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Uptake and stimulus-evoked release of [^{3}H]-γ-aminobutyric acid by myenteric nerves of guinea-pig intestine

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1 Following preloading with [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA), in the presence of β-alanine to inhibit glial uptake of the label, electrical stimulation caused a frequency-dependent release of tritium as [^{3}H]-GABA from isolated longitudinal-muscle myenteric-plexus preparations of the guinea-pig ileum and colon.

2 The electrically evoked efflux of [^{3}H]-GABA was Ca^{2+}-dependent, virtually abolished by preventing neuronal conduction with tetrodotoxin, and markedly reduced by preloading with [^{3}H]-GABA in the presence of nipecotic acid which is an inhibitor of high affinity GABA-uptake. Veratridine and KCl were less effective than electrical stimulation in evoking [^{3}H]-GABA release.

3 It is concluded that the electrically stimulated efflux of [^{3}H]-GABA originated from GABAergic neurones of the myenteric plexus which had taken up the label.

4 These results provide further evidence to support the suggestion that GABA is a transmitter in the mammalian enteric nervous system.

Introduction

Recent evidence suggests that γ-aminobutyric acid (GABA) may be a neurotransmitter in the mammalian enteric nervous system. In particular, the myenteric plexus of the guinea-pig intestine contains the GABA synthesizing enzyme glutamic acid decarboxylase (EC4.1.1.15), as well as endogenous GABA in low concentration (Jessen, Mirsky, Dennison & Burnstock, 1979), and a population of neurones in the guinea-pig myenteric plexus is selectively labelled by [^{3}H]-GABA under conditions favouring high affinity uptake, as shown by autoradiography (Jessen *et al.*, 1979; Krantis & Kerr, 1981a). Also, GABA, which has no direct effect on the smooth muscle of the intestine, stimulates intrinsic neurones of the guinea-pig enteric nervous system, an action antagonized by bicuculline, picrotoxinin, and furosemide or piretanide which block chloride-ion channels (Krantis, Costa, Furness & Orbach, 1980; Krantis & Kerr, 1981b). Furthermore, such GABA antagonism slows or prevents peristalsis (Krantis & Kerr, 1981c; Ong, 1981). All this is strongly indicative of a transmitter role for GABA in the intestine. However, even the demonstration of a high affinity uptake system for GABA is at best only supportive evidence for the identification of GABA as a transmitter (Bowery & Brown, 1972; Young, Brown, Kelly & Schon, 1973). Stronger and more direct evidence in the enteric nervous system would be provided by the demonstration of evoked release of GABA from myenteric plexus preparations.

In general, neurally-evoked release of amino acid transmitters is calcium-dependent (Rubin, 1970; Fagg & Lane, 1979), which can be taken as a criterion for transmitter identification, although such studies also depend upon the mode of stimulation and the conditions under which the efflux is evoked (Szerb, 1979). Nevertheless, calcium-dependent electrically-evoked release of [^{3}H]-GABA from brain slices has several times been demonstrated (Katz, Chase & Kopin, 1969; Srinivasan, Neal & Mitchell, 1969; Szerb, 1979), which prompted us to attempt a corresponding study in the enteric nervous system. The fact that [^{3}H]-GABA is also taken-up into glia by a high-affinity uptake system further complicates any such study, but this can largely be overcome by the use of β-alanine which inhibits any GABA uptake into glial cells (Schon & Kelly, 1975) thus ensuring that the greater proportion of [^{3}H]-GABA, released by electrical stimulation, will be of neuronal origin. That a neural source is involved in the release of a putative transmitter from the tissue

