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# ENTERIC REFLEXES AND NITRIC OXIDE IN THE FISH INTESTINE

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## Summary

**The aim of the present study was to elucidate the possible regulation of peristalsis in the intestine of the Atlantic cod *Gadus morhua*. For this purpose, the mid intestine was dissected out and placed in a partitioned bath. Balloon distension (0.1–0.4 ml) and intramural field stimulation (8 Hz, 10 V) were carried out and the responses of the circular muscle were recorded 1.5 cm orally and anally to the stimulus using force transducers. The preparations developed spontaneous contractions propagating in the anal direction with a frequency of about one contraction per 2 min. Distension of the muscle wall with a balloon did not evoke any recordable peristaltic reflexes. Intramural stimulation caused a contraction oral to the stimulation and a relaxation anal to the stimulation in most cases. Tetrodotoxin abolished the responses to electrical stimulation in both directions. Atropine reduced and methysergide abolished the oral contractions caused by electrical stimulation. Administration of the nitric oxide synthesis inhibitor L-$N^G$-nitro-arginine methyl ester (L-NAME) abolished the anal relaxation caused by electrical stimulation and augmented the oral contractions. The results indicate the presence in teleost fish intestine of an ascending excitatory peristaltic reflex which involves a cholinergic–serotonergic pathway and a descending inhibitory reflex involving a nitrergic pathway. These observations suggest a high degree of conservation of peristaltic mechanisms during vertebrate evolution.**

Key words: intestine, gut motility, enteric reflex, nitric oxide, L-NAME, teleost fish, Atlantic cod, *Gadus morhua.*

## Introduction

Peristalsis can be elicited in several mammals by distension of an isolated segment of the gut (Costa and Furness, 1976; Tonini and Costa, 1990; Grider and Jin, 1994). The peristaltic reflexes have, therefore, been considered to be entirely enteric (e.g. Crema, 1970; Furness and Costa, 1987). The reflexes controlling the circular muscle layer can be divided into an orally directed (ascending) excitatory pathway and an anally directed (descending) inhibitory pathway. Both pathways consist of sensory neurones, one or several interneurones, and motor neurones.

Orally projecting cholinergic and non-cholinergic neurones releasing substance P or other tachykinins participate in the ascending excitatory pathway and often constitute the final motor neurone affecting the muscle cell (Barthó and Holzer, 1985; Holzer, 1989; Tonini and Costa, 1990; Allescher *et al.* 1992; Holzer and Maggi, 1993; Grider and Jin, 1994). In the descending inhibitory reflex, anally projecting neurones, believed to have ATP, vasoactive intestinal peptide (VIP) and nitric oxide as transmitters, are involved (Burnstock, 1972; Grider and Jin, 1994). Physiological studies are supported by immunohistochemistry and neuronal tracing of the projections of acetylcholine-, substance-P- and VIP-containing neurones to the circular muscle in the guinea pig small intestine (Brookes *et al.* 1991).

Although the fish gut has been studied extensively both *in vivo* and *in vitro*, and the effects of several neurotransmitters have been established (elasmobranchs, Andrews and Young, 1988; teleosts, Jensen and Holmgren, 1985; Kitazawa *et al.* 1990; Karila *et al.* 1993; for a recent review, see Jensen and Holmgren, 1994), little information has been gathered about the participation of these neurotransmitters in the reflex pathways (elasmobranchs, Andrews and Young, 1993; teleosts, Grove and Holmgren, 1992*a*,*b*) and, to our knowledge, no analysis of the peristaltic reflex has been carried out in any non-mammalian species. The aims of the present study were therefore to elucidate the mechanisms underlying the peristaltic reflexes in the teleost fish intestine and to relate the results to previous findings from an evolutionary point of view. For this purpose, we used a partitioned bath previously described by Tonini and Costa (1990). Using this apparatus, the stimulation site and the recording sites can be separated and drugs can be administered independently to the different compartments.

