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INDIRECT EFFECTS OF AMINO-ACIDS ON SYMPATHETIC GANGLION CELLS MEDIATED THROUGH THE RELEASE OF γ-AMINOBUTYRIC ACID FROM GLIAL CELLS

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1 All experiments were performed on rat isolated desheathed superior cervical ganglia maintained in Krebs solution containing amino-oxyacetic acid (10 μM) at 25°C.

2 Influx rates of γ-amino-*n*-butyric acid (GABA) were measured by incubating ganglia in 0.5 μM [^{3}H]-GABA for 30 minutes. Influx was inhibited by 50% on adding 14.3 μM unlabelled GABA, 59.2 μM β-alanine (BALA) or 424 μM β-amino-*n*-butyric acid (BABA).

3 Efflux of [^{3}H]-GABA into non-radioactive solution superfused over ganglia previously incubated for 60 min in 1 μM [^{3}H]-GABA was measured. The mean resting efflux rate coefficient (k) was $0.64 \pm 0.05 \times 10^{-3}$ min^{-1}. Addition of high concentrations of unlabelled GABA, BABA or BALA to the superfusing solution increased k by (maximally) 3.6–4.3 times; half-maximal increases occurred at the following concentrations: GABA, 16 μM; BALA, 85 μM; BABA, 606 μM. Replacement of external Na^+ with Li^+ or TRIS increased the resting value of k and inhibited acceleration by external amino acids. Prior incubation in 1 μM [^{3}H]-GABA with 1 mM unlabelled GABA increased resting k 1.5 times, but did not alter the peak rate coefficient produced by external amino acids.

4 Neuronal depolarization produced by the amino acids was measured with surface electrodes. Pre-incubation in 1 mM GABA for 60 min potentiated low-amplitude responses to BALA or BABA but not those to GABA or 3-aminopropanesulphonic acid (a potent agonist with low affinity for the GABA carrier). Omission of external Na^+ reduced responses to BABA but increased those to GABA.

5 Incubation in 1 mM GABA for 60 min (as required to potentiate BABA or BALA actions) increased the amount of GABA in the tissue from 0.21 to 0.73 mmol/kg wet weight. Autoradiographs in which labelled GABA was used indicated that uptake into neuroglial cells was responsible for this accumulation.

6 It is suggested that: (i) BALA and BABA are substrates for the inward GABA carrier responsible for GABA entry into ganglionic glial cells; (ii) they accelerate efflux by inhibiting carrier-mediated re-accumulation of effluent GABA by the glial cells; (iii) interstitial GABA concentrations are thereby increased to a level capable of depolarizing adjacent neurones; and (iv) this, rather than direct GABA-receptor activation, accounts for the depolarization produced by low concentrations of BALA and BABA. Potentiation of their depolarizing action after pre-incubation in 1 mM GABA is suggested to result from the increased amount of intracellular GABA available for release, and is quantitatively compatible with this increase; inhibition in Na^+-free solution is due to their inability to inhibit re-accumulation of GABA under these conditions.

7 A model for the action of carrier substrates is described in an Appendix. Calculations based thereon yield increments in interstitial GABA concentration in the presence of carrier substrates compatible with those determined experimentally (up to 1 μM at rest or 3.4 μM after pre-incubation in GABA).

Introduction

There is no evidence to suggest that the central inhibitory neurotransmitter γ-aminobutyric acid

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(GABA) subserves any such function in sympathetic ganglia. Nevertheless, sympathetic ganglia share with brain two properties associated with GABA.

First, GABA increases chloride permeability in sympathetic neurones (Adams & Brown, 1975). This leads to a membrane depolarization (De Groat, 1970; Bowery & Brown, 1974). Although the direction of the potential change is opposed to that usually recorded in central neurones (Dreifuss, Kelly & Krnjević, 1969), the ionic permeability change is the same and available evidence suggests a close similarity between the receptors at the two sites with respect to ligand specificity.

Second, neuroglial cells surrounding sympathetic ganglion cells and fibres accumulate exogenous GABA through a high-affinity carrier-mediated transport system (Bowery & Brown, 1972; Young, Brown, Kelly & Schon, 1973). This would appear to be broadly representative of a glial cell transport system elsewhere in the peripheral nervous system (Schon & Kelly, 1974) and in the brain (Henn & Hamberger, 1971; Schon, Beart, Chapman & Kelly, 1975; Iversen & Kelly, 1975).

The present experiments arose out of certain observations concerning the efflux of GABA from glial cells in sympathetic ganglia (Bowery & Brown, 1972; Bowery, 1974). When ganglia are pre-incubated with radioactively-labelled GABA and then washed with non-radioactive solution, the accumulated radioactivity leaves the ganglion at a slow steady rate. The rate of release is then increased on adding unlabelled GABA to the washing solution. A similar acceleration may be produced by other compounds which are transported into the glial cells via the GABA-carrier, such as β-alanine (BALA: Bowery & Brown, 1972; Bowery, Brown & Yamini, 1975, and unpublished observations; see also Schon & Kelly, 1975).

This raised two questions: (1) Could the substrate-accelerated release of GABA from the glial cells generate a sufficiently high interstitial concentration of GABA to depolarize the adjacent neurones? (2) Since there is some similarity (though not identity) between the structural requirements for a depolarizing agonist and a carrier substrate, to what extent might GABA-mimetic effects be generated indirectly through the release of GABA rather than through a direct effect on the neuronal receptors?

We have attempted to probe these questions using two compounds, β-alanine (BALA) and β-amino-*n*-butyric acid (BABA): both possess a higher affinity for the carrier than for the receptor, and so appeared prospectively suited for discerning such indirect GABA-mimetic effects. In this paper we describe first their interaction with the transport of labelled GABA in isolated sympathetic ganglia of the rat, and second some experiments on their depolarizing action on the ganglion. A model for the action of these compounds, and some calculations of both changes in GABA efflux rates and changes in interstitial GABA concentrations arising therefrom, are described in an Appendix. A brief note on the action of BABA has been published (Bowery, Brown & Marsh, 1975).

Methods

Superior cervical ganglia were isolated from rats (200–300 g, either sex) anaesthetized with urethane (1.5 g/kg). The connective tissue sheath was removed from the ganglion (and its attached pre- and postganglionic nerve trunks) and the preparation was maintained in Krebs solution at 25°C bubbled with 95% O_2 and 5% CO_2. The composition of the Krebs solution was (mM) NaCl 118, KCl 4.8, $CaCl_2$ 2.52, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4$ 1.19, D-glucose 11 (pH 7.4). Na^+-free TRIS solution was prepared by replacing NaCl and $NaHCO_3$ with 143 mM TRIS-base (tris (hydroxymethyl) aminomethane) and adding 118 mequiv/l HCl to pH 7.4 (bubbled with 100% O_2).

Influx of GABA

The rate of inward GABA transport was measured from the amount of radioactivity accumulated after incubation in [^{3}H]-GABA. Isolated ganglia were incubated in Krebs solution at 25°C (at least 0.5 ml per ganglion) containing 10 μM amino-oxyacetic acid (AOAA) to inhibit transamination of accumulated GABA and 0.1 μM [^{3}H]-GABA. After 30 min the ganglia were removed, rinsed briefly (about 1 s) in non-radioactive solution, weighed and tissue radioactivity measured as described previously (Bowery & Brown, 1972). Inhibitors were added simultaneously with [^{3}H]-GABA. The concentration of [^{3}H]-GABA in the tissue was calculated in terms of the specific activity of [^{3}H]-GABA in the surrounding medium, as mmol/kg wet wt, and divided by the concentration in the medium (mmol/l), to yield a tissue/medium ratio. Saturable, carrier-mediated influx was calculated after subtracting the tissue/medium ratio (0.7) obtained at 5 mM external GABA to correct for the small (~5%) component of apparently non-saturable uptake (Bowery, 1974). Tritium retained in the presence of AOAA was >95% unchanged [^{3}H]-GABA (Bowery & Brown, 1972; Walsh, Bowery, Brown & Clark, 1974). Uptake is linear with time to 30 min, so provides an approximation to the initial influx velocity.

Efflux of GABA

Ganglia were incubated in [^{3}H]-GABA solution and then superfused with non-radioactive solution at a constant rate of 0.5 ml/minute. Both incubation and superfusion fluids contained 10 μM AOAA, such that effluent ^{3}H was unchanged [^{3}H]-GABA (see below).

Effluent superfusate was collected at 2 min intervals and its radioactivity measured. After completing the period of superfusion the residual radioactivity in the ganglion and nerve trunks was also determined. The rate of effluent radioactivity could then be expressed as the efflux rate coefficient, that is, the amount of radioactivity collected during a 2 min period divided by the arithmetic mean of the total radioactivity present in the tissue at the beginning and end of the same collection period. The latter was found by cumulatively summing the effluent radioactivity from the end of that collection period onwards with the residual radioactivity in the ganglion. Since the rate coefficient (k) so measured is very small compared with the collection period and with the rate of clearance from the extracellular space (see Brown & Scholfield, 1974) it approximates closely to that given by the differential equation

$$k = -\frac{dC_{in}}{dt} \cdot (C_{in})^{-1} \ (\text{min}^{-1})$$

assuming single compartment kinetics, where C_{in} is the intracellular GABA concentration. Contamination of effluent [^{3}H]-GABA by tritiated metabolites was assessed in separate control experiments. Several ganglia were incubated for 4 h in 5 μM [^{14}C]-GABA in the presence of AOAA and then incubated in 4 ml Krebs solution at 25°C (bubbled continuously with 95% O_2 and 5% CO_2) for up to 17 hours. The incubation fluid was concentrated by evaporation, solutes redissolved in 200 μl de-ionized water and subjected to paper electrophoresis (Walsh *et al.*, 1974). When AOAA (10 μM) was present in the effluent medium, >98% of radioactivity was identified as [^{3}H]-GABA. Hence, we assume that measured radioactivity during experimental runs also refers to unchanged [^{3}H]-GABA.

