Br. J. Pharmac (1983), **78**, 221–231

Stimulation-evoked release of [^{3}H]-noradrenaline by 1, 10 or 100 pulses and its modification through presynaptic α_2-adrenoceptors

Ian Marshall

Department of Pharmacology & Therapeutics, The Middlesex Hospital Medical School, London, W1P 7PN

1 Mice isolated vasa deferentia were loaded with 1-[7, 8-^{3}H]-noradrenaline and subsequently field stimulated with 1, 10 or 100 pulses (2 ms pulse width, 1 Hz). The tritium overflow was separated into [^{3}H]-noradrenaline and its ^{3}H-metabolites.

2 The resting release of tritium contained about 7% [^{3}H]-noradrenaline, 33% [^{3}H]-3, 4-dihydroxyphenylglycol ([^{3}H]-DOPEG) and 60% ^{3}H-non-catechols with usually less than 1% [^{3}H]-dihydroxymandelic acid ([^{3}H]-DOMA). The proportion of the tritium as [^{3}H]-noradrenaline increased with stimulation train length to 35% with 100 pulses; this increase in [^{3}H]-noradrenaline was associated with falls in [^{3}H]-DOPEG and ^{3}H-non-catechols. Generally the proportional increase in [^{3}H]-noradrenaline on stimulation was about 10× total tritium when compared with the resting release.

3 The fractional release of [^{3}H]-noradrenaline per pulse was independent of train length, averaging about 6×10^{-6}. This was reduced by the α_2-adrenoceptor agonist clonidine (0.3–30 nM) with an IC_{50} of 4.8 nM (10 pulses at 1 Hz).

4 The α_2-adrenoceptor antagonist, yohimbine (10–100 nM), did not alter the fractional release of [^{3}H]-noradrenaline elicited by 1 pulse. The antagonist did not change the amount or composition of the resting or evoked tritium overflow. However, yohimbine (1–100 nM) increased the fractional release of [^{3}H]-noradrenaline per pulse for trains of 10 or 100 pulses (1 Hz) in a concentration-dependent fashion. An increase above controls was significant only with 100 pulses and yohimbine, 30 nM.

5 The results show that the release of noradrenaline during trains of pulses in the mouse vas deferens can be regulated through presynaptic α_2-adrenoceptors. There was no evidence of inhibition by noradrenaline of its own release following a single pulse.

Introduction

It has been proposed that presynaptic α_2-adrenoceptors are associated with sympathetic nerve terminals (see review, Langer, 1981). When noradrenaline is released by nerve stimulation it may combine with the α_2-adrenoceptors and, through a negative feedback system, inhibit its own subsequent release.

The operation of this negative feedback mechanism on a pulse to pulse basis has been difficult to investigate because of the small quantities of noradrenaline released by a single pulse. However, proponents of the α_2-adrenoceptor hypothesis argue that α-adrenoceptor antagonists should not increase the release of noradrenaline by a single stimulus as there would be no agonist at these receptor sites at the time of stimulation (Langer, 1981). Recent work by Kalsner has examined this and other parts of the α_2-adrenoceptor hypothesis (e.g. Kalsner, 1979; 1980). A single electrical pulse evoked an increase in the twitch response of the guinea-pig vas deferens and in the overflow of tritium (from tissues preloaded with [^{3}H]-noradrenaline) in the presence of the α-adrenoceptor antagonist phenoxybenzamine (Kalsner, 1979). It was concluded that these findings should lead to a re-appraisal of the presynaptic receptor hypothesis.

Some problems surrounding the interpretation of the Kalsner experiments have been discussed by Starke (1981). For example, while phenoxybenzamine has been useful in the past, it is not the best available tool for testing the validity of the presynaptic receptor hypothesis. A drug selective for α_2- rather than α_1-adrenoceptors and which did not alter the neuronal or extra-neuronal uptake of norad-

0007-1188/83/010221-11-$01.00

renaline would be preferable. Additionally, a range of drug concentrations rather than the single one of 33 μM (Kalsner, 1979; 1980) would be an improvement. Finally, measurement of tritium as an index of transmitter release might hide a change in the proportion of noradrenaline to its various metabolites and produce a misleading result, especially with a drug like phenoxybenzamine which inhibits the uptake mechanisms.

Consequently in the present work, the α_2-adrenoceptor antagonist yohimbine was used, which has been shown to be selective for this receptor in a number of tissues (Starke, 1977) including the vas deferens of the mouse (Marshall, Nasmyth, Nicholl & Shepperson, 1978a). After preloading this tissue with [^{3}H]-noradrenaline, different concentrations of yohimbine have been studied on the overflow of [^{3}H]-noradrenaline (separated from its ^{3}H-metabolites) following a single pulse or trains of 10 and 100 pulses. With these trains it would be expected that there would be feedback of released noradrenaline onto α_2-adrenoceptors and hence increased [^{3}H]-noradrenaline overflow in the presence of yohimbine. The results of the experiments described here suggest that noradrenaline may regulate its own release via α_2-adrenoceptors with trains of pulses, but provide no evidence for this regulation following a single pulse. A preliminary account of some of this work has already appeared (Baker & Marshall, 1982).

Methods

Loading of vasa with [^{3}H]-noradrenaline

Male T.O. strain mice (34–40 g) were killed by stunning and cervical dislocation. The two vasa deferentia were dissected out and placed in a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. After the freeing of mesentery and blood vessels two vasa were placed in a flask containing Krebs with ascorbic acid 110 μM, disodium ethylenediaminetetra-acetic acid (EDTA) 30 μM and 17-β-oestradiol 30 μM and bubbled with 95% O_2 and 5% CO_2. Preliminary experiments showed that inhibition of the extraneuronal uptake mechanism by oestradiol led to an increased neuronal uptake of noradrenaline. After 5 min at 37°C, 1-[7,8^{3}H]-noradrenaline (50 Ci mmol^{-1}) was added to give a final volume of 1.0 ml (concentration 590 nM) and incubation continued for 30 min. Under these conditions the uptake of [^{3}H]-noradrenaline was significantly reduced by either decreasing the Na^+ concentration to 40 mM or by cocaine, 10 μM. After incubation, 2 pairs of similarly treated vasa were removed, tied together in parallel and placed under 1 g tension in a 1.0 ml glass bath. For the rest of the experiment the Krebs contained the ascorbic acid and the EDTA but not the oestradiol. The tissues were maintained at 37°C and aerated with a fine stream of 95% O_2 and 5% CO_2. The bath was drained and refilled every 10 min for 90 min to remove the loosely bound label. In some experiments these samples were collected to examine the pattern of tritium efflux.

