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The determination of presynaptic pA_2 values of yohimbine and phentolamine on the perfused rat heart under conditions of negligible autoinhibition

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1 Rat isolated perfused hearts with the right sympathetic nerves attached were loaded with [^{3}H]-(−)-noradrenaline. The nerves were stimulated with up to 40 trains of 10 pulses every min at 1 Hz, and the evoked increases of [^{3}H]-noradrenaline overflow into the perfusate, of right atrial tension development and ventricular beating frequency were measured.

2 Oxymetazoline inhibited the evoked transmitter overflow (IC_{50}: 10 nM) and decreased the postsynaptic responses in a concentration-dependent manner. It behaved as a full agonist in abolishing the evoked transmitter overflow.

3 Yohimbine up to 1 μM neither enhanced the evoked [^{3}H]-noradrenaline overflow nor the postsynaptic parameters. Phentolamine (1 μM) caused a transient, minor ($< 30\%$) increase in [^{3}H]-noradrenaline overflow.

4 Yohimbine (0.03–1.0 μM) and phentolamine (0.1–5.0 μM) shifted to the right the concentration-response curve of oxymetazoline for the inhibition of [^{3}H]-noradrenaline overflow in response to nerve stimulation without depressing the maxima. The pA_2 values were 7.82 and 7.52, respectively.

5 Yohimbine (0.1 μM) also antagonized the decrease induced by oxymetazoline in the postsynaptic responses to nerve stimulation.

6 The results confirm the existence of presynaptic inhibitory α_2-adrenoceptors at the adrenergic nerve fibres of the rat heart *in vitro*. Under the stimulation and perfusion conditions selected, the released endogenous transmitter apparently does not activate a negative feedback mechanism, thus permitting the determination of pA_2 values.

Introduction

Receptors with a pharmacological function are reliably classified by the affinities of antagonists. A large difference in the equilibrium affinity constant (K_B) of an antagonist tested against the same agonist in various organs strongly argues for the existence of different receptor subtypes (Furchgott, 1972). A measure of the affinity of a given antagonist is its pA_2 value (Arunlakshana & Schild, 1959) which, if the law of a bimolecular mass interaction between agonist and antagonist with the receptor is observed, represents the equilibrium dissociation constant of the antagonist-receptor complex.

pA_2 values of α-adrenoceptor antagonists for the presynaptic adrenoceptors at the sympathetic nerves were determined in superfused pulmonary artery strips (Starke, Montel, Gayk & Merker, 1974) and brain slices (Wemer, van der Lugt, de Langen & Mulder, 1979). The organs were incubated with [^{3}H]-noradrenaline, and the inhibition of the tritium overflow by exogenous agonists was measured. An increase, however, of the evoked transmitter overflow by the antagonists indicated that a feedback inhibition by released endogenous agonist was already present. Thus, the effect of the exogenous agonist must have interfered with that of the endogenous agonist. This appears similarly true for the studies in which the twitch response of the rat vas deferens was used as a measure of presynaptic receptor activation, since α-antagonists frequently enhance the twitch response (for review see Wilkberg, 1979, Doxey & Roach, 1980).

However, the modulation of the [^{3}H]-

noradrenaline overflow in response to nerve stimulation is the most stringent indicator for presynaptic receptors (Starke, 1977). In the following paper we give pA_2 values for phentolamine and yohimbine antagonizing the oxymetazoline-induced inhibition of [^{3}H]-noradrenaline overflow from the rat heart sympathetic nerves. The pA_2 values were obtained under conditions where yohimbine did not enhance the stimulation-evoked overflow, thus indicating a lack of feed-back inhibition by endogenous transmitter in the rat perfused heart.

Methods

Perfusion of rat hearts and stimulation of the [^{3}H]-noradrenaline release after labelling

A detailed description of the preparation and the labelling procedure has been given previously (Fuder, Rink & Muscholl, 1982a). The method and some modifications will be mentioned only briefly. Hearts of male Wistar rats (230–315 g) with the right sympathetic nerves attached were perfused via the coronary arteries according to the Langendorff technique at a rate of 7 ml/min. The perfusion medium (Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, D-glucose 5.6, and (+)-ascorbic acid 0.057) was gassed with 5% CO_2 in O_2 and kept at 34.4°C. The perfusion pressure was recorded continuously. It was 48–113 mmHg immediately before the first nerve stimulation in the absence of a drug and tended to increase in the following 50 min by 5–34 mmHg. The mean increases did not differ between treatment groups. A severe tissue oedema as the cause of the increased resistance can be ruled out by the observation that the water content was 81 ± 1.7% of the heart wet weight after 6 min of perfusion (mean ± s.e.mean; $n = 4$, number of hearts), and 82 ± 1.6% ($n = 4$) after perfusion for 3 h. A decrease in ventricular systolic tension development is accompanied by an increase in perfusion pressure (Fuder *et al.*, 1982a), and the deterioration of the mechanical function of the heart in the course of the experiment might result in increased vascular resistance. Except for [^{3}H]-noradrenaline during the labelling procedure, the drugs present in the medium did not cause significant changes in the perfusion pressure.

The $CaCl_2$ content of the perfusion medium was reduced to 0.45 mM for 30 min, starting 10 min before addition of [^{3}H]-noradrenaline. (−)-[7-^{3}H]-noradrenaline (2.7 Ci/mmol, sp. act.) was infused for 10 min at a final concentration in the aorta of 16.3 nM. The first nerve stimulation was started 60 min after the end of the infusion, i.e. 50 min after changing back to perfusion medium containing 1.8 mM $CaCl_2$. The interval ensured a nearly monophasic exponential decline of unstimulated ^{3}H-release.

