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Electrically Stimulated Relaxation is not Mediated by GABA in Cat Lower Esophageal Sphincter Muscle

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This study examined the effect of Gamma-Amino butyric acid (GABA) and selective GABA receptor related drugs on the electrically stimulated relaxation in the lower esophageal sphincter muscle (LES) of a cat. Tetrodotoxin (10^{-6} M) suppressed the electrically stimulated (0.5-5 Hz) relaxation of the LES. However, guanethidine (10^{-6} M) and atropine (10^{-6} M) had no effect indicating that the relaxations were neurally mediated *via* the nonadrenergic and noncholinergic (NANC) pathways. NG-nitro-L-arginine methyl ester (10^{-4} M, L-NAME) also inhibited the relaxant response but did not completely abolish the electrically stimulated relaxation with 60 % inhibition, which suggests the involvement of nitric oxide as an inhibitory transmitter. This study examined the role of GABA, an inhibitory neurotransmitter, on neurally mediated LES relaxation. GABA (10^{-3}-10^{-5} M, non selective receptor agonist), muscimol (10^{-3}-10^{-5} M, GABA-A agonist), and baclofen (10^{-3}-10^{-5} M, GABA-B agonist) had no significant effect on the electrically stimulated relaxation. Moreover, bicuculline (10^{-5} M, GABA-A antagonist) and phaclofen (10^{-5} M, GABA-B antagonist) had no inhibitory effect on the electrically stimulated relaxation. This suggests that GABA and the GABA receptor are not involved in the electrically stimulated NANC relaxation in the cat LES.

Key words: Lower esophageal sphincter, GABA, Electrical field stimulation, Nonadrenergic noncholinergic neuron, Nitric oxide, Relaxation

INTRODUCTION

The lower esophageal sphincter (LES) normally controls the opening and closing of the gastroesophageal junction to prevent gastric reflux but allow swallowing (Yuan *et al.*, 1998). The specialized thickened circular smooth muscle of the LES has a high resting tone, which is mediated by myogenic and neurogenic mechanisms. However, it receives strong inhibitory innervation that allows it to relax during swallowing or belching (Seelig and Goyal, 1978; Brookes *et al.*, 1996).

It was reported that the vasoactive intestinal peptide (VIP) and nitric oxide (NO) are two of the main inhibitory neurotransmitters in the LES (Goyal *et al.*, 1980; Biancani *et al.*, 1984; Tottrup *et al.*, 1991; Jury *et al.*, 1992; Yamato *et al.*, 1992). One group suggested that the observed relaxation in the cat LES evoked by electrical field stimulation (EFS) or nicotine might be due to the release of NO and VIP or VIP-like peptides with the involvement of other transmitters in relaxation (Kortezova *et al.*, 1996).

Gamma-Amino butyric acid (GABA) is an established mediator of rapid inhibitory transmission in the central nervous system and is also found in the peripheral tissues of rodents and humans (Akinci and Schofield, 1999). It was suggested that GABA is present in the enteric nervous system as a neurotransmitter of interneurons (Williamson *et al.*, 1996).

This study investigated the characteristics of LES relaxation induced by EFS and examined the possibility that GABA may be a nonadrenergic and noncholinergic (NANC) neurotransmitter or that the GABA receptor pathway might be involved in the EFS-induced relaxation of LES.

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MATERIALS AND METHODS

Drugs

The tetrodotoxin citrate, muscimol and phaclofen were purchased from Tocris cookson Ltd. (Langford, UK). The guanethidine monosulfate (1:1), N^G-nitro-L-arginine methyl ester (L-NAME), GABA, baclofen, (-)-bicuculline methobromide and the other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO U.S.A.).

Measurements of in vitro LES tone

Adult cats of either gender weighing between 2.5 and 5 kg were used in this study. The cats were anesthetized with ketamine (50 mg/mL/kg). After opening the esophagus and stomach, the LES preparation was made. The preparations, 2 mm wide and 6-7 mm long, were cut with a razor blade held in a metal block. The preparations were then sliced into 4-5 minor strips, and silk-ligatures were tied at both ends. The muscle strips were mounted in separate 1 ml muscle chambers. One wire was fixed and the other was attached to a force transducer (FT03 Grass Instruments Co., Quincy, Mass., U.S.A.). The changes in the isometric force were recorded on a polygraph (Grass model 79, Grass Instruments Co., Quincy, Mass., U.S.A.).

