell, 1982). It is also known that presynaptic dopamine receptors are present on sympathetic nerve terminals (Langer, 1973; McCulloch, Rand & Story, 1973).

While in some tissues dopamine is equally as effective as noradrenaline at inhibiting release (Rand et al., 1973) in others, such as the guinea pig vas deferens (Ambache & Zar, 1971), it is
found to be about 10 times less effective than noradrenaline. In tissues where it is equipotent to noradrenaline it is thought to act on dopamine receptors, while in the others it is said to have an action on prejunctional α-receptors.

Since the potency of dopamine is at best equal to noradrenaline in terms of a prejunctional action, it would need to be released in relatively high proportions compared with noradrenaline in order to have a significant physiological effect.

While Bell et al (1984) have shown that the ratio of release of dopamine:noradrenaline is higher than the total dopamine:noradrenaline ratio in presynaptic stores, dopamine still makes up only 6% of the total catechol release in the rat vas deferens and 1.3% in the guinea pig vas deferens. It is difficult to see how this could be large enough to have any physiological significance.

2. Prostaglandins

Adrenergic stimulation is known to cause the release of prostaglandins of the E series (Davies et al., 1968; Ferriera et al., 1973). The balance of evidence suggests that they are released from postjunctional rather than prejunctional membrane (see Hedqvist, 1981). Prostaglandins of the E series can be shown to inhibit noradrenaline and dopamine-β-hydroxylase release from sympathetic nerves (Hedqvist, 1969; Hedqvist & Brundin, 1969; Johnson et al., 1971; Wennmalm, 1971) and also inhibit the electrical response to nerve stimulation (Sjöstrand, 1972; Cunnane, 1979).

Gillespie (1980) after considering the available evidence has suggested that the mechanism of prostaglandin inhibition is completely independent from α-antagonism.

Although the action of prostaglandin, if it occurred
physiologically would be trans-synaptic, inhibition rather than prejunctional autoinhibition (Hedqvist, 1981) and although prostaglandins are known not to affect all sympathetic nerves (Hedqvist, 1977; Westfall, 1977), direct evidence suggesting that modulation occurs physiologically is probably stronger for prostaglandins than it is for any other chemical.

It can be shown that when prostaglandin synthesis is blocked during sympathetic nerve stimulation by drugs such as indomethacin, transmitter release is increased (Hedqvist, Stjärne & Wennmalm, 1971; Kuriyama & Makita, 1984). Similarly when prostaglandin synthesis is increased by addition of arachidonic acid, the precursor of prostaglandins, transmitter release is decreased (Frame & Hedqvist, 1975).

3. Adenosine

Adenosine prejunctional inhibitory receptors are known to exist (Hedqvist & Fredholm, 1976). If ATP is a co-transmitter in sympathetic nerves (e.g. Sneddon & Westfall, 1984) its breakdown product adenosine, could act prejunctionally to inhibit release. Although adenosine is equipotent to ATP prejunctionally, it is present in most body fluids in a higher concentration than ATP (Fredholm, 1981).

Fried (1981) has shown that the ratio of noradrenaline to ATP stored in small granular vesicles could be as high as 60:1. Since neither ATP nor adenosine are 60 times more potent than noradrenaline it is difficult to see, as was the case with dopamine, how it can have a physiological effect.

Adenosine is, however, released from postsynaptic sites too during sympathetic nerve stimulation. It could then function as a trans-synaptic modulator (Fredholm, 1981).
4. Neuropeptide Y

Recent improved radioimmunoassay techniques have shown that a peptide, neuropeptide Y (NPY) co-exists with noradrenaline in both the CNS and throughout the periphery in adrenergic nerves including those which innervate the vas deferens (Emson & de Quidt, 1984).

It has been shown that NPY inhibits sympathetic neurotransmitter release in a number of preparations (Lundberg et al., 1982; Lundberg & Stjärne, 1984). Its particularly potent effects on transmission in the vas deferens have led to the suggestion that it may be involved, physiologically, in a local inhibitory feedback loop (Emson & de Quidt, 1984).

Thus a number of chemicals released during sympathetic nerve stimulation may be involved in modulating transmitter release. One also has to bear in mind a possible contribution from circulating hormones such as adrenaline and angiotensin.

The situation can perhaps be highlighted by reference to a recent paper by Kuriyama & Makita (1984). These authors used a variety of pharmacological manipulations to examine the modulation of transmitter release in the mesenteric artery of two species, the guinea pig and the rabbit.

In the guinea pig mesenteric artery they found that noradrenaline had an inhibitory action on α receptors and a facilitatory action on β receptors. Prostaglandins inhibited transmitter release while purines had no effect. In the rabbit mesenteric artery they found no α receptors, noradrenaline inhibited release via β receptors, while prostaglandins and purines both inhibited transmitter release. Indomethacin was found to enhance transmitter release, suggesting that endogenous prostaglandins inhibit release physiologically.
Interestingly, the prostaglandin effect was the only one common to the two species.

Hypothetically, such systems of modulation would seem to be very useful to a nervous system whose function, under normal conditions, is to maintain tonic control of the muscle it innervates.

Such systems could, for example, be used to keep transmitter secretion to a minimum by ensuring an even spread of release throughout the preparation. Additionally, such systems would transfer the 'burden' of tonic control away from the CNS to a large extent.

As stated before, none of these systems of modulation have been proven to exist. Part of the problem is undoubtedly due to the techniques generally used to examine sympathetic transmission. Measurement of transmitter overflow and end organ responses are excellent methods of showing changes in responsiveness of systems caused by drugs. They are, however, a long way from measuring the activity of individual sympathetic varicosities.

A major part of this thesis will, therefore, use the technique of Blakeley & Cunnane (1979) to monitor release of transmitter from individual release sites from pulse to pulse during trains of stimuli. This will be done in the presence of α-adrenoreceptor agonists and antagonists, in order to investigate the most favoured of the proposed forms of modulation - modulation via prejunctional α-adrenoreceptors - and also in the absence of drugs in an attempt to discover evidence of physiological modulation of transmitter release, regardless of what causes it, from pulse to pulse.
Summary of the objectives of this thesis

Many experiments have been carried out in an attempt to understand the sympathetic transmission process. Many ideas and hypotheses exist which provide possible explanations for different features of the transmission process. In this introduction, however, it has been shown that few of these hypotheses remain unchallenged or can explain all the available experimental evidence.

In this thesis a recently developed technique (Blakeley & Cunnane, 1979) will be employed to look at sympathetic transmission in a novel manner and hopefully to provide new ideas as to the mechanism of the transmission process. The technique allows investigation of transmitter release from a relatively small number of release sites on sympathetic nerve endings innervating the rodent vas deferens.

While it is known that transmitter release at this junction is packeted, the nature of release from individual release sites (monoquantal or multiquantal) and how release from a particular release site is related to release from surrounding release sites is not known. Therefore the first part of this thesis will involve a detailed study of transmitter release from a few release sites close to the recording cell in an attempt to build up a model of the patterns of packeted transmitter release which affect a single smooth muscle cell.

Once such a model is established, the second part of this thesis will use the model to aid understanding of a number of known features of the sympathetic transmission process. The existence, or not, of a system of physiological control of transmitter release will be fully considered, and the processes of facilitation of transmitter release and neuronal uptake of transmitter will also be looked at.
Finally, an attempt will be made to extend the technique used to observe transmitter release from individual release sites of the rodent vas deferens to individual release sites of sympathetically innervated blood vessels.
CHAPTER 2

GENERAL METHODS
CHAPTER 2

General methods

This chapter describes the different preparations and the general experimental and analytical methods used throughout this thesis.

Many methods of analysis and experimental procedures used were peculiar to one chapter only. These are described in the methods section of the relevant chapters.

Preparations used

(a) Mouse vas deferens

Male C57BL6 mice (18-25g) were killed by cervical dislocation and the right vas deferens rapidly removed. It was pinned at approximately resting length to a layer of Sylgard gel (Dow Corning Corporation) on a perspex slide. This was then mounted in a 6ml recording chamber.

The prostatic end of the vas was passed into a pair of platinum bipolar ring electrodes (separation 0.5mm) connected to a Bell isolated stimulator unit.

(b) Guinea pig mesenteric arteries and arterioles

Preparations of mesenteric arteries and arterioles used in this study were made from segments of small intestine of guinea pigs of either sex (200-500g).

A segment of intestine, complete with mesenteric arteries, was pinned to a layer of Sylgard gel on a perspex slide in a dissecting dish.

(i) arteries. A suitable length of artery was selected, freed from its branches, and dissected away from the remainder of the intestine at its point of entry into the circular muscle layer. It was pinned securely to the Sylgard gel and the remainder of the intestine was removed.
(ii) arterioles. This preparation is essentially the same as that described by Hirst (1977).
A strip of the muscle mucosa was removed revealing the mesenteric blood vessels on the inner side of the intestine. A suitable arteriole tree was selected, and all other arteries running into the mesentery were cut leaving only the artery supplying the selected arteriole tree intact. The intestinal mucosa was then freed from the connective tissue sheet (containing the arteriole tree). When all the mucosa had been removed (an important step in order to stop future spontaneous contractions), the connective tissue was pinned securely to the Sylgard gel.

In both dissections the perspex slide was then mounted into the recording chamber.

(c) Rat tail artery

A strip of caudal artery (2-3cm) was dissected out from the proximal end of tails from rats of either sex (120-200g). The strip was pinned at approximately resting length to a layer of Sylgard gel on a perspex slide and then mounted in the recording chamber.

Stimulation of blood vessels was carried out either by drawing them into a suction electrode or, if the vessel was large enough, passing it through a pair of platinum bipolar ring electrodes. Both types of electrodes were attached to a Bell isolated stimulator unit.

For all preparations the recording chamber was continually perfused at a rate of 3ml/min with oxygenated (95% O₂:5% CO₂) Krebs solution, pH 7.4. The composition of the Krebs solution (in mM) was NaCl 118.4; NaHCO₃ 25; KCl 4.7; CaCl₂ 2.1; MgCl₂ 1.3;
NaH$_2$PO$_4$ 1.13; and glucose 11.1. The temperature of the recording chamber was maintained at between 35 and 36°C.

**Apparatus**

The recording chamber was fastened to a soft iron, goat myograph stand in such a way that the perspex slide could easily be placed in it without damage to the preparation.

The myograph stand was isolated from vibrations using Muffelite vibro dampers (K150, K300 cementation Muffelite). The recording chamber was illuminated from the rear and the preparation viewed using an inverted Zeiss microscope (magnification 15.75-100).

The Krebs solution, described above, was actively pumped into the recording chamber using a Watson-Marlow flow pump. The Krebs then drained out passively from the chamber by flowing through a tapered length of capillary matting into a drainage beaker.

The temperature of the Krebs solution in the recording chamber was maintained at 35-36°C using a Tempette circulator (TE7) which passed warm liquid paraffin around the side of the recording chamber. The temperature was monitored using a Telemax glass bead thermistor.

Intracellular electrical recordings were made with glass microelectrodes pulled by a W.P.1. microelectrode puller, model PUL-1, from glass (Clark EMI) which had an outside diameter of 1.2mm and fused inner filaments. These microelectrodes were filled by immersion in 3M KCl and when used they were mounted into a W.P.1. microelectrode holder. This connected the electrode to the recording apparatus. Also attached to the recording apparatus was a Ag:AgCl pellet which was placed in the recording chamber to act as the indifferent electrode.
The microelectrode could be manoeuvred into a suitable position using a rough 3 direction manipulator and then more accurately positioned using a Huxley micromanipulator (Huxley, 1961).

The membrane potentials of the smooth muscle cells were recorded using these microelectrodes (resistance 25-45M) and the electrical signals obtained were amplified using a Dagan 8100 single electrode voltage clamp in bridge clamp mode. The resistance of the electrodes could be measured easily using the Z test function of the Dagan 8100.

Many of the features of the Dagan 8100 will be considered in more depth in Chapter 4 (see also Figure 4.1).

The electrical signals obtained were recorded on a Racal Storage 4D tape recorder (tape speed 3.75in/sec) with frequency response d.c. to 3kHz.

Cell penetrations were accepted only if:

1. the cell penetration was abrupt.
2. the resting membrane potential did not become more positive once the electrode had sealed.

and for the mouse vas deferens:

3. spontaneous excitatory junction potentials were recorded.
4. following submaximal hypogastric nerve stimulation the resting membrane potential returned to the same or a more negative value (after Blakeley & Cunnane, 1979).

The electrical signals were continually displayed on a cathode ray storage oscilloscope (Tektronix 5103N) and also monitored by a digital voltmeter.
Detection of discrete events

Junction potentials were elicited by submaximal field stimulation. The first time differential of the junction potentials revealing discrete events was obtained by two methods:

(a) During the experiments the signal was continuously differentiated using an operational amplifier differentiator circuit (Fig. 2.1) whose frequency response was severely limited (<500Hz) in order to reduce output noise to a reasonable level (Blakeley & Cunnane, 1979).

The differential traces produced were stored on tape along with the junction potentials. Later they were replayed onto a Tektronix dual beam oscilloscope (Type 502A) and filmed by a Grass Kymograph camera (speed of film 10mm sec). The stimulus artefact, beginning of the discrete event and peak amplitude of the discrete event were digitised by hand (see Figure 2.2) using a Hewlett-Packard 9111A graphics tablet connected to a Research Machines 380Z microcomputer. The digitised values obtained were then stored on floppy disks.

(b) After the experiments, the recorded junction potentials were digitised (sampling frequency 5kHz) using a digitimer NL900 board attached to a Research Machines 380Z microcomputer. The digitised signal was then smoothed by averaging successive pairs of samples and differentiated by calculating differences between averages of two variable sized running sample windows displaced by one sample interval. Hard copies of the digitised traces were printed out on a JJ Instruments PL5 recorder.
Figure 2.1. The differentiator circuit.
Figure 2.2. Definition of digitised parameters.
Analysis of discrete events

Latency and time to peak histograms were obtained for all the discrete events occurring within a train of junction potentials. The discrete events were then grouped into families according to the criteria defined fully in Chapter 3.

Amplitude, rise time and rise rate distributions were then obtained for the events in each family. In those cells where events appeared superimposed upon a slow background, the background was subtracted by computer before compiling the amplitude distributions.

Drugs Used

Yohimbine Hydrochloride (Sigma)

Clonidine Hydrochloride (Catapres, Boehringer, Ingelheim)

Desmethylimipramine Hydrochloride (Geigy)
CHAPTER 3

BASIC PROPERTIES OF DISCRETE EVENTS
CHAPTER 3

Introduction

Stimulation of the sympathetic nerves innervating the rodent vas deferens causes the release of transmitter. This produces a depolarisation of the postjunctional membrane termed the excitatory junction potential (ejp) (Burnstock & Holman, 1961). The ejp reflects both the action of transmitter close to the recording electrode and electrotonically conducted depolarisation from remote sites of transmitter action. During such conduction, however, the amplitude of these depolarisations is attenuated and their time course slowed. Only transmitter released close to the recording electrode will therefore produce a rapid rate of depolarisation.

The rising phase of this ejp is not a smooth continuous event, and a train of constant intensity stimuli produces a series of ejps whose rising phase varies markedly from one stimulus to the next. If the first time differential (dV/dt) of the ejp is obtained these discontinuities in the rising phase can be revealed as transient peaks in the rate of depolarisation which have been termed discrete events (d.e.s) (Blakeley & Cunnane, 1979). These d.e.s. represent rapid effects of transmitter and can thus be attributed to local transmitter action from release sites close to the recording electrode.

The object of this chapter is to define some of the fundamental properties of d.e.s. and from these, outline possible models of the sympathetic transmitter release mechanism. In subsequent chapters experimental and theoretical procedures will be carried out in an attempt to describe d.e.s. more accurately and to determine which model of sympathetic transmitter release is most likely to be correct.
Basic Observations

The resting membrane potentials of the cells of the mouse vas deferens used in all treatments, performed during the work described in this thesis, are summarised in Table 3.1. In control conditions membrane potentials ranged from -61 to -88 mV with a mean value (± s.d.) = -72 ± 4 mV (n=350).

Typical excitatory junction potentials (ejps) recorded in response to field stimulation at a moderate intensity and 2 Hz frequency are shown in Figure 3.1. In order to avoid muscle contraction (and consequent dislodging of the recording microelectrode) all stimulation was carried out at a submaximal intensity and at a frequency low enough (normally 2 Hz) to avoid summation of successive ejps.

Association of Discrete Events

Differentiation of a train of ejps produces a set of waveforms which vary greatly from stimulus to stimulus. Discrete events are recorded as intermittent peaks in these waveforms which vary in latency, amplitude and time course (see Figure 3.2). It is now well established that these d.e.s. represent, in some way, the action of transmitter intermittently released from single release sites (Blakeley & Cunnane, 1979; Blakeley, Cunnane & Petersen, 1982; Cunnane & Stjärne, 1982). The difficulty arises in attributing particular events, or groups of events, to particular release sites.

The problem can be divided into two parts. Firstly, the acceptable limits of variation must be defined, within which pairs of events are deemed to be identical. Secondly, it has to be decided if groups of identical events can be associated together in any way to aid analysis, and if so what the physiological equivalent of such an association would be.
Table 3.1.

Membrane potentials of smooth muscle cells of the mouse vas deferens used in this study

<table>
<thead>
<tr>
<th>[Ca]₀ (mM)</th>
<th>Drug (M)</th>
<th>N</th>
<th>Vm (±S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>-</td>
<td>350</td>
<td>-71.9 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>yohimbine 10⁻⁶</td>
<td>8</td>
<td>-70.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>yohimbine 10⁻⁷</td>
<td>16</td>
<td>-72.7 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>yohimbine 5x10⁻⁹</td>
<td>9</td>
<td>-71.3 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>clonidine 10⁻⁷</td>
<td>9</td>
<td>-74.7 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>clonidine 10⁻⁸</td>
<td>7</td>
<td>-73.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>clonidine 5x10⁻⁹</td>
<td>22</td>
<td>-73.5 ± 1.5</td>
</tr>
<tr>
<td>1.1</td>
<td>-</td>
<td>43</td>
<td>-70.6 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>yohimbine 10⁻⁷</td>
<td>13</td>
<td>-67.2 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>clonidine 5x10⁻⁹</td>
<td>8</td>
<td>-74.0 ± 2.4</td>
</tr>
<tr>
<td>1.6</td>
<td>-</td>
<td>16</td>
<td>-69.9 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>yohimbine 10⁻⁷</td>
<td>8</td>
<td>-68.9 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>clonidine 5x10⁻⁹</td>
<td>9</td>
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</tr>
<tr>
<td>4.2</td>
<td>-</td>
<td>12</td>
<td>-74.0 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>yohimbine 10⁻⁷</td>
<td>7</td>
<td>-73.1 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>clonidine 5x10⁻⁹</td>
<td>7</td>
<td>-73.0 ± 2.8</td>
</tr>
</tbody>
</table>
Figure 3.1.

Typical eJPs recorded in mouse vas deferens in response to a train of submaximal nerve stimulation at 2Hz.

Calibration bars 1 sec, 5mV.
Figure 3.2.

Superimposed discrete events evoked by a train of twenty-five stimuli at 2Hz. Traces with no discernable discrete events at any latency have not been included.
Acceptable limits of variation

(i) Latency and time to peak

In other preparations, notably the skeletal neuromuscular junction, it has been shown that a variable latency can occur between the arrival of an action potential in the nerve terminal and the occurrence of a postsynaptic response (Barrett & Stevens, 1972; Datyner & Gage, 1980). It has been shown that the depolarisation/secretion coupling mechanism is mainly responsible for this rather than the diffusion of transmitter across the synapse (Katz & Miledi, 1963).

It seems reasonable to suppose that a similar "jitter" will occur in sympathetic nerves. Thus, in this study, discrete events whose latency and time to peak are within the same 2msec bands are defined as having indistinguishably different time courses from each other.

(ii) Amplitude

The limit of acceptable variation in amplitude is set by the noise of the recording system. In most cases the noise is between 0.1 and 0.2 V/sec.

The limits of acceptability for events to be identical are shown in Figure 3.3. In an average cell a train of 200 stimuli will elicit up to twenty different groups of events, each group containing anything from 1 to 50 indistinguishable members.

Families of Discrete Events

Figure 3.4 shows a typical set of amplitude, latency, and time to peak histograms, for d.e.s. from a single cell. The amplitude histogram of d.e.s., irrespective of latency, is negatively skewed and multimodal. There does not, however, appear to be any obvious relationship between the modes and
Figure 3.3.
Superimposed discrete events selected from a train of twenty-five stimuli at 2Hz to illustrate limits of acceptability. Bars show latency, peak time and amplitude criteria within which events are considered to be indistinguishable.
Figure 3.4.

Typical (A) latency, (B) time to peak and (C) amplitude histograms for all discrete events recorded in a train of stimuli at 2Hz in a single cell.

Bin size = 0.5msecs in (A) and (B) and 0.08 V/sec in (C).
indeed it is difficult to tell precisely where the modes are.

In this cell, as in many others, a large proportion of the d.e.s. appear to have the same latency and time to peak (within the limits defined above). Clearly events differing in latency come from different terminal nerve fibres and therefore different release sites.

The corollary, that events with the same latency represent transmitter release from the same fibre is not, however, necessarily true. Nevertheless, selection of events with a common latency and time to peak will eliminate events that are definitely due to release of transmitter from sites associated with a different nerve fibre. This should help in the classification of discrete events.

Figure 3.5(a & b) shows the latency and time to peak histograms from a single cell. The bands drawn down these histograms have been used to select out discrete events which have the same latency and time to peak. Figure 3.6(a & b) shows the amplitude histogram of the discrete events from this cell (a) before and (b) after the selection criteria have been applied. Figure 3.6(b) shows quite clearly that the amplitude histogram has become multimodal with modes spaced regularly along the amplitude axis.

The responses that are selected out in this way are termed a 'family' of discrete events. A reconsideration of Figure 3 shows this selection procedure in another way by looking at superimposed discrete events recorded from the same cell. It is clear that events peak at different times but there is one obvious family of responses which start and peak within a very narrow time band.

Figure 3.7 shows by way of example the same selection procedure performed on two other cells.

When families have been used for analysis in any of the
Figure 3.5.
(a) latency and (b) time to peak histograms for all discrete events recorded in a train of stimuli at 2Hz in a single cell. Vertical bars indicate selection of indistinguishable latency and time to peak discrete events.

Bin size = 0.5msecs in (a) and (b).
Figure 3.6.

Amplitude histograms of discrete events in Figure 3.5.
(a) before and (b) after selection.
Bin size = 0.1V/sec. in (a) and (b).
Figure 3.7(a)

Amplitude histograms showing similar selection procedure in two other cells.

Bin size = 0.05V/sec in (a) and 0.08V/sec in (b).

In both (a) and (b), the top histograms show amplitudes before selection while the bottom histograms show amplitudes after selection.
Figure 3.7(b).
experiments reported in this study a similarly stringent selection procedure has been performed on each group of d.e.s. used.

**Physiological equivalents of families of discrete events**

In order to use families of discrete events to gain a better understanding of the mechanisms of sympathetic transmitter release it is important to consider what possible models of transmitter release could produce such families.

The syncytial nature of smooth muscle and its multiple innervation (see Chapters 1 & 4) makes possible an almost infinite number of schematic models which could represent mechanism of sympathetic transmitter release.

However, other factors can be taken into account. The structural evidence presented in Chapter 1 suggests that transmitter is released from presynaptic vesicles in uniformly sized packets. The amplitude histograms of a family of d.e.s. is multimodal and negatively skewed. The modes are generally quite distinct from each other and spaced evenly along the abscissa. These observations suggest that transmitter is released in a packeted or quantal manner at a single latency from one or a few release sites close to the recording electrode.

Five possible types of models of packeted transmitter release could adequately describe such observations on families of d.e.s.

These models are outlined below and can be seen diagramatically in Figure 3.8.
Figure 3.8.

Schematic models of transmitter release sites representing a family of discrete events. See text for details. (page 46)
Model 1: Multiquantal release from a single varicosity, on to a single cell. The amplitude distribution reflects a Poisson distribution of uniformly sized packets released in variable integer numbers (0,1,2,3......) after each stimulus.

Model 2: Monoquantal release from a number of varicosities each acting on a different cell. The amplitude distribution reflects attenuation of the response between cells. The largest d.e.s. would be due to transmitter action on the recording cell. The next largest mode due to transmitter acting on a cell coupled to the recording cell etc.

