Articles

γ-Aminobutyric Acid Transport in Reconstituted Preparations from Rat Brain: Coupled Sodium and Chloride Fluxes†

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ABSTRACT: Transport of γ-aminobutyric acid (GABA) is electrogenic and completely depends on the presence of both sodium and chloride ions. These ions appear to be cotransported with γ-aminobutyric acid through its transporter [reviewed in Kanner, B. I. (1983) *Biochim. Biophys. Acta 726*, 293-316]. Using proteoliposomes into which a partially purified γ-aminobutyric acid transporter preparation was reconstituted, we have been able—for the first time—to provide direct evidence for sodium- and chloride-coupled γ-aminobutyric acid transport. This has been done by measuring the fluxes of $^{22}Na^+$, $^{36}Cl^-$, and [^{3}H]GABA. These fluxes have the following characteristics: There are components of the net fluxes of sodium and chloride that are γ-aminobutyric acid dependent. The sodium flux is chloride dependent; i.e., when Cl^- is replaced by inorganic phosphate or by SO_4^{2-}, γ-aminobutyric acid dependent sodium fluxes are abolished. The chloride flux is sodium dependent; i.e., when Na^+ is replaced by $Tris^+$ or by Li^+, γ-aminobutyric acid dependent chloride fluxes are abolished. Thus, the γ-aminobutyric acid dependent sodium and chloride fluxes appear to be catalyzed by the transporter. Using these fluxes we have attempted to determine the stoichiometry of the process. We measured the initial rate of sodium-dependent γ-aminobutyric acid fluxes and that of γ-aminobutyric acid dependent sodium fluxes. This yields the stoichiometry between sodium and γ-aminobutyric acid (2.58 ± 0.99). Similarly, we measured the stoichiometry between chloride and γ-aminobutyric acid, which is found to be 1.27 ± 0.12. These results are consistent with those obtained previously when a thermodynamic approach was used [Radian, R., & Kanner, B. I. (1983) *Biochemistry 22*, 1236-1241]. The half-maximal effect obtained when the γ-aminobutyric acid concentration dependence of Cl^- and Na^+ transport is determined is much higher (70-80 μM) than the known K_m of this system (K_m = 4-10 μM). Reexamination of the kinetics of γ-aminobutyric acid transport reveals that there are two transport systems for it. The first one has a high affinity, K_m = 2.5-4 μM, and the other one has a low affinity, K_m = 200-600 μM. The low-affinity γ-aminobutyric acid transport was characterized. It was found that this process, just as the high-affinity process, is electrogenic, and it is absolutely dependent on Cl^- and Na^+. The sodium, chloride, and γ-aminobutyric acid fluxes probably originate from the low-affinity transporter.

The role of high-affinity neurotransmitter uptake is to remove them from the synaptic cleft and thereby to terminate the overall process of synaptic transmission (Iversen, 1975; Kuhar, 1973). One of the important and abundant transporters in rat brain is the one for γ-aminobutyric acid (GABA)[1] [reviewed in Kanner (1983)]. This system, which is located in the synaptic plasma membrane (Kanner, 1980), transports GABA by an electrogenic process that is absolutely dependent on sodium and chloride (Kanner, 1978). There is now a wealth of evidence—albeit indirect—that GABA is cotransported with both sodium and chloride ions [Radian & Kanner, 1983; Kanner & Kifer, 1981; reviewed in Kanner (1983)]. Several other neurotransmitter transporters also have a requirement for ions in addition to sodium, and in many cases this additional ion appears to be transported as well as by those transporters [reviewed in Kanner (1983)], for example, glutamate and serotonin.

If we assume that GABA is transported in its predominant form (the zwitterion), cotransport of it together with sodium and chloride in an electrogenic fashion would require a complex stoichiometry. This will be $n Na^+ : m Cl^- :$GABA, with $n > m$. We have tried to determine this stoichiometry in synaptic plasma membrane vesicles using a thermodynamic method and found values consistent with $n > 1$ and $m = 1$ (Radian & Kanner, 1983). This method, which yields minimal values, had to be used since the direct measurements—comparing initial flux rates of GABA with those of sodium and chloride—could not be done. The reason for this is the heterogeneous nature of the membrane vesicles. Part of them are leaky, and therefore the apparent rate of $^{22}Na^+$ flux is about 50-fold higher than the V_{max} for GABA (Kanner, 1980).

