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# ENTERIC GABA-CONTAINING NERVES PROJECTING TO THE GUINEA-PIG INFERIOR MESENTERIC GANGLION MODULATE ACETYLCHOLINE RELEASE

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SUMMARY

1. The effect of GABA and GABA receptor-modulating drugs on release of [$^3$H]acetylcholine was studied in the guinea-pig inferior mesenteric ganglion.

2. GABA caused a dose-dependent increase in [$^3$H]acetylcholine release during stimulation of the lumbar colonic nerves. Muscimol (10 $\mu$M) and diazepam (5 $\mu$M) also increased [$^3$H]acetylcholine release during stimulation of the lumbar colonic nerves whereas baclofen (10 $\mu$M) had no effect.

3. Bicuculline (20–100 $\mu$M) and picrotoxin (50 $\mu$M) alone reduced [$^3$H]acetylcholine release during electrical stimulation of the lumbar colonic nerves whereas phaclofen (300 $\mu$M) had no effect.

4. Bicuculline (100 $\mu$M) significantly decreased whereas diazepam (5 $\mu$M) significantly increased distension-induced [$^3$H]acetylcholine release.

5. Colonic distension significantly increased [$^3$H]GABA release in the inferior mesenteric ganglion compared to basal periods when the colon was not distended. Distension-induced release of [$^3$H]GABA resulted from active neuronal transmission from the colon to the inferior mesenteric ganglion, since perfusion of the inferior mesenteric ganglion with tetrodoxin (1 $\mu$M) reduced basal release of [$^3$H]GABA and abolished distension-evoked increases in the release of [$^3$H]GABA.

6. In contrast to its excitatory effects on peripheral colonic afferent cholinergic nerves, exogenous GABA caused a dose-dependent decrease in [$^3$H]acetylcholine release during electrical stimulation of the central lumbar splanchnic nerves. Baclofen (10 $\mu$M) also inhibited [$^3$H]acetylcholine release whereas muscimol (10 $\mu$M) or diazepam (5 $\mu$M) had no effect. Phaclofen (300 $\mu$M) antagonized the inhibitory effects of exogenous GABA (10 $\mu$M) and of baclofen (10 $\mu$M). Bicuculline (100 $\mu$M), picrotoxin (50 $\mu$M) and phaclofen (300 $\mu$M) alone had no effect on [$^3$H]acetylcholine release during splanchnic nerve stimulation.

7. Phaclofen (300 $\mu$M) increased [$^3$H]acetylcholine release during simultaneous electrical stimulation of the lumbar colonic nerves and splanchnic nerves and when $GABA_A$ receptors were blocked by bicuculline (20 $\mu$M).

8. The data suggest that $GABA_A$ receptors facilitate release of acetylcholine from

peripheral cholinergic mechanosensory nerves projecting from the colon to the inferior mesenteric ganglion and that $GABA_B$ receptors inhibit release of acetylcholine from central cholinergic nerves. Enteric GABA-containing nerves projecting to the inferior mesenteric ganglion are mechanosensory. Endogenous release of GABA may act on $GABA_A$ receptors to facilitate peripheral cholinergic mechanosensory transmission and/or on $GABA_B$ receptors to inhibit central cholinergic transmission.

## INTRODUCTION

GABA-like immunoreactive nerves project from the colon via the lumbar colonic nerves to the inferior mesenteric ganglion (Hills, King, Mirsky & Jessen, 1988). Using electrophysiological techniques, two effects of GABA have been observed. Exogenous GABA depolarizes sympathetic neurones by acting on postsynaptic $GABA_A$ receptors (Hills *et al.* 1988; Stapelfeldt, Parkman & Szurszewski, 1993). Also, endogenously released GABA facilitates fast nicotinic synaptic input by acting on $GABA_A$ receptors located presynaptically on colonic mechanosensory afferent nerves (Stapelfeldt *et al.* 1993).

The purpose of this study was to examine the presynaptic site of GABA action more closely by directly measuring *in vitro* release of [$^3$H]acetylcholine from both peripheral afferent and central preganglionic cholinergic nerves while GABA was present by exogenous application or by release from endogenous stores. In addition, [$^3$H]GABA release in the inferior mesenteric ganglion was measured to determine the physiological stimulus activating the GABA-containing nerves projecting to the inferior mesenteric ganglion. Some of the results were previously communicated (Parkman, Stapelfeldt & Szurszewski, 1990; Parkman & Szurszewski, 1991).

## METHODS

Male guinea-pigs (250–450 g) were killed by a sharp blow to the head and exsanguinated (approved by the Animal Care and Use Committee of this Institution). After the abdominal cavity was opened by a mid-line incision, the inferior mesenteric ganglion, its central and peripheral nerve trunks and an attached segment of distal colon were rapidly dissected from the animal and placed in a dissecting dish containing oxygenated (97 % $O_2$–3 % $CO_2$) Krebs–Ringer buffer of the following composition (mM): $Na^+$, 137·4; $K^+$, 5·9; $Ca^{2+}$, 2·5; $Mg^{2+}$, 1·2; $Cl^-$, 134; $HCO_3^-$, 15·5; $H_2PO_4^-$, 1·2 and glucose 11·5. Adherent adipose and connective tissue were removed. In some experiments a segment of distal colon was left attached to the lumbar colonic nerves and in other experiments the colon was removed. The distal colon was removed when studying the effect of GABA and GABA receptor-modulating drugs on electrically evoked release of [$^3$H]acetylcholine. In these experiments, two ganglia were securely pinned down to the Sylgard base (Dow Corning Corporation, Midland, MI, USA) of a small chamber (250 $\mu$l) which was continuously perfused (3 ml $min^{-1}$) with Krebs–Ringer buffer at 37 °C. The lumbar colonic and/or splanchnic nerves of each ganglion were attached to bipolar platinum wire electrodes connected to an electrical stimulator (Grass S88, Grass Instruments, Quincy, MA, USA). The preparations were superfused with Krebs–Ringer buffer containing 0·01 % collagenase (Type 1A, Sigma Chemical Co., St Louis, MO, USA) for 10 min followed by a 20 min rinse with Krebs–Ringer buffer alone (Stapelfeldt & Szurszewski, 1989) to facilitate diffusion of released [$^3$H]acetylcholine out of the ganglion and into the superfusate. It was shown in a previous study that similar treatment of the inferior mesenteric ganglion did not affect fast or slow synaptic transmission (Stapelfeldt & Szurszewski, 1989). Preparations were loaded with 750 nM [$^3$H]choline (80 Ci $mmol^{-1}$, Amersham Corporation, Arlington Heights, IL, USA) for 180 min. The lumbar colonic or splanchnic nerves were electrically stimulated (0·5 Hz, 0·5 ms pulse duration, 20 V) during the first 90 min of the loading period because electrical stimulation increases

the uptake of [$^3$H]choline and subsequent synthesis of [$^3$H]acetylcholine (Friesen & Khatter, 1971). After loading, the preparations were washed for 30 min with Krebs–Ringer buffer containing 50 $\mu$M physostigmine and 10 $\mu$M hemicholinium-3 (Sigma Chemical Co.) (control solution).

