Inhibition of transient LES relaxations and reflux in ferrets by GABA receptor agonists

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Introduction

Transient lower esophageal sphincter (LES) relaxation is the major mechanism of gastroesophageal reflux. This study used an established ferret model to evaluate GABA\(_a\) receptor agonists' ability to reduce triggering of transient LES relaxations. One hundred sixty manometric/pH studies were performed on 18 conscious ferrets. In untreated animals, intragastric infusion of 25 ml glucose (pH 3.5) led to 2.0 ± 0.6 reflux episodes over the first 30 min. Twenty-nine of forty-seven reflux episodes occurred during transient LES relaxation, and 18 occurred after downward drifts (≤ 1 mmHg/s) in basal LES pressure. The GABA\(_a\) receptor agonists baclofen (7 \(\mu\)mol/kg ip), CGP-44532, and SKF-97541 (both ED\(_{50} \leq 0.3 \) \(\mu\)mol/kg) reduced reflux episodes and transient LES relaxations. The putative peripherally selective GABA\(_b\) receptor agonist 3-aminopropylphosphinic acid (80–240 \(\mu\)mol/kg) was ineffective, as was the GABA\(_a\) receptor agonist muscimol (5 \(\mu\)mol/kg). Baclofen's inhibition of transient LES relaxations and reflux was unaffected by low-affinity GABA\(_a\) receptor antagonists CGP-35348 and CGP-36742 at 100 \(\mu\)mol/kg but was reversed by higher-affinity CGP-54626 and CGP-62349 (0.7 \(\mu\)mol/kg) or by CGP-36742 at 200 \(\mu\)mol/kg. Therefore, GABA\(_a\) receptor inhibition of reflux shows complex pharmacology. Our other and our data indicate the therapeutic potential for these drugs.

Lower esophageal sphincter; gastroesophageal reflux; gastric distension; GABA\(_a\) receptors

In recent years there has been increased research interest in novel pharmaceutical therapies for gastroesophageal reflux disease that act by reducing the rate of postprandial transient lower esophageal sphincter (LES) relaxations (7, 8, 24, 29, 32, 34). These events present an important target for many reasons: they are the major mechanism of gastroesophageal acid reflux, they are a specific feature of LES motility, and they are likely to be mediated via specific neural pathways. Therefore, selective interference with their mechanisms of triggering and control are unlikely to be accompanied by undesirable effects on motility or secretion elsewhere in the gastrointestinal tract.

GABA\(_a\) receptors are coupled via membrane G proteins and are present at many sites within the central and peripheral nervous systems. They have been exploited clinically in the treatment of spasticity by virtue of their inhibitory effect on muscle spindle afferent input to stretch reflexes (3, 11). Inhibitory GABA\(_a\) receptors are also abundant presynaptically on vagal afferent terminals in the dorsal medulla oblongata (9), although they also mediate slow postganglionic synaptic inhibition (10). GABA\(_a\) and GABA\(_b\) receptors, on the other hand, are located mainly postsynaptically and are coupled directly to chloride channels. GABA\(_b\) receptor agonists have been shown to exert inhibitory effects on transmitter release in vagal afferent terminals in the dorsal medulla oblongata (9), although they also mediate slow postganglionic synaptic inhibition (10). GABA\(_a\) and GABA\(_b\) receptors, on the other hand, are located mainly postsynaptically and are coupled directly to chloride channels. GABA\(_b\) receptor agonists have been shown to exert inhibitory effects on transmitter release in vagal afferent terminals in the dorsal medulla oblongata (9), although they also mediate slow postganglionic synaptic inhibition (10). GABA\(_a\) and GABA\(_b\) receptors, on the other hand, are located mainly postsynaptically and are coupled directly to chloride channels. GABA\(_b\) receptor agonists have been shown to exert inhibitory effects on transmitter release in vagal afferent terminals in the dorsal medulla oblongata (9), although they also mediate slow postganglionic synaptic inhibition (10). GABA\(_a\) and GABA\(_b\) receptors, on the other hand, are located mainly postsynaptically and are coupled directly to chloride channels. GABA\(_b\) receptor agonists have been shown to exert inhibitory effects on transmitter release in vagal afferent terminals in the dorsal medulla oblongata (9), although they also mediate slow postganglionic synaptic inhibition (10). GABA\(_a\) and GABA\(_b\) receptors, on the other hand, are located mainly postsynaptically and are coupled directly to chloride channels. GABA\(_b\) receptor agonists have been shown to exert inhibitory effects on transmitter release in vagal afferent terminals in the dorsal medulla oblongata (9), although they also mediate slow postganglionic synaptic inhibition (10). GABA\(_a\) and GABA\(_b\) receptors, on the other hand, are located mainly postsynaptically and are coupled directly to chloride channels. GABA\(_b\) receptor agonists have been shown to exert inhibitory effects on transmitter release in vagal afferent terminals in the dorsal medulla oblongata (9), although they also mediate slow postganglionic synaptic inhibition (10). GABA\(_a\) and GABA\(_b\) receptors, on the other hand, are located mainly postsynaptically and are coupled directly to chloride channels.