## Materials and methods

Atlantic cod (*Gadus morhua*) of either sex weighing between 250 and 1200 g were supplied by local fishermen on

the Swedish west coast. A total of 122 fish were used. They were kept in aerated, circulating sea water at 10 °C and were not fed in captivity. The animals were killed by a blow to the head and the mid intestine (8–12 cm long) was dissected out and washed in cod Ringer's solution containing (in mmol $l^{-1}$): NaCl, 150.1; KCl, 5.2; $MgSO_4$, 1.8; $CaCl_2$, 1.9; $NaH_2PO_4$, 1.9; $NaHCO_3$, 7.0; and glucose, 5.6 (Karila *et al.* 1993). The gut segment was pulled through holes in the two latex diaphragms forming the walls between the three compartments of a Perspex organ bath. The dorsal side of the intestine was attached with heart clips to Grass FT03 transducers, one in the oral and one in the anal compartment, for the recording of circular muscle tension on a Grass Polygraph model 7 or 79. The opposite side of the intestine was fixed with surgical thread to a stainless-steel rod in the bottom of the organ bath. Along with the Grass polygraph recordings, the tension signals were sampled at 1 Hz and recorded as means every 5 s on a personal computer running AD/DATA (Professor P. Thorén, Department of Physiology, Karolinska Institute). The intestine was cannulated to allow flushing of the lumen. In experiments measuring the spontaneous rhythmic activity (amplitude, frequency and speed of propagation of the contractions), transducers were placed 1.5 cm apart in the same compartment. In experiments using distension as a stimulus, a latex balloon was placed in the lumen of the intestine about 1.5 cm from the recording site. Platinum wire electrodes were placed deep enough within the muscle wall to reach the myenteric plexus in experiments using intramural electrical stimulation. The electrodes were placed in the intermediate compartment in between, and approximately 1.5 cm from, the recording sites. In preliminary experiments, voltage–response and frequency–response curves were constructed and in the following experiments the chosen stimulations were submaximal in voltage and maximal in frequency. The bath was filled with cod Ringer's solution (10 °C) bubbled continuously with a mixture of air/$CO_2$ (99.7 %/0.3 %) to maintain the pH at 7.8 and the preparation was mounted with an initial tension of 5 mN. The Ringer's solution in the compartments and the lumen was replaced every 45 min. The preparations were left for 1 h before the experiments. During this time, the preparations relaxed and adopted a baseline tonus of about 2 mN.

### *Drugs*

The following drugs were used: atropine sulphate ($10^{-6}$ mol $l^{-1}$; Sigma Chemical Company, USA), methysergide ($10^{-6}$ mol $l^{-1}$; Sandoz), L-$N^G$-nitro-arginine methyl ester (L-NAME; $10^{-3}$ mol $l^{-1}$; Sigma), sodium nitroprusside (Na-NP; $0.5\times10^{-4}$ mol $l^{-1}$; Sigma) and tetrodotoxin ($10^{-6}$ mol $l^{-1}$; Sigma).

The drugs were dissolved according to the suppliers' recommendations and diluted to the final concentrations with cod Ringer's solution. The drugs (except L-NAME) were used at concentrations previously found to block selectively the action of the respective agonist in the cod intestine (Jensen and Holmgren, 1985).

### *Statistics*

The data are presented as means ± S.E.M. Wilcoxon's signed-ranks test for paired observations was used to evaluate the statistical significance. Differences where $P\leqslant0.05$ were regarded as statistically significant. In experiments where the effect of the addition of antagonist was tested, the mean tension of the spontaneous activity before the addition of antagonist over a period of 190 s was compared with the mean tension over a 190 s period after the full effect of the added antagonist had been reached. To measure the effect of electrical stimulation, the difference in mean tension over a pre-stimulation period (190 s) and the mean tension over a fixed period during (oral response) or immediately after (anal response) stimulation (30 s) was calculated. The sampling periods for the recordings were chosen from where the response was most apparent in preliminary experiments. The average of two or three repetitive stimulations was used in every experiment. For statistical evaluation, the response to electrical stimulation was compared before and after the addition of antagonist.

