GABA_A receptor-mediated stimulation of non-adrenergic non-cholinergic neurones in the dog ileocolonic junction

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- 1 The inhibitory effects of γ -aminobutyric acid (GABA), the GABA_A receptor agonist homotaurine and the GABA_B receptor agonist ($\pm$)-baclofen were investigated on circular muscle strips of the dog terminal ileum and ileocolonic junction.
- 2 In the presence of atropine, GABA and homotaurine induced concentration-dependent relaxations, similar to the non-adrenergic non-cholinergic (NANC)-mediated relaxations evoked by electrical stimulation or by acetylcholine. The ileocolonic junction was more sensitive to GABA and homotaurine than the ileum. ($\pm$)-Baclofen had no effect. Cross desensitization only occurred between GABA and homotaurine.
- 3 The GABA_A receptor antagonist bicuculline shifted the concentration-response curves to GABA and homotaurine to the right. The maximal relaxation to GABA remained unaffected.
- 4 GABA-induced relaxations were not inhibited by timolol, guanethidine, domperidone, hexamethonium and desensitization to ATP, but were abolished by tetrodotoxin.
- 5 Bicuculline, and pretreatment with GABA or ($\pm$)-baclofen had no effect on the NANC-evoked relaxations to electrical stimulation and acetylcholine.
- 6 In conclusion, GABA stimulates GABA_A receptors located on inhibitory NANC neurones in the dog ileocolonic junction. Our results suggest that it is unlikely that GABA is the final inhibitory NANC neurotransmitter.

Introduction

Since the proposal that γ -aminobutyric acid (GABA) may be a neurotransmitter in the vertebrate peripheral nervous system (Jessen *et al.*, 1979), evidence has accumulated demonstrating the presence of GABA receptors in the gastrointestinal tract (Krantis *et al.*, 1980; Giotti *et al.*, 1983; 1985; Ong & Kerr, 1983; Maggi *et al.*, 1984). By use of selective agonists and antagonists (Bowery *et al.*, 1981; Giotti *et al.*, 1983), two subclasses of GABA receptors can be distinguished: GABA_A receptors, stimulated by GABA, homotaurine (3-amino-1-propane sulphonic acid) or muscimol and inhibited by bicuculline (competitively) and picrotoxin (non-competitively). GABA_B receptors are stimulated by GABA and ($-$)-baclofen, and are blocked by phaclofen (Bowery *et al.*, 1981; Hill & Bowery, 1981; Dutar *et al.*, 1988).

Several lines of evidence suggest that GABA affects the motility of intestinal preparations mainly by altering the release of acetylcholine from intrinsic cholinergic neurones; stimulation of GABA_A receptors may increase acetylcholine output (Krantis *et al.*, 1980; Giotti *et al.*, 1983; Ong & Kerr, 1983) whereas GABA_B receptor stimulation may inhibit acetylcholine release (Ong & Kerr, 1983; Giotti *et al.*, 1983; 1985). In addition, evidence is available to suggest that GABA stimulates inhibitory intramural non-adrenergic non-cholinergic (NANC) neurones in the rat duodenum (Maggi *et al.*, 1984), and in the guinea-pig and rabbit distal colon (Krantis *et al.*, 1980; Giotti *et al.*, 1985; Tonini *et al.*, 1989).

Recently, we demonstrated that electrical stimulation and nicotinic receptor stimulation by acetylcholine or 1,1-dimethyl-4-phenylpiperazinium (DMPP) activates NANC neurones in the dog ileocolonic junction resulting in the release of an as yet unknown, inhibitory neurotransmitter

(Pelckmans *et al.*, 1989). Several substances have been proposed as putative NANC inhibitory neurotransmitters, including GABA (Burnstock, 1986).

Since preliminary experiments provided evidence for GABA-induced relaxations in the dog ileocolonic junction (Boeckxstaens *et al.*, 1988), the present study was designed to investigate further the effects of GABA in this preparation and to characterize the receptors involved. Some of the results have been presented to the British Pharmacological Society (Boeckxstaens *et al.*, 1990c).

Methods

Tissue preparation

Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg⁻¹, i.v.) and a laparotomy was performed. The ileum and the colon were resected 10 cm above and 3 cm below the ileocolonic junction respectively. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ileocolonic junction by means of sharp dissection. Circular muscle strips were cut, approximately 1.5 cm long and 0.3 cm wide and mounted in organ baths (25 ml) (Pelckmans *et al.*, 1989) filled with modified Krebs-Ringer solution (mm: NaCl 118.3, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, CaEDTA 0.026 and glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O₂ and 5% CO₂.