The following protocol was used to measure release of [$^3$H]acetylcholine during electrical nerve stimulation. Perfusion was stopped for 5 min, during which time the nerves were either not stimulated or stimulated (2 or 5 Hz, 30 V, 0·5 ms pulse duration, 5 min duration). The superfusate was collected at the end of each period of stopped flow, the volume measured, and immediately placed on ice for subsequent determination of the concentrations of [$^3$H]choline and [$^3$H]acetylcholine. Between incubation periods, the ganglia were superfused for 5 min with control solution. There were four periods during which perfusion was stopped. During the first and third collection periods, the preganglionic nerves were not stimulated in order to measure basal ($B_1$ and $B_2$) release whereas preganglionic nerves were stimulated during the second and fourth collection periods ($S_1$ and $S_2$). When receptor antagonists were studied, they were added to the control solution during the $B_2$ and $S_2$ periods and continued through the $S_2$ period. When receptor agonists were studied, they were present during only the $S_2$ period. Using this approach, the effects of agonists and antagonists were compared using each preparation as its own control (Barnes, Barnes, Costall, Naylor & Tyers, 1989).

Thin-layer chromatographic techniques were used to quantitate the concentrations of [$^3$H]choline and [$^3$H]acetylcholine in each perfusate. Sixty microlitres of the effluent samples and 6 $\mu$l standards of [$^3$H]choline and [$^3$H]acetylcholine were placed on cellulose thin-layer chromatography plates (Whatman International Ltd, Maidstone, Kent). [$^3$H]Choline and [$^3$H]acetylcholine were separated by ascending chromatography in a solvent of *n*-butanol: ethanol: acetic acid: water (8:2:3:1 by volume) (Wikberg, 1977). Isolated [$^3$H]-labelled compounds were localized and quantified using a radiodensitometry scanner (Radiomatic Instruments and Chemical Co., Tampa, FL, USA). The amounts of [$^3$H]choline and [$^3$H]acetylcholine in the standards were quantitated using liquid scintillation spectroscopy (Beckman LS 7000 $\beta$ Counter, Beckman Instruments, Fullerton, CA, USA).

A segment of distal colon remained attached to the lumbar colonic nerves when studying the effect of colonic distension on release of [$^3$H]acetylcholine or [$^3$H]GABA in the inferior mesenteric ganglion. In these experiments, two inferior mesenteric ganglia each with an attached segment of distal colon (10–12 cm) were placed in a two-compartment organ bath. The inferior mesenteric ganglia were pinned to the Sylgard base of the central compartment (250 $\mu$l). The attached segments of colon were placed in the surrounding second compartment and cannulated for subsequent distension. The mesentery of each preparation containing the lumbar colonic nerves was draped over the wall separating the two compartments and covered with moist strips of tissue paper to prevent drying. Both compartments were separately perfused with oxygenated Krebs–Ringer buffer solution. Similar loading conditions were used to measure [$^3$H]acetylcholine release to those in the inferior mesenteric ganglia–nerve preparations described above, except that the preganglionic nerves were not stimulated electrically during [$^3$H]choline loading to avoid activating efferent nerve release of noradrenaline in the colon which could interfere with afferent input to the inferior mesenteric ganglion (Crowcroft, Holman & Szurszewski, 1971). There were four collection periods during which perfusion was stopped for 2 min, and both colon segments were either undistended ($B_1$ and $B_2$, basal periods) or distended ($CD_1$ and $CD_2$) simultaneously with 7 ml of air. GABA receptor-modulating drugs in control solution were selectively added to the ganglion compartment during the $B_2$ and $CD_2$ periods, while the colon compartment continued to be perfused with normal Krebs–Ringer buffer solution.

Release of [$^3$H]GABA from the inferior mesenteric ganglia was measured by a method similar to that used to measure GABA release from the central nervous system (Raiteri, Bonanno & Fedele, 1989). After perfusion with collagenase, the ganglia were perfused with Krebs–Ringer buffer containing $\beta$-alanine (1 mM), an inhibitor of glial cell GABA uptake (Schon & Kelly, 1975) and amino-oxyacetic acid (AOAA, 10 $\mu$M), an inhibitor of GABA breakdown (Neal & Starr, 1973). The ganglia were loaded with [$^3$H]GABA (0·5 $\mu$M, 60 Ci mmol$^{-1}$, Amersham Corporation) in Krebs–Ringer buffer containing $\beta$-alanine and AOAA. After loading, the ganglia were washed for 30 min with Krebs–Ringer buffer containing $\beta$-alanine, AOAA, and the GABA uptake inhibitor SK & F 89976A (30 $\mu$M) (Yunger, Fowler, Zarevics & Setler, 1984; Raiteri *et al.* 1989) (control solution). The effluent was collected after 2 min of stopped flow, during which the colons were either

distended or undistended. The amount of [$^3$H]GABA in the effluent was quantitated using thin-layer chromatography and radiodensitometry with radiolabelled standards.

The following drugs were used: AOAA, baclofen, $\beta$-alanine, $\gamma$-aminobutyric acid (GABA), muscimol, picrotoxin, tetrodotoxin (all obtained from Sigma Chemical Co., St Louis, MO, USA), bicuculline (Serva, Westbury, NY, USA), phaclofen (Cambridge Research Biochemicals, Valley Stream, NY, USA) and diazepam (Elkins Sinn, Inc., Cherry Hill, NJ, USA). The GABA uptake inhibitor SK & F 89976A was provided by SmithKline Beecham Pharmaceuticals and NOVA Pharmaceutical Corporation. The drug concentrations cited in the text are the final concentrations delivered to the tissue. In some experiments, a solution low in calcium (1 M) and high in magnesium (15 nM) was used to block transmitter release (Nield, 1978).

The data are expressed as means $\pm$ standard error of the mean (S.E.M.). Statistical analysis was performed using analysis of variance and Student's $t$ test. Values of $P < 0.05$ were considered significant.

## RESULTS

### *Effect of lumbar colonic nerve stimulation on release of [$^3$H]acetylcholine*

A radiodensitometric profile of the measured counts of tritium in the superfusate after separation of choline metabolites by ascending chromatography is shown in Fig. 1. In the absence of nerve stimulation, there was a single peak which co-migrated with the [$^3$H]choline standard. The additional peak produced by electrical stimulation of the lumbar colonic nerves co-migrated with the [$^3$H]acetylcholine standard and represented released [$^3$H]acetylcholine. This second peak was not found when the cholinesterase inhibitor physostigmine was omitted from the superfusing Krebs–Ringer buffer solution, confirming that it was [$^3$H]acetylcholine. The presence of a [$^3$H]acetylcholine peak was dependent on nerve impulse propagation along the lumbar colonic nerves and was not the result of a non-specific effect of field stimulation of the two ganglia because this peak was abolished either after the lumbar colonic nerves were cut between the stimulating electrodes and the inferior mesenteric ganglia ($n = 4$), by tetrodotoxin (1 $\mu$M, $n = 5$), or by a low-$Ca^{2+}$–high-$Mg^{2+}$ Krebs–Ringer buffer solution ($n = 4$).

[$^3$H]Acetylcholine release was quantitated by calculating the area under the curve of the [$^3$H]acetylcholine peak and comparing this to the area under the curve of known standards of [$^3$H]acetylcholine. A comparison of the amount of [$^3$H]acetylcholine released in the inferior mesenteric ganglion during stimulation of the lumbar colonic and splanchnic nerves is shown in Fig. 2. Stimulation of the lumbar colonic nerves at 2 Hz significantly ($P < 0.01$) increased the amount of [$^3$H]acetylcholine in the perfusate during the first stimulation period compared to the immediately preceding basal period when the lumbar colonic nerves were not stimulated (Fig. 2A). During the second period of stimulation ($S_2$), [$^3$H]acetylcholine release was significantly ($P < 0.01$) greater than the preceding basal period and was similar ($P > 0.05$) to the release observed during the first period of stimulation ($S_1$). Release of [$^3$H]acetylcholine during the third stimulation period ($S_3$), although greater ($P < 0.01$) than the preceding basal period, was significantly less ($P < 0.05$) than either of the first two stimulation periods. This probably represents run-down of releasable [$^3$H]acetylcholine during the third stimulation period. The release of radiolabelled neurotransmitters can be expressed as a ratio of the release during different stimulation periods, thereby allowing each preparation to serve as its own control (Barnes *et al.* 1989). In the present studies, the $S_3/S_2$ ratio ($0.74 \pm 0.11$) was significantly less ($P < 0.05$) than the $S_2/S_1$ ratio ($0.96 \pm 0.12$). Because of this

apparent run-down of releasable [$^3$H]acetylcholine in the third stimulation period, only the first two stimulation periods ($S_2$ and $S_1$) were used to compare release of [$^3$H]acetylcholine.