Stimulation was provided via two parallel platinum-iridium electrodes using a Grass S48 stimulator (64 V, 2 ms pulse width). Contractions of the vasa were recorded isometrically with a Grass FT.03 transducer attached to a Grass polygraph.

Clonidine was added to the bath 30 s before stimulation, by means of a micrometer syringe. Yohimbine was added to the reservoir of Krebs solution and allowed to remain in contact with the tissues for at least 20 min before its effect on the resting or stimulation-induced overflow of [^{3}H]-noradrenaline was determined.

Single pulse experiments

In these experiments the vasa received single pulses separated by intervals of at least 15 min. To obtain a constant basal release the bath was filled and then emptied 40 s later (see Results) and the eighth such sample was collected as the prestimulation sample. After 30 s of the ninth period the single pulse was delivered and the bath emptied 10 s later. This was the stimulation sample. Following this the bath was emptied and refilled at 5 min intervals for either 10 or 20 min. Then the cycle of seven 40 s 'wash outs' followed by the prestimulation and stimulation samples was repeated. In some experiments all 10 single pulses were controls. In others only the first 4 were controls while the 5th and 6th were in the presence of yohimbine 10 nM, the 7th and 8th with yohimbine 30 nM and the 9th and 10th with yohimbine 100 nM present in the Krebs solution. In each experiment, results from the 4 controls and each pair with yohimbine were averaged.

The prestimulation and stimulation samples in all experiments (1, 10 or 100 pulses) were collected into tubes containing 1.5 ml of sodium acetate 0.2 M (pH 8.6), (−)-noradrenaline 11.4 μM, 3,4-dihydroxyphenylglycol 11.4 μM and disodium EDTA 27 μM and kept at 4°C up to 24 h for separation of the tritium into [^{3}H]-noradrenaline and its ^{3}H-metabolites.

Ten pulse experiments

The bath was filled and emptied 50 s later 5 times to obtain a constant basal release before the prestimula-

tion sample was collected. In the following 50 s stimulation period, 10 pulses at 1.0 Hz were given, beginning 30 s after the bath was filled. Following the collection of the stimulation sample, the bath was emptied and refilled at 5 min intervals for 15 min. There were 7 stimulations in these experiments, either all controls or the last 5 in the presence of 5 concentrations of clonidine or yohimbine (in order of increasing concentration). In this latter type of experiment the results of the first 2 stimulations (controls) were averaged.

One hundred pulse experiments

Three preliminary 135 s 'wash outs' were carried out before collection of the prestimulation sample. Thirty seconds after the start of the stimulation period 100 pulses at 1.0 Hz were applied to the bath. Collection of the stimulation sample was followed by a 15 min resting period as described above. There were 7 stimulation periods which followed the same pattern as in the 10 pulse experiments (see above).

Separation of [^{3}H]-noradrenaline and its ^{3}H-metabolites

The tritium which had overflowed from the vasa into the bath fluid was separated by column chromatography on alumina (prepared by the method of Anton & Sayre, 1962) and Dowex 50 W × 4 columns into [^{3}H]-noradrenaline, [^{3}H]-3,4-dihydroxyphenylglycol (DOPEG), [^{3}H]-dihydroxymandelic acid (DOMA) and the remaining ^{3}H-non-catechol (or ^{3}H-*O*-methylated) metabolites by the method of Graefe, Stefano & Langer, 1973. The DOMA fraction was counted on the alumina (after elution of the noradrenaline and DOPEG) and the Dowex was also counted for radioactivity to check on the complete elution of the [^{3}H]-noradrenaline. All samples were suspended in Insta-Gel (1:1 ratio) and counted for tritium in a liquid scintillation spectrometer. Efficiency of counting was determined by the automatic external standard channels ratio method.

At the end of each experiment the vasa were removed from the bath, blotted dry, weighed and homogenized in 2.0 ml distilled water with a Polytron. This sample was suspended in Insta-Gel and the tritium in the tissue counted as described above.

Calculation of results

The amount of tritium as [^{3}H]-noradrenaline or its ^{3}H-metabolites have been expressed in nCi. The total tritium in any sample is the sum of the individual fractions. The overflow of [^{3}H]-noradrenaline induced by electrical stimulation was calculated by subtracting its basal release (measured in the prestimulation sample) from that in the stimulation sample. This evoked release of [^{3}H]-noradrenaline was expressed as a fraction of the total tritium in the tissue at the time of stimulation (calculated using the radioactivity in the tissue at the end of the experiment with the addition of the tritium lost during resting periods and later stimulations). The fractional release of [^{3}H]-noradrenaline has been expressed per pulse of electrical stimulation to facilitate comparison of results from a single pulse or trains of 10 or 100 pulses.

Results are expressed as mean±s.e.mean. The significance of differences was assessed by means of a paired *t* test because each experiment includes its own control measurements. *P* values of less than 0.05 were considered significant.

Drugs

The following drugs and chemicals were used: alumina (Woelm), (−)-ascorbic acid (Sigma), clonidine hydrochloride (Catapres, Boehringer Ingelheim), (±)-3,4-dihydroxyphenylglycol (Sigma), disodium EDTA (BDH), Dowex 50 W × 4 (Sigma), Insta-Gel (Packard), (−)-noradrenaline bitartrate (Sigma) and sodium acetate (BDH). 17-β-Oestradiol (Sigma) was dissolved in polyethylene glycol 200 (BDH) 2 mg/ml and an appropriate volume added to the Krebs solution. Yohimbine hydrochloride (Sigma) was dissolved in distilled water (up to 10 mM) by stirring at 40°C. 1-[7,8^{3}H]-noradrenaline hydrochloride (specific activity 45–50 Ci mmol^{-1}, Radiochemical Centre, Amersham) was diluted with deoxygenated distilled water to 5.9 μM and stored at 4°C.

