Mechanism of Carbon Monoxide-Induced Relaxation in the Guinea Pig Ileal Smooth Muscle

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(Received 9 November 2000/Accepted 11 December 2000)

ABSTRACT. The mechanism of carbon monoxide (CO)-induced relaxation were investigated in the guinea-pig ileum. CO (10%) inhibited the 40 mM KCl-induced contraction. This effect was antagonized by ODQ (1 µM), a soluble guanylate cyclase inhibitor. In contrast, CO did not inhibit the 40 mM KCl-induced increase in cytosolic Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_i\)). Cumulative addition of KCl induced a graded increase in both [Ca\(^{2+}\)]\(_i\) and muscle tension. In the presence of CO, the increase in muscle tension was attenuated whereas the increase in [Ca\(^{2+}\)]\(_i\), was only slightly decreased. Thus, the [Ca\(^{2+}\)]\(_i\)-tension relationship constructed by cumulative addition of KCl shifted downwards in the presence of CO. Using the patch clamp, CO was found to have little effect on the peak Ba currents (I\(_{\text{Ba}}\)) when voltage was stepped from –60 mV to 0 mV. From these results, we conclude that CO inhibits contraction of guinea-pig ileum mainly by the decrease in the sensitivity of contractile elements to Ca\(^{2+}\) via a cyclic GMP-dependent pathway but not by the inhibition of L-type Ca\(^{2+}\) channel.

KEY WORDS: calcium channel, carbon monoxide, guinea-pig, intestinal smooth muscle, relaxation.

Inhibitory nonadrenergic, noncholinergic (NANC) neurons play an important role in the physiological control of gastrointestinal motility. These neurons are involved not only in gastric receptive relaxation and peristaltic movement throughout the gastrointestinal tract, but also in the relaxation of tonically contracted sphincters [1, 17, 27]. The transmitter(s) involved in the NANC relaxation of gastrointestinal preparations is species- and tissue-dependent and several candidates have been proposed including ATP [4], vasoactive intestinal peptide [14] and nitric oxide (NO) [19, 22].

Recently carbon monoxide (CO) has been suggested to play a role in neuronal transmission in the gastrointestinal tract as an inhibitory NANC transmitter [5, 16, 21]. Like NO, CO is an endogenously produced gas molecule [3, 7, 25, 26]. CO is produced in mammalian tissue via action of heme oxygenase, which is the rate limiting enzyme in degradation of heme. Heme is oxidatively cleaved by heme oxygenase to yield CO and biliverdin [13]. Heme oxygenases has been detected in various type of tissues including smooth muscle [12]. CO has been shown to relax vascular tissues [6, 11], cultured vascular smooth muscle cells [18], certain types of visceral smooth muscles such as ileal smooth muscles from guinea pig [24] and circular smooth muscle strips of feline lower esophageal sphincter [16].

To elucidate the mechanism of CO-induced relaxation in intestinal smooth muscle, we examined the effects of CO on Ca\(^{2+}\) channel activity and Ca\(^{2+}\) sensitivity of contractile elements.

MATERIALS AND METHODS

Tissue preparations: Male guinea-pigs (300–400 g) were killed by a sharp blow to the neck and exsanguination. A section of ileum was isolated and placed in a physiological salt solution (PSS). Lumen of ileum was cleaned gently with PSS. A 7 mm diameter glass pipette was inserted in the lumen of the ileum and longitudinal muscle layer was separated from the underlying circular muscle layer. Segments of longitudinal muscle layer, about 10 mm long, was used for experiments.

Measurements of muscle tension: Muscle strip was attached to a holder under a resting tension of 0.5 g. After equilibration for 1 hr in PSS, each strip was repeatedly exposed to 40 mM KCl solution until the responses became stable. The high K\(^+\) solution was prepared by replacing NaCl with equimolar KCl. Muscle contraction was recorded isometrically with a force-displacement transducer and recorded with a pen-recorder.

