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In pursuit of the α-adrenoceptor: a fine-structural and electron autoradiographic study using ^{3}H-phenoxybenzamine and smooth muscle from the cat and guinea-pig

J. D. P. GRAHAM*, CAROLINE IVENS†, J. D. LEVER†, R. McQUISTON†, AND T. L. B. SPRIGGS*

Departments of Anatomy† and Pharmacology, University of Wales, Cardiff, S. Wales*

Summary

1. Electron microscopical evaluation of sets of serial fine sections of pancreatic arteriolar muscle of the cat showed no significant difference between the number of pinocytotic vesicles per unit length of smooth muscle cell membrane in 'synaptic' and 'non-synaptic' regions.

2. With high resolution autoradiography, ^{3}H-phenoxybenzamine was found to be distributed over the cytoplasm and nuclei of smooth muscle cell profiles of guinea-pig vas deferens, although no localization in relation to discrete morphological organelles was evident.

3. 'Specific protection' of α-adrenoceptors using 606 μM noradrenaline resulted in a significant diminution in the binding of ^{3}H-phenoxybenzamine as evidenced by a decrease in silver grain concentrations over smooth muscle cell profiles in autoradiographs.

4. The significance of these findings in relation to the site and distribution of α-adrenoceptors is discussed.

Introduction

Examination of electron micrographs of the synaptic region of the adrenergic nerves in close apposition to smooth muscle in several organs from several species (Lever, Graham, Irvine & Chick, 1965; Esterhuizen, Graham, Lever & Spriggs, 1967, 1968; Graham, Lever & Spriggs, 1968a; Lever, Spriggs & Graham, 1968) did not reveal any morphological subsynaptic specialization which could with confidence be attributed to an organized receptor area comparable, for example, to that of the motor end plate region of skeletal muscle. However, it was observed that the plasma membrane on the adventitial aspect of arteriolar smooth muscle cells had significantly more pinocytotic vesicles than did the endothelial aspects of the muscle membrane (Lever *et al.*, 1965).

The present paper reports the results of an attempt to assess by means of serial sections whether the number of pinocytotic vesicles present at smooth muscle surfaces opposite terminal areas of adrenergic axons was significantly greater that at non-synaptic muscle membranes. In addition, an attempt was made to locate the α-adrenoceptor (Ahlquist, 1948) by using high resolution autoradiography after

exposing tissues to tritium labelled phenoxybenzamine (Phb), a drug which abolishes unsurmountably those actions of noradrenaline conventionally attributable to the occupation of α-adrenoceptors (see review by Graham, 1962). A short account of the distribution of ^{3}H-Phb in the pancreatic arteriolar wall of the cat has been published (Graham, Lever & Spriggs, 1968b).

Methods

Electron microscopy

One millimetre cubes of freshly excised pancreas of the cat were fixed for 4 h at 4° C in 2·0% glutaraldehyde buffered to pH 7·2 with cacodylate buffer. The specimens were post-fixed with 1% osmium tetroxide, dehydrated and embedded in araldite. After locating arterioles, serial fine sections were obtained, mounted on grids, stained with lead citrate solution (Reynolds, 1963) and examined in an Elmiskop 1 electron microscope. A total of seven arterioles, from four cats, were serially sectioned and each series contained sixteen to thirty sections. In three of the arterioles suitable synaptic regions were traced throughout the entire series of sections.

Synaptic regions of terminal adrenergic axons and smooth muscle were identified according to previously established criteria (Graham, Lever & Spriggs, 1969); each synapse was photographed in each section of the series in which it could be located. Photographic prints were prepared at a final magnification of ×30,000.

The following criteria were applied in the identification of 'synaptic' smooth muscle membrane: (a) presence of nerve terminal area within 4,000 Å of muscle membrane; (b) absence of Schwann cell or fibroblast processes in the synaptic interval; (c) the length of 'synaptic' muscle membrane was defined to be within 4,000 Å of the nearest apposed aspect of naked axolemma of a terminal axon (see Fig. 1); (d) lengths of muscle membrane showing myofibril attachments (dense bodies) were excluded.

