Carbon dioxide-induced inhibition of mechanical activity in gastrointestinal smooth muscle preparations isolated from the guinea-pig

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Abstract

Mechanical responses of smooth muscle elicited by application of CO₂-gas bubbled physiological salt solution (CO₂-gas solution) were investigated in isolated stomach antrum and colon preparations of the guinea-pig. Circular smooth muscle preparations of both colon and stomach were spontaneously active with periodic generation of phasic contractions. In colonic preparations, the CO₂-gas solution produced a biphasic response, with an initial small transient contraction followed by a sustained inhibition of phasic contractions. Removal of the CO₂-gas solution allowed a slow recovery of the spontaneous contractions over a period of about 40 min. The recovery developed with a similar time course irrespective of the length of time exposed to CO₂-gas solution. The inhibitory responses elicited by CO₂-gas solution were not modulated by atropine, Nω-nitro-L-arginine or neostigmine. Atropine-sensitive excitatory responses of smooth muscle elicited by transmural nerve stimulation or exogenously applied acetylcholine were attenuated or abolished in the presence of CO₂-gas solution. In stomach preparations, the CO₂-gas solution elicited a tri-phasic response, with an initial transient relaxation followed by a transient contraction and then a sustained inhibition of the rhythmic contractions. The peak amplitude of the transient contraction was about 2.5 times larger than the spontaneous phasic contractions. The pH of the CO₂-gas solution was reduced to about 6. Application of pH 6 solution again produced a tri-phasic response, as was the case for the CO₂-gas solution, however the amplitude of the transient contraction was only about 0.4 times that of the spontaneous contractions. The re-appearance of the abolished phasic contraction was quicker with the pH 6 solution (about 1.8 min) than it was for the CO₂-gas solution (about 6 min). The inhibitory responses elicited by the CO₂-gas solution could be simulated only partly by the acidified solution, and a possible involvement of additional factors in the inhibition elicited by CO₂-gas solution was considered.

Key words: CO₂ gas, intestinal smooth muscle, spontaneous contraction, acetylcholine, acidification
Introduction

Gastrointestinal smooth muscle is spontaneously active, with rhythmic generation of phasic contractions that are triggered mainly by interstitial cells of Cajal (ICC) distributed in the wall (Sanders, 1996; Huizinga et al., 1997; Komuro, 2006). The properties of the ICC distributed in the wall of the digestive tract are heterogeneous. The ICC distributed in the myenteric plexus layer between the circular and longitudinal smooth muscle layers (myenteric ICC, ICC-MP) pace the activity of smooth muscle in different regions of the tract. The intramuscular ICC (ICC-IM) found in both the circular and longitudinal smooth muscle layers of the stomach and colon of many species, or the deep muscular ICC (ICC-DMP) distributed in the circular smooth muscle layer close to the mucosal layer of the small intestine, also contribute to the spontaneous activity of the smooth muscle. In the colon, a group of ICC distributed in the submucosal layer (submucosal ICC, ICC-SMP) also take part in the production of the spontaneous contraction of the smooth muscle, in addition to the role of the ICC-MP and ICC-IM in many laboratory animals such as the guinea-pig (Kobayashi et al., 1996; Nahar et al., 1998), dog (Kobayashi et al., 1995), mouse (Yoneda et al., 2004) and rat (Plujà et al., 2001; Kato et al., 2009).