In contrast to the marked increase in release of [$^3$H]acetylcholine during electrical stimulation of the lumbar colonic nerves, there was only a minimal ($0{\cdot}1 < P < 0{\cdot}2$)

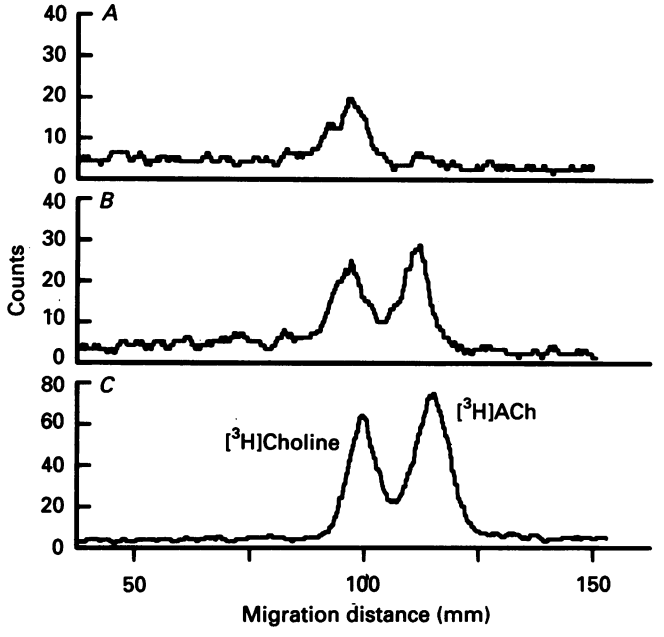


Fig. 1. Thin-layer chromatographic separation of [$^3$H]choline from [$^3$H]acetylcholine in the perfusate collected from the chamber containing two inferior mesenteric ganglion preparations. This radiodensitometry profile shows the measured counts of tritium in the perfusate *versus* the migration distance along the thin-layer chromatography plate. In the basal state without nerve stimulation (*A*), a single peak migrated with the [$^3$H]choline standard. In *B*, electrical stimulation (2 Hz, 5 min) of the lumbar colonic nerves (LCNs) produced an additional peak that co-migrated with the [$^3$H]acetylcholine standard and represented released [$^3$H]acetylcholine. Migration of standards of [$^3$H]choline and [$^3$H]acetylcholine is shown in *C*.

increase in the release of [$^3$H]choline during stimulation of the lumbar colonic nerves ($32{\cdot}4 \pm 9{\cdot}6 \times 10^3$ d.p.m. per two ganglia $(5\text{ min})^{-1}$) compared to basal release ($25{\cdot}0 \pm 15{\cdot}0 \times 10^3$ d.p.m. per two ganglia $(5\text{ min})^{-1}$).

*Effect of exogenous GABA and GABA receptor agonists on release of [$^3$H]acetylcholine during lumbar colonic nerve stimulation*

GABA caused a dose-dependent increase in the release of [$^3$H]acetylcholine during lumbar colonic nerve stimulation (Fig. 3*A*). At the highest dose tested, GABA (10 $\mu$M) significantly increased ($P < 0{\cdot}05$) [$^3$H]acetylcholine release by 74% compared to the control preparations. This suggests that presynaptic GABA receptors were

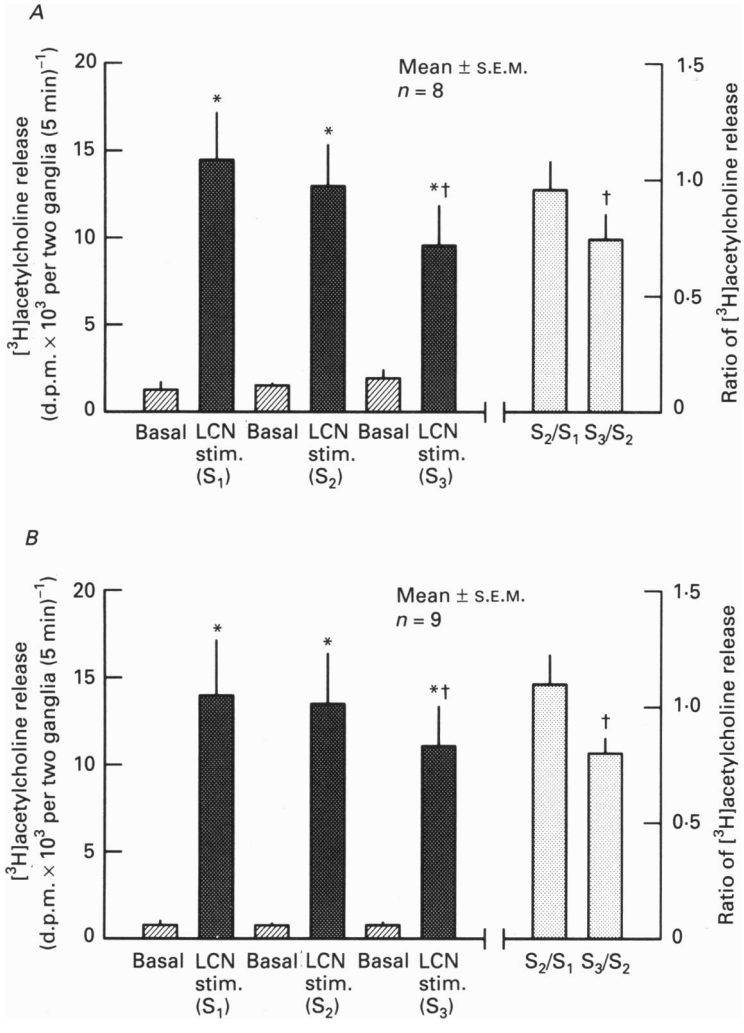


Fig. 2. Release of [$^3$H]acetylcholine in the inferior mesenteric ganglion during stimulation of peripheral lumbar colonic (*A*) or central splanchnic (*B*) preganglionic nerves at 2 Hz for 5 min. Results are expressed as disintegrations per minute per two ganglia per 5 min collection period as calculated by measuring the area under the curve of the [$^3$H]acetylcholine peak of the radiodensitometry profile (cf. Fig. 1). In *A*, the amount of [$^3$H]acetylcholine released into the perfusate during the first stimulation period ($S_1$) of the lumbar colonic nerves was significantly increased over the basal period when the nerves were not stimulated. During a second stimulation period ($S_2$), release was also greater than the intervening basal period. Release during the third stimulation period ($S_3$), although greater than the preceding basal period, was less than the first two stimulation periods, and the $S_3/S_2$ ratio was significantly less than the $S_2/S_1$ ratio. Similar results were obtained for stimulation of the central splanchnic nerves (*B*). $*P < 0{\cdot}01$; $\dagger P < 0{\cdot}05$.