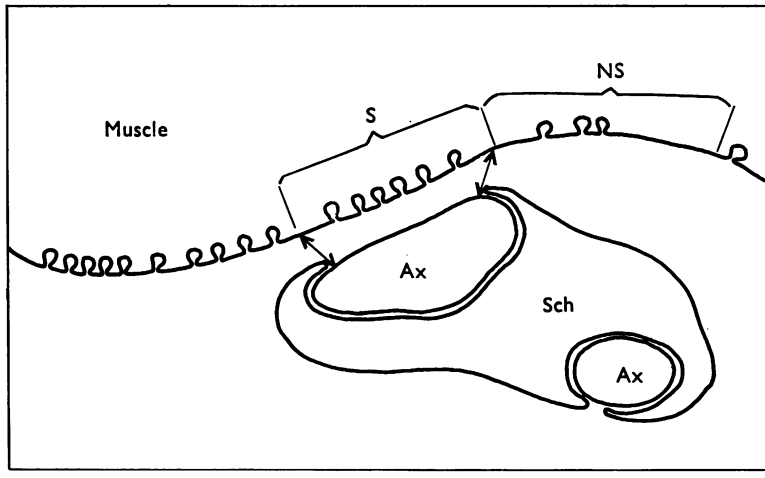

FIG. 1. Measurements used in delineating 'synaptic' (S) and 'non-synaptic' (NS) muscle membrane. Ax, axon; Sch, Schwann cell. ⟷, Interval of 4,000 Å between naked axolemma and muscle membrane. For each individual electron micrograph the length of 'non-synaptic' membrane used for pinocytotic vesicle counts was made equal to the length of 'synaptic' membrane.

For control measurements, 'non-synaptic' membrane profiles were used from muscle surfaces which were not apposed to nerve terminal areas. The length of 'non-synaptic' membrane was measured so as to be equal in length to the synaptic membrane for each micrograph. Areas of plasma membrane were selected as 'non-synaptic' only if they were shown to be so in continuity through the series. Pinocytotic vesicles showing continuity with the muscle membrane were counted, and expressed as attached pinocytotic vesicles per unit length muscle membrane.

Incubation of guinea-pig vas deferens with ^{3}H-Phb

Male guinea-pigs, 150–200 g body weight, were stunned and bled. Vasa deferentia were exsected, stripped of their mesenteric investments, and incised longitudinally to facilitate access of incubation fluid to the lumen of the organ. Specimens were incubated in appropriate solutions based on Krebs solution (composition NaCl, 118 mM; KCl, 4·69 mM; $CaCl_2$ 2·52 mM; $MgCl_2$, 1·15 mM; NaH_2PO_4, 1·17 mM; $NaHCO_3$, 24·4 mM; dextrose, 11·1 mM) gassed with 5% CO_2 in O_2 at 37° C as follows:

Control: incubated in Krebs solution for 1 min and then in Krebs solution containing ^{3}H-Phb (3·29 μM; 0·52 Ci/mmol) for 15 minutes.

Test: incubated in Krebs solution containing noradrenaline (NA) 606 μM for 1 min and then in Krebs solution containing NA, (606 μM) plus ^{3}H-Phb (3·29 μM) for 15 minutes.

Control and test material was subsequently rinsed in three washes of normal Krebs solution each of 1 min duration before specimens were taken for autoradiography.

Autoradiography

Specimens of guinea-pig vas deferens prepared as above were fixed and embedded in the same way as the pancreatic material. Fine sections were cut and processed for high resolution autoradiography according to the method of Lever, Spriggs & Graham (1968). The autoradiographs were stored for 3–6 months before being developed photographically and examined in the electron microscope. Photographs of smooth muscle were taken and printed at ×24,000 magnification. Montages were assembled and silver grain distribution expressed as grains per unit area over cellular elements and over intercellular background.

Drugs. Noradrenaline bitartrate, ^{3}H-phenoxybenzamine hydrochloride.

Results

Pinocytotic vesicles

In most sets of serial sections of arteriolar wall examined, the density of attached pinocytotic vesicles was greater for 'synaptic' than for 'non-synaptic' muscle membrane surfaces (Fig. 2). However, in no instance was this difference statistically significant (Table 1).

