ACTIONS OF NO DONORS AND ENDOGENOUS NITRERGIC TRANSMITTER ON THE LONGITUDINAL MUSCLE OF RAT ILEUM *IN VITRO*: MECHANISMS INVOLVED

Abstract

The aim of this work has been to characterize and to compare the responses of the rat ileal longitudinal muscle to the nitric oxide (NO) donors, sodium nitroprusside (SNP) and morpholinosydnonimine hydrochloride (SIN-1). SNP (10^{-5}- 10^{-3}M) caused a contraction followed by a relaxation, both components being concentration-dependent. In contrast, SIN-1 (10^{-5}-10^{-4}M) caused a relaxation followed by a contraction. Neither the neural blocker tetrodotoxin (TTX) nor atropine were able to change the response to SNP, whereas nifedipine abolished its contractile component. In contrast, TTX and nifedipine diminished both the relaxation and the contraction in response to SIN-1, whereas atropine decreased only the contractile component. The specific guanylate cyclase inhibitor oxadiazolo-quinoxalin-1-one (ODQ) decreased the relaxation induced by SNP but did not modify that caused by SIN-1. The K^+ channel blockers charybdotoxin, apamin and tetraethylamonium were unable to modify the response to SNP. In contrast, both TEA and apamin significantly decreased the relaxation induced by SIN-1. The relaxation resulting from electrical field stimulation (EFS) of enteric nerves in non-adrenergic non-cholinergic conditions is mainly but not exclusively nitrergic, as incubation with the NO synthase inhibitor L-NNA markedly decreases such relaxation. EFS-induced relaxation is also sensitive to ODQ. We conclude that SNP acts mainly on smooth muscle cells activating L-type Ca^{2+} channels, which result in contraction, and activates the soluble guanylate cyclase, which results in relaxation. In contrast SIN-1 has mixed - neuronal and muscular - effects, the contraction being caused both by acetylcholine release from neurons and by direct activation of L-type Ca^{2+} channels on smooth muscle cells. SIN-1-induced relaxation is cGMP-independent and it is likely to occur as a consequence of both, neuronal release of inhibitory transmitter(s) and by activation of apamin sensitive K^+ channels. The effect of the nitrergic transmitter released from enteric nerves is different from those caused by SIN-1 but shows similarities with those caused by SNP.

Introduction

In the gut, stimulation of non-adrenergic non-cholinergic (NANC) inhibitory neurons results in relaxation. Electrophysiological studies support the hypothesis that NANC transmitters released from nerve endings cause the inhibitory junction potentials (IJP) [1] recorded in gastrointestinal smooth muscle cells from several segments and species. The precise nature of the inhibitory transmitter released upon stimulation of such nerves has been thoroughly studied. A number of substances have been proposed as NANC transmitters; among them ATP, VIP and PACAP [2, 3, 4, 5]. Since the endothelium derived relaxing factor (EDRF) was first described, a large number of biochemical, histochemical and pharmacological studies have suggested that, in most cases, enteric NANC inhibitory transmission was dependent on the activation of the enzyme nitric oxide synthase (NOs) which was found to be present in nerves [6, 7, 8]. Thus, NOs inhibitors became important tools to progress in this field [9]. As the chemical entity of the transmitter was first assumed to be NO, a number of substances commonly referred to as NO donors or NO adducts were also used [10]. Addition of NO donors has been shown to cause hyperpolarization and smooth muscle relaxation [3, 11], though in some instances such effects do not take place [11, 12]. In addition it has been shown that not all NO donors are actually equivalent, but the controversy is not yet resolved [10, 13, 14, 15]. Though there is not yet a complete agreement on the precise nature of the nitrergic mediator, it is obvious that it has some particular features. It can easily diffuse through the plasma membrane and has a short half-life, which suggests it may be a gas such as NO. These features actually allow to nitrergic transmitter to spread to adjacent structures. Thus, when this transmitter is released from the enteric nervous system, one should not expect it to target the muscle cell exclusively, but rather to affect also enteric neurons and interstitial cells of Cajal (ICC) located in the close vicinity.

It is generally assumed that the effect of nitrergic transmitter result from stimulation of soluble guanylate cyclase [16]. Activation appears to result from NO binding to the heme group of this enzyme [17]. The resulting increases of cytosolic cGMP activate the ultimate effectors. It has also been described that some of the actions of NO are not related to cGMP but result from the interaction of either NO or NO derivatives with other cellular structures such as enzymes (catalase, cyclooxygenase, cytochromes) [18] and proteins.

Covalent modifications of the K^+ channel structure itself have also been suggested to be an important mechanism of action [19]. Other studies have also suggested that NO released from different sources interacts with different targets including soluble guanylate cyclase, K^+ and Cl^- channels and even gap junctions [20]. Finally, due to its high chemical reactivity NO might form a variety of compounds depending on the chemical background to which it is released [10].

The aim of this work has been to analyze the mechanisms that mediate the effects of two widely used NO donors on the longitudinal smooth muscle of the rat ileum *in vitro*. Additionally, the effects of the blockade of mechanisms implicated in the responses to EFS have been analyzed in order to compare responses provoked by exogenous NO donors and endogenous release of nitrergic transmitter.

Methods

Drugs and solutions: The following substances were purchased: atropine, nifedipine, tetraethylamonium (TEA), tetrapentylammonium chloride (TPEA), Nω-nitro-L-arginine (L-NNA) and phentolamine from Sigma (St. Louis, MO); sodium nitroprusside (SNP), 3-morpholinosydnonimine hydrochloride (SIN-1) apamin, charybdotoxin (ChTX), tetrodotoxin (TTX) and propranolol-HCl from RBI (Natick, MA). Oxadiazolo-quinoxalin-1-one (ODQ) was purchased from TOCRIS. Composition of Krebs solution was (in mM): 115.48 NaCl, 4.61 KCl, 2.5 $CaCl_2$, 1.16 $MgSO_4$, 1.14 NaH_2PO_4, 21.9 $NaHCO_3$, 10.09 glucose, pH 7.4. All substances were dissolved in distilled water to make stock solutions, except in case of nifedipine which was dissolved in 50% ethanol as a 10^{-2}M stock solution. SIN-1 and SNP were prepared as aqueous solutions immediately before use. The volume added to the muscle bath never exceeded 5% of its total volume.

Tissue preparation: We used Sprague-Dawley rats (300-350 g), 8-10 weeks old, kept at a constant temperature (22-23ºC) and with lighting cycle of 12h light/12h dark. The day before the experiments, animals were fasted overnight but allowed *ad libitum* access to water. Rats were euthanasied by stunning and immediate decapitation. The Ethical Committee of the Universitat Autònoma de Barcelona has approved this procedure. After exsanguination the abdomen was immediately opened and segments of 2 cm of ileum

were removed and placed in previously bubbled (95%O_2 / 5%CO_2) Krebs solution. Whole full thickness segments of ileum were placed in longitudinal direction in a 10ml muscle bath, filled with pre-aerated Krebs solution at 37ºC. The upper end of the preparation was tied to an isometric transducer (Harvard UF-1) and preloaded with 1-1.5g. Tissue was allowed to equilibrate for 1h until a stable baseline was attained. Data corresponding to mechanical activity were digitized (coupled to an ISC-16 A/D converter card, 25 samples/s) and displayed, stored and analyzed in a PC Pentium computer using Datawin 2 software (Panlab-Barcelona).

Experimental procedures and data analysis: At the start of each experiment 30mM KCl was added to the bath and the contraction was considered as a reference response (RR). At the end of the experiment, the response to 30mM KCl was measured again. The amplitude of contraction was expressed as a percent of the initial KCl reference response (%RR). All drugs were preincubated for 10 min before NO donor addition, except when indicated. Only one drug per tissue was added to the bath, unless indicated. The number of repetition (n) stands for the number of experiments performed with tissue samples taken from different animals. Statistical analysis was performed using Student's t test when two groups were compared, or ANOVA test (when more than two groups were matched) followed by Bonferroni *post-hoc* test. Differences were considered to be significant when $P<0.05$. All data are expressed as mean ± SEM.

Responses to NO donors in the basal state: The response to sodium nitroprusside (SNP; 10^{-5}-10^{-3}M), and 3-morpholinosydnonimine (SIN-1; 10^{-5}-10^{-4}M), was obtained under control conditions. To estimate the time-course of the response, challenges with SNP (10^{-4}-10^{-3}M) and SIN-1 (10^{-5}- 10^{-4}M) were made for three times at one hour intervals. In the rest of the experiments the concentration of NO donors inducing maximal effect was used (10^{-3}M SNP and 10^{-4}M SIN-1).

Effects of neural, muscarinic and L-type calcium channel blockade: The responses to SNP and SIN-1 were measured in the absence and in the presence of the neural blocker TTX (10^{-6}M), the muscarinic antagonist atropine (10^{-6}M) and the L-type Ca^{2+} channel blocker nifedipine (10^{-6}M). Ethanol, at the concentration used to dissolve nifedipine, was devoid of measurable mechanical effects.

Effects of potassium channel blockers: TEA (10^{-2}M), apamin (10^{-6}M) and charybdotoxin (10^{-7}M) were added to the organ bath 10 min prior addition of the NO donors SIN-1 and SNP, and the responses were compared with those obtained before drug addition. Moreover, the effect of SIN-1 was measured in presence of a combination of TTX + apamin (10^{-6}M each).

Effects of soluble guanylate-cyclase and nitric oxide synthase inhibition: The effects of SNP and SIN-1 were measured in the presence of the soluble guanylate cyclase inhibitor ODQ ($3x10^{-6}$M) and the nitric oxide synthase inhibitor L-NNA (10^{-3}M), which were added 30 min before NO donors.

Responses to electrical field stimulation: EFS was performed under NANC conditions (atropine, phentolamine and propranolol, 10^{-6}M each) through platinum electrodes connected to the pulse generator (Harvard-double channel stimulator). Inhibitory responses were obtained by application of repetitive electrical stimuli with the best result obtained with following parameters: 0.1Hz, 0.3 ms, 40V. Control responses were calculated as the mean amplitude of individual relaxations occurred within 5 min period of stimulation at beginning of experiments. When the effects of drugs were to be tested they were incubated for 10 min, unless indicated, without any interruption of EFS. To measure drug effects the mean value of relaxation occurred within the 10 min period rights after incubation were considered. For ODQ ($3x10^{-6}$ M) and L-NNA (10^{-3}M) the incubation period was extended to 30 min.

Results

Response to NO donors. SNP induced a response consisting of quick concentration-dependent contraction followed by a relaxation. In contrast, SIN-1 induced an initial relaxation followed by a contraction. Three consecutive challenges with SNP or SIN-1, made at 1h intervals, produced similar responses with no evidence of any desensitization. Addition of TTX, atropine or nifedipine prior to NO donors always resulted in a slight decrease of strip tone.

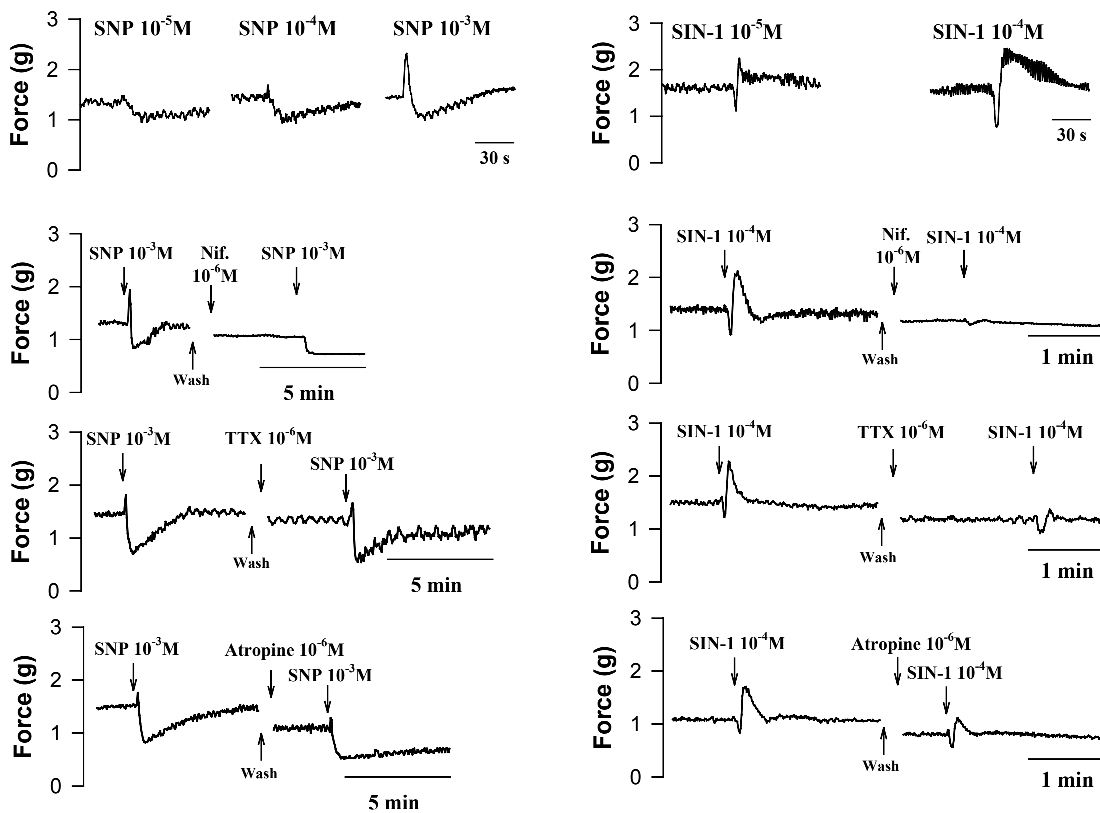

Figure 1. Recordings showing the effects elicited by SNP (left) and SIN-1 (right) in control conditions and illustrating the changes induced in such responses in the presence of nifedipine (Nif.), tetrodotoxin (TTX) and atropine.

TTX or atropine did not modify the amplitude of contractile and relaxant responses elicited by SNP 10^{-3}M (Fig. 1). In contrast, nifedipine abolished the contractile component and decreased the amplitude of the relaxant component of the response to SNP 10^{-3}M (Table 1). Duration of contractile or relaxant response to SNP 10^{-3}M (contraction: 4.06±0.37 seconds and relaxation 146.3±18.2 seconds, n=7) did not significantly change in presence of TTX or atropine. In presence of nifedipine, the duration of the SNP-induced contraction did not decrease while relaxation was longer lasting and the preparation did not completely recover the basal tone.

In case of SIN-1, TTX reduced the amplitude of both components of the response to SIN-1 10^{-4}M whereas atropine only decreased the contraction. The presence of nifedipine significantly reduced the amplitude of relaxation and the contraction induced by SIN-1. These results are illustrated in Figure 1 and summarized in Table 1. Duration of the

contraction induced by SIN-1 10^{-4}M significantly increased in presence of atropine or TTX (control 16.6±1.56 *vs.* + atropine 22.9±1.8 second, n=6, P<0.04; control 16.21±1.97 *vs.* + TTX 21.5±1.97 seconds, n=6, P<0.01), whereas the SIN-1-induced relaxation did not change. In presence of nifedipine, the duration of the SIN-1-induced relaxation was increased (control 3.91±0.23 *vs.* + nifedipine 6.82±1.2 second, n=6, P<0.01), while the duration of the SIN-1 contraction was not modified.

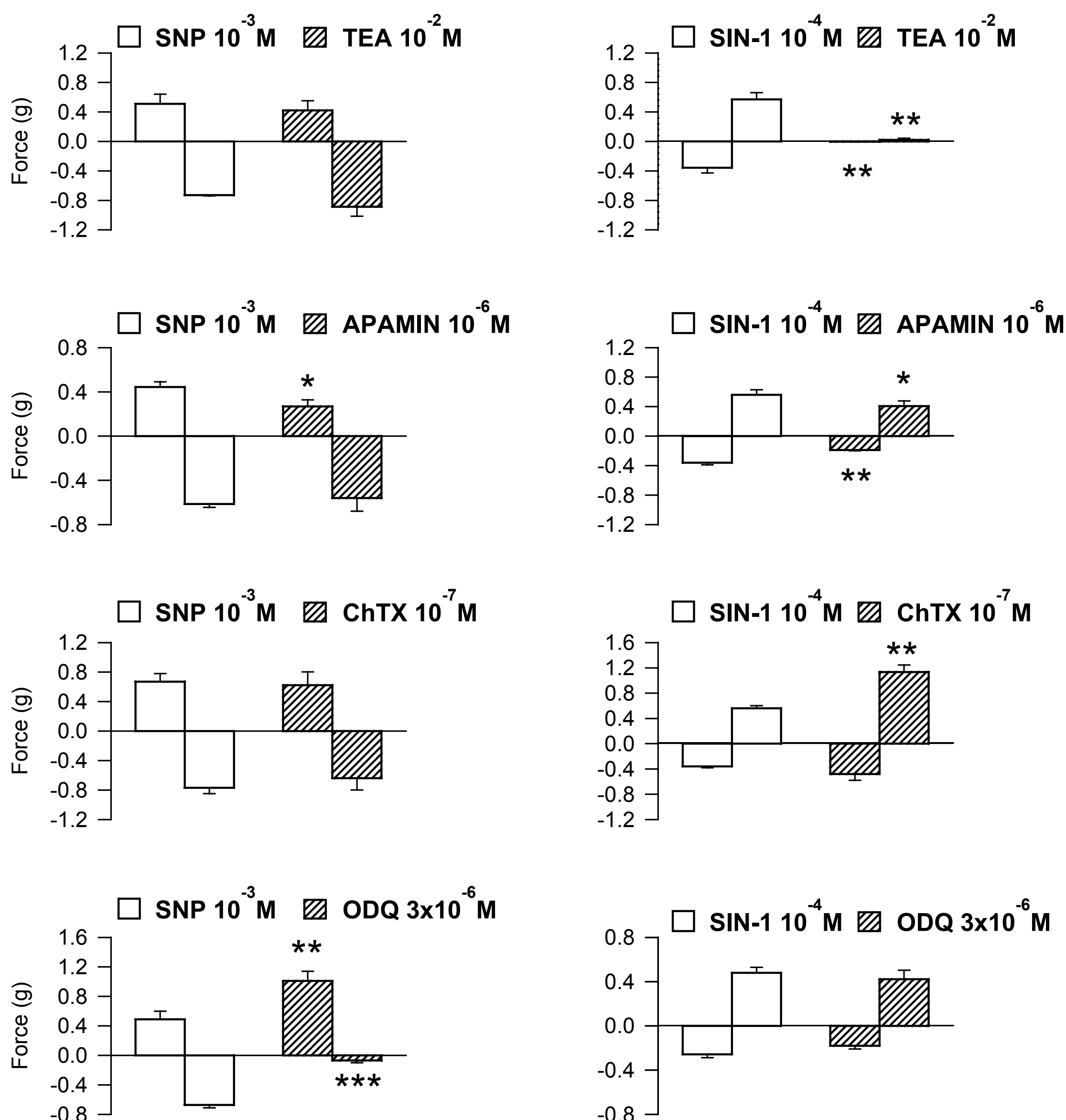

Figure 2. Influence of incubations with the non-selective K^+ channel blocker TEA, the small- and big- conductance Ca^{2+}-activated K^+ channel blocker (apamin and charibdotoxin respectively) and the guanylate cyclase inhibitor ODQ, on the responses (contraction and relaxation) elicited by the NO donors SNP (left) and SIN-1 (right). Data presented mean ± S.E.M. * P<0.05; ** P<0.01; *** P<0.001.

