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The effects of yohimbine on presynaptic and postsynaptic events during sympathetic nerve activation in cattle iris: a critique of presynaptic receptor theory

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1 The effects of presynaptic α-adrenoceptor blockade on both the efflux of ^{3}H-transmitter and on the magnitude of the effector response were measured simultaneously in a smooth muscle preparation which responds to field stimulation with noradrenergic β-receptor-mediated relaxation.

2 In the presence of atropine, the circular muscle of cattle iris relaxes in response to noradrenaline and to field stimulation at 2 Hz with 10, 20, 50 and 100 pulses.

3 Yohimbine (3×10^{-6} M), a potent presynaptic α-adrenoceptor antagonist, increased the stimulation-induced efflux of tritium to about 2.0 times control values and, contrary to theory, did so to a similar extent regardless of pulse number and with apparent indifference to the synaptic concentration of transmitter, as confirmed by the varying size of the postsynaptic response.

4 In most cases, yohimbine had no significant effect on the magnitude of the relaxations to nerve stimulation.

5 It is concluded that negative feedback regulation of transmitter release, if it functions at all, and this itself seems doubtful, would not have a substantial impact on the size of the effector response.

Introduction

The hypothesis that neurotransmitter release is regulated presynaptically, at the nerve terminals, is being subjected to increasingly close scrutiny. Yet there is another dimension of the problem that has not been satisfactorily addressed; namely the quantitative relationship between elevations in stimulation-induced transmitter efflux brought about by presynaptic antagonists and the changes induced by them in the magnitude of effector responses. The correspondence between these two parameters of drug action is of particular interest in assessing the purported consequences of negative feedback control of transmitter release. This is so because it tests the sometimes encountered, but not well founded, assumption that a doubling in stimulation-induced transmitter efflux, which is the amount of increase usually observed with presynaptic α-adrenoceptor antagonists, is matched by a correspondingly discernible change in the size of the effector response. The preparation used here to measure simultaneously the efflux of ^{3}H-transmitter and the mechanical response is the circular iris muscle of cattle, a smooth muscle which responds to sympathetic nerve activation, at a modest frequency, with β-receptor-mediated relaxation, and therefore allows the question to be examined.

Methods

Tissue preparation

Eyes were removed rapidly, after slaughter, from beef cattle of either sex, immersed in oxygenated ice-cold Krebs solution and transported to the laboratory (total time approximately 20 min). The iris sphincter (circular) muscles were then dissected out and tied at each end as described by Kern (1970) and either mounted under 1 g tension in 15 ml muscle chambers at 37°C or suspended in a superfusion apparatus as described below. The irides for superfusion experiments, after mounting under 1 g tension, were incubated individually for 60 min in 4.0 ml of oxygenated (5% CO_2 and O_2) Krebs-Henseleit (Krebs) solution (NaCl 115.3, KCl 4.6, $CaCl_2$ 2.3,

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$MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, glucose 7.8 and disodium ethylene diamine tetra-acetic acid 0.03 mM) containing (−)-[7,8-^{3}H]-noradrenaline (3.25 μCi/ml, 2.2×10^{-7} M). The preparations were then superfused continuously by pump with warmed (37°C) and oxygenated Krebs solution at a flow rate of 5 ml/min and a 90 min period allowed for equilibration before the start of the experiments. Mechanical responses were recorded isometrically by means of force-displacement transducers and a Grass polygraph.

Stimulation parameters

The irides were suspended between platinum wire electrodes fixed vertically on opposite sides of the tissue and they were stimulated (Grass model S5) at 2 Hz with 10, 20, 50 or 100 biphasic pulses of 1 ms duration and at supramaximal voltage.

