Br. J. Pharmac. (1986), **88**, 807–814

Interactions between the effects of yohimbine, clonidine and $[Ca]_o$ on the electrical response of the mouse vas deferens

A.G.H. Blakeley, A. Mathie[1] & S.A. Petersen

Department of Physiology, University of Leicester, Leicester LE1 7RH

1 Excitatory junction potentials (e.j.ps) were recorded from mouse vas deferens and resolved into families of 'discrete events' (d.es) reflecting intermittent release of packets of transmitter from one or a few sites. Within families d.es vary in amplitude between a few preferred values unaffected by any treatments used in these experiments.

2 As $[Ca]_o$ is raised from 1.1 to 4.0 mM there is a rise in d.e. amplitude due to an increase in the frequency of large events and a decrease in that of small.

3 At all $[Ca]_o$ clonidine reduces d.e. amplitude by increasing failures and small events and decreasing large events. Yohimbine has opposite effects. Both drug effects are concentration-dependent in the range 5×10^{-9}–10^{-6}M.

4 As $[Ca]_o$ is raised from 1.1 to 4.0 mM, and therefore more natural agonist is released, clonidine becomes more effective at altering d.e. amplitude whereas yohimbine becomes less so.

5 With very low frequency stimulation yohimbine elevates e.j.p. amplitude only if $[Ca]_o$ is below 1.6 mM.

6 These results are not easily compatible with the notion that yohimbine breaks a 'negative feedback' control of transmitter release.

Introduction

The release of transmitter from sympathetic nerves can be modified by α-adrenoceptor agonists and antagonists acting prejunctionally (Langer, 1977; Starke, 1977; Westfall, 1977; Gillespie, 1980). Whether these agents act at some physiologically important site involved in a negative feedback loop controlling transmitter release has, however, been questioned (Kalsner, 1979; 1981; Holman & Surprenant, 1980; Angus & Korner, 1980; Blakeley *et al.*, 1982; see also Rand *et al.*, 1982 and Kalsner, 1982).

Transient accelerations in the rate of depolarisation during excitatory junction potentials (e.j.ps) evoked in rodent vasa deferentia have been observed and termed discrete events (d.es) (Blakeley & Cunnane, 1979a,b). These d.es provide a more precise temporal definition than e.j.ps of the underlying fast postjunctional electrical events which follow transmitter release. They occur intermittently at a few fixed latencies and at any one latency vary in amplitude between a few preferred values. D.es have been grouped into families on the basis of latency and 'time to peak' (Blakeley *et al.*, 1984b,c). It has been suggested that a family of d.es represents the secretion of transmitter from one or a small number of release sites of the sympathetic innervation close to the recording electrode (Blakeley *et al.*, 1984b).

In this study both e.j.ps and families of d.es have been used to examine the effects of prejunctional modulation of transmitter release by drugs acting on prejunctional α-adrenoceptors and to see if this modulation interacts with a system of endogenous negative feedback. To this end, the relationship between the α-adrenoceptor agonist clonidine and α-adrenoceptor antagonist yohimbine and the amount of endogenous transmitter present has been measured when the amount of endogenous transmitter in the junction has been varied by altering the extracellular calcium concentration (Rubin, 1970; Rahamimoff, 1970; Kirpekar, 1975) and by using very low frequency

[1]Present address: MRC Receptor Mechanisms Group, Department of Pharmacology, University College, Gower Street, London, WC1E 6BT

stimulation (Kalsner, 1979; Kuriyama & Makita, 1983).

Preliminary accounts of these findings have already been published (Blakeley *et al.*, 1983; 1984a).

Methods

Male C57BL/6 mice (18–25 g) were killed by cervical dislocation and the right vas deferens rapidly dissected. The tissue was mounted at approximately resting length in a 6 ml organ bath perfused at 3 ml min^{-1} with Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25.0, NaH_2PO_4 1.1, KCl 4.7, $CaCl_2$ 2.1, $MgCl_2$ 1.2, glucose 11.1, maintained at a temperature of 35–36°C. In certain experiments the $CaCl_2$ concentration was altered to 1.1, 1.6 and 4.0 mM. No osmotic compensation was made. The prostatic end of the vas was passed into a pair of platinum ring electrodes (separation 0.5 mm) connected to a Bell isolated stimulator unit.

