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RELAXATION OF SMOOTH MUSCLE FOLLOWING CONTRACTION ELICITED BY SYMPATHETIC NERVE STIMULATION *in vivo*

R. ECCLES & A.G. MacLEAN
Department of Physiology, University College, Cardiff CF1 1XL

1 A method for studying *in vivo* the process of neuroeffector transmission in the nictitating membrane and nasal blood vessels of the cat is described.

2 Administration of desmethylimipramine or cocaine caused increases in both the amplitude and duration of the nasal and membrane responses which may be explained by inhibition of neuronal uptake of noradrenaline.

3 Phenoxybenzamine depressed the responses to nerve stimulation, but had little effect on the relationship between response amplitude and rate of recovery.

4 The relationship between response amplitude and rate of recovery is discussed and related to the sigmoid shape of a log concentration-response curve.

Introduction

The relaxation of smooth muscles after exposure to excitatory neurotransmitters is widely accepted to be dependent on the rate of inactivation of these agents within the tissue (Kalsner & Nickerson, 1968; Draskoczy & Trendelenburg, 1970; Trendelenburg & Henseling, 1976), and upon this assumption the mechanisms responsible for the inactivation of noradrenaline after its release from nerve terminals have been investigated *in vitro* (Brandão & Guimarães, 1974; Bell & Grabsch, 1976).

Much of the analysis of the relaxation of tissues has relied on the measurement of $T_{\frac{1}{2}}$, the time required for a response to recover to half its peak amplitude, to provide an estimate of the rate of inactivation of agonist in the tissue. This has recently been criticized by Trendelenburg & Henseling (1976), who suggested that some parameter which was independent of response amplitude might be more acceptable as a measure of the rate of loss of agonist from the biophase. The present experiments are an attempt to establish and use such a parameter in the investigation of neuroeffector transmission *in vivo*.

In the nictitating membrane, neuronal uptake is believed to be an important process for terminating the action of noradrenaline (Trendelenburg, 1971a, b), while in blood vessels, the role played by neuronal uptake may depend on the density and morphology of the adrenergic innervation (Verity, 1971; Bevan & Su, 1973; Brandão, 1976). By recording nasal vasomotor responses and simultaneous contractions of the nictitating membrane upon stimulation of the internal carotid nerve (Eccles & Wallis, 1976), comparative studies of sympathetic neuroeffector transmission in these two tissues may be made under identical conditions.

In the present experiments the relationship between response amplitude and 'rate of recovery' was investigated by analysing the effects of phenoxybenzamine, desmethylimipramine and cocaine upon responses to nerve stimulation.

Methods

Cats of either sex weighing 1.5 to 3.0 kg were anaesthetized with pentobarbitone sodium given initially in a dose of 40 mg/kg intraperitoneally and subsequently infused into the femoral vein at a rate of approximately 8 mg kg^{-1} h^{-1} for the duration of the experiment. The trachea was cannulated and the cats breathed spontaneously.

Placed supine, the cats were fixed in a rigid head frame by tapered rods inserted in the external auditory meatuses. The head was held horizontally by means of a metal rod, which passed through the mouth and rested behind the canine teeth of the upper jaw. The cats were maintained at a constant temperature (38°C) by means of a rectal probe and a Homeothermic Blanket Control Unit (Electro-Physiological Instruments Ltd.).

