Biochimica et Biophysica Acta, 731 (1983) 54–62
Elsevier

BBA 71661

EFFLUX AND EXCHANGE OF γ-AMINOBUTYRIC ACID AND NIPECOTIC ACID CATALYSED BY SYNAPTIC PLASMA MEMBRANE VESICLES ISOLATED FROM IMMATURE RAT BRAIN

BARUCH I. KANNER, ANNIE BENDAHAN and RODICA RADIAN

Laboratory of Neurochemistry, Institute of Biochemistry, Hadassah Medical School, The Hebrew University, Jerusalem (Israel)

(Received September 27th, 1982)
(Revised version received January 12th, 1983)

Key words: Nipecotic acid; γ-Aminobutyrate transport; (Brain plasma membrane)

The mechanism of γ-aminobutyric acid translocation in synaptic plasma membrane vesicles from rat brain has been probed by comparing the ion dependency of net efflux with that of exchange. Furthermore the question has been asked if the same mechanism operates for other solutes translocated by this transporter. Dilution-induced efflux of γ-aminobutyrate from the membrane vesicles is about 3-fold stimulated by externally added γ-aminobutyrate. Half maximal stimulation is obtained at a γ-aminobutyrate concentration similar to the K_m for γ-aminobutyrate influx. This stimulation (exchange) is dependent on external sodium but not on external chloride. In contrast to this γ-aminobutyrate influx is absolutely dependent on the simultaneous presence of sodium and chloride ions (Kanner, B.I. (1978) Biochemistry 17, 1207–1211), while efflux is dependent on the presence of these two ions on the inside (Kanner, B.I. and Kifer, L. (1981) Biochemistry 20, 3354–3358). Nigericin stimulates dilution-induced efflux of γ-aminobutyrate from potassium loaded vesicles to a larger extent than external γ-aminobutyrate. γ-Aminobutyrate further enhances the nigericin-induced stimulation, provided that the vesicles are not preloaded with chloride. Nipecotic acid is transported with the same features as γ-aminobutyrate and the two solutes behave similar with respect to the ion dependence of net flux and exchange. A model for the translocation cycle is proposed in which at least one of the translocated sodium ions binds to the transporter in its 'outside' conformation after chloride and the solute have bound previously. Conversely, the solute is released from its 'inside' conformation prior to chloride and at least one of the sodium ions.

Introduction

The use of membrane vesicles derived from the synaptic plasma membrane from rat brain cortex [1] has contributed significantly to our understanding of the mechanism of γ-aminobutyrate transport across this membrane. In these membrane vesicles only the high-affinity transport system for γ-aminobutyrate is expressed. Kinetic studies indicate that this neuronal system [1] displays simple Michaelis-Menten kinetics with a K_m of about 2.5 μM [2]. γ-Aminobutyrate influx is electrogenic and is absolutely dependent on both external sodium and chloride ions [2]. It is driven by the inward gradients of these two ions [2]. Efflux of γ-aminobutyrate from these vesicles requires internal sodium and chloride ions [3]. The effect of chloride ions in this system cannot be attributed to charge compensation [2,4], and therefore it appears that this γ-aminobutyrate transporter catalyses co-transport of γ-aminobutyrate, sodium and

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; solvent system 1, *n*-butanol/methanol/acetic acid/water (40:40:5:5, v/v).

chloride. Stoichiometry measurements indicate that the most likely stoichiometry is two sodium and one chloride ion per γ-aminobutyrate molecule transported [4]. Studies with membrane vesicles containing other transport systems indicate that, also there, additional ions are co-transported with the solute and sodium. Examples are the transport of serotonin in platelet membranes [5–7] and glutamate transport in synaptic plasma membrane vesicles from rat brain [8,9] renal brush border vesicles [10,11] and in membrane vesicles from rat liver [12].

Study of the ion dependence of influx and efflux of solutes provides information on the question which ions are co-transported with the solute. Furthermore, the ability of solute to trans-stimulate efflux of solute through a transport system as a function of the ions present can provide information on the binding order of the solutes and the ions to the transporter. In this communication we describe a study where this principle is applied to the (Na^+ and Cl^-)-dependent γ-aminobutyrate transporter from the rat brain synaptic plasma membrane. Furthermore we want to ask the question, if the same results are obtained when other solutes, translocated by this transporter, are used.

Nipecotic acid is an excellent candidate to fulfill the role of another solute translocated by the γ-aminobutyrate transporter. This compound is transported in a sodium dependent manner in brain slices [13], peripheral glial cells [14] and astroglial cells [15] and it seems that in these tissues nipecotic acid is transported by the same transporter as γ-aminobutyrate, since the latter inhibits nipecotic acid transport. In the case of brain slices, it has been shown that release of nipecotic acid can be provoked by either external GABA or by external nipecotic acid [13] providing further evidence that the same transport system is shared by the two solutes.