In these preparations the membrane potentials of smooth muscle cells at or near the surface of the vas deferens more than 2 mm from the point of stimulation were recorded using glass microelectrodes (resistance 25–45 MΩ, filled with 3 M KCl). Cell penetrations were accepted only if they satisfied previously defined criteria (Blakeley & Cunnane, 1979a,b). The resting membrane potential was always more than 60 mV negative. The potentials were amplified via a Dagan 8100 single electrode voltage clamp, used in either switched or bridge mode and recorded on a Racal Store 4D tape recorder (tape speed 3.75 in s^{-1}, frequency response d.c. to 2.5 kHz).

Junction potentials were elicited by submaximal field stimulation through the ring electrodes around the prostatic end of the vas. In the experiments using discrete events the intensity of stimulation was adjusted for each cell until d.es were clearly observed. Discrete events were obvious in about 80% of cells penetrated (see also Blakeley & Cunnane, 1979a,b). The intensity was then increased by a further 50% and left unchanged during experimental observations. The intensity was, however, always restricted to levels which reliably elicited junction potentials with amplitudes no greater than 25 mV.

At the beginning of each experiment with low frequency stimulation the stimulus intensity was increased until e.j.ps with amplitudes 10–15 mV were reliably elicited. The intensity was then unchanged for all further observations on that preparation. All results obtained in the presence of yohimbine were expressed as a percentage of control values obtained in the same preparation. The frequency of stimulation was 2 Hz in the normal experiments and 0.075 Hz in the low frequency experiments.

Detection of discrete events

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 whose frequency response was severely limited (<500 Hz) in order to reduce output noise to a reasonable level (Blakeley & Cunnane, 1979a,b).

(b) After the experiments the recorded junction potentials were digitised (sampling frequency 5 kHz) using a Digitimer NL900 board attached to a Research Machines 380 Z microcomputer. The digitised signal was first smoothed by averaging successive pairs of samples and then differentiated by calculating differences between averages of two variable sized running sample windows displaced by one sample interval (see Blakeley *et al.*, 1984b).

Analysis of discrete events

Latency and 'time to peak' histograms were obtained for all the d.es occurring within a train of e.j.ps. The d.es were then grouped into 'families' according to previously defined stringent latency and time to peak criteria (Blakeley *et al.*, 1984b).

Amplitude, rise time and rise rate distributions were then obtained for the events in each family. In those cells where events were superimposed upon a slow background the background was subtracted by computer before compiling the amplitude distributions. Drugs were applied to preparations via the superfusing fluid for at least 10 min before recording from cells. In a few cases penetrations were maintained whilst the drug washed in.

Statistical methods

All statistical tests were carried out using a standard Student's t test and all data are presented as mean ± s.e.mean of at least 6 observations.

Drugs

Yohimbine hydrochloride (Sigma), and clonidine hydrochloride (Catapres, Boehringer, Ingelheim) were used.

Results

Effects of clonidine and yohimbine on the facilitated discrete events

The effects of clonidine and yohimbine on the ex-

citatory junction potential and d.es in any one family are shown in Figure 1. It can be seen that clonidine decreases and yohimbine increases the probability of occurrence and mean d.e. amplitude.

Figure 2a shows an amplitude histogram of a typical family of d.es and Figure 2b shows the effect of clonidine on it. The distribution remains multi-modal after clonidine with the modes remaining in the same place on the abcissa scale and the spacing between the modes remaining constant. Although the preferred values of d.e. amplitude are unchanged large d.es are observed less frequently and small ones more frequently.

