Local application of drugs to sympathetic nerve terminals: an electrophysiological analysis of the role of prejunctional α -adrenoceptors in the guinea-pig vas deferens

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1 Focal extracellular recording techniques were used to study the effects of clonidine, yohimbine and tyramine on the intermittent transmitter release mechanism in the guinea-pig vas deferens *in vitro*. Drugs were applied locally to the varicosities located within the recording electrode. Statistical methods were used to determine whether noradrenaline (NA) acts locally to inhibit secretion from the same or a closely related release site (local regulation) on an impulse-to-impulse basis.

2 The α -adrenoceptor agonist, clonidine, inhibited transmitter release, an effect reversed by the α -adrenoceptor antagonist, yohimbine. Yohimbine alone increased action potential-evoked transmitter release, findings consistent with the idea that transmitter release is regulated through prejunctional α -adrenoceptors.

3 The indirectly acting sympathomimetic, tyramine, powerfully inhibited evoked transmitter release, an effect reversed by both yohimbine and phentolamine. The inhibitory effects of tyramine were greatly reduced in tissues taken from animals pretreated with reserpine. Clonidine powerfully inhibited transmitter release in reserpinized tissues showing that prejunctional α -adrenoceptors were functionally intact. The inhibitory effects of tyramine on transmitter release are therefore mediated indirectly through the release of endogenous NA.

4 Paradoxically, when transmitter release from a small population of varicosities on a single nerve fibre was studied in the absence of α -adrenoceptor antagonists, no evidence was found for local regulation of transmitter release.

5 The intermittent character of the transmitter release process makes it difficult to envisage how impulse-to-impulse regulation could occur. Furthermore, it is unlikely that NA will accumulate to any appreciable extent in the vicinity of the secreting varicosity.

6 The pharmacological evidence clearly supports the view that NA released from sympathetic nerve terminals by nerve impulses modulates subsequent transmitter release. However, the evidence does not support the view that released NA acts locally to inhibit secretion from recently activated varicosities.

Introduction

The idea that transmitter release from postganglionic sympathetic nerve terminals can be modulated through prejunctional α -adrenoceptors is widely accepted (see Starke, 1987) but the exact mechanism of inhibition remains to be established. Studies of the effects of α -adrenoceptor agonists and antagonists on transmitter release from electrically field stimulated tissues *in vitro* provides good evidence for the existence but little information on the anatomical location of prejunctional α -adrenoceptors. In the majority of studies, nerves are field-stimulated 'supramaximally' so that synchronous activation of many release sites occurs. This may well be an artificial situation which tends to exaggerate the importance of α -autoinhibition in regulating transmitter release.

It is usually assumed that transmitter released from one site, presumed to be the varicosity, acts locally to inhibit subsequent release from the same or a closely related site. However, electrophysiological studies at the sympathetic neuroeffector junction have shown that transmitter release from individual varicosities is highly intermittent and monoquantal (Blakeley & Cunnane, 1979; Hirst & Neild, 1980; Cunnane & Stjärne, 1984; Brock & Cunnane, 1987; 1988; Åstrand & Stjärne, 1988), findings which make it highly unlikely that transmitter will accumulate in the vicinity of its site of release. Furthermore, if release from a particular varicosity occurs only once every hundred stimuli, it is difficult to envisage how local regulation of the releasing varicosity could occur.

Studies of α -autoinhibition are further complicated by the recent finding that several transmitters are secreted from sympathetic nerve terminals. Indeed, there is good reason to believe that the transmitter responsible for the excitatory junction

potential (e.j.p.) and therefore the excitatory junction current (e.j.c.) in the rodent vas deferens is adenosine 5'-triphosphate (ATP) rather than NA. However, previous studies have shown that the e.j.p. is modulated by drugs in a manner consistent with the α -autoregulation hypothesis (Blakeley *et al.*, 1981; Illes & Starke, 1983; Brock *et al.*, 1990).

