GABA immunoreactivity and ^3H -GABA uptake in mucosal epithelial cells of the rat stomach

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SUMMARY GABA, best known as a neurotransmitter in the central nervous system, is also present in various peripheral tissues including the gastrointestinal tract, where there is strong evidence that GABA acts as a transmitter in some intrinsic myenteric neurones. Several studies indicate that the gastric mucosa is one of the sites of action of GABA in the gut. Highly specific anti-GABA antibodies have been used to detect endogenous GABA in the mucosa of the rat gastrointestinal tract, and ^3H -GABA uptake followed by autoradiography has been used to localise cells with uptake sites for exogenous GABA. It was found that although GABA immunoreactive nerve fibres are essentially absent from this site, some mucosal cells are strongly GABA-immunoreactive. These cells are common in the pyloric stomach and upper part of the small intestine. The autoradiographic experiments provide evidence that these cells also possess high-affinity GABA uptake sites. These observations raise the possibility that in the gastrointestinal tract GABA acts as a gut hormone in a subpopulation of mucosal endocrine cells, in addition to its role as an enteric neurotransmitter.

GABA which is a major neurotransmitter in the vertebrate brain and spinal cord, is also present in many neural and non-neural tissues outside the central nervous system (CNS).¹ Within one system in the periphery, the myenteric plexus of the gastrointestinal tract, there is now strong evidence that GABA acts as a neurotransmitter in a population of peripheral neurones,²⁻⁷ while the functional role of the GABA present in various non-neural tissues remains obscure. In the rat pancreatic islets GABA is found in the B endocrine cells, possibly indicating a hormonal function,⁸ although neither GABA release nor uptake has yet been demonstrated in these cells. The presence of immunohistochemically detectable glutamic acid decarboxylase (GAD), the major GABA synthesising enzyme in central GABAergic neurons, in some small intensely fluorescent (SIF) cells in rat sympathetic ganglia might also relate to a local hormonal role for GABA at this site.⁹

The present paper deals with the localisation of endogenous and exogenous GABA in another tissue containing endocrine cells – that is, the gastrointestinal mucosa, a tissue which contains a variety of hormones and regulatory peptides.¹⁰⁻¹² Highly specific anti-GABA antibodies have been used to detect endogenous GABA in the mucosa of the rat gastrointestinal tract, and ^3H -GABA uptake and autoradiography has been used to determine whether selective uptake and retention of exogenous ^3H -GABA occurs in mucosal cells. We find that although GABA immunoreactive nerve fibres are essentially absent from this site,¹³ a subpopulation of mucosal cells are strongly GABA-immunoreactive. These cells are most common in the pyloric stomach and upper part of the small intestine. The autoradiographic experiments provide evidence that these cells also possess high affinity GABA uptake sites.

Methods

ANIMALS

Male or female rats weighing 90–120 g were killed by a blow on the head. Segments of the gastrointestinal

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Fig. 1 Immunofluorescence micrographs showing the mucosa in the pyloric region of the rat stomach after application of anti-GABA antibodies. [M] marks the position of the mucosal muscle. In (a), many strongly GABA-immunostained mucosal cells are seen in the glandular part of the mucosal epithelium, while fewer immunoreactive cells are present in a comparable area in (b). The cells have various shapes, presumably depending on the plane of section through each cell. ($\times 300$)

tract were placed in oxygenated Krebs's solution, cut open, pinned down on Sylgard plates and all visible intestinal contents rinsed off. The tissues were then fixed in 4% paraformaldehyde, 0.5% glutaraldehyde in phosphate buffered saline (PBS) for three hours. After washing in PBS, tissues were treated with sodium borohydride (0.5 mg/ml) made up in PBS at pH 8.8 for a total of 90 minutes in six different changes of solution, in order to reduce background fluorescence caused by glutaraldehyde. After further washes in PBS, the preparations were cryoprotected in 30% sucrose/PBS overnight. Cryostat sections (5–10 μ m) were thawed onto gelatin coated glass slides and air dried for several hours. The sections were treated with Triton X-100 (0.15%) in PBS for 30 minutes before the immunohistochemical procedure. The primary antiserum, diluted 1:4000 to 1:5000, was applied to the sections on the glass slide in a moist chamber, and left overnight. Subsequent to three washes in PBS, biotinylated donkey antirabbit

immunoglobulin (Amersham International plc) (1:100) was applied for one hour. After further washes, streptavidin fluorescein or streptavidin Texas Red (Amersham International plc) (1:100), was applied for 20–30 minutes, after which time the sections were again washed in PBS. The sections were mounted in Citifluor (City University, London) and viewed with a fluorescence microscope. The antiserum and immunochemical reagents were diluted in a solution containing: fetal calf serum (10%); sodium azide (0.02%); lysine (0.1 M), in PBS. All procedures were carried out at room temperature except where stated.