In this article we demonstrate that nipecotic acid is actively transported into the membrane vesicles, via the GABA transporter with similar characteristics as γ-aminobutyrate itself. Efflux and exchange measurements indicate that the steps involved in the translocation cycle can be described by a limited number of mechanisms and that the mechanism is the same for γ-aminobutyrate and for nipecotic acid.

Experimental Procedures

Methods

Preparation of membrane vesicles. Membrane vesicles from 14-day-old female rats were prepared from the isolated and purified synaptosomes upon osmotic shock and stored as described [2]. The vesicles were preloaded [2] prior to the transport assays with 0.1 M potassium phosphate + 1 mM $MgSO_4$, pH 6.8, unless indicated otherwise in the legends to the figures. Upon loading the vesicles were centrifuged for 20 min at $27000 \times g$ and taken up in a small volume of the loading solution. Subsequently 10-μl aliquots were diluted ($t = 0$) into 190 μl of the influx solution usually containing 0.1 M NaCl and 0.5 μCi of γ-amino[2,3-^{3}H]butyrate (29.3 Ci/mmol). At the indicated times the reactions were stopped by addition of 2 ml of ice-cold 0.15 M NaCl, filtered, washed and counted [2]. Protein was determined according to the method of Lowry et al. [16].

Influx of nipecotic acid. This was performed exactly as described [2] except that instead of radioactive γ-aminobutyrate each assay tube contained 0.5 μCi of D,L-[3,4-^{3}H]nipecotic acid, 40 Ci/mmol (62.5 nM in the assay).

Efflux from actively loaded vesicles. This was performed exactly as described previously [3]. Prior to active loading with γ-aminobutyrate, the vesicles were preloaded with 0.1 M potassium phosphate + 1 mM $MgSO_4$, pH 6.8 unless indicated otherwise in the figure legend (Fig. 5). Loading was performed by diluting the vesicles into a medium containing 0.1 M NaCl and radioactive γ-aminobutyrate and a subsequent incubation of 5 min. Efflux was induced by 20-fold dilution into the efflux solutions indicated in the legends to the figures. As pointed out under Results, carry over of ions from the undiluted samples does not affect any of our conclusions. The reaction was carried out at room temperature (as was influx) since it is known that in synaptomes no loss of amino acids, accumulated at 27°C, occurs during the washing procedure which takes place at 0–4°C [17]. This was verified to be also correct in the case of the membrane vesicles. The absolute values of the levels of accumulation and the rates of efflux were variable from batch to batch of vesicles. The pertinent characteristics of influx as well as efflux,

however, were the same, independent of the batch of vesicles. Therefore, representative experiments are presented. Experimental observations were verified by repeating the experiments three or four times.

Materials

γ-Amino[2,3-^{3}H]butyrate was obtained from New England Nuclear and DL-[3,4-^{3}H]nipecotic acid from Amersham. Valinomycin and CCCP were from Sigma, and nigericin was a generous gift of Dr. R.J. Hosley from Eli Lilly.

Results

Nipecotic acid is translocated by the γ-aminobutyrate transporter

The data presented in Fig. 1 illustrate the uptake of D,L-[3,4-^{3}H]nipecotic acid into membrane vesicles from rat brain. An artificial sodium and also chloride ion gradient is created by diluting (at time zero) the membrane vesicles loaded with potassium phosphate 20-fold into a NaCl solution containing D,L-[3,4-^{3}H]nipecotic acid. In this experiment approx. 45 pmol of nipecotic acid per mg of protein was taken up (Fig. 1A). The accumulated radioactivity represents unmodified nipecotic acid, since it has been found to cochromatograph (upon release from the filters with acid) with authentic nipecotic acid (solvent system I). Using the determined value of the total intravesicular volume of 7.4 μl/mg protein [2] the minimal calculated internal nipecotic acid concentration for the experiment described in Fig. 1A is 6.1 μM. Since the external concentration is 62.5 nM, this represents a concentration gradient of about 100-fold. Similar concentration gradients with this preparation have been obtained for uptake of γ-aminobutyrate [2] and L-glutamate [8]. Fig. 1A also shows that accumulation of nipecotic acid absolutely requires external sodium and chloride ions. The same phenomenon has been described for γ-aminobutyrate accumulation in the same preparation [2]. The critical importance of the ion gradients as driving forces for nipecotic acid transport is further underlined by the use of ionophores. Nigericin, an ionophore which under the experimental conditions is expected to exchange external sodium for internal potassium (thereby eliminating the sodium gradient) strongly inhibits nipecotic acid uptake (Fig. 1B). The ionophore valinomycin on the other hand, causes an approx. 40% stimulation (Fig. 1B). This ionophore is under experimental conditions ($[K^+]_{in} > [K^+]_{out}$) expected to enhance the magnitude of the membrane potential (interior negative). In line with this is the inhibitory effect of the proton conductor CCCP (Fig. 1B) which will diminish the membrane potential. These effects of valinomycin and CCCP on the membrane potential have been directly verified using the lipophilic tritium labelled tetraphenyl phosphonium ion [4]. Thus transport of nipecotic acid, just as γ-aminobutyrate transport appears to be an electrogenic process, driven by an interior negative membrane potential. The dependence of the process on chloride can not be explained by its ability to serve as a permeant anion. In the absence of external chloride, but in the presence of valinomycin, a condition where the membrane potential is even more negative, there is no nipecotic acid accumulation (data not shown).

