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Nitroxergic Nerve Relaxes Rat Gastric Smooth Muscle by NO-cGMP Pathway

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This study was undertaken to investigate an involvement of nitroxergic innervation in gastric smooth muscle of rat. Isometric tension study, the measurement of single cell length, NADPH diaphorase stain of smooth muscle layers and neuronal nitric oxide synthase (nNOS) western blotting were performed. Sodium nitroprusside (SNP), a nitric oxide donor, relaxed the muscle strips precontracted by acetylcholine (ACh) in a concentration-dependent manner. Pretreatment of L-arginine decreased the contraction induced by electric field stimulation (EFS). Pretreatment of N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, increased the EFS-induced contractions. LY 83583, a guanylate cyclase (GC) inhibitor, reversed the inhibitory actions of L-arginine on the muscle contractions. The effects of L-Arginine, L-NAME and LY 83583 on ACh-induced contractions were not significant. L-arginine reduced the EFS-induced contraction in circular muscle, whereas L-NAME enhanced the EFS-induced contraction in longitudinal strips. By EFS, the phasic contractions appeared approximately 20~25 seconds later. L-NAME significantly shortened the delay time to about 2~3 seconds. In single cell study, ACh contracted gastric smooth muscle cells, SNP relaxed the cells, and the latter also inhibited the ACh-induced contraction. LY 83583 enhanced the ACh-induced contraction and antagonized SNP-induced relaxation. NADPH diaphorase activity was assessed by a histochemistry, nitroblue tetrazolium (NTB) staining. Positive staining was observed in both circular and longitudinal muscle layers. L-arginine increased the staining, while L-NAME decreased the staining. Western blotting for nNOS proved the presence of nNOS in rat gastric smooth muscle. EFS and additional Ca^{2+} increased nNOS protein expression. These results suggest that in rat stomach, both circular and longitudinal muscle layers are innervated with nitroxergic nerves which relax the gastric smooth muscle via NO-cGMP pathway.

Key Words: Gastric smooth muscle, Nitroxergic nerve, NO-cGMP pathway

INTRODUCTION

Nitroxergic nerve is a component of the enteric nervous system, which is a functional regulator of complexity in gastrointestinal (G-I) tract. Nitric oxide (NO) is released from non-adrenergic non-cholinergic (NANC) nerves to relax the smooth muscle of G-I tract of various animals (Boeckxstaens et al, 1991; Barbier and Lefebvre, 1993; Bayguinov & Sanders, 1993; Shuttleworth et al, 1993). Bayguinov & Sanders (1993) & Selemidis et al (1997) have showed some evidences that NO acts as an inhibitory neurotransmitter.

Stomach is a very important portion of elementary tract. It is innervated by autonomic nervous system and is mainly being regulated by enteric nervous system (Arakawa et al, 1997; Yuan et al, 1997; Pfannkuche et al, 1998).

There are some reports that the nitroxergic innervation may be contributing to the relaxations of gastric smooth muscles in rat (Lefebvre et al, 1995) and

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pig (Lefebvre & Vandekerckhove, 1998).

In rat stomach, early findings described that NO mediated the NANC response in gastric fundus (Li & Rand, 1990; Boeckxstaens et al, 1992), and that NO is an NANC neurotransmitter in rat stomach (Forster & Southam, 1993; Kamata et al, 1993). Recently, Kamata et al (1997) reported that NO is a possible transmitter from NANC nerve in the circular muscle of rat stomach, and Nakamura et al (1998) & Hosoda et al (1998) reported that NO is produced from myenteric plexus in rat stomach. In 1999, Jarvinen et al detected the regional differences of distribution of NOS-positive neurons from myenteric plexus or rat G-I tract.

In the present study, we tried to prove the existence of nitroxergic nerve fibers in gastric smooth muscle by NADPH-diaphorase evaluation by a histochemical method, and to observe the alterations of NOS activity by the drugs, which inflences the NO production. We also investigated the alterations of NOS activity from nerve fibers by electric field stimulations and subsequent measurement of nNOS activity.

METHODS

Isometric tension study

Sprague-Dawley rats weighing 200~250 g were sacrificed by decapitation. The stomach was isolated, and the surrounding tissue was cleared in 0~4°C Krebs-Henseleit buffer solution. Stomach was opend along greater curvature, and the mucosa was peeled off. The muscle layer was prepared as strips of 15×2 mm for isometric tension study. Each preparation was attached to a holder and mounted in the Biancani's isolated muscle bath containing 1 ml of physiologic salt solution (PSS) bubbled with 95% O_2 and 5% CO_2 mixture resulting in a pH of 7.4 at 37°C. The PSS (modified Krebs-Henseleit buffer) had the following composition (mM): NaCl 120, KCl 4.6, KH_2PO4 1.17, $NaHCO_3$ 23.8, $CaCl_2$ 1.8, $MgSO_4$ 1.2, Glucose 10. Muscle tensions were measured by isometric tension transducer (force displacement transducer, FT-03, Grass) to record on polygraph (Grass, Model 79E) and a computerized data acquisition system (BioPac, Model MP-100) connected to Macintosh personal computer (Acknowlege 881 v. 3.0 for Macintosh).

