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RESEARCH PAPER

The role of $GABA_A$ receptors in the control of transient lower oesophageal sphincter relaxations in the dog

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Background and purpose: Transient lower oesophageal sphincter relaxations (TLESRs) are triggered by activation of mechanosensitive gastric vagal afferents and are the major cause of gastroesophageal reflux and therefore an important target for therapeutic intervention in gastroesophageal reflux disease (GERD). Activation of the metabotropic $GABA_B$ receptor has shown to inhibit TLESRs. The aim of the present study was to assess the role of the ionotropic $GABA_A$ receptor in the regulation of TLESRs.
Experimental approach: TLESRs were quantified using Dentsleeve manometry in dogs, and $GABA_A$ agonists were given i.v. prior to gastric distension. Immunohistochemistry and RT-PCR were used to localize $GABA_A$ receptors in the dog nodose ganglion, the source of vagal afferents which initiate TLESRs.
Key results: The prototypical $GABA_A$ agonist muscimol produced a dose-dependent inhibition of TLESRs ranging from 19 to 56%. The two other $GABA_A$ agonists evaluated, isoguvacine and 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridin-3-ol (THIP), as well as the $GABA_A$ positive allosteric modulator diazepam, had no major effects on TLESRs. Evaluation of higher doses was limited by emesis (THIP and isoguvacine) or restlessness/sedation (diazepam). Of the predominant $GABA_A$ receptor subunits (α, β and γ components), α and β but not γ were detected in the dog nodose ganglion by RT-PCR, while immunohistochemistry in addition demonstrated nerve fibres expressing the γ subunit.
Conclusions and implications: The present observations demonstrate that $GABA_A$ receptors exert an inhibitory control of TLESRs. These results warrant further studies using $GABA_A$ isoform-selective agonists to define the identity of receptors involved.
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Keywords: gastroesophageal reflux disease; $GABA_A$; muscimol; TLESR; dogs

Abbreviations: GERD, gastroesophageal reflux disease; LES, lower oesophageal sphincter; PBS, phosphate-buffered saline; THIP, 4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridine-3-ol; TLESR, transient lower oesophageal sphincter relaxation

Introduction

Gastroesophageal reflux disease is one of the most common disorders of the gastrointestinal tract and is characterized by symptoms of heartburn, regurgitation and retrosternal pain (McDougall *et al*., 1998; Valle *et al*., 1999). Transient lower oesophageal sphincter relaxation (TLESR) is the major mechanism underlying gastroesophageal reflux, both in healthy volunteers and in gastroesophageal reflux disease (GERD) patients (Holloway, 2000). Pharmacological inhibition of TLESRs is therefore a potential target for the treatment of GERD. TLESRs are triggered by gastric distension, leading to a vagally mediated reflex pathway involving mechanosensitive gastric vagal afferents, integrative brainstem centres and vagal efferents to the lower oesophageal sphincter (LES) (Mittal *et al*., 1995).

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the central nervous system, acting through either ionotropic $GABA_A$ and $GABA_C$ or metabotropic $GABA_B$ receptors. The $GABA_{A/C}$ receptors are ligand-gated ion channels, which induce rapid synaptic inhibition upon activation. This contrasts to the $GABA_B$ receptor, which couples to G-proteins and produces longer

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lasting inhibitory signals (Berthele *et al*., 2001). Inhibitory GABA$_B$ receptors are present on gastric mechanoreceptors (Smid *et al*., 2001) and on vagal afferent terminals in the dorsal medulla, and have shown to inhibit transmitter release in vagal nuclei (Brooks *et al*., 1992). Previous studies showed that the GABA$_B$-receptor agonist baclofen significantly reduces the rate of TLESRs in dogs, ferrets and humans (Blackshaw *et al*., 1999; Lehmann *et al*., 1999; Lidums *et al*., 2000; Zhang *et al*., 2002). Baclofen also reduces the number of reflux episodes and reflux symptoms in GERD patients (Ciccaglione and Marzio, 2003; Koek *et al*., 2003; Vela *et al*., 2003). However, the side-effect profile of baclofen makes it less attractive for clinical use. It is unknown if ionotropic GABA$_A$ receptors play a role similar to that of GABA$_B$ receptors.

γ-Aminobutyric acid-A receptors are also widely expressed in the central and peripheral nervous systems, and mediate fast postsynaptic inhibition. The GABA$_A$ receptor complex is a pentameric assembly of subunits forming a chloride channel and, depending on subunit configuration, benzodiazepine, barbiturate and neuroactive steroid sites (Mehta and Ticku, 1999). The various subunits of the GABA$_A$ receptors are α1–α6, β1–β3, γ1–γ3 and δ (Macdonald and Olsen, 1994). Each GABA$_A$ receptor consists of a combination of various subunits. Coexpression of α-, β- and γ-subunits is required for the formation of a fully functional GABA$_A$ receptor (Saha *et al*., 2001). GABA and other directly acting GABA$_A$-receptor agonists bind specifically to a recognition site located between an α and a β subunit, whereas the benzodiazepine ligands bind to an allosteric site located between an α- and a γ-subunit (Ebert *et al*., 1994; Krogsgaard-Larsen *et al*., 2004). However, for the formation of the GABA$_A$ receptor, colocalization of these three types of subunits is not an absolute requirement. It is known that mice lacking the γ2-subunit were able to express functional GABA$_A$ receptors, but were lacking the benzodiazepine-binding site (Gunther *et al*., 1995). Other studies on recombinant GABA$_A$ receptors also showed that the combination of the α1- and β2-subunits produces functional GABA$_A$ receptors, but the γ2-subunit is required to express benzodiazepine binding (Pritchett *et al*., 1989; Pregenzer *et al*., 1993). Positive GABA$_A$ modulators, like benzodiazepines, facilitate GABA-mediated Cl^- flux and have sedative, anxiolytic and anticonvulsant effects.