Results

Basal tritium efflux

During the 90 min after setting up the vasa in the bath, there was a loss of over 500 nCi of tritium. The initial high rate of efflux of tritium (Figure 1), presumably representing loosely bound label, was seen in spite of the presence of oestradiol in the [^{3}H]-noradrenaline uptake Krebs solution, which was added with the intention of greatly reducing extraneuronal uptake. The rate of tritium efflux fell to a much slower rate after 30 min.

The amount of tritium in the tissue at the end of the 90 min was determined in groups of 6 experiments. In single pulse experiments this was 0.21 ± 0.02 μCi mg^{-1} vas deferens, in 10 pulse experiments 0.19 ± 0.02 μCi mg^{-1} and 100 pulse experiments 0.17 ± 0.01 μCi mg^{-1}.

In preliminary experiments after the 90 min, a 40 s

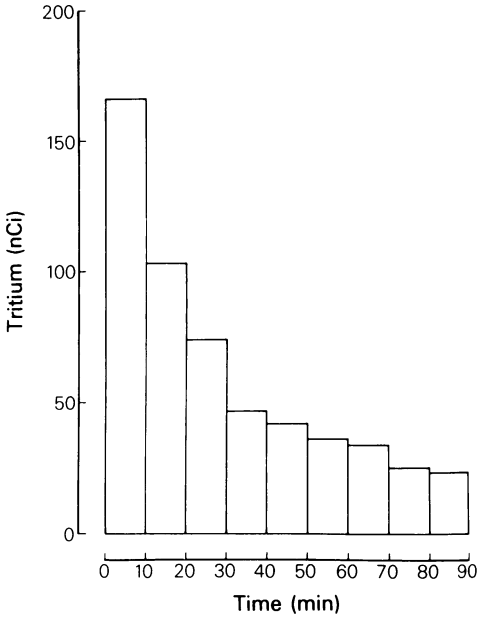

Figure 1 An example of the initial 90 min resting release of tritium from mouse vas deferens preloaded with [^{3}H]-noradrenaline.

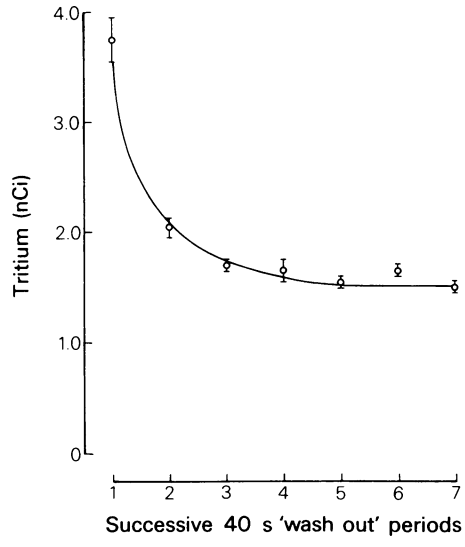

Figure 2 Efflux of tritium from the mouse vas deferens in seven successive 40 s periods (between filling and emptying the tissue bath) which were preceded by 5 min intervals. The results are the mean from 10 experiments; s.e.mean shown by vertical lines.

prestimulation sample was collected followed by a single pulse in the next 40 s. The latter sample usually contained only about half the tritium of the prestimulation sample. This led to the realization that changes in the duration of the collection period (e.g. from 10 min to 40 s) altered the resting efflux of tritium. To ensure a stable basal release in 1 pulse experiments, 7 successive 40 s 'wash outs' were carried out (Figure 2) after the usual 5 min resting periods and before a prestimulation sample was collected. In 10 pulse experiments (50 s collection period) 5 initial 'wash outs' were adequate while with 100 pulses (135 s collection period) 3 'wash outs' established a reasonably constant resting efflux before collection of the prestimulation sample.

The length of the stimulation interval was partly determined by the length of stimulation. The short period of 5 or 10 s after stimulation before emptying the bath would not allow time for all the evoked [^{3}H]-noradrenaline and its ^{3}H-metabolites to diffuse from the vasa into the surrounding Krebs solution. However, the paramount consideration was to keep the interval short so that there was a minimum basal overflow to compare with the greatest change following stimulation. This compromise was made so that changes in overflow with a single pulse could be measured reliably and reproduceably. Preliminary experiments suggested about 70% of the stimulation evoked release of [^{3}H]-noradrenaline by 100 pulses was collected with the present experimental design.

In the prestimulation samples the proportions of different fractions of the tritium was similar in all experiments (Tables 1, 2 & 3). [^{3}H]-noradrenaline represented 6–8% of the total tritium, [^{3}H]-DOPEG was about one third and ^{3}H-non-catechols about 60% (Figure 3). For the sake of comparison [^{3}H]-DOMA has also been included in this figure although its very low occurrence often made it difficult to measure reliably.