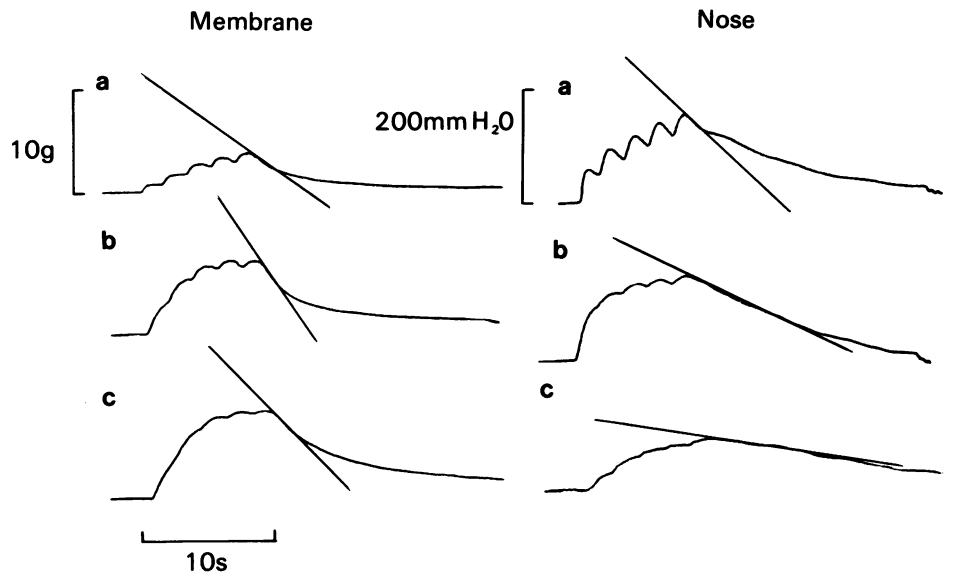

Figure 1 Nasal vasoconstrictor responses and contractions of the cat nictitating membrane elicited on stimulation of the internal carotid nerve for 10 s periods at a frequency of 0.5 Hz: (a) control, (b) after 0.2 mg/kg desmethylimipramine (DMI), (c) after a further 1.0 mg/kg DMI. The figure also illustrates how the initial rate of recovery (IRR) of responses was determined.

Recording of vasomotor responses from the nose and contractions of the nictitating membrane

Vasomotor responses from the mucosa of the nasal cavity were recorded by a plethysmographic technique, using a pressure transducer (Statham P23 Db) to record reductions in pressure from the sealed nasal cavity (Eccles & Wilson, 1974). Isometric contractions of the nictitating membrane, from an initial tension of 3 to 5 g, were recorded by means of a cotton thread passed through the outer edge of the membrane and connected to a strain gauge (Devices type 25.T.02). Nasal and nictitating membrane responses were displayed on a rectilinear pen recorder (Physiograph Six). Arterial blood pressure and respiratory movements were also recorded.

At the end of the experiment the transducers monitoring the end organ responses were calibrated *in situ*. The nasal pressure transducer was calibrated in mmH_2O by means of a water manometer connected to a side arm of the transducer. The strain gauge monitoring the contractions of the nictitating membrane was calibrated by suspending weights from the arm of the transducer.

Experimental procedure

The cervical sympathetic nerve was sectioned and the superior cervical ganglion dissected free. The internal carotid nerve was mounted on bipolar platinum electrodes and the skin around the wound in the neck sewn to a metal loop to form a pool of liquid paraffin over the nerve and electrodes. A supramaximal stimulus voltage was determined (usually between 25–35 V using a Grass S.8. stimulator and SIU5 isolation unit). The nerve was stimulated at frequencies from 0.5 to 50 Hz for periods of 10 s with a pulse duration of 0.5 milliseconds. A frequency-response curve was obtained and then drug solutions were injected via a cannula in the cephalic vein and washed in with 1 ml of 0.9% w/v NaCl solution (saline). A rest period of 20 min intervened before repeating the periods of stimulation. After subsequent drug injections a 20 min period was again allowed for the drug to exert its effects before repeating the period of stimulation. A period of 5–10 min was allowed for the tissues to recover between periods of stimulation.

Drugs

Pularin (heparin injection, B.P.) at a concentration of 40 u/ml was added to the saline in the pressure transducer and femoral artery cannula. Desmethylimipramine hydrochloride (donated by Geigy Pharmaceuticals), was given in 1 ml of saline, as was cocaine hydrochloride. Phenoxybenzamine was injected in 0.5 ml of a 4 : 1 mixture of propylene glycol and ethanol. The dose of drugs injected is expressed in mg/kg of the salt.