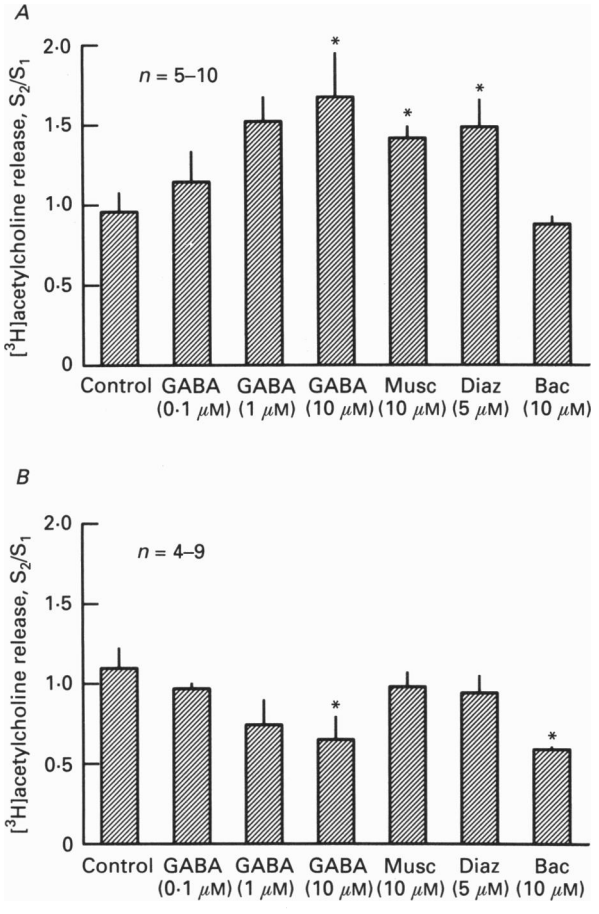


Fig. 3. Effect of GABA, GABA receptor agonists, and GABA receptor-modulating drugs on [$^3$H]acetylcholine release in the inferior mesenteric ganglion during preganglionic nerve stimulation at 2 Hz for 5 min. *A* shows the effect of exogenous GABA on [$^3$H]acetylcholine release during peripheral lumbar colonic nerve stimulation. In control preparations, with control solution perfusing the ganglia during the first two stimulation periods, the $S_2/S_1$ ratio was $0{\cdot}96 \pm 0{\cdot}12$. In different preparations, exogenous GABA was added during the second stimulation period ($S_2$), and the [$^3$H]acetylcholine release was compared to the amount released during the first stimulation period ($S_1$) in normal solution. GABA caused a dose-dependent increase in the release of [$^3$H]acetylcholine during lumbar colonic nerve stimulation, as shown by dose-dependent increases in the $S_2/S_1$ ratio. Muscimol had a similar facilitatory effect to GABA whereas baclofen had no effect. Diazepam also significantly facilitated [$^3$H]acetylcholine release. *B* shows the effect of exogenous GABA on [$^3$H]acetylcholine release during central splanchnic nerve stimulation at 2 Hz for 5 min. Exogenous GABA significantly decreased acetylcholine release. Muscimol and diazepam had no effect on [$^3$H]acetylcholine release during central nerve stimulation. Baclofen had a similar inhibitory action to GABA, suggesting that inhibitory $GABA_B$ receptors were present on central splanchnic cholinergic nerves. Musc, muscimol; Diaz, diazepam; BAC, baclofen. $*P < 0{\cdot}05$.

located on the lumbar colonic nerves and that their activation facilitated release of [$^3$H]acetylcholine.

*Subtype of GABA receptor*

Muscimol (10 $\mu$M), a $GABA_A$ receptor agonist, significantly ($P < 0.05$) increased release of [$^3$H]acetylcholine during lumbar colonic nerve (LCN) stimulation compared to periods of stimulation when muscimol was absent (Fig. 3*A*). Baclofen (10 $\mu$M), a $GABA_B$ receptor agonist, had no effect, suggesting that the presynaptic GABA receptors located on peripheral lumbar colonic nerves were of the $GABA_A$ receptor subtype. Diazepam, which is reported to have no intrinsic activity of its own, enhances $GABA_A$ synaptic transmission during release of endogenous GABA (Study & Barker, 1981; Twyman, Rogers & Macdonald, 1989). In the present study, diazepam (5 $\mu$M) facilitated release of [$^3$H]acetylcholine by 55% ($P < 0.05$) during LCN stimulation (Fig. 3*A*). This result suggested that diazepam interacted with endogenously released GABA during lumbar colonic nerve stimulation to facilitate release of [$^3$H]acetylcholine. To determine if endogenous GABA released during electrical stimulation of the lumbar colonic nerves could facilitate release of acetylcholine, experiments were performed with electrical stimulation of the lumbar colonic nerves in the presence of GABA receptor antagonists (without adding exogenous GABA to the perfusate).

*Effect of GABA receptor antagonists on release of [$^3$H]acetylcholine during LCN stimulation*

Bicuculline (20–100 $\mu$M), a $GABA_A$ receptor antagonist, significantly ($P < 0.05$) reduced [$^3$H]acetylcholine release during lumbar colonic nerve stimulation (5 Hz, 5 min, Fig. 4*A*). Picrotoxin (50 $\mu$M), a blocker of ligand-gated chloride channels of $GABA_A$ receptors, also significantly ($P < 0.05$) reduced [$^3$H]acetylcholine release (Fig. 4*A*). Phaclofen (300 $\mu$M), a $GABA_B$ receptor antagonist (Kerr, Ong, Prager, Gynther & Curtis, 1987), had no effect on [$^3$H]acetylcholine release (Fig. 4*A*). The inhibitory effects of bicuculline and picrotoxin on release of [$^3$H]acetylcholine suggest that stimulation of the lumbar colonic nerves evoked release of endogenous GABA and that endogenously released GABA acted on $GABA_A$ receptors located presynaptically on peripheral cholinergic nerves to facilitate release of acetylcholine.

In the above experiments, electrical stimulation of the LCN was used to evoke release of [$^3$H]acetylcholine and presumably of endogenous GABA. Considerable evidence exists to support the hypothesis that afferent cholinergic nerves in the lumbar colonic nerves are mechanosensory (Crowcroft *et al.* 1971). To help characterize the physiologic function of the GABA-facilitating effect on peripheral afferent cholinergic synaptic input to the inferior mesenteric ganglion, the effect of GABA receptor-modulating drugs was studied during colonic distension.

*Effect of GABA receptor-modulating drugs on release of [$^3$H]acetylcholine during colonic distension*

In these experiments a segment of distal colon was attached to the inferior mesenteric ganglion via the lumbar colonic nerve trunk. During the first period of colonic distension, there was a significant ($P < 0.01$) increase in release of

[$^3$H]acetylcholine (Fig. 5, $CD_1$, upper panel). A second period of colonic distension ($CD_2$) led to a similar increase in [$^3$H]acetylcholine release compared to the absence of colonic distension (Fig. 5, $B_2$, upper panel). Bicuculline (100 $\mu$M), selectively added to the ganglion compartment prior to the second distension period, significantly