^{3}H-Phb distribution

One hundred and eighty-six micrographs were obtained using three grids from each of two specimens of vas deferens taken from each of five guinea-pigs. The

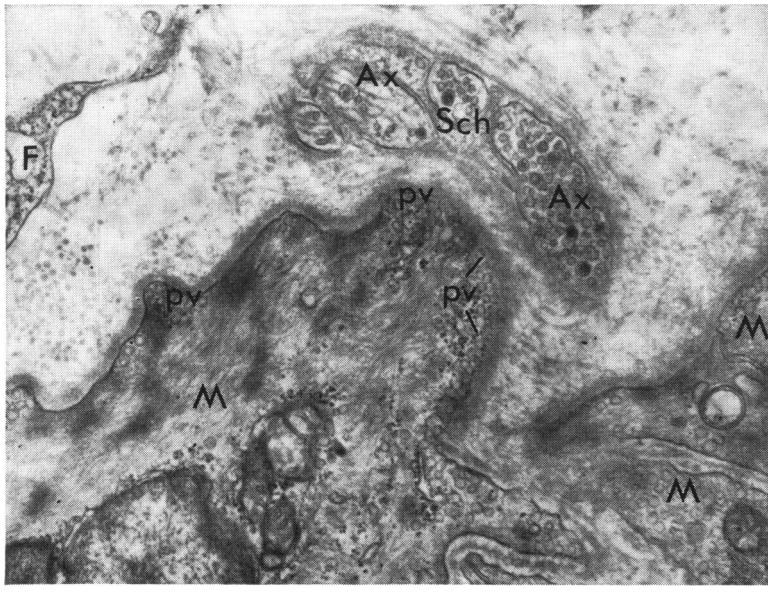

FIG. 2. Electron micrograph of terminal adrenergic axons (Ax) related to arteriolar smooth muscle (M) of the pancreas of cat. A large number of vesicles are close to, and in contact with, that portion of the muscle membrane which is closest to the axons. However, statistical quantitation from serial sections of synaptic regions show pinocytotic vesicles to occur with similar frequency on both 'synaptic' and 'non-synaptic' muscle membranes. Sch, Schwann cell; F, fibroblast process; pv, pinocytotic vesicles. ×22,800.

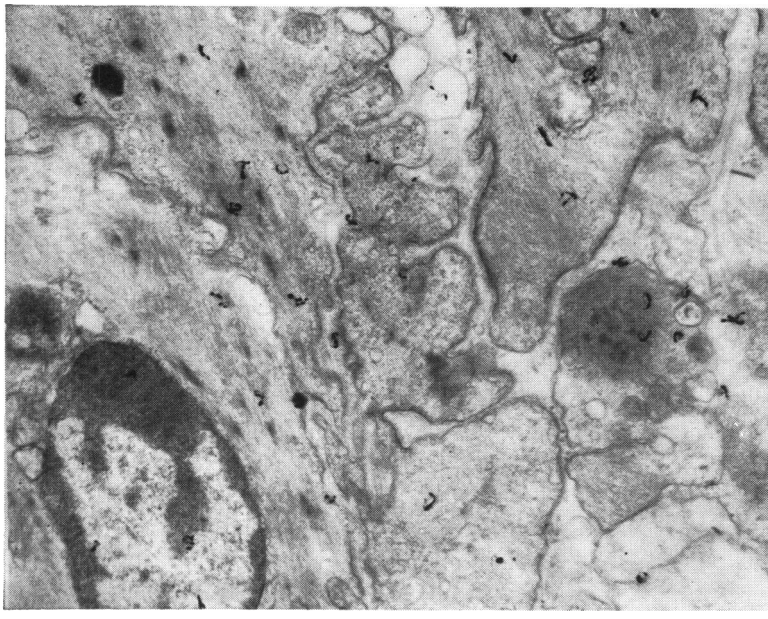

FIG. 3. Electron autoradiograph of smooth muscle from the vas deferens of guinea-pig following incubation of the muscle with ^{3}H-Phb. The distribution of silver grains, implicating the presence of bound ^{3}H-Phb shows no localization in relation to muscle membranes or to organelles. ×24,000.

concentration of silver grains over smooth muscle cytoplasm was greater than over smooth muscle nuclei or extracellular space as shown in Table 2. No specific concentration of silver grains was detectable in relation to discrete morphological structures of the smooth muscle cell (Fig. 3).