Effects of potassium channel blockers: The presence of TEA 10^{-2}M, apamin 10^{-6}M or ChTX 10^{-7}M did not reduce the relaxation in response to SNP 10^{-3}M. Apamin caused a decrease of the SNP induced contraction. Both the contractile and the relaxant components of the response to 10^{-4}M SIN-1 were abolished by TEA 10^{-2}M. Apamin decreased the relaxation and the contraction caused by SIN-1. The combination of TTX 10^{-6}M plus apamin 10^{-6}M provoked additional reduction of the contractile component of SIN-1 response (control 28.6±3.34 %RR *vs.* TTX + apamin 8.88±1.35 %RR; P<0.02, n=8), whereas the relaxation did not suffer further decrease. Charybdotoxin did not affect the SIN-1 induced relaxation but caused a twofold increase in its contractile effect. Figure 2 and Table 1 summarizes these results.

Table 1

Effect of nifedipine, TTX, atropine, TEA, apamin, ChTX or ODQ on SNP 10^{-3}M and SIN-1 10^{-4}M-induced response.

	%RR	Nifedipine 10^{-6} M	TTX 10^{-6} M	Atropine 10^{-6} M	TEA 10^{-2} M	Apamin 10^{-6} M	ChTX 10^{-7} M	ODQ 3 x 10^{-6} M
SNP 10^{-3}M	Contraction	21.1±0.9 *vs.* 0.5±0.2 P<0.001, n=6	19.6±3.64 *vs.* 19.2±4.4 n.s, n=6	19.0±2.64 *vs.* 13.67±3.68 n.s, n=7	20.33±4.1 *vs.* 17.8±4.6 n.s, n=6	20.0±1.6 *vs.* 11.7±1.8 P<0.04, n=6	23.9±2.5 *vs.* 21.1±4.7 n.s, n=6	19.8±4.2 *vs.* 39.2±4.7 P<0.02, n=7
	Relaxation	-23.0±2.1 *vs.* -15.8±1.6 P<0.006, n=6	-30.0±2.0 *vs.* -32.9±2.8 n.s, n=6	-29.6±2.4 *vs.* -25.9±1.9 n.s, n=7	-33.5±6.3 *vs.* -42.9±7.7 n.s, n=6	-29.7±5.1 *vs.* -26.3±6.4 n.s, n=6	-28.5±2.7 *vs.* -22.3±4.2 n.s, n=6	-27.9±2.6 *vs.* -3.1±1.3 P<0.001,n=7
SIN-1 10^{-4}M	Relaxation	-10.9±1.9 *vs.* -3.2±0.5 P<0.015, n=7	-16.3±1.2 *vs.* -6.4±1.6 P<0.003, n=6	-15.7±2.2 *vs.* -13.4±1.5 n.s, n=9	-13.9±2.9 *vs.* -0.1±0.01 P<0.005, n=6	-15.2±1.3 *vs.* -8.2±0.7 P<0.003,n=6	-14.3±0.5 *vs.* -18.8±3.4 n.s, n=6	-11.0±1.4 *vs.* -7.8±1.3 n.s, n=9
	Contraction	24.8±1.7 *vs.* 1.9±0.8 P<0.001, n=7	28.4±4.3 *vs.* 13.2±0.8 P<0.008, n=6	27.7±1.0 *vs.* 17.6±2.3 P<0.014, n=9	21.7±3.0 *vs.* 0.8±0.1 P<0.004, n=6	24.3±4.1 *vs.* 17.4±3.5 P<0.02, n=6	22.4±0.9 *vs.* 45.5±4.6 P<0.007,n=6	20.1±1.7 *vs.* 17.6±2.9 n.s, n=9

Figures given are the mean values of % RR ± SEM obtained in control conditions and in the presence of the drug indicated. Number of repetitions (n). Non-significant (n.s.)

Effects of soluble guanylate-cyclase and nitric oxide synthase inhibition: The presence of ODQ (3x10^{-6}M) resulted in a significant reduction of the relaxation induced by SNP, whereas there was a twofold increase in the contractile component of the response. In the case of SIN-1, no significant changes were observed upon ODQ addition. These results are also shown in Figure 2 and Table 1. Preincubation with L-NNA (10^{-3}M) did not change neither the contractile nor the relaxant components of the response to SNP or SIN-1.

Responses to electrical field stimulation. Under NANC conditions every electrical stimulus of the preparation resulted in a transient contraction followed by a relaxation. This

relaxation was significantly decreased by TTX 10^{-6}M (-15.1±1.66 *vs.* -4.2±0.52 %RR; P<0.001; n=7) and by nifedipine (-16.82±3.3 *vs.* -1.84±0.35 %RR; P<0.01; n=3). Neither SNP nor SIN-1 modified the response induced by EFS. TEA, 10^{-2}M provoked a four fold increase in the relaxation induced by EFS (-11.7±1.8 *vs.* -39.05±3.2 %RR; P<0.01; n=5). TPEA 10^{-4}M decreased the EFS induced relaxation by about 80% (-13.77±2.34 *vs.* -2.7±1.07 %RR; P<0.01; n=5). This drug also caused a dramatic reduction in the contraction in response to KCl, which was added at the end of the experimental protocol. Charybdotoxin 10^{-7}M caused a significant increase in the EFS-induced relaxation (-14.63±1.48 *vs.* -27.35±3.2 %RR; P<0.01; n=9), whereas apamin 10^{-6}M caused a marked decrease which averaged 45% (-15.07±1.29 *vs.*-8.19±1.22 %RR; P<0.001; n=11). The presence of L-NNA 10^{-3}M resulted in a 60% decrease of the EFS-induced relaxation (-14.48±1.22 *vs.* -6.3±0.77 %RR; P<0.001; n=14). The reduction of the EFS-induced relaxation induced by ODQ 3x10^{-6}M averaged 63% (-18.7±2.8 *vs*. -6.92±1.34 %RR; P<0.001; n=8). Figure 3 shows recordings obtained in the above mentioned conditions. Combinations of L-NNA plus ODQ, L-NNA plus apamin or ODQ plus apamin did not result in statistically significant additive reductions of the EFS-induced relaxation.

Discussion

This study shows that the response to NO donors is not uniform but strongly dependent on the chemical entity of the NO donor. The first interesting finding is the opposite sequence of events in the responses to SNP and SIN-1. As we describe here, SNP elicits first a contraction and then a relaxation, similar to the response induced by EFS and freshly dissolved NO [11]. An opposite pattern of response is reported in the guinea pig longitudinal ileum [21]. Though a complete explanation for this difference is not unequivocally determined, it could be due to the activation of different mechanisms of action (see below).

When nifedipine, TTX or atropine are added to the bath, the duration of the relaxation in response to SNP tends to increase. This can be explained by a blockade of the tonic release of excitatory neurotransmitters (TTX), a blockade of the muscarinic receptor that impairs the contractile effects of tonically released acetylcholine (atropine) or the blockade

of Ca^{2+} channels, which impairs the cell to reach the Ca^{2+} level required for contraction (nifedipine).

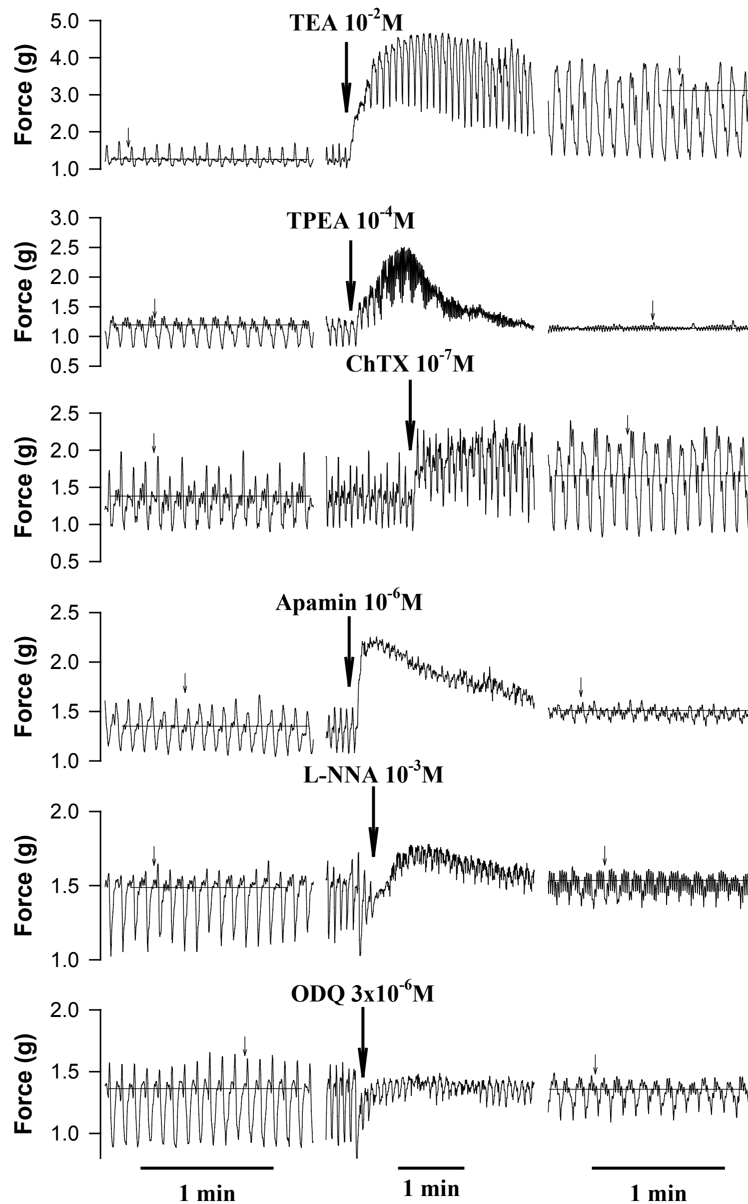

Figure 3. Recordings showing the responses of the rat longitudinal ileum to repetitive electrical stimuli (40V, 0.1 Hz, 0.3 ms). Control responses are shown on the left side. The central and right segments of the recordings show immediate and steady responses to the non-selective K^+ channel blockers TEA and TPEA; the small conductance Ca^{2+}-activated K^+ channel blocker apamin; the big conductance Ca^{2+}-activated K^+ channel blocker charybdotoxin; the NO synthase inhibitor L-NNA and the guanylate cyclase inhibitor ODQ, respectively. Steady responses were obtained after 10 min incubation for all drugs except for L-NNA and ODQ, in which cases a 30 min incubation period was necessary. Small arrows indicate the precise moment in which a particular electrical stimulus was given and the horizontal lines indicate the tone of the preparation.

We have first studied to what extent the responses to both NO donors are affected by the L-type Ca^{2+} channel blocker nifedipine. It has been clearly shown that this dihydropyridine abolishes the contractile response to SNP or SIN-1 while the relaxation is significantly decreased. Decreased relaxation in response to NO donors could be provoked by lower basal tone of the preparation. This indicates that the nitrergic element released upon NO donor addition is able to open L-type Ca^{2+} channels to elicit contraction. The response induced by SNP in the presence of ODQ shows that the contractile component is present and even increased in magnitude, what suggests that the inhibition of soluble guanylate cyclase somehow disturbs the balance between contractile and relaxant mechanisms.

In the presence of atropine a decrease in the contractile component of the response to SIN-1 was observed, suggesting that ACh release contribute to the contraction elicited by SIN-1. TTX decreases both the relaxation and the contraction induced by SIN-1 providing additional support to the concept that this substance has significant neural effects. The relaxation induced by SIN-1 was always shorter-lasting than that induced by SNP but occurred earlier. This relaxation has been shown to be completely independent of guanylate cyclase activation (insensitive to ODQ) and dependent on the activation of K^+ channels (sensitive to TEA). The decreased contractile effect of SIN-1 in presence of TEA could be caused by the lack of “rebound” contraction, resulting from a disturbed balance between contractile and relaxant component. The small conductance Ca^{2+}-activated K^+ channel blocker apamin decreased but not abolished the relaxation. This type of K^+ channels are usually seen as effectors of the relaxant and hyperpolarizing effects of other NANC inhibitory transmitters such as PACAP and ATP, though in some cases they have been found to mediate the effects of the nitrergic neurotransmitter [22]. Thus, in the canine small intestine NO has been reported to be the only transmitter responsible for the inhibitory junction potentials induced by EFS and apamin sensitive K^+ channels appear to be partly responsible for such effect [23, 4]. In the present study, though apamin inhibited SIN-1-induced relaxation to about 50% of the control response it did not change the relaxation caused by SNP. We also assayed the effects of the big conductance Ca^{2+} -activated K^+ channel blocker charybdotoxin. This substance was unable to affect the relaxation caused by SIN-1 but doubled its contractile effect. This might suggest that such channels are not involved in the neurotransmitter-induced hyperpolarization but rather in the repolarization of the cell after a depolarization. In such a case, the picture could be that

SIN-1, acting on enteric nerves, induces ACh release which causes depolarization. If the presence of charybdotoxin affects repolarization, the excitability of muscle cells is increased, so depolarization in response to minor stimuli might occur resulting in a potentiation of the contractile response. Other studies carried out in our laboratory have shown that charybdotoxin does not change IJPs induced by EFS in the rat colon but modifies its spontaneous mechanical activity (increase in amplitude of contractions and decrease in frequency) (data not shown). It is somehow conflicting, however, that when the tissue is challenged with SNP in the presence of charybdotoxin no significant changes occur in the contractile component of the response.

The fact that SIN-1 induced relaxation is insensitive to ODQ is in contrast with results obtained by other groups with other NO donors. Thus, Ekblad & Sundler have described that both the relaxation and the contraction induced by sodium-nitroso-N-acetylpenicillamine (SNAP) is dependent on the activation of soluble guanylate cyclase [13]. On the other hand, there is increasing evidence suggestive of direct effects of NO-related compounds on cell membrane structures such as ionic channels and receptors. It was shown that freshly dissolved NO rapidly increased Ca^{2+} dependent K^{+} channel activity in rabbit aortic smooth muscle cells [19]. This effect was independent of cGMP, Mg^{2+} and ATP and resulted from chemical modification of channel proteins. Thus, the concept that soluble guanylate cyclase activation is the common clue to understanding all actions of nitric oxide seems now obsolete.

Concerning the experiments in which the preparation underwent EFS, some interesting findings have been noticed. First, incubation with L-NNA showed that most but not all the relaxation is due to the release of a nitrergic transmitter. Relaxation recorded after incubation with ODQ showed a profile very similar to those observed in presence of L-NNA. Thus the nitrergic transmitter released by enteric neurons in this preparation does need to activate guanylate cyclase to exert its relaxing effect. This is in good agreement with results obtained in the rat stomach, where ODQ reverses the relaxation induced by EFS [24]. In contrast, Ekblad and Sundler [13] have reported that ODQ does not modify the response induced by EFS in the same preparation, though the discrepancy might well result from the use of markedly different EFS parameters (4V, 1ms, 4-20 Hz). As no additive effects were seen when the tissue was incubated with ODQ and L-NNA

simultaneously, we may assume that the nitrergic transmitter released from nerves targets exclusively the soluble guanylate cyclase. Another interesting finding is that TEA, a widely used specific non-selective K^+ channel blocker, actually causes a marked increase in the EFS-induced relaxation. Similar results have been found in the rat colon [25] and the guinea pig colon [26]. In these preparations it has been shown that increased relaxation may be due to a neuronal effect of TEA. TEA appears to be more effective on neurons that on smooth muscle cells, as its effect is prevented by TTX addition. So, TEA may depolarize inhibitory neurons and provoke smooth muscle relaxation. [25]. TEA also increases the amplitude of IJPs induced by EFS. In contrast, the TEA analog TPEA causes depolarization and blockade of the IJP, resulting that TPEA is acting preferentially on smooth muscle. Thus it is able to cause smooth muscle depolarization and a complete blockade of EFS induced relaxation, in agreement with the results shown here [25].

The existence of a L-NNA resistant component of the EFS-induced relaxation lead us to consider the existence of, at least, a second transmitter which could use apamin sensitive K^+ channels as a target. The fact that apamin is able to decrease the EFS-induced relaxation gave support to this concept. We tested also the combination of apamin + LNNA or apamin + ODQ in an attempt to unmask two different transmitters and/or effector mechanisms. Never such combinations result in additive effects on the EFS-induced relaxation.

It has also been described that in some preparations such as the rat gastric fundus, incubation with NO donors may result in a prejunctional inhibition of nitric oxide synthase [27]. This mechanism could be considered as a feedback regulation of nitrergic transmitter release. However, we have not found evidence of the existence of such a mechanism in the rat ileum, since SNP and SIN-1 did not change the relaxation provoked by EFS and L-NNA did not change the response to SNP and SIN-1.

In summary, our results suggest that SNP provokes first a contraction by opening L-type Ca^{+2} channels, an action which takes place earlier than the increase in cGMP responsible for relaxation. On the other side SIN-1 provokes firstly a relaxation in a cGMP-independent manner, which involves activation of apamin sensitive K^+ channels. The fact that nifedipine is able to decrease SIN-1 induced relaxation may be explained by the fact that apamin

sensitive K^+ channels are actually Ca^{2+}-dependent K^+ channels. Thus, its activation requires a rise in intracellular Ca^{2+} levels, which is difficult to achieve in the presence of a blocker of L-type Ca^{2+} channels such as nifedipine. The SIN-1-induced contraction is provoked, at least partially by ACh release form nerve endings, which is consistent with the fact that contraction in response to SIN-1 occurs later than relaxation. In view of the fact that TTX decreases SIN-1 induced relaxation and that apamin does not completely annihilate this response, a neural effect of this substance releasing inhibitory transmitters cannot be excluded. Regarding the response due to the release of endogenous nitrergic transmitter its drug sensitivity is similar to that of SNP rather than that of SIN-1.

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DIFFERENTIAL CHANGES IN KCl, ACETYLCHOLINE, SUBSTANCE P AND ELECTRICALLY- INDUCED CONTRACTILITY OF INTESTINAL SMOOTH MUSCLE FROM *TRICHINELLA SPIRALIS* INFECTED RATS

Abstract

We examined the changes in worm-positive (jejunum) and worm-free (ileum) intestinal segments of rats infected with *Trichinella Spiralis*. To investigate the relationship between structural and functional changes in smooth muscle, we measured the thickness of longitudinal and circular muscle layer of rat jejunum and ileum. Mechanical responses to KCl 30mM, acetylcholine (ACh) 10^{-8}-10^{-4}M, substance P (SP) 10^{-9}-10^{-5}M and to electrical field stimulation (EFS) of jejunal and ileal longitudinal muscle strips were studied in muscle bath. This experimental procedure was performed in controls (day 0) and on day 2, 6, 14, 23 and 72 post-infection (PI). After *T. spiralis* infection an inflammation of the mucosal and submucosal layers of jejunum was observed, whereas in the worm-free ileum there was not any inflammatory infiltrate. Increases in smooth muscle thickness of both jejunum and ileum were correlated to increased responses to depolarizing agent KCl and to ACh but not to SP. Contractions in response to SP were decreased on day 14-23 PI in jejunum and from day 6-14 PI in ileum. EFS induced contractions were transiently decreased in the jejunum (day 2PI) but in the ileum the contractile responses were decreased until the end of the study period (day 72 PI). These results support the hypothesis that alterations in intestinal smooth muscle function do not require the presence of the parasite and that the absence of histopathological signs of inflammation do not warrant intact motor function. Changes in motor responses after *T. spiralis* infection are not only due to smooth muscle damage but also to disturbances in specific receptor-mediated mechanisms.

Introduction

Numerous clinical studies have reported that altered gastrointestinal motility, cramps and diarrhea are common features following a wide variety of intestinal inflammations. Frequently such motor changes persist unexpectedly long after the recovery of the normal structure of the mucosa, and in some instances they affect not only the inflamed area but

also remote regions of the gastrointestinal tract (Bergin et al. 1993; Vermillion et al. 1993).

