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THE EFFECT OF AMITRIPTYLINE ON PRESYNAPTIC MECHANISMS IN NORADRENERGIC NERVES

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1 Electrically evoked and resting overflow of tritium was measured from mouse vas deferens previously incubated with [^{3}H]-(−)-noradrenaline.

2 At low concentrations (1.6×10^{-7} to 4×10^{-6} M) amitriptyline increased only the evoked tritium overflow while higher concentrations increased both evoked and resting overflow.

3 In the presence of atropine (1×10^{-6} M) amitriptyline still produced an increase in evoked tritium overflow.

4 In the presence of a concentration of cocaine (1.1×10^{-5} M) which produced a maximal blockade of the uptake of exogenous noradrenaline the application of amitriptyline still produced an increase in evoked tritium overflow.

5 In the presence of a concentration of phentolamine (1×10^{-5} M) that produced complete blockade of the presynaptic α-adrenoceptors, amitriptyline still produced an increase in evoked tritium overflow.

6 In the presence of cocaine and phentolamine together the effect of amitriptyline on evoked overflow was abolished.

7 It is concluded that amitriptyline may raise synaptic levels of noradrenaline by blocking presynaptic α-adrenoceptors controlling noradrenaline release and by blocking its uptake into sympathetic neurones.

Introduction

The biochemical lesion(s) underlying the clinical condition of depression are poorly understood but it is generally accepted that drugs which modify the handling of transmitter amines in the central nervous system are capable of alleviating the clinical problem. Noradrenaline in particular has been implicated in the mechanism of action of the tri- and tetra-cyclic antidepressants since all these agents are capable of inhibiting noradrenaline uptake if a sufficient concentration of them is applied to the test tissue. However, the relationship between clinical potency and ability to inhibit noradrenaline uptake *in vitro* is poor since the clinically effective doses vary over only a narrow range while noradrenaline uptake blocking potencies vary over a 170 fold range (Barth, Manns & Muscholl, 1975). Although this could be accounted for by various pharmacokinetic considerations, some antidepressants appear to be able to produce effective relief in doses which do not interfere with noradrenaline uptake, at least at peripheral sites (Fann, Davis, Janowsky, Kaufman, Griffith & Oates, 1972; Ghose, Coppen & Turner, 1976). Furthermore, the correlation between clinical improvement and the production of a blockade of noradrenaline uptake is poor (Ghose & Coppen, 1977).

It has recently become apparent that uptake of noradrenaline is by no means the only factor involved in the control of noradrenaline concentrations in the synaptic cleft. Noradrenaline release is now known to be under the control of a variety of mechanisms both in peripheral tissues and in the central nervous system (Langer, 1977; Starke, 1977). Presynaptic α-adrenoceptors, presynaptic β-adrenoceptors, muscarinic receptors, prostaglandin receptors and others may well play a part in the control of noradrenaline release though the evidence for a physiological role for all these mechanisms is not strong.

The tri- and tetra-cyclic antidepressants are not only capable of inhibiting noradrenaline uptake (Barth *et al.*, 1975) but also possess α-adrenoceptor blocking properties (Brodie, Dick, Kielholz, Pöldinger & Theobald, 1961; Scriabine, 1969; Hughes, Kneen

& Main, 1974) and are able to inhibit the actions of acetylcholine at muscarinic receptors (Rathbun & Slater, 1963; Gupta, Gupta & Bhargava, 1967; Andrasi & Borsy, 1968). These drugs could therefore modify the concentration of noradrenaline in the synaptic cleft not only through an action on noradrenaline re-uptake but also by modification of the functional state of the mechanisms controlling noradrenaline release.

This paper describes an investigation into this possibility in a peripheral tissue (mouse vas deferens) utilizing measurements of tritium overflow in response to electrical stimulation after incubation of the tissues with [^{3}H]-(−)-noradrenaline.

