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GABA Efflux from Synaptosomes: Effects of Membrane Potential, and External GABA and Cations

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Summary. Presynaptic GABAergic nerve terminals accumulate γ-aminobutyric acid (GABA) by a sodium-dependent carrier mechanism in which two Na^+ are co-transported with one GABA. We have examined the influence of external GABA and cations on GABA efflux from ^{3}H-GABA loaded rat brain synaptosomes, to determine whether or not the carriers can also mediate GABA efflux. We observed that, in Ca-free media (to minimize Ca-dependent, evoked release), external GABA promotes GABA efflux when the medium contains Na^+, but *inhibits* GABA efflux in the absence of Na^+. The efflux of GABA into Ca-free media is stimulated by depolarization (either with veratridine or increased external K^+). These data, and published data on the internal Na^+ dependence of GABA efflux into Ca-free media, indicate that exiting GABA is cotransported with Na^+. The stimulatory effect of depolarization is consistent with efflux of Na^+ along with the uncharged GABA. The (carrier-mediated) efflux is also stimulated when the carriers cycle inward with Na^+ + GABA. The inhibitory effect of GABA in Na^+-free media implies that GABA can bind to unloaded carriers and that the carriers loaded only with GABA cycle very slowly, if at all. Our data, and data from the literature, can be fitted to a simple model with sequential binding of solutes: external GABA binds to the carrier first, and only the free or fully-loaded (with $2Na^+ + 1$GABA) carriers can cycle. Other binding sequences and random binding, do not fit the experimental observations.

Key Words gamma-aminobutyric acid · GABA · synaptosomes · sodium-GABA co-transport · carrier-mediated transport · presynaptic nerve terminals

Introduction

Synaptosomes (pinched-off presynaptic nerve endings), prepared by differential and sucrose gradient centrifugation of mammalian brain homogenates, appear to be a valid model system for studying the functional properties of nerve terminals (Bradford, 1975; Blaustein, Kendrick, Fried & Ratzlaff, 1977). Synaptosomes can maintain a negative-inside resting potential that behaves like a potassium diffusion potential (Blaustein & Goldring, 1975). These terminals contain sodium channels that respond to a variety of Na channel-specific neuropharmacological agents such as tetrodotoxin (TTX), veratridine (VER) and scorpion toxin (Blaustein, 1975; Krueger & Blaustein, 1980).

Synaptosomes prepared from mammalian brain are heterogeneous and contain a mixture of cholinergic, dopaminergic, GABAergic, etc., terminals. The terminals can accumulate various transmitters and putative transmitters by "high-affinity" Na-dependent uptake mechanisms (e.g., Iverson, 1973; Kuhar, 1973); presumably, many terminals reaccumulate, selectively, the transmitters that they release (*see* Kuhar, 1973; Hedqvist & Stjärne, 1969). These reuptake mechanisms appear to serve two important functions (*cf.* Iversen, 1971): (i) They help to terminate transmitter action on the postsynaptic cells, and (ii) They help to conserve the transmitter molecules so that the molecules can be recycled. In order to fulfill these functions, these nerve terminal transport systems must be able to mediate net transmitter uptake. Indeed, Roskoski (1978; and *see* Ryan & Roskoski, 1977; Pastuszko, Wilson & Erecinska, 1981) has shown that synaptosomes can engage in net accumulation of γ-aminobutyric acid (GABA) and L-glutamate.

The properties of the GABA transport system have been studied extensively. GABA uptake requires external Na (Na_o), and the transport system utilizes energy from the transmembrane Na electrochemical gradient to power transport (Martin, 1976; Blaustein & King, 1976; Kanner, 1978). The Na_o-dependent

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GABA uptake is inhibited by depolarization, indicating that the uptake may involve the entrance of net positive charge (Blaustein & King, 1976); this would be expected if one or more Na ions enter with a GABA molecule.

If GABA accumulation is powered by the Na electrochemical gradient, we would expect a "symmetrical" transport system that also mediates the exit of GABA by a Na_i-dependent process. Indeed, Haycock, Levy, Denner and Cotman (1978) and Sandoval (1980) have obtained evidence for a Ca_o-independent, Na_i-dependent GABA efflux from synaptosomes that is promoted by depolarization. Recent observations by Schwartz (1982) indicate that Na-coupled GABA efflux may play a physiological role in horizontal cells of the toad retina.

In the present study we investigated the effects of membrane potential, external cations and GABA, on GABA efflux from synaptosomes. The data suggest that GABA efflux involves the exit of net positive charge, presumably because Na ions exit with the uncharged GABA. The data also provide evidence for a carrier model in which GABA and Na bind sequentially to the transport system. A preliminary report of these results has been published (Nelson & Blaustein, 1978).

Materials and Methods

Solutions

The standard physiological solution (145 Na + 5 K) contained (in mmol/liter): NaCl, 145; KCl, 5; $MgCl_2$, 2.6; NaH_2PO_4, 1.2; glucose, 10; and HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid), 20. The solution was titrated to pH 7.65 at 22 °C with Tris (hydroxymethyl) aminomethane base. In many instances, some or all of the NaCl was replaced by LiCl (e.g., 85 Na + 5 K contained 60 mM LiCl) or by KCl (e.g., 85 Na + 55 K contained 55 mM KCl and 10 mM LiCl); the sum of NaCl + KCl + LiCl was always 150 mM.

Veratridine (Aldrich, Milwaukee, WI) was dissolved in a few drops of 1 N HCl, and diluted with 145 Na + 5 K (retitrated to pH 7.65 with Tris base); 200 nM tetrodotoxin (TTX; Calbiochem, Palo Alto, CA) and 1 mM aminooxyacetic acid (Eastman, Rochester, NY) were directly dissolved in 145 Na + 5 K.