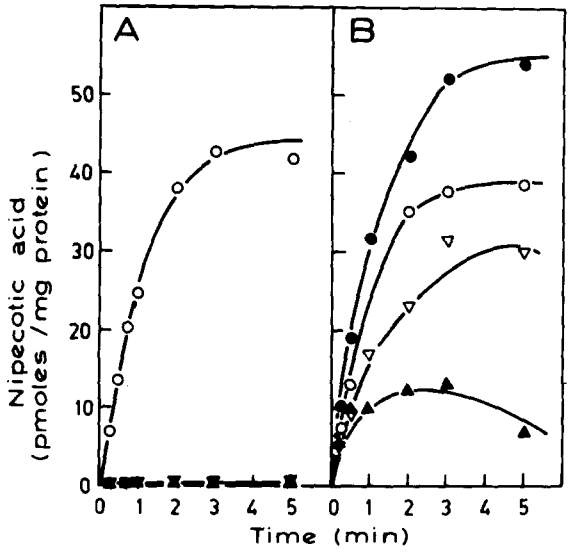

Fig. 1. Effect of external ions and ionophores on nipecotic acid transport. (A) The transport media contained 0.5 μCi of D,L-[3,4-^{3}H]nipecotic acid and 0.1 M NaCl (○——○); 0.1 M LiCl (▽——▽) or 0.1 M sodium phosphate, pH 6.8 (▲——▲). 62 μg of membrane protein was used per time point. (B) The transport media contained in addition to 0.5 μCi of D,L-[3,4-^{3}H]nipecotic acid and 0.1 M NaCl: nothing (○——○); 2.5 μM valinomycin (●——●); 5 μM CCCP (▽——▽); 5 μM nigericin (▲——▲).

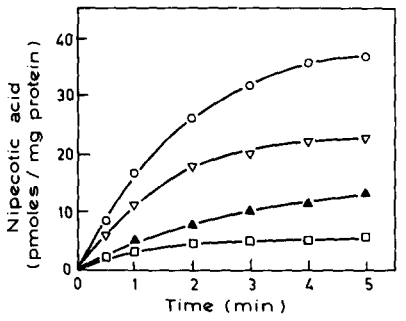

Fig. 2. Effect of transmembrane ion gradients on nipecotic acid transport. Influx was measured using 0.1 M NaCl + 1 μCi of D,L-[3,4-^{3}H]nipecotic acid (40 Ci/mmol) as external medium. Membrane vesicles were preloaded in the following media (amount of protein/assay point given in parenthesis): (○——○), $Na^+ + Cl^-$ gradients, 80 mM lithium phosphate, pH 6.8 (62 μg); (▽——▽), Cl-gradient, 80 mM sodium phosphate, pH 6.8 (70 μg); (▲——▲), Na^+ gradient, 0.1 M LiCl + 2 mM lithium phosphate, pH 6.8 (54 μg); (□——□), no gradients, 0.1 M NaCl + 2 mM sodium phosphate, pH 6.8 (54 μg).

Thus the effect of chloride is due to a specific interaction with the transporter. Internal potassium is not required, since when replaced by lithium ions, uptake is not much affected (Fig. 2).

Just as previously shown for the γ-aminobutyrate transport [2] the simultaneous presence of two gradients across the membrane appears to be required for high rates and extents of nipecotic acid uptake: (1) a Na^+ concentration gradient (out > in) and (2) a Cl^- concentration gradient (out > in) (Fig. 2). When NaCl is present on both sides of the membrane, hardly any uptake at all can be detected. In the absence of a sodium gradient, but when a chloride gradient is present, significant nipecotic acid uptake is observed. Furthermore uptake is also observed when only a sodium gradient is present. With both ion gradients present, higher levels of nipecotic acid are obtained than with either gradient alone (Fig. 2).