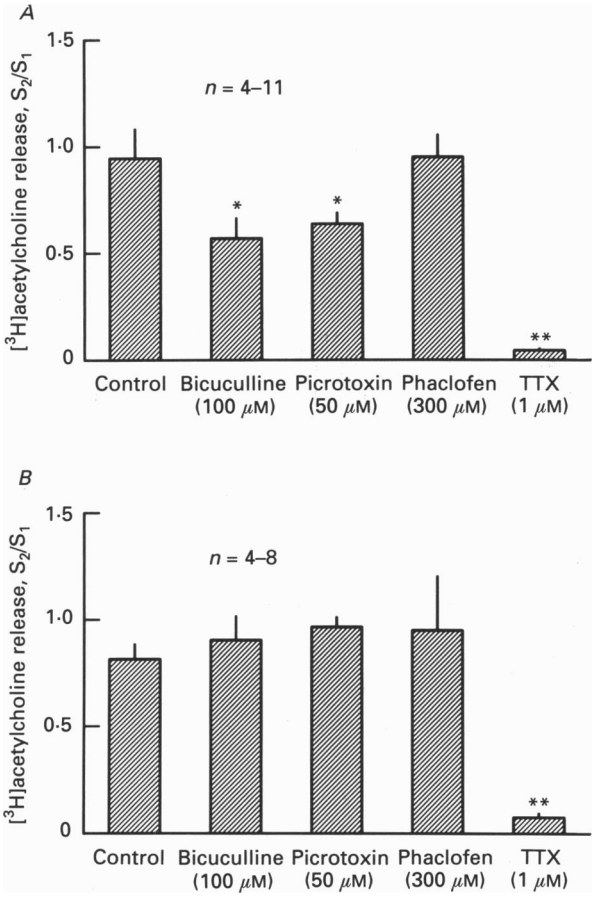


Fig. 4. Effect of GABA receptor antagonists on [$^3$H]acetylcholine release during preganglionic nerve stimulation at 5 Hz for 5 min. During electrical stimulation of the peripheral lumbar colonic nerve (*A*), bicuculline significantly reduced [$^3$H]acetylcholine release. Picrotoxin had a similar inhibitory effect. Phaclofen had no effect on [$^3$H]acetylcholine release. Tetrodotoxin nearly abolished [$^3$H]acetylcholine release during nerve stimulation. In contrast, during electrical stimulation of the central splanchnic nerve (*B*), bicuculline, picrotoxin or phaclofen had no effect on [$^3$H]acetylcholine release. TTX, tetrodotoxin. See text for further details. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

($P < 0{\cdot}05$) reduced distension-evoked release of [$^3$H]acetylcholine (Fig. 5, middle panel). In contrast, diazepam (5 $\mu$M) significantly ($P < 0{\cdot}05$) facilitated distension-evoked release of [$^3$H]acetylcholine (Fig. 5, lower panel). These results suggest that endogenous GABA was released in the inferior mesenteric ganglion during

colonic distension and that the endogenously released GABA acted on presynaptic $GABA_A$ receptors to facilitate [$^3$H]acetylcholine release from colonic mechanosensory nerves.

*Effect of colonic distension on release of [$^3$H]GABA*

Colonic distension increased [$^3$H]GABA in the perfusate (Figs 6 and 7). This increased release of [$^3$H]GABA during colonic distension was quantitated by

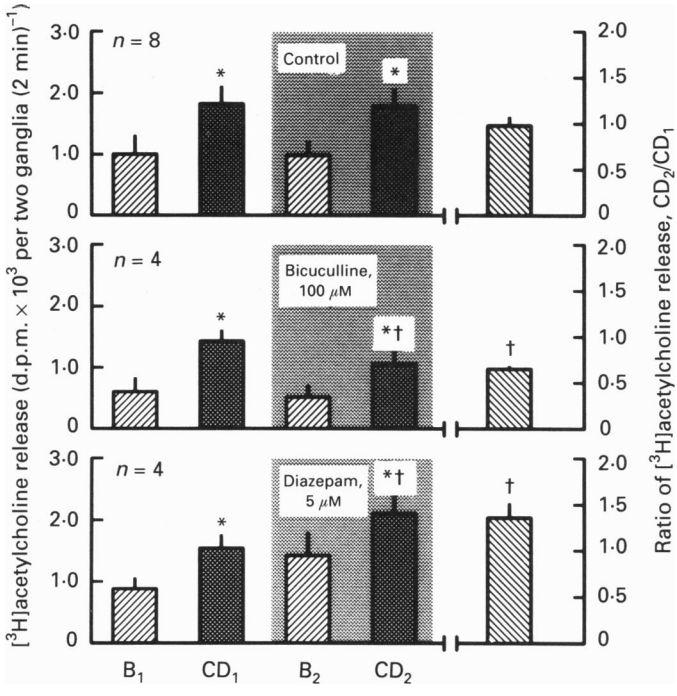


Fig. 5. Effect of $GABA_A$ receptor-modulating drugs on [$^3$H]acetylcholine release during colonic distension. The top panel shows the effect in control solution of colonic distension on release of [$^3$H]acetylcholine in the inferior mesenteric ganglion. Distension-evoked release of [$^3$H]acetylcholine was similar during the first ($CD_1$) and second distension ($CD_2$) periods. The effect of bicuculline and diazepam are shown in the middle and bottom panels, respectively. In these two panels, values for $CD_1$ represent release when control solution was present. Values for $B_2$ and $CD_2$ were obtained with bicuculline (middle panel) or diazepam (bottom panel) present in the ganglion compartment. Bicuculline (100 $\mu$M) reduced whereas diazepam (5 $\mu$M) enhanced distension-evoked [$^3$H]acetylcholine release. *$P < 0.05$ *versus* basal; †$P < 0.05$ *versus* CD.

measuring the area under the [$^3$H]GABA peaks (Fig. 7). The first distension period ($CD_1$) significantly ($P < 0.05$) increased [$^3$H]GABA in the perfusate (Fig. 7*A*). A second distension period ($CD_2$) increased [$^3$H]GABA release but the increase was not significantly different from the preceding basal period (Fig. 7*A*). Release of [$^3$H]GABA resulted from active neuronal transmission from the colon to the inferior mesenteric ganglion, since perfusion of the ganglia with tetrodotoxin (1 $\mu$M) reduced

basal release of [$^3$H]GABA and abolished distension-evoked release of [$^3$H]GABA (Fig. 7*B*). Thus, GABA-containing nerves projecting from the colon to the inferior mesenteric ganglion were mechanosensory.

*Release of [$^3$H]acetylcholine from lumbar splanchnic nerves*

To determine whether GABA receptors were located presynaptically on central preganglionic cholinergic nerves innervating inferior mesenteric ganglion neurones,

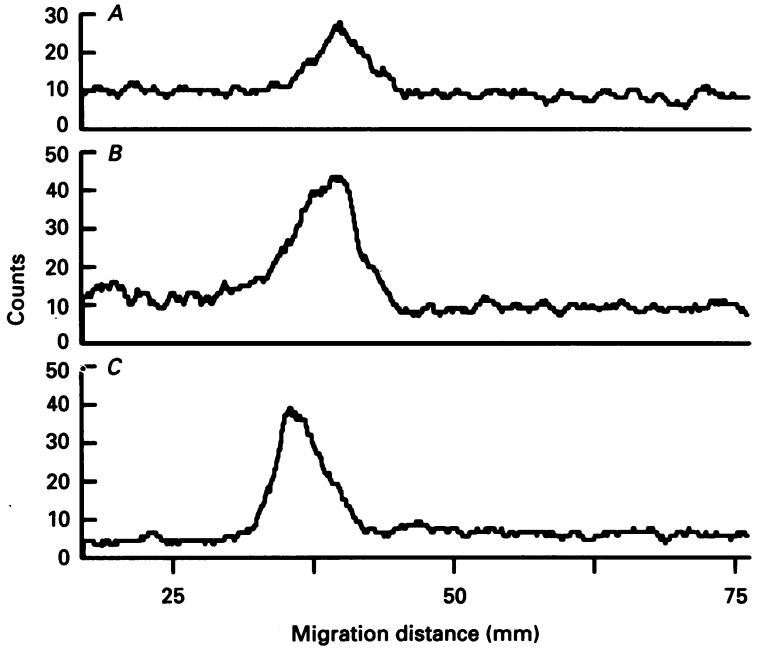


Fig. 6. Release of [$^3$H]GABA from the inferior mesenteric ganglion during colonic distension. This radiodensitometry profile shows the radioactive counts *versus* the migration distance along the thin-layer chromatography plate. In the basal state (*A*), with the colon undistended, the small peak of radioactivity, which co-migrated with the [$^3$H]GABA standard, represented continuing release of [$^3$H]GABA. During colonic distension (*B*), [$^3$H]GABA release was markedly increased by comparison with [$^3$H]GABA release with the colon undistended. Migration of [$^3$H]GABA standard is shown in *C*.