Table 3 illustrates the results of the specific protection test. In these experiments, some vas deferens specimens were exposed to 606 μM NA before ^{3}H-Phb incubation since Furchgott (1954) and others have shown that NA occupies the α-receptors thereby preventing access of halogenoalkylamine to these receptors. It was confirmed in the present experiments that NA in a concentration of 606 μM reduced the α-adrenoceptor blocking activity of 3·29 μM Phb by 60–80%. The number of silver grains per unit area of smooth muscle in the NA treated specimens was significantly lower than in the controls which were not pre-incubated with NA. The autoradiographs, however, did not reveal any recognizable difference in the silver grain distribution in relation to morphological features between the 'protected' and 'unprotected' specimens.

Discussion

The presence of pinocytotic vesicles on muscle membranes can be taken to indicate regions of considerable metabolical activity. The fact that the aggregation of these vesicles is not significantly greater opposite the synaptic cleft makes it unlikely that they constitute the morphological basis of a specialized subsynaptic adrenoceptor

TABLE 1. *Representative results showing the number of attached pinocytotic vesicles per unit length of membrane of arteriolar smooth muscle from the pancreas of the cat*

No. micrographs in series	Attached pinocytotic vesicles/unit length 'Synaptic' membrane	'Non-synaptic' membrane	P
22	3·33±1·13*	2·14±0·95	0·9
16	1·69±0·44	1·57±0·42	0·7
13	1·68±0·87	1·30±0·15	0·2

* S.E. of mean. P values obtained using Student t-test.

TABLE 2. *The density of autoradiographic silver grains over vas deferens of guinea-pig treated with ^{3}H-phenoxybenzamine*

	Extra-cellular space	Smooth muscle cytoplasm	Smooth muscle nucleus
Number of profiles examined	33	186	24
Mean grains/unit area	4·96	8·7	7·3
Standard error of mean	0·95	0·75	0·95
Statistical significance	$P<0·001$		$P<0·1$

TABLE 3. *Comparison of the density of autoradiographic silver grains over smooth muscle profiles of vas deferens of guinea-pig treated with ^{3}H-phenoxybenzamine*

	Control (C) (not pre-incubated with NA)	Test (T) (pre-incubated with 606 μM NA)
No. micrographs	50	65
Mean grains/unit area	27	10·1
Standard error of mean	1·1	1·1
Statistical significance	C>T $P<0·001$	

area in any way comparable to the motor endplate on skeletal muscle. In the guinea-pig and cat (Graham *et al.*, 1968b) and in both organs (pancreatic arteriolar smooth muscle and vas deferens) there is a degree of concentration of silver grains indicative of localization of phenoxybenzamine over the cytoplasm and nuclei of the smooth muscle, but there is no obvious concentration in relation to any given organelle. In trying to interpret the diffuse scatter of silver grains one has to allow for four possibilities. (1) The general background of spontaneously produced silver grains. This is taken account of by counts over extracellular space free from cells such as fibroblasts. (2) A portion of any dose of phenoxybenzamine is probably bound to nonspecific sites (Graham, 1960a, 1964). Halogenoalkylamines bind to nucleotides and this may account for the presence of silver grains over the profiles of nuclei. An apparent species difference can be discerned as there are more silver grains (presumably non-adrenergic sites) on the nuclei of guinea-pig vas deferens muscle than on the nuclei of cat arteriolar muscle. (3) Phenoxybenzamine occupies receptors for histamine, 5-hydroxytryptamine, and (in high enough concentration) for acetylcholine (Graham, 1960b). These specific receptors may well be localized in a manner which confuses the attempt to localize α-adrenoceptor sites. Nevertheless the specific protection experiment (Furchgott, 1954) produced a statistically significant difference in the number of grains per unit area over vas deferens muscle cytoplasm, being less in that muscle which was protected by pre-treatment with noradrenaline (Table 3). This might indicate that some of the silver grains are markers of α-adrenoceptor sites. Of interest in this context is the absence of silver grain concentration in relation to cell membranes, which is consistent with the suggestion (Paton, 1967; Ariens & Simonis, 1969) that the α-adrenoceptor is inside the cell rather than on the membrane.