γ-Aminobutyric acid-A agonism has been found to excite (Zagorodnyuk *et al*., 2002), inhibit (Yuan *et al*., 1998) or have no effect (Smid *et al*., 2001) on vagal afferents, the key initiators of TLESRs. Whether these contradictory results are due to species or methodological differences is unknown, but they warrant studies on the effects of peripherally restricted agonists on TLESRs. On the other hand, GABA$_A$ receptors mediate inhibition in the dorsal vagal complex, the central relay station translating afferent signalling into efferent firing producing TLESRs. It can therefore be speculated that central GABA$_A$ agonism may modify the peripheral actions of GABA$_A$ agonists.

These hypotheses were tested by assessing the potential for peripheral agonistic effects by determining GABA$_A$ receptor subunit expression in the dog nodose ganglion (NG), the origin of vagal afferents. Furthermore, to characterize peripheral and central involvement of GABA$_A$ receptors in the regulation of TLESRs in dog, the effects of two centrally acting GABA$_A$ agonists, muscimol and THIP (4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridine-3-ol), and the non-selective, peripherally acting agonist isoguvacine, as well as the positive GABA$_A$ modulator diazepam were studied. The selection of a benzodiazepine and THIP was based on the finding that the former selectively enhances the function of synaptic α1β2γ2S GABA$_A$ receptors, whereas the latter preferentially activates extrasynaptic α4β3δ GABA$_A$ receptors (Krogsgaard-Larsen *et al*., 2004).

Materials and methods

Animals

All animal procedures were approved by the Ethical Committee for Animal Experiments of the Göteborg region. Adult male and female Labrador retrievers were used in the experiments. Cervical oesophagostomies were made and the dogs were accustomed to rest in a Pavlov stand after recovery from surgery. Before each experiment, the dogs were fasted overnight, with free access to water. A washout of at least 3 days was allowed between experiments.

Protocol for in vivo *experiments*

The dogs were intubated with a water-perfused Dentsleeve multilumen catheter to record gastric, LES and oesophageal pressures. An antimony pH electrode was placed 3 cm above the lower oesophageal sphincter to measure acid reflux episodes, and a water-perfused catheter was placed in the hypopharynx to measure swallows.

Transient lower oesophageal sphincter relaxations were stimulated by infusion into the stomach through the central lumen of the assembly of a acidified liquid nutrient (30 ml kg^{-1}), followed by air insufflation (100 ml min^{-1}) to maintain gastric pressure between 9 and 11 mm Hg.

The number of TLESRs was measured during a 45-min period starting from the infusion of the liquid. TLESRs were defined by a rapid decrease of LES pressure (>1 mm Hg s^{-1}) to a value <2 mm Hg above gastric pressure and a duration of >1 s, without any pharyngeal signal, <2 s before onset (Lehmann *et al*., 1999).

Muscimol, isoguvacine and diazepam were administered as intravenous boluses (muscimol and isoguvacine 0.5 ml kg^{-1}; diazepam 1 mg kg^{-1}, containing 5 mg ml^{-1}) 10 min before start of the experiment. THIP was administered according to an infusion protocol of 1 ml kg^{-1} infused over 30 min. The infusion started 15 min before the infusion of liquid into the stomach. Except for TLESRs and acid-reflux episodes, basal LES pressure, duration of TLESR, latency time from start to first TLESR and swallowing rate were determined. Basal LES pressure was defined as the average pressure between the sleeve and the intragastric pressure during the 45-min period. All swallow- and TLESR-related LES pressure changes were excluded.

Acid exposure was expressed as the percentage of the 45-min period during which oesophageal pH was <4. Reflux

episodes were defined as a drop in pH >1 unit within 5 s. The average pH in the 15 s following nadir pH should be <4.

Immunohistochemistry

Primary antibodies. Various antibodies used against different GABAA receptor subunits were as follows: mouse monoclonal anti-$GABA_A$-α (Biosite AB, Täby, Sweden), mouse monoclonal anti-$GABA_A$-α1 (Chemicon International Inc. and Boehringer, Ingelheim, Germany), goat polyclonal anti-$GABA_A$-α2 and anti-$GABA_A$-α3 (Santa Cruz Biotechnology Inc.), mouse monoclonal anti-$GABA_A$-β (Chemicon International Inc., Temecula, California, USA) and rabbit polyclonal anti-$GABA_A$-γ2 (Chemicon International Inc.).

Immunolabelling procedure. Fresh dog brain tissue and dog nodose ganglia were harvested and fixed in formaldehyde (formalin 10%) for at least 12 h. The tissue was dehydrated in increasing concentrations of alcohol and xylene, and then embedded in paraffin and sectioned at 4 μm using a microtome (Leica Microsystems, Wetzlar, Germany). Tissue sections were deparaffinized and rehydrated through xylene, graded ethanol series, distilled water and phosphate-buffered saline (PBS). Different pretreatments tried as antigen retriever were as follows: no pretreatment, trypsin, high-pressure boiling with Diva or Borg solution in a Decloaker (Biocare Medical, CA, USA) or microwave boiling with a citrate buffer solution (0.01 M, pH 6). Sections were then incubated for 5 min in 3% hydrogen peroxidase (Merck, Darmstadt, Germany) in distilled water, washed twice in PBS and preincubated with 10% normal donkey serum in PBS for 20 min at room temperature, and then incubated with primary antibodies to one of the $GABA_A$ receptor subunits. The antibodies were diluted in ChemMate antibody diluent (Dako Cytomation, Glostrup, Denmark) 1:25 (α and γ) and 1:75 (β), and incubations were performed overnight at room temperature. The sections were washed three time in PBS and then incubated in biotinylated donkey anti-rabbit, anti-mouse or anti-goat IgG (Jackson Immunoresearch, West Grove, PA, USA; dilution 1:500) for 1 h at room temperature. After washing the sections three times in PBS, the avidin–biotin complex (ABC) solution (Vectastain Elite Standard kit; Vector Laboratories, Burlingame, CA, USA) was applied for 45 min at room temperature. Following rinsing in PBS, immunoreactivity was visualized using 3-amino-9-ethylcarbazole for 10–20 min at room temperature. Finally, sections were counterstained with Mayer's haematoxylin and coverslipped using a water-based mounting media (Quick Mount, Daido Sangyo, Japan).