Methods

Measurement of resting and evoked tritium overflow

Male mice (20 to 30 g; T.O. strain) were killed by a blow on the head, the vas deferens was removed and was cleared of mesentery in cold physiological saline (NaCl 118, KCl 4.75, $CaCl_2$ 2·54, KH_2PO_4 0.93, $NaHCO_3$ 25 and glucose 11 mM also containing ascorbic acid 57, disodium ethylenediaminetetra-acetic acid 27 and β-oestradiol 3.7 μM; gassed with 5% CO_2 in O_2). Ligatures were attached to each end of the tissue which was then incubated at 37°C for 10 min in 1.0 ml of physiological saline. After this time 10 μCi of [^{3}H]-(−)-noradrenaline (final concentration 1.09 μM) was added to the tissue bath and the incubation continued for a further 45 minutes. The tissue was then removed from the bath, mounted between parallel platinum wire electrodes (made of 1 mm diameter platinum wires held 5 mm apart) and placed in a second tissue bath maintained at 37°C and aerated with a fine steam of 5% CO_2 in O_2. The tissue bath was drained and refilled with 1.0 ml of physiological saline every 2 min for the rest of the experiment and the effluent was collected at each 2 min interval in a liquid scintillation vial to which was added 10 ml of a liquid scintillation solution (naphthalene 100 g, PPO 7 g, POPOP 0.5 g, methanol 50 ml and dioxan to 1000 ml). The samples were counted for tritium in a liquid scintillation counter and correction for quenching was applied by an external standard channels ratio method.

The ligature at the top of the tissue was attached to an isotonic lever system (load 100 mg) and after allowing the tissue to equilibrate and wash for 50 min, electrical stimulation was applied at 20 min intervals (15 V, 2 ms pulse width, 2.5 Hz, 180–220 mA for 45 s starting 15 s after a change of bath fluid). Up to 4 periods of electrical stimulation were applied and appropriate drugs were added to the bulk of the physiological saline and were allowed to remain in contact with the tissue for 20 min before their effect on resting or evoked overflow of tritium was determined.

At the end of the experiment the tissue was removed from the tissue bath, wrapped in a small square of Kleenex tissue and combusted in a Packard Tri-Carb Oxidiser (model 305). Recovery of radioactivity from the combustion process was 94.3 ± 0.4% (mean ± s.e. mean; $n = 6$) as determined by combustion of known amounts of [^{3}H]-(−)-noradrenaline on tissue paper. The results from the combusted samples were not corrected for this recovery.

Measurement of [^{3}H]-(−)-noradrenaline uptake

Groups of vasa deferentia were incubated at 35.5°C for 10 min in physiological saline as above from which the β-oestradiol had been omitted and to which various concentrations of cocaine were added as appropriate. [^{3}H]-(−)-noradrenaline (5.95×10^{-7} M; sp. act. 0.2 to 1.0 Ci/mmol) was then added and the tissues were incubated for a further 10 minutes. At the end of this time the tissues were removed from the incubation solution, washed briefly in fresh physiological saline, further washed for two 10 min periods in 100 ml of physiological saline, blotted dry, weighed, combusted as described above and the tritium content expressed in terms of ng noradrenaline per g (wet weight) of tissue (uncorrected for recovery of radioactivity from the combustion process) on the assumption that all the tritium retained by the tissue was as [^{3}H]-noradrenaline.

Drugs

L-Ascorbic acid (BDH), amitriptyline hydrochloride (Roche), atropine sulphate B.P., cocaine hydrochloride B.P., disodium ethylenediaminetetra-acetic acid (BDH), (−)-noradrenaline bitartrate (Sigma), and phentolamine mesylate (Ciba). β-Oestradiol (Sigma) was dissolved in ethanol (2 mg/ml) and an appropriate volume of the ethanolic solution was added to the physiological saline. [^{3}H]-(−)-noradrenaline was obtained from The Radiochemical Centre, Amersham at a specific activity of 9.1 Ci/mmol.

Statistical procedures

Where appropriate all results are given as mean ± standard error (±s.e. mean) and tests for statistical significance utilized Student's t test.