Effect of stimulation

The response of the vas deferens to a single pulse was a biphasic contracture with peaks at 280 and 680 ms. With a train of either 10 or 100 pulses at 1 Hz there was a peak within 4 pulses and then the amplitude of the responses declined. The fractional release of [^{3}H]-noradrenaline per pulse elicited by a single pulse was $6.39 \pm 0.76 \times 10^{-6}$ (mean±s.e.mean), 10 pulses $5.91 \pm 0.89 \times 10^{-6}$ and 100 pulses $7.41 \pm 0.86 \times 10^{-6}$ with no significant difference between them. Although the fall off in basal tritium efflux (Figure 1) continued throughout the experiments there was no significant change in the fractional release of [^{3}H]-noradrenaline as the experiments progressed. In a few experiments when calcium was omitted from the bathing Krebs solution there was no response of the tissue to stimulation with 100 pulses

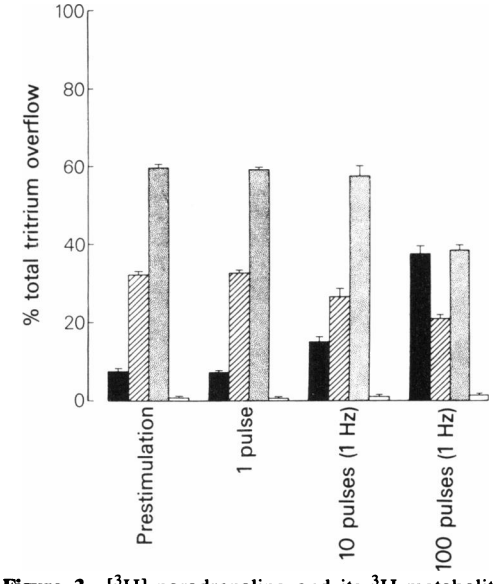

Figure 3 [^{3}H]-noradrenaline and its ^{3}H-metabolites overflowing in the prestimulation period or in response to 1, 10 or 100 pulses (1 Hz) from the mouse vas deferens. The overflow of [^{3}H]-noradrenaline (solid column) [^{3}H]-dihydroxyphenylglycol (hatched columns), ^{3}H-non-catechol (stippled columns) and [^{3}H]-dihydroxymandelic acid (open columns) are shown as a percentage of the total tritium in each sample. Each column represents the mean from at least 5 experiments; s.e.mean shown by vertical lines.

and no change in the basal release of [^{3}H]-noradrenaline or tritium.

With a single pulse the total tritium rose by 0.07 nCi (less than 4% above the prestimulation value) which was not significant while the [^{3}H]-noradrenaline fraction which increased by only 0.04 nCi (33% increase) was significant (Table 1). The difference was due to non-significant changes in [^{3}H]-DOPEG and ^{3}H-non-catechols which between them contributed most of the tritium. In the stimulation sample following 1 pulse [^{3}H]-noradrenaline represented only about 8% of the total tritium released (Figure 3).

A much greater increase in the proportion of [^{3}H]-noradrenaline in the tritium overflow (15%) was seen with 10 pulses. Here the [^{3}H]-noradrenaline increased by 100% over the prestimulation value, while total tritium increased by only 13% (Figure 3 and Table 2). Both these changes were significant.

Following stimulation with 100 pulses at 1 Hz, [^{3}H]-noradrenaline increased by 900% on the basal release to form more than 35% of the total tritium overflow which itself increased over the prestimulation level by about 75% (Table 3). Comparison of the distribution of the tritium released by 1 or 100 pulses showed that as the proportion of [^{3}H]-noradrenaline increased (8% to 35%) there was a decline in both [^{3}H]-DOPEG (from 33 to 21%, $P<0.001$) and in ^{3}H-non catechols (from 59 to 39%, $P<0.001$) (Figure 3).

Effect of yohimbine

The fractional release of [^{3}H]-noradrenaline and the maximum tension in response to a single pulse was not significantly increased by yohimbine up to 100 nM (Figure 4). It is probable that higher concentrations of yohimbine exert effects on α_1-adrenoceptors. The proportions of the basal or

Table 1 The effect of yohimbine on the metabolism of [^{3}H]-noradrenaline in prestimulation samples or after a single pulse of field stimulation (2 ms pulse width) in the mouse vas deferens

	Prestimulation (PS[1]) *or stimulation* (S[1])	*Total tritium*[2] (nCi)	*[^{3}H]-NA* (nCi)	*[^{3}H]-DOPEG* (nCi)	*^{3}H-non-catechols* (nCi)
Control	PS	2.04 ± 0.14	0.12 ± 0.01	0.69 ± 0.05	1.22 ± 0.09
	S	2.11 ± 0.14	0.16 ± 0.01	0.72 ± 0.05	1.24 ± 0.07
Yohimbine 10 nM	PS	2.02 ± 0.15	0.09 ± 0.01	0.75 ± 0.06	1.18 ± 0.09
	S	1.97 ± 0.11	0.11 ± 0.01	0.69 ± 0.05	1.15 ± 0.06
Yohimbine 30 nM	PS	1.88 ± 0.08	0.09 ± 0.01	0.68 ± 0.04	1.10 ± 0.05
	S	1.86 ± 0.06	0.12 ± 0.01	0.65 ± 0.03	1.08 ± 0.04
Yohimbine 100 nM	PS	1.76 ± 0.06	0.08 ± 0.01	0.65 ± 0.02	1.03 ± 0.04
	S	1.76 ± 0.06	0.11 ± 0.01	0.63 ± 0.02	1.01 ± 0.04

Values of radioactivity are given in nanocuries (nCi) as mean ± s.e.mean from at least 5 experiments.
[1] These periods are of 40 s duration.
[2] Total tritium is the sum of the other fractions with the addition of a small amount of [^{3}H]-dihydroxymandelic acid (DOMA) not shown in the Table.

Table 2 The effect of yohimbine on the metabolism of [^{3}H]-noradrenaline in prestimulation samples or after 10 pulses (1 Hz) of field stimulation in the mouse vas deferens

	Prestimulation (PS[1]) or stimulation (S[1])	*Total tritium[2]* (nCi)	*[^{3}H]-NA* (nCi)	*[^{3}H]-DOPEG* (nCi)	*^{3}H-non-catechols* (nCi)
Control	PS	3.24±0.19	0.29±0.06	1.12±0.09	1.80±0.09
	S	3.65±0.15	0.60±0.07	1.11±0.08	1.96±0.11
Yohimbine 1 nM	PS	2.83±0.22	0.19±0.06	1.04±0.11	1.59±0.08
	S	3.24±0.16	0.40±0.02	1.13±0.13	1.67±0.07
Yohimbine 3 nM	PS	2.88±0.32	0.17±0.04	1.11±0.19	1.59±0.14
	S	3.18±0.25	0.41±0.01	1.15±0.12	1.60±0.13
Yohimbine 10 nM	PS	2.66±0.22	0.13±0.03	0.96±0.10	1.55±0.11
	S	3.09±0.36	0.43±0.06	0.98±0.18	1.65±0.15
Yohimbine 30 nM	PS	2.53±0.20	0.13±0.01	0.84±0.17	1.55±0.12
	S	3.07±0.25	0.46±0.06	0.92±0.17	1.66±0.13
Yohimbine 100 nM	PS	2.37±0.18	0.10±0.06	0.89±0.12	1.38±0.07
	S	3.00±0.32	0.54±0.02	0.97±0.17	1.47±0.12