Under an initial tension of 2 g, preparations were perfused with PSS for 60 min and then equilibrated for 60 minutes or longer in the muscle bath. Being subjected to electrical field stimulations, the preparations were mounted between two parallel platinum wire electrodes (0.7 mm in diameter) in muscle baths. Electrical field stimulations to the strips were performed using an electric stimulator (Nihon-Kohden SEN-3201) delivering single square wave pulses (20 msec duration, 60 VDC).

Muscle strips were first contracted by acetylcholine (ACh), and then the relaxations by sodium nitroprusside (SNP), an NO donor, were observed.

Effects of L-arginine, an NO precursor, N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, LY 83583, a guanylate cyclse inhibitor, on the contractile effect of ACh and on the electric field stimulation were investigated.

Measurements of single cell length

Fine chops (less than 2×2 mm) of gastric smooth muscle were digested in PSS containing 0.08 mg/ml soybean trypsin inhibitor (Sigma biochemical), 1.2 mg/ml of collagenase (Worthington Biochemical, Type 2) and 2% bovine serum albumin at 36°C. The solution was aerated by continuous breeze of 95% O2/5% CO_2 mixed gas on the surface, and the pH was adjusted at 7.4. At the end of the digestion period, the tissue was poured over a 450 μm nylon mesh (Tekto, Elmsford, NY), rinsed with collagenae-free PSS to remove any trace of collagenase, and then incubated in this PSS in shaking (2 cm/sec) water bath at 36°C for 12~15 minutes for the cells dissociate freely.

Every 1 ml of the cell suspension was transfered into 5 ml test tubes. Various concentrations of acetylcholine (ACh) were added into the tubes so that the muscle cells contracted. To fix the contracted cells, diluted acrolein was added as 1% of final concentration in 5 seconds after the administration of ACh. The contractions responding to ACh were observed under the presence of sodium nitroprusside (SNP) or LY 83583.

Acrolein-fixed cells were transfered onto slide glasses, and the length of cells were measured with a microscaler. On a phase-contrast microscope, the cells showing a bright halo around the cell body and repelling trypan blue were selected as healthy vital cells (Fig. 6). A CCTV camera (Hidacchi VK-C 50) was employed to catch the images of cells on a phase-contrast microscope. The image sent to a

microscaler (FOR·A, IV-550) was displayed on a TV screen where the measuring lines produced by the microscaler were also displayed. The average length of 50 cells from one slide was accepted as the value of one case.

NADPH diaphorase staining

The NADPH diaphorase stain was used to localize nitric oxide synthase-containing enteric neurons.

L-arginine 200 mg/Kg or L-NAME 10 mg/kg was administered to each group of 6 rats intraperitoneally, 20 hours before sacrifice. On sacrifice, rats were anesthetized with intraperitoneal thiopental sodium (40 mg/Kg). Chest was opened, and 300 ml of saline was infused via a catheter into the left ventricle to be drained out of the open slit on the right atrial wall so that the saline washed out the blood from tissues. Then 500 ml of 2% paraformaldehyde was perfused to fix the stomach. Stomach was isolated and immersed in the 2% paraformaldehyde at 4°C for 2 hours. Fixed gastric tissue was rinsed with phosphate buffered saline (PBS) and stored in 25% sucrose aqueous solution at 4°C for 12 hours. For staining, 4 mm thick cryosections were made. The cryosections on slide glasses were treated with the mixture of 970 μl of 0.2% triton X-100 in PBS, 30 μl of 1% nitroblue tetrazolium (NBT) aqueous solution and 1 ml PBS containing 2 mg of NADPH, and were incubated in 100% humidity and 37°C for 2 hours. All sections were rinsed with PBS and distilled water then sealed with canada balsam, and were observed with light microscope.

nNOS western blotting

Stomachs were isolated from Sprague-Dawley rats. The stomach was isolated, and the surrounding tissue was cleared in 0~4°C Krebs-Henseleit buffer solution. Stomach was opened along greater curvature, and the mucosa was peeled off. The muscle layer was chopped into pieces of 2×2 mm. Those muscle chops were treated with L-arginine, L-NAME, ACh, or Ca^{2+} according to the protocols, and they were stimulated with electric field stimulation (60 VDC, 20 msec, 20 Hz) for 1 minute or 3 minutes to stimulate the nNOS. Stimulated muscle chops were sonicated in the PBS with a sonicator (Vibra Cell, Sonics and Materials, Inc.) and centrifused by 12,000 g at 4°C. From supernatent, total protein was assayed (Protein Assay Kit, BIO-RAD). 100 mg of protein was loaded on the SDS-PAGE gel, and electrophoresis (150 V, 3 hr) was done, transfered to nitrocellulose membrane (Protran, Schleicher & Schuell), and treated with 5% non fat dry milk in PBS for 1 hour.