## Results

Most preparations developed spontaneous rhythmic activity during the initial recovery period (Figs 1A, 2). The frequency of the contractions in preparations left undisturbed for over 1 h was 0.52±0.05 $min^{-1}$ ($N$=8). A comparison of recordings from transducers 1.5 cm apart showed that the contractions moved in the anal direction at 3.5±1.0 cm $min^{-1}$ ($N$=5). The addition of tetrodotoxin, atropine or the serotonin (5-HT) antagonist methysergide (all $10^{-6}$ mol $l^{-1}$) to all compartments caused, in most cases, a decrease in the mean tension of the preparation by reducing or abolishing the spontaneous contractions (Figs 1C, 2A,B, 3). Administration of the nitric oxide synthesis inhibitor L-NAME ($10^{-3}$ mol $l^{-1}$) increased the basal tone and/or the frequency of the

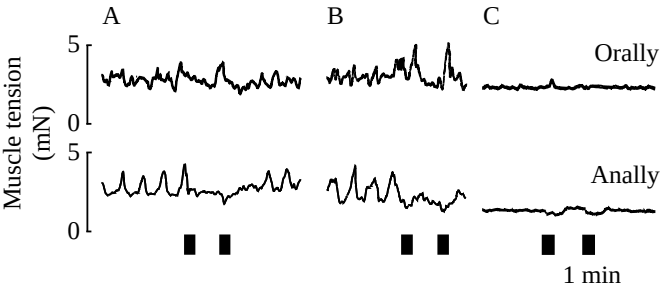


Fig. 1. Original tracings of *in vitro* circular smooth muscle activity in the intestine of the Atlantic cod *Gadus morhua*. (A) Oral reflex contraction and anal reflex relaxation, following electrical stimulation (10 V, 1.0 ms, 8 Hz, 1 min) in the intermediate compartment at the bars. (B) Methysergide ($10^{-6}$ mol $l^{-1}$) added to the intermediate bath before the electrical stimulations had no obvious effect on the responses. (C) Both the mean tension of the preparation and the responses to electrical stimulation were affected by the addition of methysergide ($10^{-6}$ mol $l^{-1}$) to all compartments.

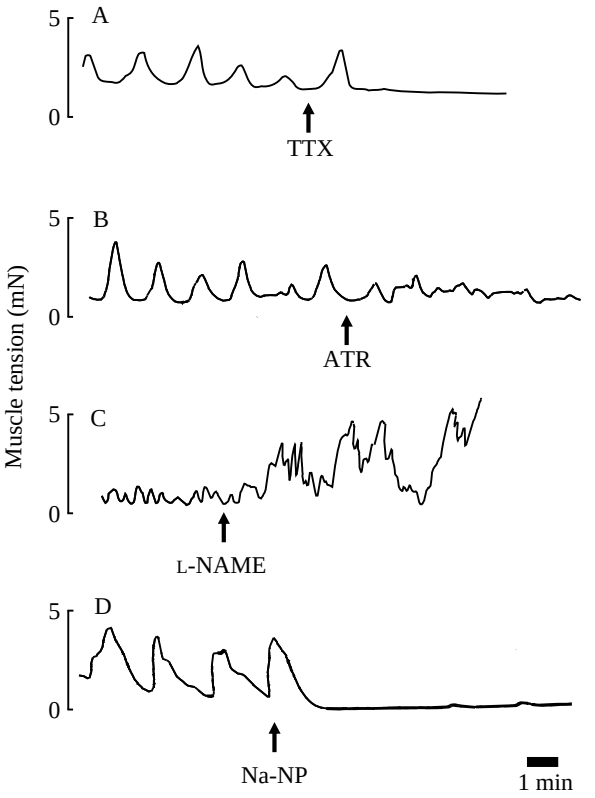


Fig. 2. Original tracings of circular smooth muscle activity in the intestine of the Atlantic cod *Gadus morhua* showing the response to addition of (A) tetrodotoxin (TTX, $10^{-6}$ mol $l^{-1}$), (B) atropine (ATR, $10^{-6}$ mol $l^{-1}$), (C) L-$N^G$-nitro-arginine methyl ester (L-NAME, $10^{-3}$ mol $l^{-1}$) and (D) sodium nitroprusside (Na-NP, $0.5\times10^{-4}$ mol $l^{-1}$). TTX and ATR caused a reduction in the spontaneous activity. L-NAME caused an increase in the spontaneous activity. The nitric oxide donor Na-NP caused a complete relaxation of the circular smooth muscle.