The high incidence of motor disturbances during intestinal inflammation has prompted a large number of experimental studies aiming to set up experimental models useful to study such disorders. It has been reported that changes in muscle function are similar regardless of the model used to induce inflammation, i.e. *T. spiralis* infection or administration of different chemical compounds as trinitrobenzenesulfonic acid (TNBS), mitomycin and acetic acid (Grossi et al. 1993). Changes that occur during the intestinal phase of infection with *T. spiralis* in the rat have been well studied. The nematode *T. spiralis* is an intraepithelial parasite that preferentially inhabits the proximal small intestine of rats (Dick and Silver 1980). In the rat, *T. spiralis* infection is a relatively mild and long-lasting inflammatory process, during which, the only remarkable symptoms are a decrease in food intake and body weight. In this model survival of the animals is warranted for long periods and the inflammation induced by the parasite, though noticeable, is moderate in comparison to that induced by chemical compounds such as TNBS acid. When *T. spiralis* larvae are given orally, the parasite inhabits the duodenum and the jejunum but it does not reach the ileum (Dick and Silver 1980). Thus, in our study specimens of jejunum were taken as representative of inflamed worm-positive, and specimens of ileum were considered as representative of non-inflamed worm-free segments. In the enteric phase of the infection, the presence of adult worms and larvae in the mucosal and submucosal layers causes an inflammatory response and functional changes in the motility of small intestine (Castro et al. 1976; Palmer et al. 1984; Vermillion and Collins 1988). *In vivo*, myoelectric activity is altered (Palmer et al. 1984) and the rate of intestinal transit is increased (Castro et al. 1976), which might be viewed as an extension of the immune response leading to the expulsion of the parasite. *In vitro*, contractility of the smooth muscle is increased (Vermillion and Collins 1988) and the function of certain enteric nerves is depressed (Collins et al. 1989). In addition, the thickness of the muscle layers is increased due to both hypertrophy and hyperplasia, which may additionally alter gastrointestinal motility, since increased smooth muscle mass may exacerbate muscle contraction and amplify the effect of excitatory stimuli (Blennerhassett et al. 1992). Changes in propulsive intestinal activity have also been observed in denervated gut segments, suggesting that elements intrinsic to the intestinal wall, *i. e.* enteric nerves and smooth muscle cells, play a crucial role (Alizadeh et al. 1987). In worm-free ileal segments

of *T. spiralis* infected rats, numerous functional and morphological changes have also been observed (Jacobson et al. 1995; Marzio et al. 1990). Thus, the local presence of the parasite is not required for the systemic response and the motor changes are not restricted to the site of inflammation. Other groups have also reported specific changes in contractility, which are not related to the responses to depolarizing agents but to specific agonists such as motilin and acetylcholine (Depoortere et al. 1999).

Accordingly, we have used healthy controls and *T. spiralis* infected rats 1- to study the time-course of morphological (inflammation and hypertrophy) and contractile changes at different times of post-infection; 2- to correlate structural with functional changes and 3- to compare worm-positive inflamed (jejunum) *vs.* worm-free non-inflamed (ileum) areas of the small intestine. We selected four stimuli: KCl, acetylcholine (ACh), substance P (SP) and, finally, contraction elicited by electrically field stimulation (EFS).

Methods

Trichinella spiralis larvae preparation: The larvae were obtained from CDI mice infected 30-90 days before, according to the method described by Castro and Fairbairn (Castro and Fairbairn 1969). Briefly, larvae were isolated from mice skeletal muscle by digestion with the standard 1% pepsin-1% HCl solution, and rats were infected by oral administration of 7.500 *T. Spiralis* larvae suspended in 1ml of 0.9% NaCl solution.

Animal model of Trichinella Spiralis infection: We used male Sprague-Dawley rats (300-350 g), 8-10 weeks old, kept at a constant temperature (22-23ºC) and with lighting cycle of 12h-light/12h dark. The day before the experiment, animals were fasted overnight but allowed *ad libitum* access to water. Rats were euthanasied on days 2, 6, 14, 23 and 72 post-infection (PI) by stunning and immediate decapitation. After exsanguination, the abdomen was immediately opened and 2 cm segments of median ileum and jejunum (5 cm distal to the ligament of Treitz) were removed. The tissue was placed in previously bubbled (95%O_2 / 5%CO_2) Krebs solution. The Ethical Committee of the *Universitat Autònoma de Barcelona* has approved this procedure.

Histopathological study: Samples of jejunum and ileum were taken from control or *T.*

spiralis infected rats on days 2, 6, 14, 23 and 72 post-infection (PI) (n=6). The samples were processed for histopathology, stained with hematoxylin and eosin and finally viewed under microscope (x 400). A standard scoring based on inflammatory cell infiltration was used to evaluate the inflammatory process. The level of inflammation was classified as none - (no infiltration), + (mild), ++ (moderate) and +++ (severe, intense infiltration) depending on the extent of inflammatory cell infiltration at mucosal, submucosal and muscular level. The same samples were also used to measure the thickness of both circular and longitudinal muscle layers. Individual data of thickness were obtained from the average value of four measurements per preparation made with an ocular micrometer. All observations were performed in a simple blind protocol.

Muscle bath experiments: Whole full thickness segments of ileum or jejunum were placed in longitudinal direction in a 10ml muscle bath, filled with pre-aerated Krebs solution at 37ºC. The upper end of the preparation was tied to an isometric transducer (Harvard UF-1) and preloaded with 1-1.5g. Tissue was allowed to equilibrate for 1h until a stable baseline was attained. Data corresponding to mechanical activity were displayed, analyzed and stored in a computer using Datawin-2 software (Panlab-Barcelona), coupled to an ISC-16 A/D card (25 samples/s). At the start of each experiment 30mM KCl was added to the bath and this contraction was considered as a reference response (RR). At the end of the experiment, the response to 30mM KCl was measured again in order to assess preparation responsiveness. The amplitude of contractions corresponding to cumulative concentration-response curves for ACh (10^{-8}-10^{-4}M) and SP (10^{-9}-10^{-5}M) and to EFS-induced responses were expressed both in grams and as a percent of the initial KCl reference response (%RR). Data were fitted to a Michaelis-Menten equation using non-linear regression. The equation was used to estimate Emax and pD_2 (negative logarithm of the concentration that induces 50% of the maximal contraction). Emax and pD_2 obtained on days 2, 6, 14, 23 and 72 PI were compared to control tissue.

Responses to electrical field stimulation (EFS): EFS was performed through platinum electrodes connected to a *SRI* pulse generator (Harvard double channel stimulator). Excitatory responses were obtained by application of repetitive stimuli (10Hz, 0.3 ms, 40V) with duration of 15s in both control and infected tissues. Peak responses were calculated as percentage of the KCl (%RR). The responses elicited by electrical stimulation were also

measured in the presence of the nitric oxide donor SNP 10^{-3}M (5 min after its addition to the bath solution) and in non-adrenergic non-cholinergic (NANC) conditions (atropine, phentolamine and propranolol, each at 10^{-6}M added 10 min before). In each case, the amplitude of the response obtained on days 2, 6, 14, 23 and 72 PI were compared to control tissue.

Data analysis: Statistical analysis was performed using ANOVA test followed by Bonferroni post-hoc test when we compared data obtained on days 2, 6, 14, 23 and 72 PI to data obtained from control tissue. We estimate the correlation between structural (hypertrophy) and functional changes (Emax values obtained from KCl and ACh and SP concentration-response curves). Differences were considered to be significant when $P<0.05$. All data are expressed as mean ± SEM. The number of repetitions (n) stands for the number of experiments performed with samples taken from different animals.

Drugs and solutions: The following substances were purchased: atropine, acetylcholine (ACh), substance P (SP) and phentolamine from Sigma (St. Louis, MO); sodium nitroprusside (SNP), tetrodotoxin (TTX) and propranolol-HCl from RBI (Natick, MA). Composition of Krebs solution was (in mM): 115.48 NaCl, 4.61 KCl, 2.5 $CaCl_2$, 1.16 $MgSO_4$, 1.14 NaH_2PO_4, 21.9 $NaHCO_3$, 10.09 glucose, and pH 7.4. All substances were dissolved in distilled water to make stock solutions. SNP was prepared as aqueous solutions immediately before use. The volume that was added to the bath never exceeded 5% of its total volume.

Results

Evolution of infected animals: A significant decrease in food intake and body weight was observed during the first 2 weeks after infection. Loss in body weight averaged 1.2% per day from 2-12 days PI. Afterwards, both parameters were back to normal.

Histopathological study: T. Spiralis infection induced an intense inflammatory response with mixed infiltrate of neutrophil and eosinophil cells in the jejunum. Cell infiltration affected the mucosal and submucosal layers of the jejunum from day 2 PI until day 23 PI with no signs of inflammation at the muscular layers. Severe inflammation scores were

observed from 2-14 days PI and a mild inflammation was present on day 23 PI (Table 1). In contrast, no signs of inflammation were seen in the worm-free ileum throughout the experimental period.

T. Spiralis infection provokes trophic changes of circular and longitudinal smooth muscle layers of both jejunum and ileum. Increased thickness of jejunal and ileal muscle layers was noted from day 2 PI until day 72 PI with the most prominent changes corresponding to samples from days 14 and 23 PI. Figure 1 illustrates the time course of these alterations. Throughout the experimental period we did not note any increase in the intercellular space.

Table 1

Severity of mucosal inflammation classified according to neutrophil and eosinophil cell infiltration.

	Control	**2PI**	**6PI**	**14PI**	**23PI**	**72PI**
Jejunum	-	+++	+++	+++	+	-
Ileum	-	-	-	-	-	-

Values are expressed *according to cell infiltration: - no infiltration,* + mild, ++ moderate and +++ severe infiltration.

Responses to KCl, ACh and SP: The amplitude to 30mM KCl-induced response at the start and end of each experiment was similar. In *T. Spiralis* infected rats increased contractile responses to 30 mM KCl were noted in both jejunum and ileum from day 6 to day 23 PI (Fig. 2-upper panel). On day 23 PI this was increased for more than twofold in both tissues. A similar time-course in the responses to ACh was observed (Fig. 2-median panel). An increase in the Emax response to ACh was observed between 6 and 23 PI in the jejunum and on days 6 and 23 in the ileum. At the end of the study period (72 days PI) the Emax value of ACh was partly recovered. A different time-course was observed in the response to SP, indicated by the fact that Emax response to SP was increased only on day 23 PI (Fig 2-lower panel).

In order to correlate morphological and functional changes we plotted smooth muscle thickness *vs.* the Emax values to KCl, ACh and SP. A significant linear correlation was found between KCl response and muscle thickness both in the jejunum (r^2=0.74; $p<0.02$) and in the ileum (r^2=0.67; $p<0.04$) (Fig. 3). Similarly, the Emax value to ACh also displays

linear correlation with muscle thickness in the jejunum (r^2=0.92; p<0.002) and ileum (r^2=0.81; p<0.01). In contrast, no lineal correlation was observed between Emax value in response to SP and muscle thickness in the jejunum (r^2=0.25) and ileum (r^2=0.008) (Fig. 3).

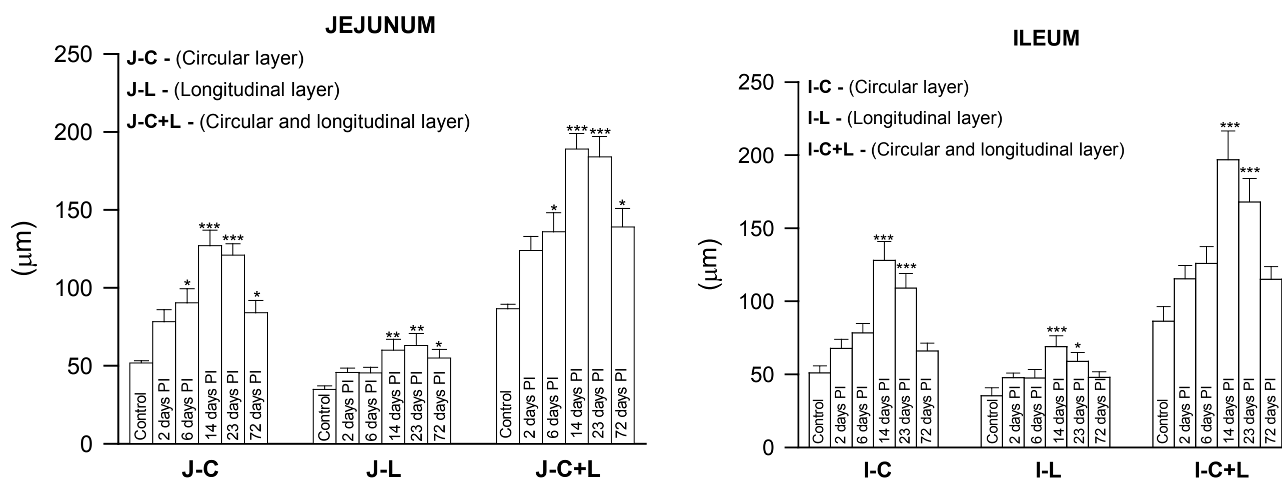

Figure 1. Thickness of longitudinal (L) and circular (C) muscle layers of jejunum and ileum in control conditions and on different times post-infection. Data are expressed in µm (means±SEM); *P<0.05, **P<0.01, *** P<0.001 compared to healthy animals; n=6.

In view of the correlation between smooth muscle thickness and KCl Emax value, we normalized our data using KCl as a reference response (%RR). The Emax values of ACh, calculated from ACh dose-response curves, expressed as %RR are plotted in figure 4 (Fig. 4). The pD_2 value is decreased for jejunal samples on days 6 and 14 PI, while pD_2 value of ileal samples was not significantly changed (Fig. 4).

Regarding the response to SP 10^{-9}-10^{-5}M expressed as %RR, a significant decrease in Emax values were found on days 14 and 23 PI for jejunal samples and on days 6 and 14 PI for ileal preparations. Both tissues were less sensitive to SP as indicated by the fact that pD_2 values were significantly decreased on days 6, 14 and 23 PI for jejunal tissue and on day 23 PI for ileal tissue (Fig. 4).

Response to EFS: Under control conditions application of electrical stimuli (10Hz, 0.3 ms, 40V, 15 s) resulted in a transient relaxation followed by a contraction in the jejunum and ileum, both being TTX-sensitive. The contraction averaged 70.82±4.75 %RR for the jejunum

and 77.73±6.55 %RR for the ileum (Fig. 5). The response of the jejunum was decreased on day 2 PI but later the contraction returned to normal values. The incubation with the nitric oxide donor SNP 10^{-3}M did not modify the responses to EFS neither in controls nor in tissue form infected rats at any day of PI period (data not shown). The NANC component of the jejunal response to EFS remained unchanged (Fig. 5). In the ileum the responses to EFS were markedly decreased starting from day 2 PI. The NANC contractile component of ileal response to EFS was also significantly decreased from day 6 PI to day 23 PI (Fig. 5).

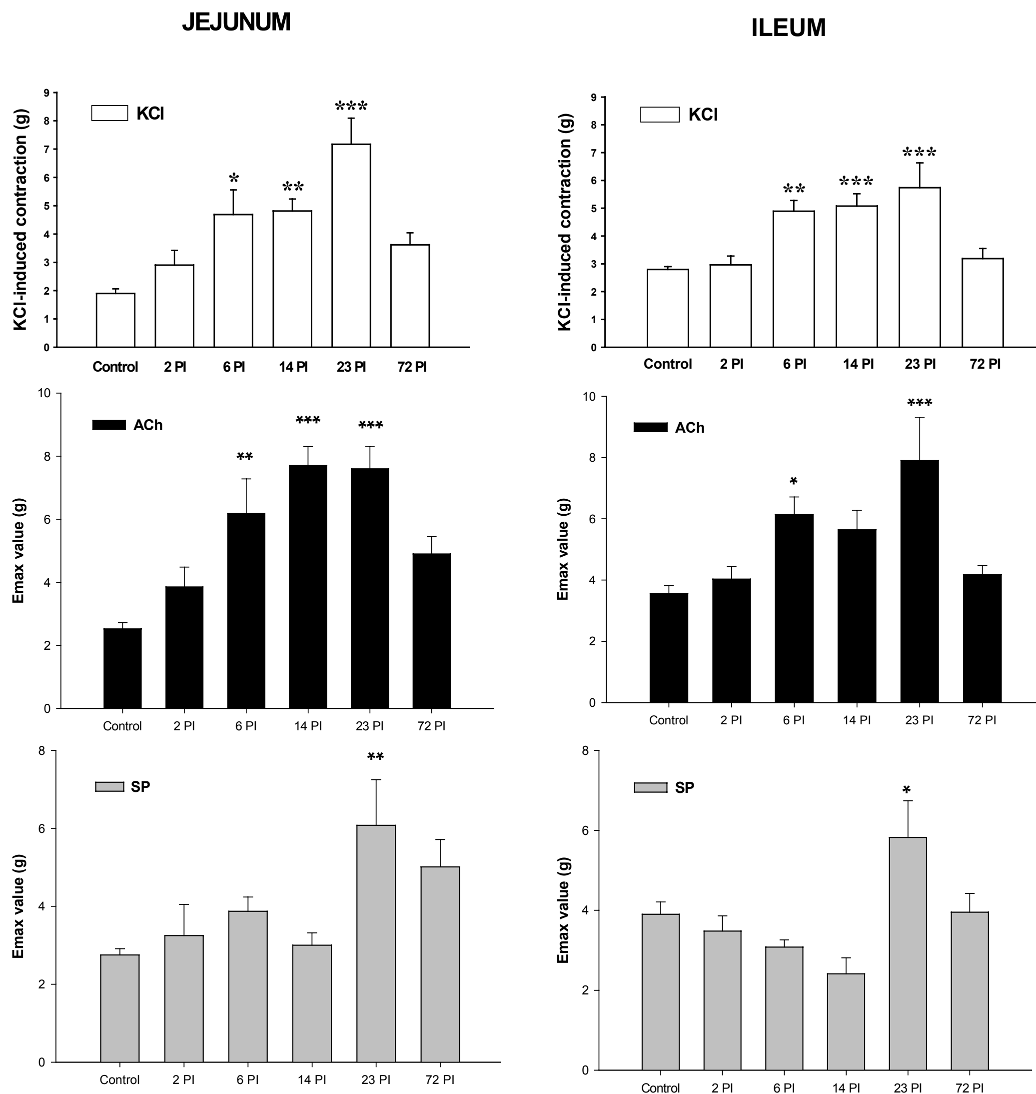

Figure 2. Responses of jejunum (left) and ileum (right) to 30mM KCl (upper panel), Emax response to ACh (mid panel) and SP (lower panel) in healthy (control) and *T. spiralis*-infected rats. Data are expressed as a mean±SEM of 7 different animals; *P<0.05; **P<0.01; ***P<0.001 indicates differences between control responses and infected rats.

Discussion

In the *T. spiralis* infected rats histopathological signs of inflammation were restricted to the mucosal and submucosal layers of the jejunum. Neither the ileum nor the muscular layers of the jejunum were affected. Inflammation in the jejunum appeared on day 2 and lasted until day 23 PI. This consideration was based on our histopathological findings and is in agreement with the statements given by other groups, which have used the same animal model (Castro and Fairbairn 1969; Marzio et al. 1990; Tanovic et al. 1999).