Tritium-labeled GABA was obtained from New England Nuclear (Boston, MA); nonradioactive GABA and EGTA (ethyleneglycol bis-β-aminoethyl N,N'-tetraacetic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). All salts were reagent grade.

Preparation of Synaptosomes

Synaptosomes were prepared from rat brain homogenates by a modified Gray and Whittaker (1962) differential and discontinuous sucrose density gradient procedure (Blaustein et al., 1978).

Synaptosomes that had been equilibrated in physiological salt solution (Blaustein, Ratzlaff, Kendrick & Schweitzer, 1978) were centrifuged at 5 °C for 10 min at 15,000 × g. To load the synaptosomes with labeled GABA, the pellets (synaptosomes) were resuspended in 145 Na + 5 K containing 1 μM GABA; ^{3}H-GABA was added to give a specific activity of ~300 μCi/μmole GABA. The suspensions, containing approximately 6 mg synaptosome protein in 10 ml were incubated for 15 min at 30 °C to load the terminals with ^{3}H-GABA. Loading was terminated by adding 4 vol of ice-cold Na-free (145 mM Li or 0.32 M sucrose) solution to the incubation tube. This suspension was then centrifuged at 15,000 × g for 7 min at 5 °C. The supernatant was decanted, and the pellet was rinsed twice with cold Na-free solution and kept on ice.

These ^{3}H-GABA-loaded synaptosomes were resuspended in a small volume (~0.8 mg protein in 0.2 ml) of ice-cold Na-free (sucrose) solution and then transferred to a large volume (5 ml) of incubation solution at 30°. After the appropriate incubation period (0 – 5 min), GABA efflux was terminated by vacuum filtration of aliquots of the synaptosome suspensions on prewashed 0.3 μm pore diameter Millipore (Bedford, MA) filters. Each filter was washed with two 4-ml aliquots of ice-cold Na-free solution containing 1 mM GABA. The ^{3}H-GABA content of the synaptosomes trapped on the filters was determined by liquid scintillation spectrophotometry: GABA efflux was determined by difference between the amount of ^{3}H-GABA in the synaptosomes at 0-time and the amount remaining after a 1–5 min incubation period. Protein was measured by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951).

Results

Effects of External GABA and Sodium on GABA Efflux

In synaptosomes, GABA uptake from solutions with low GABA concentrations (<10 μM) is strongly dependent upon external Na (Martin & Smith, 1972; Martin, 1973). This Na-dependent uptake is consistent with the view that Na is co-transported into the terminals with GABA. Furthermore, when synaptosomes are loaded with radioactive GABA, GABA efflux is stimulated by external GABA in the presence of external Na (e.g., Simon, Martin & Kroll, 1974; Raiteri, Federico, Coletti & Levi, 1975; and *see* Fig. 1). The GABA-stimulated GABA effux may be evidence that the same transport system can move GABA in *both* directions across the plasma membrane.

Figure 1 shows data from a representative experiment in which we tested the effects of external Na and GABA on GABA efflux. Although we also found that external GABA stimulated ^{3}H-GABA efflux in the presence of external Na (left), note that GABA *reduced* ^{3}H-

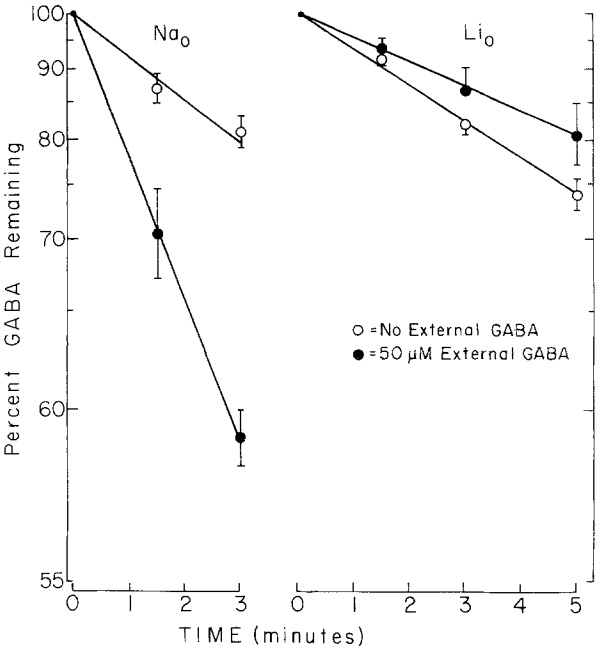

Fig. 1. Effects of external Na and GABA on GABA efflux from $^3H-$GABA-loaded synaptosomes. In the presence of external Na (left-hand graph), the addition of 50 μM unlabeled GABA to the medium (●) markedly increased GABA efflux. When all of the external Na was replaced by Li (right-hand graph), the addition of 50 μM GABA *reduced* GABA efflux. Each symbol represents the mean ±SEM of 4 determinations. These data (and the data in Fig. 2) were fitted to least squares regression lines of the form: y=antilog $(m \cdot x + 2.0)$, where y=percent of GABA remaining in the terminals, x=incubation time, and m =slope of the regression line; the correlation coefficient r^2, was also calculated. For the left-hand graph, $m = -0.031$ and $r^2 = 0.97$ for the open circles; $m = -0.079$ and $r^2 = 0.97$ for the solid circles. The values of the right-hand graph are: $m = -0.027$ and $r^2 = 0.99$ (open circles); $m = -0.017$ and $r^2 = 1.00$ (solid circles)

GABA efflux when the Na was replaced by Li (right). In the absence of external GABA, replacement of external Na by Li had no significant effect on ^{3}H-GABA efflux. One possible explanation for the inhibition of efflux by external GABA in Na-free media (*see* Discussion) is that $GABA_o$ may bind to the transport system and impede carrier cycling because Na is absent.