The nitrocellulose membrane carrying the protein bands were reacted to the primary antibody, nNOS antibody, which was diluted by 1 : 2,000 in 0.1% BSA with PBS containing 0.05% Tween 20, and reacted to for 1 hour; then the secondary antibody, anti-rabbit HRP (horse radish peroxidase), which was diluted by 1 : 4,000 in 0.1% BSA and 0.05% Tween 20 PBS for 1 hour. Nitrocellulose membrane was subsequently reacted by electrochemiluminescence (Renaissance Western Blot Chemiluminescence reagent, NEN life science products) for 1 minute, and X-ray film was exposed to this membrane to measure the nNOS protein band.

Drugs used and data analysis

Sodium nitroprusside, acetylcholine, L-arginine, L-NAME, soy bean trypsin inhibitor and bovine serum albumin were ordered from Sigma Biochemical, LY 83583 was ordered from RBI, and nNOS antibody was ordered from Serotec. Protein assay kit, protein size marker, filter paper, non fat dry milk, SDS, electrophoresis unit, electrotransfer unit and power supply were ordered from BIO-RAD.

Data were evaluated by Student's t-test (unpaired), but the paired t-test was used to evaluate a data when the data were obtained from the same strips which showed a subsequent changes by additions of some drugs. Values were considered as significantly different from control when p-value was less than 0.05.

RESULTS

Effect of SNP on ACh-induced contraction

Circular muscle strips contracted by ACh 10^{-5} M (1.7±0.3 g) were relaxed by SNP in a concentration-dependent manner. SNP at concentrations of 10^{-6}, 10^{-5}, 10^{-4} M and 10^{-3} M significantly ($p<0.05$) relaxed the muscle strips to 0.8±0.3 g, 0.6±0.3 g, 0.3±0.3 g, and 0.1±0.3 g, respectively (Fig. 1).

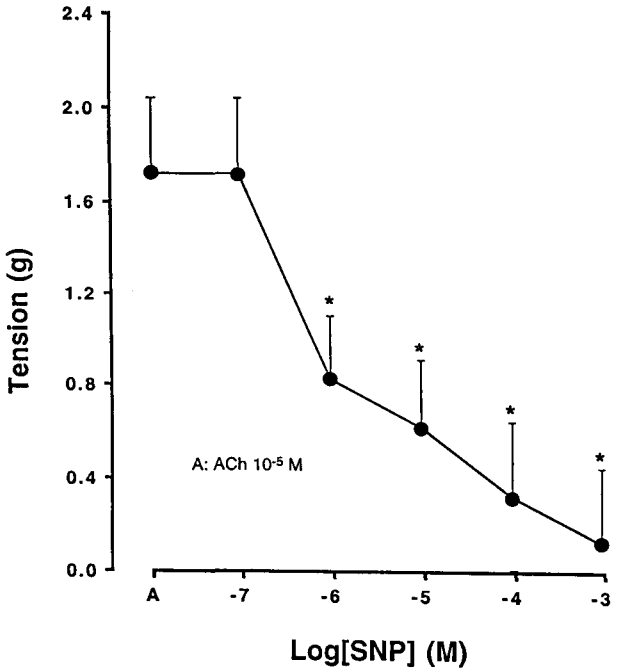

Fig. 1. Effect of sodium nitroprusside on the acetylcholine-induced contraction of rat gastric smooth muscle strips. Values express Mean±S.E.M. (n=6) A: acetylcholine 10^{-5} M, SNP: sodium nitroprusside. *$p<0.05$ compared with ACh 10^{-5} M.

Effects of NO release alterations and inhibition of guanylate cyclase on ACh-induced contractions

Both circular and longitudinal strips were contracted by additions of ACh concentration-dependently. In circular muscle, incubation in a high concentration of L-Arg (10^{-4} M for 20 minutes) tended to reduce the ACh-induced contraction, pretreatment of a high concentration of L-NAME (10^{-4} M for 20 minutes) tended to enhance the ACh-induced contraction, and pretreatment of L-arginine (10^{-4} M) and high concentration of LY 83583 (10^{-6} M) tended to enhance the ACh-induced contraction (Fig. 2A). These influences were not significant.

Longitudinal muscle responded to ACh much weaker than circular muscle, and the response to ACh was not significantly affected by L-Arg, L-NAME or LY 83583 (Fig. 2B).