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can be confirmed by preventing neural activity with tetrodotoxin (TTX), and by the use of specific inhibitors to prevent neuronal uptake of transmitters. For GABA, such neuronal uptake inhibitors are L-2, 4-diamino-*n*-butyric acid (L-DABA) and (±)-*cis*-3-aminocyclohexane-carboxylic acid (ACHC) (Iversen & Kelly, 1975; Bowery, Jones & Neal, 1976), whilst nipecotic acid inhibits both glial and neuronal GABA-uptake (Schousboe, Thorbek, Herz & Krogsgaard-Larsen, 1979). In addressing the problem of GABA as a transmitter in the enteric nervous system we have now studied uptake inhibition, calcium-dependence, and tetrodotoxin-sensitivity of release, in order to show that [^{3}H]-GABA can be accumulated by longitudinal-muscle myenteric-plexus preparations of both guinea-pig ileum and colon, and subsequently released by appropriate electrical stimulation. A preliminary account of some of the results has already been given (Krantis, 1982). Also, [^{3}H]-GABA released by electrical stimulation of preloaded cat colon preparations has recently been shown to be Ca^{2+}-dependent and tetrodotoxin-sensitive (Taniyama, Kusunoki, Saito & Tanaka, 1982). The present results confirm this in the guinea-pig ileum and colon, and further show that [^{3}H]-GABA release is virtually abolished if uptake of [^{3}H]-GABA is prevented by nipecotic acid during preloading of the plexus.

Methods

Guinea-pigs of either sex weighing 250–400 g were stunned by a blow to the head and bled. Segments of ileum and of the proximal portion of the distal colon were removed and placed in Krebs solution of the following composition [mM]: Na^+ 151.0, K^+ 4.7, Ca^{2+} 2.8, Mg^{2+} 0.6, Cl^- 143.7, $H_2PO_4^-$ 1.3, HCO_3^- 16.3, SO_4^{2-} 0.6, glucose 7.7. The solution was bubbled continuously with a gas mixture of 95% O_2 and 5% CO_2, and maintained at 36°C. Lengths of longitudinal muscle coat (3 cm) with attached myenteric plexus were then dissected from the segments and kept in the Krebs solution to which had been added amino-oxyacetic acid (AOAA), 2×10^{-5} M to inhibit GABA metabolism (Wallach, 1961), and β-alanine, 10^{-3} M to prevent GABA-uptake by glial cells (Schon & Kelly, 1975). This medium was used in all subsequent manipulations.

All procedures were run in duplicate using adjacent segments of dissected intestine, and were repeated at least twice. The pieces of tissue were preincubated for 10 min in 350 µl of the perfusion medium after which [^{3}H]-GABA was added to give a final concentration of 5×10^{-9} M GABA, and incubation was continued for a further 20 min. The tissues were then removed, blotted of excess incubating medium and suspended between platinum stimulating elec-