experiments measuring release of [$^3$H]acetylcholine during electrical stimulation of central lumbar splanchnic nerves were done without an attached segment of colon. Electrical stimulation of the splanchnic nerves at 2 Hz significantly ($P < 0{\cdot}01$) increased the amount of [$^3$H]acetylcholine in the perfusate during the first ($S_1$) and second ($S_2$) periods of stimulation compared to the immediately preceding basal periods when the splanchnic nerves were not stimulated (Fig. 2*B*). As with electrical stimulation of the lumbar colonic nerves, release of [$^3$H]acetylcholine during the third period of stimulation ($S_3$), although greater ($P < 0{\cdot}01$) than the preceding basal period was significantly ($P < 0{\cdot}05$) less than either of the first two stimulation periods (Fig. 2*B*).

*Effect of exogenous GABA and GABA receptor agonists on release of [$^3$H]acetylcholine during stimulation of the lumbar splanchnic nerves*

In contrast to the results obtained during stimulation of the peripheral lumbar colonic nerves, exogenous GABA significantly ($P < 0{\cdot}05$) decreased release of

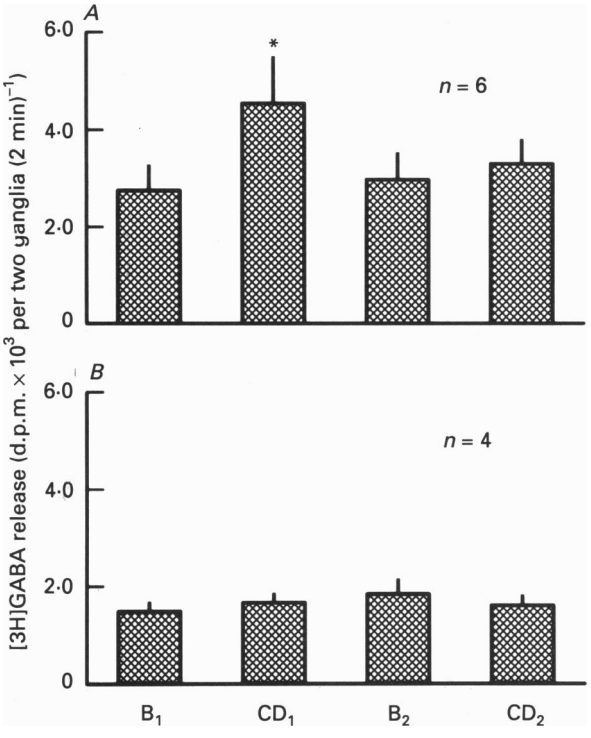


Fig. 7. Effect of colonic distension on [$^3$H]GABA release in the inferior mesenteric ganglion. In (*A*), the first distension period ($CD_1$) significantly increased [$^3$H]GABA release in the perfusate compared to the immediately preceding basal period when the colon was not distended ($B_1$). A second distension period ($CD_2$) also increased the [$^3$H]GABA release but the increase was not significant compared to the preceding basal period ($B_2$). In *B*, tetrodotoxin (1 $\mu$M) present throughout $B_1$, $CD_1$, $B_2$ and $CD_2$ reduced basal [$^3$H]GABA release and abolished distension-evoked [$^3$H]GABA release. Values on the ordinate were calculated by measuring the area under the curve of the [$^3$H]GABA peak of the radiodensitometry profile (cf. Fig. 6). *$P < 0{\cdot}05$.

[$^3$H]acetylcholine (Fig. 3*B*) during central lumbar splanchnic nerve stimulation (2 Hz, 5 min). At the highest dose tested, GABA (10 $\mu$M) decreased [$^3$H]acetylcholine release by 40% (Fig. 3*B*). This suggests that inhibitory GABA receptors were located presynaptically on central lumbar splanchnic nerves. The subtype(s) of GABA receptor(s) accounting for this inhibitory effect was investigated.

Muscimol (10 $\mu$M) and diazepam (5 $\mu$M) had no significant effect on [$^3$H]acetylcholine release during stimulation of the lumbar splanchnic nerves (Fig. 3*B*). Baclofen (10 $\mu$M) significantly ($P < 0{\cdot}05$) reduced [$^3$H]acetylcholine release (Fig. 3*B*).

Phaclofen (300 $\mu$M) abolished the inhibitory effects of GABA (10 $\mu$M) and baclofen (10 $\mu$M). These data strongly support the hypothesis that $GABA_B$ receptors were located presynaptically on central splanchnic nerves, and when activated inhibited release of [$^3$H]acetylcholine.

*Effect of GABA receptor antagonists on release of [$^3$H]acetylcholine during lumbar splanchnic nerve stimulation*

Previous immunohistochemical studies in the guinea-pig inferior mesenteric ganglion show that GABA-like immunoreactive nerves in the inferior mesenteric ganglion arise from enteric, myenteric neurones and that there are no central GABA-containing nerves projecting to the inferior mesenteric ganglion (Hills *et al.* 1988). Release of [$^3$H]acetylcholine during splanchnic nerve stimulation (5 Hz, 5 min) was unaffected by bicuculline (100 $\mu$M), picrotoxin (50 $\mu$M) or phaclofen (300 $\mu$M) (Fig. 4*B*), supporting the immunohistochemical observations that there are no GABA-containing nerves in the central splanchnic nerves.

*Effect of simultaneous stimulation of lumbar colonic and splanchnic nerves*

The possibility exists that GABA released from peripheral lumbar colonic nerves, in addition to activating $GABA_A$ receptors located on peripheral, colonic afferent cholinergic nerves, may also activate central $GABA_B$ receptors located on cholinergic nerves in the splanchnic nerves to inhibit central cholinergic input to the inferior mesenteric ganglion. To test this hypothesis, experiments were performed in preparations without a segment of attached colon and with stimulating electrodes on both the peripheral lumbar colonic and central splanchnic nerves. Bicuculline (20 $\mu$M) was continuously present during both $S_1$ and $S_2$ periods to antagonize the effect of endogenously released GABA on peripheral $GABA_A$ receptors. Under these conditions, phaclofen (300 $\mu$M) significantly increased ($P < 0{\cdot}05$) [$^3$H]acetylcholine release by 25% during the $S_2$ period. Since phaclofen did not alter [$^3$H]acetylcholine release during individual stimulation of either the lumbar colonic or splanchnic nerves, the facilitatory effect observed when the lumbar colonic and splanchnic nerves were simultaneously stimulated suggests that release of GABA from peripheral lumbar colonic nerves acted on $GABA_B$ receptors to inhibit release of [$^3$H]acetylcholine from splanchnic nerves.

## DISCUSSION

The results suggest that $GABA_A$ and $GABA_B$ receptor subtypes are located on different populations of presynaptic cholinergic nerves innervating the guinea-pig inferior mesenteric ganglion and that their activation leads to a facilitation and inhibition, respectively, of acetylcholine release.