Effects of NO release alterations and inhibition of guanylate cyclase on the contractions by electric field stimulation (EFS)

Both circular and longitudinal strips contracted in

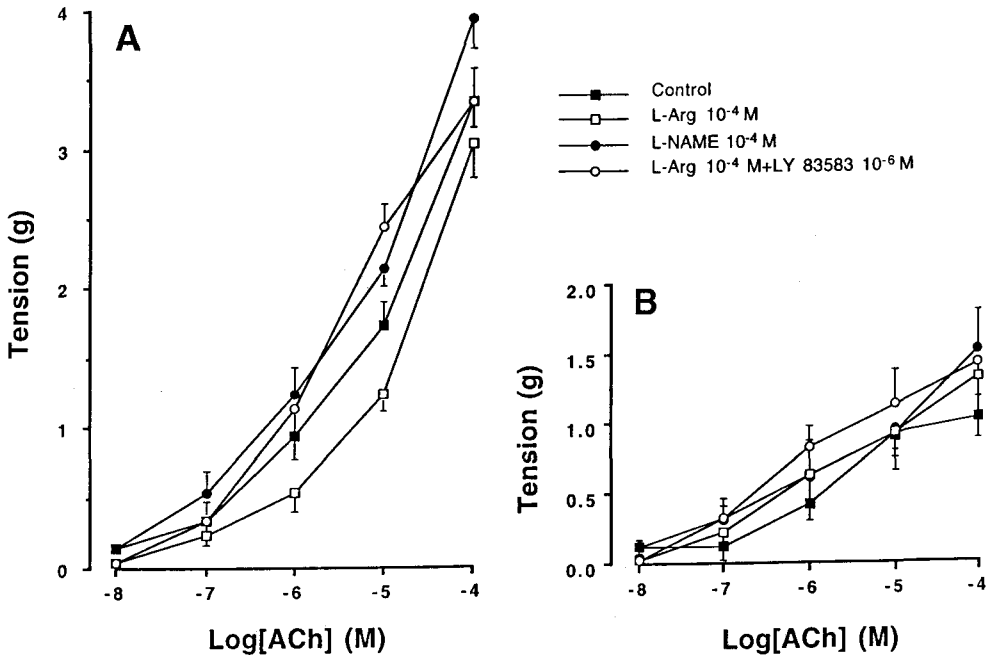

Fig. 2. Effects of L-arginine, L-NAME and LY 83583 on the acetylcholine-induced contractions of rat gastric smooth muscle strips. Values express Mean±S.E.M. (n=6). Panel A: circular muscle. Panel B: longitudinal muscle. ACh: acetylcholine, L-Arg: L-arginine.

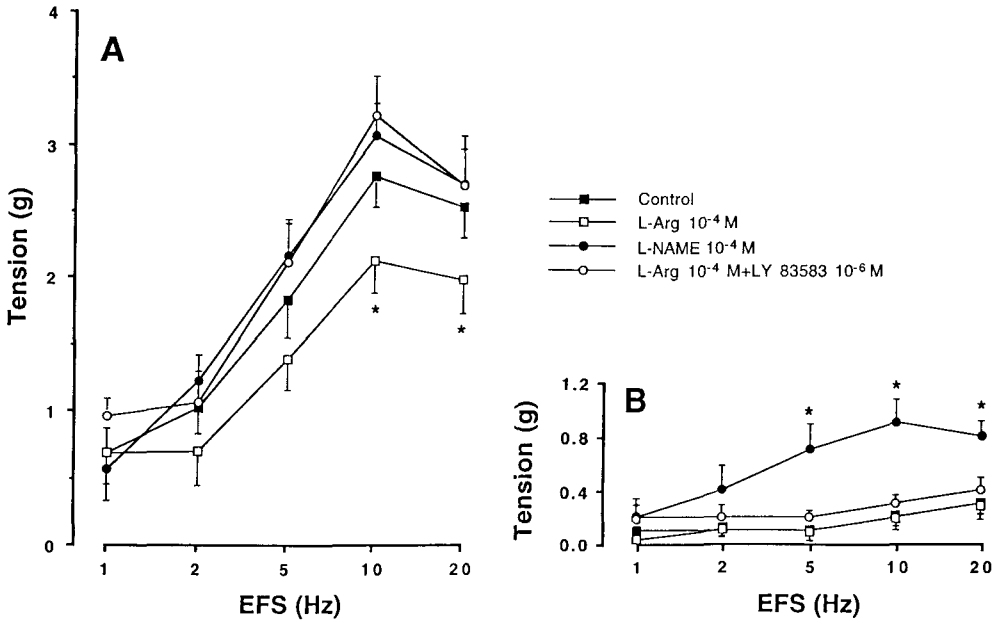

Fig. 3. Effects of L-arginine, L-NAME and LY 83583 on the electric field stimulation (EFS; 60 VDC, 20 msec square wave duration, 20 sec train duration)-induced rat gastric smooth muscle strips. Values express Mean±S.E.M. (n=6). Panel A: circular muscle. Panel B: longitudinal muscle. L-Arg: L-arginine. *$p<0.05$ compared with control.