Values of radioactivity are given in nanocuries (nCi) as means ± s.e.mean from at least 5 experiments.
[1] These periods are of 50 s duration.
[2] Total tritium is the sum of the other fractions with the addition of a small amount of [^{3}H]-dihydroxymandelic acid (DOMA) not shown in the Table.

Table 3 The effect of yohimbine on the metabolism of [^{3}H]-noradrenaline in prestimulation samples or after 100 pulses (1 Hz) of field stimulation in the mouse vas deferens

	Prestimulation (PS[1]) or stimulation (S[1])	*Total tritium[2]* (nCi)	*[^{3}H]-NA* (nCi)	*[^{3}H]-DOPEG* (nCi)	*^{3}H-non-catechols* (nCi)
Control	PS	6.07±0.38	0.43±0.02	1.98±0.16	3.62±0.24
	S	10 55±0.45	3.98±0.28	2.18±0.12	4.08±0.20
Yohimbine 1 nM	PS	5.87±0.40	0.43±0.11	2.21±0.18	3.19±0.26
	S	10.20±0.52	3.95±0.33	2.36±0.19	3.80±0.16
Yohimbine 3 nM	PS	6.15±0.17	0.40±0.05	2.34±0.11	3.37±0.14
	S	9.11±0.58	3.05±0 39	2.34±0.09	3.43±0.21
Yohimbine 10 nM	PS	5.56±0.17	0.38±0.06	2.08±0.13	3.03±0.07
	S	9.87±0.55	3.61±0.28	2.45±0.31	3.67±0.23
Yohimbine 30 nM	PS	5.88±0.05	0.37±0.03	2.08±0.13	3.40±0.14
	S	10.98±0.05	4.48±0.17	2.52±0.05	3.75±0.18
Yohimbine 100 nM	PS	6.22±0.28	0.38±0.08	2.45±0.22	3.34±0.22
	S	12.38±0.66	4.98±0.62	2.80±0.12	4.27±0.24

Values of radioactivity are given in nanocuries (nCi) as mean ± s.e.mean from at least 5 experiments.
[1] These periods are of 135 s duration.
[2] Total tritium is the sum of the other fractions with the addition of a small amount of [^{3}H]-dihydroxymandelic acid (DOMA) not shown in the Table.

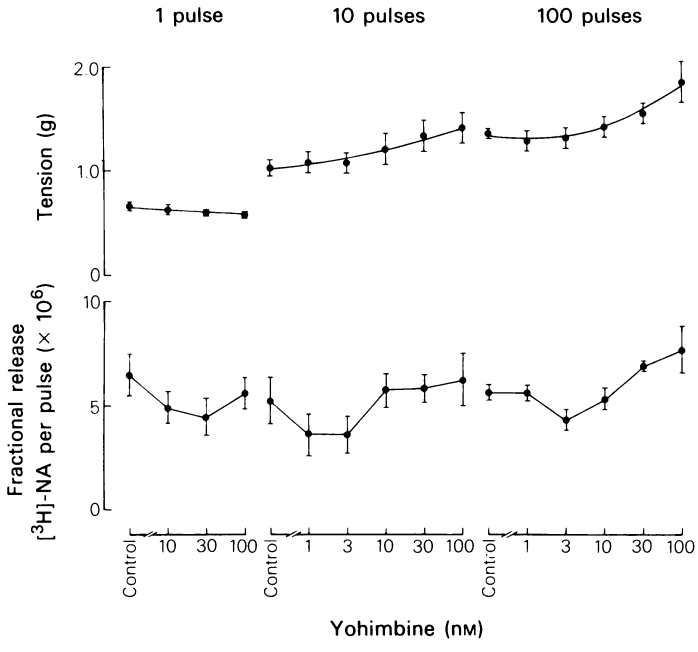

Figure 4 The effect of yohimbine on maximum tension development (above) and the fractional release per pulse of [^{3}H]-noradrenaline (below) following 1, 10 or 100 pulses (1 Hz). Each point represents the mean from at least 5 experiments; s.e.means shown by vertical lines.

stimulated overflow of [^{3}H]-noradrenaline and its ^{3}H-metabolites were unaltered by yohimbine (Table 1). This suggests that in concentrations up to 100 nM yohimbine does not alter either the uptake or metabolism of [^{3}H]-noradrenaline. The total tritium did not change on stimulation but [^{3}H]-noradrenaline always increased (Table 1).

Yohimbine produced a concentration related increase in the maximum tension developed by vasa in response to a train of 10 pulses at 1 Hz (Figure 4). The response was significantly potentiated by yohimbine 10, 30 and 100 nM. The fractional release of [^{3}H]-noradrenaline per pulse was not changed by yohimbine from the control values. However, there were significant increases when comparing the effects of the highest concentrations of yohimbine 30 and 100 nM against the lowest, 1 and 3 nM (Figure 4). In controls the proportion of [^{3}H]-noradrenaline in the total tritium doubled on stimulation (compared with the prestimulation sample) and this was increased to 3 fold by yohimbine 10 nM and to 5 fold by yohimbine 100 nM (Table 2). The changes in tritium were more modest, the control increase of 13% over basal release rose to 16% and 27% with yohimbine 10 nM and 100 nM respectively. The reason for the more modest change in tritium was because that of [^{3}H]-noradrenaline was partly offset by a decrease in ^{3}H-non catechols from a rise of 9% on stimulation in controls to only 6.5% in the presence of yohimbine, 100 nM.