Recently, we have solubilized, purified, and reconstituted the GABA transporter (Radian & Kanner, 1985; Radian et al., 1986). The resulting proteoliposomes are very tight to ions (Radian & Kanner, 1985), and thus proteoliposomes, containing GABA transporter preparations of various degrees of purity, may be suitable to measure direct fluxes. Since the pure transporter is partly inactivated and very dilute, we have chosen to do these measurements with a partially pure transporter [70P_I fraction (Radian & Kanner, 1985)], which has not undergone any inactivation and is very concentrated (Radian & Kanner, 1985), enabling us to measure fluxes on

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[1] Abbreviations: GABA, γ-aminobutyric acid; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate.

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samples containing relatively many transporters. This latter feature is very important due to the low V_{max} of GABA transport in this fraction. We will show that with these proteoliposomes we can demonstrate—for the first time—GABA- and chloride-dependent sodium transport as well as GABA- and sodium-dependent chloride transport.

Experimental Procedures

Materials

[2,3-^{3}H]GABA was obtained from the Negev Nuclear Center, and $^{22}Na^+$ and $^{36}Cl^-$ were from Amersham. Nigericin was a gift of Dr. R. J. Hosley of Eli Lilly. From Sigma Chemical Co. we obtained valinomycin, cholate that was recrystallized from 70% ethanol (Kagawa & Racker, 1971), and asolectin, which was acetone extracted (Kagawa & Racker, 1971). Brain lipids were prepared from bovine brain according to the method of Folch et al. (1957). The cation exchanger Dowex 50-X2, 100–200 dry mesh (2% cross-linking), and the anion exchanger Dowex 1-X8, 100–200 dry mesh (8% cross-linking), were from Aldrich. Sephadex G-50, fine, was from Pharmacia.

Methods

Reconstitution. The $70P_I$ fraction, a partially purified GABA transporter preparation (Radian & Kanner, 1985), was reconstituted as described (Radian & Kanner, 1985) except that the amount of protein was increased 2.5-fold (50 μL of $70P_I$) while the total volume remained constant.

Preparation of Dowex for Ion-Transport Assays. This was basically done as described (Gasko et al., 1976). To prepare cation exchanger, 300 mL of 1 M Tris was added to 100 g of Dowex 50-X2. After 2 h of stirring, the supernatant was decanted, and the resin was washed repeatedly with distilled water until the pH of the supernatant attained 8–8.5. The Dowex was poured into 1-mL Pasteur pipets that were washed with 2 mL of water and then with 1 mL of 10 mM Tris-HCl, pH 7.4, 450 mM glycerol, and 1% bovine serum albumin. Anion exchanger was prepared by addition of 10 volumes of distilled water to the Dowex 1-X8 and stirring. Subsequently, it was exposed to 4 volumes of 2 N NaOH. After washing with water, until a pH value of 7 was reached, the resin was exposed to 5 volumes of 2 N acetic acid. Subsequently, it was washed again with water until the pH reached 7. The resin was poured into 1-mL Pasteur pipets that were washed with 2 mL of distilled water and then with 10 mM Tris–acetate, pH 7.4, 450 mM glycerol, and 1% bovine serum albumin.

Transport Assays. (*A*) *$^{22}Na^+$ Transport.* Reconstituted proteoliposomes (10 μL) were incubated for the indicated times with 100 μL of this solution of the following composition: 140 mM LiCl, 10 mM NaCl, 5 μM valinomycin, and 3.5 μCi of $^{22}Na^+$ with and without 1 mM of GABA. The reactions were stopped by applying 100 μL of reaction mixture to an ice-cold Dowex column using the air pressure obtained by a fitting syringe. This was followed by two 1-mL portions of of 10 mM Tris-HCl, pH 7.4, and 450 mM glycerol. The effluent of the columns was collected in scintillation vials, 14 mL of scintillation fluid was added, and the samples were counted. The results were corrected for by substracting "zero-time" values obtained by adding 10 μL of proteoliposomes to the columns, followed by 100 μL of incubation mixture and 2 × 1 mL of wash fluid, as above.

(*B*) *$^{36}Cl^-$ Transport.* Reconstituted proteoliposomes (10 μL) were incubated with 100 μL of a solution containing 100 mM NaP_i, pH 6.8, plus 5 μM valinomycin and 1 μCi $H^{36}Cl$ with and without 1 mM GABA. Transport was done as above except that anion exchanger was used and the washing solution contained 10 mM Tris–acetate, pH 7.4, and 450 mM glycerol.

(*C*) *[^{3}H]GABA Transport.* This was measured by incubating 10 μL of proteoliposomes into 150 mM NaCl (unless indicated otherwise in the legends to the figures), 1 or 50 mM GABA, 4 μCi of [2,3-^{3}H]GABA, and 5 μM valinomycin. Reactions were terminated by membrane filtration as described (Radian & Kanner, 1985).