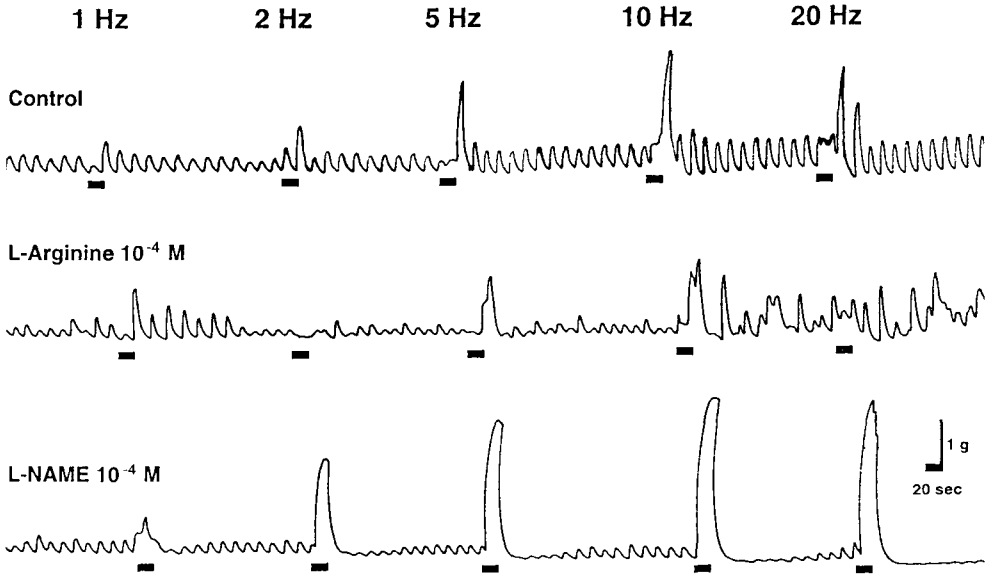

Fig. 4. Responses of circular muscle strips to electric field stimulation (EFS; 60 VDC, 20 msec square wave duration, 20 sec train duration) in the presence of the modifiers of nitric oxide production on rat gastric smooth muscle.

response to EFS. In circular muscle, L-Arg suppressed the contraction curve, where the maximal response was 2.1±0.3 g that is significantly ($p<0.05$) different fron control (2.7±0.2 g). L-NAME and LY 83583 tended to increase the maximal contractions (Fig. 3A). In longitudinal muscle, contractions by EFS were much weaker than circular muscle, but L-NAME enhanced the EFS-induced contraction dis-

tinctly, where the maximal contractions around 0.8 g was significantly ($p<0.05$) different from control of 0.4 g. L-arginine 10^{-4} M or LY 83583 10^{-6} M did not show a significant difference (Fig. 3B).

Effects of NO release alterations on the timing of EFS-contractions

There was a time delay when EFS hit the muscle. In both circular and logitudinal muscles, EFS of 1,

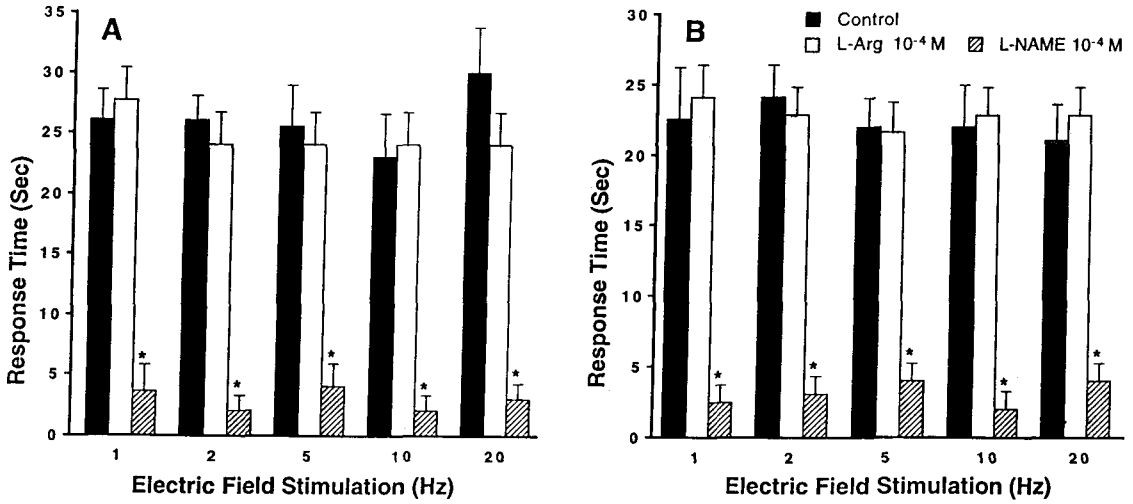

Fig. 5. Effects of L-arginine and L-NAME on the response time (RT) from electric field stimulation (EFS; 60 VDC, 20 msec square wave duration, 20 sec train duration) to contraction of rat gastric smooth muscle strips. Values express Mean±S.E.M. (n=6) Panel A: circular muscle. Panel B: longitudinal muscle. L-Arg; L-arginine. *$p<0.05$ compared with control.

