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Mexiletine inhibits nonadrenergic noncholinergic lower oesophageal sphincter relaxation in rabbits

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Abstract

Nonadrenergic noncholinergic (NANC) nerves are known to be nitrergic and to have an important role in the regulation of gastrointestinal motility and function. Cardiac antiarrhythmic therapy in humans is accompanied by a high incidence of gastrointestinal side-effects. We investigated the effect of mexiletine, a class Ib antiarrhythmic drug, on NANC lower oesophageal sphincter relaxation. Mexiletine concentration dependently inhibited the NANC relaxation induced by 30 mM KCl ($EC_{50} = 4.4 \times 10^{-6}$ M); the production of 3′,5′-cyclic guanosine monophosphate (cGMP) after KCl stimulation was concentration dependently decreased. The relaxation induced by the exogenous nitric oxide (NO) donor, diethylamine NONOate (10^{-5} M), was not inhibited by mexiletine, and the cGMP production after diethylamine NONOate application was not altered. Mexiletine did not alter the activity of NO synthase. These findings suggest that mexiletine inhibits NANC relaxation via NO-cGMP pathway modulation, possibly by inhibiting myenteric nitrergic neurotransmission in the lower oesophageal sphincter in rabbits.

Keywords: Oesophageal sphincter, lower; Smooth muscle; Mexiletine; Nitric oxide (NO); Nitric oxide (NO) synthase; 3′,5′-Cyclic guanosine monophosphate

1. Introduction

Mexiletine, which is classified as a class Ib antiarrhythmic drug (Vaughan Williams, 1984), is frequently used in the therapy of premature ventricular arrhythmias and/or ventricular arrhythmias refractory to other antiarrhythmic drugs (Monk and Brogden, 1990). Cardiac antiarrhythmic therapy in humans, however, is accompanied by a high incidence of gastrointestinal side-effects including nausea, vomiting, and abdominal discomfort (Campbell, 1987), and the incidence is reportedly as high as 38% (Morganroth, 1987). Previous investigation has revealed that the alterations of gastric motor activity by mexiletine might be one of the causes of gastrointestinal side-effects (Bielefeldt and Bass, 1991). However, little is known about the modifications of physiologic gastrointestinal motility and/or contractility by mexiletine.

The nonadrenergic noncholinergic (NANC) nerve-mediated response is known to be nitrergic (Rand, 1992; Sanders and Ward, 1992; Tottrup et al., 1992) and to have important roles in mediating peristaltic waves, inhibitory responses, and/or relaxing mechanisms of the gastrointestinal tract (Sanders and Ward, 1992). The functional role of the L-arginine–nitric oxide (NO) pathway in mediating NANC lower oesophageal sphincter relaxation has been demonstrated (Tottrup et al., 1991a,b). Recently, the nitrergic transmitter has been reported to be a stable NO-releasing molecule because various superoxide generators could reduce relaxations in response to exogenous NO but did not affect nitrergic-stimulated relaxation (Gibson et al., 1995; Rand and Li, 1995).

We previously reported that the NANC lower oesophageal sphincter relaxation in rabbits was mediated by NO or NO-related substances endogenously released from the myenteric plexus and by the activation of low-conductance K_{Ca} and K_{ATP} channels of smooth muscle (Kohjitani et al., 2001) and, further, that the intravenous anaesthetics, ketamine and midazolam, inhibited NANC relaxation via

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NO-3′,5′-cyclic guanosine monophosphate (cGMP) modulation (Kohjitani et al., 2001). In recent years, NO has been revealed to mediate physiologic responses of the oesophagus and lower oesophageal sphincter (Brookes, 1993) and to be associated with the swallowing-induced peristaltic response of the oesophageal body and lower oesophageal sphincter (Hirsch et al., 1998). Therefore, drugs affecting the NANC nerves, including anaesthetic agents, may alter physiologic lower oesophageal sphincter motility.