Materials and Methods

Recording Methods

The recording assembly consisted of a pH electrode and micromanometric assembly, which were secured together with Parafilm. This was introduced via an esophagostomy and held in place by a Neoprene harness around the animal's upper thorax and neck. The micromanometric assembly was built from a 10-lumen silicone rubber extrusion (2.0 mm OD) and incorporated a reverse-perfused sleeve sensor to monitor LES pressure. A side hole at the distal margin of the sleeve monitored intragastric pressure, and side holes located at the proximal sleeve margin and 25, 50, and 75 mm more proximal monitored esophageal body activity. Micromanometric side holes were perfused with degassed distilled water at 0.02 ml/min and the sleeve sensor was perfused at 0.04 ml/min. A central
channel (0.75 mm ID) with a side hole 15 mm beyond the distal margin of the sleeve served for gastric nutrient infusion. The pH electrode (0.8 mm OD; Synectics Medical, Stockholm, Sweden) was positioned 10 mm above the proximal end of the sleeve sensor and was referenced to an Ag/AgCl electrode. Swallows were detected with a microphone that was attached to the collar and positioned over the hyoid bone. Manometric and pH outputs were amplified with Synectics Polygraf preamplifiers and were acquired to disk using Labview-based software. This software was also used for off-line analysis of pH events and display of traces. Audio signals from the throat microphone were converted to a software time marker and integrated with other analog inputs.

The recording chamber was composed of a plastic cylinder 20 cm tall × 25 cm ID mounted on a freely rotating platform. This apparatus allowed monitoring of animals in their normal postprandial body position. The micromanometric assembly and electrical wires were protected from bite damage by a 40-cm-long steel coil spring exiting the recording chamber via a hole in the center of the chamber’s clear Perspex lid.

The recording methods above are described in more detail in our previous work (5).

Animals and Experimental Protocol

Experiments were performed on adult female ferrets (0.45–0.75 kg body wt) obtained from the Institute of Medical and Veterinary Science, Adelaide. Animals were acclimatized to handling and laboratory conditions soon after weaning and were trained to wear a harness. Chronic lateral cervical mucosa-to-skin esophagostomies were constructed on reaching adulthood in 18 ferrets under isoflurane (2–4% inhalation) anesthesia. Manometric studies were begun at least 1 mo after surgery.

Animals were fasted overnight before studies. On the day of study, each ferret was injected with saline (1 ml/kg ip) or drug treatments, intubated with the pH/manometric assembly, placed into the recording chamber, and allowed to adapt to study conditions for 10 min before data acquisition was commenced. All drugs used were demonstrated to have effects that outlasted the study period in preliminary acute studies. After 30 min of baseline data acquisition, a gastric load was introduced via the manometric assembly over 2 min. This load consisted of 25 ml of 10% dextrose, which was buffered to pH 3.5 to provide a reproducible signal for the pH electrode when reflux occurred. During reflux episodes, the pH recording was never seen to drop below 3.5, providing an indication that treatments did not promote acid secretion beyond the buffering capacity of the infused solution.