Endoscopy has been introduced for surgical operation or biopsy of intestinal tissues in the clinic, due to its low impact and low damage to the patients. But this requires an elevated pressure within the body cavity to enlarge it and allow for an increased viewing field of the operating area to facilitate the operation (Bretthauer et al., 2003; Yamamoto, 2007; Dellon et al., 2009; Uraoka et al., 2011). The elevation of pressure in the body cavity has been carried out by injecting air at high pressure, however the alternative use of CO$_2$ gas is gaining attention for many reasons, particularly because of the resulting facilitated tissue repair after the operation (Bretthauer et al., 2003; Dellon et al., 2009; Uraoka et al., 2011). Application of CO$_2$ gas has been introduced for the abdominal insufflation during laparoscopic surgery, due to its low cost, nonflammability, chemical stability and low risk of venous gas embolism (Mann et al., 1997; Zhu et al., 2010). The use of CO$_2$ gas during anesthesia has also been introduced to facilitate tissue repair (Suzuki et al., 2010). No significant changes have been found in the morphology of the intestinal wall as a result of the infusion of CO$_2$ gas into this body cavity (Ordemann et al., 2004). However, the mechanism involved in this facilitated tissue recovery as a result of using CO$_2$ gas is still unclear. An increased blood flow or dilation of the intestine as a result of the increased level of CO$_2$ has been observed (Yasumasa et al., 2006), while an elevated release of some bioactive substances such as adenosine by CO$_2$ has also been reported (Deshpande et al., 1999).

It is reasonable to understand that the introduction of CO$_2$ gas may reduce the oxygen tension, and that this will produce an acidification of body fluid. Hypoxia elicits inhibition of the activity of intestinal smooth muscle, possibly as a result of the reduced energy supply due to the inhibition of ATP synthesis in mitochondria (Ishida and Shibata, 1982; Nasu et al., 1982; Pettersson, 1985; Hayashi et al., 1986; Ishida and Takagi-Ohta, 1996). Both inhibition of the release of Ca$^{2+}$ from internal stores during the stimulation of receptors at the membrane (Testini et al., 2002) and the inhibition of Ca-channels at the plasma membrane (Rekalov et al., 1997) also occur under hypoxic conditions. Modulation of the activity of smooth muscle in response to a change in pH is also known in different visceral smooth muscle tissues (Wray, 1982).
Attempts were made to investigate the properties of the mechanical responses elicited by stimulation with CO₂ in circular smooth muscle preparations isolated from the proximal colon and middle stomach (antrum) of the guinea-pig. Experiments were carried out to investigate the properties of mechanical responses elicited by stimulation with CO₂-gas saturated Krebs solution (CO₂-gas solution) in circular smooth muscle preparations. The results have indicated that CO₂-gas solution had strong inhibitory actions on colonic and gastric smooth muscle preparations, as the contractions produced spontaneously or those evoked by endogenous and exogenous acetylcholine were strongly attenuated. The possible causal effects of acidification by changing the pH of the bathing solution on the inhibition of smooth muscle activity were also investigated.

**Materials and Methods**

Male guinea-pigs, weighing 200–300 g, were anesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (sevoflurane, Maruishi Pharm., Co. Ltd., Osaka, Japan), and exsanguinated by decapitation. All animals were treated ethically according to the guiding principles for the care and use of experimental animals in the field of basic sciences, as approved by The Experimental Animal Committee of Nagoya City University Medical School. The stomach and colon were isolated from the animal, and stored in Krebs solution at room temperature.

The upper part of the distal colon, close to the flexure region between the proximal and distal colon (proximal colon), was excised as a 1–2 cm long segment which was opened by cutting vertically. Circular muscle strip preparations, about 1 mm wide and 1.5 cm long, were made by cutting parallel to the running direction of the circular muscle. Two types of preparation were made: (i) with mucosal and submucosal layer removed (submucosal layer removed preparations) and (ii) with all layers attached (intact preparations). The stomach was excised, opened by cutting along the lesser curvature and the mucosal layer removed. Circular smooth muscle preparations (with longitudinal smooth muscle layer attached), about 1 mm wide and 1.5 cm long, were prepared from the antral region.

Both ends of each of these preparations were tied with fine threads, and suspended vertically in the center of a cylindrical recording chamber (diameter 10 mm, 20 mm deep). Preparations were superfused with oxygenated Krebs solution (warmed to 36.5°C), at a constant flow rate of about 3 mL/min. One thread was anchored to the bottom of the chamber, while the other end was connected to the lever of a force-transducer (TB-612T, Nihon-Kohden, Tokyo, Japan). The isometric force changes produced by the circular muscle were recorded through a pre-amplifier (AP-621G, Nihon Kohden, Tokyo, Japan), digitized using P-clamp (Axon Instruments, Foster City, CA, USA) and stored on a personal computer for later analysis.