The pharmacological action of class I antiarrhythmic drugs has been shown to block the Na^+ current of cardiac myocytes and/or myocardium, which inhibits depolarization of muscle cells (Shirayama et al., 1991), to activate K_{ATP} channel of muscle cells resulting in a shortening of action potential duration (Sato et al., 1995), and to block fast Na^+ channels which leads to the decrease in action potential duration and an increase in the effective refractory period of ventricular myocardium (Costard-Jackle and Franz, 1989). The mechanism of action of pain relief by mexiletine, which has also been reported to be useful in the treatment of peripheral neuropathic pain (Chabal et al., 1992), is attributed to the selective inhibition of the depolarized Na^+ current resulting in the suppression of spontaneous discharge of the afferent neural activity from injured neurons or neuroma (Chabal et al., 1989). These pharmacological characteristics of mexiletine that affect ion channels and intracellular signaling indicate that it might modify the NO-mediated lower oesophageal sphincter relaxation.

Therefore, in the current study, we investigated the effects of mexiletine on KCl-induced NANC relaxation and the possible mechanism of this modulation of NANC transmission using isolated muscle strips from rabbit lower oesophageal sphincter.

2. Materials and methods

2.1. Materials

The experimental protocol was approved by the Okayama University Animal Use Committee. Twenty-eight adult male Japanese White rabbits weighing between 2 and 3 kg were anaesthetized with thiopental sodium (50 mg/kg i.v.) and killed by exsanguination. The lower part of the oesophagus and stomach was immediately isolated. The oesophagogastric junction was opened along the longitudinal axis, and the lower oesophageal sphincter was excised by sharp circular cutting, making strips approximately 2 mm wide and 5 mm long. The mucosa was removed.

2.2. Isometric tension recording

The strips were vertically fixed between two hooks under a resting tension of 1.0 g, and the hook anchoring the upper end was connected to a force–displacement transducer. Changes in the isometric tension of circular muscle were recorded. The strips were suspended in a thermostatically controlled (37.0 ± 0.5 °C) 20 ml organ bath containing Krebs–Ringer solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 1.19 mM $MgSO_4$, 11 mM glucose). The bath fluid was aerated with a mixture of 95% O_2 and 5% CO_2 to keep the pH between 7.35 and 7.45. Before the start of the experiments, the strips were allowed to equilibrate for 60 min in the Krebs solution which was replaced every 15 min. In this series of experiments, drugs were applied directly to the organ bath by micropipette.

NANC relaxation was induced by KCl (30 mM) in the presence of atropine (3×10^{-6} M), an acetylcholine receptor antagonist, and guanethidine (3×10^{-6} M), an adrenoceptor antagonist. Atropine and guanethidine were added as pretreatment for at least for 10 min. After NANC relaxation was obtained, we washed out the organ bath using Krebs–Ringer solution, and atropine and guanethidine were added consecutively to obtain the next NANC response. The application of 30 mM KCl induced a transient relaxation, which was followed by a sustained strong contraction that might have been due to muscle membrane depolarization (Kohjitani et al., 2001). This transient relaxation was considered to be a neural response as it was abolished by pretreating with tetrodotoxin or by extracellular Ca^{2+} depletion (Kohjitani et al., 2001). As the transient relaxation was still observed in the presence of atropine and guanethidine, this relaxation was also considered to be a NANC response (Kohjitani et al., 2001). The effects of mexiletine on the NANC relaxation and 10^{-5} M diethylamine NONOate-induced relaxations were analyzed. Mexiletine was added for 10 min at concentrations of 10^{-7}–3×10^{-5} M.

