Further Studies Concerning Possible Transmitters from NANC Nerves in the Circular Muscle of the Rat Stomach Fundus

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Abstract

We examined the characteristics of the non-adrenergic, non-cholinergic (NANC) inhibitory response of the circular muscle of the rat stomach fundus to transmural nerve stimulation or high K⁺. Treatments with isotonic high K⁺ (20mM), nitric oxide (NO) and sodium nitroprusside (SNP) all elevated cyclic GMP levels in the rat stomach fundus in the presence of atropine and guanethidine. Isotonic high K⁺-induced formation of cyclic GMP was completely inhibited by tetrodotoxin (TTX) or N⁶-nitro-L-arginine (L-NNA). The K⁺ also increased cyclic AMP levels and this response was completely inhibited by TTX. Dose-dependent relaxation of the fundus in response to SNP was shifted to the right by a prior incubation with high concentration of SNP (10⁻⁴ M) for 2 hrs. Incubating the fundus with SNP for 2 hrs significantly inhibited NO-induced cyclic GMP formation. Relaxation responses to transmural stimulation (1 Hz or 30 Hz), isotonic high K⁺ and NO were significantly reduced by a prior incubation with SNP. Isotonic high K⁺ (20mM)-induced relaxation of circular muscle strips was not completely inhibited by combined treatment with 10⁻⁴ M L-NNA, 5×10⁻⁴ M oxyhemoglobin and anti-VIP (1:200). These results suggest that NO as well as VIP is possible transmitter from NANC nerves in the circular muscle of the rat stomach fundus and there should be one or more inhibitory mediators other than VIP and NO.

Key words: NANC; rat fundus; nitric oxide; cyclic GMP; VIP; cyclic AMP.

Introduction

Considerable evidence suggests that vasoactive intestinal polypeptide (VIP) acts as one of inhibitory non-adrenergic, non-cholinergic (NANC) neurotransmitters in the rat stomach (Kamata et al., 1988; De Beurme and Lefebvre, 1988; Li and Rand, 1990; D'Amato et al., 1992; Curro and Preziosi, 1997). Recently, evidence has been accumulating to indicate that nitric oxide (NO), which accounts for the biological properties of endothelium-derived relaxing factor (Moncada et al., 1991), may also be the transmitter in the rat stomach fundus (Boeckx-
staens et al., 1991; Kamata et al., 1993; Shimamura et al., 1993; Holzer-Petsche and Moser, 1996). In longitudinal strips of the rat fundus, NO and VIP complement each other in relaxing the smooth muscle, in that VIP is mainly involved in the relaxation induced by high frequency or long pulse stimulation of NANC nerves and NO in relaxation by low frequency or short pulse stimulation (Boeckxstaens et al., 1992; D'Amato et al., 1992). On the other hand, anatomical studies showed the presence of NADPH diaphorase–positive nerve in the circular muscle layer and positive nerve fibers as well as cell bodies in the myenteric plexus of the rat stomach fundus (Foster and Southam, 1993). Indeed, we have reported that the density of NO-containing nerves in the stomach fundus is richer in circular than in longitudinal muscle (Kamata et al., 1993).

In series of studies concerning characteristics of relaxation responses of the fundus to electrical stimulation of NANC nerves, we have examined methylene blue, an inhibitor of soluble guanylate cyclase, on the relaxation of the fundus to the electrical stimulation in the presence of atropine and guanethidine. Unexpectedly, methylene blue itself relaxed circular muscle of the stomach fundus (Kamata et al., 1996). In the present study, after treatment the fundus strips with high concentration of SNP (2 hrs), we examined relaxation response of circular muscle strips of the fundus to the electrical stimulation, isotonic high K⁺ or NO.

Materials and Methods

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University which is accredited by the Ministry of Education, Science, Sports and Culture, Japan.

Mechanical response

Male Wistar rats, 200–280 g in weight, were killed by decapitation after the animals were anesthetized with ether. The stomach was excised, and fundus strip preparations of approximately 20 mm in length and 2 mm in width were made essentially by the our methods (Kamata et al., 1988, 1993). Each strip was cut out along the circular muscle, and the mucous layer was completely removed to avoid reflex activity. The fundus strips were suspended in a 10 ml organ bath which contained modified Krebs Ringer solution at 37°C, aerated with 95% O₂ and 5% CO₂. The tension loaded on each strip was 1.0 g. Contractions and relaxations were recorded isotonically with an isotonic transducer connected to an ink-writing recorder (Nihon Kohden, Tokyo, Japan). The composition of the modified Krebs Ringer solution was as follows (mM): NaCl 120; KCl 4.7; CaCl₂ 2.0; MgCl₂ 1.2; NaHCO₃ 25; KH₂PO₄ 1.2; and glucose 14. Transmural nerve stimulation to the muscle was applied through two parallel platinum rings separated by 8 mm. An electronic stimulator was used to deliver the rectangular pulses. Responses were obtained with pulses of 50 mA strength and 1 ms duration. The number of pulses in each train was 20. When transmural nerve stimulation, isotonic high K⁺ (20 mM), NO or SNP was applied to the fundus, the tissue was preincubated with 10⁻⁶ M serotonin (EC₁₀₀) to maintain sustained contraction and 10⁻⁶ M atropine and 10⁻⁶ M guaneth-
NANC nerves in rat fundus

