Br. J. Pharmac. (1982), **76**, 177–183

THE EFFECTS OF NORADRENERGIC DENERVATION ON MUSCARINIC RECEPTORS OF SMOOTH MUSCLE

T. BENNETT, T.Y. LOT & P.G. STRANGE*

Department of Physiology and Pharmacology and Department of Biochemistry*, The Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 Changes in the response to acetylcholine of expansor secundariorum muscles from chicks have been analysed by pharmacological techniques and by [3-^{3}H]-quinuclidinyl benzilate ([3-^{3}H]-QNB) binding to quantify the muscarinic receptor population.

2 The expansor secundariorum muscle responded to acetylcholine up to the age of 30 days; the response declined thereafter. This developmental decrease in response to acetylcholine was prevented by surgical denervation.

3 In chicks aged less than 25 days, denervation did not affect the sensitivity of the expansor muscle to acetylcholine. In older chicks (above 40 days) denervation gradually restored the sensitivity of the expansor muscle to acetylcholine. Responses of the expansor muscle were always abolished by atropine (1 μM) indicating they were mediated by muscarinic receptors.

4 Binding studies with [3-^{3}H]-QNB showed that changes in response of expansor muscles to acetylcholine were primarily due to changes in the muscarinic receptor population.

5 It is suggested that the noradrenergic innervation of the expansor muscle influences the number of muscarinic receptors expressed in the tissue.

Introduction

In 8–12 week old chicks it has been shown, pharmacologically, that the expansor secundariorum muscle (ESM), a smooth muscle in the wing of birds, is innervated by postganglionic noradrenergic nerves only (Buckley & Wheater, 1968). The noradrenergic innervation has been confirmed by fluorescence histochemistry (Bennett & Malmfors, 1970). However, it has been reported that the ESM also possesses muscarinic receptors in chicks younger than 10 days post-hatching; these receptors disappear by day 40 post-hatching (Kuromi & Hasegawa, 1975). The noradrenergic innervation of the ESM appears to regulate the muscarinic receptors, since denervation or sympathectomy with guanethidine prevents the normal developmental decrease in the responsiveness of the ESM to acetylcholine (Kuromi & Hagihara, 1976).

In the present work the changes in the sensitivity of the ESM to acetylcholine have been analysed by pharmacological techniques. Ligand-binding studies were undertaken to quantify the muscarinic receptor population and the results compared with the pharmacological findings.

Methods

Male chicks (Light Sussex and Rhode Island Red Cross) were used in the present study.

Denervation of the expansor secundariorum muscle (ESM)

The ESM is innervated by a branch of the radial nerve which becomes more superficial, and hence visible, as it approaches the distal end of the humerus (Buckley & Lwin, 1968). While the chicks were under ether anaesthesia, the skin above the nerve close to the ESM of the left wing was incised and the nerve cut 1–2 cm from the muscle with a pair of sterile scissors. The wound was sealed with an antiseptic spray dressing. The ESM from the opposite wing was left intact to serve as the control. The chicks were killed later to study the effects of denervation.

Organ bath studies

Chicks were killed by an overdose of ether and the ESM quickly dissected out. The ESM was freed of adherent tissue, except for the tendon and a piece of secondary feather at the base of the muscle. The feather was tied to a pin held in a perspex block and suspended in a 20 ml organ bath. The tendon was threaded 3 cm from the feather and tied to one arm of an isotonic transducer connected to a flat bed pen recorder (Servoscribe).

The organ bath contained 20 ml of freshly prepared physiological saline (composition (mM): sodium chloride 118, potassium chloride 4.7, mag-

0007-1188/82/050177-07 $01.00

nesium sulphate 2.4, sodium dihydrogen orthophosphate 1, sodium bicarbonate 30, glucose 11.1 and calcium chloride 2.5) gassed with a mixture of 95% O_2 and 5% Co_2. The bath was maintained at 37°C by means of a water jacket connected to a thermostatically controlled pump (Churchill). Tissues were set up under a resting tension of 1 g and left for 30 min to equilibrate.

Drug solutions were always freshly prepared in such concentrations that the addition of 0.2 ml gave the final desired bath concentration (M). Ascorbic acid was added to dilute solutions of acetylcholine (ACh). The drug contact times were 45 s for ACh and 15 s for potassium. Atropine was applied 5 min before ACh when it was used.