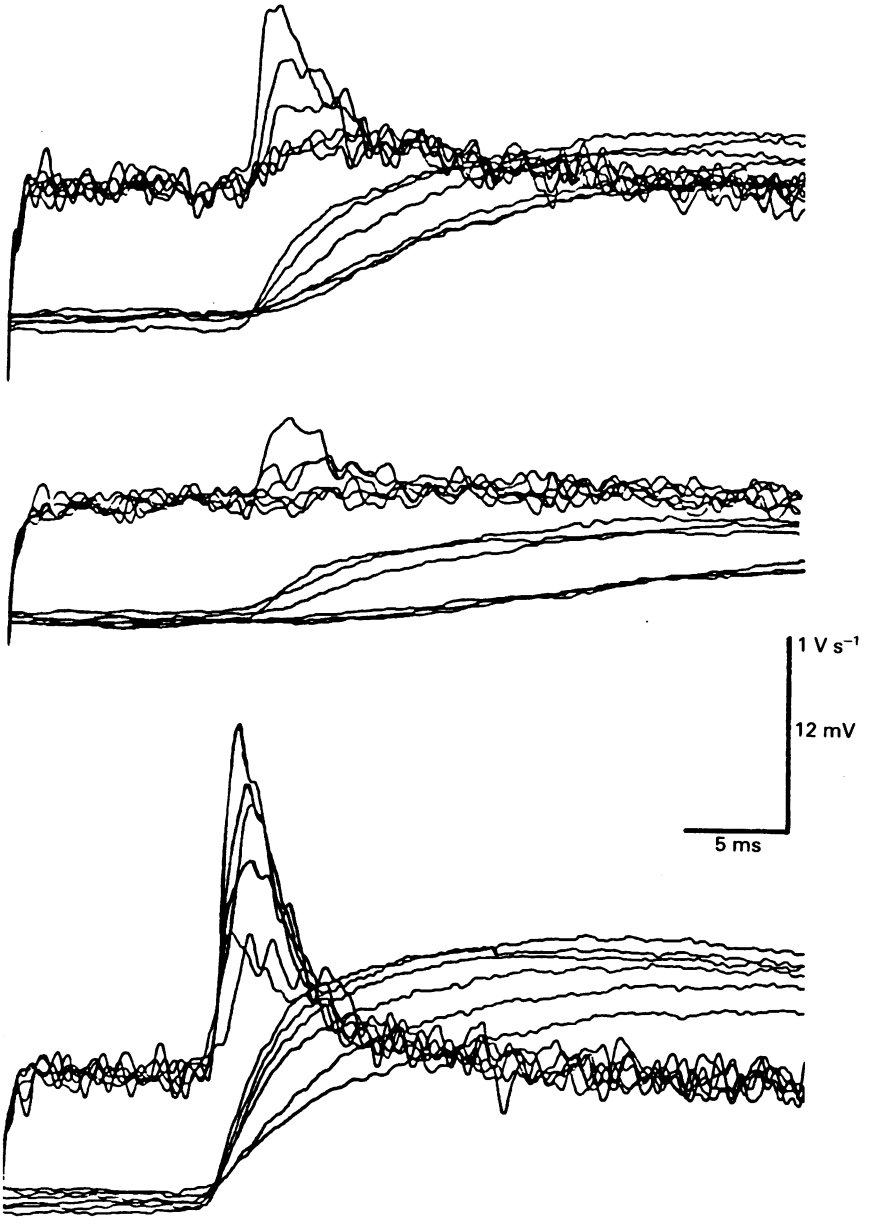

Figure 1 An illustration of the effects of clonidine (10^{-8}M) (middle traces) and yohimbine (10^{-7}M) (lower traces) on excitatory junction potentials ('quiet' trace) and discrete events ('noisy' trace) elicited in smooth muscle cells of the mouse vas deferens (control, upper traces). Typical records from different cells are shown in each case.

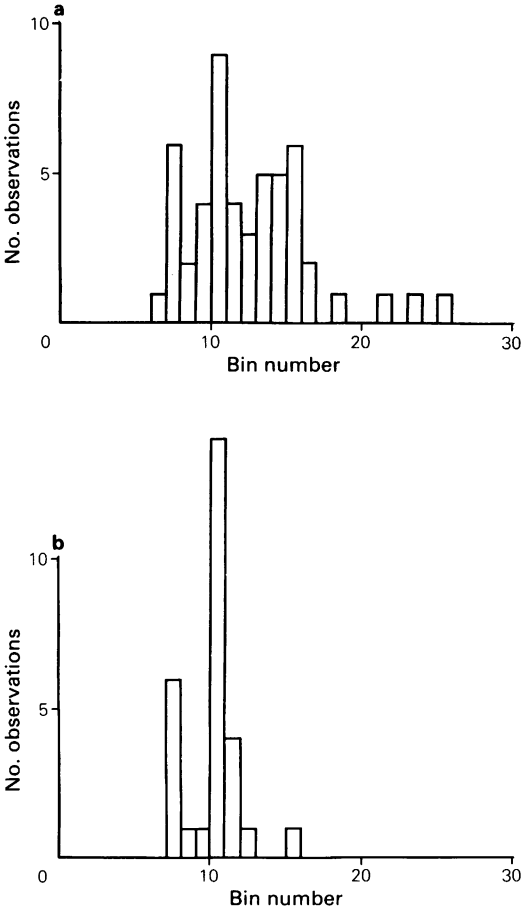

Figure 2 The effect of clonidine on the amplitude distribution of a family of discrete events in a single cell. (a) Control; proportion of failures (Pf) = 0.50. (b) Same family after addition of clonidine (10^{-8}M); Pf = 0.72. Bin size = 0.05 V s^{-1} in both (a) and (b).