The ionic composition of the Krebs solution was as follows (mM): Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134 and glucose 11.5. The solution, kept in a 1 L volume bottle, was aerated with O₂ containing 5% CO₂, and the pH of the solution, measured by a pH meter (Seven Easy S20, Mettler-Toledo International Inc., Switzerland), was maintained at 7.2–7.3. The CO₂-gas saturated Krebs solution was prepared by bubbling the Krebs solution with 100% CO₂ gas for more than 1 hr, prior to muscle preparations being stimulated by superfusing with this CO₂-gas solution for a period of time. Krebs solution with a pH of 6.0 was prepared by
adding 10N HCl drop-wise to the Krebs solution, while bubbling with a 95% O\textsubscript{2}/5% CO\textsubscript{2} gas mixture.

Chemicals used were acetylcholine chloride (ACh), atropine sulphate, N\textsuperscript{\textominus}-nitro-L-arginine (L-NA) and neostigmine bromide. All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MI, USA). These chemicals were dissolved first in distilled water at concentrations which were more than 1,000 times higher than those used in the experiments, and then further diluted with Krebs solution to prepare the desired concentrations. The addition of these drugs did not alter the pH of the Krebs solution.

Experimental values were expressed as the mean value ± standard deviation (S.D.). Statistical significance was tested using the Student’s \(t\)-test, and probabilities of less than 5\% (\(P<0.05\)) were considered to be significant.

Results

**Mechanical activity of intestinal smooth muscle preparations in response to CO\textsubscript{2} stimulation**

Circular smooth muscle preparations isolated from the proximal colon of guinea-pigs were spontaneously active, with intact muscle preparations generating periodic bursts of phasic contractions, while submucosal layer removed preparations periodically generated large amplitude phasic contractions (Fujimoto \textit{et al.}, 2010). Figure 1A shows the mechanical responses of a submucosal layer removed colonic preparation during stimulation with CO\textsubscript{2}-gas solution for 3 min. Application of CO\textsubscript{2}-gas solution elicited a transient small contraction which was followed by the cessation of spontaneous contractions. The transient small contractile responses were variable,
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and they were sometimes absent (or below detectable size). The resting tension of the muscle was often reduced during stimulation with CO₂-gas solution. Removal of the CO₂-gas solution allowed a recovery of spontaneous contractions, with a delay of about 5 min, and as a consequence a quiescent period of 4–8 min occurred before the re-appearance of spontaneous contractions. The recovery of spontaneous phasic contractions following the inhibition developed slowly after the removal of the CO₂-gas solution, during which period the frequency and amplitude of the phasic contractions were successively increased. It took from 12 to 45 min (23.9 ± 11.4 min, n=25) before complete recovery of the spontaneous contractions. The frequency of the spontaneous contractions returned to that before application of CO₂-gas stimulation, following the recovery of the amplitude. The inhibitory responses of the colonic smooth muscle to the stimulation with CO₂-gas solution were reversible, and similar responses could be elicited several times with repeated stimulation (data not shown). Intact preparations of the proximal colon periodically generated bursts of irregular contractions, as reported previously (Fujimoto et al., 2010), and stimulation with CO₂-gas solution elicited responses similar to those observed in the submucosal layer removed preparation (data not shown).

Attempts were made to investigate some properties of the inhibition of spontaneous contraction by CO₂-gas solution. When submucosal layer removed colonic smooth muscle preparations were exposed to CO₂-gas solution for 1 min, spontaneous contractions were inhibited for about 5 min (Fig. 2A), while the inhibition was doubled by increasing the exposure to CO₂-gas solution for 5 min (Fig. 2B). The time period of the inhibition was increased to about 15 min by the exposure to CO₂-gas solution for 10 min (Fig. 2C). The time period of the inhibition of spontaneous contraction was plotted as a function of time exposed to the CO₂-gas solution, and the results indicated that these two factors were linearly related (Fig. 3A). The quiescent period of