Recently a focal extracellular recording technique has been developed which can monitor transmitter release from a small population of varicosities located on a single sympathetic axon on an impulse-to-impulse basis (Brock & Cunnane, 1987; 1988). This approach has been used to study the effects on transmitter release of the local application of the α -adrenoceptor agonist, clonidine, the α -adrenoceptor antagonist, yohimbine and the indirectly acting sympathomimetic amine, tyramine. Statistical techniques were also used to determine whether local regulation of transmitter release occurs when α -adrenoceptor antagonists are not present in the physiological saline.

Methods

Extracellular recording

Male guinea-pigs (Duncan Hartley, 300–400 g) were killed by a blow to the head and bled. Vasa deferentia were removed and individual preparations pinned to the Sylgard (Dow Corning) covered base of a 4 ml Perspex organ bath, which was mounted on the stage of a Zeiss ACM microscope. The organ bath was perfused continuously at 1–3 ml min⁻¹ with Krebs solution of the following composition (mM): NaCl 118.4, NaHCO₃ 25.0, NaH₂PO₄ 1.13, CaCl₂ 1.8, KCl 4.7, MgCl₂ 1.3 and glucose 11.0. The solution was gassed with a

mixture of 95% O₂ and 5% CO₂ (to pH 7.4) and maintained at 36–37°C. Postganglionic sympathetic nerve fibres were excited by electrical stimulation of the hypogastric nerve trunk close to the prostatic end of the vas deferens. The stimulus parameters were varied as required throughout the experiment (pulse width 0.01–0.5 ms, 7–30 V).

A window was cut in the connective tissue sheath enveloping the vas deferens, and a beveled glass electrode (tip diameter ~50 µm) filled with Krebs solution applied to the muscle surface with slight suction (~20–30 mmHg, seal resistance <1 MΩ). A sintered Ag/AgCl pellet in the organ bath served as the indifferent electrode. Electrical activity was recorded through an a.c. amplifier (Neurolog NL104, low frequency cut-off 0.1 Hz) and the output fed through a low pass filter (Neurolog NL125) with the cut-off set at 3 kHz. Drugs were applied by internal perfusion of the suction electrode (25–50 µl min⁻¹).

Reserpine pretreatment

Guinea-pigs received a single dose of 5 mg kg⁻¹ reserpine (i.p.) 18 h before the experiment, a procedure which depleted the NA content of the vas deferens below levels detectable by high performance liquid chromatography.

Data collection and analysis

A system based on a Zenith 386 microcomputer and a Data Translation DT2801A analogue to digital converter card was used to digitize and analyse electrophysiological signals previously recorded on magnetic tape (see Brock & Cunnane, 1988). The signal detection level was set at about twice the noise level of the recording system which was usually about 10 µV peak-to-peak.

The stochastic features of electrically evoked transmitter release were analysed by the correlogram method (see Rotshenker & Rahamimoff, 1970). A correlogram gives an estimate of the probability of occurrence of an e.j.c. at any time after a given e.j.c. A correlogram is constructed by calculating the number of e.j.c.s (f_x) occurring 1, 2, 3, etc. pulses following each e.j.c. recorded during a train of stimuli. The expected number of e.j.c.s in each interval class (f) was determined by averaging the number of e.j.c.s (f_x) occurring 16 to 25 pulses (1 Hz) or 91 to 100 pulses (4 Hz) following each e.j.c., during which period it was assumed that e.j.c.s occurred independently. The different periods of sampling to determine f_x were chosen to ensure that comparative periods of time after the occurrence of an e.j.c. were analysed. The probability of e.j.c.s occurring 1, 2, 3 etc. pulses following an e.j.c. relative to that expected if e.j.c.s occurred independently of one another was determined by dividing f_x by f . If e.j.c.s occur independently (i.e. randomly) then the relative probability of occurrence is one.

Drugs

Clonidine hydrochloride (Sigma), phentolamine mesylate (Ciba), reserpine (Sigma), tyramine hydrochloride (Sigma) and yohimbine hydrochloride (Sigma). Reserpine was made up in 20% (w/v) ascorbic acid. The remaining drugs were made up as concentrated stock solutions in distilled water and serially diluted in Krebs solution to the required final concentration.

