Effects of vitamin E deficiency on autonomic neuroeffector mechanisms in the rat caecum, vas deferens and urinary bladder

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1. Modified sucrose-gap, standard organ-bath techniques and transmitter release studies were used to examine neuromuscular transmission in the caecum, vas deferens and urinary bladder in normal rats and in rats maintained for 12 months on a diet free of vitamin E.

2. In the caecum circular muscle, non-adrenergic, non-cholinergic inhibitory junction potentials were absent from 48 and 15% of preparations from vitamin E-deficient and control animals, respectively. Cholinergic excitatory junction potentials were absent from 83 and 8% of vitamin E-deficient and control preparations, respectively. Responses to applied noradrenaline (0·1–30 μM), α,β-methylene ATP (3–100 μM) and acetylcholine (0·1–30 μM) were attenuated or absent in vitamin E-deficient tissues. Responses to applied KCl were similar in both groups. Release of [^{3}H]noradrenaline or endogenous acetylcholine could not be evoked from vitamin E-deficient tissues.

3. In contrast, in isolated preparations of the vas deferens and urinary bladder, neuromuscular transmission by adrenergic, cholinergic and purinergic components were unaffected by long-term vitamin E deficiency.

4. In conclusion, vitamin E deficiency causes dysfunction of autonomic neuroeffector mechanisms in the smooth muscle of the rat caecum, at both a pre- and postjunctional level. The lesions in autonomic transmission mechanisms brought about by long-term vitamin E deficiency were found only in the caecum; no changes in sympathetic neuromuscular transmission were observed in the vas deferens, or in parasympathetic neuromuscular transmission in the urinary bladder.

It has become established that vitamin E (α-tocopherol) is essential for normal neuronal physiology, and that deficiency of vitamin E results in neuropathic changes (for reviews see Harding, 1987; Muller & Goss-Sampson, 1990; Sokol, 1990). The neuropathic changes that develop in chronically vitamin E-deficient animals are very similar to those that develop in man. The clinical symptoms of areflexia, altered gait, decreased sentience, paresis of oculomotor muscles, and retinopathy found in humans with chronic vitamin E deficiency (Pentschew & Schwarz, 1962; Landrieu, Selva, Alvarez, Ropert & Metral, 1985; Sokol *et al.* 1985) are well documented in the vitamin E-deficient rat (Ringsted, 1935; Malamud, Nelson & Evans, 1949; Machlin, Filipski, Nelson, Horn & Brin, 1977).

The neuropathological changes that occur during vitamin E deficiency develop in the characteristic fashion of a central–peripheral distal axonopathy (dying-back neuropathy), with posterior spinal nerve roots being affected before anterior roots, sensory neurones being affected before motoneurones, and changes in the central nervous system developing before changes in the peripheral nervous system (Malamud *et al.* 1949; Pentschew & Schwarz, 1962; Machlin *et al.* 1977; Nelson, Fitch, Fischer, Broun & Chou, 1981; Towfighi, 1981; Goss-Sampson, Kriss & Muller, 1990).

Autonomic neuropathies induced by vitamin E deficiency have been comparatively less extensively investigated than the central, sensory and somatomotor neuropathies, and

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consequently much less is known about them. In rats deficient in vitamin E for 12–24 months there is a large increase in urinary output of catecholamines, especially noradrenaline and adrenaline, which implies a raised level of sympathetic activity (Nakashima & Esashi, 1986, 1987), but these animals do not respond to various stressful stimuli with further increased urinary output of catecholamines. Sympathetic control of the cardiovascular system does not appear to be affected by vitamin E deficiency: in vitamin E-deprived rats and monkeys there is no change in resting heart rate or blood pressure (Filer, Rumery, Yu & Mason, 1949; Hubel, Griggs & McLaughlin, 1989). However, the endogenous noradrenaline content of the heart is reduced by up to 50% (Nakashima & Esashi, 1986, 1987). Since nearly all the noradrenaline in the heart is contained in sympathetic neurones, with none contained in the intrinsic cardiac neurones, and only a small proportion contained in small intensely fluorescent (SIF) cells (Nielsen & Owman, 1968; Hassall & Burnstock, 1986), the decrease in noradrenaline content of the heart may represent a sympathetic neuropathy. However, in an ultrastructural study of the sympathetic coeliac–superior mesenteric ganglion no dystrophy was found in vitamin E-deficient rats above that found in age-matched control animals (Schmidt, Coleman & Nelson, 1991).

The aim of the present study was to examine the effects of long-term (12 months) vitamin E deficiency on autonomic neuromuscular transmission in the caecum, vas deferens and urinary bladder. This selection includes organs that are innervated by cholinergic and non-adrenergic, non-cholinergic enteric neurones (caecum), adrenergic and purinergic sympathetic neurones (vas deferens), and cholinergic and purinergic parasympathetic neurones (urinary bladder). To this end a variety of *in vitro* techniques were employed, including standard organ-bath pharmacology, a modified sucrose-gap, measurement of transmitter content and measurement of evoked transmitter release.

METHODS

Animal model of vitamin E deficiency

The model has been described in detail (Goss-Sampson *et al.* 1990), and its main features are as follows. Male Wistar rats (post-weanlings, 21–23 days old), obtained from Charles River Ltd (Margate, UK), were maintained on a vitamin E-deficient diet (Machlin/Draper-HLR No. 814, supplied by Dyets, PA, USA) comprising tocopherol-stripped corn oil (10%), glucose (65%), salt mix (4%), vitamin-free casein (20%), and full vitamin supplementation (excluding vitamin E). The diet was sterilized by irradiation (1·5 Mrads). The vitamin E-content of the diet was determined as being less than 1 ng g^{-1}. Control animals were fed the same diet with the addition of α-tocopherol acetate (40 mg (kg diet)$^{-1}$). Animals were allowed free access to food and water. The two groups of animals were kept on their respective diets for 12 months. The animals were killed by an overdose of sodium pentobarbitone (Sagatal, RMB Animal Health, Dagenham, UK) and cardiac puncture. At the time of death the control animals were significantly ($P < 0·001$) heavier than the vitamin E-deficient animals, being (mean ± S.E.M.) 780 ± 38·8 g ($n = 18$) compared with 523 ± 33·6 g ($n = 18$).

Preparation of the caecum

A modified single sucrose-gap technique (Hoyle, 1987) was used to record changes in membrane potential and membrane resistance in preparations of strips of smooth muscle isolated from the caecum. Mechanical activity was recorded simultaneously. The caecum was removed, opened up along the line of its mesenteric attachment, and washed in several changes of cool Krebs solution (approximately 12 °C). The caecum was then gently pinned out onto wax at room temperature (22 ± 1 °C), and from the middle 2 cm, strips of smooth muscle 200–500 μm wide and approximately 10 mm in length were dissected free from the underlying mucosa and submucosa along the orientation of the circular muscle fibres. The preparations were left in Krebs solution at room temperature for at least 2 h before being mounted in the sucrose-gap chamber, and were allowed to equilibrate in this apparatus for a further 15–30 min. The superfusing Krebs solution was maintained at 35 ± 0·5 °C, and had the following composition (mM): NaCl, 133; KCl, 4·7; NaH_2PO_4, 1·4; $MgSO_4$, 0·6; $NaHCO_3$, 16·3; $CaCl_2$, 2·5; glucose, 7·7. This solution was gassed continually with 95% O_2–5% CO_2, and had a pH of 7·2–7·3.

Mechanical activity of the smooth muscle was recorded via a Gould Metrigram isometric force transducer. Electrical activity of the smooth muscle was recorded using calomel electrodes across agar bridges (4% agar w/v, 4 M KCl). The signals were fed to a high impedance unity gain amplifier. Electrical and mechanical signals were displayed on a Gould ink-writing chart recorder.