Protein Determination. This was done with the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).

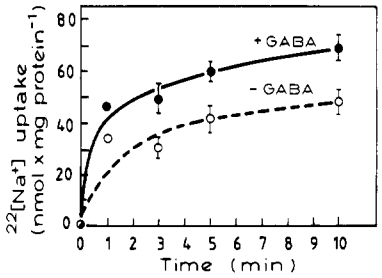

FIGURE 1: GABA-dependent $^{22}Na^+$–Na^+ exchange. Proteoliposomes were reconstituted by using the $70P_I$ fraction. The "in" medium contained 100 mM NaCl and 20 mM NaP_i, pH 6.8, and the protein concentration was 1.55 mg/mL. The proteoliposomes (10 μL) were diluted into 100 μL of 10 mM NaCl, 140 mM LiCl, and 3.5 μCi of carrier-free $^{22}Na^+$ with (●) or without (○) 1 mM GABA. Transport was measured as described under Experimental Procedures. The error bars indicate the standard deviation.

Results

GABA-Dependent $^{22}Na^+$–Na^+ Exchange. The GABA transporter has a relatively low V_{max}. This V_{max} is even lower at the suboptimal concentrations of sodium and chloride required to measure GABA-dependent $^{22}Na^+$ and $^{36}Cl^-$ fluxes, respectively. The rate of exchange may be faster than net flux, since it involves fewer steps (Kanner et al., 1983) and may thus be lacking the rate-limiting step for net flux. Therefore, we first tried to measure $^{22}Na^+$–Na^+ exchange. This exchange—an electroneutral process as opposed to net flux—when catalyzed by the GABA transporter should be dependent on GABA and chloride, the two cosubstrates. The $70P_I$ fraction—a GABA transporter preparation partially purified by ammonium sulfate fractionation (Radian & Kanner, 1985)—was reconstituted into liposomes by using the highest possible protein/lipid ratio. The proteoliposomes, which were formed in a sodium-containing medium, took up $^{22}Na^+$ from the external medium, and this uptake was stimulated by GABA (Figure 1). The GABA-dependent part of $^{22}Na^+$–Na^+ exchange was found to be dependent on chloride, and the chloride was required on the outside, i.e., the same side where GABA was present (data not shown). When this was the case, the GABA-dependent uptake was almost equal to the GABA-independent component. To determine whether the $^{22}Na^+$ taken up was in fact transported into the proteoliposomes, exchange was allowed to proceed for 10 min, and subsequently the proteoliposomes were diluted 10-fold into a medium containing unlabeled sodium in the presence or absence of the ionophore nigericin, which under these conditions will exchange internal with external sodium. It can be seen that nigericin caused an immediate exit of $^{22}Na^+$ from the proteoliposomes whether it had been taken up in the presence or absence of GABA (Figure 2). The release in the absence of the ionophore was much slower. Thus, all the $^{22}Na^+$ taken up, including the GABA-dependent portion, is transported into the proteoliposomes.

GABA-Dependent Net $^{22}Na^+$ Flux. After it was established that GABA can promote $^{22}Na^+$–Na^+ exchange via its trans-

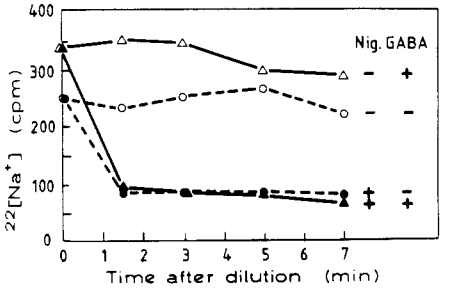

FIGURE 2: Efflux of $^{22}Na^+$, previously accumulated via exchange, by nigericin. Proteoliposomes (10 μL, 16 μg of protein) were loaded with $^{22}Na^+$ via exchange with or without GABA exactly as described in Figure 1. Subsequently, 100 μL of reaction mixture was diluted 10-fold in a solution containing 150 mM NaCl with or without 5 μM nigericin. At various times 100-μL aliquots of the diluted reaction mixture were taken and the $^{22}Na^+$ content of the proteoliposomes was determined as described under Experimental Procedures. (△) With GABA, at the loading stage, without nigericin; (○) without GABA and nigericin; (▲) with GABA and nigericin; (●) without GABA but with nigericin.