The right sympathetic nerves were stimulated postganglionically (Fuder, Siebenborn & Muscholl, 1982b) by platinum ring electrodes at a current strength of 20–30 mA adjusted individually to obtain maximal postsynaptic responses. The current was controlled by a constant current unit and the impulse flow was monitored by an oscilloscope. The pulses were delivered by a Grass S 11 Stimulator adjusted to produce trains of 10 pulses (1 ms pulse duration) at a frequency of 1 Hz and at 1 min intervals between the first pulses of two subsequent trains. Up to 40 trains were applied.

Before, during and after release was induced, 2 min samples of the perfusate were collected into vials containing 1 M HCl (to adjust the pH to 2–3) and 1 mg ascorbic acid. After removing 1 ml of the fluid to determine the total tritium content, a mixture of unlabelled noradrenaline (NA), 3-methoxy-4-hydroxymandelic acid (VMA), 3-methoxy-4-hydroxy-phenylglycol (MOPEG), normetanephrine (NMN), 3,4-dihydroxy-phenylglycol (DOPEG) and 3,4-dihydroxymandelic acid (DOMA) was added as carrier for the column chromatography. [^{3}H]-noradrenaline was separated from its tritiated metabolites according to Graefe, Stefano & Langer (1973). Since NMN represents only 5% or less of the total tritium (Fuder *et al.*, 1982b), no attempts were made to separate it from MOPEG plus VMA.

The values were corrected for minor cross contaminations (Fuder *et al.*, 1982a) and expressed as pmol referring to the specific activity of [^{3}H]-noradrenaline infused. Tritium was measured by scintillation counting. The basal [^{3}H]-noradrenaline overflow, determined from two samples both before the start and after the end of nerve stimulation, did not differ significantly although a tendency to a small decline was visible in the majority of the experiments. The values of spontaneous [^{3}H]-noradrenaline overflow before and after the stimulation series were displayed graphically, and for each collection period an interpolated individual basal overflow was subtracted from the total [^{3}H]-noradrenaline content to calculate the stimulation-evoked overflow. Since 2 min samples were collected, each contained the amounts of noradrenaline released by two consecutive pulse trains. More than 95% of the noradrenaline is washed out within 1 min after the end of the stimulation. Thus, the contamination of noradrenaline in a given collection period by that released by the preceding pair of trains is small.

Characterization of presynaptic α-adrenoceptors

The inhibition by exogenous agonists of the stimulation-evoked [^{3}H]-noradrenaline overflow was considered a measure of activation of inhibitory presynaptic receptors. A similar procedure was previously used to assess the affinity constants of atropine-like drugs towards inhibitory presynaptic muscarinic receptors in the rat heart (Fuder *et al.*, 1982a).

In one series of control experiments the decline in the evoked [^{3}H]-noradrenaline overflow upon repeated stimulation was established, and the effect of nerve stimulation for each collection period was expressed as a percentage of the sum of stimulation-evoked [^{3}H]-noradrenaline overflow of the fifth plus sixth train (SNS 5 + 6). To test the effects of the α-adrenoceptor antagonists yohimbine (0.1 and 1 μM) and phentolamine (1 μM) on the evoked overflow, the perfusion fluid for the hearts was changed to one containing the drug at the respective concentration 10 s before the seventh pulse train and kept for the following 26 trains (13 collection periods). The mean evoked overflow (expressed as % of SNS 5 + 6) in each period was compared with that in the absence of a drug. In a third series, oxymetazoline at concentrations increasing by a factor of 10 was added to the medium. Each concentration (up to 4) was maintained for 4–8 min at which time the respective maximum effect (both in the absence and presence of an antagonist) had been attained.

The reversibility of the decrease was always checked to preclude artefacts due to decay of the nerve function. The concentration-response curves were constructed by dividing the individual overflow (% of SNS 5 + 6) at the maximum oxymetazoline effect (for each concentration) by the mean relative overflow observed in the absence of any drug in the respective sample of perfusate, and by expressing the decrease as % of the maximum inhibition. The individual concentration of half-maximal inhibition (IC_{50}) was estimated graphically from a semilogarithmic plot. The geometric mean IC_{50} was calculated and used to determine the dose-ratio (defined as IC_{50} of oxymetazoline in the presence over the IC_{50} in the absence of antagonist).

Concentration-response curves for oxymetazoline were also obtained in the presence of yohimbine (0.03, 0.1, 1 μM) and phentolamine (0.5, 1.0, 5.0 μM). Except at the lowest concentration of yohimbine (added 40 min before the nerve stimulation) the antagonists were added 20 min before the first nerve stimulation and kept throughout. As indicated above, the respective individual IC_{50} in the presence of the antagonist concentrations was estimated and for each heart a dose-ratio was calculated.

The pA_2 values for the antagonists were determined according to Arunlakshana & Schild (1959) using the equation: $pA_2 = pA_x + \log(\text{dose-ratio} - 1)$; pA_x being the − log molar antagonist concentration and pA_2 being the − log K_B of the receptor-antagonist-complex.

Postsynaptic responses to nerve stimulation

The right atrial and ventricular tension development was recorded transversely (Fuder *et al.*, 1982a). The preload was adjusted individually to obtain optimal recordings (atria: 1.2–1.6 mN, ventricles: 2–7 mN). A rate meter was triggered from the ventricular action and the ventricular beating frequency was recorded. The values of atrial tension development and heart rate before and 6–10 min after the train series (i.e. under resting conditions) could be connected by a line in the recording, and a basal value for each stimulation period was subtracted from the maximum stimulation-evoked response. Thus, in addition to the relevant direct parameter of [^{3}H]-noradrenaline release, indirect postsynaptic parameters for transmitter release were obtained. In the rat heart, however, the transverse recording method yields satisfactory tracings only in about half of the experiments because the ventricular contractions often distort the atrial tension recordings. For these technical reasons the number of observations was smaller than for the noradrenaline overflow, and results are given for only a few treatment groups. We evaluated the postsynaptic responses to nerve stimulation only with regard to the effects of yohimbine 0.1 μM, since the postsynaptic blockade by high concentrations of phentolamine of the postsynaptic responses to nerve stimulation and exogenous noradrenaline in the guinea-pig heart (Langer, Adler-Graschinsky & Giorgi, 1977) prevents a meaningful conclusion on the underlying presynaptic mechanisms.

