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AN INHIBITORY EFFECT OF ATROPINE ON RESPONSES OF THE VAS DEFERENS OF THE MOUSE TO FIELD STIMULATION

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1 Atropine is shown to impair responses of the vas deferens of the mouse to field stimulation by acting at a site proximal to the smooth muscle cells.

2 The inhibitory effect of atropine is prevented by desmethylimipramine and reversed by dexamphetamine, and appears similar to the adrenergic-neurone blockade of guanethidine.

3 Electronmicroscopical studies show the presence in vas of presumptive noradrenergic axons which have acetylcholinesterase reaction product associated with their axolemmae.

4 These results are discussed in relation to the controversial hypothesis of a 'cholinergic link' in noradrenergic transmission.

Introduction

Previous pharmacological studies of the vas deferens have been confined largely to organs obtained from guinea-pigs or rats and the innervation of these structures is considered to be adrenergic in nature (Huković, 1961; Birmingham & Wilson, 1963; Graham, Al Katib & Spriggs, 1968; Swedin, 1971; Furness, 1974). The existence of a physiologically important cholinergic component in the innervation was largely accounted for by the discovery of ganglionic relays in what had hitherto been considered as classical post-ganglionic sympathetic nerves (Sjöstrand, 1962; Graham *et al.*, 1968). Postganglionic neurotransmission is not sensitive to hemicholinium (Bentley & Sabine, 1963) and is resistant to blockade by atropine (Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Graham *et al.*, 1968). There remain, however, phenomena which are inconsistent with the concept that the vas is a completely noradrenergically innervated tissue. Axons which show a positive reaction for acetylcholinesterase have been reported to be present in the vas of the guinea-pig (Robinson, 1969); the response of the vas to postganglionic nerve stimulation is potentiated by anticholinesterase drugs (Birmingham, 1966) and the sensitivity of the vas to noradrenaline (Ambache & Zar, 1970) and to α-adrenoceptor blockade is very low (Bentley & Smith, 1967).

In the present study, in which the vas deferens of the mouse was used, atropine exerted an inhibitory effect on responses to field stimulation. The possibility of a physiologically significant cholinergic component in the innervation of the vas of mouse has been investigated by means of pharmacological and morphological techniques.

Methods

Mature male albino mice, T.O. strain, weighing 25-35 g and over four weeks old were killed by cervical dislocation and both vasa deferentia removed. Single organs were suspended in Huković's (1961) solution of the following composition (mM): NaCl 113, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.1, $NaHCO_3$ 25.0, glucose 11.5, bubbled with 5% CO_2 in O_2 at 32° C. The tissue was subjected to 300 mg applied tension and allowed to equilibrate for 30-40 minutes. Isometric responses were monitored on a Devices 2 channel pen recorder via a 2 oz dynamometer UFI strain gauge. Isotonic contractions were recorded on smoked paper with a frontal writing lever or on chart paper with an ink writing 'Follograph' (EOS Industrial Electronics Ltd. see Tonks & Williams, 1972). The magnification used in each isotonic method was 10 to 15 times.

Field stimulation (Birmingham & Wilson, 1963) of the vas, suspended between two parallel platinum wire electrodes immersed in a 50 ml bath, was by means of an SR1 6053 stimulator delivering rectangular pulses of 0.3 ms duration at frequencies of 30 Hz and of supramaximal voltage every 3.25 minutes. An oscilloscope was used to confirm the uniformity and reproducibility of the

stimulation parameters under experimental conditions with the electrodes totally immersed in the bathing fluid.

Inhibitory drugs

The response to field stimulation was measured 20 min after exposure of the vas to the drug, at which time the inhibition was maximal.

Results are expressed in terms of percentage inhibition which was calculated as:

$$100 - \left(\frac{\text{size of response 20 min after exposure to drug}}{\text{size of (control) response before exposure to drug}} \times 100 \right).$$

The percentage inhibition caused by the inhibitory drug in the presence of dexamphetamine (Dex) or desmethylimipramine (DMI) was calculated as:

$$100 - \left(\frac{\text{size of response 20 min after exposure to guanethidine or atropine}}{\text{size of (control) response before exposure to Dex or DMI}} \times 100 \right)$$

Mean percentage inhibition with standard error was calculated for each dose of drug. The correlation coefficient, regression coefficient, and the corresponding regression line was determined for each experimental condition.

Responses to noradrenaline or acetylcholine

In experiments not involving field stimulation, tissues were suspended in 10 ml baths. Noradrenaline (NA), acetylcholine (ACh) or potassium was left in contact with the tissues for 0.5 min every 3 minutes. Bathing fluid was replaced at 1.5 min intervals. When testing the response to NA, ACh or potassium in the presence of an inhibitor a 15 min period elapsed before addition of agonist during which time the bathing fluid + inhibitor was changed every 1.5 minutes.