Model 3: Monoquantal release from a number of varicosities on a single axon onto a single cell. The amplitude distribution reflects a binomial distribution of uniformly sized packets released from a variable number of release sites (0,1,2,3....) after each stimulus.

Model 4: As (3) except that the variation in amplitude is due to the distance of the release site from the recording electrode and not due to coincident release of transmitter from more than one site.

Model 5: Monoquantal release from a number of varicosities each on a different axon onto a single cell. Variation in amplitude could be caused in the same way as either model (3) or model (4).

It should be noted that all these models ignore the contribution of transmitter released from sites giving d.e.s. which do not fit into families.

In each of the models these could be depicted as single varicosities on axons running close to the recording cell which have a significantly different latency to the axon or
The object of the next four chapters is to carry out experimental and theoretical procedures designed to test which of the five proposed models is most likely to be correct.

The results obtained will be discussed in Chapter 8.

Some other observations on families of discrete events

(i) **Disappearance of families of d.e.s.**

In cells where long trains of stimuli (generally >300) are analysed, families of discrete events are seen to disappear. When this occurs, a second family with different latency, time to peak, and amplitude characteristics often takes its place. This phenomenon is illustrated in Figure 3.9. Figure 3.9(b) shows the amplitude histogram of the initial family and its occurrence distribution while Figure 3.9(a) shows the amplitude histogram which takes its place together with its occurrence distribution. In this example the first family appears to fire normally for about the first 120 stimuli. It then disappears and the second appears.

**Physiological importance**

The physiological importance of this effect will be discussed fully in Chapter 9 where patterns of transmitter release and the effect a given release has on subsequent release will both be looked at with a view to investigating the existence (or not) of a system of negative feedback.

**Experimental importance**

From an experimental point of view this observation has been discussed now because many future experimental observations and conclusions are based on the proportion of failures of occurrence of a family member d.e. Obviously disappearance of a family would distort such results.
Figure 3.9. (A & B)

top: Amplitude histograms of two different families of discrete events recorded in a single cell in response to trains of stimulation at 2Hz. Bin size = 0.1 v/sec in both.

bottom: The lower histograms in each case show the occurrence distributions of the two families. Stimulus group 1 = stimuli 1-20. Stimulus group 2 = stimuli 21-40 etc.
Figure 3.9(B).
Thus, in future experiments, the majority of trains of stimuli used were restricted to between 100 and 200 shocks. Before the cell was further analysed the responses were divided into blocks of 50 and the proportion of failures for each block was calculated so as to ensure no significant change occurred from one block to the next.

(ii) Poisson distribution and non Poisson failures

At a number of chemical synapses the release of a small variable number of packets of transmitter can be reasonably described by Poisson statistics (Fatt & Katz, 1952; Katz, 1966; McLachlan, 1978).

In a Poisson process, where the mean probability of release (m) is low, on some occasions, invasion of the release site by an action potential will not elicit the release of transmitter from that site. These are termed 'Poisson failures'.

In families of d.e.s. in the mouse vas deferens the amplitude distributions obtained resemble a Poisson distribution (see for example Figure 3.5b). However, too many failures of occurrence exist to allow application of Poisson statistics to the distributions by any of the conventional methods (cf. McLachlan, 1978). This suggests that a proportion of the failures that occur are due to some failure before a Poisson release process is entered. It is tempting to hypothesise that these failures represent failure of action potential invasion of the particular varicose terminal region responsible for the generation of that particular family.

Such a feature of families of d.e.s. in the mouse vas deferens is comparable with non-Poisson failures of release found at other junctions, e.g. guinea pig vas deferens (Blakeley, Cunnane & Petersen, 1982), crustacean neuromuscular junction (Bittner & Harrison, 1970).

The Poisson nature of the amplitude distribution and non
Poisson failures will be examined in more detail in Chapter 7.
CHAPTER 4

BIOPHYSICAL STUDIES
CHAPTER 4

Introduction

This chapter discusses three approaches, mainly theoretical, attempted to try to understand more fully the electrical properties of smooth muscle cells and, consequently what the expected electrical response of a single cell to transmitter action (both direct and on a coupled cell) would be.

The three approaches are:-

a) A study of the passive electrical properties of the mouse vas deferens in response to intracellular current injection.

b) The design of a mathematical model consisting of an accurate representation of the electrical equivalent circuit for a smooth muscle bundle which can undergo conductance changes similar to those caused by transmitter action.

c) An attempt to 'voltage clamp' the recording cell with a single microelectrode to gain more insight into the currents which act on any one cell of the smooth muscle bundle.

The underlying aim of all these approaches is to define more closely what the 'discrete event' represents.

Impedance Measurements

Introduction

In order to understand fully the response of a bundle of smooth muscle cells to the action of a chemical transmitter it would be useful to develop the electrical equivalent circuit of the muscle cell bundle. To do that, the passive electrical
The passive electrical properties of smooth muscle must be known.

Smooth muscle consists of many short fibres (~400µ in length) connected together by 'nexi' or 'tight junctions' (see Chapter 1), which cause it to behave like an electrical syncytium. This structure of smooth muscle is entirely responsible for its passive electrical properties.

The passive electrical properties of smooth muscle (with particular reference to the rodent vas deferens).

The passive electrical properties of smooth muscle can be shown to vary markedly depending on what type of stimulation is employed (Tomita, 1970; Bennett, 1972).

a) Stimulation of a strip of smooth muscle with large external electrodes, for example, (Tomita, 1966a,b, 1967a; Abe & Tomita, 1968) shows that the muscle has cable-like properties. Cable properties are normally associated with long, thin projections such as nerve cells and certain types of muscle fibres where the potential change developed at the point of stimulation decays only in the longitudinal direction along the cable. Thus it is perhaps surprising that smooth muscle has cable properties as its structure is very different from other structures which possess such properties. However, the observation can be explained if it is assumed that large external electrodes induce a large polarisation which causes the muscle bundle to be transversely equipotential about the area of stimulation. Potential would then only decay in the longitudinal direction away from the stimulating electrode.

Using such a technique, in the guinea pig vas deferens, Tomita (1967b) has calculated a time constant for this preparation ($T_m$) of 100msecs and a length constant of 2.1mm. Bywater and Taylor (1980), after applying correction factors
to allow for the fact that the relative lengths of tissue in the stimulating and recording compartments affects the time course of the electrotonic potential, found $T_m = 270\text{msecs}$ and $\lambda = 0.86\text{mm}$ for the same preparation.

b) Intracellular stimulation, using a microelectrode inserted into a single smooth muscle cell, produces a quite different effect. Current injected into a cell by an intracellular electrode will escape in all three dimensions via the low resistance pathways between cells. In this case both the space and time constants will be markedly less than those obtained when external polarisation is used (Tomita, 1970; Bennett, 1972). Intracellular measurements of resistance (input resistance) were first reported for cells of the vas deferens by Hashimoto, Holman & Tille (1966) who showed that the guinea pig vas deferens had a variable input resistance from 15-37MΩ.

Further studies by Holman, Taylor & Tomita (1977) have apparently shown two populations of cells, one type with input resistance 10-30MΩ and a time constant 1-1.5msecs and the other type with input resistance 20-50MΩ and time constant 5-15msecs.

In the same paper they showed that mouse vas deferens cells could also be split into two types, one of high input resistance and one low, with input resistances varying from 20-200MΩ and time constants 3-25msecs.

All these measurements were carried out using a single microelectrode impaled into a smooth muscle cell. Current was passed using a bridge circuit.

**Impedence Measurements: a.c. current analysis**

By passing an alternating current through an intracellular electrode into a cell it is possible to measure the input
impedance of that cell (Tasaki & Hagiwara, 1957) at any
given frequency. If the response to sinusoidal current over
a wide range of frequencies is measured, much information
about the membrane impedance, particularly the reactive
component, is obtained.

In a number of other types of excitable cell the
variation in impedance with frequency has been measured. The
values obtained were then compared with the impedance changes
with frequency seen for a number of different R/C network
models, in order to find the RC model which was most similar
to the observed results. This model was then taken as being
the electrical equivalent circuit for that particular
excitable cell (see Falk & Fatt, 1964; Jack, Noble & Tsien,
1975). Bennett (1972) has suggested that such a procedure
would provide much information as to the electrical equivalent
circuit of smooth muscle, however, no rigorous experimental
observations have so far been reported for this type of
muscle.

The major aim of this part of the chapter, then, is to
carry out a series of impedance measurements, at different
frequencies in order to find an electrical equivalent circuit
for smooth muscle. This study will also compare impedance
and resistance measurements obtained from a number of cells
to see if there is any correlation between membrane impedance
and the presence or absence of prominent discrete events.

**Methods**

The general equipment used in experiments described
throughout this chapter was described in Chapter 2.

All resistance and impedance measurements were carried
out using a Dagan single electrode voltage clamp system,
Model 8100 (see Figure 1). This allowed measurements to be
carried out using two different techniques: bridge clamp
or switch current clamp.

a) Bridge clamp.

In this mode the Dagan 8100 functions as a standard d.c. intracellular preamplifier with bridge current injection capability. The bridge clamp can be seen in Figure 4.1 - amplifier A1. The impedance recorded by the microelectrode is used as the unknown positive input into A1 and can thus be measured accurately.

Current injected into the microelectrode can be controlled either by using the current control provided (where current can be selected to .02% resolution) or by using an external source. When an external source is used a ten volt signal supplied to the Dagan results in 100nA current at the microelectrode.

The impedance of a cell is measured, therefore, by passing a known current and measuring the imbalance created in the bridge (A1). Before the cell is penetrated, impedance generated in the recording electrode when current is passed through it, can be balanced out using the offset voltage control (Figure 4.1).

b) Switched clamp

In the switched clamp mode, the Dagan 8100 switches rapidly between current injection and voltage recording using the same microelectrode. The current passed during the injection phase is controlled by the 'error signal' generated by comparison of the voltage recorded in the non-injection phase and the reference voltage.

In order to obtain the best results in this mode the switching frequency, which has a range of between 500Hz and 20kHz, should be set as high as possible. There are two main reasons for this:
Figure 4.1.
Circuit diagram of Dagan Model 8100 single electrode voltage clamp.
(i) **Optimisation of recording.** In this study the response of the preparation to intracellular current sine wave pulses was measured using sine waves up to a frequency of about 800Hz, i.e. 1 sine wave every 1.25msecs. The switch cycle should be much shorter than .25msecs in order to have sufficient number of cycles per wave to properly represent it. Greater than five cycles (i.e. a frequency >4kHz) should be sufficient.

(ii) **Optimisation of current passing.** When a current pulse is passed into the cell, it is important that the time constant of the switching cycle is small compared with the membrane time constant. In this way the current pulses will be reasonably smoothed to change the membrane capacitance during stimulation. The membrane time constant of the mouse vas deferens is low because of the low resistance pathways between the smooth muscle cells. It has been found to vary between 3 and 25msecs with a mean of about 7-8msecs (Holman, Taylor & Tomita, 1977).

Thus, again, a switching frequency greater than 4kHz would be desirable particularly in cells with a small membrane time constant.

Unfortunately, the frequency of switching is limited by stray capacitance at the input connector which distorts the signal and causes the loss of high frequency components of the signal.

**Stray or residual capacitance**

The stray capacitance present varies from microelectrode to microelectrode. During stimulation a voltage builds up at the probe input connector. This voltage decays during the recording half of the duty cycle to a steady state value.
The actual voltage measurement by the Dagan takes place during the last 10μsecs of the recording period (see Figure 4.2). If the switching frequency and the stray capacitance are both sufficiently high so that the voltage is sampled before the transient has decayed then an incorrect reading will occur.

Two methods have been used to try to overcome the problem.

a) **Shielding**

The microelectrode holder was encased in a piece of hollow brass tubing connected to a driven shield. This driven shield is kept at the same potential as that which exists at the input and should reduce negative capacitance.

b) **Capacity compensation control** (Figure 4.1 - amplifier A2)

This function of the Dagan 8100 allows stray capacitance to be 'balanced out'. In simplified terms this control works by measuring the capacitative current generated by the stray capacitance during switching and then passing an equal and opposite current - 'negative capacitance' - in compensation.

Before impalement of a cell, each microelectrode was tested using adjustments of the switching frequency, and capacity compensation control to find the highest switching frequency which gave a clear output signal for that microelectrode.

The maximum switching frequencies that could be obtained were of the order of 5kHz. For most electrodes the frequency could be raised above 4kHz and the maximum was never lower than 3kHz.

The switched clamp system, developed originally by Bennecke & Lindemann (1974a, b) has the great advantage over the bridge system that it works independently of any
Figure 4.2.

Duty cycle of switch clamp.

(a) Theoretical switching pattern of Dagan switch clamp.

(b) Voltage recording made in last 10μsec of voltage record phase.

(c) Schematic representation of stray capacitance inhibiting correct voltage recording. The switching frequency is too fast with this amount of stray capacitance for the steady-state true voltage to be reached in the voltage record phase.

R = voltage record. S = current stimulation.
microelectrode impedance. Thus, changes in microelectrode impedance after cell penetration, should not affect measurements using this technique.

Voltage clamp experiments were also carried out using the Dagan single electrode voltage clamp system (Model 8100).

The system works principally as described above for the switch circuit current clamp, in that a single electrode switches between passing current and measuring voltage. Switching frequency was maximised to 5kHz as described above.

The successful clamping of a cell requires fine adjustment of a number of different controls. The switching frequency and capacity compensation were set up as described before. Two other variable controls required to be set.

a) Gain control. The gain control sets the overall clamp gain and can vary between 5 and 5000. If the gain is much different from its maximum value it produces less than 100% clamping. However, high gain often causes oscillations and unclear waveforms.

b) Phase control. This control adds a high frequency component to the clamping circuit. It is essentially a stability control designed to stop oscillation while maintaining maximum clamp speed and maximum gain.

In order to clamp cells successfully these two parameters had to be preset using the electrical model shown below.

```
          22MΩ
           |
          ---
           |
            0.01μF
```

The electrical components in this model were chosen in order to mimic as closely as possible the combined serial impedance of the recording electrode and the impaled cell.
Once the voltage clamp was capable of clamping the model without oscillating it was possible to start clamping cells. The cells were clamped at a predetermined holding potential initially as close as possible to their resting membrane potential.

The potential could then be hyperpolarised and depolarised. It was never clear what the actual altered value of the membrane potential was, however, because of the inherent problems, described later, in trying to voltage clamp an electrical syncytium like smooth muscle.

**Resistance Measurements**

Membrane resistance measurements (R) were carried out in both bridge clamp and switch clamp modes by passing a step function current of 0.3nA and measuring the potential change produced once it reached a steady level. The potential was then divided by the input current to give R.

**Impedence Measurements**

Membrane impedance measurements (Z) were also carried out using both bridge clamp and switch clamp modes.

A pure sine wave alternating current was passed into the cell at varying frequencies, using a Gould Advance signal generator, Model J4A to externally control the current, and the output voltage sine wave was recorded. In bridge clamp the amplitude of the input current was 1nA ptp. In switch clamp the amplitude was doubled to 2nA ptp. This was because the switch clamp operated on a 50% duty cycle, i.e. half the time passing current, half recording voltage. Therefore a 2nA ptp input current gave a mean actual current passed of 1nA ptp in switch clamp mode.

a) The impedance value Z was obtained by measuring the
the amplitude of the elicited voltage sine wave and dividing it by the input current.

b) The phase difference between the output voltage and input current was calculated using Lissajous figures.

The voltage signal was placed on the vertical amplifier of a Tektronix C.R.O. (Type 5113) and the current on the horizontal amplifier. For convenience the amplitude of the two signals was made equal by altering the X and Y gain. The combination of the two traces produced an ellipse which fitted within the defined boundaries of the X and Y axis (see Figure 4.3). The sine of the phase angle between the two signals is equal to the ratio of the Y-axis intercept \( Y_1 \) to the maximum vertical deflection represented by \( Y_2 \).

\[
\text{sine } \theta = \frac{Y_1}{Y_2} = \frac{X_1}{X_2} \quad \text{(where in this study } 0 \leq \theta \leq 90)\]

Calculation of resistance and reactance

From the values of impedance \( Z \) and phase angle \( \theta \), the resistive and reactive components of the impedance can be separated using the equations:

\[
R \quad \text{(resistance)} = Z \cos \theta \\
I \quad \text{(reactance)} = jZ \sin \theta \quad \text{where } j \text{ signifies that } I \text{ is a complex number (in this study } j \text{ can be ignored)}
\]

Results

1. **Comparison of bridge and switch clamp**

   The input impedance of cells was measured by the two methods, bridge clamp and switch clamp, described above. Table 4.1 shows mean values obtained at 3 different frequencies of alternating current together with the input resistance measured using a step current function. The values obtained are not significantly different when comparing one
Figure 4.3.

Lissajous figure. Allows computation of the phase angle between two signals of the same frequency: \( \sin \theta = \frac{x_1}{x_2} = \frac{y_1}{y_2} \).
Table 4.1

Comparison of bridge clamp vs switch clamp

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Bridge</th>
<th>Switch clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>d.c.</td>
<td>16.3 ± 2.1 (11)</td>
<td>15.5 ± 1.8 (21)</td>
</tr>
<tr>
<td>100</td>
<td>12.7 ± 2.2 (11)</td>
<td>13.7 ± 2.1 (46)</td>
</tr>
<tr>
<td>200</td>
<td>11.3 ± 1.7 (11)</td>
<td>10.2 ± 2.4 (37)</td>
</tr>
<tr>
<td>300</td>
<td>10.9 ± 1.9 (11)</td>
<td>9.2 ± 2.1 (35)</td>
</tr>
</tbody>
</table>
technique with the other. As the techniques work by different methods to each other, it is difficult to suggest an error which could be constant to both. This implies that both techniques are probably fairly accurate at these frequencies.

**Frequency limitations of the two techniques**

Figure 4.4 shows the results obtained in a single cell when the impedance was measured over a wide range of frequencies using each method in turn.

In switch clamp mode, the maximum switching frequency which can be used in this set up is 5kHz (see Methods). Any input sine wave with a frequency greater than about 800Hz to 1kHz would lead to few samples (<6) per sample and consequently inaccurate measurements particularly of the phase angle. The frequency limitation of this technique is therefore 800Hz.

In Figure 4.4 it can be seen that the results obtained for the two techniques deviate at frequencies greater than 400Hz. As there is no reason to suppose inaccuracies in the switch clamp at such a low frequency and it is known that the impedance of the microelectrode distorts results obtained in bridge mode when the frequency is high (Bennett, 1972) it is most likely that the bridge circuit is giving distorted readings at frequencies greater than 400Hz.

2. **Phase angle and impedance locus**

By measuring the phase difference between the input current and the output voltage it is possible to divide the impedance measurements obtained into a resistive component and a separate reactive component (see Methods). The results obtained are summarised in Table 4.2.

The construction of an impedance locus diagram from sets of data expressed as separate resistive and reactive components
Figure 4.4.

Comparison of impedance, at varying frequencies, of a single cell in the mouse vas deferens measured in (A) bridge mode and (B) switch clamp mode of the Dagan 8100.
Table 4.2

Effect of frequency variation on cell impedance

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>N</th>
<th>Z (MΩ)</th>
<th>θ (°)</th>
<th>R</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>18</td>
<td>14.3 ± 2.4</td>
<td>26.3 ± 4.1</td>
<td>12.8</td>
<td>6.3</td>
</tr>
<tr>
<td>75</td>
<td>16</td>
<td>14.1 ± 2.2</td>
<td>25.9 ± 4.3</td>
<td>12.7</td>
<td>6.2</td>
</tr>
<tr>
<td>100</td>
<td>46</td>
<td>13.7 ± 2.1</td>
<td>26.9 ± 2.5</td>
<td>12.2</td>
<td>6.2</td>
</tr>
<tr>
<td>200</td>
<td>37</td>
<td>10.2 ± 2.4</td>
<td>29.5 ± 2.7</td>
<td>8.9</td>
<td>5.0</td>
</tr>
<tr>
<td>300</td>
<td>35</td>
<td>9.2 ± 2.1</td>
<td>39.7 ± 3.4</td>
<td>7.1</td>
<td>5.8</td>
</tr>
<tr>
<td>400</td>
<td>11</td>
<td>9.3 ± 2.2</td>
<td>43.5 ± 2.8</td>
<td>6.7</td>
<td>6.4</td>
</tr>
<tr>
<td>500</td>
<td>10</td>
<td>8.8 ± 2.0</td>
<td>44.3 ± 3.2</td>
<td>6.3</td>
<td>6.1</td>
</tr>
<tr>
<td>600</td>
<td>10</td>
<td>8.5 ± 1.7</td>
<td>44.4 ± 5.5</td>
<td>6.1</td>
<td>5.9</td>
</tr>
<tr>
<td>700</td>
<td>9</td>
<td>7.1 ± 1.5</td>
<td>40.8 ± 7.5</td>
<td>5.4</td>
<td>4.6</td>
</tr>
</tbody>
</table>
has been used to deduce an equivalent electrical circuit for other types of muscle (Falk & Fatt, 1964; Jack, Noble & Tsien, 1975). A similar plot of resistance vs reactance for the results obtained in this study can be seen in Figure 4.5.

Many cells can be represented by a simple electrical equivalent consisting of a resistance ($R_M$) and a capacitance ($C_M$) connected in parallel. The total impedance $Z_M$ will equal $R_M - 1/\omega^2 C_M$. When an impedance locus diagram plotting the real (resistive) against the imaginary (reactive) parts of the impedance is drawn, the imaginary component is equal to 0 both when $R = 0$ ($\omega = \infty$) and $R = R_M$ ($\omega = 0$). Between these values, the locus of $Z_M$, as $\omega$ is varied from 0 to $\infty$, is described by a semi-circle (Jack, Noble & Tsien, 1975).

The results in this study (Figure 5) do not appear to be fitted by a semi-circle. The $I$ value obtained when $R = 9 \text{M}\Omega$ is significantly less than those obtained when $R = 12 \text{M}\Omega$ or $7 \text{M}\Omega$.

Falk & Fatt (1964) have shown that the impedance locus of skeletal muscle can be fitted by two semi-circles. They attributed the cause of this to the T-system of the muscle which adds an extra resistance and capacitance to the basic electrical equivalent circuit.

There is no T-system as such in smooth or cardiac muscle (e.g. Sommer & Johnson, 1968; Burnstock, 1970, 1979), however an extra resistance and capacitance can be added to the electrical equivalent circuit if it is assumed that there is resistive and capacitative coupling between cells in an axial direction (Freygang & Trautwein, 1970; Jack, Noble & Tsien, 1975).

Johnson & Sommer (1967) derived a model of cardiac muscle fibres and their interconnections forming a three dimensional network. The structure of this muscle is very similar to smooth
Figure 4.5.
Impedence locus diagram for cells of the mouse vas deferens. R = resistive component. I = reactive component. See text for details.
The arrowed point has a reactive value which is significantly less than the two starred points.
muscle. These authors showed that the electrical equivalent circuit of their model had the same impedance function as the network model of skeletal muscle described by Falk & Fatt (1964).

Bennett (1972), on the other hand, has assumed that the coupling between cells is entirely resistive. Consequently he stated that the membrane impedance of smooth muscle is equivalent to a parallel resistance and capacitance and that the impedance locus would therefore be semi-circular. However, no experimental evidence to substantiate this hypothesis has been reported and the mathematical equations used by Bennett (1972) are inconsistent with the equations derived by Koide (1966) and upon which Bennett's model was based.

The results obtained in this study are impossible to fit with a semi-circle. It is possible that they could be fitted by 2 semi-circles, however the lack of information at high frequencies makes such a definite conclusion inappropriate.

Thus, while the results obtained using the switch clamp recording method in this study are in broad agreement with those produced by other studies, the frequency limitations are such that they do not allow any extension of existing hypotheses and, therefore, they do not allow a definite electrical equivalent circuit for smooth muscle to be chosen.

3. Relationship between input impedance and the presence or absence of prominent d.e.s.

In a number of cells the input impedance at 3 frequencies was compared with the existence or otherwise of prominent d.e.s. Cells were divided into three groups: cells with distinct families of d.e.s.; cells with few d.e.s. and cells with no d.e.s. at all. In the cells without d.e.s. the stimulus intensity was continually increased until it reached a level
which caused muscle contraction and dislocation of the recording microelectrode.

The results obtained are shown in Table 4.3. There was found to be no significant difference in input impedance between cells of each of the groups.