The intramural nerves of the preparations were stimulated electrically via parallel Pt wire electrodes (approximately 0·5 mm diameter) 3 mm apart, above and below the preparation (Hoyle, 1987), using a Grass S48 stimulator and a Grass stimulus isolation unit. The usual protocol was to apply a single pulse of electrical field stimulation every 30 s with a field strength of 150 V, evoking either a transient depolarization, i.e. an excitatory junction potential (EJP), or a transient hyperpolarization, i.e. an inhibitory junction potential (IJP). The response to electrical field stimulation varied according to the pulse width, which had an overall range of 0·005–0·3 ms. Details of pulse widths employed are given in the Results section.

Because evoked IJPs tend to mask any EJP, excitatory transmission was also studied in the presence of apamin (30 nM), which blocks the potassium ion channel involved in the generation of the IJP, thus allowing EJPs to be unmasked (Banks *et al.* 1979; Shuba & Vladimirova, 1980). The EJPs were abolished by atropine (0·3 μM), indicating that they are indeed cholinergic. In the rat caecum IJPs are non-adrenergic, non-cholinergic in nature, being resistant to atropine, guanethidine, or adrenoceptor blockers (Manzini, Hoyle & Burnstock, 1986; Hoyle, Reilly, Lincoln & Burnstock, 1988); they may be predominantly purinergic since they are inhibited by the P_2-purinoceptor antagonist Reactive Blue 2 (Manzini *et al.* 1986), but they do not appear to involve nitric oxide because N^G-nitro-L-arginine methylester (L-NAME, 50 μM) has no effect on them (C. H. V. Hoyle, unpublished observations).

Electrotonic potentials were generated in the smooth muscle membrane by passing anodal or cathodal current through a pair of electrodes, one in contact with the preparation but isolated from the environment of the tissue by a silicone grease seal and the other remote from the preparation immersed in the superfusing solution (Hoyle, 1987). With these electrodes changes in the resistance (or conductance) of the smooth muscle membrane could be monitored by passing anodal pulses of constant current (usually 5–15 μA, 1–2 s) at regular intervals (every 30 s).

Drugs were applied acutely via a multiport tap, used to change the superfusing solution from normal Krebs solution to one containing a known concentration of the drug to be applied, for a known contact time. Concentration–response relationships were established for acetylcholine, noradrenaline and α,β-methylene ATP; the contact time for each applied concentration of acetylcholine and noradrenaline was 60 s, and α,β-methylene ATP was applied for 90 s. These times were approximately 33% longer than that required to reach a maximum effect. α,β-Methylene ATP was chosen because it is a stable analogue of ATP, resistant to degradation by ecto-ATPase (Welford, Cusack & Hourani, 1987), and is a better agonist than ATP for constructing concentration–response relationships in this preparation (see Hoyle *et al.* 1988). After applying a given concentration the drug was washed out and the next concentration was applied when the preparation had restabilized, usually after 10–15 min. Only one drug was tested per preparation, and the order of application of particular concentrations was randomized. At the end of the study, the preparation was challenged with isotonic KCl (160 mM). When atropine (0·3 μM) or apamin (30 nM) were applied to a preparation, their wash-in was followed by observing the response of the tissue. Both these agents appeared to have their maximal effects within 10–15 min.

Noradrenaline assay

Samples of caecum were dissected as above and stored in liquid nitrogen until assay. The tissues were homogenized in 500 μl of perchloric acid (0·1 M) containing sodium bisulphite (0·4 mM) and 12·5 ng of an internal standard, dihydroxybenzylamine (DHBA). After centrifugation, the supernatants were subjected to alumina extraction as described previously (Keller, Oke, Mefford & Adams, 1976). Noradrenaline and DHBA were measured by high performance liquid chromatography with electrochemical detection. Separation was carried out on a C-18 reverse phase column, using a mobile phase of NaH_2PO_4 (0·1 M) containing ethylenediaminetetraacetate (0·1 mM), heptane sulphonate (5 mM), and 10% (v/v) methanol (pH 5·0). Quantitation was achieved with a Coulochem detector used at the following potentials: guard cell, +0·35 V; detector 1, +0·35 V; detector 2, −0·30 V. Levels of noradrenaline were corrected for recovery using the DHBA internal standard. The results were expressed as micrograms per gram of wet tissue.

Transmitter release studies

Preparations of the caecum were made as described above, except that they were approximately 15 mm long and 1 mm wide. These were attached to an electrode holder that consisted of two Pt rings approximately 2 mm in diameter and 1 cm apart, through which the preparation was threaded. Using silk ligatures, one end of the preparation was attached to the foot of the electrode holder, and the other end was attached to a force-displacement transducer. The preparation was continuously superfused with Krebs solution (composition as above) maintained at 36 ± 0·5 °C at a rate of 1 ml min^{-1}. The preparation could be stimulated electrically, using a Grass SD9 stimulator (16 Hz, 0·3 ms, 40 V, 60 s). The superfusate could be collected dropwise.

In the experiments carried out to measure the evoked release of acetylcholine the Krebs solution also contained hemicholinium (1 μM) to prevent reuptake of choline derived from released acetylcholine. The preparation was first allowed to equilibrate for 1 h. Then three consecutive 3 min samples were collected before electrical field stimulation (EFS) was applied for 1 min. The superfusate was collected during the stimulation period and for the subsequent 2 min. The samples of collected superfusate were assayed luminometrically, using a modification of the assay for acetylcholine (Israel & Lesbats, 1982) as described by Milner *et al.* (1989).

To measure the evoked release of noradrenaline, the preparations were first incubated in [^{3}H]noradrenaline (0·14 nM, specific activity 37 Ci $mmol^{-1}$) for 2 h at 36 ± 0·5 °C, and were then washed in three changes of Krebs solution before being mounted in the electrode holder where they were left to equilibrate for a further 90 min. Three consecutive 3 min samples of superfusate were collected before EFS was applied for 1 min. The superfusate was collected during this stimulation and for the subsequent 2 min. Samples were added in a ratio of 1:3 to Optiphase mp (LKB, Loughborough, UK) scintillation cocktail, and ^{3}H radioactivity was counted using a Beckman 7500 liquid scintillation counter. After the final sample collection the preparations were blotted, weighed and dissolved in Optisolv (LKB) tissue solubilizer to measure their total [^{3}H]noradrenaline content. Results were expressed as the rate of noradrenaline release per unit mass (i.e. in pmol (mg tissue)$^{-1}$ min^{-1}). In a separate series of experiments the superfusing Krebs solution contained tetrodotoxin (0·3 μM), but otherwise the protocol for noradrenaline release was identical.

Measurement of ATP release was attempted, using a luciferin–luciferase assay (Kirkpatrick & Burnstock, 1987). However, the levels measured were around the limit of detection, probably due to the rapid degradation of ATP by this tissue (C. H. V. Hoyle, unpublished observations), and hence this series of experiments was not pursued.

Preparation of the vas deferens and urinary bladder

The animals were killed as described above, and both the vasa deferentia were removed and cleaned of extraneous connective tissue. Segments approximately 15 mm long, centred around the mid-point, were mounted in 10 ml organ baths containing Krebs solution (composition as above), gassed with 95% O_2–5% CO_2 and maintained at 37 ± 0·5 °C. Using silk ligatures one end of the vas deferens was attached to a rigid support in the bath, and the other end to a force-displacement transducer (Grass FT0C3). Electrical field stimulation was facilitated by two Pt rings, through which the vas deferens was threaded, connected to a Grass SD9 stimulator. The preparations were given an initial load of 1 g and were allowed to equilibrate for at least 45 min before frequency–response relationships were established (EFS 0·5–32 Hz, 0·3 ms, supramaximal voltage, train duration 30 s, train interval 5 min). Four frequency–response relationships were established on each preparation. The first was established in normal Krebs solution; the second after 30 min equilibration in the presence of yohimbine (an α_2-adrenoceptor antagonist, 1 μM); the third after additional incubation (30 min) with either prazosin (an α_1-adrenoceptor antagonist, 1 μM) or α,β-methylene ATP (which

desensitizes and blocks P_{2X}-purinoceptors, 10 μM); the fourth in the presence of all three antagonists. Finally the preparations were treated with tetrodotoxin (1 μM) to qualify the EFS, and then challenged with KCl (120 mM).