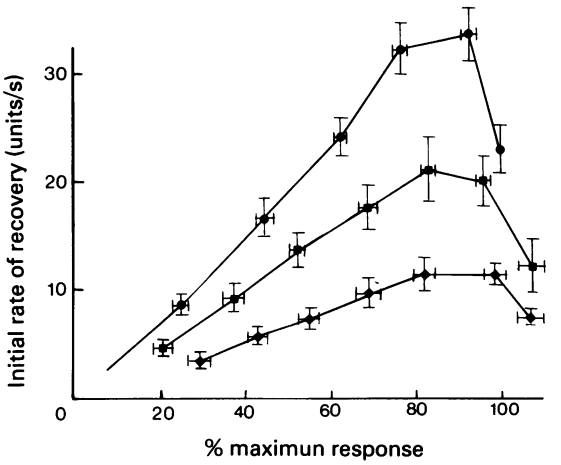

Figure 2 The relationship between the initial rate of recovery and the amplitude of nictitating membrane responses at various frequencies of nerve stimulation; left to right 0.5, 1, 2, 5, 10, 20 and 50 Hz. Control (●), after 0.2 mg/kg desmethylimipramine (DMI) (■), after a further 1.0 mg/kg DMI (◆). Bars indicate ± s.e. mean, $n = 11-16$.

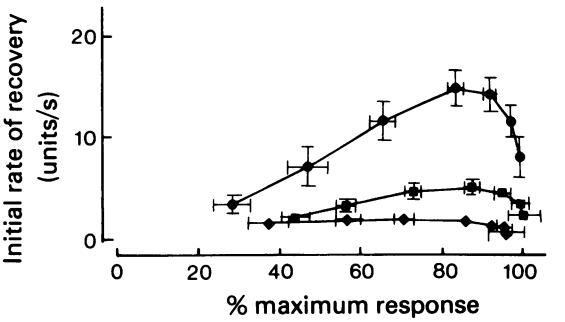

Figure 3 The relationship between the initial rate of recovery and the amplitude of the nasal vasoconstrictor responses at various frequencies of nerve stimulation; left to right 0.5, 1, 2, 5, 10, 20 and 50 Hz. Control (●), after 0.2 mg/kg desmethylimipramine (DMI) (■), after a further 1.0 mg/kg DMI (◆). Bars indicate ± s.e. mean, $n = 4-11$.

Results

Control

Graded nasal vasoconstrictor responses and contractions of the nictitating membrane were elicited on stimulation of the internal carotid nerve at frequencies between 0.5 and 50 hertz. At a frequency of 0.5 Hz (Figure 1) the responses to individual stimuli were apparent in both the nasal and the membrane recordings.

The initial rate of recovery (IRR) of the responses was determined by measuring the gradient of a tangent which was fitted to the initial portion of the recovery curve (Figure 1). This method was found to be reproducible, involving very little error when applied to recordings over 1 cm in amplitude, provided that a suitable recording speed (0.5 cm/s) was used. The IRR is expressed in units/s, where 100 units equals the amplitude of the maximum control response of that tissue.

Figures 2 and 3 illustrate the relationship between the amplitude and the IRR of responses. Under control conditions, up to an amplitude of 80–90% max., there is a linear relationship between the amplitude of responses and the IRR, but for responses larger than 80% max. the IRR decreases with

Table 1 Effects of desmethylimipramine (DMI) on the time required ($T_{\frac{1}{2}}$) for nasal and membrane responses to decline to half their peak amplitudes

Nasal

Frequency (Hz)	1	5	20	
Control	6.8 ± 1.0	5.5 ± 0.7	7.0 ± 0.8	$n = 11$
DMI (0.2 mg/kg)	9.4 ± 1.3	13.8 ± 1.9	24.1 ± 3.8	$n = 6$
DMI (1.2 mg/kg)	21.6 ± 2.1	34.6 ± 4.1	58.9 ± 7.1	$n = 6$

Membrane

Frequency (Hz)	1	5	20	
Control	2.7 ± 0.3	2.0 ± 0.2	2.2 ± 0.2	$n = 18$
DMI (0.2 mg/kg)	3.7 ± 0.6	3.3 ± 0.5	4.2 ± 0.5	$n = 9$
DMI (1.2 mg/kg)	8.9 ± 1.7	8.9 ± 1.7	21.0 ± 4.1	$n = 9$

Values are means ± s.e. mean.

