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EVIDENCE THAT ADRENERGIC NERVES ARE RESPONSIBLE FOR THE ACTIVE UPTAKE OF NORADRENALINE IN THE GUINEA-PIG ISOLATED TRACHEA

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1 6-Hydroxydopamine (50 mg/kg, i.p.) was given to guinea-pigs to destroy the adrenergic nerve terminals in the trachea.

2 The destruction was demonstrated by fluorescence histochemistry, which showed a marked loss of beaded fluorescent terminal fibres and by electrical transmural stimulation of the isolated atropinized trachea, which showed a marked reduction of dilator responses.

3 Such tracheae showed greatly reduced uptake-with-retention of (−)-[^{3}H]-noradrenaline in incubation experiments and the efflux curve of radioactive material showed a selective but incomplete reduction in the volume of the slowly exchanging compartment.

4 It is concluded that much, but perhaps not all, of the uptake-with-retention occurs into adrenergic nerves.

Introduction

Foster & O'Donnell (1972) examined the properties of the uptake-with-retention of radioactive material occurring when the guinea-pig trachea was exposed to (−)-[^{3}H]-noradrenaline. Though lacking direct evidence, they concluded that the uptake occurred into adrenergic nerves since (a) adrenergic nerves can be demonstrated in the guinea-pig trachea pharmacologically (Foster, 1964) and histochemically (O'Donnell & Saar, 1973) and (b) the properties of the uptake resembled those of established intraneuronal uptake of noradrenaline, namely, sodium dependence, susceptibility to ouabain, saturability, energy dependence and susceptibility to inhibition by cocaine and desipramine (cf. Iversen, 1970).

The experiments described in this paper were carried out in an attempt to obtain more direct evidence that the radioactive material firmly retained in trachea after incubation in (−)-[^{3}H]-noradrenaline 50 nM had been taken up into adrenergic nerves. The approach adopted used 6-hydroxydopamine (6-OHDA) as a tool to destroy the functional integrity of the adrenergic nerve terminals in the trachea (O'Donnell & Saar, 1974). The uptake-with-retention in tracheae from animals pretreated with 6-OHDA was compared with that in controls and the effectiveness of the destruction was checked both histochemically, by the fluorescence technique of Falck & Hillarp (Falck, 1962), and pharmacologically, by transmural stimulation to excite adrenergic nerves and so elicit relaxant responses.

Methods

Female guinea-pigs (250-560 g) were used. A dose of 50 mg/kg 6-OHDA was given intraperitoneally to 12 animals 24 h before examining the trachea. Control animals, matched with these for body weight, received 0.9% w/v NaCl solution (saline).

Fluorescence histochemistry and uptake-with-retention

The laryngeal ring from each of six control animals and six animals pretreated with 6-OHDA was taken for fluorescence histochemistry as described by O'Donnell & Saar (1973). Before freeze-drying the rings were incubated in α-methylnoradrenaline 1 μM for 30 minutes. The remainder of the trachea was cut into rings and the uptake-with-retention of radioactive material measured according to the method of Foster & O'Donnell (1972) by incubating for 15 min in (−)-[^{3}H]-noradrenaline 50 nM (and ascorbic acid 0.6 mM) and washing for

30 min to clear the interstitial fluid of radioactive material.

Transmural stimulation and efflux

Each trachea from six control and six 6-OHDA pretreated guinea-pigs was set up, in Krebs solution containing atropine (350 nM), for transmural stimulation essentially according to the method of Foster (1964), although changes in intraluminal pressure, rather than volume, were recorded (Farmer & Coleman, 1970). A 20 min equilibration period at atmospheric pressure was allowed, plus a further 10 min after closing the system. Each trachea was transmurally stimulated (positive electrode in lumen) with 40 mA pulses, width 0.5 ms for 7 seconds. Relaxant responses were produced by three stimulation trains at each of 1.25, 2.5 and 5 Hz given at 4 min intervals and then by three trains at each of 10 and 20 Hz given at 5 min intervals. The response to transmural stimulation was measured as the mean change in intraluminal pressure obtained with the second and third trains of stimuli in each sequence. The trachea was then cut into rings and allowed to equilibrate in fresh Krebs solution containing atropine (350 nM) at 37° C for 1 h before transfer to 4 ml of incubation medium also containing (−)-[^{3}H]-noradrenaline (50 nM) and ascorbic acid (0.6 mM) for 15 minutes. The efflux of radioactive material on repeated washing of tissue was then measured, essentially by the method described by Foster & O'Donnell (1972) but following it for the longer time of 75 minutes.

