



Depressant effects of hypoxia and hypoglycaemia on neuro-effector transmission of guinea-pig intestine studied *in vitro* with a pharmacological model

¹A.D. Corbett & G.M. Lees

Department of Biomedical Sciences, University of Aberdeen, Marischal College, Aberdeen, AB9 1AS

1 Since intermittent ischaemia may play an important role in the aetiology of Inflammatory Bowel Disease, particularly Crohn's Disease, a pharmacological model of neuronal ischaemia was applied to guinea-pig isolated intestinal preparations to mimic the acute effects of reduced blood flow on intestinal motility.

2 Neuro-effector transmission and smooth muscle performance were examined in myenteric plexus-longitudinal muscle preparations of guinea-pig ileum exposed to sodium cyanide (NaCN), in order to inhibit oxidative phosphorylation, or to iodoacetic acid (IAA), to block glycolysis. Comparisons were made with the effects due to simple deprivation of oxygen or glucose.

3 Depressions of cholinergic neuro-effector transmission induced by hypoxia or NaCN (effective concentration range 0.1–3 mM), given as separate treatments, singly or repetitively over 60–90 min, were apparent within 30 s and were reversible. The maximum inhibition was 90% and the IC₅₀ for NaCN was 0.3 mM. A conspicuous component of these inhibitions was prejunctional.

4 Non-cholinergic neuro-effector contractions were inhibited by up to 90% by anoxia or NaCN but recovery was incomplete and slower than with cholinergic contractions.

5 Glucose-free solutions also caused a reversible failure of cholinergic neuro-effector transmission but of slower onset. In contrast, IAA (0.06–1 mM) abolished contractions irreversibly, apparently by a direct depressant effect on smooth muscle contraction. Unlike NaCN, IAA caused an initial potentiation of electrically-induced contractions, partly by a prejunctional potentiation of cholinergic neuro-effector transmission.

6 It is concluded that a disruption of intestinal activity in pathological conditions associated with intestinal ischaemia may result from disturbances in the function of enteric neurones.

Keywords: Intestinal ischaemia; *in vitro* pharmacological model; guinea-pig myenteric neurones; neuro-effector transmission; sodium cyanide; iodoacetic acid; hypoglycaemia; hypoxia

Introduction

It has long been established that intestinal motility and secretion are dependent on the activity of enteric nerves and that Inflammatory Bowel Disease (IBD) is associated with alterations in bowel function. It seems reasonable to assume, therefore, that the activity of enteric neurones might be altered in IBD, especially in Crohn's Disease, which probably results from multifocal intestinal infarction (Wakefield *et al.*, 1989; 1991; Shepherd, 1991; Hudson *et al.*, 1992; 1994; Osborne *et al.*, 1993; Pounder, 1994). The question arises, therefore, whether ischaemic episodes cause changes in neuronal activity.

Despite the substantial body of knowledge about the physiology and pharmacology of enteric neurones, there is still surprisingly little information concerning the effects of ischaemic episodes on the properties of enteric neurones or on neuro-effector transmission to the effector tissue (last reviewed by Kosterlitz & Lees, 1964). Pioneering investigators of the effects of intestinal ischaemia focused their attention on alterations in the biochemistry and contraction of smooth muscle (Garry, 1928; see also Day & Vane, 1963). In order to differentiate between the effects of substances acting directly or indirectly on the contractions of intestinal smooth muscle, Day & Vane (1963) used the technique of transmural electrical stimulation (Paton, 1955; 1957) of isolated preparations of guinea-pig intestine to study nerve-mediated responses of smooth muscle. In the course of their experiments, they found that oxygen deprivation blocked electrically-induced and certain

drug-induced contractions of guinea-pig ileum at a time when responses to directly-acting agents were scarcely affected.

Since a pharmacological model of cerebral ischaemia had recently been described (Reiner *et al.*, 1990), this new approach for studying possible neuronal effects of intestinal infarction or short periods of hypoxia or hypoglycaemia on the function of enteric neurones seemed worthy of re-examination. The model was used to examine the effects of stimulated ischaemia on neuro-effector transmission in guinea-pig small intestine, by monitoring the responses of the longitudinal muscle to electrical stimulation of intrinsic nerves and to various drugs under different metabolic conditions. Myenteric plexus-longitudinal muscle preparations were chosen for their convenience, rather than an analysis of neuro-effector transmission to the mucosa, and the fact that information on the effects of anoxia and hypoglycaemia on intestinal smooth muscle was available.

Preliminary accounts of the results have been published (Corbett & Lees, 1993; Lees *et al.*, 1993).

Methods

Guinea-pig small intestine

Male guinea-pigs (Dunkin Hartley strain; weighing 200–650 g) were killed by cervical dislocation and exsanguination. With the exception of the terminal 10–12 cm of ileum, which was discarded, the small intestine was isolated and the lumen gently flushed with Krebs solution 34–37°C to remove any luminal contents. Myenteric plexus-longitudinal muscle preparations were made from 12–20 cm segments of whole in-

¹ Author for correspondence at: Department of Biological Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow G4 0BA.

testine by the method of Kosterlitz *et al.* (1970). Briefly, a segment of small intestine was slipped onto a glass rod and the longitudinal muscle, with the adherent myenteric plexus, was separated from the circular muscle layer by means of tangential strokes with fine cotton wool dampened with Krebs solution.

Myenteric plexus-longitudinal muscle preparations were suspended in Krebs solution at 37°C in 3 ml organ baths under a tension of 1 g. To examine pre- and post-junctional effects, isometric contractions (Pye-Ether transducer) were induced by drugs or by electrical field stimulation according to the method of Paton (1963). Unless stated otherwise, stimuli of supramaximal voltage (0.5 ms duration at 0.1 Hz, delivered from Grass S48 or S88 stimulators) were displayed on a Grass 4-channel pen recorder. In a few experiments, contractions were recorded isotonicity (under a tension of 1 g) by means of a Washington Type 2 transducer (Harvard Apparatus) or quasi-auxotonically with a Grass FT 03 force-displacement transducer; all mechanical responses were displayed on the pen recorder. The indirect (i.e. nerve-mediated), cholinergic nature of the electrically-induced contractions was established by use of hyoscine (100 nM) and tetrodotoxin (100 nM).