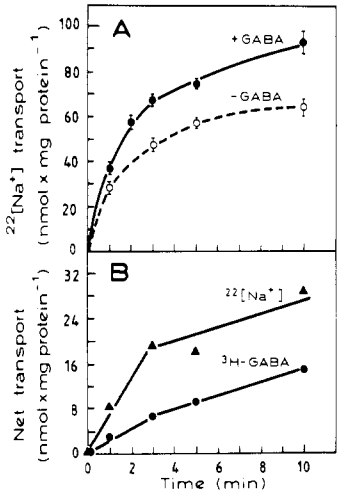

FIGURE 3: GABA-dependent $^{22}Na^+$ fluxes as compared to GABA fluxes. (A) Proteoliposomes (10 μL, 18 μg of protein) containing 120 mM KP_i, pH 6.8, as internal medium were diluted into 100 μL of a reaction medium of the following compositions: 10 mM NaCl, 140 mM LiCl, 5 μM valinomycin, and 3.5 μCi of carrier-free $^{22}Na^+$ with (●) or without (○) 1 mM GABA. The reactions were stopped at the indicated times as described Experimental Procedures. (B) GABA-dependent $^{22}Na^+$ flux (▲) is the difference of the two experiments shown in (A). GABA fluxes (●) were determined as follows: 10 μL of the same proteoliposomes were diluted with 100 μL of a reaction mixture containing 10 mM NaCl, 140 mM LiCl, 5 μM valinomycin, 1 mM GABA, and 4 μCi of [^{3}H]GABA. Reactions were terminated at the indicated times as described under Experimental Procedures. The points shown were those corrected for by subtracting values obtained in the presence of 20 mM unlabeled GABA. The error bars in (A) indicate the standard deviation.

porter, the next step was to see whether it can also support net $^{22}Na^+$ fluxes. As can be seen in Figure 3A—where the same experiment was done as in Figure 1 except that the proteoliposomes were prepared in a potassium-containing rather than a sodium-containing medium—there is a GABA-dependent of $^{22}Na^+$ uptake. GABA influx is an electrogenic process that is strongly stimulated by an interior negative membrane potential (Kanner, 1978; Radian & Kanner, 1985; Radian et al., 1986). Addition of valinomycin to the potassium-containing proteoliposomes not only strongly stimulates GABA influx, its presence is required to detect the GABA-dependent sodium flux (Table I). Furthermore, the process requires chloride (Table I) notwithstanding that the proteoliposomes contained potassium and valinomycin was present. Thus, chloride is not exerting its effect by charge compensation following the positive charge inside, since this function would

Table I: Effect of Valinomycin and External Chloride on GABA-Dependent $^{22}Na^+$ Fluxes

addition[a]	$^{22}Na^+$ flux [nmol (mg of protein)$^{-1}$]	
	-GABA	+1 mM GABA
none	104.5 ± 10.5	108.0 ± 6.6
valinomycin	102.9 ± 5.9	134.5 ± 14.3
external ion[b]		
Cl^-	53.3 ± 9.7	86.5 ± 6.6
SO_4^{2-}	58.5 ± 9.0	50.0 ± 6.4

[a] Proteoliposomes (10 μL, 12.3 μg of protein), loaded with 120 mM KP_i, pH 6.8, were diluted into 100 μL of 10 mM NaCl, 140 mM LiCl, and 3.5 μCi of carrier-free $^{22}Na^+$ with or without 5 μM valinomycin in the presence or absence of GABA, as indicated. Transport was measured for 5 min. [b] The KP_i-loaded proteoliposomes (15 μg of protein) were added to 100 μL of either the above valinomycin-containing influx medium (with external chloride) or 5 mM Na_2SO_4, 70 mM Li_2SO_4, 75 mM sucrose, 5 μM valinomycin, and 3.5 μCi of carrier-free $^{22}Na^+$ in the presence or absence of GABA as indicated. Transport was measured for 5 min.

Table II: Substrate Specificity for GABA-Dependent $^{22}Na^+$ Flux[a]

substrate	$^{22}Na^+$ flux [nmol (mg of protein)$^{-1}$]
none	59.8 ± 0.3
GABA	84.5 ± 4.5
D-glutamate	65.4 ± 4.2
D-glucose	60.7 ± 8.9

[a] Proteoliposomes (10 μL, 21 μg of protein), loaded in 120 mM KP_i, pH 6.8, were incubated for 3 min with 100 μL of 10 mM NaCl, 140 mM LiCl, 5 μM valinomycin, and 3.5 μCi of carrier-free $^{22}Na^+$. Substrates were added to a final concentration of 1 mM. Transport was measured as described under Experimental Procedures.

be fulfilled much better by exit of potassium mediated by valinomycin. These observations parallel those observed with GABA fluxes (Kanner, 1978; Radian & Kanner, 1983). These observations strongly suggest that the GABA-dependent component is occurring via its transporter. Again, the $^{22}Na^+$ counts accumulated could be discharged by nigericin (data not shown), and thus the process represents net GABA-dependent $^{22}Na^+$ flux. It also shows specificity for GABA as it could not be replaced by glucose or l-glutamate (Table II). The inability of glutamate to support the process is to be expected, since the l-glutamate transporter is fractionated away in the ammonium sulfate step to purify the GABA transporter (data not shown).