Radioassay

Samples (1 ml) of washings or of the clear supernatant of a 0.4 N perchloric acid homogenate of trachea were added to 10 ml of phosphor, which had the composition: 333 ml Triton-X 100; 5.5 g PPO (2,5-diphenyl-oxazole); 0.1 g dimethyl-POPOP (1,4-di-(2(4-methyl-5-phenyl oxazolyl))-benzene); toluene (A.R.) to 1 litre. The automatic external standard channels ratio method was used to correct for quenching.

Drugs

6-OHDA was obtained from Hassle, Sweden. Doses are given in terms of the hydrochloride. (−)-Noradrenaline-[7-^{3}H] (5.6 Ci/mmol, radiochemical purity greater than 97%) was obtained from the Radiochemical Centre, Amersham. Other drugs and chemicals were from commercial sources.

Statistical analysis

The measure of variation of the mean quoted is the standard error. Student's *t*-test (2 tailed) was used to assess the probability that two means did not differ. Correlation was examined by the non-parametric Kendall rank correlation coefficient (Siegel, 1956).

Results

The mean tracheal content of radioactive material after 15 min incubation with (−)-[^{3}H]-noradrenaline and 30 min washing was 70.6 ± 12.3 pmol/g in the control group, while in the 6-OHDA pretreated group it was 20.1 ± 2.4 pmol/g ($P < 0.005$).

Beaded, terminal fibres were seen in the sections from five of six control tracheae but were absent from the sections of one, which also gave the smallest uptake-with-retention (38.5 pmol/gram). Beaded terminal fibres were absent from sections from five of the 6-OHDA pretreated tracheae but two or three faint beaded strands were present in one, which also gave the largest uptake-with-retention (30.5 pmol/gram). The probability (P) that this combination, or one more extreme, would arise by chance is 0.04 (Fisher's exact test).

The mean dilator response to each frequency of stimulation was significantly smaller in tracheae from 6-OHDA-treated animals than in the control tracheae, indeed at the lower three frequencies the mean response in the 6-OHDA group did not differ significantly from zero (Figure 1).

In the efflux experiments the tracheal content of radioactive material at all times after incubation was lower in each of the 6-OHDA pretreated group than in any of the controls. Curve stripping (Riggs, 1963) revealed three exponential components to the decay of tissue radioactivity with time in both groups (Table 1). The effect of 6-OHDA was selectively directed to the most slowly exchanging compartment, whose apparent volume (C) was reduced while its efflux rate constant (γ) was increased.

The fall in intraluminal pressure caused by transmural stimulation at 5 Hz and the size of this slowly exchanging compartment estimated as the intercept (C) at the beginning of washout seemed to be related to each other (Figure 2). The estimated volume of distribution of slowly exchanging radioactive material at zero response was 0.7 ml/g which corresponds with an uptake-with-retention (30 min •washing) of 23 pmol/gram.

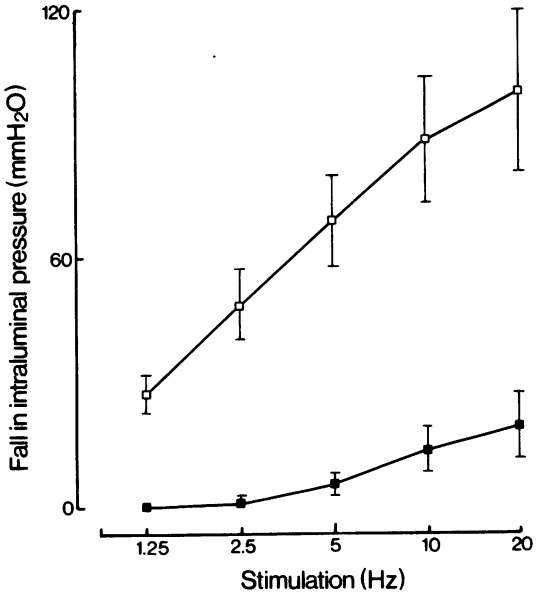

Figure 1 Effect of 6-hydroxydopamine (6-OHDA) pretreatment on the dilator response to transmural stimulation of atropinized trachea. Ordinates — mean fall in intraluminal pressure (mm water). Vertical bars show s.e. mean. Abscissae — frequency of stimulation (Hz) applied for 7 seconds. (□) Control, (■) 6-OHDA pretreated trachea.