idine to avoid cholinergic and adrenergic effects throughout the experiments. The fundus strips were placed in an organ bath that contained Krebs Ringer solution, equilibrated with 95% O₂ and 5% CO₂, and left for 60 min at 37°C. The relaxation responses to transmural nerve stimulation, isotonic high K⁺ (20 mM), NO and SNP were expressed as a percentage of the contractile response of the circular muscle of the fundus induced by 10⁻⁶ M serotonin in the presence of 10⁻⁶ M atropine and 10⁻⁶ M guanethidine.

Measurement of cyclic nucleotides

When the response of the fundus strips to transmural nerve stimulation, isotonic high K⁺ (20 mM), 10⁻⁴ M NO or 10⁻⁶ M SNP reached a plateau, the strips were frozen immediately and transferred to vials that contained trichloroacetic acid (6%) and 3-isobutyl-1-methylxanthine (10⁻⁶ M). The cyclic nucleotides contents in the sample were measured using commercially available radioimmunoassay kits (Yamasa Cyclic AMP assay kit and Yamasa Cyclic GMP assay kit, Yamasa Corp., Choshi, Japan). The recovery rate of the cyclic GMP contents in each column was calculated by counting the radioactivity of [³H]cyclic GMP using a liquid scintillation counter, and the values for the cyclic AMP and cyclic GMP contents obtained from the radioimmunoassay were corrected using the recovery rate. The recovery rate of the cyclic GMP content in each column ranged from 85 to 90%. Protein was measured by the method of Lowry et al. (1951). To investigate the influence of N⁶-nitro-L-arginine (L-NNA) or tetrodotoxin (TTX) on isotonic high K⁺-induced cyclic GMP formation, fundus strips were incubated with 10⁻⁵ M L-NNA or 10⁻⁶ M TTX for 20 min.

Drugs

Atropine sulfate, 5-hydroxytryptamine creatine sulfate, 3-isobutyl-1-methylxanthine and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Guanethidine sulfate was purchased from Tokyo Kasei Pharmaceutical Co. Ltd. (Tokyo, Japan). Rabbit antiporcine VIP antiserum (anti-VIP) was purchased from Funakoshi Pharmaceutical Co. Ltd. (Tokyo, Japan). Pure hemoglobin (oxyhemoglobin) was prepared according to the method described by Martin et al. (1985). Responses to NO were obtained by addition of the NaNO₂ solution adjusted to pH 2 (Furchgott, 1988). The concentration mentioned in the text and figures are those of freshly prepared acidified NaNO₂. Isotonic high K⁺ (20 mM) solution was prepared by replacing the NaCl with KCl.

Statistical analysis

Data are mean ± S.E. Statistical differences were determined by Dunnet’s test for multiple comparison, after a one-way analysis of variance and a probability level of P < 0.05 was regarded as significant.
Results

Relaxation and cyclic nucleotides formation in response to $K^+$, electrical stimulation, NO or SNP

Consistent with our earlier results (Kamata et al., 1993), isotonic high $K^+$ (20 mM) produced long-lasting relaxation in circular muscle strips of the fundus (Fig. 1). The $K^+$ (20 mM)-induced relaxation was markedly suppressed by $10^{-5}$ M L-NNA and was changed to contraction by $10^{-6}$ M TTX (data not shown). NO application by administration of acidified nitrite ($10^{-4}$ M) also relaxed circular muscle strips of the fundus (Fig. 1).

As shown in Fig. 2, the level of cyclic GMP in the fundus was significantly increased by the addition of 20 mM $K^+$ (control, 53.7±8.4 fmol/mg protein, $n=4$; 20 mM $K^+$, 104.7±8.6 fmol/mg protein, $n=4$). The effect of $K^+$ was abolished by $10^{-6}$ M TTX (22.3±2.1 fmol/mg protein, $n=4$) or $10^{-5}$ M L-NNA (6.9±1.9 fmol/mg protein, $n=4$). Cyclic GMP levels were also significantly increased by $10^{-4}$ NO (80.1±3.9 fmol/mg protein, $n=4$) or $10^{-6}$ M SNP (78.8±2.8 fmol/mg protein, $n=4$) (Fig. 2).