Each animal was studied on a minimum of 4 and a maximum of 10 occasions, with at least 1 wk between each study so that data were obtained in intraperitoneal saline control experiments (two per animal) in alternate weeks with drug treatment experiments. This was to minimize the influence of any chronic variations in esophageal motility on drug treatment data. Drug doses were given in random order. Antagonists were administered simultaneously with agonists where applicable.

The procedures performed in this study were approved by the Animal Ethics Committees of the Royal Adelaide Hospital and Institute of Medical and Veterinary Science, Adelaide. For details of postoperative care, see Blackshaw et al. (5).

Drugs

Compounds used were baclofen and muscimol hydrobromide (Sigma); 3-aminopropylphosphinic acid (3-APPi), SKF-97541, CGP-35348, CGP-36742, CGP-44532, CGP-54626, and CGP-62349 (Astra Zeneca, R&D Möln达尔).

Data Analysis

Manometry. Basal LES pressure was referenced to mean intragastric pressure. No differences were revealed on statistical comparison if atmospheric pressure was used as the reference. Measurements were taken as the visual mean of end-expiratory pressure over the entire basal recording period (using a compressed display time base) and during the first 30 min after intragastric infusion, after exclusion of the effects of swallowing and transient LES relaxation on LES pressure. Drug effects on basal LES pressure were assessed as differences between postinfusion rather than preinfusion pressures so that compounds would have had 40–70 min to reach maximal activity. Transient LES relaxations were defined similarly to those in humans (20) as rapid (>1 mmHg/s) drops in LES pressure to 2 mmHg or less above gastric pressure for >5 s, with no associated swallow signal. Swallow-induced relaxations were defined as drops in LES pressure occurring within 2 s before or after a swallow was detected. A drift was scored when there was a slow (<1 mmHg/s) decrease in LES pressure to <2 mmHg above gastric pressure that remained at its nadir for >10 s. Acid reflux during transient lower esophageal sphincter (LES) relaxation. Bottom channel marks swallows, as determined with throat microphone. A few seconds before reduction in esophageal pH, a transient LES relaxation begins with an abrupt drop in LES pressure to gastric pressure several seconds before swallowing or esophageal peristalsis occurs. Relaxation lasts for ∼7 s. The pH drops to 4 during this sphincter relaxation, at which point a synchronous esophageal pressure wave and swallow are initiated, and LES pressure rises to 30 mmHg. Esophageal pH rises after clearance of refluxed acid by a primary peristaltic wave associated with swallow. B: group data for number of transient LES relaxations (TLESR) over 30 min before infusions and over first 30 min after gastric infusions in control experiments. Occurrence of TLESR increased significantly (++P < 0.01; n = 36).

Fig. 1. A: manometric and pH recording in conscious ferret in a control experiment 1 min after intragastric glucose infusion showing reflux during transient lower esophageal sphincter (LES) relaxation.
reflux was scored when intraesophageal pH dropped to pH 4 or below for >4 s.

Behavioral indexes. Behavior was scored by one investigator (E. Staunton) who was in visual contact with animals throughout each study. A scale of 0–5 was used to quantify alertness and mobility during baseline and postinfusion periods of each study. A value of 5 represented normal fasted behavior, and 0 represented unrousable catatonia (which was never encountered).

Statistics. Differences between groups of data were assessed using Student's paired t-test. Data are expressed as means ± SE as all data displayed a normal distribution (Kolmogorov-Smirnov test).