Total GABA

In a few experiments endogenous concentrations of GABA and several other amino acids, and net changes in GABA levels following incubation in GABA solution, were measured by a dansylation method. After an appropriate period of incubation, individual ganglia were weighed and homogenized in 100 μl of distilled water containing a known number of d/min of ^{14}C-labelled aspartate, glutamate, glutamine, glycine, alanine and GABA and [^{35}S]-taurine as internal standards. An equal volume of acetone was then added and the mixture evaporated to dryness by blowing compressed air over the surface of each sample. The amino acids were dissolved in 7 μl of a solution of sodium hydroxide (pH 11.3) and reacted with [^{3}H]-5-dimethylaminonaphthalene-sulphonyl chloride ([^{3}H]-dansyl chloride) at 40°C in the dark for 30 min (Briel & Neuhoff, 1972). The radioactive dansyl-amino acids were separated from each other and from contaminants by thin layer chromatography (Joseph & Halliday, 1975) and the radioactivity estimated by scintillation spectrometry. The yield of each dansyl-amino acid was ascertained by estimating the recovery of the internal standards and results have been expressed as mmol/kg wet tissue weight.

Autoradiographs

Ganglia were incubated in [^{3}H]-GABA solution with added AOAA, rinsed for 60 min in non-radioactive solution, fixed with glutaraldehyde, embedded in paraffin, sectioned at 5 μm and autoradiographs prepared as described by Young *et al.* (1973). Glutaraldehyde binds 30–60% of accumulated [^{3}H]-GABA; the retained label shows a distribution pattern comparable to that obtained using freeze-dried frozen sections (see Young *et al.*, 1973).

Depolarization

Ganglion cell responses to GABA and analogues were monitored as surface-depolarization recorded as described by Bowery & Brown (1974) with a modification for continuous superfusion (Brown & Marsh, 1975). Potential changes were recorded continuously with time on a potentiometric chart recorder. Agonists were applied by superfusion for 4 min at 20–30 min intervals, to minimize desensitization.

Compounds

[^{3}H-2,3]-γ-aminobutyric acid (10 Ci/mmol) was obtained from New England Nuclear. Purity (> 95%) was checked as described previously (Bowery & Brown, 1972). [^{3}H]-dansyl chloride (8 Ci/mmol, uniformly labelled) was obtained from New England Nuclear and ^{14}C- and ^{35}S-labelled amino acids were obtained from the Radiochemical Centre. Unlabelled compounds were obtained as follows: γ-aminobutyric acid (4-amino-*n*-butyric acid, GABA), BDH; β-alanine (3-aminopropropionic acid, BALA), BDH; DL-β-amino-*n*-butyric acid (DL-3-aminobutanoic acid, β-aminobutyric acid, BABA), Sigma; 3-amino-propanesulphonic acid (3-APS), K & K Laboratories. Methylbicuculline was synthesized by J.F. Collins according to the method of Johnston, Beart, Curtis, Game, McCulloch & MacLachlan (1972).

Results

The results are divided into two sections. Part A concerns interaction of β-alanine (BALA) and β-amino-*n*-butyric acid (BABA) with the inward glial-cell carrier for GABA, as determined by their effects on the influx and efflux of [^{3}H]-GABA; part B concerns the depolarization of the neurones by BALA

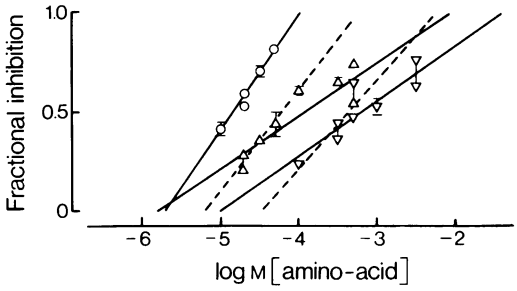

Figure 1 Effect of unlabelled γ-aminobutyric acid (GABA, ○), β-alanine (BALA, △) and β-amino-*n*-butyric acid (BABA, ▽) on the influx of [^{3}H]-GABA into rat isolated superior cervical ganglia. Ganglia were incubated for 30 min at 25°C in 0.5 μM [^{3}H]-GABA in the presence of 10 μM amino-oxyacetic acid, with or without unlabelled amino acid. Tissue concentrations of [^{3}H]-GABA were corrected for infiltration of the extracellular space and for non-saturable cellular uptake (see Methods section) to determine the saturable influx rate. Influx rate in the presence of unlabelled amino acid was expressed as a fraction of pooled controls ($n=26$), to give fractional inhibition of uptake (ordinates). Abscissa scale gives the log molar concentration of unlabelled amino acid. Points show single determinations or means ± s.e. (bars) where $n \nless 3$. Lines are least-squares regression coefficients for the full range of amino acid concentrations (———) or for concentrations corresponding to fractional inhibition $\ngtr 0.6$ (- - - -, see text).

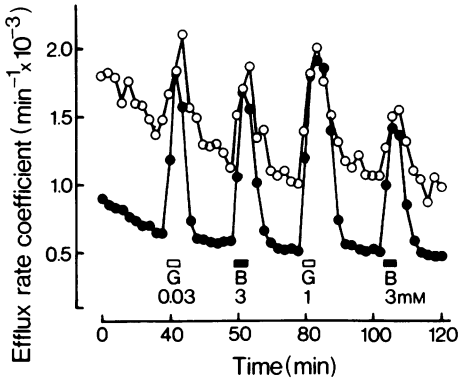

Figure 2 Effects of 4 min applications of unlabelled γ-aminobutyric acid (G) and β-amino-*n*-butyric acid (B) on the rate coefficient for efflux of tritium from paired rat superior cervical ganglia previously incubated in 1 μM [^{3}H]-GABA for 180 min (●) or 1 mM [^{3}H]-GABA for 60 min (○). All solutions contained 10 μM amino-oxyacetic acid. Ordinates: rate coefficient (min^{-1} x 10^{-3}). Abscissae: time after completing pre-incubation in [^{3}H]-GABA (min).

and BABA and its variation under conditions where the internal GABA concentration is changed or their interaction with the carrier is suppressed.

Part A

Transport of GABA

BALA and BABA inhibited the influx of GABA and accelerated its efflux.

1. *Inhibition of [^{3}H]-GABA influx.* Figure 1 shows the auto-inhibition of [^{3}H]-GABA uptake by unlabelled external GABA and the hetero-inhibition of uptake by BALA (β-alanine) and BABA (β-aminobutyric acid). As expected, the slope of the least-squares regression line (0.58) for auto-inhibition of [^{3}H]-GABA influx accords with the maximum slope (0.59) predicted for competitive inhibition in a monomolecular substrate-carrier reaction (see, for example, Goldstein, Aranow & Kalman, 1968). The regression slopes for hetero-inhibition are much less. The probable reason for this is that hetero-inhibition was incomplete, in the sense that the amount of [^{3}H]-GABA accumulated was reduced by only 70–80% even at very high (5 mM) inhibitor concentrations. (By definition, see Methods section, auto-inhibition of saturable uptake was complete at 5 mM GABA.) On restricting data analysis to the effects of low inhibitor concentrations ($\ngtr 100$ μM BALA, $\ngtr 300$ μM BABA) the regression slopes (0.51 and 0.45 respectively, interrupted lines in Figure 1) approached more closely those for competitive hetero-inhibition.

From these modified regression lines, mean concentrations producing 50% inhibition (IC_{50}) were: GABA, 14.3 μM; BALA, 59.2 μM; and BABA, 423.5 μM. For competitive inhibition $IC_{50} = K_i(1 + S/K_s)$, where K_i is the equilibrium dissociation constant of the inhibitor-carrier complex, S is the substrate (GABA) concentration, and K_s is the equilibrium dissociation constant of the substrate-carrier complex. Since S (0.1 μM) is much less than K_s ($=K_m$, 7 μM: Bowery & Brown, 1972), these IC_{50} values should approximate to K_i.

2. *Acceleration of [^{3}H]-GABA efflux.* When ganglia were pre-incubated in [^{3}H]-GABA (1 μM for 180 min) and then superfused with non-radioactive Krebs solution, addition of unlabelled GABA, BALA or BABA to the superfusion fluid increased the rate at which tritium appeared in the effluent superfusate (see Figures 2 and 4). The augmented efflux rate coefficient attained a maximum level 4 min after adding the amino acid, remained elevated for up to 60 min during the continued presence of the amino acid, and then subsided to the control level within 10 min of

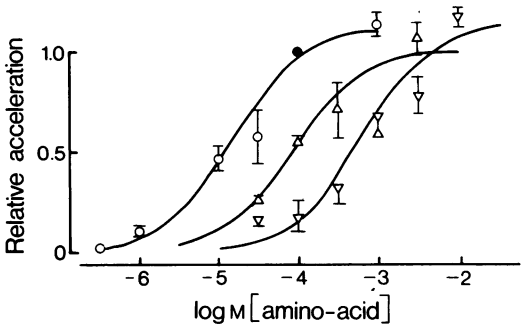

Figure 3 Mean dose-response curves for the acceleration of [^{3}H]-γ-aminobutyric acid (GABA) efflux produced by unlabelled GABA (○), β-alanine (BALA, △) and β-amino-n-butyric acid (BABA, ▽) determined from 6 experiments of the type shown in Figure 2. In each experiment a series of concentrations of GABA and either BALA (3 experiments) or BABA (3 experiments) were applied, and the increment in rate coefficient was calculated as $\Delta k/k^{rest}$, where Δk is the difference between the rate coefficients before and during application of amino acid and k^{rest} is the resting rate coefficient (see text). Ordinates show this increment expressed as a fraction ($=f'$) of that produced by 100 μM GABA (●). Points are means ± s.e. ($n=6$ for GABA, 3 each for BALA and BABA). Curves are drawn according to the hyperbolic expression $f' = f'_{max}S/(K_E+S)$ where S is the amino acid concentration and f_{max} and K_E are constants. Values for f_{max} and K_E were derived from least-squares regressions for the linear transformation $f' = f'_{max} - K_E(f'/S)$ (Dowd & Riggs, 1965). The nature of the curve is discussed further in the Appendix.

removing the amino acid. With 4 min exposure periods, the effect of a single concentration of an amino acid could be repeated fairly consistently if applied at 20 min intervals.

Concentration-dependence The acceleration of [^{3}H]-GABA release increased with augmenting concentrations of external amino acid in the manner shown in Figure 3. In these experiments a random series of concentrations of unlabelled amino acid was administered with a contact time of 4 min and dose-intervals of 20 min, starting 45 min after the completion of the preincubation period, when the efflux rate coefficient had descended to a fairly steady value (mean $k^{rest} = 0.64 \pm 0.05 \times 10^{-3}$ min^{-1}). The peak rate coefficient (k') attained in the presence of each concentration (S) of amino acid was expressed as a multiple of the immediately-preceding resting value, and the increment expressed in the form $(k'/k^{rest}) - 1$ ($=\Delta k$). All such increments were expressed as a fraction (f') of that produced by 100 μM GABA, to minimize variations between preparations.

