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# EVIDENCE AGAINST THE UNITARY HYPOTHESIS OF AGONIST AND ANTAGONIST ACTION AT PRESYNAPTIC ADRENOCEPTORS

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**1** The concept that presynaptic receptors regulate noradrenergic transmitter release via a system of inhibitory receptors mediating negative feedback relies on a supposed association between increases in stimulation-induced efflux of [$^3$H]-noradrenaline by antagonists and blockade by them of the inhibitory effects of exogenous noradrenaline.

**2** It was shown in guinea-pig ureter, that yohimbine ($3 \times 10^{-7}$ M), a presumed selective presynaptic antagonist, increased transmitter efflux substantially at 1 Hz and 5 Hz with 100 pulses, purportedly representing antagonism of the inhibitory effect of locally released noradrenaline but did not reduce the inhibitory effect of exogenous noradrenaline ($1.8 \times 10^{-6}$ M or $1.8 \times 10^{-7}$ M) except in one case.

**3** Additionally, the inhibitory effect of oxymetazoline ($1.0 \times 10^{-7}$ M or $1.0 \times 10^{-8}$ M) on stimulation-induced efflux was in no way antagonized by yohimbine ($3 \times 10^{-7}$ M).

**4** It is concluded that the increased efflux of [$^3$H]-noradrenaline produced by antagonists and the decreased efflux produced by exogenous agonists may represent actions at different loci and that the hypothesis of presynaptic feedback regulatory sites is still not substantiated.

## Introduction

Probably the most impressive evidence used to support the hypothesis that presynaptic receptors regulate noradrenergic transmitter release via negative feedback is the purported association between enhancement of stimulation-induced transmitter efflux by α-adrenoceptor antagonists and the blockade by them of agonist-induced inhibition of efflux. The linkage between these two effects is necessary to prove that each is an expression of the same event, namely α-receptor blockade; the one effect representing blockade of the inhibitory response to endogenously released and the other to exogenously applied agonist. The literature is surprisingly scanty and uninformative on this pivotal issue of the presynaptic consequences of antagonists both for noradrenaline released by nerves and for noradrenaline added from outside (Starke, 1972; Starke, Endo & Taube, 1975a). For this reason, experiments were done by my laboratory to determine if the enhancement and the inhibition of transmitter efflux by drugs might not represent two discrete actions, at separate loci, rather than a single action at a common locus. The tissue selected for this study was the guinea-pig ureter, a paired organ, yielding stimulation-induced increases in $^3$H-transmitter efflux which are substantially and reliably elevated in the presence of α-antagonists. The adrenoceptor antagonist selected for this initial study was yohimbine, since it is reputed to be one of the most selective presynaptic blocking agents now available (Starke, Borowski & Endo, 1975b; Anden, Grabowska & Strombom, 1976; Doxey, Smith & Walker, 1977) and both noradrenaline and oxymetazoline were used as agonists.

## Methods

Ureters were removed from guinea-pigs, desheathed and incubated for 60 min in 4.0 ml of oxygenated (5% $CO_2$ in $O_2$) Krebs-Henseleit (Krebs) solution containing ethylenediamine tetraacetic acid (disodium salt; 0.03 mM) to retard heavy metal catalyzed oxidation of catecholamines (Kalsner, 1979a) and (−)-[7,8-$^3$H]-noradrenaline (10 μCi/ml, $7.6–10.0 \times 10^{-7}$ M) then washed with fresh Krebs solution and mounted under 1 g tension between platinum wire electrodes. Tissues were superfused continuously with warmed (37°C) and oxygenated Krebs solution at a flow rate of 5 ml/min. Cocaine hydrochloride (3 or 10 μg/ml; 8.8 or 29 μM; usually the latter) and normetanephrine hydrochloride (2.2 μg/ml; 10 μM) were routinely present in the Krebs solution to block neuronal and extraneuronal uptake processes. Yohimbine hydrochloride, oxymetazoline hydrochloride and (−)-noradrenaline bitartrate, when used, were made up in appropriate stock concentrations and added directly to the Krebs solution-reservoir.

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After a 90 min equilibration period each tissue was stimulated transmurally with a train of 100 pulses of 1 ms duration and supramaximal voltage twice at each of the two test frequencies (1 and 5 Hz) with a 10 min interval between tests. One of each pair of ureters was then exposed to either the agonist (15–30 min) or antagonist (20–30 min) alone, followed, without washout, by a repetition of the stimulation cycle in both the control and treated preparations. Alternately, both drugs were present together during the second stimulation period in specific protocols described below.