Effects of Potassium and Veratridine on GABA Efflux

GABA is a zwitterion and is uncharged at neutral pH. Therefore, since GABA is co-transported with Na, the influx of GABA should involve the net entry of positive charge. This would have two important consequences: (i) GABA influx should depolarize the terminals slightly, because of the electrogenic effect (i.e., the inward current flow across the membrane resistance), and (ii) Conversely GABA influx should be influenced by the electrical potential across the plasma membrane: depolarization should slow GABA entry, while hyperpolarization should speed it up. Indeed, GABA uptake into synaptosomes (Blaustein & King, 1976) and into resealed synaptic plasma membrane vesicles (Kanner, 1978) is inhibited by depolarizing agents such as veratridine and high external potassium concentrations.

The Na electrochemical gradient across the plasmalemma appears to be the driving force for net transport of GABA (*cf.* Kanner, 1978). This may indicate that the transport system is symmetric, in that it also can mediate the exit of GABA along with Na (i.e., a net outward movement of positive charge). Under these circumstances, depolarization should *stimulate* the efflux of GABA through the Na-coupled transport system. Indeed, Haycock et al. (1978) and Sandoval (1980) have reported that GABA efflux into Ca-free media is stimulated by raising the extracellular K concentration ($[K]_o$). As shown in Fig. 2, we confirmed this observation. In our experiments (and *see* Sandoval, 1980), the Ca-free medium (with 0.5 mM EGTA) was used to minimize the contribution of Ca_o-dependent evoked GABA release (*cf.* Cotman, Haycock & White, 1976). If the stimulating effect of elevated $[K]_o$ on GABA release is primarily a consequence of its depolarizing action, then the effect should be mimicked by agents that depolarize the terminals by different means. This hypothesis was explored by testing the effect of veratridine, an alkaloid that depolarizes nerve terminals by opening TTX-sensitive Na channels (Krueger & Blaustein, 1980). Figure 2 also shows that the efflux of ^{3}H-GABA is stimulated by veratridine and that this stimulated efflux is blocked by the application of TTX (*also see* Haycock et al., 1978).

One possible explanation for these results is that high $[K]_o$ and veratridine both open Na channels (by different means) and increase Na influx and, thereby, the internal Na concentration ($[Na]_i$). A rise in $[Na]_i$ may then increase GABA efflux through the transport system. Haycock et al. (1978) demonstrated that Ca_o-independent K_o- and veratridine-stimulated GABA efflux requires internal Na. They concluded that the Na-coupled GABA transport does, indeed, mediate this efflux. However, we

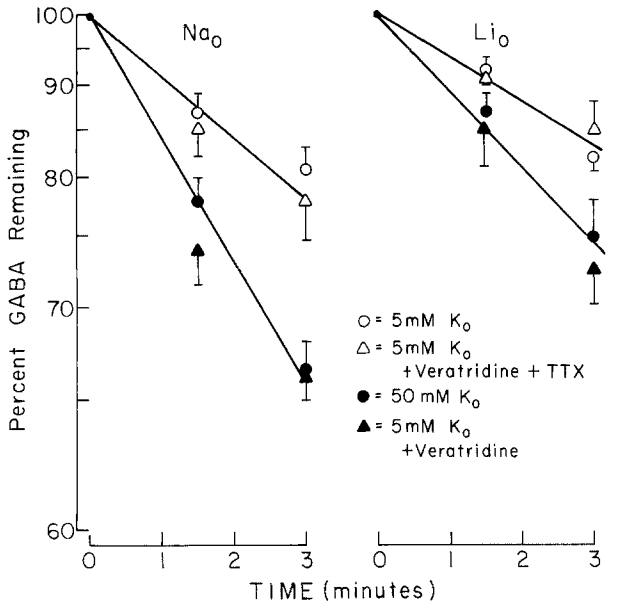

Fig. 2. Effects of depolarization on GABA efflux from ^3H-GABA-loaded synaptosomes in the presence (left-hand graph), and absence (right-hand graph) of external Na. Depolarization of the synaptosomes, either with media containing 50 mM K (●) or with 75 μM veratridine (▲) increased the rate of GABA efflux from the synaptosomes, whether the media contained Na (left-hand graph) or not (right-hand graph). In this experiment, the external Na was replaced by Li. When TTX was added to the media containing veratridine (△) the stimulation of GABA efflux was blocked - presumably because the terminals are not depolarized under these circumstances (*cf.* Blaustein & Goldring, 1975; Krueger & Blaustein, 1980). Each symbol represents the mean of 4 determinations ±SEM. Regression lines were calculated for these data (*see* Fig. 1 legend). Since the slopes of the regression lines calculated for pairs of open or closed symbols were not significantly different from one another ($p > 0.1$), the data have been lumped together. For the left-hand graph, $m = -0.034$, and $r^2 = 0.95$ for the open symbols; $m = -0.060$ and $r^2 = 0.95$ for the solid symbols. The values for the right-hand graph are: $m = -0.028$, $r^2 = 0.99$ (open symbols), and $m = -0.046$, $r^2 = 0.99$ (closed symbols)

have observed that both veratridine and K-rich media also stimulate GABA efflux into Na-free Li-containing media (Fig. 2). Both veratridine and K-rich media should also depolarize the synaptosomes in Na-free Li-containing media; the veratridine should depolarize these terminals because Li passes through TTX-sensitive "Na" channels about as well as Na (e.g., Moore et al., 1966). However, $[Na]_i$ will not increase under these circumstances. Furthermore, K-rich media probably do not stimulate Na influx in synaptosomes because most Na channels appear to be inactivated in these preparations (Krueger & Blaustein, 1980); in fact, increased $[K]_o$ should reduce $[Na]_i$ by stimulating Na efflux through the Na–K exchange pump (e.g., Baker et al., 1969). Thus, the stimulating effects of the depolarizing agents on GABA efflux can be attributed to a direct effect of the membrane potential change on Na-coupled GABA efflux, rather than to a rise in $[Na]_i$.