Drugs and radiochemicals

The drugs used and their sources were: cocaine hydrochloride (BDH Ltd.), normetanephrine hydrochloride (Sigma Chemical Co.), yohimbine hydrochloride (Nutritional Biochemicals Corp.), atropine sulphate monohydrate (Calbiochem), (−)-noradrenaline bitartrate (Sigma Chemical Co.), propranolol hydrochloride (Aldrich Chemical Co.), and tetrodotoxin (Sigma Chemical Co.). The radioisotope (−)-[7,8-^{3}H]-noradrenaline hydrochloride (specific activity 15 Ci/mmol) was obtained from Amersham Corporation. It was diluted to a stock concentration of 100 μCi/ml (6.7×10^{-6} M) in ascorbic acid (50 μg/ml) and stored at 4°C in 5 ml aliquots under nitrogen gas. To obtain a final concentration of 3.25 μCi/ml (2.2×10^{-7} M) in the incubation medium, 0.13 ml of this stock solution was added to 3.87 ml of Krebs solution. The cocaine (3 μg/ml; 8.8×10^{-6} M), normetanephrine (2.2 μg/ml; 1×10^{-5} M) and atropine (0.3 μg/ml; 4.3×10^{-7} M) were dissolved directly in the Krebs solution and maintained throughout all the experimental procedures.

Efflux of [^{3}H]-noradrenaline

The efflux of [^{3}H]-noradrenaline from the preparations was determined by counting 1.0 ml aliquots of the 15.0 ml superfusate collected in vials by a fraction collector which rotated once every 3 min. Stimulation was always begun at the start of a 3 min collection period. The aliquots were then transferred to vials containing 10 ml of Aqueous Counting Scintillant (Amersham) and counted in a Beckman LS-230 counter with automatic external standardization to determine efficiency.

Basal efflux is expressed as disintegrations per minute (d/min) and referred to as the total radioactivity detected in the 3 min sample collected immediately before stimulation. Stimulation-induced efflux was calculated as the difference between basal efflux and the total d/min detected in the 3 min samples collected during and immediately after stimulation.

Mean data on efflux and mechanical response are presented with their standard errors and Student's paired *t* test was used for all intra-iris comparisons, with the unpaired test used for comparisons between groups; *P* values of less than 0.05 were considered significant. Other details of the experimental protocols were described previously (Kalsner & Chan, 1979).

Results

Responses to exogenous noradrenaline and nerve stimulation

The circular muscle of the iris develops spontaneous tone over the initial period of 1–2 h after mounting in muscle chambers containing Krebs-Henseleit solution. Following an equilibration period of 120 min, the addition of noradrenaline to the irides in sequentially increasing concentration, from 1 ng to 0.1 μg/ml (3.1×10^{-9} to 3.1×10^{-7} M), with washout of the muscle baths between each response, gave progressively larger relaxations (Figure 1b). It was established previously that responses to the catecholamine are blocked by β-adrenoceptor antagonists and that

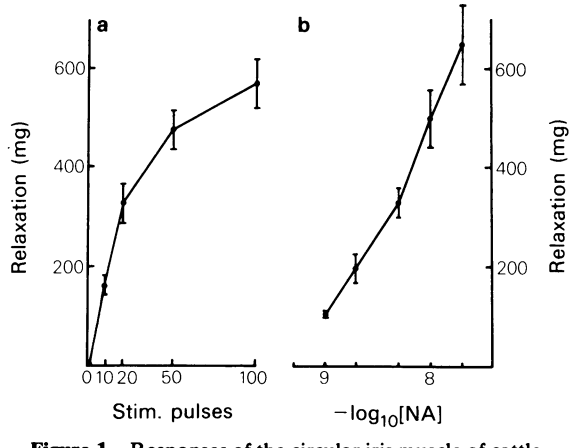

Figure 1 Responses of the circular iris muscle of cattle to field stimulation at 2 Hz (a) and to exogenous noradrenaline (NA) (b). Number of values are 18 and 4 respectively. Noradrenaline is referred to in terms of the concentration of the base in the bathing medium in g/ml.