The urinary bladder was removed, and transected at the level of entry of the ureters. From the corpus two strips of detrusor approximately 1 cm long and 1–2 mm wide were dissected free from the mucosa. Using silk ligatures these preparations were mounted in the organ baths in the same manner as the vasa deferentia. An initial load of 1 g was applied, and after equilibration for at least 45 min three frequency–response relationships were established (EFS 0·5–32 Hz, 0·3 ms pulse-width, supramaximal voltage, applied until the contraction reached a maximum and had declined by approximately 33%, train interval 5 min). The first was in the absence of any agents; the second was after incubation for 30 min in the presence of either atropine (1 μM) or α,β-methylene ATP (10 μM); the third was in the presence of both antagonists. Finally, tetrodotoxin (1 μM) was added to qualify the EFS, and the preparations were challenged with KCl (120 mM).

Release studies and evaluation of noradrenaline content were not carried out on either the vas deferens or urinary bladder because there were no indications that vitamin E deficiency had caused any changes (see below).

Drugs

Atropine sulphate was obtained from Antigen Ltd (Roscrea, Ireland). Acetylcholine chloride, α,β-methylene ATP, apamin, noradrenaline (± L-arterenol bitartrate), prazosin and yohimbine were obtained from Sigma Chemical Co. The Krebs salts were all obtained from BDH. Noradrenaline was dissolved in ascorbic acid (at a concentration of 0·01 times the molar concentration of noradrenaline), acetylcholine was dissolved in distilled water. Prazosin was also dissolved in distilled water, to produce a stock solution of 1 mM, which needed to be sonicated to achieve proper dissolution. Yohimbine was dissolved in 50% ethanol to produce a stock solution of 10 mM, which was also sonicated.

Statistical analyses

Comparisons between means of groups were carried out using Student's t test. Comparisons between frequency distributions were carried out using χ^2 tests. In constructing the frequency-distribution histograms of junction potential amplitude the bin-widths were based on a Fibonacci sequence. (A Fibonacci sequence is one in which a given term is the sum of the preceeding two terms.) The Fibonacci sequence is a natural growth sequence and rises less steeply than an integral logarithmic progression. Statistical significance was determined at the 5% level. Some groups of data were normalized to 100%, fitted to stimulus–response curves using probit analysis and averaged horizontally (Finney, 1971). Data are presented as means ± standard error of the mean (n) or mean with 95% confidence limits, where, unless otherwise stated, the number of replicates, n, also refers to the number of animals.

RESULTS

Caecum – electrical stimulation of the intramural nerves

In the absence of any agents, EJPs were seen in response to single pulses of EFS in three control preparations (from a population of 34 preparations from 14 animals), but were never seen in the preparations from vitamin E-deficient animals (45 preparations, 16 animals). The EJPs were only recorded when the pulse width was short (20–40 μs) and were maximal within this range; at longer pulse widths an IJP would dominate the overall response.

In the presence of atropine (0·3 μM) in preparations from both the control group and the vitamin E-deficient group, single pulses of electrical field stimulation could evoke IJPs. The maximum amplitude of IJPs in control preparations was significantly greater ($P < 0·001$) than that in preparations from vitamin E-deficient animals, being 6·4 ± 0·57 mV ($n = 34$, from 14 animals) as opposed to 2·8 ± 0·55 mV ($n = 45$, from 16 animals). The rate of failure to evoke IJPs in tissues from vitamin E-deficient animals was significantly greater than that in the control group: the frequency distribution of IJP amplitude (Fig. 1) shows the pronounced tendency of IJPs to be of lower maximum amplitude in the former group.

When the electrical field and stimulation frequency were kept constant (150 V, 0·033 Hz), the IJP amplitude was dependent upon the duration of the applied stimulus (pulse width). Stimulus–response curves were constructed (Fig. 2), and in addition to the reduced maximum response in vitamin E-deficient tissues the IJP-producing neurones appeared to be less sensitive to electrical stimulation because the stimulus–response curve for these tissues lay to the right of that of the control group. The pulse width that evoked an IJP of half-maximal amplitude was 33 (26·5, 41·0) μs in the control group, and 48 (37·8, 60·9) μs (mean with 95% confidence limits in parentheses) for the vitamin E-deficient group ($P < 0·02$).

Apamin (30 nM) abolished evoked IJPs in all preparations. In control tissues EJPs were revealed in eleven out of twelve (92%) preparations from six animals, and these were abolished by atropine (0·3 μM) (Fig. 3), but EJPs became apparent in only two out of twelve (17%) preparations from vitamin E-deficient animals.

When EFS was applied at 16 Hz (0·03 ms pulse width, 10 s) there was a biphasic response, with a transient maximum hyperpolarization being followed by a reduced more-or-less steady level of hyperpolarization (Fig. 3). All control preparations responded with a hyperpolarization whose mean maximum was 7·7 ± 1·09 mV and whose steady-state hyperpolarization was 3·4 ± 0·85 mV ($n = 22$ from 11 animals). In preparations from vitamin E-deficient animals the maximum was significantly ($P < 0·01$) lower at 2·1 ± 0·58 mV, as was the steady-state level of 0·9 ± 0·61 mV ($P < 0·01$) ($n = 24$ from 12 animals).

Following application of apamin, in response to EFS at 16 Hz (0·03 ms, 10 s) (Fig. 3) a mean maximum hyperpolarization of 8·8 ± 1·55 mV ($n = 6$) was converted to a depolarization of 14·6 ± 2·79 mV in control tissues, and a hyperpolarization of 1·8 ± 1·29 mV ($n = 7$) was converted to a depolarization of 2·42 ± 2·42 mV in the vitamin E-

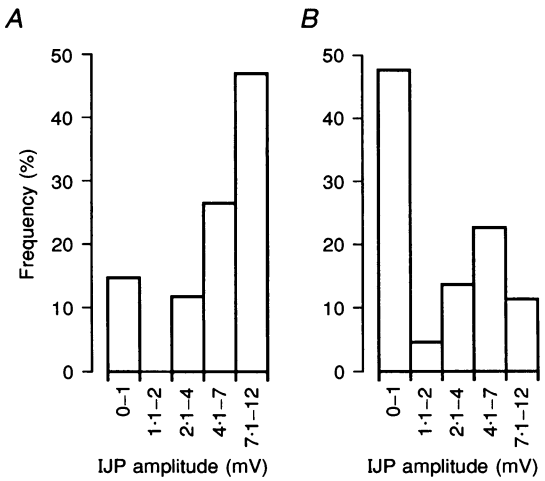

Figure 1. Frequency distribution of maximum IJP amplitudes in control and 12-month vitamin E-deficient rats

Frequency distribution of maximum IJP amplitudes in preparations of the circular muscle of the caecum isolated from control (*A*) and 12-month vitamin E-deficient (*B*) rats. The bin widths are based on a modified Fibonacci sequence (a natural growth sequence). The control population is made up of 34 preparations from 14 animals, and the vitamin E-deficient population is 45 preparations from 16 animals. The distributions are significantly different ($P < 0{\cdot}01$, χ^2 test).

deficient group. In this latter group, in the absence of apamin only two of the seven preparations responded with a hyperpolarization, and in the presence of apamin only one of these preparations responded with a depolarization. In all tissues the depolarization that occurred during EFS was abolished by atropine (0·3 μM).