With a train of 100 pulses yohimbine, 30 and 100 nM, significantly potentiated responses to stimulation (Figure 4). Here, the fractional release of [^{3}H]-noradrenaline per pulse was significantly increased above the controls by yohimbine 30 nM, the greatest rise (about 40%) being seen with yohimbine 100 nM. Concentration-dependent effects on the fractional release of [^{3}H]-noradrenaline were obtained (Figure 4). As in all other experiments yohimbine did not consistently change the basal release of tritium in the prestimulation sample. In control stimulations [^{3}H]-noradrenaline and total tritium rose by 900% and 75% over the basal release and these increased further in the presence of yohimbine, (e.g. at 100 nM to 1300% and 100% respectively). The increase in [^{3}H]-noradrenaline was accompanied by significant increases in [^{3}H]-DOPEG (yohimbine 30 and 100 nM) and sometimes by greater increases in ^{3}H-non-catechols than seen in controls (yohimbine 10 and 100 nM) (Table 3).

Effect of clonidine

Clonidine significantly reduced the fractional release of [^{3}H]-noradrenaline per pulse (by 10 pulses at 1.0 Hz) with an IC_{50} (concentration to halve the

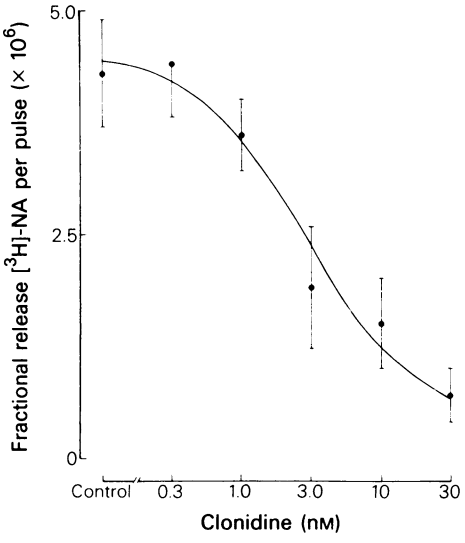

Figure 5 The effect of clonidine on the fractional release per pulse of [^{3}H]-noradrenaline following 10 pulses (1 Hz) in the mouse vas deferens. Each point represents the mean from at least 5 experiments; s.e.means shown by vertical lines.

control value) of 4.8 nM (Figure 5). The decrease in stimulated [^{3}H]-noradrenaline overflow was the major effect of increasing clonidine concentration, with little change in the amount of metabolites formed (Table 4) or their percentage of the tritium overflow.

Discussion

In the present experiments the overflow of [^{3}H]-noradrenaline from the isolated vas deferens of the mouse was studied; this tissue has a very dense sympathetic innervation and a high concentration of noradrenaline (Sjöstrand, 1965) making it suitable for studying the release of the catecholamine.

The overflow of radioactivity has been separated into [^{3}H]-noradrenaline and various metabolite fractions. This is time consuming but has the advantage that [^{3}H]-noradrenaline (cf. tritium) is more sensitive to changes in experimental conditions. For example, separation allows a consistent change in [^{3}H]-noradrenaline to be measured following a single electrical pulse while there was no significant increase in total tritium above the prestimulation level. Alternatively, with a train of 100 pulses, [^{3}H]-noradrenaline rose by 900% (3.5 nCi) above the prestimulation level while total tritium increased by 75% (4.5 nCi).

In addition to the increased sensitivity of [^{3}H]-noradrenaline to stimulation, separation of the tritium has another advantage. When drugs are added to the preparation, the proportions of different metabolite fractions in the total tritium can provide indirect evidence of changes in noradrenaline uptake

Table 4 The effect of clonidine on the metabolism of [^{3}H]-noradrenaline in prestimulation samples or after 10 pulses (1 Hz) of field stimulation in the mouse vas deferens

	Prestimulation (PS[1]) or stimulation (S[1])	*Total tritium[2]* (nCi)	*[^{3}H]-NA* (nCi)	*[^{3}H]-DOPEG* (nCi)	*^{3}H-non-catechols* (nCi)
Control	PS	3.33 ± 0.33	0.39 ± 0.08	1.07 ± 0.16	1.84 ± 0.18
	S	3.69 ± 0.35	0.71 ± 0.10	1.05 ± 0.16	1.88 ± 0.13
Clonidine 0.3 nM	PS	2.69 ± 0.13	0.19 ± 0.03	0.92 ± 0.09	1.54 ± 0.11
	S	3.88 ± 0.63	0.54 ± 0.10	1.29 ± 0.25	2.01 ± 0.28
Clonidine 1.0 nM	PS	2.50 ± 0.29	0.17 ± 0.03	0.92 ± 0.12	1.49 ± 0.11
	S	3.32 ± 0.52	0.45 ± 0 08	1.04 ± 0.25	1.79 ± 0.20
Clonidine 3.0 nM	PS	2.47 ± 0.29	0.18 ± 0 04	0.90 ± 0.12	1.37 ± 0.14
	S	3.22 ± 0.52	0.35 ± 0.09	1.19 ± 0.21	1.64 ± 0.23
Clonidine 10 nM	PS	2.33 ± 0.21	0.13 ± 0.03	0.91 ± 0.07	1.28 ± 0.13
	S	2.38 ± 0.25	0.26 ± 0.07	0.96 ± 0.18	1.13 ± 0.12
Clonidine 30 nM	PS	2.63 ± 0.13	0.12 ± 0.02	0.93 ± 0.07	1.55 ± 0.20
	S	3.09 ± 0.27	0.16 ± 0.06	1.12 ± 0.11	1.72 ± 0.13

Values of radioactivity are given in nanocuries (nCi) as mean ± s.e.mean from at least 5 experiments.
[1] These periods are of 50 s duration.
[2] Total tritium is the sum of the other fractions with the addition of a small amount of [^{3}H]-dihydroxymandelic acid (DOMA) not shown in the Table.

or metabolism. Measurement of total tritium will increase the likelihood of misinterpreting such changes as changes in release.