Ligand binding studies

Chicks were killed by an overdose of ether, the ESM quickly removed, cleared of adherent tissue and placed in ice cold HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane-sulphonic acid) – phosphate-saline-buffer with a composition as used by Golds, Przyslo & Strange (1980). ESMs pooled from chicks were homogenized in ice cold HEPES-phosphate-saline buffer (100–120 mg tissue wet wt./5 ml buffer) with a glass-glass homogenizer.

The suspension (0.25 ml) was used immediately to measure [3–^{3}H]-quinuclidinyl benzilate ([3–^{3}H]-QNB) binding by incubating at 30°C for 60 min with 0.5 nM (–)-[3–^{3}H]-QNB in the presence (non-specific binding) or absence (total binding) of (±)-atropine 20 μM in a final volume of 1.0 ml in quadruplicate. The assay was terminated by the addition of 2 ml of ice-cold buffer, and the membranes were collected by rapid filtration over GF/B (Whatman) filters and rinsed with 4 ml (2 × 2 ml) buffer. The filters were placed in vials containing 0.2 ml water; 3 ml of Fiso Fluor '1' scintillant (Fisons, Loughborough, Leics.) was added and the radioactivity determined by liquid scintillation spectrometry. Specific binding was determined by subtracting non-specific binding from total binding. Nonspecific binding was less than 50% of the total binding.

The protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using boving serum albumin as a standard and for experimental samples employing a precipitation step with trichloroacetic acid (100 g/l final).

Drugs

(–)-[3–^{3}H]-QNB (43 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks. Atropine sulphate, bovine serum albumin and N-2-hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid were obtained from Sigma (London) Chemical Co. Ltd, Poole, Dorset. Acetylcholine bromide was obtained from British Drug Houses.

Statistical analysis

Regression lines with confidence limits were calculated for the linear portions of log concentration-response curves. The significance of differences in slope was used as a measure of parrallelism of the two lines.

Log concentration limits at 50% of the maximum response were used in the analysis of the significance of concentration differences.

Maximum responses were compared by Student's unpaired *t* test.

Results

Organ bath studies

ACh contracted the expansor secundariorum muscle (ESM) from chicks up to 30 days post-hatching in a dose-related fashion. The response of the ESM from chicks above 30 days to ACh rapidly declined (Figure 1).

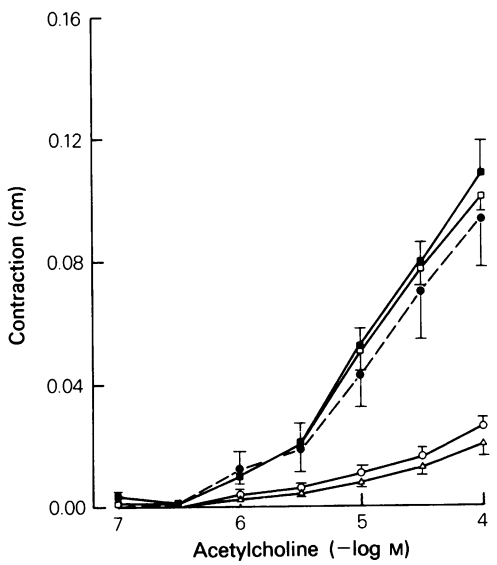

Figure 1 Mean contractions of the expansor secundariorum muscle (ESM) ($n = 5$ in each group) to graded increases in the concentration of acetylcholine (ACh); vertical lines show s.e.mean. Ordinate scale: contraction in centimetres; abscissa scale: final bath concentration (M) of ACh on a log scale. ESM from 15 day old chicks (■); ESM from 20 day old chicks (□); ESM from 30 day old chicks (●); ESM from 35 day old chicks (○); ESM from 40 day old chicks (△).

ESM from 23–25 days old chicks denervated 14–16 days previously Denervation did not significantly change the response of the ESM to ACh (Figure 2a, Table 1).

ESM from 52 day old chicks denervated for different periods The ESM from 52 day old chicks was virtually unresponsive to ACh (Figure 2b). The maximum response of the ESM from 52–54 day old chicks denervated 32–34 days previously was approximately 11 times that of the control muscle (Figure 2b). This finding indicates that denervation prevents the developmental decrease in the responsiveness of the ESM to ACh as previously reported