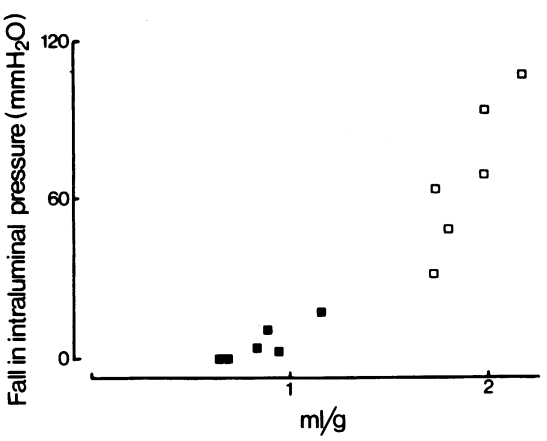

Figure 2 The relationship between response to transmural stimulation and tissue radioactivity in atropinized tracheae. Ordinates — mean fall in intraluminal pressure (mm water), obtained with transmural stimulation at 5 Hz for 7 seconds. Abscissae — apparent volume of slowly exchanging compartment of tissue radioactivity after incubation with 50 nM (−)-[^{3}H]-noradrenaline for 15 minutes. (□) Control tracheae correlate with $P < 0.05$; (■) 6-OHDA pretreated tracheae correlate with $P < 0.1$. All twelve tissues correlate with $P < 0.001$.

Table 1 Separation of exponents from the efflux curve $Y_t = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$, where Y_t = radioactive material content of 1 g of trachea, t = time of washing

Exponential decay	*Control*	*6-OHDA*	*2P*
Very fast			
A (ml/g)	1.24 ± 0.10	1.41 ± 0.16	0.6
α (min^{-1})	1.083 ± 0.073	1.158 ± 0.122	0.6
Fast			
B (ml/g)	0.87 ± 0.01	1.07 ± 0.08	0.06
β (min^{-1})	0.104 ± 0.003	0.118 ± 0.008	0.15
Slow			
C (ml/g)	1.90 ± 0.07	0.85 ± 0.08	<0.0001
γ (min^{-1})	0.0055 ± 0.003	0.0158 ± 0.0019	0.003

The very fast exponential decay ($t_{\frac{1}{2}}$ c 0.6 min) probably represents radioactive incubation medium adhering to the tissue on transfer to the first washing tube. The fast phase ($t_{\frac{1}{2}}$ c 6 min) represents radioactive material in the interstitial space of the tissue and the slow one ($t_{\frac{1}{2}}$ c 120 min) 'cellular' radioactivity (Foster & O'Donnell, 1972).

Discussion

The primary objective of this study was to obtain evidence that the radioactive material which was firmly retained in the isolated trachea after exposure to a low concentration of (−)-[^{3}H]-noradrenaline had been taken up into adrenergic nerves. This objective was attained. Pretreatment with 6-OHDA was demonstrated both histochemically and by transmural stimulation to cause loss of the functional integrity of adrenergic nerves of the trachea. Moreover, preparations so treated

retained significantly less radioactive material than controls after incubation with 50 nM (−)-[^{3}H]-noradrenaline.

There is evidence that 6-OHDA was not completely effective in destroying all adrenergic nerves in that (a) beaded fluorescent fibres were seen in one pretreated trachea, (b) dilator responses to the higher frequencies of transmural stimulation were reduced but not abolished, (c) the uptake-with-retention of radioactive material was reduced but not abolished, and (d) the apparent volume of the most slowly exchanging compartment of tissue radioactivity, which is its basis, was reduced but not to zero. It is a matter for speculation whether the residual dilator responses to transmural stimulation were mediated by residual adrenergic nerves or by non-adrenergic nerves (Coleman, 1973). However O'Donnell & Saar (1974) have shown that higher doses of 6-OHDA always cause loss of beaded fluorescent fibres from the trachea, but do not further reduce its uptake-with-retention of radioactive material.

However the question arises does all the uptake-with-retention represent radioactive material taken up into adrenergic nerves. None of the treatments shown in previous publications to inhibit significantly uptake-with-retention of radioactivity has abolished it; a residuum varying from approximately 10 pmol/g (ouabain, Foster & O'Donnell, 1972; and guanethidine, Foster, 1968) to 17 pmol/g (phenoxybenzamine, Foster, 1968) has always remained despite treatment with apparently maximally effective concentrations of inhibitors of uptake.

O'Donnell & Saar (1973) have suggested, on the basis of histochemical evidence, that after incubation with a high concentration of noradrenaline the noradrenaline which is bound to cartilage (extraneuronal, extracellular) does not wash away quickly enough to be dissipated in 30 minutes. Experiments to test the idea that this is the origin of the residuum are in progress.

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