Models of ischaemia

The following conditions were used as models of severe hypoxia ('anoxia'), hypoglycaemia and ischaemia:

Anoxia (a) 'Simple anoxia' achieved by oxygen deprivation; usually 95% N₂/5% CO₂ (commercial nitrogen-carbon dioxide mixtures were used, so traces of oxygen may have been present) but gas mixtures of 5–15% O₂ were also used, each with 5% CO₂ to maintain pH or (b) sodium cyanide (NaCN), as inhibitor of oxidative phosphorylation. No particular precautions, such as sealing the organ bath (Schmitt & Nicoll, 1933; Day & Vane, 1963; Allen *et al.*, 1989), seemed to be required to exclude oxygen from gaining access to the bathing solutions.

Hypoglycaemia (a) 'Hypoglycaemia' achieved by glucose-free solutions or (b) iodoacetic acid (IAA) as inhibitor of the glycolytic enzyme, 3-phosphoglycerate dehydrogenase.

Ischaemia (a) Combined use of oxygen deprivation and glucose-free solutions or (b) NaCN plus IAA.

Drugs

The composition of the Krebs solution was as follows (mM): NaCl 118, KCl 4.75, CaCl₂ 2.54, NaH₂PO₄ 1.1, MgSO₄ 1.2, NaHCO₃ 25.0 and glucose 11.1; the pH was 7.4 when gassed with 95% O₂/5% CO₂. Salts were dissolved in either single-distilled water or deionised water without prior boiling (Day & Vane, 1963). Stock solutions of acetylcholine chloride (BDH) were dissolved in 5% NaH₂PO₄ and dilutions were made with Krebs solution acidified with HCl to pH 4.0. Atropine methylnitrate (Sigma), carbachol (carbamoyl-β-methylcholine chloride; BDH), ethyleneglycol-bis-(β-aminoethyl ether) N,N,N,N-tetra-acetic acid (EGTA), histamine acid phosphate (BDH), hyoscine hydrobromide (Sigma), phentolamine mesylate (Ciba), neostigmine bromide (Sigma), (±)-propranolol hydrochloride (I.C.I.) and tetrodotoxin (Sigma) were dissolved in an isosmotic modified Krebs solution lacking phosphate and bicarbonate. Sodium cyanide (NaCN; BDH) and iodoacetic acid (IAA; Sigma) were dissolved in Krebs solution gassed with 95% O₂/5% CO₂; solutions of NaCN and IAA were prepared immediately before use. The nitric oxide synthesis inhibitors N^G-nitro-L-arginine (L-NOARG; Sigma) and N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma) were added to the bathing Krebs solution.

Statistical analysis

Data are expressed as mean ± s.e. mean. Differences between the means were determined by Student's paired *t* test; *P* values of 0.05 or less were considered significant.

Results

Effects of 'anoxia' on cholinergic neuro-effector transmission

Electrical field stimulation (0.1 Hz) of myenteric plexus-longitudinal muscle preparations of guinea-pig small intestine caused reproducible contractions (2.37 ± 0.14 g, $n = 12$) which were inhibited, 97–100%, by the addition of the muscarinic cholinergic antagonists hyoscine or atropine (1 μM; $n = 6$). In 7 preparations, NaCN (0.1–3.0 mM) caused a concentration-dependent, readily-reversible, inhibition of the electrically-evoked contractions (Figure 1a); this depression was not preceded by a period of potentiation of the twitch-like contractions. Although the IC₅₀ value was estimated as 0.3 mM (range 0.19–0.41) the effective concentrations are likely to have been considerably less than these because continuous bubbling (with 95% O₂/5% CO₂) of such dilute solutions of NaCN in the organ bath blows off HCN, a product of hydrolysis (Schmitt & Nicoll, 1933). A striking feature of the inhibition due to NaCN was the rapidity with which it produced its effect, often within 10–20 s and maximal usually within 60 s despite the continuous presence of oxygen. The maximum inhibition produced by NaCN was 90%; the NaCN-insensitive contractions were completely antagonized by atropine (1 μM, $n = 3$). When preparations ($n = 4$) were later gassed with 95% N₂/5% CO₂ instead of 95% O₂/5% CO₂, a reversible depressant effect of comparably rapid onset was observed but it was usually, though not always, preceded by a brief period of potentiation (up to 20%) of the responses to supramaximal electrical stimuli; the reversal was equally complete and swift (Figure 1b). During continuous exposure to 1 mM NaCN for 75 min ($n = 3$), the inhibition reached a maximum (90%) in about 100 s and slowly waned to only 50% after 12 min,

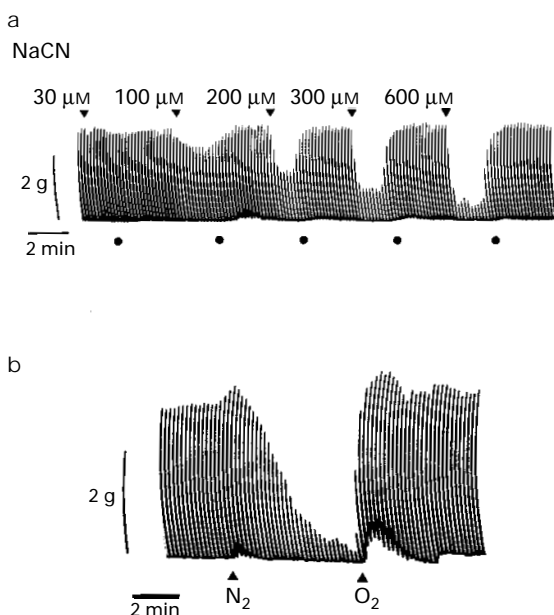


Figure 1 Effects of 'anoxia' on cholinergic, isometric contractions of a guinea-pig myenteric plexus-longitudinal muscle preparation evoked by supramaximal electrical field stimulation at 0.1 Hz. (a) Concentration-related inhibitory effect of NaCN. NaCN was added at ▼ and washed out at ●. (b) Effects of N₂ substitution for O₂ (see Methods). Note rapidity of recovery of the twitch-like contraction on re-introduction of oxygen. This is a typical example of an experiment which was repeated four times.