Extraction of ^{3}H-compounds from the heart

Most of the hearts (mean wet weight at the end of the perfusion: 0.94 ± 0.01 g, $n = 59$) were cut into pieces and immersed three times in 10 ml $HClO_4$ (0.4 M) for more than 30 min each. After 2 periods of extraction, only 1.6 ± 0.36% ($n = 5$) of the tritium remained in the pieces as shown by measuring tritium in the extract after homogenization and centrifugation (Fuder *et al.*, 1982a) of the pre-extracted pieces of heart. Using the present procedure, about 94% of the tritium was recovered as unchanged noradrenaline.

The sum of the tritium determined from aliquots of the three extracts and expressed in terms of [^{3}H]-noradrenaline of the above specific activity was

137 ± 7.9 pmol/g wet weight ($n = 54$). The following drugs were used: oxymetazoline hydrochloride (Merck), phentolamine mesylate (Ciba-Geigy), yohimbine hydrochloride (Roth). The drugs were dissolved in distilled water. (−)-[7-^{3}H]-noradrenaline (NEN) was diluted with 0.9% w/v NaCl solution (saline) immediately before the infusion.

Statistics

Results are given as the mean ± s.e.mean or with the 95% confidence limits, and statistical significance was examined by use of Student's *t* test and, if more than one group of treatments was compared with one control group, by analysis of variance followed by Dunnett's test (Dunnett, 1964). Regression lines of the Arunlakshana-Schild plots were calculated by the least squares method. Slopes of the lines in the plots were tested for difference from unity according to Sachs (1974). The 95% confidence limits of the pA_2 values were calculated according to Documenta Geigy (1973).

Results

Effects of sympathetic nerve stimulation

The basal overflow of [^{3}H]-noradrenaline in two subsequent collection periods both before and after nerve stimulation accounted for roughly 15% of the total tritium (Table 1). Neither yohimbine nor phentolamine affected the basal overflow when added before nerve stimulation, in agreement with earlier reports on the effect of phentolamine on the resting [^{3}H]-noradrenaline overflow from the rabbit pulmonary artery (Starke *et al.*, 1974). Oxymetazoline did not suppress the evoked overflow below the interpolated (see Methods) basal overflow. The evoked [^{3}H]-noradrenaline overflow in the absence of a drug varied depending on the effectiveness of nerve stimulation and was 0.284 ± 0.053 pmol/2 min ($n = 12$) in the sample containing SNS 5 + 6 (which was always arbitrarily set as 100%). This corresponded to a 7 ± 1.5 fold increase in transmitter overflow ($n = 12$). Thus [^{3}H]-noradrenaline made up about 50% of the sum of the metabolites plus noradrenaline.

The contribution of ^{3}H-metabolites was derived from representative experiments in the absence of drugs (Table 1). MOPEG plus VMA plus NMN on one hand, and DOPEG on the other hand, represented approximately 40% each, and DOMA 4% of the sum of all the metabolites under resting conditions. The percentage contribution was only half as much in the sample of SNS 5 + 6. As shown previously for stronger stimuli (180 pulses, 3 Hz, Fuder *et al.*, 1982b) the nerve stimulation in the perfused rat heart is not followed by considerable increases in the overflow of metabolites. Most of the stimulation-evoked tritium release appears to be due to an increase in unmetabolized noradrenaline (Table 1). This is also true for all the other collection periods with nerve stimulations either before or after SNS 5 + 6 (not shown).

In the experiments with agonist and/or antagonists the pattern of metabolites was checked only occasionally to preclude changes in the rates of metabolism. The total tritium content of the samples, how-

Table 1 ^{3}H-compounds (pmol/2 min) in the overflow of the rat isolated heart before, during and after sympathetic nerve stimulation (SNS) in the absence of drugs

Time before or after SNS 1 (min)	Before SNS 4–2	Before SNS 2–0	SNS 5 + 6 4–6	After SNS 40–42	After SNS 42–44
MOPEG + VMA + NMN ($n = 3$)	0.11 + 0.014	0.12 + 0.017	0.13 + 0.035	0.10 + 0.001	0.09 + 0.002
DOPEG ($n = 5$)	0.12 + 0.028	0.12 + 0.028	0.13 + 0.033	0.11 + 0.025	0.11 + 0.026
DOMA ($n = 3$)	0.01 + 0.001	0.01 + 0.002	0.01 + 0.002	0.01 + 0.002	0.01 + 0.003
Noradrenaline ($n = 12$)	0.05 + 0.006	0.05 + 0.005	0.31 + 0.051*	0.04 + 0.009	0.04 + 0.009

The perfused rat hearts were loaded with [^{3}H]-(−)-noradrenaline (16.3 nM) for 10 min. The first nerve stimulation (SNS 1) started after a washout period of 60 min. SNS consisted of successive trains of 10 pulses at 1 Hz and 1 min intervals. The overflow was collected in 2 min samples. The ^{3}H-metabolites were separated from [^{3}H]-noradrenaline by column chromatography. The values *before SNS* represent the collection periods from 4–2 min and 2–0 min before SNS 1. The ^{3}H-compounds of *SNS 5 + 6* (as reference sample for all the subsequent stimulations) represent values which are typical for all the following collection periods (not shown). Samples *after SNS* were collected 0–2 min and 2–4 min after the last SNS. Each value is a mean ± s.e.mean. The number of observations is given by *n*. The asterisk indicates a significant difference from the sample 2 − 0 min before SNS ($P < 0.0001$). MOPEG = 3-methoxy-4-hydroxy-phenylglycol; VMA = 3-methoxy-4-hydroxymandelic acid; NMN = normetanephrine; DOPEG = 3, 4-dihydroxy-phenylglycol; DOMA = 3, 4-dihydroxymandelic acid.