ANTISERA

The antiserum to GABA (Code no GABA7) was raised in rabbits and recognises a condensation product of GABA and glutaraldehyde. The antiserum has been extensively characterised^{14–16} and was kindly donated by Dr Peter Somogyi.

SPECIFICITY OF THE IMMUNOHISTOCHEMICAL REACTION

Specificity was tested by replacing the primary antiserum with non-immune rabbit serum at the same dilution as the anti-GABA antiserum, with anti-GABA antiserum adsorbed with GABA before immunolabelling or with anti-GABA antiserum adsorbed with irrelevant antigens. To obtain serum adsorbed with GABA, the diluted antiserum was subjected to solid phase adsorption, using the antigen coupled to polacrylamide beads, essentially as described by Somogyi *et al.*¹⁶ To obtain sera adsorbed with irrelevant antigens, aliquots of anti-GABA antiserum diluted to twice the final staining concentration were incubated at 4°C overnight with equal amounts of vasoactive intestinal polypeptide (VIP), substance P (SP), L-enkephalin (L-ENK), somatostatin (SOM), or cholecystokinin (CCK). The concentration of all peptides in these incubation

Fig. 2 Fluorescence micrograph of the pyloric region of rat gastric mucosa treated with antibodies against GABA. One immunolabelled cell is present carrying a narrow process. ($\times 600$)

Fig. 3 Autoradiograph of the rat pyloric mucosa after incubation in $2 \times 10^{-6} \text{ M } ^3\text{H}$ -GABA. [M] marks the position of the mucosal muscle. Five heavily labelled cells lie scattered in the glandular mucosa. ($\times 300$)

mixtures was 10^{-5} M . The peptides were purchased from Cambridge Research Biochemicals Ltd, UK (VIP, SP, L-ENK, CCK) and Calbiochem Biochemicals and Immunochemicals, San Diego, USA (SOM).

AUTORADIOGRAPHY

Gut segments were rinsed as described above, mucosal segments dissected free of the muscularis externa, and then incubated in oxygenated Krebs' solution containing ^3H -GABA at a concentration of $2 \times 10^{-6} \text{ M}$ ($100 \mu\text{Ci/ml}$) or $2 \times 10^{-7} \text{ M}$ ($10 \mu\text{Ci/ml}$) (57 Ci/mmol [$2,3\text{-H}^3$ GABA], Amersham International plc) for 35 minutes at 37°C. The tissues were then washed briefly in Krebs' solution. Tissues which were to be processed for autoradiography only, were fixed

in 5% glutaraldehyde in PBS for two hours, dehydrated and embedded in resin (Durcupan ACM, Fluka). One micron sections were dried on to gelatin coated glass coverslips, dipped in Ilford K-5 emulsion diluted 1:1 with water at 42°C, gelled on an ice cold metal plate and allowed to dry at room temperature. After four to 21 days exposure at 4°C the slides were developed with Ilford Contrast FF developer. In some instances consecutive sections were dried on to separate slides, stained briefly with toluidine blue and kept for comparison with the autoradiographic sections. Tissues which were to be processed for both autoradiography and immunohistochemistry were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde in PBS after incubation in ^3H -GABA, treated with borohydride, processed for immunohistochemistry and dipped in photographic emulsion as described above.