possibly due to the declining effective concentration of NaCN. Cessation of exposure to NaCN led to complete recovery of the twitch within 2 min, 90% recovery being achieved within 1 min. Repeated exposures (for up to 10 min during periods of 60–90 min) to either simple anoxia (see Methods; $n=4$) or to NaCN (0.1–1 mM; $n=3$) did not lead to a change in the magnitude or character of the inhibition, which remained reversible. No inhibition of electrically-evoked contractions was observed acutely or with prolonged exposures (1–2 h) to oxygen-deficient medium (5–15% O₂/5%CO₂; $n=7$).

Effects of 'anoxia' on non-cholinergic neuro-effector transmission

Since the electrically-induced, cholinergic contractions could not be completely suppressed by NaCN, similar experiments were performed in the presence of hyoscine or atropine (1 μ M) to test the susceptibility of non-cholinergic (effectively also non-adrenergic) contractions to this inhibitor of oxidative phosphorylation. The magnitude of the non-cholinergic contractions was 70–120% that of the cholinergic contractions at 0.1 Hz ($n=3$). First, it was established that isometric contractions induced by short trains of supramaximal pulses (10 or 25 Hz for 2 s at 30 s intervals) remained virtually constant in amplitude over >90 min. Such contractions were inhibited by simple anoxia or NaCN (1 mM) within 90–120 s. The max-

imum depression was 80% at 10 Hz and 90% at 25 Hz ($n=4$); recovery was incomplete and was much slower than with cholinergic contractions, 50% recovery taking about 20 min.

Effects of 'anoxia' on smooth muscle contractions induced by exogenous agents

In the absence of electrical stimulation, the effects of simple anoxia or NaCN-treatment (or both) on smooth muscle contractions induced by acetylcholine (ACh), carbachol, histamine and potassium-rich solutions were investigated in 22 preparations. As previously shown (Schmitt & Nicoll, 1933; West *et al.*, 1951; Paton, 1961), it was observed that, under normal conditions, contractions (isometric, quasi-auxotonic or isotonic) induced by each agonist were nearly always biphasic, a second, slow, large contraction following the initial fast response (first or 'spike' phase; <20 s to peak) to the agonist, which was always applied for at least 1 min; in some instances, tension was maintained for the duration of the exposure to the agonist but at a value less than the initial level. The time taken to reach this plateau depended on the concentration of agonist applied but was usually complete within 2 min after the commencement of exposure to the agonist, except in the case of higher concentrations (30–40 mM) of KCl, which required 7–8 min to reach a maximum. These second ('tonic') phase contractions induced by carbachol or histamine were not affected by tetrodotoxin (1 μ M) at a time when electrically-induced contractions of the same preparations were <2% of control values; spike contractions also were unaffected by tetrodotoxin. Alterations were made to the availability of calcium in the bathing solution to examine the contribution of extracellular calcium ions to the tonic phase. When calcium was omitted from the Krebs solution, the tonic phase contractions induced by ACh, histamine or KCl were greatly reduced ($\geq 50\%$). Furthermore, in the case of histamine, they were absent when 0.5 mM EGTA was added to the calcium-free Krebs solution ($n=3$). Under anoxic conditions, the peak tension generated by ACh (control, 3.8 ± 0.6 g; $n=5$) was not depressed (NaCN 1 mM; 3.5 ± 0.4 g) but the tonic phase of the contractile response was much more markedly affected

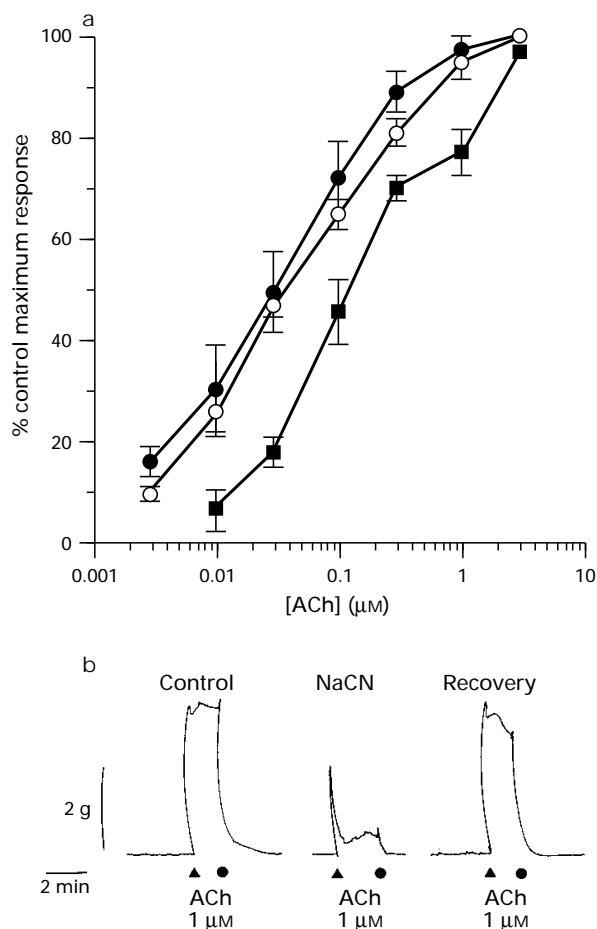


Figure 2 Effects of NaCN on isometric contractions of guinea-pig myenteric plexus-longitudinal muscle preparations. (a) Effect of NaCN on concentration-response curve to exogenously administered acetylcholine (ACh). (●) Control; (■) in the presence of 1 mM NaCN; (○) recovery. Each point is the mean \pm s.e.mean (vertical lines) of seven observations. (b) Change in shape of response to ACh in the presence of NaCN (1 mM). Note the reversible depression of the second (tonic) phase of contraction. This is a typical example of an experiment which was repeated six times. ACh was added at \blacktriangle and washed out at \bullet .