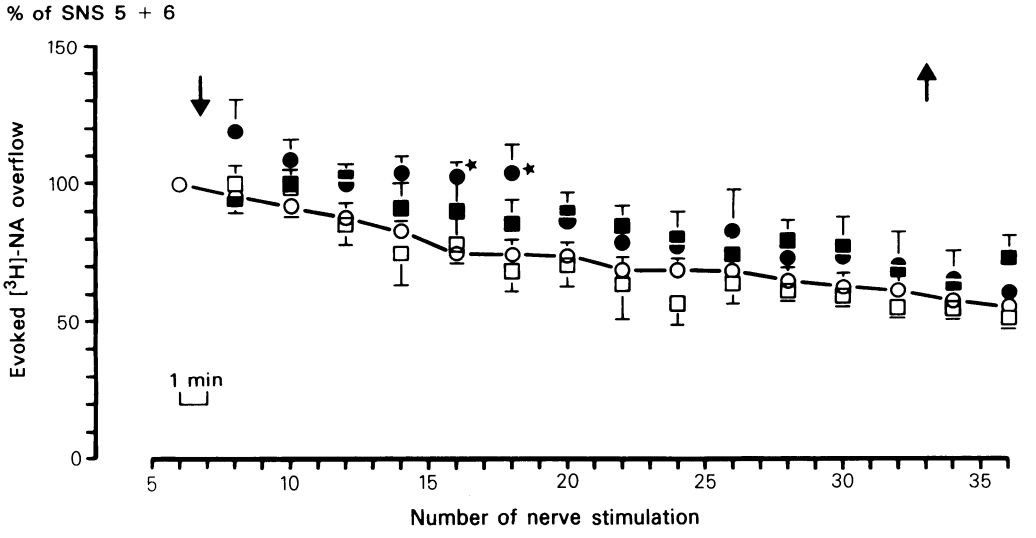

Figure 1 The effects of antagonists on the evoked [^{3}H]-noradrenaline ([^{3}H]-NA) overflow in response to sympathetic nerve stimulation (SNS, trains of 10 pulses at 1 Hz and 1 min intervals) in the perfused rat heart. For labelling procedure see Methods. The overflow was collected in 2 min samples. Perfusion with drugs started before SNS 7 and ended after SNS 32 as indicated by arrows. The overflow in a given sample is expressed as a percentage of SNS 5 + 6. Symbols: no drug (○, connected by a line, $n = 7$, number of observations), yohimbine 0.1 μM (□, $n = 3$) and 1 μM (■, $n = 5$), phentolamine 1 μM (●, $n = 4$). Each point represents a mean value and the vertical lines show s.e.mean. Asterisks indicate significant differences from the corresponding sample without drug (Dunnett's test: * $P < 0.05$). The absolute values of stimulation-evoked overflow at SNS 5 + 6 (pmol [^{3}H]-noradrenaline/sample) in the absence of a drug did not differ significantly from any group where antagonists were added.

ever, was always determined, although it represented a far less sensitive parameter of transmitter release than [^{3}H]-noradrenaline which is concentrated about 14 fold by the column chromatography.

Effects of antagonists on the evoked [^{3}H]-noradrenaline overflow

Yohimbine 0.1 and 1 μM did not significantly enhance the evoked [^{3}H]-noradrenaline overflow during an exposure time of 26 min (Figure 1). In contrast, phentolamine 1 μM caused a small and transient increase in [^{3}H]-noradrenaline in the perfusate which became significant only for the periods of 8–12 min exposure time, but not afterwards. It is unlikely that this effect of phentolamine is due to blockade of an inhibitory α-adrenergic feedback mechanism since yohimbine failed to increase the overflow of [^{3}H]-noradrenaline. When yohimbine 1 μM or phentolamine 5 μM (as the highest concentrations of antagonists in the experiments for the presynaptic Arunlakshana-Schild plot) was added 24 min before the control stimulation period, SNS 5 + 6 caused an evoked overflow of 0.18 ± 0.033 ($n = 3$) and 0.22 ± 0.023 ($n = 3$) pmol, respectively. This is only 63 and 77% of that observed in the absence of drugs, so that no increase in transmitter overflow can be derived from these values. These results indicate that the increase by antagonists of the evoked [^{3}H]-noradrenaline overflow, if present at all, must be small under our stimulation conditions in the perfused rat heart.

Effects of oxymetazoline on the evoked [^{3}H]-noradrenaline overflow

Oxymetazoline decreased the evoked [^{3}H]-noradrenaline overflow in a concentration-dependent manner (Figure 2). The inhibition was slowly reversible (not shown). The maximum inhibition observed was $87 \pm 7.5\%$ ($n = 5$) at 0.5 μM and 100% ($n = 2$) at 5 μM. The geometric mean of the IC_{50} was 10 nM (4–23.5 nM, 95% confidence limits, $n = 5$). Phentolamine (0.1–5 μM) shifted the concentration-response curve to the right without depressing the maxima (Figure 2). Yohimbine (0.03–1 μM), like phentolamine, shifted the concentration-response curve of oxymetazoline to the right in a nearly parallel fashion without depression of the maxima. The shifts by phentolamine and yohimbine of the IC_{50} for oxymetazoline are illustrated by presynaptic Arunlakshana-Schild plots (Figure 3). The slopes of the regression lines are not different from unity. The pA_2 value of yohimbine is