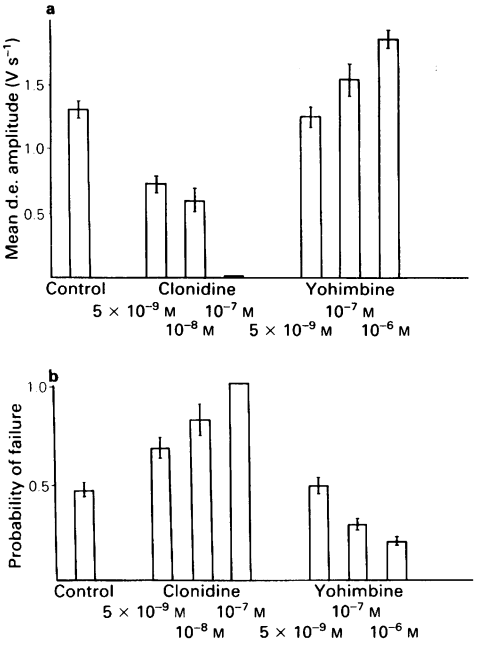

Figure 3 The concentration-dependent effects of clonidine and yohimbine on (a) mean amplitude and (b) probability of failure of discrete events (d.es) from a single family in a number of cells ($n > 6$ for each column). In both (a) and (b) all concentrations of clonidine shown had a significant effect as did 10^{-7}M and 10^{-6}M yohimbine ($P < 0.05$; Student's t test).

Similarly, yohimbine is found to alter the shape and not the modes of the amplitude distribution of a family of d.es. Figure 3a shows that the effects of clonidine and yohimbine on d.e. amplitude are concentration-dependent, and Figure 3b shows a similar concentration-dependence of the probability of occurrence. Details of these changes have been described elsewhere (Blakeley *et al.*, 1984b).

Changes in clonidine and yohimbine effects to varying [Ca]$_o$

Figures 4(a,b) and 5(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, at concentrations chosen (from Figure 3) to have a mid range effect. Figure 4a shows that as the [Ca]$_o$ is increased the mean d.e. amplitude is significantly increased in the control situation and in the presence of yohimbine, but remains unchanged in the presence of clonidine. Figure 4b shows that the proportion of failures is significantly reduced in all three cases as the [Ca]$_o$ is increased.

Figure 5(a and b) expresses the results in Figure 4(a and b) as a percentage of their respective control value. As the [Ca]$_o$ is increased the proportionate effect of yohimbine is decreased and clonidine increased significantly in terms of both mean d.e. amplitude and proportion of failures.

Effects of yohimbine on unfacilitated transmitter release

Figure 6 shows the effect of various concentrations of yohimbine at three different [Ca]$_o$ on unfacilitated