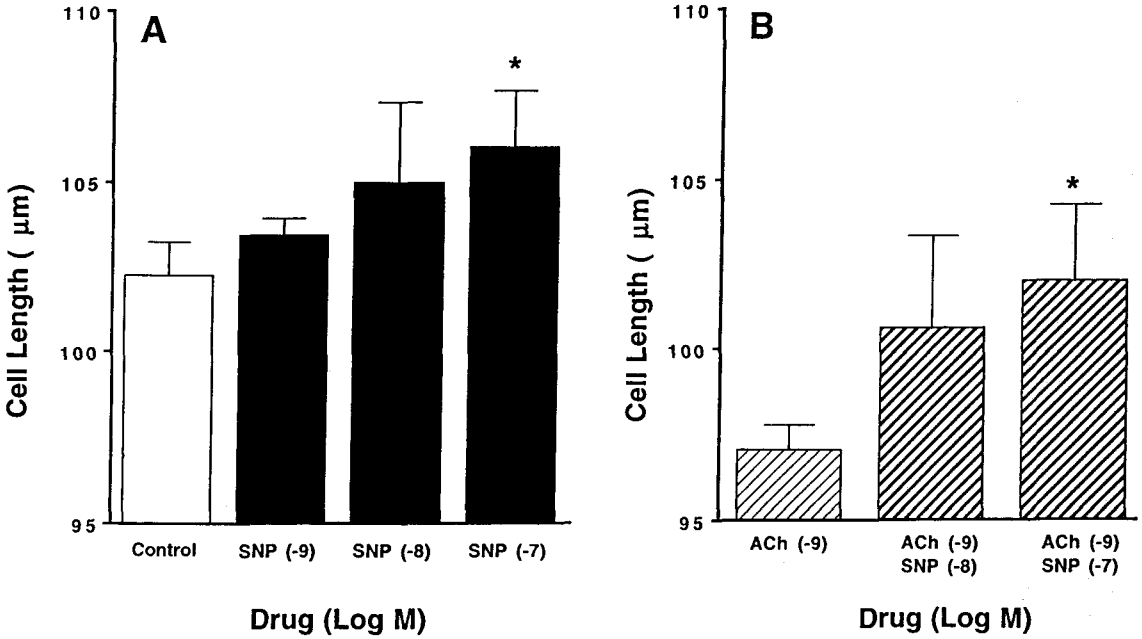

Fig. 6. Effects of acetylcholine and sodium nitroprusside on the length of smooth muscle cells isolated from rat stomach. Values express Mean±S.E.M (n=6). ACh: acetylcholine. SNP: sodium nitroprusside. *$p<0.05$ compared with control (A), ACh 10^{-9} M (B).

2, 5, 10 and 20 Hz gave the muscle about 20~25 seconds delay until the contraction appeared. L-Arg did not affect it, but L-NAME reduced the delay-time less than 5 seconds ($p<0.05$)(Fig. 4, 5A, 5B).

Effect of nitroprusside on ACh-induced contraction in single cells

Single cells from gastric smooth muscle contracted to ACh upto 97 ± 0.8 μm and it was significantly shorter than control (102 ± 1.0 μm) ($p<0.05$).

Cells treated with SNP by 10^{-9} M, 10^{-8} M and 10^{-7} M relaxed upto 103 ± 0.5 μm, 105 ± 2.3 μm and 106 ± 1.7 μm ($p<0.05$) (Fig. 6A). SNP 10^{-8} M and 10^{-7} M inhibited the contraction induced by ACh 10^{-9} M ($p<0.05$) (Fig. 6B).

Effect of LY 83583 on the actions of ACh and SNP in single cells

LY 83583 enhanced the ACh-induced contraction. The contractions by ACh 10^{-9} M alone was 97 ± 0.8 μm. The contraction by simultaneous additions of ACh 10^{-9} M with LY 83583 10^{-8} M was 97 ± 1.3 μm, with LY 83583 10^{-7} M was 95 ± 1.1 μm, and with LY 83583 10^{-6} M was 93 ± 1.2 μm ($p<0.05$) (Fig 7A).

LY 83583 inhibited the relaxant effect of SNP. The cell lengths treated by SNP 10^{-8} M was 105 ± 2.3 μm, and simultaneous addition of LY 83583 10^{-8} M, 10^{-7} M and 10^{-6} M shortened the cell lengths to 101 ± 2.5 μm, 100 ± 3.0 μm and 98 ± 2.1 μm ($p<0.05$), respectively (Fig. 7B).

Effects of EFS and NO release alterations on nNOS expression

NADPH diaphorase-histochemistry showed positive reactions on both circular and longitudinal muscle layers. The reactivity was increased by L-arginine 200 mg/Kg and was decreased by L-NAME 10 mg/Kg (Fig. 8).

The expression of nNOS was increased by EFS. EFS for 1 minute produced $159\pm38\%$ of control, and EFS for 3 minutes produced $169\pm13\%$ of control. EFS for 3 minutes in the presence of L-Arg 10^{-5} M produced $162\pm37\%$ of control, and EFS 3 minutes in the presence of L-NAME 10^{-5} M produced $120\pm24\%$ of control. Addition of Ca^{2+} 100 mM produced nNOS $196\pm35\%$ ($p<0.05$) of control (Fig. 9).