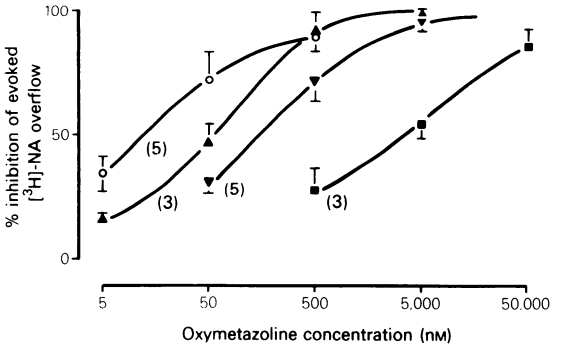

Figure 2 The effects of oxymetazoline on the [^{3}H]-noradrenaline ([^{3}H]-NA) overflow in response to stimulation (trains of 10 pulses, 1 Hz, 1 min intervals) of the extrinsic sympathetic nerves in the rat heart. From any one heart, one cumulative concentration-response curve was obtained by adding oxymetazoline at the respective concentration to the perfusion medium. The onset of the oxymetazoline effect was followed and equilibrium conditions were observed for the agonist in the absence and presence of the antagonist. The overflow in the sample with the maximum inhibition for a given concentration was expressed as % of SNS 5 + 6 and divided by the mean ratio for the corresponding sample in the absence of the agonist. The inhibition was expressed as % of maximum inhibition. If present, phentolamine perfusion started 26 min before oxymetazoline was added for the first time. Symbols: (○, Controls) no antagonist; (▲) 0.1, (▼) 0.5, (■) 5 μM phentolamine. Mean of the number of observations given in parentheses; s.e.mean shown by vertical lines.

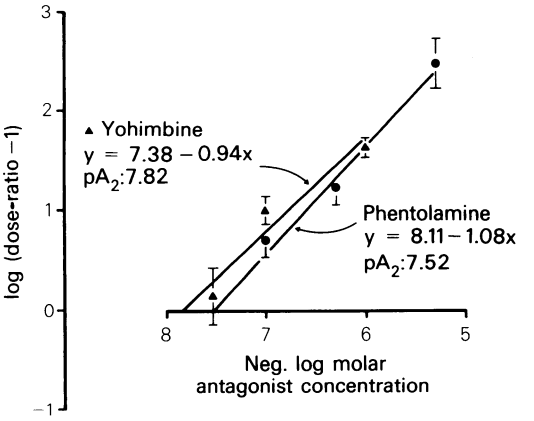

Figure 3 The antagonism by phentolamine and yohimbine of the oxymetazoline-induced inhibition of evoked [^{3}H]-noradrenaline overflow in the perfused rat heart (Arunlakshana-Schild plot for presynaptic α-adrenoceptors). Abscissa scale, negative log antagonist concentration (M); ordinate scale, log (dose-ratio − 1), dose-ratio defined as an individual IC_{50} of oxymetazoline in the presence of the respective antagonist concentration over the geometric mean IC_{50} in its absence (10 nM). Symbols (each derived from 3–5 hearts): (▲) yohimbine, (●) phentolamine, means, with s.e.mean as vertical lines. Equations of the best fit line computed by linear regression analysis of the individual values are inserted (correlation coefficients, $P < 0.001$). The slopes of the lines do not differ from unity.

7.82 (8.8–7.4, $n = 11$), and that of phentolamine, 7.52 (8.3–7.2, $n = 10$).

Postsynaptic responses to sympathetic nerve stimulation

Right atrial tension development and ventricular rate of some of the hearts in which the overflow of [^{3}H]-noradrenaline was determined were recorded (Figures 4 and 5). The resting tension development before the 6th, 10th, and 32nd nerve stimulation was 0.65 ± 0.13, 0.64 ± 0.13, and 0.55 ± 0.11 mN (each $n = 8$), respectively, when no drug was added, and 0.84 ± 0.18, 0.81 ± 0.17, and 0.65 ± 0.11 mN (each $n = 4$), respectively, when yohimbine 0.1 μM was added after the 6th stimulation. The resting beating frequency before the 6th, 10th, and 32nd nerve stimulation was 185 ± 12, 184 ± 12, and 184 ± 13 beats per min (each $n = 8$), respectively, when no drug was added, and 170 ± 6, 170 ± 6, and 173 ± 11 beats per min (each $n = 4$), when yohimbine 0.1 μM was added after the 6th nerve stimulation. Thus, this low concentration of yohimbine did not affect either the basal tension development or the basal heart rate.

The increases above resting values by nerve stimulation of the atrial muscular activity were similar in the absence (Figure 4a) and presence of yohimbine (either when preperfused for 26 min or when added before SNS 7, both in Figure 4b). Oxymetazoline significantly decreased the response to nerve stimulation (Figure 4a). Higher concentrations of oxymetazoline were needed for the same degree of inhibition in the presence of yohimbine 0.1 μM (Figure 4b). The effect of oxymetazoline was slowly reversible (Figures 4 and 5).

Apparently yohimbine 0.1 μM did not enhance to a significant extent the frequency-response to nerve stimulation although a tendency to a slight increase existed (Figure 5). As expected, oxymetazoline 0.5 μM nearly abolished the frequency-response (Figure 5a) and yohimbine 0.1 μM antagonized this effect (Figure 5b). The postsynaptic responses, thus, confirm the results of the [^{3}H]-noradrenaline overflow studies obtained in one and the same heart: Although oxymetazoline abolished both [^{3}H]-noradrenaline overflow and the postsynaptic responses upon sympathetic nerve stimulation, yohimbine did not enhance either the pre- or postsynaptic parameters. Nevertheless, yohimbine antagonized the inhibitory