Analysis of covariance (Snedecor, 1956) was used to determine whether regression lines were parallel and coincident with one another.

Electron Microscopy

Mice were injected intravenously with vehicle (0.2 mg ascorbic acid/ml 0.9% w/v NaCl solution or 6-hydroxydopamine (6-OHDA) 0.2, 0.4 or 1 mmol/kg, killed 24 h later by cervical dislocation and both vasa deferentia removed.

Routine investigation One mm cubes of epididymal end of vas tissue were immersed in 1% osmium tetroxide, buffered to pH 7.5 with barbitone acetate for 1 h at 4° C, dehydrated and subsequently embedded in araldite. Fine sections were stained with lead citrate (Reynolds, 1963) and viewed with a Phillips 300 electron microscope.

Demonstration of acetylcholinesterase (AChE) Specimens were fixed in gluteraldehyde and incubated for 4 h at pH 5.5 with acetylthiocholine in the presence of 2×10^{-4} M ethopropazine (a pseudocholinesterase inhibitor) after the method of Lewis & Shute (1966). When physostigmine (2×10^{-4} M), a non-specific anticholinesterase was used in place of ethopropazine, no acetylcholinesterase reaction product was visible in any of the specimens examined.

Results

Field stimulation

Inhibition of responses to field stimulation was induced by each of the following drugs; guanethidine, phentolamine, pronethalol, lignocaine, atropine or hyoscine, when present in the bath in appropriate concentration (Figure 1). The inhibitory effect of lignocaine was readily reversed on washing out the bath, whereas the effects of the other drugs were not alleviated after repeatedly washing out the bath over a 30 min period. A relationship which did not differ from linearity ($P < 0.001$) was found between the log dose of each drug used and its inhibitory activity. There was no significant difference ($P > 0.05$) between regression coefficients and the regression lines may be considered to be parallel. A significant difference in elevation indicates that the lines are not coincident ($P < 0.01$).

Responses to noradrenaline or acetylcholine

In the presence of guanethidine (2.0×10^{-6} M) or pronethalol (7.5×10^{-5} M) the response of the vas to NA (6×10^{-6} M) was unaffected or potentiated and that to ACh (2×10^{-4} M) was unmodified. Lignocaine (6.9×10^{-4} M) increased the response

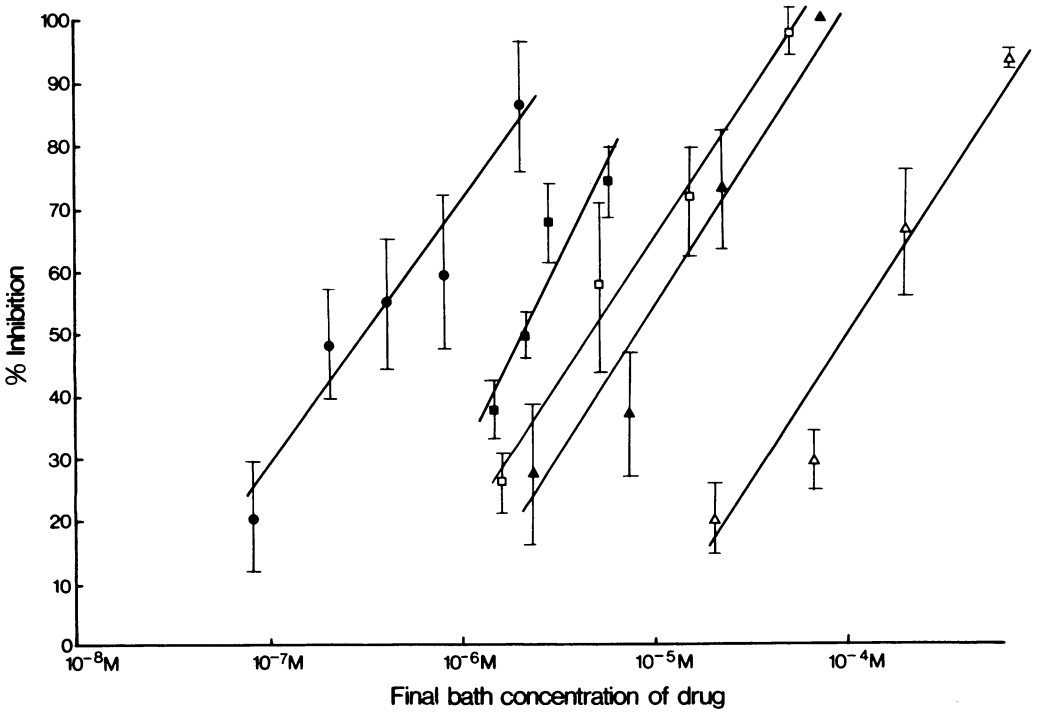

Figure 1 The percentage inhibition of the response of the vas deferens of the mouse to field stimulation in the presence of guanethidine (●), atropine (■), phentolamine (□), pronethalol (▲), or lignocaine (△). Each point is the mean ± s.e. of the values obtained from at least 5 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis.

