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Functional evidence for different roles of $GABA_A$ and $GABA_B$ receptors in modulating mouse gastric tone

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ABSTRACT

The aims of the present study were to investigate, using mouse whole stomach *in vitro*, the effects of γ-aminobutyric acid (GABA) and GABA receptor agonists on the spontaneous gastric tone, to examine the subtypes of GABA receptors involved in the responses and to determine the possible site(s) of action.

GABA induced gastric relaxation, which was antagonized by the $GABA_A$-receptor antagonist, bicuculline, potentiated by phaclofen, $GABA_B$-receptor antagonist, but not affected by 1,2,5,6-Tetrahydropyridin-4-yl methylphosphinic acid hydrate (TPMPA), $GABA_C$-receptor antagonist. Muscimol, $GABA_A$-receptor agonist, mimicked GABA effects inducing relaxation, which was significantly reduced by bicuculline, Nω-nitro-L-arginine methyl ester (L-NAME), inhibitor of NO synthase or apamin, inhibitor of small conductance Ca^{2+}-dependent K^+ channels, which blocks the purinergic transmission in this preparation. It was abolished by tetrodotoxin (TTX) or L-NAME plus apamin. Baclofen, a specific $GABA_B$-receptor agonist, induced an increase in the gastric tone, which was antagonized by phaclofen and abolished by TTX or atropine. Bicuculline, but not phaclofen or TPMPA, *per se* induced an increase in gastric tone, which was prevented by L-NAME. In conclusion, our results suggest that GABA is involved in the regulation of mouse gastric tone, through modulation of intrinsic neurons. Activation of $GABA_A$-receptors mediates relaxation through neural release of NO and neurotransmitters, activating Ca^{2+}-dependent K^+ channels, likely purines, while activation of $GABA_B$-receptors leads to contraction through acetylcholine release.

1. Introduction

In the last years, evidence has been accumulated on the presence, action and distribution of γ-aminobutyric acid (GABA) receptors and GABAergic neurons in the mammalian gastrointestinal tract. However, although GABA meets the criteria to be considered a transmitter of enteric neurons, little attention has been given to its possible pharmacological and physiological role in the modulation and control of gut functions, including motility. Indeed, GABA and GABA synthesizing enzyme have been identified within enteric nerves (Tanaka, 1985; Williamson et al., 1995) as well as a high-affinity uptake system (Saffrey et al., 1983). Tetrodotoxin- and calcium-dependent release of GABA has been demonstrated (Taniyama et al., 1982). Moreover, immunohistochemistry and pharmacological studies have revealed the presence of $GABA_A$, $GABA_B$ and $GABA_C$ receptors in the myenteric neurons (Minocha and Galligan, 1993; Krantis et al., 1995; Fletcher et al., 2001; Bayer et al., 2002; 2003; Zizzo et al., 2007). Thus, it appears that GABA is able to modulate the activity of interneurons within the gastrointestinal wall. However, the activation of the different types of GABA receptors can lead to variable effects on intestinal motility depending on the region of the gut examined or the animal species studied.

In particular, in the intestine, the activation of ionotropic $GABA_A$ receptors may stimulate cholinergic excitatory and non adrenergic non cholinergic (NANC) inhibitory motor neurons, leading to contraction or relaxation of the smooth muscle, respectively (Frigo et al., 1987; Boeckxstaens et al., 1990; Minocha and Galligan, 1993; Kaputlu and Şadan, 1996; Bayer et al., 2002; 2003; Zizzo et al., 2007). Metabotropic $GABA_B$ receptor activation has been reported to be related to presynaptic inhibition of acetylcholine release through the blockade of voltage-sensitive calcium channels (Cherubini and North, 1984; Marcoli et al., 2000). Moreover, ionotropic $GABA_C$ receptor activation can increase the nitric oxide (NO) release from NANC inhibitory neurons (Zizzo et al., 2007).