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 every 3 min. The aliquots were 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/min (d/min) and referred to as the total radioactivity detected in the 3 min sample collected immediately before stimulation. Transmural stimulation was always begun at the onset of a 3 min collection period. Stimulation induced efflux was calculated as the difference between basal efflux and the total d/min in the 3 min sample collected during and immediately after stimulation. Each stimulation frequency was repeated twice and the efflux values averaged. Mean data are presented with their standard errors and Student's $t$ test was used for all comparisons; a $P$ value of less than 0.05 was considered significant.

## Results

### *Noradrenaline and yohimbine*

Noradrenaline ($1.8 \times 10^{-6}$ M) present during the second run ($S_2$) in one of each set of ureters, produced a substantial reduction in the stimulation-induced efflux of tritium, when second run values were compared to those of the first run (Table 1). The inhibition was somewhat less at 5 Hz than at 1 Hz; that is a 56.0% versus an 88.6% reduction in tritium efflux. This inhibition of efflux presumably represents activation of inhibitory presynaptic α-sites by the exogenous amine, diminishing exocytotic extrusion of transmitter.

In preliminary experiments with yohimbine it was established that a concentration of $3 \times 10^{-7}$ M present during the second run produced the maximal increase in stimulation-induced tritium efflux obtainable with the alkaloid. For example, efflux was increased to $3.7 \pm 0.72$ and $3.4 \pm 0.27$ times initial ($S_1$) control values for $3 \times 10^{-7}$ and $3 \times 10^{-6}$ M yohimbine at 1 Hz with 100 pulses in 6 matched sets of ureters.

The protocol selected to assess directly the relationship between yohimbine-induced enhancement of tritium efflux and noradrenaline-induced inhibition was to obtain first the control values for stimulation-induced efflux at 1 Hz and 5 Hz in both ureters taken from the same animal and then to administer yohimbine ($3 \times 10^{-7}$ M) followed 15 min later, without washout, by noradrenaline ($1.8 \times 10^{-6}$ M), to one of each pair. Fifteen minutes later and in the presence of the drugs the stimulation

**Table 1** Actions and interactions of yohimbine ($3 \times 10^{-7}$ M) and noradrenaline ($1.8 \times 10^{-6}$ M) on stimulation-induced $^3$H-transmitter efflux in guinea-pig ureter

| *Experimental group (n)* | *Hz* | *Transmitter efflux* ($\times 10^3$ d/min) 1st run ($S_1$) | 2nd run ($S_2$) | *Efflux ratio* $S_2/S_1$ | *% inhibition of efflux by noradrenaline* |
|---|---|---|---|---|---|
| a) Control (6) | 1 | 3.69±0.71 | 4.12±0.78 | 1.12±0.08 | |
| Noradrenaline (6) | 1 | 4.54±0.72 | 0.48±0.18 | 0.12±0.04** | (a) 88.6±5.4 |
| Control (6) | 5 | 5.36±1.02 | 5.21±1.08 | 0.96±0.06 | |
| Noradrenaline (6) | 5 | 5.47±0.90 | 2.11±0.70 | 0.42±0.04** | (b) 56.0±3.4 |
| b) Yohimbine (8) | 1 | 4.48±0.23 | 23.38±1.56 | 5.26±0.34 | |
| Yohimbine + noradrenaline (8) | 1 | 5.19±0.39 | 4.09±0.91 | 0.89±0.24** | (c) 84.3±3.5 |
| Yohimbine (8) | 5 | 5.86±0.26 | 25.52±1.76 | 4.36±0.24 | |
| Yohimbine + noradrenaline (8) | 5 | 6.78±0.71 | 10.84±0.95 | 1.81±0.30** | (d) 59.5±5.4 |

**$P < 0.001$ compared with ratio for corresponding group without noradrenaline. Ureters were exposed to noradrenaline ($1.8 \times 10^{-6}$ M) or to yohimbine ($3 \times 10^{-7}$ M) or to both drugs together after the initial period of stimulation at 1 Hz and 5 Hz with 100 pulses. This was followed, without washout, by a repetition of the stimulations ($S_2$). (a) vs (c) NS; (b) vs (d) NS.

cycle was repeated. Yohimbine alone induced a considerable increase in the efflux of tritium at both test frequencies in the preparations treated only with the alkaloid, as expected, and the effect was greater at 1 than at 5 Hz; namely ratios of $S_2$ versus $S_1$ of 5.3 and 4.4 times initial control values (Table 1).