Results

Extracellular recording

A detailed description of the pattern of electrical activity recorded by use of the focal extracellular recording method has been described elsewhere (Brock & Cunnane, 1988). For the purpose of the present inquiry it is useful to state briefly the key features of the transmitter release mechanism. When a small suction electrode is applied to the surface of the vas

deferens, in the absence of stimulation, spontaneous excitatory junction currents (s.e.j.cs) are recorded. Following electrical field stimulation of the hypogastric nerve trunk with trains of pulses at 0.1–4 Hz, stimulus-locked excitatory junction currents (e.j.c.) are evoked intermittently, the amplitude and time course of which are similar to those of spontaneous excitatory junction currents (s.e.j.cs). Evoked events are always preceded by a non-intermittent nerve impulse. For a number of reasons we believe that the nerve impulse preceding the e.j.c. is the extracellular equivalent of the nerve terminal action potential (Brock & Cunnane, 1988). Intermittence therefore results from a low probability of transmitter release in the invaded varicosity. Examples of nerve impulses and e.j.cs are shown in Figure 1. Negative-going e.j.cs reflect the quantal release of transmitter from varicosities located under the recording electrode. Positive-going activity reflects the depolarization of smooth muscle cells outside the recording electrode (see Brock & Cunnane, 1988).

Local application of clonidine and yohimbine

Clonidine (0.1 µM) applied by internal perfusion of the focal recording electrode (6 experiments) markedly reduced the number of e.j.cs recorded within 5–10 min of application (Figure 2a,b). Inhibition of transmitter release by clonidine occurred without any apparent alteration in the nerve terminal impulse (Figure 1a) and was readily reversed by wash. Clonidine did not significantly alter the size or the frequency of occurrence of s.e.j.cs. Yohimbine (0.1–1 µM) applied by the

Figure 1 Effects of local application of clonidine on evoked electrical activity in the guinea-pig vas deferens. (a) Nerve impulses and excitatory junction currents (e.j.cs) evoked by trains of 100 stimuli at 1 Hz before and 10 min after the addition of clonidine (0.1 µM). Clonidine inhibited the occurrence of e.j.cs without altering the nerve terminal impulse (NTI). (b) Histogram showing the number of e.j.cs recorded during trains of 100 pulses at 1 Hz delivered at 5 min intervals before, and during the addition of clonidine (0.1 µM) and yohimbine (0.1–1 µM). The control value is the mean number of e.j.cs recorded during 5 periods of stimulation (vertical bar shows s.e.mean). The inhibitory effect of clonidine was reversed by yohimbine.

Figure 2 Inhibition of transmitter release by locally applied tyramine in the guinea-pig vas deferens. (a) Nerve impulses and excitatory junction currents (e.j.cs) evoked by trains of 50 stimuli at 2 Hz before and 10 min after the addition of tyramine (100 μ M). Tyramine inhibited transmitter release without altering the nerve terminal impulse. (b) Histogram showing the number of e.j.cs recorded during trains of 100 pulses at 2 Hz delivered at 5 min intervals before, and during the addition of tyramine (100 μ M) and phentolamine (1 μ M). The control value is the mean number of e.j.cs recorded during 5 periods of stimulation (vertical bar shows s.e.mean). The inhibitory effect of tyramine was reversed by phentolamine.

same route (4 experiments) reversed the inhibitory effects of clonidine (Figure 1b). The effects of yohimbine alone, when applied by internal perfusion of the suction electrode, were also examined. At a concentration of 0.1 μ M, which previously had been shown to antagonize the effects of clonidine (Figure 1b), yohimbine failed to cause a consistent increase in the size or number of e.j.cs (7 experiments). We have subsequently found, however, that at higher concentrations yohimbine increases the frequency of occurrence of e.j.cs during trains of stimuli, and this effect is enhanced by desipramine which prevents the released NA from being taken up into sympathetic nerve terminals (Brock *et al.*, 1990).