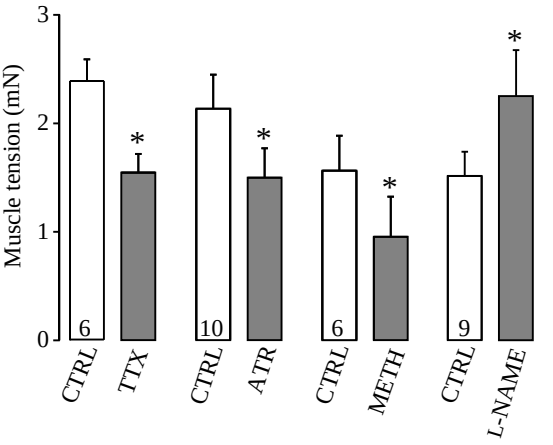


Fig. 3. The mean spontaneous tension (± S.E.M., *N* values are given within columns) in untreated (CTRL) and antagonist-treated preparations of the intestine of the Atlantic cod *Gadus morhua*. Tetrodotoxin (TTX, $10^{-6}$ mol $l^{-1}$), atropine (ATR, $10^{-6}$ mol $l^{-1}$) and methysergide (METH, $10^{-6}$ mol $l^{-1}$) reduced the mean tension of the preparations whereas L-$N^G$-nitro-arginine methyl ester (L-NAME, $10^{-3}$ mol $l^{-1}$) increased the mean tension. The asterisks denote a significant difference in mean tension before and after the addition of antagonists ($P\leqslant0.05$).

spontaneously occurring contractions, resulting in an elevated mean tension (Figs 2C, 3).

### *Mechanical stimulation*

Distension of the muscle wall using a balloon filled with water (0.1–0.4 ml; *N*=24) did not trigger a recordable peristaltic reflex in either the oral or the anal direction.

### *Electrical stimulation*

Intramural electrical stimulation (10 V, 1.0 ms, 8 Hz, 30–60 s) in the intermediate compartment caused in most cases a transient contraction oral to the stimulation site (in the proximal bath) and a more pronounced relaxation anal to the stimulation site (in the distal bath) (Fig. 1A,B). The responses occurred after a delay of 10–60 s (the excitatory response being the first to occur) and lasted for between 20 s and 2 min. The inhibitory response lasted longer and often reduced the subsequent spontaneous contraction (see the anal tracing in Fig. 1A).

Tetrodotoxin added to the intermediate compartment (the site for electrical stimulation) blocked or reduced the response to electrical stimulation in both directions (Fig. 4). The addition of atropine to the intermediate compartment had no effect on the response to electrical stimulation (Fig. 5A), whereas the addition of atropine to all compartments reduced the effect of stimulation on the oral side, but not on the anal side (Fig. 5B).

Addition of methysergide to the intermediate compartment only had no effect on the response to electrical stimulation (Figs 1B, 6A), whereas addition of methysergide to all compartments reduced or abolished the response both orally and anally (Figs 1C, 6B). In the oral compartment, when the oral contraction was reduced but not abolished (*N*=2), the addition of atropine abolished the residual contraction.

L-NAME, when added to all three compartments, abolished the anal inhibitory response to electrical stimulation, but enhanced the oral excitatory response compared with that under control conditions (Fig. 7).

The nitric oxide donor Na-NP ($0.5\times10^{-4}$ mol $l^{-1}$) mimicked the inhibitory response to electrical stimulation in the descending reflex by reducing muscle tone and eliminating spontaneous activity (Fig. 2D).

## Discussion

This study demonstrates, for the first time, a polarised enteric reflex in the fish intestine which can be affected by various antagonists.

The speed of propagation of the spontaneously occurring contractions lies in the same range as the speed of the migrating myoelectric (or motor) complex (MMC) measured in