Fig. 2. Mechanical responses of a guinea-pig proximal colon smooth muscle preparation (mucosal layer removed) during stimulation with CO₂-gas solution. CO₂-gas solution was applied for 1 min (A), 5 min (B) and 10 min (C) (at the bar shown under each trace). All traces were obtained from the same preparation.
time resulting from the stimulation with CO₂-gas solution (equal to the time for re-appearance of spontaneous contraction after cessation of the stimulation, recovery time) was also plotted as a function of the duration of the exposure to the CO₂-gas solution, and it was found that the recovery time was constant, irrespective of the duration of exposure (Fig. 3B). Thus, it appeared that the stimulation with CO₂-gas solution for 1 min was sufficient to produce the maximum inhibition of spontaneous activity in the smooth muscle preparations.

Stimulation of circular smooth muscle preparations isolated from the antrum of the guinea-pig stomach with CO₂-gas solution elicited a response with three phases, an initial transient relaxation, followed by a transient contraction and then a sustained relaxation with an absence of spontaneous contractions. Figure 1B shows the responses of an antral smooth muscle preparation exposed to CO₂-gas solution for 3 min. Rhythmic contractions were immediately stopped and a transient
relaxation was elicited. The transient contraction followed with a peak amplitude larger than that of the spontaneous contractions (2.48 ± 1.10 times larger than spontaneous contraction, n=9), and the time required to reach the peak amplitude was 2.49 ± 0.85 min (n=9). This contraction was elicited soon after the beginning of the superfusion with the CO₂-gas solution. Continued stimulation with the CO₂-gas solution produced a sustained relaxation of the antral smooth muscle, with no spontaneous rhythmic contractions. A comparison of the time course of the initial relaxation and that of the sustained relaxation suggested that both relaxations were a continuous single process, and it appeared that the sustained relaxation which started immediately in response to the application of CO₂-gas solution was interrupted by the transient contraction. The inhibition of the spontaneous contractions by the CO₂-gas solution reversed slowly after the removal of the CO₂-gas solution, but required 30–60 min (mean time, 40.97 ± 10.16 min, n=9) for a complete recovery. The frequency of the spontaneous contractions tended to increase during the recovery phase.

Effects of N-\textsuperscript{\textomega}nitro-L-arginine, neostigmine and atropine on spontaneous phasic contractions

The possible involvement of enteric nerve excitation in causing the inhibition of the spontaneous activity by the CO₂-gas solution was examined in colonic smooth muscle preparations. Intestinal smooth muscle is innervated mainly by cholinergic excitatory and nitrergic inhibitory nerves (Hoyle and Burnstock, 1989; Furness, 2000), as has been shown in the colon of the guinea-pig (Fujimoto et al., 2010). Experiments were carried out to observe the effects of N-\textsuperscript{\textomega}nitro-L-arginine (L-NA, an inhibitor of nitric oxide biosynthesis), neostigmine (an inhibitor of acetylcholine esterase) and atropine (muscarinic receptor antagonist) on the responses produced by the application of CO₂-gas solution for 3 min. In intact colonic smooth muscle preparations, the amplitude of the spontaneous contractions was enhanced by 3 × 10⁻⁵ M L-NA or 10⁻⁷ M neostigmine, while it was attenuated by 10⁻⁶ M atropine, as reported previously (Fujimoto et al., 2010). The inhibitory responses produced by the CO₂-gas solution were elicited either in the absence or presence of L-NA (Fig. 4B), neostigmine (Fig. 4C) or atropine (data not shown). Quantified data showed that the time required for the recovery from the stimulation with CO₂-gas solution was not significantly altered by L-NA (control, 7.06 ± 2.62 min; in L-NA, 7.99 ± 3.95 min; n=8; P>0.05), neostigmine (control, 7.44 ± 2.30 min; in neostigmine, 7.32 ± 3.34 min; n=8; P>0.05) and atropine (control, 8.18 ± 1.91 min; in atropine, 7.38 ± 1.73 min; n=4; P>0.05). These results suggested that the inhibition of the spontaneous contraction by CO₂-gas solution was not causally related to the enhanced release of nitric oxide (NO) or reduced release of acetylcholine from enteric nerves.