Negative controls included sections that were incubated in the presence of negative control immunoglobulin fraction from non-immunized rabbits or goats in the same dilution as the primary antibodies, or as antigen–antibody preabsorption experiments with the native antigen preincubated at 4 °C for 24 h with the diluted antibody solution. Dog cerebellum was used as positive control.

Images were captured with the image programme Picsara (Euromed Networks, Stockholm, Sweden) from the light microscope, using a Sony 3CCD video camera. The images were imported into Adobe Photoshop or Microsoft Photo Editor for minor adjustments of brightness, contrast and sharpness.

Table 1 Primers used in PCR experiments

Subunit	*Primer sequence*	*Primer length*
α1f	5′-AATTTGGCCAGGGGTGAC-3′	18
α1r	5′-GAAAGCTATTCTTGACAGTCGGTC-3′	24
α1 probe	5′-TGGCTTAGCCACGATTGCTAAAAGTGC-3′	27
β2f	5′-GATGTGGAGAGTCCGGAAAAAG-3′	22
β2r	5′-CTTTCAGGAGTCTATCCACTGTCTCTT-3′	27
β2 probe	5′-TGTGCGCAGAGTGTCAATGACCCTAGTAA-3′	29
β3f	5′-CAAAGAATGACCGTTCCAAG-3′	20
β3r	5′-TGAGTTGTCAAAGGGTCGTG-3′	20
β3 probe	5′-CATGAGCATCCACCCGATTGC-3′	21
γ1f	5′-CAACAAACTTCGCCCAGATA-3′	20
γ1r	5′-TTTAAACGACTGTCAAACCAGG-3′	22
γ1 probe	5′-TGGGATCAACTGGTCCAATGCTG-3′	23

f = forward; PCR = polymerase chain reaction; r = reverse.

Reverse transcription-PCR

Nodose ganglion and cerebellum (positive control) were taken from the same dog. RNA was prepared (TRIzol reagent; Invitrogen, Carlsbad, California, USA) and reverse transcribed into cDNA using oligo(dT) and random primers (iScript cDNA Synthesis kit; BioRad, Hercules, California, USA). The cDNA was amplified by reverse transcription-PCR (RT-PCR) (Taqman Universal PCR Master Mix; Applied Biosystems, Foster City, California, USA) using primer pairs and probes for $GABA_A$ receptor subunits α1, β2, β3 and γ1 (Eurogentec SA, Seraing, Belgium). All primer sequences are listed in Table 1. Real-time PCR was performed using a 7500Real Time PCR System (Applied Biosystems). Product specificity of the PCR products was confirmed by agarose gel electrophoresis.

Data analysis

Each dog served as its own control. All variables were calculated on the basis of the mean of five preceding control experiments. Data are presented as mean ± s.e.mean. Statistical analysis was performed using paired Student's *t*-tests. A *P*-value <0.05 was considered statistically significant.

Drugs

All compounds were from Tocris, Bristol, UK, and were dissolved in physiological saline (0.9% NaCl), except for diazepam, which was from Dumex-Alpharma, Copenhagen, Denmark, and was delivered as a solution, which was directly used at a dose of 3.5 μmol kg^{-1} (1 mg kg^{-1}). The doses of muscimol, which were tested, were 8.2 μmol kg^{-1} (1 mg kg^{-1}), 0.82 μmol kg^{-1} (0.1 mg kg^{-1}), 0.27 μmol kg^{-1} (0.033 mg kg^{-1}) and 0.082 μmol kg^{-1} (0.01 mg kg^{-1}). A dose of 8.2 μmol kg^{-1} (1.3 mg kg^{-1}) and 5.7 μmol kg^{-1} (1 mg kg^{-1}) was used for isoguvacine and THIP, respectively.

Results

In vivo *experiments*

TLESRs. The $GABA_A$ agonist muscimol produced a dose-dependent inhibition of TLESRs over the range of doses used (Figure 1; $n = 6$ for all doses). Surprisingly, at the lowest and

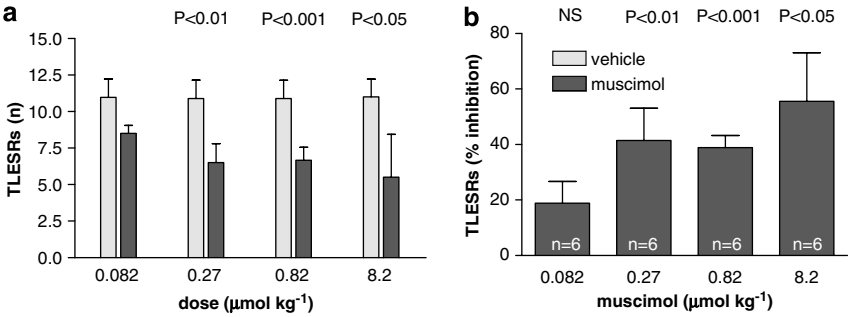

Figure 1 Dose-dependent effect of muscimol on the occurrence of TLESRs. The effect is expressed in mean values (**a**) and as percent inhibition of control (**b**). The number of TLESRs was significantly reduced at all doses except the lowest dose of muscimol. *P*-values are shown above each set of experiments (paired Student's *t*-test; $n=6$ for each dose). TLESR, transient lower oesophageal sphincter relaxation.