Results

Effect of electrical stimulation on tritium overflow

The resting overflow of tritium from the tissue (which

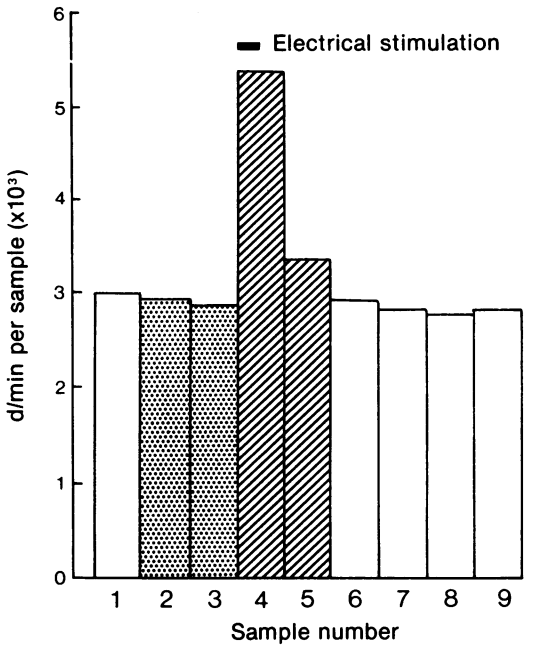

Figure 1 Effect of electrical stimulation on the tritium overflow from a mouse vas deferens previously incubated with [^{3}H]-(−)-noradrenaline. Samples, consisting of the bath fluid surrounding the tissue (1.0 ml), were collected at 2 min intervals. The tritium content of the two samples immediately before the application of electrical stimulation (stippled) were averaged to provide a measure of resting overflow. The tritium in samples 4 and 5 (hatched) in excess of that expected from the resting overflow was taken as a measure of evoked overflow. The tritium content of this particular tissue at the time of stimulation was 7.46×10^5 d/minute.

was taken as the average of the tritium content of the two samples collected immediately before a period of electrical stimulation) was increased by electrical stimulation both in the collection period in which electrical stimulation was applied and in the subsequent period (Figure 1). Analysis of the areas under the histogram shows that at least 96% of the tritium overflowing as a result of electrical stimulation appeared in these two samples and evoked overflow was therefore taken as the tritium in these two samples which was in excess of that expected from the resting overflow. In untreated tissues this evoked overflow of tritium represented only a small fraction of that in the tissue (approximately 4.4×10^{-5} of the total tritium content per shock) as combustion of the tissues at the end of the experiment showed that they contained large amounts of tritium (4.7 to 11.6×10^5 d/min per tissue).

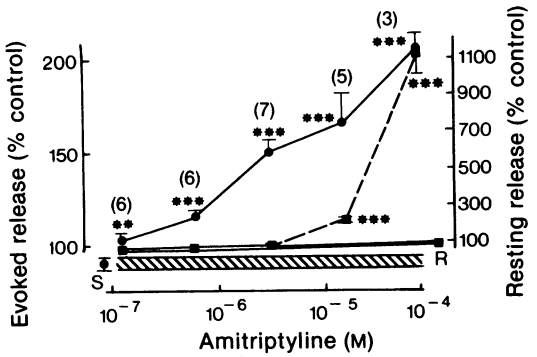

Figure 2 The effect of various concentrations of amitriptyline on the resting overflow (R; ■---■) and electrically evoked overflow (S; ●) of tritium from mouse vas deferens previously incubated with [^{3}H]-(−)-noradrenaline. Note that resting and evoked overflow are plotted on different scales and that corresponding values in the absence of amitriptyline are shown by the bars at the bottom of the graph. The points represent means of the overflow expressed as a percentage of overflow in a preceding control period for each tissue. Vertical lines show s.e. means. The number of tissues contributing to each group is shown in parentheses and statistically significant differences from untreated tissues (Students' t test) are shown as follows: $^{**}P < 0.01$ but >0.001; $^{***}P < 0.001$.

In untreated tissues the tritium overflowing as a result of the first period of electrical stimulation (S1) was variable and was often considerably larger than overflow evoked by subsequent periods of stimulation. For this reason samples obtained during this period were discarded. The second period of stimulation (S2) was used as a control period for each tissue and resting overflow (R) and electrically evoked overflow (S) in later periods were expressed as a percentage of these control values for each tissue. In untreated tissues the evoked overflow in the third and fourth periods of stimulation (S3 and S4) were $90.9 \pm 2.1\%$ and $91.4 \pm 7\%$ (±s.e. mean, $n = 7$) of the control values respectively and were not significantly different ($P > 0.8$). Corresponding values for the resting overflow (R3 and R4) were $86.8 \pm 1.5\%$ and $75.7 \pm 3.6\%$ ($n = 7$) of control resting overflow and were significantly different ($P < 0.02$).

Effect of amitripytline on tritium overflow

After 20 min exposure to amitriptyline (up to 4×10^{-6} M) resting overflow of tritium was not significantly altered but at higher concentrations (2 and 10×10^{-5} M) a statistically significant increase in resting overflow was observed of nearly 12 fold at