2.3. Assay of cGMP

For the radioimmunoassay of cGMP, mexiletine (10^{-6} M, 10^{-5} M, 3×10^{-5} M) was applied to lower oesophageal sphincter strips for 10 min in the presence of atropine and guanethidine, and the strips were frozen in liquid nitrogen 2 min after the application of KCl. In a previous study, we measured the content of cGMP for time intervals of 10 s, 30 s, 1 min, 2 min, and 3 min between the application of KCl and freezing of the muscle strips in liquid nitrogen. The maximal content of cGMP was obtained with the time interval of 2 min (Kohjitani et al., 2001). In the control study, using another strip from the same animal, an equivalent volume of KCl was applied in the presence of atropine and guanethidine. The effect of increasing concentrations of mexiletine on diethylamine NONOate-induced cGMP production was tested similarly. The strips were frozen in liquid nitrogen 3 min after the application of diethylamine NONOate, at which time the maximal relaxation could be obtained. The strips were then homogenized in 6% volume-to-volume ratio (V/V) trichloroacetic acid. The homogenate was centrifuged at 3000 rpm for 10 min, the supernatant fractions were subjected to ether extraction

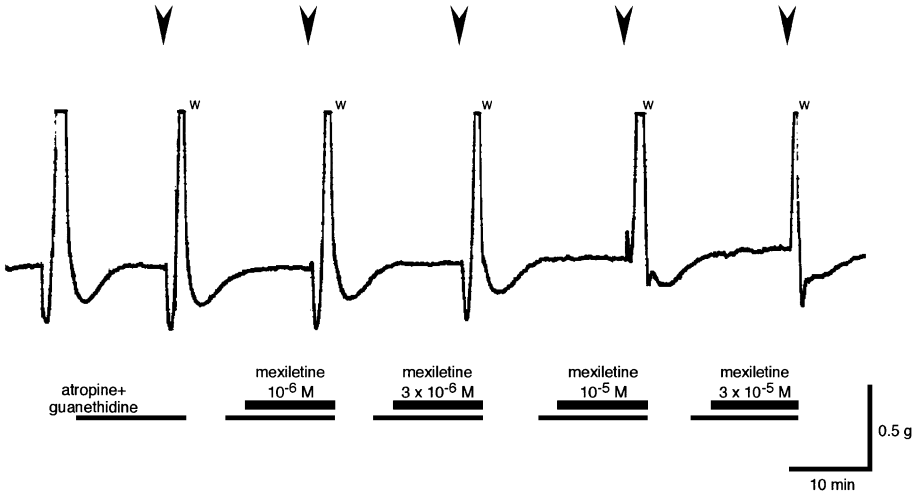

Fig. 1. The effect of increasing concentrations of mexiletine on 30 mM KCl-induced relaxation of a strip from the lower oesophageal sphincter in the presence of atropine and guanethidine (nonadrenergic noncholinergic [NANC] relaxation) is shown. The NANC relaxation is inhibited in a concentration-dependent manner by pretreatment with mexiletine. To magnify the relaxation, the component of KCl-induced contraction is overscaled. Arrows indicate the application of KCl. Upper horizontal bars indicate the presence of mexiletine. Lower horizontal bars indicate the presence of atropine and guanethidine. "w" indicates washout.

and subsequent succinylation, and the pellet was analyzed for protein content. cGMP in each sample was radioimmunoassayed using a Yamasa assay kit® (Yamasa Shoyu, Chiba, Japan). Levels of cGMP in tissues were expressed as femtomoles per mg protein.