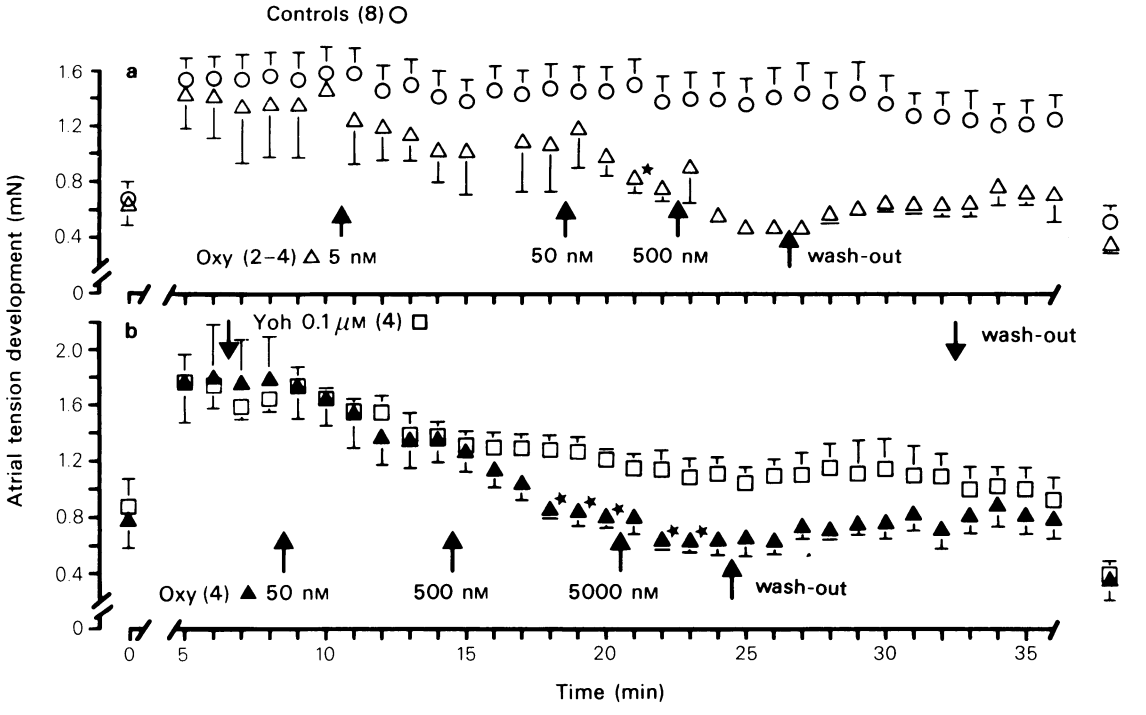

Figure 4 The effects of sympathetic nerve stimulation (see Figure 1) on the right atrial tension development (in mN) of the perfused rat heart. The time (min) indicates simultaneously the number of the train applied in this period. Symbols (mean, with s.e.mean as vertical lines) at 0 and beyond 36 represent spontaneous values before and 6–10 min after the train series, symbols in between are increases (above resting state) in response to nerve stimulation in the presence and/or absence of oxymetazoline and/or yohimbine: (○, Controls) no drug; (△) oxymetazoline (Oxy) in the absence and (▲) in the presence of yohimbine (Yoh) (0.1 μM preperfused for 26 min); (□) yohimbine 0.1 μM. Drugs at the respective concentrations were added and washed out as shown by arrows. Figures in parentheses are the numbers of observations ($n < 3$, if no s.e. is shown). Significant differences between corresponding values of the same panel (only tested before washout) are indicated by asterisks ($P < 0.05$). Basal values between nerve stimulations were unaffected (see Results) and omitted for clarity.

effects of oxymetazoline on pre- and postsynaptic responses to nerve stimulation.

Discussion

The perfused rat heart as a tool to study adrenergic release mechanisms

Perfused organs with the microcirculation intact are used only rarely in the current research on transmitter release from the autonomic nervous system (Starke, 1977; Gillespie, 1980), although they appear to provide more physiological conditions than incubated tissues. The rat isolated perfused heart with the sympathetic nerves attached is a useful tool to study the presynaptic modulation of the adrenergic transmitter release (Fuder *et al.*, 1982a, b; and present paper). Both, [^{3}H]-noradrenaline overflow and postsynaptic responses to nerve stimulation can be used as parameters of presynaptic activity. However, we must emphasize that the perfused rat heart differs considerably from commonly used tissue preparations in two aspects: (i) lack of major extraneuronal and intraneuronal metabolism of [^{3}H]-noradrenaline released in response to nerve stimulation (Fuder *et al.*, 1982a,b; Table 1 present paper); (ii) elimination of released transmitter from the synaptic cleft by neuronal reuptake (roughly 50%) and by very rapid washout into the perfusion fluid (Fuder *et al.*, 1982b). Both observations are readily explained by short diffusion distances for the transmitter to reach the capillaries, and by the effective drainage of the 'biophase' (the spaces around the terminal fibre and/or other parts of the nerve where release modulating processes occur). As we have suggested earlier (Fuder *et al.*, 1982a) the low metabolism is in part due to labelling of the rat heart with minute concentrations of [^{3}H]-noradrenaline. This is confirmed by