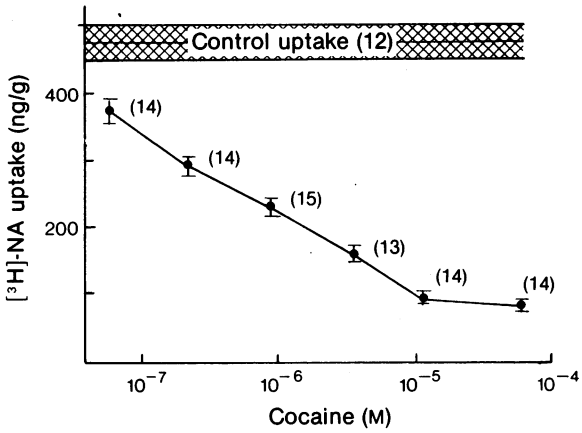

Figure 3 Uptake of tritium (expressed as ng noradrenaline per g wet weight of tissue) in groups of mouse vasa deferentia incubated for 10 min with [^{3}H]-(−)-noradrenaline (5.95×10^{-7} M) in the presence or absence of various concentrations of cocaine. The values shown are means and the number of tissues contributing to each group is shown in parentheses. Vertical lines show s.e. means.

the higher concentration (Figure 2). Electrically evoked overflow was significantly increased compared with untreated tissues at all the concentrations of amitriptyline tested and the size of the increase was concentration-dependent and up to 2 fold at the highest concentration (Figure 2).

Effect of atropine on tritium overflow and response to amitriptyline

Exposure of the tissues to atropine (1×10^{-6} M) for 20 min did not change either the resting overflow of tritium ($P > 0.8$) or the evoked overflow of tritium ($P > 0.3$) when compared with untreated tissues (Table 1). In the presence of this concentration of atropine the application of amitriptyline (4×10^{-6} M) did not affect the resting overflow of tritium ($P > 0.5$) but did increase the electrically evoked overflow when compared with tissues treated with atropine alone ($P < 0.001$) (Table 1). The increase in the evoked overflow produced by amitriptyline was about 84% of the control overflow (in stimulation period S2) and was comparable to that produced by the application of amitriptyline alone ($\approx 60\%$).

Effect of cocaine on noradrenaline uptake, tritium overflow and on the response to amitriptyline

In concentrations from 5.9×10^{-8} M to 5.9×10^{-5} M cocaine inhibited the uptake of tritium by vasa deferentia incubated with [^{3}H]-(−)-noradrenaline (Figure 3). The inhibition of tritium uptake was concentration-dependent and reached a maximum at 1.1×10^{-5} M cocaine since the uptake of [^{3}H]-(−)-noradrenaline in the group of tissues incubated with this concentration of cocaine was not significantly different from that in the group incubated with 5.9×10^{-5} M ($P > 0.05$).

After 20 min exposure to cocaine at a concentration of 1.1×10^{-5} M the resting overflow of tritium from

Table 1 Effect of atropine (1×10^{-6} M), cocaine (1.1×10^{-5} M) or phentolamine (1×10^{-5} M) on the resting (R) and electrically evoked (S) overflow of tritium from mouse vas deferens previously incubated with [^{3}H]-(−)-noradrenaline and the effect of amitriptyline (4×10^{-6} M) in the presence of these drugs

	Overflow			Overflow	
First treatment	*resting* (R3)	*evoked* (S3)	*Second treatment*	*resting* (R4)	*evoked* (S4)
Untreated	86.8 ± 1.5	90.9 ± 2.1	Untreated	75.7 ± 3.6	91.4 ± 1.7
Atropine	89.8 ± 2.8	95.2 ± 4.4	Atropine	84.0 ± 5.6	88.2 ± 3.1
Atropine	82.8 ± 2.9	94.8 ± 3.5	Atropine + amitriptyline	87.7 ± 3.8	172.3 ± 11.2***
Cocaine	86.3 ± 2.1	98.5 ± 8.2	Cocaine	80.3 ± 3.8	94.3 ± 9.9
Cocaine	91.2 ± 2.9	105.5 ± 6.0*	Cocaine + amitriptyline	92.8 ± 4.9	151.0 ± 9.1***
Phentolamine	107.5 ± 5.5**	302.2 ± 27.3***	Phentolamine	98.3 ± 6.4	254.2 ± 25.6
Phentolamine	104.0 ± 1.6***	308.5 ± 23.8***	Phentolamine + amitriptyline	113.5 ± 7.0	332.3 ± 19.9*

The table shows the resting and evoked overflow at stimulation periods 3 and 4 expressed as a percentage of the overflow at period 2 for each tissue. The figures are means (± s.e. mean) and 6 tissues contributed to each group except for the untreated group where $n = 7$. Levels of statistical significance for differences between means (Student's t test: *$P < 0.05$ but >0.01; **$P < 0.01$ but >0.001; ***$P < 0.001$) are shown in R3 and S3 for comparisons with the untreated group. In R4 and S4 comparisons are between the groups exposed to a drug with and without additional amitriptyline.

vasa deferentia was unchanged in comparison with control tissues ($P > 0.5$) and although electrically evoked overflow was marginally increased, this effect achieved statistical significance in only one of the treated groups (Table 1). Since the two groups were treated in an identical manner to this point in the experiment it is possible to calculate a combined mean for the evoked release (102.0 ± 4.9%; $n = 12$) though comparison with untreated tissues still does not show a statistically significant difference ($P < 0.2$ but >0.1).