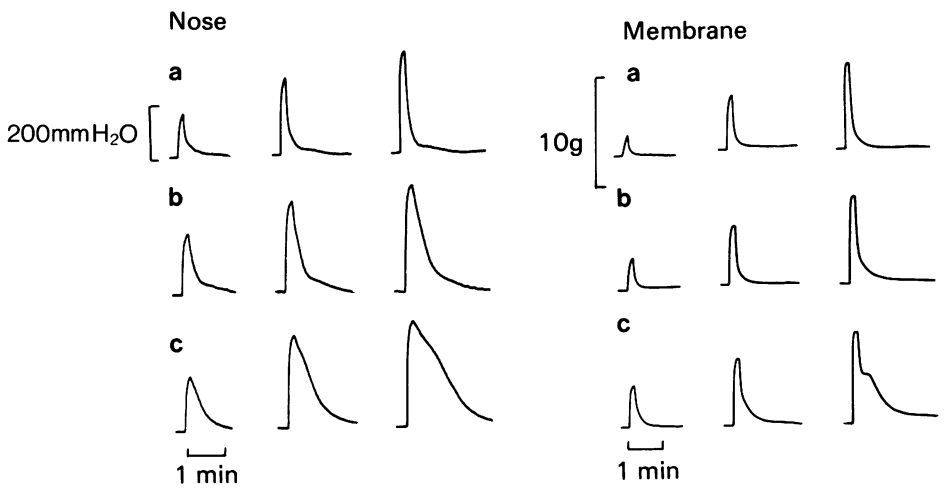

Figure 4 Nasal vasoconstrictor responses and contractions of the nictitating membrane elicited on stimulation of the internal carotid nerve for 10 s periods at frequencies of 1, 5 and 20 Hz (left to right). Left panel: nasal responses, right panel: membrane responses. (a) Control, (b) desmethylimipramine (DMI) 0.2 mg/kg, (c) DMI 1.2 mg/kg.

increasing amplitude. Throughout the frequency range the IRR of the nictitating membrane responses was faster than that of the nasal responses. For example, after a response to a frequency of 10 Hz, under control conditions, the membrane relaxed at a rate of 32.7 ± 2.4 units/s (s.e. mean $n=6$) and the nasal response at 14.2 ± 1.7 units/s (s.e. mean, $n=11$).

The recovery was also assessed by measuring the time taken for the tissue to relax to half of its contracted state. This value was designated $T_{\frac{1}{2}}$ and Table 1 presents the $T_{\frac{1}{2}}$ measurements for both nasal and nictitating membrane responses at frequencies of 1, 5 and 20 hertz. The membrane has a shorter $T_{\frac{1}{2}}$ than the nasal corresponding to its faster rate of relaxation. The responses of the nasal vasculature and nictitating membrane to stimulation at 1, 5 and 20 Hz are shown at a slow recording speed in Figure 4.

In one experiment in which bilateral recordings were made, both the $T_{\frac{1}{2}}$ of responses and the relationship between response amplitude and IRR were found to remain steady over a 4 h period. This was the longest period over which any experiment was performed.

Desmethylimipramine

After administration of desmethylimipramine (DMI) 0.2 mg/kg the absolute amplitude of both the nasal and nictitating membrane responses was increased, while the IRR corresponding to a given amplitude was reduced. This removed the recovery-amplitude curves downwards and to the right (Figures 2 and 3). Administration of an additional 1.0 mg/kg DMI caused a further increase in the duration of the nasal and membrane responses and consequently a further downward shift of the recovery-amplitude curves.