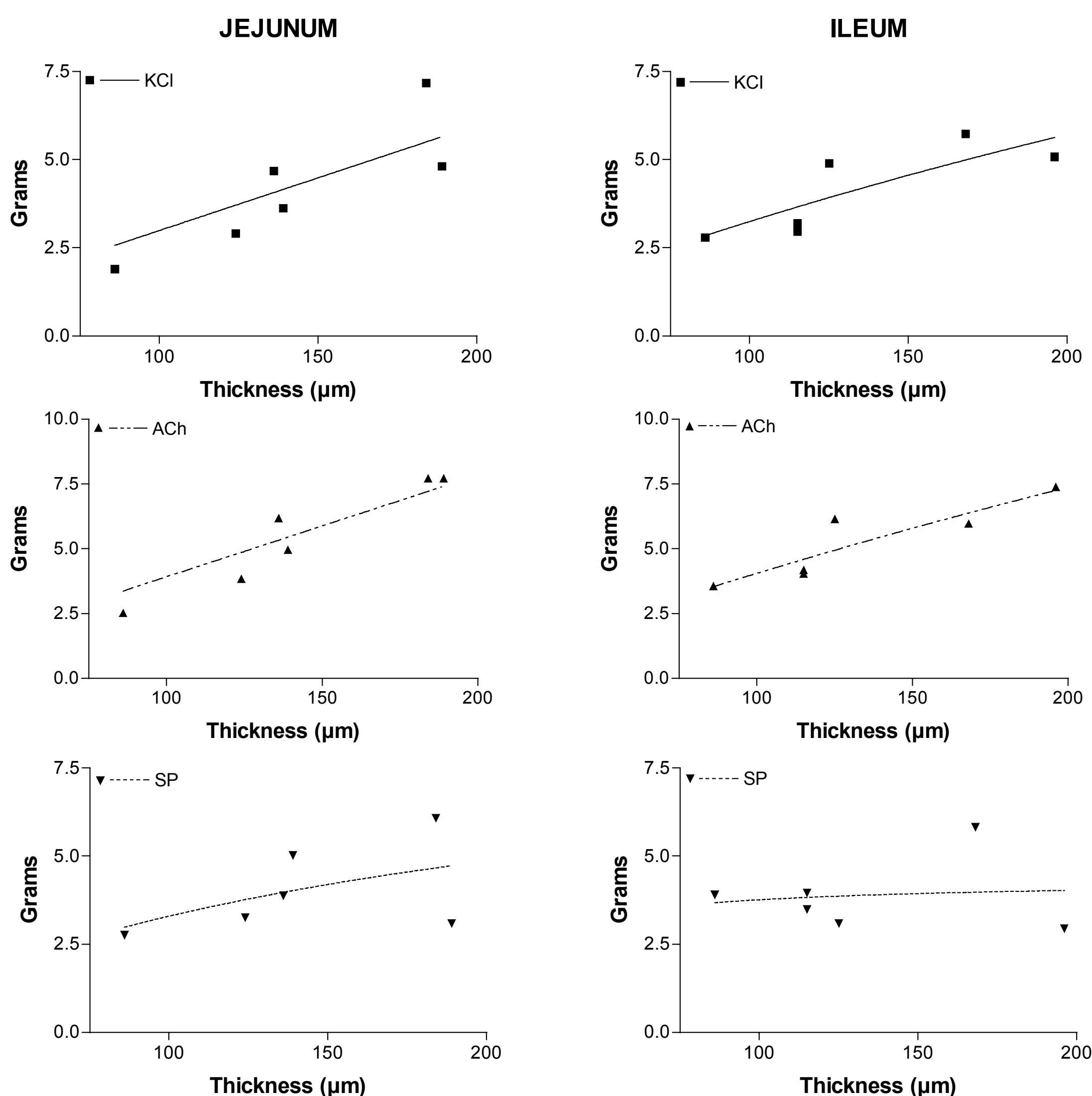

Figure 3. Correlation between full muscle thickness (μm) and KCl (upper panel), ACh (mid panel) and SP (lower panel) response (g) of jejunum (left) and ileum (right). Notice the presence of correlation between muscle thickness, KCl and ACh response and the absence of correlation in case of SP.

Indeed, mucosal inflammation is well correlated with the presence of parasites and remission of histopathological inflammatory evidence occurs soon after parasite eviction, on day 17 to 23 PI. Muscle thickness was increased from day 6 until day 72 PI in the inflamed jejunum. This indicates that an inflammation, primarily located in the mucosa and submucosa has a powerful mitogenic effect on the underlying smooth muscle. However, both inflamed and non-inflamed areas were hypertrophied and both the longitudinal and the circular layers appear to be affected. Blennerhassett *et al.* have also reported that hyperplasia and hypertrophy, associated with *T. spiralis* infection, are responsible for increased smooth muscle thickness of rat small intestine (Blennerhassett et al. 1992). In that study both jejunum and ileum displayed a similar pattern of muscle hyperplasia and hypertrophy (preserved by day 23 PI), although the non-inflamed ileum showed a less pronounced trophic response. Moreover, other studies have reported that presence of edema, production of extracellular matrix or increases in collagen do not contribute to the apparent increase in tissue mass (Blennerhassett et al. 1992; Weisbrodt et al. 1994). Thus, a systemic host's inflammatory reaction might be responsible for the increase in muscle thickness observed in the jejunum and ileum.

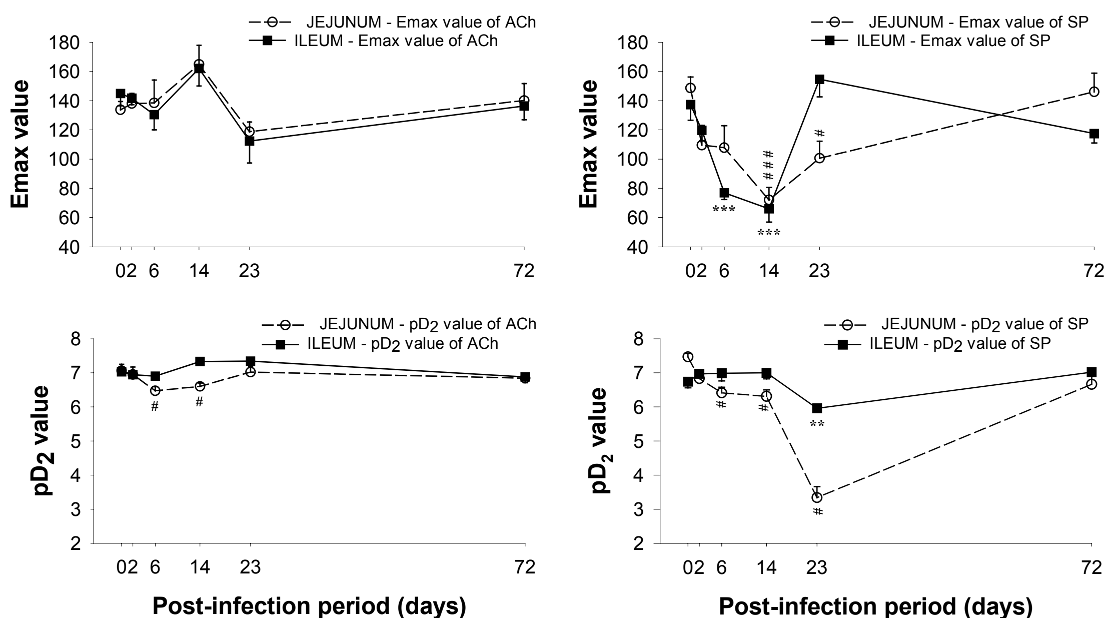

Figure 4. Time-course of responses to ACh and SP using KCl as a reference response (%RR) from jejunum and ileum. Upper panels show the changes in Emax values and lower panels show the evolution of pD_2. Data represent the mean±SEM (n=7). Symbol # for jejunum and symbol * for ileum indicate differences from non-infected rats: # *P<0.05; ## **P<0.01; ### ***P<0.001.

Contractility results from ileum and jejunum (see figure 2) evidenced that both jejunum and ileum are similarly affected. In both tissues an increase in the response to KCl and ACh was observed suggesting that local inflammation is not the causal factor of these changes. An increase in contractility has also been found in jejunal segments surgically isolated from rest of the gut before *T. spiralis* infection, suggesting the presence of a systemic mechanism (Marzio et al. 1990). It has been reported that in rats, in which colitis has been induced by intrarectal administration of *T. spiralis* larvae, the motility of the non-inflamed ileum is decreased (Jacobson et al. 1995). Additionally, intestinal inflammation may result in an increase in actin synthesis in smooth muscle, so we cannot exclude that some of the changes observed in the present study could result from increased contractile protein content (Weisbrodt et al. 1994). Taken together, these data suggest that impaired function in non-inflamed areas is a generalized feature of different types of intestinal inflammation. Thus, the host response, and not local mechanisms due to the presence of the parasite, appear to be responsible for the structural and functional changes observed in the jejunum and worm-free ileal segments. The increased propulsive activity might be also viewed as an adaptive change associated with the host immune system, enabling it to act as an “extension” of the immune system in evicting the parasite from the intestine (Castro et al. 1976).

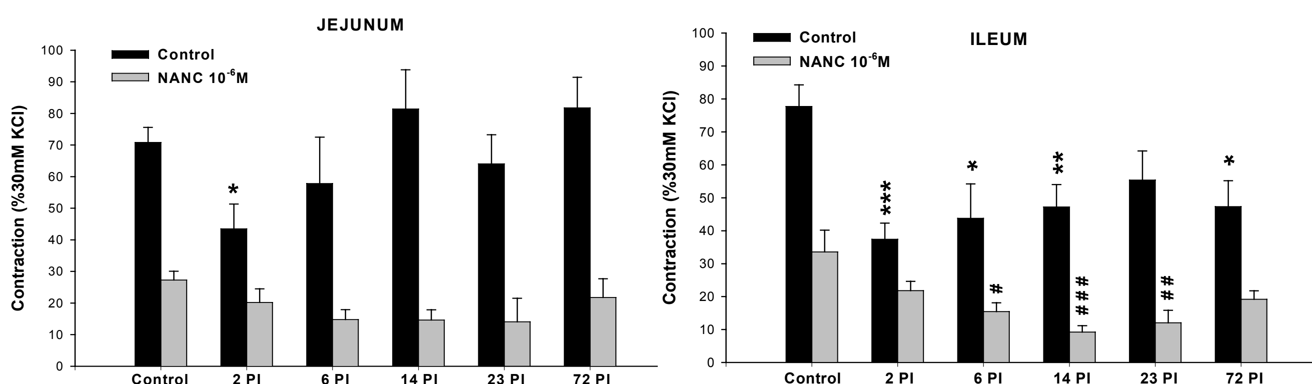

Figure 5. Contractile responses of jejunal (left) and ileal (right) preparations to EFS (40V, 10 Hz, 0.3 ms, 15s) under control or NANC conditions. Data represent mean values ±SEM (n=7) expressed in percentage of KCl-induced reference response (%RR), Symbol * for control Krebs and symbol # for NANC conditions indicate differences between healthy (control) and infected rats: # *P<0.05; ## **P<0.01; ### ***P<0.001.

The contractile responses to KCl and ACh were increased from day 6-23 PI, while an increase in SP response was observed only on day 23 PI. The fact that SP response

showed significantly increased Emax value only on day 23 PI could be due to the mixed influence of a decreased contraction in response to SP, possibly provoked by alteration of SP receptor sensitivity, counterbalanced by the increased muscle thickness.

In other species, as NIH Swiss mice, intestinal smooth muscle contractility to carbachol and KCl was increased for more than 40 days after infection with *T. spiralis* (Barbara et al. 1997). In agreement with the results of the present study there is an increased responsiveness of jejunal longitudinal muscle to carbachol and 5-HT of *Nippostrongylus brasiliensis* infected rats (Farmer et al. 1983; Fox-Robichaud and Collins 1968). In contrast to these results, Marzio *et al.* have reported that muscle from worm-free ileum of *T. spiralis* infected rats displays decreased contractility to ACh and 5-HT compared with control (Marzio et al. 1990). However, the same group found that trophic changes in non-inflamed ileal segments of *T. spiralis* infected rats were less pronounced compared to the jejunum, what may explain this result (Blennerhassett et al. 1992). Thus, a systematic normalization of contractility data should help us understand the discrepancies reported in those studies. Since the contractile capacity of the muscle layer is directly proportional to the muscle mass, we tried to discriminate motor changes related to muscle thickness from those related with alterations at the receptor level to test the hypothesis that the changes in intestinal contractility are also agonist-specific. We found that changes in thickness of muscle layers correlated to KCl-induced contractions both in the jejunum and ileum. Thus, the increase in contractility due to depolarization induced by KCl might be a consequence of the hypertrophy and/or hyperplasia found in this tissue. Regarding the response to ACh, a correlation with an increased thickness in both jejunum and ileum were found, suggesting that the increase in the response, observed during the course of infection, is probably due to the increased muscular mass. In contrast, we did not observe such correlation between SP response and muscle thickness.

Different methods to normalize the contractile responses for the amount of muscle mass are used in the literature, which may lead to conflicting results (Moreels et al. 2001). In view of the relationship found between muscle thickness and response to KCl, we used the response to KCl as a reference response (%RR) to normalize contractility data. We did not find significant changes in Emax values in the ACh response neither in the jejunum nor in the ileum, although slight decreases in pD_2 values were observed on days 6 and 14 PI

in the jejunum. In contrast, Emax values obtained with SP were significantly decreased on days 14 and 23 PI for jejunal samples and on days 6 and 14 PI for ileal samples. Decreases in pD_2 values were observed for both jejunum and ileum though in the case of jejunum this difference was particularly evident (6-23 PI). This effect might be due to an increased release of SP or an altered sensitivity to SP during *T. spiralis* infection (Goldhill et al. 1995; Swain et al. 1992). Alternatively, conformational changes involved in the interaction between the SP receptors and its effector system may also be affected. Moreover, down-regulation of neural endopeptidase in the inflamed rat intestine causing an increased bioavailability of SP might be responsible of SP receptor desensitization (Hwang et al. 1993). In contrast, to the results observed in the small intestine, in rats in which colitis was provoked by intrarectal administration of *T. spiralis* larvae or by chemical agents, a decreased contractility to ACh, SP or KCl is generally reported (Depoortere et al. 1999; Myers et al. 1997). Furthermore, the occurrence of such functional changes appeared to be unrelated to the agent used to induce colitis (Depoortere et al. 1999; Grossi et al. 1993; Jacobson et al. 1995; Myers et al. 1997).

The contractile response induced by EFS is mainly due to ACh and SP release from enteric motor neurons. In the jejunum the response to EFS is transiently decreased on day 2 PI, reaching normal values between 6-72 days PI. This is an unexpected result because a decrease in the response to EFS until day 28 in mice infected with *T. Spiralis* has been reported (Barbara et al. 1997). We do not have an explanation for this discrepancy but the time course of the recovery from inflammation might be responsible of this discordance. Another explication might be that different animal model were used (mice *vs.* rata). In the present study we found that contractile responses to EFS were decreased in non-inflamed ileum throughout the whole study period. This might be due to pre-junctional mechanisms including both ACh and SP. Our results suggest that cholinergic post-junctional mechanisms are not modified in the ileum since the response to exogenously added ACh is not apparently altered. In contrast, a decrease in ACh release from enteric motor neurons has been reported in the *T. spiralis* infected rat (Collins et al. 1992). Thus, a pre-junctional impairment of ACh release might explain the results observed here. Additionally, a decrease in the response to exogenously added SP may contribute to the decreased response to EFS observed in NANC conditions. Although SP-like-immunoreactivity is not modified in myenteric neurons of non-inflamed ileum, we cannot rule out the possibility that

SP release from myenteric neurons is decreased (Swain et al. 1992). It is interesting to notice the difference in the contractile response to EFS between jejunum and ileum. Similarly, EFS-induced relaxations of jejunum and ileum from *T. spiralis* infected rats caused a significantly decreased relaxation only in the ileum on days 14-23 PI, while jejunal strips did not display any significant change during the studied period (72 days) (Tanovic et al. 2002). The reason underlying such regional differences has not been provided so far, but an impairment of both inhibitory and excitatory nerve mediated responses might be a putative explanation.

In some preparations such as the rat gastric fundus, incubation with nitric oxide donors may result in a prejunctional inhibition of nitric oxide synthase activity (De Man et al. 1995). This mechanism could be considered as a feedback regulation of nitrergic transmitter release, which may change the balance between excitatory and inhibitory inputs. Baccari *et al.* also showed that muscular contractions evoked by ACh were not influenced by SNP, although SNP was able to depress the EFS-elicited cholinergic contractions, suggesting a putative neuro-modulatory role for NO (Baccari et al. 1995). However, we have not found any evidence supporting the existence of such a mechanism in the rat jejunum or ileum, since SNP did not change the contraction provoked by EFS neither in tissues from control nor from infected animals.

In summary, we have provided evidence that changes provoked by *T. spiralis* infection occur in both worm-positive inflamed (jejunum) and worm-free non-inflamed (ileum) tissue. In both cases an increased thickness of both circular and longitudinal layers occurs reaching a maximum between days 14 and 23 PI. The response to KCl and ACh correlated with an increased thickness in both jejunum and ileum, suggesting that the increase in the response during infection is probably due to the increased muscular mass. In contrast, we did not observe such correlation in case of SP-induced response. Though jejunal and ileal segments exhibited similar increases in smooth muscle layer thickness and contractility, the more prominent functional motor change was a long-lasting decrease of the ileal contractility in response to EFS. Our work shows that an impaired neural function and/or long-lasting damage of the excitation-contraction coupling might also occur in the non-inflamed ileum of *Trichinella spiralis* infected rats.

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CHANGES IN THE INHIBITORY RESPONSES TO ELECTRICAL FIELD STIMULATION OF INTESTINAL SMOOTH MUSCLE FROM *TRICHINELLA SPIRALIS* INFECTED RATS

Abstract

Functional motor changes and morphological alterations have been associated with intestinal inflammation. The aim of this work was to study functional motor changes in inflamed and non-inflamed intestinal segments of *Trichinella spiralis* infected rats. Thickness of muscle layers and cell infiltration during infection were also evaluated. Segments of rat jejunum and ileum were placed in organ bath and relaxations of the longitudinal muscle in response to electrical field stimulation (EFS) were recorded. During the post-infection (PI) period EFS-induced relaxations in ileum were decreased. Maximal decreases in relaxation were found on day 14-23 PI for ileum, whereas non-significant changes were observed in jejunal samples throughout the experimental period. The sensitivity of the EFS-induced relaxations to the NO synthase inhibitor Nω-nitro-L-arginine (L-NNA) and to the soluble guanylate cyclase inhibitor oxadiazolo-quinoxalin-1-one (ODQ) was decreased on day 14 PI for jejunum, whereas in the ileum it lasted from day 14-23 PI. The sensitivity of EFS-induced relaxations to apamin (a small conductance calcium activated potassium channel blocker) disappeared between day 6-23 PI for both jejunum and ileum. In contrast, the sensitivity of the EFS-induced relaxations to the K^+ channel blockers tetraethylamonium (TEA) and tetrapenthylammonium (TPEA) chloride was similar for healthy tissue and for tissue obtained form infected animals. Distribution and density of NADPH-diaphorase positive neurons was similar in tissue obtained form healthy and infected animals. In conclusion, intestinal inflammation induces functional and structural changes in both worm-free and worm-positive intestinal segments. Increased muscle thickness was similar for both inflamed and noninflamed segments but the most prominent functional changes i.e. a long-lasting decrease of EFS-induced relaxation was found in non-inflamed ileal segments.

Introduction

Intestinal inflammation is commonly associated with changes in gut physiology including

increased secretion [1] and motor abnormalities [2, 3, 4, 5]. The motor disturbances are related to digestive symptoms such as diarrhea, abdominal pain and constipation [6]. The symptoms result from complex interactions between the immune system, the neuroendocrine system and the enteric neuromuscular system [7]. The high incidence of motor disturbances has prompted a large number of studies aiming to set up experimental models mimicking such pathological conditions. The *Trichinella spiralis* infected rat has been widely used for such purpose [1-7].