Isometric tension recording

One end of each muscle strip was connected to a metal rod while the other end was attached to a strain gauge transducer (Statham UC₂) for continuous recording of the isometric tension. After the muscle strips were brought to the optimal point of their length-tension relationship (Vanhoutte &

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Leusen, 1969), they were washed three times and then allowed to equilibrate for at least 45 min before experimentation.

Experimental protocols

All experiments were performed on muscle strips maximally contracted with noradrenaline (Pelckmans *et al.*, 1990) ($30 \mu\text{M}$; $2.0 \pm 0.3 \text{ g}$ in the ileum and $2.8 \pm 0.4 \text{ g}$ in the ileocolonic junction, mean $\pm$ s.e.mean; n = at least 6) and in the presence of $0.3 \mu\text{M}$ atropine. Concentration-response curves to GABA, homotaurine and ($\pm$)-baclofen were constructed in a non-cumulative manner: only one concentration of agonist was administered during each noradrenaline-induced contraction and was left in contact with the tissue for 5 min. After each noradrenaline-induced contraction, the muscle strips were washed at least three times with an interval of 5 min, resulting in a cycle time of at least 20 min. This protocol was selected to avoid tachyphylaxis.

To investigate cross-desensitization, the preparations were incubated with the respective agonists (0.3 mM) 10 min before the administration of noradrenaline. Again, only one agonist was administered on the same noradrenaline-induced contraction. In these experiments, nitroglycerin ($1 \mu\text{M}$) was used as nonselective smooth muscle relaxant. In addition, the effect of various antagonists on the relaxation evoked by a submaximally-effective concentration of GABA (0.1 mM) was assessed after a 15 min preincubation period.

In another series of experiments, the effects of bicuculline, tetrodotoxin (TTX) and GABA desensitization were studied on relaxations to electrical stimulation (2–16 Hz, 1–2 ms), acetylcholine ($30 \mu\text{M}$), GABA (0.1 mM) and nitroglycerin ($1 \mu\text{M}$). These stimuli were applied during the same noradrenaline-induced contraction. The muscle strips were washed at least three times with an interval of 5 min between each contraction. Electrical impulses (rectangular waves, 2–16 Hz, 1–2 ms, 200 mA, 9 V) were delivered by a Grass stimulator and a direct current (d.c.) amplifier for 10 s (Verbeuren *et al.*, 1983; Pelckmans *et al.*, 1989).

Drugs used

The following drugs were used: acetylcholine chloride, adenosine 5'-triphosphate (ATP), γ -aminobutyric acid (GABA), hexamethonium bromide, homotaurine (3-amino-1-propane sulphonic acid) (Sigma Chemical Co., St. Louis, MO., USA), atropine sulphate (Federa, Brussels, Belgium), ($\pm$)-baclofen, guanethidine monosulphate (Ciba-Geigy, Switzerland), domperidone (Janssen Pharmaceutica, Beerse, Belgium), nitroglycerin (Merck, Darmstadt, F.R.G.), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), tetrodotoxin (Janssen Chimica, Beerse, Belgium) and timolol maleate (Merck Sharpe and Dohme Research Lab., Rahway, N.J., USA).

All drugs were administered as aqueous solutions except baclofen and bicuculline which were dissolved in 0.1 M citric acid. Ascorbic acid (0.6 mM) was added to the stock solution of noradrenaline. These were prepared on the day of experimentation and administered in volumes not exceeding 0.5% of the bath volume. The stock solution of tetrodotoxin (1 mM , in sodium citrate, pH: 4.8) was stored at -20°C .

Presentation of results and statistical analysis

The relaxations are expressed as percentage decrease of the noradrenaline-induced contraction or as a percentage of the relaxation of the respective agonist in control solution. The results are shown as mean $\pm$ s.e.mean for the number of dogs indicated.

The negative logarithm of the concentration of agonist (pD_2 or $-\log \text{ED}_{50}$) that produced a half-maximal response was calculated by linear regression analysis (Tallarida & Murray, 1981).

For statistical analysis, a two tailed Student's *t* test for

paired or unpaired observations was used. *P* values of less than 0.05 were considered to be significant.