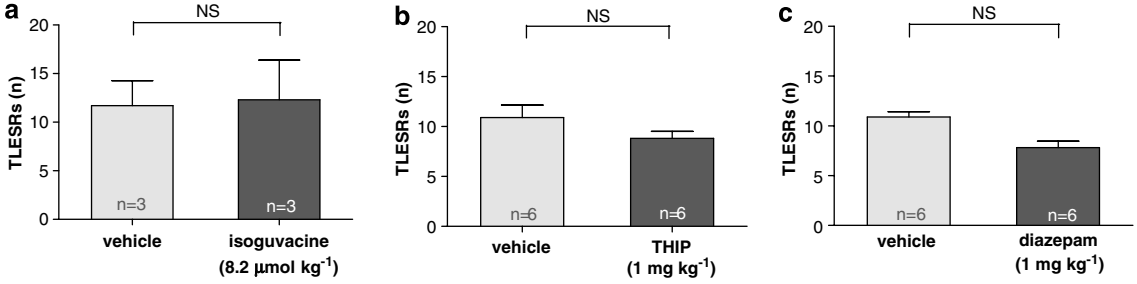

Figure 2 Isoguvacine (**a**), THIP (**b**) and diazepam (**c**) had no significant effect on the number of TLESRs. THIP, 4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridine-3-ol; TLESR, transient lower oesophageal sphincter relaxation.

highest dose of muscimol no side effects occurred, whereas at the intermediate doses emesis occurred just after drug administration. No sedative effects were seen after muscimol. The two other GABA$_A$ agonists evaluated, isoguvacine (8.2 μmol kg^{-1}) and THIP (5.7 μmol kg^{-1}), as well as the GABA$_A$-positive allosteric modulator, diazepam (3.5 μmol kg^{-1}), had no major effect on TLESRs at the doses tested (isoguvacine, 0 ± 12% change; THIP, 15 ± 9% inhibition; diazepam, 29 ± 12% inhibition; Figure 2), and were limited by emesis (isoguvacine and THIP) or restlessness and sedation (diazepam).

Basal LES pressure. Basal LES pressure was variably affected by muscimol (Figure 3), reaching statistical significance only after one intermediate dose (0.82 μmol kg^{-1}; $P<0.04$). The other compounds showed no significant effect on basal LES pressure.

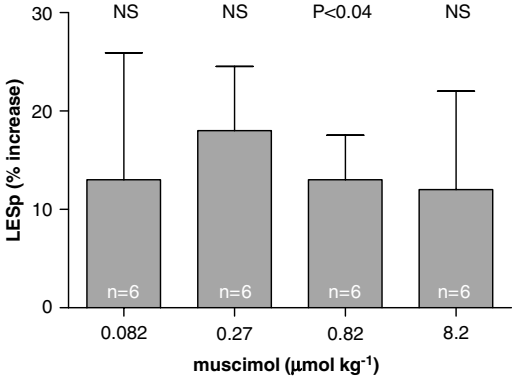

Figure 3 Effect of muscimol on basal LES pressure (LESp). The effects were small and highly variable; only at 0.82 μmol kg^{-1} was there a significant increase in LESp ($P<0.04$; paired Student's *t*-test; $n=6$ for each dose). LES, lower oesophageal sphincter.

Reflux and other parameters. Reflux episodes were significantly reduced by 25 ± 8.8% after 0.27 μmol kg^{-1} muscimol (controls 3.2 ± 0.37; muscimol 0.27 μmol kg^{-1} 2.4 ± 0.4; $P<0.03$). The other doses of muscimol, isoguvacine, THIP and diazepam showed no significant effect on reflux episodes (data not shown). Swallowing rate and latency time to the first TLESR were not significantly affected by any of the tested compounds (data not shown).

Immunohistochemistry

We were unable to detect any immunoreactivity, in either NG or cerebellum, with the GABA$_A$-α and -α1-specific antibodies. Immunoreactivity of the GABA$_A$-α2 and -α3 subunits was observed scattered throughout the dog NG, where it was localized in the cell bodies (Figure 4). The immunoreactivity in the cerebellum (positive control tissue) of GABA$_A$-α2 and -α3 was found in the granular cell layer and in the fibres of the white matter. The β-subunit was intensely found in the granular cell layer of the cerebellum, but not in the NG. GABA$_A$-γ2 immunoreactivity in the NG was confined to fine varicose fibres surrounding the somata. This staining was not seen using the IgG-negative controls (Figure 5). In the cerebellum, weak γ2 immunoreactivity was found in the Purkinje cells and some staining was seen in the endothelial cells of the brain and NG.