In order to estimate the Na^+/GABA stoichiometry, it is necessary to compare GABA-dependent sodium fluxes and sodium-dependent GABA fluxes measured under identical conditions. A problem encounted in this regard was that sodium flux experiments had to be terminated by passage of the reaction mixture over Tris–Dowex columns rather than membrane filtration (too high background), while the latter method had to be used for the GABA fluxes. Probably the reason for this is that GABA—while being a zwitterion—probably has a lower binding affinity to the Dowex resin than Tris. Therefore, the fluxes of $^{22}Na^+$ and [^{3}H]GABA were done under identical conditions but the termination was done differently. GABA transport was corrected for binding by subtracting values obtained in the presence of 20 mM unlabeled GABA (Figure 3B). As shall be shown later (Table V) values obtained in such a fashion are very similar to those obtained in the absence of either sodium or chloride. Thus, the GABA transport as measured in Figure 3B is fully sodium (as well as chloride) dependent. The stoichiometry calculated from the experiment depicted in Figure 3B was 3.0. However, there is quite a large variation in this value from experiment to experiment. A value of 2.58 ± 0.99 (SD) was found,

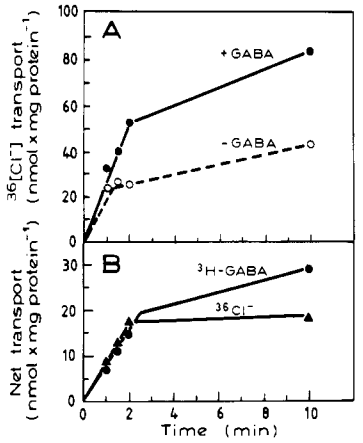

FIGURE 4: GABA-dependent $^{36}Cl^-$ fluxes, as compared to GABA fluxes. (A) Proteoliposomes (10 μL, 17 μg of protein) containing 120 mM KP_i, pH 6.8, as internal medium were diluted into 100 μL of a reaction medium of the following composition: 100 mM NaP_i, pH 6.8, 5 μM valinomycin, and 1 μCi of $^{36}Cl^-$ (equivalent to 22 mM HCl) with (●) or without (○) 1 mM GABA. The reactions were stopped at the indicated times as described under Experimental Procedures. (B) GABA-dependent $^{36}Cl^-$ flux (▲) is the difference of the two experiments shown in (A). GABA fluxes (●) were determined as follows: 10 μL of the same proteoliposomes were diluted in 100 μL of a reaction mixture containing 100 mM NaP_i, pH 6.8, 5 μM valinomycin, 22 mM HCl, 1 mM GABA, and 4 μCi of [^{3}H]GABA. Reactions were terminated at the indicated times as described under Experimental Procedures. The points shown were those corrected for by subtracting values obtained in the presence of 50 mM unlabeled GABA.

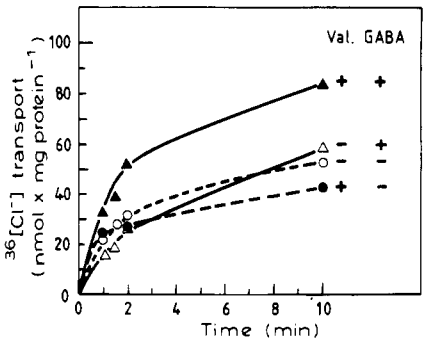

FIGURE 5: Effect of valinomycin on GABA-dependent $^{36}Cl^-$ fluxes. Proteoliposomes (10 μL, 16 μg), containing 120 mM KP_i, pH 6.8, as internal medium, were diluted into 100 μL of a medium containing 100 mM NaP_i and 1 μCi of $^{36}Cl^-$ with (●) or without (○) 5 μM valinomycin or with 1 mM GABA with (▲) or without (△) 5 μM valinomycin. At the indicated times reactions were stopped as described under Experimental Procedures.

summarizing the data from five independent experiments. This is due to the difficulty in accurately estimating the GABA-dependent sodium flux, as it was relatively low compared to the background—the GABA-independent component. Thus, while it is quite clear that the Na^+/GABA stoichiometry is greater than 1, we do not know if it is 2 or 3.