Fura-2 loading and simultaneous measurements of tension and [Ca\(^{2+}\)]\(_i\): [Ca\(^{2+}\)]\(_i\) was measured according to the method described by Kwon et al. [9] using the fluorescent Ca\(^{2+}\) indicator, fura-2. Muscle strips were exposed to the acetoxyethyl ester of fura-2 (fura-2/AM, 5 µM) in the presence of 0.02% cremophor EL for 5–6 hr at room temperature (22–24°C). The muscle strip was illuminated alternatively (48 Hz) with 340 nm and 380 nm light, and the ratio of 500 nm fluorescence induced by 340 nm excitation (F340) and that induced by 380 nm excitation (F380) were detected with a spectrophotometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). Increase in the ratio due to 40 mM KCl was considered as a reference response (100%).

Preparation of cells for patch clamp analysis: The muscle layers of ileum were cut into small pieces and placed in a Ca\(^{2+}\)-free PSS. Then, the Ca\(^{2+}\)-free PSS was replaced by a

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PSS containing 30 µM Ca²⁺ (low Ca²⁺ PSS) followed by a 30 min incubation at 37°C in a low-Ca²⁺ PSS containing collagenase (0.3 mg/ml), papain (0.3 mg/ml) and bovine serum albumin (1 mg/ml). After the enzyme digestion, tissue fragments were suspended in a PSS containing 0.5 mM Ca²⁺. Cell suspension was placed on glass cover-slips and stored in a moist atmosphere at 4°C. Experiments were carried out at room temperature (22–24°C).

Whole-cell voltage clamp: Whole-cell membrane current was recorded at room temperature using standard patch-clamp techniques. Patch pipette had a resistance of 3–6 MΩ when filled with a pipette solution. Membrane currents were amplified with an Axopatch 1C voltage-clamp amplifier (Axon Instrument). Command pulses were applied using an IBM-compatible computer and pCLAMP (version 5.5) software. The data were filtered at 2 kHz and displayed on an oscilloscope (Tektronix), a computer monitor and a pen recorder (Universal Oscillograph, Harvard).

Solutions: PSS had following composition (mM): NaCl 126; KCl 6; CaCl 2 2; MgCl 2 1.2; glucose 14; HEPES 10.5 (pH was adjusted to 7.2 with NaOH). PSS was saturated with 100% O₂ at 37°C. Inward currents were isolated by the suppression of K⁺ currents using pipette solution containing (mM): CsCl 134, glucose 14, EGTA 0.05, HEPES 10.5 and Na₂ATP 4 (pH was adjusted to 7.2 with CsOH). In the experiments to measure Ca²⁺ currents, CaCl₂ in the bath solution was replaced by BaCl₂.

Preparation of CO solution: CO was applied by superfusing the preparations with CO-containing PSS which was prepared by bubbling PSS in a container of about 100 ml volume with a gas mixture of 10 vol. % CO and 90 vol. % O₂. The resulting 10% CO-containing PSS was applied by changing the solution in the recording chamber. The estimated CO concentration was 100% CO at 37°C. Inward currents were isolated by the suppression of K⁺ currents using pipette solution containing (mM): CsCl 134, glucose 14, EGTA 0.05, HEPES 10.5 and Na₂ATP 4 (pH was adjusted to 7.2 with CsOH). In the experiments to measure Ca²⁺ currents, CaCl₂ in the bath solution was replaced by BaCl₂.

Statistics: Results of the experiments are expressed as mean ± S.E.M. Unpaired Student’s t-test was used for statistical analysis of the results and the number of preparations taken from separate animals was indicated by n. P values less than 0.05 were considered to be significantly different.

RESULTS

CO caused a concentration-dependent relaxation in the 40 mM KCl-induced contraction, and the maximal relaxation was achieved at 10% (Table 1). CO (10%) had no effect on the basal tension of the guinea-pig ileal muscle strips. After CO was applied to the tissues precontracted with 40 mM KCl, a sustained relaxation was induced (Fig. 1A). In the presence of CO, the 40 mM KCl-induced contraction was inhibited by 39.8 ± 5.8% (n=6). To know if the CO-induced relaxation is mediated by the activation of a cyclic GMP signal transduction pathway, the effect of ODQ, a soluble guanylate cyclase inhibitor, was examined. ODQ (1 µM) had no effect on either the resting

