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Tachykinergic neurotransmission is enhanced in duodenum from dystrophic (*mdx*) mice

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1 Duodenal longitudinal muscle of *mdx* mice, an animal model for Duchenne muscular dystrophy, showed a decrease in the electrically evoked nonadrenergic, noncholinergic (NANC) inhibitory responses associated with a reduction of the participation of nitric oxide (NO). In this study, we investigated whether the impairment of NO could also lead to alterations in the NANC excitatory transmission.

2 Nerve-evoked responses consisted of an inhibitory phase followed, at the end of stimulation, by an excitatory response characterised by an increase in amplitude of the spontaneous contractions. In *mdx* mice, the amplitude of the nerve-evoked contractions was significantly higher than in normals.

3 N_ω-nitro-L-arginine methyl ester (L-NAME) or 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylyl cyclase, increased the amplitude of the nerve-evoked contractions only in normals, being ineffective in *mdx* mice. Apamin, a blocker of Ca^{2+}-dependent potassium channels, failed to affect the nerve-evoked contractions.

4 In both models, substance P and neurokinin A produced concentration-dependent contractions, reduced by tachykinin NK_1 and NK_2 receptor antagonists, respectively. Moreover, NK_1 and NK_2 receptor antagonists reduced the amplitude of the nerve-evoked contractions.

5 Sodium nitroprusside (SNP) reduced the amplitude of nerve-evoked contractions similarly in normal and *mdx* mice. ODQ, but not apamin, prevented the SNP-induced effects. SNP did not affect the contractions induced by exogenous tachykinins.

6 The results suggest that NO can exert an inhibitory modulatory role on tachykinergic excitatory transmission *via* activation of guanylyl cyclase in mouse duodenum. In *mdx* mice, the impairment of NO function leads to an increase in the nerve-evoked contractions.
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Abbreviations: DMD, Duchenne muscular dystrophy; EFS, electrical field stimulation; L-NAME, N_ω-nitro-L-arginine methyl ester; NANC, nonadrenergic, noncholinergic; NKA, neurokinin A; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside; SP, substance P; SR 140,333, ((*S*)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxy-phenylacetyl)-piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2] octane, chloride); SR 48,968, ((*S*)-*N*-methyl-*N*[4-(4-acetylamino-4-phenylpiperi-dino)-2-(3,4-dichloro-phenyl)butyl]benzamide; TTX, tetrodotoxin

Introduction

We are currently interested in studying the gastrointestinal motor dysfunctions in *mdx* mice, an animal model for Duchenne muscular dystrophy (DMD). Duchenne patients and *mdx* mice lack dystrophin, a membrane associated protein present in normal skeletal, cardiac and smooth muscle cells and in some neurones (Hoffman *et al*., 1987). Different hypotheses have been put forward to explain the mechanism by which the lack of dystrophin causes muscle dysfunction. In particular, a role for nitric oxide (NO) in the pathogenesis of muscular dystrophy has been proposed, because the lack of dystrophin in human DMD and in *mdx* mice causes neuronal nitric oxide synthase (nNOS) to disappear from its normal position at the sarcolemma. It becomes cytoplasmic, with a consequent decrease in its activity (Brenman *et al*., 1995). In *mdx* mice, changes in NO function have been reported to have a strong impact on motor and electrical patterns in different gastrointestinal segments, and alterations in the nNOS isoform have been found in the smooth muscle of the colon from dystrophic mice (Azzena & Mancinelli, 1999; Mulè *et al*., 1999, 2001; Baccari *et al*., 2000; Serio *et al*., 2001; Mulè & Serio, 2002; Zizzo *et al*., 2003). The duodenum of *mdx* mice showed functional motor alterations referable to a reduction in the nitrergic control associated to defects in the mechanisms necessary to transduce NO signals (Zizzo *et al*., 2003).

NO may act as an inhibitory neurotransmitter in the gastrointestinal tract either directly on the smooth muscle cells or indirectly by inhibiting the release of excitatory neurotransmitters such as acetylcholine and tachykinins. There are, however, tissue and species differences in the neuromodulatory effects of NO. NO-mediated inhibition of excitatory

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neurotransmitter release has been reported in the ileum and colon of guinea-pig (Wiklund *et al*., 1993; Kilbinger & Wolf, 1994; Hebeiss & Kilbinger, 1998; Mizhorkova *et al*., 2001), in dog ileum (Hryhorenko *et al*., 1994) and in mouse ileum (Mang *et al*., 2002). NO or nitric oxide synthase (NOS) inhibitors do not modify the electrically evoked acetylcholine release from the colon of guinea-pig and dog (Ward *et al*., 1996; Rae *et al*., 1998), and from stomach preparations of man (Leclere & Lefebvre, 2002), pig (Leclere & Lefebvre, 2001) and guinea-pig (Milenov & Kalfin, 1996).

Previous investigations showed that the duodenum of *mdx* mice does not relax in response to electrical field stimulation (EFS). In fact, in place of relaxation observed in normal animals, EFS caused just a frequency-dependent inhibition of the spontaneous activity. Moreover, block of NOS with N_{ω}-nitro-L-arginine methyl ester (L-NAME) abolished the inhibitory response in normal animals, while it only partly reduced that in mdx preparations. Thus, in *mdx* mice there is a reduction in the nonadrenergic, noncholinergic (NANC) relaxation associated with a reduction of the participation of NO (Zizzo *et al*., 2003). In addition, in both preparations, after the initial inhibitory response termination 'off' or rebound contractions could be observed, but these were not systematically analysed. Poststimulus excitatory responses have been described in a variety of the intestinal preparations and a role for tachykinins in their genesis has been assessed (Lecci *et al*., 2002). In particular, tachychinergic excitatory nerve-evoked responses have been described in mouse stomach (Mulè & Serio, 2002) and ileum (Goldhill *et al*., 1995; Saban *et al*., 1999).