Nevertheless, internal Na does appear to have an effect: Haycock et al. (1978) showed that, when terminals were depleted of (internal) Na, the depolarization-promoted GABA efflux was reduced. Furthermore, we have observed that the stimulation of GABA efflux by depolarizing agents is reduced when external Na is replaced by Li (Fig. 2); under these circumstances, $[Na]_i$ should be reduced. Both observations are consistent with the view that reducing $[Na]_i$ slows GABA efflux.

Discussion

The Na-GABA Co-Transport System Appears to Mediate both GABA Influx and Efflux

As noted above, there is now substantial evidence that the accumulation of GABA by presynaptic nerve terminals is powered by the Na electrochemical gradient and is voltage-sensitive (Martin, 1973; Blaustein & King, 1976; Kanner, 1978). In the present report, we provide evidence that the Na-coupled transport system in nerve terminals mediates the exit, as well as the entry, of GABA along with Na. This view is based on three observations: (i) Depolarization promotes Ca_o-independent GABA efflux, (ii) This GABA efflux appears to require internal but not external Na, and (iii) GABA efflux into Ca-free media is inhibited when the carriers are prevented from cycling because GABA, but *not* Na, is present in the incubation medium.

The possible implications of our findings deserve mention. One important consideration concerns the roles of the Na-GABA co-transport system in physiological (depolarization-evoked) GABA release and in GABA re-uptake. With a normal Na electrochemical gradient across the plasmalemma and a normal resting potential, this transport system should be able to maintain a low GABA concentration in the synaptic cleft. When the GABAergic terminals are depolarized, GABA uptake should be inhibited and efflux through this system should be promoted. This does not rule out the possibility that the primary mode of GABA release in most GABAergic neurons (but *see* Schwartz, 1982) is Ca-dependent, and involves the depolarization-evoked exocytosis of the

contents of synaptic vesicles that store GABA. However, the voltage dependence of the Ca-independent Na-GABA co-transport system may indicate that this transport system helps to maintain a high GABA concentration in the synaptic cleft during depolarization, and then mediates rapid presynaptic re-accumulation when the terminals are repolarized.

A second consideration that deserves comment pertains to the numerous studies that have been carried out on GABA binding to "GABA receptors" in synaptic membranes (e.g., Enna & Snyder, 1975, 1977; Lester & Peck, 1979). Our observations clearly indicate that GABA binds, with high affinity, to the Na-GABA co-transport system in the absence of external Na. Thus, considerable caution must be exercised in GABA binding studies, in order to distinguish Na-independent binding to postsynaptic receptors from binding to the presynaptic Na-GABA co-transport system (Lester & Peck, 1979). In fact, GABA binding to synaptic membranes in the absence of Na, exhibits a complex kinetics that may be indicative of multiple binding sites (Winkler, Nicklas & Berl, 1978); perhaps the binding of GABA to autoreceptors and/or to the Na-dependent carriers in the absence of Na (*see* below) may contribute to this behavior.

A Model for Na-GABA Co-Transport

In the ensuing discussion, we present a kinetic model of the Na-GABA co-transport system that appears to account for all the observed effects of external cation and GABA concentrations, and membrane potential, on GABA influx and efflux.

The relationship between external GABA concentration ($[G]_o$) and GABA uptake, at a constant external Na concentration ($[Na]_o$) can be described by a simple Michaelis-Menten expression; i.e., it can be fitted to a rectangular hyperbola (*cf.* Martin, 1976, and *see* Fig. 5). This indicates that one GABA molecule binds to one carrier. However, the relationship between $[Na]_o$ and GABA influx is sigmoid, which implies that more than one Na ion is required to activate the uptake of one GABA molecule. The available evidence indicates that the stoichiometry is 2Na:1GABA (*cf.* Blaustein & King, 1976; Martin, 1976). In this respect, the carrier-mediated transport of Na and GABA is analogous to an enzymatic reaction with three substrates (e.g., two external Na ions and one GABA molecule), and three products (two internal Na ions and the GABA).

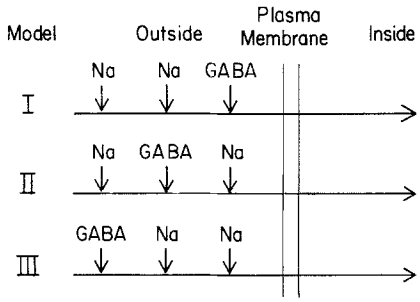

Fig. 3. Diagram illustrating the binding sequences (reading left to right) for Na and GABA in the three sequential models (I, II and III, respectively)

Two different general mechanisms of substrate binding to the carriers should be distinguishable, namely, an ordered and a random type. Possible ordered mechanisms include: (i) Binding of both Na ions before the GABA, (ii) Binding of one Na ion before, and one after the binding of GABA, and (iii) Binding of GABA before either Na ion. These three situations are considered, respectively, in models I, II and III (*see* Figs. 3 and 4). The other general mechanism, random binding, represents a combination of the three ordered mechanisms. However, we show (*see* below) that only one of the ordered mechanisms fits the experimental data; thus, the random mechanism appears to be ruled out and will not be considered further.