Effects of CO₂-gas solution on acetylcholine-induced responses

Acetylcholine (ACh) is an excitatory agonist for colonic smooth muscle (Fujimoto et al., 2010). In submucosal layer removed preparations, application of 10⁻⁶ M ACh for 3 min produced an excitatory response, an increase in frequency of the phasic contractions with a sustained elevation of the resting tension (Fig. 5B). The ACh-induced excitatory response could be elicited in preparations in which spontaneous contraction were absent after exposure to the CO₂-gas solution, however the amplitude and frequency of the ACh-induced phasic contractions were markedly
Fig. 4. Effects of L-NA and neostigmine on mechanical responses produced by CO$_2$-gas solution in an intact circular muscle preparation isolated from the proximal colon of a guinea-pig. CO$_2$-gas solution was applied for 3 min in the absence (A) and presence of $3 \times 10^{-5}$ M L-NA (B) or $10^{-7}$ M neostigmine (C). All responses were recorded from the same preparation.

Fig. 5. The effects of CO$_2$-gas solution on the mechanical responses produced by exogenously applied acetylcholine (ACh) in a circular smooth muscle preparation isolated from the proximal colon of a guinea-pig. Responses were produced by application of CO$_2$-gas solution for 10 min (A), $10^{-4}$ M ACh for 3 min (B) and $10^{-4}$ M ACh for 3 min in the presence of CO$_2$-gas solution (C). All responses were recorded from the same preparation.
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The effects of CO2-gas solution on the mechanical responses elicited by transmural nerve stimulation (TNS) were investigated in mucosal layer removed colonic preparations. In these preparations, TNS (10 pulses at 10 Hz frequency) elicited an enhanced amplitude of phasic contraction which was abolished by 10-7 M atropine, confirming that this muscle received a cholinergic excitatory nerve (Fujimoto et al., 2010). Figure 6A shows the mechanical responses produced by repeated application of TNS every 5 min, while stimulation of muscle with CO2-gas solution diminished and the ACh-induced sustained contractions were absent (Fig. 5C). Quantified data showed that in control preparations, the frequency of the spontaneous contractions (2.08 ± 0.10 times/min, n=10) was increased to 5.68 ± 0.69 times/min (n=10) by 10^-6 M ACh, but it was increased only to 4.03 ± 1.28 times/min (n=10) in the presence of CO2-gas solution; this value was significantly low compared to the control, but still significantly higher than that observed in the absence of CO2-gas solution. The peak amplitude of spontaneously generating phasic contractions at 48.58 ± 1.36 mN (n=10) was not significantly enhanced by ACh (47.62 ± 3.04 mN, n=10, P>0.05). However, in the presence of CO2-gas solution, the amplitude of phasic contractions elicited during the ACh-stimulation (13.05 ± 4.82 mN, n=10) was significantly smaller than in the absence of CO2-gas solution. Thus, CO2-gas solution strongly attenuated the ACh-induced excitatory responses of colonic smooth muscle.
solution for 3 min. The TNS-induced response (Fig. 6B) was abolished during the inhibition of rhythmic contraction by CO₂-gas solution (Fig. 6C). The TNS-induced response recovered slowly with successive increase in amplitude of spontaneous contractions, and it required 20–30 min for the complete recovery (Fig. 6, D and E).

Comparison of the responses elicited by CO₂-gas solution with low-pH solution

It was reasonable to consider that bubbling Krebs solution with pure CO₂ gas, instead of O₂ containing 5 % CO₂ gas, may alter the pH of the solution. Attempts were made to measure the pH of the solution during application of CO₂ gas. When the Krebs solution had been bubbled with pure CO₂ gas for 1 h, the pH of the solution was found to be lowered to about 6 (6.01 ± 0.15, n=5), and the value remained unchanged after bubbling the Krebs solution with CO₂ gas for up to 3 h (data not shown).