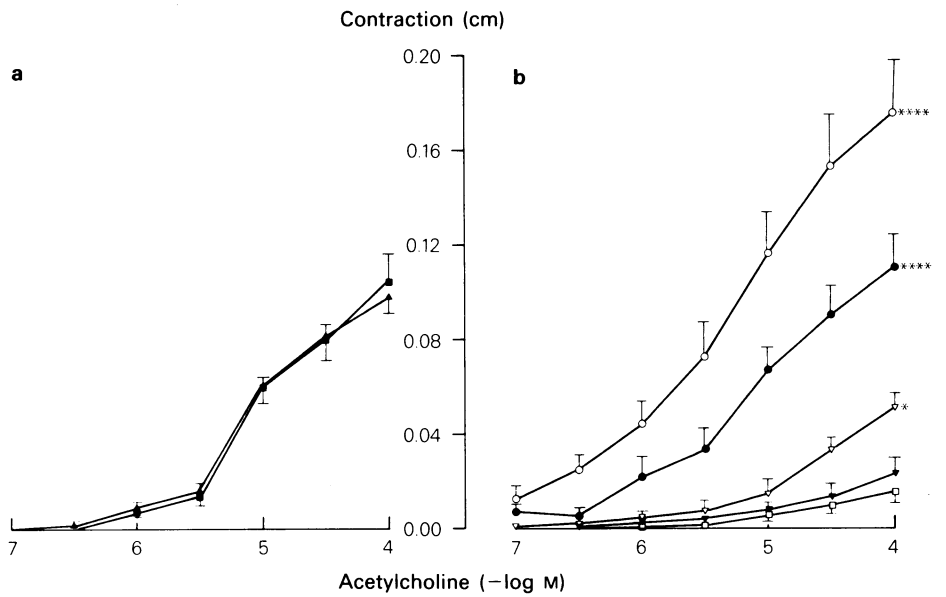

Figure 2 Mean contraction of the expansor secundariorum muscle (ESM) ($n = 6$ in each group) to graded increases in concentration of acetylcholine (ACh); vertical lines show s.e.mean. Ordinate scale: contraction in centimetres; abscissa scale: final bath concentration (M) of ACh on a log scale. (a) Normal ESM from 23–25 day old chicks (■); ESM from 23–25 day old chicks denervated 14–16 days previously (▲); (b) Normal ESM from 52–54 day old chicks (□); ESM from 52–54 day old chicks denervated 32–34 days previously (○); ESM from 55 day old chicks denervated 3 days previously (▼); ESM from 56–57 day old chicks denervated 4–5 days previously (▽); ESM from 66–68 day old chicks denervated 14–16 days previously (●).
****$P < 0.001$; *$0.05 > P > 0.02$; Student's unpaired t test (compared to the control).

Table 1 Acetylcholine (ACh) sensitivities of the expansor secundariorum (ESM) muscle of the chick

Age of chicks (days)	*Treatment*	*ED_{50} of ACh (mol)*
23–25	Control	$1.0 \pm 1.2 \times 10^{-5}$
23–25	Denervated 14–16 days previously	$8.1 \pm 1.2 \times 10^{-6}$
52–54	Denervated 32–34 days previously	$4.7 \pm 1.3 \times 10^{-6}$**
56–57	Denervated 4–5 days previously	$1.4 \pm 1.5 \times 10^{-5}$
66–68	Denervated 14–16 days previously	$7.0 \pm 1.2 \times 10^{-6}$

Values obtained from 6 animals in each group.
** $0.02 > P > 0.01$ compared to 23–25 day old control by Student's unpaired t test.
Note that ED_{50} values were determined using responses to ACh 1×10^{-4}M as maximum values since higher concentrations did not significantly change the responses; values are given ± s.e.mean.

(Kuromi & Hagihara, 1976). The ESM from 52–54 day old chicks denervated 32–34 days previously was most responsive to ACh since it developed the greatest maximum response (Figure 2) and was most sensitive (Table 1) to ACh.

The ESM from 55 day old chicks denervated 3 days previously was as unresponsive to ACh as the control (Figure 2b). Compared to the control there was an approximately 3 fold increase in the maximum response to ACh of the ESM denervated for 4–5 days, this further rose to a 7 fold increase when the ESM was denervated for 14–16 days (Figure 2b). Associated with these changes in maximum response there was also a change in sensitivity of the ESM to ACh. The ESM denervated 14–16 days previously was approximately twice as sensitive to ACh as the ESM denervated 4–5 days previously; this difference was significant ($0.01 > P > 0.001$) (Table 1). The ESM from 56–57 day old chicks denervated 4–5 days previously was the least sensitive to ACh (Table 1). These findings indicate that ESMs from older animals are virtually unresponsive to ACh but denervation gradually restores their sensitivity and responsiveness to ACh.