Evidence for Ordered Binding of Na and GABA to the Carrier

The derivations of the kinetic expressions for GABA transport by the three ordered binding models are given in the Appendix. Expressions are only provided for binding at the external face of the plasmalemma. Because insufficient data are available, we are unable to determine the sequence of binding or dissociation at the internal face of the membrane; thus, we cannot yet distinguish between glide symmetry and mirror symmetry (*cf.* Hopfer & Groseclose, 1980; Hopfer & Liedtke, 1981). However, we can use the kinetic expressions and experimental data to distinguish among the three ordered mechanisms, with respect to binding at the external face of the membrane.

The numerator in Eq. (A8) (*see* Appendix) shows that the *apparent* maximal rate of GABA transport (J^*_{GABA}) in Model I (both Na ions bind before GABA) is given by the expression:

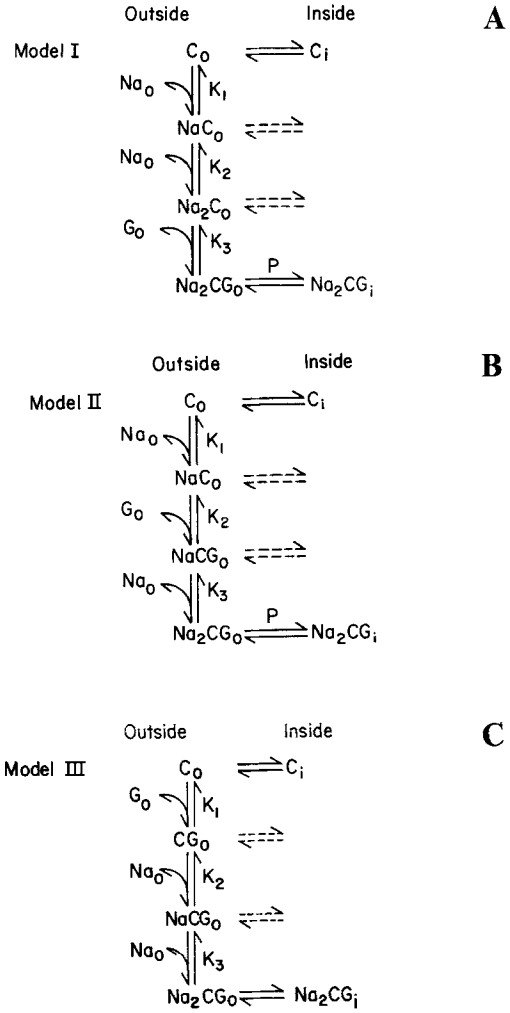

Fig. 4. Kinetic reaction schemes for models I, II and III. In these models, C_o and C_i represent the free carriers at the external and internal membrane faces, respectively. Na_o and G_o are, respectively, external Na and GABA. Na_2CG_o and Na_2CG_i represent the fully-loaded carriers at the external and internal faces of the membrane, respectively. The partially-loaded carriers are all "dead-end" products (i.e., they do not participate in translocation). Note that the dissociation constants for the three reactions, K_1, K_2 and K_3, are not identical in all of the models (*see* Table 1). Further details are given in the text

$$J^*_{GABA} = J_{GABA(max)}(1), \tag{A9}$$

where $J_{GABA(max)}$ is defined in the Appendix (Eq. (A4)). In other words, according to this model, J^*_{GABA}, the maximum rate of GABA uptake is independent of the Na concentration, $[Na]_o$; only the apparent half-saturation for GABA uptake (*see* bracketed terms in the denominator of Eq. (A8)) is dependent upon the Na concentration. The lack of effect of $[Na]_o$ on J^*_{GABA} is inconsistent with experimental observation (e.g., *see* Martin, 1973, and Fig. 5).

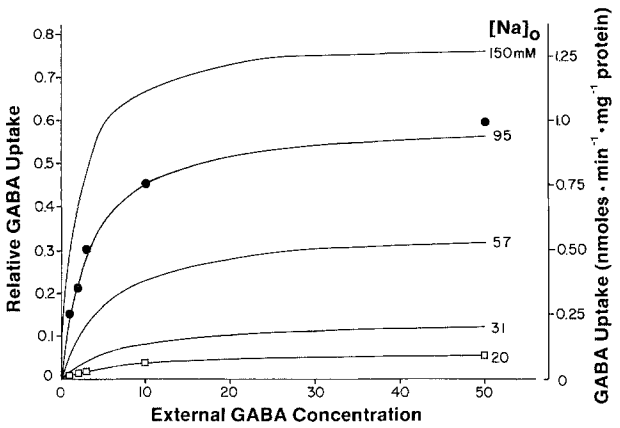

Fig. 5. Effect of GABA concentration on GABA uptake by synaptosomes at several external Na concentrations. The curves were calculated from Eq. (3) with $J_{GABA(max)} = 1.0$ and the values of $[Na]_o$ indicated on the graph. Filled circles and open squares are data from Figs. 3 and 5 of Martin (1973) (*see* right-hand ordinate scale)

Thus, Model I can be excluded from further consideration.

In contrast, the expressions for J^*_{GABA} in Models II and III (Appendix Eqs. (A13) and (A16), respectively) both show a dependence on $[Na]_o$.

For Model II

$$J^*_{GABA} = J_{GABA(max)} \left(\frac{[Na]_o}{[Na]_o + K_3} \right) \tag{A13}$$

and for Model III

$$J^*_{GABA} = J_{GABA(max)} \left(\frac{[Na]_o^2}{K_2 K_3 + K_3 [Na]_o + [Na]_o^2} \right). \tag{A16}$$

This dependence on the external Na concentrations is consistent with experimental observations which show, for example, that a fivefold increase in $[Na]_o$ can increase J^*_{GABA} for GABA uptake five- to tenfold (*see* Fig. 5 and Martin, 1973). The fact that J^*_{GABA} actually shows a greater-than-linear dependence on $[Na]_o$ appears to be evidence in favor of Model III (in which GABA binds before either Na ion).