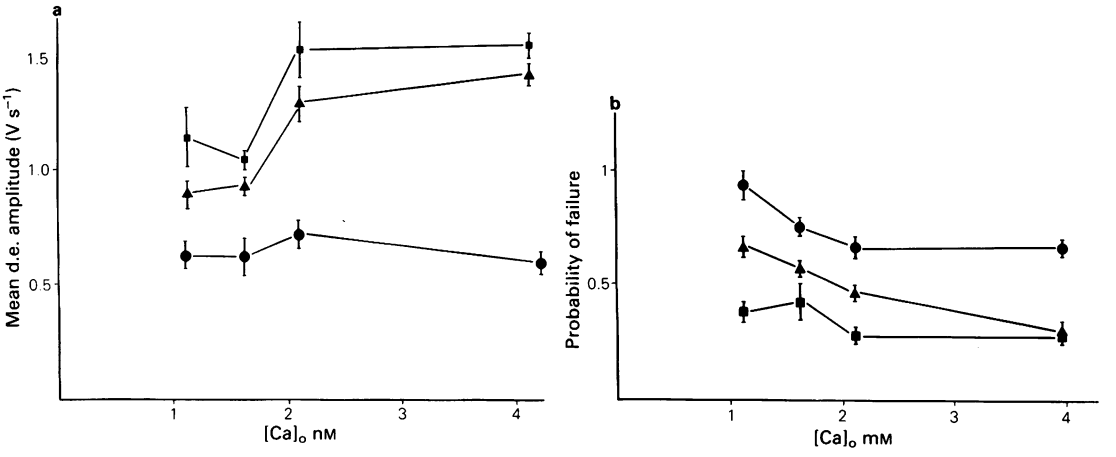

Figure 4 Variations with $[Ca]_o$ in the effect of clonidine (10^{-8}M) and yohimbine (10^{-7}M) on (a) the mean amplitude and (b) the probability of failure of discrete events (d.es) from a single family. (▲) Control; (●) clonidine; (■) yohimbine. As $[Ca]_o$ was increased the mean d.e. amplitude increased significantly ($P < 0.05$; Student's t test) in control and yohimbine treated cells but not in the presence of clonidine. The probability of failure was significantly reduced in all 3 cases ($P < 0.05$). $n \geq 6$ for each point.

e.j.p. amplitude elicited by very low frequency stimulation (<0.1 Hz). At 2.1 mM $[Ca]_o$ yohimbine has no effect. However, at 1.6 and 1.1 mM $[Ca]_o$ yohimbine significantly enhances the unfacilitated e.j.p. amplitude. The experiments were carried out on e.j.ps rather than d.es because it was difficult to obtain trains of stimuli, at such a low frequency in a single cell, which were long enough for accurate analysis of d.e. family distributions.

For one particular cell, however, Figure 7 shows the effect of yohimbine on a family of d.es when the $[Ca]_o$ was 1.1 mM. 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

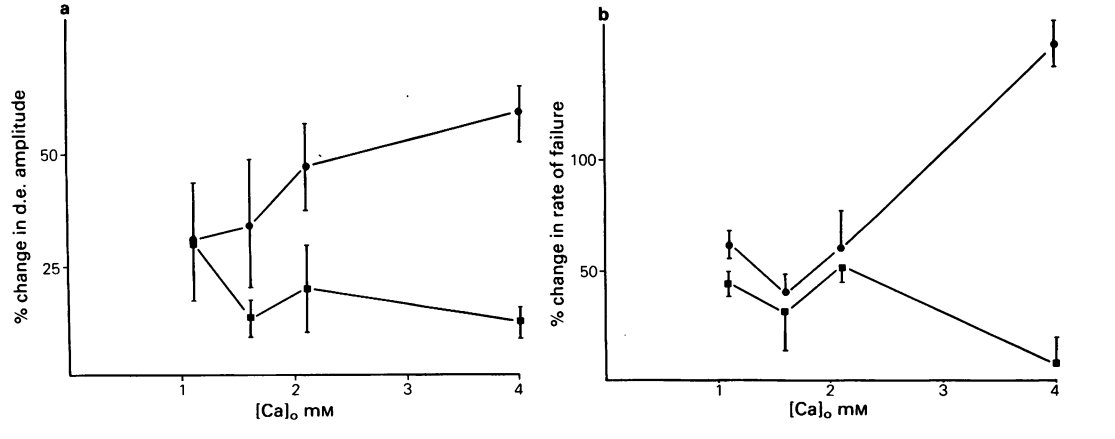

Figure 5 Percentage changes in (a) mean amplitude and (b) probability of failure of discrete events (d.es) from a single family following clonidine 10^{-8}M (●) or yohimbine 10^{-7}M (■) at different $[Ca]_o$. Both reductions and elevations are represented positively to emphasise a point in the text. In both (a) and (b) as the $[Ca]_o$ is increased the proportionate effects of yohimbine are significantly increased and the proportionate effects of clonidine significantly decreased ($P < 0.05$). $n \geq 6$ for each point.

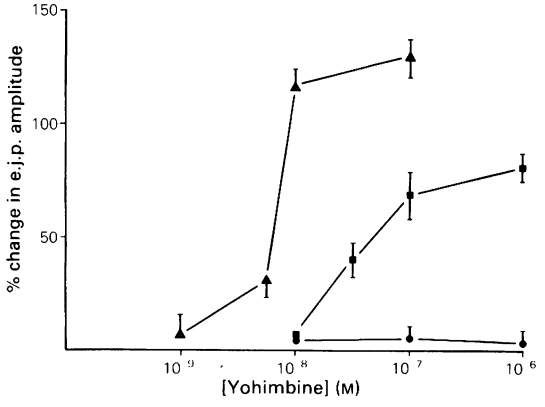

Figure 6 The effects of yohimbine (10^{-9}–10^{-6}M) on unfacilitated excitatory junction potential (e.j.p.) amplitude at different $[Ca]_o$, (●) 2.1, (■) 1.6 and (▲) 1.1 mM. Stimulation frequency 0.075 Hz. When the $[Ca]_o$ was lowered from 2.1 mM to 1.6 mM, yohimbine 5×10^{-8}M significantly increased unfacilitated e.j.p. amplitude. When $[Ca]_o$ was 1.1 mM, yohimbine 10^{-8}M significantly increased unfacilitated e.j.p. amplitude. $n = 10$ for each point.

distribution and the spacing between the modes remain constant.