2.4. Assay of nitric oxide synthase activity

For the assay of NO synthase activity, the lower oesophageal sphincter strip was quickly excised from another animal, cleaned of connective tissue, and frozen in liquid nitrogen until the assay. A commercial NO synthase quantitative assay kit (Calbiochem®, San Diego, CA) was used. The frozen lower oesophageal sphincter strip was homogenized in 20 vol. of homogenization buffer (pH 7.4, 25 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA), and the crude homogenates were centrifuged at 4 °C for 5 min at 15,000 rpm and the supernatants were collected. Supernatant samples (10 μl) were added to reaction buffer (40 μl) of the following composition: pH 7.4, 25 mM Tris–HCl buffer, 3 μM tetrahydrobiopterin, 1 μM flavin adenine dinucleotide, 1 μM flavin mononucleotide, 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 1 μCi of L-[^{3}H]arginine. Mexiletine at a final concentration of 10^{-6}, 10^{-5} or 3×10^{-5} M was added to each tube. Samples were incubated for 30 min at 30 °C and the reaction was discontinued by the addition of ice-cold (2 °C) stop buffer (pH 5.5, 50 mM HEPES, 5 mM EDTA). To obtain free L-[^{3}H]citrulline for the determination of enzyme activity, equilibrated resin was added to eliminate excess L-[^{3}H]arginine. The supernatant was assayed for L-[^{3}H]citrulline, using a liquid scintillation counter (Wallac 1414 WinSpectral, Turku, Finland). Enzyme activity was expressed as picomoles citrulline per milligram protein per minute. NO synthase activity in the positive control was measured in the presence of 0.6 mM $CaCl_2$ and rat cerebellum extract instead of lower oesophageal sphincter samples. NO syn-

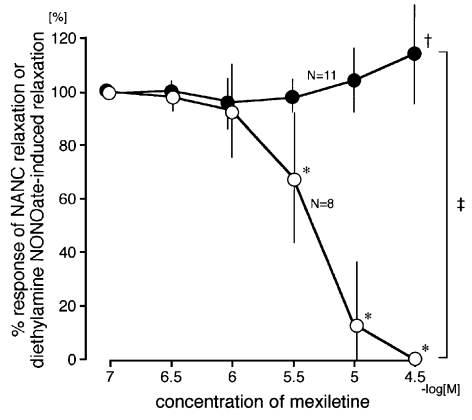

Fig. 2. The concentration–response relationship of mexiletine effects on 30 mM KCl-induced relaxation in the presence of atropine and guanethidine (nonadrenergic noncholinergic [NANC] relaxation; open circle) or on 10^{-5} M diethylamine NONOate-induced relaxation (closed circle) of strips from the lower oesophageal sphincter are shown. Mexiletine inhibited the NANC relaxation in a concentration-dependent manner; the diethylamine NONOate-induced relaxation was augmented by 3×10^{-5} M mexiletine. The curve shows percent response of each relaxation in the absence of mexiletine. Each point represents the mean for tissues from three to five animals; vertical lines show SDs. "N" = number of strips. *Significantly different from the value for KCl-induced NANC relaxation in the absence of mexiletine. †Significantly different from the value for diethylamine NONOate-induced relaxation in the absence of mexiletine. ‡Significant difference between the NANC and diethylamine NONOate-induced response groups.

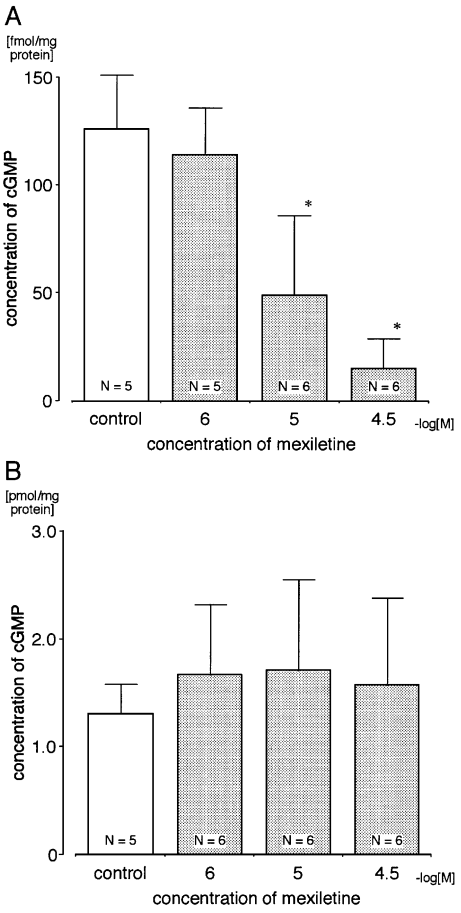

Fig. 3. Effects of 10^{-6}, 10^{-5} or 3×10^{-5} M mexiletine on 30 mM KCl (A)-stimulated 3′,5′-cyclic guanosine monophosphate (cGMP) production in the presence of atropine and guanethidine, and on 10^{-5} M diethylamine NONOate (B)-stimulated cGMP production in strips from the lower oesophageal sphincter are shown. For the control study, mexiletine was replaced by distilled water in the presence of atropine and guanethidine. Each bar represents the mean for tissues from three to four animals; vertical lines show SDs. "N" = number of strips. *Significantly different from the value in the absence of mexiletine.