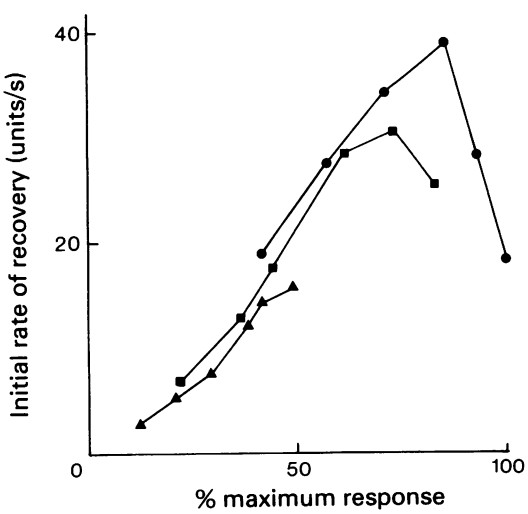

Figure 5 The relationship between the initial rate of recovery and the amplitude of the nictitating membrane responses at various frequencies of stimulation; left to right 1, 2, 5, 15, 25 and 40 Hz. Control (●), after 0.25 mg/kg phenoxybenzamine (Pbz) (■), after a further 0.25 mg/kg Pbz (▲).

After the second dose of DMI the relaxation of the nictitating membrane was distinctly biphasic, and at

frequencies above 10 Hz a temporary arrest of the relaxation was sometimes seen (Figure 4). This was occasionally observed at frequencies of 50 Hz after the lower dose of DMI.

The membrane response to stimulation at 0.5 Hz (Figure 1) showed an increase in its IRR after 0.2 mg/kg DMI. However, the IRR of this response was reduced after the injection of a further 1.0 mg/kg DMI. As the dose of DMI increased there was fusion of the responses to the 5 individual stimuli delivered at this frequency.

Cocaine

In three experiments administration of cocaine 1 mg/kg caused an increase in the amplitude of the membrane and nasal responses and a reduction in the corresponding IRR similar to that observed with DMI. The dose of 1 mg/kg cocaine caused a change in the nictitating membrane response similar in extent to that caused by DMI 0.2 mg/kg; yet the same dose of cocaine caused as large a depression in the IRR of the nasal response as that seen after a cumulative dose of 1.2 mg/kg DMI, indicating that the nasal vasculature is very sensitive to the effects of cocaine.

Phenoxybenzamine

In six experiments responses of the nictitating membrane were determined before and after phenoxybenzamine (Pbz) was given in two separate doses of 0.25 mg/kg. After Pbz the amplitude of the response to each frequency of stimulation was depressed, and the fastest IRR was developed at a higher frequency of stimulation. The IRR-amplitude curves overlapped considerably and were not displaced to the right as occurred with DMI and cocaine. The results of one experiment are shown in Figure 5; the points representing the 1 Hz response (control), the 5 Hz response (after 0.25 mg/kg Pbz), and the 40 Hz response (after an additional 0.25 mg/kg Pbz) all lie close together, indicating that they were similar both in amplitude and IRR.

In two experiments in which nasal responses were recorded, Pbz (0.25 mg/kg) was found to have little effect on the amplitude of response developed at the higher frequencies of stimulation. However, these responses did recover more slowly after Pbz.

The administration of the solvent for Pbz (1:4, ethyl alcohol:propylene glycol) in the volumes used in these experiments had no significant effect on the responses.

Discussion

The responses of the nictitating membrane are directly related to changes in tension of the smooth muscle in the tissue while the nasal responses are recorded as reductions in pressure brought about by a reduction in the volume of nasal erectile tissue (Bojsen-Møller & Fahrenkrug, 1971; Änggård & Edwall, 1974). Despite these differences in the tissues and the recording techniques used, the curves relating the initial rate of recovery (IRR) to the amplitude of responses are similar in shape, suggesting that some common property of neuroeffector transmission determines this relationship. The inactivation of noradrenaline is mainly accomplished by neuronal uptake in tissues with a dense innervation such as the nictitating membrane (Haefely, Hurlimann & Thoenen, 1964; Esterhuizen, Graham, Lever & Spriggs, 1968; Trendelenburg, 1971a), and this is considered to be the major factor determining relaxation of densely innervated vascular smooth muscle (Hughes, 1972; Bevan & Su, 1973; Brandão & Guimarães, 1974; Brandão, 1976).

The present results demonstrate that there is a linear relationship between the IRR and the amplitude of the nictitating membrane and nasal responses at amplitudes below 80% of maximum, while the IRR becomes progressively slower as amplitudes exceed 80% of maximum.