Results

Effect of GABA, homotaurine and ($\pm$)-baclofen

During a noradrenaline ($30 \mu\text{M}$)-induced contraction and in the presence of $0.3 \mu\text{M}$ atropine, both GABA and the GABA_A receptor agonist homotaurine ($10 \mu\text{M}$ – 1 mM) produced concentration-dependent relaxations in the dog terminal ileum and ileocolonic junction (Figure 1). The ileocolonic junction was more sensitive to GABA and homotaurine than the terminal ileum, as demonstrated by the pD_2 ($-\log \text{ED}_{50}$) values (Table 1). The potency of GABA in relaxing the ileocolonic junction was greater than that of homotaurine, whereas in the ileum, no statistically significant differences could be demonstrated (Figure 1, Table 1). In six experiments, the GABA_B receptor agonist ($\pm$)-baclofen (0.6 mM) failed to evoke relaxations in the terminal ileum or ileocolonic junction.

Effect of bicuculline on GABA- and homotaurine-induced relaxations

In both the terminal ileum and ileocolonic junction, the GABA_A receptor antagonist bicuculline (3 – $10 \mu\text{M}$) shifted the concentration-response curves to GABA and homotaurine to the right (Figure 2). In the ileocolonic junction, bicuculline had no effect on the maximal relaxation evoked by 1 mM GABA. Bicuculline had no effect on the spontaneous activity of the preparations.

Figure 1 Concentration-response curves for the relaxant effect of GABA (●) and homotaurine (○) on circular muscle strip preparations of the dog terminal ileum (a) and ileocolonic junction (b). The experiments were performed during a noradrenaline ($30 \mu\text{M}$)-induced contraction and in the presence of $0.3 \mu\text{M}$ atropine. The results are shown as mean for 6 experiments and expressed as the percentage decrease of the noradrenaline-induced contraction; vertical bars show s.e.mean. *P < 0.05, significantly different from homotaurine; Student's *t* test for unpaired observations.

Table 1 pD_2 ($-\log \text{ED}_{50}$) values of GABA and homotaurine in the canine ileum and the ileocolonic junction

Agonist	Ileum	Ileocolonic junction
GABA	$4.04 \pm 0.15^\dagger$	$4.59 \pm 0.11^*$
Homotaurine	$3.68 \pm 0.10^\dagger$	3.94 ± 0.06

The results are shown as mean $\pm$ s.e.mean for at least 5 experiments.

* *P* < 0.05, GABA versus homotaurine in the ileocolonic junction.

† *P* < 0.05, ileum versus ileocolonic junction; Student's *t* test for unpaired observations.

Figure 2 Effect of $3\ \mu\text{M}$ (●) and $10\ \mu\text{M}$ (Δ) bicuculline on the concentration-response curve to (a) GABA and (b) homotaurine (control, ○) in the canine terminal ileum (i) and ileocolonic junction (ii). The experiments were performed during a noradrenaline ($30\ \mu\text{M}$)-induced contraction and in the presence of $0.3\ \mu\text{M}$ atropine. The results are shown as mean for 6 experiments and are expressed as a percentage of the relaxation to $1\ \text{mM}$ GABA (a) or homotaurine (b) in control solution; vertical bars show s.e.mean. These relaxations (expressed as percentage decrease of the noradrenaline-induced contraction) were $98 \pm 9\%$ and $84 \pm 6\%$ respectively in the ileum, and $101 \pm 6\%$ and $97 \pm 10\%$ respectively in the ileocolonic junction. * $P < 0.05$, significantly different from control, Student's t test for paired observations.

Effect of desensitization to GABA, homotaurine and ($\pm$)-baclofen on relaxations to GABA, homotaurine and nitroglycerin

The relaxations to GABA ($0.3\ \text{mM}$ in the ileum and $0.1\ \text{mM}$ in the ileocolonic junction, $n = 5-7$) and homotaurine ($0.3\ \text{mM}$, $n = 5-7$) were abolished after preincubation with $0.3\ \text{mM}$

GABA or homotaurine. However, the relaxations to nitroglycerin remained unchanged. Incubation with $0.3\ \text{mM}$ baclofen had no effect ($n = 6$) on the spontaneous activity of the preparations or on any of the relaxations studied (Table 2). Desensitization was readily reversed upon removal of the agonist.

Effect of various substances on GABA-induced relaxations

Timolol ($3-10\ \mu\text{M}$), guanethidine ($3\ \mu\text{M}$), domperidone ($10\ \mu\text{M}$), hexamethonium ($0.1-0.5\ \text{mM}$) and desensitization to ATP (achieved by successive administrations of $30\ \mu\text{M}$ ATP) did not affect the relaxations to GABA ($0.1\ \text{mM}$) in the terminal ileum and in the ileocolonic junction (Table 3).