The muscles were initially stretched to 2.5 g in order to bring them to close to the conditions for the development of the optimal force, and were equilibrated for 2 h while being perfused continuously with oxygenated Krebs. The tissues were maintained in a Krebs buffer with the following composition (mM): NaCl 116.6, $NaHCO_3$ 21.9, NaH_2PO_4 1.2, KCl 3.4, $CaCl_2$ 2.5, glucose 5.4 and $MgCl_2$ 1.2. During this time, the tension in the LES strips increased, becoming almost level at 2 h, whereas the tension in the esophageal strips decreased rapidly and became stabilized at less than 0.5 g. The solution was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 at pH 7.4 and 37°C.

Electrical field stimulation (EFS)

The strips were stimulated with pulse trains, 80 V in amplitude and 10 seconds in duration, with a pulse duration of 0.5 milliseconds at a frequency of 0.5-5 Hz using a stimulator (model S88; Grass Instruments) through platinum wire electrodes placed longitudinally on either side of the strips. After the LES strips showed a stable resting tone, the frequency-response relationship (0.5-5 Hz) was determined. The rings were washed three times and allowed to equilibrate for 50 min after EFS in order for the strips to recover completely from the relaxant responses to EFS. The amplitude of the relaxation induced by EFS is expressed as a percentage of the 10 iM SNP-produced relaxation, which was taken as 100%.

Assessment of drug responses

The control responses of EFS on the resting tension of the LES were investigated. The response to EFS was confirmed to be neuronal in origin by pretreating some of the preparations with tetrodotoxin (TTX, 10^{-6} M) for 15 min. The strips were pretreated with atropine (10^{-6} M) and guanethidine (10^{-6} M) for 15 min in order to determine if sympathetic and cholinergic neurotransmission were involved in the response to EFS. The effects of the GABA and GABA receptor agonists and antagonists on the basal tone of LES smooth muscle were assessed by measuring the tension in LES strips that had been exposed to these compounds. The strips were also pretreated with GABA (10^{-3}-10^{-5}M), muscimol ($GABA_A$ receptor agonist, 10^{-3}-10^{-5} M), baclofen ($GABA_B$ receptor agonist, 10^{-3}-10^{-5}M) for 5 min in order to determine if the GABAergic pathway is postsynaptically involved in the response to EFS. The involvement of the endogenously released relaxants in the responses to EFS were investigated by constructing frequency-response curves in the presence or absence of the following antagonists and enzyme inhibitors: 1) N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4}M, NOS inhibitor) for 20 min to determine if the nitrergic nerves are involve; 2) a 10 min pretreatment with bicuculline ($GABA_A$ receptor antagonist, 10^{-5} M) and phaclofen ($GABA_B$ receptor antagonist, 10^{-5} M) to determine if the nerve-released GABA is involved (Tohda *et al.*, 1998; Devlin and Schlosser, 1999; Hebeiss and Kilbinger, 1999; Kilbinger *et al.*, 1999).

Data analysis

The data is expressed as the mean ± SEM of different experiments. An analysis of variance was used to determine the statistical significance of the differences between the groups. If a significant difference was found, Duncan's multiple range test was used to differentiate the differences between the groups. A p value < 0.05 was considered significant.

RESULTS

Characteristics of the EFS-induced relaxation of LES

The muscle strips from the LES maintained their tone at rest and were relaxed when stimulated by EFS (Fig. 1). EFS (0.5-5 Hz) induced a frequency-dependent relaxation, which was suppressed by tetrodotoxin (10^{-6}M). However, neither atropine (10^{-6}M) nor guanethidine (10^{-6}M) had a significant effect on the EFS-induced relaxation of the LES tone (Fig. 2). A pretreatment with L-NAME (10^{-4}M) significantly inhibited the EFS-induced LES relaxation (Fig. 3).