Effects of locally applied tyramine

It was interesting to determine whether endogenous NA released locally by the indirectly acting sympathomimetic amine, tyramine, could activate prejunctional α -adrenoceptors and thus inhibit quantal transmitter release. In 8 experiments tyramine (10–100 μ M) caused a marked inhibition of release without altering the nerve terminal impulse (Figure 2a,b). The inhibitory effect of tyramine was reversed by introducing yohimbine (1 μ M) or phentolamine (1 μ M) into the recording suction electrode, suggesting that the inhibition was mediated through prejunctional α -adrenoceptors. It is possible that tyramine was directly stimulating prejunctional α -adrenoceptors at the concentrations used (10–100 μ M). However, in reserpinized tissues (4 experiments) the inhibitory actions of tyramine (100 μ M) on transmitter release were absent or markedly reduced, whereas the local application of

Figure 3 Lack of effect of tyramine (100 μ M) on electrically evoked transmitter release in reserpinized guinea-pig vas deferens (5 mg kg⁻¹ i.p., 18 h earlier). The histogram shows the number of excitatory junction currents (e.j.cs) recorded during trains of 100 pulses at 1 Hz delivered at 5 min intervals before, and during the addition of both tyramine and clonidine. The control value is the mean number of e.j.cs recorded during 4 periods of stimulation (vertical bar shows s.e.mean). Note that although tyramine was without effect in the reserpinized tissue, clonidine (0.3 μ M) powerfully inhibited transmitter release.

clonidine (0.1–0.3 μ M) still markedly inhibited transmitter release (Figure 3). These observations demonstrate that tyramine inhibits electrically evoked transmitter release by releasing endogenous NA from sympathetic nerve terminals.

Does transmitter release inhibit subsequent evoked release?

Previous studies have clearly shown that exogenously applied drugs can modify the transmitter release process but have failed to demonstrate that the endogenous transmitter can regulate its own release locally in the absence of prejunctional α -adrenoceptor antagonists (Blakeley *et al.*, 1982). Using the focal extracellular recording technique, we have tried to establish whether the occurrence of an e.j.c. alters the probability of observing subsequent e.j.cs by analysing the stochastic features of the transmitter release process (see Methods). Correlograms were constructed for trains of 500 pulses delivered at various frequencies (0.5–4 Hz) in five separate experiments. In no case was there any evidence for a decreased probability of occurrence of evoked transmitter release immediately following an e.j.c. Indeed, the occurrence of an e.j.c. had no obvious effect on the probability of observing subsequent e.j.cs, suggesting that transmitter release events occur independently of one another. Examples of two correlograms constructed from data obtained in a single attachment are shown in Figure 4. It is clear that the relative probability that an e.j.c. would occur in the next 20 pulses following an e.j.c., during trains of 500 stimuli delivered at 1 and 4 Hz, is unchanged. If α -autoinhibition were operating locally to any appreciable extent, then one would expect previous transmitter release to inhibit subsequent release in the field of the recording electrode.

Discussion

The results of the present inquiry have a bearing on the mechanisms involved in α -autoinhibition. While the pharmacological evidence clearly supports the hypothesis that trans-

Figure 4 Correlograms of excitatory junction current (e.j.c.) interval data for trains of 500 stimuli at 1 and 4 Hz in a single attachment in the guinea-pig vas deferens. The continuous line is the curve predicted if e.j.cs occur independently and the broken lines represent the 95% confidence limits of this prediction. The occurrence of an e.j.c. does not inhibit the occurrence of subsequent e.j.cs on an impulse-to-impulse basis.

mitter release is regulated by the activation of prejunctional α -adrenoceptors, surprisingly little is known about the precise mechanisms involved in the regulation of release at the level of the individual varicosity. To clarify further these mechanisms the secretory activity of a small group of varicosities located on a single sympathetic axon has been studied, in the absence and presence of α -adrenoceptor agonists and antagonists.