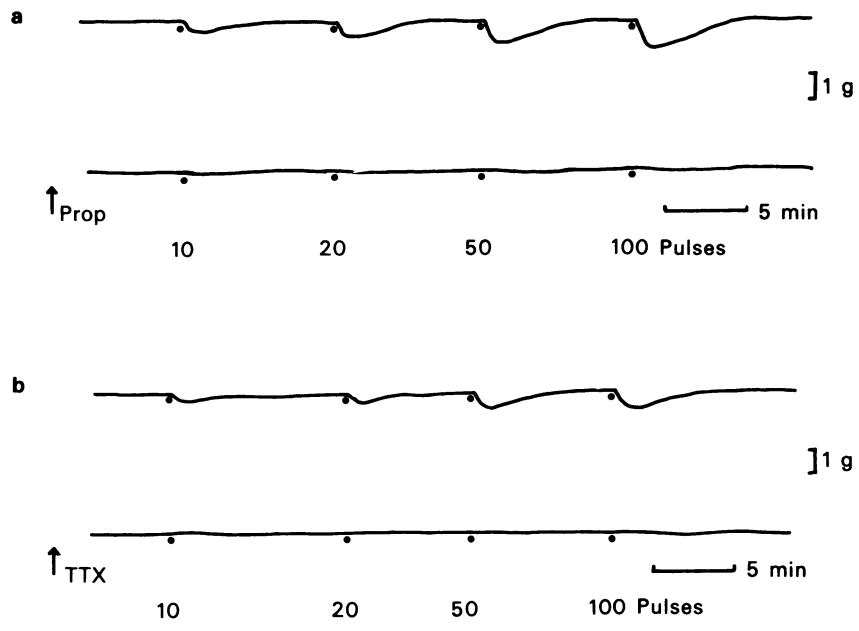

Figure 2 Responses of the circular iris muscle of cattle to field stimulation with 10, 20, 50 and 100 pulses. (a) Responses in the absence (top trace) and then of the same preparation in the presence (lower trace) of propranolol (Prop 5×10^{-6} M). (b) Responses in the absence (upper trace) and then of the same preparation in the presence (lower trace) of tetrodotoxin (TTX 1×10^{-6} M).

the adrenoceptors involved are of the β-type (Schaeppi & Koella, 1963; Kalsner, 1978).

Irides mounted in a superfusion apparatus, as described in Methods, and treated with atropine to block cholinergic influences, relax in response to field stimulation (Figures 1a and 2). Relaxations increased progressively in size when preparations were stimulated successively, at 9 min intervals, with 10, 20, 50 and 100 pulses at a constant frequency of 2 Hz. Repetition of the pulse number-response curves after a 30 min interval yielded slightly, but not significantly, greater responses confirming the suitability of iris preparations to test the link between presynaptic and postsynaptic events.

The responses to field stimulation were much attenuated or entirely prevented by treatment with the β-adrenoceptor blocking agent propranolol (5×10^{-6} M) and no functional population of postsynaptic α-receptors was revealed (Figure 2a). A 30 min exposure of the iris to tetrodotoxin (1×10^{-6} M) abolished relaxations to stimulation with 10 to 100 pulses (Figure 2b). These two procedures confirmed mediation of responses by noradrenergic nerves. The effect of yohimbine on the size of the effector response was determined by obtaining initial response curves to 10, 20, 50 and 100 pulses on two irides (S_1) and then exposing one of them to yohimbine (3×10^{-7} or 3×10^{-6} M) for 30 min followed, without washout of the antagonist, by repetition of the stimulation cycle (S_2) (10, 20, 50 and 100 pulses at 9 min intervals) in both the treated and the untreated muscles. Yohimbine itself had no detectable effect on the tone of the smooth muscle.

Yohimbine (3×10^{-7} M) increased only slightly the peak magnitude of the relaxations in response to field stimulation (Table 1). Statistical significance was reached, in fact, only in one case, with 20 pulses, where the increased relaxation (S_2/S_1 ratio), compared to matching controls, was in the order of 30%. A 10 fold higher concentration of yohimbine still had only minor effects on relaxation magnitudes and the relaxations were not, in any case, significantly different (S_2/S_1 ratios) from matching control preparations or from those obtained in the presence of yohimbine (3×10^{-7} M).