of the vas to NA or ACh but responses returned to control values after washing the lignocaine from the tissue bath. Phentolamine (5.3×10^{-5}M) abolished completely the response to NA which was not restored after repeated washings of the tissue with phentolamine-free Huković solution. The response to ACh was unaffected by the presence of phentolamine.

The response of the vas to ACh (1×10^{-4}M) was abolished in the presence of atropine (2.9×10^{-6}M) whereas that to potassium (2×10^{-2}M) and NA appeared unaffected. The log dose-response curve for NA in the presence of atropine (2.9×10^{-6}M) was further investigated (Figure 2) in 12 vasa and found not to differ from linearity in the concentration range 3×10^{-7} to 1.2×10^{-4}M (correlation coefficient, $r = 0.96$, $d.f. = 130$, $P < 0.001$; regression coefficient, $b = 30.18$) and was compared with that for NA alone ($r = 0.93$, $d.f. = 117$, $P < 0.001$, $b = 32.56$). There was no significant difference ($P > 0.05$) in the slopes or elevations of these two lines.

The antagonism of drug-induced blockade of nerve transmission

DMI (9.4×10^{-8}M) or dexamphetamine (2.7×10^{-6}M) added to the bath prior to guanethidine inhibited the development of guanethidine-induced blockade (Figure 3). Atropine-induced blockade similarly was prevented by prior administration of DMI or dexamphetamine (Figure 4).

For each preparation the percentage inhibition caused by the inhibitory drug in the presence of DMI or dexamphetamine was calculated. A relationship which did not differ from linearity ($P < 0.001$) was found between this percentage inhibition and concentration of guanethidine or atropine in the bath (Figures 3 and 4). An analysis of covariance carried out on the regression lines showed no significant difference ($P > 0.05$) between the slopes of the six regression lines, and significant ($P < 0.01$) shifts of the log dose-response curve for guanethidine to the right in the

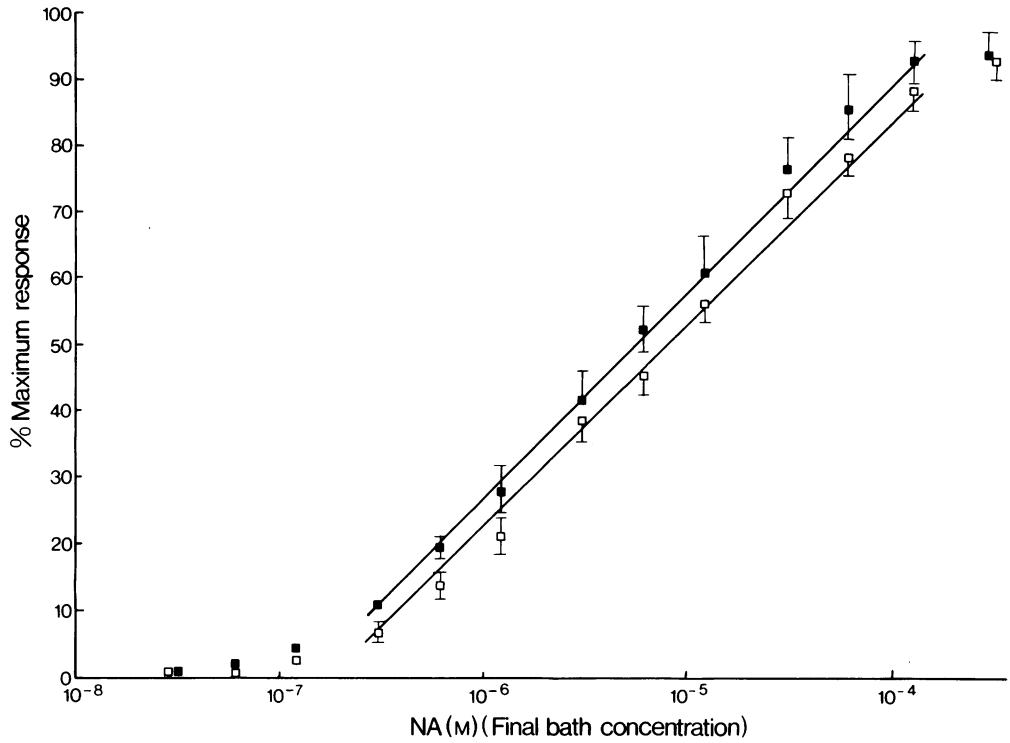

Figure 2 The log dose-response curve for noradrenaline (NA) alone (■), and NA in the presence of atropine (□, 2.9×10^{-6}M) in the vas deferens of the mouse. Each point is the mean ± s.e. from 12 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis in the dose range 3×10^{-7} M to 1.2×10^{-4} M for NA.