Experiments were carried out to compare the effects of stimulation with CO₂-gas solution with those with low-pH solution in smooth muscle preparations isolated from the antrum of the guinea-pig stomach. As shown in Figure 7A, application of CO₂-gas solution for 10 min produced a triphasic response. Stimulation of this preparation with the pH 6 solution again produced the triphasic response, with an initial transient relaxation with inhibition of spontaneous contractions, followed by a transient contraction and then inhibition of spontaneous contractions with a sustained relaxation (Fig. 7B). However, the amplitude of the transient contraction generated as the second phase was much smaller than that produced by the CO₂-gas solution. Quantification of the amplitude of the second phase contraction indicated that the CO₂-gas solution produced a transient contraction that was 2.48 ± 3.12 (n=9) times greater relative to the amplitude of the spontaneous contractions, while the pH 6 solution produced a transient contraction that was 0.39 ± 0.05 (n=3)

Fig. 7. Mechanical responses produced by CO₂-gas solution (A) and pH 6 solution (B) in a circular smooth muscle preparation isolated from the antrum of the guinea-pig stomach. Both responses were recorded from the same preparation.
times the amplitude of the spontaneous contractions, with the latter being significantly smaller than the former. The time required to reach the peak amplitude of contraction was 2.49 ± 0.85 min (n=9) for the CO_{2}-gas solution and 2.49 ± 0.12 min (n=3) for the pH 6 solution; these two values were not significantly different. The time required for the recovery of the spontaneous contractions following the inhibition was also different between these two types of stimulation; spontaneous contraction appeared within 1–3 min (1.81 ± 0.3 min, n=5) after the removal of the pH 6 solution, while it required 5–30 min (11.8 ± 3.5 min, n=7; \( P < 0.05 \)) after the removal of CO_{2}-gas solution. The recovery from the inhibition produced by CO_{2}-gas solution developed slowly requiring 20–50 min (40.97 ± 10.16 min, n=9), while the inhibition produced by the pH 6 solution recovered quickly within 8–12 min (9.55 ± 0.73 min, n=3; \( P < 0.05 \)), with an associated generation of a rebound contraction. Thus, the inhibitory responses of the stomach smooth muscle preparations to the CO_{2}-gas solution were not identical to those elicited by the acidified solution. The responses elicited by the pH 6 solution were reversible, and repeated stimulation produced similar responses (data not shown).