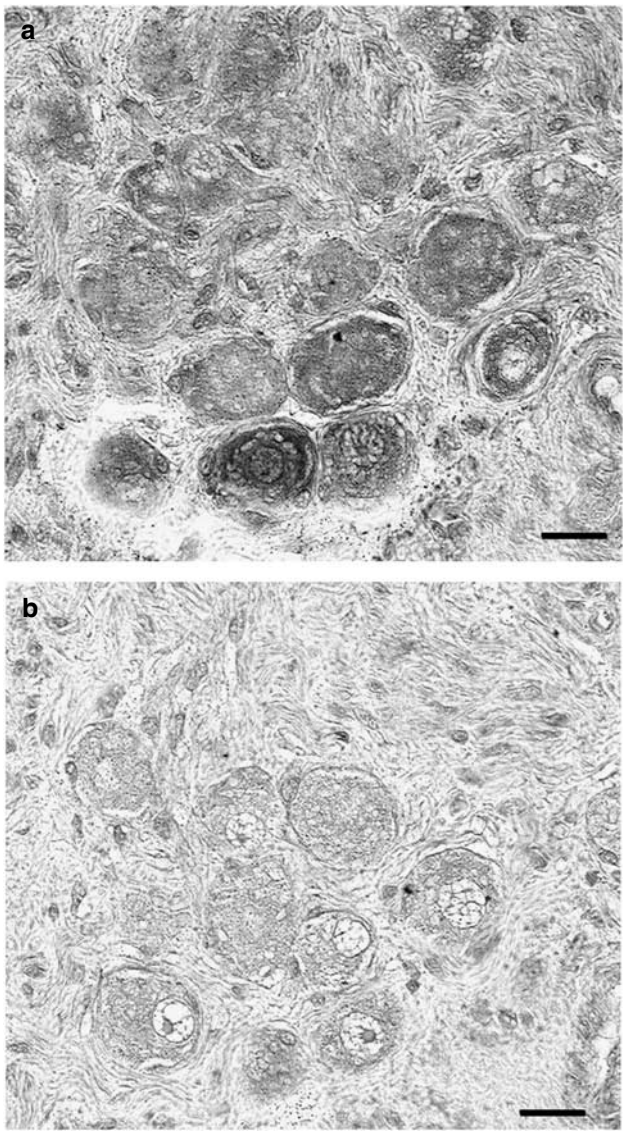

Figure 4 (**a**) Photomicrograph showing the dog NG, with cell bodies staining positively for $GABA_A$-receptor α3-subunits. (**b**) Preabsorption control experiments for $GABA_A$ α3-subunits. Scale bar = 50 μm. $GABA_A$, γ-aminobutyric acid-A; NG, nodose ganglion.

Reverse transcription-PCR

γ-Aminobutyric acid-A subunits α1, β2 and β3, but not γ1, were detected in the dog NG by PCR analysis. mRNA expression of these $GABA_A$ subunits in the dog cerebellum was used as positive control (Figure 6).

Discussion

In the present study, we investigated the effects of $GABA_A$ agonists on the occurrence of TLESRs and the expression of $GABA_A$ subunit receptors in the NG of the dog. This study showed a significant and dose-dependent inhibition, up to 56%, of TLESRs by the centrally acting $GABA_A$ agonist muscimol in the dog. This suggests that the use of $GABA_A$-receptor agonists may be a novel strategy in the treatment of GERD. However, muscimol was less effective in reducing TLESRs than the $GABA_B$-receptor agonist baclofen, which previously showed an up to 90% reduction of postprandial TLESRs in dogs (Lehmann *et al*., 2002). The maximal effect of baclofen has been reproduced in several independent studies in our laboratory (Lehmann *et al*., unpublished observations), and so it was not deemed necessary to include a separate baclofen dose group in the current study. If the difference between muscimol and baclofen in terms of efficacy can be generalized to their respective targets, it appears that $GABA_B$-receptor agonists would offer a more attractive option than $GABA_A$-receptor agonists in the quest for novel drugs for treatment of GERD.

Muscimol has been reported to inhibit TLESRs in ferrets, but only at a dose ($10\,\mu mol\,kg^{-1}$), which induced sedation (Blackshaw *et al*., 1999). In the current experiments, reduction of TLESRs could not be secondary to sedation since this side effect was not seen at the doses used. In contrast to the effect of muscimol, the less potent $GABA_A$ agonist THIP (Kemp *et al*., 1986) and the peripherally restricted $GABA_A$ agonist isoguvacine (Krogsgaard-Larsen *et al*., 1981) showed no effect on the occurrence of TLESRs. The emetogenic effects of both THIP and isoguvacine limited further studies with higher doses. THIP has been administered in baboons at a dose ranging from 0.25 to $8.0\,mg\,kg^{-1}$, intravenously (Meldrum and Horton, 1980). However, in our study, emesis occurred at doses higher than $1\,mg\,kg^{-1}$, even in the absence of intubation and administration of a high gastric load. THIP has potent anxiolytic and analgesic properties in man, but is known to have approximately 8–10 times lower affinity for $GABA_A$ receptors compared with muscimol (Meldrum and Horton, 1980; Huckle, 2004; Vyazovskiy *et al*., 2005). Isoguvacine has poor penetration into the CNS (Rode *et al*., 2005) and acts as a peripherally restricted $GABA_A$ agonist (Krogsgaard-Larsen *et al*., 1981). However, the hypothesis that a peripherally restricted agonist would augment or reduce the number of TLESRs by activating or inhibiting vagal afferents could not be confirmed in our study. This may suggest that a central but not peripheral site of action of $GABA_A$ agonists plays a critical role in the control of TLESRs. Although there was a tendency towards inhibition of TLESRs, the positive $GABA_A$ modulator diazepam had no statistically significant effect. In analogy with mice devoid of the γ2-subunit, the lack of γ2-subunit expression in vagal afferents in our study may render them insensitive to benzodiazepines (Gunther *et al*., 1995; Mehta and Ticku, 1999), and therefore an effect of diazepam on the rate of TLESRs through binding to vagal afferent terminals might be not anticipated.