Therefore, the present study was performed in mouse duodenum to investigate whether NANC excitatory motor transmission is modulated by NO and whether it can be affected in *mdx* mice due to the impairment of the NO function.

In particular, we analysed and compared the 'off' contractile activity in response to NANC electrical nerve stimulation, the effects induced by L-NAME, sodium nitroprusside (SNP) and 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) on NANC contraction and the role of tachykinins in normal and *mdx* mice.

Methods

Tissue preparation

Experiments, authorised by the Ministero della Sanità (Rome, Italy), were performed on dystrophic (*mdx* mutants; C57BL/10Sn-Dmd/J) adult (12 months old) male mice and their genetically related C57 control animals (Harlan, Italy) killed by cervical dislocation. The abdomen was immediately opened and segments of duodenum were removed and placed in Krebs solution (mM: NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1). The contents of the excised segments were gently flushed out and the segments (20 mm in length) were drawn over a glass rod and the mucosal layer was gently removed. The segments were suspended in 10 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained at 37°C. The distal end of each segment was tied to an organ holder and the proximal end was secured with a silk thread to an isometric force transducer (FORT 10, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy). Longitudinal preparations were subjected to an initial tension of 200 mg and were allowed to equilibrate for at least 30 min in the Krebs solution before starting the experiments. Krebs solution contained atropine (1 μM) and guanethidine (1 μM), to establish NANC conditions, indomethacin (10 μM) to increase the reactivity of the muscle to contractile agents (Maggi *et al*., 1994), and phosphoramidon (1 μM) (*N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp) and captopril (1 μM) ((2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline) to inhibit peptidases.

Mechanical activity was digitised on an A/D converter, visualised, recorded and analysed on a personal computer using the PowerLab/400 system (Ugo Basile. Italy). EFS was applied by a Grass S88 (Grass Instruments Co., Quincy, MA, U.S.A.) electrical stimulator through a stimulus isolation unit (SIU5) using direct coupling. Square-wave pulses (0.5 ms, 2–32 Hz, supramaximal voltage) were delivered in 10-s train *via* a pair of platinum plate electrodes placed in parallel with the tissue.

Experimental protocols

At the beginning of each experiment, the preparation was challenged with 80 mM KCl for 2 min until reproducible responses were obtained. The amplitude of the KCl response was 387.0 ± 63 mg ($n = 20$) in normal and 382.5 ± 72 mg ($n = 20$) in *mdx* mice. Frequency–response curves to EFS were obtained first in control condition by stimulating the tissues with individual trains delivered at 15 min intervals. Subsequently, antagonists were added to the organ baths and, after a period of 20–30 min, curves to EFS were repeated. Tachykinergic receptor agonists were tested in a noncumulative manner (leaving 45 min between consecutive additions to avoid tachyphylaxis) and they were allowed to be in contact with the tissue for 3 min. Preliminary experiments showed that a second curve to the agonist obtained in these experimental conditions was reproducible. Tachykinergic receptor antagonists were added to the bath and a new curve to agonist was produced after 40 min contact time. Each preparation was tested with a single antagonist, except when otherwise stated.

Drugs

Drugs used were apamin, atropine sulphate, guanethidine monosulphate, ODQ, indomethacin, neurokinin A (NKA), L-NAME, (2*S*)-1-(3-mercapto-2-methylpropionyl)-L-proline (captopril), *N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-Leu-Trp (Phosphoramidon), SNP, substance P (SP), tetrodotoxin (TTX), all purchased from Sigma (Sigma-Aldrich, Inc., St Louis, U.S.A.). ((*S*)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxy-phenyl-acetyl)-piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octane, chloride) (SR 140,333) and ((S)-*N*-methyl-*N*[4-(4-acetyl-amino-4-phenylpiperi-dino)-2-(3,4-dichloro-phenyl)butyl]benzamide (SR 48,968) were from Sanofi Recherche (Montpellier Cèdex, France). Indomethacin solution was prepared daily in 2% sodium carbonate solution, and the pH was adjusted to 7.4. ODQ, and SR 48,968 and SR 140,333 were dissolved in dimethylsulphoxide (DMSO). All the other drugs were dissolved in distilled water. The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions in Krebs and were added to the organ bath.

Control experiments using DMSO alone showed that it has no effect on the tissue responses studied.

Statistical analysis

All data are given as means ± s.e. *n* indicates the number of animals from which the intestinal segment was taken. The excitatory responses to EFS were normalised to the amplitude of the spontaneous contractions set to 100%, being a nerve-evoked increase in the amplitude of the spontaneous contractions. The contractile responses to tachykinergic receptor agonists were expressed as per cent of the response to KCl (80 mM). Agonist responses were fitted to sigmoid curves (Prism 4.0, GraphPAD, San Diego, CA, U.S.A.), and EC_{50} values (with 95% CLs) were determined from these curves. Moreover, antagonist potency was expressed as the negative logarithm of the concentration of the antagonist required to cause a two-fold rightward shift of the agonist dose–response curve (pA_2 value), calculated by nonlinear regression analysis of the individual dose–response curves using Prism 4.0. Statistical analysis was performed by means of Student's *t*-test or analysis of variance, followed by Bonferroni's test, when appropriate. A probability value of less than 0.05 was regarded as significant.