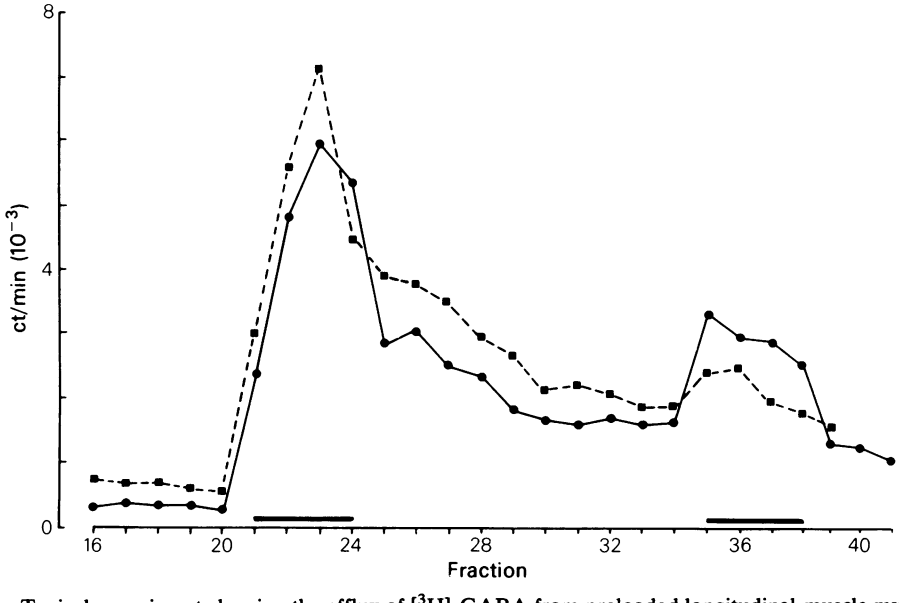

Figure 1 Typical experiment showing the efflux of [^{3}H]-GABA from preloaded longitudinal-muscle myenteric-plexus preparations, evoked by electrical stimulation: (●) guinea-pig ileum; (■) guinea-pig colon. In this and all subsequent figures each point is the radioactivity of a 2 ml fraction collected over 30 s; stimulation (20 Hz, 3 ms duration, 60 V) was applied for 2 min at the bar above the abscissa scale which shows the fraction number. Amino-oxyacetic acid, 2×10^{-5}, and β-alanine, 10^{-3} M, were present throughout. Each experiment was run in duplicate and repeated at least twice.

trodes in twin glass superfusion chambers containing 2 ml of the superfusion medium. The tissues were washed repeatedly (total 40 ml) over an equilibration period of 55 min, to establish a basal efflux of [^{3}H]-GABA, after which superfusion was commenced and 2 ml fractions collected from each chamber at 30 s intervals. Electrical stimulation (Grass S48 stimulator) was applied for 2 min periods, using 60 V and 3 ms pulse duration at various frequencies, generally 20 Hz.

The superfusate fractions were then each added to 10 ml of a Triton/toluene scintillator, and the radioactivity measured by liquid scintillation spectrometry. Radioactivity has been expressed as ct/min rather than d/min since variations in counting efficiency between samples was negligible.

To test the influence of Ca^{2+} on the stimulated efflux of tritium, the Krebs solution was substituted by a Ca^{2+}-free superfusion medium containing 0.1 mM EGTA and buffered with Tris (3 mM), the remaining constituents being as in the Krebs solution. This solution also contained AOAA and β-alanine. After being stimulated, the tissues were returned to the normal Krebs solution and [^{3}H]-GABA efflux in response to electrical stimulation again observed. Compounds used were: β-alanine, amino-oxyacetic acid (AOAA), EGTA, nipecotic acid, tetrodotoxin (TTX), (all Sigma), veratridine (Sandoz), GABA (Calbiochem, Sigma), and [^{3}H]-GABA (66 Ci mmol^{-1}; 2,3-[^{3}H]-GABA) Radiochemical Centre, Amersham).

Results

Uptake and release of [^{3}H]-GABA

The majority of experiments were conducted on preparations from the ileum. After loading with [^{3}H]-GABA, the efflux of tritium from the superfused preparations was followed over the washing period. There were two components in the unstimulated efflux of tritium: an initial, rapid washout phase with high tritium content, declining over some 30–40 min, followed by a slower, relatively constant efflux level, here termed the basal efflux. Once this basal efflux was achieved, generally within one hour, electrical field stimulation of the preparation induced an immediate increased release of tritium. The stimulated efflux of tritium reached a maximum within 2 min of stimulation at 20 Hz. These conditions were found to be optimal and used in all subsequent manipulations. On cessation of electrical stimulation the release of radioactivity again declined towards the basal efflux level. Succeeding periods of stimulation at 20 Hz were always less effective in provoking further tritium release (Figure 1).

Preparations from the distal colon treated in the same way displayed similar efflux characteristics (Figure 1), but we did not investigate this further.

Neither KCl, 56 mM, nor veratridine, 5×10^{-5} M, were effective in stimulating [^{3}H]-GABA efflux from the myenteric plexus, beyond an approximate doubling of counts with KCl depolarization and an almost indetectable increase with veratridine stimulation (not shown). Consequently we used electrical stimulation as the preferred method for evoking [^{3}H]-GABA release from the plexus preparations in order to show the effects of GABA-uptake inhibition, TTX treatment, and calcium-free solutions.

Tetrodotoxin (10^{-6} M), which would block all neural activity when added to the perfusion medium, was effective in abolishing the stimulated release of [^{3}H]-GABA from ileal preparations (Figure 2). These tests were run on duplicate, separate, tissues with and without TTX, since difficulties were encountered in washing-out the TTX rapidly enough to permit demonstration of stimulated [^{3}H]-GABA efflux from the washed, TTX-treated tissues.