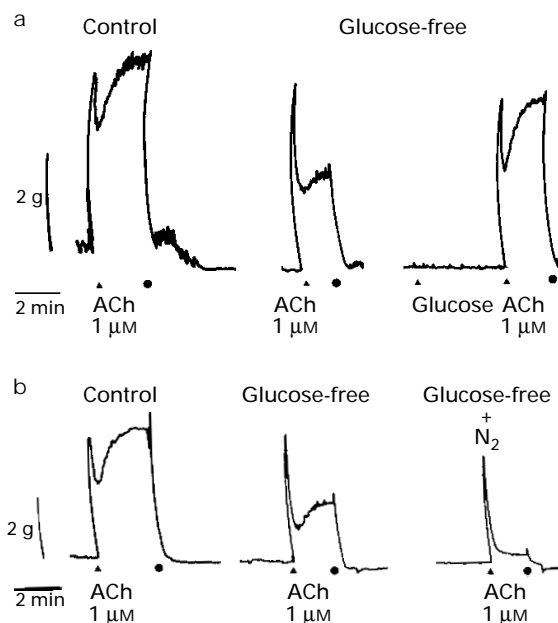


Figure 3 Effects of glucose-free conditions and anoxia on ACh-induced isometric contractions of a guinea-pig myenteric plexus-longitudinal muscle preparation. (a) Effects of glucose deprivation on ACh-induced contractions and the rapid recovery on re-introduction of glucose. (b) Effects of glucose and oxygen deprivation on ACh-induced contractions. These are typical examples of experiments repeated at least twice. ACh was added at \blacktriangle and washed out at \bullet .

than the spike phase (Figure 2). In the presence of 1 mM NaCN the EC_{50} value for ACh was increased from $0.04 \mu\text{M}$ ($pEC_{50} = 7.39 \pm 0.04$) to $0.14 \mu\text{M}$ ($pEC_{50} = 6.90 \pm 0.09$; $P < 0.01$) and since the spike response to ACh was usually the bigger of the two contractile phases there was little depression in the amplitude of spike contractions matching the responses to supramaximal electrical stimulation (Figure 2a). There was, however, a failure either to increase tension during the tonic phase of the response or to maintain it, despite continuing

exposure to the agonist (Figure 2b). In the presence of 1 mM NaCN, the peak tension of the tonic phase contractions induced by ACh at concentrations giving 70% maximal contractions was only 0.48 ± 0.36 g, compared to 3.52 ± 0.2 g ($n = 3$; $P < 0.05$), under control conditions. Similar depressions were observed with glucose-free solutions (Figure 3a), except that the onset of the inhibition was very much slower and was dependent on the contractile activity of the tissue; several near-maximum contractile responses were required before re-