In addition, general sedation is thought to reduce the number of TLESRs (Dent *et al*., 1980; Cox *et al*., 1988). In our study, however, sedation occurred with diazepam, but no significant reduction in TLESRs was seen. It should be noted that the onset of sedation in the dogs followed a period of restless behaviour, and any effect of sedation on TLESRs could be masked. However, TLESRs were seen throughout the total study time after diazepam, and they peaked during the first 20 min as in control experiments. It would be important to assess whether there is a $GABA_A$ tone controlling TLESR by using receptor antagonists. However,

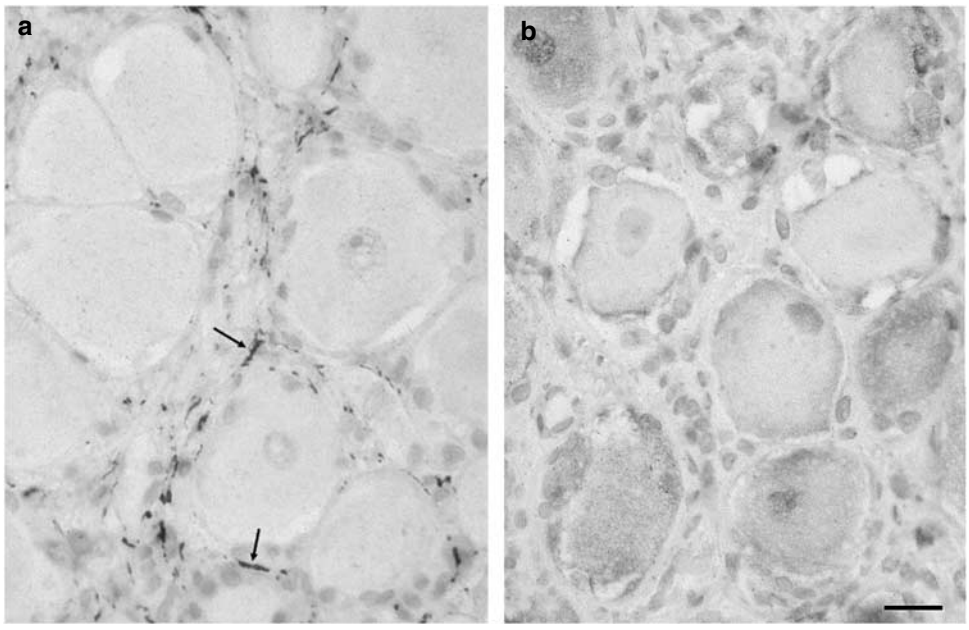

Figure 5 (**a**) Photomicrograph showing the dog NG, with fibres staining positively for GABA$_A$-receptor γ2-subunits (arrows). (**b**) IgG control experiment for GABA$_A$ γ2-subunits. Scale bar = 20 μm. GABA$_A$, γ-aminobutyric acid-A; NG, nodose ganglion.

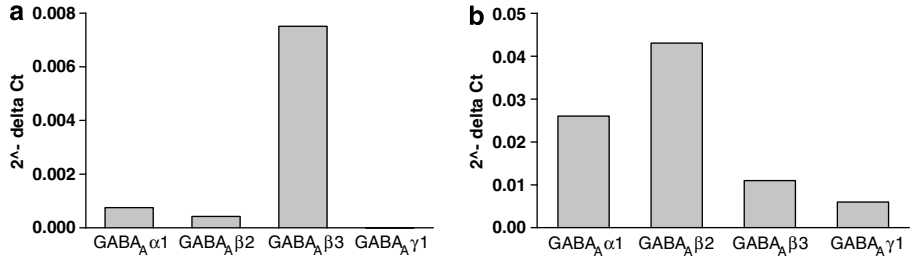

Figure 6 Results of RT-PCR, with the different GABA$_A$ receptor subunits expressed in dog NG (**a**) and cerebellum as positive control (**b**). There is abundant expression of β3-subunits, but lack of γ1-subunit expression in the dog NG. GABA$_A$, γ-aminobutyric acid-A; NG, nodose ganglion; RT-PCR, reverse transcription-PCR.

GABA$_A$-receptor antagonists can trigger epileptic seizures and ethical considerations prevent such experiments in dogs.

Muscimol has no effect on basal LES pressure in ferrets (Blackshaw *et al*., 1999), but it slightly increased LES pressure in dogs. This effect is likely to be mediated in the hindbrain, since muscimol has been shown to elevate basal LES pressure in cats upon hindbrain microinjection (Washabau *et al*., 1995). In the management of GERD, stimulation of LES pressure is considered to have a beneficial effect in patients who display long periods of low or no LES pressure, which possibly promote reflux. There were discrepancies between the effect of muscimol on TLESRs and acid reflux episodes, since the two highest doses significantly attenuated TLESRs but not reflux. However, this difference is probably more apparent than real, as the number of reflux episodes was quite low and changes were therefore difficult to identify. Also, the pHmetric method used failed to detect superimposed reflux,that is, reflux occurring shortly after a preceding reflux episode, which already has acidified the distal oesophagus.

The distribution of various GABA$_A$ subunits in the NG has to our knowledge not been described before in any species, including the dog. Interestingly, GABA itself has been detected immunocytochemically in the feline NG (Stoyanova, 2004). Its function there, if any, is obscure. Immunohistochemical studies on GABA$_A$ subunits in peripheral ganglia have been performed in a few cases. For instance, *in situ* hybridization showed expression of α2, β2 and γ2 in dorsal root ganglia of rats (Persohn *et al*., 1991) and all GABA$_A$ receptors subunits were detected in rat spinal ganglia by immunohistochemistry (Yamamoto *et al*., 2002). Although the present study focused on another peripheral ganglion, the NG, published data support the presence of GABA$_A$-receptor subunits in peripheral ganglion cells. We showed immunoreactivity with the α2- and α3-subunits in the cell bodies of the dog NG, and with γ2 in pericellular fibres. In line with this, additional RT-PCR showed α1, β2 and β3, but no γ1-subunit expression in the dog NG. We were unable to detect any immunoreaction with the α- and α1-specific antibodies in our paraffin-embedded material. This is surprising as the predominant GABA$_A$-receptor subunit combination throughout the brain is composed of α1β2/3γ2 (Fritschy and Mohler, 1995; Mohler *et al*., 1995; Nusser *et al*., 1995). The fact that most other studies have been