Results

Nerve-evoked excitatory responses: normal vs mdx *mice*

Duodenal segments, in the presence of atropine, guanethidine, indomethacin and peptidase inhibitors, exhibited spontaneous mechanical activity, consisting of rhythmic changes in isometric tension with a similar amplitude (Table 1).

In normal mice, EFS produced a transient initial relaxation of longitudinal muscle (Zizzo *et al*., 2003), followed by TTX-sensitive, frequency-dependent, excitatory response, which consisted in an increase in amplitude of the spontaneous contractions (Figure 1). At 32 Hz, the maximal amplitude of the nerve-evoked contractions reached about 180% of the spontaneous contraction amplitude.

As we have previously reported, in *mdx* mice, the inhibitory effects to EFS consisted just in the suppression of the phasic activity, and a frank relaxation was never observed (Zizzo *et al*., 2003). Instead, the excitatory response to EFS consisted in slowly developing increase in muscular tone with superimposed phasic contractions. The amplitude of the nerve-evoked contractions was significantly higher than in normal animals (Figure 1). At 32 Hz, the increase in the tone was 52.2 ± 7.0% ($n = 20$) of the response to KCl (80 mM) and the amplitude of the nerve-evoked contractions reached about 204% of the spontaneous contraction amplitude.

Nerve-evoked excitatory responses: effects of nitrergic drugs

L-NAME (100 μM), inhibitor of NOS, caused no change in the amplitude of the spontaneous contractions in both types of animals (Table 1). In normal animals, L-NAME (100 μM) increased the amplitude of the nerve-evoked contractions at all frequencies tested, but the tonic component of the response observed in *mdx* mice was not unmasked (Figure 2). In *mdx* mice, L-NAME (100 μM) did not significantly increase the response to EFS, leaving unaltered either the amplitude of the nerve-evoked contractions at all frequencies tested (Figure 2),

Table 1 Amplitude of the spontaneous contractions in control conditions and in the presence of L-NAME, ODQ, apamin or NK_1 and NK_2 receptor antagonists

	Spontaneous activity (mg)						
	Control (n = 20)	*L-NAME* (n = 7)	*ODQ* (n = 7)	*Apamin* (n = 5)	*SR 140,333* (n = 7)	*SR 48,968* (n = 7)	*SR 140,333 + SR 48,968* (n = 7)
Normal	186.7 ± 9.6	189.5 ± 7.2	184 ± 16.3	192.3 ± 6.3	175.6 ± 16.7	184.9 ± 17.0	174.8 ± 15.0
mdx	208.6 ± 14.4	212.4 ± 10.4	217.7 ± 17.5	223.5 ± 15.3	192.3 ± 10.1	212.3 ± 11.4	190.5 ± 16.4

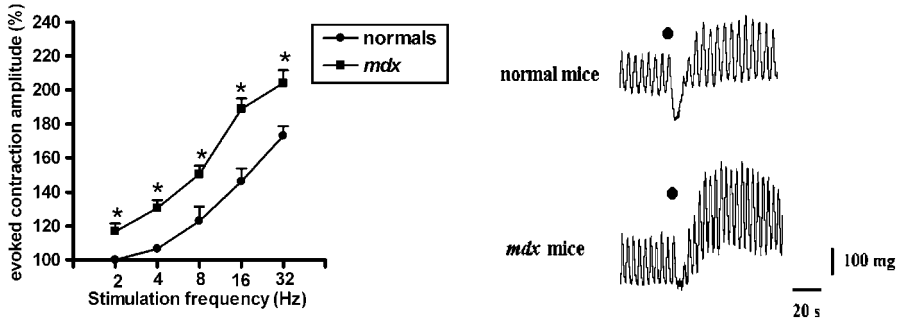

Figure 1 Nonadrenergic, noncholinergic (NANC) responses evoked by electrical field stimulation (EFS) in normal and *mdx* longitudinal duodenal muscle preparations. Left: relationship between stimulation frequency and amplitude of the nerve-evoked contractions. All values are means ± s.e. ($n = 20$ for normal and for *mdx* mice) and are normalised to the amplitude of the spontaneous contractions set to 100%. *Significantly different from the normal animals. Right: typical tracings showing the NANC-evoked responses induced by EFS in duodenal segments from normal and *mdx* mice (0.5 ms, 32 Hz, supramaximal voltage for 10 s). Note that in *mdx* duodenum the nerve-evoked excitatory response is enhanced compared to normal animals.