thase activity in the presence of 1 mM N^G-nitro-L-arginine-methyl-ester (L-NAME) served as a negative control.

2.5. Drugs

The following drugs were used: potassium chloride from Nacalai Tesque (Kyoto, Japan); atropine sulfate, guanethidine hydrochloride, mexiletine hydrochloride, and diethylamine NONOate from Sigma (St. Louis, MO). Diethylamine NONOate was prepared in a gas-tight vial each time at the time of the experiment. All other drugs were dissolved in distilled water and handled in siliconized glassware.

2.6. Statistical analysis

The results were expressed as mean values ± standard deviations. One-way analysis of variance was used to examine the differences in effects among the concentrations of mexiletine, and the Bonferroni test was used as a post hoc comparison to test for statistical significance of differences between control values vs. drug-treated ones. The paired *t*-test was used to test the significance of the difference between the NANC-induced and the diethylamine NONOate-induced response groups. For all statistical tests, a P value less than 0.05 was regarded as significant.

3. Results

3.1. Effects of mexiletine on the NANC and diethylamine NONOate-induced lower oesophageal sphincter relaxations

Fig. 1 shows a typical tension record displaying a decrease in the NANC relaxation with increasing concentrations of mexiletine. The application of mexiletine did not affect the resting tension except for a slight elevation (baseline elevated 0.1–0.2 g) at 3×10^{-5} M. Mexiletine concentration dependently reduced the KCl-induced NANC response ($EC_{50} = 4.4 \times 10^{-6}$ M) (Fig. 2); the diethylamine NONOate-induced relaxation was not affected but was significantly augmented by 3×10^{-5} M mexiletine (Fig. 2; $P < 0.05$), comparing each of the relaxations without mexiletine taken as 100%. A significant difference was observed between the NANC-induced and the diethylamine NONOate-induced response groups, which were pretreated with mexiletine. The suppression of the NANC response by mexiletine was significant at concentrations of 3×10^{-6} M ($P < 0.05$), 10^{-5} M ($P < 0.01$), and 3×10^{-5} M ($P < 0.01$).

3.2. Effects of mexiletine on cGMP production and NO synthase activity

The cGMP production stimulated by KCl in the presence of atropine and guanethidine was decreased by the increasing concentrations of mexiletine (Fig. 3A); the decrease was significant at 10^{-5} M ($P < 0.01$) and 3×10^{-5} M ($P < 0.01$). Diethylamine NONOate-induced production of cGMP, however, was not affected by mexiletine (Fig. 3B). The sensitivity of our NO synthase activity assay is indicated in Table 1. We could measure the NO synthase activity

Table 1
The sensitivity of the assay for measuring the activity of nitric oxide synthase

Negative control (L-NAME)	Positive control (cerebellum extract)	Positive control + L-NAME	Control (lower oesophageal sphincter)
68.9 ± 14.2 ($N = 3$)	624.0 ± 136.9 ($N = 3$)	67.5 ± 13.9 ($N = 3$)	144.8 ± 28.1 ($N = 6$)

Units are based on pmol citrulline/mg protein/min. "N" indicates the number of samples. L-NAME = N^G-nitro-L-arginine-methyl-ester.