<table>
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<tr>
<th>Condition</th>
<th>Relaxation (%)</th>
<th>n</th>
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<tbody>
<tr>
<td>O₂-100%, KCl-40 mM</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>O₂-95%, CO-5%, KCl-40 mM</td>
<td>11.4 ± 3.3*</td>
<td>7</td>
</tr>
<tr>
<td>O₂-95%, N₂-5%, KCl-40 mM</td>
<td>1.2 ± 0.8</td>
<td>5</td>
</tr>
<tr>
<td>O₂-90%, CO-10%, KCl-40 mM</td>
<td>36.4 ± 6.7*</td>
<td>5</td>
</tr>
<tr>
<td>O₂-90%, N₂-10%, KCl-40 mM</td>
<td>4.5 ± 3.4</td>
<td>5</td>
</tr>
<tr>
<td>O₂-80%, CO-20%, KCl-40 mM</td>
<td>43.2 ± 7.1*</td>
<td>5</td>
</tr>
<tr>
<td>O₂-80%, N₂-20%, KCl-40 mM</td>
<td>15.3 ± 4.3</td>
<td>5</td>
</tr>
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</table>

Muscle tension are expressed by relative values taking the value in the resting ileum as 0% and the high K⁺-stimulated ileum as 100%. Each value represents mean ± S.E.M. n, number of experiments. * p<0.05 compared with the corresponding control under same concentrations of N₂.

Fig. 1. The inhibitory effect of ODQ (1 µM), a soluble guanylate cyclase inhibitor, on the CO-induced relaxation in guinea-pig ileum. (A) Record of isometric contraction showing that ODQ suppressed the CO (10%)-induced relaxation of longitudinal strip precontracted with 40 mM KCl. When the muscle tension induced by 40 mM KCl reached a steady state level, CO (10%) was added. (B) Summary of the effect of ODQ on the relaxation induced by CO (10%) (n=6). The tonic contractions of intestinal tissues induced by KCl before and after the application of CO were represented by open and solid columns, respectively. ** p<0.01.
tension level or the KCl-induced tonic contraction (Fig. 1A). In the tissues pretreated with ODQ for 20 min, however, the CO-induced relaxation of the muscle precontracted with KCl (40 mM) was reduced significantly from 39.8 ± 5.8% to 5.2 ± 2.1% (p<0.05, n=6) (Figs. 1A and 1B).

To examine whether the inhibitory effect of ODQ on the 40 mM KCl-induced contraction was due to the blockade of the cyclic GMP pathway, the effect of R8-8-Br-PET-cGMP, the protein kinase G inhibitor at 10 µM as well as the interaction of R8-8-Br-PET-cGMP with ODQ were further studied. This R8-8-Br-PET-cGMP pretreatment significantly reduced the CO-induced relaxation from 40.4 ± 6.6 to 15.1 ± 4.2 (n=6). The inhibitory effect of ODQ on the CO-induced relaxation was not potentiated by R8-8-Br-PET-cGMP.

As shown in Fig. 2, high K+ (40 mM) induced sustained increases in the muscle tension and [Ca2+]i in the muscle loaded with a Ca2+ indicator, fura-2. Approximately 5 min after the application of the KCl, the levels of muscle tension and [Ca2+]i, reached a plateau. In the presence of KCl, addition of CO inhibited the muscle tension to 43.3 ± 7.3% (n=5) without changing the plateau [Ca2+]i. The effect of CO was abolished by washout (not shown).

Cumulative addition of KCl induced concentration-dependent increase in both muscle tension and [Ca2+]i (Fig. 3) and there is a positive correlation between [Ca2+]i and muscle tension. CO inhibited the 40 mM KCl-stimulated muscle tension without changing [Ca2+]i, resulting in the downward shift of the [Ca2+]i-tension relationship.

The effects of CO on Ca2+ currents through the voltage-dependent Ca2+ channels were examined in single ileal smooth muscle cells (Fig. 4A). When Ba2+ was used instead of Ca2+ as the charge carrier, Ba2+ currents (I_{Ba}) was elicited by depolarization from a holding potential of -60 mV to a test potential of 0 mV for 80 ms at 0.1 Hz. Application of CO showed no effect on the peak I_{Ba} (Figs. 4A and 4B).