RESULTS

Basal LES Pressure

In control experiments, mean basal LES pressure was 23.8 ± 1.8 mmHg above mean intragastric pressure during the 30-min preinfusion period. As reported in our previous study (5), in the baseline period, rhythmic positive fluctuations in LES pressure occurred in phase with upper gastric pressure waves. These increases in LES pressure were 5–30 mmHg in amplitude and had a basic frequency of approximately eight per minute. Rhythmic patterns of motility of this type were no longer seen after gastric infusions. During intragastric infusions, a gradual increase in gastric and LES pressure was often seen, which rapidly attenuated within 10 min after completion of the infusion. Mean LES pressure over the 30-min period after intragastric infusion did not show any significant change vs. preinfusion. Neither pre- nor postinfusion patterns of intraluminal pressures were affected by any of the drug treatments used, other than those associated with transient LES relaxations and swallowing.

Transient LES Relaxations

Transient LES relaxations were rare during the basal period but were more frequent either during or within a few minutes of completion of the intragastric load in untreated animals (Fig. 1). Sixty-five percent of transient LES relaxations occurred during the first 10 min after intragastric infusions, as we have shown previously (5). This relative distribution was unaffected by any of the drug treatments.

The GABA<sub>B</sub> receptor agonist baclofen (1–7 µmol/kg ip at start of baseline period) dose dependently reduced the number of transient LES relaxations, such that at 7 µmol/kg they occurred rarely (Fig. 2A). The selective GABA<sub>B</sub> receptor agonist CGP-44532 did not have a statistically significant effect on the rate of transient LES relaxations at 0.3 µmol/kg or 0.65 µmol/kg, but this was against a background of highly variable control studies (Fig. 2B). When statistical analysis was performed on both treatment groups together, there was a significant effect (P < 0.001). SKF-97541, a drug chemically related to CGP-44532, dose dependently inhibited transient LES relaxations. At a dose of 0.7 µmol/kg, this compound completely abolished transient LES relaxations in all experiments (Fig. 2C). The putative peripherally selective GABA<sub>B</sub> receptor agonist 3-APPi (80 and 240 µmol/kg) had little or no effect (Fig. 2D), as did the selective GABA<sub>A</sub> receptor agonist muscimol at a dose of 5 µmol/kg (Fig. 2E), whereas 10 µmol/kg muscimol significantly reduced transient LES relaxations in association with marked side effects (see Behavior).

The effects of baclofen (7 µmol/kg) on transient LES relaxations were unaffected by coadministration of the low-affinity GABA<sub>B</sub> receptor antagonists CGP-36742 or
CGP-35348 at 50–100 µmol/kg but were reversed by the high-affinity GABA<sub>B</sub> receptor antagonists CGP-54626 (7 µmol/kg) or CGP-62349 (0.7 µmol/kg) or by CGP-36742 at 200 µmol/kg (Fig. 3). All of these antagonists were without effect on transient LES relaxations when administered alone (data not shown).

Relationship of pH Events to Manometry

All animals showed evidence of gastroesophageal reflux after nutrient infusion in control experiments. Twenty-nine of forty-seven acidification episodes (62%) occurred during transient LES relaxation, and 18 (38%) occurred after gradual (<1 mmHg/s) downward drifts in basal LES pressure to ~2 mmHg above intragastric pressure. Clearance of refluxed acid occurred because of repeated primary peristalsis, which restored normal esophageal pH usually within 2 min of the episode's onset. Not all transient LES relaxations were associated with reflux. Esophageal acidification occurred during 29 of 49 (59%) transient LES relaxations after glucose infusions.

Effects of agonist and antagonist drugs on the occurrence of reflux episodes followed similar patterns to those on the occurrence of transient LES relaxations (Figs. 4 and 5). No treatment was seen to disproportionately affect reflux episodes without changing the rate of transient LES relaxations.

Swallowing

The GABA<sub>B</sub> receptor agonist baclofen (7 µmol/kg ip at start of baseline period) reduced the rate of spontaneous swallowing (Fig. 6). The selective GABA<sub>B</sub> receptor agonists CGP-44532 and 3-APPi had little or no effect on swallowing. SKF-97541 substantially reduced the rate of swallowing only at the highest dose administered (0.7 µmol/kg). The selective GABA<sub>A</sub> receptor agonist muscimol was also ineffective at a dose of 5 µmol/kg, although inhibition was seen at a dose of 10 µmol/kg.