To assess the magnitude of the inhibitory effect of noradrenaline it was essential that the percentage inhibition of efflux in the presence of the antagonist be compared with similar measurements done in the absence of antagonist. As shown clearly the catecholamine had a pronounced inhibitory effect on efflux in the presence of yohimbine and the percentage of the agonist-induced inhibition did not significantly differ from that obtained when the antagonist was not present (Table 1). In fact, the catecholamine eliminated entirely the yohimbine-induced increase in efflux at 1 Hz. Inhibition of efflux by noradrenaline was 88.6% in the absence and 84.3% in the presence of antagonist at 1 Hz and the values at 5 Hz were also almost identical to each other being 56.0% and 59.5% (Table 1).

When the concentration of noradrenaline was raised to $6.0 \times 10^{-6}$ M, a significantly greater inhibitory effect on stimulation-induced efflux was detected at 5 Hz but not at 1 Hz; namely reductions of $77.2 \pm 1.1\%$ (3 ureters) and $88.2 \pm 2.0\%$ (3 ureters) respectively, compared to matching control values. To ensure further that the present results were not obtained with a supramaximal concentration of agonist, the above-described protocol with yohimbine was repeated with a 10 fold lower concentration of noradrenaline (Table 2). The catecholamine at $1.8 \times 10^{-7}$ M decreased efflux significantly less than it did at $1.8 \times 10^{-6}$ M, at both of the test frequencies (Table 2). Yohimbine slightly but significantly antagonized the inhibitory effect of noradrenaline at the lower of the two test frequencies, as determined by comparison of percentage inhibition values (Table 2).

A still smaller concentration of noradrenaline ($1.8 \times 10^{-8}$ M) only slightly inhibited stimulation-induced efflux by $22.5 \pm 3.4\%$ (6 ureters) and $16.9 \pm 5.6\%$ (6 ureters) at 1 and 5 Hz. This confirmed that the concentrations employed in the present study were indeed on the steep portion of the concentration-effect curve. It was also established that the concentrations of noradrenaline required to inhibit stimulation-induced efflux of [$^3$H]-noradrenaline in ureters by 50% at 1 and 5 Hz are $6.6 \times 10^{-8}$ M and $9.7 \times 10^{-7}$ M.

*Oxymetazoline and yohimbine*

In other experiments, the inhibitory effect of oxymetazoline, which is regarded as a potent presynaptic agonist, was studied to assess its susceptibility to antagonism by yohimbine. The protocols employed were similar to those already described for noradrenaline. Ureters were exposed to oxymetazoline alone for 15 min before $S_2$; to yohimbine alone for 30 min before $S_2$, and when combination was used the agonist was present during the final 15 min of the 30 min exposure to yohimbine before $S_2$. All drug exposures were maintained throughout $S_2$. As shown in Tables 3 and 4, yohimbine ($3 \times 10^{-7}$ M) produced,

**Table 2** Actions and interactions of yohimbine ($3 \times 10^{-7}$ M) and noradrenaline ($1.8 \times 10^{-7}$ M) on stimulation-induced $^3$H-transmitter efflux in guinea-pig ureter

| *Experimental group (n)* | *Hz* | *Transmitter efflux* ($\times 10^3$ d/min) 1st run ($S_1$) | 2nd run ($S_2$) | *Efflux ratio* $S_2/S_1$ | *% inhibition of efflux by noradrenaline* |
|---|---|---|---|---|---|
| a) Control (7) | 1 | 6.52±0.97 | 5.80±0.82 | 0.90±0.04 | |
| Noradrenaline (7) | 1 | 6.67±0.50 | 1.60±0.14** | 0.25±0.03** | (a) 71.2±4.7 |
| Control (8) | 5 | 8.29±1.14 | 7.79±1.06 | 0.95±0.03 | |
| Noradrenaline (8) | 5 | 9.16±0.60 | 5.74±0.51* | 0.62±0.02** | (b) 34.5±3.1 |
| b) Yohimbine (7) | 1 | 7.12±1.23 | 23.33±3.29 | 3.48±0.40 | |
| Yohimbine + noradrenaline (7) | 1 | 7.47±0.84 | 16.27±2.47* | 2.02±0.31* | (c) 41.9±10.0 |
| Yohimbine (7) | 5 | 9.93±1.75 | 25.58±3.96 | 2.61±0.16 | |
| Yohimbine + noradrenaline (7) | 5 | 9.08±1.19 | 18.23±2.79* | 2.07±0.32* | (d) 25.2±10.7 |

**$P<0.001$ (*$P<0.05$) compared with ratio for corresponding group without noradrenaline. Ureters were exposed to noradrenaline ($1.8 \times 10^{-7}$ M) or to yohimbine ($3 \times 10^{-7}$ M) or to both drugs together after the initial period of stimulation at 1 Hz and 5 Hz with 100 pulses. This was followed, without washout, by a repetition of the stimulations ($S_2$). (a) vs (c) $P<0.05$; (b) vs (d) NS.