Results

IMMUNOHISTOCHEMISTRY

Application of GABA antibodies to 5–10 μm fixed frozen sections from the rat stomach revealed a population of strongly immunoreactive mucosal

cells. The number of GABA immunopositive cells varied considerably from sample to sample, although tissue was always taken from the lowest third of the stomach and in the region of the lesser curvature, suggesting that within this area the GABA-containing cells may be unevenly distributed (Fig. 1). These cells could not be seen in the surface epithelium but were confined to the epithelium of the gastric glands. They appeared scattered throughout the glandular epithelium and always occurred singly. The shape of individual cells varied greatly, presumably depending on the plane of section. In many cases they could be seen to give rise to a narrow, tapering process (Fig. 2). GABA-immunoreactive mucosal cells were most frequent in the lower third of the stomach and in the jejunum/duodenum, although they could be found in the ileal mucosa and in the body of the stomach; they were not found in the mucosa of the distal colon.

No immunostained cells were seen in the gastric mucosa if the GABA antiserum had been adsorbed with GABA coupled to sepharose beads before staining, while adsorption with VIP, SP, L-ENK, SOM, or CCK, all at 10^{-5} M, did not affect the immunostaining. Immunostained mucosal cells were

Fig. 4 (a): Autoradiograph of the rat gastric mucosa after incubation in 2×10^{-7} M ^3H -GABA. One autoradiographically labelled cell is present (arrow). (b): Bright field micrograph of an adjacent plastic section (sections a and b are 1 μm thick) stained with toluidine blue. The cell which is labelled with autoradiographic grains in (a) is clearly visible in (b) (arrow). ($\times 500$)

Fig. 5 Autoradiograph (a) and bright field micrograph of adjacent section stained with toluidine blue (b) from the rat gastric mucosa; similar to Fig. 4. The autoradiographically labelled cell seen in (a) (arrow) is clearly visible in (b) (arrow). ($\times 500$)

not present in experiments in which the GABA antiserum was replaced by non-immune rabbit serum.

AUTORADIOGRAPHY

To test the possibility that the GABA cells in the gastric mucosa possessed high affinity uptake sites for GABA, such as those found in the membrane of GABAergic neurones and of glial cells, pieces of gastric mucosa were incubated in 2×10^{-6} M ^3H -GABA (100 $\mu\text{Ci}/\text{ml}$) for 35 minutes at 37°C , fixed, embedded in resin and processed for autoradiography. The samples were developed after four, 13, and 21 days of exposure. It was found that while the majority of mucosal cells were completely unlabelled, some cells were densely covered by autoradiographic grains (Figs 3–6). In other experiments the mucosal pieces were incubated in a 10-fold lower concentration of ^3H -GABA (10 $\mu\text{Ci}/\text{ml}$) and the samples developed at four, 13, and 21 days. In this case, only a few grains were seen over mucosal cells after four days of exposure, while heavy grain labelling was again seen over some mucosal cells in

samples developed after 13 and 21 days. Like the GABA-immunolabelled mucosal cells, the autoradiographically labelled cells were restricted to the glandular epithelium and were not detected in the mucosa of the distal colon. Although the autoradiographic grains did not reveal cell shape with the accuracy of immuno-staining, some grain-labelled cells appeared to give rise to a tapering process (Fig. 6).

Experiments were also carried out in which mucosal pieces were incubated in 2×10^{-6} M ^3H -GABA, fixed, sectioned in a cryostat and immunolabelled using GABA antibodies before being processed for autoradiography. The samples were developed after four or six days. This revealed four populations of cells in the glandular epithelium: the majority of cells, which were neither immunolabelled nor showed autoradiographic grains, many cells which were both immunolabelled and covered with autoradiographic grains, and other cells which were either immunolabelled or grain labelled. In double labelled cells it was often hard to detect the immunolabelling beneath the cover of autoradiographic

Fig. 6 Autoradiograph from the rat gastric mucosa after incubation in 2×10^{-7} M 3 H-GABA. Two heavily labelled cells are present, one of which (arrow) shows a tapering process. ($\times 600$)

grains. In most of these cells the immunofluorescence could only be seen as a narrow halo around the grain-covered area (Fig. 7) and it seemed clear that in most cells with dense grain cover, immunofluorescence, even if present, would be completely obscured. Double labelling could therefore only be detected in cells with medium to low grain density.