The effects of baclofen on swallowing were partly reversed by coadministration with the selective GABA<sub>B</sub> receptor antagonists CGP-35348, CGP-36742, CGP-54626, or CGP-62349 in a dose-dependent manner. Antagonist potency was different from that seen against baclofen's action on transient LES relaxations, such that CGP-36742 was effective at both doses of 50 and 200 µmol/kg. All antagonists were without effect on swallowing when administered alone.

Although the velocity and distance of propagation of peristaltic waves were not measured in this study, visual inspection of tracings yielded no obvious drug effects. Subtle changes may have occurred that were beyond the resolution of our recording system, which displayed a minimum of 1 min of raw data per screen/page.

Behavior

Baclofen had no significant effects on alertness and mobility at a dose of 1 µmol/kg but significantly reduced both at a dose of 7 µmol/kg (P < 0.05). Other GABA<sub>B</sub> receptor agonists did not influence behavior. GABA<sub>B</sub> receptor antagonists reversed baclofen's effects on behavioral indexes generally in parallel with swallowing (data not shown), such that all GABA<sub>B</sub> receptor antagonists were effective in reversing effects of baclofen. The GABA<sub>A</sub> receptor agonist muscimol had mild inhibitory effects on behavior at 5 µmol/kg, and these became marked and reached statistical significance at 10 µmol/kg (P < 0.0001).

DISCUSSION

This study is the first, alongside those in humans (22) and dogs (21), to demonstrate that GABA<sub>B</sub> receptor
agonists are potent inhibitors of gastroesophageal reflux and that this occurs through a reduction in the rate of transient LES relaxations.

Other treatments previously documented to reduce the rate of transient LES relaxations and reflux episodes include anesthesia (14), morphine (29), atropine (24), a cholecystokinin A antagonist (8), and a nitric oxide synthase inhibitor (7). With the exception of anesthesia, none of these treatments was equivalent to GABA\textsubscript{B} receptor agonists in terms of the magnitude of their effect on the occurrence of transient LES relaxations. Although behavioral side effects of the selective GABA\textsubscript{B} receptor agonist baclofen were observed at the higher dose in this study, these were unlikely to be mainly responsible for its effect on transient LES relaxations and reflux, because other GABA\textsubscript{B} receptor agonists were not as effective.

Fig. 4. Effects of GABA receptor agonists on occurrence of reflux episodes. Baclofen (A), CGP-44149 (B), SK&F-97541 (SK&F; C), 3-APPPi (D), and muscimol (E) are shown. Doses are given per kilogram body weight. *P < 0.05 and **P < 0.01 vs. control.

Fig. 5. Effects of combination treatment of baclofen with GABA\textsubscript{B} receptor antagonists on occurrence of reflux episodes. CGP-36742 (CGP36; A), CGP-35348 (CGP35; B), CGP-54626 (CGP54; C), and CGP-62349 (CGP62; D) are shown. Doses are given per kilogram body weight. *P < 0.05 vs. control.
agonists were capable of producing inhibition of transient LES relaxations without accompanying behavioral effects. From these data and preliminary findings in humans (22) and dogs (21), in which lower doses of baclofen were effective in reducing transient LES relaxations and reflux, GABA\textsubscript{B} receptor agonism appears promising as a novel strategy in the management of gastroesophageal reflux disease.