This is an important observation. Discrete events simply reflect an increased rate of depolarisation of a smooth muscle cell (in response to transmitter action) compared to the normal response. It might be argued that prominent d.e.s. reflect peculiarities in the recording cell's response to depolarisation whether it is caused by direct transmitter action on it or by current spread from surrounding cells. The results obtained in this study suggest that despite the heterogenicity of smooth muscle cell response to nerve stimulation the cells themselves appear to be homogenous in terms of their membrane conduction properties. Thus the heterogenicity of the responses of the cells to non-stimulation is probably due to some other factor most likely the extent of effective innervation to each cell. Variation in response could then arise either from variations in the density of innervation from cell to cell or because the innervation to cells with few or no d.e.s. was unable to be stimulated either because of nerve damage during dissection or nerve 'death' with time after the dissection.

4. **Comparison of the Resistance Measurements in this study with other studies.**

The resistance measurements obtained in this study (Table 4.1) were similar to those previously reported for the guinea pig vas deferens (Hashimoto, Holman & Tille, 1966), but lower than those previously reported for the mouse vas deferens
Table 4.3

Comparison of cell impedance with nature of observed d.e.s.

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Prominent d.e.s. (n=13)</th>
<th>Few d.e.s. (n=5)</th>
<th>No. d.e.s. (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>11.9 ± 2.0</td>
<td>13.6 ± 2.4</td>
<td>13.8 ± 3.5</td>
</tr>
<tr>
<td>200</td>
<td>9.5 ± 1.8</td>
<td>12.4 ± 1.5</td>
<td>9.0 ± 3.2</td>
</tr>
<tr>
<td>300</td>
<td>8.7 ± 1.8</td>
<td>11.7 ± 1.9</td>
<td>8.4 ± 4.3</td>
</tr>
</tbody>
</table>
Holman, Taylor & Tomita, 1977). Furthermore unlike the results described by these authors, the results in this study could not be split into two groups suggesting two different populations of cells. Perhaps the answer to this disparity lies a recent paper by Illes and Starke, 1983). These authors suggest that the superficial cells of the mouse vas deferens have a high input resistance (>30MΩ) and low membrane potential (<65mV) while deep muscle cells have a low input resistance (<30MΩ) and high membrane potential (>65mV). As the mean membrane potential of cells used in this study was 72mV and only very rarely were cells found with membrane potentials ≤ 65mV, this study did not include observations of the high resistance, low membrane potential cells seen in other studies.

Mathematical Model

The primary object of the impedance measurements was to try to obtain an electrical equivalent circuit for the smooth muscle of the mouse vas deferens in order to help design a mathematical model.

This has not been possible because of the frequency limitations of the measuring techniques.

Instead, a mathematical model has been developed into which any parameters for membrane resistance/capacitance, coupling resistance/capacitance, number of coupling etc., can be fitted.

Mathematical models of smooth muscle

A number of previous models have been devised to try to describe the electrical behaviour of a three dimensional syncytium.

Most of them have been limited extensions of cable
models (e.g. George, 1961; Yayo, 1965) which could not adequately describe a 3-dimensional phenomenon. However, Bennett (1972) was the first to accurately model a three dimensional network and Purves (1976) developed his "discrete" and "ladder" models from this work of Bennett (1972).

Mathematical Model

The mathematical model developed in this study is based on the model of Bennett (1972) and the "discrete" and "ladder" models of Purves (1976).

It consists of a driver cell (see Figure 4.6) (equivalent to the cell which is normally recorded from experimentally) coupled to six elements. Each of these six elements is coupled to a further six elements and so on.

Each cell consists of an identical membrane resistance, membrane capacitance, coupling resistance and coupling capacitance which are preset before each run of the computer model. The number of times coupling occurs out from the recording cell is also preset before each run.

The capacitance between each cell is not something that has been included in the previous models of Bennett (1972) and Purves (1976). However, it has been suggested by other authors to exist in a three dimensional syncytium (Freygang & Trautwein, 1970; Jack, Noble & Tsien, 1975). A coupling capacitance has, therefore, been included in this model. It is given a numerical value proportional to the value of membrane capacitance. The proportion being the same as the ratio of cell membrane in close apposition to a neighbouring cell compared with the total cell membrane. In the case of the mouse vas deferens this ratio is said to be 1:60 (see Lane & Rhodin, 1964a).
Figure 4.6 (cont.)

The driver cell is coupled to 6 elements (illustrated on the top right). Each element is, in turn, coupled to 6 further elements (illustrated on the bottom right) and so on.

\[ R_1 C_1 = \text{coupling resistance } \alpha \text{ capacitance.} \]
\[ R_2 C_2 = \text{membrane resistance } \alpha \text{ capacitance.} \]
\[ R_T = \text{variable resistor representing conductance change caused by transmitter action.} \]
\[ I = \text{cell membrane currents.} \]
\[ V_{O'} V_n V_{n+1} = \text{cell membrane potentials.} \]
Figure 4.6.

Diagram of the driver cell and how it is coupled to the elements and a typical element cell used in the construction of the Mathematical Model of a bundle of smooth muscle cells.
**Simplified model algorithm**

The model works by an *iterative* method because the analysis is carried out on a digital computer. The number of iterations and the interval between each one is set before the program is run. The smaller the iteration interval the more accurate the model.

On each iteration the program enters a loop which calculates the impedance of each cell and consequently the membrane potential of each cell starting from the outermost cell and working in to the driver cell. The impedance of each cell is determined by what is happening in the cell coupled to it, i.e. the cell's output impedance. If no conductance changes occur in any cells, the output impedance of each cell remains constant and consequently the membrane potential of each cell remains constant.

A conductance change, roughly equivalent in time course and size to transmitter action on a cell, can be added to (a) the driver cell, (b) to all other cells apart from the driver cell, or (c) to all cells in the model. The conductance change is described by the equation:

\[ G(t) = G_0 e^{-\alpha t} \] (where \( G_0 \) and \( \alpha \) are constants)

Also

\[ G(t)_{\text{max}} = 25 \times 10^{-9} \Omega^{-1} \] (where \( t = 1 \times 10^{-3} \text{ secs} \))

A graph of this conductance change with time can be found in the appendix.

Such a conductance change in the driver cell, for example, will cause a change in the output impedance of the cell coupled to it with time. This will in turn change the output impedance of the cell coupled to that one and so on. Each iteration will therefore detect changes in the membrane potential of each cell with time. The first time differential of the membrane potential is also calculated.
The various parameters for resistances and capacitances were similar to those used by Bennett (1972, 1973) and Purves (1976).

They were generally as follows:

- membrane resistance = 3000Ω
- membrane capacitance = 60pF
- coupling resistance = 70Ω

The coupling capacitance was chosen as 1pF (see above) and the number of times coupling occurred out from the driver cell was generally limited to six. The source of the mathematical equations and the computer program developed for the model are listed in the Appendix.

Methods

The computer program was run on a Research Machines 380Z computer using an extended Basic program with high-resolution graphics. Final copies of the results were obtained using an Epson RX80 F/T printer.

Results

Figure 4.7 shows the response of the driver cell after a typical 'run' of the computer model. In this example and all others in this study, the parameters listed above for resistances, capacitances and number of couplings are used. In this case there is no alteration in the conductance of surrounding cells, only the driver cell. The conductance change in the driver cell is multiplied by 0,1,2,3,4 & 5 to show the effect of between 0 and 5 equally sized 'packets' acting on the driver cell.

Figure 4.8(a-d) shows the effect that various conductance changes in surrounding cells have on the response of the driver cell to a constant conductance change in it.
Figure 4.7.

Response of the mathematical model to transient conductance changes. In this example there is no alteration in the conductance change of surrounding cells. The change in the driver cell is multiplied by 0, 1, 2, 3, 4 and 5. Bottom trace is the potential changes in the driver cell. Top trace is the first time differential \((dV/dt)\) of the bottom trace.
Figure 4.8.
Response of the mathematical model to transient conductance changes. The change in the driver cell is the same as in Figure 4.7. Each example (a - d) shows the effect of additional variable depolarisations in surrounding cells:

(a) surround depolarisation = 1/5 size, 1/5 decay time of driver cell depolarisation.
(b) surround depolarisation = same size, same decay time as driver cell depolarisation.
(c) surround depolarisation = 3 x size, same decay time as driver cell depolarisation.
(d) surround depolarisation = same size, 1/5 decay time as driver cell depolarisation.

In each example, bottom trace is the potential change in the driver cell. Top trace is the first time differential (dV/dt) of the bottom trace. The conductance change in the driver cell is multiplied by 0,1,2,3,4 and 5.
Figure 4.9.

Response of the mathematical model to transient conductance changes. The change in the driver cell is the same as in Figure 4.7 multiplied by 0 + 5. The potential of the surrounding cells is clamped at various levels.

(a) -70mV.
(b) -65mV.
(c) -60mV.

In each example the bottom trace is the potential change in the driver cell. The top trace is the first time differential of the bottom trace.
It is quite clear that varying the conductance in surrounding cells sufficiently affects the output impedance of the driver cell to alter its response to a constant conductance change affecting it.

Figure 4.9(a-c) shows this effect in another way by showing that holding the potential in surrounding cells at different values alters the response of the driver cell to a constant conductance change occurring in it. Once again the change in the output impedance of the driver cell has a significant effect on its response.

Fig. 4.10 shows the effect of a conductance change in the driver cell and the response this generates in a cell coupled to the driver cell, a cell coupled to that cell and so on. It shows the huge attenuation of the response across a cell coupling.

Discussion

A comparison of Figures 4.7, 4.8 and 4.9 shows that the response of the driver cell to a constant conductance change is very much dependent upon changes in impedance of surrounding cells.

This suggests, for example, that an identical packet of transmitter acting on the driver cell (recording cell) will produce a different response depending on whether the surrounding cells are depolarised or not. This has serious consequences when considering the comparison of spontaneous and evoked discrete events in a three dimensional syncytium. The first time differential of the evoked response is also clearly different in the two cases.

It could also be argued that the response of the driver cell will vary from pulse to pulse due to variations in the conductance of cells coupled to the recording electrode. In
the case of the mouse vas deferens where every cell is directly innervated (see Chapter 1) this effect should be minimised as the average quantal content of all the cells coupled to the driver cell should remain fairly constant from pulse to pulse. Comparison of pulse to pulse evoked release is probably, therefore, a valid procedure while comparisons of spontaneous and evoked d.e.s. is much more doubtful.

The huge attenuation in conductance change across a cell coupling suggests that discrete events probably solely represent transmitter acting directly on the recording cell. To a certain extent this suggestion is backed up by the results of Figure 8 where the response to equally sized multiples of the unitary conductance change provides a set of first time differential responses very similar to those seen experimentally. Thus five equal sized packets of transmitter will produce the same response regardless of where on the smooth muscle cell they act.

**Voltage-Clamp experiments**

The difficulties in trying to 'voltage-clamp' multicellular preparations, particularly using microelectrodes, are many fold and it has been suggested that it has never been carried out completely successfully (Johnson & Lieberman, 1971).

Multicellular preparations from heart and smooth muscles have small diameters which makes penetration and holding of the cells impossible with anything other than a high resistance microelectrode. Unfortunately, these electrodes have difficulty passing current (Atwell & Cohen, 1977). The usual method for clamping multicellular preparations is therefore the "sucrose gap technique" (Beeler & McGuigan, 1978)
and this is particularly true in the case of smooth muscle (Bolton, 1975, Bolton, et al., 1980).

The major problem which faces any method of voltage clamping smooth muscle is series resistance. This series resistance can be made up of a number of components:

(a) electrode resistance.
(b) resistance due to the endothelium.
(c) resistance due to bulk extracellular solution.
(d) cleft resistance due to extracellular solution between cells.
(e) resistance of cells coupled to the recording cell by low resistance pathways.

The single electrode chopped current clamp (Brennecke & Lindemann, 1974a,b) used in this study can be shown to eliminate problems a to c (Atwell & Cohen, 1977). However, the problems of cleft resistance and low resistance connections between cells remains.

These problems can normally be kept to a minimum by using a combined sucrose gap technique where the cells within the recording node are, in theory, kept isopotential. Bolton (1975) has however shown that the node width must be kept $<0.15\text{mm}$ for uniform potential but must be $>0.5\text{mm}$ for the cells in the node to respond in the same manner to electrical stimuli as if they were kept in normal conditions. Thus it has proven almost impossible to obtain an adequate voltage clamp system for smooth muscle.

In this study a quantitative analysis of the different components of the membrane current was not required. Rather a qualitative separation of currents occurring in the recording cell from those that didn't, seemed desirable.

Thus the problem of non-uniformity of potential throughout the syncytium can be turned to an advantage. Using the
single electrode clamp of Brennecke & Lindemann (1974a,b) a single electrode was used both to record voltage and pass current (see Methods). Clearly, because of the low resistance pathways between cells, the impaled cell is the only cell which can be controlled completely by the clamp. Thus the 'voltage clamp' can be used to try to differentiate currents arising in the clamped cell from those arising elsewhere.

Another feature of a multicellular syncytium is that a conductance change in a single cell can be shown to have a much slowed and reduced effect on a cell coupled to it (see Figure 4.10). Thus the major influence on the recording cell in "voltage clamp" experiments will be from currents arising in that cell.

Methods

See Chapter 2 and the beginning of this chapter.

Results

Figure 4.11 shows two typical examples of current traces recorded in 'clamped' cells. These cells can be divided into two groups; those with and those without a 'fast' component.

Figure 4.11(a) shows an example where the stimulus intensity was relatively low when the 'fast' component appeared. For the cell in Figure 4.11(b), however, the stimulus intensity had to be much larger to initiate a 'fast' component and the surrounding transmitter action was therefore much larger leading to a large 'slow' component in the current trace.

In both examples, however, the separation between 'fast' and 'slow' components is quite clear. The fast component reaches its maximum amplitude well before the slow
Figure 4.10.

The effect of a conductance change in the driver cell on the potential of that cell (cell 0) and cells 1, 2, 3 → N couplings out from the driver cell.
Figure 4.11.

(a) Example of fast currents in a voltage 'clamped' cell of the mouse vas deferens evoked by nerve stimulation.

(b) Example of both fast and slow currents in a different voltage 'clamped' cell of the mouse vas deferens. Bottom trace is the potential of the cell during stimulation.

In this figure and the next two, the recorded currents, although inward currents, travel in a positive direction. This is because the currents are being used in a qualitative rather than a quantitative manner and such a representation facilitates comparison with ejps and d.e.s. in Figure 4.12.

Calibration bars = 20msec, 0.1nA, 10mV.
component, it occurs intermittently and when it occurs it varies markedly in amplitude.

Figure 4.12 shows a comparison of (a) currents and (b) ejps and d.e.s. recorded in the same cell. The current trace consists of mainly a fast component which varies markedly in amplitude from pulse to pulse. This fast current has a similar latency to the d.e.s. shown in Figure 4.12(b). The slower ejps of Figure 4.12(b) can be seen to produce a very small 'slow' component on the current trace (Figure 4.12(a)).

Comparison of the relative proportion of occurrence of d.e.s. and fast currents in this cell showed them to be similar ($P_{\text{d.e.s}} = 0.46$, $P_{\text{fast currents}} = 0.51$). In five cells analysed, the proportion of d.e.s. found was not to be significantly different (student's t-test) to the proportion of fast currents ($P_{\text{d.e.s.}} = 0.48 \pm 0.05$; $P_{\text{fast currents}} = 0.54 \pm 0.08$).

Altering the holding potential of the impaled cell

Cells, once clamped could be depolarised or hyperpolarised by altering the holding potential. Because of the properties of smooth muscle it was difficult to determine the actual value to which the membrane potential was changed, however, the potential could be altered by quite large amounts without oscillation of the clamp occurring.

Figure 4.13 shows the effect of depolarisation on the components of the current response to stimulation. The values given for depolarisation are simply what the holding potential 'dial' was set to and are not an accurate measure of the cell membrane potential. Nevertheless, they give some idea of the size of potential change applied.

Depolarisation appears to cause a graded reduction in the size of the 'fast' current component with apparently no
Figure 4.12.

(A) Fast currents  (B) ejps and d.e.s. evoked in a single cell of the mouse vas deferens by field stimulation of the sympathetic innervation.

Calibration bars: 20ms; t→b 0.2nA; 0.5V/s; 5mV.
Figure 4.13.

Effect of altering clamp potential on currents evoked in a single cell of the mouse vas deferens.

(A) -20mV.

(B) -40mV.

(C) -60mV. (≈ resting potential).

Calibration bars: 20msec; 0.1nA; 5mV.

In each example: Upper trace = current record

Lower trace = voltage record.
effect on the 'slow' component.

Discussion of 'clamp' experiments

Voltage 'clamp' of a smooth muscle cell by a single electrode reveals a 'fast' and a 'slow' component in the current response to nerve stimulation. The fast component peaks (has maximum amplitude) before the equivalent potential change recorded in the same cell in current clamp mode. The 'fast' current is therefore out of phase with the voltage (it leads the voltage).

The fast current occurs intermittently, has varying amplitude in response to constant nerve stimulation and it can be removed in a graded manner when the cell is depolarised.

The slow component has maximum amplitude at the same time as the equivalent potential change recorded in the cell. The slow potential occurs in response to every stimulus, has relatively fixed amplitude and is unaffected by depolarisation.

All these observations combined with a knowledge of the capabilities and limitations of voltage clamping any 3 dimensional syncytium suggests that the 'fast' component possibly represents direct clamping of the potential change induced by transmitter acting on the recording cell while the 'slow' component represents indirect clamping of the potential change induced by transmitter acting on cells coupled to the recording cell.

These fast currents are similar to d.e.s. in a number of ways. They occur intermittently, they have a very similar frequency of occurrence, they reach maximum amplitude before the potential change in the cell and their amplitude varies markedly from pulse to pulse.
It seems reasonable to conclude that the 'fast' currents and discrete events are a measure of the same thing - namely transmitter action on the recording cell. These current traces also show directly for the first time that the electrical response of the cell to direct stimulation can be separated from the electrical response generated in that cell because of stimulation to surrounding cells and can thus be used as further validation for the use of discrete events to analyse transmitter acting close to the recording electrode.

Recently, Finkel, Hirst & van Helden (1984) used a single electrode voltage clamp system to investigate currents generated by nerve stimulation, in short segments of arterioles. The current they recorded had only one component similar in form to the fast component obtained in this study. The segments of arteriole used in their investigation are small enough to be adequately voltage clamped so that transmitter action on one cell will cause an identical potential change in each cell in the segment (Bolton, 1975, Bolton, et al., 1980; Hirst & Neild, 1978).

Thus these results support the conclusions drawn in this study that the fast component of the current represents transmitter action on the recording cell being directly clamped and therefore that the potential change in the recording cell due to transmitter acting on surrounding cells is indirectly clamped.
CHAPTER 5

VARIATION IN STIMULUS INTENSITY
CHAPTER 5

Introduction

It has previously been noted, in sympathetically innervated smooth muscle studies, that the excitatory junction potentials (ejps) elicited by electrical nerve stimulation have an amplitude which is graded, proportional to the strength of stimulation (Burnstock & Holman, 1961; Orlov, 1962; Bell, 1969; Furness, 1970 and Hirst, 1977). Consideration of the structural studies of Hillarp (1959, 1960), the discovery of low resistance connections (nexuses) between smooth muscles cells (Dewey & Barr, 1962, 1964; Lane & Rhodin, 1964b; Burnstock, 1979), and the discovery of regions of close contact (70-200Å) between nerve and muscle cells (Lane & Rhodin, 1964a; Richardson, 1962; Merrillees, et al., 1963; Merrillees, 1968) led to the hypothesis that the increase in amplitude of the ejp with stimulus strength is due to an increase in the number of postganglionic axons stimulated (Furness, 1970) rather than an increase in transmitter concentration in the synapse due to each nerve ending releasing more transmitter (Rosenblueth, 1950).

Assuming this hypothesis to be correct many workers have estimated the number of axons contributing to the recorded ejp, in sympathetically innervated smooth muscle, by varying the stimulus intensity in a stepwise manner and observing how many jumps in ejp amplitude occur.

The figures obtained vary greatly from preparation to preparation: 3 to 4 axons in mesenteric arterioles (Hirst, 1977), 4 to 6 in uterine artery (Bell, 1969) and up to 15 to 22 axons in the mouse vas deferens (Furness, 1970).

Because of the electrical properties of smooth muscle (see Chapter 4) these figures represent both axons releasing
transmitter which acts directly on the recording cell and axons acting on adjacent cells coupled to the recording cell.

The object of this chapter is to test whether all the members of a family of discrete events are associated with a single axon or not.

At first sight, the experiments to test this question seem easy. The nerves could be stimulated at an intensity below threshold for a jump in ejp amplitude then at an intensity above threshold for a jump in ejp amplitude. Eventually a family of d.e.s. would exist above threshold but not below, if they are associated with one axon.

It is possible, indeed likely, however that more than one axon has a similar threshold stimulus intensity particularly in a preparation like the mouse vas deferens where the innervation is so dense (see Chapter 1, also Furness, 1970). A more accurate study of the threshold of an axon is thus required.

It can occasionally be shown (Furness, 1970; Holman, Taylor & Tomita, 1977) that large variations in amplitude of ejps in the mouse vas deferens can occur from stimulus to stimulus at a fixed stimulus intensity. Presumably, such variation arises because the strength of stimulation used is in the middle of a threshold range, for initiating action potentials in one or a few axons. Furness (1970) has suggested that this effect could be obtained quite often by careful adjustments of the strength of stimulation.

Accordingly, in this chapter, a series of experiments were carried out to characterise this threshold property more fully and then use it to try to determine whether family member d.e.s. are associated with a single axon or not.
Methods

The general methods used for collecting and analysing data in this chapter were as described in Chapter 2.

Method for finding a threshold range

In order to find a threshold range for one or a group of axons the stimulus intensity was increased until it reached a level which caused a significant jump in ejp amplitude. Careful adjustment of the stimulus intensity about this point invariably revealed an intensity where intermittent jumps in the ejp amplitude in a given train of stimuli occurred. The stimulus intensity was then varied at random to levels ±5-10% of this intensity in order to obtain the "threshold curve" for a particular jump in ejp amplitude (see Results).

Randomisation of stimulus intensity

In the study of discrete events and their variation with stimulus intensity, the stimulus intensity was randomised in order to overcome variations due to fluctuations in axon excitability. Five intensities were chosen as follows:

C = stimulus intensity by trial variation around threshold range as described above.
A = C - 0.2V (same pulse width)
B = C - 0.1V (same pulse width)
D = C + 0.1V (same pulse width)
E = C + 0.2V (same pulse width)

The stimulus intensities were applied in the following pattern:

B C A D E
A D C E B
E B D C A
D A E B C
C E B A D
Each intensity was applied 25 times at 2Hz followed by a gap of 5secs before moving on to the next. The first five stimuli in each train were ignored when the results were analysed, to avoid complications due to facilitation.

In experiments where ejps were studied the trains of stimuli used had intensities which were also applied in random order.

Results

The graded threshold of jumps in ejp amplitude

Figure 5.1 shows an amplitude histogram of ejps elicited by field stimulation in the mouse vas deferens. It is typical of responses that can be recorded in most cells and shows the graded jumps that occur in ejp amplitude as the stimulus intensity is increased.

Figure 5.2 considers this process more closely. Here, the graded threshold for the firing of the large ejp spreads over a 16% variation in the intensity of applied stimulus. The threshold range was found using the technique described in the methods and the variation in stimulus intensity was applied in a random order (3.45, 3.50, 3.30, 3.60, 3.55) in order to overcome fluctuations in axon excitability.

A graph of the proportion of large ejps plotted against stimulus intensity in the threshold range is shown in Figure 5.3. The rise in proportion of large ejps is linear over the threshold range.

Figures 5.2 and 5.3 allow two deductions. Firstly, this range of stimulus is the threshold for only one axon, as some intermediate ejp amplitudes would be predicted to occur statistically (see Fig. 5.2) if more than one axon had this range of stimulus intensity for threshold.
Figure 5.1.

Effect of increasing stimulus intensity on ejp amplitude in a single cell. Each point represents a mean of 10 pulses at 2Hz; 0.3msecs pulse width.
Figure 5.2.

Effect of increasing stimulus intensity on trains of eips in a single cell.

(A) 3.45V.  (B) 3.50V.  (C) 3.30V.  (D) 3.60V.  (E) 3.55V.