In some tissues, after incubation with apamin and atropine there was a small hyperpolarization (e.g Fig. 3*C*). This was not examined in any detail, and the possibility that it is due to nitric oxide remains to be explored. Also, in the presence of atropine (0·3 μM) there was a phase of depolarization that arose at the end of the stimulation period. This non-adrenergic, non-cholinergic phase was very variable in terms of amplitude, action potential discharge, and duration, and was not analysed in detail. However, this delayed phase of depolarization was present in all the control preparations but only in those vitamin E-deficient preparations that had displayed non-adrenergic, non-cholinergic inhibitory transmission.

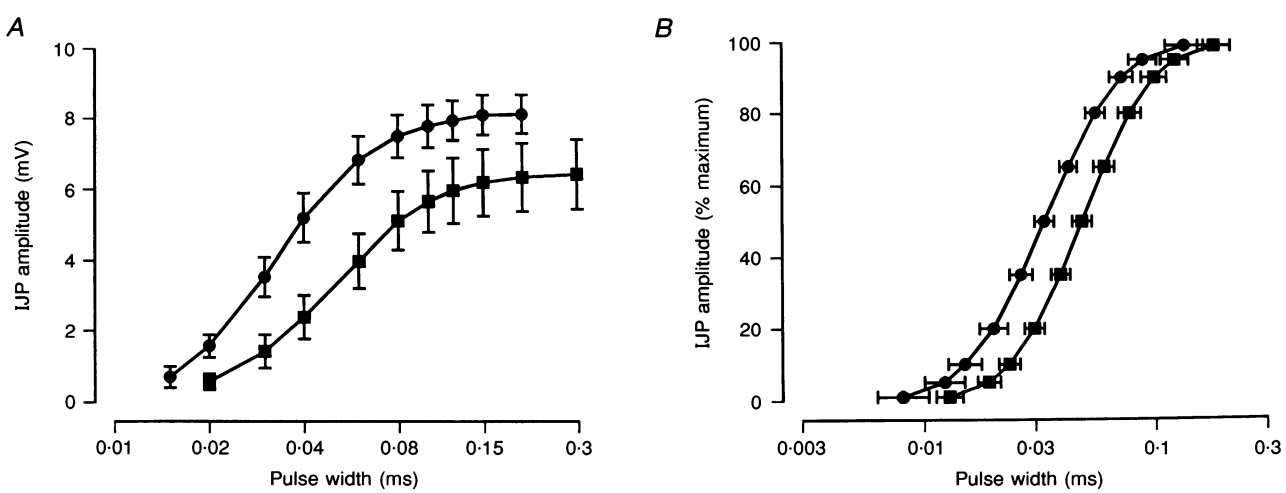

Figure 2. Dependency of amplitude of IJP on pulse width of applied electrical field stimulation

The abscissa shows the pulse width (ms) on a logarithmic scale, and the ordinate shows the amplitude of the IJP. In *A*, IJP amplitude is given in absolute values (mV), and in *B* the IJP amplitude is expressed relative to its own maximum. In *B*, the curves were constructed following probit transformation and horizontal averaging. Note that at any given point the response in control tissues (●) is greater than that in the vitamin E-deficient tissues (■).

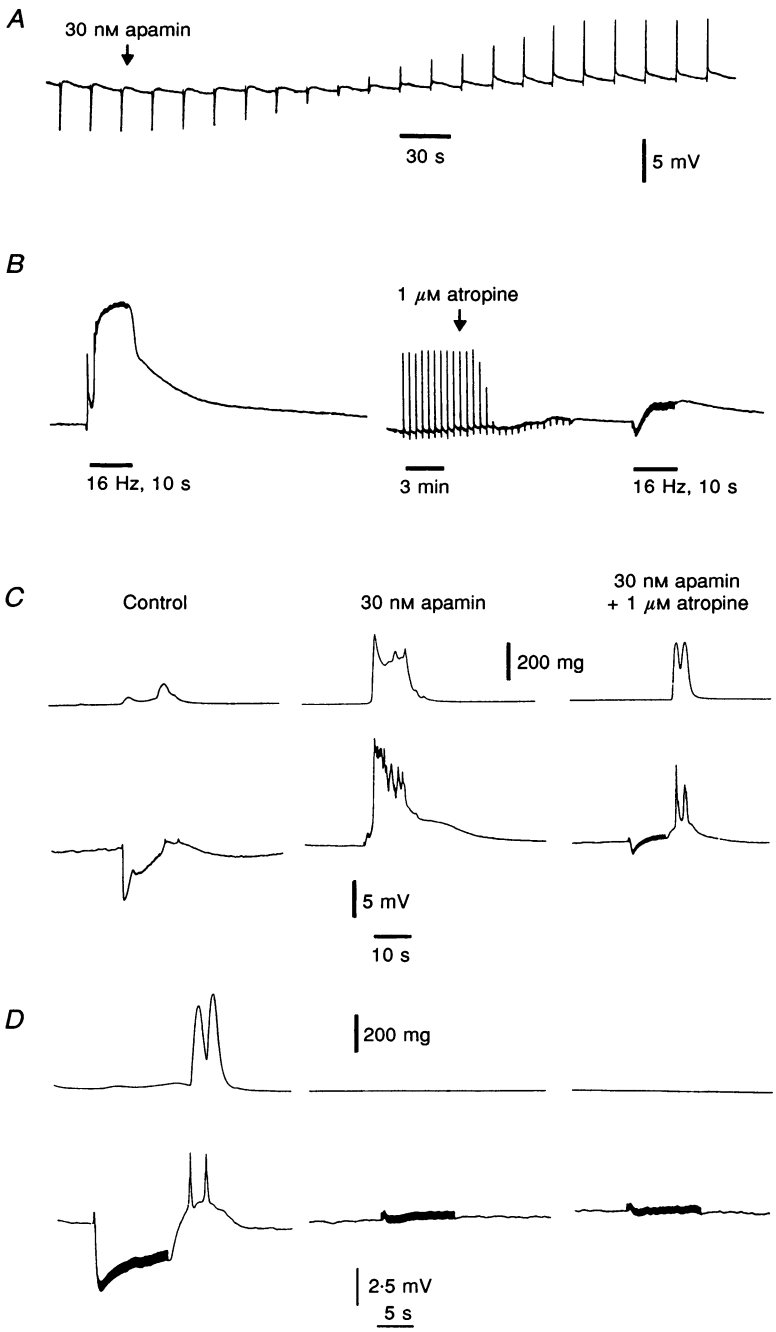

Figure 3. Sucrose-gap recordings of membrane potential of rat caecum circular muscle, showing the effects of apamin (30 nM) and atropine (1 μM) on neuromuscular transmission

In *A*, in normal Krebs solution, IJPs were evoked every 30 s by single pulses of EFS (0·07 ms, 150 V); apamin was applied at the arrow, and converted the response to EFS from an IJP to an EJP. In *B*, in the presence of apamin, EFS applied for 10 s at 16 Hz (0·03 ms, 150 V) evoked depolarization, and single pulses (to the right of the break in the trace) evoked EJPs. Atropine (applied at arrow) abolished the EJPs and severely attenuated the response to EFS at 16 Hz. *C* shows simultaneous recording of mechanical activity (upper trace) and membrane potential in a control preparation stimulated at 16 Hz, 0·03 ms, 10 s in normal Krebs solution, then in the presence of apamin (30 nM), and finally in the presence of apamin plus atropine (1 μM). *D* is a pair of recordings as in *C* from a vitamin E-deficient animal. Note the lack of response in the presence of apamin and in the presence of apamin plus atropine.

