Effect of Modulators of Neural Transmission on Mechanical Activity in Colon of Dystrophic (mdx) and Normal Mice

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Abstract
Patients with Duchenne muscular dystrophy (DMD) exhibit some disorder in colonic movement with atrophy and fibrosis of visceral smooth muscles. To explore the origin of the abnormal mechanical activity of colon in DMD patients, the effect of neural modulators on intraluminal pressure was examined in the proximal and distal segments of the colon of mice with muscular dystrophy (mdx), an animal model of DMD, and compared with the control. Many, though not all, of proximal colons from both mdx and control mice exhibited cyclic contractions (CCs) spontaneously, while most of the distal colons of both types of mice were quiescent. Hexamethonium (0.05 – 5 mM), apamin (250 nM) and Nω-nitro-L-arginine (NOLA) (0.01 – 1 mM) either increased the frequency of spontaneous CCs or induced CCs in quiescent colons. The frequency of the CCs in the presence of any of the drugs was less than 8 cpm in all of the colon groups. Sodium nitroprusside (SNP) (5 µM) and ATP (0.1 mM) decreased the frequency of CCs. Carbamylcholine (0.5 – 50 µM) produced a transient contraction with or without generation of CCs. There was no difference in the response to these drugs except the NOLA, among the colon groups. The frequency of CCs was 2.3 ± 0.3 cpm (mean ± sem, n=14) in mdx distal colon at 0.1 mM NOLA, which was significantly lower than that in the control distal colon (3.7 ± 0.6 cpm, n=13, p<0.05). No difference was observed in the response to NOLA between proximal colons from mdx and control mice. The results show some disorder in the NO synthase activity in the mdx distal colon. In addition, hexamethonium (5 mM) reduced the SNP-sensitive active tone by generating powerful CCs, suggesting that there is an interaction between the mechanisms underlying the CCs and the active tone.

Key words
colon, Duchenne muscular dystrophy, mdx mouse, nitric oxide, peristalsis

INTRODUCTION
Patients with Duchenne muscular dystrophy (DMD) suffer a progressive wasting of skeletal and cardiac muscle fibers, leading to death. This wasting is related to the lack of dystrophin, a cytoskeletal protein normally found throughout the cytoplasmic face of the plasma membrane in muscle fibers 1–3). DMD patients also exhibit dysphagia and constipation with atrophy and fibrosis of visceral smooth muscles.
muscles lacking dystrophin\(^4\)\(^5\). However, little is known about the cause of gastrointestinal disorders in DMD.

In the colon, peristalsis triggered by a local inflation migrates along its length. The migration of peristalsis is due to neural activity in the myenteric plexus\(^6\)\(^7\)\(^8\), since paralysis of the smooth muscles by some drugs has little effect on the electrical events underlying the peristalsis. Pharmacological and/or immunohistochemical studies suggest that nitric oxide (NO) and ATP are inhibitory neurotransmitters; both NO and ATP hyperpolarize the membrane of circular smooth muscles\(^9\)\(^10\). It has been shown that in the mouse colon, hexamethonium, a cholinergic blocker, and tetrodotoxin depolarize the membranes of circular smooth muscles to such an extent that cyclic slow depolarization, which underlies the peristalsis, disappears almost completely\(^11\). Under experimental conditions in which the cyclic slow depolarization is obscured by the depolarization of smooth muscles, however, it is difficult to deduce the pacemaker mechanism responsible for the peristalsis. Studies such as those done with strips of rat colon\(^12\) in which cyclic contractions (CCs) of the strip are modulated by some pharmacological agents are necessary for understanding the pacemaker mechanism. Since tetrodotoxin increases the frequency and amplitude of CCs in the rat colon, in contrast to the effect in the mouse colon, the results obtained with the rat colon can not be extended to mouse colon.

Studies with the muscular dystrophy (mdx) mouse, an animal model of DMD, suggest some abnormal mechanical activity in the mdx colon; frequent retrograde peristalsis disturbs the analward transportation of the contents in the distal colon and unusual development of active tone in the proximal colon\(^13\)\(^14\)\(^15\). Enhancement of NO production by L-arginine reduces the frequency of spontaneous peristalsis in both retrograde and anterograde directions, indicating an important role of NO in initiating the peristalsis in the mouse colon. Though unusual development of tone, which is probably due to increased Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels in the circular smooth muscle, has recently been reported in the proximal segment of the mdx colon\(^15\)\(^16\), the abnormality in the peristalsis, especially in the pacemaker mechanism, has not been well characterized in the mdx colon.