The nematode *Trichinella spiralis* is an intraepithelial parasite that preferentially inhabits the rat proximal small intestine [8]. In the enteric phase of the infection, the presence of adult worms and *larvae* in the mucosal and submucosal layers causes an inflammatory response and functional changes in the motility of small intestine [4, 6, 9]. Although microscopic observations confirm that the intestinal inflammatory process is overcome within 3 weeks, some neural and muscular alterations can persist for longer periods. *In vivo*, myoelectric activity is altered [4] and the rate of intestinal transit is increased [6]. *In vitro*, contractility of the smooth muscle is increased [9] and the function of certain enteric nerves is depressed [10].

Available data indicate that changes in muscle functionality are similar regardless of the model used to induce inflammation, *i.e. T. spiralis* infection or administration of trinitrobenzenesulfonic acid [TNBS], mitomycin or acetic acid [11]. Moreover, changes in propulsive intestinal activity have also been observed in denervated gut and worm-free ileal segments, clearly suggesting that elements intrinsic to the intestinal wall - enteric nerves and smooth muscle cells - play a crucial role [12, 13, 14].

Stimulation of intrinsic non-adrenergic non-cholinergic [NANC] inhibitory neurons results in intestinal smooth muscle relaxation. Electrophysiological studies have supported the hypothesis that NANC transmitters such as nitric oxide, ATP, VIP and PACAP released from nerve endings cause inhibitory junction potentials and relaxations, recorded in gastrointestinal smooth muscle cells from different segments and species [15, 16, 17, 18, 19]. It is generally assumed that the effect of inhibitory neurotransmitters may involve a variety of effector mechanisms such as soluble guanylate cyclase, K^+ and Cl^- channels and even gap junctions [20].

In view of the lack of information about the changes caused by inflammation on inhibitory responses, we have investigated the time-course of the changes in the relaxations induced by EFS in inflamed segments of jejunum and non-inflamed worm-free segments of ileum taken from *Trichinella spiralis* infected rats. The time-course of histopathological evidence of inflammation and smooth muscle thickness has also been measured.

Methods

Drugs and solutions: The following substances were purchased: atropine, tetraethylammonium (TEA), tetrapenthylammonium chloride (TPEA), Nω-nitro-L-arginine (L-NNA), phentolamine, Triton X-100, NADPH and nitroblue tetrazolium from Sigma (St. Louis, MO); sodium nitroprusside (SNP), apamin, tetrodotoxin (TTX) and propranolol-HCl from RBI (Natick, MA). Oxadiazolo-quinoxalin-1-one (ODQ) was purchased from TOCRIS. Composition of Krebs solution was (in mM): 115.48 NaCl, 4.61 KCl, 2.5 $CaCl_2$, 1.16 $MgSO_4$, 1.14 NaH_2PO_4, 21.9 $NaHCO_3$, 10.09 glucose and pH 7.4. All substances were dissolved in distilled water to make stock solutions, except in case of SNP that was prepared as aqueous solutions immediately before use.

Histopathological study: We performed a histopathological monitoring of the intestinal inflammation during the infection. Samples of jejunum and ileum that were taken from healthy or *T. spiralis* infected rats on days 2, 6, 14, 23 and 72 post-infection (PI) (n=6) were stained with hematoxylin and eosin and examined under the microscope (x 400). A standard scoring based on the inflammatory cell infiltration (scale from 0-4) was used to evaluate the inflammatory process. The level of inflammation was classified as none 0 (no infiltration), 2 (mild), 3 (moderate) and 4 (severe, intense infiltration). The same samples were also used to measure the thickness of both circular and longitudinal muscle layers (x 400). Individual data of thickness were obtained from the average value of four measurements per preparation. All observations were performed in a simple blind protocol.

Tissue preparation: We used male Sprague-Dawley rats (from Iffa-Credo; 300-350 g), 8-10 weeks old, kept at a constant temperature (22-23ºC) and with lighting cycle of 12h light/12h dark. Animals were specific pathogen free when purchased and during the experimental period they were periodically checked for absence of intestinal parasites. The

day before the experiments, animals were fasted overnight but allowed *ad libitum* access to water. Rats were euthanasied by stunning and immediate decapitation. The Ethical Committee of the Universitat Autònoma de Barcelona has approved this procedure. After exsanguination the abdomen was immediately opened and segments of 2 cm of jejunum (5 cm distal to the ligament of Treitz) and median ileum were removed and placed in previously bubbled (95%O_2 / 5%CO_2) Krebs solution. Whole full thickness segments of jejunum and ileum were placed in longitudinal direction in a 10ml organ bath, filled with pre-aerated Krebs solution at 37ºC. The upper end of the preparation was tied to an isometric transducer (Harvard UF-1) and preloaded with 1-1.5g. Tissue was allowed to equilibrate for 1h until a stable baseline was attained. Data corresponding to mechanical activity were digitized (coupled to an ISC-16 A/D converter card, 25 samples/s) and displayed, stored and analyzed in a PC Pentium computer using Datawin 2 software (Panlab-Barcelona).

Trichinella spiralis infection: The larvae were obtained from CDI mice infected 30-90 days before, according to the method described by Castro and Fairbairn [21]. Briefly, larvae were isolated from skeletal muscle by digestion with the standard 1% pepsin-1% HCl solution and rats were infected by oral administration of 7,500 *T. Spiralis* larvae suspended in 1ml of 0.9% NaCl solution.

Experimental procedures: Data corresponding to EFS-induced relaxations were expressed in absolute values (g) and as a percent of the 10^{-3}M SNP inhibitory response in order to distinguish specific changes. EFS was always performed under non-adrenergic non-cholinergic (NANC) conditions (atropine, phentolamine and propranolol each added at 10^{-6}M). Inhibitory responses in both healthy and infected rats were obtained by application of repetitive electrical stimuli through platinum electrodes connected to a pulse generator (Harvard double channel stimulator). The best results in relaxation were obtained with the following parameters: 0.1Hz, 0.3 ms and 40V. Control responses were calculated as the mean values of amplitudes of individual relaxations occurred within a 5-10 min period of stimulation at the beginning of the experiments. When the effects of drugs were to be tested they were incubated for 10 min without any interruption of EFS. To measure the effects of different drugs the mean relaxations of a 10-min interval, right after the incubation period, were considered. For ODQ (3x10^{-6}M) and L-NNA (10^{-3}M) the incubation

period was extended to 30 min, while in case of SNP it was 5 min. Only one drug per tissue was added to the bath, unless indicated. The volume added to the organ bath never exceeded 5% of its total volume.

Experimental protocols: Under NANC conditions EFS-induced relaxations were measured in the presence of the neural blocker TTX (10^{-6}M) in healthy and *T. spiralis*-infected rats. The effects of EFS were also measured in the presence of the soluble guanylate cyclase inhibitor ODQ ($3x10^{-6}$M) and the nitric oxide synthase (NOS) inhibitor L-NNA (10^{-3}M) that were incubated for 30 min prior EFS. The nitric oxide (NO) donor SNP (10^{-3}M) was added to the bath in order to estimate the effect of NO on EFS-induced relaxations. The potassium channel blockers TEA (10^{-2}M), TPEA (10^{-4}M) and apamin (10^{-6}M) were added and the responses obtained were compared with those before drug addition both in healthy and infected rats.

NADPH-diaphorase histochemistry: in order to study changes in NADPH-diaphorase-activity, segments of the jejunum and ileum were taken from healthy and infected rats on 2, 6, 14, 23 and 72 days PI (n=6). The intestinal segments were opened longitudinally and fixed in 4% para-formaldehyde. Preparations of longitudinal muscle layer with the myenteric plexus attached (LMMP) were incubated in phosphate buffered saline (PBS) containing 0.3% triton x-100, 1 mM NADPH and 0.1 mM nitroblue tetrazolium at 37ºc for 45 min. After several washes, preparations were mounted on glass slides and examined under the microscope (x200). The density of staining was estimated as the number of positive ganglion found in six fields from each slide by a blind observer.

Data analysis: Statistical analysis was performed using Student's *t* test when two groups were compared or ANOVA test, when more than two groups were matched, followed by Bonferroni *post-hoc* test. Differences were considered to be significant when $P<0.05$. All data are expressed as mean ±SEM. The number of repetitions (n) stands for the number of experiments performed with tissue samples taken from different animals.

Results

Evolution of infected animals. A significant decrease in food intake and body weight was

observed during the first 2 weeks after infection. Loss in body weight averaged 1.2% per day from 2-12 days PI. Afterwards, both parameters were back to normal.

Table 1

Thickness of longitudinal and circular muscle layers of jejunum and ileum from healthy animals and from *T. spiralis* infected rats at different times post infection.

	Healthy		2 PI		6 PI		14 PI		23 PI		72 PI	
	Circular	Longi-tudinal	Circular	Longi-tudinal	Circular	Longi-tudinal	Circular	Longi-tudinal	Circular	Longi-tudinal	Circular	Longi-tudinal
Jejunum	51.8 ± 1.42	34.8 ± 2.34	78 ± 7.7	45.7 ± 2.8	91± 8.9 *	45.5 ± 3.55	106± 23.3 ***	51.4± 12.1 **	121± 7.31 ***	63.2± 7.7 **	84± 8.32 *	55± 5.5 *
Ileum	51 ± 4.84	35.2 ± 5.55	67.7 ± 6.25	47.7 ± 3.15	78.3 ± 6.5	47.5 ± 5.81	127± 13 ***	68.9± 7.5 ***	110± 10 ***	59± 6 *	66.8 ± 5.37	48.4 ± 3.75

Data expressed in µm (means±SEM). *P<0.05, **P<0.01, *** P<0.001 compared to healthy animals; n=6. Post-infection (PI) is expressed in days.

Histopathological study: T. spiralis infection induced a mixed inflammatory infiltrate in jejunum with neutrophil and eosinophil cells. Cell infiltration was present in the jejunal mucosa and submucosa from 2-23 day PI with no signs of inflammation at the muscular layers. Severe inflammation scores were observed from 2-14 day PI and a mild inflammation was present on day 23 PI. No signs of inflammation were seen in the worm-free ileum throughout the experimental period. In contrast, *T. spiralis* infection provokes trophic changes of circular and longitudinal smooth muscle layers of both intestinal areas. Increased thickness of jejunal and ileal muscle layers was noted from day 2 PI until day 72 PI with the most prominent changes corresponding to tissues taken on days 14 and 23 PI (Table 1).

Responses to electrical field stimulation. Under NANC conditions every electrical stimulus (0.1 Hz, 0.3ms and 40V) resulted in a transient contraction followed by a relaxation in jejunum and ileum (Fig. 1). In *T. spiralis* infected rats a decrease in EFS induced relaxation of jejunum and ileum was observed. To unmask alterations due to hypertrophy from increases in motor responses due to changes provoked at the receptor or channel level, the results were expressed directly (in grams) and as percentage of the relaxation caused by SNP respectively. In infected rats the relaxation in response to 10^{-3}M SNP was

significantly increased from day 14-23 day PI in both jejunum and ileum (Fig. 2) and such increases were found to be parallel to those found in muscle thickness. The responses to EFS (expressed in grams and % of SNP) were significantly decreased on days 14-23 PI for ileal samples (Fig. 3).

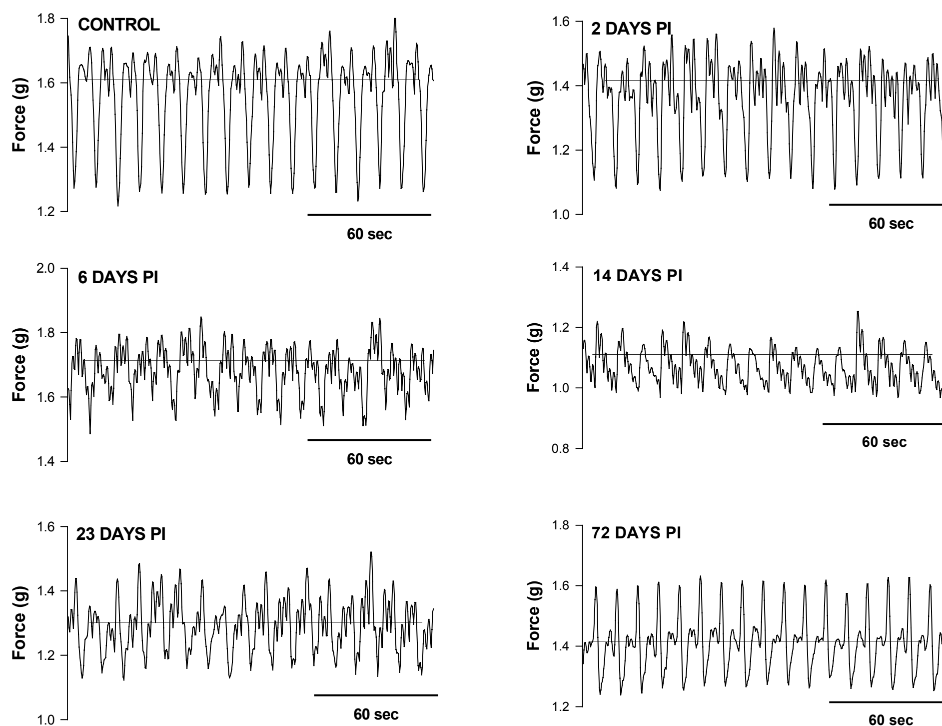

Figure 1. Representative recordings showing the responses of the rat longitudinal ileum to repetitive electrical stimuli (0.1 Hz, 0.3ms and 40V) in healthy tissue and tissue taken from infected animals during an experimental period of 72 days. Data are expressed in grams. The horizontal lines indicate the basal tension of the preparation.

TTX 10^{-6}M induced a significant decrease (70-80%) in relaxations of jejunal and ileal tissue samples taken from both healthy and infected rats. The presence of the NO donor SNP (10^{-3}M) did not result in significant changes in the EFS-induced relaxations neither in healthy controls nor in infected rats (Fig. 4).

In healthy tissue the presence of L-NNA 10^{-3}M or ODQ $3x10^{-6}$M significantly decreased the EFS-induced relaxations. During the post-infection period the inhibitory effects of L-NNA or ODQ in infected tissue were smaller due in part to the general decrease in relaxations in the post-infection period. In spite of this we could discriminate significantly smaller inhibitory effect of L-NNA and ODQ on day 14PI in jejunal- and from day 14-23PI in ileal specimens, in comparison with their own controls (Fig. 5). Combinations of L-NNA plus

ODQ as well as L-NNA plus apamin or ODQ plus apamin did not result in statistically significant additive reductions of the EFS-induced relaxation (Table 2)

The potassium channel blocker TEA 10^{-2}M provoked a four-fold increase in the relaxation induced by EFS, while TPEA 10^{-4}M decreased the EFS-induced relaxation in about 80% in both healthy ileum and jejunum. The addition of such drugs to tissue taken from *T. Spiralis* infected rats resulted in effects that were similar to those seen in healthy tissue (Table 3).

Table 2

Effect of combinations of L-NNA plus ODQ as well as L-NNA plus apamin to EFS-induced relaxation.

	Healthy		2 PI		6 PI		14 PI		23 PI		72 PI	
	L-NNA +ODQ	L-NNA + APA	L-NNA +ODQ	L-NNA + APA	L-NNA +ODQ	L-NNA + APA	L-NNA +ODQ	L-NNA + APA	L-NNA +ODQ	L-NNA + APA	L-NNA +ODQ	L-NNA + APA
Jejunum	36.2± 10	26.5± 8.3	38± 6.7	25.2± 5.8	28.4± 4.6	22.5± 3.5	30.3± 3.2	33.5± 2.1	21± 6.2	18± 3.6	13.5± 3.5	14.3± 2.8
Ileum	15± 3.3	18.2± 1.2	25.7± 5.6	23.7± 3.15	20± 3.38	17.5± 4.82	20.6± 3.26	19± 5.7	15± 6.2	16.8± 6.2	18± 5.37	14.3± 3.38

Effects of L-NNA 10^{-3}M plus ODQ $3x10^{-6}$M and L-NNA 10^{-3}M plus apamin 10^{-6}M on jejunal and ileal responses to EFS (40V, 0.1Hz, 0.3ms) in healthy and *T. spiralis* infected rats. Figures represent mean values ±SEM expressed as percentages of relaxation responses to 10^{-3}M SNP; n=8. Post-infection (PI) is expressed in days.

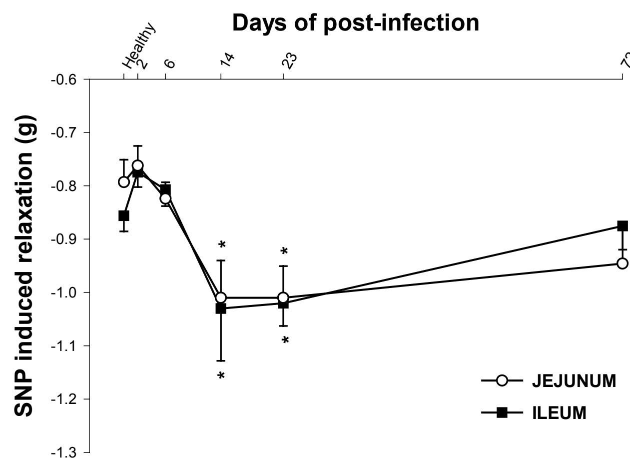

Figure 2. Relaxation in response to 10^{-3}M SNP displayed by jejunal and ileal segments of healthy and *T. spiralis*-infected rats on different days PI. Data are expressed in grams (mean ± SEM); n=6; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ indicates significant differences *vs.* healthy tissue taken from non-infected rats.

The small conductance Ca^{2+}-activated K^{+} channel blocker apamin (10^{-6}M) caused a marked decrease of the EFS-induced relaxation in both healthy jejunum and ileum. In tissue from infected animals the sensitivity of EFS induced relaxations to apamin disappeared from days 6-23 PI (Table 3).

Table 3

Sensitivity of the EFS-induced relaxation to drugs.

	%R/ SNP	Healthy	2PI	6PI	14PI	23PI	72PI
Jejunum	+ APAMIN	50±6.35 vs. 29±3.3 58% ***; n=8	68.4±13 vs. 30±5.6 43.8%***; n=8	69±15 vs. 57±12 n.s. ; n=8	63±13 vs. 51±11 n.s. ; n=7	56.4±6.3 vs. 55±13 n.s. ; n=7	46±2.5 vs. 25±7.1 54.3%*; n=9
	+ TEA	45±7.3 vs. 192±25 426%***; n=7	68±7.3 vs. 154±6 226%**; n=8	69±5.8 vs. 180±5 260%***; n=8	45.6±2.9 vs. 162±28 355%***; n=8	61±7 vs. 179±72 293%***; n=8	39±7.2 vs. 183±14 469%***; n=8
	+ TPEA	62.8±2.3 vs. 8.2±4.2 13%***; n=6	52±6.1 vs. 6.7±0.04 12.8%***; n=6	38.5±9 vs. 5.7±1.4 14.8%**; n=6	86.8±24 vs. 3.7±0.8 4.26%***; n=6	78±3.64 vs. 6.8±2.5 8.7%***; n=6	43±6.4 vs. 3.68±1.25 8.55%***; n=8
Ileum	+ APAMIN	48±5.62 vs. 26±4.9 54.1%***; n=9	58±9.1 vs. 20±1.46 34.4%*; n=7	43±11 vs. 39±9.4 n.s. ; n=8	24±8.2 vs. 25±6 n.s. ; n=9	29±3.5 vs. 26±3.9 n.s. ; n=8	39.2±4.9 vs. 29±2.1 73%**; n=9
	+ TEA	48.5±4.8 vs. 101±12 208%***; n=8	52±10 vs. 195±29 375%***; n=7	53±12 vs. 131±22 247%***; n=8	43±6 vs. 93±10 216%***; n=7	33±10 vs. 191±16 328%***; n=8	39±4.7 vs. 166±22 425%***; n=8
	+ TPEA	37±6.5 vs. 7.6±3 20%**; n=6	37.5±2.1 vs. 7±1.37 18.6%**; n=6	31±12 vs. 2.1±0.8 6.7%***; n=6	26±9 vs. 3.3±0.1 12.6%***; n=6	34±13 vs. 6.1±1.1 18%***; n=6	36±5 vs. 2.5±0.6 6.9%***; n=6

Values are given as residual relaxation when the indicated drugs were added (paired data). Residual responses were calculated whenever a significant difference was found between relaxation before and after drug addition. Number of repetitions (n). Post-infection (PI) is expressed in days. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared to control.