Table 1 Quantitation of the inhibitory effect of guanethidine, atropine, hyoscine or lignocaine on the response of the vas deferens of the mouse to field stimulation, and the effect of dexamphetamine thereon.

Drug	*Final bath conc. M*	*Inhibited response as % control response*	*Response after dexamphetamine (1.1×10^{-5} M) as % control response*
Guanethidine	8.1×10^{-8}	79.4 ± 8.4 (6)	102.0 ± 5.5 (6)*
	2.0×10^{-7}	51.9 ± 8.8 (6)	102.5 ± 14.9 (6)**
	4.0×10^{-7}	45.4 ± 10.2 (6)	90.8 ± 6.7 (6)**
	8.1×10^{-7}	40.2 ± 12.2 (6)	93.5 ± 8.9 (6)**
	2.0×10^{-6}	13.5 ± 10.2 (5)	90.8 ± 13.2 (5)**
Atropine	1.4×10^{-6}	62.2 ± 4.0 (7)	105.5 ± 10.3 (7)**
	2.2×10^{-6}	50.4 ± 3.6 (6)	79.0 ± 8.0 (6)**
	2.9×10^{-6}	32.2 ± 6.4 (6)	87.4 ± 9.2 (6)**
	5.8×10^{-6}	25.6 ± 5.5 (6)	77.7 ± 3.1 (6)**
Hyoscine	1.4×10^{-6}	63.1 ± 5.4 (3)	91.2 ± 2.9 (2)**
Lignocaine	2.1×10^{-5}	80.0 ± 5.4 (6)	93.6 ± 27.48 (3)
	6.9×10^{-5}	70.9 ± 4.9 (6)	82.3 ± 9.78 (4)
	2.1×10^{-4}	43.6 ± 6.7 (6)	59.1 ± 1.52 (2)

Significant differences between columns 2 and 3 were calculated using Student's *t* test (Saunders & Fleming, 1957) $*P < 0.05$; $**P < 0.01$.

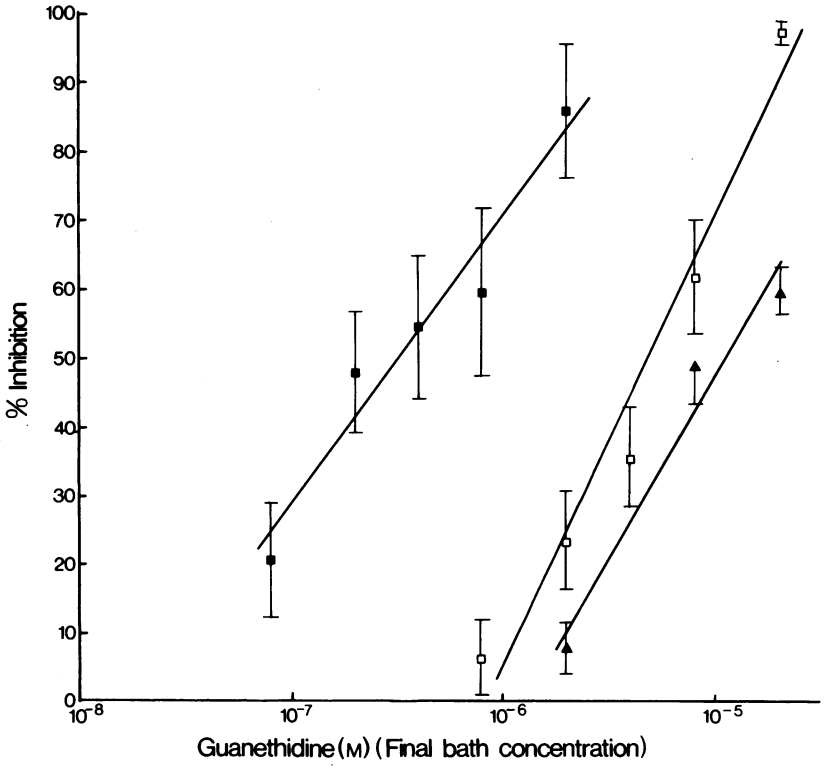

Figure 3 The inhibition by dexamphetamine (2.7×10^{-6}M) or desmethylimipramine (DMI) (9.4×10^{-8}M) of the guanethidine-induced blockade of responses of mouse vas to field stimulation. Responses are plotted as the percentage inhibition of the maximum response to field stimulation. Each point is the mean ± s.e. from not less than 4 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis. (■), Guanethidine alone; (□) DMI + guanethidine; (▲) dexamphetamine + guanethidine.

presence of DMI or dexamphetamine. The shifts in the log dose-response curve for atropine in the presence of DMI or dexamphetamine are also significant ($P < 0.01$).