In all cases: frequency 2Hz, pulse width 0.3msecs.

Calibration bars: 5 secs; 6mV.
Figure 5.3.

Graph of the proportion of large events vs stimulus intensity over a range of stimuli for the cell shown in Figure 5.2.
Secondly, the linearity of the threshold response (Fig. 5.3) would seem to rule against fluctuations in excitability causing a "fuzzed threshold" and rather that some definite process is occurring which can be overcome by increasing the intensity of stimulation.

The cause of this graded threshold will be considered in the discussion. Whatever the cause, however, it is an interesting phenomenon, which is easily reproducible (see Table 5.1).

Because the threshold for firing of a single axon can be so easily characterised in the mouse vas deferens it should be possible to answer the question posed in the introduction as to whether a family of discrete events are associated with a single axon.

Threshold of discrete events

A similar "threshold" study to that carried out on ejps was carried out on the discrete events (d.e.s.) associated with the ejps - Figures 5.4 and 5.5. show a typical example. The "threshold range" for a particular family of d.e.s. was found as described in the methods. Then, as before, the stimulus intensity was varied in a random manner.

It can be seen that as the stimulus intensity is increased, the number of discrete events occurring within a given latency band (i.e. a family of discrete events) increases. However, although the number of observations changes (Pf falls) the shape of the distribution remains the same (Fig. 5.5). The distributions in Figure 5.5 were analysed by counting the number of events in each mode of the distribution and comparing distributions using a $\chi^2$ test. A $\chi^2$ value of 2.96 was calculated, with 9 degrees of freedom. Thus, it can be said that statistically there is a greater than 95% chance that
Table 5.1

Effect of variation in stimulus intensity on ejp amplitude

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>stim (V)</th>
<th>3.2</th>
<th>3.3</th>
<th>3.35</th>
<th>3.4</th>
<th>3.45</th>
<th>3.5</th>
<th>3.55</th>
<th>3.6</th>
<th>3.8</th>
<th>4.0</th>
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<tbody>
<tr>
<td></td>
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<td>0</td>
<td>0.02</td>
<td>0.02</td>
<td>0.10</td>
<td>0.38</td>
<td>0.50</td>
<td>0.95</td>
<td>1</td>
<td>1</td>
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<tr>
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<td>2.7</td>
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</tr>
<tr>
<td></td>
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<td>0.31</td>
<td>0.75</td>
<td>0.90</td>
<td>1.0</td>
<td></td>
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</tr>
<tr>
<td>3</td>
<td></td>
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<td>2.6</td>
<td>2.7</td>
<td>2.8</td>
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<td></td>
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<td>( P_L )</td>
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<td>0.08</td>
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<tr>
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<td>5</td>
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</tr>
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<td>( P_L )</td>
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<td>( P_L )</td>
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<td>0.02</td>
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<td>0.29</td>
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<td>7</td>
<td></td>
<td>3.66</td>
<td>3.76</td>
<td>3.86</td>
<td>3.96</td>
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<tr>
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<td>( P_L )</td>
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<td>0.03</td>
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</tr>
<tr>
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<td>5.45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>( P_L )</td>
<td>0.23</td>
<td>0.16</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>3.96</td>
<td>4.01</td>
<td>4.06</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( P_L )</td>
<td>0.03</td>
<td>0.09</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

In all cells the pulse width was kept constant.

\( P_L \) = proportion of large ejps.
Figure 5.4.

Example of d.e.s. from a single cell at various stimulus intensities. Each figure = 20 superimposed traces.

(A) 2.30V. (B) 2.40V. (C) 2.50V. (D) 2.60V.

In all cases: 0.35msecs pulse width; frequency = 2Hz.

Calibration bars: 20msecs; 1V/s.
Figure 5.5.

Amplitude histogram of family member d.e.s. at varying stimulus intensities.

(a) 2.0V Pf = 0.823.
(b) 2.20V Pf = 0.803.
(c) 2.30V Pf = 0.663.
(d) 2.40V Pf = 0.691.

Pf = proportion of failure of occurrence of a family member d.e.

In all cases pulse width = 0.3msecs; frequency = 2Hz.
there is no difference between the four distributions.

The results of a more rigorous study are shown in Table 5.2. In this study 5 cells were stimulated at 5 different intensities applied in a random manner. The method for finding a threshold range to begin with was again carried out as described in the methods section. The method used to randomise the applied stimulation intensity is also described in the methods section.

Using a Friedman analysis of variance test for the five cells in Table 5.2, the proportion of failures of occurrence of a family member d.e. was found to decrease as the stimulus intensity increased (p<0.01) while the mean amplitude of a family member d.e. was found not to significantly vary (0.3>P>0.2) as the stimulus intensity increased.

Discussion

The jump in ejp amplitude associated with the firing of a terminal nerve fibre has a 'fuzzed' threshold over a range of stimuli rather than an all-or-none threshold.

There are two main reasons why this may occur.

a) Feature of the stimulation process

In order to excite a nerve, it is necessary to depolarise its membrane potential to a critical level over a sufficiently large area of its membrane. The type of stimulation employed in this study, field stimulation of the postganglionic hypogastric nerve fibres, like most types of stimulation, usually elicits a depolarising current which is nonuniform over any significant length of the nerve fibre. Thus, in order to obtain a propagating action potential the inward current generated at any particular point in the fibre as the membrane potential depolarises towards threshold often has to supply
Table 5.2

Effect of variation in stimulus intensity on d.e.s.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Pf</th>
<th>Mean d.e. amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.817 ± 0.04</td>
<td>0.81 ± 0.08</td>
</tr>
<tr>
<td>B</td>
<td>0.803 ± 0.03</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>C</td>
<td>0.716 ± 0.06</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>D</td>
<td>0.661 ± 0.06</td>
<td>0.94 ± 0.05</td>
</tr>
<tr>
<td>E</td>
<td>0.628 ± 0.05</td>
<td>0.97 ± 0.07</td>
</tr>
</tbody>
</table>

For definition of A – E see text.
local circuit current to regions surrounding that point.

The effect of stimulation on whether an excitable tissue reaches threshold for an action potential or not is a delicate balance between the relative activation of Na$^+$ and K$^+$ conductances. Such a balance, superimposed upon the nonuniformity of depolarisation along the fibres (described above), could easily cause a 'fuzzed' threshold of firing a propagating action potential over a range of stimulus intensities.

b) **Property of the terminal axon region**

The electrical properties of axons in their terminal region are poorly understood, mainly because of the technical difficulties involved in studying such small fibres. Recently, it has been suggested that in a number of systems the nerve action potential develops an increasingly large Ca$^{++}$ component as it reaches the terminal region of the nerve (e.g. Salzberg, et al., 1983; Cunnane & Stjarne, 1984).

The hypogastric nerve fibres innervating the mouse vas deferens are already varicose before they enter into the muscle bundles and Cunnane & Stjarne (1984) have shown that even at such a proximal region of the fibres a calcium component exists in the nerve action potential. Cunnane & Stjärne have also suggested that these fibres may have a low safety factor for transmission.

It may be that such fibres require a critical concentration of calcium to be reached intracellularly, before propagation of an action potential occurs, and that stimulation around threshold intensity causes the calcium concentration to vary sufficiently from pulse to pulse to cause intermittent failure of conduction.

It is important to note that the strict linearity of the threshold response (see Fig. 5.3), even when variations in
stimulus intensity are applied in a random order suggests that
the "fuzzed threshold" is not due to random fluctuations in
the excitability level of the fibre. Whatever the cause of the
"fuzzed threshold", it provides a useful mechanism for
separating out events associated with a single axon because,
in examples like that shown in Figure 5.2, one can be certain
that only one axon is responsible for the jump in ejp amplitude.

This study has shown that while the proportion of
occurrence of a family member d.e. varies with the stimulus
intensity when it is around threshold for the firing of an
axon, the shape of the d.e. amplitude distribution and the mean
family d.e. amplitude remains the same. Thus, it can be said
that all the members of a family of discrete events are
associated with a single terminal axon.
CHAPTER 6

SPONTANEOUS TRANSMITTER RELEASE
CHAPTER 6

Introduction

Spontaneous excitatory junction potentials (sejps), reflecting the spontaneous discharge of transmitter from nerve varicosities, have been recorded at a number of sympathetic neuromuscular junctions (e.g. Burnstock & Holman, 1962, 1966; Bennett, 1972, Cheung, 1982a).

At the skeletal neuromuscular junction much information about the chemical transmission process has been obtained by analysing spontaneous potentials and their relationship to evoked release of transmitter by nerve stimulation (see Chapter 1). Interpretation of the results of similar experiments carried out in smooth muscle is made difficult by the syncytial structure of smooth muscle (see Chapter 4) and because this type of muscle is multiply innervated (see for example Burnstock, 1979).

These properties of smooth muscle result in the amplitude histograms of sejps being markedly skewed with the majority of potentials barely distinguishable from the recording noise (Holman, 1970). Furthermore, ejps are found to have a much longer time course than sejps.

Experimental approaches to overcome such problems in sympathetically innervated smooth muscle

(i) Hirst and Neild (1978) have shown that the complicating factors above can be virtually eliminated by using very short segments of arterioles (see Chapter 1). In these preparations sejps initiated in muscle cells at varying distances from the recording electrode will be little attenuated, indeed Hirst and Neild (1978) found the sejps had, in these preparations, unimodal amplitude histograms. They also found considerable
overlap between sejps and the smallest evoked response. 
Hirst & Neild (1980) have suggested from these observations 
that evoked transmitter release from sympathetic nerves is 
both packeted and muMquantal.

(ii) The use of discrete events (Chapter 3) allows 
comparison of spontaneous and evoked transmitter release from 
varicosities close to the recording electrode. There should, 
therefore, be little difference in time course between 
detectable spontaneous and evoked d.e.s.

Blakeley & Cunnane (1979) showed that, occasionally, 
selected spontaneous and evoked d.e.s. could be matched exactly. 
Cunnane & Stjärne (1982) found a match between many spontaneous 
and evoked d.e.s. (including the largest evoked d.e.s. which 
occurred) could be made in certain 'simple' cells. From these 
results, Cunnane & Stjärne have suggested that transmitter 
release is packeted but monoquantal from any one varicosity 
in the nerves innervating all cells.

In order to try to understand better the relationship 
between spontaneous and evoked release, and consequently gain 
more insight into the mechanisms of transmitter release, this 
study has compared spontaneous and evoked d.e. amplitude 
distributions in a number of cells regardless of the complexity 
of their innervation.

Methods

The general methods used in these experiments are 
outlined in Chapter 2.

Other methods

For comparison of spontaneous and evoked d.e.s., cells, 
when penetrated, were left unstimulated for at least 5 minutes 
(more if the spontaneous potential frequency was low) then 
stimulated at 2Hz at a stimulus intensity which was varied until
d.e.s. were reliably elicited by a proportion of the stimuli.

Spontaneous potentials required a trigger pulse before they could be analysed by computer or filmed. This was done by running the taped record backwards and passing the recorded signal through a Schmidt trigger. This device picked up the positive going junction potentials and sent out a trigger pulse at a set time after the sejp was noted. The trigger pulse was recorded on another channel of the tape recorder. When the tape was run forward again the trigger pulse could be used to trigger either an oscilloscope for display or the computer for analysis.

Results

Spontaneous excitatory junction potentials

In these experiments spontaneous excitatory junction potentials (sejps) were recorded from the smooth muscle cells of the mouse vas deferens (Figure 6.1). The frequency of occurrence of sejps were found to vary between cells: in the case of Figure 6.1 from 9/min to 138/min (the mean frequency of occurrence calculated for 12 cells = 33/min). The amplitude of the individual potentials was also seen to vary greatly and the frequency of smaller potentials was always much greater than that of the larger ones, i.e. the distribution is negatively skewed with amplitudes running down to the level of the recording noise.

A typical amplitude histogram is shown in Figure 6.2. Examples of sejps and their associated d.e.s. can be seen in Figure 6.3. The time course of the spontaneous d.e.s. is very similar to that of evoked d.e.s. (see also Blakeley & Cunnane, 1979).
Figure 6.1.

(A - C) Spontaneous eJPs recorded from 3 different cells.

Calibration bars: 5secs; 4mV.
Figure 6.2.

Amplitude histogram of sejps recorded from a single cell.
Figure 6.3.

Typical sejps (lower trace) with associated d.e.s. (upper trace) recorded from a single cell.

Calibration bars: 20msecs; 0.5V/s; 4mV.
Comparison of spontaneous and evoked d.e.s.

The amplitude histograms of d.e.s. from sejps and ejps were compared in a number of cells to see if there was any consistent relationship between the two. Typical histograms from two cells are shown in Figure 6.4. Table 6.1 summarises the results of all the cells studied. The table shows, that for the majority of cells the mean spontaneous d.e. amplitude is smaller than the mean evoked d.e. amplitude. The modes of the d.e. amplitudes coincide however (see Figure 6.4) suggesting that an analogy can be drawn with the Mg$^{++}$ poisoned skeletal neuromuscular junction (e.g. del Castillo & Katz, 1954a) and that the modes represent the quantal release of transmitter from one or a few varicosities close to the recording electrode (see Chapters 3 and 8). This would suggest that the unit mode of sejp d.e.s. in figure 6.4(a) and in 8 of the cells in Table 6.1 is comparable with the smallest evoked d.e. mode and probably reflects the release of a single quantum of transmitter (e.g. Katz, 1966, 1969).

Giant sejps

Giant sejps (>20mV) have been observed in many sympathetically innervated smooth muscles (Burnstock & Holman, 1961; Blakeley & Cunnane, 1979; Hirst & Neild, 1980; Kuriyama & Makita, 1983). Their origin is unknown, however they are generally thought to be different in origin to normal sejps. A number of authors (e.g. Burnstock, 1970; Thureson-Klein & Stjärne, 1981) have correlated their occurrence with the existence of large granular vesicles (LGVs) hypothesising that the spontaneous release of the contents of an LGV would cause a giant sejp.

In this study giant sejps were found to occur very rarely (3 occurrences in 12 cells analysed) and they have been ignored in the sejp amplitude distributions used above.
Figure 6.4.

Comparison of spontaneous and evoked d.e. amplitude histograms in two single cells. In each example the lowest graph gives the sejp amplitude distribution for that cell.
Table 6.1

Comparison of spontaneous and evoked d.e. amplitude distributions

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<th>Cell</th>
<th>sejp mean d.e. amp (V/sec)</th>
<th>ejp mean d.e. amp (V/sec)</th>
<th>comment on d.e. distribution</th>
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<td>1</td>
<td>0.83</td>
<td>1.09</td>
<td>spon. = smallest evoked</td>
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<td>2</td>
<td>0.67</td>
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<td>6</td>
<td>0.50</td>
<td>0.87</td>
<td>&quot;</td>
</tr>
<tr>
<td>7</td>
<td>0.61</td>
<td>0.80</td>
<td>&quot;</td>
</tr>
<tr>
<td>8</td>
<td>0.76</td>
<td>0.93</td>
<td>&quot;</td>
</tr>
<tr>
<td>9</td>
<td>0.75</td>
<td>0.58</td>
<td>spon. &gt; evoked</td>
</tr>
<tr>
<td>10</td>
<td>0.61</td>
<td>0.62</td>
<td>spon. = evoked</td>
</tr>
<tr>
<td>11</td>
<td>0.93</td>
<td>1.03</td>
<td>spon. = evoked*</td>
</tr>
<tr>
<td>12</td>
<td>1.11</td>
<td>0.96</td>
<td>spon. = evoked*</td>
</tr>
</tbody>
</table>

* distributions of both are multimodal but largest evoked > largest spon. in both cells.
Figure 6.5 shows an example of a giant sejp recorded with a fast time base. The giant sejp has an initial fast rise time (reflected in the fast component of the d.e.) which then slows down (second component of d.e. trace). This second component is similar to the background mass response often seen when looking at the differential of an ejp.

The falling phase of the giant sejp has a half decay time of approximately 45msecs. This is about twice as long as that normally seen for sejps in the mouse vas deferens (see Holman, 1970) and is similar to that seen for ejps.

These observations suggest that giant sejps are much more like an ejp in both time course and amplitude than a normal sejp and leads to the alternative suggestion that giant sejps represent the spontaneous firing of an action potential along an axon in the terminal region.

It has previously been established that sejps occur in the presence of a tetrodotoxin concentration sufficient to block nerve action potentials (Tomita, 1967a; Hashimoto, Holman & McLean, 1967). If the hypothesis above is correct one would expect giant sejps to disappear in the presence of tetrodotoxin. They occur so rarely, however, that it is doubtful if their absence would have been noted in these studies if they had disappeared.

Thus, the above hypothesis is a plausible alternative explanation to the idea that giant sejps represent the release of the contents of large granular vesicles.
Figure 6.5.
An example of a giant sejp (lower trace) with associated discrete event (upper trace).

Calibration bars: 20msecs; 0.5V/s; 4mV.
Discussion

The spontaneous release of transmitter from nerve terminals has been extensively studied at the skeletal neuromuscular junction (e.g. Katz, 1969) and its comparison with release due to nerve stimulation has been a major factor in the development of the quantal hypothesis (del Castillo & Katz, 1954a,b; Martin, 1966).

Similar correlations between spontaneous and evoked release have been attempted at other synapses but in sympathetically innervated smooth muscle the syncytial coupling of the muscle cells and the problems of multiple innervation have made results difficult to interpret (e.g. Bennett, 1972).

In this study a technique which eliminates many of the problems caused by these features has been used to compare spontaneous and evoked release.

The observations made show that spontaneous d.e.s. are generally similar in amplitude and time course to the smallest evoked d.e.s. that occur in the multimodal evoked d.e. amplitude histogram and consequently that larger d.e.s. are made up of integer multiples of this initial unit. These results are in broad agreement with those of Hirst & Neild (1980) who used a different technique to eliminate the syncytial problems of smooth muscle. They are also similar to the observations which led del Castillo & Katz (1954a, b) to formulate the quantal hypothesis for skeletal neuromuscular transmission.

There are, however, a number of inherent problems in the electrophysiological comparison of spontaneous and evoked potentials.
1. The use of intracellular recording techniques requires that submaximal stimulation must be used in order to avoid action potential generation in the smooth muscle and consequent dislodging of the microelectrode. It follows, therefore, that there will always be sites releasing spontaneous transmitter which will not release any due to nerve stimulation.

2. A number of distinct differences between spontaneous and evoked release in terms of their sensitivity to \([Ca^{++}]_o\) (Fatt & Katz, 1952; Hubbard, 1973), osmotic pressure (Hubbard, Jones & Landau, 1968) and modification by various other treatments (Liley, 1966a; Birks & Cohen, 1968; Alnaes & Rahamimoff, 1975; McLachlan, 1977) exist. Consequently, it has been suggested that spontaneous and evoked release are each activated by a different mechanism such as release of a different intracellular store of calcium (Kharasch, Mellow & Silinsky, 1980; Illes & North, 1982).

3. The electrical responses recorded, particularly the d.e.s. used in this study, include an element of noise in their waveform (see Chapter 3). This means that particular waveforms cannot be attributed to particular release sites with any great confidence. Furthermore, occasional identity between events will not prove that other slightly different events reflect the activity of a different site nor does it imply that identical waveforms reflect transmitter release from the same site.

4. The results obtained from the mathematical model developed in Chapter 4 suggest that the response to a unit amount of transmitter acting at a particular point on a smooth muscle cell will depend on the membrane potential of that cell and the cells surrounding it. That is to say, because of the
electrical properties of smooth muscle, the response of a cell to an identical amount of transmitter released from an identical site will vary depending on the level of activity in surrounding cells and that consequently an evoked and a spontaneous d.e. reflecting an identical amount of transmitter release from an identical site will be different in amplitude and shape.

The first two problems, although they are important to consider when interpreting results, still allow valid conclusions to be drawn from comparison of spontaneous and evoked d.e.s. in the same cell. The second two suggest that release of a single unit of transmitter from a single site will produce a different d.e. waveform each time it occurs either due to variability caused by noise or due to variation in the response of the smooth cell due to varying activity in surrounding cells.

This would seem to seriously question the validity of comparing individual spontaneous and evoked d.e.s. particularly if such a procedure is carried out to try to associate spontaneous and evoked release from a particular site. This, in turn, suggests that only some form of loose quantitative comparison between spontaneous and evoked distributions remains a valid method of analysis and that even in this case great care has to be taken with interpretation of results.

Within the framework of such limitations, the results in this chapter confirm that distinct modes in the evoked d.e. amplitude histogram exist (see Chapter 3) and shows that a relationship between the modes of spontaneous and evoked d.e. amplitude histograms exists such that (in the majority of cells studied) the unit mode of spontaneous d.e.s. is of similar size to the smallest mode of evoked d.e.s.
CHAPTER 7

FACILITATION OF TRANSMITTER RELEASE
AND VARIATION IN [Ca]₀
CHAPTER 7

Introduction

Facilitation, a process which increases transmitter release by successive impulses in a train over a relatively brief time span (Magleby & Zengel, 1976), has been observed at many synapses.

The process is also found to occur at many sympathetic junctions (e.g. Burnstock, Holman & Kuriyama, 1964; Speden, 1964; Hirst & Neild, 1978) but not all (Surprenant, 1980). However, in the preparation used here, the mouse vas deferens, its occurrence is well established (Furness, 1970; Bennett & Florin, 1975; Blakeley, Cunnane & Petersen, 1981; Blakeley, et al., 1984).

Facilitation is known to cause an increase in quantal release (Dudel & Kuffler, 1961; del Castillo & Katz, 1954a; Liley, 1956b; Martin & Pilar, 1964) rather than an increase in quantal size or postsynaptic sensitivity (del Castillo & Katz, 1954c; Burnstock, Holman & Kuriyama, 1964; Kuffler & Yoshikami, 1975).

Since the amount of transmitter released is related to $[Ca^{++}]_o$ (see Rubin, 1970; Rahamimoff, 1970; Kirpekar, 1975) an exact relationship between epp amplitude and $[Ca^{++}]_o$ has been established at the skeletal neuromuscular junction. The initial observation by del Castillo & Katz (1954b) that the amplitude of the epp was $Ca^{++}$ dependent was confirmed by Dodge and Rahamimoff (1967) who measured epp amplitude at a series of different calcium concentrations. At low calcium concentrations this variation is found to be non linear and the number of packets of transmitter released was found to be
proportional to the fourth power of the calcium concentration, i.e. \( epp \propto [CaX]^4 \) or \( epp = K[CaX]^4 \).

The idea that calcium joined to a presynaptic receptor to form a calcium/receptor complex was first put forward by del Castillo & Katz (1954a). Katz & Miledi (1968) postulated that facilitation occurs because some calcium/receptor complex is necessary for vesicles to bind to the membrane and release their quantum of transmitter. In a train of stimuli the calcium/receptor complex \((CaX)\) was said to form more quickly than it broke down therefore the residue from one stimulation remains when the next one occurs. This idea became known as the 'residual calcium hypothesis'.

Younkin (1974) showed that the relationship between epp amplitude and calcium, described above, correctly predicted the growth of facilitation. Similar studies of a number of sympathetic neuroeffector junctions (Itoh & Tajima, 1979; Tashiro, Gallagher & Nishi, 1976; Kuriyama & Makita, 1983) including the mouse vas deferens (Bennett & Florin, 1975) have also been able to show a relationship between ejp amplitude and \([Ca^{++}]_o\) and have successfully predicted the growth of facilitation based on the residual calcium hypothesis.

While general agreement now exists that facilitation occurs because of an accumulation of residual calcium somewhere in the presynaptic terminal (e.g. Katz & Miledi, 1968; Zucker & Lara Estrella, 1983), this calcium accumulation may occur at more than one site in the presynaptic terminal and therefore the residual calcium hypothesis, as it stands, may be an oversimplification.

It has been shown in the mouse vas deferens (see Chapter 3), the guinea pig vas deferens (Blakeley, Cunnane & Petersen, 1982) and at the crayfish neuromuscular junction (Bittner & Harrison, 1970) that a greater proportion of failures of release of
transmitter occurs than would be predicted by Poisson statistics. These have been termed non-Poisson failures and are suggested as being due to failure of invasion of the action potential into presynaptic terminals (see Chapter 3, Blakeley, Cunnane & Petersen, 1982; McLachlan, 1978).

Thus a process like facilitation is provided with two sites of action - the depolarisation/secretion coupling mechanism and the extent to which varicosities are invaded. Stjärne (1978) and Alberts, Bartfai & Stjärne (1981) have shown that facilitation affects both these processes to varying degrees depending on the \([\text{Ca}^{++}]_o\).