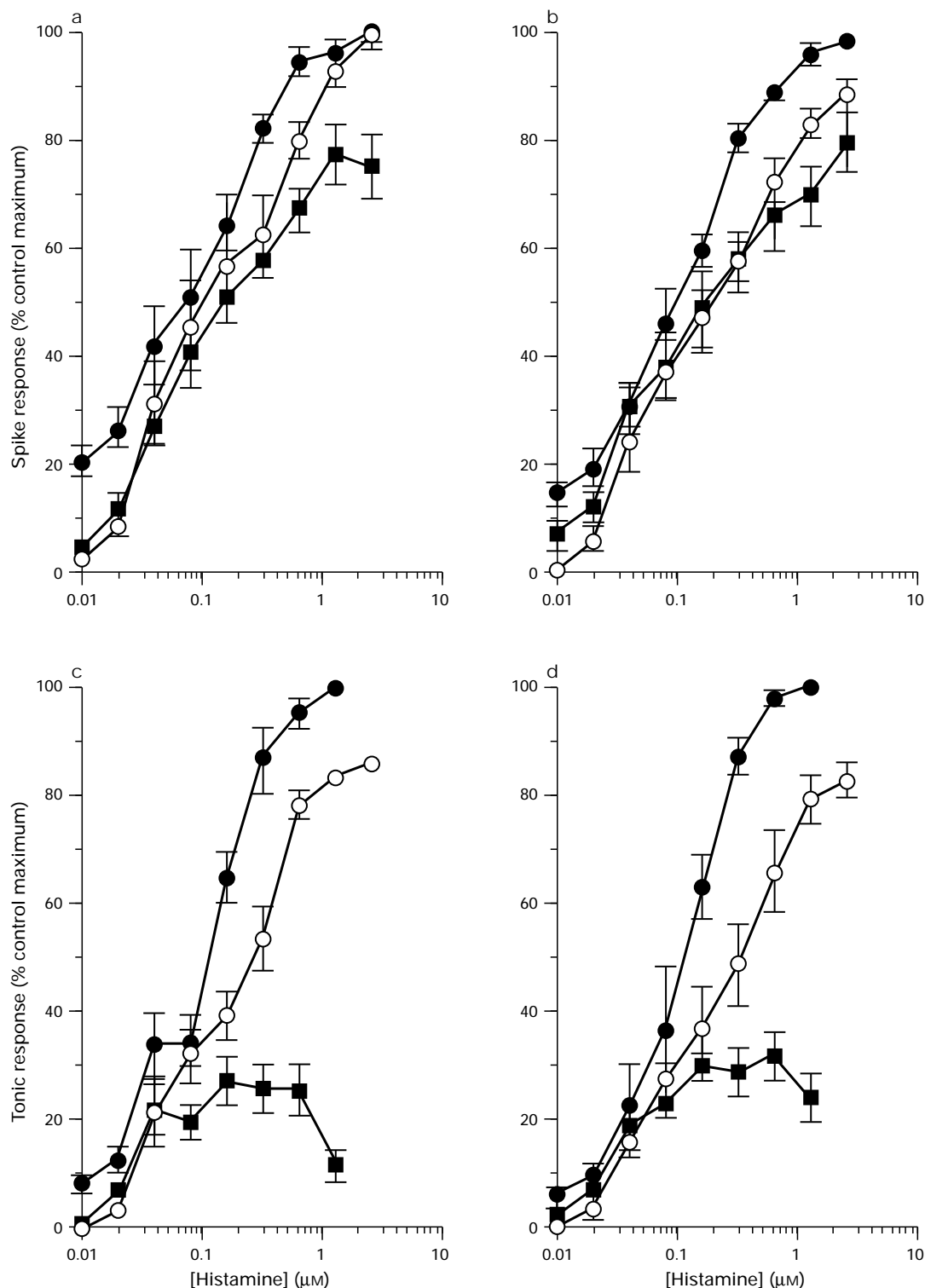


Figure 4 Effect of anoxia on the two phases (a and b, spike response; c and d tonic response) of the histamine-induced contractor responses of myenteric plexus-longitudinal muscle preparations of guinea-pig ileum in the absence (a and c) and presence (b and d) of the nitric oxide synthase inhibitor N^G -nitro-L-arginine (L-NOARG). (●) Control; (■) simple anoxia; (○) recovery. Each point is the mean \pm s.e. mean (vertical lines) of five observations.

producible inhibitions of the tonic responses were obtained. Furthermore, the tonic responses were further depressed when deprived of both oxygen and glucose (Figure 3b). On re-introducing either glucose or oxygen and on discontinuing exposure to NaCN recovery was complete and rapid (Figures 2b and 3).

With respect to histamine ($n=5$) and KCl (10 and 30 mM; $n=3$), the spike phase, as well as the tonic component, of isometric contractions were reversibly depressed by NaCN (1 mM) or simple anoxia (Figure 4a and c). The maximal tonic component of the histamine-induced contractions was decreased by 69% from 2.50 ± 0.23 g to 0.78 ± 0.12 g ($P < 0.05$), whereas the maximal spike phase was reduced by only 23% from 3.86 ± 0.36 g to 2.98 ± 0.40 g ($P < 0.05$).

Since isometric contractions are metabolically demanding, it was anticipated that anoxia might have less effect on responses recorded under isotonic or auxotonic conditions. In a separate series of experiments, the peak amplitudes of spike contractions with ACh ($n=4$) and carbachol ($n=3$) as agonists were unchanged under anoxic conditions, whereas the tonic phase of isometric, but neither isotonic nor auxotonic, contractions was markedly depressed ($n=6$). Thus, with respect to the tonic phase, the effect of anoxia on smooth muscle made to contract isometrically differed from that under other conditions, findings that were confirmed in adjacent preparations ($n=6$) from the same animal made to contract isometrically and isotonically. In addition, the post-junctional inhibitory effect of NaCN (1 mM) on histamine-evoked contractions was only apparent when the tissue was made to contract isometrically (Figure 5). Under isometric conditions, NaCN increased the EC_{50} for histamine from $0.11 \mu\text{M}$ ($pEC_{50} = 6.98 \pm 0.06$) to $0.65 \mu\text{M}$ ($pEC_{50} = 6.19 \pm 0.06$; $P < 0.01$) whereas it was unchanged under isotonic conditions.

Effects of 'hypoglycaemia' on cholinergic neuro-effector transmission and smooth muscle contractions induced by exogenous agonists

Exposure of myenteric plexus-longitudinal muscle preparations to glucose-free Krebs solution resulted in a gradual reduction in the amplitude of electrically-stimulated contractions over 30–60 min depending on the stimulus frequency. At 0.1 Hz the contractions were undiminished after 30 min in glucose-free Krebs solution; even after 60 min continuous stimulation the twitches were still $>90\%$ of their original size. After 30 min stimulation at 0.2 and 0.4 Hz the contractions were reduced by 50% and 80%, respectively; continuing stimulation for 60 min reduced the contractions by no more than 80%. In glucose-free Krebs solution, the electrically-evoked contractions were abolished when the preparations were either bubbled with 95% $N_2/5\%$ CO_2 or NaCN (1 mM) was added. Contractions recovered within 1–2 min on readmission of glucose ($n=3$). Since the principal deleterious effects of ischaemia are attributable to both hypoxia and hypoglycaemia, the effects of an irreversible inhibitor of glycolysis, iodoacetic acid (IAA; 0.03–0.5 mM) were examined alone and in combination with NaCN. The threshold concentration for a detectable effect of IAA alone on responses to electrical field stimulation at 0.1 Hz was 0.1 mM ($n=7$ preparations); however, even at this concentration, the inhibition was complete and irreversible (Figure 6a). Over the range 0.01–20 Hz, the higher the concentration or the stimulus frequency, the more rapid was the decline in evoked tension in each of 4 preparations tested. However, at low frequencies of stimulation (0.01–0.2 Hz) but not at frequencies of ≥ 0.4 Hz, the twitches induced by supramaximal electrical field stimulation were initially enhanced, by up to 40% (maximal at 0.2 Hz; Figures 6a and 7). Three principal mechanisms by which such a potentiation could occur were investigated. The possible mechanisms of disinhibition of α -adrenoceptor-mediated reduction in ACh release, or β -adrenoceptor-mediated depression of the sensitivity of smooth muscle to ACh, were excluded by the finding that, in three experiments, the IAA-induced potentiation oc-

curred in the simultaneous presence of phentolamine (0.1–10 μM) and (\pm)-propranolol (0.5 μM), in concentrations demonstrated (by the method of Kosterlitz *et al.*, 1970) in these preparations to have produced a profound block of α - and β -adrenoceptors, respectively. When neostigmine (20–60 nM) was used to produce a potentiation of the twitch at 0.1 Hz, there was no further potentiation in the presence of IAA (0.1–0.3 mM). On the other hand, at the time of maximal potentiation of the twitch by IAA, the peak amplitude of the response to ACh was either unchanged or less potentiated than the twitch response ($n=3$). Furthermore, in other preparations, when IAA had reduced the twitch by 10%, there was no depression of the first phase of contractions induced by ACh or its stable analogue, carbachol, only a reduction, in the secondary, plateau phase. Thus, even if IAA may have been acting non-selectively to inhibit cholinesterase activity, IAA-induced potentiation of the twitch appeared to have been due to a prejunctional facilitation of cholinergic transmission and not mainly due to an increased post-junctional sensitivity to ACh. As expected, continued exposure to IAA resulted in an inhibition of both phases that was irreversible (by washing). A non-specific post-junctional facilitation seemed to be ruled out by the consistent observation that isometric contractions