In the presence of 1.1×10^{-5} M cocaine the application of amitriptyline (4×10^{-6} M) produced a slight increase in resting overflow though this effect only approached statistical significance ($P < 0.1$ but >0.05). Electrically evoked overflow was still increased however in comparison with tissues treated with cocaine alone ($P < 0.01$) and the magnitude of this increase in release was approximately 56% of the control overflow (stimulation period S2) and was comparable to the increase in evoked overflow produced by this concentration of amitriptyline alone (≈60%) (Table 1).

Effect of phentolamine on tritium overflow and response to amitriptyline

After 20 min exposure to phentolamine (3×10^{-8} to 1×10^{-5} M) resting overflow of tritium was not altered when compared with untreated tissues ($P > 0.6$ in all cases) except with phentolamine at a concentration of 1×10^{-5} M where a small (20%) increase in resting overflow was seen ($P < 0.001$) (Figure 4). Electrically evoked overflow of tritium was increased at all the concentrations of phentolamine tested and a maximal increase of about 3 fold was produced at 3×10^{-6} M (Figure 4).

In the presence of 1×10^{-5} M phentolamine the application of amitriptyline (4×10^{-6} M) produced a further increase in electrically evoked overflow of tritium when compared with tissues treated with phentolamine alone ($P < 0.05$) (Table 1). The magnitude of the increase produced by amitriptyline was about 75% of the control release and was comparable to the size of the increase produced by the application of amitriptyline alone (≈60%).

Effect of phentolamine and cocaine on response to amitriptyline

After 40 min exposure to a mixture of phentolamine (1×10^{-5} M) and cocaine (1.1×10^{-5} M) the resting overflow and evoked overflow of tritium were 88.8 ± 1.1% and 85.5 ± 1.2% ($n = 6$) respectively of the corresponding values after 20 min exposure. The inclusion of amitriptyline (4×10^{-6} M) for the last 20 min of this time period gave values for resting

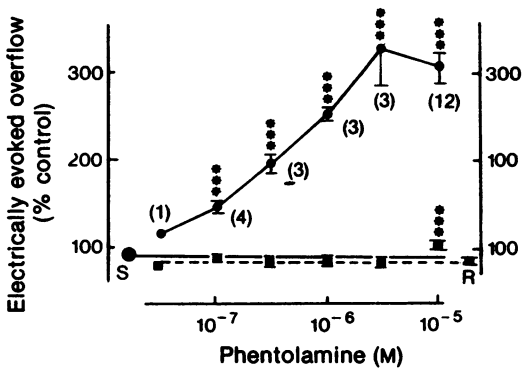

Figure 4 Effect of various concentrations of phentolamine on resting and electrically evoked overflow of tritium from mouse vas deferens previously incubated with [^{3}H]-(−)-noradrenaline. The resting overflow (R; ■——■) and electrically evoked overflow (S; ●) are plotted on the same scales and the values for these parameters in the absence of phentolamine are indicated by the bars at the bottom of the graph. The points represent means of the overflow in the presence or absence of phentolamine expressed as a percentage of the overflow in a preceding control period for each tissue. Vertical lines show s.e. means. The number of tissues contributing to each point is shown in parentheses. Statistically significant differences between means and the corresponding values in untreated tissues (Student's t test) are shown as follows: $^{***}P < 0.001$.

overflow of 118.0 ± 3.6% and for evoked overflow of 77.7 ± 2.9% ($n = 6$). Clearly amitriptyline increased resting overflow of tritium ($P < 0.001$). However, electrically evoked overflow was not increased (in contrast to the effect of amitriptyline alone or in the presence of the other drugs individually) and a slight fall in evoked overflow was apparent though this effect did not achieve statistical significance when compared with the tissues in which amitriptyline was absent ($P < 0.1$ but >0.05).