Efflux of ^{3}H-transmitter

The iris muscles also responded to field stimulation with 10, 20, 50 and 100 pulses at 2 Hz with increases in the efflux of ^{3}H-transmitter. The effect of yohimbine on the stimulation-induced efflux of tritium was assessed simultaneously in the same preparations and during the same experimental protocols described above for the study of relaxation rates. As shown in Table 2, yohimbine (3×10^{-7} M) increased the efflux

Table 1 Mechanical responses to field stimulation at 2 Hz in the absence and presence of yohimbine[a] in the circular iris muscle of cattle

Experimental group	*No. of pulses*	*No. of values*	*Relaxation* (g) *1st stim. period* (S_1)	*2nd stim. period* (S_2)	*Peak response ratio* (S_2/S_1)	*Treated group as % of control*†
Yohimbine (3×10^{-7} M)						
Control	10	12	0.16 ± 0.02	0.22 ± 0.03	1.47 ± 0.24	(a) 123.5 ± 17.0
Yohimbine	10	12	0.23 ± 0.04	0.31 ± 0.04	1.60 ± 0.21	
Control	20	12	0.33 ± 0.04	0.33 ± 0.04	1.05 ± 0.07	(b) 130.4 ± 14.0
Yohimbine	20	12	0.39 ± 0.05	0.48 ± 0.05	1.30 ± 0.09*	
Control	50	12	0.48 ± 0.04	0.47 ± 0.04	0.98 ± 0.04	(c) 110.7 ± 6.1
Yohimbine	50	12	0.54 ± 0.05	0.58 ± 0.06	1.07 ± 0.03	
Control	100	12	0.57 ± 0.05	0.52 ± 0.05	0.92 ± 0.03	(d) 111.6 ± 4.2
Yohimbine	100	12	0.65 ± 0.06	0.65 ± 0.06	1.01 ± 0.03	
Yohimbine (3×10^{-6} M)						
Control	10	6	0.22 ± 0.04	0.28 ± 0.06	1.28 ± 0.16	(e) 110.3 ± 10.6
Yohimbine	10	6	0.25 ± 0.07	0.33 ± 0.09	1.34 ± 0.08	
Control	20	6	0.33 ± 0.06	0.41 ± 0.08	1.24 ± 0.07	(f) 117.4 ± 12.3
Yohimbine	20	6	0.34 ± 0.08	0.46 ± 0.10	1.44 ± 0.15	
Control	50	6	0.54 ± 0.10	0.63 ± 0.12	1.17 ± 0.07	(g) 111.3 ± 10.4
Yohimbine	50	6	0.54 ± 0.08	0.67 ± 0.09	1.29 ± 0.11	
Control	100	6	0.75 ± 0.13	0.77 ± 0.13	1.03 ± 0.08	(h) 118.3 ± 9.0
Yohimbine	100	6	0.69 ± 0.09	0.81 ± 0.09	1.19 ± 0.06	

[a]Yohimbine, when given, was administered in the interval between S_1 and S_2 as described in text.
*$P < 0.05$ compared to corresponding control group. All other S_2/S_1 response ratios, in the presence of yohimbine, are not significantly different (NS) from their matched control groups.
†Obtained by comparing individually the S_2/S_1 ratio for control and treated irides from the same experiment and expressing the treated value as a percentage of the control value. (a) vs (e), NS; (a) vs (d), NS; (e) vs (h), NS; (b) vs (f), NS.

of tritium most at 20 pulses, about 130% above controls, 78% above at 100 pulses, and to a lesser extent at the two other pulse trains. With the 10 fold higher concentration of yohimbine the stimulation-induced efflux over the entire range of pulse trains was increased to approximately twice control values, and the magnitude of this effect did not vary significantly with pulse train length (Table 2). For example, the percentage increases at 10 pulses and at 100 pulses did not differ significantly from each other.