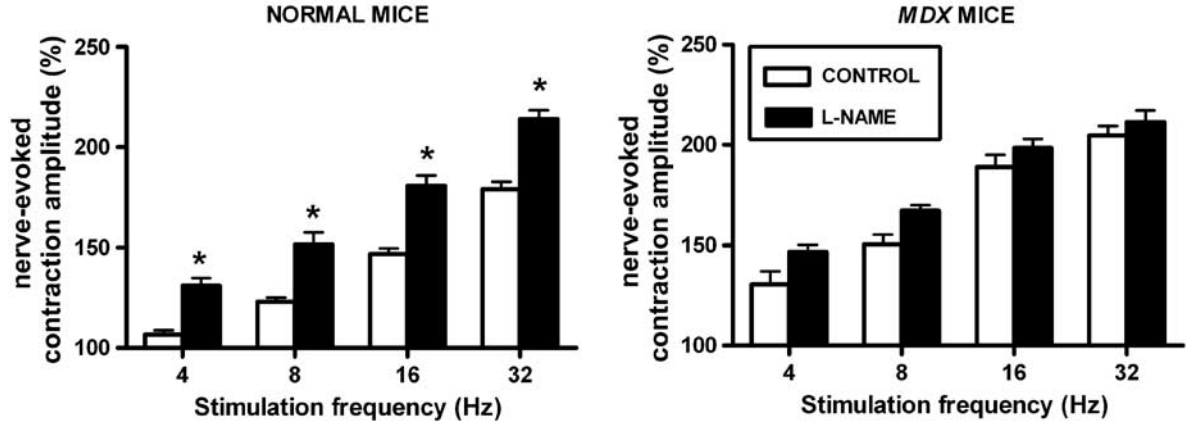

Figure 2 Histograms showing the effects of L-NAME (100 μM) on the amplitude of the contractions evoked by EFS at different stimulation frequencies (0.5-ms pulse duration, supramaximal voltage for 10 s) in normal and *mdx* longitudinal duodenal muscle preparations. Data are means$\pm$s.e. ($n=7$ for normal and for *mdx* mice) and are normalised to the amplitude of the spontaneous contractions set to 100%. *$P<0.05$ when compared to the respective control.

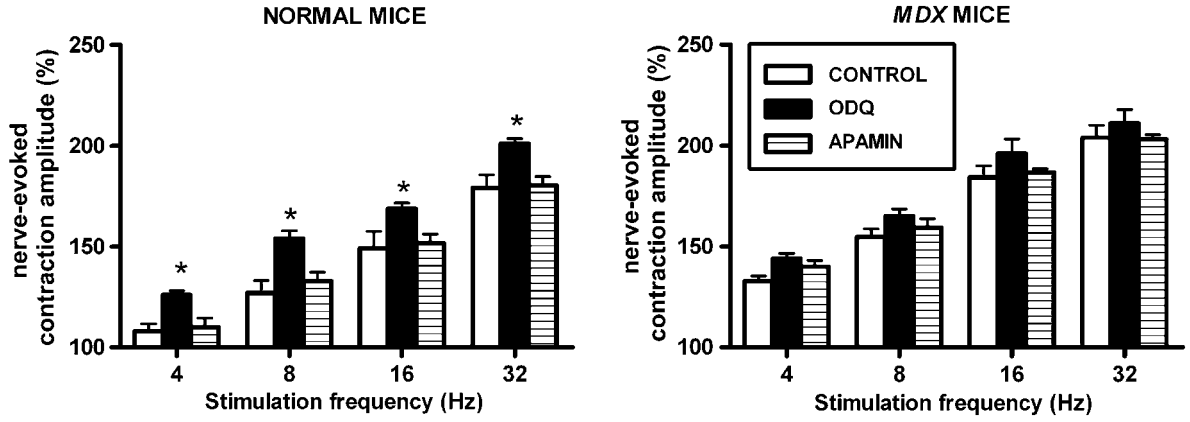

Figure 3 Histograms showing the effects of ODQ (1 μM, $n=7$) or apamin (0.1 μM, $n=5$) on the excitatory responses evoked by EFS at different stimulation frequencies (0.5-ms pulse duration, supramaximal voltage for 10 s) in normal and *mdx* longitudinal duodenal muscle preparations. Data are means$\pm$s.e. and are normalised to the amplitude of the spontaneous contractions set to 100%. *$P<0.05$ when compared to the respective value obtained in the control.

and the underlying increase in the tone (at 32 Hz the increase in the tone was 61.3$\pm$5.0% ($n=7$) of the response to 80 mM KCl in the presence of L-NAME). The amplitude of the nerve-evoked contractions obtained in normal animals after L-NAME was not significantly different from that observed in *mdx* mice.

Since we have previously demonstrated that in mouse duodenum, NO-induced inhibitory effects are mediated in part by activation of soluble guanylyl cyclase and in part by increasing the open probability of apamin-sensitive Ca^{2+}-dependent potassium channels (Serio *et al*., 2003), we tested the effects of either ODQ, an inhibitor of soluble guanylate cyclase, or apamin on nerve-evoked responses. Both ODQ (1 μM) and apamin (0.1 μM) did not modify the amplitude of the spontaneous contraction in both types of animals (Table 1). In normal animals, ODQ and apamin had different effects on the excitatory responses. In fact, just ODQ increased the amplitude of nerve-evoked contractions, apamin was ineffective (Figure 3). The tonic component of the response to EFS observed in *mdx* mice was not unmasked by ODQ. In *mdx* mice, in contrast with normal animals, ODQ (1 μM) did not significantly increase the amplitude of nerve-evoked contractions and, once again, apamin was without any effect (Figure 3). The tonic component of the response to EFS was not affected by either ODQ or apamin (at 32 Hz the increase in the tone was 59.4$\pm$7.5% ($n=7$) and 51.9$\pm$6.4% ($n=5$) of the response to 80 mM KCl in the presence of ODQ or apamin, respectively).