**Discussion**

The present experiments showed that the CO_{2}-gas solution elicited a biphasic response in smooth muscle preparations isolated from the proximal colon of the guinea-pig, with an initial transient contraction and following inhibition of spontaneously generated phasic contractions. The inhibition of spontaneous phasic contractions by CO_{2}-gas solution was maintained for a long period of time compared to the length of the application of CO_{2}-gas solution. For example, application of CO_{2}-gas solution for 1 min caused an inhibition of spontaneous activity for 5 min (Fig. 2). Furthermore, the time required for the recovery from the inhibition was similar irrespective of the length of time the preparation was exposed to the CO_{2}-gas solution. These results suggest that an exposure of a preparation to CO_{2}-gas solution for 1 min is sufficient to alter the state of the intracellular mechanism so as to block the generation of spontaneous activity. In the proximal colon, the rhythmic activity of the phasic contractions has been suggested to be paced by ICC-MP distributed in the myenteric layer (Plujà et al., 2001; Kato et al., 2009; Fujimoto et al., 2010). Therefore, it is possible to suggest that the CO_{2}-gas solution inhibits the pacemaker activity of the ICC-MP. Alternatively, inhibition of the contractile mechanism in smooth muscle cells could be responsible for the abolition of the phasic contractions. It is also possible that the activities of both ICC-MP and smooth muscle cells are inhibited by the CO_{2}-gas solution. In considering the successive increase in the amplitude of the phasic contractions, with a similar or a slightly higher frequency, during the recovery from the inhibition produced by CO_{2}-gas solution (see Figs. 1 and 2), it is reasonable to consider that the abolition of the phasic contractions in the CO_{2}-gas solution is mainly due to the inhibition of the contractile mechanisms in the smooth muscle cells. If this is the case, exposure to the CO_{2}-gas solution for 1 min is sufficient to inhibit the excitation-contraction coupling mechanism in smooth muscle cells for around 5 min. This means that the activity of smooth muscle cell is very sensitive to the CO_{2}-gas solution. The CO_{2}-gas solution is anoxic as well as being an acidic solution. It remains unclear which component is responsible for the actions of CO_{2}-gas solution on colonic smooth muscle cells.
The inhibitory responses of colonic smooth muscle preparations during stimulation with the CO₂-gas solution were not altered by atropine, neostigmine or L-NA. The circular smooth muscle of the colon has a rich distribution of cholinergic excitatory and nitrergic inhibitory nerves (Hoyle and Burnstock, 1989; Furness, 2000), and excitation of any of these nerves could modulate the activity of the spontaneous phasic contractions (Fujimoto et al., 2010). The results of the present experiments suggest that neither an enhanced release of ACh from cholinergic nerves or of NO from nitrergic nerves was involved in the inhibition produced by the CO₂-gas solution. An enhanced release of adenosine by CO₂ from enteric nerve preparations has been reported (Deshpande et al., 1999). Adenosine has inhibitory actions on the release of substance P (Broad et al., 1992; Broad et al., 1993; Moneta et al., 1997) and also of ACh (Lee et al., 2001) from enteric nerves. As both of these transmitter substances produce excitatory responses in intestinal smooth muscle (Hoyle and Burnstock, 1989; Furness, 2000), the enhanced release of adenosine may attenuate the activity of smooth muscle. Adenosine itself has inhibitory actions on smooth muscle, through the enhanced production of cyclic AMP (Giron et al., 2008; Matsuda and Miller, 2010). Thus, most of the factors involved in the generation of excitatory responses in intestinal smooth muscle cells may be attenuated by the enhanced release of adenosine. However, the present experiments showed that none of the inhibitory responses produced by the CO₂-gas solution were altered by either atropine, neostigmine or L-NA. These results suggest that neuronal factors may not be involved in the inhibition elicited by the CO₂-gas solution.

In the colonic smooth muscle preparations, the time required for the recovery of the spontaneous contractions following the inhibition produced by the CO₂-gas solution was constant irrespective of the length of time they were exposed to the solution, and a 1 min exposure to the CO₂-gas solution was sufficient to produce the maximum inhibitory response in these preparations. When considering the experimental apparatus used (volume of the tissue chamber, about 2 mL; speed of perfusion, about 3 mL/min), the results are rather unexpected, since 1 min may be nearly the minimum time required to change the solution in the experimental chamber from control to CO₂-gas solution. These results suggest that the modulation of the intracellular smooth muscle mechanism is unexpectedly quick as the CO₂ tension of the solution is elevated. If this is the case, the slow recovery from the inhibition elicited by the CO₂-gas solution may be to the result of a different mechanism to that of the onset of the inhibitory response. The rhythmic activity of intestinal smooth muscle is produced mainly by the ICC-MP (Sanders, 1996; Huizinga et al., 1997), and the periodicity may be produced by the metabolic activity of mitochondria in ICC-MP (Suzuki et al., 2006). Thus, it is reasonable to consider that the inhibition of spontaneous contraction by the CO₂-gas solution is not directly related to the inhibition of mitochondrial activity in ICC-MP. The activity of ICC-MP is enhanced in an anoxic solution (Nakamura et al., 2009), suggesting that the acidification of the solution may be responsible for the inhibition produced by the CO₂-gas solution.

Comparison of the responses produced by the CO₂-gas solution between the stomach antrum and the proximal colon indicates that the initial transient contraction was much more marked in the former compared to the latter. Although the transient relaxation was elicited first in response to the application of CO₂-gas solution, it appears that the sustained relaxation without spontaneous contractions started before the generation of the transient contraction during the application of the
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CO₂-gas solution. The reason for the difference in the size of the initial transient contraction between the stomach and colon preparations remains unclear. It was also considered that these differences may have been the result of the methods used in the evaluation, i.e., the amplitude was expressed as relative to the amplitude of the spontaneously generated phasic contractions, and the absolute value of the force developed by the CO₂-gas solution was not so much different between the stomach and colon preparations. Some additional examination may be required to understand this issue.