The per pulse efflux of transmitter, calculated in the initial runs (S_1) with 24 preparations changed only slightly between 10, 20 and 50 pulses but declined significantly with 100 pulses. The values were 1.27 ± 0.09, 1.20 ± 0.08, 1.09 ± 0.08, and 0.87 ± 0.04 d/min × 10^3, with increasing train length. It was also established from the data given in Table 2 that the per pulse efflux of transmitter did not vary significantly between 10 and 100 pulses during S_2 in yohimbine (3×10^{-6} M)-treated tissues or in the matching set of control irides. In the latter group, although per pulse efflux was generally insensitive to train length, a tendency to a reduction with 100 pulses was again noted.

The basal or spontaneous efflux of tritium, measured for 3 min immediately prior to each stimulation, declined significantly between S_1 and S_2. It was 20.5 ± 0.55 and 14.8 ± 0.38 d/min × 10^3 in the 18 control preparations yielding an S_2/S_1 ratio of 0.72. The S_2/S_1 ratio for basal efflux in tissues treated with yohimbine, between runs, did not differ from untreated controls. The ratios were 0.72 and 0.71 for the lower and the higher antagonist concentrations.

Discussion

Few available reports on the blockade of noradrenergic presynaptic receptors provide information about what is happening simultaneously, during nerve stimulation, to both the end organ response and to [^{3}H]-noradrenaline efflux. Such information is necessary if we are to assess the credibility of the postulate that a process of presynaptic negative feedback attenuates the size of the effector response

Table 2 Stimulation-induced efflux of transmitter in the absence and presence of yohimbine in cattle circular iris[a]

Experimental group	*No. of pulses*	*No. of values*	*Transmitter efflux* 1st stim. period (S_1) ($\times 10^3$ d/min)	2ns stim. period (S_2)	*Efflux ratio* (S_2/S_1)	*Treated group as % of control*†
Yohimbine (3×10^{-7} M)						
Control	10	12	13.48 ± 1.29	12.58 ± 0.96	0.97 ± 0.07	(a) 152.3 ± 11.0
Yohimbine	10	12	12.01 ± 1.33	16.12 ± 1.38	1.49 ± 0.16**	
Control	20	12	25.78 ± 2.61	22.68 ± 1.90	0.89 ± 0.06	(b) 230.0 ± 31.9
Yohimbine	20	12	22.10 ± 1.80	41.20 ± 4.28	1.93 ± 0.22**	
Control	50	12	53.58 ± 4.57	51.17 ± 3.73	0.97 ± 0.03	(c) 142.5 ± 9.3
Yohimbine††	50	11	55.51 ± 6.48	72.53 ± 5.37	1.39 ± 0.10**	
Control	100	12	92.50 ± 7.40	85.60 ± 6.20	0.93 ± 0.03	(d) 177.9 ± 5.9
Yohimbine	100	12	81.80 ± 4.44	134.72 ± 8.59	1.65 ± 0.06**	
Yohimbine (3×10^{-6} M)						
Control	10	6	5.97 ± 1.05	6.12 ± 0.69	1.09 ± 0.08	(e) 190.5 ± 41.2
Yohimbine	10	6	6.01 ± 0.95	10.47 ± 1.43	1.94 ± 0.31*	
Control	20	6	9.76 ± 0.80	11.15 ± 1.05	1.14 ± 0.05	(f) 166.3 ± 11.5
Yohimbine	20	6	11.05 ± 1.12	21.06 ± 2.96	1.89 ± 0.15**	
Control	50	6	25.52 ± 1.96	25.52 ± 2.66	0.98 ± 0.03	(g) 229.5 ± 22.2
Yohimbine	50	6	26.63 ± 1.76	61.54 ± 9.33	2.26 ± 0.22**	
Control	100	6	45.85 ± 4.21	47.09 ± 5.12	1.02 ± 0.04	(h) 230.7 ± 16.8
Yohimbine	100	6	47.01 ± 2.86	110.60 ± 11.89	2.34 ± 0.17**	

[a]Efflux values obtained from same tissues whose mechanical response is shown in Table 1. Yohimbine, when given, was administered in the interval between S_1 and S_2 as described in text.