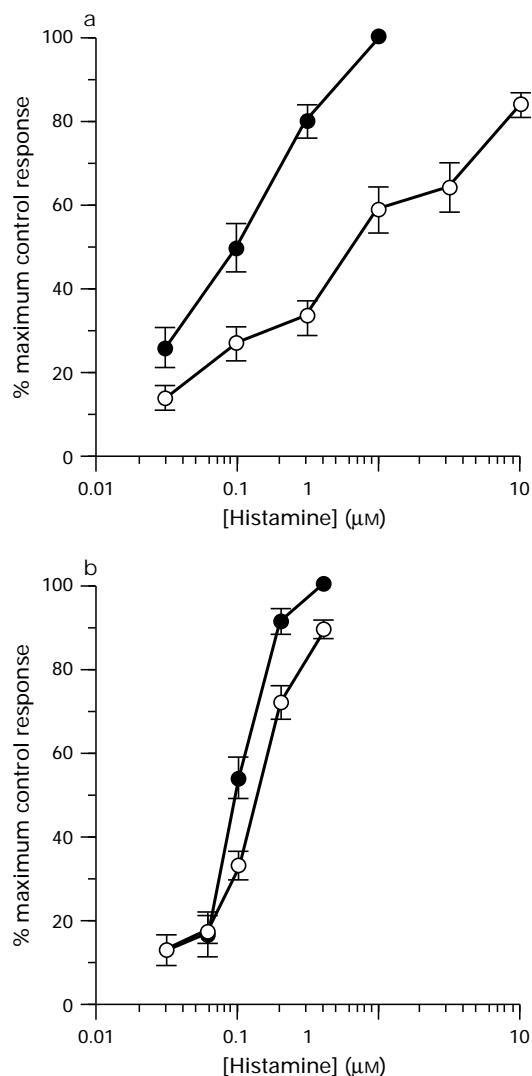


Figure 5 Comparison of the effects of NaCN on isometric and isotonic contractions of the small intestine. A post-junctional inhibitory action of NaCN was apparent when the smooth muscle was made to contract (a) isometrically but not (b) isotonic in response to histamine. (●) Control; (○) NaCN (1 mM). Each point is the mean \pm s.e. mean (vertical lines) of three observations.

evoked by histamine were not potentiated by IAA. Two additional effects of IAA seem noteworthy: first, histamine-induced contractions declined faster than those evoked by carbachol ($n=4$). Secondly, in the presence of IAA (0.1 mM) in six preparations, electrically-induced contractions (0.1 Hz) were still large (~ 2 g; 60% control) at a time when histamine no longer caused a contraction.

Effects of 'ischaemia' on cholinergic neuro-effector transmission and drug-induced smooth muscle contractions

In 6 preparations, subthreshold concentrations of IAA (0.03–0.06 mM) combined with previously just threshold concentrations of NaCN (0.1–0.2 mM) were found to induce a profound ($>70\%$ inhibition) block of neuro-effector transmission (Figure 6b) that was irreversible not only under anaerobic but also in aerobic conditions. When the electrically-induced contractions were depressed by about 50%, there was a marked reduction in the maximum contraction that could be elicited by either carbachol or histamine; complete blockade of neuro-effector transmission was associated with the virtual absence of drug-induced smooth muscle contractions.

Role of NO in neurotransmission deficits caused by 'ischaemia'

Since cerebral ischaemia is associated with the production of nitric oxide which is neurotoxic, the possibility was considered that nitric oxide might be involved in producing deficits in neuro-effector transmission after ischaemic insults. To investigate this, preparations were exposed to inhibitors of nitric oxide synthase for 20 min before being made hypoxic. Simple anoxic conditions caused a significant depression of both phases of the maximum isometric contractions induced by histamine (0.01–10 μM ; Figure 4a and c). L-NOARG (0.1–0.3 mM), which had no effect on histamine-induced contractions under normoxic conditions, failed to prevent the depression of contractions of either the first or the second phase elicited in the absence of oxygen ($n=5$; Figure 4b and d). In another experiment, L-NAME (0.1 mM) was also ineffective in blocking the depressions in histamine-induced contractions during simple anoxia. The depressant effects of NaCN on electrically-evoked contractions were similarly unaffected by L-NOARG (0.1 mM).

Discussion

Since the first descriptions of the effects of anoxia on the contractions of intestinal smooth muscle many years ago, little attention has been paid to the possibility of important effects of hypoxia and hypoglycaemia on the function of enteric neurones, even though these neurones, particularly cholinergic neurones, are intimately involved in the regulation of intestinal motility and secretion. There is evidence that (a) anoxia can preferentially depress cholinergic neural pathways involved in intestinal reflexes (Schaumann *et al.*, 1953; Kosterlitz & Robinson, 1959) and (b) ACh biosynthesis in neurones is acutely dependent on oxidative phosphorylation (Gibson & Duffy, 1981). Furthermore, Szurszewski & Steggerda (1968) demonstrated that 4 h of hypoxia decreases the contractile frequency of intact jejunal segments in anaesthetized dogs presumed to be by an action on the myenteric plexus. Other pointers to there being potentially important deleterious effects of severe hypoxia or ischaemia on enteric neurones are provided by studies of the physiology and pharmacology of ACh release. Glucose deprivation or anoxia decreased the spontaneous release of ACh from the guinea-pig myenteric plexus prepara-