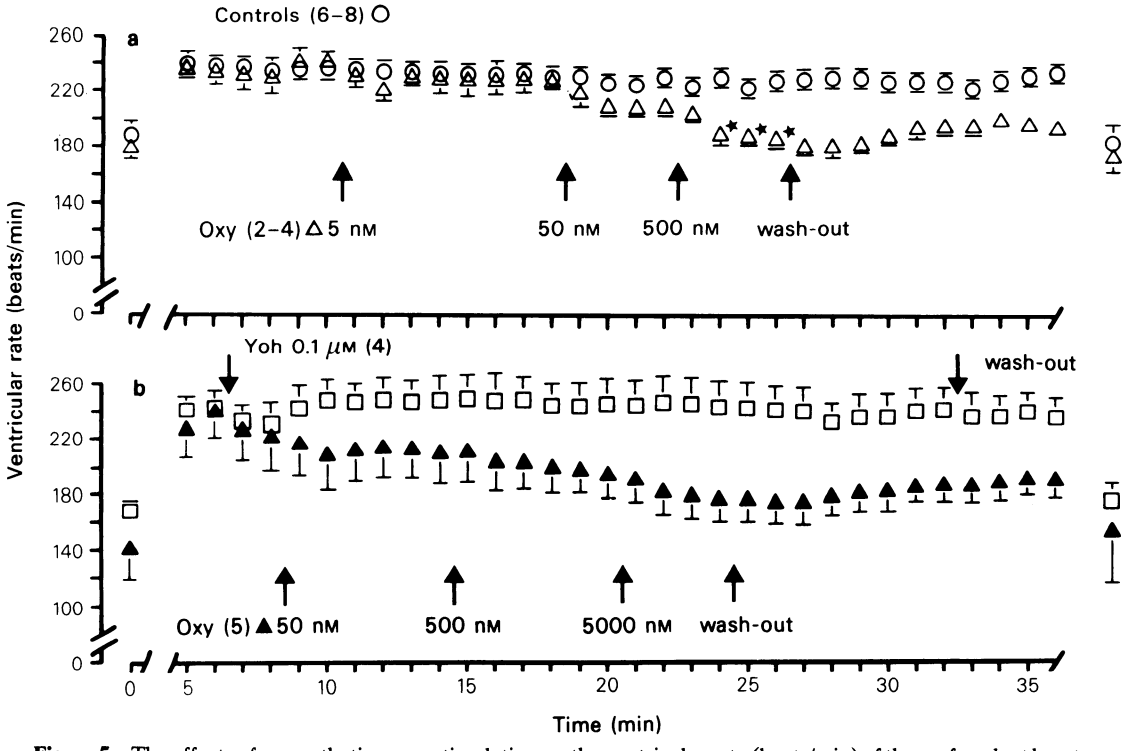

Figure 5 The effects of sympathetic nerve stimulation on the ventricular rate (beats/min) of the perfused rat heart. For explanation see Figure 4.

the finding of Majewski, Hedler, Steppeler & Starke (1982) that in the perfused rabbit heart a high rate of formation of DOMA is a consequence of the contact of the organ with high concentrations of exogenous noradrenaline.

Autoinhibition in the perfused heart has been demonstrated previously. Thus in the isolated heart of the rabbit, phentolamine enhanced the noradrenaline overflow evoked by stimulation of the sympathetic nerves with 300 pulses at 5 Hz at concentrations (1 μM) that did not block the neuronal reuptake (Starke, Montel & Wagner, 1971). The frequency-response of the rabbit isolated perfused heart to nerve stimulation with 15 pulses at 3.2 Hz was potentiated by yohimbine, but not that with about 5 pulses at 0.32 Hz (Carr & Fozard, 1981). However, the modulation of the frequency-response reflects, at best, the degree of autoinhibition occurring on nerve fibres innervating the sinus node. In the perfused guinea-pig heart, α-adrenoceptor antagonists enhanced the [^{3}H]-noradrenaline release evoked by field-stimulation with 180 pulses at 1 Hz (Wakade & Wakade, 1981). In the presence of desipramine, an inhibitor of neuronal reuptake, yohimbine and phentolamine increased the [^{3}H]-noradrenaline release evoked by 16 pulses at 0.125, 0.5, 2, and 10 Hz, but not at 30 Hz (Wakade & Wakade, 1982). It was concluded that the autoinhibition is effective over a wide range of stimulation frequencies. This may be true when the neuronal reuptake as an important elimination pathway for released transmitter is blocked as in the latter experiments. Blockade of the neuronal reuptake enhanced the autoinhibition (Story, McCulloch, Rand & Standford-Starr, 1981) probably by increasing the biophase concentration of noradrenaline. Our results do not contradict those given above, since fewer pulses were used to stimulate release and since the neuronal reuptake was not blocked.

It is tempting to speculate that the biophase concentrations of transmitter in a tissue lacking thorough perfusion may be elevated for a longer time period than in perfused organs. Thus, upon similar stimuli of release, the biophase concentration of transmitter in the synaptic cleft of a perfused tissue may be lower than in an incubated or superfused tissue. Hence, one would expect that the α_2-adrenergic autoinhibition which depends on a threshold concentration of an agonist (Langer, 1977), requires different stimuli for a non-perfused tissue and a perfused organ. Depend-

ing on the technique, the borderline conditions for the activation of the autoinhibition could therefore be different.

In the isolated heart of the rat sympathetic nerve stimulation with 180 pulses at 3 Hz caused an increase in the atrial tension development by 150–200% and an increase in the beating frequency to up to 300 beats/min (Fuder *et al.*, 1982a). Nerve stimulation with 10 pulses at 1 Hz increased the atrial tension development by roughly 100% and the beating frequency to up to about 250 beats/min under conditions comparable to those used in the paper quoted above (Figures 4 and 5). Rather small amounts of [^{3}H]-noradrenaline appeared in the perfusate (0.14 pmol/10 pulses, Table 1) compared to stimulation with 180 pulses at 3 Hz (about 1 pmol/180 pulses) within the same time interval of 1 min (Fuder *et al.*, 1982b). Thus, from the magnitude of the transmitter overflow as well as from the postsynaptic responses of the heart, the stimulation by 10 pulses at 1 Hz can be interpreted as a moderate to mild (certainly submaximal) stimulus for transmitter release.

Threshold concentration for the activation of the autoinhibition

Recently, in the mouse vas deferens the presence of autoinhibition of [^{3}H]-noradrenaline overflow evoked by 10 field pulses at 1 Hz was demonstrated with yohimbine (Baker & Marshall, 1982). Similarly, the evoked tritium overflow from the guinea-pig vas deferens (previously loaded with [^{3}H]-noradrenaline) by trains of 10 pulses at 1 Hz was enhanced by phenoxybenzamine (Kalsner, 1980). In the isolated field-stimulated atria of guinea-pig the tritium overflow induced by 4 pulses at 0.25–1 Hz was increased by phentolamine 3 μM (Story *et al.*, 1981). These and other results (for review see Gillespie, 1980) show that the autoinhibition in various non-perfused organs can readily be demonstrated at 1 Hz (or lower frequency) with 10 or fewer pulses, in contrast to the present results. The disagreement with the above results is unlikely to arise from the different mode of pulse application (field pulses vs. stimulation of the extrinsic nerves with orthodromic propagation). Evidence of an adrenergic feedback inhibition was derived from studies using accelerans nerve stimulation (Starke *et al.*, 1971, Langer *et al.*, 1977) as well as from studies using field stimulation (Story *et al.*, 1981). Hence, one explanation may be that the rapid removal of transmitter from the biophase by the perfusion stream (and the neuronal reuptake) prevents the threshold concentration for the activation of the autoinhibition being reached.