The aim of the present study was to explore the origin of the frequent retrograde peristalsis in mdx colon. For this purpose, colons isolated from mdx mice were separated into proximal and distal segments and the changes in their intraluminal pressure in response to several pharmacological agents which modulate neural activity were determined and compared with their controls. The results showed that hexamethonium, N\(^\omega\)-nitro-L-arginine (NOLA), and apamin induced CCs in colonic segments, while sodium nitroprusside (SNP) diminished them. There was neither quantitative nor qualitative difference in the response to these agents, except to NOLA, between proximal and distal colons from mdx mice, or between mdx colon and control colon. In the presence of NOLA, the frequency of CCs was lower in the distal colon from mdx than from control mice, suggesting a lower sensitivity to NOLA in the pacemaker mechanism for the CCs in the former compared with the latter. In addition, it was found that active tone developed in both mdx and control colons was reduced markedly by powerful CCs induced by hexamethonium.

**MATERIALS AND METHODS**

**General**

Male normal (C57BL/10 ScSn) and mdx (C57BL/10 mdx) mice at the ages of 3 – 5 months were used. They were housed and fed as described previously\(^17\). They were treated in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan.

The animals were anesthetized with diethyl ether and sacrificed by cervical dislocation. The abdomens were immediately opened and the entire
colon was removed. The contents of the isolated colon were gently flushed out with Krebs solution. The colon was separated into proximal, central and distal segments, each of which was about 1.5 cm long. Colonic segments were mounted horizontally in an organ bath (4 × 5 × 4 cm) with Krebs solution kept at 37˚C and oxygenated continuously with 95% O₂ – 5% CO₂ gas. Krebs solution had the following composition (in mM): NaCl 120, KCl 5.0, CaCl₂ 2.5, MgCl₂ 1, NaH₂PO₄ 1, NaHCO₃ 25, and glucose 11 at pH 7.2 – 7.4.

The distal end of each colonic segment was tied around the mouth of an L-shaped tube connected to a pressure transducer (Nihon Koden, TP603T, Tokyo) with the other end ligated with a silk thread. The mechanical activity of isolated colons was detected as changes of their intraluminal pressure, which are mainly generated by contractile activity of circular muscles, and stored in a microcomputer by using an A/D converter (Keyence, NR110, Tokyo). The preparations were allowed to equilibrate for at least 1 hour before starting a series of experiments. Before the experiment, the intraluminal pressure was increased to about 10 cmH₂O. Most of the data were obtained from proximal and distal segments of the colon, and a few from central segments. Since the data from central segments were quite similar to those from distal ones, they were combined and treated as data from the distal colon. Since the smooth muscle contracted under high pressure without generating a prominent contractile ring in colonic segments under the present experimental conditions, it was difficult to determine which contraction in the colonic segment was related to the peristaltic movement. Therefore, we regarded cyclic changes of intraluminal pressure with a sufficient magnitude as CCs in the colon.

**Drugs**

The following drugs were used: ATP, apamin, carbamylcholine chloride, hexamethonium chloride, NOLA, and SNP. They were purchased from Sigma Chemicals (St. Louis, MO). All drugs were prepared as stock solutions in distilled water except for NOLA, which was dissolved in Krebs solution. A small amount of stock solution was introduced to the organ bath to obtain the required final dose while stirring. Drugs were tested for 15 – 20 min at each dose in order from low to high doses. Two or three kinds of drugs were tested in each preparation. Unless otherwise stated, the preparation was washed with Krebs solution for about 30 min before and after the test of a drug.

**Data analysis and statistics**

The frequency of CCs was measured during the last 10-min period of time following drug administration when a steady state was reached. One-way or two-way ANOVA followed by Scheffe’s F test was employed to detect any significant differences between the groups as required. Data are expressed as means ± sem (n = number of preparations). Statistical significance was determined at the 5% level (p<0.05).

**RESULTS**

Many, though not all, of proximal colons from both mdx and control mice exhibited CCs spontaneously, while more than half of distal colons from both types of mice were quiescent. The quiescent colons produced CCs in response to hexamethonium, NOLA and apamin. Since the present study was aimed at characterizing the neural mechanism responsible for CCs in the mdx as well as control colon, attention was focused on the effect of drugs on the frequency rather than the strength of CCs. The frequency of CCs was observed to be lower than 8 cpm in all colons examined and lower than 4 cpm in about two-thirds of them. Since there was little difference in the distribution or the mean of the frequency of spontaneous CCs among the four colon groups (i.e., proximal and distal colons from mdx and control mice), the CCs were not divided into subgroups depending on their frequency.