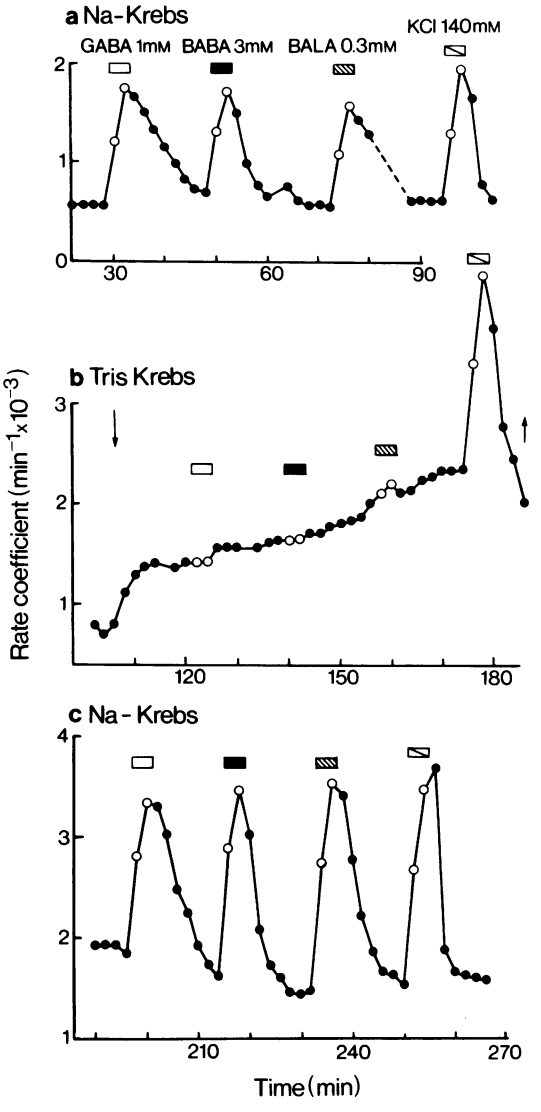

Figure 4 Effect of replacing external Na^+ ion with TRIS on the acceleration of [^{3}H]-γ-aminobutyric acid (GABA) efflux produced by GABA (1 mM), β-amino-n-butyric acid (BABA, 3 mM), β-alanine (BALA, 0.3 mM) and KCl (140 mM, added to solution). (a), (b) and (c) represent continuous time records of the efflux rate coefficient k (min^{-1} x 10^{-3}, ordinates) into: (a) and (c) normal Krebs solution; and (b) Na^+-free, 150 mM TRIS solution (substituted and removed at the arrows). All solutions contained 10 μM amino-oxyacetic acid. The ganglion had been previously incubated for 180 min in 1 μM [^{3}H]-GABA. Temperature, 25°C. (During this rather prolonged exposure to TRIS solution the rate coefficient showed a progressive increase after an initial rise and did not return to its control level on restoring Na^+: responses to shorter exposures were fully reversible.)

The fractional increments varied as a hyperbolic function of S such that $f'/f'_{max} = S/(S+K_E)$, where K_E is the concentration of amino acid producing a half-maximal acceleration of efflux (see Appendix). Regression analysis of a least-squares linear transform (see legend to Figure 3) yielded values for K_E of 16, 85 and 606 μM for GABA, BALA and BABA respectively (Table 1). A fourth amino acid with a much more pronounced depolarizing action, 3-aminopropanesulphonic acid (3-APS, see Bowery & Brown, 1974) showed a K_E of 341 μM (Table 1, omitted from Figure 3 for clarity). All four amino acids produced comparable maximal increments in the fractional efflux rate coefficients f_{max}: when reconverted to absolute rate coefficients these ranged from means of 2.05 to 2.30×10^{-3} min^{-1}, a 3.2 to 3.6-fold increase over the mean resting rate coefficient. The mean tissue ^3H concentration at the end of the pre-loading period in 1 μM [^3H]-GABA was 19.5 ± 3.8 μmol/kg tissue, representing some 9.5% labelling of the endogenous GABA (see Table 2 below).

External Na^+ ions. Replacement of external Na^+ with TRIS produced a sustained increase in the efflux rate coefficient, comparable to that produced by a high concentration of an amino acid (Figure 4). Subsequent addition of amino acid (in TRIS solution) then failed to accelerate the efflux any further, but addition of K^+ continued to stimulate efflux in TRIS solution as in normal Na^+ solution (see Bowery & Brown, 1972). Both the resting rate coefficients and the increments on adding an amino acid were fully restored within 20 min of restoring normal Na^+, providing the exposure time to TRIS did not exceed 30 minutes.

Table 1 Kinetic constants for the acceleration of [^3H]-γ-aminobutyric acid (GABA) efflux by unlabelled GABA, β-alanine (BALA), β-amino-*n*-butyric acid (BABA) and 3-aminopropanesulphonic acid (3-APS)

	K_E (μM)	k_{max} ($min^{-1} \times 10^{-3}$)
GABA	16.0 ± 4.4	2.27 ± 0.09
BALA	85.3 ± 21.5	2.10 ± 0.09
3-APS	341 ± 64	2.05 ± 0.08
BABA	606 ± 115	2.30 ± 0.10

Constants (means ± s.e.) were derived from least-squares regression lines for the linear expression $f = f_{max} - K_E (f/S)$ (see legend to Figure 3) where f is the normalized fractional increment in efflux rate coefficient in the presence of amino acid at concentration S, f_{max} is the maximal increment at $S = \infty$, and K_E is the concentration of amino acid producing half-maximal acceleration in efflux rate. The extrapolated true maximal rate coefficient k_{max} (min^{-1}) was calculated from f_{max} and the average resting rate coefficient ($= 0.635 \times 10^{-3}$ min^{-1}).

Part B

Depolarization

The objective of these experiments was to find out if the accelerated efflux of GABA produced by BABA or BALA, as described above, led to a depolarization of the ganglionic neurones. This required some means of distinguishing between a depolarization produced by the release of GABA and that resulting from a direct action of the amino acids on the neuronal GABA-receptors. Two approaches were attempted: (1) to increase the amount of GABA available for release, so as to exaggerate indirect effects mediated through such release; and (2) to inhibit amino acid-driven exit.

1. *Effect of increasing endogenous GABA* Ideally, it would be more helpful to remove endogenous GABA and thereby eliminate the possibility of its release. However, we have so far been unable to devise a satisfactory method for depleting the tissue of GABA, and so have been forced to adopt the more indirect approach of artificially increasing ganglionic GABA levels. This may be readily achieved by incubating the tissue in a high concentration of exogenous GABA.

Table 2 Effect of incubating ganglia* in 1 mM γ-aminobutyric acid (GABA) for 60 min in the absence (*a*) and presence (*b*) of 10 μM amino-oxyacetic acid (AOAA) on their content of GABA

		a −AOAA	*b* +AOAA
(1)	−GABA	0.153 ± 0.017 (1.21)	0.205 ± 0.015 (1.63)
(2)	+GABA	0.318 ± 0.031 (2.52)	0.729 ± 0.053 (5.79)
(3)	+GABA / −GABA	2.08 ± 0.13	3.56 ± 0.14

Numbers give measured GABA concentrations (mean ± s.e., $n = 4$) expressed as mmol/kg wet weight; figures in brackets are calculated concentrations of GABA in the neuroglial cells, based on the premises that (i) GABA is confined to glial cells and (ii) glial cells comprise 13% of tissue volume (D.L. Tamarind, personal communication from examination of electron micrographs). Ratios +GABA/−GABA are mean ratios measured in paired contralateral ganglia from the same rats (mean ± s.e., $n = 4$).

* Ganglia were incubated overnight at 4°C in Krebs solution, pre-equilibrated for 3 h in Krebs solution at 25°C (with or without AOAA), incubated for a further 60 min in the absence or presence of 1 mM GABA, washed for 30 min in Krebs solution, homogenized and amino acid levels determined by micro-dansylation (see Methods section).

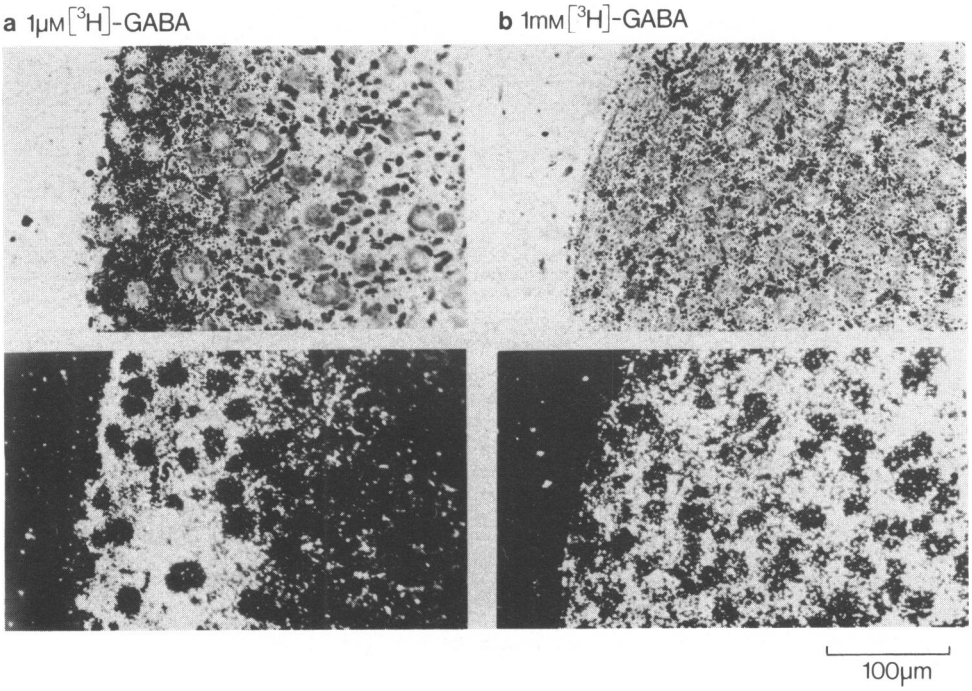

Figure 5 Autoradiographs of sections of rat superior cervical ganglia previously incubated for 60 min in (a) 1 μM [^{3}H]-γ-aminobutyric acid (GABA) and (b) 1 μM [^{3}H]-GABA with 1 mM unlabelled GABA added. Upper photographs were taken under transmitted light, lower photographs (of the same field) under incident light. Exposure times: 28 days. Scale, 100 μm.