GABA-Dependent Net $^{36}Cl^-$ Flux. GABA-dependent $^{36}Cl^-$ uptake could be measured by using Dowex (acetate) columns to terminate the reaction (Figure 4A). Since the process requires external sodium and is specific for GABA (Table III), it appears that it is catalyzed by the GABA transporter. This idea is reinforced by its valinomycin dependence (Figure 5). Diffusion of $^{36}Cl^-$ through the membrane would be inhibited by an interior negative membrane potential, i.e., valinomycin addition under the conditions of the experiment. As a matter of fact, there is some inhibition of the GABA-independent component of $^{36}Cl^-$ uptake (Figure 5). However, the GABA-dependent component is strongly stimulated by valinomycin. Thus, it can be concluded that this process is catalyzed by the GABA transporter and due to transport into the proteoliposomes.

Table III: Cation and Substrate Dependence of $^{36}Cl^-$ Fluxes

(A) Cation Dependence[a]		
external ion	$^{36}Cl^-$ flux [nmol (mg of protein)$^{-1}$]	
	-GABA	+1 mM GABA
Na^+	36.6 ± 0.4	52.5 ± 3.4
Li^+	29.5 ± 3.0	34.9 ± 4.8
$Tris^+$	36.4 ± 8.7	38.1 ± 4.7
(B) Substrate Dependence[b]		
substrate	$^{36}Cl^-$ flux [nmol (mg of protein)$^{-1}$]	
none	25.4 ± 1.3	
GABA	52.1 ± 6.2	
D-glutamate	23.4 ± 0.6	
D-glucose	24.3 ± 9.7	

[a] Proteoliposomes (10 μL, 16.6 μg of protein) loaded in 120 mM KP_i, pH 6.8, were incubated for 3 min in 100 μL of 100 mM NaP_i, pH 6.8, 1 μCi of $^{36}Cl^-$ (equivalent to 22 mM HCl), and 5 μM valinomycin with or without 1 mM GABA or they were incubated in similar media in which LiP_i or Tris-P_i (both pH 6.8) replaced the NaP_i. Transport was measured as described under Experimental Procedures. [b] Transport was measured as above for 10 min with the substrates added to a final concentration of 1 mM.

The Cl^-/GABA stoichiometry is estimated from the ratio of GABA-dependent $^{36}Cl^-$ flux and chloride-dependent [^{3}H]GABA flux. Also here there is the restriction that the termination of the reaction is done under different conditions, although the two measurements are done under the same conditions. In the case of chloride transport, the GABA-dependent component is relatively large (Figure 4A), and therefore the quantitation of the stoichiometry is relatively accurate (Figure 4B). A value of 1.27 ± 0.12 (SD) was found, summarizing the data from four independent experiments. Thus, there is much less variation than with the determination of Na^+/GABA. Since there is no inherent reason for underestimates with this method, it is apparent that one chloride ion is translocated per GABA molecule.

Two Kinetically Distinct GABA Transporters. In the GABA-dependent sodium and chloride flux experiments, very high GABA concentrations (1 mM) have been used. However, the K_m for GABA uptake under optimal conditions is between 2 and 4 μM, and even in the presence of low concentrations of either sodium and chloride, it is about 10–15 μM (Kanner et al., 1983). Thus, we examined the concentration dependence of the ability of GABA to stimulate sodium and chloride fluxes. From the experiments depicted in Figure 6 it can be seen that the concentration of GABA required to give half-maximal $^{22}Na^+$ (Figure 6B) or $^{36}Cl^-$ (Figure 6A) fluxes is substantially higher—around 80 μM. A strict comparison is not possible since these are measurements of extent, and a kinetic measurement with suboptimal GABA concentrations is technically difficult (low signal to noise ratio). Under the assumption that the affinity for GABA influx is higher than that for GABA-dependent sodium and chloride flux, a possible explanation might be that there are (kinetically, at least) two types of GABA transport—one with high and one with low affinity. In order to check this possibility, the kinetics of GABA transport were measured over an extended GABA concentration range, and the results were translated to Eadie–Hofstee plots (Figure 7). The experimental points are well fitted by two straight lines (Figure 7A–C), suggesting the presence of a low-affinity GABA transport system in addition to the high-affinity one. As an approximation, at each of the higher concentrations the initial velocity was corrected for that

Table IV: Kinetic Parameters for High- and Low-Affinity GABA Transport

		high affinity		low affinity	
ionic condition	preparation	K_m (μM)	V_{max} [pmol min^{-1} (mg of protein)$^{-1}$]	K_m (μM)	V_{max} [pmol min^{-1} (mg of protein)$^{-1}$]
low sodium	proteoliposomes	13.8	108	933	2014
low chloride	proteoliposomes	7.7	400	753	2380
optimal	proteoliposomes	2.2	1700	170	3350
optimal	membrane vesicles	6.3	1700	163	1935

[a] Low sodium, low chloride, and optimal ionic conditions are those described in the legend to Figure 7 (parts A, B, and C, respectively). K_m was calculated from the slopes of the plots of high- and low-affinity transport, and V_{max} was derived from the intercepts with the ordinate. The data on the proteoliposomes are a summary of those from Figure 7.