*$P < 0.05$; **$P < 0.01$.

†Obtained by comparing individually the S_2/S_1 ratio for control and treated irides from the same experiment and expressing the treated value as a percent of the control value. (a) vs (d), NS; (e) vs (h), NS; (a) vs (e), NS; (b) vs (f), NS.

††One sample lost during experiment.

during the course of neurotransmission. Regardless of the mechanism by which agents such as yohimbine, a presumed potent presynaptic α-receptor antagonist, acts to increase transmitter efflux, and this is currently under vigorous debate elsewhere (e.g. Kirpekar, 1975; Bevan, 1978; Gillespie, 1980; Starke, 1981; Kalsner, 1982a,b), the assumption of quantitative parity between presynaptic and postsynaptic events needs to be tested.

The present work showed that yohimbine (3×10^{-6} M) increased the efflux of tritium during field stimulation of irides about 2 fold, and did so over a range of pulse trains administered at the moderate frequency of 2 Hz. A lower concentration of antagonist had somewhat less effect except at 20 pulses. However, the magnitudes of the effector responses, which ranged in untreated irides from very small with 10 pulses to fairly sizeable relaxation with 100 pulses, were generally not detectably increased by yohimbine at either of the two concentrations. In only one case (20 pulses), and with the lower concentration of antagonist, was any significant enhancement of response size detected and it may simply reflect a chance statistical occurrence. However, it should be noted that the efflux of tritium by yohimbine (3×10^{-7} M) was also increased the most at 20 pulses.

A doubling in stimulation-induced transmitter efflux, a value often seen with presynaptic antagonists in diverse sympathetically innervated systems, clearly failed to magnify response size correspondingly in the iris and this observation probably stems from the nature of the relationship between drug and receptors described by Clark (1937), and well known to pharmacologists. The sigmoid shape of the dose-response curve on a semi-logarithmic plot of agonist concentration against response, coupled with the slopes often encountered for agonists in autonomic effector systems, indicates that substantive increases in response magnitude generally require multiple dose increments and this relationship appears to hold also for noradrenaline released from nerves during stimulation.

Calculations based on the concentration-response curve to noradrenaline in iris (Figure 1) reveal that a doubling of response magnitude at 10 pulses (160 mg

relaxation) or at 20 pulses (330 mg relaxation) requires agonist concentration multiples of 3.1 and 4.4 and to increase the response at 50 pulses (600 mg), even by 25%, demands a 1.8 fold multiple in the applied concentration of agonist, confirming the analysis made above. However, it is imperative to note that it is not possible at present to establish the temporal-spatial kinetics of transmitter in the neuroeffector cleft during progressive modifications to the pulse train length, and it is unlikely that responses to exogenous agonist have anything more than a very circumstantial resemblance to local events in the terminal region of sympathetic nerves during neurotransmission. Thus, empirical demonstrations of transmitter efflux and response relationships, as provided here, are essential to assess directly the extent of the correspondence. It appears from the present work with the smooth muscle of the iris that negative feedback regulation of release, if indeed the effects of yohimbine are to be assigned to its inhibition, has an impact on the dimensions of the effector response which can only be described as trivial. It is clear, however, that other suitable preparations also need to be examined.

There are some other reports in the literature which bear on the issue, although it is not generally practical to assess the postsynaptic consequences of presynaptic blockade in autonomic effector systems, the major limitation being that mechanical responses are generally mediated by excitatory α-receptors which are blocked by the very same agents which are active presynaptically. In one relevant study of that kind it was sought to demonstrate the physiological relevance of presynaptic receptors by establishing that yohimbine (3×10^{-7} M), 'enhances the overflow of tritium and the smooth muscle contraction induced by transmural sympathetic nerve stimulation' (Starke, Borowski & Endo, 1975). An analysis of these authors' data (their Figure 1) shows that the maximal increment in efflux of ^{3}H-transmitter at 2 Hz was 161% above controls but contractions were increased in size only by a maximum of 20%. Additionally, at their other test frequency of 4 Hz the increase in tritium efflux was 109% accompanied by only an 11% increase in the effector response.