DISCUSSION

Enteric nervous system plays a very important role in regulations of gastric motor function (Abrahamsson, 1980; Allescher & Daniel, 1994; Salzman, 1995). To accommodate the intake of food or liquid, gastric

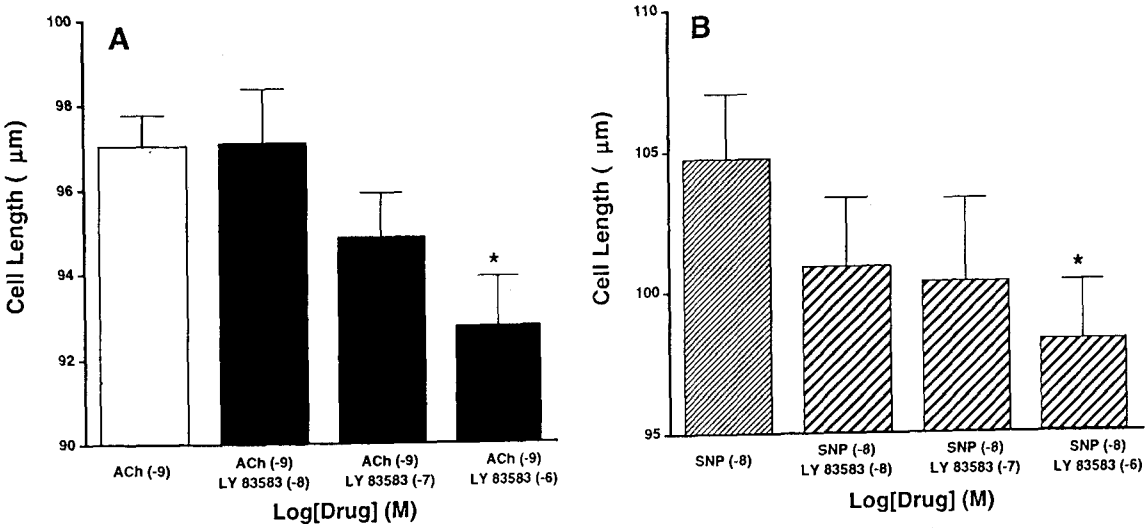

Fig. 7. Effects of LY 83583 on the acetylcholine-induced contraction and sodium nitroprusside-induced relaxation of smooth muscle cells isolated from rat stomach. Values express Mean±S.E.M. (n=6) ACh: acetylcholine. SNP: sodium nitroprusside. *$p<0.05$ compared with ACh 10^{-9} M or SNP 10^{-8} M.

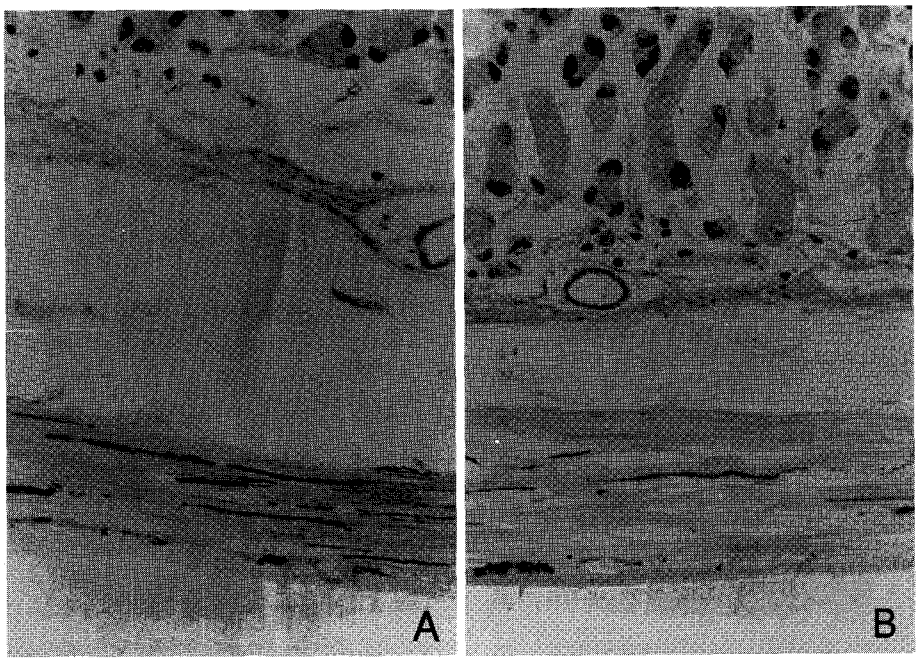

Fig. 8. Effects of L-arginine and L-NAME on the NADPH diaphorase activity of rat stomach. Panel A: L-arginine 200 mg/kg. Panel B: L-NAME 10 mg/kg (×200).