In the controls the fractional release of [^{3}H]-noradrenaline per pulse remained constant (about 6×10^{-6}) with varying train length. This parallels the constant value for tritium overflow reported in the mouse vas deferens for different train lengths (160–1920 pulses) or frequencies of stimulation (1–16 Hz; Farnebo & Malmfors, 1971). The constant fractional release contrasts with the increasing proportion of the tritium overflow present as [^{3}H]-noradrenaline. This rose from about 8% on stimulation with a single pulse to over 35% with a train of 100 pulses at 1 Hz. The increase in [^{3}H]-noradrenaline and the fall in the proportion of [^{3}H]-DOPEG and ^{3}H-non-catechols suggests that neuronal and extra-neuronal uptake may be reduced during depolarization with a consequent fall in metabolism. Similar findings have been reported by other workers (e.g. Häggendal & Malmfors, 1969, Dubocovich & Langer, 1976).

The presence of presynaptic α_2-adrenoceptors in the mouse vas deferens was suggested from studies using selective agonists and antagonists on the post-junctional twitch response elicited by field stimulation (Marshall *et al.*, 1978a). In the present experiments the selective α_2-adrenoceptor agonist clonidine (Starke, Montel, Gayk & Merker, 1974) inhibited the overflow of [^{3}H]-noradrenaline by electrical pulses at concentrations which do not alter the uptake of the amine (unpublished observations). There was an 87% inhibition of the fractional release of [^{3}H]-noradrenaline with clonidine 30 nM but no significant change in the fractional release of tritium, in confirmation of earlier findings (Marshall, Nasmyth & Shepperson, 1978b). This may explain the inability of clonidine to reduce the fractional release of tritium in the guinea-pig vas deferens reported earlier by Stjarne (1975).

Further evidence for the presence of these presynaptic receptors was found by the use of yohimbine, a selective α_2-adrenoceptor antagonist (Starke, Borowski & Endo, 1975). The overflow of [^{3}H]-noradrenaline (100 pulse stimulation) was significantly increased above controls with yohimbine, 30 nM, which is consistent with noradrenaline feeding back onto α_2-adrenoceptors to inhibit its own release. In other cases with 10 or 100 pulses, yohimbine did not significantly increase the fractional release above controls although in both situations there were significantly higher values with the higher concentrations of antagonist compared with lower ones. The magnitude of the increase in [^{3}H]-noradrenaline overflow is small (around 40%) compared with the rise often reported for phentolamine or phenoxybenzamine in many tissues (Langer, 1981) and the difference may partly reflect the blockade of uptake by these latter adrenoceptor antagonists.

Three important factors influencing changes in noradrenaline release in the presence of α_2-adrenoceptor antagonists may be frequency, train length and tissue differences. Recent experiments using the field stimulated guinea-pig atria loaded with [^{3}H]-noradrenaline showed no change in tritium efflux in the presence of phentolamine, 3 μM, with a train of 4 pulses (2 Hz) but there was an increase with the longer train of 16 pulses (Story, McCulloch, Rand & Stanford-Starr, 1981). However, in the atria stimulated at 1 Hz, the frequency used in the present experiments, phentolamine did increase tritium efflux evoked by only 4 pulses.

The suggestion that noradrenaline released by one pulse can feed back onto α_2-adrenoceptors to inhibit its own release by that same stimulus is controversial (see Introduction). Unlike the original work suggesting this idea, based on experiments with phenoxybenzamine in the guinea-pig vas deferens (Kalsner, 1979; 1980) the present experiments used the more selective α_2-adrenoceptor antagonist, yohimbine. This has been studied in a range of concentrations which themselves had no effect on the basal overflow of [^{3}H]-noradrenaline and its ^{3}H-metabolites (unlike phenoxybenzamine, Kalsner, 1979). Further, these concentrations of yohimbine do not alter the uptake of [^{3}H]-noradrenaline into the vas deferens of the mouse (Shepperson, 1979). There was no significant change in the fractional release of [^{3}H]-noradrenaline by a single pulse in the presence of yohimbine. This does not support the suggestion that the catecholamine can regulate its own release during one pulse and the lack of demonstrable feedback with some longer pulse trains is in agreement with these findings. Because yohimbine is so much more selective for α_2-adrenoceptors than is phenoxybenzamine (which has a higher affinity for α_1-adrenoceptors (Dubocovich & Langer, 1974), inhibits neuronal and extra-neuronal uptake and has a reserpine-like action (see Starke, 1981)) it is likely that some or all of the increase in tritium overflow reported by Kalsner (1979) originates through a mechanism independent of this particular feedback system.

If noradrenaline released by a single pulse does not inhibit its own release there is an important practical consequence. One of the problems when measuring the effects of drugs on presynaptic α_2-adrenoceptors is their competition for these sites with endogenous noradrenaline. Use of the single pulse technique may eliminate (or at least greatly reduce) such an interaction.

It is difficult to know how far changes in [^{3}H]-noradrenaline overflow on stimulation mirror changes in the overflow of the endogenous amine and yet this information is of vital importance in inter-