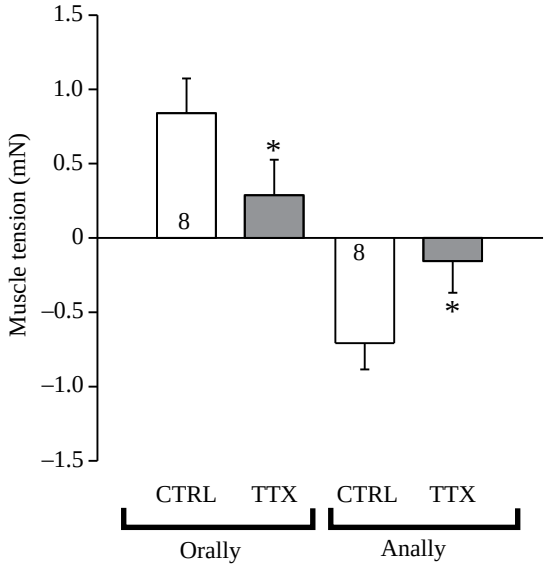


Fig. 4. Changes in mean tension (± S.E.M., *N* values are given within columns) of the circular muscle of the intestine of the Atlantic cod *Gadus morhua* during (oral response) or immediately after (anal response) electrical stimulation. In the control situation (CTRL), there was an oral contraction and an anal relaxation. After tetrodotoxin (TTX, $10^{-6}$ mol $l^{-1}$) had been added to the intermediate compartment (the site for stimulation), there was a significant reduction in the response to electrical stimulation as indicated by the asterisks ($P \leqslant 0.05$).

mammalian intestine: guinea pig colon, 3.6–4.8 cm $min^{-1}$; cat small intestine, about 6 cm $min^{-1}$; dog small intestine, 3.5–6.2 cm $min^{-1}$ (Szurszewski, 1969; Perkins, 1971; Costa and Furness, 1976), and the pattern we observed could be the fish analogue of the MMC. In mammals, there is a difference between herbivores and carnivores (or between continuous *versus* discrete meal feeders) in the activity of the MMC. In carnivores, the propagation is seen in the unfed state only (Bueno and Ruckebusch, 1978; Wingate, 1981), whereas in herbivores the waves of activity are also seen in the fed state (Wingate, 1981). In birds, there are no observed differences between herbivores and carnivores, with waves of activity occurring in both the fed and the fasting states (Clench *et al.* 1989; Mueller *et al.* 1990). In the teleost intestine, there also seemed to be regular activity irrespective of the feeding status (although the fish were not fed in captivity, chyme was still present in some specimens before the experiments). The fish differed from both mammals and birds in that most contractions seemed to occur without intervening quiescent periods, at least *in vitro*. As with the MMC in mammals, the progression of the spontaneously occurring contractions in the teleost intestine is tetrodotoxin-sensitive and thus dependent on the enteric nervous system (Sarna *et al.* 1981; see Torsoli and Severi, 1993).

The mean tension of the resting activity was lowered by atropine, as previously reported from pressure monitoring *in vitro* in the cod intestine (Jensen and Holmgren, 1985), but some preparations were little affected, suggesting the participation of non-cholinergic MMC-like mechanisms as well as the cholinergic pathways. Atropine-resistant MMCs have been described in the rat small intestine (Al-Saffar, 1984). In mammals, 5-HT may be the non-cholinergic agonist as it affects various parameters of the MMC. For example, infusion of 5-HT increases the propagation velocity of the MMC in the opossum (Coelho *et al.* 1986). Furthermore, destruction of 5-HT-containing neurones in the rat disrupts the MMC (Piñeiro-Carrero *et al.* 1991). As judged from the decrease in mean tone after the addition of methysergide, 5-HT may be a transmitter in the non-cholinergic MMC-like activity in the teleost intestine as well.

The inability to elicit peristaltic reflexes by distending the teleost intestine suggests that distension is not the normal