The GABA efflux data provide additional means to distinguish between Models II and III. In the absence of external GABA, external Na has no effect on GABA efflux (Martin, 1973). However, as shown in Fig. 1, in the absence of external Na, external GABA *inhibits* GABA efflux. The most straightforward expla-

nation for these findings is that GABA can bind to the carriers in the absence of Na, although the carriers are then unable to cycle.

Estimation of Model III Kinetic Parameters

In sum, the aforementioned considerations lend strong support to the view that the Na-GABA co-transport system involves the ordered binding of GABA first, and then two Na ions, at the external face of the plasmalemma. Further support is provided by the fact that we can obtain a set of consistent kinetic parameters from Model III that appears to fit the experimental observations.

Model III can be described by a Michaelis-Menton type of rate expression (*see* Eqs. (A15) and (A16)),

$$J_{GABA}=\frac{J^*_{GABA}\cdot[G]_o}{K_G+[G]_o} \qquad (A5)$$

where J_{GABA} is the rate of (Na_o-dependent) GABA uptake. The apparent maximal rate of GABA uptake, J^*_{GABA}, is given by Eq. (A16) (above). The apparent half-saturation, K_G, is given by (*see* denominator in Eq. (A15)):

$$K_G=\left(\frac{K_1K_2K_3}{K_2K_3+K_3[Na]_o+[Na]_o^2}\right). \qquad (1)$$

K_1, K_2 and K_3 are the dissociation constants for the binding of GABA and the two Na ions, respectively, to the carrier (*see* Fig. 4 and Appendix). $[Na]_o$ and $[G]_o$ are, respectively, the external Na and GABA concentrations.

On the assumption that $K_2K_3 \gg K_3[Na]_o$, Eq. (1) can be simplified to:

$$K'_G=\left(\frac{K_1K_2K_3}{K_2K_3+[Na]_o^2}\right). \qquad (1a)$$

Also from Eq. (A16), we have:

$$J^*_{GABA}=J_{GABA(max)}\left(\frac{[Na]_o^2}{K_2K_3+[Na]_o^2}\right). \qquad (2)$$

Initial estimates of the model parameters appear to validate the simplifying assumption that led to Eq. (1a).

With this simplification, estimates of the dissociation constants for Model III can be obtained in several ways:

Method (1). The Ratio of J^*_{GABA} at High and Low $[Na]_o$ ($[Na_1]_o$ and $[Na_2]_o$, Respectively). According to Eq. (2),

$$\frac{J^*_{GABA}\text{ at }[Na_1]_o}{J^*_{GABA}\text{ at }[Na_2]_o}=\frac{[Na_1]_o^2(K_2K_3+[Na_2]_o^2)}{[Na_2]_o^2(K_2K_3+[Na_1]_o^2)}.$$

For example, we can use the observed J^*_{GABA} values of Martin (1973, Fig. 4) for $[Na]_o=95$ and 19 mM to solve for K_2K_3:

$$\frac{J^*_{GABA}\text{ at 95 mM Na}}{J^*_{GABA}\text{ at 19 mM Na}}=\frac{10^3\text{ pmol/mg protein}\times\text{min}}{10^2\text{ pmol/mg protein}\times\text{min}}$$

$$\frac{10}{1}=\frac{(95)^2\{K_2K_3+(19)^2\}}{(19)^2\{K_2K_3+(95)^2\}}.$$

The solution is:

$$K_2K_3\simeq 6\times10^3\text{ mM}^2.$$

Method (2). The Ratio of K'_G at High and Low $[Na]_o$. As shown by Martin (1973), the apparent affinity of the transport system for GABA is increased by increasing $[Na]_o$. Then, from Eq. (1a), above, we have:

$$\frac{K_G\text{ at }[Na_1]_o}{K_G\text{ at }[Na_2]_o}=\frac{K_2K_3+[Na_2]_o^2}{K_2K_3+[Na_1]_o^2}.$$

Substituting in data from Martin (1973, Fig. 5):

$$\frac{K'_G\text{ at 95 mM Na}}{K'_G\text{ at 19 mM Na}}=\frac{4\,\mu\text{M}}{8.5\,\mu\text{M}}=\frac{K_2K_3+(19)^2}{K_2K_3+(95)^2}.$$

Solving for K_2K_3 gives:

$$K_2K_3\simeq 7\times10^3\text{ mM}^2.$$

Thus, both methods yield similar results.

Equation (1a) can also be used to estimate K_1. At low $[Na]_o$ (e.g., 19 mM),

$$K_2K_3\gg[Na]_o^2.$$

That is:

$$6\times10^3\text{ mM}^2\gg 361\text{ mM}^2.$$

Then, substituting into Eq. (1a) at low $[Na]_o$, we have:

$$K'_G\simeq K_1\left(\frac{K_2K_3}{K_2K_3}\right).$$

Thus,

$K'_G \simeq K_1 \simeq 8.5\,\mu M.$

The explicit GABA influx expression for Model III (*see* Eqs. (A15), (A16), (1) and (2)) can then be written as:

$$J_{GABA} = \frac{J_{GABA(max)} \left(\frac{[Na]_o^2}{6 \times 10^3\,mM^2 + [Na]_o^2} \right) \cdot [G]_o}{\left(\frac{50\,mM^3}{6 \times 10^3\,mM^2 + [Na]^2} \right) + [G]_o}. \quad (3)$$

This model fits the experimental observations (e.g., Martin, 1973, and Blaustein & King, 1976) on the voltage dependency of the transport system, and shows the effects of $[Na]_o$ on the apparent maximal rate of GABA transport and on the apparent affinity (i.e., half-maximal rate of transport) (*see* Fig. 4). For example, when Eq. (3) is used to graph GABA efflux at several different values of $[Na]_o$, the curves are sections of rectangular hyperbolae (Fig. 5). We see that lowering $[Na]_o$ substantially decreases J^*_{GABA}, and slightly increases K'_G (Eq. (1a)). This is consistent with the aforementioned observations of Martin (1973, Fig. 5), as well as our own, unpublished data.