performed on frozen material could explain this difference in observed immunoreactivity. Presence of the α1-subunit was nevertheless indicated by RT-PCR, and we can therefore speculate that several α-subunits are present in the NG along with β-subunits, especially β-3, according to the RT-PCR experiments. However, our immunohistochemical studies did not demonstrate convincing β2 staining in the NG. A possible explanation for this could be the fact that the antibodies used were not directed against dog GABA$_A$. An interesting finding is the immunohistochemical localization of the γ2-subunit in pericellular nerve fibres rather than in the ganglion cells of the NG. Previous studies have demonstrated the presence of such pericellular fibres in the NG of the monkey, rabbit and pigeon (Katz and Karten, 1980; Ling *et al.*, 1992). Also GABA$_B$ receptor-1a immunoreactivity has been found on fibres surrounding the nerve cell bodies in guinea pig NG (Zagorodnyuk *et al.*, 2002).

In conclusion, the present study revealed the expression of GABA$_A$ receptor subunits in the dog NG. In addition, we showed the involvement of GABA$_A$ receptors in the control of TLESRs. The GABA$_A$-receptor agonist muscimol reduced the rate of TLESRs by 19–56% depending on dose. The potential of GABA$_A$ agonists as inhibitors of TLESRs, and therefore as future anti-reflux agents, depends on their therapeutic margin. Whereas the currently available GABA$_A$-stimulating drugs registered for other indications carry a side-effect profile not compatible with their use in GERD, emerging GABA$_A$ subunit-selective compounds (Basile *et al.*, 2004) may be useful in this context. Therefore, further studies are warranted in which the effects of GABA$_A$ subtype-selective compounds on TLESRs are assessed.

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Conflict of interest

ACJR, KC, SP, MA, LB, JJ and AL are all full-time employees of AstraZeneca. GEB has participated as an investigator in clinical studies sponsored by AstraZeneca.

References

Basile AS, Lippa AS, Skolnick P (2004). Anxioselective anxiolytics: can less be more? *EurJ Pharmacol* **500**: 441–451.

Berthele A, Platzer S, Weis S, Conrad B, Tolle TR (2001). Expression of GABA(B1) and GABA(B2) mRNA in the human brain. *Neuroreport* **12**: 3269–3275.

Blackshaw LA, Staunton E, Lehmann A, Dent J (1999). Inhibition of transient LES relaxations and reflux in ferrets by GABA receptor agonists. *Am J Physiol* **277**: G867–G874.

Brooks PA, Glaum SR, Miller RJ, Spyer KM (1992). The actions of baclofen on neurones and synaptic transmission in the nucleus tractus solitarii of the rat *in vitro*. *J Physiol* **457**: 115–129.

Ciccaglione AF, Marzio L (2003). Effect of acute and chronic administration of the GABA B agonist baclofen on 24 h pH metry and symptoms in control subjects and in patients with gastro-oesophageal reflux disease. *Gut* **52**: 464–470.

Cox MR, Martin CJ, Dent J, Westmore M (1988). Effect of general anaesthesia on transient lower oesophageal sphincter relaxations in the dog. *Aust N Z J Surg* **58**: 825–830.

Dent J, Dodds WJ, Friedman RH, Sekiguchi T, Hogan WJ, Arndorfer RC *et al.* (1980). Mechanism of gastroesophageal reflux in recumbent asymptomatic human subjects. *J Clin Invest* **65**: 256–267.

Ebert B, Wafford KA, Whiting PJ, Krogsgaard-Larsen P, Kemp JA (1994). Molecular pharmacology of gamma-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different alpha, beta, and gamma receptor subunit combinations. *Mol Pharmacol* **46**: 957–963.

Fritschy JM, Mohler H (1995). GABAA-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* **359**: 154–194.

Gunther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F *et al.* (1995). Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **92**: 7749–7753.

Holloway RH (2000). The anti-reflux barrier and mechanisms of gastro-oesophageal reflux. *Baillieres Best Pract Res Clin Gastroenterol* **14**: 681–699.

Huckle R (2004). Gaboxadol. Lundbeck/Merck. *Curr Opin Investig Drugs* **5**: 766–773.

Katz DM, Karten HJ (1980). Substance P in the vagal sensory ganglia: localization in cell bodies and pericellular arborizations. *J Comp Neurol* **193**: 549–564.

Kemp JA, Marshall GR, Woodruff GN (1986). Quantitative evaluation of the potencies of GABA-receptor agonists and antagonists using the rat hippocampal slice preparation. *Br J Pharmacol* **87**: 677–684.

Koek GH, Sifrim D, Lerut T, Janssens J, Tack J (2003). Effect of the GABA(B) agonist baclofen in patients with symptoms and duodeno-gastro-oesophageal reflux refractory to proton pump inhibitors. *Gut* **52**: 1397–1402.

Krogsgaard-Larsen P, Frolund B, Liljefors T, Ebert B (2004). GABA(A) agonists and partial agonists: THIP (gaboxadol) as a non-opioid analgesic and a novel type of hypnotic. *Biochem Pharmacol* **68**: 1573–1580.

Krogsgaard-Larsen P, Schultz B, Mikkelsen H, Aaes-Jorgensen T, Bogeso KP (1981). THIP, isoguvacine, isoguvacine oxide, and related GABA agonists. *Adv Biochem Psychopharmacol* **29**: 69–76.

Lehmann A, Antonsson M, Bremner-Danielsen M, Flärdh M, Hansson-Bränden L, Kärrberg L (1999). Activation of the GABA(B) receptor inhibits transient lower esophageal sphincter relaxations in dogs. *Gastroenterology* **117**: 1147–1154.