TABLE I

KINETIC CONSTANTS FOR γ-AMINOBUTURATE (GABA) AND NIPECOTIC ACID TRANSPORT

The reactions were terminated after 30 seconds. The data were plotted according to Lineweaver-Burk and the appropriate constants were calculated. The K_i of a solute on transport of the other was determined by measuring initial rates of this transport at varying concentrations in the presence and absence of one or more concentrations of unlabelled competing solute. Each measurement was performed at least three times and from the results the mean ± S.D. was calculated.

	K_m (μM)	K_i on other solute (μM)	V_{max} (pmol·mg^{-1}·min^{-1})
GABA	2.8 ± 0.4	3.0 ± 0.3	1750 ± 350
Nipecotic acid	9.1 ± 1.0	8.5 ± 1.4	4700 ± 1400

Transport of nipecotic acid is a saturable process which exhibits Michaelis-Menten kinetics. A K_m of 9.1 μM and a V_{max} of 4700 pmol/min per mg protein are obtained (Table I). It is of interest to note that γ-aminobutyrate inhibits nipecotic acid transport competitively with an apparent K_i of 3.0 μM. This value is very close to the affinity of γ-aminobutyrate for the transport (K_m is 2.8 μM, Table I). Furthermore unlabelled nipecotic acid competitively inhibits γ-aminobutyrate transport. The apparent K_i of 8.5 μM is in very good agreement with the K_m value for nipecotic acid transport (Table I). These results indicate that these two solutes are transported by the same transporter.

Efflux and exchange of γ-aminobutyrate and nipecotic acid

Dilution induced efflux of γ-aminobutyrate from actively loaded vesicles is measured in the experiment depicted in Fig. 3. Membrane vesicles previously loaded with potassium phosphate are diluted into a medium containing sodium chloride and γ-amino[2,3-^{3}H]butyrate and incubated for 5 min. Under these conditions accumulation of γ-aminobutyrate occurs until a steady-state plateau is reached [2]. At this time, the reaction mixture is diluted 20-fold with buffered NaCl, resulting in a slow effelux (Fig. 3). Unlabelled γ-aminobutyrate at 50μM, which is about 20-fold the K_m, when added to the efflux medium, converts efflux into exchange [3]. This causes an about 3-fold stimulation of loss of unlabelled γ-aminobutyrate from the vesicles (Fig. 3). This stimulation is due to a direct effect of external γ-aminobutyrate on the transporter, since the concentrations of γ-aminobutyrate which give rise to half-maximal stimulation of efflux (2–4 μM) are quite similar to the apparent K_m of value for γ-aminobutyrate influx

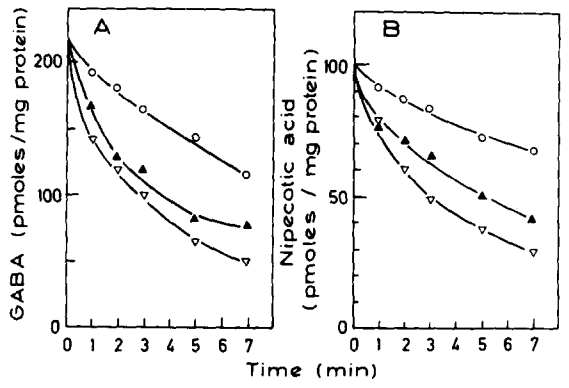

Fig. 3. Effect of external γ-aminobutyrate (GABA) and nipecotic acid on efflux of γ-aminobutyrate and nipecotic acid. Membrane vesicles were actively loaded and dilution induced efflux was measured. (A) Efflux of γ-aminobutyrate. 27 μg of membrane protein was used per assay point. (B) Efflux of nipecotic acid 29.5 μg of membrane protein was used. The efflux medium contained 90 mM NaCl + 10 mM sodium phosphate, pH 6.8, and the following additions: none (○——○); 100 μM nipecotic acid (▲——▲); γ-aminobutyrate either 20 μM (A) or 50 μM (B) (▽——▽).

(Table I). These observations indicate that exchange of internal with external γ-aminobutyrate is more rapid than net γ-aminobutyrate efflux and are consistent with a mechanism in which (a) slow step(s) distinct from γ-aminobutyrate translocation is rate-determining for net transport but is not obligatory for exchange. As indicated previously, this is not due to build up of a membrane potential during efflux which would limit the process [3].

If nipecotic acid is transported via the same transporter, dilution induced efflux of γ-aminobutyrate should also be accelerated by external nipecotic acid. This is indeed observed, although this stimulation is significantly smaller than that caused by γ-aminobutyrate (Fig. 3A). It is of interest to note that also the concentrations of nipecotic acid which give rise to half maximal stimulation of efflux are quite similar to the apparent K_m values for the transport of these solutes (data not shown). Furthermore it is of interest to note that the same features are observed for dilution induced efflux of nipecotic acid (Fig. 3B). Thus external γ-aminobutyrate as well as external nipecotic acid stimulates. Also in this case γ-aminobutyrate is more effective than nipecotic acid, although both are used at saturating levels (Fig. 3B).