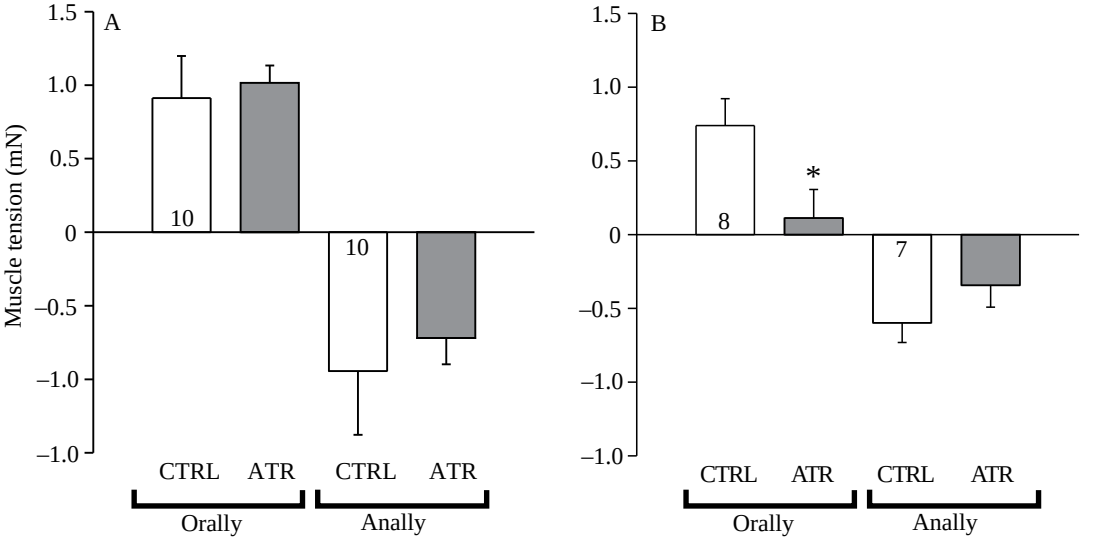


Fig. 5. Changes in mean tension (± S.E.M., *N* values are given within columns) of the circular muscle of the intestine of the Atlantic cod *Gadus morhua* during (oral response) or immediately after (anal response) electrical stimulation. In the control situation (CTRL), there was an oral contraction and an anal relaxation. (A) Atropine (ATR, $10^{-6}$ mol $l^{-1}$) added at the site for stimulation (the intermediate compartment) had no effect. (B) After addition of ATR ($10^{-6}$ mol $l^{-1}$) to all compartments, the oral contraction was antagonised whereas the anal response was unaffected. The asterisk denotes a significant difference in the response to electrical stimulation before and after the addition of ATR ($P \leqslant 0.05$).

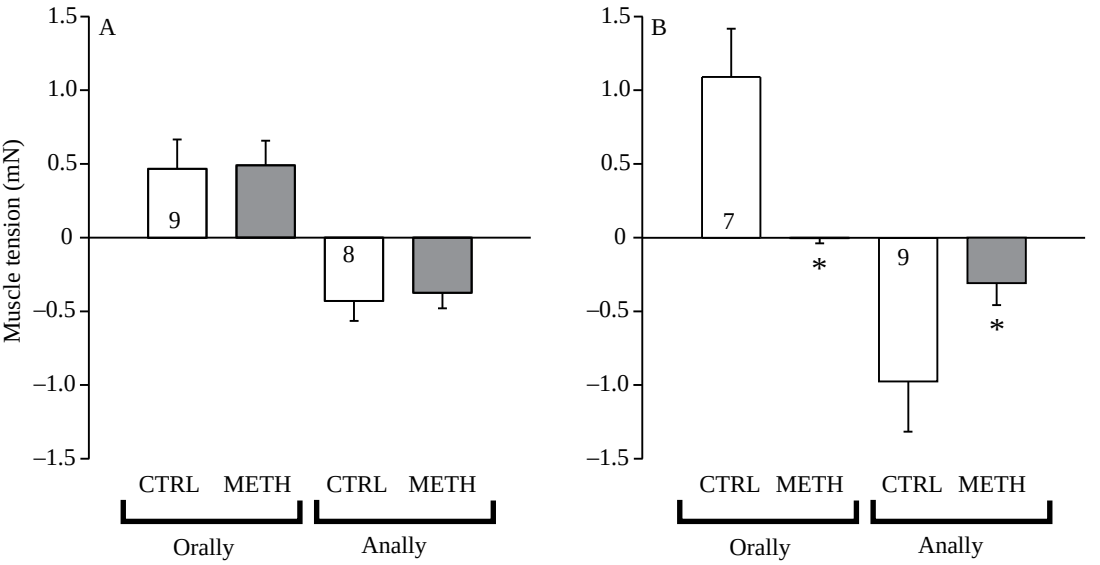


Fig. 6. Changes in mean tension (± S.E.M., *N* values are given within columns) of the circular muscle of the intestine of the Atlantic cod *Gadus morhua* during (oral response) or immediately after (anal response) electrical stimulation. In the control situation (CTRL), there was an oral contraction and an anal relaxation. (A) Both the oral and the anal responses were unaffected by addition of methysergide (METH, $10^{-6}$ mol $l^{-1}$) to the intermediate compartment. (B) Addition of METH ($10^{-6}$ mol $l^{-1}$) to all compartments abolished the oral contraction and decreased the anal relaxation. The asterisks denote a significant difference in the response to electrical stimulation before and after the addition of METH ($P \leqslant 0.05$).