$GABA_A$ receptors were present on peripheral cholinergic nerves projecting to the inferior mesenteric ganglion because exogenously applied GABA and the $GABA_A$ receptor agonist muscimol but not the $GABA_B$ receptor agonist baclofen enhanced release of [$^3$H]acetylcholine during electrical stimulation of the peripheral lumbar colonic nerves. Moreover, in the absence of exogenous GABA, bicuculline or pic-

rotoxin significantly reduced, whereas diazepam significantly increased [$^3$H]acetylcholine release. The effect of these $GABA_A$ receptor-modulating drugs is consistent with the notion that endogenous GABA was released from colonic afferent nerves and that it facilitated peripheral cholinergic input. Previous electrophysiological experiments in which intracellular recordings were made from sympathetic neurones in the inferior mesenteric ganglion suggest that endogenously released GABA acts only to modulate nicotinic cholinergic transmission because bicuculline decreases and diazepam increases the amplitude and frequency of nicotinic EPSPs without affecting the amplitude or duration of slow EPSPs or changing the resting membrane potential (Stapelfeldt *et al.* 1993). GABA receptors present in other peripheral ganglia and in the central nervous system modulate cholinergic transmission. In some areas of the guinea-pig brain, GABA enhances release of acetylcholine (Bianchi, Tanganelli, Marzola & Beani, 1982). However, in most areas of the brain and spinal cord, GABA inhibits release of acetylcholine (Beani, Bianchi, Siniscalchi, Sivilotti, Tanganelli & Veratti, 1984). In the rat superior cervical ganglion, activation of presynaptic $GABA_A$ receptors increases the frequency of nicotinic synaptic input (Galvan, Grafe & ten Bruggencate, 1980). In the enteric nervous system, both $GABA_A$ and $GABA_B$ receptor subtypes are present on cholinergic motor neurones. $GABA_A$ receptors enhance whereas $GABA_B$ receptors inhibit cholinergically mediated contractions (Krantis & Kerr, 1981; Giotti, Luzzi, Spagnesi & Zilletti, 1983; Grider & Makhlouf, 1992).

The present study provides evidence for presynaptic $GABA_B$ receptors on central splanchnic nerves. Exogenous GABA and the $GABA_B$ receptor agonist, baclofen, reduced [$^3$H]acetylcholine release during central splanchnic nerve stimulation. These results are in agreement with previous observations which suggested the existence of $GABA_B$ receptors on nerve terminals in the central nervous system (Bowery *et al.* 1980) and in bullfrog sympathetic ganglia (Kato & Kuba, 1980). The results of the present study also suggest that colonic afferent GABA nerves can decrease central cholinergic synaptic input to sympathetic neurones in the inferior mesenteric ganglion. In experiments in which the peripheral lumbar colonic nerves were electrically stimulated to release endogenous GABA, the amount of [$^3$H]acetylcholine released during simultaneous stimulation of central splanchnic nerves and peripheral lumbar colonic nerves when the $GABA_B$ receptor antagonist, phaclofen, was present was greater than the amount of [$^3$H]acetylcholine released in the absence of phaclofen. The increase in [$^3$H]acetylcholine release was not due to electrical activation of central preganglionic nerves containing GABA, as is the case in the superior cervical ganglion (Dobo, Kasa, Wenthold, Joo & Wolff, 1989), because no GABA-containing nerves have been found in central splanchnics projecting to the guinea-pig inferior mesenteric ganglion (Hills *et al.* 1988). The possibility remains that $GABA_B$ receptors were located on GABA-containing colonic afferent nerves and that the $GABA_B$ receptors function as autoreceptors to inhibit release of GABA (Raiteri *et al.* 1989). However, increased release of acetylcholine would have been expected during lumbar colonic nerve stimulation in the presence of phaclofen secondary to increased release of GABA. Such an effect was not observed.

The physiological importance of the enteric GABAergic nerves projecting from the colon to the inferior mesenteric ganglion was investigated by measuring the release

of radiolabelled neurotransmitters during colonic distension. Similar to peripheral cholinergic nerves, GABAergic nerves innervating the inferior mesenteric ganglion were also mechanosensory, since colonic distension released [$^3$H]GABA in the inferior mesenteric ganglion. During colonic distension, $GABA_A$ receptor antagonism decreased release of [$^3$H]acetylcholine, suggesting that the mechanosensory GABA-ergic nerves facilitate acetylcholine release from cholinergic mechanosensory nerves.

In addition to presynaptic GABA receptors, previous electrophysiological studies provide evidence for the existence of postsynaptic $GABA_A$ receptors (Adams & Brown, 1975; Hills *et al.* 1988; Stapelfeldt *et al.* 1993). In these previous experiments, exogenous administration of GABA evoked a depolarization of the membrane potential sufficient to block postsynaptic transmission. Since a postsynaptic depolarization was not observed when GABA was released from endogenous stores (Stapelfeldt *et al.* 1993), it seems reasonable to suggest that under *in vivo* conditions, the presynaptic effect of GABA may be physiologically more relevant. The functional advantage of postsynaptic $GABA_B$ receptors may be a self-protecting mechanism limiting transmission during high-intensity activation of colonic GABA-afferent nerves. Although our studies show that GABA was released in the inferior mesenteric ganglion during colonic distension, our studies do not directly demonstrate the origin of the released GABA. Hills *et al.* (1988) have demonstrated GABA-containing nerves projecting from the colon via the lumbar colonic nerves to the inferior mesenteric ganglion. In an analogous fashion, our studies suggest that GABA release was due to active neuronal transmission from the colon to the inferior mesenteric ganglion since tetrodotoxin abolished distension-evoked [$^3$H]GABA release. Previous studies have suggested that glial cells in sympathetic ganglia may accumulate radiolabelled GABA thereby serving as a protective mechanism from effects of excessive extracellular GABA (Young, Brown, Kelly & Schon, 1973; Bowery, Brown, White & Yamini, 1979*b*). Efflux of radiolabelled GABA may then represent a 'leak' secondary to high intracellular GABA concentrations after exogenous GABA loading (Bowery, Brown & Marsh, 1979*a*). However, our experiments demonstrating release of [$^3$H]GABA were performed using $\beta$-alanine during the loading period, which minimizes GABA uptake into glial cells while having no effect on the neuronal uptake of radiolabelled GABA (Schon & Kelly, 1975; Bowery *et al.* 1979*a*), further supporting the concept that the release of [$^3$H]GABA in response to colonic distension was from GABA-containing nerves.

From a clinical viewpoint, these studies suggest that benzodiazepines may act peripherally to influence colonic motility because diazepam, a commonly prescribed drug, increased afferent mechanosensory cholinergic input to peripheral sympathetic ganglia. An increase in cholinergic input would be expected to reflexly increase inhibitory sympathetic outflow to the colon, reducing colonic motility and intraluminal pressure. Of note, benzodiazepines have been found to inhibit gastrointestinal transit (Birnbaum, Ben-Menachem & Schwartz, 1970). The effect of diazepam described in the present study may explain its therapeutic effect in irritable bowel syndrome (Ritchie & Truelove, 1979).

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## REFERENCES

ADAMS, P. R. & BROWN, D. A. (1975). Actions of γ-aminobutyric acid on sympathetic ganglion cells. *Journal of Physiology* **250**, 85–120.

BARNES, J. M., BARNES, N. M., COSTALL, B., NAYLOR, R. J. & TYERS, M. B. (1989). 5-$HT_3$ receptors mediate inhibition of acetylcholine release in cortical tissue. *Nature* **338**, 762–763.