Ion dependence of trans-stimulation of efflux by external γ-aminobutyrate and nipecotic acid

In the experiment depicted in Fig. 4, the ability of external γ-aminobutyrate to stimulate dilution-induced γ-aminobutyrate efflux is examined as a function of the external ion composition. In sodium chloride containing media the efflux of γ-aminobutyrate is slow and a clear stimulation by external γ-aminobutyrate is observed (Fig. 4A). If the chloride is replaced by phosphate, dilution-induced efflux is also stimulated by external γ-aminobutyrate (Fig. 4B). On the other hand, when the sodium ions are omitted the ability of external γ-aminobutyrate to stimulate is lost (Fig. 4C). The same result is obtained when both sodium and chloride ions are replaced (data not shown). The small amount of carried over Na^+ or Cl^- from the undiluted sample (5 mM upon a 20-fold dilution of a solution containing 100 mM) does not affect our conclusions. At 5 mM of either Na^+ or Cl^-, influx of γ-aminobutyrate is almost totally absent (data not shown). In addition, with regard to sodium ions this effect has also been examined in synaptosomes [17]. At 5 mM of external Na^+ virtually to stimulation of efflux by external γ-aminobutyrate is observed in these studies. Exactly the same ion dependence is observed when unlabelled nipecotic acid is used instead of γ-

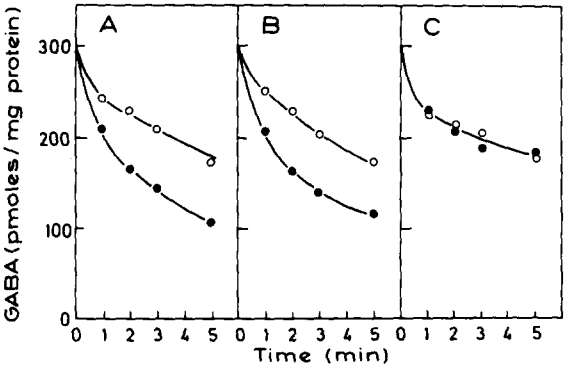

Fig. 4. Effect of external ions on the stimulation by external γ-aminobutyrate (GABA) on efflux of γ-aminobutyrate from actively loaded vesicles. Vesicles were loaded and efflux was performed with the following efflux solutions. (A) 90 mM NaCl + 10 mM sodium phosphate, pH 6.8. (B) 100 mM sodium phosphate, pH 6.8. (C) 90 mM LiCl + 10 mM lithium phosphate, pH 6.8. Open symbols, no further additions; closed symbols, + 20 μM γ-aminobutyrate. 23.5 μg protein was used per assay point.

aminobutyrate (data not shown). Similar results are also observed on trans-stimulation by γ-aminobutyrate of efflux of nipecotic acid (data not shown). These results indicate that the interaction of external γ-aminobutyrate or nipecotic acid with the transporter under exchange conditions is different from that during net influx. The later requires not only external sodium but also external chloride ions (Ref. 2; Fig. 1A).

It has been shown that efflux of γ-aminobutyrate requires both internal sodium and chloride ions [3] and it is likely that during dilution induced efflux upon active loading, one or both of these internal ions limit the efflux process. A possible explanation of the stimulation by external γ-aminobutyrate or nipecotic acid is that under exchange conditions internal sodium and chloride are not required. The results of the experiment depicted in Fig. 5 are consistent with this possibility. It can be seen that when vesicles preloaded with potassium phosphate are diluted into sodium chloride containing media, addition of nigericin (which is expected to cause an increase of the internal sodium ion concentration) causes strong stimulation of efflux (Fig. 5A). This stimulation is stronger than that by γ-aminobutyrate. It is of interest to note that when the same experiment is repeated with vesicles preloaded with sodium phosphate, nigericin hardly stimulates (Fig. 5B). This result indicates that when the vesicles are preloaded with a given ionic species, reequilibration with external ions during the uptake phase is relatively small. In the case of sodium preloaded vesicles (Fig. 5B), external γ-aminobutyrate stimulates stronger than nigericin. The stimulation by γ-aminobutyrate (Fig. 5B) is possibly due to the fact that in the presence of nigericin, internal chloride is still expected to be rate limiting. Support for this comes from the experiment in Fig. 5C with vesicles preloaded with potassium chloride. Both external γ-aminobutyrate and nigericin stimulate, but unlike with the chloride-less vesicles the combination of the two does not further stimulate (Fig. 5C). Thus when internal chloride and sodium are both present at sufficiently high levels, external γ-aminobutyrate does not accelerate efflux anymore. The dependence of the efflux process on internal sodium and chloride (Fig. 5 and Ref. 3) as well as the fact that these vesicles do not equilibrate with γ-aminobutyrate in the absence of sodium [2] indicate that efflux (in the absence of γ-aminobutyrate) is transporter-mediated. Similar results have also been obtained for efflux of nipecotic acid.