Equation (A15) (with the same simplification, that $K_2 K_3 \gg K_3 [Na]_o$) can be rearranged to give:

$$J_{GABA} = \frac{J_{GABA(max)}}{1 + \left(\frac{\bar{K}_{Na}}{[Na]_o} \right)^2} \quad (4)$$

where $\bar{K}_{Na}$ is given by:

$$\bar{K}_{Na} = \sqrt{\frac{K_1 K_2 K_3 + K_2 K_3 [G]_o}{[G]_o}}. \quad (4a)$$

Equation (4) enables us to examine the relationship between $[Na]_o$ and J_{GABA}, as shown in Fig. (6). The curves described by this expression (the Hill equation, with $n=2$) are consistent with the observed $2\,Na^+ : 1\,GABA$ stoichiometry (e.g., Blaustein & King, 1976; Martin, 1973, 1976). Although the fit of the theoretical curves to the experimental data is imperfect, note that, for both the data and the model, $\bar{K}_{Na}$ increases as $[GABA]_o$ is reduced, as is the case experimentally (e.g., Martin, 1973, Fig. 6, and

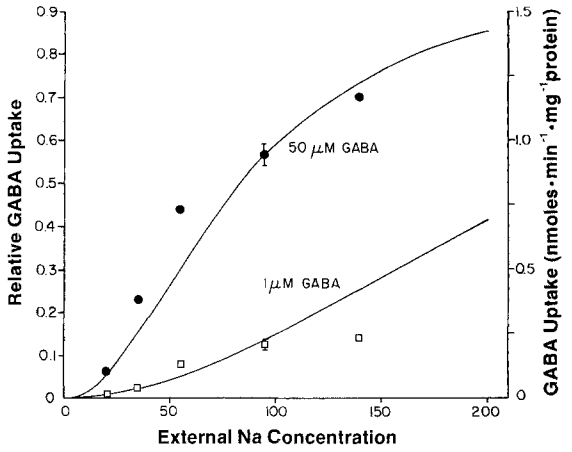

Fig. 6. Effect of $[Na]_o$ on GABA uptake by synaptosomes. The curves were calculated from Eq. (4) with $K_1 = 8.5\,\mu M$, $K_2 K_3 = 6 \times 10^3\,mM^2$ and $J_{GABA(max)} = 1.0$. The filled circles and open squares are data from Fig. 6 of Martin (1973) (*see* right-hand ordinate scale). Note that, at 100 mM Na, the calculated ratio of GABA uptakes (50 μM GABA: 1 μM GABA) is 3.9 - similar to ratio obtained by Martin

M.T. Nelson & M.P. Blaustein, *unpublished data*). In sum, Model III appears to be consistent with a large variety of experimental observations.

Recently, Wheeler and Hollingsworth (1979) examined several kinetic models for GABA transport in synaptosomes. Their models $P-1$, $P-2$ and $P-3$ are identical to our models I, II and III, respectively. They, too, concluded that: (i) binding of substrates to the carriers is sequential, (ii) free C may bind GABA to form the "dead-end product", CG, and (iii) only fully-loaded carriers (Na_2CG) can transport GABA. They favored a model similar to our model I (both Na ions bind to the carriers before GABA), but including a step identical to the first step in our model III ($C + G \rightleftharpoons CG$) to produce the dead-end product CG. This (more complex) model produced the smallest errors when fit to their data by computer simulation. However, as we have shown, model I does not allow for any influence of $[Na]_o$ on the apparent maximum rate of GABA transport (*see* Fig. 6). Furthermore, in the model favored by Wheeler and Hollingsworth, NaC and Na_2C should be dead-end products when Na, but not GABA, is present in the external medium. But we found no such effect: in the absence of (external) GABA, removal of external Na did not stimulate GABA efflux (*see* Figs. 1 and 2). Thus, our model III appears to be most consistent with the available data on the Na-depen-

dent, carrier-mediated transport of GABA in synaptosomes.

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Appendix

The GABA transport system in rat brain presynaptic terminals appears to have a Na/GABA stoichiometric ratio of 2:1. In addition, the partially-loaded carriers cannot transport GABA (i.e., at $[Na]_o \to 0$, the carrier-mediated GABA influx$\to$0). Thus, the Na ions must be essential for GABA-loaded carrier translocation. This transport system can be modelled with the three simple ordered binding sequences diagrammed in Fig. 3. The expanded kinetic models for these three sequences are shown in Fig. 4 and the dissociation constants for the substrate binding reactions are listed in Table 1.

For all three models, the product of the three dissociation constants, $K_1K_2K_3$, is given by:

$$K_1K_2K_3 = \frac{[Na]_o^2 \cdot [C]_o \cdot [G]_o}{[Na_2CG]_o} \quad (A1)$$

The GABA influx, J_{GABA}, is:

$$J_{GABA} = P \cdot [Na_2CG]_o \quad (A2)$$

$$= P \cdot \left(\frac{[Na]_o^2 \cdot [C]_o \cdot [G]_o}{K_1K_2K_3}\right). \quad (A3)$$

where P is the "permeability coefficient" for the fully-loaded carrier complex, Na_2CG. The maximal influx, $J_{GABA(max)}$, is:

$$J_{GABA(max)} = P \cdot C_T \quad (A4)$$

where C_T is the total (external) carrier concentration (*see* below).