Story *et al.* (1981) were not able to show a significant autoinhibition with 4 pulses at 2 Hz and 0.125 Hz. The lack of autoinhibition at the higher frequency was explained by a delay until the mechanism becomes operational at 2 Hz. It was suggested that the lack of feedback at 0.125 Hz was attributable to a maximum persistence of the activation up to 4–8 s. The latter finding could as well be explained by a low biophase concentration of transmitter below the threshold concentration for the activation of the autoinhibition at the time of the application of the next pulse.

One argument for a low biophase concentration of released transmitter is the high intrinsic activity of oxymetazoline in depressing the [^{3}H]-noradrenaline overflow. In the rabbit isolated heart, a negative correlation was observed between the ability of oxymetazoline to suppress the noradrenaline overflow and the amount of transmitter released (Starke, 1972). A similar negative correlation between the inhibition by clonidine of the stimulation-evoked tritium overflow and the amount of tritium in the overflow was found in rabbit brain cortex slices previously incubated with [^{3}H]-noradrenaline (Reichenbacher, Reimann & Starke, 1982).

In contrast to the present results, oxymetazoline caused less than 50% inhibition of the evoked [^{3}H]-noradrenaline overflow when the rat heart sympathetic nerves were stimulated with 180 pulses at 1 Hz (Fuder, Spemann & Wiebelt, 1982c).

Characterization of presynaptic adrenoceptors

The pA_2 values obtained from evoked postsynaptic responses and overflow studies under conditions when the antagonists enhance the response by their own have been reviewed by Wikberg (1979) and by Doxey & Roach (1980). The aim of the present study was to characterize the presynaptic α-adrenoceptors with antagonists. A direct functional parameter (transmitter release) was used. We tried to eliminate possible pitfalls, either the underestimation of the pA_2 value due to summation of the actions of the endogenous noradrenaline plus the exogenous agonist, or the overestimation of the pA_2 due to summation of the actions of the exogenous partial agonist, acting also as an antagonist, plus the antagonist to be tested.

The reliability of the results from the overflow study depends mainly on the relation between transmitter in the overflow and the transmitter actually released. Since neuronal and extraneuronal metabolisms are unimportant, no special care had to be taken to block these pathways. Neuronal uptake, however, is an important factor. Nevertheless, we deliberately did not add a drug to block the neuronal uptake since there is some evidence for an inhibition of the oxymetazoline or clonidine effect at presynaptic receptors by drugs causing inhibition of the neuronal up-

take (cocaine, desipramine and amphetamine) in brain slices (Pelayo, Dubocovich & Langer, 1980) and in the cat spleen (Dubocovich & Langer, 1981). Most probably, the neuronal reuptake will affect the relation between release and overflow to a similar degree over the whole range of the concentration-response curve, since the mild stimuli presently applied cannot release massive transmitter amounts to saturate the reuptake process.

The antagonism between phentolamine or yohimbine and oxymetazoline as documented in the Arunlakshana-Schild plots (Figure 3) is in agreement with a competitive interaction at the presynaptic receptor site. The pA_2 values of yohimbine observed for α-adrenoceptors at cholinergic nerves were 7.78 (Drew, 1978) and 7.66 (Grundström, Andersson & Wikberg, 1981) in the guinea-pig ileum, and 7.85 in the trachea (Grundström *et al.*, 1981). These constants, derived from postsynaptic responses to cholinergic nerve stimulation, closely agree with our pA_2 value of 7.82 obtained by measuring the [^{3}H]-noradrenaline overflow. The pA_2 values of yohimbine at postsynaptic receptor sites in aortae of species other than rat are 6.6 (guinea-pig, Grundström *et al.*, 1981), 6.7 (rabbit, Furchgott, 1955) and 6.8–6.9 (cat, dog and hamster, Ruffolo, Waddell & Yaden 1982). Thus, the affinity of yohimbine for presynaptic receptors appears to be at least one log unit higher than for the classical postsynaptic α-adrenoceptors.

At postsynaptic adrenoceptors the pA_2 values of phentolamine range between 6.86 and 8.39 (for review see Wikberg, 1979). Our pA_2 of 7.52 agrees with the majority (7.5–7.9) of the listed values. This accords with the idea that phentolamine does not discriminate between pre- and postsynaptic adrenoceptors (Starke, 1981).

The characterization of presynaptic adrenoceptors in the present study is exclusively based on overflow studies. The postsynaptic responses to nerve stimulation, however, closely correlate with the results of the transmitter determinations. Both parameters demonstrate the inhibition of transmitter release by oxymetazoline and its antagonism by yohimbine. Similarly, a lack of an effect of yohimbine on either parameter is a strong argument for a lack of autoinhibition under the present conditions. The frequency- response indicates a slight (though insignificant) increase in beating frequency in response to nerve stimulation by yohimbine. It is well known that the adrenergic innervation is very dense around the sinus node and the amounts of transmitter released per g tissue would be the highest in this area. Thus, if a drug antagonizes autoinhibition on the heart, the frequency-response to sympathetic nerve stimulation would be the parameter most likely to be affected. The increase in frequency response to 15 pulses at 1 Hz by phentolamine in the pithed rat *in vivo*, however, is small (Drew, 1980). Our *in vitro* data can be reconciled with those *in vivo* data because of the higher number of pulses used in the *in vivo* study, causing a higher biophase concentration of noradrenaline in the densely innervated sinus node area.

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