In contrast, the function of GABA receptors in the intrinsic nerves of the stomach has not been much studied and it is still poorly understood. The majority of the studies have been

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performed in *in vivo* conditions and GABA has been shown to exhibit complex effects, depending on the types of receptors involved (see Tsai, 2005 for review). Therefore, the aims of the present work were to investigate the effects of GABA and GABA receptor agonists on the spontaneous gastric tone *in vitro*, to examine the subtypes of GABA receptors involved in the responses and to determine the possible site(s) of action. For this purpose, mouse whole stomach was used in order to examine the muscle function under conditions where the influence of external factors is removed, but the muscle performs in a manner analogous to its *in vivo* capacity and it is able to relax in absence of contractile agents (Mulè and Serio, 2003; Mulè et al., 2005).

2. Methods

2.1. General

Adult male mice (C57BL/10SnJ; weighing 25.5 ± 0.5 g), obtained from Harlan Laboratories (San Pietro di Natisone-Udine, Italy) were used for the study. Animals were housed in standard conditions under a constant light–dark cycle, constant temperature (22 ± 1 °C) and humidity (55 ± 5%), with free access to food and water. Experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 and were approved by Ministero della Sanità (Rome, Italy). Animals were fed ad libitum prior to use. Animals were killed by cervical dislocation. The abdomen was immediately opened, the esophagus was tied just below the lower esophageal sphincter, and the entire stomach was excised.

The stomach was mounted in a custom designed organ bath (volume = 5 ml), which was continuously perfused with oxygenated (95% O_2 and 5% CO_2) and heated (37 °C) Krebs solution with the following composition (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 11.1. The pyloric end was cannulated and connected to a standard pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, USA). The mechanical activity, monitored as changes of endoluminal pressure, was recorded on ink-writer polygraph (Grass model 7D, Grass Medical Instruments). Preparations were allowed to equilibrate for about 60 min before starting the experiment.

2.2. Experimental protocol

At the beginning of each experiment the preparation was challenged either with 3 μM sodium nitroprusside (SNP) or with 0.1 μM carbachol (CCh) for 2 min until reproducible responses were obtained. Drugs were added to the bath serosally. The amplitude of the relaxant response to SNP was 2.4 ± 0.3 cm H_2O ($n = 15$) while the amplitude of the contractile response to CCh was 2.2 ± 0.2 cm H_2O ($n = 15$).

In a first set of experiments, concentration–response curves to GABA receptor agonists were obtained non-cumulatively by adding increasing concentrations of agonists in volumes of 50 μl after switching off the perfusion. Each concentration of agonist was applied at 20 min intervals for 3 min and then washed out. This time was selected to avoid the development of tachyphylaxis. Each preparation was tested with a single agonist. Concentrations of GABA receptor agonists were determined from literature (Marcoli et al., 2000; Bayer et al., 2002; Zizzo et al., 2007).

Due to the difficulty to obtain a second concentration–response curve in the same stomach, in different gastric preparations, a sub-maximal dose of agonist was tested in the presence or in the absence of bicuculline (10 μM), phaclofen (10 μM) or 4-cis-aminocrotonoic acid (CACA) (10 μM), $GABA_A$-, $GABA_B$- and $GABA_C$-selective GABA receptor antagonist, respectively. All the antagonists were allowed to maintain contact with the tissue for at least 30 min before testing agonists.

To examine the mechanism of action responsible for the effects mediated by $GABA_A$ receptor a sub-maximal concentration of agonist (30 μM) was tested in presence of tetrodotoxin (TTX) (1 μM), a voltage-dependent Na^+-channel blocker, or Nω-nitro-L-arginine methyl ester (L-NAME) (300 μM), an inhibitor of NO synthase, apamin (0.1 μM), a blocker of small conductance Ca^{2+}-dependent K^+ channels, or L-NAME and apamin. Similarly the $GABA_B$ agonist (30 μM) was tested in the presence of TTX (1 μM) or atropine (1 μM).

2.3. Drugs

The following drugs were used and stock solutions were prepared using distilled water or as indicated below. The working solution was prepared fresh on the day of the experiment by diluting the stock solution in Krebs. Apamin, atropine, baclofen, bicuculline, carbachol (CCh), gamma aminobutyric acid (GABA), Nω-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), 1,2,5,6-Tetrahydropyridin-4-yl methylphosphinic acid hydrate (TPMPA), 4-cis-aminocrotonic acid (CACA), all purchased from Sigma (Sigma–Aldrich, Inc., St. Louis, USA); muscimol and tetrodotoxin citrate (TTX) were purchased from Ascent (Ascent scientific Ltd., Bristol, UK); phaclofen was from Tocris (Tocris Cookson Ltd., Avonmouth, UK). Bicuculline was dissolved in dimethyl sulphoxide (DMSO) and phaclofen in 0.1 N NaOH. Drugs were added to the organ bath in volumes of less than 1.0% of the bathing solution. Control experiments using solvents alone showed that they have no effect on the spontaneous mechanical activity.