The object of this chapter is to use discrete events to try to understand better where facilitation acts and consequently at what site or sites in the sympathetic terminal nerve net accumulation of residual calcium is important. The effect that variation in \([\text{Ca}^{++}]_o\) has on both normal trains of stimuli and the facilitation process will also be looked at.

These experiments should give further insight into the mechanism of sympathetic neurotransmission.

Methods

The general methods used in this chapter for recording and analysing data were as described in Chapter 2.

Stimulus trains applied to look at facilitation

In the facilitation experiments described in this chapter the intensity of stimulation applied was varied until a family of discrete events could be reliably elicited. The stimulation intensity was then increased until it was supramaximal for that family.
The preparation was then left for 30 secs unstimulated. Following that, a 12.5 second cycle was applied consisting of 5 stimuli at 2Hz followed by a 10 second gap to allow decay of facilitation (Blakeley, Cunnane & Petersen, 1981; Blakeley, et al., 1984) at the preset intensity of stimulation.

Changes in \([\text{Ca}^{++}]_o\)

The extracellular calcium concentration was altered by perfusing Krebs solutions with different concentrations of \(\text{Ca}^{++}: 1.1, 1.6, 2.1\) and 4.2mM. The \([\text{Mg}^{++}]_o\) concentration was kept constant at 1.3mM in all solutions.

In all studies, the preparation was allowed 10 minutes to adapt to a new \([\text{Ca}^{++}]_o\) before any further recordings were made.

Results

(1) Development of facilitation

Figure 7.1 shows a typical example of the development of facilitation in ejps in the mouse vas deferens, while Figure 7.2 shows the mean amplitude of successive ejps following the first 5 stimuli in a train at 2Hz. There is a steady increase in amplitude so that the 5th ejp is some 28% larger than the first. This increase is statistically significant \((p<0.001\) Friedman analysis of variance), however it is much less than the 6 fold (600%) increase seen in the guinea pig vas deferens at a similar frequency of stimulation (Burnstock, Holman & Kuriyama, 1964; Holman, 1970).

Figure 7.3 shows some examples from a single cell of trains of 5 stimuli elicited at 2Hz separated by sufficient time (10secs) to allow decay of facilitation (Blakeley, Cunnane, & Petersen, 1981; Blakeley, et al., 1984). The figure shows
Figure 7.1.

Typical examples of ejp facilitation.

Calibration bars: 1sec; 8mV.
Figure 7.2.

Change in ejp amplitude during facilitation in a single cell. (n = 10 pulses for each point).
Figure 7.3.

Superimposed ejp/d.e.s. for first five pulses in a train. Number beside d.e.s. (top traces) indicates what stimulus in train produced them.

Calibration bars: 20msecs; 0.5V/s; 8mV.
ejps on a fast time scale together with their associated d.e.s.

Amplitude distributions for a family of d.e.s. in a single cell, elicited by either the first or fifth stimulus in a train of five are shown in Figure 7.4 while the combined data from all the cells studied at the control calcium concentration of 2.1mM are shown in Figures 7.5 and 7.6.

Within a particular family, events become larger as facilitation develops and the proportion of stimuli which fail to elicit a member of the family is reduced. Both these trends are statistically significant with, in each case p<0.01 (Friedman two-way analysis of variance).

Looking at the mean d.e. amplitude of the events, however, does not fully describe the changes (in amplitude) that occur. The amplitude distribution in Figure 7.4 can be seen to be assymmetric for both first and fifth stimuli, however, the skew occurs in opposite directions. That is to say, while the modes of the distribution remain constant, a preponderance of large events due to the fifth stimuli replaces the preponderance of small events that occur due to the first stimuli. This can be shown in Figure 7.7 where a 'skew index' has been plotted. This 'skew index' has been calculated as the difference between the percentage of total observations on either side of the mean value.

From Figures 7.4, 7.5 and 7.6 it can also be seen that the high proportion of failures of occurrence of a family are inconsistent with the number of large events in that family that occur if the release process were described by a Poisson process (see McLachlan, 1978). This feature will be discussed more fully later in this chapter.
Figure 7.4.

Change in amplitude distribution of a family of events during facilitation. \([Ca]_o = 2.1\text{mM}\). (A) Amplitude distribution of all events in a family irrespective of the degree of facilitation \((P_f = 0.524)\). (B) Amplitude distribution of family members evoked by 1st stimulus \((P_f = 0.548)\). (C) Amplitude distribution of family members evoked by 5th stimulus \((P_f = 0.452)\).

Bin size = 0.05V/sec.
Figure 7.5.

Change in mean d.e. amplitude during facilitation.

(n = 15 for all points).
Figure 7.6.
Change in the proportion of failures during facilitation.
(n = 15 for all points).
Figure 7.7.
Skew index changes during facilitation (n = 15). The skew index is calculated as the difference between the percentage of total observations on either side of the mean.
(2) **Changes in the number of families during facilitation**

Most cells have only one or two clearly defined families of events together with a few other events at odd latencies (see Chapter 3). Table 7.1 shows how the number of families and the number of 'non-family' events vary during facilitation. There is no significant change in the number of families but a small increase in the number of independent events observed.

(3) **[Ca]₀ and neurotransmitter secretion**

As the secretion of neurotransmitters require the presence of Ca⁺⁺ in the extracellular fluid (e.g. Rubin, 1970; Kirpekar, 1975) and a direct relationship has been found between [Ca⁺⁺]₀ and the amount of neurotransmitter released, defined by ejp amplitude, in the mouse vas deferens (Bennett & Florin, 1975), the relationship between [Ca⁺⁺]₀ and d.e. amplitude and probability of occurrence has been investigated both on trains of stimuli and in the process of facilitation.

Figure 7.8 shows the amplitude distribution of a single family of discrete events elicited by trains of (a) 151 and (b) 150 stimuli at 2Hz in bath concentrations of 2.1 and 1.1mM calcium. The events are significantly larger in high-calcium Krebs solution (p<0.01, Wilcoxon signed rank test). This increase occurs because of a rise in the number of large events and a reduction in the number of small with, however, no change in the position of the modes of the distribution.

Table 7.2 summarises the changes of discrete event amplitude and the probability of occurrence from several cells as the [Ca]₀ is altered.
Table 7.1

Family and non family events during facilitation

\([\text{Ca}^{++}]_0 = 2.1\text{mM}, n = 15 \text{ in all cases}\)

<table>
<thead>
<tr>
<th>stimulus number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean no. of latencies</td>
<td>1.6</td>
<td>2.07</td>
<td>2.27</td>
<td>2.40</td>
<td>2.40</td>
</tr>
<tr>
<td>Mean no. of families</td>
<td>0.73</td>
<td>0.87</td>
<td>0.93</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean no. of non family d.e.s.</td>
<td>0.87</td>
<td>1.20</td>
<td>1.33</td>
<td>1.40</td>
<td>1.40</td>
</tr>
</tbody>
</table>
Figure 7.8.

Changes in the amplitude distribution of a family of events when the $[\text{Ca}^{++}]_o$ is raised from (a) 1.1mM to (b) 2.1mM.

Pf: (a) = 0.65. (b) = 0.46. Bin size = 0.05V/sec.
Table 7.2

Effects of different [Ca$^{++}$] on facilitated d.e. amplitude and failures

<table>
<thead>
<tr>
<th>[Ca$^{++}$] (mM)</th>
<th>N</th>
<th>mean d.e. amp (V/s)</th>
<th>Pf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>8</td>
<td>0.88 ± 0.07</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>1.6</td>
<td>11</td>
<td>0.92 ± 0.04</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>2.1</td>
<td>22</td>
<td>1.28 ± 0.08</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>4.2</td>
<td>6</td>
<td>1.4 ± 0.07</td>
<td>0.35 ± 0.04</td>
</tr>
</tbody>
</table>
(4) \([\text{Ca}^+]\) and facilitation

Figure 7.9 shows that facilitation of d.e.s. still occurs when the \([\text{Ca}^+]\) is lowered to 1.1mM and the amplitude distributions of a family evoked by either the first or fifth stimuli in a train are compared. Table 7.3 gives details of variations in the mean size of families of d.e.s. and their probability of occurrence during facilitation at two different calcium concentrations.

In both \([\text{Ca}^+]\), mean d.e. amplitude and proportion of failures are significantly different from first to fifth stimulus and for any given stimulus in the train, including the first the mean d.e. amplitude is significantly higher and the proportion of failures significantly lower in the high \([\text{Ca}^+]\).

(5) Poisson distributions and non-Poisson failures

In Chapter 3 it was noted that although the amplitude distributions of families of d.e.s. resembled a Poisson distribution, there were too many failures of occurrence compared with the number of modes of the distribution for application of Poisson statistics. Theoretical Poisson distributions are calculated by first determining the Poisson variable 'm'. In a typical d.e. amplitude distribution application of either of the two conventional techniques for calculating 'm', namely (a) division of the mean evoked event size by the mean spontaneous event size, or (b) estimation of 'm' from the proportion of failures \((\text{pf} = e^{-m})\) (McLachlan, 1978; Blakeley, Cunnane & Petersen, 1982) fails to provide a theoretical distribution to fit the observed one, because of the excess failures in the observed distribution.

If the Poisson variable 'm' is calculated by a method
Figure 7.9.

Changes in the amplitude distribution of a family of events during facilitation when [Ca]₀ is 1.1mM.

(a) amplitude distribution of family members evoked by first stimulus (Pf = 0.617). (b) amplitude distribution of family members evoked by fifth stimulus (Pf = 0.617).

Bin size = 0.05V/s.
<table>
<thead>
<tr>
<th>Stimulation number</th>
<th>( \bar{r} )</th>
<th>( \bar{e} )</th>
<th>( \bar{z} )</th>
<th>( \bar{N} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>2.4</td>
<td>1.37</td>
<td>1.48</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Table 7.3

Changes in d.e. during facilitation when [Ca++] = 2.1 and 1.1 mM

mean d.e. amp/sec

[Ca++] = 1.1 mM

6

[Ca++] = 2.1 mM

15
which only uses the non zero part of the distribution, it is possible to apply Poisson statistics. Such a method, although not as accurate as the more usual methods for calculating Poisson distributions given above, allows separation of Poisson and non-Poisson failures.

The equation used to calculate the proportion of occurrence of any unit multiple in a Poisson distribution is:

\[ P_X = \frac{m^x}{x!} e^{-m} \] ...........(1) (Katz, 1966)

where \( P_X \) = the proportion of occurrence of the unit \( x \)
\( x \) = the unit multiple
\( m \) = the mean quantal content.

Now \( P_X = \frac{nx}{N} \) ............(2)

where \( nx \) = number of events of size \( X \)
\( N \) = total number of events in the Poisson distribution.

Thus \( \frac{nx}{N} = \frac{m^x}{x!} e^{-m} \)

and \( nx = \frac{Nm^x e^{-m}}{x!} \) ............(3)

In this case both \( m \), the mean quantal content and \( N \), the number of events in the Poisson require to be calculated. Using two different \( nx \) values (e.g. \( n_1 + n_2 \) or \( n_2 + n_3 \) etc) from the observed distributions, \( m \) and \( N \) can be calculated using simultaneous equations from equation (3).

In each distribution of this study \( m \) was calculated from four different combinations of two \( nx \) values and averaged. Then \( N \) was calculated from four different combination of two \( nx \) values and averaged. Then \( N \) was calculated four times by substituting the average \( m \) value into four different equations for \( nx \) and again averaged.
A theoretical distribution was then formed (see Katz, 1966) and compared with the observed distribution using the derived Poisson variable 'm'. The number of failures to enter the Poisson process - non-Poisson failures - was also obtained.

Figure 7.10 shows an example of such a calculation. Figure 7.10a shows the actual amplitude distribution observed for a family of d.e.s. Figure 7.10b shows this distribution described as modes. The lower two diagrams are predicted distributions. Figure 7.10c predicts the Poisson distribution using the proportion of failures while Figure 7.10d uses the method outlined above. It can clearly be seen that the second method gives a much closer fit between observed and predicted distributions.

(6) Effect of variation in \([Ca^{++}]_o\) on Poisson and non-Poisson failures

Earlier in this chapter it was shown that both facilitation and variation in \([Ca^{++}]_o\) altered the shape of the d.e. amplitude distribution by changing its quantal content per pulse. It was decided to consider Poisson and non-Poisson failures individually to see if facilitation and variation in \([Ca^{++}]_o\) affects more than one process. Clearly, because the Poisson distribution is altered the number of Poisson failures will also be altered. What is not clear, however, is the effect these manipulations have on non-Poisson failures.

The trains of stimuli obtained during the facilitation experiments are not long enough for an accurate separation of the two components of the proportion of failures. It is possible with trains of stimuli obtained in different \([Ca^{++}]_o\),
Figure 7.10.
Calculation of Poisson distributions - see text for details.
(a) Amplitude distribution of a typical family of d.e.s.
(b) Distribution in (a) described as modes (Nf = 47).
(c) Predicted distribution from failures.
(d) Predicted distribution from method in text
   (Predicted total Nf = 49; Poisson Nf = 13; Non Poisson Nf = 36).
However the method used to separate Poisson and non-Poisson failures (described above) depends upon sizeable numbers in at least three or four modes of the amplitude distribution to give accurate results. In many cells when the calcium concentration was 1.1 or 1.6mM it was therefore impossible to carry out an accurate separation of the two components. Figure 7.8 showed a family d.e. distribution from a single cell in the presence of 1.1 and 2.1mM $[\text{Ca}^{++}]_o$. Table 7.4 shows the results obtained when these distributions were analysed as described above and the two components of the proportion of failures separated.

In this particular example the proportional non-Poisson failures in the total distribution was seen to fall from 43% to 35% as the $[\text{Ca}^{++}]_o$ was raised from 1.1 to 2.1mM. Much more obviously from this example the number of Poisson failures has also fallen (from 34 to 5) due to the alteration in quantal content. This latter effect had previously been observed by looking at the amplitude distribution in Figure 7.8.

Table 7.5 summarises the results obtained when other cells in varying $[\text{Ca}^{++}]_o$ were analysed in the same way.

A large reduction in non-Poisson failures was only obtained when the $[\text{Ca}^{++}]_o$ was raised to 4.2mM. It is difficult to assess the effect of $[\text{Ca}^{++}]_o$ on non-Poisson failures accurately, however, because of the small number of cells suitable for analysis in this way, the relative accuracy of the method for obtaining the predicted distributions in the first place and the fact that the effect of $[\text{Ca}^{++}]_o$ variation on non-Poisson failures seems to be much smaller than that on the Poisson failures due to the alteration in the quantal content of the family.
Table 7.4

Effect of $[Ca^{++}]_o$ on Poisson and non Poisson failures in a single cell

<table>
<thead>
<tr>
<th>$[Ca^{++}]_o$</th>
<th>$N_{stim}$</th>
<th>$N$ in Poisson</th>
<th>$N_f$ (Poisson)</th>
<th>$N_f$ (non Poisson)</th>
<th>$%f$ (non Poisson)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1mM</td>
<td>151</td>
<td>86</td>
<td>34</td>
<td>65</td>
<td>43</td>
</tr>
<tr>
<td>2.1mM</td>
<td>150</td>
<td>98</td>
<td>5</td>
<td>52</td>
<td>35</td>
</tr>
</tbody>
</table>
Table 7.5

Variation of non Poisson failures with $[\text{Ca}^{++}]_0$

<table>
<thead>
<tr>
<th>$[\text{Ca}^{++}]_0$</th>
<th>N</th>
<th>% non Poisson failures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>3</td>
<td>50.0 ± 1.8</td>
</tr>
<tr>
<td>1.6</td>
<td>3</td>
<td>48.9 ± 3.1</td>
</tr>
<tr>
<td>2.1</td>
<td>5</td>
<td>46.4 ± 8.4</td>
</tr>
<tr>
<td>4.2</td>
<td>3</td>
<td>19.8 ±10.3</td>
</tr>
</tbody>
</table>
Discussion

By looking at the release of transmitter from one or a few release sites of the sympathetic nervous system the process of facilitation has been examined in a novel manner.

Using this technique, an attempt has been made to find out the relative effects that the facilitation process and varying $[Ca^{++}]_o$ has on the depolarisation/secretion coupling mechanism and the extent to which varicosities are invaded.

**Effect of facilitation and varying $[Ca^{++}]_o$ on depolarisation/secretion coupling mechanism**

As facilitation develops, discrete events in a particular family get larger. The modes of the amplitude distribution do not change so there is an increased proportion of large to small events. The likeliest explanation of this observation is that there is an increase in the number of packets of transmitter released that is to say there is an increase in the mean quantal content.

This change is analogous to what is seen at the skeletal neuromuscular junction where facilitation acts on the depolarisation/secretion coupling mechanism (Mallart & Martin, 1967; Rahamimoff, 1968).

Increasing $[Ca^{++}]_o$ has the same effect on the mean quantal content of families of d.e.s. Therefore it has been shown that both facilitation and increasing $[Ca^{++}]_o$ enhances the depolarisation/secretion coupling mechanism. It is probable therefore, that accumulation of residual $Ca^{++}$ is occurring at this site during facilitation and it is this that increases the mean quantal content.
Effects of facilitation and varying $[\text{Ca}^{++}]_o$ on invasion of varicosities

The proportion of failures of occurrence of a d.e. family member is made up of two components. (a) Poisson failures which is the number of failures which would be expected if the release process was controlled by one mechanism (e.g. the depolarisation/secretion coupling mechanism) and the statistics of transmitter release could be described by a Poisson process. (b) Non-Poisson failures; if the proportion of failures is too large to be solely attributed to Poisson failures, some other process (failure of invasion) must be occurring to prevent this excess proportion of failures entering the Poisson release process.

Facilitation and increasing $[\text{Ca}^{++}]_o$ causes a decrease in the proportion of failures. The relative decrease in the two components of the proportion of failures is difficult to measure (impossible in the facilitation experiments) because the only technique which can be used to separate them requires large numbers of observations and a well defined multimodal distribution (see Results).

The results that have been obtained suggest that most of the decrease in the proportion of failures seen during facilitation and raised $[\text{Ca}^{++}]_o$ is due to a decrease in Poisson failures (i.e. because of the increase in mean d.e. amplitude). A small decrease in the proportion of non-Poisson failures also occurs but it is difficult to be sure how significant this is because of the errors which are generated in the measuring technique.

Thus, facilitation and increasing $[\text{Ca}^{++}]_o$ seem to have an effect on the invasion process. However, this appears to be a smaller effect than that which they have on the depolarisation/
secretion coupling mechanism.

Other observed effects of facilitation

Once facilitation has developed more non-family discrete events are observed at varying latencies. Some of these events associate into pairs of similar time course and related in amplitude. They may reflect the release of transmitter from sites previously silent either by promotion of depolarisation/secretion coupling or invasion. Alternatively, they may be due to the development, during facilitation, of large 'multiquantal' releases from families remote from the recording electrode whose release had previously been lost in the noise of the recording system.

The principle observations in this study; that facilitation and increasing $[Ca^{++}]_o$ enhance the activity of the depolarisation/secretion coupling mechanism and (probably to a lesser extent) promote invasion of the varicosities compare well with the observations of Alberts, et al (1981), who, on the basis of noradrenaline overflow studies, suggested that at physiological $[Ca^{++}]_o$ levels promotion of depolarisation/secretion coupling rather than invasion is the main effect of facilitation.
CHAPTER 8

DETERMINATION OF A MODEL FOR THE RELEASE OF SYMPATHETIC TRANSMITTER FROM INDIVIDUAL RELEASE SITES
CHAPTER 8

Trains of discrete events elicited in response to repetitive field stimulation of the nerves innervating the mouse vas deferens and representing the packeted release of transmitter from release sites close to the recording cell are heterogeneous both in terms of amplitude and time course. In Chapter 3 association of discrete events was carried out to reduce discrete events to a small, more practical, number of groups. This was done in two ways. Firstly, limits of latency, time to peak and amplitude of discrete events were defined, within which discrete events were said to be indistinguishable from each other. Secondly, discrete events which fell into narrow latency and time to peak bands were defined, regardless of amplitude, as being members of a single family of discrete events.

Five possible models of transmitter release were then described, each of which could produce a family of discrete events with variable amplitude but constant latency and time to peak, similar to the observed results. In Chapters 4-7 a number of experiments were carried out to try to define which of the five models of transmitter release is most likely to be correct.

Summary of observations on discrete events

(A) General observations

(1) Impedence Measurements

Cells with prominent discrete events were found to have the same membrane impedance as those without. This observation shows that the presence of prominent d.e.s. is probably a feature of the innervation of the cell rather than
their presence reflecting the existence of a population of specialised muscle cells which respond differently to transmitter action.

(2) Mathematical model

The mathematical model developed to mimic the response of a bundle of smooth muscle cells to 'packets' of transmitter causing postjunctional conductance changes produced two important pieces of information:

(a) Integer multiples (0, 1, 2, 3) of a unit of conductance change acting on the recording cell produced potential changes and a first time differential of the potential change very similar to ejps and d.e.s. observed experimentally.

(b) Attenuation of conductance changes between cells is so large that detection of discrete events due to transmitter action on cells adjacent to the recording cell would be unlikely.

(3) Voltage-clamp experiments

When smooth muscle cells of the mouse vas deferens were voltage clamped the current changes in response to nerve stimulation consisted of two components: fast and slow. It is most likely that the fast currents reflect current generated by a conductance change in the recording cell itself, while slow currents represent current due to conductance changes in neighbouring cells conducted to the recording cell via the low impedance pathways between cells. Fast currents are found to have a similar frequency of occurrence to d.e.s. recorded in the same cells. In some cells their amplitude varies in a stepwise manner.
(4) **Comparison of spontaneous and evoked discrete events**

Spontaneous d.e.s. were found to occur with varying frequency in every cell of the mouse vas deferens recorded from in this study. In most cells analysed, they were found to be similar in amplitude and time course to the smallest amplitude mode of evoked d.e.s. in the same cell.

(B) **Observations on families of discrete events**

(1) **Amplitude distributions**

Discrete events in a particular family are defined as having a constant latency and time to peak. The amplitude of discrete events in a given family is however variable. Amplitude histograms are found to be multimodal. The modes are quite distinct from each other and generally evenly spaced along the abscissa indicating that the size difference between each mode is constant. The distribution is negatively skewed, i.e. there is a preponderance of small amplitude d.e.s.

(2) **Variation in stimulus intensity**

The stimulus intensity was varied around values which were threshold for the firing of a particular terminal nerve fibre. Such experiments caused the proportion of occurrence of a family of discrete events to vary with stimulus intensity while the amplitude distributions remained the same.

The results suggest that the varicosity or varicosities responsible for the generation of a family of discrete events are located on a single terminal nerve fibre.

(3) **Facilitation and variation in \([Ca^{++}]_o\)**

A comparison of facilitated and unfacilitated d.e.s. and a comparison of d.e.s. elicited in varying \([Ca^{++}]_o\) was carried out.

Unfacilitated d.e.s. had a mean d.e. amplitude which was
less than that of facilitated d.e.s. This arose because the amplitude distribution of the d.e.s. had altered. The distributions became more negatively skewed. However, the modes of the distributions remained in the same place on the abscissa and the spacing between the modes remained equal.

Similarly, changing the $\left[Ca^{++}\right]_o$ altered the shape of the d.e. amplitude distributions and not the size of the individual modes or the spacing between them. In low $\left[Ca^{++}\right]_o$ the distributions were at their most negatively skewed with mean d.e. amplitude at its lowest value. In high $\left[Ca^{++}\right]_o$ the mean d.e. amplitude was increased and although the amplitude distributions remained negatively skewed they were much less so. Both these manoeuvres altered the 'quantal content' per pulse of the varicosity or varicosities responsible for generating the family of d.e.s. rather than the quantal size.

**Which model(s) best satisfy the experimental observations?**

The five different models described in Chapter 3 are shown again in Figure 8.1.

The pros and cons of the experimental observations above have been considered for each model. They allow 3 of the models to be rejected while the 2 remaining models cannot be rejected. The reasons are given below.

**Models which can be rejected**

**Model 2:** Monoquantal release from a number of varicosities each acting on a different smooth muscle cell

The observation from the mathematical model that attenuation between cells would make transmitter action on an adjacent cell to the recording cell undetectable using discrete events is complemented by the 'voltage clamp' studies which
Figure 8.1.