Presynaptic α-receptor antagonism is more profitably examined in a system which responds postsynaptically through β-receptors and a few such reports, with cardiac tissue only, are available. In a study with guinea-pig isolated atria it was concluded that phentolamine increased substantially both transmitter efflux and the chronotropic response to accelerans nerve stimulation (Langer, Adler-Graschinsky & Giorgi, 1977). However, the conditions used to study the two parameters differed widely. Efflux was examined with 240 pulses at 4 Hz during S_2 and S_3 whereas chronotropism was studied separately with 5 pulses at 0.5 Hz during S_5–S_9. An interesting observation by the authors should also be noted. The sensitization of chronotropism is shown still to be fully present (101.6% of peak sensitization values) 30 min after washout of phentolamine (0.31 μM) but in S_3 of the efflux study, done 44 min after washout of the antagonist (the only comparable measurement), enhancement had already declined markedly from 3.7 to 1.9 times control values.

In some other studies the effect of presynaptic receptor antagonists on parameters such as heart rate or blood pressure have been studied *in vivo* but without concomitant analysis of transmitter efflux and with conflicting results (Antonaccio, Halley & Kerwin, 1974; Docherty & McGrath, 1979; Graham, Stephenson & Pettinger, 1980; Robie, 1980; Drew, 1980). For example, in a detailed analysis Drew found that phentolamine had only very modest effects on heart rate in anaesthetized rats during sympathetic nerve stimulation and concluded that 'the physiological importance of the presynaptic α-inhibitory feedback system in regulating end-organ responses, rather than noradrenaline release, remains unclear'.

Interestingly, the amount of the enhancement of transmitter efflux by yohimbine reported here, about a doubling, regardless of pulse number, is typical of values encountered with a wide assortment of tissues and species and with several different antagonists encompassing a range of stimulation parameters (Kalsner, 1982b). For example, to take one widely employed tissue, phenoxybenzamine increased tritium overflow to 265% of control values in the guinea-pig vas deferens when stimulated with 450 pulses at 5 Hz and by 231% with 900 pulses at 10 Hz (Hedqvist, 1972). Similarly, 600 pulses at 10 Hz increased efflux to 152% of controls (Stjarne, 1973a) and with 300 pulses at 5 Hz, efflux was raised by the haloalkylamine to 210% of controls (Stjarne, 1973b). At the other end of the frequency-pulse number spectrum, Kalsner (1978a) found that the haloalkylamine enhanced output to a single pulse in the vas deferens to 195% of controls or to four pulses by a similar extent and again 10 and 50 pulses at 0.5 to 10 Hz were also increased from 192% to a maximum of 330% of controls (Kalsner, 1979b).

In previous reports from my laboratory the point was made that the profile of antagonist effect on stimulation-induced efflux with changes in frequency and pulse number did not conform to the expectations of negative feedback (Kalsner, 1982a,b). Such a paramount anomaly, which strains the credibility of presynaptic theory was observed again in the present work with yet another antagonist (yohimbine) and a different tissue (iris). It appears that yohimbine increases efflux of ^{3}H-transmitter to approximately the

same extent regardless of the stimulation parameters, and seemingly indifferent to the concentration of transmitter in the neuroeffector gap. This was confirmed by examining the size of postsynaptic response with increasing pulse number. Recent work from other laboratories has also raised concerns about negative feedback regulation of release by previously released transmitter (e.g. Robie, 1980; Drew, 1980; Fitzgerald, Watkins & Dollery, 1981; Blakeley, Cunnane & Petersen, 1982). For example, Blakeley and associates using an elegant electrophysiological approach to examine the release of transmitter from single sites in rat vas deferens found, in the absence of drugs, 'no evidence of any inhibition of release at the same release site from 500 ms to 3 s after release, and also no evidence of inhibition on nearby sites from 7 ms after release'. It is clear that the matter of functional presynaptic receptors is not yet settled.

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