The reversal of drug-induced blockade of nerve transmission

The blockade induced by guanethidine, atropine or hyoscine was reversed significantly ($P < 0.05$) by the addition of dexamphetamine (1.1×10^{-5}M) to the bath. Dexamphetamine did not reverse the inhibitory effect of lignocaine, phentolamine or pronethalol. In Table 1 responses, measured 20 min after guanethidine (or atropine) and responses measured 20 min after the additional administration of dexamphetamine, have been expressed as percentage of control (pre-guanethidine or pre-atropine) responses.

Electron microscopy

Axons containing small dense-core vesicles (25-60 nm) in tissue fixed with glutaraldehyde and post-osmicated are characteristic of noradrenergic axons and accounted for 85% of the total axon population. In tissues fixed solely with OsO_4, only 41% of axons possessed small dense-core vesicles (Table 2). However, previous workers have reported that OsO_4 is not a good fixative for dense-core vesicles (Richardson, 1962; Tranzer, Thoenen, Snipes & Richards, 1969). In tissue pretreated with 6-OHDA presumptive noradrenergic axons show small dense-core vesicles even with OsO_4 fixation or (with larger doses) clumping of axoplasmic contents to form dense osmiophillic masses (Table 2).

In tissue that had been incubated with acetylthiocholine, fixed in gluteraldehyde and

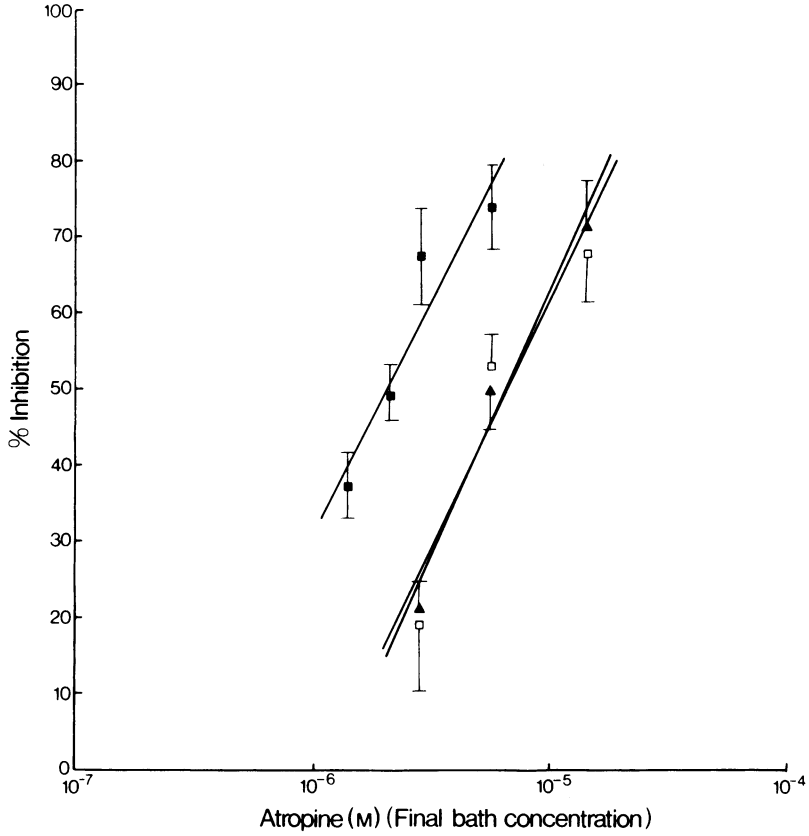

Figure 4 The inhibition by dexamphetamine (2.7×10^{-6}M) or desmethylimipramine (9.4×10^{-8}M) of the atropine-induced blockade of responses of mouse vas to field stimulation. Responses are plotted as a percentage inhibition of the maximum response to field stimulation. Each point is the mean ± s.e. from not less than 5 vasa. The lines are drawn through co-ordinates calculated by the method of least squares analysis. (■) Atropine alone; (□) DMI + atropine; (▲) dexamphetamine + atropine.

Table 2 Analysis of electronmicrographs: the percentage of total axons having the axonal characteristic described in column 1.