Table V: Ion Dependence and Ionophore Sensitivity of Low-Affinity GABA Transport

condition	low-affinity GABA transport [nmol min^{-1} (mg of protein)$^{-1}$]
control	12.9
+10 mM GABA	2.2
+5 μM nigericin	3.6
no sodium	2.3
no chloride	2.5
no sodium + 10 mM GABA	1.2
no chloride + 10 mM GABA	1.5
no chloride + 5 μM nigericin	2.7
control without valinomycin	4.0

[a] Transport was measured by using proteoliposomes (10 μL, 16 μg of protein) preloaded with 120 mM KP_i, pH 6.8, which were diluted into 100 μL of 150 mM NaCl, 1 mM GABA (40 cpm/pmol), and 5 μM valinomycin. For the conditions "no sodium" and "no chloride" the 150 mM NaCl was replaced by 150 mM LiCl or 150 mM sodium glucuronate, respectively. Other additions are indicated in the table at their final concentrations.

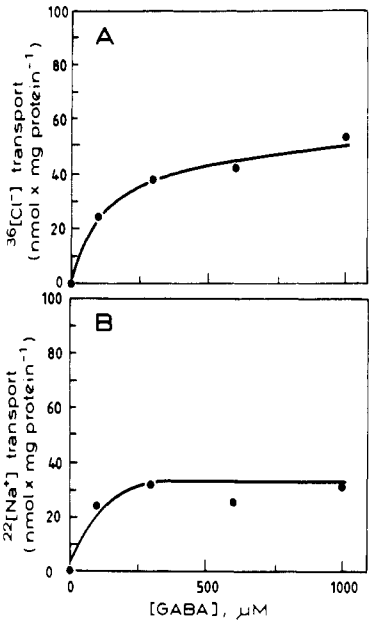

FIGURE 6: Dependence of $^{22}Na^+$ and $^{36}Cl^-$ fluxes on the GABA concentration. (A) GABA-dependent $^{36}Cl^-$ fluxes were determined for 10 min as in Figure 4 except that the GABA concentration was varied as indicated on the abscissa. (B) GABA-dependent $^{22}Na^+$ fluxes were determined for 5 min as in Figure 3 except for the variation in the GABA concentration.

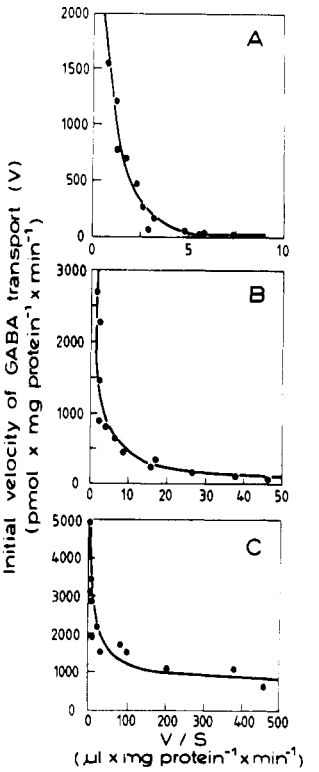

FIGURE 7: Kinetics of sodium- and chloride-dependent GABA fluxes. The proteoliposomes used (10 μL per assay point), which contained 120 mM KP_i, pH 6.8, as internal medium, were diluted into 100 μL of reaction mixture, of which the compositions are given below, containing 1.27–2000 μM GABA with a specific radioactivity between 5512 and 165 cpm/nmol. The data were corrected for nonspecific binding by subtracting values obtained in parallel experiments in which the unlabeled GABA concentration was raised to 50 mM. The time of the assay was 3 min (the reaction is still linear with time). Reactions were stopped as described under Experimental Procedures. (A) The reaction mixture contained 10 mM NaCl, 140 mM LiCl, and 5 μM valinomycin. Per time point 21 μg of protein was used. (B) The reaction mixture contained 100 mM NaP_i, pH 6.8, 22 mM HCl, and 5 μM valinomycin. Per time point 28.5 μg of protein was used. (C) The reaction mixture contained 150 mM NaCl and 5 μM valinomycin. Per time point 18 μg of protein was used.