The effects of clonidine and yohimbine on the e.j.c. are similar to those previously reported both for NA release and for the e.j.p. (see Starke, 1987). Clonidine powerfully inhibited electrically evoked transmitter release, an effect reversed by yohimbine. Moreover, yohimbine alone potentiated transmitter release (Brock *et al.*, 1990). These findings are in accord with the hypothesis that NA inhibits transmitter release by activating prejunctional α -adrenoceptors. It should be stressed that α -autoinhibition is not the major cause of intermittent transmitter release, since α -adrenoceptor antagonists only increase release by about one to two fold, i.e. on average from one, to two or three secretory events per hundred stimuli per varicosity (Brock *et al.*, 1990).

The e.j.p. (and therefore the e.j.c.) in many sympathetically innervated tissues including the guinea-pig vas deferens is probably mediated by ATP or a related purine nucleotide

rather than by NA (see White, 1988). E.j.ps can be recorded in tissues taken from animals pretreated with reserpine to deplete NA stores (Burnstock & Holman, 1961; Sneddon & Westfall, 1984) and when NA synthesis was also inhibited with α -methyl *p*-tyrosine (Cunnane & Manchanda, 1989). Much evidence suggests that NA and ATP are co-released from the same sympathetic nerve terminal and therefore it is reasonable to assume that the e.j.c. is an indirect measure of the quantal release of NA. It should be noted that Ellis & Burnstock (1989) have recently argued on the basis of overflow studies that NA and ATP may be stored and/or released separately. Nevertheless, the present pharmacological observations support the view that ATP release is modified by exogenous α_2 -adrenoceptor agonists and endogenous NA (see below). In the mouse vas deferens and in vascular smooth muscle, the e.j.p. is similarly modified by α_2 -adrenoceptor agonists and antagonists (Blakeley *et al.*, 1981; Illes & Starke, 1983; Ramme *et al.*, 1987).

In a previous investigation no evidence was found for local regulation of transmitter release in the absence of drugs when release from a small population of varicosities was studied (Blakeley *et al.*, 1982). Similarly, the occurrence of an e.j.c. had no apparent inhibitory effect on the occurrence of subsequent e.j.cs. Although not rigorously investigated, there was some evidence to suggest that pairs of e.j.cs, with a similar amplitude and time course, were evoked within a few pulses of one another during trains of stimuli even though release was intermittent (see Brock & Cunnane, 1988). Similar observations have been made for discrete events in this tissue (Cunnane & Stjärne, 1984). Discrete events and e.j.cs of similar amplitude and time course probably represent transmitter release from the same or a closely related group of transmitter release sites. Therefore, following transmitter release there appears to be, if anything, a transient increase in the probability of subsequent transmitter release from the same site rather than the expected inhibition. We have not yet investigated whether β -adrenoceptors are involved in this apparent enhancement of transmitter release. Thus when a focal extracellular electrode is used to monitor transmitter release on an impulse-to-impulse basis from a few release sites no evidence for local α -autoinhibition can be found.

Further evidence against the idea of local regulation of release is provided by the intermittent nature of the transmitter release mechanism. It is difficult to envisage how appreciable concentrations of transmitter could accumulate locally if release from a particular varicosity only occurs once or twice per 100 stimuli. It might seem surprising that a transient high concentration of transmitter at the releasing varicosity would fail to activate the local prejunctional receptors. One explanation might be that the interval between successive releases from the same varicosity is sufficiently long to allow the transmitter release mechanism to recover from any transient inhibitory effects produced by the released transmitter. Further, intermittence makes it difficult to envisage how local regulation could occur on an impulse-to-impulse basis if there are long intervals between successive secretory events. There can be no doubt however that α -adrenoceptor antagonists increase electrically evoked transmitter release during short trains of low frequency stimuli (Story *et al.*, 1981; Blakeley *et al.*, 1981; Illes & Starke, 1983; Brock *et al.*, 1990).