Tachykinin-induced contractions: normal vs mdx *mice*

In normal animals, NK_1 receptor-preferring natural tachykinin, SP (10 nM–1 μM) produced concentration-dependent contractions with an EC_{50} value of 45 nM (95% CL 21–97 nM), reaching an E_{max} value of 74.9$\pm$2.5% of response to KCl ($n=7$). NK_2 receptor-preferring natural tachykinin, NKA (10 nM–1 μM) produced concentration-dependent contractions with an EC_{50} value of 28 nM (95% CL 11–76 nM), reaching an E_{max} value of 79.9$\pm$2.9% of response to KCl ($n=6$). There was no significant difference between EC_{50} or E_{max} values between SP and NKA, or between their concentration–response curves ($P>0.05$). In *mdx* animals, addition of SP

(10 nM–1 μM) produced concentration-dependent contractions with an EC_{50} value of 91 nM (95% CL 34–241 nM), reaching an E_{max} value of 65.3±3.2% of response to KCl ($n=7$). NKA (10 nM–1 μM) produced concentration-dependent contractions with an EC_{50} value of 24 nM (95% CL 8.8–65 nM), reaching an E_{max} value of 85.6±3.0% of response to KCl ($n=6$). Statistical analysis showed that there was no significant difference between EC_{50} or E_{max} values of SP or NKA, or of their concentration–response curves ($P>0.05$) between normal and *mdx* mice.

Nerve-evoked excitatory responses: effects of NK_1 and NK_2 receptor antagonists

To assess the involvement of tachykinins in the excitatory transmission, we tested the effects of NK_1 and NK_2 receptor antagonists on the nerve-evoked contractions in response to EFS, both in normal and *mdx* mice. At first, in order to choose the antagonist concentration we analysed, in normal animals, the effects of the NK receptor antagonists on the concentration–response curves to the agonists. The selective NK_1 receptor antagonist, SR 140,333 (1 nM–0.1 μM), produced a concentration-dependent rightward shift of the dose–response curve to SP ($pA_2=8.28\pm0.05$) (Figure 4), whereas the selective NK_2 receptor antagonist, SR 48,968 (1 nM–0.1 μM), produced a concentration-dependent rightward shift of the dose–response curve to NKA ($pA_2=8.31\pm0.02$) (Figure 4). Conversely, SR 48,968 did not modify the response to SP ($EC_{50}=64$ nM (95% CL 19–211 nM) in the presence of SR 48,968, $n=4$, $P>0.05$) and SR 140,333 did not modify the contractions in response to NKA ($EC_{50}=45$ nM (95% CL 18–140 nM) in the presence of SR 140,333, $n=4$, $P>0.05$).

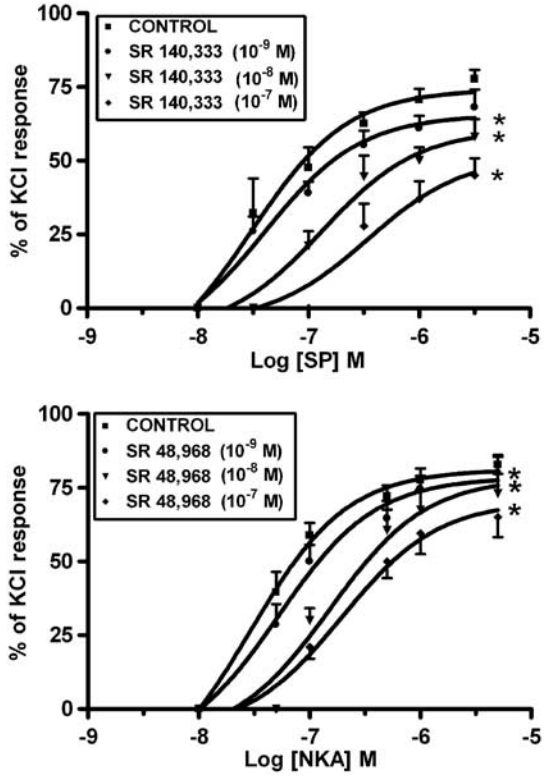

Figure 4 Normal mice. Upper pannel: concentration–response curves to SP in the absence or in the presence of different concentrations of SR 140,333, NK_1 receptor antagonist. Lower panel: Concentration–response curves to NKA in the absence or in presence of different concentrations of SR 48,968, NK_2 receptor antagonist. Data are means±s.e. of at least four experiments and are expressed as per cent of the response to KCl (80 mM). *$P<0.05$ when the concentration–response curves were compared to that obtained in control condition.

Either SR 140,333 (0.01 μM) or SR 48,968 (0.01 μM), alone or in combination, had no effect on the amplitude of the spontaneous contractions (Table 1). Indeed, SR 140,333 (0.01 μM) or SR 48,968 (0.01 μM) significantly reduced the amplitude of the nerve-evoked contractions in responses to EFS at all stimulus frequencies tested in both normal and *mdx* mice in the presence or in the absence of L-NAME (Figure 5). Moreover, in *mdx* mice, the tonic component of the response to EFS was slightly affected by either SR 140,333 or SR 48,968 (0.01 μM) (at 32 Hz the increase in the tone was 39.7±6.5% ($n=7$) and 43.9±7.2% ($n=7$) of the response to 80 mM KCl in the presence of SR 140,333 or SR 48,968, respectively). The blockade of both NK_1 and NK_2 receptors by co-administration of the selective antagonists resulted in a further reduction of the contractile responses evoked by EFS (Figure 5). Moreover, the increase in the tone observed in the NANC responses in *mdx* mice was abolished by co-administration of the NK_1 and NK_2 receptor antagonists.