Potassium was used to examine the possibility that the restoration of the sensitivity of the ESM to ACh following denervation was a non-specific effect. Although denervation decreased ($0.01 > P > 0.001$) the response of the ESM to the largest dose of potassium used (Figure 3a), the sensitivity of the muscle to potassium was unaffected (Figure 3b). These findings suggest that changes in the response of the ESM to ACh are specific and this was confirmed by the finding that responses of control or denervated ESMs to ACh were always completely abolished by atropine (1 μM).

[3–^{3}H]-quinuclidinyl benzoate binding results

The binding study results are summarized in Table 2. In membrane preparations of the ESM from 23 day old chicks, moderate levels of [3–^{3}H]-QNB binding were found. Other results are compared with this level of binding.

Membrane preparations of ESMs from the left or right wing of 57 day old chicks bound 0.9 fmol [3–^{3}H]-QNB/mg protein each. Since the ESM from the left wing was always denervated leaving that from the right wing as the control, differences in the amount of binding described subsequently are not due to basic differences between the ESM from left and right wings.

The amount of [3–^{3}H]-QNB bound by the ESM decreased with age. The difference was most marked between ESMs from 23 day old chicks and those from chicks above 50 days of age. This finding fits with the observed, normal development decrease in the responsiveness of the ESM to ACh with increasing age of the chicks.

In the ESMs from 23 or 57 day old chicks, denervation 3 days previously reduced [3–^{3}H]-QNB binding by 0.6 fmol/mg protein each. Compared to their contralateral controls, this represented an 8% reduction in binding for 23 day old chicks and 67% reduction for 57 day old chicks. These differences in reduction of binding as proportions of the controls are due to differences in the total [3–^{3}H]-QNB bound at both ages.

The ESM from 54 day old chicks bound 14% of the [3–^{3}H]-QNB bound by the ESM from 23 day old chicks; denervation 34 days previously caused an approximately 4 fold increase in the binding. The ESM from 68 day old chicks bound 11% of the

Table 2 [3–^{3}H]-quinuclidinyl benzilate ([3–^{3}H]-QNB) binding by the expansor secundariorum (ESM) of the chick

Age of chicks (days)	*Treatment*	*[3–^{3}H]-QNB specifically bound (fmol/mg protein)*
23	Control	7.4 ± 0.06 (100 ± 0.8)
	ESM denervated 3 days previously	6.8 ± 0.08 (91.9 ± 1.1)
	ESM denervated 14 days previously	7.1 ± 0.09 (95.9 ± 1.2)
54	Control	1.0 ± 0.15 (13.5 ± 2.0)
	ESM denervated 34 days previously	4.3 ± 0.64 (58.1 ± 8.6)
57	Control	0.9 ± 0.09 (12.2 ± 1.2)
	ESM denervated 3 days previously	0.3 ± 0.03 (4.1 ± 0.4)
68	Control	0.8 ± 0.06 (10.8 ± 0.8)
	ESM denervated 14 days previously	4.0 ± 0.30 (54.1 ± 4.1)

Note that values in parentheses represent binding by ESMs expressed as percentages of the [3–^{3}H]-QNB bound by control ESMs from 23 day old chicks. The values are given as the mean (± s.e.mean) of at least 3 determinations.

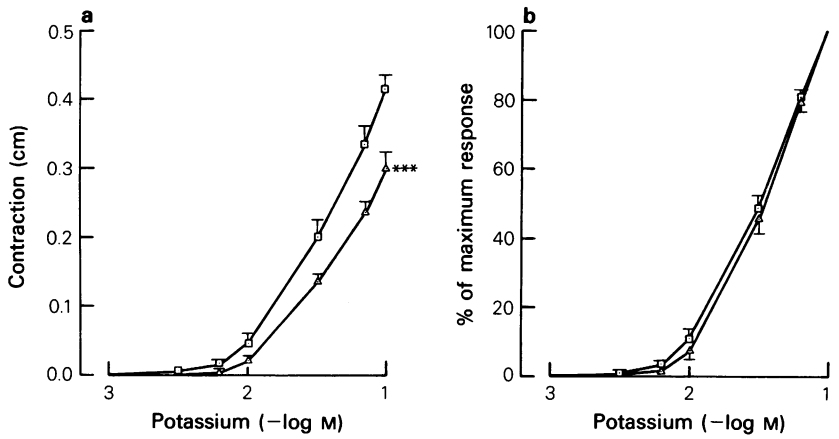

Figure 3 Mean contraction of the expansor secundariorum muscle (ESM) ($n = 6$ in each group) to graded increases in the concentration of potassium; vertical lines show s.e.mean. Abscissa scale: final bath concentration of potassium (M) on a log scale. Normal ESM from 66–68 day old chicks (□); ESM from 66–68 day old chicks denervated 14–16 days previously (△). (a) Ordinate scale: contraction in centimetres. ***$0.01 > P > 0.001$; Student's unpaired t test. (b) Ordinate scale: contraction as a percentage of the maximum response.