Effect of GABA desensitization, bicuculline and TTX on relaxations to electrical stimulation, acetylcholine, GABA and nitroglycerin

GABA ($0.1\ \text{mM}$)-induced relaxations are qualitatively similar to the NANC relaxations evoked by electrical stimulation ($2-16\ \text{Hz}$, $1-2\ \text{ms}$) and acetylcholine ($30\ \mu\text{M}$), as shown for the ileocolonic junction in Figure 3. Desensitization to GABA abolished the GABA-induced relaxations, whereas those to electrical stimulation and acetylcholine remained unaffected. Similarly, bicuculline ($10\ \mu\text{M}$) only reduced the relaxations to GABA. Preincubation with $0.3\ \text{mM}$ ($\pm$)-baclofen had no effect on any of the relaxations studied. TTX ($0.3\ \mu\text{M}$) abolished the relaxations to electrical stimulation, acetylcholine and GABA, but not those to nitroglycerin ($1\ \mu\text{M}$) (Figure 3).

Table 3 Effect of non-GABA antagonists on GABA ($0.1\ \text{mM}$)-induced relaxations in the canine ileum and ileocolonic junction

Antagonist	Ileum (% of control)	Ileocolonic junction (% of control)
Timolol ($10\ \mu\text{M}$)	99 ± 7	96 ± 5
Guanethidine ($3\ \mu\text{M}$)	105 ± 3	107 ± 5
Domperidone ($10\ \mu\text{M}$)	99 ± 5	103 ± 8
Hexamethonium ($0.5\ \text{mM}$)	104 ± 3	105 ± 6
ATP desensitization	98 ± 7	99 ± 6

The experiments were performed during a noradrenaline ($30\ \mu\text{M}$)-induced contraction and in the presence of $0.3\ \mu\text{M}$ atropine. Results are shown as mean $\pm$ s.e.mean for 5-7 experiments and expressed as a percentage of the control values. These control relaxations (expressed as percentage decrease of the noradrenaline-induced contraction) are $63 \pm 8\%$ in the ileum and $76 \pm 6\%$ in the ileocolonic junction.

Table 2 Effect of pretreatment with GABA ($0.3\ \text{mM}$), homotaurine or ($\pm$)-baclofen on GABA-, homotaurine-, and nitroglycerin-induced relaxations

	GABA pretreatment (% of control)	Homotaurine pretreatment (% of control)	($\pm$)-Baclofen pretreatment (% of control)
Ileum			
GABA ($0.3\ \text{mM}$)	0*	0*	104 ± 3
Homotaurine ($0.3\ \text{mM}$)	0*	0*	93 ± 9
Nitroglycerin ($1\ \mu\text{M}$)	99 ± 3	97 ± 5	93 ± 5
Ileocolonic junction			
GABA ($0.1\ \text{mM}$)	0*	0*	98 ± 3
Homotaurine ($0.3\ \text{mM}$)	0*	0*	109 ± 8
Nitroglycerin ($1\ \mu\text{M}$)	98 ± 8	101 ± 6	97 ± 6

The experiments were performed during a noradrenaline ($30\ \mu\text{M}$)-induced contraction and in the presence of $0.3\ \mu\text{M}$ atropine. Results are shown as mean $\pm$ s.e.mean for 5-7 experiments and are expressed as a percentage of the control values. These control relaxations (expressed as percentage decrease of the noradrenaline-induced contraction) are in the ileum: GABA $67 \pm 7\%$, homotaurine $51 \pm 5\%$, nitroglycerin $64 \pm 5\%$; in the ileocolonic junction: GABA $56 \pm 4\%$, homotaurine $46 \pm 5\%$, nitroglycerin $62 \pm 8\%$.

* $P < 0.05$, significantly different from control, Student's t test for paired observations.

Figure 3 Typical example of the relaxant effects of electrical stimulation (2–16 Hz, 1–2 ms), acetylcholine (ACh, 30 μ M), GABA (0.1 mM) and nitroglycerin (NTG, 1 μ M) on circular muscle strips of the dog ileocolonic junction. GABA desensitization abolished and bicuculline (10 μ M) reduced the relaxation to GABA, but had no effect on the relaxations to electrical stimulation and acetylcholine. Tetrodotoxin (TTX) abolished all relaxations, except those to nitroglycerin. The experiments were performed during a noradrenaline (NA, 30 μ M)-induced contraction and in the presence of 0.3 μ M atropine. Similar results were obtained in five additional experiments in both the terminal ileum and ileocolonic junction.