Treatment with isotonic high $K^+$ (20 mM) increased significantly cyclic AMP levels in the fundus and this response was completely abolished by $10^{-6}$ M TTX (control, 1.8 pmol/mg protein, $n=4$; 20 mM $K^+$, 2.4±0.1, $n=4$) (Fig. 3). NO ($10^{-4}$ M) or SNP ($10^{-6}$ M) had without effects on cyclic AMP levels in the fundus (NO, 1.7±0.1 pmol/mg protein, $n=4$; SNP, 1.6±0.1 pmol/mg protein, $n=4$) (Fig. 3).

![Graph](image-url)

Fig. 1. Typical responses to isotonic high $K^+$ (20 mM) or NO ($10^{-4}$ M) in circular muscle strips of the rat stomach fundus which had been contracted with $10^{-6}$ M serotonin (5-HT) in the presence of $10^{-4}$ M atropine and $10^{-6}$ M guanethidine.
Fig. 2. Effects of various agents on the cyclic GMP formation in the presence of 10^{-6} M serotonin (5-HT), 10^{-6} M atropine and 10^{-6} M guanethidine. Isotonic high K^{+} (20 mM) (K^+), 10^{-4} M NO or 10^{-4} M SNP was applied to the fundus. Fundus strips were incubated with 10^{-4} M L-NNA or 10^{-6} M TTX for 20 min and then 20 mM K^{+} was applied. *P<0.05, ***P<0.001. P<0.001, K^{+} vs. K^+ + TTX or L-NNA.

Fig. 3. Effects of various agents on the cyclic AMP formation in the presence of 10^{-6} M serotonin (5-HT), 10^{-6} M atropine and 10^{-6} M guanethidine. Isotonic high K^{+} (20 mM) (K^+), 10^{-4} M NO or 10^{-4} M SNP was applied to the fundus. Fundus strips were incubated with 10^{-6} M TTX for 20 min and then 20 mM K^{+} was applied. **P<0.01. P<0.01, K^{+} vs. K^+ + TTX.

**Relaxation and cyclic GMP formation after desensitization of soluble guanylate cyclase**

After the fundus was incubated with 10^{-4} M SNP for 2 hrs, we examined the SNP-induced relaxation. As shown in Fig. 4, the SNP-induced dose-dependent relaxation of circular strips of the fundus was markedly shifted to the right after incubation with SNP. The relaxation responses of the circular muscle strips to transmural nerve stimulation (1 Hz, 30 Hz), isotonic
Fig. 4. Changes in dose-response curve for the relaxation induced by sodium nitroprusside (SNP) in circular muscle strips of the fundus that had been incubated with $10^{-4}$ M SNP for 2 hrs. Each data point on the graph represents the mean±S.E. of six experiments; the vertical lines indicate the S.E. and are only included when they exceeded the dimensions of the symbols used. *P<0.05, **P<0.01.

Fig. 5. Changes in relaxation responses induced by transmural nerve stimulation (1 Hz, 30 Hz), isotonic high K+ (20 mM) and $10^{-4}$ M NO in circular muscle strips of the fundus that had been incubated with $10^{-4}$ M SNP for 2 hrs. Each data point on the graph represents the mean±S.E. of ten experiments; the vertical lines indicate the S.E. *P<0.05, **P<0.01.

high K+ (20 mM) or $10^{-4}$ M NO were all inhibited after incubation with $10^{-4}$ M SNP for 2 hrs (Fig. 5). As shown in Fig. 6, the $10^{-4}$ M NO-induced accumulation of cyclic GMP was significantly decreased after incubating the fundus with $10^{-4}$ M SNP for 2 hrs (control, 74.4±6.5 fmol/mg protein, $n=4$; NO, 110.1±3.2 fmol/mg protein, $n=4$; NO (SNP 2 hrs), 64.9±8.1 fmol/mg protein, $n=4$).

Effects of combined treatment with L-NNA, oxyhemoglobin and anti-VIP

As illustrated in Fig. 7, isotonic high K+ (20 mM)-induced relaxation of circular muscle
Fig. 6. Changes in cyclic GMP formation in response to $10^{-4}$ M NO in the fundus that had been incubated with $10^{-4}$ M SNP for 2 hrs. **$P<0.01$.** $P<0.01$, NO vs. NO, SNP 2 hrs incubation.

Fig. 7. Effect of L-NNA ($10^{-5}$ M) plus oxyhemoglobin ($5 \times 10^{-5}$ M) plus anti-VIP (1:200) on the isotonic high K$^+$ ($20$ mM)-induced relaxation of circular muscle strips which had been contracted with $10^{-6}$ M serotonin in the presence of $10^{-6}$ M atropine and $10^{-6}$ M guanethidine.

strips was not completely inhibited by combined treatment with $10^{-5}$ M L-NNA, $5 \times 10^{-5}$ M oxyhemoglobin and anti-VIP (1:200).