Responses to applied acetylcholine

In preparations from control animals, acetylcholine (0·3–30 μM) caused concentration-dependent depolarizations (Fig. 4*A*) which were usually accompanied by action potential discharge and a contractile response (Fig. 5). The IJP amplitude increased during the depolarization, and so did the resistance of the smooth muscle membrane, as determined by the increase in amplitude of applied analectrotonic potentials. In contrast, in most of the preparations from vitamin E-deficient animals, acetylcholine was almost inert over the same concentration range, or may even have induced a small hyperpolarization instead of the expected depolarization (Figs 4*A* and 5).

Responses to applied noradrenaline

In preparations from control animals, noradrenaline (0·3–30 μM) caused a concentration-dependent hyperpolarization of the smooth muscle cells, a decrease in the amplitude of the IJP and a decrease in the smooth muscle membrane resistance (Figs 4*B* and 5). In preparations from vitamin E-deficient animals, noradrenaline was usually without significant effect over the same concentration range. In cases where it did cause hyperpolarization it was markedly less potent than in the control tissues (Figs 4*B* and 5).

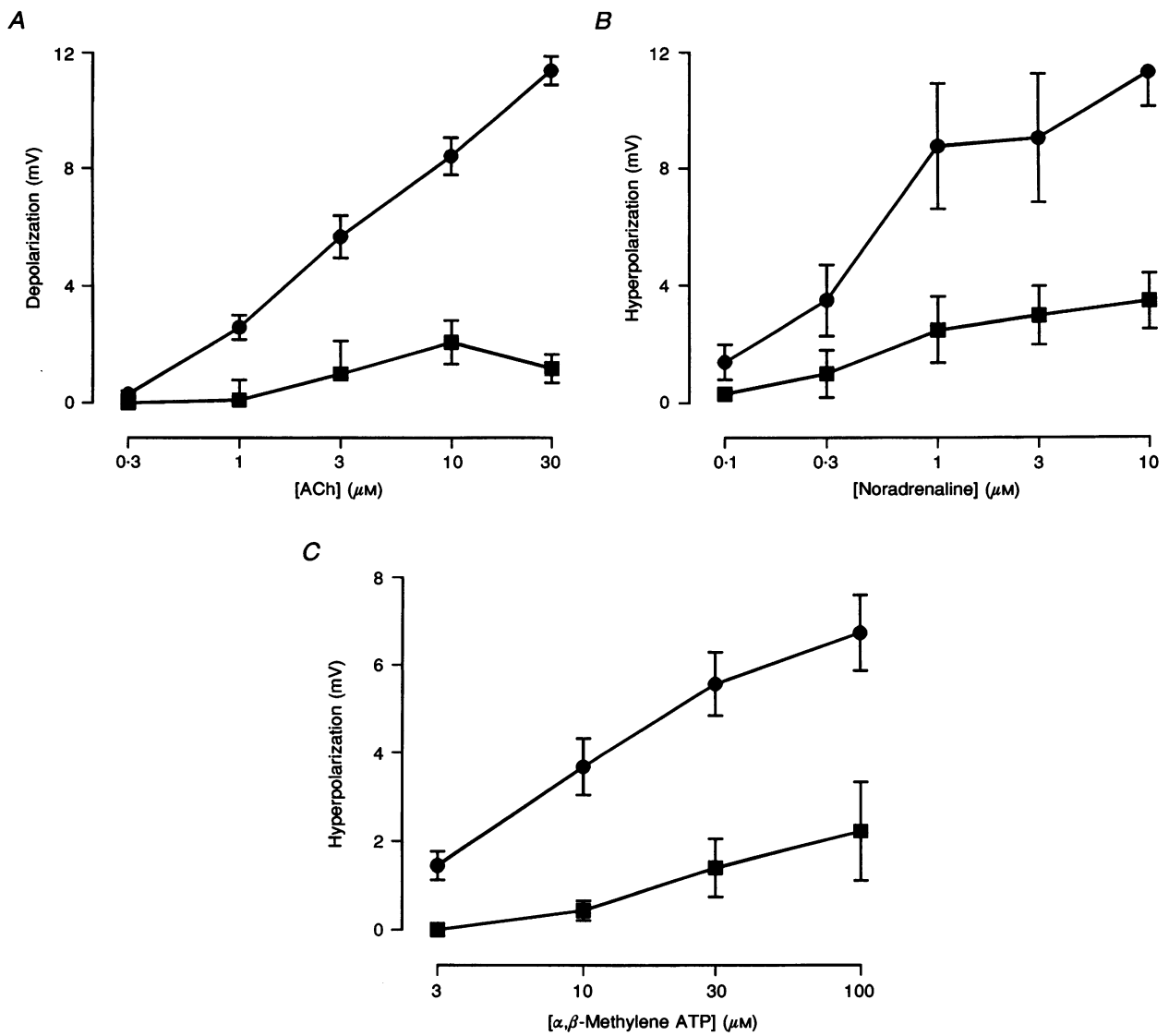

Figure 4. Concentration–response curves to ACh (*A*), noradrenaline (*B*) and α,β-methylene ATP (*C*) in preparations from the caecum of control (●) and vitamin E-deficient (■) animals

The abscissa shows the concentration of ACh, noradrenaline or α,β-methylene ATP on a logarithmic scale, and the ordinates show the level of depolarization induced by ACh, or of hyperpolarization evoked by noradrenaline and α,β-methylene ATP. Points show mean ± S.E.M. from 5 or 6 animals in each group. Preparations from vitamin E-deficient animals in which there was no response to the applied agonist have not been included (2 or 3 for each agonist).