The fact that the IRR-amplitude curves were similarly displaced by by known inhibitors of neuronal uptake, DMI (Titus & Spiegel, 1962; Callingham, 1967) and cocaine (Iversen, 1963; Osswald & Branco, 1973), suggests that the 'initial rate of recovery' is related to the rate of elimination of released transmitter. Furthermore, the effect of phenoxybenzamine, which depressed the response to all frequencies of stimulation without altering the relationship between response amplitude and IRR, suggests that this relationship is a consequence of some fundamental property of the tissue, such as the shape of the agonist concentration-response curve. Figure 6 is such a hypothetical curve which shows that, for a given fractional change in concentration of agonist per unit time (represented by a fixed increment on the log concentration axis, δC), there will be a greater change in the amplitude of response R_2 which lies on the linear portion of the curve, than of either R_1 or R_3 lying near the lower and upper extremes respectively. If the processes terminating the activity of agonist in the neuroeffector gap exhibit first order kinetics throughout the range of agonist concentration, then the rate of recovery of response R_2 will be faster than the rate of recovery of R_1 or R_3. For responses close to maximum, the rate of recovery must be slower since the amplitudes of the responses correspond to the upper curved part of the sigmoid dose-response curve (Trendelenburg & Henseling, 1976).

The use of the response curve in Figure 6 to explain the relationship between response amplitude and IRR cannot be applied rigidly as the concentration of noradrenaline may vary throughout the tissue. An overall tissue response is therefore only approximately related to a particular noradrenaline concentration.

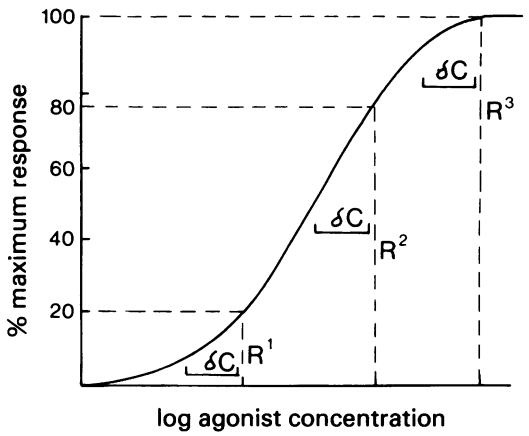

Figure 6 Hypothetical log concentration-response curve for nasal and membrane responses. (See text for explanation.)

The increase in amplitude of the nasal and membrane responses caused by DMI or cocaine may be explained by an inhibition of the neuronal uptake of noradrenaline which by depressing the rate of removal of neurotransmitter would increase the concentration of noradrenaline in the neuroeffector gap. The amplitude of the responses was most obviously increased at the low frequencies of stimulation probably because at these frequencies the concentration of noradrenaline is normally limited by neuronal uptake between stimuli. This is suggested by the fusion of responses to individual stimuli which occurred after DMI and cocaine.

The membrane responses elicited by stimulation at 0.5 Hz had a faster IRR after administration of the low dose of DMI which may also be explained by reference to Figure 6. If response R_1 represents the membrane response to 0.5 Hz stimulation in the absence of DMI, then after DMI (0.2 mg/kg) the response is of larger amplitude (e.g. represented by R_2) and now recovers from a point which is higher up on the concentration-response curve. This results in a faster initial rate of relaxation.

After a cumulative dose of 1.2 mg/kg DMI, the recovery of the nasal responses was greatly prolonged and the IRR-amplitude curve was flattened. This may be explained by an almost complete block of neuronal uptake. The great sensitivity of the nasal vasoconstrictor response to DMI and cocaine indicates that neuronal uptake of noradrenaline is a major factor in determining the rate of loss of noradrenaline from the neuroeffector gap in this tissue. This is compatible with the finding that the nasal mucosa of the cat has a very dense adrenergic innervation (Dahlström & Fuxe, 1965; Ånggård & Densert, 1974).

An alternative explanation for the slow recovery of responses larger than 80% max. under control conditions might be that the inactivation processes function less efficiently at high noradrenaline concentrations. It is conceivable that at frequencies above 15 Hz, and therefore possibly outside the physiological frequency range (Folkow, 1952; Iggo & Vogt, 1960), the concentration of transmitter released into the tissue might be higher than that required to saturate the neuronal uptake process (Mellander, 1960).