Thus, when ganglia were incubated in 1 mM GABA for 60 min at 25°C the total tissue level was increased by 108% as measured in paired contralateral ganglia, from 0.15 ± 0.02 to 0.32 ± 0.03 mmol/kg tissue (Table 2). When metabolism of GABA was inhibited throughout with 10 μM amino-oxyacetic acid (AOAA), a much greater increase of 256% in GABA concentration occurred following such incubation, from 0.21 ± 0.02 to 0.73 ± 0.05 mmol/kg tissue. Further analysis between paired ganglia confirmed that addition of AOAA alone (without added GABA) might produce an increase of some 55 (±20)% in GABA levels from 0.16 to 0.23 mmol/kg tissue. Addition of either GABA or AOAA or both did not materially affect the tissue concentrations of the other amino acids measured. Average concentrations of these were mmol/kg wet weight, mean ± s.e., $n=24$): glycine, 2.58 ± 0.16; taurine, 3.14 ± 0.20; α-alanine, 1.40 ± 0.07; aspartate, 2.51 ± 0.12; glutamate, 4.85 ± 0.26; glutamine, 1.36 ± 0.10. (It should be pointed out that these values do not necessarily represent fresh tissue levels, but rather those obtaining under the experimental conditions used to measure depolarization, in which the ganglia were first incubated overnight at 4°C (see below)).

When the incubation fluid contained 1 mM [^{3}H]-GABA the tissue [^{3}H]-GABA concentrations, expressed at the specific activity of the incubation medium, were 0.15 ± 0.02 and 0.59 ± 0.05 mmol/kg tissue in the absence and presence of AOAA respectively (mean ± s.e., $n=4$ in each case). These values correspond quite well with the increments in total GABA in Table 2, suggesting that the bulk of accumulated [^{3}H]-GABA represents net uptake rather than exchange for endogenous GABA.

Autoradiographs (Figure 5) indicated this accumulated radioactivity to be extraneuronal in location. The cellular distribution pattern resembles that observed after incubating in a much lower (1 μM) concentration of [^{3}H]-GABA, which has been previously judged (from simultaneous electron microscope autoradiographs) to represent accumulation in neuroglial cells (see Young *et al.*, 1973).

Resting efflux rate coefficients tended to be rather greater in ganglia previously incubated in 1 mM GABA than in 1 μM GABA (see Figure 2). In 4 pairs of ganglia compared directly in this manner, mean resting rate coefficients ($min^{-1} \times 10^{-3}$, ± s.e.) were: (a) 1 μM [^{3}H]-GABA, 0.73 ± 0.10; (b) 1 mM [^{3}H]-GABA, 1.09 ± 0.07; b/a, 1.47 ± 0.19. (The rate coefficient

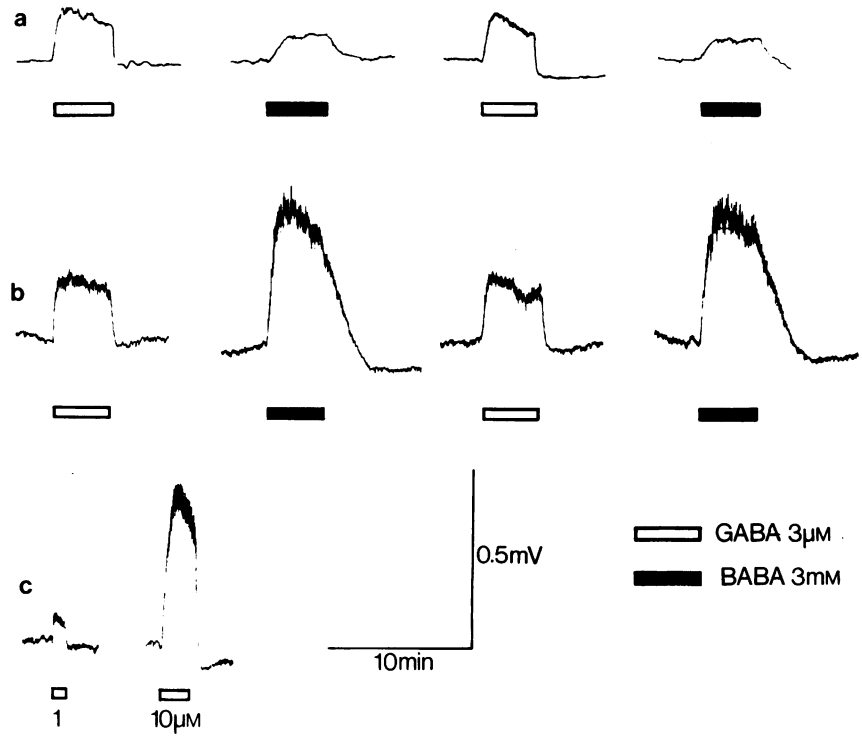

Figure 6 Records of surface depolarizations in a rat isolated superior cervical ganglion produced by 4 min applications of 3 μM γ-aminobutyric acid (GABA, open bars) and 3 mM β-amino-*n*-butyric acid (BABA, closed bars) (a) before, and (b) and (c) after superfusing with 1 mM GABA for 60 min, followed by 60 min washing. All solutions contained amino-oxyacetic acid (10 μM). Amino acids were applied at 30 min intervals. Calibrating GABA doses (c) were given at the end of the experiment for 1 min periods. Scales: 0.5 mV, 10 min.

tends to diminish with time, more rapidly at higher loading concentrations, so the mean values taken through a prolonged efflux run are not strictly comparable; initial maximum values may have more theoretical significance, but cannot be measured in practice: see Appendix.) More importantly, increasing the loading concentration does not alter the peak rate coefficient attained in the presence of a high concentration of GABA or BABA (see Figure 2).

Depolarization after increasing endogenous GABA levels. Figure 6 illustrates the effect of increasing endogenous GABA concentrations, by incubating the ganglion in 1 mM external GABA, on the depolarizing actions of BABA and GABA itself. It also serves to indicate the experimental design used in subsequent experiments of this type, described below. First, responses to 2–4 applications of BABA and GABA were recorded in a ganglion which had not been previously exposed to GABA but which had been incubated overnight at 4°C. The standard concentration of BABA used, 3 mM, was such as to produce a substantial acceleration of GABA efflux (see Figure 3). Initially, this concentration produced a very low amplitude depolarization, and was matched (approximately) by the standard concentration of 3 μM GABA (i.e. a just-suprathreshold concentration, well below the ED_{50} value of 12.5 μM, cf. Bowery & Brown, 1974). (The very low amplitude responses were, of course, helpful in detecting increments after loading, but necessitated very stable recording conditions; overnight incubation facilitated this, by allowing the initial 'injury potential' to dissipate.) The agonists were applied alternately for 4 min periods at 30 min intervals: the exposure time corresponded to that required for a maximal increment in measured [^{3}H]-GABA release, and the interval was sufficient to prevent desensitization of the response to the subsequent dose of agonist. After obtaining stable control responses, the ganglion was exposed to 1 mM GABA solution for 60 min, washed in GABA-free solution for a further 60 min, and the agonists re-applied. Finally, responses to other concentrations of GABA were usually obtained, so that the responses to

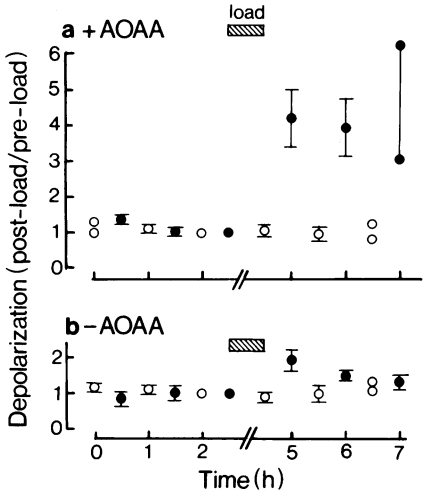

Figure 7 Effect of loading with γ-aminobutyric acid (GABA) (by superfusing with 1 mM GABA solution for 60 min) on depolarizations produced by 3 μM GABA (○) and 3 mM β-amino-*n*-butyric acid (BABA, ●). Each point gives the mean and s.e. mean of 4 experiments in the presence (upper graph) and absence (lower graph) of amino-oxyacetic acid (AOAA) in the superfusing solution. Depolarization amplitudes are expressed as a fraction of those produced by each agonist immediately prior to loading.

BABA could be approximately calibrated in terms of the equally-effective concentration of GABA. (This procedure was not satisfactory when the GABA dose-equivalents were less than 1–3 μM, since this is at the extreme lower limit of the GABA log dose–response curve.)

In the experiment illustrated in Figure 6 (performed in the continuous presence of AOAA) incubation in 1 mM GABA clearly increased the effect of BABA without producing any corresponding change in the response to 3 μM GABA. This differential effect was a consistent observation, as shown in Figure 7. An enhanced response to BABA was also consistently observed in the absence of AOAA, though of much smaller voltage-amplitude (Figure 7b).

Calibrated in terms of the responses to GABA, the effect of 3 mM BABA corresponded to that produced by between 1 and 4 μM GABA under control (pre-loading) conditions. Following loading the increased response corresponded to an additional 2 to 8 μM GABA in the presence of AOAA and <2 μM in the absence of AOAA (the lower amplitude responses here precluding accurate calibration). (In this respect Figure 6 illustrates a rather extreme increment, cf. Figure 8.)

The relative amplitudes of the depolarizations produced by the standard concentrations of 3 mM BABA and 3 μM GABA prior to loading varied appreciably between experiments, from 0.3 to 1.5 ($n=8$). This ratio showed no consistent difference in ganglia incubated in the presence or absence of AOAA. Further, addition of AOAA without added GABA during the course of an experiment did not enhance the effect of BABA (3 experiments).

Antagonism The depolarization produced by BABA after loading was blocked *pari-passu* that by GABA with methylbicuculline, indicating an effect solely upon GABA-receptors (cf. Bowery & Brown, 1974; Bowery, Brown & Collins, 1975).