Discussion

Effects of clonidine and yohimbine

The results obtained in this study demonstrate concentration-dependent modulation of transmitter release by clonidine and yohimbine. These drugs produce changes in families of d.es which are most easily explained by assuming they alter the 'quantal content' of a packeted release process. Within a family, therefore, changes in mean amplitude of d.es reflect changes in the mean number of packets of transmitter released. The proportion of failure of occurrence of a family member d.e. in a train of stimuli is too large, under normal conditions, to be accounted for by a normal Poisson release process (Blakeley *et al.*, 1984b,c), the excess failures representing a failure of activation of the release process which generates family member d.es. The alteration in the proportion of failures caused by yohimbine and clonidine reveals changes in the probability of activation of the release process. Thus, these drugs not only alter the quantal content of the release process when it is activated, but also alter the probability of activation itself.

These two mechanisms offer different potential sites of prejunctional modulation and it is tempting to

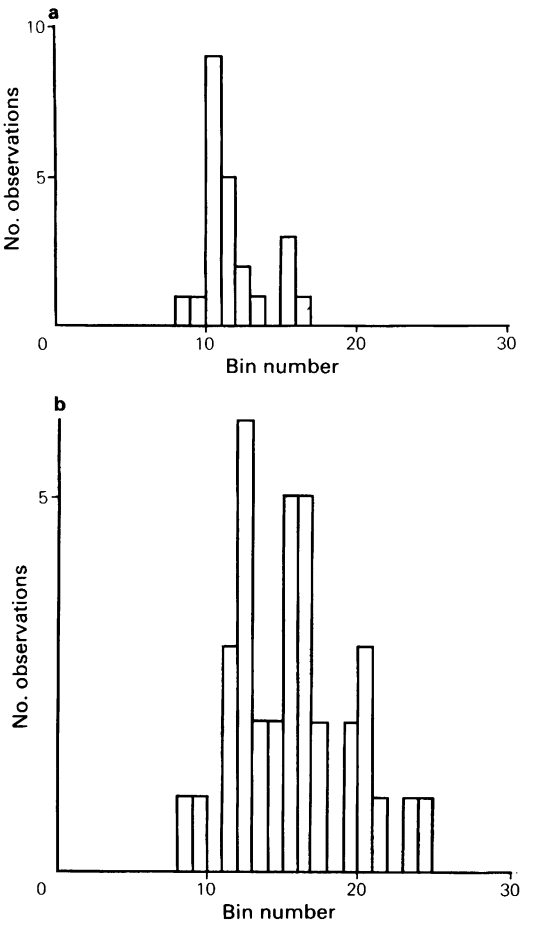

Figure 7 The effect of yohimbine on the amplitude distribution of a family of discrete events evoked in a single cell by very low frequency stimulation (< 0.1 Hz) at $[Ca]_o$ 1.1 mM. (a) Control; proportion of failures (Pf) = 0.68. (b) Same family after addition of yohimbine (10^{-7}M); Pf = 0.45. Bin size = 0.05 V s^{-1} in both (a) and (b).

compare them with the two sites of 'α-autoinhibition' suggested by Alberts *et al.* (1981) – namely, (a) invasion of the varicosity by an action potential, and (b) the depolarization/secretion coupling mechanism in the varicosity.

It should be noted that these drugs affect neither the modes of the amplitude distribution nor the spacing between the modes within a particular family indicating that their action is, indeed, prejunctional. Recent studies have cast doubt whether noradrenaline is responsible for the postjunctional electrical changes, but for this study the identity of the transmitter

responsible for eliciting e.j.ps and d.es is not important. The transmitter released from postganglionic sympathetic nerves which elicits a fast electrical response in the effector cell has been suggested to be adenosine 5'-triphosphate (ATP) released as a co-transmitter with noradrenaline (Sneddon *et al.*, 1982; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1984). If this is the case, in the mouse vas deferens, the amplitude of e.j.ps will still be proportional to the amount of noradrenaline released and the occurrence of a d.e. will reflect the release of noradrenaline from a particular release site.