**NOLA**

Figure 1 shows examples of the effect of NOLA...
on proximal and distal colons from mdx and control mice. In general, NOLA either induced CCs in quiescent colons or increased the frequency of ongoing CCs when they existed. Figure 2 shows the frequency of CCs at doses of 0.01, 0.1, and 1 mM NOLA in proximal and distal colons from mdx and control mice. Though the frequency of CCs depended on the dose of NOLA in some preparations (Fig. 2), there was no significant dependence of the CC frequency on the dose of NOLA in any of the colon groups. The frequency of CCs at a given dose of NOLA was lower in the mdx distal colon than in the control distal colon at 0.01 and 0.1 mM (p<0.05) and mdx proximal colon at 0.1 (p<0.05) and 1.0 mM (p<0.001). Since, as described previously, there was no significant difference in the frequency of CCs among the colon groups, and since NOLA depresses NO production, the present result suggests that the mdx distal colon is less sensitive to the inhibitor of NO production than the mdx proximal and control distal colons.

**Hexamethonium**

Hexamethonium induced CCs in all of the colons examined at a dose of 0.5 mM or even at a dose of 0.05 mM in some preparations (Fig. 3). This result indicates that blockade of the nicotinic receptor of cholinergic neurons by hexamethonium is quite effective in generating CCs.

Hexamethonium at a dose of 5 mM either raised the frequency of the ongoing CCs without affecting greatly their magnitude or increased the magnitude of CCs with reduced frequency. A typical example of the latter is shown in Fig. 4B. The transition of the form of CCs suggests that an inhibition of outputs from the cholinergic neuron accelerates the pacemaker activity for CCs to a great extent.
SNP (5 µM) was administered to colons in the presence of hexamethonium (0.5 mM) to examine the effect of SNP on CCs. SNP decreased both the frequency and amplitude of the CCs in many of the colons examined (Figs. 3C and D) and abolished the CCs completely in some colons (Figs. 3A and B). Table 1 shows the frequency of CCs before and after treatment with SNP in mdx and control colons. This result suggests that acceleration of NO production by SNP inhibits the activity of the pacemaker for CCs. There was no significant difference in the frequency of CCs in the presence of SNP between mdx colon and the control.

**ATP, apamin and carbamylcholine**

ATP (0.1 mM) administered to colons in the presence of hexamethionium (0.5 mM) decreased the frequency of CCs in about half of the colons (Figs. 5B and D) but had little effect on CCs in the other half (Figs. 5A and C) with both mdx and control colons. ATP did not abolish the CCs completely in any of the colons examined, suggesting a weaker inhibitory effect of ATP on the CCs than SNP (cf. Figs. 3 and 5). On the other hand, apamin (250 nM) applied to colons after ATP and in the presence of hexamethionium (0.5 mM) increased the frequency of CCs (Fig. 5). These results suggest that ATP or its related substances have some inhibitory effect on the

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**Table 1** Frequency of cyclic contractions (CCs) before and after treatment with sodium nitroprusside (SNP) in mdx and control colons.

<table>
<thead>
<tr>
<th>Type</th>
<th>Proximal (n)</th>
<th>Distal (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>4.10 ± 0.60</td>
<td>2.40 ± 0.34</td>
</tr>
<tr>
<td>After</td>
<td>2.58 ± 0.25*</td>
<td>1.57 ± 0.41</td>
</tr>
<tr>
<td><strong>Mdx</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>3.42 ± 0.21</td>
<td>2.39 ± 0.62</td>
</tr>
<tr>
<td>After</td>
<td>0.83 ± 0.54**</td>
<td>0.90 ± 0.58*</td>
</tr>
</tbody>
</table>

Significant difference before and after treatment with SNP; *p<0.05, **p<0.01. No significant difference between mdx and control colons after treatment with SNP.
pacemaker activity.

Carbamylcholine induced a transient contraction dose-dependently in the range of 0.5 – 50 µM in all of the colons examined (Fig. 6). Carbamylcholine appeared to have little effect on the ongoing CCs (Fig. 6C), while it induced CCs in some of the quiescent colons from mdx and control mice (Fig. 6A).