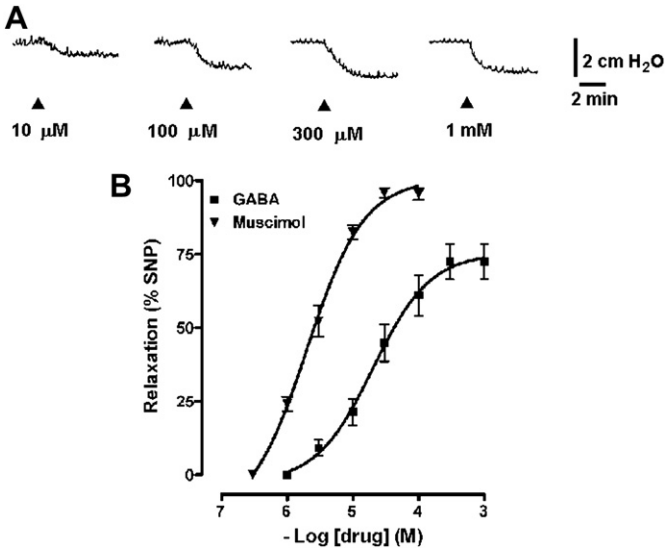

Fig. 1. A: Original tracings showing the relaxation induced by increasing concentrations of GABA on isolated mouse gastric preparations. B: Concentration–response curves for the relaxation induced by GABA ($n = 5$) or muscimol ($n = 6$). Data are means ± SEM and are expressed as a percentage of the response induced by 3 μM SNP in the same tissue.

2.4. Data analysis and statistical tests

Relaxant or contractile effects to GABA receptor agonists were expressed as a percentage of the response induced by SNP (3 μM) or CCh (0.1 μM), respectively. The concentration (EC_{50}) with 95% confidence intervals (CIs) producing half maximum response was calculated using Prism 4.0, GraphPad (San Diego, CA, USA). All data are expressed as mean values ± SEM. The letter *n* indicates the number of

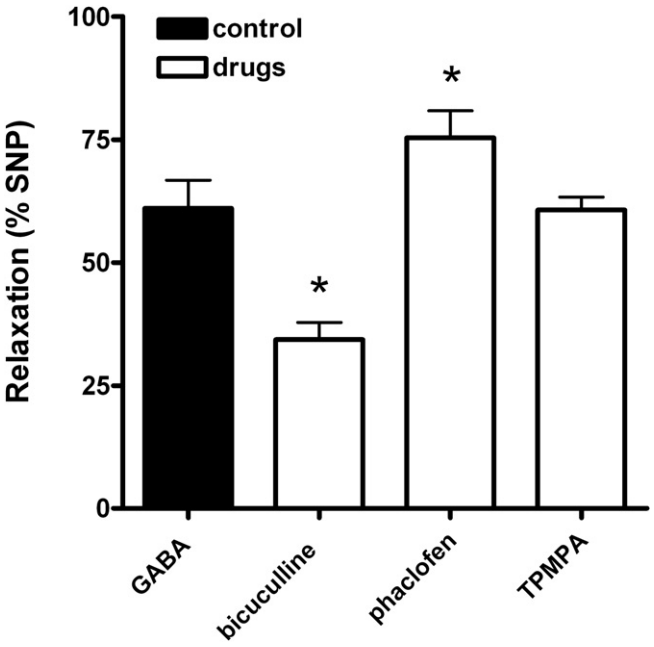

Fig. 2. Gastric relaxation induced by GABA (100 μM) before and after pretreatment with bicuculline (10 μM; $n = 5$), phaclofen (10 μM; $n = 5$), TPMPA (10 μM; $n = 4$). The response is expressed as a percentage of the response induced by 3 μM SNP in the same tissue. Data are means ± SEM. $^*P < 0.05$ compared to control.