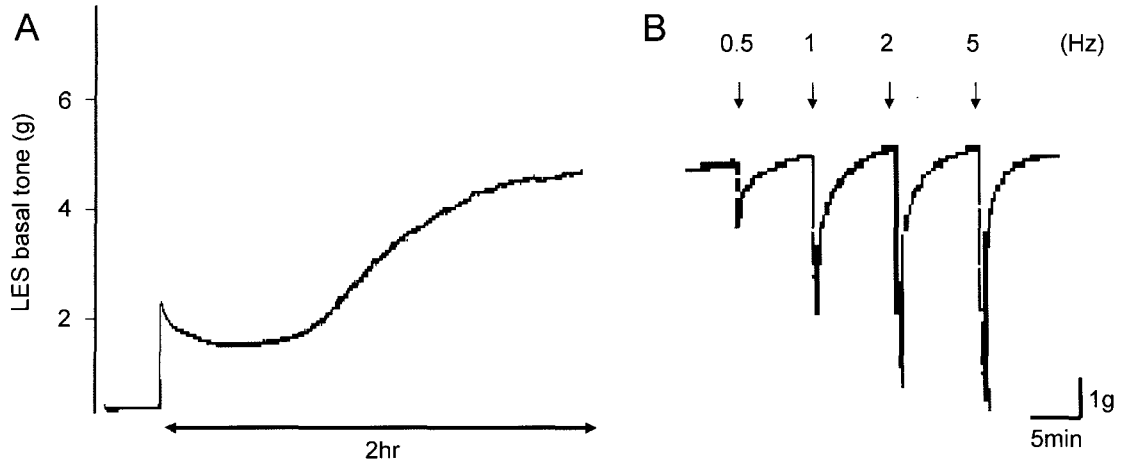

Fig. 1. The response of the LES to various EFS frequencies. *A*: The LES muscle strips were equilibrated for 2hrs in a warmed oxygenated Krebs solution prior to experimentation. The strips generated a tone at rest. *B*: Original records of the frequency-response relationship for the EFS-induced relaxation of LES.

Effect of GABA on the EFS-induced relaxation of LES

The LES muscle strips were exposed to GABA (a non selective receptor agonist; 10^{-3}-10^{-5} M), muscimol (a $GABA_A$ receptor agonist; 10^{-3}-10^{-5}M), baclofen (a $GABA_B$ receptor agonist; 10^{-3}-10^{-5}M), bicuculline (a $GABA_A$ receptor antagonist, 10^{-5}M), and phaclofen (a $GABA_B$ receptor antagonist, 10^{-5}M). None had any effect on the basal tone of the LES muscle strips (data not shown). In order to determine if the GABA pathway affects the EFS-induced relaxation, the LES muscle strips were exposed to GABA, muscimol and baclofen for 5 min prior to electrical stimulation. The exogenous GABA, muscimol and baclofen had no significant effect on the EFS-induced LES relaxation (Fig. 4). In addition, bicuculline and phaclofen did not have any significant influence on the EFS-induced relaxation of LES (Fig. 5).

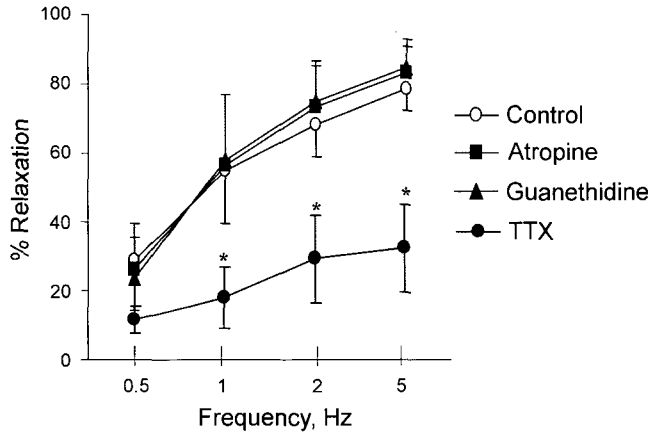

Fig. 2. Effect of tetrodotoxin (TTX), atropine and guanethidine on the EFS-induced relaxation of the LES. EFS-induced relaxation was significantly suppressed by TTX(10^{-6} M), indicating that the response is mainly mediated neuronally in origin. The EFS-induced relaxation of strips from the cat LES was not abolished by either guanethidine(10^{-6} M) or atropine(10^{-6} M), indicating that the response is nonadrenergic noncholinergic in nature. The values are expressed as the mean ± S.E.M.(n=4). *P<0.05 vs. control

DISCUSSION

The lower esophageal sphincter relaxes when its intrinsic inhibitory innervation is stimulated. Most investigators examining the mechanism of LES relaxation focused on the relaxation that occurs during the stimulation of the intrinsic esophageal nerves (Uc *et al.*,