When the ileal tissue was treated with the neuronal GABA-uptake inhibitor, nipecotic acid (10^{-3} M) before and during loading with [^{3}H]-GABA, the subsequent electrically-evoked efflux of tritium was markedly reduced (Figure 3).

Calcium dependence of stimulated [^{3}H]-GABA release

When the ileal tissue was preloaded with [^{3}H]-GABA, washed, and then superfused with Ca^{2+}-free

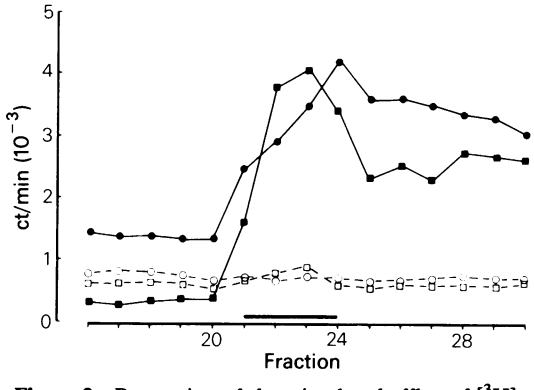

Figure 2 Prevention of the stimulated efflux of [^{3}H]-GABA, but not the resting efflux, from preloaded myenteric-plexus preparations of the guinea-pig ileum by tetrodotoxin (TTX). Segments of dissected ileum were taken at random and electrically stimulated as follows: (●, ■) normal perfusion medium; (○, □) in the presence of TTX 10^{-7} M. Stimulation of each tissue for 2 min was applied at the bar above the abscissa scale. The experiment was repeated twice with the same result.

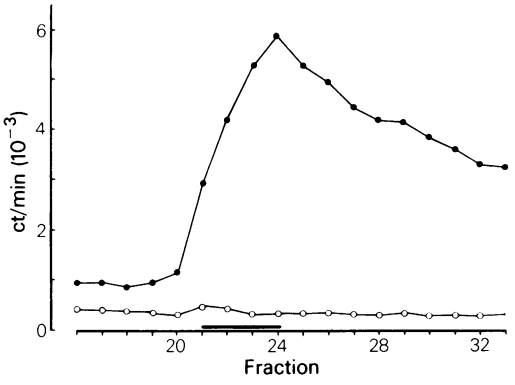

Figure 3 Reduction of the stimulated efflux of [^{3}H]-GABA from myenteric-plexus preparations of the guinea-pig ileum by inhibition of high affinity uptake of [^{3}H]-GABA during preloading: (●) normal incubation and perfusion medium; (○) in the presence of nipecotic acid, 10^{-3} M, before and during preloading. Electrical stimulation for 2 min was applied at the bar above the abscissa scale. The experiment was run in duplicate and repeated twice with the same result.

medium containing EGTA, 0.1 mM, the spontaneous efflux of tritium was unchanged, but electrical stimulation no longer evoked any additional efflux. Upon replacing the superfusion medium with that containing a normal Ca^{2+} concentration, electrical stimulation again evoked an appreciable efflux of tritium (Figure 4).

Discussion

The spontaneous output of tritium fell during the washing period, first rapidly then more slowly. Carry-over of [^{3}H]-GABA from the incubating medium, as well as washout from non-neural sources, would account for the bulk of the initial high-level tritium efflux which, despite the presence of AOAA to prevent metabolism of [^{3}H]-GABA (Wallach, 1961), probably consists not only of [^{3}H]-GABA but its metabolites, including tritiated water, for AOAA is not entirely effective in blocking GABA catabolism (Gardner & Richards, 1981). Nevertheless, once the basal efflux was reached, the bulk of the radioactivity appearing, and that recovered during electrical stimulation of the tissue, was very probably [^{3}H]-GABA since it has been found to be the major component in similar studies on other tissues, particularly the colon (Taniyama *et al.*, 1982). Homocarnosine was found by Jessen *et al.* (1979) in plexus preparations following incubation with [^{3}H]-glutamate, but whether exogenous GABA is similarly incorporated into this dipeptide by the plexus under the conditions of our experiments using [^{3}H]-GABA is not known. We therefore consider it most likely that the majority of the efflux tritium was [^{3}H]-GABA released from neurones by the stimulation, although the use of t.l.c. with various solvent systems would provide more sure, chromatographic, evidence for this.