Schematic models of transmitter release sites representing a family of discrete events. See text for details (also Chapter 3). (pages 107-109.)
strongly suggest that discrete events represent transmitter action on the recording cell only. These results are sufficient to allow this model to be discarded. However, a number of the other observations such as the equal spacing between the modes of evoked d.e. amplitude distributions and the lack of large mejps are equally difficult to explain with this model.

Model 4: Monoquantal release from a number of varicosities each acting on the same cell. Amplitude variation due to distance of transmitter action on recording cell

The observation that families of discrete events have an amplitude distribution with modes equally spaced along the abscissa is difficult to explain with such a model. It could happen, however, if the sites of transmitter action were equally spaced away from the recording electrode, although this would be unlikely to occur in many cells.

Much more difficult to explain is the lack of large spontaneous d.e.s. to match the large evoked d.e.s. and also the observation that increasing [Ca++]_o increases the number of large d.e.s. but decreases the number of small d.e.s.

In addition to the information from these experiments it is generally accepted that the decay of potential with distance from a source of current in a 3-dimensional syncytium occurs at the coupling between cells and little, if any, drop in potential occurs within a particular cell (see for example, Tomita, 1970). The fact that this model is unable to explain all these observations makes it very unlikely to be correct.
Model 5: Monoquantal release from a number of varicosities each on a difference nerve fibre. Amplitude variation due to co-incident release of transmitter from more than one varicosity;

The very definition of families - that they consist of d.e.s. which all have identical latency and time to peak (within the technical limits of this study) casts doubts on a model which consists of a number of nerve fibres. The results obtained by variation in stimulus intensity, showing that all varicosities contributing to the family are on a single nerve fibre renders this model impossible.

Models which cannot be rejected

Model 1: Multiquantal release from a single varicosity onto a single smooth muscle cell

Model 3: Monoquantal release from a number of varicosities on a single nerve fibre each acting on the same cell. Amplitude variation due to co-incident release.

Unfortunately all the experimental observations made in the previous chapters can be explained by both Model 1 and Model 3. Indeed it is impossible to design an experiment using the postjunctional response to nerve stimulation which will distinguish between the two. It can be argued that as far as the muscle cell is concerned, both models effectively describe a single latency, multiquantal release site.

Information from other studies

Structural

The structural evidence reviewed in Chapter 1 shows that
the rodent vas deferens is densely innervated. Each smooth muscle cell of the mouse vas deferens is in close contact with at least one nerve fibre (e.g. Yamauchi & Burnstock, 1969).

Merrillees (1968) has shown that although less than half the cells of the guinea pig vas deferens are in close contact with a nerve fibre, between 0 and 35 varicosities are within 500Å of each cell. Merrillees (1968) has also described lengths of individual nerve fibres running close to muscle (within 500Å) for 10-60μm (10-20 varicosities).

This structural evidence from the rodent vas deferens also fails to distinguish between the two possible models of transmitter release.

**Contributory evidence from other junctions**

It is now generally accepted that transmitter is released in packets or quanta at all chemical synapses so far studied. Examination of the statistics of transmitter release, i.e. the distribution of the number of packets released each impulse, has been used by a number of workers at other junctions to try to understand the release process better and to suggest physical correlates of the various statistical parameters.

Martin (1955) showed that Poisson statistics could be applied at the amphibian neuromuscular junction under normal conditions of release when many quanta were released each impulse. As a result of this study the quantal release of transmitter at all synapses was originally assumed to obey Poisson statistics.

Later experiments, however, at certain other synapses, in particular the crayfish neuromuscular junction, under normal conditions failed to reveal a release process which could be
fitted by a Poisson model (e.g. Bittner & Harrison, 1970; Johnson & Wernig, 1971). Instead a binomial distribution was found to fit the observed results more accurately (Johnson & Wernig, 1971; Zucker, 1973). This led McLachlan (1978) to state that normal release at all junctions seems to occur by binomial sampling from a finite population rather than random selection from a large population of available quanta in a Poisson manner and that Martin's results could be described by a Poisson distribution purely by chance. Previously, del Castillo & Katz (1954b) had suggested that binomial statistics may well apply, at the skeletal neuromuscular junction, when release is normal and that the Poisson model they observed under abnormally low release conditions was just an extreme form of this binomial distribution.

In other words McLachlan (1978) believed that the number of available quanta for release per stimulus, the releasable pool of transmitter, is smaller than would be predicted by a Poisson distribution.

Vere-Jones (1966) had previously suggested two main possible physical correlates of the number of quanta available for release (n). It could either be the number of vesicles in the presynaptic membrane, or the number of discrete sites which could be occupied by, at most, one quantum.

Zucker (1973) had calculated from extracellular recordings of the crayfish neuromuscular junction that 'n' is a measurable number of releasable quanta with a value of between 2 and 4. This correlates well with the studies of Atwood & Morin (1970) who had shown that presynaptic membrane thickenings; active zones (Couteaux & Pecot-Dechavassine, 1974), associated with clusters of vesicles, are spaced every few microns along
a terminal and that an extracellular electrode would be capable of recording from 2 to 4 of these thickenings. Thus, Zucker (1973) suggested that the number of releasable quanta is equal to the number of active zones, which in turn implies that release, per impulse is monoquantal from each active zone. Similarly, Wernig (1972; 1975) at both the crayfish and frog neuromuscular junction found that n is related to the length of activated nerve terminal and the number of vesicle attachment sites.

Birks, Huxley & Katz (1960) and McMahon, et al. (1972) showed, at the frog neuromuscular junction, that vesicles cluster at regions of membrane thickenings while Hubbard & Kwanbunbumpen (1968) showed that the incidence of vesicle fusion was highest at these thickenings running round the surface of the nerve terminal.

Birks, et al. (1960) also estimated that an extracellular electrode at this junction would record from between 7 and 20 active zones. This is a similar number to the 'n' value calculated by Katz & Miledi (1965a) from just such a method of recording as being between 4 and 13.

Wernig & Stirner (1977) took this idea of one quantum per active zone per impulse a stage further by suggesting that a quantum of transmitter actually represented the release of transmitter from one active zone which was made up of 2 to 15 subunits (vesicles) discharged simultaneously. Heuser, et al. (1979) however, found no evidence to support such a view that active zones act in an all-or-none manner. They were able to show that one quantum of transmitter was equivalent to one vesicle opening (see Chapter 1). It is interesting to note that 4-AP used in these experiments to enhance release and make vesicle openings easier to see, increased release of
transmitter by 2 orders of magnitude per pulse (from 200 to 5000 quanta). This is not consistent with the other estimates (Martin, 1955, above) of the number of quanta available for release.

Under normal release conditions, Heuser, et al. (1979) found it difficult to visualise vesicle openings and while they stated that one quantum was the equivalent of one vesicle rather than one active zone, they were unable to determine whether one active zone was capable of releasing only one vesicle per stimulus. They did show, however, when release was increased in the presence of 4-AP that no detectable pattern of vesicular release within an active zone could be found and that vesicles released transmitter completely independently of activity in surrounding vesicles. This lack of a relationship of release between neighbouring vesicles in an active zone, suggested to Heuser, et al. (1979) that the active zones had no real functional significance in terms of release of transmitter and that a nerve might just as well have one single active zone in a longitudinal direction rather than a series of short transverse ones.

McLachlan (1978), on the other hand, has pointed out that the amphibian neuromuscular junction has \( \approx 300 \) active zones, \( \approx 1200 \) associated vesicles and under normal conditions, a quantal content (m) of \( \approx 200 \). Trying to accommodate these different orders of magnitude, McLachlan states that "it seems simplest to infer that normally, at most, only one quantum is released from a release site in response to a nerve impulse".

At present, therefore, the physical correlate of the statistical parameter 'n' is still unclear. It may represent either the number of vesicles available for release or, alternatively, the number of active zones (release sites).
The measurement of epps in spinal motor neurones evoked by impulses in single Ia axons fluctuate in amplitude (Redman & Walmsley, 1983). At this junction the density of innervation of the motorneurones by the Ia afferent axons is much lower than that at the neuromuscular junction. Each ending of a Ia afferent is made up of a number of boutons. These boutons are similar to active zones both in terms of their size and the spacing between each. However, there are many fewer boutons in a Ia terminal than there are active zones in a motor nerve terminal.

By a combination of electrophysiological and morphological techniques it can be shown that release from a single bouton is all-or-none, i.e. one quantum per bouton (Jack, Redman & Wong, 1984) and that the number of quanta released, per pulse, never exceeds the number of boutons (Redman & Walsmsley, 1983).

Thus for this junction at least, release from any particular release site has been shown to be both monoquantal and intermittent. Cunnane & Stjärne (1982) have recently suggested, on the basis of experiments using discrete events, that release from any particular varicosity is monoquantal and extremely intermittent during trains of constant intensity nerve stimulation in certain cells.

However, in order to explain the observed d.e. amplitude distribution obtained in this study, purely in terms of coincident monoquantal release of transmitter from individual varicosities, the degree of intermittency from a single varicosity seen in this study must be less than the figure quoted by Cunnane & Stjärne (1982).
Conclusion

The structural similarity between active zones (at the skeletal neuromuscular junction), synaptic boutons (at spinal motorneurones) and varicosities (at sympathetic neuromuscular junctions) (see, for example, McLachlan, 1978) suggests that the mechanism of transmitter release may be the same at each of these junctions and thus (as has been found at the junction between Ia afferents and spinal motorneurones) monoquantal and intermittent from a particular 'varicosity'.

At the sympathetic neuromuscular junction it has not been conclusively proven that such a situation exists and the issue will probably not be resolved at this junction until direct electrical recording of the response of a single varicosity to nerve stimulation becomes technically possible.

At present, therefore, a family of discrete events must be considered as representing the packeted release of transmitter from one or a few varicosities on a single nerve fibre behaving effectively, as far as the smooth muscle cell is concerned, as a single release site.
CHAPTER 9

MODULATION OF TRANSMITTER RELEASE
Introduction

The object of this chapter is to examine the effects of prejunctional modulation on families of discrete events to try to discover evidence for a system of inhibitory control of transmitter release.

The release of transmitter from sympathetic nerves can be modified by α-adrenoreceptor agonists and antagonists acting prejunctionally. Whether these agents act at some physiologically important site involved in controlling transmitter release has, however, been questioned (see Chapter 1).

Therefore, firstly, the relationship between the effects of the α₂-agonist clonidine, the α₂-antagonist, yohimbine and the concentration of endogenous transmitter in the junctional cleft has been examined to see if the relationship could be predicted by assuming these drugs interacted with an existing physiological control system.

Secondly, the release of endogenous transmitter from individual release sites in the absence of drugs has been examined by considering trains of discrete events evoked by nerve stimulation and observing if there are any short term local inhibitory effects upon the release from a single site from pulse to pulse.

These two approaches may provide new information as to the physiological mechanism, if any, of prejunctional inhibitory modulation of transmitter release.
Methods

The general methods used for recording and analysing data were as described in Chapter 2.

Trains of d.e.s. 100-250 pulses long due to submaximal electrical stimulation at 2Hz were used for analysis in all conditions, except the low frequency experiments, described in this chapter.

Trains of ejps 10-30 pulses long due to electrical stimulation at 0.075Hz were used for analysis of the effects of low frequency stimulation. For every preparation a submaximal stimulation intensity was chosen which reliably elicited a constant amplitude ejp in the absence of any drug. The stimulus intensity was then held constant for that particular preparation and all further results obtained in the presence and absence of yohimbine were expressed as a percentage of the initial control value.

The patterns of stimulation used in facilitation experiments were as described in the Methods section of Chapter 7.

Survivorship analysis

One method for examining whether negative feedback occurs is to measure how release from a single release site is related to subsequent release from that same site with respect to time.

Once release occurs, three things may occur: the probability of subsequent release may be (a) unchanged, (b) inhibited, or (c) facilitated.

These alternatives can be clearly distinguished between using a logarithmic survivorship curve (Nelson, 1964).

The number of stimuli between successive family member
Figure 9.1.
Idealised survivorship curve.
discrete events, in a train of stimuli can be recorded. When the whole train has been analysed, the number of times that each "gap length" (between successive family member d.e.s.) occurs is calculated. These numbers are tabulated and then cumulated from the largest to the smallest gap length. The successive cumulations are then tabulated below their respective gap length and the logarithms of these values plotted on the ordinate of a graph which has the number of stimuli between successive family members on the abscissa (see Petersen, 1975).

If the probability of subsequent release from a single site is unchanged by a release from that site, the slope of the curve will be a straight line. If subsequent release is inhibited, the slope of the curve will be initially shallow then steep. Finally, if subsequent release is facilitated, the slope of the curve will be initially steep then shallow (see Figure 9.1).

Changes in bathing medium

Changes in the bathing medium such as altering [Ca^{++}]_o or the addition of yohimbine or clonidine were carried out by perfusing the organ bath with the new solution and allowing 30 minutes for equilibration.

In single cells studies where the effects of changing the bathing medium were measured both before and after the change, the equilibration interval was reduced to between 15-30 minutes depending upon the stability of the penetration.

Results

(A) Effects of clonidine and yohimbine on trains of evoked discrete events

The effects of varying doses of clonidine and yohimbine have been measured, both on the probability of occurrence and on the mean amplitude of the discrete events in any one family. The results are shown in Figures 9.2 and 9.3. It can be seen that clonidine (10^{-9} - 10^{-7}M) decreases the probability of occurrence and mean d.e. amplitude significantly in a dose
Figure 9.2.

The effect of various concentrations of clonidine and yohimbine on the mean amplitude of a family of d.e.s. All concentrations of clonidine used and $10^{-7}$M & $10^{-6}$M yohimbine had a significant effect ($p<0.05$ student's t-test). In each case $n>6$. 
Figure 9.3.

The effect of various concentrations of clonidine and yohimbine on the proportion of failures of occurrence of a family member d.e. All concentrations of drug used had a significant effect (p<0.05 student's t-test). In all cases n>6.
dependent manner while yohimbine \((5 \times 10^{-9} - 10^{-6}\text{M})\) significantly increases the probability of occurrence and mean d.e. amplitude in a dose dependent manner.

Figure 9.4(a) shows an amplitude distribution of a typical family of d.e.s. and Figure 9.4(b) shows the effect of clonidine on that family. The distribution remains multi-modal with the modes remaining in the same place on the abscissa and the spacing between the modes remains constant. This indicates that the action of the drug is prejunctional and that the 'quantal size' is unaltered. Although the preferred values of d.e. amplitude are unchanged by clonidine, large d.e.s. are observed less frequently and small ones more frequently.

Similarly, yohimbine is found to alter the shape and not the modes of the amplitude distribution of a family of d.e.s. (Figure 9.5(a) and (b)).

(B) Changes in clonidine and yohimbine effects when \([\text{Ca}^{++}]_o\) is altered

Figures 9.6(a & b) and 9.7(a & b) show the effects of varying the calcium concentration on mean d.e. amplitude of a family and on the proportion of failures of occurrence of that family in the control situation and in the presence of clonidine and yohimbine.

Figure 9.6(a) shows that as the \([\text{Ca}^{++}]_o\) is increased the mean d.e. amplitude is significantly increased in the control and yohimbine conditions but remains unchanged in the presence of clonidine. Figure 9.6(b) shows that the proportion of failures is significantly reduced in all three cases as the \([\text{Ca}^{++}]_o\) is increased.

Figures 9.7(a) and 9.7(b) express the results shown in Figures 9.6(a) and 9.6(b) as a percentage of their respective
Figure 9.4.

Effect of clonidine (5 \times 10^{-9} \text{M}) on a family of d.e.s. in a single cell.

(a) control (Pf = 0.50).
(b) + clonidine (Pf = 0.69).

Bin size = 0.05V/sec.
Figure 9.5.
Effect of yohimbine ($10^{-7}$M) on a family of d.e.s. in a single cell.

(a) control ($P_f = 0.46$).
(b) + yohimbine ($P_f = 0.39$).
Bin size = $0.05$V/sec.
Figure 9.6.

The effects of clonidine ($5 \times 10^{-9}$ M) and yohimbine ($10^{-7}$ M) on (a) the mean amplitude and (b) the proportion of failures of occurrence of a family of d.e.s. in varying $[\text{Ca}^{++}]_o$.

- Clear bars = control.
- Striped bars = addition of yohimbine ($10^{-7}$ M).
- Dotted bars = addition of clonidine ($5 \times 10^{-9}$ M).

As $[\text{Ca}^{++}]_o$ is increased (from 1.1 - 4.2 mM) mean d.e. amplitude significantly increases ($p<0.05$ student's t-test) in control and yohimbine cases but not clonidine. The proportion of failures is significantly reduced in all 3 cases ($p<0.05$ student's t-test).
Figure 9.7.

The effects of the drugs shown in Figure 9.6. expressed as a percentage of the control value at their respective calcium concentration.

(a) mean d.e. amplitude.  (b) proportion of failures.

Clear bars = control.
Striped bars = addition of yohimbine ($10^{-7}$M).
Dotted bars = addition of clonidine ($5 \times 10^{-9}$M).

As $[\text{Ca}^{++}]_o$ is increased (from 1.1 - 4.2mM) the proportionate effects of yohimbine are significantly increased while the proportionate effects of clonidine are significantly decreased ($p<0.05$ student's $t$-test).
EFFECTS OF CALCIUM ON MEAN AMPLITUDE

EFFECTS OF CALCIUM ON THE NUMBER OF FAILURES
control value. From this method of expressing the results, it can be seen that as the \([Ca^{++}]_o\) is increased, the proportionate effect of yohimbine is significantly decreased while the proportionate effect of clonidine is significantly increased both in terms of mean d.e. amplitude and proportion of failures.

(C) The effect of clonidine and yohimbine on facilitation

\(\alpha\)-adrenoreceptor agonists and antagonists have previously been shown to affect both the development and the decay of facilitation of excitatory junction potentials in the rodent vas deferens (Blakeley, Cunnane & Petersen, 1981; Blakeley, et al., 1984). In this study their effects on mean family d.e. amplitude and proportion of failure of family occurrence were measured in two different calcium concentrations during the development of facilitation.

Changes in discrete events mirror the alterations in ejp amplitude when the \([Ca^{++}]_o\) is 2.1mM. Figure 9.8(a & b) shows changes in discrete event amplitude and probability of occurrence during facilitation in the presence of yohimbine (10^-7M) or clonidine (5 x 10^-9M).

Clonidine significantly decreases mean discrete event amplitude and increases the probability of failure in both the facilitated and unfacilitated condition. Yohimbine increases mean discrete event amplitude and reduces the probability of failure significantly only for discrete events following fourth and fifth stimuli, i.e. once facilitation has developed.

When the \([Ca^{++}]_o\) is lowered the effects of the drugs are altered (Figure 9.9(a & b)). The depressing effect of clonidine on discrete event amplitude is proportionately less and in the
Figure 9.8.

Effects of yohimbine ($10^{-7}$M) and clonidine ($5 \times 10^{-9}$M) on (a) mean d.e. amplitude and (b) proportion of failures during facilitation when $[Ca^{++}]_o = 2.1$ mM.

Control $n = 15$; yohimbine $n = 5$; and clonidine $n = 3$. 
Figure 9.9.

Effects of yohimbine ($10^{-7}$M) and clonidine ($5 \times 10^{-9}$M) on (a) mean d.e. amplitude and (b) proportion of failures when $[Ca^{++}]_o = 1.1$ mM.

Control $n = 6$; yohimbine $n = 6$; clonidine $n = 4$. 
unfacilitated state there is no significant effect on the probability of failure.

Conversely, yohimbine now elevates mean discrete event amplitude and reduces the probability of failure following all stimuli in a train including the first.

To look at this effect more closely a study of the effect of various concentrations of yohimbine in various $[\text{Ca}^{++}]_o$ on unfacilitated transmitter release was carried out.

D) Effects of yohimbine on unfacilitated transmitter release

Figure 9.10 shows the effect of various concentrations of yohimbine at three different $[\text{Ca}^{++}]_o$ on unfacilitated ejp amplitude elicited by very low frequency stimulation ($<0.1\text{Hz}$).

At 2.1mM $[\text{Ca}^{++}]_o$ yohimbine has no effect. However, if the $[\text{Ca}^{++}]_o$ is lowered to 1.6 or 1.1mM yohimbine significantly enhances unfacilitated ejp amplitude.

The experiments were carried out on ejps rather than discrete events because it is difficult to obtain long trains of stimuli at a very low frequency in a single cell. Consequently, analysis can be carried out much more easily on ejps which do not vary from stimulus to stimulus under constant conditions unlike discrete events whose amplitude varies markedly from stimulus to stimulus and thus analysis of a whole distribution is required.

For one particular cell, however, Figure 9.11 shows the effect of yohimbine on a family of discrete events when $[\text{Ca}^{++}]_o$ was 1.1mM. It can be seen that the mean d.e. amplitude is increased and the proportion of failures reduced. It is also apparent that the modes of the distribution and the spacing between the modes remains constant.
Figure 9.10.

The effect of various concentrations of yohimbine and 
$[\text{Ca}^{++}]_0$ on the amplitude of ejps elicited by constant 
intensity, low frequency stimulation.
Figure 9.11.

The effect of yohimbine ($10^{-7}$M) on a family of d.e.s. in a single cell elicited by low frequency stimulation when $[\text{Ca}^{++}]_0 = 1.1\text{mM}$.

(a) control $\text{Pf} = 0.68$.

(b) + yohimbine $\text{Pf} = 0.45$.

Bin size $= 0.05\text{V/sec}$. 
E) Patterns of transmitter release and endogenous control

Figure 9.12 shows the pattern of occurrence of discrete events which have been classified as all belonging to the same family in a single cell. How are the occurrence of family member discrete events related to each other with respect to their position in the stimulus train? If a discrete event occurs the occurrence of a second may be unaffected, facilitated or inhibited.

One of the best methods of investigating such a question is to consider the number of failures that occur between each d.e. This can be represented by a logarithmic survivorship curve (see Methods). Figure 9.13 shows the application of a logarithmic survivorship curve to a typical family of discrete events in control conditions and in the presence of clonidine and yohimbine. In all three cases the slope of the curve is initially steep then it becomes shallow. That is to say, it initially deviates from what would be predicted if there was no relationship between the occurrence of one d.e. and the occurrence of a second.

The curve deviates in such a way as to suggest that the probability of a second discrete event occurring a short time after a first one, is increased (Slater, 1975; Petersen, 1975). There seems therefore to be some form of 'short term facilitation' within a family when a family member occurs. This is emphasised in Table 9.1 where the probability of a second discrete event occurring 1 or 2 pulses after a first one is significantly raised.

It is possible that during this short term facilitation of transmitter release, the amplitude of discrete events, reflecting the amount of transmitter released by each pulse, is also altered.
Figure 9.12.

Occurrence distribution of family member d.e.s. in a train of stimuli.

Clear box = failure.

Shaded box = occurrence.
Figure 9.13.

Survivorship curves for families of S.e.s. in 3 different cells.

Filled circles = control.
Clear circles = clonidine (5 x 10^{-9} M).
Clear triangles = yohimbine (10^{-7} M).

The area between the dotted lines is the area where one cannot be certain if short term facilitation exists (see text and compare this figure with Figure 9.1).
Table 9.1

Pulse to pulse occurrence of family member d.e.s.

| [Ca\(^{++}\)]\(_o\) = 2.1mM | 7 | 0.533 ± 0.05 | 0.559 ± 0.04 | <0.05 |
| + clonidine (5x10\(^{-9}\)M) | 6 | 0.474 ± 0.06 | 0.54 ± 0.07 | <0.05 |
| + yohimbine (10\(^{-7}\)M) | 5 | 0.765 ± 0.05 | 0.776 ± 0.05 | <0.1 |

Mean \(P_o\) = mean probability of occurrence of a family member d.e.

Observed \(P_o\) (n+1 or n+2) = observed probability of occurrence of a family d.e., 1 x 2 pulses after a family member d.e. occurs.
It has previously been shown that the amplitude of a discrete event is unaffected by the number of preceding stimuli which have failed to evoke it, and also that transmitter release from one site does not influence release from nearby sites (Blakeley, Cunnane & Petersen, 1982). Neither of those results, however, provide an answer to the question of modulation from pulse to pulse of a particular family of discrete events during short term facilitation.