Pretreatment of mice	None	None	6-OHDA 0.2 mmol/kg 24 h	6-OHDA 0.4 mmol/kg 24 h	6-OHDA 1 mmol/kg 24 h
Fixation of vas	Glutaraldehyde post OsO_4	OsO_4	OsO_4	OsO_4	OsO_4
Axonal characteristic					
1. Small dense-core vesicle	85.0	41.4	77.2	82.3	8.4
2. Small electron-translucent vesicle	9.3	50.0	10.0	6.1	7.2
3. Signs of degeneration	–	–	8.4	3.6	78.4
4. None of above characteristics	5.7	8.6	4.4	8.0	–

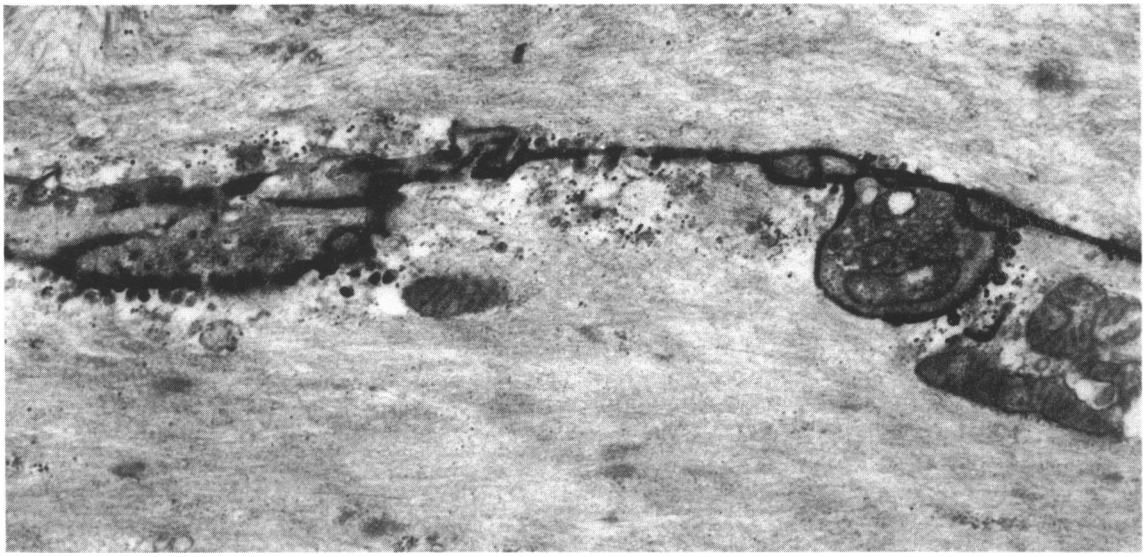

Figure 5 Electronmicrograph of glutaraldehyde-fixed vas incubated with acetylthiocholine. Two axons having an electron dense acetylcholinesterase reaction product associated with their axolemmae possess small vesicles (ca. 50 nm diameter) which contain electron-dense material. Such vesicles in glutaraldehyde-fixed tissue are indicative of noradrenergic nerves.

Table 3 The distribution of acetylcholinesterase (AChE) reaction product around axons in control and chemically sympathectomized vasa

Axon characteristics	*No. axons, with characteristics shown in column 1, as percentage of total axons*	
	*Control vasa**	*Chemically sympathectomized vasa*†*
1. Intact containing small dense-core vesicles with AChE reaction product	29.5	0
2. Degenerating with AChE reaction product	0	16.7
3. Intact with electron translucent vesicles and AChE reaction product	7.5	7.8
4. Intact with electron-translucent vesicles and *no* AChE reaction product	1.8	5.0
No. of axons studied	161	258

* Fixation with glutaraldehyde and post-osmicated
† Mice given 6-OHDA 1 mmol/kg intravenously 24 h previously.