For convenience in fitting the experimental data, we will employ the Michaelis-Menton forms of the rate equations for all three models:

$$J_{GABA} = \frac{J^*_{GABA} \cdot [G]_o}{K_G + [G]_o} \quad (A5)$$

where J^*_{GABA} is the *apparent* maximum GABA influx, and K_G is the apparent half-saturation "coefficient" for external GABA.

In the subsequent sections, we derive the Michaelis-Menton forms of the influx equation for each of the three models. Unfortunately, insufficient data are available to justify modelling of GABA efflux as well.

Model I (Fig. 4A). Both Na Ions Bind before GABA

In this case,

$$C_T = [C]_o + [NaC]_o + [Na_2C]_o + [Na_2CG]_o. \quad (A6)$$

Table 1. Dissociation constants for the three substrate binding reactions in kinetic models I–III (*see* Fig. 4)

Dissociation constant	Model I (Fig. 4*A*)	Model II (Fig. 4*B*)	Model III (Fig. 4*C*)
K_1	$\frac{[Na]_o \cdot [C]_o}{[NaC]_o}$	$\frac{[Na]_o \cdot [C]_o}{[NaC]_o}$	$\frac{[C]_o \cdot [G]_o}{[CG]_o}$
K_2	$\frac{[Na]_o \cdot [NaC]_o}{[Na_2C]_o}$	$\frac{[NaC]_o \cdot [G]_o}{[NaCG]_o}$	$\frac{[Na]_o \cdot [CG]_o}{[NaCG]_o}$
K_3	$\frac{[Na_2C]_o \cdot [G]_o}{[Na_2CG]_o}$	$\frac{[Na]_o \cdot [NaCG]_o}{[Na_2CG]}$	$\frac{[Na]_o \cdot [NaCG]_o}{[Na_2CG]_o}$

Multiplying J_{GABA} (Eq. (A3) by C_T/C_T, and substituting Eq. (A6) in the denominator, upon rearrangement of terms, gives:

$$J_{GABA} = \frac{P \cdot C_T(1)[G]_o}{\left(\frac{K_1K_2K_3}{[Na]_o^2} + \frac{K_2K_3}{[Na]_o} + K_3\right) + [G]_o}. \quad (A7)$$

Then, substituting in $J_{GABA(max)}$ (Eq. (A4) yields the Michaelis-Menten form of the influx expression (*see* Eq. (A5)):

$$J_{GABA} = \frac{J_{GABA(max)}(1)[G]_o}{\left(\frac{K_1K_2K_3}{[Na]_o^2} + \frac{K_2K_3}{[Na]_o} + K_3\right) + [G]_o} \quad (A8)$$

where the *apparent* maximum GABA influx, J^*_{GABA}, is:

$$J^*_{GABA} = J_{GABA(max)}(1). \quad (A9)$$

Model II (Fig. 4B). One Na Ion Binds before GABA

In model II, C_T is given by:

$$C_T = [C]_o + [NaC]_o + [NaCG]_o + [Na_2CG]_o. \quad (A10)$$

Therefore, multiplying J_{GABA} (Eq. (A3)) by C_T/C_T, and substituting Eq. (A10) into the denominator, and rearranging terms gives:

$$J_{GABA} = \frac{P \cdot C_T \left(\frac{[Na]_o}{[Na]_o + K_3}\right)[G]_o}{\left(\frac{K_1K_2K_3}{[Na]_o^2 + [Na]_oK_3} + \frac{K_2K_3}{[Na]_o + K_3}\right) + [G]_o}. \quad (A11)$$

The Michaelis-Menten form (*see* Eq. (A5)) of this expression is obtained by substituting for $P \cdot C_T$ (Eq. (A4)):

$$J_{GABA} = \frac{J_{GABA(max)} \left(\frac{[Na]_o}{[Na]_o + K_3}\right)[G]_o}{\left(\frac{K_1K_2K_3}{[Na]_o^2 + [Na]_oK_3} + \frac{K_2K_3}{[Na]_oK_3}\right) + [G]_o}. \quad (A12)$$

In this model, the *apparent* maximum GABA influx, J^*_{GABA}, is:

$$J^*_{GABA} = J_{GABA(max)} \left(\frac{[Na]_o}{[Na]_o + K_3} \right). \quad (A13)$$

Model III (Fig. 4C). GABA Binds First

When GABA binds before the two Na ions, the total (external) carrier concentration is:

$$C_T = [C]_o + [CG]_o + [NaCG]_o + [Na_2CG]_o. \quad (A14)$$

In this case, multiplying J_{GABA} (Eq. (A3)) by C_T/C_T, and substituting Eq. (A14) into the denominator gives, upon rearrangement and substituting from Eq. (A4):

$$J_{GABA} = \frac{J_{GABA(max)} \left(\frac{[Na]_o^2}{K_2K_3 + K_3[Na]_o + [Na]_o^2} \right) [G]_o}{\left(\frac{K_1K_2K_3}{K_2K_3 + K_3[Na]_o + [Na]_o^2} \right) + [G]_o}. \quad (A15)$$

Thus, if GABA binds first, the *apparent* maximum GABA influx, J^*_{GABA}, is:

$$J^*_{GABA} = J_{GABA(max)} \left(\frac{[Na]_o^2}{K_2K_3 + K_3[Na]_o + [Na]_o^2} \right). \quad (A16)$$

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