preting results on the α_2-adrenoceptor negative feedback mechanism. In the vas deferens there is some evidence supporting a similarity between the overflow of labelled and unlabelled catecholamine on stimulation. Firstly, in the rabbit vas deferens, tissues loaded with 59 nM [^{3}H]-noradrenaline showed a greater specific activity of overflowing amine compared with that in the tissue (Hughes, 1973). With the higher concentration of 590 nM (as employed in these experiments) there was no difference between the two specific activities. It was concluded that there was homogeneous labelling of tissue stores of noradrenaline following incubation with 590 nM but not with 59 nM (Hughes, 1973). Secondly, the fractional release per pulse of endogenous noradrenaline from the mouse vas deferens (1 Hz, 120 pulses) measured with a radioenzymic assay was 3.1×10^{-6} (Shepperson, 1979), and in the rabbit vas deferens 2.2×10^{-6} measured by bioassay (2 Hz, Hughes, 1972). These values are similar to those for [^{3}H]-noradrenaline in the present experiments (about 6.5×10^{-6}) and in our earlier studies (5.9×10^{-6}, Marshall *et al.*, 1978b). Thirdly, there was no significant change in the fractional release per pulse of [^{3}H]-noradrenaline in the present work over 2–3 h (although there was a continuous decline in the basal release of tritium). This suggests that [^{3}H]-noradrenaline is not redistributed within the neurone during the experiment. Fourthly, values for the fractional release per pulse of tritium from different experiments using mouse vasa preloaded with [^{3}H]-noradrenaline are similar in spite of differences in experimental protocol e.g. 3.5×10^{-5} (Farnebo & Malmfors, 1971), 4.4×10^{-5} (Hughes, 1978), 3.8×10^{-5} (Marshall *et al.*, 1978b) and approx. 5×10^{-5} in the present experiments. In summary, the evidence suggests there is enough similarity between the overflow of endogenous noradrenaline and its radiolabelled counterpart to allow measurement of the latter to generate useful hypotheses about the regulation of noradrenaline release via presynaptic α_2-adrenoceptors.

This research is supported by the Medical Research Council. The excellent technical assistance of Miss Susan Baker is gratefully acknowledged.

References

ANTON, A.H. & SAYRE, D.F. (1962). A study of the factors affecting the aluminum oxide-trihydroxyindole procedure for the analysis of catecholamines. *J. Pharmac. exp. Ther.*, **138,** 360–375.

BAKER, S. & MARSHALL, I. (1982). The release of [^{3}H]-noradrenaline from the mouse vas deferens by trains of 1–100 electrical pulses. *Br. J. Pharmac.*, Proc. Suppl., **75,** 155P.

DUBOCOVICH, M.L. & LANGER, S.Z. (1974). Negative feed-back regulation of noradrenaline release by nerve stimulation in the perfused cat's spleen: Differences in potency of phenoxybenzamine in blocking the pre- and post-synaptic adrenergic receptors. *J. Physiol.*, **237,** 505–519.

DUBOCOVICH, M.L. & LANGER, S.Z. (1976). Influence of the frequency of nerve stimulation on the metabolism of ^{3}H-norepinephrine released from the perfused cat spleen: Differences observed during and after the period of stimulation. *J. Pharmac. exp. Ther.*, **198,** 83–101.

FARNEBO, L.-O. & MALMFORS, T. (1971). ^{3}H-noradrenaline release and mechanical response in the field stimulated mouse vas deferens. *Acta physiol. scand.*, Suppl. **371,** 1–18.

GRAEFE, K.H., STEFANO, F.J.E. & LANGER, S.Z. (1973). Preferential metabolism of (−)-(^{3}H)-norepinephrine through the deaminated glycol in the rat vas deferens. *Biochem. Pharmac.*, **22,** 1147–1160.

HÄGGENDAL, J. & MALMFORS, T. (1969). The effect of nerve stimulation on the uptake of noradrenaline into the adrenergic nerve terminals. *Acta physiol. scand.*, **75,** 28–32.

HUGHES, I.E. (1978). The effect of amitriptyline on presynaptic mechanisms in noradrenergic nerves. *Br. J. Pharmac.*, **63,** 315–321.

HUGHES, J. (1972). Evaluation of mechanisms controlling the release and inactivation of the adrenergic transmitter in the rabbit portal vein and vas deferens. *Br. J. Pharmac.*, **44,** 472–491.

HUGHES, J. (1973). Differential labelling of intra-neuronal stores with different concentrations of (−)-[^{3}H]-noradrenaline. *Br. J. Pharmac.*, **47,** 428–430.

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

KALSNER, S. (1980). Limitations of presynaptic adrenoceptor theory: The characteristics of the effects of noradrenaline and phenoxybenzamine on stimulation-induced efflux of (^{3}H)-noradrenaline in vas deferens. *J. Pharmac. exp. Ther.*, **212,** 232–239.

LANGER, S.Z. (1981). Presynaptic regulation of the release of catecholamines. *Pharmac. Rev.*, **81,** 337–362.

MARSHALL, I., NASMYTH, P.A., NICHOLL, C.G. & SHEPPERSON, N.B. (1978a). α-adrenoceptors in the mouse vas deferens and their effects on its response to electrical stimulation. *Br. J. Pharmac.*, **62,** 147–151.

MARSHALL, I., NASMYTH, P.A. & SHEPPERSON, N.B. (1978b). Pre-synaptic α-adrenoceptors and [^{3}H]-noradrenaline overflow from the mouse vas deferens. *Br. J. Pharmac.*, **62,** 382–383P.

SHEPPERSON, N.B. (1979). Presynaptic alpha-adrenoceptors and noradrenaline in neurotransmission in the mouse isolated vas deferens. *Ph.D. Thesis, University of London.*

SJÖSTRAND, N.O. (1965). The adrenergic innervation of

the vas deferens and the accessory male genital glands. *Acta physiol. scand.*, **63,** Suppl. 257.

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

STARKE, K. (1981). Presynaptic receptors. *A Rev. Pharmac. Tox.*, **21,** 7–30.

STARKE, K., BOROWSKI, E. & ENDO, T. (1975). Preferential blockade of presynaptic α-adrenoceptors by yohimbine. *Eur. J. Pharmac.*, **34,** 385–388.

STARKE, K., MONTEL, H., GAYK, W. & MERKER, R. (1974). Comparison of the effects of clonidine on pre- and post-synaptic adrenoceptors in the rabbit pulmonary artery. *Naunyn Schmiedebergs Arch. Pharmac.*, **285,** 133–150.

STJARNE, L. (1975). Clonidine enhances the secretion of sympathetic neurotransmitter from isolated guinea pig tissues. *Acta physiol. scand.*, **93,** 142–144.

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

(Received July 29, 1982.
Revised September 27, 1982.)