Influence of SNP on nerve-evoked excitatory responses and on exogenous tachykinin contractions

To study the influence of exogenous NO on the excitatory responses to EFS and on tachykinin-induced contractions, we analysed the effects of SNP at submaximal dose (Zizzo *et al*., 2003). In normal mice, SNP (10 μM) significantly reduced the amplitude of the nerve-evoked contractions (Figure 6). A low concentration of ODQ (0.1 μM), which *per se* did not appreciably increase the amplitude of the nerve-evoked contractions, prevented the effects induced by SNP (Figure 6). In *mdx* mice, in the presence of SNP (10 μM), the amplitude of nerve-evoked contractions was reduced as in the normal animals, and pretreatment with ODQ (0.1 μM) prevented the effects induced by SNP on the nerve-evoked contractions. Lastly, the inhibitory effect induced by SNP was not prevented by apamin both in normal and *mdx* mice (Figure 6).

Moreover, we tested the responses to SP and NKA in the presence of SNP (10 μM). In both animals, the responses to SP (1 μM) and to NKA (1 μM) were not significantly different from those observed before addition of SNP (Figure 7).

Discussion

The results of the present study indicate that, in mouse duodenum, NO exerts an inhibitory modulatory role on tachykinergic excitatory transmission, *via* activation of guanylyl cyclase, and in *mdx* mice the impairment of NO pathways leads to increased tachykinergic muscular contractions in response to EFS.

NO is an inhibitory neurotransmitter that causes inhibition of intestinal motility. This action is not confined just to its direct action on the smooth muscle cells, but NO may decrease gastrointestinal motility also acting indirectly by inhibiting the