stimulus triggering peristalsis and that receptors other than mechanoreceptors (e.g. chemoreceptors) form the sensory link of the reflex. The normally fluid consistency of the chyme in the cod intestine may not cause local distension of the intestinal wall in such a way that mechanosensory peristaltic reflexes are triggered. Another explanation for the lack of response is the need for intact extrinsic pathways. In the rat colon, it has recently been shown (Grider and Jin, 1994) that the sensory neurones activated by mucosal stimulation are intrinsic whereas the neurones activated by muscle stretch are extrinsic and probably originate from the dorsal root ganglia. However, in the rat, both types of reflexes could be stimulated in acutely isolated preparations, suggesting that viable collaterals from the extrinsic neurones synapse onto intrinsic interneurones.

Both the ascending and the descending reflexes elicited by electrical intramural stimulation were abolished by tetrodotoxin, indicating that this method caused selective local stimulation of nerves. The ability of TTX, when confined to the intermediate bath, to block the oral and anal effects also indicates that the electrical stimulation is limited to the intermediate bath. That the oral and anal responses to electrical stimulation are of different types further confirms that the appropriate reflexes are elicited by this method.

Electrical stimulation may excite all types of neurones (sensory, interneurones and motor neurones), as opposed to distension as a stimulus, and the results are therefore complicated to interpret. Since the oral response to electrical stimulation is affected by atropine, muscarinic receptors are involved in the ascending reflex. These are probably situated mainly on the muscle, since the addition of atropine to the intermediate bath ought to have revealed the presence of any muscarinic receptors in neurone–neurone synapses in the intermediate bath; however, the possibility that some are present in neurone–neurone synapses more distant from the stimulation site in the ascending pathway cannot be completely ruled out. No muscarinic receptors could be detected in the descending reflex.

The reduction of the oral excitatory contraction was more obvious with methysergide than with atropine, suggesting an important role for 5-HT in the ascending reflex. Indeed, numerous 5-HT-immunoreactive neurones are found in the cod intestine (Jensen and Holmgren, 1985), and in a study in two flatfish species, 5-HT was implicated in the stimulation of

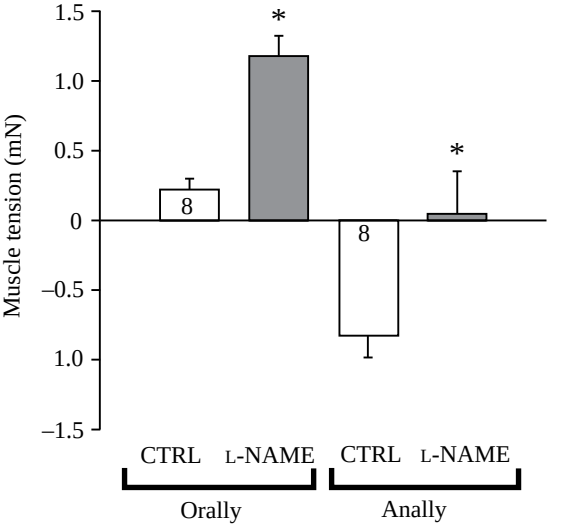


Fig. 7. Changes in mean tension (± S.E.M., *N* values are given within columns) of the circular muscle of the intestine of the Atlantic cod *Gadus morhua* during (oral response) or immediately after (anal response) electrical stimulation. In the control situation (CTRL), there was an oral contraction and an anal relaxation. L-$N^G$-nitro-arginine methyl ester (L-NAME, $10^{-3}$ mol $l^{-1}$, added in all compartments) potentiated the oral contraction and abolished the anal relaxation. The asterisks denote a significant difference in the response to electrical stimulation before and after the addition of L-NAME ($P \leqslant 0.05$).