β-Alanine BALA also accelerates the release of [^{3}H]-GABA (Figure 2) and, like BABA, showed an increased depolarizing action after loading in 1 mM GABA (3 experiments). An example is illustrated in Figure 8, using a standard concentration of 0.3 mM BALA. Here the increment in response to BALA was equivalent to an additional 1.3 μM GABA, as against 2.2 μM GABA for the increment in BABA response.

3-Aminopropanesulphonic acid (3-APS) This compound is a very potent GABA-receptor agonist, about 3 times more potent than GABA (Curtis, Phillis & Watkins, 1961; Bowery & Brown, 1974). Thus, 1 μM 3-APS produced a depolarization as great as 3 μM GABA. At such concentrations, 3-APS produces negligible acceleration of [^{3}H]-GABA efflux (EC_{50}, 341 μM; Table 1). Hence, if the increased depolarization produced by BABA or BALA after loading the ganglion with GABA was due to an increased release of GABA rather than to some change in receptor sensitivity, no such augmentation of 3-APS effects would be expected: this was verified in 3 ganglia, all showing the previously-described augmentation of BABA responses.

Dose-response curves A further attempt to quantitate the post-loading increases in depolarization was made from dose-response curves to GABA-mimetics. The dose-response curves for GABA and 3-APS were not materially altered by prior incubation with 1 mM GABA, except for a slight (not significant) reduction at very high response levels (Figures 9a and b). In contrast, curves for BABA and BALA show a significant sensitization at the lower end, with convergence or desensitization at the upper extremity (Figures 9c and d; although the curve for BABA is restricted to concentrations $\leqslant 30$ mM, it shows the same tendency to converge at the higher response levels as that to BALA). This type of shift is contrary to that expected for receptor sensitization, but accords with that predicted for a limited increment in GABA produced by a release process. The increments in interstitial GABA required to account for the shift could be estimated by calibrating the responses to

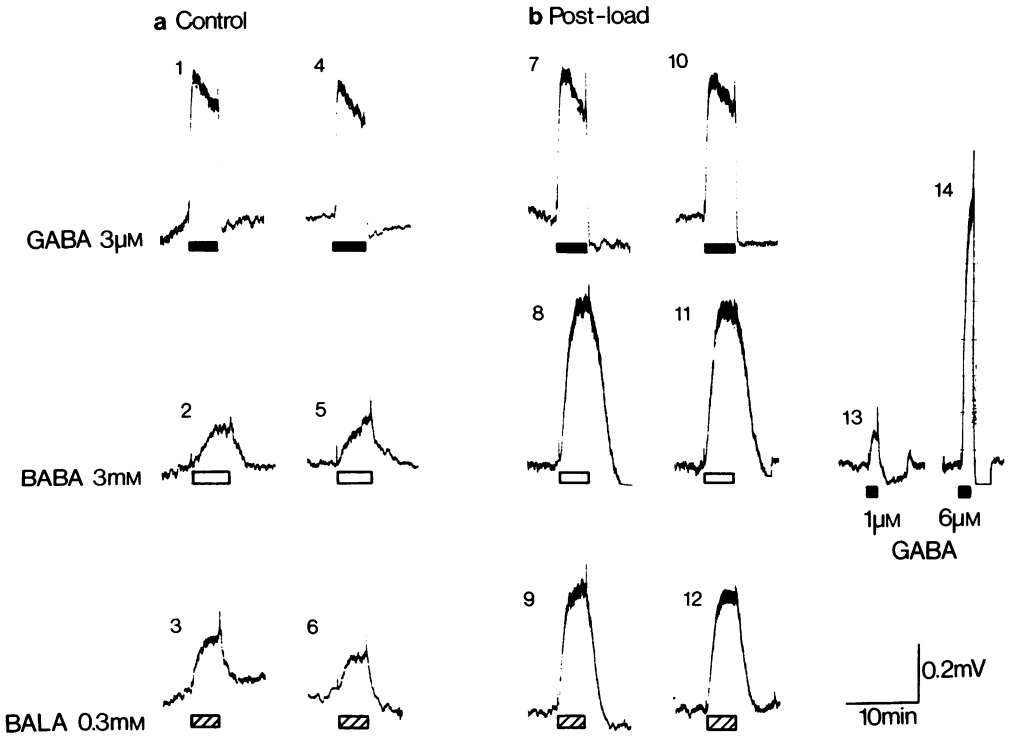

Figure 8 Depolarizing responses to γ-aminobutyric acid (GABA, 3 μM) β-amino-*n*-butyric acid (BABA, 3 mM) and β-alanine (BALA, 0.3 mM) (above downwards) recorded (a) before and (b) after loading for 1 h with 1 mM GABA (amino-oxyacetic acid present). Agonists were added for 4 min periods at 30 min intervals in the sequence indicated. Responses to final calibrating doses of GABA are shown on the extreme right. Calibration: 0.2 mV, 10 min. (Numbers indicate order of responses.)

BABA and BALA in terms of the equivalent concentrations of GABA, derived from the GABA dose-response curves. Results showed a maximum increment at carrier-saturating concentrations of the two agonists (cf. Figure 3) of about 2–3 μM interstitial GABA.

2. *Effect of inhibiting the GABA-carrier* One method of inhibiting the substrate-stimulated release of [^{3}H]-GABA is to remove external Na^+ ions, by replacement with TRIS or Li^+ (see Figure 4 above). Since the depolarizing action of GABA itself is not dependent upon Na^+ ions (Bowery, 1974; Adams & Brown, 1975), it appeared feasible to use Na^+-replacement as a means of eliminating indirect GABA-releasing effects. Unfortunately replacing external Na^+ caused very large shifts in the standing potential difference between the two recording electrodes, presumably reflecting differential rates of change of the liquid junction potentials. Further, after some time (60 min or more) in TRIS solution responses to GABA itself became attenuated, a feature previously observed using Li^+ (Bowery, 1974). These problems placed serious constraints upon the interpretation of replacement experiments. Nevertheless, in 2 experiments the electrode potentials were restored sufficiently rapidly for reasonably clear effects to be seen. These are summarized in Figure 10. A clear depression of the response to BABA was observed at a time when the response to GABA was appreciably enhanced. It may be noted that this effect occurred both before and after loading the ganglion with GABA, suggesting that, even in the unloaded situation, the effect of BABA is dependent upon activation of the GABA-carrier.

Discussion

The central observation to this paper is that the depolarizing actions of the GABA-mimetic agents β-aminobutyric acid (BABA) and β-alanine (BALA) on the isolated superior cervical ganglion of the rat are potentiated following a period of exposure to a high

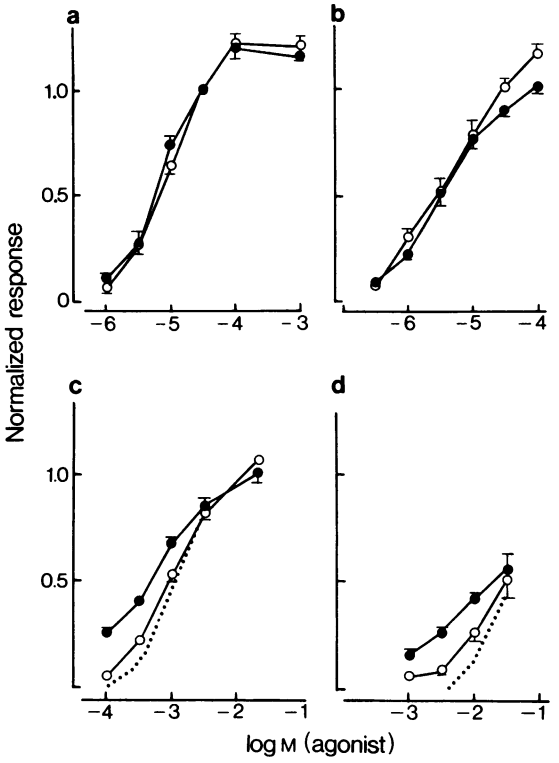

Figure 9 Dose-response curves to (a) γ-aminobutyric acid (GABA), (b) 3-aminopropanesulphonic acid (3-APS), (c) β-alanine (BALA) and (d) β-amino-*n*-butyric acid (BABA) measured before (○) or after (●) incubation in 1 mM GABA (amino-oxyacetic acid present throughout). Responses were normalized with respect to those produced by 30 μM GABA (= 1%) to permit comparison between different preparations. Each point is the mean of 4 ganglia; bars = s.e. mean. The dotted lines (. . . . . .) in (c) and (d) show calculated responses in a tissue totally depleted of GABA (i.e. in the absence of GABA-release), based on calculated interstitial GABA concentrations in Figure 12.

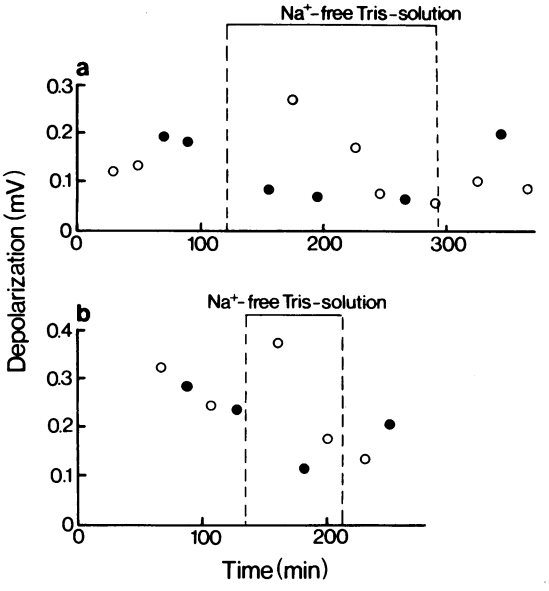

Figure 10 Effects of replacing external Na^+ ions with TRIS on the depolarizing responses of two rat isolated ganglia to 3 μM γ-aminobutyric acid (GABA, ○) and 3 mM β-amino-*n*-butyric acid (BABA, ●), (a) without prior incubation in GABA and (b) after incubation for 60 min in 1 mM GABA. (Amino-oxyacetic acid present.) Ordinates: mV peak depolarization; abscissae: time from first test responses.

concentration of GABA. Our interpretation of this is that BABA and BALA release GABA from the neuroglial cells into the interstitial spaces, and that the depolarization of the ganglion cells which they produce results (in low doses at least) from the action of the released GABA rather than from their direct action on the neuronal GABA-receptors. The increase in the depolarization after exposure to GABA then results from the accumulation of GABA in the glial cells: extra GABA is therefore available for release.