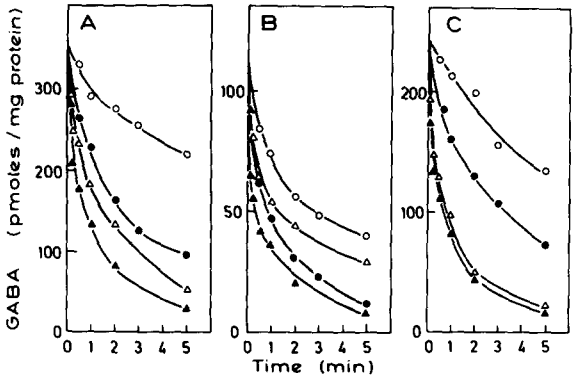

Fig. 5. Effect of nigericin and external γ-aminobutyrate (GABA) on efflux of γ-aminobutyrate from actively loaded vesicles. Vesicles were loaded with: (A) 0.1 M potassium phosphate, pH 6.8; (B) 0.1 M sodium phosphate, pH 6.8, or (C) 90 mM KCl + 10 mM potassium phosphate, pH 6.8. After active loading dilution induced efflux was initiated with 1 ml of 90 mM NaCl + 10 mM sodium phosphate, pH 6.8, and either of the following additions: none (○——○); 50 μM γ-aminobutyrate (●——●); nigericin 5 μM (△——△); 50 μM γ-aminobutyrate + 5 μM nigericin (▲——▲).

Discussion

The data described in this article provide strong evidence for the idea that nipecotic acid and γ-aminobutyrate are transported by the same transporter in synaptic plasma membrane vesicles from rat brain. Just like γ-aminobutyrate transport [2], transport of nipecotic acid also displays the absolute and specific requirement both for sodium as well as for chloride (Fig. 1A), is electrogenic (Fig. 1B) and is energized by the inward gradients of both sodium and chloride ions (Fig. 1B and Ref. 2). Furthermore γ-aminobutyrate and nipecotic acid competitively inhibit each other's transport and the apparent K_i for each solute is in close agreement with the apparent K_m for transport of this solute (Table I). Dilution induced efflux of one solute is trans-stimulated by one or the other solute. These latter data have also been observed for slices from rat brain [13] although there it was reported that the affinity of nipecotic acid is greater

than that of γ-aminobutyrate. Exactly the opposite result is observed in our experiments (Table I). Although the reason for this discrepancy is not known, it should be remembered that in slices, measurements of transport are complicated by many factors, e.g. $(Na^+ + K^+)$-ATPase, possible sequestration of the solute into storage organelles and considerable pools of endogenous solutes. Also it is possible that the discrepancy is due to developmental changes [18]. The identical behavior of γ-aminobutyrate and nipecotic acid in efflux and exchange (Figs. 3, 4, and 5) indicate that these two solutes are transported not only by the same transporter but also by the same mechanism.

With regard to the mechanism of translocation, the results obtained previously [2,3] and in this article are compatible both with an ordered or a random mechanism. However, the results put quite a lot of restrictions on to either mechanism. This is illustrated for the case of an ordered mechanism in Fig. 6. This model can account for all the data accumulated. The solute(s) stands for γ-aminobutyrate or nipecotic acid. Since the same results are obtained for both solutes, we shall, for the sake of simplicity, use γ-aminobutyrate. It is proposed that the first step during influx is the binding of chloride to the external face of the transporter (step 1). This is followed by the binding of γ-aminobutyrate (step 2) and two sodium ions (step 3). This results in the formation of the translocation complex (CNa_2SCl). Upon translocation (step 4), the γ-aminobutyrate is released first (step 5) followed by sodium and chloride (step 6). Finally the unloaded transporter reorients so that the binding sites for γ-aminobutyrate, sodium and chloride become again exposed to the outside (step 7). Now a new influx cycle can be initiated. Efflux occurs via the same route, but in the opposite direction (clockwise).

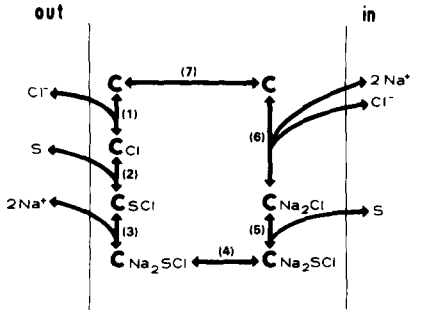

Fig. 6. Proposed model for the translocation cycle catalysed by the γ-aminobutyrate transporter. S, γ-aminobutyrate or nipecotic acid.