**Table 3** Actions and interactions of yohimbine ($3 \times 10^{-7}$ M) and oxymetazoline ($1.0 \times 10^{-7}$ M) on stimulation-induced $^3$H-transmitter efflux in guinea-pig ureter

| *Experimental group (n)* | *Hz* | *Transmitter efflux* ($\times 10^3$ d/min) 1st run ($S_1$) | 2nd run ($S_2$) | *Efflux ratio* $S_2/S_1$ | *% inhibition of efflux by noradrenaline* |
|---|---|---|---|---|---|
| a) Control (2) | 1 | 10.06±2.92 | 9.95±3.72 | 0.96±0.09 | |
| Oxymetazoline (3) | 1 | 12.33±2.96 | 3.65±1.47 | 0.28±0.06* | (a) 70.1±4.2 |
| Control (2) | 5 | 12.16±2.86 | 12.05±2.54 | 1.00±0.03 | |
| Oxymetazoline (3) | 5 | 15.60±2.20 | 13.42±2.73 | 0.85±0.05* | (b) 23.3±2.3 |
| b) Yohimbine (4) | 1 | 7.13±1.25 | 22.19±3.26 | 3.16±0.21 | |
| Yohimbine + oxymetazoline (4) | 1 | 8.07±1.46 | 9.20±2.41 | 1.11±0.12* | (c) 64.7±3.1 |
| Yohimbine (4) | 5 | 11.18±1.94 | 27.99±3.18 | 2.58±0.17 | |
| Yohimbine + oxymetazoline (4) | 5 | 11.62±2.96 | 20.43±5.34 | 1.76±0.05* | (d) 30.9±3.7 |

*$P<0.05$ compared with the ratio for the corresponding group without oxymetazoline. Ureters were exposed to oxymetazoline ($1.0 \times 10^{-7}$ M) or to yohimbine ($3 \times 10^{-7}$ M) or to both drugs together after the initial period of stimulation ($S_1$) at 1 Hz and 5 Hz with 100 pulses. This was followed, without washout, by a repetition of the stimulations ($S_2$). (a) vs (c) NS; (b) vs (d) NS.

by itself, a large increase in stimulation-induced efflux of [$^3$H]-noradrenaline but was not able to block the inhibitory effects of oxymetazoline ($1.0 \times 10^{-7}$ M or $1.0 \times 10^{-8}$ M), even though the lower concentration produced only a very modest reduction in transmitter efflux. In two other preparations it was determined that oxymetazoline, at the slightly lower concentration of $3.4 \times 10^{-9}$ M had a negligible effect on efflux; namely reductions of 15.1% and 0.0% at 1 and 5 Hz, confirming that the agonist effects produced were on the steep portion of the concentration-response curve.

*Spontaneous efflux*

The basal or spontaneous efflux of tritium, measured immediately before each stimulation and averaged, declined very slightly but significantly between first and second runs in the group of untreated ureters represented in Table 1. The values were 9.3± $1.7 \times 10^3$ and $6.7 \pm 1.2 \times 10^3$ d/min, respectively. In ureters exposed to noradrenaline ($1.8 \times 10^{-6}$ M) between runs, an intrastrip comparison of ratios showed that the extent of the decline was slightly but significantly less: the mean ratio of spon-

**Table 4** Actions and interactions of yohimbine ($3 \times 10^{-7}$ M) and oxymetazoline ($1.0 \times 10^{-8}$ M) on stimulation-induced $^3$H-transmitter efflux in guinea-pig ureter