The primary idea for the present experiments came from the clinical evidence that the application of CO₂ gas used to expand the internal cavity during endoscopy facilitates the post-operative recovery (Brethauer et al., 2003; Dellon et al., 2009; Zhu et al., 2010; Uraoka et al., 2011). Infusion of CO₂ gas into the abdominal cavity increases the intestinal circulation and reduces the intestinal tension: the former may be produced by the dilatation of vascular smooth muscle and the latter by a reduction of the resting tension of the intestinal smooth muscle, possibly due to the acidification (Testini et al., 2002; Yasumasa et al., 2006). It is reasonable to consider that bubbling Krebs solution with pure CO₂ gas will reduce both the oxygen tension and pH of the solution. Thus, there are at least two factors in the stimulation with the CO₂-gas solution, stimulus as an acidified solution and stimulus as a hypoxic solution. In many types of smooth muscle preparations, acidification of the solution results in a relaxation of the preparation and inhibition of agonist-induced contractions (Wray, 1988; Naderali et al., 1997; Duquette and Wray, 2001; Pierce et al., 2003). Hypoxia induces different responses in different gastrointestinal smooth muscle preparations; in the depolarized guinea-pig taenia coli, anoxia produced by replacing O₂ with N₂ enhances muscle contractions (Nasu et al., 1982; Ishida and Takagi-Ohta, 1996), while N₂-gas replaced anoxic solution produced strong inhibition of mechanical activities in smooth muscle preparations isolated from both the human stomach (Hayashi et al., 1986) and the rabbit colon (Pettersson, 1985). In smooth muscle preparations isolated from the guinea-pig stomach, however, anoxic condition produces differential responses with the inhibition of smooth muscle activity and an enhancement of the activity of ICC-MP (Nakamura et al., 2009).

We found in the present experiments that the pH of Krebs solution was lowered to about 6 in CO₂-gas solution, and that the application of a pH 6 solution produced a sustained inhibition of spontaneous contractions in stomach smooth muscle preparations. Thus, the inhibition of the spontaneous contractions by the CO₂-gas solution could be simulated by acidification of the solution. However, comparison of the responses of preparations to the CO₂-gas solution with those to the pH 6 solution indicated that these two stimuli were not identical. Although both solutions evoked a transient contraction at the beginning of the application, the amplitude of the transient contraction was much larger for the CO₂-gas solution than for the pH 6 solution. The recovery from the inhibition was also different; it required a very long time (about 40 min) for the CO₂-gas solution while it was only a short period of time (less than 2 min) for the pH 6 solution, before the appearance of spontaneous contractions. Thus, it seems likely that the mechanism of the inhibition of spontaneous contraction differs between the CO₂-gas solution and the pH 6 solution.

Even though the responses of gastric smooth muscle were different between the CO₂-gas solution and the pH 6 solution, the strong inhibition of rhythmic activity was produced by either
stimulus. This result strongly suggests that the main inhibitory actions of the CO₂-gas solution are produced by acidification of the solution, but may not be caused by hypoxia. The differences in the responses of the smooth muscle preparations to the CO₂-gas solution and to the pH 6 solution may involve hypoxia as the modulator or the synergist of the inhibitory responses produced by acidification in gastric smooth muscle.

It is summarized that in gastrointestinal smooth muscle preparations, the CO₂-gas solution produces a transient contraction and strong inhibitory responses, and the latter included the abolition of spontaneous contractions and attenuation of ACh-induced contractions. As a part of these responses could be simulated by the pH 6 solution, acidification of the solution may be one of the important factors that induces the inhibition. However, the responses elicited by the CO₂-gas solution were not identical to those elicited by the pH 6 solution, suggesting an involvement of additional factors.

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