The principal lines of experimental evidence supporting this interpretation may be summarized as follows:

(a) In concentrations required to depolarize the ganglion both BABA and BALA produce a substantial acceleration of [^{3}H]-GABA efflux (Table 3). In contrast, 3-APS, whose effect is not

Table 3 Comparison of the effects of amino acids on (a) γ-aminobutyric acid (GABA) receptors and (b) GABA efflux

	a *Receptor* ED_{50} *(μM)*	*b* *Carrier* K_E *(μM)*	*c* *a/b*
3-APS	3.7	341	0.011
GABA	12.5	16	0.78
BALA	1,300	85	15.24
BABA	>30,000	606	>50

Values in (a) are ED_{50} concentrations for the depolarization of isolated ganglia not previously exposed to GABA, deduced from the data of Bowery & Brown (1974) and Bowery (1974); values in (b) are the K_E values in Table 1 for acceleration of [^{3}H]-GABA efflux. BALA = β-alanine; BABA = β-amino-*n*-butyric acid; 3-APS = 3-aminopropanesulphonic acid.

potentiated after GABA-loading, only accelerates GABA efflux in concentrations well above those required to depolarize the neurones.

(b) The shift in the dose-response curves to BABA and BALA after loading accord with the effect of a limited increment in agonist (GABA) concentration, rather than a general receptor-sensitization.

(c) Potentiation was enhanced when metabolism of GABA was inhibited with amino-oxyacetic acid, according with the greater amount of GABA accumulated.

(d) The potentiated response to BABA was reduced in Na^+-free solution, which inhibits efflux-acceleration by BABA but does not itself prevent neuronal depolarization by GABA (see also Adams & Brown, 1975).

Mechanism of efflux acceleration

It seems clear from the relative abilities of the different amino acids employed in this study to inhibit [^{3}H]-GABA uptake and accelerate [^{3}H]-GABA efflux (compare Figures 1 and 2) that the latter arises through an interaction with the inward GABA-carrier. In fact, β-alanine appears to serve as a substrate for the glial GABA-carrier (Schon & Kelly, 1975; Bowery & Brown, unpublished observations), and one might suppose the same to apply to BABA.

There are essentially two ways in which a substrate for the inward carrier might accelerate GABA-efflux: accelerated exchange diffusion or inhibition of a continuous re-accumulation process. The former implies that GABA is transported in both directions across the cell membrane by the same carrier, but that the outward transport rate is normally submaximal; then addition of a substrate to the outside and its consequent inward transport may increase the rate of turnover of the carrier and thereby accelerate the exit rate (see, for example, Stein, 1967). With re-accumulation it is envisaged that GABA leaves the cells at a constant rate and that a proportion of the effluent GABA is then re-accumulated from the pericellular fluid by the inward carrier; occupation of the external carrier sites by another substrate (or by unlabelled substrate if the exit of labelled material is considered) will reduce the rate of re-accumulation and produce an increased overflow into the external medium (a 'leak-and-pump' situation: Willbrandt & Rosenberg, 1963).

In both cases the increased efflux rate depends, in the first instance, on the degree of saturation of the inward carrier, and hence will show a similar dependence upon the external substrate concentration. On other grounds, however, we tend to view a reuptake-inhibition process as the most likely system responsible for efflux acceleration in the ganglion. Two pieces of experimental information favour this. First, the efflux rate is also increased in Na^+-free solution to a comparable extent to that attained on saturating the external carrier (Figure 4): Na^+-free solution inhibits inward transport and would slow the carrier turnover rate rather than accelerate it. Second, unless the internal face of the carrier was very unsaturated increasing the intracellular GABA concentration should reduce the rate coefficient for carrier-mediated exit of GABA, whereas the opposite tendency (if anything) occurred (see Results section and Figure 2). This suggests that the exit process *per se* tends to follow diffusion kinetics, i.e. is governed by a constant rate coefficient, independent of intracellular concentration. (This need not imply that exit is due to true diffusion; more likely, exit is mediated through a carrier whose Michaelis constant is sufficiently in excess of the internal GABA concentration as to render the rate of exit directly proportional to $[GABA]_{in}$ within the narrow range of experimental variation in $[GABA]_{in}$ exployed in our experiments.)

Quantitative considerations

In the Appendix some estimates have been made of the increments in interstitial GABA concentration expected to accompany accelerated efflux of intracellular GABA. These calculations are based on the above mechanistic interpretation that the accelerated efflux of GABA results from inhibition of re-accumulation, but would, in fact, also be valid for the alternate situation where re-accumulation was negligible and efflux was increased by accelerated exchange diffusion. The results predict that carrier-saturating concentrations of BABA or BALA would increase the interstitial GABA concentration by 1.04 μM in the resting (unloaded) state and by 3.41 μM if the ganglion had been previously loaded with GABA by pre-incubation in GABA solution as described above. The potentiation would therefore correspond to an additional 2.37 μM interstitial GABA. These values accord very closely with those (2–3 μM) estimated from the modification of the dose-response curves. Notwithstanding the many simplifications used in the calculations, this argument suggests that the concept is numerically reasonable, at the very least.

A corollary to this is that, even without prior loading with GABA, the depolarizing responses to low concentrations of GABA and BALA may be attributed entirely to the release of GABA. This results from the not-negligible resting intracellular GABA concentrations in the tissue. Thus, if the responses attributable to the calculated increments in interstitial GABA are subtracted, the dose-response curves for BABA and BALA are shifted further to the right (interrupted lines in Figure 9): this corrected curve would indicate the response attributed to direct

receptor-activation. (It may be noted that the increment in interstitial [GABA] resulting from carrier-saturation is so low as not to affect materially the responses at which previous potency measurements were made (Bowery & Brown, 1974): these latter would reflect solely the direct action of the analogues on the neural receptors.) Clearly, it would be helpful to obtain experimental corroboration of this indirect component by depleting the tissue of its normal GABA levels. This we have so far been unable to achieve. However, some support is given by the observation that the response to low BABA concentrations in normal (unloaded) ganglia is reduced in Na^+-free solution (Figure 10).

Implications

The indirect GABA-mimetic action described in this paper is not likely to be an important mechanism of drug action on sympathetic ganglia *per se* since, while detectable in terms of depolarization, the amount of GABA released is too low to affect transmission (see De Groat, 1970; Adams & Brown, 1975). Instead the experiments might be viewed as suggesting a possible mechanism of drug action in the brain, where GABA fulfils the role of a natural inhibitory neurotransmitter. In this context the following considerations may be relevant:

(a) The endogenous GABA concentrations in the brain exceed those attained in ganglia, even after the latter have been enhanced by prior loading. Maximal values quoted include 9.7 mmol/kg (Fahn & Coté, 1968) and 4.3 mmol/kg (Balcom, Lenox & Meyerhoff, 1975), i.e. 5–10 times those attained in ganglia (cf. Table 2).

(b) Addition of carrier-substrates has been observed to accelerate the efflux of GABA from central nervous system preparations (Cutler, Hammerstad, Cornick & Murray, 1971; de Belleroche & Bradford, 1972; Simon, Martin & Kroll, 1974; Levi & Raiteri, 1974; Raiteri, Federico, Colethi & Levi 1975). When measured under comparable experimental conditions the rate coefficients for resting and substrate-stimulated efflux of [^{3}H]-GABA from isolated mammalian olfactory cortex slices are equal to or exceed those for the corresponding release from ganglia (Brown & Scholfield, unpublished observations). Taken in conjunction with (a), and assuming comparable clearance rates, this would imply that the interstitial GABA levels in brain attained during such release might exceed those in ganglia by an order of magnitude. (Substrate-stimulated release from brain has been attributed to accelerated exchange-diffusion, rather than inhibited reuptake; however, this would not affect calculations of interstitial GABA concentrations, see Appendix.)

(c) It seems reasonable to assume that the sensitivity of central neurones to GABA is no less than that of ganglionic neurones (see Adams & Brown, 1975). Further, for equivalent degrees of receptor-activation GABA would exert a more profound depressant action on central transmission by virtue of the neuronal hyperpolarization (cf. Dreifuss *et al.*, 1969 with Adams & Brown, 1975). Thus, if (from (a) and (b) above) a carrier-substrate raised interstitial GABA concentrations to 10–20 μM, appreciable inhibition of excitatory transmission would be anticipated.

Some complexity is introduced by the fact that, in the brain, GABA is present in both glial cells and inhibitory nerve terminals. The precise partition of endogenous GABA between these two loci is rather uncertain. Intra-terminal concentrations have been estimated at 60 mM (Fonnum, 1973) and 12.5 mM (Patel, Johnson & Balazs, 1974). The glial 'pool' may account for 1.3 mmol/kg brain (Patel *et al.*, 1974) or 0.9 mmol/kg pineal (Schon *et al.*, 1975); if corrected for the glial volume, a glial concentration of at least 5–10 mM seems probable. Both pools appear subject to substrate-stimulated release, but the substrate-specificities of the carriers differ. As in ganglia, BALA is an effective substrate for the glial carrier (Schon & Kelly, 1975; Schon *et al.*, 1975) and causes an accelerated efflux of [^{3}H]-GABA from olfactory cortical slices (Brown & Scholfield, unpublished observations). L-2,4-Diaminobutyric acid (DABA) is a good substrate for the synaptosomal (presumed neural) carrier (Iversen & Johnston, 1971; Simon & Martin, 1973; Kelly, Dick & Schon, 1975), and accelerates GABA-efflux from brain synaptosomes (de Belleroche & Bradford, 1972; Simon *et al.*, 1974). BABA has also been reported to accelerate the release of GABA from synaptosome preparations (de Belleroche & Bradford, 1972). BABA, DABA and BALA would therefore all be potential indirect GABA-mimetics in brain, albeit releasing GABA from different loci.