Lehmann A, Bremner-Danielsen M, Bränden L, Kärrberg L (2002). Inhibitory effects of GABA(B) receptor agonists on swallowing in the dog. *Eur J Pharmacol* **448**: 67–70.

Lidums I, Lehmann A, Checklin H, Dent J, Holloway RH (2000). Control of transient lower esophageal sphincter relaxations and reflux by the GABA(B) agonist baclofen in normal subjects. *Gastroenterology* **118**: 7–13.

Ling EA, Yick TY, Ng GL, Wong WC (1992). Immunocytochemical localisation of substance P in vagal ganglion cells and pericellular arborisations in the monkey. *J Anat* **181**: 61–71.

Macdonald RL, Olsen RW (1994). GABAA receptor channels. *Annu Rev Neurosci* **17**: 569–602.

McDougall NI, Johnston BT, Collins JS, McFarland RJ, Love AH (1998). Three- to 4.5-year prospective study of prognostic indicators in gastro-oesophageal reflux disease. *Scand J Gastroenterol* **33**: 1016–1022.

Mehta AK, Ticku MK (1999). An update on GABAA receptors. *Brain Res Rev* **29**: 196–217.

Meldrum B, Horton R (1980). Effects of the bicyclic GABA agonist, THIP, on myoclonic and seizure responses in mice and baboons with reflex epilepsy. *Eur J Pharmacol* **61**: 231–237.

Mittal RK, Holloway RH, Penagini R, Blackshaw LA, Dent J (1995). Transient lower esophageal sphincter relaxation. *Gastroenterology* **109**: 601–610.

Mohler H, Benke D, Benson J, Luscher B, Fritschy JM (1995). GABAA-receptor subtypes *in vivo*: cellular localization, pharmacology and regulation. *Adv Biochem Psychopharmacol* **48**: 41–56.

Nusser Z, Roberts JD, Baude A, Richards JG, Somogyi P (1995). Relative densities of synaptic and extrasynaptic GABAA receptors on cerebellar granule cells as determined by a quantitative immunogold method. *J Neurosci* **15**: 2948–2960.

Persohn E, Malherbe P, Richards JG (1991). *In situ* hybridization histochemistry reveals a diversity of GABAA receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. *Neuroscience* **42**: 497–507.

Pregenzer JF, Im WB, Carter DB, Thomsen DR (1993). Comparison of interactions of [3H]muscimol, t-butylbicyclophosphoro[35S]thionate, and [3H]flunitrazepam with cloned gamma-aminobutyric acidA receptors of the alpha 1 beta 2 and alpha 1 beta 2 gamma 2 subtypes. *Mol Pharmacol* **43**: 801–806.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR *et al*. (1989). Importance of a novel GABAA receptor subunit for benzodiazepine pharmacology. *Nature* **338**: 582–585.

Rode F, Jensen DG, Blackburn-Munro G, Bjerrum OJ (2005). Centrally-mediated antinociceptive actions of GABA(A) receptor agonists in the rat spared nerve injury model of neuropathic pain. *Eur J Pharmacol* **516**: 131–138.

Saha S, Sieghart W, Fritschy JM, McWilliam PN, Batten TF (2001). Gamma-aminobutyric acid receptor (GABA(A)) subunits in rat nucleus tractus solitarii (NTS) revealed by polymerase chain reaction (PCR) and immunohistochemistry. *Mol Cell Neurosci* **17**: 241–257.

Smid SD, Young RL, Cooper NJ, Blackshaw LA (2001). GABA(B)R expressed on vagal afferent neurones inhibit gastric mechanosensitivity in ferret proximal stomach. *Am J Physiol Gastrointest Liver Physiol* **281**: G1494–G1501.

Stoyanova II (2004). Gamma-aminobutiric acid immunostaining in trigeminal, nodose and spinal ganglia of the cat. *Acta Histochem* **106**: 309–314.

Valle C, Broglia F, Pistorio A, Tinelli C, Perego M (1999). Prevalence and impact of symptoms suggestive of gastroesophageal reflux disease. *Dig Dis Sci* **44**: 1848–1852.

Vela MF, Tutuian R, Katz PO, Castell DO (2003). Baclofen decreases acid and non-acid post-prandial gastro-oesophageal reflux measured by combined multichannel intraluminal impedance and pH. *Aliment Pharmacol Ther* **17**: 243–251.

Vyazovskiy VV, Kopp C, Bosch G, Tobler I (2005). The GABAA receptor agonist THIP alters the EEG in waking and sleep of mice. *Neuropharmacology* **48**: 617–626.

Washabau RJ, Fudge M, Price WJ, Barone FC (1995). GABA receptors in the dorsal motor nucleus of the vagus influence feline lower esophageal sphincter and gastric function. *Brain Res Bull* **38**: 587–594.

Yamamoto Y, Matsubara A, Ishii K, Makinae K, Sasaki A, Shinkawa H (2002). Localization of gamma-aminobutyric acid A receptor subunits in the rat spiral ganglion and organ of Corti. *Acta Otolaryngol* **122**: 709–714.

Yuan CS, Liu D, Attele AS (1998). GABAergic effects on nucleus tractus solitarius neurons receiving gastric vagal inputs. *J Pharmacol Exp Ther* **286**: 736–741.

Zagorodnyuk VP, D'Antona G, Brookes SJ, Costa M (2002). Functional GABAB receptors are present in guinea pig nodose ganglion cell bodies but not in peripheral mechanosensitive endings. *Auton Neurosci* **102**: 20–29.

Zhang Q, Lehmann A, Rigda R, Dent J, Holloway RH (2002). Control of transient lower oesophageal sphincter relaxations and reflux by the GABA(B) agonist baclofen in patients with gastro-oesophageal reflux disease. *Gut* **50**: 19–24.