contributed by the high-affinity system by using the slope from the right-hand part of the graphs. The resulting velocities were replotted according to the Eadie–Hofstee method. This yielded a single straight line—probably reflecting the low-affinity system—and the kinetic parameters were derived by linear regression (Table IV). With the present analysis we cannot rule out alternative possibilities, that is, three distinct transport systems, but it is clear that there is a high-affinity system and one (or more) low-affinity system(s). The latter has (have) an affinity 30–100-fold lower than that of the high-affinity system. This kinetic behavior is observed not only under optimal conditions, i.e., 150 mM NaCl (Figure 7C), but also under the conditions that were used for the sodium flux [10 mM NaCl + 140 mM LiCl (Figure 7A)] or for the chloride flux [100 mM NaP_i + 22 mM HCl (Figure 7B)]. The phenomenon is not an artifact caused by the reconstitution, since it was also observed in membrane vesicles (Table IV). Not only was there a huge decrease of affinity of the low-affinity system as compared to the high-affinity one, but the V_{max} values of the two systems are also different, except when optimal sodium and chloride are present (Table IV). From these data, it is clear that when $^{22}Na^+$, $^{36}Cl^-$, or [^{3}H]GABA

transport is measured in the presence of 1 mM GABA, mainly the low-affinity system is measured.

As can be seen in Table V, the low-affinity system is also sodium as well as chloride dependent. Excess (10 mM) unlabeled GABA inhibits the process strongly, although some [^{3}H]GABA is still taken up, indicating that this may be due to binding. That this is the case is further supported by the observation that this level of uptake is similar to that without 10 mM GABA in the absence of either sodium or chloride (Table V). It is also similar to that without 10 mM GABA in the presence of nigericin, an ionophore that under these conditions will collapse the electrochemical gradient of sodium (Table V). Furthermore, the GABA (10 mM) sensitive process is sensitive to increases in the medium osmolarity, whereas the resistant component is not (data not shown). Low-affinity GABA transport appears to be electrogenic since it is strongly stimulated by valinomycin (Table V).

Discussion

All the previous mechanistic studies on sodium- and chloride-dependent GABA transport from rat brain indicated that those occur via coupled transport with both sodium and chloride [reviewed in Kanner (1983)]. Most of the predictions of such cotransport could be verified by using membrane vesicles derived from the synaptic plasma membrane except for the GABA-dependent sodium and chloride fluxes. In membrane vesicles the background (GABA-independent) ion uptake is too high, probably because vesicles leaky to ions are present in such heterogeneous preparations. Using ion impermeant liposomes into which a partially purified GABA transporter preparation was reconstituted (Radian & Kanner, 1985), we have now been able to measure GABA-dependent sodium and chloride fluxes (Figures 3 and 4). Those fluxes are apparently catalyzed by the GABA transporter since they are chloride (Table I) and sodium dependent (Table III), respectively, and are electrogenic—stimulated by a negative membrane potential on the trans side (Table I and Figure 5).

As outlined in the introduction, the stoichiometry expected would be at least two sodium ions per chloride and GABA. Observations consistent with this have been obtained in membrane vesicles, where the stoichiometry was determined by using a thermodynamic approach (Radian & Kanner, 1983). Further information on this is now available from the direct flux measurements. On the Na^+/GABA stoichiometry we cannot yet decide whether this is 2 or 3. The decision of this issue will have to await a purification procedure of the GABA transporter that will yield not only pure but fully active transporter. On the other hand, the Cl^-/GABA stoichiometry is clearly 1. Thus, it appears that the data obtained with the kinetic method are in agreement with those obtained with the thermodynamic method. Since the different GABA transport systems are operating under the conditions of the kinetic measurements (Figures 6 and 7; Table IV), future research will have to be devoted to the question if both transport systems display the same stoichiometry. This is very likely to be the case since the low-affinity system is also both sodium and chloride dependent and electrogenic (Table V). Although low-affinity GABA uptake in synaptosomes has already been reported, it has not been characterized very well (Wood & Sidhu, 1986). The existence of two transport systems for GABA, at least kinetically, is of particular interest in view of the possibility that they may be interrelated. If so, there is the possibility that one could be converted into the other, for instance by phosphorylation/dephosphorylation. This might constitute a cellular mechanism by which the transporter could be regulated; conversion of the high-affinity to the low-affinity form might very effectively stop reuptake of GABA from the synaptic cleft. The high-affinity GABA transport has been purified to homogeneity in a reconstitutively active form, and antibodies against it have been prepared (Radian et al., 1986). The pure transporter is partly inactivated (Radian et al., 1986), making it difficult to see if this preparation also exhibits low-affinity transport. Yet there are clearly experimental possibilities to shed light on this question in future studies.

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