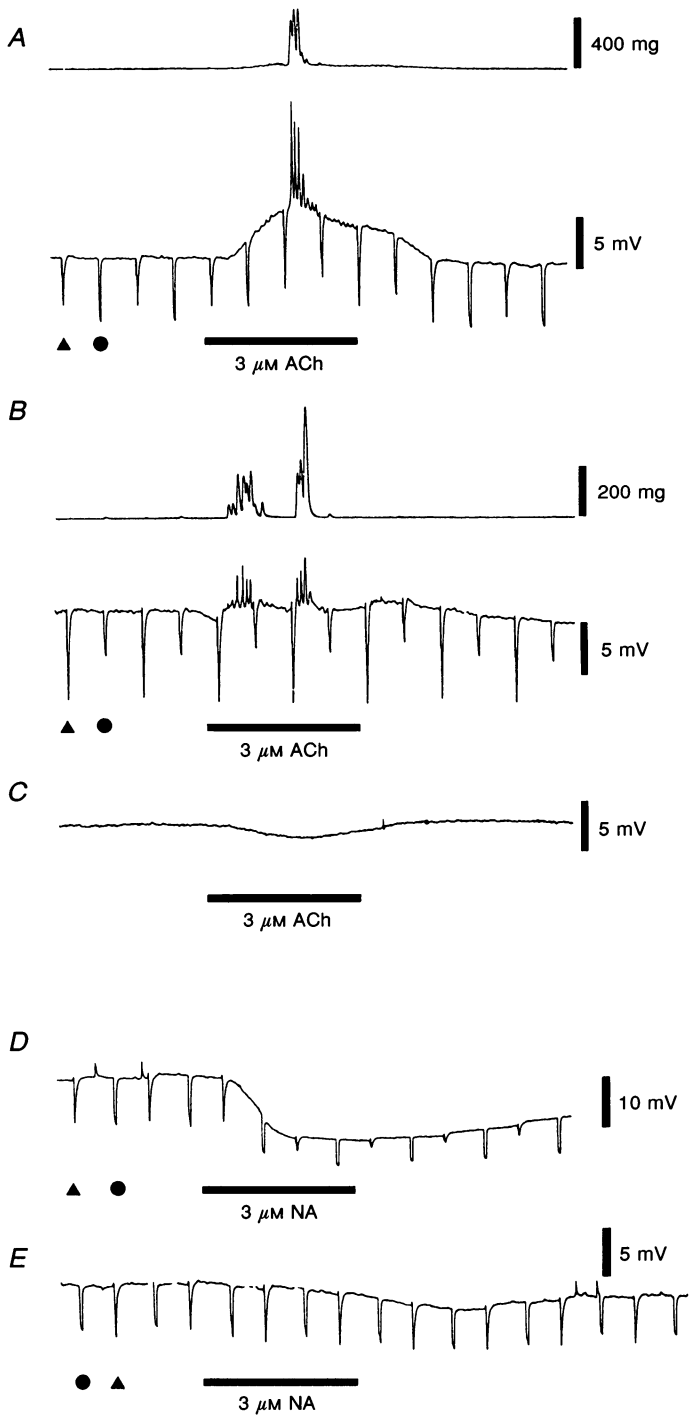

Figure 5. Sucrose-gap recordings of the effects of ACh and noradrenaline on preparations of the caecum from control and 12-month vitamin E-deficient rats

Lower trace, electrical; upper trace, mechanical. *A*, control preparation: IJPs (▲) were evoked by single pulses of EFS every 30 s, and in between consecutive IJPs a constant current pulse was applied (●) to monitor changes in the smooth muscle membrane resistance. ACh (3 μM) was applied for 60 s, as indicated by the bar. Note the substantial depolarization, action potential discharge and consequent contractile activity. *B*, a preparation from a vitamin E-deficient animal. In this recording, which was amongst the best from this group in terms of IJP amplitude and responsiveness to ACh, IJPs were evoked (▲) and the membrane resistance was monitored (●) as above. Note the lower level of depolarization at which action potentials were generated. *C*, recording of membrane potential only, from a vitamin E-deficient animal, in

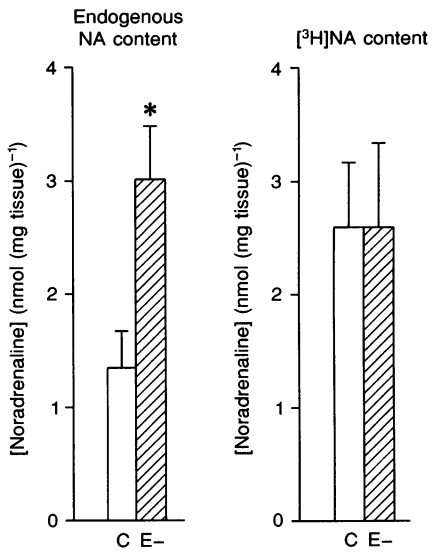

Figure 6. Noradrenaline content of rat caecum circular muscle from control and 12-month vitamin E-deficient animals

The panel on the left shows the endogenous noradrenaline content in control (C, □) and vitamin E-deficient (E−, ▨) tissues. Bars show S.E.M.; $n = 8$ for both groups; $*P < 0{\cdot}01$, significant difference between groups. The panel on the right shows the ^3H content following incubation with [^{3}H]noradrenaline (0·14 nM, specific activity 37 Ci mmol^{-1}, for 2 h at 36 ± 0·5 °C) in control (C, □) and vitamin E-deficient (E−, ▨) tissues. Bars show S.E.M.; $n = 6$ for both groups; no significant difference.

Responses to applied α,β-methylene ATP

In preparations from control animals, α,β-methylene ATP (3–100 μM) caused a concentration-dependent hyperpolarization of the smooth muscle cells, a decrease in the amplitude of the IJP and a decrease in smooth muscle membrane resistance. In preparations from vitamin E-deficient animals, α,β-methylene ATP was usually without effect over the same concentration range. In cases where it did cause hyperpolarization it was markedly less potent than in the control tissues (Fig. 4*C*).

Responses to applied KCl

The depolarizations induced by KCl (160 mM) were averaged for all the preparations from the same animal. The mean depolarization due to KCl was 28·7 ± 2·43 mV ($n = 14$) for the control group and 23·4 ± 2·33 mV ($n = 16$) for the vitamin E-deficient group: these values are not significantly different.

Noradrenaline content

The mean noradrenaline content in the muscle layers of the caecum from control rats was 1·33 ± 0·26 nmol (g tissue)$^{-1}$ ($n = 8$). In the muscle layers from the caecum of the vitamin E-deficient animals the level of noradrenaline was more than doubled, being 2·96 ± 0·46 nmol (g tissue)$^{-1}$ ($n = 8$) (Fig. 6). This increase was statistically significantly different ($P < 0{\cdot}01$). Following loading with [^{3}H]noradrenaline (0·14 nM; specific activity, 37 Ci mmol^{-1}) for 2 h at 36 ± 0·5 °C, the amount of radioactivity taken up into the tissue was almost identical in both groups (Fig. 6).

Evoked release of acetylcholine and [^{3}H]noradrenaline

In preparations of the caecum from control animals, EFS evoked release of acetylcholine and [^{3}H]noradrenaline that was significantly above the basal levels of unstimulated release (Figs 7 and 8). In caecal preparations from vitamin E-deficient animals there was no significant release of either acetylcholine or [^{3}H]noradrenaline evoked by the same stimulus (Figs 7 and 8). Tetrodotoxin (0·3 μM) had no effect on basal release of noradrenaline in either of the two groups, but abolished the evoked release in the control group.

Vas deferens and urinary bladder

There were no significant differences between the frequency– response relationships established in vasa deferentia from the two groups of animals (Fig. 9). In both groups the EFS-evoked response was frequency dependent (0·5–32 Hz), yohimbine potentiated the responses to higher frequencies (16 and 32 Hz), prazosin inhibited responses at all frequencies, and α,β-methylene ATP

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which IJPs could not be evoked, and in which ACh caused a small hyperpolarization rather than depolarization. *D*, recording of the membrane potential of a preparation from a control animal. IJPs were evoked (▲) and the resistance of the smooth muscle membrane was monitored (●) as described above. Noradrenaline (NA, 3 μM) was applied for 60 s, as indicated by the bar, and caused a large hyperpolarization. Note the reduction in IJP amplitude and decrease in membrane resistance. *E*, a preparation from a vitamin E-deficient animal which was a very good one from this group in terms of IJP amplitude and responsiveness to noradrenaline. IJPs were evoked (▲), and the membrane resistance was monitored (●) as above. Noradrenaline (NA, 3 μM), applied as indicated, caused only a relatively small hyperpolarization, accompanied by small reductions in IJP amplitude and membrane resistance.

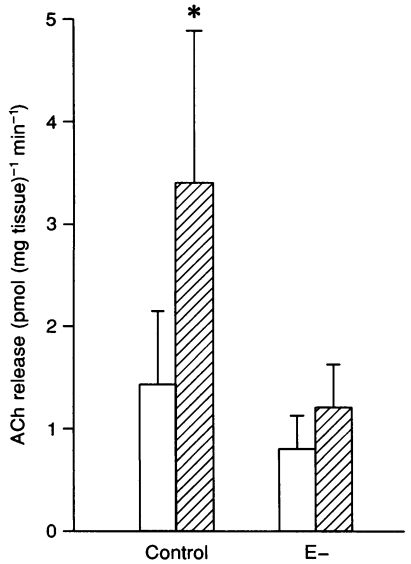

Figure 7. Release of ACh evoked by EFS from rat caecum circular muscle from control and 12-month vitamin E-deficient rats

Open columns show basal, prestimulus, levels; hatched columns show levels of release evoked by EFS (16 Hz, 0·3 ms, 40 V, 60 s). Bars show S.E.M.; $n = 6$ for both groups. In control tissues the evoked release was significantly greater than the basal level ($P < 0·05$, *), but in the vitamin E-deficient group (E–) the level of released ACh was not significantly greater than basal.

inhibited responses at lower (< 8 Hz) frequencies (Fig. 9). In the presence of all three antagonists there was a small residual response that was abolished by tetrodotoxin.