We are therefore left with the following observations: (1) exogenous agonists readily inhibit transmitter release locally, (2) antagonists reveal inhibition of transmitter release by endogenous NA and (3) the inhibition escapes detection when release is recorded on an impulse-to-impulse basis in the absence of drugs. It was therefore of some interest to determine whether the endogenous NA released from sympathetic nerve terminals by locally applied tyramine could activate prejunctional α -adrenoceptors and thus inhibit transmitter release. Ambache *et al.* (1972) were the first to demonstrate the inhibitory effects of tyramine on electrically evoked contractions of the guinea-pig vas deferens. In the present experiments it has clearly been demonstrated that locally applied

tyramine readily inhibits electrically evoked transmitter release, indirectly, by releasing endogenous NA. The inhibitory actions of tyramine were greatly attenuated in tissues taken from reserpinized animals but clonidine still powerfully inhibited transmitter release showing that reserpine had not interfered with prejunctional α -adrenoceptor transduction mechanisms. Interestingly, in reserpinized tissues, yohimbine did not potentiate the amplitude of the e.j.ps evoked by trains of stimuli presumably because α -autoinhibition was no longer operative (Brock *et al.*, 1990). Thus endogenous NA released from nerves by tyramine can inhibit transmitter release locally but action potential evoked NA release apparently cannot.

Some further explanation is required for the paradoxical inability of NA released by nerve impulses to inhibit transmitter release locally. First, the neurotransmitter may act at sites remote from its own site of release. It is important to remember that the question addressed by the statistical approach was – does previous release inhibit subsequent release from the same or closely related release sites? It does not address the question whether the absolute probability of release at the release sites under investigation is determined by the influence of NA spreading from neighbouring varicosities (whose activity is not detected by the recording electrode). Thus, one can account for the effects of locally applied yohimbine by assuming that it removes the lateral inhibition produced by the spread of NA from distant release sites. The idea of lateral inhibition of transmitter release fits well with the intermittent nature of the transmitter release process, since the biophase concentration of NA 'seen' by the prejunctional α -adrenoceptors will depend on the amount of NA diffusing from the sites of transmitter release and not on the concentration in the junctional cleft. Second, prejunctional α -adrenoceptors may have an anatomical location at some point remote from the sites of transmitter release, for example at some point upstream or downstream from the site of release (Stjärne, 1989). However, the local application of clonidine and the local release of NA by focally applied tyramine both powerfully inhibited transmitter release, suggesting that α -adrenoceptors are located close to the sites of transmitter release and certainly within the recording electrode ($\sim 50 \mu\text{m}$

diameter). Third, NA and ATP may not be co-released. If one abandons the assumption that NA and ATP are co-released from the same nerve terminal then these electrophysiological studies, which measure ATP release, will give no indication as to the prejunctional site(s) of action of NA. What these results clearly demonstrate is that ATP (or a related breakdown product) does not regulate its own release locally. The present and previous results do, however, clearly demonstrate that the release of ATP is regulated by endogenous NA.

A working hypothesis which encompasses most observations can be briefly stated: when a particular varicosity releases a packet of transmitter, the probability of subsequent release from that varicosity may be enhanced and this effect overrides local α -autoinhibition. The released NA spreading to varicosities not yet activated stimulates prejunctional α -adrenoceptors decreasing the probability that these varicosities will release transmitter when invaded by a nerve action potential. Thus the concept of lateral inhibition rather than local inhibition of transmitter release emerges, one function being to limit the overall magnitude of transmitter release from the entire nerve terminal network. (Sjörne, 1981; 1989; Starke, 1987). It may well be that the terminal network of the sympathetic nerve fibres has a complex three dimensional branching anatomical arrangement the function of which is not yet fully appreciated.

In conclusion, postganglionic sympathetic nerve terminals are endowed with α -adrenoceptors which limit transmitter release during short trains of stimuli. Local regulation of transmitter release by previously released NA was not detected. It would seem that α -autoinhibition operates at a point remote from the immediate site of transmitter release (lateral inhibition), presumably by reducing the probability that varicosities will release transmitter when invaded by the nerve action potential. However, the interpretation of some of these observations would have to be reviewed if it can be demonstrated that NA and ATP are released independently of one another and this is a question for future study.

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