[3-^{3}H]-QNB bound by the ESM from 23 day old chicks, denervation 14 days previously gave an approximately 5 fold increase in [3-^{3}H]-QNB binding sites.

Discussion

The expansor secundariorum muscle (ESM) from chicks up the age of 10 days post-hatching has been shown to be sensitive to acetylcholine (ACh). The sensitivity to ACh decreases progressively with age and disappears by day 40 post-hatching (Kuromi & Hasegawa, 1975). This finding was confirmed by results from the present study based on the response of the ESM isolated from chicks of different ages. However, it was found during the present study that the responsiveness of the ESM to ACh was maintained up to the age of 30 days post-hatching.

The ESM is innervated by postganglionic noradrenergic nerves only (Buckley & Wheater, 1968; Bennett & Malmfors, 1970). Results from the present study indicate that noradrenergic denervation of the ESM from young chicks prevents the developmental decrease in its response to ACh. In the older animals, noradrenergic denervation gradually restored the responsiveness of the ESM to ACh. Thus the noradrenergic innervation of the ESM appears to influence its response to ACh. This finding is consistent with a previous suggestion based on the observation that denervation or chemical sympathectomy with guanethidine prevented the developmental decrease in the responsiveness of the ESM to ACh (Kuromi & Hagihara, 1976). The mechanisms by which the noradrenergic innervation of the ESM influence its responsiveness to ACh have not previously been reported and hence were investigated during the present study.

Changes in smooth muscle sensitivity to agonist drugs following denervation have previously been described. Since, in some investigations, it has been observed that chronic denervation increases the sensitivity of smooth muscle to different agonists it has been suggested that it is a non-specific effect. According to this suggestion, denervation causes partial depolarization of smooth muscle cells (guinea-pig vas deferens) or changes the threshold for electrical activation (rat vas deferens), both resulting in supersensitivity to agonists acting by different mechanisms (Fleming, 1978). Results from the present study indicate that changes in the sensitivity of the ESM to ACh were not due to a non-specific effect since the denervated muscle was not supersensitive to potassium.

One possible explanation of the supersensitivity is a change in the number of ACh receptors. This proposal is based on the observation that the responses of control or denervated ESMs were always abolished by atropine, a known muscarinic receptor antagonist. The proposal was further strengthened when results of pharmacological studies were correlated with [3-^{3}H]-QNB binding studies used to quantify the muscarinic receptor population of the ESMs. Thus ESMs from 23 day old chicks were

sensitive to ACh and showed a moderate level of [3-^{3}H]-QNB binding sites while the ESM from chicks above 50 days old were virtually unresponsive to ACh and had very few binding sites. These data suggest that the developmental decrease in the response of the ESM to ACh could be attributed to a reduction in its postsynaptic muscarinic receptor population. Further support for this suggestion arises from the observation that denervation of the ESM from 54 day old chicks gradually restored its sensitivity to ACh and caused an increase in the number of [3-^{3}H]-QNB binding sites.

Since it has been shown that surgical denervation of the ESM for 3 days causes degeneration of its noradrenergic nerve terminals without affecting the postsynaptic receptors (Geffen & Hughes, 1972) this provides a means of assessing the number of presynaptic receptors in the ESM. In the present study it was observed that there was a reduction in [3-^{3}H]-QNB binding of 0.6 fmol/mg protein when ESMs from either 23 or 57 day old chicks were denervated 3 days previously. This indicates that the ESM possesses presynaptic receptors whose population does not appear to change with age.