Discussion

It is well appreciated that tritium overflow from a tissue previously incubated with [^{3}H]-(−)-noradrenaline represents the difference between released tritium and that taken back up into the tissue (mainly as noradrenaline through the uptake$_1$ process). The tritium overflowing into the bathing fluid is present as noradrenaline and as metabolic products (Starke, 1977) but the proportion of these metabolic products originally existing as noradrenaline in the synaptic cleft and therefore contributing to the activation of receptors is unknown. No attempt has been made to

separate the tritium into identified molecular species; therefore it must be remembered throughout this work that alterations in tritium overflow may not quantitatively reflect alterations in noradrenaline concentrations in the synaptic cleft.

Amitriptyline affected both the resting overflow of tritium and the electrically evoked overflow of tritium and there was a clear distinction between the concentrations of amitriptyline required to produce effects on these two processes. The massive rise in resting overflow produced by high concentrations of amitriptyline is probably of little direct relevance to the mechanism of action of this drug in man, as the concentration levels required are never even approached except in the grossest overdose. In clinical use, amitriptyline plasma levels range up to 300 ng/ml (1×10^{-6} M; Braithwaite & Widdop, 1971; Jørgensen, 1975) and allowing for 90% plasma protein binding (Bôrga, Azarnoff, Forshell & Sjöqvist, 1969; Glassman, Hurwic & Perel, 1973) this represents a free drug concentration of about 1×10^{-7} M, a level very close to that found to produce a significant increase in tritium overflow in response to electrical stimulation. However, direct comparisons of effective concentrations must be made with caution as peripheral noradrenergic neurones may differ in their sensitivity to those in the central nervous system and tissue binding of amitriptyline during chronic administration may also be a complicating factor.

The increase in evoked overflow of tritium cannot be mediated through an ability of amitriptyline to produce blockade of presynaptic muscarinic receptors since the effect was still observed in the presence of a concentration of atropine nearly 3 orders of magnitude above the pA_2 for this drug at classical muscarinic receptors ($pA_2 = 8.9$). Furthermore, atropine itself produced no effect on evoked overflow whereas amitriptyline did. This observation does not necessarily imply that under these conditions there is no contribution from muscarinic receptors to the control of noradrenaline release since it must be borne in mind that other controlling mechanisms will still be operative (e.g. the presynaptic α-receptors) and any increase in noradrenaline release could well be compensated for by increased negative feedback through other controlling pathways.

In the presence of cocaine at a concentration which produced a maximal effect on the uptake of exogenous noradrenaline, amitriptyline still produced a rise in evoked overflow of tritium which was of comparable size to that produced by amitriptyline alone. It seems unlikely therefore that the cause of the increased evoked overflow could be uptake blockade, although amitriptyline will produce a blockade of uptake of exogenous noradrenaline at this concentration (Harper & Hughes, 1977). Furthermore, cocaine alone produced little effect on evoked overflow although it must be remembered that the high concentration of cocaine found to be necessary to produce maximal blockade of exogenous noradrenaline uptake may produce some local anaesthetic action (Starke, Wagner & Schümann, 1972) which may complicate conclusions which could be drawn from this observation. In addition, a compensation for any increase in synaptic noradrenaline concentrations may have taken place through other mechanisms controlling release.

Phentolamine applied alone produced a marked increase in evoked overflow of tritium which was concentration-related and up to 300% of control overflow, illustrating the major role of the presynaptic α-adrenoceptor in the control of noradrenaline release. The effect reached a maximum at 3×10^{-6} M and in the presence of 1×10^{-5} M phentolamine all presynaptic control of noradrenaline release mediated through α-adrenoceptors should be eliminated. Under these conditions amitriptyline still produced a rise in evoked release of tritium which was comparable in size to that produced by amitriptyline alone. This could be interpreted as evidence that the effect of amitriptyline is independent of the presynaptic α-adrenoceptors. However, in the absence of this major control mechanism a blockade of noradrenaline uptake by amitriptyline could now give rise to increased synaptic levels of noradrenaline (and therefore increased overflow of tritium) unopposed through α-adrenoceptor mediated control of noradrenaline release. For this reason the effects of amitriptyline were investigated in the presence of both phentolamine and cocaine and under these conditions the stimulant effect of amitriptyline on evoked overflow was eliminated completely and a small reduction in tritium overflow was produced though this effect did not achieve statistical significance. The evidence suggests that amitriptyline increases the evoked overflow of tritium by blocking the uptake of noradrenaline into the sympathetic neurones and by blocking presynaptic α-adrenoceptors. Both of these phenomena could be of importance in the effect of the drug on the central nervous system.

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