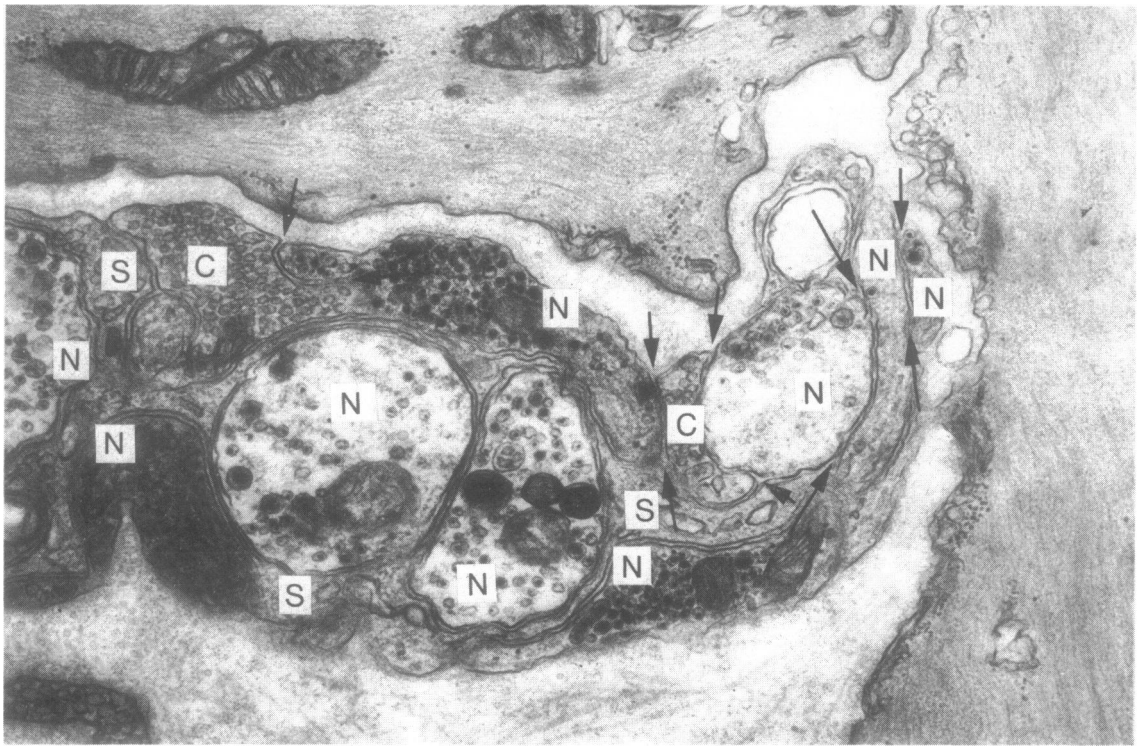

Figure 6 Electron micrograph of axon bundle in vas taken from mouse treated 1 h previously with 6-hydroxydopamine 0.2 mmol/kg. Presumptive noradrenergic axons (N) show conspicuous dense core vesicles, whereas presumptive cholinergic axons (C) contain electron-translucent vesicles. Possible synaptic relationships between axons are indicated by arrows, the interval between axolemmae being ca. 20 nm and devoid of Schwann cell processes (S).

post-osmicated, acetylcholinesterase (AChE) reaction product was observed on the axolemma of most nerves containing small electron-translucent vesicles in both control or chemically sympathectomized vasa (Table 3, line 3). Such AChE positive axons constituted 7-8% of the total number of axons investigated, and are designated as presumptive cholinergic axons.

A χ^2 test (Seigal, 1956) showed that there was no difference in the population of AChE positive axons containing electron-translucent vesicles ($P > 0.05$) as between normal and sympathectomized vasa.

Because of the small sample number of AChE negative axons containing electron translucent vesicles in control vasa (Table 3, line 4) it was not possible to carry out χ^2 tests on this sample.

In addition to axons containing electron-translucent vesicles, AChE reaction product was observed on the axolemmae of axons containing small dense-core vesicles (Figure 5); 29.5% of the total number of axons studied in control vasa were AChE positive and contained small dense-core vesicles. In sympathectomized vasa 16.7% of the total showed signs of degeneration and were also AChE positive (Table 3). A χ^2 test showed that normal and sympathectomized vasa differed with respect to the number of presumptive noradrenergic axons which were also AChE positive ($P < 0.05$). Nerve bundles (containing 5.6 ± 0.24 axons/bundle) in the extracellular spaces within muscle cell bundles contained some axons which were densely packed with vesicles and mitochondria and others which contained only neurotubules. The Schwann cell sheath was often incomplete leaving some axolemmae naked in parts. Axons in bundles were frequently in close apposition (20 nm) to other axons within that bundle, a situation in which synaptic transmission could occur. In a survey of 475 axons in bundles, 84 presumptive noradrenergic axons and 8 presumptive cholinergic axons were juxtaposed to

neighbouring axons and although no specializations of axolemmae were observed the relationships appeared potentially synaptic (Figure 6).

Discussion

The effective motor innervation of the vas deferens of the mouse appears to be exclusively noradrenergic in nature (Furness, 1974; Jones & Spriggs, 1975). The ability of guanethidine to block completely the response of the vas to field stimulation contributes to this hypothesis. An apparent inconsistency arises, however, with the present finding that atropine impairs responses to field stimulation. Although the concentrations of atropine used in the present study may appear large, they are comparable with the concentrations of ACh (2.8×10^{-6}M to 4.4×10^{-4} M) necessary to elicit contractions of the mouse vas (Jones & Spriggs, 1971) and do not impair responses to potassium (Jones, 1974) or to noradrenaline (Figure 2). Two possibilities arise:

1 ACh is an important motor transmitter acting at muscarinic receptors on smooth muscle of the vas. In support is the observation that presumptive cholinergic axons (AChE positive axons with electron-translucent vesicles) are present in the vas. However, such axons constitute less than 10% of the total axon population and although they survive chemical sympathectomy with 6-OHDA, transmural stimulation no longer elicits any increase in tension of the vas (Jones & Spriggs, 1975).