During the present study, it was observed that denervation of the ESM increased its response to all doses of ACh tested. This might indicate that the receptors were more accessible to ACh compared to control muscles. This proposal is consonant with the mechanism of denervation supersensitivity in skeletal muscle. It has been shown electrophysiologically that nicotinic receptors of skeletal muscle are localized to the neuromuscular junction. Following denervation, the receptors spread to other parts of the muscle fibre membrane resulting in an increase in the number of receptors and their accessibility to exogenously administered agonist drugs (Fleming, 1978; Mathers & Thesleff, 1978). It is of interest that proliferation of nicotinic receptors started from the 4th day following denervation (Mathers & Thesleff, 1978). This fits with results from the present study since significant increases in the response of the ESM from 52 day old chicks to ACh started 4–5 days following denervation. However, it remains to be determined whether the muscarinic receptors of the ESM behave in a similar fashion to the nicotinic receptors of skeletal muscle.

There are some indications from the present study that other factors also contributed to the observed changes in response of the ESM to ACh following denervation. Thus, ESMs from 68 day old chicks denervated 14 days previously showed an approximately 5 fold increase in [3-^{3}H]-QNB binding sites but an approximately 7 fold increase in the maximum response to ACh. Also, ESMs from 68 day old chicks denervated 14 days previously only bound 54% of the [3-^{3}H]-QNB bound by ESMs from 23 day old chicks but the preparations did not differ significantly in their sensitivity or maximum responses to ACh. Furthermore, the ESM from 54 day old chicks denervated 34 days previously bound approximately 4 times the amount of [3-^{3}H]-QNB bound by the contralateral control muscle but showed an approximately 11 fold increase in the maximum response to ACh. In addition, the ESM from 54 day old chicks denervated 34 days previously bound 58% of the [3-^{3}H]-QNB bound by the muscle from 23 day old chicks but it was more responsive and developed a greater maximum contraction to ACh. One possible explanation of the greater increase in maximum response compared to the increase in [3-^{3}H]-QNB binding is that denervation causes an increase in muscle mass (Campbell *et al.*, 1977; Lot & Bennett, 1981, unpublished) which might contribute to the maximum response, while [3-^{3}H]-QNB binding is expressed per mg of protein which does not take into account changes in muscle mass. However, expressing [3-^{3}H]-QNB binding in this way does indicate that the changes seen were due to changes in binding sites per cell rather than to changes in numbers of cells.

In summary, the present study has shown that noradrenergic nerves can influence the muscarinic receptors of smooth muscle. This interaction is not well documented at present but some of the factors responsible for the interaction have been described.

References

BENNETT, T. & MALMFORS, T. (1970). The adrenergic nervous system of the domestic fowl (*Gallus domesticus L.*). *Z. Zellforsch*, **106**, 22–50.

BUCKLEY, G.A. & LWIN, S. (1970). The expansor secundariorum of the domestic fowl: a smooth muscle-nerve preparation without cholinergic receptors. *Br. J. Pharmac.*, **39**, 245P.

BUCKLEY, G.A. & WHEATER, L.E. (1968). The isolated expansor secundariorum – a smooth muscle preparation from the wing of the domestic fowl. *J. Pharm. Pharmac.*, **20** (Suppl.), 114S–121S.

CAMPBELL, G.R., GIBBINS, I., ALLAN, I. & GANNON, B. (1977). Effects of long-term denervation on smooth muscle of the chicken expansor secundariorum. *Cell Tiss. Res.*, **176**, 143–156.

FLEMING, W.W. (1978). The trophic influence of autonomic nerves on eletrical properties of the cell membrane in smooth muscle. *Life Sci.*, **22**, 1223–1228.

GEFFEN, L.B. & HUGHES, C.C. (1972). Degeneration of sympathetic nerves *in vitro* and development of smooth muscle supersensitivity to noradrenaline. *J. Physiol.*, **221**, 71–84.

GOLDS, P.R., PRZYSLO, F.R. & STRANGE, P.G. (1980). The binding of some antidepressant drugs to brain muscarinic receptors. *Br. J. Pharmac.*, **68,** 541–549.

KUROMI, H. & HAGIHARA, Y. (1976). Influence of sympathetic nerves on development of responsiveness of the chick smooth muscle to drugs. *Eur. J. Pharmac.*, **36,** 55–59.

KUROMI, H. & HASEGAWA, S. (1975). Changes in acetylcholine and noradrenaline sensitivity of chick smooth muscle wholly innervated by sympathetic nerve during development. *Eur. J. Pharmac.*, **33,** 41–45.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193,** 265–275.

MATHERS, D.A. & THESLEFF, S. (1978). Studies on neurotrophic regulation of murine skeletal muscle. *J. Physiol.*, **282,** 105–114.

(Received November 17, 1981.
Revised January 4, 1982.)