Tone

Reduction of basal pressure upon administration of SNP (5 µM) was regarded to indicate active tone (Fig. 3A and D). SNP-sensitive tone was observed in 2 out of 6 preparations of mdx proximal colon, while no SNP-sensitive tone was observed in control proximal colons (n=5). On the other hand, about two-thirds of the preparations of mdx (6 out of 9 preparations) and control (10 out of 14 preparations) distal colons developed SNP-sensitive tone. It was also noticed that especially in distal colons from control and mdx mice, generation of powerful CCs in response to application of either hexamethonium (5 mM) or NOLA was accompanied by lowering of the basal pressure from which the CCs rose (Figs. 1D, 4A, and 5B). This may mean that the existing basal pressure reflects an active tone. The lowering of the basal tone was most prominent when the CCs were powerful. This result suggests that there is an interaction between the mechanisms underlying the active tone and the CCs.

DISCUSSION

The present study indicated that there was no apparent difference in the effect of pharmacological agents, except NOLA, on the generation of CCs between mdx and control colons. The difference in the responsivity to NOLA between mdx and control colons was not qualitative but quantitative. There-
fore, we first consider the pacemaker mechanism for CCs which seems to be common to colons from mdx and control mice, and subsequently consider the difference in the responsivity to NOLA between them.

**Neural mechanism underlying the generation of CCs**

The present study showed that in the mouse colon, enhancement of NO synthesis by SNP inhibited the generation of CCs, while its depression by NOLA induced the generation of CCs, in agreement with previous reports\(^1\). NO has been viewed as the main non-adrenergic, non-cholinergic inhibitory neurotransmitter\(^9\)\(^\text{10}\). Therefore, it is likely that the pacemaker receives inhibitory inputs from NO-releasing neurons (NO neurons). On the other hand, hexamethonium, a blocker of the nicotinic receptor, was a potent initiator of the CCs (Figs. 3 and 6), suggesting that the cholinergic neuron is presynaptic to a neuron which suppresses the activity of the pacemaker. The NO neuron may be postsynaptic to the cholinergic neuron.

Apamin, an inhibitor of the ATP-activated K-channel, reduces inhibitory junction potentials in the circular smooth muscle in the mouse colon\(^1\)\(^\text{18}\). Since apamin increased the frequency of CCs in the present study (Fig. 4D), it is thought that the pacemaker receives synaptic inputs which are inhibited by apamin. In the rat colon, exogeneous ATP causes a slight hyperpolarization in the circular smooth muscle and reduces the frequency of CCs\(^1\)\(^\text{2}\). In the present study with the mouse colon, exogeneous ATP decreased the frequency of CCs. Therefore, it is likely that neurons which release ATP or a related substance have a function that suppresses the pacemaker activity, though their function may be minor compared with that of the NO neuron.

Carbamylcholine, an agonist of the muscarinic receptor, produced a transient dose-dependent contraction by its direct action on the receptors on the smooth muscle membrane. If there is a reflex mechanism from the smooth muscle or the surrounding tissues to enteric nerves, contraction of the smooth muscle would induce CCs. Actually, generation of CCs by carbamylcholine was observed in some of the colons examined (Fig. 6A), though carbamylcholine had little effect on CCs in many of them. Under the present experimental conditions, in which the intraluminal pressure was raised to about 10 cmH\(_2\)O before the start of the experiment, contraction of the smooth muscle might occur with a small amount of change in the diameter of the colonic segment. This may be one of the reasons why carbamylcholine had little effect on CCs in many colons. It is also possible that the reflex mechanism might be numbed or fully activated before the administration of carbamylcholine, since, as described above, the colon was pre-loaded with a high intraluminal pressure. Development of active tone in many of the colons suggests that the reflex
mechanism was working in our preparations.

We speculate that there was a functional connection among neurons in the pacemaker mechanism based on the results of the present study, as shown in Fig. 7. The neural inputs to the smooth muscle have been deduced from a previous report \(^{11}\). That is, the circular smooth muscle receives inhibitory inputs mediated by NO and possibly ATP. Though it is not certain whether or not the same NO neuron innervates both the pacemaker and the smooth muscle, it is convenient to assume that the same NO neuron innervates both of them. Here, the NO neuron is assumed to release ATP in addition to NO, since co-localization of ATP and NO has been reported in neurons of the myenteric plexus in the rat colon \(^{19}\). The NO neuron is assumed to receive excitatory inputs from cholinergic neuron via nicotinic receptor, as discussed previously in this section. The effect of hexamethonium on the generation of CCs suggests that the cholinergic neurons (Tonic type neuron in Fig. 7) fire spontaneously and vigorously, supporting a steady firing of NO neurons induced through the nicotinic receptor. Such a mechanism explains why inhibition of the function of the NO neuron at pre- as well as post-synaptic sites by drugs brings about the CCs. The pacemaker, when released from inhibition by NO neurons, generates a periodic burst of discharges which causes CCs in the smooth muscles. Though it is uncertain whether or not the activated pacemaker inhibits the activity of NO neurons via interneurons, the interneuron is incorporated in the model. The neural mechanism for development of tone is out of consideration in the model.