2. Atropine impairs sympathetic transmission and/or smooth muscle contractility in the vas of the mouse. A post-synaptic action is unlikely as the response of the vas to potassium remained unimpaired by the presence of atropine in concentrations which inhibited contractions induced by field stimulation; responses to exogenous NA were similarly unaffected.

Muscarinic receptors on adrenergic neurones in the heart have been reported by Lindmar, Löffelholz & Muscholl (1968). These receptors, when stimulated by ACh antagonize the release of NA. The activity of atropine described in this paper cannot be explained by the presence on noradrenergic neurones of this type of muscarinic receptor.

Percentage inhibition of responses to field stimulation/log dose plots for atropine and guanethidine are parallel, not only to each other, but also to similar plots for the local anaesthetic lignocaine, the α-adrenoceptor blocker phentolamine and the β-adrenoceptor blocker pronethalol. The inhibitory effect of atropine was persistent, as was that of guanethidine, in contrast to that of lignocaine which was readily reversed on washing out the bath.

Dexamphetamine is effective in reversing the adrenergic neurone blockade of guanethidine and similar drugs in many tissues (Day & Rand, 1963; Spriggs, 1966) including the vas of the mouse. In the present experiments dexamphetamine also reversed the inhibitory effect of atropine, but not that of lignocaine, phentolamine or pronethalol. Thus the inhibitory action of atropine is more like that of guanethidine than that of any of the other drugs used. This similarity was upheld in further experiments in which pretreatment of the vas with the uptake$_1$ inhibitor DMI significantly reduced the inhibitory effect of atropine as it did that of guanethidine (Figures 3 and 4). The possibility arises that atropine may be interfering with a 'cholinergic link' in noradrenergic transmission (Burn & Rand, 1959). Most of the evidence used in support of this controversial hypothesis would favour the involvement of 'nicotinic-like' rather than 'muscarinic-like' receptors (Burn & Rand, 1959, 1965; Cabrera, Torrance & Viveros, 1966; Rand & Wilson, 1967). On the other hand, Kadzielawa & Gumulka (1967a, b) and more recently Lederer, Rand & Wilson (1970) have claimed similarities between the adrenergic-neurone blocking activity and the anti-muscarinic potency of some compounds which they have investigated. However, we are not satisfied that their experimental design or the statistical appraisal of their results justifies such conclusions.

The finding that almost 40% of presumptive noradrenergic axons (i.e. axons containing dense-core vesicles in gluteraldehyde-fixed tissue or after 6-OHDA treatment) in the vas of the mouse showed a positive reaction for AChE is compatible with the 'cholinergic-link' hypothesis. However, acceptance of this hypothesis leaves unresolved the ability of atropine (5.8×10^{-6}M) to cause 75% inhibition of responses to field stimulation when only 40% of the presumptive noradrenergic axons show a positive reaction for AChE.

It has been argued that the presence of AChE is a prerequisite for a functional cholinergic mechanism and in earlier work on noradrenergic neurones (Esterhuizen, Graham, Lever & Spriggs, 1968; Graham, Lever & Spriggs, 1968) the absence of AChE was considered to militate against the presence of a functional cholinergic mechanism. The present findings of AChE in relation to presumptive noradrenergic axons has prompted us to undertake firstly, a more rigorous assessment of the specificity of this AChE and secondly, autoradiographic evidence that these axons do take up [^{3}H]-noradrenaline. However, we consider that there is now sufficient evidence for the

'classical noradrenergic neurone' to undergo careful scrutiny in each different tissue, and that one should be cautious in making or interpreting generalized statements concerning noradrenergic nerves; there are now reports in the literature of tissues in which (1) *no* noradrenergic axons show AChE 'staining' (pancreatic arterioles, Graham *et al.*, 1968; and nictitating muscles, Esterhuizen *et al.*, 1968, of cat) (2) *some* noradrenergic axons show AChE 'staining' (hypogastric and splenic nerves of cat, Lever, Spriggs, Graham & Ivens, 1970; vas of rat, Mottram, Ivens, Lever & Presley, 1973; vas of mouse, this paper) (3) *all* noradrenergic axons show AChE 'staining' (pineal of rat, Eranko, Rechardt, Eranko & Cunningham, 1970).

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