NADPH-diaphorase staining: LMMP preparations (devoid of the hypertrophied circular muscle layer) of jejunum and ileum showed a preserved network of NADPH-diaphorase-positive neurons (26±5 NADPH-positive neurons per preparation). In the LMMP preparations a well-organized network of NADPH-diaphorase-positive neurons was visible in specimens taken from both healthy and *T. spiralis* infected rats. No difference was observed in the distribution or the number of neurons in the tissues of healthy and infected rats on any day within the studied period.

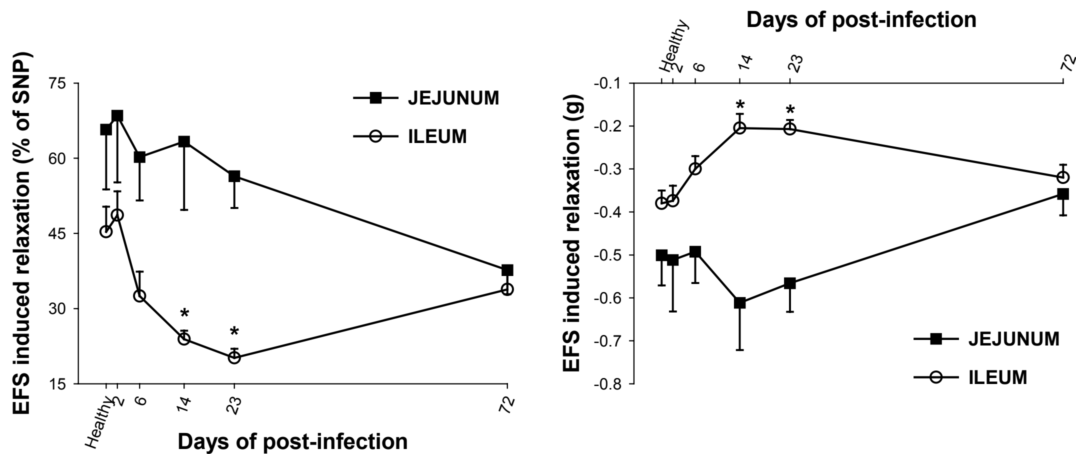

Figure 3. Time-course of the responses of jejunal and ileal samples to EFS (0.1 Hz, 0.3ms and 40V) during the experimental period. Figures represent mean values ±SEM expressed in percentage of relaxation induced by 10^{-3}M SNP (left) and in grams (right); n=7; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ indicates significant differences *vs.* healthy tissue taken from non-infected rats.

Discussion

The primary aim of this work has been to assess to what extent the occurrence of inflammation in the jejunum induces morphological and functional changes in this region and in other worm-free areas of the small intestine, and to study the time-course and the main features of these changes. The inflammation induced by *T. spiralis* is moderate, as the only remarkable symptoms are transient decreases in food intake and body weight. Thus, the *T. spiralis* infected rat has been assumed to be a good model mimicking situations of mild intestinal inflammation. When *T. spiralis* larvae are given orally, the parasite inhabits the duodenum and the jejunum but does not reach the ileum [8]. Thus, in our study, specimens of jejunum and ileum were taken as representative samples of inflamed worm-positive and non-inflamed worm-free segments, respectively. This consideration was based on our histopathological findings and is in agreement with the statement given by other groups that have used the same animal model [13, 14].

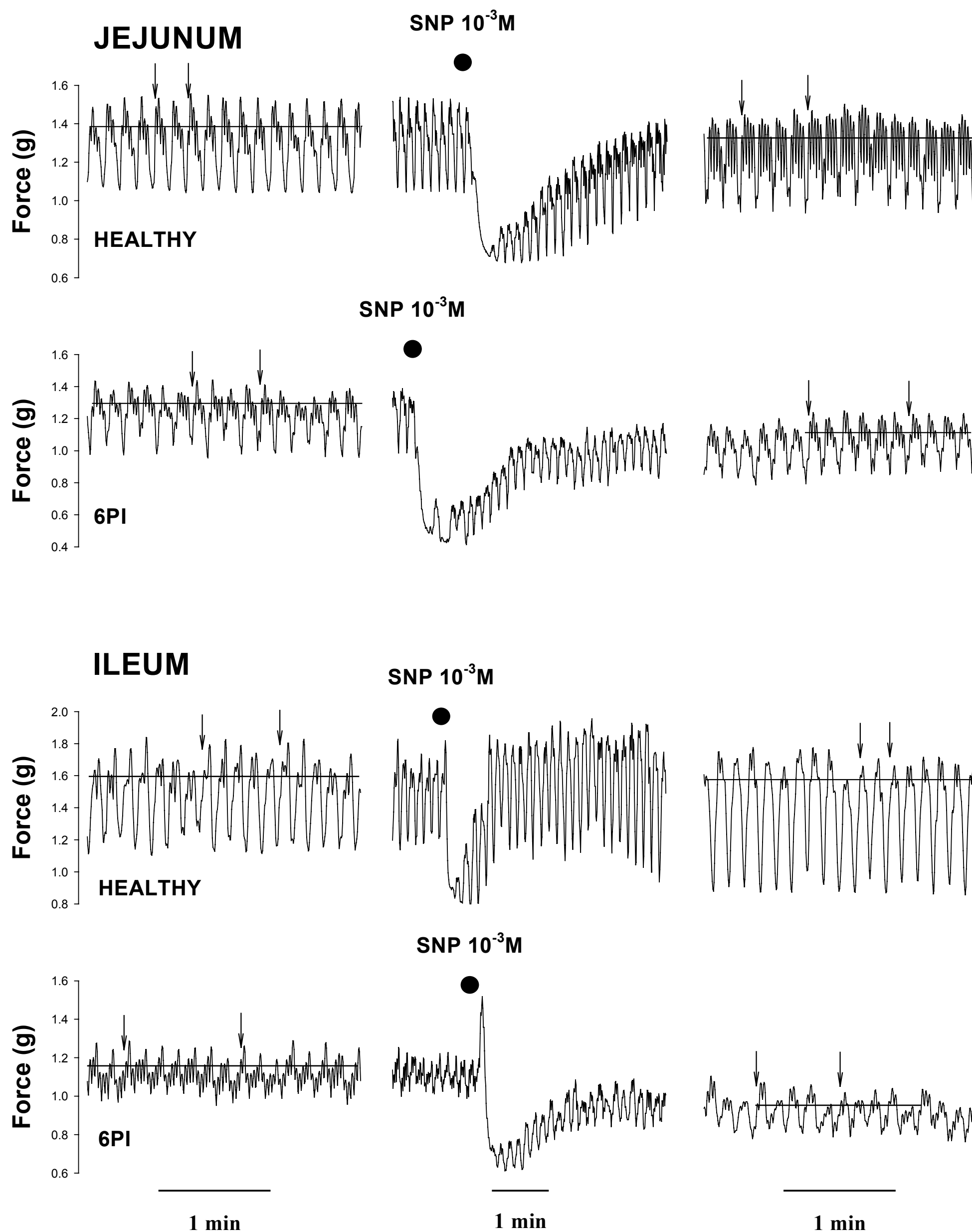

Figure 4. Recordings showing the responses of the rat longitudinal jejunum and ileum to repetitive electrical stimuli (40V, 0.1Hz, 0.3ms) before and after addition of 10^{-3}M SNP. Recordings correspond to responses exhibited by tissue taken from non-infected rats (healthy) and from infected rats on day 6 PI, which represents the peak of inflammation. Control responses of healthy and infected rats are shown on the left side. The central and right side of the recordings show immediate and steady response to the 10^{-3}M SNP. Steady responses were obtained after 5 min incubation. Arrows indicate some of the precise moments when electrical stimuli were given and the horizontal lines indicate the tone of the preparation. Dots indicate the addition of SNP.

Infection with *T. spiralis* generates restricted mucosal and submucosal inflammation in the jejunum and duodenum [13]. Indeed, mucosal inflammation is well correlated with the presence of parasites and remission of histopathological inflammatory evidence occurs soon after parasite eviction. In contrast, increased muscle thickness appears to be more generalized and lasts longer [22].

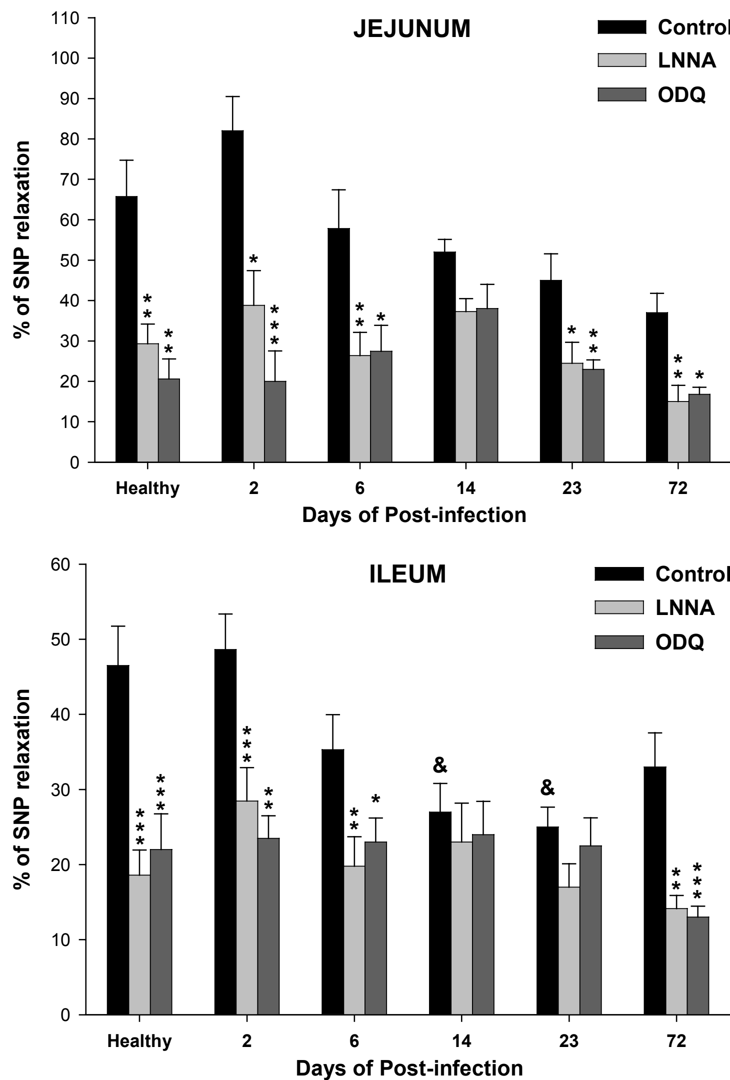

Figure 5. Effects of L-NNA 10^{-3}M and ODQ $3x10^{-6}$M on jejunal and ileal responses to EFS (40V, 0.1Hz, 0.3ms) in healthy and *T. spiralis* infected rats. Figures represent mean values ±SEM expressed as percentages of relaxation in response to 10^{-3}M SNP; n=8; & P<0.05 indicates significant differences between control responses in samples taken from healthy rats *vs.* control responses displayed by tissue from infected animals (different days of post-infection). *P<0.05; **P<0.01; ***P<0.001 indicates significant differences between responses in control conditions and in presence of L-NNA 10^{-3}M or ODQ $3x10^{-6}$M.

In the present study muscle thickness did not return to normal values until day 72 PI. Interestingly, both inflamed and non-inflamed areas undergo this thickening and both the longitudinal and the circular layer appear to be affected. In another study, significant hypertrophy and hyperplasia in both smooth muscle layers of rat jejunum associated with intestinal inflammation with *T. spiralis* has also been reported [23]. These findings indicate that even when the inflammatory reaction is primarily located in the mucosa and submucosa, a strong trophic response is initiated. This response may be attributed to locally released inflammatory mediators with powerful trophic effects on the underlying smooth muscle, which may account, at least in part, for the altered smooth muscle contractility.

There is evidence showing that functional changes are unexpectedly persistent after the remission of morphological evidence of inflammation, and in some cases remote areas that are not close to the inflammatory focus are also affected [13, 22, 24]. In NIH Swiss mice, intestinal smooth muscle contractility to carbachol and KCl was increased for more than 40 days after infection with *T. spiralis,* although mucosal morphology had rapidly returned to normality [25]. In the jejunum of *T. spiralis* infected rat, ^{3}H-norepinephrine release was significantly decreased for 100 days PI, showing persistent alterations with rapid onset and very slow recovery of neuromuscular function [26].

Many efforts have been devoted to describe changes caused by inflammation on contractile activity and also on excitatory neurotransmitter release. In contrast, there is little information about the consequences of inflammation on inhibitory responses. Gastrointestinal motility cannot be evaluated considering excitatory responses alone, as relaxation and contraction of the intestinal smooth muscle are coordinated to allow progression of intestinal contents. In our studies we have tried to discriminate the functional changes in the intrinsic innervation from those that are due to altered inputs from extrinsic innervation, i.e. changes in norepinephrine release. Thus, all our experiments were carried out in non-adrenergic non-cholinergic conditions. The rat ileum has been shown to relax upon application of appropriate electrical stimuli (0.1 Hz, 0.3 ms, 40 V), a response that has already been characterized in healthy rat intestine [27]. In this preparation, incubation with the NO synthase inhibitor LNNA or with the soluble guanylate

cyclase inhibitor ODQ results in a marked- though incomplete - reduction of relaxation. Thus, relaxation is mainly due to the release of a nitrergic transmitter, which in turn targets the soluble guanylate cyclase of the smooth muscle cells and provokes relaxation. It must be pointed out that guanylate cyclase activation is not the only mechanism leading to relaxation. For instance some NO donors have been shown to cause relaxation independently of this mechanism [27]. In any case the response to exogenously added NO donors has often been used as a standard relaxation, in the same way that the response to KCl has often been used as a standard to refer contractile responses [28].

We have studied the relaxation that follows addition of SNP, a NO donor, throughout the post-infection period and have found that the response is not steady. There is an increase in the relaxation elicited by this substance, particularly between days 14-23. This increased relaxation is found for both jejunum (inflamed area) and ileum (non-inflamed area). Regarding EFS-induced relaxation, which has been found to be markedly reduced in the post-infection period, a different situation was found in jejunum compared to ileum. We found that in the jejunum the reduction in EFS induced relaxation was not significant. In contrast, in the ileum there was a much more pronounced reduction in the EFS-induced relaxation despite the fact that the response to exogenously added SNP is significantly increased. As shown in figure 5, during the days in which a more pronounced reduction (14 and 23 PI) of EFS-induced relaxation was found, the sensitivity of such response to both ODQ and L-NNA was lost. Thus, smooth muscle responsiveness to NO is increased (the relaxation caused by SNP is more pronounced) but the release of endogenous nitrergic transmitter is impaired (the EFS induced relaxation is less pronounced and insensitive to LNNA and ODQ). As no additive effects were seen when the tissue (from healthy as well as infected animals) was incubated with ODQ and L-NNA simultaneously, we assumed that the nitrergic transmitter released from nerves targets exclusively the soluble guanylate cyclase in the smooth muscle. This is in good agreement with results obtained in the rat stomach, where ODQ reverses the relaxation induced by EFS [29].

Another interesting finding was that TEA, a widely used specific non-selective K^+ channel blocker, actually causes a marked increase in the EFS-induced relaxation and a marked smooth muscle cell hyperpolarization. Similar results have been found in the rat [30] and the guinea pig [31] colon. In these preparations it has been shown that TEA induced

smooth muscle hyperpolarization is due to neuronal depolarization resulting in massive inhibitory transmitter release, as shown by the fact that TTX prevents such smooth muscle hyperpolarization (unpublished data). So, TEA may depolarize inhibitory neurons and provoke smooth muscle relaxation [30]. In contrast, the TEA analog TPEA causes depolarization and blockade of the IJP, acting preferentially on smooth muscle [30]. Its ability to cause smooth muscle depolarization and a complete blockade of EFS induced relaxation is in agreement with the results shown here.

Regarding apamin, a selective blocker of the small conductance Ca^{+2}-activated potassium channels, it provoked a significant decrease of EFS induced relaxation. During inflammation the sensitivity of EFS induced relaxation to apamin is lost from day 6 to day 23 for both jejunum and ileum. These changes suggest that functional changes in calcium activated potassium channels contribute to some extent to the impaired relaxation found during the post infection period or alternatively that the release of inhibitory transmitters which exert their effects through such channels is impaired.

It has been described that in some preparations such as the rat gastric fundus, incubation with nitric oxide donors may result in a prejunctional inhibition of nitric oxide synthase activity [32]. This mechanism could be considered as a feedback regulation of nitrergic transmitter release. However, we have not found evidence of the existence of such a mechanism in the rat jejunum or ileum, since SNP did not change the relaxation provoked by EFS in both healthy and infected animals.

It has also been suggested that NO itself might act as inflammatory mediator [33]. Cytokines or bacterial lipopolysaccharides are able to stimulate inducible nitric oxide synthase (iNOS) expression, which may result in the synthesis of cytotoxic amounts of NO [33, 34]. The expression of iNOS occurs early after exposure to the inflammatory stimulus. Thus, increased immunoreactivity to iNOS has been found in intestine as early as 2h after oral administration of *T. Spiralis* larvae (Torrents and Vergara; submitted). In contrast, no changes in NADPH-*diaphorase* staining were found when the animals were sacrificed on 2-72 days PI. This suggests a prompt remission of increased iNOS activity despite the presence of the enzyme for longer periods. Thus, decreased inhibitory response to EFS in the post-infective period might be even attributed to a decreased release of NO from

nerves but not to a decreased responsiveness of smooth muscle to NO. The fact that the NADPH diaphorase staining does not appear to be altered and the reported increase in the relaxation caused by exogenously added NO donor supports this view.

In summary, our results have provided evidence that changes provoked by *T. spirals* infection occur in both inflamed worm-positive and non-inflamed worm-free tissue. The most prominent feature found is a long-lasting inhibition of the small intestine relaxations in response to EFS, suggesting an impaired neural function. The sensitivity of the response to L-NNA and ODQ suggests an impaired release of nitrergic transmitter. The altered sensitivity to apamin may be suggestive of an impairment of the release of other inhibitory neurotransmitters or of a functional change affecting small conductance calcium activated potassium channels present in smooth muscle cells. Finally, though jejunal and ileal segments exhibited similar increases in smooth muscle layer thickness, the most prominent functional motor changes were found in the non-inflamed ileum of *Trichinella spiralis* infected rats.

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DISCUSSION

A wide variety of NO donors have been used for the investigation of different biological processes that are assumed to be triggered by nitrergic neurotransmitter. Thus, we first analysed the kinetics of NO release and the effects induced by two NO donors, sodium nitroprusside (SNP) and 3-morpholino-sydnonimine-hydrochloride (SIN-1). Our primary aim here was to establish the most appropriate choice of exogenous NO source.