**Low NOLA sensitivity in mdx distal colon**

NOLA induced CCs in mdx as well as control colons, while SNP, a NO donor, inhibited CCs. This result is consistent with those of previous reports \(^{12,14}\) at least qualitatively. The frequency of CCs in the presence of NOLA was lower in mdx distal colon than in control colon (Fig. 2). This suggests a disorder in the activity of NO synthase in the NO neuron of the mdx distal colon (see the model in Fig. 7). In skeletal muscle, the NO synthase is normally anchored by dystrophin and localized at a proper position \(^{20-22}\). Deficiency of dystrophin re-localizes the NO synthase. Since dystrophin is also localized in neurons \(^{23,24}\), it seems possible that NO synthase in nerve terminals of the NO neuron is disordered especially in mdx colon. Such a disorder in the localization of NO synthase may be responsible for the observed difference in the sensitivity to NOLA between the mdx distal colon and the control.

Mancinelli *et al.*\(^{13}\) reported a frequent retrograde peristalsis which disturbs analward transport of colon contents, especially in the distal colon of “female” mdx mice. This may mean that the distal colon isolated from “female” mdx mice has a higher pacemaker activity than the control. The lower sensitivity to NOLA of the mdx distal colon compared with the control in the present study may have no direct relation to the observation made by Mancinelli *et al.*, however, since these authors reported retrograde peristalsis only in colons isolated from “female”, not “male”, mdx mice, and since the present results were obtained with colons isolated from “male” mdx mice. **Tone**

Under the present experimental conditions in which the colon was pre-loaded with a high pressure, tone might be developed via reflex from the smooth muscle to the enteric nerves. In the present study, the proximal colon from mdx mice developed an SNP-sensitive tone, while the control proximal colon did not. This result agrees with a previous report\(^{15}\). There was no difference in the tendency to develop tone in the distal colon between mdx and control mice in the present study, suggesting that there was no difference in the mechanism of generation of tone between the distal colons from mdx mice and their control.

In the present study, powerful CCs induced by hexamethonium markedly reduced the tone. One possibility is that powerful contraction generated by
spikes in the circular smooth muscles may be followed by relaxation as a result of some inherent property of the smooth muscle. Another possibility is that the neural mechanism underlying the CCs interacts with the mechanism responsible for the development of tone. Since, however, electrical events which underlie the peristalsis travel spontaneously over the colonic segments in which contraction of the smooth muscles is inhibited with nifedipine, contraction of the smooth muscle is not required for the travelling of peristalsis. The independence of the travelling of peristalsis from the generation of tone suggests that the latter possibility is unlikely.

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抄 録
筋ジストロフィー症マウス大腸の機械的活動に対する
神経伝達修飾薬の効果

荻原 恭子

デュシェンヌ型筋ジストロフィー患者で見られる大腸蠕動運動異常の原因を探る目的で、その動物モデルである筋ジストロフィー症（mdx）マウス大腸を口側と肛門側に分離し、各々の機械的活動に対する神経伝達修飾薬の効果を調べ対照と比較した。多くの口側腸管では周期的収縮（cyclic contractions: 以下 CCs と略す）が自発的に生じたが、肛門側腸管では CCs の発生は数少なかった。Hexamethonium (0.05 〜 5 mM), apamin (250 nM), Nω-nitro-L-arginine (以下 NOLA と略す) (0.01 〜 1 mM) の投与は標本の収縮活動を活発にしたのに対し, sodium nitroprusside (5 μM) や ATP (0.1 mM) は CCs の頻度を減少させた。Carbamylcholine (0.5 〜 50 μM) は一過性の収縮を引き起こしたが, CCs に対しては顕著な効果はなかった。0.1 mM NOLA 存在下での mdx の肛門側腸管の CCs の頻度は 2.3 ± 0.3 cpm (mean ± sem, n=14)で，対照 (3.7 ± 0.6 cpm, n=13) よりも有意に小さかった (p<0.05)。NOLA 以外の薬物に対しては, mdx の肛門側腸管は対照と同様の反応を示した。Mdx の口側腸管の薬物反応は対照と同様であった。以上の結果は mdx の肛門側腸管の一酸化窒素合成が異常なことを示唆する。また, mdx 腸管では持続的収縮の自発的発生が顕著であり, この収縮は hexamethonium による CCs の発生で抑制されたので, CCs と持続的収縮の発生機構の間には相互作用があると考えられる。

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