In the urinary bladder strips, there were also no significant differences between the frequency–response relationships of the two groups (Fig. 10). Atropine inhibited responses at all frequencies, while α,β-methylene ATP caused a greater inhibition at lower rather than higher frequencies (Fig. 10). In the presence of both these agents the residual response was abolished by tetrodotoxin.

For both the vas deferens and the urinary bladder, the contractile responses to KCl were not significantly different between the two groups of animals.

DISCUSSION

The results presented show that chronic vitamin E deficiency in rats causes neuropathic and myopathic changes in the autonomically innervated circular muscle of the caecum, but not in the sympathetic or parasympathetic responses of the vas deferens and urinary bladder, respectively.

The reduced probability of being able to record either an EJP or an IJP in the preparations from vitamin E-deficient animals implies that there was a dystrophy of both the cholinergic excitatory and the non-adrenergic, non-cholinergic inhibitory intrinsic nerves. The presence of an autonomic neuropathy was supported by the compromised release of acetylcholine and noradrenaline from the

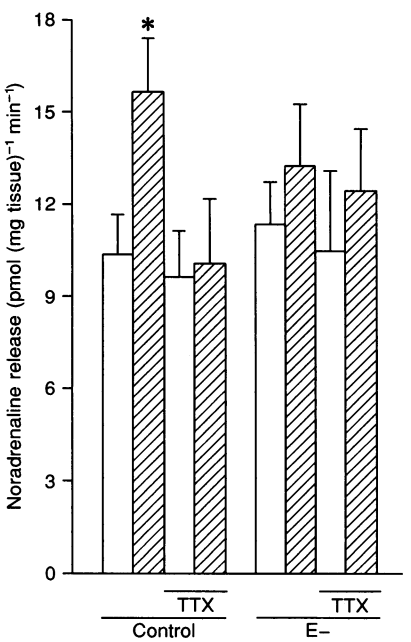

Figure 8. Release of [^{3}H]noradrenaline evoked by EFS from rat caecum circular muscle from control and 12-month vitamin E-deficient rats

Open columns show basal, prestimulus, levels; hatched columns show levels of release evoked by EFS (16 Hz, 0·3 ms, 40 V, 60 s). Bars show S.E.M.; $n = 6$ for both groups. In control tissues the evoked release was significantly greater than the basal level ($P < 0·02$, *), but in the vitamin E-deficient (E–) group the level of released ^{3}H was not significantly greater than basal. In the presence of TTX (0·3 μM) EFS did not evoke significant release in either group.

vitamin E-deficient tissues. However, the greatly diminished responses to applied acetylcholine, noradrenaline and α,β-methylene ATP indicate that there were also dysfunctional transduction mechanisms in the smooth muscle. Nevertheless, the smooth muscle membrane potential must have been approximately the same in the two groups because the depolarization evoked by KCl in vitamin E-deficient tissues was similar to that in control tissues.

Vitamin E is an important lipid-soluble antioxidant that acts as a quenching agent of free radicals, thus inhibiting radical chain reactions. One of its major functions is to protect polyunsaturated fatty acids from peroxidation (McCay, 1985). Polyunsaturated fatty acids are components of biological membranes, and their peroxidation by reactive free radicals alters the chemicophysical properties of the membrane, principally resulting in a loss of membrane fluidity and integrity, and an increase in ionic permeability (Slater, 1984; Halliwell & Gutteridge, 1985). Also, reactive free radicals have deleterious effects on cells by covalently binding to membrane-bound proteins, such as enzymes and receptors (Muakkassah-Kelly, Andersen, Shih & Hochstein, 1983; Slater, 1984; Halliwell & Gutteridge, 1985; Doelman, Kramer, Timmerman & Bast, 1988). For example, in the related model of ascorbate-induced lipid peroxidation a decrease in 5-hydroxytryptamine (5-HT) binding to subpopulations of 5-HT receptors in rat striatal membranes occurs (Muakkassah-Kelly *et al.* 1983). Thus in vitamin E deficiency, when there is decreased protection of lipids from peroxidation, there is the potential for several types of neuroeffector mechanisms to become subfunctional.

Effects of vitamin E deficiency on a cholinergic system have been reported previously. In isolated mesenteric blood vessels from chronically vitamin E-deficient rats the relaxant responses to acetylcholine are attenuated (Hubel *et al.* 1989), and it has been shown that the cholinergic responses in this vascular bed are endothelium dependent (Ralevic & Burnstock, 1988). Whether the altered

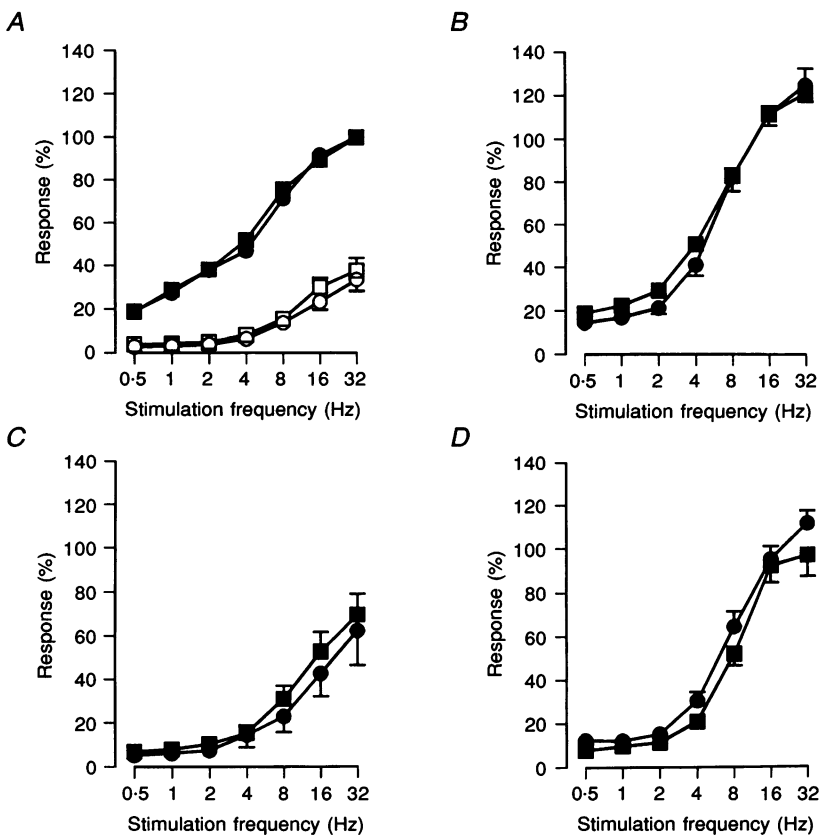

Figure 9. Frequency–response curves of rat vas deferens from control and 12-month vitamin E-deficient rats

A, in normal Krebs solution (filled symbols) and in the presence of prazosin (1 μM), yohimbine (1 μM) and α,β-methylene ATP (10 μM) (open symbols). In this and all other panels control animals are denoted by circles, and vitamin E-deficient animals by squares. *B*, in the presence of yohimbine (1 μM). *C*, in the presence of prazosin (1 μM). *D*, in the presence of α,β-methylene ATP (10 μM). There were no significant differences between the two groups at any point. Responses are expressed relative to the response evoked by EFS at 32 Hz in normal Krebs solution. Points show mean ± S.E.M., unless occluded by symbol; $n = 8$ for both groups in *A*, and $n = 4$ for both groups in *B*, *C* and *D*.

responses are due to a damaged endothelium or to a change in receptor binding or transduction was not established (Hubel *et al.* 1989). Furthermore, muscarinic receptors in the rat trachea are susceptible to oxidative stress induced by hydrogen peroxide (Doelman *et al.* 1988), hence it is likely that subsensitive responses to acetylcholine could develop during long-term vitamin E deficiency.