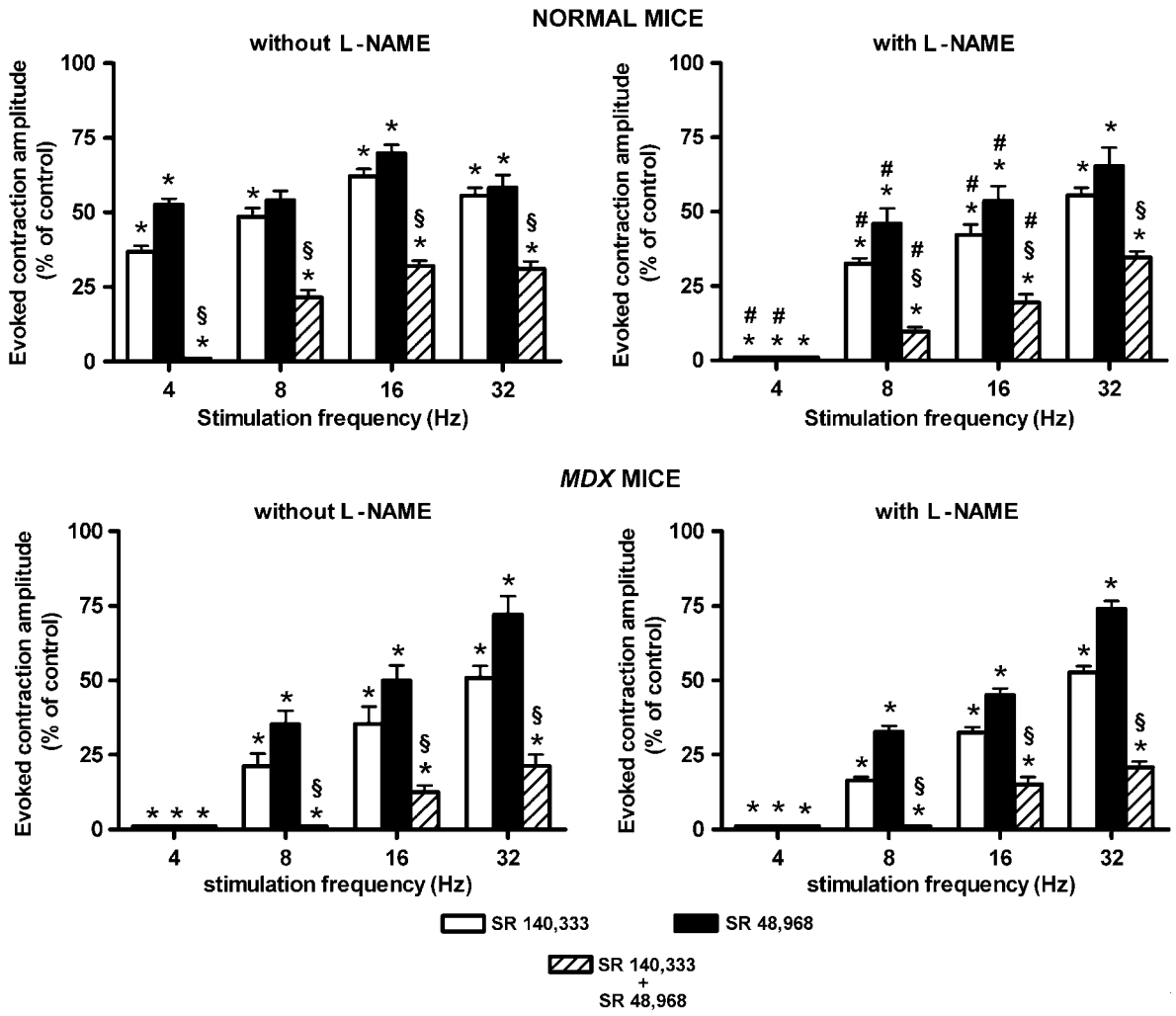

Figure 5 Histograms showing the effects of SR 140,333 (0.01 μM, $n=7$), NK_1 receptor antagonist, and SR 48968 (0.01 μM, $n=7$), NK_2 receptor antagonist, alone or in combination ($n=5$) on the responses evoked by EFS at different stimulation frequencies (0.5-ms pulse duration, supramaximal voltage for 10 s) in the presence or in the absence of L-NAME (100 μM) in normal and *mdx* longitudinal duodenal muscle preparations. Data are means ± s.e. and are expressed as a percentage of the respective control, taken as 100%. $^{*}P<0.05$ when compared to the respective control. $^{\S}P<0.05$ when compared to SR 140,333 or SR 48,968 alone. $^{\#}P<0.05$ when compared to the respective values without L-NAME.

release of excitatory neurotransmitters such as acetylcholine (Wiklund *et al*., 1993; Hryhorenko *et al*., 1994; Kilbinger & Wolf, 1994; Hebeiss & Kilbinger, 1998; Mizhorkova *et al*., 2001) and tachykinins (Wiklund *et al*., 1993; Yunker & Galligan, 1996). In particular, in mouse ileum, NO inhibits cholinergic transmission and nNOS is the enzymatic source of NO-mediating inhibitory action (Mang *et al*., 2002).

Different regions of the gastrointestinal tract of *mdx* mice show motor alterations referable to a reduction in the nitrergic control (Mulè *et al*., 1999; Serio *et al*., 2001; Mulè & Serio, 2002; Zizzo *et al*., 2003), and the expression of nNOS isoform is altered in the smooth muscle of colon from dystrophic mice (Mulè *et al*., 2001). In *mdx* mice duodenum, there is a reduction of NANC relaxation associated with a reduced participation of NO (Zizzo *et al*., 2003). Therefore, our working hypothesis was that the impairment of NO system could interfere with the function of NANC excitatory transmission. Consequently, in the present study we analysed and compared the contractile responses to NANC nerve stimulation in normal vs *mdx* duodenal segments. In both tissues, EFS evoked a neurogenic excitatory response, significantly higher in *mdx* mice than in normal mice. Interestingly, in *mdx* mice, NOS inhibition with L-NAME failed to affect the evoked contractions, while in normal animals it increased significantly the EFS-induced contractions, whose amplitude reached that of the *mdx* tissue. Therefore, endogenous NO, released concurrently by field stimulation, markedly interfere with excitatory NANC neurotransmission in normal animals. We have previously showed that, in mouse duodenum, relaxations to NO released endogenously by enteric nerves or to exogenous SNP are mediated in part by cGMP-dependent mechanisms and in part by activation of apamin-sensitive Ca^{2+}-dependent potassium channels (Serio *et al*., 2003). Indeed, the NO modulation of NANC excitatory response is likely mediated *via* activation of guanylyl cyclase. In fact, although both ODQ and apamin significantly antagonised the EFS-induced relaxation (Serio *et al*., 2003), only ODQ enhanced the amplitude of nerve-evoked contrac-

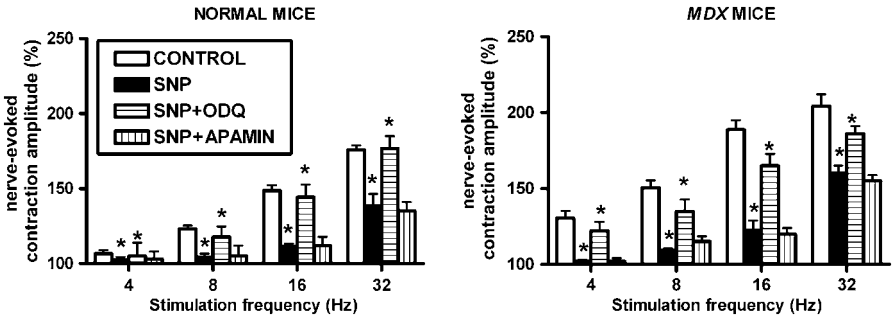

Figure 6 Histograms showing the effects of SNP alone (10 μM), and after pretreatment with ODQ (0.1 μM) or apamin (0.1 μM) on the NANC excitatory responses evoked by EFS at different stimulation frequencies (0.5-ms pulse duration, supramaximal voltage for 10 s) in normal and *mdx* longitudinal duodenal muscle preparations. Data are means $\pm$ s.e. ($n = 5$ for normal and for *mdx* mice) and are calculated taking into account the amplitude of the phasic contractions, which is normalised to the amplitude of the spontaneous contractions set to 100%. $^*P < 0.05$ when compared to the respective value obtained in the control.