DISCUSSION

We have shown in the guinea-pig ileum that exogenous CO reversibly inhibited the contraction induced by high K⁺-
depolarization (Fig. 1). We have also shown that CO-induced relaxation was inhibited by ODQ, a selective inhibitor of soluble guanylate cyclase. R₈-8-Br-PET-cGMP at a submaximal dose, also reduced significantly the CO-induced relaxation. It has been known that actions of cyclic GMP are 1) to activate the K⁺-channels to inhibit the voltage-operated Ca²⁺-channels [1, 2, 15], 2) to directly inhibit the voltage-operated Ca²⁺-channels [10], 3) to activate the Ca²⁺ pump on the plasma membrane [20], and 4) to decrease the sensitivity of contractile elements to Ca²⁺ [8]. CO is considered to produce effects on smooth muscle by an activation of soluble guanylate cyclase, resulting in an increase in intracellular cyclic GMP levels, and relaxation [6, 11].

This seems to be the case also in the guinea-pig ileum, where an increase of cyclic GMP concentrations after CO exposure, and an inhibiting effect of methylene blue on the CO-induced relaxation has been demonstrated [24]. R₈-8-Br-PET-cGMP is a membrane-permeable and specific inhibitor of cyclic GMP-dependent protein kinase [5]. These data indicate that the mechanism of CO-induced relaxation of guinea-pig ileum was primarily through the activation of the guanylate cyclase/cyclic GMP system.

The contraction of smooth muscle is regulated not only by [Ca²⁺], but also by Ca²⁺-sensitivity of the contractile apparatus, which is also regulated by the intracellular signaling systems. It is well known that NO-mediated relaxation of smooth muscles involves elevated cGMP and cGMP inhibit the Ca²⁺-induced contraction by the decrease in Ca²⁺ sensitivity of contractile elements [9]. Like NO, CO is also known to increase intracellular cGMP levels, this suggests that CO may also affect the Ca²⁺ sensitivity. In the present study, cumulative addition of KCl induced a graded increase in [Ca²⁺], and muscle tension. In the presence of CO, cumulative addition of KCl induced smaller contraction than in the absence of CO. On the other hand, the increase in [Ca²⁺], induced by cumulative addition of KCl was only slightly decreased in the presence of CO, and the [Ca²⁺]–tension relationship shifted downwards (Fig. 3). Since CO shifted the [Ca²⁺]–tension relationship to downwards, inhibition of high K⁺-induced contraction by CO may be attributable to the decrease in the sensitivity contractile elements to Ca²⁺.

In vascular smooth muscle, Lin and McGrath [11] reported that CO-induced relaxation is due to decrease in Ca²⁺ concentration. In our experiments with ileal muscle, however, CO decreased neither [Ca²⁺], nor the inward Ca²⁺ current (Fig. 4). These findings suggest that the inhibition of L-type Ca²⁺ currents may not be responsible for the CO-induced relaxation in the guinea-pig ileal smooth muscle and that there is a species- and/or tissue-difference in the effects of CO. In a variety of smooth muscles, hypoxia or metabolic inhibition resulted in a significant reduction in force with no change or increments in [Ca²⁺] either at rest or in activated tissues [23]. In the present experiments, we confirmed that resting tone of the ileum was not inhibited by CO. Furthermore, the fact that both ODQ and R₈-8-Br-PET-cGMP inhibited the relaxant effect of CO suggest that the effect of CO is mediated by cyclic GMP but not by metabolic inhibition. However, further investigation is needed to draw conclusion on the possible involvement of metabolic inhibition in the CO-induced relaxation.

In summary, we demonstrated that CO, through the cyclic GMP-dependent signaling pathways, inhibits smooth muscle contraction by decreasing Ca²⁺ sensitivity of contractile elements in the guinea-pig ileal smooth muscle.

ACKNOWLEDGEMENT. This work was supported by Ministry of Education in 1996.
REFERENCES