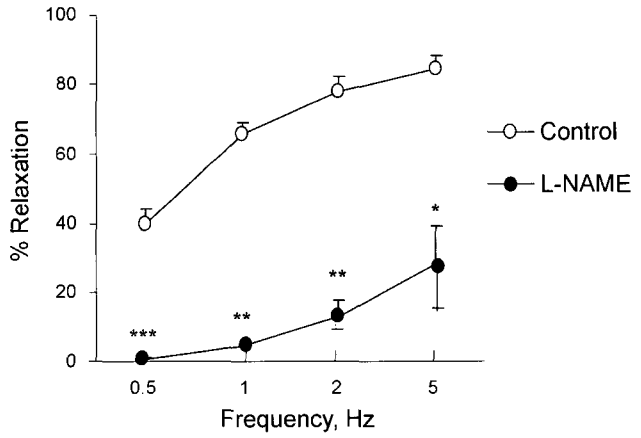

Fig. 3. Effect of L-NAME on the EFS-induced relaxation of LES. L-NAME (10^{-4} M, NOS inhibitor) significantly attenuated the LES relaxation induced by EFS. This suggests that NO is released from the neurons stimulated by EFS. The values are expressed as a mean ± S.E.M.(n=4) *P<0.05, **P<0.01, ***P<0.001 vs. control

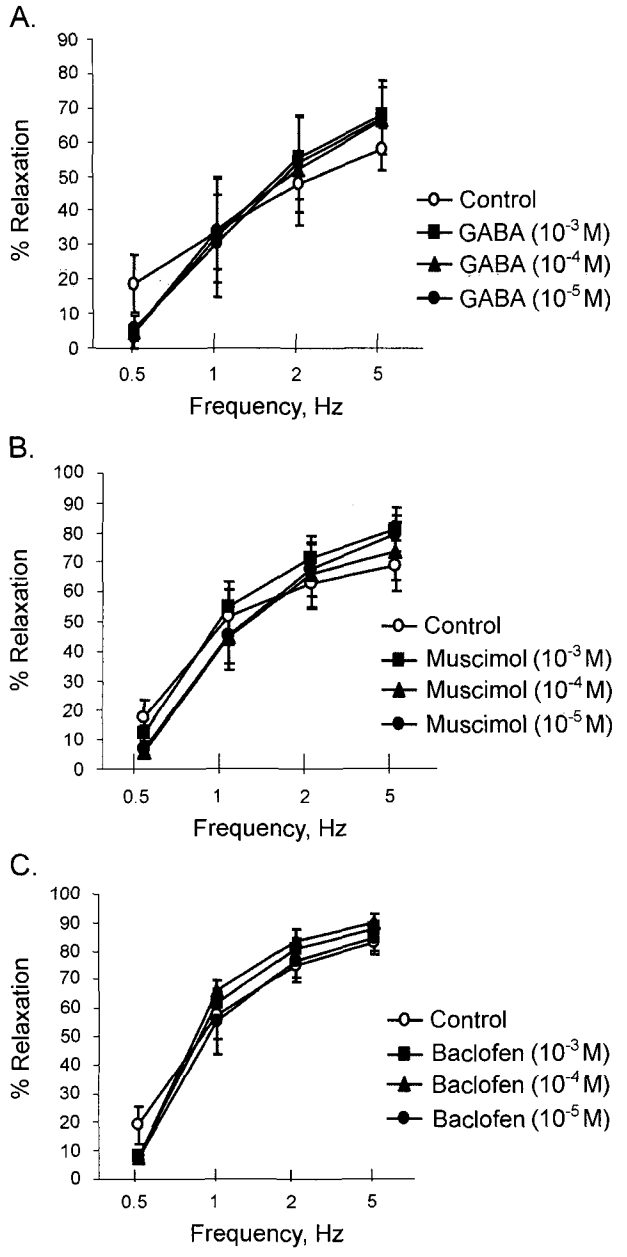

Fig. 4. Effect of GABA, muscimol, baclofen on the EFS-induced relaxation of LES. GABA(10^{-3}-10^{-5} M), muscimol(10^{-3}-10^{-5} M) and baclofen (10^{-3}-10^{-5} M) were pretreated for 5 min before the application of EFS. However, exogenous GABA(A), muscimol(B), baclofen(C) had no effect on the EFS-induced LES relaxation. The values are expressed as the mean ± S.E.M.(n=4-8)

1999). Many studies on neurotransmitter substances or mediators that may be involved in the relaxation of the LES tone have been reported. NO, VIP, adenosine 5-triphosphate (ATP), calcitonin gene-related peptide (CGRP) are neurotransmitter substances and mediators that have been suggested to contribute in some way to LES relaxation (Kortezova *et al.*, 1996; Uc *et al.*, 1997; Murr *et al.*, 1999).