Interaction between clonidine and yohimbine and the amount of transmitter released

The effect of clonidine and yohimbine at different external calcium concentrations, known to affect the amount of endogenous transmitter released (Rubin, 1970; Rahamimoff, 1970; Kirpekar, 1975), has been examined. Prejunctional modulation clearly exists (see also Langer, 1977; Starke, 1977; Westfall, 1977; Gillespie, 1980). If it is part of some endogenous 'feedback' process then it is likely that: first, the endogenous and exogenous agonist should have similar effects and show similar calcium-dependence. Second, the effects of the antagonist should be greatest when endogenous transmitter release is greatest. The release of transmitter is so rapid that there is insufficient time for a large release to displace antagonist by mass action.

As the calcium concentration was increased clonidine became more effective at lowering the quantal content and increasing the proportion of failures of occurrence of a d.e. family member. If the endogenous agonist shows a similar calcium-dependence then one would expect the effects of yohimbine, the antagonist, also to be increased. However, the opposite was found, i.e. as the calcium concentration was raised, yohimbine became if anything less effective. It seems, then, as though the endogenous agonist and clonidine have a different calcium-dependence.

E.j.ps are found to increase in amplitude (Bennett & Florin, 1975) and the quantal content of individual releases increases as external calcium concentration increases. Clearly there should have been greater autoinhibition in this case and yohimbine should have been more effective. It was not.

These results are similar to those found by Kalsner (1981) measuring [^{3}H]-noradrenaline overflow and are comparable with experiments which show the effect of α-adrenoceptor blockade to be inversely proportional to the frequency of stimulation (Stjärne & Brundin, 1977; Chan & Kalsner, 1979), and independent of the stimulus pulse duration (Kalsner, 1983).

When the stimulation frequency is kept very low there is no facilitation and the persistence of free transmitter between stimuli will be minimal (Kalsner, 1979; Kuriyama & Makita, 1983). Under these conditions with $[Ca]_o$ 2.1 mM, yohimbine was found to have no effect (see also Angus & Korner, 1981) as might be expected by the feedback hypothesis. When, however, the $[Ca]_o$ was lowered, yohimbine became effective despite there being less transmitter release, as assessed by control e.j.p. amplitude (Bennett & Florin, 1975).

Whilst clonidine and yohimbine modify release over a short time scale (see also Rand *et al.*, 1973) by changing the number of varicosities releasing transmitter and the amount each releases, it is difficult to see how the observations made in this study, by manipulations changing endogenous transmitter release, can be consistent with any notion of endogenous 'feedback' control.

This reinforces the previous conclusion, from this laboratory (Blakeley *et al.*, 1982) and others (Story *et al.*, 1981), that the endogenous agonist responsible for the e.j.p. does not exert short-term control of its own release or of the release of any co-transmitter released with it.

References

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

ANGUS, J.A. & KORNER, P.I. (1980). Evidence against presynaptic α-adrenoreceptor modulation of cardiac sympathetic transmission. *Nature*, **286**, 288–291.

BENNETT, M.R. & FLORIN, T. (1975). An electrophysiological analysis of the effect of Ca ions on neuromuscular transmission in the mouse vas deferens. *Br. J. Pharmac.*, **55**, 97–104.

BLAKELEY, A.G.H. & CUNNANE, T.C. (1979a). Packeted transmitter release in the mouse vas deferens: an electrophysiological study. *J. Physiol.*, **295**, 44P.

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

BLAKELEY, A.G.H., CUNNANE, T.C. & PETERSEN, S.A. (1982). Local regulation of transmitter release from sympathetic nerve terminals? *J. Physiol.*, **325**, 93–109.

BLAKELEY, A.G.H., MATHIE, A. & PETERSEN, S.A. (1983). Dose related effects of prejunctional modulators of sympathetic transmission in the mouse vas deferens. *Br. J. Pharmac. Proc. Suppl.*, **80**, 456P.

BLAKELEY, A.G.H., MATHIE, A. & PETERSEN, S.A. (1984a). Effects of yohimbine on unfacilitated transmitter release in the mouse vas deferens. *J. Physiol.*, **346**, 44P.