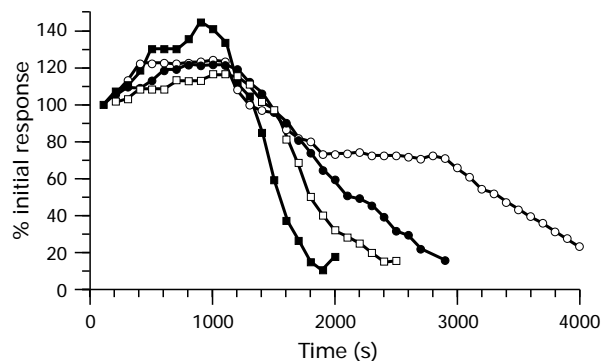


Figure 7 Typical example of the effects of iodoacetic acid (IAA; 0.1 mM) on isometric contractions of guinea-pig myenteric plexus-longitudinal muscle preparations evoked by increasing frequency of electrical stimulation; (○) 0.01 Hz; (●) 0.05 Hz; (□) 0.1 Hz; (■) 0.2 Hz.

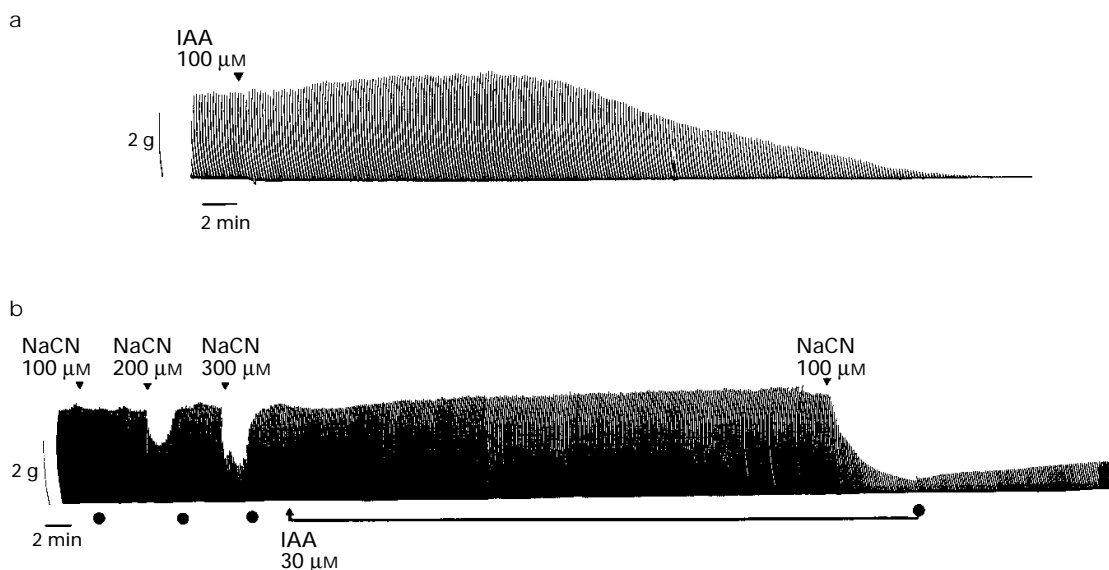


Figure 6 Effects of iodoacetic acid on cholinergic contractions of myenteric plexus-longitudinal muscle preparations of guinea-pig ileum evoked by field stimulation at 0.1 Hz. (a) Iodoacetic acid (IAA; 100 μM) alone. Note the initial potentiation of the twitch (see also Figure 7 and text) in response to IAA, followed by a complete inhibition of contractions. (b) Interactions between threshold concentrations of NaCN and IAA. NaCN was added at \blacktriangledown and washed out at \bullet ; IAA was in contact with the tissue for the duration of the bar. These are typical examples of experiments repeated at least three times.

tion, whereas combined treatment caused a seven fold increase in release as a result of elevated intracellular calcium (Larson & Martins, 1981). The electrically-evoked output per pulse of acetylcholine (ACh) from enteric neurones is maximal at low frequencies (<0.1 Hz), can correspond to about 80% of the total ACh content of the tissue and shows heavily time-dependent recovery (Kosterlitz & Waterfield, 1970), indicating that significant biosynthesis of ACh takes place in response to each electrical field stimulus. In addition, cholinergic nerve terminals are endowed with many different kinds of pharmacological receptor resulting in release of ACh being most susceptible to modulation at low frequencies (<1 Hz; Paton, 1957; Cowie *et al.*, 1968; Kilbinger, 1977).

The present study was prompted by the mounting evidence for the role of intermittent focal ischaemia of the gut in Crohn's disease (Wakefield *et al.*, 1989; Shepherd, 1991; Hudson *et al.*, 1992; 1993; Osborne *et al.*, 1993; Pounder, 1994) and by the availability of a new pharmacological model of ischaemia for electrophysiological investigations of neurones of the central nervous system (Reiner *et al.*, 1990). Our intention was to use this model to extend previous data on the effects of anoxia on neural responses in intestinal preparations and to explore the neural consequences of simulated ischaemia, rather than to examine its applicability as a model for IBD, in general, and Crohn's disease, in particular.