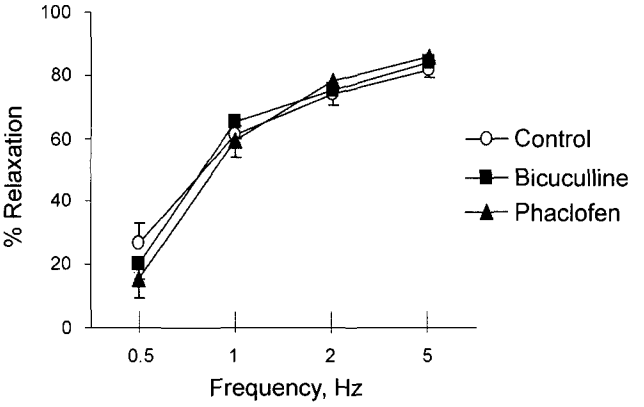

Fig. 5. Effect of bicuculline, $GABA_A$ receptor antagonists and phaclofen, $GABA_B$ receptor antagonist on the EFS-induced relaxation of LES. Bicuculline (10^{-5} M, $GABA_A$ receptor antagonist) and phaclofen (10^{-5} M, $GABA_B$ receptor antagonist) had no effect on the EFS-induced LES relaxation, suggesting that GABA does not participate in the LES relaxation. The values are expressed as the mean ± S.E.M(n=4-6)

This study examined the possibility that GABA might mediate LES relaxation. First, EFS-induced relaxation was investigated in an isolated LES of cats. The EFS-induced relaxation of the muscle strips from the cat LES was inhibited by tetrodotoxin, which suggest that this relaxation has a neurogenic origin. This relaxation was not abolished by either guanethidine or atropine, highlighting their nonadrenergic noncholinergic nature (Kortezova *et al.*, 1996). The L-NAME induced blocking of NO synthesis attenuated the TTX-sensitive EFS-induced relaxation. These findings are consistent with the evidence showing that NO is released from the neurons stimulated by EFS, which results in the relaxation of muscle strips under NANC conditions (Murr *et al.*, 1999). However, the observation of a 60 % decrease in the amplitude of the high-frequency-induced relaxation in the presence of L-NAME suggests the participation of not only NO but of other substances as well (Kortezova *et al.*, 1996).

GABA is an important inhibitory neurotransmitter within the central nervous system and is found in regions of the brainstem. There is accumulating evidence suggesting that GABA is a neurotransmitter within the enteric nervous system. These results suggest that GABA plays some role in modulating the gastrointestinal motility. Indeed, the enzyme responsible for GABA synthesis has been identified within the enteric nerves as well as in a high-affinity uptake system (Williamson *et al.*, 1995). The $GABA_B$ receptor has also been observed in the reticular formation of the brain stem (dorso vagal complex) as well as in the myenteric synapse of the LES (McDermott *et al.*, 2001).

GABA is released by the electrical stimulation of gut

preparations (Kerr and Krantis, 1983). Therefore, this study investigated the possibility of GABA involvement in the EFS-induced relaxation and in the modulation of the LES smooth muscle tone. In order to test whether or not GABA affects the basal tone of LES, the LES muscle strips were exposed to exogenous GABA, GABA agonists or antagonists. However, none of these agents had any effect on the basal tone of the LES muscle strips (data not shown). Exogenous GABA, GABA agonists such as muscimol and baclofen, GABA antagonists such as bicuculline and phaclofen also had no significant effect on the EFS-induced relaxation of LES. This suggests that GABAergic neurons or GABA receptors are not involved on the EFS-induced relaxation of LES.

In conclusion, the EFS-induced relaxation of LES is neurogenic and is mediated *via* a nonadrenergic non-cholinergic NO pathway. GABA or its receptor do not appear to be involved in EFS-induced LES relaxation

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