To do this, it is necessary to define two boundaries. The first boundary is the number of failures of occurrence of a family member that have to occur before one can be certain that the short term facilitation is over. The second is the number of failures which can occur while one can still be certain that short term facilitation is present. In ideal conditions these boundaries should be the same, however it seems fair to assume that a certain area exists where one cannot be sure of the existence or not of short term facilitation.

Both these boundaries can be obtained from an examination of Figure 9.13. It has been decided that 5 pulses are required to occur without eliciting a family member d.e. in order for short-term facilitation to be said to have passed, while no more than 3 failures should occur consecutively in order that short term facilitation can be said to still exist.

Thus trains of stimuli have been examined and the amplitude of the first three pulses following the initiation of short term facilitation measured. The results have been normalised with respect to the first pulse and are shown in Table 2. The number of 'bursts' of short term facilitation in each cell ranged from 7-22. With the exception of the starred cell in the control results (see Table 9.2), the results obtained show
Table 9.2

Pulse to pulse d.e. amplitudes

<table>
<thead>
<tr>
<th>Cell</th>
<th>Event n</th>
<th>Event n + 1</th>
<th>Event n + 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.993</td>
<td>1.333</td>
</tr>
<tr>
<td>2*</td>
<td></td>
<td>0.612</td>
<td>0.775*</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.013</td>
<td>0.928</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1.050</td>
<td>1.108</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.000</td>
<td>0.917</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.947</td>
<td>0.895</td>
</tr>
<tr>
<td>mean (except *)</td>
<td>1</td>
<td>1.001</td>
<td>0.996</td>
</tr>
</tbody>
</table>

+clonidine (5x10^-9 M)

<table>
<thead>
<tr>
<th>Cell</th>
<th>Event n</th>
<th>Event n + 1</th>
<th>Event n + 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>1.162</td>
<td>0.757</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.970</td>
<td>1.061</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.181</td>
<td>1.069</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.969</td>
<td>1.031</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.937</td>
<td>0.923</td>
</tr>
<tr>
<td>mean</td>
<td>1</td>
<td>1.044</td>
<td>0.968</td>
</tr>
</tbody>
</table>

+yohimbine (10^-7 M)

<table>
<thead>
<tr>
<th>Cell</th>
<th>Event n</th>
<th>Event n + 1</th>
<th>Event n + 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.867</td>
<td>0.800</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.824</td>
<td>0.944</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.131</td>
<td>0.920</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.902</td>
<td>0.877</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.093</td>
<td>1.150</td>
</tr>
<tr>
<td>mean</td>
<td>1</td>
<td>0.964</td>
<td>0.938</td>
</tr>
</tbody>
</table>

The mean amplitudes of certain d.e.s. (n) in each cell (see text) was normalised to 1. The mean amplitudes of d.e.s. occurring 1 and 2 pulses (n + 1, n + 2) after the first d.e.s. (n) were expressed as a fraction of the mean amplitude of those first d.e.s.
that there is no alteration in mean amplitude between pulses of family d.e.s. during short term facilitation. Neither clonidine nor yohimbine affect this result.

The starred cell is unusual. The first d.e. in a burst of short term facilitation is significantly larger than those following. An explanation for this observation is not obvious. The discrete events in this cell were among the largest ever recorded (1.875V/sec c.f. mean d.e. amplitude in general = 1.28 ± 0.08V/sec). Perhaps when transmitter release is so large some alternative form of modulation occurs. Whatever the reason this phenomenon was not observed in any other analysed cell.

No evidence of endogenous feedback control has been observed then, either in terms of whether release occurs or not, or in terms of the amplitude of that release when it occurs.

Discussion

The aim of this project was to investigate whether a system of prejunctional inhibitory modulation of transmitter release exists in the peripheral sympathetic nervous system. The investigation was effectively split into two sections. Firstly, the actions of α-adrenoreceptor agonists and antagonists were looked at in order to consider the existence or not of a system of negative feedback by released noradrenaline on subsequent transmitter release. Secondly, trains of stimuli were examined in the absence of drugs to see if any inhibitory feedback from pulse to pulse, regardless of its cause, could be shown to exist.

(A) Negative feedback by noradrenaline
Dose dependent prejunctional modulation of transmitter release by clonidine, an $\alpha_2$-adrenoceptor agonist, and yohimbine, an $\alpha_2$-adrenoceptor antagonist, has been demonstrated.

Families of discrete events, on the balance of available evidence, representing the packeted release of transmitter from a few release sites on a single nerve fibre innervating the recording cell (Chapter 8) are affected in two ways. The mean family d.e. amplitude and the proportion of occurrence of a d.e. family member are increased by yohimbine and decreased by clonidine.

It has been shown in Chapters 3 and 7 that there are two different components which make up the proportion of failure of occurrence of a family member d.e.: Poisson and non-Poisson failures. These two components are difficult to separate with any real degree of accuracy (see Chapter 7) and while the drugs are known to affect the proportion of failures it is difficult to be sure whether they affect both components.

They certainly affect the Poisson release process, simply because of their action on the amplitude distribution of discrete events. Since, in normal calcium solutions, the major component of the proportion of failures can be described as 'non-Poisson' it seems likely that any significant alteration in the proportion of failures will include an effect on the 'non-Poisson' failures.

Thus, it can be said with reasonable confidence, that these drugs affect both components of the release process and not just the Poisson component.

In Chapter 7 the observation of Poisson and non-Poisson
failures was correlated with experiments of Alberts, et al. (1981) who studying the kinetics of transmitter overflow, found that two components of the release process, which responded differently to various treatments, existed. They considered these two components of the release process to be (a) the depolarisation/secretion coupling mechanism, and (b) the invasion or not of a terminal varicosity, and found that both processes were susceptible to modulation by α-adrenoreceptor agonists and antagonists.

The observations made in this study would therefore appear to complement the results of Alberts, et al. (1981).

(2) Ionic mechanisms of action of α₂-adrenoreceptor agonists

So far two possible mechanisms of action have been proposed for α₂-adrenoreceptor agonists.

(i) Direct action on K⁺ conductance of α-agonists

Nakamura, et al. (1981) have shown that clonidine reduces excitability in terminals of locus coeruleus neurones and this effect has been shown in vivo to be due to an increase in K⁺ conductance (Aghajanian & Vandermaelen, 1982; Egan, et al. 1983) and consequent membrane hyperpolarisation. Similarly, in the periphery Morita & North (1981) have shown that α₂-agonists increase K⁺ conductance with a consequent hyperpolarisation in guinea pig myenteric neurones.

This action on K⁺ conductance could result in one or both of the following occurrences:

a) A failure to recruit release sites due to hyperpolarisation causing a selective blockade of the impulse conduction.

b) Hyperpolarisation of the nerve membrane will decrease spike duration and obviously nerve
terminal depolarisation. This would decrease the opening time of calcium channels and consequently decrease depolarisation/secretion coupling and transmitter release.

(ii) Direct inhibition of inward Ca\(^{++}\) by \(\alpha\)-agonists

It has been shown in rat sympathetic neurones that \(\alpha\)-agonists can directly inhibit the inward movement of Ca\(^{++}\) (Horn & McAffee, 1979, 1980). This would lead to a decreased availability of that ion for depolarisation/secretion coupling.

Cunnane & Stjärne (1984) have suggested that in the terminal regions of sympathetic nerves the safety factor for transmission may be low and that nerve impulse conduction becomes calcium dependent. If this is the case a direct inhibition of inward Ca\(^{++}\) by \(\alpha\)-agonists will also increase the probability that an action potential will fail to invade a particular varicosity.

Thus either of the two mechanisms above could account for the observations made in this study and the study of Alberts, et al. (1981), that \(\alpha\)-agonists and antagonists affect both invasion of a varicosity and the depolarisation/secretion coupling mechanism.

(3) Interactions between \(\alpha\)-adrenoreceptor agonists and antagonists and endogenous transmitter concentration

The actions of \(\alpha\)-adrenoreceptor agonists and antagonists on transmitter release from individual varicosities compares very well with results of other studies (e.g. Langer, 1977; Starke, 1977; Westfall, 1977; Gillespie, 1980). If the results obtained with the antagonists, yohimbine, are taken to be due to blockade of inhibition normally excited by
endogenous transmitter, then the results obtained appear to show that negative feedback occurs physiologically.

However, when experiments were carried out in which the concentration of released transmitter was varied, the effects of the drugs became much less clear.

(a) When the $[\text{Ca}^{++}]_o$ was varied in order to alter the amount of transmitter released (Rubin, 1970; Rahamimoff, 1970; Kipekar, 1975), clonidine became proportionately more effective as the $[\text{Ca}^{++}]_o$ was raised while yohimbine became proportionately more effective when the $[\text{Ca}^{++}]_o$ was lowered.

(b) When the stimulation frequency was kept sufficiently low to avoid persistence of free transmitter between stimuli (Kalsner, 1979; Kuriyama & Makita, 1983), yohimbine, as would be expected, was found to have no effect under normal $[\text{Ca}^{++}]_o$ conditions (2.1mM). When, however, the $[\text{Ca}^{++}]_o$ was lowered to 1.6 or 1.1mM, yohimbine significantly enhanced the amount of transmitter released per pulse.

The above observations, particularly concerning the antagonist yohimbine, are contrary to what might be predicted if a system of negative feedback existed.

In both instances the antagonist yohimbine becomes more effective the less endogenous agonist is present to antagonise.

The possibility that such an effect exists because the antagonist is denied access to prejunctional receptors when the concentration of transmitter in the synapse is high must be considered unlikely as such an inverse relationship between antagonist effect and transmitter concentration has also been seen when the antagonist used was an irreversible binder to $\alpha_2$ receptors such as phenoxybenzamine (see Gillespie, 1980).
Is α-autoinhibition a physiological control mechanism or a pharmacological artefact?

The results in this study have shown that the effect of the α-adrenoreceptor antagonist yohimbine on the release of transmitter from single release sites (one or a few varicosities on a single nerve fibre) is greatest when the concentration of endogenous transmitter in the synapse is low. Such an effect on the release from single sites is similar to a number of other studies where release has been measured either as whole organ response or as amount of transmitter overflow (e.g. Angus & Korner, 1981; Chan & Kalsner, 1979; Kalsner, 1979, 1981, 1983).

Such observations cannot be reconciled with any simple system of negative feedback control of transmitter release by released noradrenaline, and have led to suggestions that α-autoinhibition does not exist at all (e.g. Kalsner, 1983, 1984). If this is the case the effects of these drugs on transmitter release must be regarded as a pharmacological artefact. Such a conclusion requires that yohimbine must have an active action on transmitter release rather than purely passive inhibition of α-adrenoreceptors. This idea has received some support (see Kajiwara, Kitamura & Kuriyama, 1981; Kalsner, & Quillan, 1984).

An alternative view is that the transmission process is much more complicated than previously imagined.

For example, it is known that a number of other substances such as prostaglandins, ATP, dopamine and neuropeptide Y are released into the junctional cleft during stimulation and also have a prejunctional action inhibiting transmitter release (see Chapter 1). Any one of these substances may have a physiological role in the control of transmitter release, and
may operate optimally at different degrees of nerve activity. Thus, when the activity of the junction is altered, for example, by changing $[\text{Ca}^{++}]_o$ or the frequency of nerve stimulation, the relative activity of different physiological control systems may alter too. Experiments which modulated one control system without interfering with others present would therefore produce unpredictable results.

Similarly, if noradrenaline and ATP are released as co-transmitters and both can control subsequent nerve activity, blockade of one type of feedback control might simply enhance the activity of the other. Such a system could easily produce an inverse relationship between the amount of transmitter released and the proportionate effect of an $\alpha$-adrenoreceptor antagonist.

(B) Physiological feedback in the absence of drugs

Previous experiments in the guinea pig vas deferens have failed to reveal an inhibitory relationship either between discrete events evoked by successive stimuli or between early and late discrete events following a given stimulus (Blakeley, Cunnane & Petersen, 1982).

In this study, in the mouse vas deferens, events were associated into families, representing the release of transmitter from one or a group of varicosities on a single nerve fibre behaving, effectively, as a simple release site (see Chapter 8).

In this study, no evidence for inhibition of transmitter release from a particular family of d.e.s., from pulse to pulse has been found. Rather, the opposite occurs. Family member d.e.s. are seen to occur in bursts, i.e. there is some process of short term facilitation of transmitter release from a
particular group of varicosities after an initial release occurs. The amplitude of d.e.s. within this 'short term facilitation' is unaltered from pulse to pulse.

**Implications for release process**

Bevan, et al. (1984) discussed the possibility that transmitter release from a particular varicosity inhibits subsequent release from that varicosity for the next few pulses in a train and that such a system would explain the intermittency of release from varicosities and allow an even spread of transmitter release from different varicosities surrounding a particular cell while conserving the amount of transmitter released as much as possible.

The results of this study negate this hypothesis. However, the observation that family member d.e.s. occur in bursts and not in a completely random order allows the possibility that control of transmitter release is occurring - not at the level of the individual varicosity, but at some point further 'up' the terminal nerve fibre. Such a mechanism of control would also allow an even spread of transmitter release from all varicosities and therefore conserve the amount of transmitter release.

**Alternative mechanisms of action for an inhibitory control process**

The results of this study suggest that if an inhibitory control process exists, it would have to exert its control, not from pulse to pulse, but over a number of pulses. Such control would have to consist of an interaction between varicosities and not simply autoinhibition by released transmitter on the varicosity from which it was released.

Thus, it is possible to hypothesise two interacting systems of control. Positive control of transmitter release
from groups of varicosities to promote transmitter release from set varicosities in short bursts and secondly, a more integral inhibitory control of transmitter release, the activity of which would be dependent on the overall activity of the nervous system.

By looking at the release of transmitter from pulse to pulse from individual release sites one would observe the short term facilitation of release from single sites, but not the integral form of control over a number of pulses.

Another possible explanation for the lack of observed inhibitory control of transmitter release is that such a system only operates physiologically in abnormal conditions when transmitter release is suddenly increased. Such a situation exists during the process of facilitation (Bennett & Middleton, 1975). In this chapter α-adrenoreceptor agonists and antagonists were found to have a significant effect on the development of facilitation and they have previously been shown to also affect decay of facilitation (Blakeley, Cunnane, Maskell, Mathie & Petersen, 1984). A physiological role of prejunctional α-adrenoreceptors might therefore be to limit the control of transmitter release during facilitation.

In conclusion this chapter has shown that α-adrenoreceptor agonists and antagonists clearly modify transmitter release probably by altering both the number of sites releasing transmitter and the amount each releases. The peculiar relationship between concentration of endogenous transmitter and effectiveness of α-adrenoreceptor antagonists is, however, difficult to correlate with any physiological system of α-autoinhibition. Furthermore the lack of evidence for endogenous negative feedback on transmitter release from pulse to pulse is difficult to correlate with any notion of physiological short term inhibitory control, regardless of the nature of the modulator.
CHAPTER 10

EFFECTS OF DESMETHYLIMIPRAMINE
CHAPTER 10

Introduction

Sympathetic nerves have been shown to possess a process for taking up exogenous noradrenaline (Axelrod, 1965; Hillarp & Malmfors, 1964), which can be selectively blocked by drugs (Iversen, 1967).

While this catecholamine uptake has been shown to occur in sympathetic nerves using a number of techniques (e.g. denervation histochemistry, autoradiography, $^{3}$H-noradrenaline overflow studies) (see Iversen, 1967), electrophysiological studies seem to reveal that the uptake process itself may not be important for the termination of transmitter action.

Holman (1967, 1970) has shown that cocaine has little effect on the time course and amplitude of ejps and little effect on the time course of sejps. The records shown (Holman, 1967) suggest that the amplitude of the sejps may increase in the presence of cocaine, but Holman, herself, (1970) states that it was difficult to obtain quantitative evidence on this point. Bennett & Middleton (1975) using desmethylimipramine (DMT), one of the most potent drugs at blocking neuronal uptake available, found that the drug had no effect on sejps; no effect on ejp amplitude; but, in high concentrations of DMT, a lengthening of ejp duration which these authors correlated with a temporal dispersion of the terminal nerve action potential of a similar time course. They also found that the amplitude of the terminal nerve action potential decreased by about 10%. Thus Bennett & Middleton (1975) have suggested that the prolongation of the ejp is due to DMT slowing the conduction of some terminal nerve action potentials, increasing temporal dispersion and causing a proportion of the varicosities to release their
transmitter later than in control conditions.

From both Holman's and Bennett & Middleton's observations it would seem that neuronal uptake is not of major importance for the cessation of transmitter action.

Nevertheless, the effect of DMI on discrete events has been looked at for two reasons. Firstly, an examination of the effects of uptake blockade on transmitter release from individual release sites may provide new information as to how, if at all, the release of transmitter is affected by uptake blockade.

Secondly, the observation that DMI increases temporal dispersion in terminal nerve action potentials (Bennett & Middleton, 1975; Chang & Chuang, 1972) can be confirmed or rejected at the sympathetic neuromuscular junction by looking at the release of transmitter from varicosities situated on a few terminal nerve fibres close to the recording electrode.

Methods

The general methods of recording and analysis used in this chapter were as described in Chapter 2.

Two concentrations of DMI were used in the majority of experiments in this study. The first, $10^{-6}$ M is sufficient to be 92.5% effective in blocking neuronal uptake (Iversen, 1967). The second $10^{-5}$ M is greater than the value of 1μg/ml ($\approx 3 \times 10^{-6}$ M) stated by Bennett & Middleton (1975) as being the concentration of DMI, above which the drug affects the terminal nerve action potential.
Results

(i) **Effect of DMI on membrane potential**

The effect of $10^{-5}$ and $10^{-6}$M DMI on membrane potential is shown in Table 10.1. DM1 has no significant effect on membrane potential.

(ii) **Effect of DMI on ejps**

The effect of $10^{-5}$ and $10^{-6}$M DMI on trains of ejps has been examined. The ejps were elicited by constant intensity stimulation. Table 10.2 shows the effect of DM1 on four standard parameters of the ejp: latency, time to peak, time to ½ decay and amplitude. $10^{-6}$M DMI does not have a significant effect on any of the parameters, however, at $10^{-5}$M DMI, the duration of the ejp is lengthened. Figure 10.1 shows typical sets of superimposed ejps from a single cell in the presence and absence of $10^{-5}$M DMI.

This lengthening of the ejp duration was suggested by Bennett & Middleton (1975) as being due to temporal dispersion of the terminal nerve action potential.

By using discrete events to examine the release of transmitter from a few varicosities close to the recording cell it should be possible to investigate whether such a temporal dispersion between different nerve fibres in the terminal region occurs.

(iii) **Effect of DMI on d.e.s.**

(a) **Time course.** Table 10.3 shows the effect of $10^{-5}$ and $10^{-6}$M DMI on latency and time to peak of d.e.s. While the mean latency and time to peak values are unaltered by either concentration of DMI, the width of the distribution of both latencies and times to peak is expanded when [DMI] is $10^{-5}$M. This can be seen in columns 3 and 4 of Table 10.3 where it is expressed as the bandwidth in milliseconds within which 90% of the discrete events are found.
<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Vm ±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>350</td>
<td>-71.9 ± 2.1</td>
</tr>
<tr>
<td>+ DMI ($10^{-6}$)</td>
<td>9</td>
<td>-71.4 ± 3.0</td>
</tr>
<tr>
<td>+ DMI ($10^{-5}$)</td>
<td>40</td>
<td>-73.4 ± 2.3</td>
</tr>
</tbody>
</table>
Table 10.2

Effect of DMI on ejps recorded in the mouse vas deferens

<table>
<thead>
<tr>
<th>n</th>
<th>mean ejp</th>
<th>latency</th>
<th>time to peak</th>
<th>time to 1/2 decay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amp (mV)</td>
<td>(ms)</td>
<td>(ms)</td>
<td>(ms)</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>9.48±0.44</td>
<td>13.3±0.92</td>
<td>40.7±0.99</td>
</tr>
<tr>
<td>DMI (10^{-6}M)</td>
<td>4</td>
<td>10.2±0.48</td>
<td>14.3±0.42</td>
<td>41±1.10</td>
</tr>
<tr>
<td>DMI (10^{-5}M)</td>
<td>8</td>
<td>10.08±0.06</td>
<td>14.5±0.50</td>
<td>42.5±1.49</td>
</tr>
</tbody>
</table>

(p > 0.25) (p > 0.25) (p > 0.25) (p > 0.25) (p > 0.25) (p > 0.25) (p > 0.05)
Figure 10.1.

Ejps recorded using a fast sweep speed control (upper trace) and DMI ($10^{-5}$M) (lower trace).

Calibration bars: 20msecs; 10mV.

Dotted lines = time to $\frac{1}{2}$ decay.
### Table 10.3

**Effect of DMI on time course of d.e.s.**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>mean latency (ms)</th>
<th>mean Hp latency (ms)</th>
<th>90% latency band (ms)</th>
<th>90% time to peak band (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>14.56±0.83</td>
<td>17.10±0.8</td>
<td>3.83±0.67</td>
<td>5±0.63</td>
</tr>
<tr>
<td>DMI (10^-6 M)</td>
<td>4</td>
<td>14.56±0.83</td>
<td>17.59±0.4</td>
<td>3.53±0.87</td>
<td>5.3±0.66</td>
</tr>
<tr>
<td>DMI (10^-5 M)</td>
<td>6</td>
<td>13.8±0.81</td>
<td>18.13±0.94</td>
<td>5.8±1.17</td>
<td>7.67±1.35</td>
</tr>
</tbody>
</table>

*p>0.25* (0.25>*p>0.1*) (0.1>*p>0.05*)
Figure 10.2 shows a typical example of discrete events after the addition of DMT (10^-5 M) while Figure 10.3 shows the latency and time to peak histograms for a single cell after the addition of DMT (10^-5 M).

(b) Families of discrete events and the proportion of failures

The dispersion of d.e. latencies and times to peak perhaps poses a problem as to how to divide d.e.s. into families. Earlier in the thesis (Chapters 5 and 8) it was suggested that all the events in a family are due to transmitter released from varicosities on a single nerve fibre. If this is so, the dispersion of events in a family will be less than those not in the family. Thus the family selection procedures used here were not altered from those described in Chapter 3, that is to say d.e.s. with latencies and times to peak within a single 2msec band were said to be members of the same family of discrete events.

Table 10.4 shows (a) the proportion of complete failure of occurrence of a d.e. and (b) the proportion of failures of the major family in a particular cell in control situations and in the presence of DMI (10^-5 and 10^-6 M).

When the DMI concentration was 10^-5 M both of the proportions of failures measured were significantly raised. The rise in the proportion of failures of a family member d.e. could be explained in two ways. It could either be a direct action of DMI on the terminal nerve net to decrease the activity of family member release sites, or alternatively, the temporal dispersion of d.e.s. by DMI (10^-5 M) may cause certain family member d.e.s. to no longer be picked up by the selection procedure. If this occurred one would expect to see a rise in the number of non family d.e.s. and therefore no change in the total number of d.e.s. seen and no change in the proportion of total failures. Instead the proportion of total
Figure 10.2.

Superimposed ejps (lower trace) with their associated d.e.s. (upper trace) in the presence of DMI (10^{-5} M).

Calibration bars: 20msecs; 0.5V/s; 8mV.
Figure 10.3.
Latency (upper) and time to peak (lower) histograms for a single cell in the presence of DMT ($10^{-5}$M).
Bin size = 0.5msecs.
### Table 10.4

**Effects of DMI on families of d.e.s.**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>mean family d.e. amp(Vs)</th>
<th>P complete failure</th>
<th>Pf (family member)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22</td>
<td>1.275 ± 0.085</td>
<td>0.36 ± 0.03</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>DMI (10^-6 M)</td>
<td>4</td>
<td>1.39 ± 0.111</td>
<td>0.41 ± 0.04</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>DMI (10^-5 M)</td>
<td>6</td>
<td>1.44 ± 0.103</td>
<td>0.45 ± 0.05</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p&gt;0.1)</td>
<td>(0.05&gt;p&gt;0.01)</td>
<td>(0.01&gt;p&gt;0.02)</td>
</tr>
</tbody>
</table>
failures is found to rise by the same factor as the proportion of failures of a family member in the presence of DMI (10^-5M). This suggests that the second alternative is unlikely, and that the observed effect of DMI (10^-5M) is brought about by some direct action on the terminal nerve net decreasing the activity of all release sites.

(c) Amplitude of d.e.s.

Table 10.4 also shows the mean amplitude of family member d.e.s. before and after the addition of DMI. While the mean amplitude is slightly higher in the presence of 10^-5M DMI it is not significantly different from the control.