Net efflux (as well as influx) involves all steps (1–7) while exchange may occur via steps 2–5. The comparison between the two can give information on the mechanism of transport. Thus trans-stimulation of efflux by external γ-aminobutyrate can be explained by assuming that one of the steps 1, 6, or 7, needed in efflux but not in exchange, is the rate limiting step in efflux. The increased loss of radioactive γ-aminobutyrate during exchange conditions is not dependent on external chloride. Thus upon binding of the solutes, on the inside, translocation and release of radioactive γ-aminobutyrate to the outside (steps 6 to 2), unlabelled γ-aminobutyrate can rebind to the transporter before chloride is released (step 1) and loss of γ-aminobutyrate occurs a result of shuttling back and forth through these steps. Thus the radioactive γ-aminobutyrate can be lost from the vesicles in the absence of external chloride. On the other hand external sodium is needed for exchange to occur; thus at least one of the sodium ions should be released on the outside (step 3) before γ-aminobutyrate (step 2). The number of sodium ions participating in the translocation cycle is larger than one and is possibly two [4] and this is the reason that two sodium ions are placed in the model. For the sake of simplicity it is assumed that both sodium ions are associating and dissociating together, but in fact we have no information on the second sodium the binding of which can be accommodated anywhere in the scheme. Even the idea that one of the sodium ions is released on the outside prior to γ-aminobutyrate is not completely certain. Theoretically the alternative explanation is possible, namely that sodium is released on the outside after the solute. In that case, one would have to assume that upon release of solute, sodium is released much faster than the rebinding of solute. Since stimulation by γ-aminobutyrate does not require external chloride, sodium would have to be released between γ-aminobutyrate and chloride. But in this case, there should be quite a lot of restriction on the various rate constants. Although this may be the case, we prefer the simpler ex-

planation which proposes that at least one sodium ion is released on the outside prior to solute.

It has been established that efflux of γ-aminobutyrate requires both internal chloride and sodium [3]. An example of this is illustrated in Fig. 5A; nigericin (which increases internal sodium) stimulates the rate of loss of radioactive γ-aminobutyrate stronger than external γ-aminobutyrate. When internal sodium and chloride are no longer rate-limiting, the stimulation by external γ-aminobutyrate is also not observed anymore (Fig. 5C). Thus the model should explain that when internal sodium and chloride are rate-limiting, external γ-aminobutyrate can somehow by-pass this. The possibility that external γ-aminobutyrate is doing this by increasing internal sodium and chloride is very unlikely. The V_{max} of the transporter is 750–1500 pmol/min per mg protein and the internal volume is 7.4 μl/mg protein. Thus in one minute the internal concentration of these ions because of γ-aminobutyrate influx rises only a few tenths of mM. A more adequate explanation is to postulate that on the inside the release of γ-aminobutyrate precedes the release of sodium and chloride (Fig. 6). Thus external γ-aminobutyrate will allow this translocation complex to be formed from the outside and upon reorientation of the transporter, unlabelled GABA is released to the inside (steps 1 to 5). If radioactive γ-aminobutyrate can bind (reversal of step 5) before the sodium and chloride are lost (step 6) the radioactive γ-aminobutyrate can exit (steps 5 to 1) even when internal sodium and chloride are rate-limiting. It is clear that an opposite order (sodium and chloride closer to the transition complex than γ-aminobutyrate) can not account for these observations. One difficulty is how to explain the stimulation by external γ-aminobutyrate, when internal chloride is limiting and no chloride is present on the outside (Fig. 4B). However it should be appreciated that the translocation complex still can be formed on the inside (albeit at a slower rate; steps 6 and 5). Once this priming step has taken place, the stimulated release of radioactive γ-aminobutyrate may proceed at full speed (steps 2 to 5, back and forth). Thus there should be a small lag before the rapid release of γ-aminobutyrate, but it is likely that this is to short to be picked up experimentally.

It should be stressed that this is not the only possible model. Also a random mechanism can not be excluded. In view of the reasoning above, one would have to assume that on the outside sodium dissociates faster than γ-aminobutyrate and chloride dissociates slower. On the inside γ-aminobutyrate dissociates faster than sodium and chloride.