Discussion

The present results demonstrate that the effects of GABA on the canine terminal ileum and ileocolonic junction are due to activation of GABA_A receptors; GABA-induced relaxations were antagonized by bicuculline and mimicked by the GABA_A agonist homotaurine, but not by the GABA_B agonist ($\pm$)-baclofen. In addition, the concentration-response curves for GABA and homotaurine were parallel, reached the same maximum and were subject to cross desensitization between GABA and homotaurine. Preincubation with ($\pm$)-baclofen had no effect on the GABA- and homotaurine-induced relaxations. The relaxations to GABA were abolished by TTX, but resistant to muscarinic and adrenoceptor antagonists, suggesting that GABA stimulates GABA_A receptors on NANC inhibitory neurones.

These findings are in agreement with the observations in the guinea-pig and rabbit distal colon (Krantis *et al.*, 1980; Giotti *et al.*, 1985; Tonini *et al.*, 1989) and in the rat duodenum (Maggi *et al.*, 1984): GABA activates GABA_A receptors resulting in NANC-induced relaxations. In contrast, stimulation of the GABA_A receptors in the guinea-pig ileum (Krantis *et al.*, 1980; Kaplita *et al.*, 1982; Giotti *et al.*, 1983) and in the cat colon (Taniyama *et al.*, 1987) triggered cholinergic post-ganglionic neurones which resulted in a contractile response.

Inhibitory responses to GABA have also been attributed to GABA_B receptor activation (Kaplita *et al.*, 1982; Ong & Kerr, 1983; Giotti *et al.*, 1983; 1985; Tonini *et al.*, 1989). In our experiments however, the GABA_B agonist ($\pm$)-baclofen had no effect on the spontaneous activity of the preparations and did not induce relaxations when the muscle tension was raised with noradrenaline. It should be emphasised however that in the guinea-pig and rabbit intestine, GABA_B-mediated relax-

ation results from a reduction of resting and electrically-stimulated cholinergic nerve activity (Kaplita *et al.*, 1982; Giotti *et al.*, 1983; 1985; Ong & Kerr, 1983; Tonini *et al.*, 1989). Since our experiments were performed in the presence of atropine and since neither atropine nor TTX reduces the tone or spontaneous activity of the dog terminal ileum and ileocolonic junction, the failure to detect GABA_B receptor-mediated effects can be explained by the absence of cholinergic tone in our preparations.

In previous experiments, we demonstrated the presence of an inhibitory NANC innervation in the canine terminal ileum and ileocolonic junction; acetylcholine and the nicotinic receptor agonist 1,1-dimethyl-phenylpiperazinium induced relaxations via the stimulation of nicotinic receptors located on inhibitory NANC neurones (Pelckmans *et al.*, 1989; Boeckxstaens *et al.*, 1990b). These neurones could also be activated by electrical stimulation resulting in the release of an unknown inhibitory neurotransmitter, which is not vasoactive intestinal polypeptide, an opioid (Pelckmans *et al.*, 1989), 5-hydroxytryptamine (Boeckxstaens *et al.*, 1990a), or ATP (Boeckxstaens *et al.*, 1990b). The NANC-induced relaxations in response to electrical stimulation and acetylcholine could also be mimicked by GABA but were not affected by bicuculline and desensitization to GABA or baclofen. These experiments, therefore, provide evidence against the involvement of GABA as the final NANC neurotransmitter in dog terminal ileum and ileocolonic junction. However, the finding that TTX abolishes the relaxation to GABA suggests a neuronal involvement. GABA may, therefore, stimulate NANC inhibitory neurones to release an as yet unidentified neurotransmitter. Since ATP desensitization had no effect on GABA-evoked relaxations, it is unlikely that this neurotransmitter is ATP, contrasting with observations in the rat duodenum (Maggi *et al.*, 1984; Manzini *et al.*, 1985).

In conclusion, our results indicate that GABA produces a transient relaxation of the dog terminal ileum and ileocolonic junction through a GABA_A receptor-mediated activation of inhibitory NANC neurones. This finding adds further evidence for the presence of a NANC inhibitory innervation in this region of the canine gut. In addition, we provide evidence against GABA as the final NANC neurotransmitter. The nature of the latter remains to be determined.

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