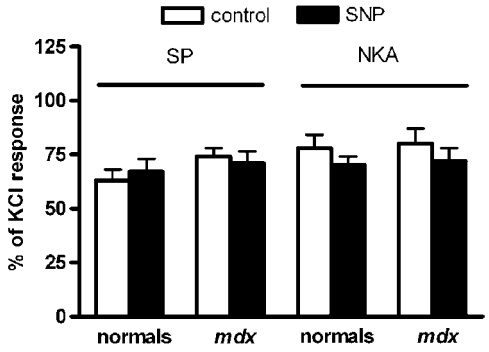

Figure 7 Histogram showing the effects of SNP (10 μM) on the contractions evoked by SP (1 μM) and by NKA (1 μM) in normal and *mdx* longitudinal duodenal muscle preparations. Data are means $\pm$ s.e. ($n = 4$ for normal and for *mdx* mice) and are expressed as per cent of the response to KCl (80 mM).

tions, being apamin ineffective. In support of this hypothesis, there is the observation that exogenous SNP decreased the evoked contractions acting through the activation of guanylyl cyclase, because its effect was antagonised by ODQ, but not apamin. A similar mechanism is involved in NO inhibition of acetylcholine release and cholinergic contractions in mouse ileum (Mang *et al*., 2002) and in NO inhibition of cholinergic and tachykinergic components of nerve-mediated contraction in guinea-pig ileum (Yunker & Galligan, 1996; Hebeiss & Kilbinger, 1998).

In *mdx* mice, neither ODQ nor apamin increased the amplitude of nerve-evoked duodenal contractions indicating that, as L-NAME, they had no facilitating role on the NANC-evoked contractions. However, when in *mdx* mice, NO is supplied exogenously is able to exert its inhibitory effects on the NANC excitatory system, as indicated by the observation that SNP decreased the evoked contractions even in *mdx* mice. We suggested that, in *mdx* mice, there is loss of the nitrergic modulation of NANC excitatory transmission, which in turn leads to an increase in the contractile responses to EFS.

In addition, the nature of the neurotransmitter/s involved in the neurogenic-evoked contractions in duodenal segments from normal animals and possible difference in respect to *mdx* mice were analysed. A role for acetylcholine, noradrenaline or prostanoids in the evoked response was ruled out because the experiments were conducted in the presence of atropine, guanethidine and indomethacin. On the other hand, in our experiments, the sensitivity to tachykinergic receptor antagonists indicates a major role for tachykinins in the genesis of nerve-evoked contractions in duodenal muscle. The tachykinins are important excitatory neurotransmitters in the enteric nervous system and are involved in the coordination of gastrointestinal motility. Three tachykinin receptors have been identified, NK_1, NK_2 and NK_3 receptors differently located on smooth muscle, interstitial cells of Cajal and neurones depending on the region and on the species considered (Holzer & Holzer-Petsche, 2001). In particular, NK_1 and NK_2 receptors are reported to mediate excitatory neuromuscular transmission in the gut (Holzer & Holzer-Petsche, 2001). Results from our experiments indicate the presence of functional NK_1 and NK_2 receptors in mouse duodenum. In fact, NK_1 receptor antagonist significantly inhibited the SP-evoked responses, without interfering with the NKA-induced contractions, and NK_2 receptor antagonist significantly inhibited the NKA responses, without interfering with the SP-induced contractions. No difference has been found in the potency of the two tachykinins. Moreover, either NK_1 receptor antagonist or NK_2 receptor antagonist is able to inhibit the contractions evoked by EFS. These effects were more evident when the modulatory role of NO on the tachykinergic component had been removed by L-NAME. In addition, coadministration of both receptor antagonists induced additive effects causing a further inhibition of the nerve-evoked contractions. Thus, these observations confirm our conclusion that at our parameters of stimulations, both tachykinergic receptors were recruited. These observations indicate that in mouse duodenum both NK_1 and NK_2 receptors play a fundamental role as NANC excitatory transmitters. It has been reported that NK_1 and NK_2 receptors modulate the responses to exogenously applied agonists also in mouse ileum, while in this region NK_1 are the primary tachykinin receptors involved in the noncholinergic excitatory neurotransmission (Saban *et al*., 1999).

In *mdx* mice, both tachykinergic receptor antagonists were able to inhibit the contractions evoked by EFS and coadministration of both receptor antagonists induced additive effects causing also the inhibition of the tonic component of the nerve-evoked responses. Thus, in *mdx* duodenum, the increase in the contractile response to EFS is due to an increase of participation of the tachykinins as excitatory neurotransmitters. The observation that there is no change in the muscular responsiveness to tachykinins in *mdx* mice suggests that the

increased contractile response to EFS is not the consequence of changes in muscular sensitivity to tachykinins; instead, it can be ascribable to the reduction of nitrergic control on the tachykinin-mediated responses. Alteration in tachykinergic transmission due to the loss of the interaction with other transmitters, one of these being NO, has been reported in other pathophysiological conditions such as inflammation (Goldhill *et al*., 1997).

Lastly, the modulator influence of NO might be on the NANC excitatory nerves or on the smooth muscle cells. From our studies, it is not possible to assess the site(s) of action of NO (i.e. pre- vs postjunctional) for a certainty. However, the observation that exogenous NO does not influence the response to tachykinins rules out the possibility of a functional antagonism strongly supporting the idea that the interaction is at the prejunctional level.

In conclusion, in *mdx* mouse duodenum, the impairment of NO pathway leads not only to a reduction of the muscular relaxation but also to an increase in the muscular contractions evoked by noncholinergic nerve stimulation due to alteration of the modulation of tachykinergic neurotransmission. Such mechanisms can explain gastrointestinal clinical manifestations observed in DMD patients, such as chronic constipation.

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