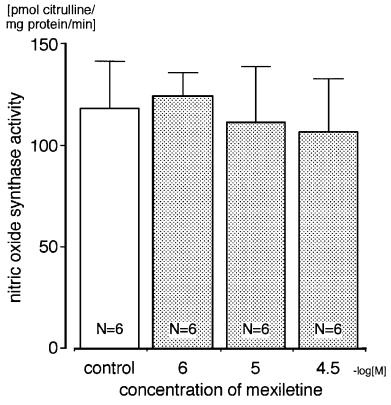

Fig. 4. The effects of mexiletine on the activity of nitric oxide (NO) synthase of supernatant samples from tissue homogenates of the lower oesophageal sphincter. The activity of NO synthase was not affected by increasing concentrations of mexiletine. Each bar represents the mean for tissues from three to four animals; vertical lines show SDs. "N" = number of strips.

of lower oesophageal sphincter smooth muscle within the assessable range. NO synthase activity was not altered by increasing concentrations of mexiletine (Fig. 4).

4. Discussion

In the current study, mexiletine concentration dependently inhibited NANC lower oesophageal sphincter relaxation, which was mediated by endogenously released NO from the myenteric plexus (Kohjitani et al., 2001) and left the diethylamine NONOate-induced relaxation unchanged (except at 3×10^{-5} M). Increasing concentrations of mexiletine significantly decreased the KCl-induced cGMP production but did not affect the diethylamine NONOate-induced cGMP production. It has been demonstrated that in vascular smooth muscle, NO reacts rapidly with superoxide anion (O_2^-), which leads to the production of peroxynitrite ($ONOO^-$) (Beckman et al., 1990) and to the loss of its vasodilating activity (Gryglewski et al., 1986), indicating that superoxide anion contributes to the instability of endothelium-derived relaxing factor (EDRF) (Gryglewski et al., 1986). The possibility that mexiletine produced superoxide anion and eliminated the relaxing effect of NO could be considered as one of the mechanisms of inhibition of NANC relaxation; however, diethylamine NONOate-induced relaxation was never inhibited in the current study and, therefore, superoxide anion is not involved in the mexiletine inhibition of NANC relaxation. The decrease in KCl-stimulated cGMP production may be due to the suppression of axonal transport and/or neurotransmitter release, including NO, from the endplate of the myenteric neurons because mexiletine has been reported to block Na^+channels (Hondeghem and Katzung, 1984) and Na^+current (Shirayama et al., 1991), and the blockade of Na^+ current in neural tissues inhibits action potential conduction (Fink and Cairns, 1984), axonal transport (Kanai et al., 2001), and synaptic transmission (Sotgiu et al., 1991).

As the activity of NO synthase was not altered by mexiletine, NO synthase does not appear to be a site of action of mexiletine. However, the data should be carefully interpreted based on two points. First, it has been demonstrated that neuronal NO synthase mediates NANC lower oesophageal sphincter relaxation in mice (Kim et al., 1999), and immunohistochemical studies revealed that the NO synthase immunoreactivity is evident at the myenteric plexus in rat intestine (Bredt et al., 1990) and at the myenteric plexus and the motor nerve terminals in mouse oesophagus (Sang and Young, 1998). Therefore, we consider that the NO synthase activity in the current study was mainly derived from myenteric neurons although we could not differentiate NO synthase activity in the nerves from that in the smooth muscle cells. Second, NO synthase activity is dependent on the influx of Ca^{2+}; mexiletine has Na^+ channel blocking properties (Hondeghem and Katzung, 1984; Shirayama et al., 1991) and thus may inhibit axonal transport and/or neurotransmitter release of the myenteric neurons. Therefore, our data for NO synthase activity modulation at high concentrations (3×10^{-5} M) of mexiletine may be less solid because Ca^{2+} influx to myenteric neurons may be entirely suppressed. This is a limitation of our experimental setups using tissue homogenates. However, 10^{-6} or 10^{-5} M mexiletine would not fully suppress myenteric neural transmission, and we should consider that mexiletine does not itself affect the ability to convert L-arginine to L-citrulline.