The diminished responses to exogenous noradrenaline could be due to a lesion similar to that of the cholinergic system. There are no studies that have previously directly addressed the question of altered adrenergic function in vitamin E deficiency, but to a greater extent than the muscarinic receptors, β-adrenoceptors in the rat trachea are susceptible to oxidative stress (Doelman *et al.* 1988). Although an α-adrenoceptor mediates the hyperpolarizations evoked by noradrenaline in the rat caecum (Hoyle *et al.* 1988) it is likely that oxidative stresses during vitamin E deficiency could lead to a subsensitivity of this subtype of receptor. The chronically increased levels of circulating catecholamines, taken to indicate enhanced activity of the sympathetic nervous system, in vitamin E-deficient rats (Nakashima & Esashi, 1986, 1987) might also induce a downregulation of peripheral adrenoceptors.

In the present study, vitamin E deficiency caused a significant increase in the noradrenaline-content of the caecum. This finding could be indicative of increased synthesis and enhanced sympathetic activity in the caecum of vitamin E-deficient rats. However, it must be emphasized that increased levels of neurotransmitters do not only occur when neural activity increases. For example, in a study of diabetic neuropathy, it was demonstrated that levels of vasoactive intestinal polypeptide (VIP) are significantly elevated in the ileum of diabetic rats (Belai, Lincoln, Milner, Crowe, Loesch & Burnstock, 1985), yet despite this increase in content, release of VIP could not be evoked by EFS of the enteric nerves in diabetic preparations, but could be evoked in control preparations (Belai, Lincoln & Burnstock, 1987). Thus an increased

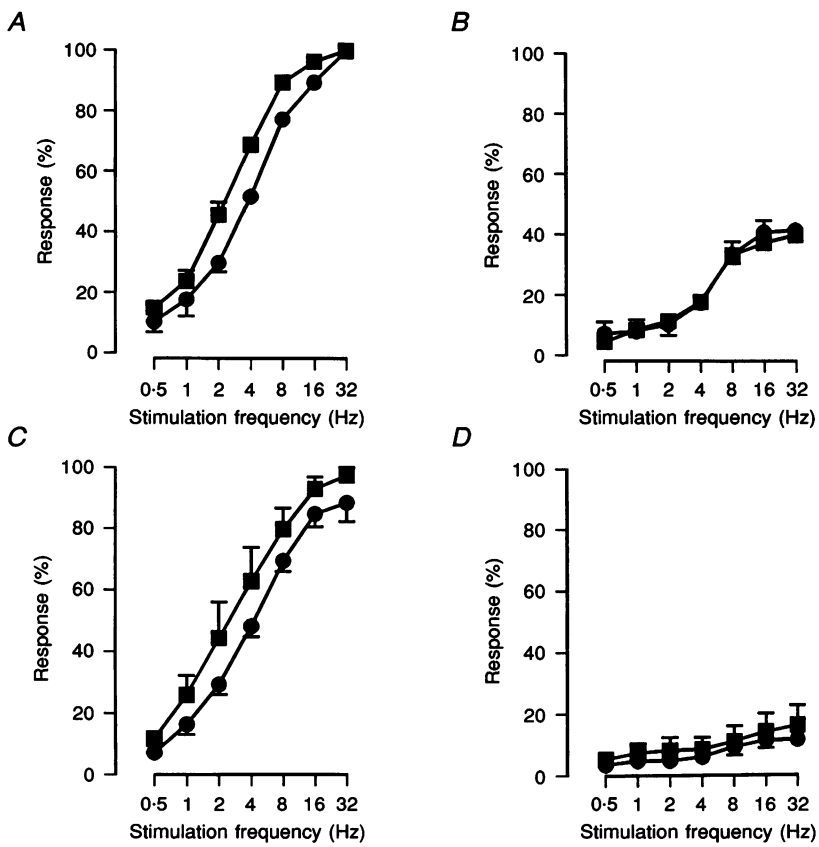

Figure 10. Frequency–response curves of rat urinary bladder from control and 12 month vitamin E-deficient rats

A, in normal Krebs solution; *B*, in the presence of atropine (1 μM); *C*, in the presence of α,β-methylene ATP (10 μM); *D*, in the presence of α,β-methylene ATP (10 μM) and atropine (1 μM). Points show mean $\pm$ S.E.M.; $n = 8$ for both groups in *A* and *D*, $n = 4$ for both groups in *B* and *C*. In all panels circles denote control animals, and squares denote vitamin E-deficient animals. Responses are expressed relative to the response evoked by EFS in normal Krebs solution at 32 Hz.

content of neurotransmitter can arise when impaired release and neurodegenerative changes occur (Loesch, Belai, Lincoln & Burnstock, 1986; Belai *et al.* 1987). The fact that both groups of tissues took up [^{3}H]noradrenaline to the same extent is interesting. Although the uptake$_1$ and uptake$_2$ systems were not examined separately, if there were a total loss of neuronal uptake sites (uptake$_1$) then a lower ^{3}H content might be expected. Thus it is possible that the elevated endogenous noradrenaline content in the vitamin E-deficient tissues was partially due to impaired neuronal release mechanisms in the presence of intact uptake mechanisms.

Taken together with the observed dysfunction of the muscarinic- and α-adrenoceptor-mediated responses, and the lack of evoked acetylcholine and noradrenaline release, it is therefore possible that the reduced effectiveness of stimulation of the non-adrenergic, non-cholinergic nerves in evoking IJPs is due to a combination of altered postjunctional P_2-purinoceptors and compromised transmitter release. For each of these systems there may be a temporal sequence of dysfunction, with decreased transmitter release causing disuse of receptors, and ultimately receptor downregulation.

From the results of the depolarization of the smooth muscle membrane by applied KCl, it appears that the smooth muscle cells of the caecum of the vitamin E-deficient rats had a similar resting membrane potential. The sucrose-gap technique does not allow absolute measurement of membrane potential, and the use of KCl in this way is rather a blunt tool; nevertheless there did not appear to be a significant difference in polarization that could account for either the reduced hyperpolarization evoked by noradrenaline or α,β-methylene ATP or the reduced depolarization evoked by acetylcholine.

The lack of effect of vitamin E deficiency on neuromuscular transmission in the vas deferens and urinary bladder is perplexing. Why should the smooth muscle of these organs, and their neural supply, be spared? Possibly the large intestine is exposed to oxidants of dietary or bacterial origin, whose effects are more detrimental when anti-oxidant protective mechanisms are compromised by vitamin E deficiency. Another possibility is that the cell membranes of these organs contain different levels of activity of protective mechanisms. By way of analogy, hepatocytes are exquisitely sensitive to free radical attack compared with intestinal epithelial cells, owing to the presence of non-esterified fatty acids amongst the epithelial membrane lipids (Balasubramanian, Nalini, Cheeseman & Slater, 1989).

In conclusion, a chronic lack (12 months) of dietary vitamin E induced dysfunctions in autonomic neuromuscular transmission in the rat caecum at both the neural and the muscular site. The autonomic neuropathy did not appear to be a general process since both the urinary bladder and vas deferens were unaffected by vitamin E deficiency.

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