Four important points emerge from our studies. First, as far as mechanical responses of the smooth muscle are concerned, the depressant effects of anoxia (produced either by the omission of oxygen or treatment of the preparation with NaCN) on cholinergic neuro-effector transmission were rapid in onset and were readily reversible. Presumably the precipitous fall in the force of contraction is the consequence of an inhibition of aerobic metabolism, due to loss of available or utilisable oxygen (Schmitt & Nicoll, 1933). As described previously (Job *et al.*, 1955; Day & Vane, 1963), contractions were preceded by a phase of facilitation, except when NaCN was used. Secondly, unlike cardiac muscle (Allen *et al.*, 1989), repeated anoxic insults did not lead to irreversible damage, as assessed by either neuro-effector transmission or smooth muscle contraction *per se*. Thirdly, a consistent finding was of a greater inhibition of the twitch-like contractions induced by electrical field stimulation than of the peak amplitude of contraction due to exogenously administered agonists, suggesting that neuronal events associated with neuro-effector transmission were acutely dependent on oxygen availability and were relatively more susceptible to oxygen deprivation than post-junctional events. Hayashi *et al.*, (1986) came to a similar conclusion for the human small intestine under anoxic conditions (95% N₂/5% CO₂). They found that the contractor response elicited by 10 mM KCl was significantly inhibited, though, contrary to our observations, contractions elicited by 30 mM KCl or by exogenously administered acetylcholine were unaffected. They also found that contractions evoked by cholinergic nerve stimulation (10 s trains) at 5 Hz were not affected under these conditions, whereas those elicited at 20 Hz, which were apparently also cholinergic since they were completely blocked by atropine (1 μ M), were significantly depressed, albeit only slightly (approximately 87% of control values). In this investigation, electrical stimulation of the myenteric plexus preparation with trains of 10 or 25 Hz in the presence of atropine, produced contractions which were inhibited by simple anoxia or NaCN. The recovery of these non-cholinergic neuro-effector contractions from anoxic insult was incomplete and slower than cholinergic contractions, possibly indicating that non-cholinergic excitatory transmission is more susceptible to anoxia than cholinergic transmission.

In anoxic conditions, the failure of guinea-pig intestinal smooth muscle to maintain the more energy-dependent isometric contractions, elicited by acetylcholine, carbachol, histamine or raised potassium concentrations, was in sharp contrast to the maintained contractions under isotonic conditions, in which oxygen requirements for the sustained shortening are thought to be less than in the absence of

drug-induced contractions (see Schmitt & Nicoll, 1933). Thus, our results confirm and extend previously published observations.

Fourthly, conditions mimicking hypoglycaemia led to the gradual depression of responsiveness of the smooth muscle to both electrical field stimulation and to the above excitatory drugs. These observations suggest that, although the rate of onset of depression of contraction of the smooth muscle was greatly accelerated when higher stimulus rates were used in the absence of glucose or during exposure to IAA (>0.1 mM), the function of neurones and smooth muscle, except when generating a high tension for a prolonged period, is probably less heavily dependent on glycolysis, than on oxidative phosphorylation.

The mechanisms responsible for neuronal death following ischaemic brain damage are associated mainly with the actions of glutamate on N-methyl-D-aspartate (NMDA) receptors (Rothman & Olney, 1986). Although NMDA receptors are present in the guinea-pig myenteric plexus (Luzzi *et al.*, 1988) they are not thought to be of importance in this tissue. In addition, nitric oxide synthesis is an important contributor to neuronal damage in cerebral ischaemia (Dawson *et al.*, 1991) whereas, the deficits in neuro-effector transmission produced by ischaemia in the gut do not appear to involve nitric oxide production since inhibitors of nitric oxide synthesis provided no protection against ischaemic insults.

The initial potentiation of the electrically-induced twitch in the presence of IAA was not observed in the absence of glucose and was seen only with very low frequencies of stimulation (0.2 Hz or less), possibly because the rate of onset of depression of contraction of the smooth muscle is greatly accelerated when the muscle is made to generate a greater tension for longer periods during higher stimulus rates in the absence of glucose or during exposure to IAA (>0.1 mM). This potentiation was presumed, at first, to be prejunctional in origin but the mechanism was not entirely clear. IAA is known to bind covalently to the sulphhydryl groups of the enzyme, glyceraldehyde-3-phosphate dehydrogenase, which is responsible for converting glyceraldehyde-3-phosphate to 1, 3-diphosphoglycerate in the glycolytic pathway; such an inhibition may not be selective. The potentiation was not attributable to blockade of α -adrenoceptor-mediated inhibition of the twitch. The possibilities have not been excluded that either acetylcholinesterase activity is inhibited or that the release of ACh is increased as was observed with simulated ischaemia on spontaneous ACh release in this tissue (Larson & Martins, 1981). Of relevance are the observations and conclusions of Eisner *et al.* (1989), who found that exposure of rat ventricular myocytes to IAA (0.5 mM) or 2-deoxyglucose, but not to glucose-free solutions, resulted in an increase in the amplitude of contraction of the fibre over a period of a few minutes before it was effectively abolished, before the development of a contracture. As hypothesized by these authors, the profound blockade of glycolysis through the inhibition of the enzyme glucose-6-phosphate transmutase may have caused a build up of sugar phosphates within the cell and effectively trapped all the cellular phosphate (Pirolo & Allen, 1986), leading first, to an increase in calcium ion sensitivity of contractile apparatus (Kentish, 1986), hence an increase in contraction, then to cell death. For the following reasons, our results suggest that such a sequence of biochemical events may also have occurred in cholinergic nerve terminals, which are preferentially activated by low-frequency electrical stimulation (Paton, 1955; 1957; 1961; Kosterlitz *et al.*, 1970). First, IAA was capable of potentiating the electrically-induced twitch-like contractions, even when these were already potentiated through blockade of prejunctional α_2 -adrenoceptors, though not when they were potentiated to a similar degree by the acetylcholinesterase inhibitor, neostigmine. Secondly, IAA potentiation of electrically-induced contractions occurred at a time when the peak amplitude of the response (a) to ACh was either unchanged or less potentiated than the twitch response at that time and (b) to histamine, acting directly on smooth muscle cells (Day & Vane,

1963; see also Kosterlitz & Lees, 1964), showed no potentiation.

It will be apparent that the pharmacological model proposed by Reiner *et al.* (1990) was not entirely appropriate for our purposes. In hippocampal neurones, ischaemia was mimicked by NaCN and IAA given together to block both oxidative phosphorylation and glycolysis, respectively; in that preparation, the combination of drugs was much more effective than either agent alone. In our preparation, IAA given alone and in suprathreshold concentrations, completely inhibited contractions of the smooth muscle. Thus, it was not possible to apply the model in its original form to neuro-effector transmission.

It is clear, however, from the present results that neuro-effector transmission is particularly vulnerable to hypoxia.

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