We observed that the responses to SNP and SIN-1 are not similar. Both SNP and SIN-1 cause biphasic responses but with opposite sequence of events. SNP elicits first a rapid contraction and then a longer lasting relaxation, similar to the response induced by electrical field simulation (EFS) and freshly dissolved NO (Barthó *et* Lefebvre, 1995). In contrast, SIN-1 provokes first a relaxation and then a contraction. We also demonstrated that SNP acts exclusively at post-junctional level, affecting only the smooth muscle cells. SNP-induced contraction involves L-type Ca^{+2} channel opening, an action that takes place earlier than the increase in cGMP, which is responsible for the relaxation. This result is also supported by data obtained by other groups, which show that the NO-induced contraction in the rat small intestine is related to extracellular calcium influx trough L-type Ca^{+2} channels, that might be activated in response to the closure of small conductance Ca^{+2}-dependent K^+ channels (Lefebvre, 1997). The strong inhibition of SNP-induced relaxation in the presence of soluble guanylate cyclase inhibitors demonstrated the implication of cGMP pathway. This is in good agreement with results obtained in the rat gastric fundus where the relaxation induced by endogenous and exogenous NO was also due to activation of soluble guanylate cyclase (Lefebvre, 1998).

In contrast, SIN-1 provokes a mixed muscular and neural effect by affecting both the enteric nerves and the smooth muscle cells. We observed first a quick cGMP-independent relaxation, which involves activation of K^+ channels, particularly, the small conductance calcium activated K^+ channels. The fact that atropine and/or TTX provoke a decrease in the contractile component induced by SIN-1, clearly demonstrates that ACh release participates in SIN-1-induced contraction. The neural blocker TTX decreases both the relaxation and the contraction induced by SIN-1 providing additional support to the concept

that this substance has significant neural effects.

Thus, the opposite sequence of events in responses to SNP and SIN-1 may be due to different mechanisms in their responses. SNP-induced contraction (L-type Ca^{+2} channels) occurs prior to relaxation (cGMP increase) and SIN-1 provokes first a quick and fading relaxation by activation of K^{+} channels followed by ACh release which, may explain the last-longer contraction (Figure 1). In view of the responses to SNP and the endogenous nitrergic transmitters in terms of temporal sequence of events and drug sensitivity, we concluded that SNP is more appropriate tool for mimicking the response of the endogenous nitrergic transmitter in the intestinal smooth muscle then SIN-1 (Figure 1).

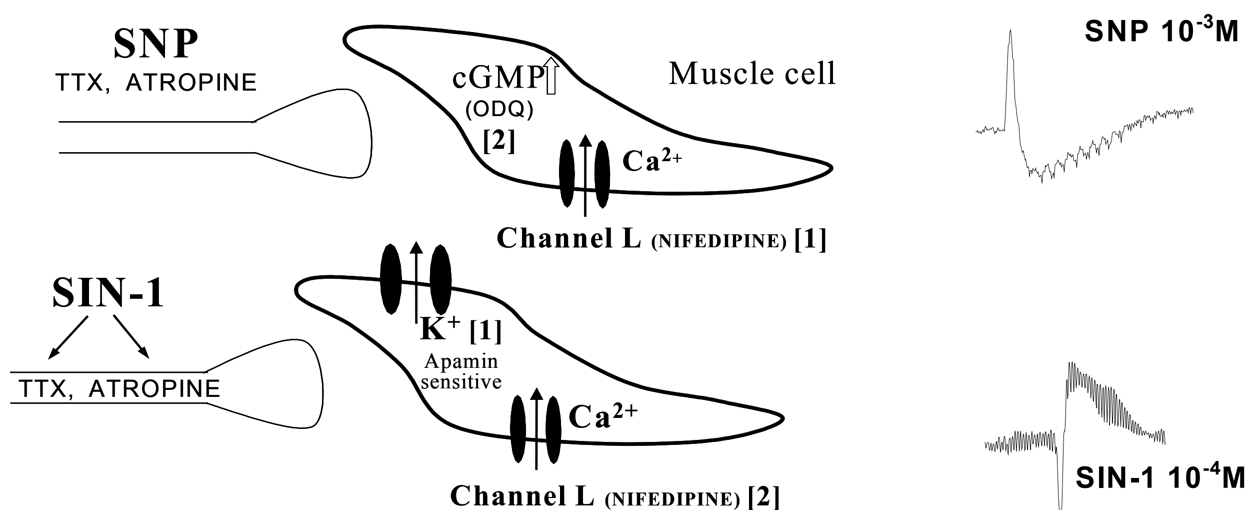

Figure 1. Mechanisms implicated in mechanical responses to sodium nitroprusside (SNP) and 3-morpholino-sydnonimine-hydrochloride (SIN-1). SNP induces first a contraction by the activation of L-type nifedipine sensitive Ca^{+2} channels **[1]**, followed by the relaxation that involves the increase of cGMP **[2]**. In contrast, SIN-1 provokes first a relaxation, which involves activation of apamin sensitive K^{+} channels **[1]**, followed by the contraction, which, at least partially, implicates ACh release from nerve endings and L-type Ca^{+2} channel activation **[2],** showing its muscular and neural effect.

A review of the literature evidences that responses to NO donors are strongly dependent on the chemical entity of the donor. Moreover, marked tissue to tissue and species to species variations have been reported (De Man *et al.*, 1995 [b]; Ekblad *et* Sundler, 1997). For instance, it has been described that in the rat ileum both relaxation and contraction induced by SNAP are dependent on the activation of soluble guanylate cyclase (Ekblad *et*

Sundler, 1997). In addition, it was reported that SNAP and other nitrosothiols as, S-nitroso-L-cysteine and S-nitrosoglutathione, induced relaxation of the canine ileocolonic junction is pharmacologically different from the NANC nerve-mediated relaxation (De Man *et al*., 1995 [b]). There is also increasing evidence that NO-related compounds may act not only at the soluble guanylate cyclase level but also they can modify membrane structures, such as ionic channels and receptors, independently of cGMP activation. Bolotina *et al* presented evidences that NO directly activated charybdotoxin-sensitive K^+ channels in cell-free membrane patches of rabbit aorta (Bolotina *et al*., 1994). One of the mechanisms which could mediate cGMP-independent NO-induced relaxation is hyperpolarization of smooth muscle cells by a direct activation of multiple K^+ channels. Koh *et al* demonstrated that NO can directly, and independently of cGMP, activate at least two types of K^+ channels (K_{NO1} and K_{NO2}) in colonic smooth muscle (Koh *et al*., 1995), while Janssen *et al* demonstrated that in the human mainstream *bronchi* NO^+ (but not $NO^\bullet$ radical) causes release of Ca^{2+} in a cGMP-independent manner, leading to activation of Ca^{2+}-activated K^+ channels (Janssen *et al*., 2000). Thus, the concept that soluble guanylate cyclase activation is not the common clue to understanding all actions of NO. Additionally, it widely accepted that NO donors and other inhibitory mediators may induce opposite effects on quiescent or precontracted tissue (Fernández *et al*., 1998). The fact that the effects of NO donors are commonly tested on precontracted preparations may be an explanation for the observed differences in the response to various NO donors. In contrast, the present study reproduced biphasic responses to SNP and SIN-1 with the same sequence of events both at a basal tension and in a precontracted state of the longitudinal muscle of the rat ileum, in good agreement with the results reported by Ekblad *et* Sundler (Ekblad *et* Sundler, 1997; Tanovic *et al*., 2000).

We also examined the kinetics of NO released by SNP and SIN-1 under conditions similar to those under which the functional studies were performed. This was carried out by measuring the nitrite levels according with the Griess method. As the half-life of NO in aqueous solutions is very short (3-5s) and the fact that authentic NO in the presence of oxygen it is converted almost exclusively in nitrite (Feelisch, 1991; Kelm *et al*., 1988), the measure of nitrite levels provides a good estimate of the time course of NO release from NO donors. Our observation was that the two NO donors tested are completely different regarding NO release kinetics, as SNP provides a high concentration of NO in a short

time, whereas SIN-1 release NO gradually. Regarding the result obtained with SNP, we previously demonstrated that incubation of rat colonic circular muscle with SNP results in a hyperpolarization and a cessation of the spontaneous cyclic mechanical activity (Pluja *et al*., 1999). These effects appear immediately upon addition of SNP and do not fade, unless a washout is performed.

A causal link has been suggested between NO overproduction and the occurrence of functional changes in contractility during inflammation. NO overproduction has been documented in inflammation and has been proposed to be one of the major events leading to functional changes (Hogaboam *et al*., 1995; Goldhill *et al*., 1995; Levine *et al*., 1998; Miampamba *et* Sharkey, 1999). For instance, in the model of TNBS-induced colitis in rat high NO production has been considered as a causal factor for the observed reduction in KCl-induced contraction (Mourelle *et al*., 1996). Thus, in this study we tried to determine whether the mere occurrence of high NO concentrations might cause the decreased contractility displayed by the tissue. After short- and long-lasting incubations with NO donors, we checked the contractile responses to KCl, acetylcholine (ACh) and substance P (SP). It is well known that KCl initiates smooth muscle contraction by membrane depolarisation and activation of calcium influx through voltage-sensitive channels on the plasmalemma without mobilization of intracellular calcium. Thus, the contraction induced by KCl was abolished in presence of nifedipine. The contraction induced by ACh and SP results from activation of muscarinic and NK1receprotrs respectively. The rise in intracellular calcium required for the contraction in response to muscarinic and NK1 tachykinin receptor activation is provided both by calcium influx through L-type Ca^{2+} channels and by calcium release from intracellular calcium stores (Maggi *et* Giuliani, 1995; Patacchini *et al*., 1997; Sims *et* Jansen, 1993). In spite of the fact that NO affects mechanisms potentially important for muscle contraction such as membrane potential, guanylate cyclase activity, L-type calcium channel and potassium channel activity (Sanders *et* Ward, 1992), we did not find any substantial change in the responses to KCl, ACh or SP in any of the functional experiments performed. As neither SNP nor SIN-1 were able to affect measurably the contractile machinery of the rat longitudinal ileal muscle implicated in responses to these agonists (Figure 2), we concluded that an increased NO level is not the causal factor of altered muscle function during inflammatory states (Tanovic *et al*., 2000). This result gets further support from other work, in which it is shown that

incubation of the rat ileum for long periods with interleukin-1-ß inhibits ACh-induced contractions independently of the rate of NO synthesis (Aubé *et al.*, 1996). Baccari *et al* have also shown that muscular contractions evoked by ACh were not influenced by SNP, although SNP was able to depress EFS-elicited cholinergic contractions, suggesting a possible neuro-modulatory role for NO (Baccari *et al.*, 1995). Others studies provide additional support to this result demonstrating that the induction of iNOS by bacterial lipopolysaccharide is unable to modify cholinergic contractions and IP_3-related calcium release (Ando *et* Daniel, 1993).

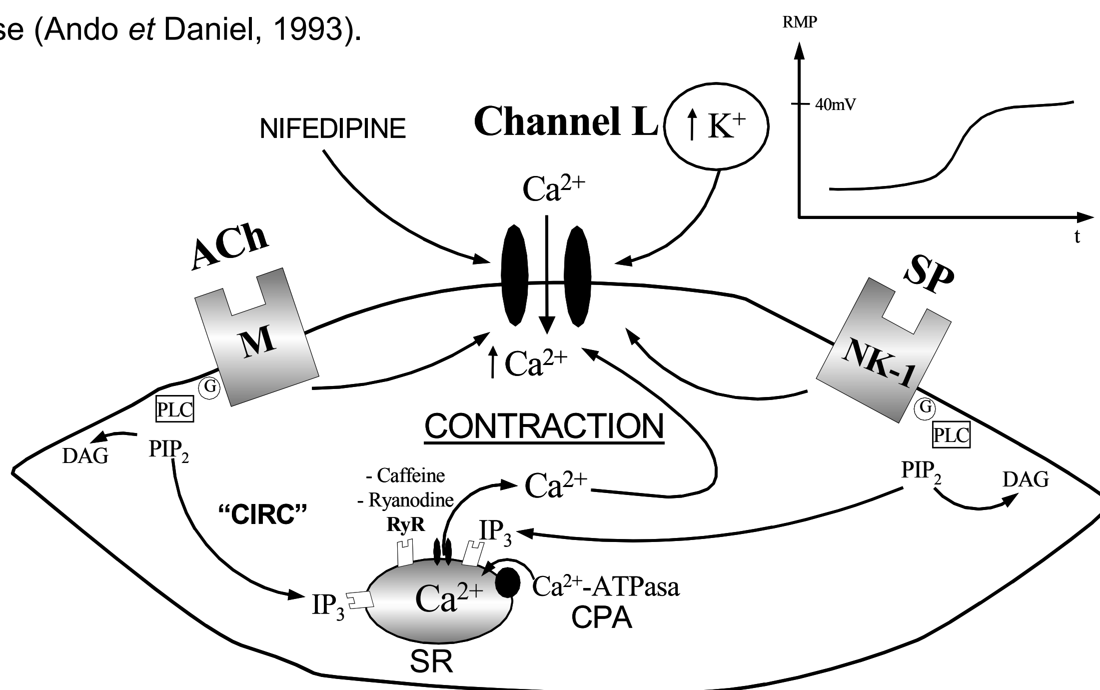

Figure 2. Different pathways involved in KCl, acetylcholine (ACh), substance P (SP) and cyclopiazonic acid (CPA)-induced responses in intestinal smooth muscle cell. KCl initiates smooth muscle contraction by the membrane depolarization (abolished by nifedipine) and Ca^{2+} influx trough voltage-sensitive channels. ACh and SP activates muscarinic (M) and NK1 receptors respectively. Activation of muscarinic and NK1 receptors induce **[1]** depolarization, that in turn activates L-type voltage-sensitive Ca^{2+} channels (extracellular Ca^{2+} influx) and **[2]** G protein mediated activation of phospholipase C (PLC), which leads to generation of two second messengers: diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which induces mobilization of Ca^{2+} from the sarcoplasmic reticulum (SR). In addition, Ca^{2+} itself opens ryanodine receptors in SR and triggers Ca^{2+}-iduce-Ca^{2+}-release (CIRC) process. CPA depletes intracellular Ca^{2+} stores by blocking the Ca^{2+}-ATPase in SR and inhibiting Ca^{2+} pumping into the SR. RMP - resting membrane potential.

The sarcoplasmic Ca^{2+}-ATPase blocker CPA induces a strong, immediate and sustained tonic contraction of the rat longitudinal ileal muscle. When the tissue was preincubated

with nifedipine, CPA could not reach a maximal contraction. Similarly, at the top of CPA-induced contraction, the addition of nifedipine caused partial and sharp decrease of the contraction. These data suggest the strong dependence of the CPA-induced contraction on extracellular Ca^{2+}. Regarding the type of Ca^{2+} channels involved in the refilling of intracellular Ca^{2+} stores, Dessy and Godfraind have shown that blockade of L-type Ca^{2+} channels inhibits changes in $[Ca^{2+}]_i$ after mobilization of intracellular Ca^{2+} stores by histamine, caffeine, ryanodine or thapsigargin in the guinea-pig ileal smooth muscle (Dessy *et* Godfraind, 1996). According by the so-called *capacitive calcium entry*, the depletion of intra-cellular Ca^{2+}stores provides a signal for activation of calcium influx through the plasma membrane (Berridge, 1995; Petkov *et* Boev, 1996 [a]; Petkov *et* Boev, 1996 [b]). For instance, Petkov *at al* also observed that nifedipine causes a sharp relaxation during the sustained contraction induced by CPA or thapsigargin (Petkov *et* Boev, 1996 [a]; Petkov *et* Boev, 1996 [b]). However, Ohta *et al* have reported that emptying of intracellular calcium stores may also activate calcium influx not associated to voltage-dependent Ca^{2+}channels in ileal smooth muscle of the rat (Ohta *et al*., 1995). Other groups have also shown that in cells isolated from the mouse anococcygeus muscle, CPA activates a transient calcium-dependent chloride current as a consequence of Ca^{2+} release from intracellular stores. This current may depolarize the cells and lead to the opening of voltage-operated L-type Ca^{2+} channels (nifedipine-sensitive component of muscle contraction) and non-selective cation conductance (nifedipine-insensitive component of Ca^{2+} entry) (Wayman *et al*., 1996).

While in our case preincubation with SNP did not modify CPA-induced response, in the cat gastric fundus preincubation with 1mM SNP or 1mM SIN-1 inhibited CPA-induced contraction. In contrast, data obtained in our lab and by Petkov *et al* indicate that the addition of SNP at the top of the CPA-induced contraction inhibits completely CPA-induced tonic contraction (Petkov *et* Boev, 1996 [a]; Petkov *et* Boev, 1996 [b]). These finding may suggest that CPA and NO-releasing agents (or cyclic GMP) interact on the same target - intracellular Ca^{2+} or the sarcoplasmatic reticulum Ca^{2+}-ATPase. Another study has reported that the EFS-induced inhibitory NANC responses (mediated by NO) of the rat anococcygeus muscle were diminished following exposure to CPA (Raymond *et al*., 1995). Thus, this may suggest that NANC relaxing response is associated to a decrease in intracellular Ca^{2+} concentration, which is generally achieved by uptake of Ca^{2+} into the SR.

Conversely, depletion of intracellular Ca^{2+} stores may attenuate the effect of NO, suggesting an involvement of functional Ca^{2+} stores in NO-induced response. Thus, in the rat ileal segments depletion of intracellular Ca^{2+} stores by CPA and carbachol strongly inhibit relaxations induced by SIN-1 and cGMP analogs (Franc *et al*., 1998).

Concerning the experiments in which the excitatory or inhibitory responses of the small intestine were induced by electrical field stimulation (EFS) some interesting findings have been observed. Excitatory responses in the jejunum and ileum were obtained by application of repetitive stimuli of 10Hz, 0.3 ms, 40V with duration of 15 s that resulted in a transient relaxation followed by a contraction. In the jejunum and ileum the contractile response induced by EFS is mainly due to ACh and SP release from enteric motor neurons. The incubation with the nitric oxide donor SNP 10^{-3}M did not modify the responses to EFS.

Electrically induced inhibitory responses were performed always under NANC conditions by application of repetitive electrical stimuli with following parameters: 0.1Hz, 0.3 ms, 40V. Under the above mentioned conditions, every electrical stimulus resulted in a transient contraction followed by a relaxation. Incubation with the NO synthase inhibitor L-NNA or with the soluble guanylate cyclase inhibitor ODQ results in a marked reduction of EFS-induced relaxation. Thus, we concluded that relaxation is mainly due to the release of a nitrergic transmitter, which in turn targets the soluble guanylate cyclase in the smooth muscle cells and provokes relaxation. This is in good agreement with results obtained by Lefebvre in the rat stomach, where ODQ reverses the relaxation induced by EFS (Lefebvre, 1998). The existence of a L-NNA resistant component of the EFS-induced relaxations led us to consider the existence of other transmitter/s. The fact that the small conductance Ca^{2+}-activated K^{+} channel blocker apamin decreased the EFS-induced relaxation gave support to this concept. Combinations of apamin + LNNA or apamin + ODQ were used in an attempt to unmask the presence of two different transmitters, but no additive effects were found. Regarding K^{+} channel blockers, we found that TEA, a specific and non-selective K^{+} channel blocker, actually causes a marked increase in the EFS-induced relaxation. Similar results have been reported in the rat colon (Plujà, 2000). In both tissues the increased relaxation has been due to a neuronal effect of TEA as preincubation with TTX reverted the enhanced relaxation observed in the presence of TEA

alone. TEA seems to be more effective on enteric neurons than on smooth muscle cell, and may cause a depolarisation of inhibitory neurons and in turn a smooth muscle relaxation. In contrast, the TEA analogue TPEA causes depolarisation and blockade of the inhibitory junction potential (IJP) in the rat colon (Plujà, 2000), showing that TPEA acts preferentially at the post-junction level. Thus, its ability to cause smooth muscle depolarisation and a complete blockade of EFS induced relaxation is in agreement with the results shown here.