It may be difficult, at present, to design definitive tests for such indirect effects in brain. One rather oblique approach might be to seek marked discrepancies between the potencies of amino acids as agonists in brain and ganglia, on the basis that (i) the GABA-receptors are structurally similar at the two sites (Bowery & Brown, 1974) but that (ii) the endogenous glial concentration of GABA is probably lower, and neural GABA absent, in the ganglion. From this viewpoint it may be of significance that DABA has only 0.1% of the activity of GABA on ganglionic neurones (Bowery & Brown, 1974) but appears to be a relatively much stronger GABA-mimetic on central neurones (Purpura, Girado, Smith, Callan & Grundfest, 1959; Curtis & Watkins, 1960; Curtis *et al.*, 1961). It would be interesting to know whether the GABA-releasing action of DABA accounts for this difference.

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APPENDIX

KINETICS OF SUBSTRATE-STIMULATED EFFLUX

D.A. BROWN & L. SAUNDERS

We have described above how the addition of unlabelled GABA or certain analogues accelerates the efflux of labelled GABA from the ganglion. In this Appendix we consider further some quantitative aspects of this acceleration, from which to derive an estimate of the changes in interstitial GABA following application of a carrier-substrate.

For these calculations we have selected, as an operational model, the situation where the exit of GABA from the cells shows a simple first-order dependence upon internal GABA concentration (within the range of variation in the latter generated in the present experiments), but where the consequent interstitial concentration and final exit rate into the surrounding medium is modified by a continuous re-accumulation process mediated by a saturable inward carrier. BABA or BALA then act as alternative substrates for the inward carrier, and accelerate efflux primarily by saturating the inward carrier so as to inhibit re-accumulation of GABA. If exit of labelled GABA is considered, external unlabelled GABA exerts a similar inhibitory effect. Reasons for selecting this model, rather than an accelerated exchange diffusion system, are given in the Discussion section; however, as pointed out below, the numerical results are likely to be similar since in either case, the acceleration depends ultimately upon the degree of saturation of the inward carrier.

Analysis

The superfused ganglion may be considered to comprise three compartments in series—the intracellular compartment X, the interstitial fluid Y and the surrounding perfusion fluid Z. Throughout the efflux collection the net flow of intracellular GABA is in the direction $X \rightarrow Z$.

Let the volumes of the compartments (m^3) be V_x, V_y and V_z respectively; let the amounts of GABA (mol) in each compartment at time t be Q_x, Q_y and Q_z; and let the concentrations of GABA (mol m^{-3} = mM) at time t be x, y and z (Q_x/V_x, Q_y/V_y, Q_z/V_z).

Since $V_z \geqslant 1000$ (V_y, V_x) and Q_z is very small, $z \ll y$ at all values of t, so that diffusional backflux from $Z \rightarrow Y$ can be neglected.

Calculation is simplified (but not invalidated, see 'Limitations', below) if the intracellular GABA initially present in the tissue is regarded as being uniformly distributed throughout the intracellular fluid, since, in this case, $V_x \approx V_y = V$ (see Brown, Halliwell & Scholfield, 1971).

At $t=0$ all of the GABA in the system is present in the intracellular compartment X. Then a conservation equation applies:

$$Q_x + Q_y + Q_z = Q_0$$

or

$$x \cdot V + y \cdot V + z \cdot V_z = x_0 V \qquad (1)$$

where the subscript (0) refers to $t=0$.

Transfer rates between compartments may be expressed as follows:

(i) *Efflux* X→Y: diffusive, governed by a single rate coefficient k_1 (see above):

$$-\frac{dx}{dt} = k_1 x \qquad (2)$$

(ii) *Influx* Y→X: a saturable function of y

$$+\frac{dx}{dt} = \frac{\alpha y}{K + y} \qquad (3)$$

where α and K are constants with units of velocity (mol m^{-3} min^{-1}) and concentration (mol m^{-3}) respectively.

(iii) *Efflux* Y→Z: follows diffusion kinetics governed by rate coefficient k_2:

$$+\frac{dz}{dt} = k_2 y \frac{V}{V_z} \qquad (4)$$

As noted above, diffusive backflux $Z \rightarrow Y$ can be neglected since $z \ll y$.

From (2), (3) and (4), the rate of change of the interstitial GABA concentration y is given by:

$$\frac{dy}{dt} = k_1 x - \frac{\alpha y}{K+y} - k_2 y \quad (5)$$

From (1):

$$x = x_0 - y - z(V_z/V) \quad (6)$$

Substituting for x in (5):

$$\frac{dy}{dt} = k_1 x_0 - (k_1 + k_2)y - \frac{\alpha \cdot y}{K+y} - k_1 z\left(\frac{V_z}{V}\right) \quad (7)$$

Calculation Equations (7) and (4) were integrated numerically at time intervals of 10^{-4} min using a two-stage fourth order Runge-Kutta design for x, y and dz/dt at $t > 0$. From these the apparent efflux rate coefficient k_{app} was calculated as:

$$k_{app} = \frac{dz}{dt} \cdot \frac{V_z}{V} / (x+y) \quad (8)$$

Constants The following values were selected:

(i) Initial overall intracellular GABA concentration $x_0 = 0.46$ mol m^{-3} (0.46 mM) unloaded and 1.6 mol m^{-3} (1.6 mM) loaded (i.e. after exposure to 1 mM external GABA for 60 min, see Table 3; AOAA is assumed to be present).

(ii) $V_z/V = 10^3$ (at a perfusion rate of 1 ml/min and ganglion volume 1 µl, i.e. assuming exchange at 1 min intervals, a minimum estimate).

(iii) $k_1 = 2.4 \times 10^{-3}$ min^{-1}. This is the projected maximum value for the measured efflux rate coefficient for [^{3}H]-GABA at infinite external concentrations of unlabelled GABA or inhibitor, when re-accumulation of [^{3}H]-GABA is totally inhibited. Then the middle term of equation (5) is zero, and equation (5) simplifies to

$$\frac{dy}{dt} = k_1 x - k_2 y \quad (9)$$

If $k_1 \neq k_2$, this integrates to

$$y = \frac{k_1 x_0}{k_2 - k_1} [\exp(-k_1 t) - \exp(-k_2 t)] \quad (10)$$

Since $k_2 \gg k_1$ (see (iv) below), at finite values of t

$$y = \frac{k_1}{k_2} \cdot x_0 [\exp(-k_1 t)] \quad (11)$$

Combining (4) and (11) and rearranging:

$$k_1 = \frac{dz}{dt} \cdot \frac{V_z}{V} \cdot (x_0)^{-1} \quad (12)$$

Comparing with the experimentally-measured rate coefficient in (8):

$$k_{app} = \frac{dz}{dt} \cdot \frac{V_z}{V} (x+y)^{-1} \quad (8)$$

and with $y \ll x$, $k_{app} \approx k_1$.

(iv) The rate coefficient for efflux from the interstitial fluid $k_2 = 0.7$ min^{-1} (estimated from the desaturation curves for extracellular ^{24}Na (Brown & Scholfield, 1974) and mannitol (Brown & Scholfield, 1972)).

(v) $\alpha = 1.11 \times 10^{-2}$ mol m^{-3} min^{-1} (the experimentally-determined maximal velocity for the carrier-mediated accumulation of [^{3}H]-GABA: Bowery, 1974). (K was varied, see Results below).

Results of calculations

Figure 11 shows how the calculated interstitial GABA concentration (y) and the rate coefficient for efflux of GABA from the tissue (k_{app}) change with time. The

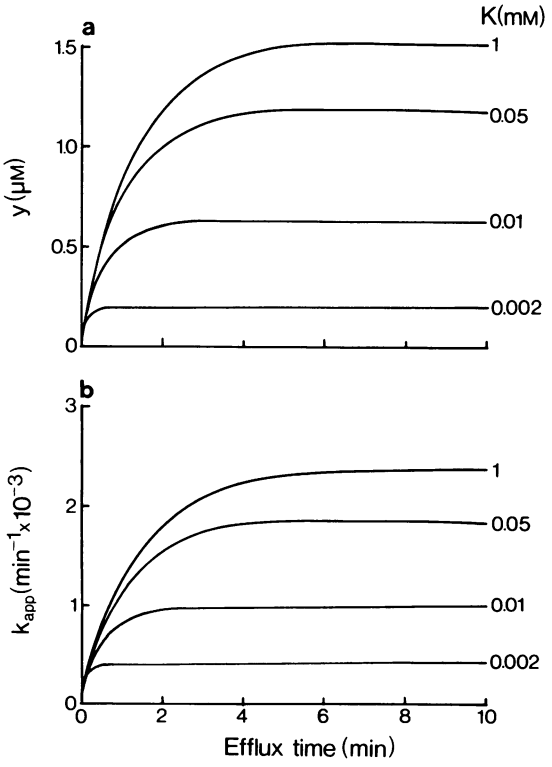

Figure 11 Calculated interstitial γ-aminobutyric acid (GABA) concentration (a) and tissue efflux rate coefficient (b) at $x_0 = 0.46$ mM (unloaded ganglion) and $K = 2$ µM – 1 mM, plotted against time.

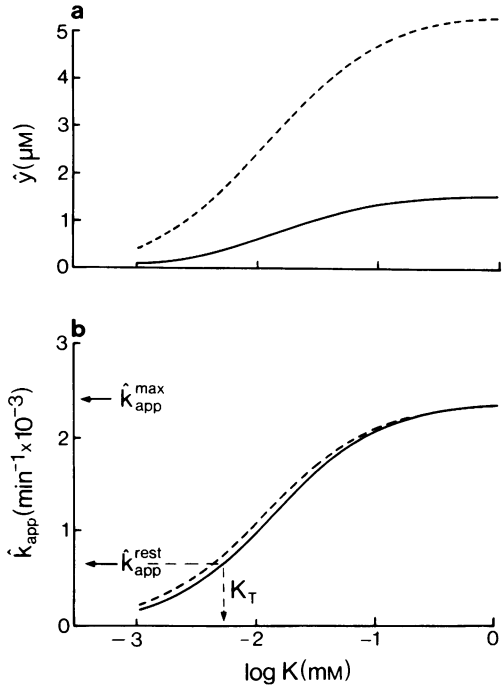

Figure 12 Calculated maximum interstitial GABA concentration (a) and tissue efflux rate coefficients (b) plotted against K (on a logarithmic scale) for unloaded ganglia (solid line, $x_0 = 0.46$ mM) and for ganglia previously loaded with GABA ($x_0 = 1.6$ mM). Arrows indicate values appropriate to the experimentally-observed resting efflux rate coefficient (0.64×10^{-3} min^{-1} at $x_0 = 0.46$ mM).

ganglion was assumed not to have been previously loaded with GABA ($x_0 = 0.46$ mM) and K was varied. The maximum value of k_{app} ($\hat{k}_{app}$), although delayed compared with $\hat{y}$, was attained by 4 min except at very high values for K (i.e. corresponding to very high external substrate concentrations, see below): hence the efflux rate coefficient measured after 4 min (see Figure 3) provides a good approximation to $\hat{k}_{app}$.