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REFERENCES

AHLQUIST, R. P. (1948). A study of adrenotropic receptors. *Am. J. Physiol.*, **153**, 586–600.

ARIENS, E. J. & SIMONIS, A. M. (1969). Adrenergic blocking agents, structure and activity. *Acta Physiol. Pharmac.*, **15**, 78–83.

ESTERHUIZEN, A. C., GRAHAM, J. D. P., LEVER, J. D. & SPRIGGS, T. L. B. (1967). The innervation of the smooth muscle of the nictitating membrane of the cat. *J. Physiol., Lond.*, **192**, 41–42*P*.

ESTERHUIZEN, A. C., GRAHAM, J. D. P., LEVER, J. D. & SPRIGGS, T. L. B. (1968). Catecholamine and acetylcholinesterase distribution in relation to noreadrenaline release. An enzyme histochemical and autoradiographic study on the innervation of the cat nicitating muscle. *Br. J. Pharmac. Chemother.*, **32**, 46–56.

FURCHGOTT, R. E. (1954). Dibenamine blockade in strips of rabbit aorta and its use in differentiating receptors. *J. Pharmac. exp. Ther.*, **111**, 265–284.

GRAHAM, J. D. P. (1960a). Farmacologia ed usi terapeutici degli antagonisti adrenomotori della serie 2-alogenoalchilamine. *Recenti Progr.*, **28**, 487–507.

GRAHAM, J. D. P. (1960b). Structure and activity in a series of 2-halogenoalkylamines. *J. Med. pharm. chem.*, **2**, 499–522.

GRAHAM, J. D. P. (1962). The 2-halogenoalkylamines. In: *Progress in Medicinal Chemistry*. Vol. II. ed. Ellis, G. P. & West, G. B., pp. 132–175. London: Butterworths.

GRAHAM, J. D. P. (1964). Distribution of (2-bromoethyl) ethyl (naphth-1-methyl-^{14}C-amine) hydrobromide in the rat. *Br. J. Pharmac, Chemother.*, **23**, 285–294.

GRAHAM, J. D. P., LEVER, J. D. & SPRIGGS, T. L. B. (1968a). An examination of adrenergic axons around pancreatic arterioles of the cat for the presence of acetylcholinesterase by high resolution autoradiographic and histochemical methods. *Br. J. Pharmac. Chemother.*, **33**, 15–20.

GRAHAM, J. D. P., LEVER, J. D. & SPRIGGS, T. L. B. (1968b). The localization of ^{3}H-phenoxybenzamine in the arterioles of the cat pancreas. *Br. J. Pharmac.*, **34**, 699*P*.

GRAHAM, J. D. P., LEVER, J. D. & SPRIGGS, T. L. B. (1969). The effect of morphine dependence on the vasicular content of adrenergic nerves in relation to arteriolar smooth muscle in the pancreas of the rat. *Br. J. Pharmac.*, **37**, 19–23.

LEVER, J. D., GRAHAM, J. D. P., IRVINE, G. & CHICK, WENDY, J. (1965). The vesiculated axons in relation to arteriolar smooth muscle in the pancreas. A fine structural and quantitative study. *J. Anat.*, **99**, 299–313.

LEVER, J. D., SPRIGGS, T. L. B. & GRAHAM, J. D. P. (1968). A formolfluorescence, fine structural and autoradiographic study of the adrenergic innervation of the vascular tree in the intact and sympathectomized pancreas of the cat. *J. Anat.*, **103**, 15–34.

PATON, W. D. M. (1967). Adrenergic receptors viewed in light of general receptor theories. *Ann. N.Y. Acad. Sci.*, **139**, 632–644.

REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.*, **17**, 208–212.

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