| *Experimental group (n)* | *Hz* | *Transmitter efflux* ($\times 10^3$ d/min) 1st run ($S_1$) | 2nd run ($S_2$) | *Efflux ratio* $S_2/S_1$ | *% inhibition of efflux by noradrenaline* |
|---|---|---|---|---|---|
| a) Control (4) | 1 | 3.96±0.32 | 3.64±0.22 | 0.93±0.05 | |
| Oxymetazoline (4) | 1 | 2.56±0.60 | 1.90±0.58 | 0.70±0.08* | (a) 25.1±7.4 |
| Control (4) | 5 | 5.12±0.18 | 5.27±0.23 | 1.03±0.06 | |
| Oxymetazoline (4) | 5 | 3.71±0.81 | 3.67±0.98 | 0.96±0.07 | (b) 8.6±5.2 |
| b) Yohimbine (6) | 1 | 3.10±0.50 | 12.31±2.37 | 3.87±0.31 | |
| Yohimbine + oxymetazoline (6) | 1 | 2.58±0.23 | 6.43±1.37 | 2.59±0.48* | (c) 31.5±13.2 |
| Yohimbine (6) | 5 | 4.63±0.99 | 13.82±2.59 | 3.22±0.17 | |
| Yohimbine + oxymetazoline (6) | 5 | 3.42±0.35 | 8.04±1.70 | 2.41±0.43* | (d) 27.5±12.8 |

*$P<0.05$ compared with the ratio for the corresponding group without oxymetazoline. Ureters were exposed to oxymetazoline ($1.0 \times 10^{-8}$ M) or to yohimbine ($3 \times 10^{-7}$ M) or to both drugs together after the initial period of stimulation ($S_1$) at 1 Hz and 5 Hz with 100 pulses. This was followed, without washout, by a repetition of the stimulations ($S_2$). (a) vs (c) NS; (b) vs (d) NS.

taneous efflux in second versus first periods of stimulation was $0.73 \pm 0.02$ and $0.82 \pm 0.04$ for the matching untreated and noradrenaline-treated ureters. Yohimbine had no significant effect on spontaneous efflux; the second run versus first run ratio was $0.83 \pm 0.04$ and in the group treated with both noradrenaline ($1.8 \times 10^{-6}$ M) and yohimbine between runs the value was $0.90 \pm 0.02$; the latter ratio was not significantly different from that of the group treated only with noradrenaline. In other experiments it was determined that oxymetazoline had no effect on basal efflux: the mean ratios for second versus first stimulation periods were $0.77 \pm 0.02$ and $0.77 \pm 0.01$ in matching untreated and oxymetazoline ($1.0 \times 10^{-7}$ M)-treated preparations.

## Discussion

From the results described here it appears likely that two discrete mechanisms and sites of action are involved for yohimbine as antagonist and noradrenaline and oxymetazoline as presynaptic agonists. The antagonist-mediated action culminates in increased transmitter efflux during stimulation and the agonist-mediated action in a reduction of efflux. Successful blockade of α-sites by the supposed selective antagonist, according to presynaptic theory, was demonstrated by the impressive magnitude of the enhancement of transmitter efflux. This was not accompanied by a commensurate, or for that matter, by any amount of antagonism of the effects of exogenous oxymetazoline and only in one instance, with 1 Hz at $1.8 \times 10^{-7}$ M, was the effect of noradrenaline even diminished. This is especially surprising since the concentration of endogenously released noradrenaline in the neuroeffector gap during stimulation is supposedly greater than that achieved by the amounts of exogenous amine used in the present experiments.

It is important to note that the inhibitory effect of noradrenaline and oxymetazoline on efflux, in the presence of yohimbine, can only be quantified with reference to efflux values for control matching preparations determined in the presence of antagonist and not by comparison with those of untreated control values. The latter comparison might, by not taking enhancement into account, yield the misleading conclusion that blockade of inhibition was successful.

Previous workers did not study in any precise way the relation between antagonist and agonist effect. Starke (1972) showed that noradrenaline (10 ng/ml) had no inhibitory effect on efflux in the presence of 1 μg/ml of phenoxybenzamine in the rabbit heart. Additionally, Starke, Montel, Gayk & Merker (1974) and Starke *et al.* (1975a) using the rabbit pulmonary artery reported that a very high concentration of phentolamine ($1 \times 10^{-5}$ M) 'shifted the presynaptic dose-response curves for noradrenaline and oxymetazoline to the right'. Enhancement by antagonists was not especially studied in these experiments and the concentrations of antagonist used may have been too high to allow separation of multiple presynaptic effects.

Contrary to the demands of theory, the two effects of enhancement and inhibition of transmitter efflux cannot be described adequately solely within a framework which depicts the relationship as that of agonist and antagonist acting at a common presynaptic adrenoceptor locus. It appears from the present study and from several other recent studies (Kalsner, 1979a,b; 1980a,b; Chan & Kalsner, 1979a,b; Kalsner & Chan, 1979; 1980; Kalsner, Suleiman & Dobson, 1980; Kalsner, 1981) that a model of perineuronal regulating sites identified as α- and β-negative and positive feedback systems is not a satisfactory explanation for the available experimental data.

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