BLAKELEY, A.G.H., MATHIE, A. & PETERSEN, S.A. (1984b). Facilitation of single release sites of a sympathetic

neuroeffector junction in the mouse. *J. Physiol.*, **349,** 57–71.

BLAKELEY, A.G.H., MATHIE, A. & PETERSEN, S.A. (1984c). Is the vesicle the unit of transmission at the sympathetic neuroeffector junction? – electrophysiological evidence. In *Catecholamines part A, Basic and Peripheral Mechanisms*, ed. Usdin, E., Carlsson, A., Dahlstrom, A. & Engel, J. pp. 65–78. New York: Alan R. Liss Inc.

CHAN, C.C. & KALSNER, S. (1979). An examination of negative feedback function of presynaptic adrenoceptors in vascular tissue. *Br. J. Pharmac.*, **67,** 401–407.

GILLESPIE, J.S. (1980). Presynaptic receptors in the autonomic nervous system. In *Handbook of Experimental Pharmacology 44, 1. Adrenergic activators and inhibitors.* ed. Szekeres, L. pp. 353–425. Berlin, Heidelberg, New York: Springer-Verlag.

HOLMAN, M.E. & SURPRENANT, A. (1980). An electrophysiological analysis of the effects of noradrenaline and α-receptor antagonists on neuromuscular transmission in mammalian muscular arteries. *Br. J. Pharmac.*, **71,** 651–661.

KALSNER, S. (1979). Single pulse stimulation of guinea-pig vas deferens and the pre-synaptic receptor hypothesis. *Br. J. Pharmac.*, **66,** 343–349.

KALSNER, S. (1981). The role of calcium in the effects of noradrenaline and phenoxybenzamine on adrenergic transmitter release from atria. No support for negative feedback of release. *Br. J. Pharmac.*, **73,** 363–371.

KALSNER, S. (1982). The presynaptic receptor controversy. *Trends Pharmac. Sci.*, **3,** 11–21.

KALSNER, S. (1983). Yohimbine and prolongation of stimulation pulse duration alter similarly ^{3}H-transmitter efflux in the heart: an alternative negative feedback hypothesis. *Br. J. Pharmac.*, **79,** 985–992.

KIRPEKAR, S.M. (1975). Factors influencing transmission at adrenergic synapses. *Prog. Neurobiol.*, **4,** 163–210.

KURIYAMA, H. & MAKITA, Y. (1983). Modulation of noradrenergic transmission in the guinea pig mesenteric artery – an electrophysiological study. *J. Physiol.*, **335,** 609–627.

LANGER, S.Z. (1977). Presynaptic receptors and their role in the regulation of transmitter release. *Br. J. Pharmac.*, **60,** 481–497.

RAHAMIMOFF, R. (1970). Role of calcium ions in neuromuscular transmission. In *Calcium & Cellular Function.* 131. ed. Cuthbert, A.W. London-Basingstoke: Macmillan.

RAND, M.J., McCULLOUGH, M.W. & STORY, D.F. (1982). Feedback modulation of noradrenergic transmission and the presynaptic receptor controversy. *Trends Pharmac. Sci.*, **3,** 8–11.

RAND, M.J., STORY, D.F., ALLEN, G.S., GLOVER, A.B. & McCULLOUGH, M.W. (1973). Pulse to pulse modulation of noradrenaline release through a pre-junctional α-receptor auto-inhibitory mechanism. In *Frontiers in Catecholamine Research.* ed. Usdin, E. & Snyder, S.H., pp. 579–581. London: Pergamon.

RUBIN, R.P. (1970). The role of calcium in the release of neuro-transmitter substances and hormones. *Pharmac. Rev.*, **22,** 389–428.

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

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

STARKE, K. (1977). Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmac.*, **77,** 1–124.

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

STJÄRNE, L. & BRUNDIN, J. (1977). Frequency dependence of ^{3}H-noradrenaline secretion from human vasoconstrictor nerves: Modification by factors interfering with α or β adrenoceptor or prostaglandin E_2 mediated control. *Acta. physiol. scand.*, **84,** 217–223.

STORY, D.F., McCULLOUGH, R.W., RAND, M.J. & STANDFORD-STARR, C.A. (1981). Conditions required for the inhibitory feedback loop in noradrenergic transmission. *Nature*, **293,** 62–65.

WESTFALL, T.C. (1977). Local regulation of adrenergic transmission. *Physiol. Rev.*, **57,** 659–728.

(Received November 21, 1985.
Revised February 25, 1986.
Accepted March 25, 1986.)