Figure 10.4 shows that the amplitude distribution of a family of d.e.s. in the presence of DMI (10^-5M) has the same characteristics as a normal family d.e. amplitude distribution. It is multimodal and negatively skewed with the modes spaced equally along the abscissa.

Discussion

At a concentration of 10^-6M, a concentration sufficient to block the uptake of noradrenaline into sympathetic nerves by 92.5% (Iversen, 1967), DMI has no effect on the membrane potential of the smooth muscle cells of the mouse vas deferens, no effect on the ejp elicited by sympathetic nerve stimulation and no effect on the d.e.s. associated with these ejps.

Thus, this study confirms the observation of previous studies that blockade of amine uptake has no effect on the electrical response to nerve stimulation and extends the observation to the electrical response evoked by transmitter release from individual release sites.

When the concentration of DMI is increased to 10^-5M, the time course of ejps is lengthened. There is also an observed
Figure 10.4.

Amplitude histogram of a family of d.e.s. selected from the cell in Figure 10.3. (Pf = 0.66).

Bin size = 0.08V/sec.
temporal dispersion of the latencies and times to peak of discrete events in a train of stimuli.

These observations are probably best explained by the hypothesis of Bennett & Middleton (1975) who suggested that such an effect was due to DMI at this concentration causing a decline in amplitude and an increased duration of the terminal compound action potential.

At this concentration DMI significantly decreased the proportion of occurrence of family member discrete events. This is most likely due to the development of local anaesthetic activity of DMI when it is used in such a high concentration as sympathetic nerves are suggested to have a low safety factor for conduction (Cunnane & Stjärne, 1984).

If the transmitter responsible for the ejp is not noradrenaline (see Chapter 1) an alternative explanation for such an effect could be that noradrenaline available in increased concentrations in the biophase is acting as an inhibitory modulator of transmitter release (e.g. Ambache, et al. 1972). This hypothesis is unlikely to be correct, however, as at $10^{-6}\text{M}$ DMI no such effect is observed.

Thus, while the block of transmitter uptake into nerve terminals by DMI appears to have no effect on transmitter release from individual release sites, two other actions of DMI have been observed.

DMI ($10^{-5}\text{M}$) causes both a dispersion of the latencies of active release sites on different nerve fibres responding to a particular stimulus and also decreases the number of times release sites are active in any train of stimuli.
CHAPTER 11

D.Es IN BLOOD VESSELS
CHAPTER 11

Introduction

The object of the experiments described in this chapter is to attempt to extend the observation of 'discrete events' from the sympathetic nerves innervating the rodent vas deferens to those innervating blood vessels.

Unfortunately there is a considerable difference in structure between the two junctions which must be fully considered.

The morphology of blood vessels is somewhat different to that of other smooth muscles such as the vas deferens. Nerves are frequently restricted to the medio-adventitial border. Thus cells other than the outermost cells must be activated either by long distance diffusion or by some form of electrical or mechanical coupling between cells (Speden, 1970). Even for the outermost cells in a muscle bundle, however, nerve/muscle separation is rarely less than 1000\(\mu\)m. More recent studies have suggested that as arteries get smaller, the neurones become progressively more clustered until at the smallest vessels they are all quite close together. The neuromuscular gap, however, still does not fall much below 1000\(\mu\)m (Bevan, et al. 1984).

A number of recent studies have used electrophysiological techniques to look at the electrical response of the vascular system. In most preparations ejps were elicited in response to nerve stimulation (e.g. Hirst, 1977; Surprenant, 1980; Cheung, 1982b). However, because of the size of the neuromuscular gap, in blood vessels, diffusion of transmitter requires to be greater than in the vas deferens, release from individual varicosities may be difficult to observe by looking at the postjunctional response.
Methods

The general methods and the dissections of the preparations used in this chapter have been described fully in Chapter 2.

Results

In the first part of this study two preparations, the guinea pig mesenteric artery and the rat tail artery have been examined to see if they possess discrete events, reflecting transmitter release from individual varicosities.

The guinea pig mesenteric artery responds, fairly typically for a blood vessel, to stimulation and drug treatment (Surprenant, 1980; Kuriyama & Makita, 1983). The rat tail artery, on the other hand, possesses cells with high sejp frequency and ejps with large amplitudes and fast time courses. These effects have been attributed to this artery having a particularly dense innervation and relatively close neuromuscular junctions (Surprenant, 1980; Cheung, 1982a,b).

Typical ejps recorded in response to submaximal nerve stimulation are shown in Figure 11.1 for (a) the rat tail artery and (b) the guinea pig mesenteric artery. The most significant feature seen in these figures is the difference in time course of the electrical response of the two preparations to nerve stimulation. The ejp in the guinea pig mesenteric artery lasts about twice as long as that in the rat tail artery, and is about four times as long as that seen in the mouse vas deferens.

Figure 11.2 shows the first time differential traces of the two cells shown in Figure 11.1. There is clearly no evidence of discrete events in the guinea pig mesenteric artery. However, there is possibly some variation in the
Figure 11.1.

Typical ejps elicited by field stimulation of sympathetic nerves innervating (a) rat tail artery (upper trace) and (b) guinea-pig mesenteric artery (lower trace).

Stimulation frequency in (a) = 1Hz.

(b) = 0.5Hz.

Calibration bars: (a) 4secs; 2mV.

(b) 8secs; 2mV.
Figure 11.2.

First time differential trace of ejps recorded in cell shown in Figure 11.1 (a) - upper trace and cell shown in Figure 11.1 (b) - lower trace.

Calibration bars: 10msecs; 0.1V/s.
differential trace obtained from ejps elicited in the rat tail artery.

In all, fourteen cells in the mesenteric artery were examined in detail and none showed any evidence of significant variation in the differential trace. Sixteen cells in the rat tail artery were examined in detail and six showed possible discrete events. Figure 11.3 shows an example of one of the best of these cells.

Clearly, while differences in the differential traces exist, they are unsatisfactory for any form of quantitative analysis at present. The signal to noise ratio in both preparations is good (see Figure 11.1). Therefore the d.e.s. are small, probably, because of the slow rise time of the ejps due to the relatively large neuromuscular gap compared with that found in the rodent vas deferens.

Prejunctional Modulation

The original aim of the second half of this chapter was to use d.e.s. generated in blood vessels to look at the release of transmitter from individual varicosities from pulse to pulse in order to see if any modulation of transmitter release occurred. Because d.e.s. were not observed at all in the mesenteric artery and were not observed with sufficient clarity in the rat tail artery it was impossible to look at the release of transmitter from individual varicosities in these two preparations.

Instead, an alternative preparation, the guinea pig mesenteric arteriole was used. It has been suggested that the neuromuscular gap in this preparation might be expected to be less than that in the other two preparations because of its smaller size (e.g. Suzuki, 1983). However, this advantage has to be balanced by the fact that the preparation is technically much more difficult to use than the previous two.
Figure 11.3.

Superimposed first time differential traces of ejps recorded in a single cell of the rat tail artery.

Calibration bars: 10secs; 0.2V/sec.
Typical ejps from this preparation are seen in Figure 11.4. Unfortunately, the signal to noise ratio is not nearly so good as what was obtained with the other two preparations, mainly because the ejps are a lot smaller, and the differential trace revealed no discrete events above the level of the recording noise.

Hirst (1977) has shown that in the mesenteric arteriole, ejps vary in amplitude from pulse to pulse, even at a stimulus intensity which is above threshold for a particular nerve fibre. A similar effect can be seen with the ejps in Figure 11.4. This variation is assumed to reflect variation in the quantal release from the varicosities close to the recording cell from pulse to pulse (Hirst, 1977).

Individual variation can be observed in this preparation and not others, presumably because arterioles are much smaller than other blood vessels and thus each cell is affected by fewer release sites. That is to say, firstly, there will be less varicosities surrounding each cell and secondly, each cell in the arteriole is coupled to less cells than an equivalent artery cell. Therefore, less potential changes will occur in the recording cell due to transmitter action on surrounding cells.

If prejunctional modulation of transmitter release occurred from pulse to pulse one might expect a particular pattern of ejp size to emerge such that a large ejp would more often than not be followed by a small ejp and vice versa. Survivorship analysis (see Chapter 9) provides an easy method for testing such a pattern.

Table 11.1 shows the effect of survivorship analysis on the cell in Figure 11.4 and three others analysed in the same way. The ejps in each cell have been divided into two groups;
Figure 11.4.
Ejps elicited by field stimulation of the sympathetic nerves innervating a single guinea pig mesenteric arteriole cell. Stimulation frequency = 2Hz.
Calibration bars: 1sec; 2mV.
Table 11.1

Pulse to pulse occurrence of 'large' ejps in mesenteric arteriole cells

<table>
<thead>
<tr>
<th>Ncells</th>
<th>Mean $P_L$</th>
<th>$P_L(n+1)$</th>
<th>$P_L(n+2)$</th>
<th>$P_L(n+3)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.282</td>
<td>0.363</td>
<td>0.429</td>
<td>0.375</td>
</tr>
</tbody>
</table>

$P_L$ = the mean probability of a large ejp occurring following a stimulus.

$P_L(n+1)$ = observation of a large ejp occurring 1 pulse after a previous large ejp.

$P_L(n+2)$, $P_L(n+3)$ = as above, 2 and 3 pulses after a large ejp.
large and small; and the pattern of large and small releases examined.

The results obtained suggest that if a large ejp occurs it is more likely to be followed by another large ejp than a small one. Thus, no evidence for inhibitory modulation of release from pulse to pulse has been obtained and it seems instead that short term facilitation of release occurs from pulse to pulse.

Discussion

A number of different studies looking at the electrical response to nerve stimulation in different blood vessels have recently been published.

In most blood vessels electrical stimulation of the nerves elicits eJPs (e.g. Hirst, 1977; Surprenant, 1980; Kuriyama & Makita, 1983; Makita, 1983). In others, however, only a slow depolarisation is observed following trains of pulses (Makita, 1983; Suzuki, 1983). In some blood vessels that produce eJPs, it has been noted that increasing the stimulus intensity to high levels can produce a slow depolarisation as well (Cheung, 1982b; Suzuki & Kou, 1983; Suzuki, 1984).

The slow depolarisation can be mimicked by addition of exogenous noradrenaline and the slow depolarisation and the depolarisation provided by exogenous noradrenaline can both be blocked by \( \alpha \)-antagonists such as phentolamine (Suzuki, 1983). The slow depolarisation appears, therefore, to be due to noradrenaline released from nerves on nerve stimulation. EJPs in many vascular smooth muscles are resistant to \( \alpha \)-adrenoceptor antagonists (e.g. Holman & Surprenant, 1980; Hirst, Neild & Silverberg, 1982; Kuriyama & Makita, 1983). Currently two alternative hypotheses exist to explain such an observation.
Either noradrenaline elicits ejps by acting on a different type of receptor (Y) (Hirst & Neild, 1981) or the transmitter responsible for the ejp is not noradrenaline and is, instead, a co-transmitter - possibly ATP (Sneddon & Westfall, 1984).

Suzuki (1983) has suggested that the morphology of the blood vessel may be important. When there is wide separation between nerve and muscle (e.g. main pulmonary artery and certain veins) phentolamine sensitive ejps occur. Conversely, relatively close contact between nerve and muscle (e.g. small arteries and arterioles) results in phentolamine insensitive ejps.

Whichever of the two alternatives above is responsible for ejps, they still can be regarded as an accurate assay of transmitter release in sympathetic nerves.

Unfortunately, discrete events have proven difficult to record in blood vessels with any clarity. This is, perhaps, not surprising because of the relatively wide separation between nerve and muscle in blood vessels. They have been shown to exist, however, and it is possible that technical improvements which increase the signal/noise ratio even further may result in d.e.s. which are sufficiently distinctive for accurate quantitative analysis. The observation that they do exist encourages speculation that many results obtained in the vas deferens about transmitter release from individual varicosities may be transferable to blood vessels, at least those with relatively close neuromuscular gaps.

**Prejunctional modulation in blood vessels**

As in other smooth muscles (see Chapters 1 and 9) doubts have been raised as to the physiological relevance of prejunctional modulation of transmitter release via adreno-receptors in blood vessels (Holman & Surprenant, 1980; Kuriyama & Makita, 1983, 1984; Kajiwara, Kitamura & Kuriyama,
The observation that prejunctional α-adrenoreceptor antagonists can affect transmitter release by a single pulse (Holman & Surprenant, 1980; Kuriyama & Makita, 1983) has led to suggestions that α-adrenoreceptor antagonists such as yohimbine may, at least in part, owe their action to an active effect on the prejunctional nerve terminal rather than purely passive inhibition of α-adrenoreceptors (Kajiwara, Kitamura & Kuriyama, 1981).

In this study evidence for prejunctional modulation has been looked for in the absence of drugs by looking at the patterns of transmitter release from a few varicosities from pulse to pulse.

No evidence for any pulse to pulse inhibition of transmitter release was found. Indeed, short term facilitation of release appeared to occur instead.

This is a similar picture to what was found in the mouse vas deferens (see Chapter 9) and implies that if modulation of release occurs, either via α-adrenoreceptors or by any other mechanism such as release of prostaglandins by nerve stimulation (Kuriyama & Makita, 1984), it must occur in a more integrated manner over a number of pulses in a train and not from pulse to pulse.
CHAPTER 12

CONSOLIDATION
CHAPTER 12

This chapter provides a consolidation of the major results obtained in this study. It also outlines some ideas as to how this area of research might develop in the future in order to provide a greater understanding of the mechanisms of sympathetic transmitter release.

1. Patterns of packeted sympathetic transmitter release

The experiments in the first half of this thesis (Chapters 3-7) were carried out with the primary aim of designing a model of the patterns of packeted transmitter release from functional varicosities surrounding a particular smooth muscle cell. The results obtained were fully considered in Chapter 8. At the end of Chapter 8, the following conclusion was reached:

A comparison of the results obtained in this study with studies of patterns of transmitter at other junctions leads to the suggestion that release may be monoquantal and intermittent from a particular varicosity. At present, however, available evidence at the sympathetic junction restricts the conclusion to saying that release from a particular release site is both packeted and intermittent; where a particular release site may be an individual varicosity releasing transmitter multiquantally, a group of varicosities on a single nerve fibre each releasing monoquantally or a combination of both.

In Chapter 10 the effects of blockade of neuronal uptake of released transmitter by DMI were examined. The results were considered fully, in terms of drug action, in the discussion of that chapter. However, they also provided
confirmation of some of the deductions about families of d.e.s. made in Chapter 8. DMI (10^(-5)M) was found to disperse the latencies of discrete events, while the latency of events in a single family of d.e.s. was found to remain the same. Bennett & Middleton (1975) had previously suggested that such a high concentration of DMI would exacerbate the difference in conduction velocities of different nerve fibres. Thus, these results support the idea that families of d.e.s. represent release of transmitter from one or a few sites on a single nerve fibre.

The nature of release from a single varicosity will only be finally resolved once it becomes technically possible to record transmitter release, ideally from one varicosity, but alternatively from a known number of varicosities. This could be done using an extracellular electrode which would record the mean quantal content of the varicosity. Ideally these figures would then be correlated with families of d.e.s. recorded in the smooth muscle cell which was innervated by the varicosity. This type of experiment is similar to previous experiments which confirmed the packeted nature of transmitter release at the skeletal neuromuscular junction (Katz & Miledi, 1965a).

2. Prejunctional modulation of transmitter release

Modulation of transmitter release from individual release sites of the sympathetic nerves innervating the mouse vas deferens was investigated in Chapter 9. The results obtained provided no evidence for any physiological system of α-autoinhibition. They also provided no evidence for any endogenous negative feedback (regardless of the nature of the modulator) on release from pulse to pulse from a single site. Instead, the results showed that a process of short term
facilitation of release from a single site exists. A similar
feature was observed in Chapter 11 for pulse to pulse release
of transmitter from sympathetic nerves innervating mesenteric
arterioles.

That short term facilitation occurs is encouraging, in
the sense that it shows that transmitter release undergoes some
form of modulation. The lack of evidence of any negative
feedback control implies, however, that inhibitory modulation,
if it exists, is a more complex phenomenon than previously
imagined. It may involve some form of integral control of a
number of release sites or alternatively be a mechanism which
operates only during abnormally increased transmitter release
such as during facilitation (see Blakeley, et al., 1984).
Furthermore, interactions between any of the potential
physiological modulators considered in Chapter 1 may also
occur.

Future studies
A number of possible experiments could be carried out in
an attempt to understand more fully the mechanisms of
prejunctional modulation.
(a) Consideration of the release of transmitter from
individual release sites in response to trains of stimuli when
the effects of each of the possible modulators and combinations
of these modulators are specifically antagonised pharmacologically
might provide much information about modulation. Interpretation
of the results of such experiments would be complicated, however,
by the possibility that other factors may modulate transmitter
release which are not yet documented.
(b) An investigation of transmitter release patterns
from individual release sites following precise patterns of
stimulation may provide information as to the nature of a possible integral control of release over a number of stimuli.

(c) An examination of the ionic changes that occur in a particular varicosity both in terms of intracellular ion concentrations and also what ion channel activity exists during nerve stimulation, though technically impossible at present may become possible in the future. Such information would provide much information as to the ionic requirements which determine whether transmitter release occurs from a particular varicosity. The results obtained could then be compared with the effects that physiological and pharmacological modulators of transmitter release have on ion channel activity and intracellular ion concentrations to see which would be most likely to facilitate or inhibit transmitter release.

(d) Finally, it is probable that transmitter release from different sympathetic nerves does not occur with any degree of synchronicity in most sympathetically innervated tissues (see Neild, 1983). More emphasis, therefore, should perhaps be placed on studying the mechanisms controlling transmitter release under more physiological release conditions.

3. Discrete events in sympathetic nerves innervating blood vessels

The preliminary study outlined in Chapter 11 showed that discrete events can be observed in blood vessels. The rising phase of ejps recorded in blood vessels is, however, slower than what is observed in the rodent vas deferens. This makes d.e.s. difficult to observe with any degree of clarity.

Future experiments using discrete events in blood vessels require either the discovery of a blood vessel with closer neuromuscular 'contacts' than those found in the guinea-pig
mesenteric arteriole (see Chapter 11) or, alternatively, technical improvements which would allow a better signal to noise ratio to be obtained in records of ejps obtained in the arteriole preparations. If either of these alternatives succeed in producing clear d.e.s. capable of being analysed, it would, perhaps, be more clinically relevant, in the future, to investigate the mechanisms of transmitter release from sympathetic nerves innervating blood vessels rather than those innervating the rodent vas deferens.
APPENDIX

The mathematical model used in this thesis was developed in collaboration with A. G. Corbett and M. K. Elassadi of the Department of Engineering, University of Leicester. The full mathematical derivations used in developing the computer program can be found in a project writeup by M. K. Elassadi (1982) entitled, "Numerical solution of the transient response of a branched ladder network".

A simplified algorithm of how the model works is given in Chapter 4 of this thesis.

The transient conductance change applied to the model (Figure 4.6) is described by the equation $G(t) = G_0 e^{-\alpha t}$ and has the following form:

The computer program was written in BASIC and one version of it is listed overleaf.
DIM DA(5,500)
30 DIM V(200),I(200),F(100),P(100),S(100)
40 INPUT "INTER CELLULAR RESISTANCE";R1
41 INPUT "INTER CELLULAR CAPACITANCE";C1
42 INPUT "MEMBRANE RESISTANCE";R2
43 INPUT "MEMBRANE CAPACITANCE";C2
45 INPUT "NO COUPLINGS";X
46 RS=0.00E-6
47 INPUT "ITERATION INTERVAL";T
48 INPUT "NO ITERATIONS";M
50 C4=0
60 N=5
64 FOR W=0 TO 5 STEP 1
65 G5 = 100000
66 G6=G5;G7=G5;W
67 IM=-2.3333333E-11
70 FOR W=0 TO N
70 V(W)=-0.07
101 C4=C4+1
102 FOR C5=0 TO 5
103 DA(C5,W)=0
106 NEXT C5
130 FOR F1=0 TO (N-1)
140 I(F1)=0
150 NEXT F1
230 A=1/C1*1/C2
270 E=A^(X/C2)
280 G9=0
285 G=0
290 FOR J=1 TO M
294 G9=(G5) »J/5»T»EXP(-20':>:0*J/5»T)
295 IF G9 = 0 THEN G9=1OE-10
296 R=1/G9
297 R1=1/C1
298 R2=1/C2
300 R=R1/R2
310 B=1/((C1*R1)+(1/R2))
320 FOR Gl=(N-2) TO 0 STEP -1
325 L6=R*B*V(Gl)+R1*V(Gl)
330 V(Gl)=V(Gl)+T»(A*V(Gl)- (1/C2) * (1/(G1+1)) +L6)
340 NEXT Gl
350 IF J<21 THEN GOTO 355
354 G=(G7) * (J-20) »T*EXP(-20':>:0*J-20) »T)
355 IF G=0 THEN G=1OE-10
360 R=1/G9
365 F=1/R2-(1/R)- (C2/(C1*R1))
370 FOR U1=N TO 1 STEF -1
380 U1=(C2/X)/U1
390 NEXT U1
4,.0 FOR H1=N TO 2 STEP -1
410 F1(H1)=1/X
420 NEXT H1
430 FOR Z1=(N-1) TO 1 STEP -1
440 F1(Z1)=V(Z1)-V(Z1+1)
460 NEXT Z1
470 S0=(F1(0)+B*C2*V(1))/X
480 FOR D1=(N-1) TO 1 STEP -1
490 F1(0)+F1(D1)*F1(D1) +F1(D1)+B*C2*V(D1)
500 S1=S1+V(D1)-S1(X-1)+S1(X-1)
510 NEXT D1
520 FOR D=(10)+500 TO (10)
530 S1=(F1(0)+B*C2*V(1))/X
570 FOR D2=(N-1)
580 S0=(C2(X-1)-C1(X-1)-B*C2*V(D2)-V(D2))
590 NEXT D
600 FOR C=0 TO 5:DA(C,J)=V(C):NEXT C
605 PRINT J
610 NEXT J
612 IF C=1 THEN GOTO 700
670 GRAPH:0;GRAPH1
675 CALL "RESOLUTION",0.2
700 FOR C3=0 TO 0
710 CALL "PLOT",0.0,2
720 FOR C=1 TO M
720 CALL "LINE",C1,1,0.0,0.0+DA(C1,C1);C1+1,1,0.0
740 NEXT C
750 NEXT C1
755 GOTO 780
760 GRAPH1;GRAPH1
770 CALL "RESOLUTION",0.2
780 FOR C3=0 TO 0
790 CALL "PLOT",0.0,0.2
900 FOR C=1 TO M
810 CALL "LINE",C1,1,0.0,0.0+DA(C1,C1);C1+1,1,0.0
920 NEXT C
930 NEXT C1
950 NEXT W
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Stimulation of the sympathetic nerves innervating the rodent vas deferens elicits excitatory junction potentials (ejps). Intermittent transient accelerations in the rising phase of these ejps, termed discrete events (d.e.s.), were observed. D.e.s. were shown to reflect packets of transmitter acting on the recording cell. They vary in latency, amplitude and time course.

D.e.s. with similar latency and time course have been associated into 'families'. Families of d.e.s., generally, have multimodal amplitude distributions with clearly defined modes. Families were shown to reflect transmitter release from a single terminal nerve fibre. Variation in [Ca]₀ altered the relative proportion of occurrence of large and small d.e.s., in a family, without affecting the modes themselves. Facilitation had the same effect. Therefore, families of d.e.s. represent the packeted, intermittent, release of transmitter from a single release site which may be either one varicosity or a group of varicosities on a single nerve fibre.

Spontaneous d.e.s. have also been observed. In most cells the amplitude distribution of spontaneous d.e.s. was unimodal and this mode was equal to the smallest evoked d.e. mode in the same cell.

Transmitter release from individual release sites could be modulated by α-adrenoreceptor agonists and antagonists. However, the effect of such drugs was not consistent with the presence of an endogenous system of modulation via prejunctional α-receptors. No evidence could be found for endogenous inhibitory modulation of release from pulse to pulse. Rather, short term facilitation appeared to occur.

Blockade of neuronal uptake of noradrenaline by desmethylimipramine had no effect on d.e.s. Higher concentrations of desmethylimipramine altered the time course of ejps and d.e.s. Finally d.e.s. were recorded in the rat tail artery. This may provide a more clinically relevant preparation for future studies.