It can be discounted that significant [^{3}H]-GABA could be released from some non-neural source as a

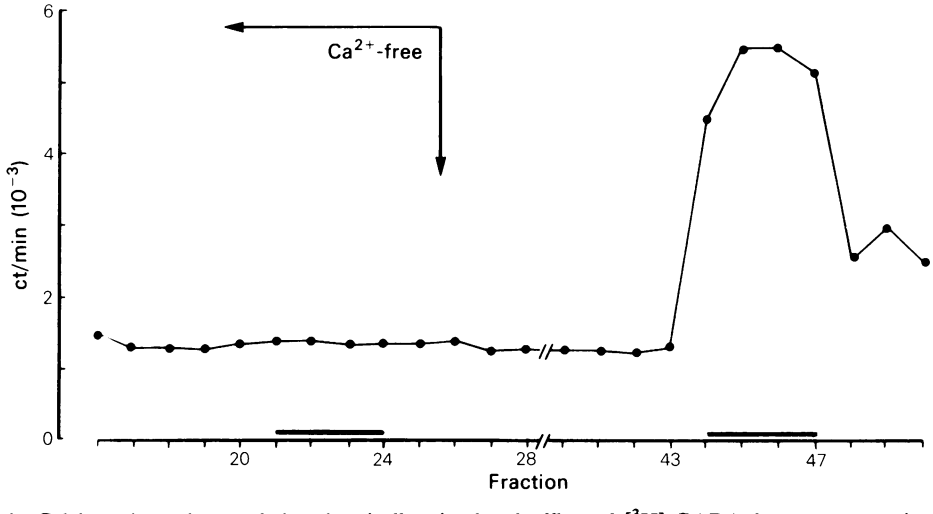

Figure 4 Calcium dependence of the electrically-stimulated efflux of [^{3}H]-GABA from a myenteric-plexus preparation preloaded and then stimulated, first in Ca^{2+}-free Krebs solution (with EGTA 3 mM), and then in normal perfusion medium. The results are typical and were repeated twice. Basal efflux was not altered by the Ca^{2+}-free medium, whereas electrical stimulation, at the first bar, was ineffective. Subsequent stimulation, second bar, in normal medium with Ca^{2+} present caused an appreciable tritium efflux.

result of smooth muscle contractions during the stimulation period since the efflux was greatly reduced by inhibiting neural [^{3}H]-GABA uptake with nipecotic acid. It is also unlikely that release from glia could contribute significantly to either the spontaneous or the stimulated efflux of [^{3}H]-GABA since β-alanine was present throughout and is an effective inhibitor of GABA-uptake by glia (Schon & Kelly, 1975).

Autoradiographic evidence has shown that the major part of [^{3}H]-GABA accumulated by ileal longitudinal-muscle myenteric-plexus preparations is incorporated into a population of neurones of the plexus under conditions of high affinity uptake (Krantis & Kerr, 1981a). It can therefore be assumed that similar accumulation of [^{3}H]-GABA had occurred in the plexus of the ileum and colon of the present study under comparable conditions of high affinity uptake. In autoradiographs there is clear evidence of a population of cell-bodies labelled with [^{3}H]-GABA in the ganglia of Auerbach's plexus, together with profuse labelling of processes in the primary, secondary and tertiary meshworks of the plexus (Krantis & Kerr, 1981a). Furthermore, L-DABA and nipecotic acid, which are inhibitors of neuronal GABA uptake, prevented this labelling in the autoradiographs (to be detailed elsewhere). In the present study nipecotic acid also greatly reduced the stimulated-efflux of [^{3}H]-GABA relative to that from untreated tissues. Such labelled neurones and processes are thus the most likely source of the [^{3}H]-GABA released from the plexus by electrical stimulation in the present study. Both tetrodotoxin and calcium-free superfusion were able to stop the stimulated efflux of [^{3}H]-GABA, again confirming its neural origin. Frequencies of stimulation above 20 Hz were less effective in evoking [^{3}H]-GABA release, which would suggest the existence of GABA autoreceptors capable of depressing output of transmitter by a prejunctional inhibitory feedback mechanism. Alternatively the release may have been depressed by some other, co-released, transmitter, or else the presynaptic fibres may simply not be able to conduct at stimulus rates above 20 Hz.