**Discussion**

We have reported that isotonic high K$^+$ ($20$ mM) is able to relax circular but not longitudinal muscle strips, the K$^+$-induced relaxation is changed to contraction by treatment with TTX, and is inhibited by treatment with L-NNA, suggesting that the K$^+$ can release NO from NANC inhibitory nerves in the circular muscle of the rat stomach fundus (Kamata et al., 1993). Consistent with our former results, isotonic high K$^+$ ($20$ mM) produced long-lasting relaxation in circular muscle strips of the fundus in the present study. NO, SNP or K$^+$ was also able to
accumulate cyclic GMP levels in the fundus and the K\(^+\)-induced cyclic GMP formation was completely inhibited by TTX or L-NNA, suggesting that NO is one of transmitters in the NANC relaxation of the rat fundus.

Isotonic high K\(^+\) (20 mM) but not NO or SNP also increased in cyclic AMP levels in the fundus and this response was completely inhibited by TTX, suggesting that the K\(^+\) may release VIP as well as NO from NANC nerves. A number of studies have suggested that VIP is a mediator of the NANC relaxation of the rat stomach fundus induced by various stimuli (Kamata et al., 1988; De Beurme and Lefebvre, 1988; Li and Rand, 1990; D'Amato et al., 1992; Takahashi and Owyang, 1995; Curro and Preziosi, 1997). VIP is known to stimulate adenylate cyclase activity and the formation of cyclic AMP in the rat stomach fundus (Kamata et al., 1988). It is most likely, therefore, that isotonic high K\(^+\)-induced relaxation of circular muscle strips and formation of cyclic AMP may be due to release of not only NO but also VIP from NANC nerves in the stomach fundus.

It has been reported that there is the high immunoreactivity of NO synthase in the myenteric plexus in the gut (Bredt et al., 1990) and cyclic GMP formation is involved in non-vascular smooth muscle relaxation elicited by NO (Sean et al., 1992; Lefebvre, 1993). VIP generates cyclic AMP as a second messenger in the rat stomach fundus (Kamata et al., 1988), whereas NO activates the soluble guanylate cyclase, leading to an increase in cyclic GMP (Sean et al., 1992). Methylene blue is known to inhibit soluble guanylate cyclase activity (Grutter et al., 1981), and inhibit the relaxation induced by both stimuli and the elevation of cyclic GMP induced by electrical stimulation and NO in the rat ileum (Kanada et al., 1992). As mentioned in Introduction, we have reported that methylene blue itself relaxed circular muscle strips of the fundus (Kamata et al., 1996). In the present study, therefore, after treatment the fundus strips with high concentration of SNP (2 hrs), we examined relaxation response of circular muscle strips of the fundus to the electrical stimulation, isotonic high K\(^+\) or NO. In the SNP-treated fundus, SNP-induced relaxation was markedly inhibited, suggesting that the soluble guanylate activity is desensitized by this treatment. In the desensitized fundus, the relaxation responses of circular muscle strips to electrical stimulation, isotonic high K\(^+\) or NO were significantly but not completely inhibited, suggesting that NO is one of transmitters of NANC nerve in the stomach fundus. These conclusion are supported by the finding that an increased production of cyclic GMP by NO was significantly antagonized by incubation of fundus with high concentration of SNP for 2 hrs.

We have reported that the transmitter released from the NANC nerves in the rat stomach is a peptide and is probably VIP or closely related peptide (Kamata et al., 1988). In our report, there are a correlation between the dose–response curves for formation of cyclic AMP and relaxation. Furthermore, an increased production of cyclic AMP by transmural nerve stimulation was completely antagonized by pretreatment with VIP antiserum (anti-VIP). Nicotine-induced relaxation in the rat stomach fundus is significantly reduced by tetrodotoxin or VIP antiserum, indicating that VIP is possibly involved in NANC relaxation of the rat stomach fundus induced by nicotine (Curro and Preziosi, 1997). In the present study, isotonic high K\(^+\) (20 mM) increased not only cyclic GMP but also cyclic AMP levels, suggesting that VIP is also possible one of neurotransmitter from NANC nerves in the rat stomach fundus.
In the circular muscle strips of the fundus pretreated with L-NNA, oxyhemoglobin, and anti-VIP, isotonic high $K^+$-induced relaxation was not completely inhibited, indicating that there should be one or more inhibitory mediators other than VIP and NO.

In conclusion, it appears that the putative transmitter released from NANC nerve in circular muscle strips of the rat stomach fundus is NO and VIP. However, one or more inhibitory mediators other than NO and VIP may be involved in the $K^+$-induced relaxation of circular muscle strips of the fundus.

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