cholinergic neurones in the intestine (Grove and Campbell, 1979). In mammals, 5-HT receptors are thought to be located on interneurones in the ascending pathway (Yuan *et al.* 1994). The presence of 5-HT receptors on interneurones could not be confirmed in the present study, since the addition of the 5-$HT_1$ and 5-$HT_2$ blocker methysergide to the intermediate bath had no effect on the oral contractions. In the cod, the descending reflex may involve receptors for 5-HT, as indicated by the reduction in the response to electrical stimulation when methysergide was added to all three baths. The response was, however, unaffected by methysergide addition to the intermediate bath. This makes it more probable that the decreased response to electrical stimulation in the anal direction is an effect of a lowered muscle tone in the preparation caused by the addition of methysergide itself. Such a lowering of muscle tone would limit the ability of the preparation to relax further during the following stimulation. Descending relaxations elicited by distension in the guinea pig colon and intestine, respectively, were not affected by methysergide (Costa and Furness, 1976) or by 5-$HT_3$ and 5-$HT_4$ receptor antagonists (Yuan *et al.* 1994). 5-HT is, however, involved in both the ascending and the descending reflexes elicited by stroking the mucosa in the mammalian intestine. These receptors are probably situated on sensory neurones or on interneurones (Neya *et al.* 1993).

The presence of neurones containing nitric oxide synthase (NOS) has been indirectly demonstrated in the cod intestine, where numerous NADPH-diaphorase reactive enteric neurones have been found (Olsson and Karila, 1995). The elevated resting tone and augmented ascending contractions recorded after the addition of the nitric oxide synthesis inhibitor L-NAME suggest an inhibitory effect on muscle tone of these nitrergic neurones in the teleost intestine. Nitric oxide synthase inhibitors have previously been reported to increase the intraluminal pressure in the rat intestine (Calignano *et al.* 1992) and the frequency of peristaltic contractions in the guinea pig ileum (Suzuki *et al.* 1994). In the rat ileum (Allescher *et al.* 1992), the excitatory response to electrical stimulation was augmented by L-NAME, indicating that nitric oxide causes a tonic inhibition. Similarly, in the rabbit stomach (Baccari *et al.* 1993) and the guinea pig ileum (Wiklund *et al.* 1993), nitric oxide synthase inhibitors potentiated the response to electrical stimulation, and it was concluded that nitric oxide had a prejunctional action on cholinergic and substance-P-like neurotransmission. Nitric oxide synthase inhibitors have also been reported to induce an organised MMC pattern in rats, even when they were fed, and to affect the motor pattern in chickens by causing, for instance, an increase in the propagation rate (Rodrígues-Membrilla *et al.* 1995).

As in our experiments, the descending relaxation in the rat intestine was decreased by nitric oxide synthesis inhibitors (Kanada *et al.* 1992), and the inhibitory response to electrical stimulation was reduced in the longitudinal muscle of the guinea pig caecum (Shuttleworth *et al.* 1991). In an electrophysiological study of circular smooth muscle in the guinea pig ileum (He and Goyal, 1993), the slow inhibitory junction potentials were blocked, suggesting a role for nitric oxide in this reflex. In the study by Olsson and Karila (1995), the majority of the myenteric neurones in the cod intestine were shown to project either anally or along the circular muscle layer, further supporting the idea that nitrergic neurones participate in the descending inhibitory pathway in the cod intestine. It is too early to speculate about the specific site of action for nitric oxide in the cod intestine, but it seems likely that there is both a tonic inhibition of neurones in the ascending pathway and the smooth muscle, and a direct relaxing effect on the smooth muscle in the descending pathway.

In conclusion, we have demonstrated the presence of a polarised enteric reflex in the teleost fish intestine. Its control involves features that are apparently common to the vertebrates since, as in mammals, the ascending excitatory reflex involves a cholinergic–serotonergic pathway and the descending inhibitory reflex involves a nitrergic pathway. Furthermore, a motility pattern probably analogous to the MMC, which, as in birds and mammals, can be affected by neuronal blockade, has been described. Taken together, these observations strongly suggest a high degree of conservation of these important mechanisms.

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