Another interesting point is that the extent of stimulation of efflux by nipecotic acid is significantly lower than that by γ-aminobutyrate (Fig. 3) while its V_{max} value for influx is about 2.5-fold higher than that of γ-aminobutyrate (Table I). The extent of stimulation by external solute is dependent on the ratio of the rate of association of this solute to the rate of dissociation of chloride on the outside. Thus the rate of association of γ-aminobutyrate to the outside face of the transporter is probably larger than that of nipecotic acid. However V_{max} for influx may be determined by entirely other parameters. One of these might be the transmembrane movement of the translocation complex. Thus during influx conditions it is not clear which step is slower, the outward translocation of the unloaded transporter or the inward translocation of the fully loaded transporter. Since net influx is electrogenic, one of the two forms of the transporter has to bear a charge on it, a negative charge on the unloaded carrier or a positive charge on the fully loaded translocation complex. Since at present we do not know which of the two translocation steps is the slower, no charges are placed in the model.

In intact synaptosomes efflux is rather slow and is in several cases probably not mediated by the γ-aminobutyrate transporter [17,19,20] (but see Ref. 21). The reason that transporter mediated efflux is not observed is probably due to the low levels of internal sodium in synaptosomes (internal sodium is required for net efflux) and also to the fact that they are polarized. Since the uptake process is electrogenic [2,4] depolarisation may also provoke transporter-mediated efflux and this may be the explanation for depolarization-induced calcium-independent release [19]. Since exchange is not expected to be very dependent on internal sodium and this also does not involve net movement of charge, it is easy to understand that this process is readily observed in intact synaptosomes.

In membrane vesicles net efflux is also slow, but when the internal ion commposition is manipulated, so that they contain both high sodium and chloride-net efflux is so fast, that external γ-aminobutyrate does not stimulate any further. It is of interest to note that very recently a similar binding order with regard to sodium and γ-aminobutyrate (chloride was not considered) to the external face of the γ-aminobutyrate transporter in synaptosomes has been deduced [21]. In the total absence of sodium, external γ-aminobutyrate inhibited efflux, probably by locking the transporter in a form which can not recycle. In our experiments we did not see a significant inhibition under such conditions, but this may be due to the carry-over of some sodium ions (final concentration 5 mM) during the dilution.

Finally the results obtained here may explain some very recent results by Pastuszko et al. [22]. These authors reported that using intact synaptosomes they obtained only a shallow dependent of γ-aminobutyrate accumulation on external chloride. Since intact synaptosomes contain substantial amounts of γ-aminobutyrate, it is possible that in fact exchange was studied. As shown here, that process does not require external chloride.

Acknowledgement

We wish to thank Drs. Hannah Rahamimoff and Benjamin Rivnay for critical reading of the manuscript. This work was supported by Grant No. NS 16708-01 from the National Inst. of Neur. and Communicative Disorders and Stroke, and by Grant No. 2151/80 of the U.S.-Israel Binational Science Foundation

References

1 Kanner, B.I. (1980) Biochemistry 19, 692–697
2 Kanner, B.I. (1978) Biochemistry 17, 1207–1211
3 Kanner, B.I. and Kifer, L. (1981) Biochemistry 20, 3354–3358
4 Radian, R. and Kanner, B.I. (1983) Biochemistry 22, 1236–1241
5 Rudnick, G. (1977) J. Biol. Chem. 252, 2170–2174
6 Nelson, P.J. and Rudnick, G. (1979) J. Biol. Chem. 254, 10084–10089
7 Keyes, S.R. and Rudnick, G. (1982) J. Biol. Chem. 257, 1172–1176
8 Kanner, B.I. and Sharon, I. (1978) Biochemistry 17, 3949–3953
9 Kanner, B.I. and Marva, E. (1982) Biochemistry 21, 6327–6330
10 Burckhardt, G., Kinne, R., Stange, G. and Murer, H. (1980) Biochim. Biophys. Acta 559, 191–201
11 Schneider, E.G. and Sacktor, B. (1980) J. Biol. Chem. 255, 7645–7649
12 Sips, H.J., De Graaf, P.A. and Van Dam, K. (1982) Eur. J. Biochem. 122, 259–264
13 Johnston, G.A.R., Stephenson, A.L. and Twichin, B. (1976) J. Neurochem. 26, 83–87
14 Minchin, M.C.W. (1979) J. Neurochem. 32, 1519–1524
15 Larsson, O.M., Krogsgaard-Larsen, P. and Schousboe, A. (1980) J. Neurochem. 34, 970–977
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
17 Simon, J.R., Martin, D.L. and Kroll, M. (1973) J. Neurochem. 23, 981–991
18 Johnston, G.A.R. and Davies, L.P. (1974) J. Neurochem. 22, 101–105
19 Raiteri, M., Frederico, R., Colletti, A. and Levi, G. (1975) J. Neurochem. 24, 1243–1250
20 Levi, G. and Raiteri, M. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2981–2985
21 Nelson, M.T. and Blaustein, M.P. (1982) J. Membrane Biol. 69, 213–223
22 Pastuszko, A., Wilson, D.F. and Erecinska, M. (1982) J. Biol. Chem. 257, 7514–7519