Somewhat surprisingly, it was found that neither veratridine nor KCl were as effective as electrical stimulation in provoking [^{3}H]-GABA efflux from ileal plexus preparations, although both have been used in the study of such efflux from other tissues (Srinivasan *et al.*, 1969; Minchin, 1979; Szerb, 1979). Whilst veratridine stimulation distinguishes neuronal from glial sources of transmitter efflux (Neal & Bowery, 1979), the more usual criterion of Ca^{2+}-dependence for neuronal release of transmitter fails when veratridine is used as the stimulus. On the contrary, veratridine causes an increased output of transmitter in the absence of Ca^{2+} (Minchin, 1979; Szerb, 1979; Cunningham & Neal, 1981). Not having investigated the influences of varying Ca^{2+} on the [^{3}H]-GABA efflux evoked by veratridine or KCl in the present study, we have no explanation for the relatively small tritium efflux they caused in the isolated intestine, particularly since KCl would be expected to release [^{3}H]-GABA from both glial and neuronal sources, but especially from neurones (Neal & Bowery, 1979).

There are conflicting reports concerning calcium dependence of transmitter output when using electrical stimulation. Although Szerb (1979) showed such dependence, Srinivasan *et al.* (1969) found a slightly increased [^{3}H]-GABA efflux from electrically-stimulated brain slices in the presence of low Ca^{2+} without chelating agent, which is to be expected since there could well be an electrically-hyperexcitable phase during progressive depletion of membrane Ca^{2+} under these conditions. In the present study this was avoided by the use of EGTA without Ca^{2+} in the perfusing medium, thus more rapidly depleting Ca^{2+} from the membranes. There is the possibility that such treatment, particularly if prolonged, might damage the tissue and prevent transmitter output, but evidently this did not occur for there was a prompt return of the stimulated [^{3}H]-GABA efflux upon restoring the Ca^{2+} content in the perfusion medium to normal. Electrical stimulation thus remains a valid method for eliciting putative transmitter output from the functional myenteric plexus, as has already been shown in the ileum for cholinergic transmission using [^{3}H]-choline (Szerb, 1976; Kilbinger & Wessler, 1980), and for [^{3}H]-5-hydroxytryptamine (Schultz & Cartwright, 1974; Jonakait, Tamir, Gintzler & Gershon, 1979), as well as for [^{3}H]-adenosine triphosphate from taenia coli of the guinea-pig (Su, Bevan & Burnstock, 1971). Since it is possible, in the present experiments, that [^{3}H]-GABA might have been accumulated as a false transmitter by some neurones of the plexus, it would be desirable to show the release of endogenous GABA from the plexus, as well as to demonstrate, by immunohistochemical localization of GAD, that the GAD found there (Jessen *et al.*, 1979) is indeed located in neurones of Auerbach's plexus. Nevertheless, the ability of nipecotic acid to prevent [^{3}H]-GABA uptake and stimulated release is strong evidence that the released [^{3}H]-GABA observed in this study had originated from neurones of the plexus, particularly since glial uptake would have been already blocked by the combined presence of β-alanine in the medium. We conclude that the [^{3}H]-GABA released from the myenteric plexus is of neural origin, the release being Ca^{2+}-dependent and tetrodotoxin-sensitive, and that GABA may be a transmitter in the mammalian enteric nervous system.

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