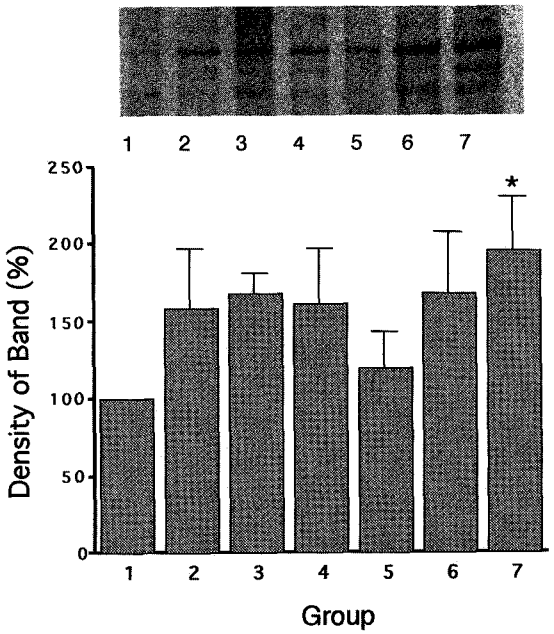

Fig. 9. Effects of electric field stimulation (60 VDC, 20 msec square wave duration, 20 sec train duration), L-arginine, L-NAME, acetylcholine and Ca^{2+} on the nNOS expression of rat gastric smooth muscle layer. Densitometric analysis of nNOS band. Values express Mean± S.E.M. (n=5). *$p<0.05$ compared with control. 1: Control, 2: EFS 1 min, 3: EFS 3 min, 4: L-arginine 10^{-5} M+EFS3 min, 5: L-NAME 10^{-5} M+EFS 3 min, 6: Acetylcholine 10^{-5} M+EFS 3 min, 7: Ca^{2+} 100 mM+EFS 3 min.

reservoir functions are important as the physiological reflex. A nitric oxide (NO) dependent non adrenergic, non cholinergic neural pathway is involved in the relaxation reflex (Arakawa et al, 1997). Uno et al (1997), by their functional study with isolated guinea pig stomach, reported that NO may be involved in the mechanisms of adaptive relaxation and receptive relaxation, and Arakawa et al (1997) emphasized that disorders of these reservoir functions result in symptoms of early satiety and anorexia, which are the major symptoms of patients with functional dyspepsia.

To clarify the existence of nitroxergic innervation and its mode of contribution to gastric relaxation will be helpful to find a therapeutic resort to treat a functional dyspepsia.

In the present study, gastric smooth muscle strips in isolated muscle chamber were relaxed by SNP, an NO donor, concentration dependently.

ACh-induced contraction of the strips were not significantly affected by L-arginine, a precursor of NO or L-NAME, NO synthase inhibitor. On the other hand, EFS-induced contractions of the muscle strips were inhibited by L-arginine, and were enhanced by L-NAME. Both L-arginine and L-NAME should have been absorbed into smooth muscle cells and nerve fibers, but these result suggest that only the nerve fibers synthesize the NO and release it by nerve im-

pulse. In these experiments, the effect of L-arginine, an relaxant action, was observed from the circular muscle strips more distinctly than from the longitudinal muscle, and the effect of L-NAME, a contractile action, was observed from the longitudinal muscle strips more distinctly than from circular muscle. In fact, the circular muscle was substantially much stronger in contraction than the longitudinal muscle. So the effects of the opposite direction might be expressed more prominently.

The EFS-induced contractions often have some time delay until they actually start to contract. The delay time was approximately 25 seconds in control group, and L-NAME abolished the delay. The nitroxergic nerve may excite and inhibit the action of cholinergic nerve by EFS, but the action of nitroxergic innervation lasts shorter than that of cholinergic innervation. This finding is in agreement with that in human sigmoid colon and anal sphinctor, an N^G-Nitro-L-Arginine-sensitive innervation inhibits the muscle contraction (Burleigh, 1992).

Single cells that are totally independent from any innervation showed that the smooth muscle cells are relaxed by NO, and the inhibition of guanylate cyclase reduced the NO-relaxation. This finding is similar to that the inhibition of NO synthesis in canine proximal colon reduced the production of cGMP (Ward et al, 1992) and that the effect of NO in canine pyloric sphinctor mimicked by 8-bromo-cyclic GMP (Bayguinov & Sanders, 1993).

Hope et al (1991) reported that neuronal NADPH diaphorase is a nitric oxide synthase. Schmidt et al (1992) reported that NOS possesses NADPH diaphorase activity in the presence of the electron acceptor nitroblue tetrazolium (NBT). NADPH diaphorase histochemistry, therefore, provides a specific histochemical marker for neurons producing nitric oxide, and this method has been used to localize nitric oxide synthase-containing enteric neurons (Conklin et al, 1995).

In the present study, we observed a strong staining reactivity to NTB in whole thickness in the muscle layer. The NADPH diaphorase activity was increased by L-arginine and decreased by L-NAME.

To make sure that the NADPH diaphorase activity is of the nitroxergic nerve, we observed the alterations of NOS activity by EFS with the quantitative measurement by Western blotting. EFS to stimulate release of NO, loading of L-arginine to increase the source of NO, or addition of calcium ion to excite the nerve endings increased the NOS expression.

These findings suggest that nitric oxide releasing nerve fibers innervate in both the circular and longitudinal muscle layers of rat stomach, and that the NO relaxes gastric smooth muscle via guanylate cyclase pathway.

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