To elicit NANC relaxation of smooth muscle, electrical field stimulation is generally employed. In our previous study, however, electrical field stimulation under various conditions never induced relaxation but did induce contractions that were sensitive to tetrodotoxin in strips from rabbit lower oesophageal sphincter (Kohjitani et al., 1996). The transient relaxation induced by KCl is considered to be a truly neural response because the relaxation component is abolished by pretreatment with tetrodotoxin or by extracellular Ca^{2+} depletion (Kohjitani et al., 2001). The KCl-induced relaxation in the presence of atropine and guanethidine is inhibited by pretreatment with N^G-nitro-L-arginine (L-NNA) or methylene blue, indicating that this relaxation is nitrergic (Kohjitani et al., 2001). Therefore, we used chemical stimulation by KCl, in the presence of atropine and guanethidine, to induce NANC relaxation.

Interest in the actions of mexiletine on vascular smooth muscle has recently increased; mexiletine has been reported to induce endothelium-independent relaxation by inhibiting Ca^{2+} entry through voltage-dependent and receptor-operated Ca^{2+} channels (Dohi et al., 1994; Fernandez del Pozo et al., 1997), to augment endothelium-independent relaxation via the activation of K_{ATP} channels in vascular smooth muscle (Kinoshita et al., 2000), and to interfere with transmembrane Na^+ influx, thereby reducing Na^+ ions available for Na^+–Ca^{2+} exchange, thus decreasing the intracellular

Ca^{2+} concentration of vascular smooth muscle (Fernandez del Pozo et al., 1997). In the current study, mexiletine at high concentrations augmented diethylamine NONOate-induced relaxation. Diethylamine NONOate induces relaxation, acting directly on smooth muscle by spontaneous NO production. Although the mechanism of this augmentation is unclear, the activation of the smooth muscle K_{ATP} channel may be associated, as reported for vascular smooth muscle (Kinoshita et al., 2000).

We have already reported the inhibitory effects of the intravenous anaesthetics, ketamine and midazolam, on NANC lower oesophageal sphincter relaxation in rabbits (Kohjitani et al., 2001). The mechanism of the inhibitory action on NANC lower oesophageal sphincter relaxation appears to differ between mexiletine and these intravenous anaesthetics. Mexiletine appears to inhibit NANC lower oesophageal sphincter relaxation via inhibition of myenteric nitrergic neurons, as exhibited in the current study. Midazolam, whose ED_{50} value for inhibiting NANC relaxation is similar to that of mexiletine (4.8×10^{-6} M; (Kohjitani et al., 2001)), significantly suppressed the NO synthase activity almost identically to negative control values at 3×10^{-5} M (Kohjitani et al., 2003). Ketamine inhibits NANC relaxation and decreases KCl-induced cGMP production via the extracellular production of superoxide anion, as ketamine inhibited the relaxation induced by exogenous NO donors and the ketamine-induced inhibition of the NANC relaxation was reversed by pretreating with superoxide dismutase (Kohjitani et al., 2003).

We had suggested earlier that ketamine and midazolam may be associated with alterations of physiologic gastrointestinal motility (Kohjitani et al., 2001). The results of the current study appear to show that the suppression of endogenous NO, which has an important role in the maintenance of physiologic oesophageal motility, is one of the reasons for mexiletine-induced upper gastrointestinal distress. The suppression of the NANC relaxation by mexiletine in the current study was found to occur almost within the clinical plasma concentration levels, which have been reported as 8×10^{-7} to 10^{-5} M (Talbot et al., 1973).

In conclusion, it is suggested that mexiletine inhibits NANC relaxation via NO-cGMP pathway modulation, possibly by inhibiting myenteric nitrergic neurotransmission in the rabbit lower oesophageal sphincter. As endogenous NO plays an important role in the maintenance of physiologic oesophageal motility, the suppression of endogenous NO of the lower oesophageal sphincter smooth muscle by mexiletine may contribute to the reported gastrointestinal side effects.

Acknowledgements

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