GABA\textsubscript{B} receptors are found predominantly in the central nervous system and in particular on the central terminals of primary afferent neurons (9, 11, 30), but they are also found on peripheral terminals of afferent and efferent fibers (12, 13, 31, 37). Antitussive actions of GABA\textsubscript{B} receptor agonists may be both peripheral and central (6), as is the case with their effects on gastric acid secretion (19), although effects of GABA\textsubscript{B} receptor agonists on acid secretion are seen only in urethane-anesthetized animals (2, 19). The site of action of GABA\textsubscript{B} receptor agonists in reducing triggering of transient LES relaxations in the present study could not be determined. A central action was manifested in the behavioral responses that were observed and perhaps also in the effects of baclofen on swallowing. However, the relative rank order of potency of antagonists in reversing baclofen's effect on transient LES relaxations was different than that in reversing its effects on swallowing. This may indicate either a peripheral site of action or a central action at sites distinct from those involved in coordination of swallowing. The latter is the more likely conclusion because 3-APPi, which was shown previously to have only peripheral effects after systemic administration (6), was ineffective in reducing transient LES relaxations or swallowing. The distinction between effects on transient LES relaxations and swallowing may be of particular significance in the light of evidence that the two events are thought to be coordinated by largely common central pathways (25). Our evidence indicates that there are subtle differences in the pharmacology of those central pathways that are not in common. To reach a conclusion on the site of action based on published peripheral or central selectivity of agonists and antagonists is further frustrated because, in contrast to the lack of effect of 3-APPi described above, the reportedly peripherally restricted antagonist CGP-54626 (18) was effective in partly reversing the effects of baclofen. Therefore, this study shows either that there are species-specific effects of these compounds or that drug efficacy may be governed by factors more complex than their access to the central nervous system. The highly potent, selective, and centrally acting GABA\textsubscript{B} receptor antagonist CGP-62349 (18) dose dependently reversed baclofen's inhibition of transient LES relaxations up to a dose of 0.7 µmol/kg, above which its effect was diminished. This action is unprecedented in the literature and was remarkably not accompanied by a similar pattern of effect on reflux episodes. We suggest, therefore, that this compound, although a highly potent and selective GABA\textsubscript{B} receptor antagonist at low doses, may exert other actions at doses in the micromolar range. The GABA\textsubscript{B} receptor antagonists were without effect when administered alone, indicating that GABA\textsubscript{B} receptors are not activated by endogenous GABA in the control of LES function.

Gastric distension is the best stimulus for transient LES relaxations (15–17), which is signaled to the...
central nervous system by vagal mechanoreceptors. It may appear, therefore, that the effect of baclofen in reducing triggering of transient LES relaxations could be secondary to an inhibitory action on gastric tone, which would reduce this signal. In fact, the reverse is true: baclofen causes large increases in gastric motility and tone in urethane-anesthetized ferrets (1, 4). This may indicate that baclofen reduces occurrence of transient LES relaxations in the face of an increased natural stimulus for them. There is a converse argument that the baclofen may have reduced transient LES relaxations and reflux by accelerating gastric emptying of the stimulus and thereby removing it. Although this cannot be ruled out in ferrets, no effects of baclofen on liquid and nutrient gastric emptying were seen in studies in conscious dogs (A. Lehmann, unpublished observations), and gastric motility was unaffected by baclofen in this study.

As far as the pathway acted on by GABA_B receptor ligands is concerned, vagal reflex pathways have been strongly implicated to play an important role in the triggering of transient LES relaxations (23). Considering the previously documented effects of GABA_B receptor agonists on other vagal reflexes (1, 35, 36), it is perhaps not therefore surprising that they had potent effects on triggering of transient LES relaxations. Electrophysiological studies in our laboratory have established very recently that GABA_B receptor agonists reduce the sensitivity of gastroesophageal vagal afferent fibers to mechanical stimuli (4, 26) and have an additional central effect on transmission of gastrointestinal afferent signals through the dorsal medulla (27, 28). The site of action of GABA_B receptor agonists in inhibiting transient LES relaxations may be either, both, or neither of these.

In conclusion, we have shown in a recently established animal model for gastroesophageal reflux that GABA_B receptor agonists directly and reversibly reduce reflux through reduction in the occurrence of transient LES relaxations. This may be achieved with minimal effects on other systems using very low doses of novel compounds. The site of action remains to be elucidated.

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