It has been reported that exposure of the rat gastric fundus to NO donors may result in feedback inhibition of NO synthase activity and/or prejunctional inhibition of NANC relaxations without affecting the postjunctional response to NO (De Man *et al.*, 1995 [a]). NO is thought to bind with high affinity to the heme group of NO synthase, forming a stable complex with a low dissociation constant (Tsai, 1994). The feedback inhibition of NO release might function as a brake on the NO release from nerve endings, allowing a fine regulation of neurotransmission and preventing excessive release of toxic amounts of NO. However, we did not found any evidence of such mechanism in the rat ileum, since long incubations with SNP and SIN-1 did not change the relaxation provoked by EFS. This is in agreement with other studies, which did not found evidence for the prejunctional inhibition of neuronal NOS by NO in the pig gastric fundus (Lefebvre *et* Vandekerckhove, 1998).

Morphological and functional alterations have been associated with intestinal inflammation in the *Trichinella spiralis* infected rats. In this model histopathological signs of inflammation were restricted to the mucosal and submucosal layers of the jejunum. Neither the ileum nor the muscular layers of the jejunum were affected. Thus, mucosal inflammation seems to be well correlated with the presence of parasites as remission of histopathological inflammatory evidences occur soon after parasite eviction on day 17-23 PI. However, in both inflamed and non-inflamed areas, thickness of the longitudinal and the circular muscle layer appears to be affected. The structural changes of the small intestine muscle layers after *T. spirals* infection resulted to be unexpectedly persistent and similar in both inflamed worm-positive and non-inflamed worm-free segments. This indicates that an inflammatory reaction primarily located in the mucosa and submucosa has a powerful trophic and mitogenic effect on the underlying smooth muscle, which may account, for the altered smooth muscle contractility. The occurrence of functional changes implicates that

an inflammatory process restricted to the mucosa and the lamina propia can influence the release of neurotransmitters from enteric neurons. As the enteric nervous system plays an important role in the organization and propagation of motor activity, it is likely that localized mucosal inflammation can result in altered gastrointestinal motility over more extensive segments.

Blennerhassett *et al* have reported that both hyperplasia and hypertrophy, associated with *T. spiralis* infection, are responsible for increased smooth muscle thickness of rat small intestine (Blennerhassett *et al*., 1992). The presence of oedema, production of extracellular matrix or increase in collagen were not significant components of the apparent increase in tissue mass (Blennerhassett *et al*., 1992; Weisbrodt *et al*., 1994). An increase in total smooth muscle-related protein specially argues against oedema as a primary factor in increased muscle thickness. In addition to the increased muscle mass there is also an increase in contractile protein concentration (Weisbrodt *et al*., 1994). The rapid onset and very slow recovery of muscle thickness may exaggerate functional motor responses and amplify the effect of excitatory stimuli masking changes at the receptor level such as down regulation of receptor proteins (Blennerhassett *et al*., 1992; Weisbrodt *et al*., 1994).

To find out whether the changes in intestinal contractility during *Trichinella spiralis* infection are agonist specific, affecting receptor mediated mechanisms, or are due to non-specific general smooth muscle damage we intended to discriminate motor changes related to muscle thickness from those related to alterations at the receptor level in response to different agonists. The excitation-contraction coupling pathway of KCl-induced contraction seems to be unaltered fallowing *T. spiralis* infection as we found that the changes in thickness of muscle layer in jejunum and ileum correlated with the magnitude of KCl-induced contraction. In view of the relationship found between increase in contractility to KCl and muscle thickness, we used the response to KCl as a reference response to normalize contractility data of ACh- and SP-induced responses (%RR). In some models, it has also been reported that inflammation may result in an increase in actin and myosin synthesis, so we cannot exclude that some of the observed changes could result from increased contractile protein content in smooth muscle (Weisbrodt *et al*., 1994).

Regarding the responses to excitatory substances as ACh and SP, we noticed that increases in KCl- and ACh-induced responses bearded a significant correlation with changes in smooth muscle thickness, suggesting that the increase in the response is probably due to the increased muscular mass. This result is in agreement with reports indicating that there is an increased responsiveness of jejunal longitudinal muscle to the muscarinic agonist carbachol and 5-HT in *Nippostrongylus barsiliensis* and *Trichinella spiralis* infected rats (Farmer *et al*., 1983; Fox-Robichaud *et* Collins, 1986; Vermillion *et* Collins, 1988). These results support the hypothesis that changes in intestinal smooth muscle arise as a result of the inflammatory process and contribute to the increased propulsive activity of the intestine associated with the enteric parasitism. In contrast to these findings, Marzio *et al* have reported that worm-free ileal segments of *T. spiralis* infected rats display decreased contractility to ACh and 5-HT compared with control, illustrating regional differences in muscle responses to the infection and the fact that the presence of the parasite in the gut lumen is not required for the observed changes (Marzio *et al*., 1990).

Though the increases in tension developed in response to ACh were correlated to those in response to KCl, we observed significant decreases in the response to SP. E_{max} values for SP expressed as %RR were found to be significantly lower in both jejunal and ileal samples. The fact that SP response expressed in grams showed significantly increased E_{max} values on day 23 PI in both tissues could be explained by the mixed influence of a decreased contraction in response to SP, possibly provoked by alteration of SP receptor sensitivity, counterbalanced by the increased muscle thickness. Temporal changes observed in the pD_2 values for SP suggest that inflammation also affects the receptor affinity or may cause changes involving the interaction between agonist-receptor and/or its effector system. Similarly, in TNBS-induced colitis in the rabbit the inhibition in the response to ACh parallels the decrease in response to KCl, whereas the response to SP and motilin decrease more slowly (Depoortere *et al*., 1999). Swain *et al* using model of *T. spiralis*-infected rats have shown that inflammation increases SP immunoreactivity (SP-IR) in myenteric nerves (LM-MP) of the jejunum through a mechanism that involves T-lymphocytes, though no increase in SP-IR was found in the worm-free and uninfected ileum (Swain *et al*., 1992). In contrast, in the study by Miller *et al* ileitis caused by TNBS in guinea pigs is associated with a marked and sustained reduction (30 days) in mucosal and

submucosal substance P content which parallels the inflammatory response (Miller *et al*., 1993). The mechanisms and consequences of substance P alteration during inflammation still remain to be determined.

Regarding EFS-induced contractions of intestinal segments of rats infected with *T. spiralis* the most remarkable signs - long-lasting contractile decreases, were found only in non-inflamed and worm-free regions of ileum. Thus, while in the jejunum the responses to EFS were decreased only on day 2 PI, the contractile responses in non-inflamed ileum were decreased throughout the whole study period. These findings show that transient mucosal inflammation may alter enteric neuromuscular function, which may persist long after mucosal recovery (Barbara *et al*., 1997). Taking into account the fact that suppression of norepinephrine or SP release was not seen in non-inflamed ileum of *T. spiralis* infected rats (Swain *et al*., 1991; Swain *et al*., 1992) the impaired release of cholinergic neurotransmitter might possibly underlay the observed changes. Indeed, Collins *et al* have reported that response induced by KCl or veratridine on muscle of *T. spiralis* infected rats is accompanied by a suppression of ^{3}H-ACh release in rat jejunum and ileum. This decrease was found to be fully reverted after 40 days PI, whereas EFS-induced responses under same conditions still remained at 50% of control values (Collins *et al*., 1989). In *T. spiralis*-infected NIH Swiss mice small intestinal contraction induced by EFS of intramural nerves was decreased by 60%, while carbachol- and KCl-induced contractions of longitudinal muscle were threefold increased (Barbara *et al*., 1997). Whereas in our study EFS-elicited contractions under NANC conditions in jejunal samples did not differ significantly from controls, a marked decrease was found in ileal tissue. Reduced NANC contractions in the ileum may possibly result from increased release of SP during the PI period which results in SP-receptor desensitisation (Goldhill *et al*., 1995). Alternatively, an increased bioavailability of SP resulting from a marked down-regulation of its clearing enzyme endopeptidase (Hwang *et al*., 1993) during inflammation might also contribute to observed changes.

In addition, it has been reported that NO was able to depress the electrically evoked release of [^{3}H]-acetylcholine acting presinaptically, suggesting a possible neuro-modulatory role of NO (Hebeiβ *et al*., 1996). However, we have not found evidence for the existence of such a mechanism in the rat jejunum or ileum, since incubation with SNP did

not change the contraction provoked by EFS in tissues from both control and infected animals.

While many studies have been devoted to describe changes in contractile activity or changes in excitatory neurotransmitter release caused by inflammation little is known about the consequences of inflammation on inhibitory responses. Thus, we studied EFS-induced relaxations in tissue from *T. spiralis* infected rats during a post-infection period of 72 days. Although EFS-induced relaxation responses were reduced in the post-infection period the changes found in jejunum were markedly different to those found in the ileum. In the non-inflamed ileum we observed a much more pronounced reduction in the EFS-induced relaxation, similarly to changes observed in EFS-induced contraction, in spite of the fact that the responses to exogenously added SNP was significantly increased. Thus, we concluded that decreased inhibitory response to EFS in the post-infective period might be attributed to a decreased release of NO from nerves but not to a decreased responsiveness of smooth muscle to NO (Tanovic *et al*., 2002). The fact that the localization of NOS activity by NADPH diaphorase staining does not appear to be altered and the reported increase in the relaxation caused by exogenously added NO donor supports this view. Crosthwaite and co-workers have also reported a significant reduction of EFS-induced relaxation of jejunal circular smooth muscle taken from *N. brasiliensis*-infected rats (Crosthwaite *et al*., 1990). During marked reduction in the EFS-induced relaxation the effects of LNNA and ODQ were less pronounced. Though responses to EFS in presence of specific non-selective K^+ channel blockers TEA and TPEA did not display any significant change, a smaller sensitivity to the selective blockers of the small and big conductance Ca^{+2}-activated K^+ channels -apamin and charybdotoxin- have been observed. These changes suggest that functional changes in Ca^{+2}-activated K^+ channels may be related to the reported changes in inhibitory responses and contribute to the impaired relaxation observed during the post-infection period. Changes in contractile activity of the jejunum and the ileum to different agents, electrically-induced contractions and relaxations, histopathological and structural changes during *T. spiralis* infection are summarized in Table 1.

Structural and functional changes in *T. spiralis* infected rats show parallel features with processes found in human gut inflammation. For instance, transient enteric infection and

inflammation may produce neuromuscular alterations that persist after recovery of mucosal integrity and may lead to development of irritable bowel syndrome (IBS). Important pathological and functional motor changes of small intestine have also been reported associated to Crohn's disease (Vermillion *et al*., 1993). Thus, there are many evidences showing that functional changes are present even after the remission of morphological changes of inflammation, which in some cases affect remote areas from the inflammatory focus (Jacobson *et al*., 1995; Marzio *et al*., 1990; Tanovic *et al*., 1999). Available data indicate that changes in propulsive intestinal activity have also been observed in denervated gut, worm-free ileal segments or surgically excluded jejunum of *T. spiralis*-infected rats, clearly suggesting that elements intrinsic to the intestinal wall play a crucial role (Alizadeh *et al*., 1987; Jacobson *et al*., 1995; Marzio *et al*., 1990). Such changes were prevented by corticosteroid administration indicating that alterations in muscle function were a consequence of the intrinsic hosts' systemic responses to the infection rather than of direct effect of the parasite. The structural and functional alterations may be also viewed as long-lasting adaptive changes of the gut, enabling it to act as an "extension" of the immune system resulting in the parasite eviction. Thus, the immune system serves to recognize foreign and harmful organisms or substances in the gut lumen and to recruit motor system to assist in the eviction of these agents from the gut (Collins, 1996). In this way the motor-sensory apparatus of the gut may be viewed as a subject of modulation by the immune system.

In contrast to changes observed in the inflamed small intestine, in *in vitro* studies using colonic muscle strips obtained from patients with ulcerative colitis or taken from animal models of colitis, support the idea that colitis is generally accompanied by a decrease in the contractility of smooth muscle to ACh, SP or KCl (Grossi *et al*., 1993). Moreover, decreased contractility of longitudinal muscle in acute colitis in rats is independent of the agent use to induce colitis(intrarectal administration of *T. spiralis* larvae or by different chemical agents) and is due to both nonreceptor and receptor-dependent mechanisms (Depoortere *et al*., 1999; Grossi *et al*., 1993). It has been reported that after dextran sulfate sodium (DSS)-induced colitis EFS-evoked cholinergic and noncholinergic contractions as well as NANC relaxation were impaired in the distal colon (Mizuta *et al*., 2000).

The impaired NANC relaxation was associated with decreased synthesis of NO caused by

reduction on nNOS in the myenteric plexus. In experimental models of colitis the motility of the non-inflamed ileum is also decreased showing that changed function in non-inflamed areas is a generalised feature of different types of intestinal inflammation (Jacobson *et al.*, 1995).

Table 1

The most prominent changes of the jejunum and the ileum observed during *T. spiralis* infection in morphological and contractile activity to different drugs and electrically-induced contractions and relaxations.

Histopathological and structural changes		**Jejunum**	**Ileum**
Mucosal and submucosal inflammation		YES 2-23 PI	NO
Muscle inflammation		NO	NO
Muscle thickness		↑ 6-72 PI	↑ 14-23 PI
Contractile response to:	**Pathway**	**Inflammation**	
		Jejunum*	**Ileum***
SNP	[1] Activation of L-type Ca^{+2} channel, [2] cGMP increase.	=	=
KCl	[1] Membrane depolarization, [2] activation of L-type Ca^{+2} channel.	=	=
ACh	[1] Activation of L-type Ca^{2+} channels, [2] Ca^{2+} release from the SR.	=	=
SP	[1] Activation of L-type Ca^{2+} channels, [2] Ca^{2+} release from the SR.	↓ 14-23 PI	↓ 6-14 PI
Response to EFS:			
Contraction	Release of ACh and SP	↓ 2 PI	↓ 2-14, 23 PI
NANC component	Release of SP	=	↓ 6-23 PI
Relaxation	Release of NO and other NANC inhibitory mediator	=	↓ 14-23 PI
+ L-NNA and/or + ODQ	Release non-nitrergic inhibitory mediator	=	=

* - correlated to muscle thickness. PI - days of post-infection. SR - sarcoplasmatic reticulum. After *Trichinella spiralis* infection an inflammation of the mucosal and submucosal layers of jejunum was observed, whereas the muscular layers of the jejunum and the ileum were not affected. In contrast, increased muscle thickness affect both inflamed and non-inflamed areas. The response to SNP, KCl and ACh correlates with an increased thickness in both jejunum and ileum, suggesting that the increases in their responses are probably due to the increased muscular mass. This correlation was not found for SP-induced response, showing that changes after *T. spiralis* infection are also due to disturbances in specific receptor-mediated mechanisms. A long-lasting inhibition of EFS-induced contractions and relaxations was the most prominent functional motor change in non-inflamed ileal segments. The lost sensitivity of EFS-induced relaxations to L-NNA and ODQ may suggests an impaired release of nitrergic transmitter, possibly mixed with altered release of one another apamin-sensitive and non-nitrergic inhibitory neurotransmitter.

The mechanism(s) involved in changes in smooth muscle contractility and thickness could be due to a number of different mechanisms. It has been proposed that infiltration of T-lymphocytes might be a causal factor for these changes since athymic rats do not develop the contractile and trophic changes in smooth muscle, normally observed in euthymic animals (Vermillion *et al*., 1991). Otherwise, impaired ^{3}H-ACh release from jejunum and worm-free ileum of *T. spiralis* infected rats (Collins *et al*., 1989; Collins *et al*., 1992) occur independently of T-cells function. Galeazzi *et al* have demonstrated a direct interaction between inflammatory cells and nerve function identifying macrophages, and non T-cells, as the cell type involved in the functional impairment of enteric cholinergic nerves in *T. spiralis*-infected mice (Galeazzi *et al*., 2000). They have demonstrated that during *T. spiralis* infection depletion of macrophage infiltration in muscle layers and myenteric plexus can prevent the functional neural changes. Additionally, the increased expression in the myenteric plexus of interleukin 1β (IL-1β) during *T. spiralis* infection has been noticed (Hurst *et al*., 1993; Main *et al*., 1993). The ability of IL-1β to suppress ACh release and, at the same time, to stimulate the synthesis of SP in the myenteric plexus of noninfected rats mark IL-1β as a putative mediator of the impaired nerve function during infection. The expression of IL-1β together with IL-1α and TNFα in athymic rats during *T. spiralis* infection suggests that these cytokines do not contribute to the trophic muscle changes or to the altered contractibility but may mediate the changes in neurotransmitter release in this model (Ishikawa *et al*., 1994).

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CONCLUSIONS

1. The responses of smooth muscle to NO donors are strongly dependent on their chemical entity. In view of the responses induced by SNP and SIN-1 and that elicited by the endogenous nitrergic transmitter we concluded that SNP is more appropriate than SIN-1 for mimicking the responses to endogenous nitrergic transmitter in the intestinal smooth muscle.

2. NO in excess is not, by itself, responsible for the altered responses to KCl, ACh and SP during intestinal inflammation. Incubation of healthy intestinal muscle with high concentrations of NO does not seem to be the cause of the altered muscle function observed during inflammation.

3. Calcium handling properties, including calcium release from intracellular stores and influx of extracellular calcium through L-type Ca^{2+} channels, are not modified in the presence of high concentrations of exogenously added NO.

4. Depletion of calcium stores in the rat ileal longitudinal muscle activates L-type Ca^{2+} channels, as shown by the fact that addition of nifedipine to CPA-precontracted muscle results in a sharp and immediate relaxation.

5. Electrical field stimulation - induced relaxation is mainly due to the release of a nitrergic transmitter, which targets the soluble guanylate cyclase of the smooth muscle cells and provokes relaxation. The fact that the selective blocker of small conductance Ca^{+2}-activated K^+ channels apamin also provoked a significant decrease of EFS induced relaxation, suggests the release of a second inhibitory neurotransmitter.

6. *T. spiralis* infection induces an inflammation of the mucosal and submucosal layers of worm-positive jejunum, whereas neither the ileum nor the muscular layers of the jejunum were affected. However, both inflamed and non-inflamed areas of jejunum and ileum were hypertrophied in both the longitudinal and the circular layers. Thus, alterations in intestinal smooth muscle function do not require the presence of the parasite and the absence of histopathological signs of inflammation does not warrant intact motor function.

7. The distribution and number of NADPH-diaphorase-positive neurons were found in LMMP of jejunum and ileum of *T. spiralis* infected rats were similar to those found in specimens form healthy controls.

8. The relaxation induced by electrical field stimulation of ileal segments of *T. spiralis* infected rats was significantly decreased in spite of the lack of inflammatory signs in this area. In contrast, in inflamed jejunal strips of such animals electrical field stimulation was unchanged.

9. The incubation with the nitric oxide donor SNP does not modify electrical field stimulation-induced relaxations and contractions neither in controls nor in tissue form infected rats.

10. Changes in motor responses after *T. spiralis* infection are not only due to smooth muscle damage but also to disturbances in specific receptor-mediated mechanisms. Increases in smooth muscle thickness of both jejunum and ileum correlates to increased responses to KCl and to ACh. In contrast, SP response is decreased in spite of increased muscle thickness.

11. The electrical field stimulation-induced ileal contractile responses (and its NANC component) were markedly decreased during the entire period studied, while only a transient decrease of contractions was observed in the jejunum of *T. spiralis* infected rats.