Discussion

The work reported here provides evidence, but does not prove, that the glandular epithelium of the rat gastrointestinal tract, in particular that of the pyloric stomach and upper small intestine, harbours a population of cells which contain high concentrations of endogenous GABA. Furthermore, highly selective and heavy autoradiographic grain labelling of cells in the mucosal epithelium is seen if the gastric mucosa is incubated in low (10^{-6} M and 10^{-7} M) concentrations of 3 H-GABA, suggesting that these cells possess high

Fig. 7 A micrograph showing a section from the rat pyloric mucosa which has been incubated with anti-GABA antibodies and treated with immunohistochemical methods (a) and processed for autoradiography (b). In the fluorescence micrograph (a) two GABA-immunostained cells can be seen, one of which is also labelled with autoradiographic grains which mostly obscure the immunostaining (arrow). In the bright field micrograph of the same field (b), only the cell labelled with autoradiographic grains is visible (arrow). ($\times 750$)

affinity GABA uptake sites. The results of experiments in which individual sections from the gastric mucosa were processed for immunolabelling and autoradiography are consistent with the view that the cells possessing GABA uptake sites, and the cells containing high concentrations of endogenous GABA are, at least in part, the same cell population (see below). These observations, when taken together with functional studies (see below), raise the possibility that in the gastrointestinal tract GABA acts as a gut hormone of a subpopulation of mucosal endocrine cells, in addition to its role as an enteric neurotransmitter.²⁻¹⁷ To test this possibility further, it will be important to determine if GABA release can be obtained from mucosal preparations, and to study whether GABA colocalises with established gut hormones such as gastrin or somatostatin.

The notion that the GABA-immunolabelled and autoradiographically labelled cells are the same cell population receives support from the fact that among neurones, high affinity GABA uptake sites and GABAergic properties are highly correlated,¹⁸⁻²⁰

from the observation that many mucosal cells showed both GABA immunoreactivity and autoradiographic grains, and from the general similarities in distribution and appearance of the cells revealed with the two methods. On the other hand it is possible that cells which accumulate ³H-GABA, but do not contain endogenous GABA, might show GABA immunoreactivity as a result of the exogenous GABA only, although studies on GABA uptake and immunolabelling in the monkey striate cortex indicate that this is not probable.²⁰ It is also clear that many cells were only immunolabelled or grain labelled, which could suggest the existence of two separate populations. An alternative explanation is equally likely, however – that is, within any one tissue section, neither the immunohistochemistry nor the autoradiography faithfully reveals the complete population of cells containing endogenous GABA or high affinity GABA uptake sites, respectively. This would result in both properties being revealed in some cells, while only one or the other was apparent in other cells, which is consistent with the observations made in the present study. Evidently more experiments are needed to determine unambiguously the degree of overlap between the immunohistochemically and autoradiographically labelled cell populations. It also remains to be established whether the GABA-uptake sites in the mucosal cells are pharmacologically more similar to neuronal or glial uptake sites. This kind of analysis in other non-neuronal cells shows that in some tissues – such as, the thyroid, the uptake sites are more akin to those in neurones, while in other tissues – for example, ovaries, they are more similar to glial uptake sites.²¹ The functional significance, if any, of these differences is not clear.

What might be the role of these putative GABA-secreting mucosal endocrine cells? Pharmacological experiments show that exogenous GABA, either indirectly or directly, affects mucosal function. Thus GABA has an anti-ulcer effect in a variety of model systems using intact animals,²² while in isolated fragments of gastric mucosa, GABA stimulates gastrin secretion.^{23,24} In this system there is evidence that GABA acts through modulation of acetylcholine release from cholinergic terminals present in the vicinity of the gastrin releasing cells. These experiments raise the possibility that physiological control of mucosal activity may be carried out in part by endogenous GABA. The present study reinforces this possibility by showing that the mucosa contains an endogenous source of GABA – namely, the GABA immunoreactive epithelial cells. This observation is particularly important, as a previous study has shown that few, if any, of the GABA containing myenteric neurons project into the mucosal layer.¹³ Based on immunohistochemical evidence, the cells

described in this study are therefore the only significant source of GABA in the rat gastric mucosa.

The implication of the present work – that is, that GABA, a major central neurotransmitter, may act in the gut as a neurotransmitter and as a local gut hormone is not without precedent, because several substances presumed to be transmitters in central neurones – for example, VIP, substance P, enkephalin, and serotonin, are also considered to have a dual role in the gut, acting as neurotransmitters and enteric hormones.²⁵

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