In Figure 12 values for $\hat{y}$ and $\hat{k}_{app}$ are plotted against K on a logarithmic scale for the two conditions where the ganglion has or has not been previously incubated in GABA ($x_0 = 1.6$ and 0.46 mM respectively). Considering first the *rate coefficient*, the following points emerge:

(i) The absolute value of $\hat{k}_{app}$ shows a hyperbolic dependence upon K such that

$$\hat{k}_{app} = \frac{\hat{k}_{app}^{max} \cdot K}{\phi + K} \qquad (13)$$

where ϕ is the value of K corresponding to $\hat{k}_{app} = 0.5\,\hat{k}_{app}^{max}$. This latter value ($2.4 \times 10^{-3}$ min^{-1}) is numerically equivalent to k_1, as predicted in equations (8) and (12).

(ii) At the mean resting rate coefficient measured experimentally for [^{3}H]-GABA efflux in the absence of external substrate (0.64×10^{-3} min^{-1} at $x_0 = 0.46$ mM), $K = 5.4$ μM. This should correspond to K_T, the Michaelis constant for GABA as a substrate for the inward carrier. In fact it is slightly lower than the values previously measured for the high-affinity influx process (7–8 μM: Bowery & Brown, 1972; Bowery, 1974) or that derived from the overall IC_{50} (14.3 μM, Figure 1). This discrepancy is probably acceptable if the following points are borne in mind: (a) k_{app}^{rest} measured experimentally showed great variation. Further, the value of 0.64×10^{-3} refers to that attained after a rather prolonged period of efflux, which, at low values of K, would tend to under-read the peak value. The latter would be attained after only a few minutes efflux (see Figure 11), but would not be open to experimental measurement because of interference by clearance of extracellular radioactivity. (b) The position of the curve also depends critically upon the maximal uptake velocity, which is subject to further measurement uncertainty.

(iii) The effect of adding an external substrate S is to increase K above K_T such that

$$K = K_T(1 + S/K_S) \qquad (14)$$

where S is the external substrate concentration and K_S is the concentration of S producing half-saturation of the carrier. (Clearly, if the efflux of radioactive GABA is measured and S is unlabelled GABA, $K_S = K_T$). Thus, when the fractional *increase* in the efflux rate coefficient for labelled GABA is plotted against the external unlabelled substrate concentration a hyperbolic curve results (Figure 13) as previously obtained experimentally (cf. Figure 3). However, it should be noted that the concentration of substrate producing a half-maximal acceleration of efflux (designated earlier as K_E) is *not* equal to K_S but (in this case) equals $4K_S$. This numerical relationship between K_E and K_S is not invariate but depends upon the value of K_T, the Michaelis constant for GABA-uptake. Thus, in the presence of a re-uptake system, the absolute value of K_S cannot be determined directly from the efflux acceleration unless K_T is known, though measurements of relative affinities for the inward carrier can, of course, be made.

(iv) The effect of increasing x_0 to 1.6 mM is to shift the curve in Figure 12 to the left (ϕ in equation (13) reduced from 15.3 to 13.3 μM) and hence to raise the resting efflux rate coefficient. This results from the increased interstitial GABA concentration (Figure 12a): in consequence a smaller proportion of effluent GABA is re-accumulated, leading to a greater overflow. At $K_T = 5.4$ μM, the resting rate coefficient is increased from 0.64 to 0.75×10^{-3} min^{-1}, but the maximum rate coefficient at high external substrate

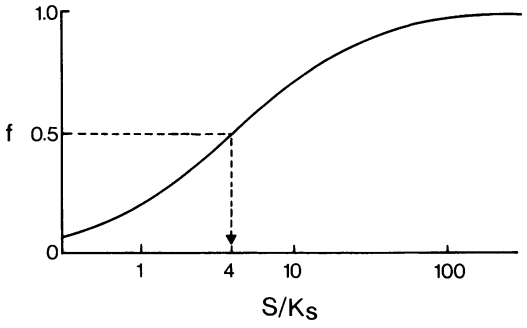

Figure 13 Transposition of data in Figure 12b to the form of Figure 3, in which the fractional increase in efflux rate coefficient $f = \Delta\hat{k}/\Delta\hat{k}^{max}$ is plotted against the external substrate concentration in units S/K_S, where

$$\Delta\hat{k} = (\hat{k}_{app}/\hat{k}_{app}^{rest}) - 1$$

and

$$\Delta\hat{k}^{max} = (\hat{k}_{app}^{max}/\hat{k}_{app}^{rest}) - 1$$

$\hat{k}_{app}^{rest}$ was taken as 0.64×10^{-3} min^{-1} and $\hat{k}_{app}^{max}$ as 2.4×10^{-3} min^{-1}. S/K_S was calculated from equation (14) assuming K_T = 5.4 μM [GABA]. The curve is hyperbolic such that

$$f = \frac{S/K_S}{4 + S/K_S}$$

concentration is unchanged. This accords with the trend of the experimental observations (see Results section).

Interstitial GABA concentrations Some values for *y* abstracted from Figure 12a are listed in Table 4. The following points seem worth emphasizing.

(i) Even in the absence of external substrate, at $K_T = 5.4$ μM maximal interstitial GABA concentrations of 0.41 and 1.64 μM may be anticipated to result from the exit of GABA from normal and preloaded tissue respectively. The latter value is not negligible in view of the neuronal sensitivity, and may account for the slight modification on the dose-response curves for GABA and 3-APS after loading.

(ii) A 4 min exposure to 300 μM BALA or 3 mM BABA might increase this interstitial concentration by some 0.9 μM in unloaded ganglia and 2.8–2.9 μM in loaded ganglia. Hence the increase in the interstitial GABA concentration produced by these agents consequent upon loading would be 1.9–2.0 μM, rising to a limit of 2.5 μM at very high concentrations. These values correspond very well with those calculated from the shift in the experimental dose-response curves after loading.

(iii) The above figures also suggest that, at low concentrations, the depolarizing actions of BABA and BALA can be accounted for entirely by the increased interstitial GABA concentration, and not by direct receptor-activation. Thus, the response to 3 mM BABA in unloaded ganglia corresponded to the action of 1 μM GABA, which accords with the increment in interstitial GABA (0.9 μM) very precisely.

Limitations

The model is simplified in that it does not take into account the anatomical distribution of GABA in the ganglion or possible gradients in the interstitial GABA concentration. Neither limitation may be too serious.

Table 4 Calculated interstitial γ-aminobutyric acid (GABA) concentrations from Figure 12

	Condition	*Interstitial [GABA]* (μM)		
		a *Unloaded* $x_0 = 0.46$ mM	*b* *Loaded* $x_0 = 1.6$ mM	*c* *b–a*
(1)*	Rest	0.41	1.64	1.23
(2)†	300 μM BALA	1.32	4.42	3.10
(3)	(2)–(1)	0.91	2.78	1.87
(4)†	3 mM BABA	1.30	4.54	3.24
(5)	(4)–(1)	0.89	2.90	2.01
(6)	At $S=\infty$, $t=4$ min	1.45	5.05	3.60
(7)	(6)–(1)	1.04	3.41	2.37
(8)	At $S=\infty$, $t=\infty$	1.55	5.32	3.77
(9)	(8)–(1)	1.14	3.68	2.54

* Assuming K_T = 5.4 μM. † After 4 min application.

(i) The GABA is not evenly distributed in the intracellular space but is (probably) confined entirely to glial cells. This would not affect calculations of average interstitial concentrations since the smaller intracellular volume will be offset by a higher concentration than that assumed on the basis of an even intracellular distribution.

(ii) It might be anticipated that higher concentrations would arise (transiently) in the narrow clefts between satellite cells and neurones than elsewhere in the interstitial space. However, this would be offset by a faster redistribution into the remaining interstitial space than that for total extracellular clearance. Further, the Schwann cells surrounding fibres may be a quantitatively much larger source of GABA than the satellite cells, since the uptake of GABA by sympathetic nerve trunks exceeds that of the ganglion itself (Bowery & Brown, 1972).

Alternative models

The congruence between predictions and observations suggests that the above concept of inhibited re-accumulation is appropriate for this system. However, it may be noted that the same approach would also encompass accelerated exchange diffusion, with certain restrictions. These would be that either x_0 is invariate and x does not change appreciably with time, or that the internal face of the outward carrier is very unsaturated: in both cases the efflux $X \rightarrow Y$ would be regarded as governed by the single rate coefficient k_1. However, the effect of varying x_0 appears incompatible with such a process in the ganglion. Thus, one explanation for accelerated exchange diffusion might be that the outward transport rate is normally submaximal because the internal substrate concentration is too low to saturate the inward face of the carrier. If no re-accumulation occurs the degree of saturation may be calculated from the ratio of the resting and maximal driven efflux rate coefficients at any given internal GABA concentration. In the ganglion at $x_0 = 0.46$ mM, this ratio is 0.27, giving a value for K_x of 1.24 mM. If now x_0 is raised to 1.6 mM, the efflux rate coefficient will fall from 0.64 to 0.39×10^{-3} min^{-1}; further, the maximum rate coefficient attained at high external substrate concentration will be only 0.69×10^{-3} min^{-1}, instead of 2.4×10^{-3} min^{-1}, since the inward-facing carrier sites show a greater degree of saturation (0.56 as against 0.27 at $x_0 = 0.46$ mM). Both effects are contrary to our experimental observations. An alternative view is that the internal face of the carrier is fully saturated but outward mobility is low, perhaps because of the low internal Na^+ concentration (see Martin, 1973). In this case, however, both resting and stimulated efflux rate coefficients should fall in proportion to the increase in x_0. These objections need not necessarily hold in other tissues, of course, and Levi & Raiteri (1974) and Raiteri *et al.* (1975) have recently suggested that accelerated exchange diffusion may be responsible for the substrate-stimulated efflux of GABA from synaptosomes.

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