BEANI, L., BIANCHI, C., SINISCALCHI, A., SIVILOTTI, L., TANGANELLI, S. & VERATTI, E. (1984). Different approaches to study acetylcholine release: endogenous ACh versus tritium efflux. *Naunyn-Schmiedeberg's Archives of Pharmacology* **328**, 119–126.

BIANCHI, C., TANGANELLI, S., MARZOLA, G. & BEANI, L. (1982). GABA induced changes in acetylcholine release from slices of guinea-pig brain. *Naunyn-Schmiedeberg's Archives of Pharmacology* **318**, 253–258.

BIRNBAUM, D., BEN-MENACHEM, J. & SCHWARTZ, A. (1970). The influence of oral diazepam on gastrointestinal motility. *American Journal of Proctology* **21**, 263–266.

BOWERY, N. G., BROWN, D. A. & MARSH, S. (1979*a*). γ-Aminobutyric acid efflux from sympathetic glial cells: effect of 'depolarizing agents.' *Journal of Physiology* **293**, 75–101.

BOWERY, N. G., BROWN, D. A., WHITE, R. D. & YAMINI, G. (1979*b*). [$^3$H]γ-Aminobutyric acid uptake into neuroglial cells of rat superior cervical sympathetic ganglia. *Journal of Physiology* **293**, 51–74.

BOWERY, N. G., HILL, D. R., HUDSON, A. L., DOBLE, A., MIDDLEMISS, D. N., SHAW, J. & TURNBULL, M. (1980). (−)Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. *Nature* **283**, 92–94.

CROWCROFT, P. J., HOLMAN, M. E. & SZURSZEWSKI, J. H. (1971). Excitatory input from the distal colon to the inferior mesenteric ganglion in the guinea-pig. *Journal of Physiology* **219**, 443–461.

DOBO, E., KASA, P., WENTHOLD, R. J., JOO, F. & WOLFF, J. R. (1989). Evidence for GABAergic fibres entering the superior cervical ganglion of rat from the preganglionic nerve trunk. *Histochemistry* **92**, 133–136.

FRIESEN, A. J. D. & KHATTER, J. C. (1971). The effect of preganglionic stimulation on the acetylcholine and choline content of a sympathetic ganglion. *Canadian Journal of Physiology and Pharmacology* **49**, 375–381.

GALVAN, M., GRAFE, P. & TEN BRUGGENCATE, G. (1980). Presynaptic actions of 4-aminopyridine and γ-aminobutyric acid on rat sympathetic ganglia *in vitro*. *Naunyn-Schmiedeberg's Archives of Pharmacology* **314**, 141–147.

GIOTTI, A., LUZZI, S., SPAGNESI, S. & ZILLETTI, L. (1983). $GABA_A$ and $GABA_B$ receptor-mediated effects in guinea-pig ileum. *British Journal of Pharmacology* **78**, 469–478.

GRIDER, J. R. & MAKHLOUF, G. M. (1992). Enteric GABA: mode of action and role in the regulation of the peristaltic reflex. *American Journal of Physiology* **262**, G690–694.

HILLS, J. M., KING, B. F., MIRKSY, R. & JESSEN, K. R. (1988). Immunohistochemical localization and electrophysiologic actions of GABA in prevertebral ganglia in guinea-pig. *Journal of the Autonomic Nervous System* **22**, 129–140.

KATO, E. & KUBA, K. (1980). Inhibition of transmitter release in bullfrog sympathetic ganglia induced by γ-aminobutyric acid. *Journal of Physiology* **298**, 271–283.

KERR, D. I. B., ONG, J., PRAGER, R. H., GYNTHER, B. D. & CURTIS, D. R. (1987). Phaclofen: a peripheral and central baclofen antagonist. *Brain Research* **405**, 150–154.

KRANTIS, A. & KERR, D. I. B. (1981). GABA induced excitatory responses in guinea-pig small intestine are antagonized by bicuculline, picrotoxin and chloride ion blockers. *Naunyn-Schmiedeberg's Archives of Pharmacology* **317**, 257–261.

NEAL, M. J. & STARR, M. S. (1973). Effects of inhibitors of gamma-aminobutyrate aminotransferase on the accumulation of $^3$H-gamme-aminobutyric acid by the retina. *British Journal of Pharmacology* **47**, 543–555.

NIELD, T. O. (1978). Slowly developing depolarization of neurones in the guinea-pig inferior mesenteric ganglion following repetitive stimulation of the preganglionic nerves. *Brain Research* **140**, 231–239.

PARKMAN, H. P., STAPELFELDT, W. H. & SZURSZEWSKI, J. H. (1990). Peripheral GABA-containing nerves projecting to the inferior mesenteric ganglion selectively facilitate colonic afferent cholinergic input. *Gastroenterology* **98**, A379.

PARKMAN, H. P. & SZURSZEWSKI, J. H. (1991). GABA is released in the inferior mesenteric ganglion during colonic distension and enhances cholinergic colonic mechanosensory input. *Gastroenterology* **100**, A480.

RAITERI, M., BONANNO, G. & FEDELE, E. (1989). Release of $\gamma$-($^3$H)aminobutyric acid (GABA) from electrically stimulated rat cortical slices and its modulation by $GABA_B$ autoreceptors. *Journal of Pharmacology and Experimental Therapeutics* **250**, 648–653.

RITCHIE, J. A. & TRUELOVE, S. C. (1979). Treatment of irritable bowel syndrome with lorazepam, hyoscine butylbromide, and ispaghula husk. *British Medical Journal* **1**, 376–378.

SCHON, F. & KELLY, J. S. (1975). Selective uptake of $^3$H-beta-alanine by glia: Association with the glial uptake system for GABA. *Brain Research* **86**, 243–257.

STAPELFELDT, W. H., PARKMAN, H. P. & SZURSZEWSKI, J. H. (1993). The electrophysiological effects of endogenous GABA in the guinea-pig inferior mesenteric ganglion. *Journal of Physiology* **471**, 175–189.

STAPELFELDT, W. H. & SZURSZEWSKI, J. H. (1989). Neurotension facilitates release of substance P in the guinea-pig inferior mesenteric ganglion. *Journal of Physiology* **411**, 325–345.

STUDY, R. E. & BARKER, J. L. (1981). Diazepam and (−)-pentobarbital: Fluctuation analysis reveals different mechanisms for potentiation of $\gamma$-aminobutyric acid responses in cultured central neurones. *Proceedings of the National Academy of Sciences of the USA* **78**, 7180–7184.

TWYMAN, R. E., ROGERS, C. J. & MACDONALD, R. L. (1989). Differential regulation of $\gamma$-aminobutyric acid receptor channels by diazepam and phenobarbital. *Annals of Neurology* **25**, 213–220.

WIKBERG, J. (1977). Release of $^3$H-acetylcholine from isolated guinea-pig ileum. A radiochemical method for studying the release of the cholinergic neurotransmitter in the intestine. *Acta Physiologica Scandinavica* **101**, 302–317.

YOUNG, J. A. C., BROWN, D. A., KELLY, J. S. & SCHON, F. (1973). Autoradiographic localization of sites of [$^3$H]$\gamma$-aminobutyric acid accumulation in peripheral autonomic ganglia. *Brain Research* **63**, 479–486.

YUNGER, L. M., FOWLER, P. J., ZAREVICS, P. & SETLER, P. E. (1984). Novel inhibitors of gamma-aminobutyric acid (GABA) uptake: anticonvulsant actions in rats and mice. *Journal of Pharmacology and Experimental Therapeutics* **228**, 109–115.