An antagonist at α-adrenoceptors, would be expected to shift to the right the agonist concentration-response curve, and this effect may be used to explain the enduring nature of the amplitude-IRR relationship after α-adrenoceptor blockade with Pbz. Provided that, over a given frequency range, the concentration of transmitter released during periods of nerve stimulation is below that required to saturate the uptake mechanisms, then at these frequencies there should be a similar fractional reduction in transmitter concentration per unit time. Under these conditions, responses whose amplitudes correspond to the steepest part of the concentration-response curve will recover more quickly than either larger or smaller responses. During exposure to Pbz, with the consequent shift of the concentration-response curve, a given amplitude of response will be generated at a higher frequency of stimulation. The ensuing relationship between IRR and response amplitude should not change, unless the shape of the agonist concentration-response curve is altered in the presence of Pbz. It seems unlikely that the doses of Pbz used in these experiments had any presynaptic actions as Langer (1974) found that in cat spleen the concentration of Pbz required to enhance transmitter release was 30 times that required to produce blockade of postsynaptic α-adrenoceptors.

A comparison of the results obtained with Pbz, DMI and cocaine indicates that the actions of these drugs in the dosages used were quite distinct. Although DMI has been reported to have some α-adrenoceptor blocking activity which might complicate the interpretation of its effects on the uptake of noradrenaline (Turker & Khairallah, 1967; Hughes, Kneen & Main, 1974), its effects were similar to those of cocaine and strikingly different from those of phenoxybenzamine. This suggests that under the present experimental conditions the effect of DMI is principally due to inhibition of the neuronal uptake process for noradrenaline.

Pbz has been reported to inhibit the extraneuronal uptake process for noradrenaline (Avakian & Gillespie, 1968; Lightman & Iversen, 1969) and such an action, which tends to prolong the activity of noradrenaline, would be expected to move the amplitude-IRR curve in a similar manner to DMI or cocaine. The failure of Pbz to have this effect suggests that either the extraneuronal uptake is not inhibited in

these studies, or that it is not a significant route of transmitter inactivation in the presence of an effective neuronal uptake mechanism.

The second component of the distinctly biphasic relaxation of the nictitating membrane responses, elicited at high frequencies of stimulation in the presence of DMI (1.2 mg/kg), may be due to the re-release of noradrenaline from a second pool which was formed during the period of nerve stimulation, for instance by binding to collagen (Avakian & Gillespie, 1968) or by uptake into smooth muscle (Gillespie, 1976). The formation of a second pool of noradrenaline in the media of rabbit aortic strips has already been demonstrated (Nedergaard & Bevan, 1971) and the efflux of noradrenaline from extraneuronal pools in smoth muscle has already been shown to affect the rate of relaxation of rabbit aortic strips (Trendelenburg & Henseling, 1976). A second component in the relaxation of the nictitating membrane has been previously studied *in vitro* after inhibition of monoamine oxidase (Trendelenburg, 1971b) and was considered to be due to leakage of noradrenaline from nerve endings after uptake. Such a leakage would not satisfactorily explain the present results, as the second slow component of relaxation was observed only after DMI and at high frequencies of stimulation. Under these conditions, neuronal uptake is inhibited and the high concentration of noradrenaline in the neuroeffector gap would more readily permit uptake of noradrenaline into smooth muscle, producing an extraneuronal pool from which it may subsequently be released. The second slow component in the relaxation of the nictitating membrane complicates the interpretation of $T_{\frac{1}{4}}$ values when the shoulder of the recovery curve is greater than 50% of the response amplitude, as this produces a disproportionate increase in the $T_{\frac{1}{4}}$ value (see $T_{\frac{1}{4}}$ of 20 Hz response after 1.2 mg/kg DMI in Table 1 and Figure 4).

Because the IRR is independent of response amplitude and complicating factors such as biphasic recovery curves, it is concluded that the graphs of IRR against amplitude of responses can provide information about the process of neuroeffector transmission which is often obscured in the presentation of results in the form of $T_{\frac{1}{4}}$.

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